Peroxisome Proliferator-activated Receptor α (PPARα) Agonist Treatment Reverses PPARα Dysfunction and Abnormalities in Hepatic Lipid Metabolism in Ethanol-fed Mice*

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Monika Fischer, Min You, Michinaga Matsumoto, and David W. Crabb‡

From the Departments of Medicine and Biochemistry and Molecular Biology, Indiana University School of Medicine and Richard Roudebush Veterans Affairs Medical Center, Indianapolis, Indiana 46202

Proper function of the peroxisome proliferator-activated receptor α (PPARα) is essential for the regulation of hepatic fatty acid metabolism. Fatty acid levels are increased in liver during the metabolism of ethanol and should activate PPARα. However, recent in vitro data showed that ethanol metabolism inhibited the function of PPARα. We now report that ethanol feeding impairs fatty acid catabolism in the liver in part via blocking PPARα-mediated responses in C57BL/6J mice. Ethanol feeding decreased PPARα/retinoid X receptor α binding in electrophoretic mobility shift assay of liver nuclear extracts. mRNAs for PPAR-regulated genes were reduced (long chain and medium chain acyl-CoA dehydrogenases) or failed to be induced (acyl-CoA oxidase, liver carnitine palmitoyl-CoA transferase, very long chain acyl-CoA synthetase, very long chain acyl-CoA dehydrogenase) in livers of the ethanol-fed animals, and ethanol feeding did not increase the rate of fatty acid β-oxidation. Wy14,643, a PPARα agonist, restored the DNA binding activity of PPARα/retinoid X receptor α, induced mRNA levels of PPARα target genes, stimulated the rate of fatty acid β-oxidation, and prevented fatty liver in ethanol-fed animals. Impairment of PPARα function during ethanol consumption contributes to the development of alcoholic fatty liver, which can be overcome by Wy14,643.

Alcohol-induced liver injury is the most common liver disease in which fatty acid metabolism is deranged. It has been traditionally held that during alcohol metabolism, the increase in cellular NADH concentration generated by alcohol and aldehyde dehydrogenases impairs β-oxidation and tricarboxylic acid cycle activity. This in turn leads to severe fatty acid (FFA) overload, increased synthesis of triacylglycerol, and steatosis, despite elevated rates of very low density lipoprotein synthesis and secretion (1, 2). Although fatty liver was thought in the past to be relatively benign, more recent studies show that fat accumulation renders the liver more susceptible to injury by other agents such as drugs and toxins, especially endotoxins (3), which are believed to be involved in the pathogenesis of alcoholic hepatitis and fibrosis (4, 5). In addition, obesity, which may potentiate the development of fatty liver, has also been shown to be an independent risk factor for cirrhosis in alcohol abusers (6). Therefore, fatty liver must be treated as an important liver disorder, possibly amenable to better therapy.

Peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear hormone receptor superfamily and a receptor for FFA (7), has been identified as a key transcriptional regulator of many genes involved in FFA oxidation systems in liver (8). Studies on PPARα null mice have shown that the function of the PPARα battery is essential for constitutive mitochondrial fatty acid catabolism (9, 10). Fasted PPARα null mice suffer from a severe impairment in hepatic FFA oxidation, resulting in a phenotype characterized by hypoglycemia, hypocarnitinaemia, elevated plasma levels of FFAs, and fatty liver (11). On the other hand, constitutive peroxisomal β-oxidation is quite independent of PPARα, but the receptor is required for induction of this system by peroxisome proliferators (9). Recent observations (11–14) point out the critical importance of PPARα in determining the severity of hepatic steatosis in fasting, in diabetes (14), and in animals fed a high fat diet (11).

Because PPARα coordinates fatty acid metabolism in the liver, and PPARα knock-out mice develop fatty liver when fasted (12–14), it is obvious to ask whether PPARα is involved in the pathogenesis of alcoholic fatty liver. Fatty acid levels are increased in the liver during the metabolism of ethanol; therefore, the PPARα battery of proteins should be induced by alcohol consumption. Although a subset of the PPARα responsive genes were reported to be induced by ethanol, such as cytochrome P450 4A1 (lauryl ω-hydroxylase) (15) and liver fatty acid-binding protein (16), many others did not change or even decreased (17). Increased generation of dicarboxylic fatty acids because of enhanced lauryl ω-hydroxylase activity and the failure of ethanol to induce acyl-CoA oxidase (18–20), the first step in peroxisomal β-oxidation, led to augmented excretion of dicarboxylic fatty acids in the urine in alcohol-fed rats (15) and in alcoholic men (21). Medium chain acyl-CoA dehydrogenase activity (22) and mRNA level (20) were decreased by ethanol feeding.

β-OHB, β-hydroxybutyrate; RT, reverse transcription; PPRE, PPAR response element; SREBP, sterol regulatory element-binding protein.
These reports suggest that the PPARα battery of fat-metabolizing enzymes is not fully induced during ethanol feeding and suggest the possibility that activation of this system would ameliorate some of the toxic effects of ethanol. Indeed, a recent study (23) showed that fenofibrate, a PPARα ligand, ameliorated the fatty liver and hepatomegaly of ethanol feeding in three of five rats and decreased serum triacylglycerol level in alcoholic patients despite the fact that they continued drinking during the fenofibrate administration. Similarly, an early study indicated that feeding rats clofibrate ameliorated the fatty liver and hepatomegaly resulting from ethanol feeding (24).

Recent data from our laboratory (25) showed that ethanol and its metabolite, acetaldehyde, inhibited the transcriptional and DNA binding activity of the PPARα receptor, inhibited PPARα activation of a reporter plasmid in hepatoma cells, and reduced the ability of Wy14,643, a potent PPARα agonist, to activate the reporter in hepatoma cells or cultured rat hepatocytes. This effect of ethanol was abolished by the alcohol dehydrogenase inhibitor 4-methylpyrazole and augmented by the aldehyde dehydrogenase inhibitor cytochrome P450, indicating that acetaldehyde was likely responsible for the effect of ethanol. Furthermore, in vitro translated PPARα exposed to acetaldehyde failed to bind DNA (26). Because some PPARα-regulated genes mentioned above are induced with ethanol treatment, it is uncertain to what extent the effect of ethanol on the expression of a simple reporter construct in a hepatoma cell can be extrapolated to the expression of hepatic genes under the control of complex promoters or what the potential contribution of PPARα dysfunction to the multiple effects of ethanol on the liver might be. Therefore, in the present paper we study the effect of chronic ethanol feeding on PPARα and RXRα levels, PPAR/RXR binding activity, levels of PPAR-regulated genes, measures of fat metabolism in mice fed alcohol chronically, and the ability of the PPARα agonist Wy14,643 to reverse the effects of ethanol.

MATERIALS AND METHODS

Animals and Diets—6–8-week-old male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). Animals were housed individually in stainless steel wire-bottom cages in a temperature- and humidity-controlled room (23 °C and 50% relative humidity) with a 12:12-h yellow light/dark cycle. Liquid diets were based upon the Lieber-DeCarli formulation (27) and provided 1 kcal/ml (prepared by DYETS, Bethlehem, PA). Protein content was constant at 18% of total calories, fat 10% (6% from cocoa butter and 4% from safflower oil), and each diet had identical mineral and vitamin content. The animals were divided into four dietary groups: (a) ethanol-containing diet (27.5% of total calories), (b) pair-fed control diet in which ethanol was replaced isocalorically with carbohydrate, (c) control diet supplemented with 0.1% Wy-14,643, and (d) ethanol-containing diet supplemented with 0.1% Wy-14,643. In the latter two groups Wy-14,643 (Biomol Research Laboratories, Plymouth Meeting, PA) was added to the diet for the last 4 weeks of liquid diet feeding, the animals were sacrificed at 11 a.m., at which time blood and liver tissue were collected. The studies were approved by the Indiana University School of Medicine Animal Care and Use Committee and the Veterans Affairs Animal Use Subcommittee.

Assays from Blood—After animals were anesthetized, blood was collected by heart puncture. Plasma was separated by centrifugation at 4 °C and stored at −20 °C. Plasma triacylglycerol and glucose were determined using kits from Roche Diagnostics. β-Hydroxybutyrate (β-OHB) was measured using a KetoSite kit (GDS Diagnostics, Elkhart, IN). FFA was determined with a NEFA C-Test (Wako Chemicals, Richmond, VA), and cholesterol was determined with Infinity cholesterol determination kits (Wako Chemicals, Richmond, VA).

Tissue Lipid Determination—Frozen sections of the liver (10 μm) were stained with Oil Red O and counterstained with hematoxylin and eosin for histology. Samples of liver were homogenized in 0.25% sucrose containing 1 mM EDTA. Lipids were extracted using chloroform/methanol (2:1, v/v) and evaporated in a Speedvac, and the pellets were dissolved in 5% fatty acid-free bovine serum albumin dissolved in water. Protein in the homogenate was assayed using protein assay reagent (Bio-Rad) to normalize the amount of lipid extracted. Colorimetric cholesterol and triacylglycerol assays were carried out as described above.

Fatty Acid β-Oxidation Activity—Fatty acid β-oxidation activity was measured according to Aoyama et al. (9). Briefly, fresh livers were homogenized and incubated with the assay medium containing 50 μM [1-14C]palmitic acid (American Radiolabeled Chemicals, St. Louis, MO) for 30 min. The reaction was stopped by adding 0.2 M perchloric acid. The mixture was centrifuged at 2000 × g for 10 min, and the unmetabolized fatty acids were removed by three extractions using 2 ml of n-hexane. Radioactive degradation products in the water phase were determined by thin-layer chromatography. Palmitate β-oxidation activity was expressed as pmol [1-14C]palmitate oxidized/mg protein/min.

Preparation and Analysis of Nuclear Extracts—Fresh livers were homogenized in 10 mM Hepes (pH 7.9), 25 mM KCl, 10 mM NaF, 0.15 mM spermine, 1 mM EDTA, 10% glycerol, 2 mM succrose, 2% protease inhibitor (Roche Diagnostics), and 0.5 mM dithiothreitol, and nuclear proteins were extracted as described by Neish et al. (28) except that 1 mM NaF and 2% protease inhibitor were added to all buffers. Western blotting was performed by fractionating 80 μg of nuclear extract protein on 10% SDS-PAGE gels. After electrophoresis onto nitrocellulose membranes, blots were blocked with Tris-buffered saline containing 0.1% Tween 20 and 5% dry milk for 1 h at room temperature and incubated overnight with each of the following polyclonal rabbit antibodies: anti-PPARα and anti-PPARγ (Geneka Biotechnology, Montreal, Quebec, Canada) and anti-RXRα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immune complexes were detected using the ECL Plus kit (Amersham Biosciences). Immunoreactive bands were quantified using Fluorimag (Amersham Biosciences). DNA binding assays were performed using the Gelshift kit according to the manufacturer’s instructions (Geneka Biotechnology, Montreal, Quebec, Canada). The following probes were used: PPAR response element (PPRE), 5′-GAAGTACGTTCAAGGATCTCATCCCT-3′; and mutant PPRE, 5′-GAAGTACGAGGATAGACATCCCT-3′. For supershift assays, anti-PPARα antibody was added (Geneka Biotechnology). Reaction mixtures containing 5-μg nuclear extracts were separated by electrophoresis (4°C) on a nondenaturing PAGE in 1% Tris/glycine electrophoresis buffer at 4 °C. The shifted bands were quantified using a PhosphorImager and ImageQuant (Amersham Biosciences) software analysis.

RNA Isolation and Quantitative RT-PCR—Total RNA was prepared from frozen liver using an RNaseasyTM Total RNA kit (Qiagen). Quantitative RT-PCR was performed using reverse transcription and co-amplification of 18S ribosomal RNA as internal control (QuantumRNA 18S internal standard kit; Ambion, Austin, TX) according to the manufacturer’s instructions. 5 μg of RNA was mixed with 2 μl of random primers (50 μM; Ambion) and RNase-free water up to 13 μl of volume, denatured at 80 °C for 5 min, and rapidly cooled on ice for 5 min. Then, 1 μl of dNTP mix (10 mM), 0.5 μl of primers (Promega), 4 μl of avian myeloblastosis virus reverse transcriptase 5× buffer (Promega), and 1 μl of avian myeloblastosis virus RT enzyme (Promega) were added to each sample. The mixtures were incubated for 1 h at 42 °C. PCR was performed in duplicate using 2 μl of the RT product in a reaction mixture containing 5 μl of 10× PCR buffer (Promega), 2 mM MgCl2, 1 μl of dNTPs (10 mM), 2 μl of gene-specific primer pair (5 μM each), 4 μl of 18 S Classic Primer and Competimer pair (Ambion), 0.25 μl of Taq DNA polymerase, 0.5 μl of [α-32P]dATP (10 μCi/μl), and nulease-free water up to 50 μl. The optimal ratio of 18 S Classic Primer:Competimer was determined for each target gene individually. PCR was performed using the following general cycle profile: 40 cycles of 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 60 s, followed by a 72 °C for 7-min extension. PCR primer pairs were designed from mouse- and rat-specific sequence data (Gen-Bank™) except for LCAD (29). PCR cycle numbers were determined for each primer pair by extensive pretesting to optimize the conditions and that the product is with in the linear range of the PCR amplification reaction. The PCR product was separated on a 6% polyacrylamide gel and quantified with a PhosphorImager and ImageQuant software (Amersham Biosciences). Data were expressed as the ratio of the mRNAs of interest to that of the internal control.

Data Analysis—All data are expressed as mean ± S.D. for the experiments, which included between four and eight animals in each group. The paired t-test was used to evaluate statistical differences between ethanol- and pair-fed mice. A p value < 0.05 was considered significant.
mice, increasing the liver/body weight ratio from 0.04. Treatment enlarged the livers of both ethanol- and pair-fed mice. A significant increase in the liver/body weight ratio. Wy14,643 co-treated animal did not show evidence of steatosis. Contrast, the liver of ethanol-fed and Wy14,643 co-treated animal did not show evidence of steatosis.

FIG. 1. Comparative liver histology of control (A), ethanol-fed (B), Wy14,643-fed (C), and ethanol + Wy14,643-fed (D) mice. Representative frozen liver sections of each group stained with Oil Red O to detect neutral lipids are shown. Fat accumulation was conspicuous in the liver of ethanol-fed animal. In contrast, the liver of ethanol-fed and Wy14,643 co-treated animal did not show evidence of steatosis.

RESULTS

Characterization of the Liver Response to the Diets and Drug Treatment—To determine the effect of ethanol on PPARα in vivo, we fed mice ethanol (27.5% of the total calories) using the Lieber-DeCarli liquid diet and a pair-feeding protocol (27) for 4 weeks. Ethanol was introduced gradually into the diet; after the animals were accommodated to the liquid diet, they were given alcohol 9% of the total calories for 2 days, then 18% for 3 days, and finally 27.5%. Two groups of mice were given 0.1% ethanol alone for the last 2 weeks of the feeding period. Ethanol feeding did not cause a significant increase in the body weight was observed in all four groups at the end of the feeding period. Ethanol feeding did not cause a significant increase in the liver/body weight ratio. Wy14,643 treatment enlarged the livers of both ethanol- and pair-fed mice, increasing the liver/body weight ratio from 0.04 ± 0.004 to 0.08 ± 0.007, a known effect of the hepatocyte hypertrophy and hyperplasia caused by PPARα in rodents (30).

Histological analysis of the livers with Oil Red O staining revealed prominent lipid accumulation in the livers of ethanol-fed animals (Fig. 1B) whereas lipid droplets were rare in the livers of the control group (Fig. 1A). Liver sections of pair-fed mice given Wy14,643 treatment looked similar to those of controls (Fig. 1C). When the ethanol-fed animals were co-treated with Wy14,643 for the last 2 weeks of the experiment, the Oil Red O staining showed no sign of hepatic steatosis (Fig. 1D).

Quantification of liver lipids was concordant with the histologic findings (Fig. 2A). Ethanol feeding increased the liver triacylglycerol content by 4-fold. Wy14,643 treatment alone did not alter the amount of triacylglycerol in the liver. In striking contrast, when Wy14,643 was co-administered for the last 2 weeks of the ethanol feeding, the triacylglycerol content was significantly decreased to the level of control animals. Cholesterol content was induced by 2-fold in the livers of ethanol-fed animals (Fig. 2B), possibly a result of activation of SREBP-1 (31). Wy14,643 itself increased liver cholesterol content by about 2-fold. Gemfibrozil, another PPARα agonist, was reported recently (32) to increase the transcriptional activity of SREBP-2, a major regulator of cholesterol biosynthesis, and strongly induced 3-hydroxy-3-methylglutaryl-CoA reductase mRNA and activity. This effect of the PPARα activator may have resulted in enhanced cholesterol production in the liver. Animals fed ethanol plus Wy14,643 had lower cholesterol in liver than those W14,643.

Effects of Chronic Ethanol Administration and Wy14,643 on Plasma Lipids and Hepatic Fatty Acid β-Oxidation—We measured plasma lipids to better understand the mechanisms by which ethanol and Wy14,643 might influence hepatic steatosis. Because the liquid diets were withdrawn which ethanol and Wy14,643 might influence hepatic steatosis. Plasma lipids were taken, the plasma FFA most likely reflects the balance between peripheral lipolysis and hepatic fat oxidation, as the liver is the major utilizer of circulating FFAs in the early postabsorptive phase. Chronic ethanol feeding significantly increased the serum levels of FFA, triacylglycerol, and β-OHB as shown in Table II. Wy14,643 entirely prevented the elevation of FFA and triacylglycerol caused by ethanol, and its administration was accompanied by an even higher level of β-OHB than ethanol alone. Chronic ethanol feeding had no effect on serum cholesterol level, whereas Wy14,643 treatment alone and in combination with ethanol feeding increased the level of cholesterol.

| Gene   | Forward primer 5'–3' | Reverse primer 5'–3' | Predicted product size (bp) | Cycle No. | GenBank™ accession No. |
|--------|----------------------|----------------------|----------------------------|-----------|------------------------|
| AOX    | cttgtgcgcggcataaggg  | caggagctcactgttacc   | 216                        | 26        | AF006688               |
| MCAD   | tggagacacttggaacgacgc| accatagactgagaacaggg | 355                        | 26        | NM007382               |
| LCAD   | aaggtttttcttggaacagcc| ggaaggaggaccggagtc   | 380                        | 28        | J05029                 |
| VLCAD  | cgtgagaggctactttgtagtg| cttggactcaacatcaactgc| 288                        | 30        | AF017176               |
| LCPT   | ccagcaagggggaattg   | tggcccaaatctctttgc   | 212                        | 26        | AF017175               |
| VLACS  | tgtgtgtgtgtgtgagcc   | tttgtgtgtgtgtgttccc  | 270                        | 22        | AJ223958               |
| L-FABP | cctggtgcctgatcgattcc| gttattggtgtgtgttccc  | 157                        | 28        | Y14660                 |
| PPARα  | agggactcctgaggaaatgc| cactggtgcctgcttcc    | 307                        | 30        | NM011344               |
| RXRα   | acatctctccaccaggtgac| tgtatttcctaggtgtgtcc | 155                        | 26        | AF374170               |
| ACC    | cactggaacgatgtgcagg | aggtgatgtgatgtgacc   |                            |           |                        |

| Gene   | Forward primer 5'–3' | Reverse primer 5'–3' | Predicted product size (bp) | Cycle No. | GenBank™ accession No. |
|--------|----------------------|----------------------|----------------------------|-----------|------------------------|
| AOX    | cttgtgcgcggcataaggg  | caggagctcactgttacc   | 216                        | 26        | AF006688               |
| MCAD   | tggagacacttggaacgacgc| accatagactgagaacaggg | 355                        | 26        | NM007382               |
| LCAD   | aaggtttttcttggaacagcc| ggaaggaggaccggagtc   | 380                        | 28        | J05029                 |
| VLCAD  | cgtgagaggctactttgtagtg| cttggactcaacatcaactgc| 288                        | 30        | AF017176               |
| LCPT   | ccagcaagggggaattg   | tggcccaaatctctttgc   | 212                        | 26        | AF017175               |
| VLACS  | tgtgtgtgtgtgtgagcc   | tttgtgtgtgtgtgttccc  | 270                        | 22        | AJ223958               |
| L-FABP | cctggtgcctgatcgattcc| gttattggtgtgtgttccc  | 157                        | 28        | Y14660                 |
| PPARα  | agggactcctgaggaaatgc| cactggtgcctgcttcc    | 307                        | 30        | NM011345               |
| RXRα   | acatctctccaccaggtgac| tgtatttcctaggtgtgtcc | 155                        | 26        | AF374170               |
| ACC    | cactggaacgatgtgcagg | aggtgatgtgatgtgacc   |                            |           |                        |

| Gene   | Accession No. | Product size (bp) | Cycle No. | GenBank™ accession No. |
|--------|---------------|-------------------|-----------|------------------------|
| AOX    | AF006688      | 216               | 26        | NM007382               |
| MCAD   | NM007382      | 355               | 26        | AJ223958               |
| LCAD   | J05029        | 380               | 28        | Y14660                 |
| VLCAD  | AF017176      | 288               | 30        | NM011344               |
| LCPT   | AF017175      | 212               | 26        | AJ223958               |
| VLACS  | 22            | 270               | 22        | Y14660                 |
| L-FABP | 28            | 157               | 28        | NM011345               |
| PPARα  | AF374170      | 307               | 30        | NM011345               |
| RXRα   | 26            | 155               | 26        | AF374170               |
| ACC    | 26            | 155               | 26        | AF374170               |
Total hepatic fatty acid β-oxidation capacity was assessed by using 14C-labeled palmitic acid (Fig. 3). This substrate was chosen, because studies on PPARα null mice showed defective mitochondrial catabolism of long chain fatty acids (9). The rate of fatty acid oxidation was similar in control and ethanol-fed mice. Wy14,643 caused significant induction in palmitic acid oxidation compared with the control and ethanol-fed groups. These results are consistent with the hypothesis that despite the elevation of plasma and presumably hepatic FFA in the ethanol-fed animals, there was no induction of fatty acid oxidizing capacity. As a result, some FFA are converted to ketone bodies, but the remainder are esterified to triacylglycerol and either stored as fat droplets or secreted as triacylglycerol-rich lipoproteins. Wy14,643 was able to induce fatty acid oxidizing capacity in the ethanol-fed animals sufficiently to dispose of them as ketone bodies or complete oxidation and therefore normalize plasma FFA and triacylglycerol levels and prevent fatty liver. This suggested that ethanol feeding subverted the normal homeostatic role of PPARα. Therefore the expression and functional capacity of PPARα were studied. Effect of Ethanol Feeding on Expression of PPARα and Its Heterodimerization Partner, RXRα, in Liver and DNA Binding Ability of PPARα/RXRα Heterodimers—The level of PPARα mRNA (Table III) and protein were not altered by ethanol (Fig. 4A). Wy14,643 increased PPARα mRNA expression by 5-fold (Table III) and significantly increased the amount of PPARα protein (Fig. 4, A and B). However, RXRα mRNA content decreased by 20% (Table III), and the level of immunoreactive RXRα protein was reduced by 60% in alcohol-fed mice, which could not be prevented by Wy14,643 (Fig. 4, A and B). Protein content of PPARγ, a receptor structurally related to PPARα that also recognizes DR-1 promoter elements, was unchanged by ethanol feeding. We attempted to quantify the levels of RXRβ and RXRγ but were unable to detect these proteins with available antibodies.

Previously, we found that the DNA binding ability of PPARα/RXRα was impaired by ethanol in cultured cells (26). To investigate whether this in vitro observation could be extrapolated to in vivo conditions, we studied the ability of nuclear extracts from liver to bind a PPAR response element (Fig. 5A). Ethanol feeding reduced the ability of PPARα/RXRα heterodimer to bind a PPRE by 40% compared with nuclear extracts from control animals. Wy14,643 treatment enhanced this binding activity by 2-fold. When ethanol-fed mice were given Wy14,643, the relative binding ability of PPARα/RXRα was 3-fold higher than in ethanol-fed animals, which restored it to higher levels than the controls (Fig. 5A), although still significantly less than observed in mice treated with Wy14,643 alone. The interpretation of this experiment was complicated by the large number of transcription factors that can bind DR-1 elements; therefore, supershift assays were performed. The amount of supershifted complexes was decreased in ethanol-fed mice. Wy14,643 co-administration increased the abundance of the supershifted complex by 2.5-fold in animals fed ethanol compared with controls (Fig. 5B). Our in vitro data suggested that the effect of ethanol on PPARα was not a nonspecific, toxic effect of alcohol, because several other members of the nuclear receptor family were not affected by ethanol (26). To see whether other transcription factors were affected in vivo we checked the DNA binding ability of HIF1α (hypoxia inducible factor-1) in the liver and found it to be unaltered by ethanol feeding (data shown).

Effects of Ethanol Feeding on Expression of PPARα Target Genes—To determine whether the decreased DNA binding abil-

Table II

| Parameters       | Control | Ethanol | Control + Wy14,643 | Ethanol + Wy14,643 |
|------------------|---------|---------|--------------------|--------------------|
| FFA (µmol/L)     | 979 ± 267 | 1548 ± 304<sup>ab</sup> | 767 ± 322<sup>d</sup> | 851 ± 178<sup>cd</sup> |
| TG (mg/dL)       | 115 ± 17  | 204 ± 39<sup>b</sup> | 108 ± 19<sup>a</sup> | 82 ± 20<sup>ab</sup> |
| Cholesterol (mg/dL) | 124 ± 11 | 128 ± 10 | 206 ± 39<sup>a</sup><sup>c,d</sup> | 147 ± 12<sup>d</sup><sup>c</sup> |
| β-OHB (mg/dL)    | 0.7 ± 0.1 | 2.0 ± 0.6<sup>d</sup> | 3.2 ± 0.8<sup>b,c,d</sup> | 3.2 ± 0.5<sup>b,c,d</sup> |
| Glucose (mg/dL)  | 247 ± 41  | 205 ± 24 | 211 ± 27 | 193 ± 34<sup>b</sup> |

<sup>a</sup> Significant difference versus control.
<sup>b</sup> p < 0.05.
<sup>c</sup> Significant difference versus alcohol-fed group.
<sup>d</sup> p < 0.001.
<sup*e</sup> Significant difference versus Wy-14,643 group.
ity of PPARα/RXRα heterodimer resulted in altered gene expression, hepatic mRNA levels of several PPARα targets were analyzed by RT-PCR (Table III). mRNAs for mitochondrial LCAD and medium chain acyl-CoA dehydrogenase (MCAD) were decreased by 30 and 40%, respectively, in the ethanol-fed group. Levels of mRNAs encoding acyl-CoA oxidase (AOX), liver carnitine palmitoyl-CoA transferase (LCPT), very long chain acyl-CoA synthetase (VLACS), and very long chain acyl-CoA dehydrogenase (VLCAD) were unchanged between ethanol-fed and control animals. Liver fatty acid-binding protein (LFABP) was the only target gene that was induced by ethanol feeding, consistent with earlier reports (16). Fourteen days of Wy14,643 treatment increased the mRNA level of the PPARα target genes 1.5–7-fold except for LCPT and VLCAD. In ethanol-fed animals, Wy14,643 effectively induced mRNAs of LCAD, MCAD, AOX, VLACS, and LFABP to levels significantly higher than that in ethanol-treated mice.

Although acetyl-CoA carboxylase (ACC) is not usually considered a PPARα-regulated gene, we also analyzed its mRNA level. ACC not only catalyzes the pace-setting step of fatty acid synthesis (34) but also plays a crucial role in the control of mitochondrial fatty acid β-oxidation through its product, malonyl-CoA (35). Ethanol feeding induced the level of ACC mRNA by 2.5-fold, and Wy14,643 prevented this induction (Table III).

**FIG. 3. Total hepatic fatty acid β-oxidation in control (pair-fed), ethanol-fed, control (pair-fed) plus Wy14,643-fed, and ethanol plus Wy14,643-fed mice.** Data are shown as mean ± S.D. of five animals in each group. Values are expressed as pmo1 [14C]palmitate oxidized/mg protein/min. A statistically significant difference was found compared with control group (a) and compared with ethanol-fed group (b) (p < 0.05).

| Target genes | Control | Ethanol | Control + Wy14,643 | Wy14,643 + Ethanol |
|--------------|---------|---------|-------------------|-------------------|
| LCAD         | 1 ± 0.1 | 0.7 ± 0.1^ab | 3.9 ± 1.8^bcd | 4.1 ± 0.6^bcd |
| MCAD         | 1 ± 0.1 | 0.6 ± 0.2^abc | 2.0 ± 0.2^cd | 1.7 ± 0.3^cd |
| AOX          | 1 ± 0.2 | 1.0 ± 0.01 | 4.3 ± 1.2^cd | 3.2 ± 1.1^cd |
| L-CPT I      | 1 ± 0.2 | 0.9 ± 0.1 | 0.7 ± 0.3 | 0.9 ± 0.9 |
| VLCAD        | 1 ± 0.1 | 0.9 ± 0.9 | 1.4 ± 0.1 | 1.5 ± 0.6 |
| VLACS        | 1 ± 0.2 | 0.9 ± 0.3 | 1.7 ± 0.4^cd | 1.6 ± 0.3^cd |
| L-FABP       | 1 ± 0.3 | 5.1 ± 2.8^cd | 7.0 ± 3.7^e | 7.7 ± 2.9^e |
| ACC          | 1 ± 0.6 | 2.5 ± 2.1^cd | 1.1 ± 0.2 | 0.8 ± 0.3^cd |
| PPARα        | 1 ± 0.3 | 1.6 ± 0.4 | 4.7 ± 1.7^cd | 3.3 ± 1.0^cd |
| RXRα         | 1 ± 0.33 | 0.8 ± 0.1^e | 1.0 ± 0.2 | 0.8 ± 0.1^e |

^a Significant difference compared to control.
^b p < 0.001 by paired t test.
^c p < 0.05.
^d Significant difference compared to ethanol-fed animals.

**DISCUSSION**

In the present paper, we demonstrated that chronic ethanol feeding inhibited DNA binding and transcriptional activity of the PPARα/RXRα heterodimer in livers of mice. Expression of some target genes involved in peroxisomal and mitochondrial fatty acid oxidation were decreased, whereas others that might have been expected to be induced were not altered by ethanol feeding. Our previous work indicated that in hepatoma cells, Wy14,643 stimulated the reporter 5-fold without ethanol and 4-fold in the presence of ethanol. Wy14,643 stimulated the reporter 4-fold without and 2-fold with ethanol in primary hepatocyte cultures (26). In the ethanol-fed mice, Wy14,643 restored the ability of PPARα/RXRα receptor complex to bind the specific response element. Furthermore, it induced mRNA levels of many PPARα target genes resulting in higher rate of fatty acid β-oxidation, normalized serum FFA and triacylglycerol levels, and prevented triacylglycerol accumulation in the livers of ethanol-fed mice. Our study indicates that incomplete activation of the PPARα battery of enzymes during ethanol consumption contributes to the development of alcohol-induced fatty liver. However, ethanol has multiple effects on liver lipid metabolism that do not involve PPARαs, and Wy14,643 has effects that are not all explained by activation of PPARαs. It was therefore expected that the interaction between these two compounds would not be straightforward.

There have been clues to this effect of ethanol in the fatty liver literature. Although a major effect of ethanol metabolism is an increased NADH/NAD+ ratio in the liver as demonstrated by numerous short term studies, this perturbation appears to abate with chronic ethanol consumption, as estimated by the lactate/pyruvate ratio in hepatic vein samples from ethanol-fed baboons (36). Because fatty liver persisted in these animals, alternative mechanisms were needed to explain this. One such mechanism is the increase in lipid synthesis driven by activation of SREBP-1 that was reported recently (31). Previous data have suggested that ethanol may impair the ability of the liver to respond to an increased level of PFA (15, 21), and we have summarized effects of ethanol on PPARα-regulated genes (17). Furthermore, we have presented evidence for ethanol-inhibition of PPARα action in vitro (26). It was crucial, therefore, to carry out experiments to directly test this hypothesis.

We have utilized mice in these experiments, as they present the opportunity for using genetically modified animals in the future to further dissect the abnormal response of the animals to ethanol. Ethanol was accepted in the diet without...
short term health effects, and the ethanol-fed animals developed histological and biochemical fatty liver (see Figs. 1 and 2). Thus, they provide a model for the early effects of chronic alcohol consumption.

We found that chronic alcohol feeding inhibited DNA binding activity of PPAR/ RXR heterodimer in liver nuclear extracts, consistent with in vitro findings (26). We saw only a modest effect of ethanol on the mRNA for these factors (a 20% decrease in the level of mRNA for RXRα, similar to that reported in rats studied using the Tsukamoto-French model (37), and no effect on PPARα mRNA), but there was a substantial decrease in RXRα protein level. It has been reported that endotoxin can cause such a decrease in mRNA and protein levels of RXR (38). Because ethanol feeding is reported to increase endotoxin in the plasma lipid profile and the rate of palmitate oxidation in MCAD was decreased. This disparate behavior of these mRNAs presumably reflects the influence of ethanol on other control regions of their promoters or on mRNA stability. Treatment of the ethanol-fed mice with Wy14,643 resulted in increased formation of dimer would be observed in the nuclear extracts of the Wy14,643-treated animals. Unless there was very tight binding of the drug to the receptor heterodimer that survived extraction, the finding suggests that the presence of Wy14,643 prevented the effect of ethanol on the receptor, which we hypothesize is a post-translational modification. Several possibilities can be suggested: binding of Wy14,643 or formation of the heterodimer might alter the conformation of the receptor and alter its susceptibility to modification. We suggested that ethylation of the receptor at lysyl residues near the zinc finger domain could be a mechanism for the effect of acetdehyde (26); conceivably activation of the receptor by Wy14,643 increased the fraction that was DNA-bound and inaccessible to acetdehyde.

Presumably, the endogenous PPARα ligands, FFAs, do not bind with sufficient affinity to prevent the effect of ethanol. Forman et al. (25) showed in the ligand-induced complex formation assay that 30 μM palmitate or linoleic acid induced complex formation only 30–40%, as well as 5 μM Wy14,643 (25). Thus, weaker ligands like FFA may not be able to prevent the actions of ethanol on the receptor, whereas high affinity ligands like Wy14,643 retain that ability. This will require additional study of receptor purified from cells exposed to ethanol or acetdehyde, in the presence or absence of Wy14, 643.

To assess the functional implications of the reduced DNA binding of the PPAR/RXR complex, we measured the levels of a number of mRNAs known to be regulated by this transcription factor. PPARα/RXRα affects genes in two ways; some mRNAs, such as LCAD and VLCAD, are strongly dependent on PPARα for constitutive expression, as judged by the reduction in mRNA in PPARα−/− animals (9). mRNAs of LCAD was decreased in the ethanol-fed animals as well (Table III). There are also a number of genes for which PPARα/RXRα is not required for basal expression, but rather for induction by peroxisome proliferators, and presumably by fatty acids. These include AOX, LCPT, LFABP, VLACS, and MCAD, which were not decreased in PPARα null mice but also not inducible by peroxisome proliferators when analyzed by immunoblotting (9). Although one would predict that they would be induced by the elevated FFA levels in the alcohol-fed livers, with the exception of fatty acid-binding protein, they were not. Moreover, mRNA of MCAD was decreased. This disparate behavior of these mRNAs presumably reflects the influence of ethanol on other control regions of their promoters or on mRNA stability. Treatment of the ethanol-fed mice with Wy14,643 resulted in induction of mRNAs of many PPARα target genes (Table III), indicating that the increased ability of PPAR/RXR to bind DNA in gel shift experiments was paralleled by induction of a number of PPAR-regulated genes.

This failure of full induction of critical mRNAs for FFA metabolism during ethanol feeding was functionally reflected in the plasma lipid profile and the rate of palmitate oxidation in liver homogenates. In the ethanol-fed animals, the plasma triacylglycerols and FFA were increased, consistent with previous work (41–43). It is interesting to note that ketone bodies (represented by β-OHB) were increased nearly 3-fold in the ethanol-fed animals. Given that rodents tend to consume most of their diet early in the dark cycle, it is likely that they had metabolized the ethanol taken in the diet many hours earlier. Therefore, the increased level of β-OHB may represent acceleration of ketogenesis that follows relief of the redox pressure of ethanol metabolism, similar to the pathogenesis of alcoholic ketoacidosis (44). This capacity for fatty acid oxidation and ketogenesis was not, however, sufficient to dispose of excess fat in the liver, possibly because the maximum rate of

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**Figure 4. Western immunoblot analysis of PPARα, PPARγ, and RXRα transcription factors from livers of mice fed a control diet (pair-fed), ethanol-containing diet, control (pair-fed) diet supplemented with Wy14,643, and ethanol-containing diet supplemented with Wy14,643.** A, three lanes of representative samples from a group of mice, 60 μg of crude nuclear extract of liver was subjected to electrophoresis and immunoblotting. Positive control, which was a nuclear extract prepared from the HL-60 human promyelocytic leukemia cell line for PPARγ, and from RXRα-transfected H4IEC3 rat hepatoma cell line for RXRα. B, results were quantified by FluorImaging. All protein levels were expressed relative to control (pair-fed) mice (mean ± S.D., n = 5 animals). *p < 0.05; **p < 0.001 by paired t test compared with control group.
oxidation, as assessed from the oxidation of palmitate in liver homogenates, was not increased. Induction of the rate-limiting enzymes for fat oxidation is apparently needed to handle the FFA load seen with ethanol feeding, as shown by the effect of Wy14,643. This compound induced mRNAs for fatty acid oxidation by mitochondrial and peroxisomal systems, increased the maximum rate of palmitate oxidation, lowered plasma triglyceride and FFA levels, and increased ketone body levels to over four times the control level. These effects were observed even in the presence of ethanol in the diet, and thus appeared to overcome the block in fat metabolism caused by ethanol. Thus, it appears that fatty liver and the other disturbances of lipid metabolism seen in the ethanol-fed mice resulted, in part, from an incomplete activation of the PPARα homeostatic system.

An additional interesting finding was that ethanol feeding induced ACC mRNA, and Wy14,643 reduced this to normal. Induction of the rate-limiting enzymes for fat oxidation is apparently needed to handle the FFA load seen with ethanol feeding, as shown by the effect of Wy14,643. This compound induced mRNAs for fatty acid oxidation by mitochondrial and peroxisomal systems, increased the maximum rate of palmitate oxidation, lowered plasma triglyceride and FFA levels, and increased ketone body levels to over four times the control level. These effects were observed even in the presence of ethanol in the diet, and thus appeared to overcome the block in fat metabolism caused by ethanol. Thus, it appears that fatty liver and the other disturbances of lipid metabolism seen in the ethanol-fed mice resulted, in part, from an incomplete activation of the PPARα homeostatic system.

These data suggest that treatment of individuals with alcoholic fatty liver with activators of PPARα may reduce the degree of fat accumulation. The fact that animals improved with Wy14,643 treatment while still receiving ethanol in the diet indicates that this effect is not simply prevention of fatty liver. Thus, such therapy might be effective even in patients unable to completely abstain. Because some of the hepatotoxicity of ethanol may be because of the presence of fatty infiltration (e.g. sensitivity to endotoxin, susceptibility to lipid peroxidation, or alterations in blood flow to the centrilobular zone with resultant hypoxia), PPARα agonist treatment might even have a benefit in preventing or ameliorating more serious forms of liver injury such as alcoholic hepatitis or fibrosis, but this will clearly require additional study. Further, this study suggests mechanisms for increased genetic risk for alcoholic liver injury. Several polymorphisms of the human PPARα gene have been described recently (46–48). Of these, the L162V variant showed decreased non-ligand-dependent transactivation activity and decreased sensitivity to low concentrations of ligand in tissue culture experiments (46). In human studies, it was reported to be associated with elevated plasma low density lipoprotein concentration and increased risk for atherosclerosis and ischemic heart disease (33, 47, 48). Our study suggests that carriers of this allele might be more susceptible to the toxic hepatic effects of ethanol.

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Peroxisome Proliferator-activated Receptor α (PPARα) Agonist Treatment Reverses PPAR α Dysfunction and Abnormalities in Hepatic Lipid Metabolism in Ethanol-fed Mice

Monika Fischer, Min You, Michinaga Matsumoto and David W. Crabb

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