Hepatocyte Retinoid X Receptor α-Dependent Regulation of Lipid Homeostasis and Inflammatory Cytokine Expression Contributes to Alcohol-Induced Liver Injury

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ABSTRACT

Hepatocyte retinoid X receptor α (RXRα)-deficient mice are more sensitive to ethanol toxicity than wild-type mice. Because RXRα-mediated pathways are implicated in lipid homeostasis and the inflammatory response, we hypothesized that a compromise in lipid metabolism and associated production of proinflammatory mediators are responsible for the hepatotoxicity observed in ethanol-treated hepatocyte RXRα-deficient mice. Wild-type and hepatocyte RXRα-deficient mice were fed ethanol-containing diets or pair-fed control diets for 6 weeks. After ethanol treatment, serum ALT levels increased significantly (4-fold) in hepatocyte RXRα-deficient mice, but not in the wild-type mice. Hepatic liver fatty acid binding protein (L-FABP) mRNA and protein levels were reduced due to RXRα deficiency. Ethanol induced L-FABP mRNA and protein in wild-type mice and provided protection against nonesterified fatty acid toxicity; however, this effect was absent in the mutant mice. Accordingly, hepatic nonesterified fatty acid level was increased in ethanol-fed mutant mice. Ethanol increased nuclear factor (NF)-κB binding activity in hepatocyte RXRα-deficient mice, but not in wild-type mice. In agreement, hepatic mRNA levels of proinflammatory cytokines and chemokines were increased to a greater extent in the mutant than in wild-type mice. Furthermore, signal transducer and activator of transcription factor (STAT) 3 and associated Bcl-xL induction was observed in ethanol-fed wild-type mice but not in ethanol-fed hepatocyte RXRα-deficient mice. Taken together, after ethanol treatment, hepatocyte RXRα deficiency results in lack of L-FABP induction, increased hepatic free fatty acids, NF-κB activation, and proinflammatory cytokines production and a lack of STAT3 activation, which in part may contribute to alcohol-induced liver damage.

In the adult liver, the nuclear receptor, retinoid X receptor α (RXRα), is the most abundant among the three RXR isoforms (α, β, and γ) (Mangelsdorf et al., 1992). RXRα dimerizes with class II nuclear hormone receptors including retinoid acid receptors, thyroid hormone receptors, vitamin D receptor, farnesoid X receptors, liver X receptors (LXRs), and peroxisome proliferator-activated receptors (PPARs) (Blumberg and Evans, 1998).

Previously, we have shown that many nuclear receptor-mediated pathways, including the lipid-sensing PPARα-mediated pathway, are compromised when hepatocyte RXRα is deficient (Wan et al., 2000a,b, 2003; Cai et al., 2002). Recent reports indicate that these nuclear receptors play important role in the inflammatory process; therefore, it is important to study how RXRα regulates inflammatory signaling and alcoholic liver disease (ALD).

Recent data from our laboratory indicated that a deficiency in the expression of RXRα in hepatocytes caused a reduction of S-adenosylmethionine (SAM) and glutathione levels, resulting in more serious alcohol-induced liver injury (Dai et al., 2003). In addition, we established that hepatocyte RXRα controls the metabolism of ethanol (Gyamfi et al., 2006).
Hepatocyte RXRα deficiency leads to induction of alcohol dehydrogenase activity and reduction of aldehyde dehydrogenase and glutathione S-transferase activities (Gyamfi et al., 2006). These changes resulted in acetaldehyde accumulation in the hepatocyte RXRα-deficient mice after ethanol ingestion (Gyamfi et al., 2006). Thus, RXRα is not only important for regulating lipid homeostasis; it also plays a crucial role in ethanol and acetaldehyde detoxification. Furthermore, hepatic retinoid content is depleted in alcoholic liver disease (Leo and Lieber, 1983). Taken together, it is highly likely that retinoid-mediated signaling pathways contribute to the protection of liver from alcohol-induced liver injury. Accordingly, RXRα could serve as a target for the prevention and treatment of alcohol-induced liver injury.

Inflammation plays an important role in the development of ALD. Nuclear receptors are known to have regulatory effects on the inflammatory process. Both PPARs and LXRα are mediators of lipid metabolism and inflammatory gene expression in cells of the artery wall including macrophages (Tontonoz and Mangelsdorff, 2003; Evans et al., 2004). Lipid accumulation in the macrophages leads to the transcriptional activation of PPARs and LXRα by providing the cell with oxidized fatty acid and oxysterol ligands, respectively (Nagy et al., 1998). Lack of PPARα and LXRα in macrophages accelerates atherosclerosis in rodents (Tangirala et al., 2002). Therefore, PPARs and LXRα are negative regulators of inflammatory gene expression in macrophages. Like hepatocyte RXRα-deficient mice, PPARα-null mice are also more susceptible to alcohol-induced liver injury (Nakajima et al., 2004).

Evidence also indicates that RXRα plays a role in the inflammatory process in the liver. For example, tumor necrosis factor (TNF) α and interleukin (IL)-1 decrease the expression of RXRα, PPARα, PPARγ, LXRα, and their cofactors in human hepatoma Hep3B cells (Kim et al., 2007). The acute phase response induced by LPS is associated with decreased hepatic proteins involved in lipid metabolism concomitant with the reduction of hepatic RXRα level in both the mouse and the hamster (Beigneux et al., 2000; Ghose et al., 2004). The down-regulation of RXRα in inflamed liver is due to nuclear export and degradation (Zimmerman et al., 2006). The RXRα-mediated pathways could also be inhibited due to direct interaction between NF-κB p65 and the RXRα DNA binding domain and thus may prevent the binding of RXRα to consensus DNA sequences (Gu et al., 2006). These findings clearly demonstrate a down-regulation of RXRα signaling during the inflammatory process. However, the role of RXRα in regulating inflammatory cytokines and its relationship with fatty acid metabolism remains to be elucidated.

Using hepatocyte RXRα-deficient mice, we investigated the role of hepatocyte RXRα in regulating lipid homeostasis and inflammatory cytokines and their impact upon ALD. We document that alcohol-induced liver injury in hepatocyte RXRα-deficient mice is associated with dysregulation of lipid homeostasis, NF-κB activation, and a robust induction of hepatic mRNA levels of IL-6, IL-1α, TNFα, macrophage inflammatory protein (MIP)-2, plasminogen activator inhibitor type-1 (PAI-1), and collagen 1α. In addition, ethanol-induced signal transducer and activator of transcription factor (STAT) 3 and Bcl-xL activation were not observed in hepatocyte RXRα-deficient mice. These findings provide the first evidence for a direct link between hepatocyte RXRα and inflammatory processes in the development of ALD.

Materials and Methods

Animals. Age-matched wild-type (of mixed genetic background of C57/Bl/6, 129/SvEvTac, and DBA-2) (Wan et al., 2000a, 2003) and hepatocyte RXRα-deficient mice (10–12 weeks old) were used in all the experiments. Hepatocyte RXRα-deficient mice were generated by specifically mutating the RXRα gene in hepatocytes using cre/lox-mediated recombination as described previously (Wan et al., 2000a). The mice were housed individually in steel microisolator cages at 22°C with a 12/12-h light/dark cycle. After 2 days of feeding mice control liquid diet, mice were randomized into ethanol- or pair-fed groups (n = 6). The ethanol-fed group was allowed free access to ethanol-containing diets with increasing concentrations of ethanol over a 7-day period as described previously (McMullen et al., 2005). The ethanol concentration was kept thereafter at 5% for an additional 5 weeks. Ethanol comprised 27.5% of the total caloric intake of mice in this group. Liquid diets were based upon the Lieber-DeCarli ethanol formulation and provide 1 kcal/ml (purchased from DYETS Inc., Bethlehem, PA). Protein content accounted for 18.9% of total calories, fat comprised 16.5% of total calories, and 64.5% of calories were derived from carbohydrates. Control mice were pair-fed diets that isocalorically substituted cornstarch for ethanol during the entire study period. All procedures were conducted in accordance with the Institutional Animal Care and Use Committee. After 6 weeks of feeding, mice were fasted overnight and sacrificed. Blood samples were centrifuged at 3000 rpm for 15 min to collect serum. Livers were rapidly excised and weighed, and then aliquots were snap-frozen in liquid nitrogen and kept at −70°C for RNA, nuclear protein, and lipid extraction or Oil Red O staining. The remaining fresh liver tissue was immediately homogenized for isolation of microsomes or fixed in 10% formalin for hematoxylin and eosin (H&E) staining.

Serum ALT Activity. Serum was stored at −70°C and used for the alanine aminotransferase (ALT) activity assay. Serum ALT activity was determined using the Liquid ALT Reagent kit (Pointe Scientific Inc., Brussels, Belgium).

Hepatic Nonesterified Fatty Acid, Triglyceride, and Cholesterol Levels. Total liver lipids were extracted from 50 to 100 mg of liver homogenate using methanol and chloroform as described previously (Folch et al., 1957; Zhou et al., 2006). Hepatic nonesterified fatty acid (NEFA) level was determined using the NEFA C test kit (Wako Pure Chemical Industries, Richmond, VA).

Hepatic triglyceride was quantified using a Triglyceride test kit (Wako Pure Chemical Industries). The hepatic cholesterol content was quantified using a Cholesterol E-test kit (Wako Pure Chemical Industries). The hepatic albumin and retinol-binding protein levels were measured colorimetrically using the Human Albumin and Retinol Binding Protein ELISA kits (BioVendor Laboratory Medicine, Inc., Heidelberg, Germany).

Oil Red O and H&E Staining of Liver Sections. Frozen liver sections (10 μm) were stained with Oil Red O and counterstained with H&E for lipid content determination. Following fixation of the livers with 10% formalin/phosphate-buffered saline, livers were sliced and stained with H&E for histological examination. Apoptotic cells in 10 random × 400 fields/liver section were counted by a pathologist blinded to the study.

Hepatic CYP2E1 Activity. Microsomal fractions were separated from fresh liver tissue as described previously (Gyamfi et al., 2006). CYP2E1 activity in liver microsomes was estimated colorimetrically by measuring the hydroxylation of p-nitrophenol to 4-nitroaniline (Bicho and Moreau, 1995).

SAM and S-Adenosylhomocysteine. The liver homogenate was prepared as described previously (Dai et al., 2003) and mixed with an equal volume of 10% trichloroacetic acid solution, then centrifuged at...
10,000 rpm for 20 min at 4°C. The supernatant was collected and stored at −80°C. The levels of SAM and S-adenosylhomocysteine (SAH) were measured as described previously (She et al., 1994).

Preparation of Cytosolic and Nuclear Extracts, Western Blot Analysis, and Enzyme-Linked Immunosorbent Assay-Based NF-κB Binding Assay. Frozen livers were homogenized in lysis buffer [10 mM HEPES, pH 7.9, 100 mM KCl, 1.5 mM MgCl2, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5% Nonidet P-40, and a protease inhibitor cocktail (Pierce, Rockford, IL)] at 4°C, followed by centrifugation at 10,000g for 30 min. The supernatant was collected as cytosolic fraction. The nuclear pellet obtained was resuspended in nuclear extraction buffer at 4°C. The suspension was kept on ice and vortexed intermittently for 30 min, followed by centrifugation at 10,000g for 10 min. The supernatant was collected as nuclear extracts. The cytosolic fraction (30 μg/lane) and liver homogenate (25–50 μg/lane) were mixed in Laemmli loading buffer containing β-mercaptoethanol, boiled for 5 min, and then subjected to Western blot analysis as described previously (Gyamfi et al., 2006). Liver homogenate and cytosolic fraction were separated by 10% or 15% SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membrane. The cytosolic fractions were immunoblotted with phosphospecific anti-STAT3 (Tyr705) (Cell Signaling Technology, Boston, MA) and liver fatty acid binding protein (L-FABP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The liver homogenate and cytosolic fraction were separated by 10% or 15% SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membrane. The cytosolic fractions were immunoblotted with phosphospecific anti-STAT3 (Tyr705) (Cell Signaling Technology, Inc.) and liver fatty acid binding protein (L-FABP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The liver homogenate and cytosolic fraction were separated by 10% or 15% SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membrane. The cytosolic fractions were immunoblotted with phosphospecific anti-STAT3 (Tyr705) (Cell Signaling Technology, Inc.) and liver fatty acid binding protein (L-FABP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Quantification of mRNA Levels Using Real-Time Polymerase Chain Reaction. Total RNA was isolated from frozen liver tissues using the TRizol reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). RNA concentration and quality were determined spectrophotometrically at 280 nm and by the A260/A280 ratio, respectively. Total RNA (1 μg) was reverse transcribed into cDNA in a total reaction volume of 50 μl with the use of 2.5 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and 6.25 ng of oligo(dT)15 (Promega, Madison, WI) as a primer. The samples were placed in a Thermo Cycler and heated to 42°C for 15 min and then to 95°C for 15 min. cDNA was then diluted 10-fold with water and subjected to real-time polymerase chain reaction (PCR) to quantify the mRNA level of MIP-2, IL-1β, IL-6, TNFα, PAI-1, collagen 1α, L-FABP, β-actin, and GAPDH. Primers and probes (Table 1) were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA). The primers and probes were designed to cross introns to ensure that only cDNA and not genomic DNA was amplified. The fluorogenic MGB probe was labeled with the reporter dye 5-carboxyfluorescein. TaqMan Universal PCR Master Mix (Applied Biosystems) was used to prepare the PCR mix. Primers and probes were added to a final concentration of 909 and 125 nmol, respectively, in a total volume of 20 μl. The amplification reactions were carried out

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in the ABI Prism 7900 sequence detection system (Applied Biosystems) with initial hold steps (50°C for 2 min, followed by 95°C for 10 min) and 40 cycles of a two-step PCR (92°C for 15 s, 60°C for 1 min). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the target gene. The comparative CT method was used to determine -fold differences between samples. The amount of mRNA was calculated using the comparative CT method, which determines the amount of target normalized to an endogenous reference. Each gene was normalized to either GAPDH or β-actin after a validation experiment to verify that efficiency of the target gene amplification is equal to that of the endogenous reference.

**Statistical Analysis.** Data are presented as means ± S.E.M. (n = 4–6). Statistical analysis was performed using one-way analysis of variance followed by post hoc tests. A P value of <0.05 was considered statistically significant.

**Results**

Ingestion of ethanol for 6 weeks had no apparent effect on body and liver weight of either genotype (Table 2). Serum ALT activity was measured as an index of hepatocyte injury. Ethanol significantly elevated serum ALT levels (4-fold) in hepatocyte RXRα-deficient mice (Fig. 1a). The increase in serum ALT levels in the mutant mice fed ethanol was 3.1-fold compared with ethanol-fed wild-type mice (Fig. 1a). In contrast, serum ALT levels were not induced by ethanol in the wild-type mice (Fig. 1a). This finding indicates that using the Lieber-DeCarli ethanol model, hepatocyte RXRα-deficient mice are more susceptible to alcohol-induced liver injury, which is consistent with our previous finding using the intragastric ethanol infusion model (Dai et al., 2003). Furthermore, apoptotic cells were found in ethanol-fed mutant mice (Fig. 1, b and d) but not noted in ethanol-fed wild-type mice. Ethanol-fed hepatocyte RXRα-deficient mice also revealed the presence of mitotic cells, suggesting that apoptosis and cell proliferation are operative to prevent severe hepatocyte injury (Fig. 1, b–d). The increase in apoptosis in ethanol-fed mutant mice might in part contribute to the observed liver injury seen in hepatocyte RXRα-deficient mice (Fig. 1, a and e).

Lipid content was evaluated by analyzing the nonesterified fatty acid (Fig. 2a), triglyceride (Fig. 2b), and cholesterol (Fig. 2c) levels in the liver. Hepatic free fatty acids levels were not different between wild-type and hepatocyte RXRα-deficient mice fed a control diet (Fig. 2a). However, ethanol treatment significantly increased hepatic free fatty acid levels in the mutant mice, but not in wild-type mice (Fig. 2a). Ethanol feeding significantly increased hepatic triglyceride and cholesterol levels in both mutant and wild-type mice (Fig. 2, b and c). The increase in hepatic triglyceride levels by ethanol was not different between the two genotypes (Fig. 2b). However, the increase in hepatic free fatty acids and cholesterol levels by ethanol was higher in hepatocyte RXRα-deficient mice compared with wild-type mice fed ethanol (Fig. 2, a and c). In addition, the basal hepatic cholesterol levels were higher in the hepatocyte RXRα-deficient mice than the wild-type mice.

L-FABP, a PPARα target-gene, binds fatty acids and stimulates their esterification, thereby attenuating fatty acid accumulation and toxicity (Shevchuk et al., 1991; Lieber, 2004). Chronic ethanol ingestion increases the concentration of L-FABP in the liver (Shevchuk et al., 1991; Lieber, 2004). The levels of L-FABP mRNA were quantified in ethanol treated wild-type and hepatocyte RXRα-deficient mice (Fig. 2d). The basal level of L-FABP mRNA level was reduced when hepatocyte RXRα was deficient due to a defective RXRα/PPARα-mediated pathway (Fig. 2d) (Wan et al., 2000a). Consistent with these results, it was observed that the protein levels of L-FABP were decreased by 50% in the untreated mutant mice (Fig. 2e). Ethanol feeding increased L-FABP mRNA and protein levels by 6- and 2.5-fold, respectively, in wild-type mice (Fig. 2, d and e). In contrast, ethanol ingestion increased neither L-FABP mRNA nor protein levels in the mutant mice (Fig. 2, d and e). Compared with ethanol-fed wild-type mice, both L-FABP mRNA and protein levels were decreased in hepatocyte RXRα-deficient mice fed ethanol (Fig. 2, d and e). This lack of L-FABP mRNA induction in the livers of the mutant mice may be responsible for the observed ethanol-induced accumulation of nonesterified fatty acids in the mutant mice after ethanol ingestion (Fig. 2a). However, a 2-fold reduction of both L-FABP mRNA and protein levels due to hepatocyte RXRα deficiency did not alter the basal nonesterified fatty acid level compared with wild-type mice (Fig. 2a).

Histological analysis of the livers by Oil Red O staining revealed lipid accumulation in the ethanol-fed mouse livers (Fig. 2, h and i), whereas lipid droplets were rare in the livers of the control group (Fig. 2, f and g). H&E staining of liver section from wild-type mice fed ethanol revealed minimal neutrophil infiltration (data not shown), whereas neutrophil infiltration was prominent in hepatocyte RXRα-deficient mice fed ethanol (Fig. 2j).

Ethanol induced CYP2E1 activity in the wild-type mice (21.1 ± 1.7 nmol/mg), compared with wild-type mice fed control diet (13.9 ± 0.7 nmol/mg). However, CYP2E1 activity was not induced by ethanol in the mutant mice (13.5 ± 1.9 nmol/mg), compared with hepatocyte RXRα-deficient mice fed the control diet (13.1 ± 1.0 nmol/mg). This finding suggests that CYP2E1 did not contribute to alcohol-induced liver injury in hepatocyte RXRα-deficient mice.

In addition to dysregulation of lipid metabolism, abnormal methionine metabolism leading to decreased SAM levels is

**TABLE 2**

Effects of ethanol on mouse body and liver weight

Data are mean ± S.E.M. for five to six mice per group.

<table>
<thead>
<tr>
<th>Parameter (unit)</th>
<th>Wild Type</th>
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<th>RXRα Knockout</th>
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<td>Control</td>
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<tr>
<td>Body weight (g)</td>
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important in the pathogenesis of alcohol-induced liver injury (Dai et al., 2003; McClain et al., 2005). SAM down-regulates the production of toxic proinflammatory cytokines while increasing the levels of beneficial anti-inflammatory cytokines (McClain et al., 2005). Hepatocyte RXRα-deficiency results in reduction of hepatic SAM level (Fig. 3a). Ethanol ingestion significantly decreased SAM levels in both wild-type and hepatocyte RXRα-deficient mice (Fig. 3a). Compared with ethanol-fed wild-type mice, SAM levels decreased in the mutant mice after ethanol ingestion (0.76-fold) (Fig. 3a). SAH levels were significantly increased by ethanol feeding of wild-type mice (Fig. 3b). The SAM/SAH ratio was significantly lower in ethanol-fed wild-type mice compared with wild-type mice fed ethanol diet. However, ethanol-fed hepatocyte RXRα-deficient mice also showed an increase in SAH levels, but this induction did not reach statistical significance (Fig. 3b). As a result, ethanol did not decrease the SAM/SAH ratio in hepatocyte RXRα-deficient mice (Fig. 3c).

SAM levels and the SAM/SAH ratio after ethanol treatment were not different between wild-type and hepatocyte RXRα-deficient mice (Fig. 3, b and c). This finding suggests that ethanol-induced ALT is not due to reduction of the SAM/SAH.

NF-κB is a central proinflammatory transcription factor known to induce proinflammatory cytokine synthesis. Ethanol increased NF-κB p65 binding activity in hepatocyte RXRα-deficient mice (1.7-fold), but not in wild-type mice (Fig. 4). The activation of NF-κB suggested increased expression of NF-κB-regulated cytokines and chemokines in the mutant mice. This finding suggests that NF-κB activity can be controlled by multiple regulatory processes including RXRα. To determine the effect of RXRα deficiency on the expression of inflammatory mediators during ethanol exposure, mRNA levels of several proinflammatory cytokines and chemokines were measured. TNFα, IL-6, and IL-1β mRNA levels were increased by 8.6-, 52.0-, and 6.9-fold, respectively, in hepatocyte RXRα-deficient mice after ethanol feeding, whereas only the IL-6 mRNA level was increased in wild-type mice after

**Fig. 1.** Serum ALT activity and hepatocyte apoptosis in wild-type and hepatocyte RXRα-deficient mice fed control or ethanol-containing diets. Male wild-type or hepatocyte RXRα-deficient mice were fed control diet (pair-fed) or ethanol for 6 weeks. ALT level (a) and assessment of apoptosis by H&E-stained liver sections (b–e) were determined. Liver sections showing apoptotic cells (200×) and insert (400×) (indicated by arrows, b); mitotic cells, 400× (indicated by arrowheads, c); and apoptotic and mitotic cells, 400× (indicated by arrows and arrowheads, d) in hepatocyte RXRα-deficient mice fed ethanol. Quantification of apoptotic cells in the H&E-stained sections and expressed as the ratio of apoptotic cells to hepatocytes counted per field (e). Data represent means ± S.E.M. (n = 4–6). §, P < 0.05 versus wild-type mice fed ethanol diet. ##, P < 0.01; #, P < 0.05 versus hepatocyte RXRα-deficient mice fed control diet.
ethanol treatment (2.5-fold) (Fig. 5, a–c). A significant decrease in the basal TNFα mRNA level was observed in hepatocyte RXRα-deficient mice compared with wild-type mice (Fig. 5a), suggesting that RXRα may be important for TNFα expression. Ethanol ingestion induced MIP-2 mRNA level by 3.5-fold in hepatocyte RXRα-deficient mice; such induction did not reach statistical significance in wild-type mice (Fig. 5d). Ethanol ingestion also caused a significantly greater induction of the profibrogenic factor PAI-1 mRNA level in hepatocyte RXRα-deficient (5.5-fold) compared with wild-type (2.3-fold) mice (Fig. 5e). A significant increase in the collagen-1α mRNA (1.6-fold) level was observed by ethanol feeding of hepatocyte RXRα-deficient mice, but not in wild-type mice (Fig. 5f). Because IL-6 mRNA levels were highly induced by ethanol in the mutant mice, we also examined protein levels. Consistent with the mRNA levels, ethanol-treated mutant mice had the highest level of IL-6 protein (Fig. 6, a and b). However, the -fold induction in protein levels was not as high in comparison with the changes at the mRNA level (Fig. 6, a and b).

The STAT3 is a transcription factor implicated in the regulation of inflammatory signaling in the liver that is potently activated by IL-6 (Hong et al., 2002a). IL-6-induced STAT3 phosphorylation is a known antiapoptotic pathway that protects the liver from ethanol-induced liver injury (Hong et al., 2002a). Because IL-6 mRNA was induced by ethanol in the livers of wild-type and hepatocyte RXRα-deficient mice, phosphorylated STAT3 was quantified by Western blot. Ethanol increased the level of phosphorylated STAT3 in wild-type mice, but not in the hepatocyte RXRα-deficient mice (Fig. 7, a and b). IL-6 is highly induced by ethanol in the hepatocyte RXRα-deficient mice, but the associated STAT-3 activation was not found. Thus, hepatocyte RXRα deficiency alters many signaling pathways, including IL-6-mediated STAT3 activation. The underlying mechanism remains to be investigated.

STAT3 is required for the expression of antiapoptotic genes including Bcl-2 and Bcl-xL. The protein levels of Bcl-2 and Bcl-xL were therefore examined by Western blot. Bcl-xL protein was significantly increased, whereas Bcl-2 protein was decreased due to RXRα deficiency. Ethanol induced the level of Bcl-xL protein (2.7-fold) in the wild-type mice, but not in the mutant mice (Fig. 8, a and b). Furthermore, ethanol decreased Bcl-2 protein level in hepatocyte RXRα-deficient mouse livers to 0.1-fold compared with the level in the mutant mice fed control diet (Fig. 8, a and c). Bcl-2 protein level in ethanol-fed wild-type was 0.6-fold compared with wild-type mice fed control diet. Although there was no difference
Methods

Data represent means ± S.E.M. (n = 5–6). *** P < 0.001; ** P < 0.01; * P < 0.05 versus wild-type mice fed control diet. §§, P < 0.01 versus wild-type mice fed ethanol diet. ###, P < 0.001 versus hepatocyte RXRα-deficient mice on control diet.

Discussion

Hepatocyte RXRα deficiency increases the susceptibility to alcohol-induced liver damage (Dai et al., 2003; Gyamfi et al., 2006). In earlier reports, we found that decreased hepatic SAM and reduced glutathione levels as well as increased alcohol clearance associated with decreased acetaldehyde elimination in hepatocyte RXRα-deficient mice contributed to a greater susceptibility to ethanol-induced toxicity in the mutant compared with the wild-type mice (Dai et al., 2003; Gyamfi et al., 2006). In the present study, we find that the pathologic events during chronic ethanol feeding of the hepatocyte RXRα-deficient mice might involve lack of L-FABP gene induction and elevated hepatic NEFA levels accompanied by NF-κB activation and markedly up-regulation of proinflammatory cytokine and chemokine gene expression. In addition, ethanol-induced STAT3 and Bcl-xL activation were suppressed due to hepatocyte RXRα deficiency. Although ethanol-fed wild-type mice had steatotic livers and higher CYP2E1 activity than ethanol-treated mutant mice, chronic alcohol feeding did not induce liver damage in the wild-type mice, most probably due to induction of the L-FABP gene, Bcl-xL protein, and minimal up-regulation of inflammatory cytokines and chemokines in wild-type mice.

Fatty acids are a major energy source and comprise an integral structural component of cell membranes as esterified phospholipids. Intracellular accumulation of free fatty acids may damage the cell membrane, thereby contributing to necrosis, inflammation, and progression to fibrosis and cirrhosis (Lieber, 2004). FABPs are proposed to function in the transport, metabolism, and storage of fatty acids (Desvergne et al., 1998) and to protect other proteins and membranes from the toxic effects of high fatty acid concentrations. Reports indicate that chronic ethanol ingestion increases concentrations of L-FABP (Shevchuk et al., 1991; Lieber, 2004). Deleterious accumulation of fatty acids in ethanol-fed female rats and in alcoholic women is the result of inadequate induction of cytosolic L-FABP, which might contribute to the sex differences in ethanol-induced toxicity (Shevchuk et al., 1991). Consistent with the published findings, we also observed induction of L-FABP mRNA and protein in wild-type mice by ethanol (Shevchuk et al., 1991; Lieber, 2004). The basal L-FABP mRNA level was significantly reduced and not inducible by ethanol in the hepatocyte RXRα-deficient mice. L-FABP is a direct PPARα target gene (Landrier et al., 2000a). Reduced L-FABP gene expres-
sion in the mutant mice was associated with elevated hepatic free fatty acids levels in the mutant mice after ethanol ingestion, which might contribute to the ethanol-induced liver injury we observed in the mutant mice. It was also observed that ethanol ingestion increased hepatic triglyceride and cholesterol levels in both mutant and wild-type mice; however, the ethanol-induced steatosis in the wild-type mice was not associated with liver injury. Our data are consistent with recent reports in the literature of a distinction between ethanol-induced steatosis and hepatocyte injury/flammation observed in PAI-1 knockouts and in the different responses of complement knockouts fed ethanol (Bergheim et al., 2006; Pritchard et al., 2007).

Our data found that the SAM level was decreased after ethanol ingestion in wild-type and hepatocyte RXRa-deficient mice. SAM is a precursor of reduced glutathione, a major cellular antioxidant, and the decrease in SAM levels may exacerbate oxidative stress induced by ethanol ingestion. Because the basal SAM level is much lower in hepatocyte RXRa-deficient mice than wild-type mice, the reduced hepatic SAM level might be another priming factor besides reduced basal L-FABP levels for increased susceptibility of hepatocyte RXRa-deficient mice to ethanol-induced liver injury.

The activation and translocation of NF-κB plays a central role both in inflammatory responses and in liver injury (Tilg and Diehl, 2000). Induction of TNFα and IL-6 genes is partially regulated through the NF-κB sequence localized in their promoter region (Drouet et al., 1991; Galien et al., 1996). Consistent with the observation that ethanol-fed hepatocyte RXRa-deficient mice had increased TNFα and IL-6 mRNA expression compared with wild-type mice fed ethanol, we observed increased NF-κB p65 activation in ethanol-fed hepatocyte RXRa-deficient mice compared with ethanol-fed wild-type mice. In agreement with the observed up-regulation in NF-κB activation, the gene expression of IL-1β, MIP-2, and PAI-1 controlled by NF-κB was coordinately induced by ethanol in hepatocyte RXRa-deficient mice, which may contribute to liver injury.

Another intriguing finding is the decreased basal TNFα gene expression and its high inducibility by ethanol in the absence of hepatocyte RXRa. TNFα is produced in Kupffer cells as well as other sinusoidal cells in the liver following alcohol administration (Thurman, 1998). TNFα promotes inflammation, leukocyte infiltration, tissue fibrosis, and cytokine production. One of the cell targets for TNFα is parenchymal cells as verified by the ability of hepatocytes to express TNFα receptors and chemokines in response to TNFα (Liu et al., 2000). If TNFα is produced by nonparenchymal cells exclusively, then the presence of cross talk between hepatocyte RXRa and Kupffer cells needs to be examined. However, because TNFα mRNA is detectable in unstimulated mouse liver, it is very likely that the TNFα gene is expressed in hepatocytes. If this is the case, our findings suggest that RXRa in hepatocyte might directly or indirectly control TNFα gene expression in hepatocytes and Kupffer cells. The likelihood of an indirect effect is high, particularly given the increase in inflammatory cells in the livers of the mutant mice after ethanol ingestion.
STAT3 mediates the expression of a variety of genes in response to cytokines and thus plays a key role in hepatocyte proliferation and apoptosis prevention after liver injury (Cressman et al., 1996; Hong et al., 2002a). In response to cytokines and growth factors, including interferons, epidermal growth factor, IL-5, IL-6, and hepatocyte growth factor, STAT3 can be activated through phosphorylation by Janus kinase and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators (Desrivieres et al., 2006). IL-6 is readily detected in patients with ALD, and its expression and protein expression were more prone to ethanol-induced apoptosis in the liver (Hong et al., 2006). IL-6 is readily detected in patients with ALD, and its gene and protein expression were more prone to ethanol-induced apoptosis in the liver (Hong et al., 2006). IL-6 is readily detected in patients with ALD, and its gene and protein expression were more prone to ethanol-induced apoptosis in the liver (Hong et al., 2006).

In wild-type or hepatocyte RXRα-deficient mice fed control or ethanol-containing diets. Liver homogenate (50 μg/lane) from ethanol and pair-fed mice were electrophoresed on 15% SDS-polyacrylamide, transferred to nitrocellulose membranes, and incubated with anti-IL-6 antibody as indicated in representative blots (a). Quantitative analysis of IL-6 level plotted after normalizing with β-actin in wild-type or hepatocyte RXRα-deficient mice fed control (pair-fed) (%) or ethanol-containing (%) diets. Densitometric analysis was performed by the Quantity One 1-D Analyzer Software (Bio-Rad Laboratories). Data represent means ± S.E.M. (n = 3–4). #, P < 0.05 versus hepatocyte RXRα-deficient mice on control diet.

that liver repair that includes regeneration, and antiapoptosis might be impaired in the hepatocyte RXRα-null mice leading to liver injury. Further investigation on how RXRα regulates STAT3 activation and inhibition of ethanol-induced apoptosis is warranted.

Retinoic acid (RA) inhibits hepatic macrophage TNFα expression and is known to have anti-inflammatory effects (Motomura et al., 2001). LG268, which is an RXR-specific ligand, inhibits TNFα mRNA expression (Motomura et al., 2001). Although anti-inflammatory action of RA was found to involve destabilization of the TNFα mRNA, our current results suggest a prominent role by the RA receptor, RXRα (Motomura et al., 2001). Our results demonstrating the important role of RXRα in inflammation is also supported by findings that PPARα-deficient mice had prolonged inflammatory response after arachidonic acid or leukotriene B4 administration (Devchand et al., 1996). PPARα-deficient mice also display an exacerbated inflammatory response to LPS as well as to chronic high-fat diet ingestion (Delerive et al., 1999; Stienstra et al., 2007). It remains to be investigated which RXRα-mediated pathway is important for alcohol-induced liver injury. Furthermore, it also remains to be elucidated whether RXRα or the heterodimeric partner of RXRα is a better target to treat and prevent the ethanol-induced inflammatory response.

In summary, the present study is the first to address the role of hepatocyte RXRα in the expression of proinflammatory cytokines and chemokines in response to alcohol inges-
tion. Taken together, our results revealed dysregulation of lipid metabolism as well as an increased inflammatory response due to hepatocyte RXRα deficiency. Reduced L-FABP mRNA and protein levels, elevated hepatic free fatty acid level and reduced hepatic SAM content might prime the hepatocyte RXRα-deficient mice to be susceptible to ethanol toxicity. Enhanced activation of NF-κB, a pleiotropic transcription factor that triggers the production of other cytokines and chemokines and suppression of STAT3 phosphorylation coupled with increased apoptosis in the mutant mice, is a critical mechanism leading to ethanol-induced liver injury in hepatocyte RXRα-deficient mice.

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Fig. 8. Immunoblot analysis of Bel-2L and Bel-2 in wild-type and hepatocyte RXRα-deficient mice fed control or ethanol-containing diets. Liver homogenate (50 μg/lane) from ethanol and pair-fed mice were electrophoresed on 15% SDS-polyacrylamide, transferred to nitrocellulose membranes, and incubated with specific antibodies as indicated in representative blots (a). Quantitative analysis of Bel-2L level (b) or Bel-2 (c) plotted after normalizing with α-actin in wild-type or hepatocyte RXRα-deficient mice fed control (pair-fed) or ethanol-containing diets. Densitometric analysis was performed by the Quantity One 1-D Analyzer Software (Bio-Rad Laboratories). Data represent means ± S.E.M. (n = 3–4). *, P < 0.05 versus wild-type mice fed control diet. §, P < 0.05 versus wild-type mice fed ethanol diet.
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