The mechanisms underlying alcoholic liver disease are not completely understood, but lipid accumulation seems to be central to the cause of this disease. The peroxisome proliferator-activated receptor α (PPARα) plays an important role in the control of lipid homeostasis, metabolism of bioactive molecules, and modulation of inflammatory responses. To investigate the roles of PPARα in alcoholic liver injury, wild-type and PPARα-null mice were continuously fed a diet containing 4% ethanol, and liver injury was analyzed. PPARα-null mice fed ethanol exhibited marked hepatomegaly, hepatic inflammation, cell toxicity, fibrosis, apoptosis, and mitochondrial swelling. Some of these hepatic abnormalities were consistent with those of patients with alcoholic liver injury and were not found in wild-type mice. Next, the molecular mechanisms of ethanol-induced liver injury in PPARα-null mice were investigated, and changes related to ethanol and acetaldehyde metabolism, oxidative stress, inflammation, hepatocyte proliferation, fibrosis, and mitochondrial permeability transition activation occurred specifically in PPARα-null mice as compared with wild-type mice. In conclusion, these studies suggest a protective role for PPARα in alcoholic liver disease. Humans may be more susceptible to liver toxicity induced by ethanol as PPARα expression in human liver is considerably lower compared to that of rodents. (Hepatology 2004;40:972–980.)
Materials and Methods

Animals and Ethanol Treatment. PPARα-null mice on a Sv/129 genetic background were produced as described elsewhere.19 These mice were treated in a specific pathogen-free facility according to Shinsu University and National Institutes of Health animal care and the Accreditation of Laboratory Animal Care guidelines. The mice were housed in a temperature- and light-controlled environment (25°C; 12-h light/dark cycle), and maintained on stock rodent chow and tap water ad libitum until 12 weeks of age. Twelve-week-old male wild-type and PPARα-null mice (26–29 g body weight) were fed an ethanol-containing liquid diet, described by Lieber and DeCarli.16 The ethanol concentration was raised gradually from 2% to 4% within 1 month. Interindividual difference in daily food consumption was lowered considerably by this acclimatization treatment; no significant difference in food intake (14 ± 1 mL/day per mouse) was found between wild-type and PPARα-null mice. Mice were fed a 4% ethanol-containing liquid diet. For controls, another group of 12-week-old mice were fed the same volume of a control liquid diet prepared by replacing ethanol in the Lieber and DeCarli diet with isocaloric sucrose.

Blood ethanol concentration was measured using the vial equilibration method20 to confirm the extent of ethanol intake and absorption. The range in blood ethanol concentrations of wild-type and PPARα-null mice were: 26 to 42 mM and 30 to 48 mM (fed for 1 month), respectively; 33 to 57 mM and 22 to 50 mM (fed for 2 months), respectively; and 31 to 47 mM and 29 to 51 mM (fed for 6 months), respectively. These results also indicate that the intake of the ethanol-containing diet during the administration period was not significantly different between either genotype. Therefore, this method was less stressful to mice compared with the Tsukamoto-French rat model where blood ethanol was maintained at similar concentrations (216 ± 120 mg/100 g) over the course of the study20 to those in this study.

In the ethanol-treated PPARα-null group, abnormal behaviors indicated by gait disturbance, shivering, blunting of response to physical irritation, and excessive periods of crouching were observed in approximately half of the mice under study. Abnormal behaviors were observed after 2 months of ethanol feeding in PPARα-null mice and usually were accompanied by a reduction in body weight. These behaviors were not seen in wild-type mice housed under identical conditions, nor in PPARα-null mice provided with the control liquid diet. The abnormal behaviors, which appeared only in ethanol-fed PPARα-null mice, were not quantified. All mice were killed by carbon dioxide asphyxiation, regardless of the existence of abnormal behaviors, for analysis of pathologies and biochemical parameters.

Morphological Studies. Small sections of liver from each mouse were fixed in 10% buffered formalin, and the sections were stained with either hematoxylin and eosin or Azan-Mallory as described earlier.21 Steatohepatitis was quantified according to the grading system,22 with minor modifications. The severity of hepatic steatosis was graded based on the percentage of hepatocytes showing lipid accumulation as follows: 0, absent; 1, mild; 2, moderate;
3, severe. Another small piece of liver was fixed in 4% paraformaldehyde and was used to determine hepatocyte apoptosis by staining using the MEBSTAIN apoptosis Kit (MBL, Nagoya, Japan). For analysis of hepatocyte proliferation, the sections were stained with mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA, Santa Cruz Biotech, Santa Cruz, CA). Ten randomly selected microscopic fields magnified ×400 were examined on each section, and the average number of PCNA-positive hepatocyte nuclei per 1,000 hepatocytes was determined for each mouse. Electron microscopy was performed as described.21

**Enzyme Assays.** Alcohol dehydrogenase activity (ADH) in cytosolic fractions and aldehyde dehydrogenase (ALDH) activity in 700 g supernatant fractions were examined by measuring the rate of NADH formation. Glutathione peroxidase (GPx) activity in 700 g supernatant fractions was measured using the GPx-340 kit (OXIS International Inc., Portland, OR). Glutathione S-transferase activity in cytosolic fractions was measured as described.23 Cu, Zn-, and Mn-superoxide dismutase (SOD) activity in 700 g supernatant fraction was measured using the method of Misra and Fridovich.24

**Immunoblot Analysis.** Immunoblot analysis was performed as described previously.25 The primary polyclonal antibodies to cell cycle regulators (p21, PCNA, cyclin-dependent kinase [CDK] 2, and CDK4), growth factors (hepatocyte growth factor-α [HGFα], transforming growth factor-α [TGFα], and TGFβ1, Bax, Bid, Bcl-2, Bcl-xL, and nuclear factor κB (NF-κB) p65 were purchased from Santa Cruz Biotech. Mouse anti-cytochrome c monoclonal antibody was obtained from BD Biosciences (Tokyo, Japan).

**Messenger RNA Analysis.** Messenger RNA (mRNA) analysis was performed by Northern blotting as mentioned previously.25 The cDNA probes used were for TGFβ1,28 PCNA,29 CDK4,30 and PPARα.31 Tumor necrosis factor-α (TNFα) and Fas ligand mRNAs were measured with real-time polymerase chain reaction technique as described.32 Forward and reverse primers were designed as follows: 5′-CCGGAGCCGGGCCGGT-3′ and 5′-GCTGGGTAAGAGATGATGAAAC-3′ for TNFα and 5′-TGTTGAGCTTCT GTTTGGAATG-3′ and 5′-TGGGGCAGCTCCTGTCATGCT-3′ for Fas ligand. Glyceraldehyde 3-phosphate dehydrogenase mRNA levels were used for calculation. The values in ethanol-treated PPARα-null mice were expressed as a relative percentage compared with those in ethanol-fed wild-type mice.

**Other Methods.** Serum aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, and TG concentrations were measured using kits purchased from Wako (Osaka, Japan). Lignoceric acid β-oxidation activity was measured as described.25 Lipid peroxides (malondialdehyde and 4-hydroxyalkenals) in whole liver homogenate were measured using LPO-586 kit (OXIS International Inc.). Serum TNFα levels were analyzed using an ELISA kit (R & D Systems, Minneapolis, MN).

**Statistical Analysis.** Analysis for significant differences for the interactive effects of two factors (PPARα gene and ethanol treatment) was performed using a two-way ANOVA. Probability levels less than 0.05 were used as the measure of significance.

**Results**

**Effects of Ethanol Treatment on Wild-Type and PPARα-Null Mice.** Hepatomegaly induced after 6 months of ethanol feeding was significantly greater in PPARα-null mice than in wild-type mice (Fig. 1A). In both wild-type and PPARα-null mice, liver TG levels increased similarly after 6 months of ethanol consumption (Fig. 1A). Interestingly, serum TG levels were significantly higher in wild-type mice after ethanol consumption compared with PPARα-null mice fed ethanol. Serum aspartate aminotransferase and alanine aminotransferase concentrations were markedly elevated in ethanol-fed PPARα-null mice, whereas γ-glutamyl transpeptidase levels were lower compared with wild-type mice fed ethanol (Fig. 1A). These results indicate that long-term ethanol consumption results in severe hepatocyte damage in PPARα-null mice and not in similarly treated wild-type mice.

Histopathological examination revealed an accumulation of large lipid droplets in hepatocytes in both genotypes that had been fed ethanol (Fig. 1B). Scores of the severity of hepatic steatosis were not different between ethanol-fed PPARα-null and wild-type mice (2.0 ± 0.0 vs. 1.6 ± 0.5; n = 12 in each group). However, marked inflammatory cell infiltration including polymorphonuclear leukocytes and histiocytes was observed around hepatocytes with large lipid droplets in all ethanol-fed PPARα-null mice (Fig. 1B). Hepatic inflammation scores were much higher in ethanol-fed PPARα-null mice than in ethanol-fed wild-type mice (2.6 ± 0.5 vs. 0.8 ± 0.4; P <.05; n = 12 in each group). Nuclear vacuolation of hepatocytes also was observed in ethanol-fed PPARα-null mice, although alcoholic hyaline was not found. Fibrosis was noted in all PPARα-null mice fed ethanol, and portal areas were expanded moderately with fibers and inflammatory cells (Fig. 1B). In these mice, apoptotic hepatocytes were observed mainly in areas with inflammatory
cell infiltration (Fig. 1B). These findings except for steatosis were very rare in ethanol-treated wild-type mice (Fig. 1B). Electron microscopic examination revealed marked mitochondrial swelling (20- to 40-fold swelling in volume, as compared with the wild-type samples) at many sites in the livers of all PPARα-null mice fed ethanol but was not observed in similarly treated wild-type mice (Fig. 1B).

The serial changes in ethanol-fed PPARα-null mice are summarized in Fig. 2. Accumulation of TG in the liver occurred rapidly at 2 to 4 weeks after ethanol intake and gradually decreased. Increased liver weight (hepatomegaly) began 4 months after commencing ethanol administration and continued to increase by 8 months after ethanol treatment. Hepatic inflammation began at 1 month, increased rapidly, and reached a maximal plateau at approximately 6 months. Interestingly, the hepatic inflammation preceded other markers showing ethanol-induced liver injury except for TG accumulation. Liver fibrosis began from 4 months of ethanol feeding and continued to increase by 8 months. In PPARα-null mice, apoptosis of hepatocytes started after 5 to 6 months of ethanol feeding.

**Alcohol and Acetaldehyde Metabolism.** To elucidate the mechanism by which ethanol ingestion induced hepatic damage in PPARα-null mice, the formation and degradation of acetaldehyde, which is known to be highly cytotoxic, was examined. Ethanol ingestion resulted in elevated levels of alcohol dehydrogenase and cytochrome P450 2E1, enzymes that catalyze the formation of acetaldehyde, to a similar extent in both PPARα-null and wild-type mice (Fig. 3A). However, the level of ALDH was markedly lower in PPARα-null mice fed ethanol, with the greatest reduction in the low-Kₘ ALDH; this was not found in ethanol-fed wild-type mice (Fig. 3A). These results suggest that PPARα-null mice have more elevated acetaldehyde levels as a result of lower ALDH than do wild-type mice.

**Hepatic Oxidative Stress.** The role of PPARα in ethanol-induced oxidative stress also was examined, because...
it was shown that increased oxidative stress may contribute to hepatic damage and inflammation.\textsuperscript{33,34} In PPAR\textalpha-null mice, ethanol treatment resulted in markedly lower levels of GPx, SOD, and catalase as compared with the wild-type mice (Fig. 3B). In contrast, ethanol treatment increased glutathione S-transferase levels in both genotypes (Fig. 3B). Furthermore, the hepatic level of lipid peroxides observed after 6 months of ethanol consumption was significantly higher in PPAR\textalpha-null mice than in wild-type mice (159.1 $\pm$ 9.8 vs. 63.9 $\pm$ 4.4 pmol/mg protein; $P < .05$; n = 8 in each group). Combined, these results suggest that hepatocytes from PPAR\textalpha-null mice are exposed to higher levels of oxidative stress than wild-type mice under conditions of chronic ethanol ingestion.

**Other Factors Associated with Inflammation.** Impaired peroxisomal degradation of prostaglandins, leukotrienes, and very long-chain fatty acids can cause cell toxicity and inflammation.\textsuperscript{5,6} To examine this possibility, peroxisomal $\beta$-oxidation was measured. Haptic peroxisomal $\beta$-oxidation of lignoceric acid (a C-24 very long-chain fatty acid) was significantly lower in ethanol-fed PPAR\textalpha-null mice than in similarly treated wild-type mice (Fig. 3C), consistent with reduced activity of catalase (Fig. 3B). Additionally, activation of the p65 subunit of NF-\kappaB was significantly greater in hepatic nuclear fractions from ethanol-fed PPAR\textalpha-null mice compared with ethanol-fed wild-type mice (Fig. 3D). Similar results were obtained for the p50 subunit of NF-\kappaB (data not shown). Combined, these results suggest that inflammation is enhanced in hepatocytes from ethanol-fed PPAR\textalpha-null mice.

**Hepatocyte Proliferation.** Given the marked hepatomegaly and fibrosis found in ethanol-treated PPAR\textalpha-null mice, changes in the expression of growth factors involved in hepatocyte proliferation, fibrosis (HGF\textalpha, TGF\textalpha, and TGF\textbeta), or both were examined.\textsuperscript{3,35} Surprisingly, constitutive expression of TGF\alpha and TGF\textbeta was significantly lower in PPAR\textalpha-null mice compared with wild-type controls (Fig. 4A). HGF\textalpha, TGF\textalpha, and TGF\textbeta levels all were higher in the PPAR\textalpha-null mice fed ethanol than in the similarly treated wild-type mice (Fig. 4A). Of all proteins associated with cell cycle regulation, PCNA, CDK2, and CDK4 were markedly higher in PPAR\textalpha-null mice, whereas p21 expression decreased by approximately 50% in both mouse genotypes after ethanol feeding (Fig. 4A).
Expression of c-Fos, c-Myc, p53, p16, CDK1, CDK5, CDK6, cyclin A, B1, D1, D2, and E remained virtually unchanged in both genotypes after ethanol treatment (data not shown). Expression of PPARα was decreased by approximately 60% in wild-type mice in response to ethanol feeding. Northern blot analysis of liver mRNA was performed for comparison with changes in the expression of several proteins. Changes in the expression of mRNAs encoding TGF-β1, PCNA, CDK4, and PPARα were consistent with changes observed at the protein level (Fig. 4B). Additionally, the average number of PCNA-positive hepatocytes per 1,000 hepatocytes was significantly increased in ethanol-fed PPARα-null mice than in ethanol-fed wild-type mice (8.9 ± 0.5 vs. 2.6 ± 0.3; P < .05; n = 8 in each group). These results suggest that in the absence of PPARα expression, ethanol ingestion causes increased signaling that leads to the stimulation of hepatocyte proliferation and fibrosis.

**Apoptosis and Mitochondrial Swelling.** To examine the mechanism underlying increased hepatic apoptosis and mitochondrial swelling in ethanol-fed PPARα-null mice, markers of mitochondrial permeability transition (MPT) activation were measured. Indeed, higher expressions of Bax and truncated Bid, two proteins that are known to contribute to MPT activation, were observed in PPARα-null mice fed ethanol compared with wild-type mice (Fig. 5). Additionally, decreased levels of Bcl-2 and Bcl-xL were observed, but this occurred in both genotypes fed ethanol (Fig. 5). Because these two proteins are known to inhibit MPT activation, their decreased expression may contribute to enhanced MPT activation. Last, increased cytosolic cytochrome c levels in PPARα-null mice fed ethanol were significantly greater than wild-type mice (Fig. 5). To explore further other mechanisms of the increased apoptosis, the mRNA levels of TNFα and Fas ligand were examined. The TNFα mRNA level was significantly higher in ethanol-treated PPARα-null mice compared with that in ethanol-treated wild-type mice (224 ± 15.9 vs. 100 ± 12.3%; P < .05). The serum TNFα level also was higher in ethanol-treated PPARα-null mice than that in ethanol-treated wild-type mice (39.3 ± 4.8 vs. 19.4 ± 2.7 pg/mL, P < .05; n = 6 in each group). These results are consistent with the increased hepatic apoptosis and mitochondrial swelling observed in ethanol-fed PPARα-null mice (Fig. 1B).

**Discussion**

PPARα-null mice fed a diet containing a low concentration of ethanol for 6 months exhibit marked symptoms similar to those observed in patients with alcoholic liver disease, including hepatomegaly, steatosis, elevated serum aspartate aminotransferase and alanine aminotransferase levels, inflammation, spotty necrosis, centrilobular fibrosis, apoptosis, and swollen or large-sized mitochondria. Mallory bodies, typical megamitochondria (giant mitochondria), and cirrhosis were not observed in ethanol-fed PPARα-null mice. However, these pathological features are found in only a low percentage of patients, in those with high alcohol consumption and predominantly in patients in terminal stage, respectively. Thus, these pathological features are not considered essential signs of alcoholic liver damage. Given that all PPARα-null mice fed ethanol developed many common symptoms of alcoholic liver disease, these findings suggest that this mouse line represents a model for human alcoholic liver injury and may be of value for studying the means for prevention and treatment of this disease. However, it should be noted that there may be significant differences between the PPARα-null mouse model and the cause of the human condition. In addition, because approximately half of PPARα-null mice showed various types of abnormal behavior, this mouse line also may be a useful model to study alcoholic neuropsychiatric disorders.

Other animal models of alcoholic liver injury have been developed. Lieber and DeCarli devised a liquid diet containing alcohol and used it to feed baboons. Using this protocol, it was shown that fibrosis occurred at an incidence of approximately 20% in baboons, whereas liver damage developed in rats fed the same diet at a much
lower rate. When Tsukamoto-French model rats were fed a high-fat diet, 40% to 60% of them developed fibrosis, accompanied with inflammation. However, treatment of rats with ethanol by this method is not an ideal animal model, because dietary fat content can strongly affect the incidence of symptoms. In addition, using this method requires higher concentrations of ethanol to increase the incidence of fibrosis. This outcome, and the additional stress caused by surgical pretreatment and forced ethanol administration, could influence results obtained with this animal model. Therefore, the PPARα-null mouse line is superior to these rat models because they develop symptoms with an incidence of 100% under natural feeding conditions and with a diet containing a lower concentration of ethanol and fat. Moreover, PPARα-null mice fed ethanol develop some manifestations of alcoholic liver damage that are similar to those typically observed in human patients. Some studies have revealed that human liver contains lower levels of PPARα than those found in mice. Peroxisome proliferation has not been observed in human liver after fibrate treatment. It is tempting to speculate that species differences in PPARα signaling can affect ethanol-induced toxicity significantly. Because ethanol consumption reduces the expression of PPARα (Fig. 4), it is reasonable to suggest that the level of hepatic PPARα in humans will be very low in chronic drinkers. This could explain why some of the features observed in PPARα-null mice are similar to those observed in human patients.

The results suggest that adverse hepatic changes are the results of increased cellular toxicity mediated by one or more of four pathways: (1) accumulation of acetaldehyde after alcohol-induced stimulation of acetaldehyde formation and the suppression of its degradation, (2) ethanol-induced reduction in levels of three enzymes (GPx, SOD, and catalase) involved in preventing intracellular oxidative stress, (3) increased activation of NF-κB target genes probably resulting from enhanced acetaldehyde levels and increased oxidative stress, and (4) reduced ability to catabolize prostaglandins, leukotrienes, and very long-chain fatty acid derivatives. The first possibility is consistent with the finding that the ALDH promoter includes a retinoid X receptor α binding site. Interestingly, a decrease in hepatic retinoid X receptor α was observed in control PPARα-null mice, and this level is further decreased (approximately 40%–50% of control PPARα-null mice) in ethanol-treated PPARα-null mice. The second pathway is supported by both the catalase and Cu, Zn-SOD promoters having PPARα response elements. Additionally, the expression of GPx is regulated by the transcription factor Nrf2 in response to the changes of oxidative stress. In the third scenario, the role of PPARα in activation of NF-κB target genes is well established. Finally, in the fourth pathway, previous work demonstrated that PPARα-null mice exhibited enhanced inflammation in response to leukotriene B4. Thus, PPARα seems to regulate the expression of the variety of proteins directly or indirectly, and all four pathways may contribute to the increased susceptibility of PPARα-null mice to alcohol-induced liver disease. In addition to these four pathways, the presence of another pathway has been demonstrated in which PPARα suppresses steatohepatitis through reducing the levels of fatty acids or TGs, substrates of lipid peroxides. The level of TG accumulation in PPARα-null mice administered ethanol for 1 to 2 months was very high and much higher than that in the similarly treated wild-type mice. Because the degree of inflammation rapidly increased at this stage, the fifth pathway may contribute significantly to the induction of steatohepatitis. However, the TG level in PPARα-null mice fed ethanol for 4 to 6 months decreased and became similar to that in wild-type mice of the same stage, probably because of a significant increase of cytosolic lipase activity and a gradual decrease in the level of glycerol-3-phosphate acyltransferase after 3 months of ethanol feeding. The degree of inflammation still increased in this period, which probably reflects continuous promotion through the first four pathways.

The hepatomegaly induced by ethanol administration to PPARα-null mice could be the result of stimulation and promotion of hepatocyte proliferation because of increased HGFα and TGFα levels. These growth factors can stimulate cell cycle progression as indicated by the observed increase in CDK2 and the simultaneous increase and decrease in CDK4/p21 in the PPARα-null mice fed alcohol. Ethanol-induced fibrosis found in PPARα-null mice may be the result of elevated TGFβ1. A marked increase in HGFα that is known to inhibit the effect of TGFβ1 could suppress a more severe course of fibrosis or the onset of cirrhosis. It is worth noting that many of the changes at the molecular level observed in PPARα-null mice administered ethanol also have been reported in human patients. Combined, these findings provide evidence that the PPARα-null mouse is a potential model for human alcoholic liver injury and indicate that PPARα may be a susceptibility gene for alcoholic liver disease. They are particularly important because there is considerable interindividual variability in PPARα levels in the human liver, and polymorphisms have also been described for the human PPARα gene.

A very large number of variables are involved in the mechanism for the onset of apoptosis and mitochondrial swelling. Although the precise mechanism underlying
the increased hepatic apoptosis resulting from ethanol treatment in PPARa-null mice can not be determined from these studies, results from the present studies could lead to clues that provide an explanation for such pathological features. Several common factors can work in concert to cause this type of liver damage. The MPT pore is thought to be one such factor, and significant differences in the MPT pore regulation proteins, decreased Bcl-2 and Bcl-xL, and increased Bax and truncated Bid were found in ethanol-fed PPARa-null mice (Fig. 5). These findings are consistent with the possibility that alterations in the MPT pore contribute to increased hepatic apoptosis and mitochondrial swelling in this genotype. In addition, it is likely that the elevated oxidative stress found in the PPARa-null mouse fed alcohol indirectly leads to alterations in these proteins resulting in opening the MPT pore and initiation of apoptosis. 37

In conclusion, the PPARa-null mouse line is a new animal model to study alcoholic liver disease because some of the symptoms, pathological features, and molecular changes associated with ethanol toxicity are similar to those observed in humans. However, it remains to be established whether this model mimics the cause of human alcohol-induced liver disease. Additionally, this study suggests a novel protective role for PPARα in preventing ethanol toxicity in liver. The possibility that differences in human liver PPARα expression, functionality, or both may have a significant impact on susceptibility to ethanol toxicity remains to be confirmed by additional experimentation.

References