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Chronic ethanol exposure downregulates hepatic expression of pregnane X receptor and *P450 3A11* in female ICR mice

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Abstract

Pregnane X receptor (PXR) is a nuclear receptor that regulates cytochrome P450 3A (CYP3A) gene transcription in a ligand-dependent manner. Ethanol has been reported to be either an inducer or an inhibitor of CYP3A expression. In this study, we investigated the effects of chronic ethanol exposure on *PXR* and *P450 3A11* gene expression in mouse liver. Female ICR mice were administered by gavage with different doses (1000, 2000 and 4000 mg/kg) of ethanol for up to 5 weeks. Hepatic *PXR* and *P450 3A11* mRNA levels were measured using RT-PCR. Erythromycin *N*-demethylase (ERND) activity was used as an indicator of CYP3A protein expression. Results showed that chronic ethanol exposure markedly decreased hepatic *PXR* and *P450 3A11* mRNA levels. Consistent with downregulation of *P450 3A11* mRNA, chronic ethanol exposure significantly decreased ERND activity in a dose-dependent manner. Additional experiment showed that chronic ethanol exposure significantly increased plasma endotoxin level and hepatic *CD14* and *TLR*-4 mRNA expression, all of which were blocked by elimination of Gram-negative bacteria and endotoxin with antibiotics. Correspondingly, pretreatment with antibiotics reversed the downregulation of *PXR* and *P450 3A11* mRNA expression and ERND activity in mouse liver. Furthermore, the downregulation of hepatic *PXR* and *P450 3A11* mRNA expression was significantly attenuated in mice pretreated with $GdCl_3$, a selective Kupffer cell toxicant. $GdCl_3$ pretreatment also significantly attenuated chronically ethanol-induced decrease in ERND activity. These results indicated that activation of Kupffer cells by gut-derived endotoxin contributes to downregulation of hepatic *PXR* and *P450 3A11* expression during chronic alcohol intoxication.

Keywords: Ethanol; Endotoxin; Pregnane X receptor; Cytochrome P450 3A; Kupffer cells

1. Introduction

The *cytochrome P450 3A* (*CYP*3*A*) is a member of the cytochrome P-450 monooxygenase superfamily, which accounts for 25–35% of the total cytochrome P450 present in adult human or rat liver (Wrighton et al., 2000). CYP3A enzymes are responsible for the oxidative metabolism of numerous clinically used drugs, including psychotropic, cardiac, analgesic, hormonal, immunosuppressant, antineoplastic and antihistaminic agents (Thummel and Wilkinson, 1998). Hepatic *CYP3A* expression is highly regulated by development,

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tissue-specific factors, hormonal influences, xenobiotics and pathophysiological mechanisms (Quattrochi and Guzelian, 2001; Goodwin et al., 2002). Recent studies found that pregnane X receptor (PXR), a member of the nuclear receptor superfamily, regulates *CYP*3*A* gene transcription in a ligand-dependent manner (Kliewer et al., 1998; Lehmann et al., 1998; Bertilsson et al., 1998).

Clinically, consumption of alcoholic beverages is associated with development of liver damage and hepatic failure from therapeutic doses of acetaminophen (APAP) (Zimmerman and Maddrey, 1995). In experimental animals, treatment with ethanol also results in increased liver damage from APAP (Nelson, 1990; Lieber, 1993). P450 2E1 has been hypothesized to have a pivotal role in alcohol-mediated increases in APAP hepatotoxicity (Lee et al., 1996). However, recent studies demonstrated that CYP3A is also involved in ethanol-mediated increases in APAP hepatotoxicity. First, CYP3A4 contributes appreciably to the formation of *N*-acetyl-*p*-aminobenzoquinone imine (NAPQI) from acetaminophen (APAP) (Thummel et al., 1993). Second, xenobiotic receptor constitutive androstane receptor (CAR), the transactivator of CYP3A gene, has been identified as a key regulator of acetaminophen metabolism and hepatotoxicity (Zhang et al., 2002). Next, short-term treatment with alcohols enhances APAP hepatotoxicity in *P450 2E1*(−/−) mice (Sinclair et al., 2000). Finally, triacetyloleandomycin, a specific inhibitor of CYP3A, protects against alcohol-mediated increases in APAP hepatotoxicity (Kostrubsky et al., 1997).

Ethanol has been reported to be either an inducer or an inhibitor of CYP3A expression. CYP3A exposure-induced P450 3A in primary cultures of human and rat hepatocytes (Kostrubsky et al., 1995; DiPetrillo et al., 2002). In the CYP3A4-expressing HepG2 cell line, incubation with ethanol increased *CYP*3*A*4 mRNA level and CYP3A activity in a dose-dependent manner (Feierman et al., 2003). Several in vivo studies indicated a relationship between CYP3A and the duration of ethanol exposure. In rats fed ethanol with the Lieber-DeCarli diet for 7–14 days, both ERND catalytic activities and immunoreactive CYP3A are increased (Roberts et al., 1995). In addition, ethanol significantly increased fentanyl *N*-dealkylatase activities in rats fed ethanol for 21 days (Feierman et al., 2003). However, in rats fed ethanol diet for 38 days, there was a significant decrease in hepatic testosterone 6β-hydroxylase activities (Badger et al., 1993). Similarly, Rowlands et al. (2000) found that CYP3A apoprotein level and testosterone 6β-hydroxylase activities decreased in rat fed ethanol diet for 42–55 days. Consistent with downregulation of CYP3A protein and testosterone 6β-hydroxylase, *CYP*3*A*2 mRNA levels were decreased by 73–83%.

In the present study, we investigated the effects of chronic ethanol administration on *PXR* and *P450 3A11* expression in mouse liver. Our results indicated that chronic ethanol exposure significantly downregulates *PXR* and *P450 3A11* mRNA levels and decreases ERND activity in mouse liver. Activation of Kupffer cells by gut-derived endotoxin contributes to downregulation of hepatic *PXR* and *P450 3A11* and ERND activity during chronic alcohol intoxication.

2. Materials and methods

2.1. Animals and treatments

Female 8- to 10-week-old ICR mice, weighing 20–22 g, were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories Inc. The animals were allowed free access to food and water at all times and were maintained on a 12-h light/12-h dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment for a period of 1 week before use. The present study consisted of three separate experiments.

2.1.1. Experiment 1

Female ICR mice were administered by gavage with different doses (1000, 2000 and 4000 mg/kg) of ethanol daily for 5 weeks. Mice from the controls were administered with saline. All mice were sacrificed. Mouse livers were excised for total RNA extraction and microsome isolation.

2.1.2. Experiment 2

Female ICR mice were randomly divided into four groups. The mice in ethanol group were administered by gavage with 4000 mg/kg of ethanol for 5 weeks. In addition to ethanol (4000 mg/kg, po), the mice in ethanol + antibiotics group were also treated with polymyxin B (150 g/(kg day), po) plus neomycin (450 g/(kg day), po) to prevent growth of intestinal bacteria, the main source of endotoxin in the gastrointestinal tract. The saline- and antibiotics-treated groups administered with either saline or polymyxin B (150 g/(kg day), po) plus neomycin (450 g/(kg day), po) served as controls. Half of the mice were anesthetized at 2 h after the last ethanol treatment. Heparinized blood samples were collected from the portal vein for endotoxin measurement. Liver samples were excised for the determination of CD14 and TLR4 mRNA. The remaining mice were sacrificed at 12 h after the last ethanol treatment. Liver

samples were excised for total RNA extraction, microsome isolation and lipid peroxidation assay.

2.1.3. Experiment 3

Female ICR mice were randomly divided into four groups. The mice in ethanol group were administered by gavage with 4000 mg/kg of ethanol for 5 weeks. In addition to ethanol (4000 mg/kg, i.g.), the mice in ethanol + $GdCl_3$ group were also administered twice weekly with $GdCl_3$ (10 mg/kg, i.v.), a selective Kupffer cell toxicant, to inactivate Kupffer cells. The saline- and $GdCl_3$-treated groups administered with either saline or $GdCl_3$ (10 mg/kg, i.v.) served as controls. Half of the mice were anesthetized at 2 h after the last ethanol treatment. Liver samples were excised for the determination of proinflammatory cytokine (TNF-α, IL-1β and IL-6) mRNA. The remaining mice were sacrificed at 12 h after the last ethanol treatment. Liver samples were excised for total RNA extraction, microsome isolation and lipid peroxidation assay.

All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

2.1.4. Isolation of total RNA and RT

A sample of liver tissue (50 mg) was collected from each mouse. Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNase-free DNase (Promega) was used to remove genomic DNA. The integrity and concentration of RNA was determined by measuring absorbance at 260 nm followed by electrophoresis on agarose gels. Total RNA was stored at −80 °C. For cDNA synthesis, total RNA (2.0 μg) from each sample was resuspended in reaction buffer (final volume, 20 μl), which contained 25 mM Tris–HCl, pH 8.3, 37.5 mM KCl, 10 mM dithiothreitol, 1.5 mM $MgCl_2$, 10 mM of each dNTP and 0.5 mg oligo$(dT)_{15}$ primer (Promega). After the reaction mixture reached 38 °C, 400 units of RT (Promega) was added to each tube and the sample was incubated for 60 min at 38 °C. Reverse transcription was stopped by denaturing the enzyme at 95 °C.

2.1.5. PCR amplification

The final PCR mixture contained 2.5 μl of cDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP mixture, 1 U of Taq DNA polymerase, 1 μM sense and antisense primers and sterile water to 50 μl. The reaction mixture was covered with mineral oil. PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed on each individual sample as an internal positive-control standard. Following primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Company (Shanghai, China), according to sequence designs previously described (Table 1). Number of cycles and annealing temperature were optimized for each primer pair. For *GAPDH*, *TLR*4 and *CD*14, amplification was initiated by 3 min of denaturation at 94 °C for 1 cycle, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for

Table 1
Oligonucleotide primers and size for PCR analysis

Name	Dir	Oligonucleotide sequence (5′-3′)	Size (bp)	References
GAPDH	F	GAG GGG CCA TCC ACA GTC TTC	340	Xu et al. (2004)
	R	CAT CAC CAT CTT CCA GGA GCG		
PXR	F	GCG CGG AGA AGA CGG CAG CAT C	254	Xu et al. (2004)
	R	CCC AGG TTC CCG TTT CCG TGT C		
P450 3A11	F	CTC AAT GGT GTG TAT ATC CCC	423	Xu et al. (2005)
	R	CCG ATG TTC TTA GAC ACT GCC		
P450 2E1	F	AGT GTT CAC ACT GCA CCT GG	125	Kwon et al. (2003)
	R	CCT GGA ACA CAG GAA TGT CC		
TNF-α	F	GGC AGG TCT ACT TTG GAG TCA TTG C	307	Chen et al. (2005)
	R	ACA TTC GAG GCT CCA GTG AAT TCG G		
IL-1β	F	TCA TGG GAT GAT GAT GAT AAC CTG CT	502	Chen et al. (2005)
	R	CCC ATA CTT TAG GAA GAC ACG GAT T		
IL-6	F	CTG GTG ACA ACC ACG GCC TTC CCT A	600	Chen et al. (2005)
	R	ATG CTT AGG CAT AAC GCA CTA GGT T		
TLR4	F	AAT TCC TGC AGT GGG TCA AG	601	Itoh et al. (2003)
	R	AGG CGA TAC AAT TCC ACC TG		
CD14	F	ACA TCT TGA ACC TCC GCA AC	500	Itoh et al. (2003)
	R	AGG GTT CCT ATC CAG CCT GT		

1 min. For *P450 3A11*, amplification was initiated by 3 min of denaturation at 94 °C for 1 cycle, followed by 25 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min. For *P450 2E1*, amplification was initiated by 3 min of denaturation at 94 °C for 1 cycle, followed by 25 cycles at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min. For *PXR*, amplification was initiated by 3 min of denaturation at 94 °C for 1 cycle, followed by 45 cycles at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 1 min. For TNF-α and IL-1β, amplification was initiated by 3 min of denaturation at 94 °C for 1 cycle, followed by 30 cycles each of denaturation at 94 °C for 45 s, annealing of primer and fragment at 60 °C for 45 s and primer extension at 72 °C for 1 min. For IL-6, amplification was initiated by 3 min of denaturation at 94 °C for 1 cycle, followed by 35 cycles each of denaturation at 94 °C for 45 s, annealing of primer and fragment at 60 °C for 45 s and primer extension at 72 °C for 1 min. A final extension of 72 °C for 10 min was included. The amplified PCR products were subjected to electrophoresis at 75 V through 1.5% agarose gels (Sigma, St. Louis, MO) for 45 min. The pBR322 DNA digested with Alu I was used for molecular markers (MBI Fermentas). Agarose gels were stained with 0.5 mg/ml ethidium bromide (Sigma, St. Louis, MO) TBE buffer.

2.1.6. Preparation of liver microsomes

Liver microsomal fraction was prepared by homogenization and differential centrifugation (Haugen and Coon, 1976). All procedures were conducted at 4 °C. Tissue was homogenized in four volumes of Tris–chloride buffer, pH 7.4, containing 150 mM potassium chloride and 1 mM EDTA, with a Polytron homogenizer and centrifuged at 10,000 × *g* for 20 min. The supernatant was collected and centrifuged at 211,000 × *g* for 40 min. The microsomal pellet was resuspended and washed in sodium pyrophosphate buffer, pH 7.4, containing 1 mM EDTA and centrifuged again at 211,000 × *g* for 40 min at 4 °C. The washed microsomal pellet was resuspended in a Tris–chloride buffer, pH 7.4, containing 20% glycerol, with a ground glass tissue grinder and stored at −80 °C. Protein concentrations of microsome samples were measured according to the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.1.7. CYP3A catalytic activity

Erythromycin *N*-demethylase (ERND) activity was used as an indicator of CYP3A expression. ERND was measured according to the method of Werringloer (1978) with a 45 min incubation containing erythromycin (4 mM), NADPH (0.5 mM) and microsomal fraction (0.4 mg). The rate of formaldehyde formation was determined spectrophotometrically at 412 nm using the Nash reagent. Measurement for ERND catalytic activity was repeated twice.

2.1.8. Endotoxin measurement

The heparinized blood samples were centrifuged at 150 × *g* for 10 min. Platelet-rich plasma samples were diluted 1:10 and heated to 75 °C for 10 min to denature endotoxin-binding proteins that interfere with the assay (Rivera et al., 1998). Tubes used for sample collection, storage and assay preparation were borosilicate glass heated to 200 °C overnight to destroy endotoxin. Endotoxin was measured kinetically using a chromogenic test based on the limulus amebocyte lysate assay (BioWhittaker). Pyrogen-free water and pooled normal rat plasma were used as controls. The concentration of endotoxin in each sample was calculated from a standard curve prepared for each assay.

2.1.9. Lipid peroxidation assay

Hepatic lipid peroxidation was quantified by measuring thiobarbituric acid-reactive substance (TBARS) as described previously (Ohkawa et al., 1979). Liver tissue was homogenized in 9 volumes of 50 mmol/l Tris–HCl buffer (pH 7.4) containing 180 mmol/l KCl, 10 mmol/l EDTA and 0.02% butylated hydroxytoluene. To tissue homogenate (0.2 ml) were added sodium dodecyl sulfate (8.1%, 0.2 ml), acetic acid (20%, 1.5 ml), thiobarbituric acid (0.9%, 1.5 ml) and distilled water (0.6 ml) and mixture was vortexed. The reaction mixture was placed in a water bath at 95 °C for 1 h. After cooling on ice, 1.0 ml of distilled water and 5.0 ml of butanol/pyridine mixture (15:1, v/v) were added and vortexed. After centrifugation at 10,000 × *g* for 10 min, absorbance of the resulting lower phase was determined at 532 nm. The TBARS concentration was calculated using 1,1,5,5-tetraethoxypropane as standard.

2.1.10. Statistical analysis

The *PXR*, *P450 3A11* and *P450 2E1* levels were normalized to *gapdh* mRNA level in the same samples. The *PXR*, *P450 3A11* and *P450 2E1* levels of the control were assigned as 100%. Quantified data from analysis of plasma endotoxin, TBARS, RT-PCR and ERND assay were expressed as means ± S.E.M. at each point. ANOVA and the Student–Newmann–Keuls post hoc test were used to determine differences between the treated animals and the control and statistical significance.

3. Results

3.1. Body weight growth

Most animal studies with alcohol use some kind of gastric feeding protocol to examine the effects of chronic administration, such as continuous intragastric enteral feeding protocol of Tsukamoto et al. (1990). In this study, ethanol was administered by gavage. To ensure that controls obtain a similar calorific intake as ethanol-treated animals, we compared the body weight growth among different groups. Results are presented in Fig. 1. There were no significant differences in body weight growth among groups.

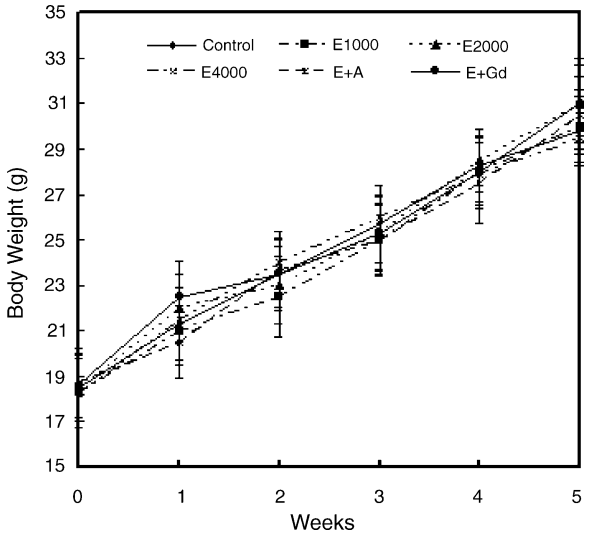

Fig. 1. Body weight growth among different groups. Female ICR mice were used in this study. Body weights were measured once a week. Data were expressed as means ± S.E.M. of 12 mice.

3.2. Downregulation of PXR and P450 3A11 mRNA and ERND activity

The effects of ethanol on *PXR* and *P450 3A11* mRNA are presented in Fig. 2A and B. Results showed that

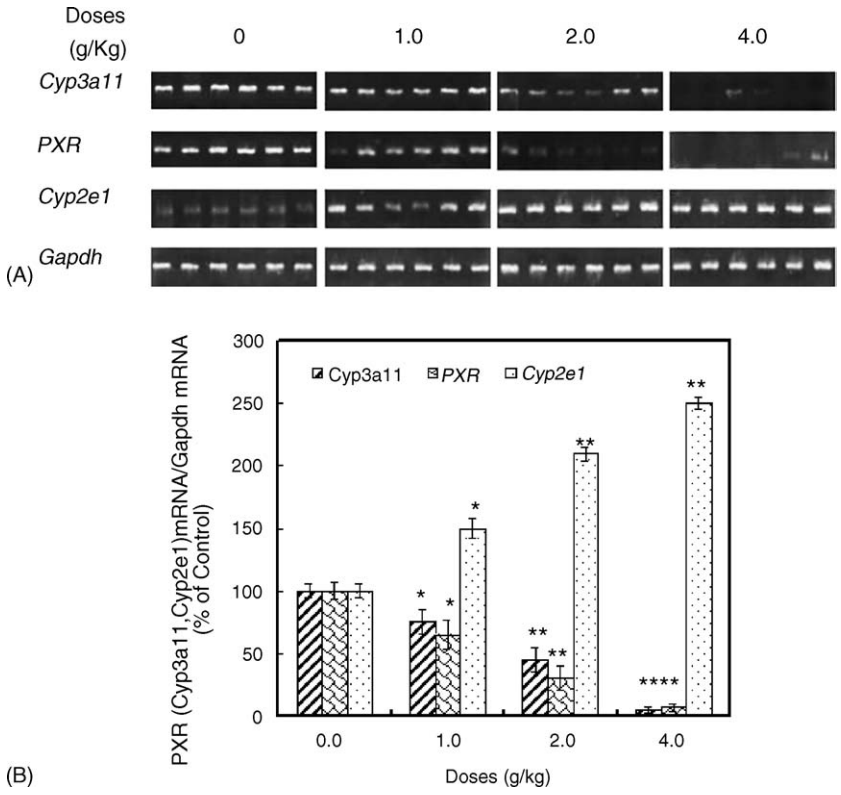

Fig. 2. The effects of ethanol on *PXR* and *P450 3A11* mRNA levels in mouse liver. (A) Mice were administered with different doses of ethanol (1.0–4.0 g/kg, i.g.) for 5 weeks. Livers were excised and total RNA was extracted. *PXR*, *P450 3A11* and *P450 2E1* mRNA levels were determined using RT-PCR. (B) Quantitative analysis of *PXR*, *P450 3A11* and *P450 2E1* mRNAs on four individual mouse liver RNA samples at each point was performed. The *PXR*, *P450 3A11* and *P450 2E1* mRNA levels were normalized to *Gapdh* mRNA level in the same samples. The *PXR*, *P450 3A11* and *P450 2E1* mRNA levels of the control were assigned as 100%. The number of mice at each point is 12. $^{*}P < 0.05$; $^{**}P < 0.01$ as compared with control group.

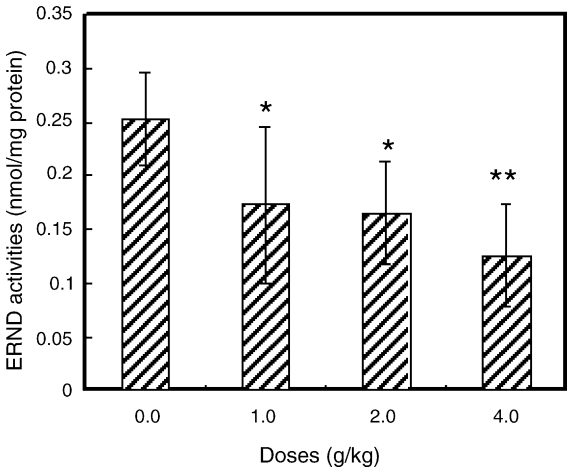

Fig. 3. The effects of ethanol on ERND catalytic activities in mouse liver. Mice were administered with different doses of ethanol (1.0–4.0 g/kg, i.g.) for 5 weeks. Livers were excised and microsomes were isolated from livers at 12 h after the last ethanol treatment. Data were expressed as means ± S.E.M. of nine mice. $^{*}P < 0.05$ as compared with the control; $^{**}P < 0.01$ as compared with the control.

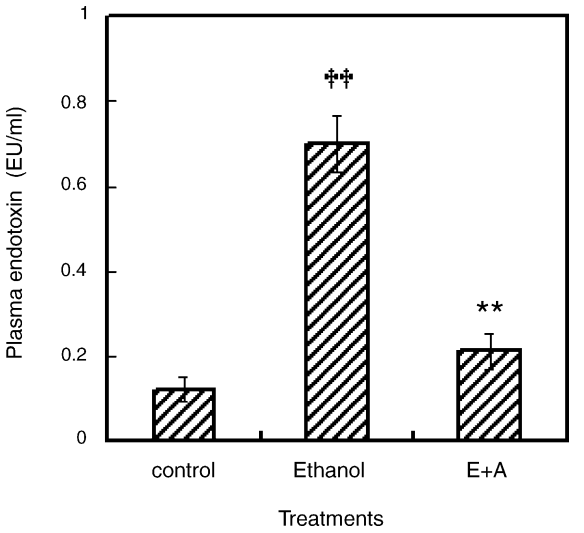

Fig. 4. Effects of antibiotics on ethanol-induced plasma endotoxin elevation. Mice were administered with ethanol (4.0 g/(kg day), i.g.) for 5 weeks in the ethanol group. Mice were administered with ethanol (4.0 g/(kg day), i.g.) and polymyxin B (150 g/(kg day), i.g.) plus neomycin (450 g/(kg day), i.g.) in the ethanol + antibiotics group. Mice were anesthetized at 2 h after last ethanol administration. Heparinized blood samples were collected from the portal vein for endotoxin measurement. Data were expressed as means ± S.E.M. of 12 mice. $^{\dagger\dagger}P < 0.01$ as compared with the control; $^{**}P < 0.01$ as compared with ethanol-treated group.

chronic ethanol exposure markedly decreased *PXR* and *P450 3A11* mRNA level in mouse liver. The effects of ethanol on ERND activity are presented in Fig. 3. Results showed that chronic ethanol exposure significantly inhibited ERND activity in a dose-dependent manner. To exclude the effect of ethanol-induced liver injury on *PXR* and *P450 3A11* expression and ERND activity, we also investigated the effects of ethanol on *P450 2E1* mRNA in mouse liver. In contrast, chronic ethanol exposure significantly increased *P450 2E1* mRNA expression in mouse liver (Fig. 2A and B).

3.3. Role of endotoxin on downregulation of PXR and P450 3A11 mRNA and ERND activity

The effects of chronic ethanol administration on plasma endotoxin levels are presented in Fig. 4. As expected, plasma endotoxin levels significantly increased in ethanol-treated group as compared with the control. In addition, *CD*14 and *TLR*-4 mRNA expression was upregulated during chronic alcohol intoxication (Fig. 5).

To test the possible role of endotoxin in chronically ethanol-induced downregulation of *PXR* and *P450 3A11* expression and ERND activity, mice were pretreated with polymyxin B (150 g/(kg day), po) plus neomycin (450 g/(kg day), po) to prevent growth of intestinal bacteria. As expected, treatment with antibiotics significantly decreased plasma endotoxin concentration (Fig. 4) and hepatic *CD*14 and *TLR*-4 mRNA level (Fig. 5).

The effects of pretreatment with antibiotics on chronically ethanol-induced downregulation of *PXR* and *P450 3A11* gene expressions are presented in Fig. 6A and B. Results showed that the downregulation of *PXR* and *P450 3A11* mRNA was reversed by gastrointestinal sterilization. In addition, pretreatment with antibiotics also attenuated chronically ethanol-induced repression of ERND catalytic activities in mouse liver (Fig. 7).

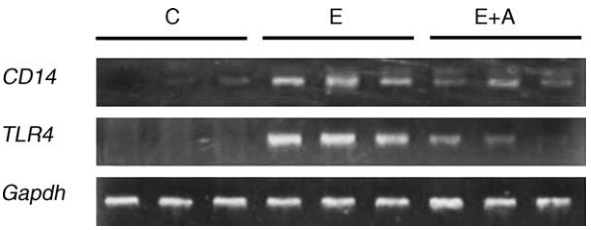

Fig. 5. Effects of antibiotics on ethanol-induced CD14 and TLR-4 mRNA expressions. Mice were administered with ethanol (4.0 g/(kg day), i.g.) for 5 weeks in the ethanol group. Mice were administered with ethanol (4.0 g/(kg day), i.g.) and polymyxin B (150 g/(kg day), i.g.) plus neomycin (450 g/(kg day), i.g.) in the ethanol + antibiotics group. Livers were excised and total RNA was extracted at 2 h after the last ethanol treatment. Hepatic CD14 and TLR-4 mRNA levels were determined using RT-PCR.

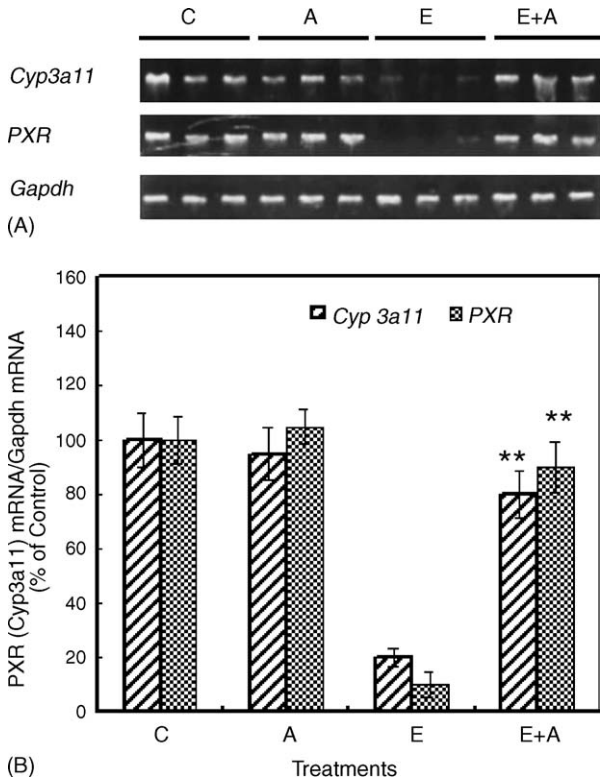

Fig. 6. Effects of antibiotics on ethanol-induced downregulation of *PXR* and *P450 3A11* mRNA expressions. Mice were administered with ethanol (4.0 g/(kg day), i.g.) for 5 weeks in the ethanol-treated group. Mice were administered with ethanol (4.0 g/(kg day), i.g.) and polymyxin B (150 g/(kg day), i.g.) plus neomycin (450 g/(kg day), i.g.) in the ethanol + antibiotics group. (A) Livers were excised and total RNA was extracted at 12 h after the last ethanol treatment. *PXR* and *P450 3A11* mRNA levels in mouse livers were determined using RT-PCR. (B) Quantitative analysis of *PXR* and *P450 3A11* mRNA on 12 individual mouse liver RNA samples at each point was performed. The *PXR* and *P450 3A11* mRNA levels were normalized to *Gapdh* mRNA level in the same samples. The *PXR* and *P450 3A11* mRNA level of the control was assigned as 100%. $^{**}P < 0.01$ as compared with the control; $^{\ddagger\ddagger}P < 0.01$ as compared with the ethanol group.

3.4. Effects of $GdCl_3$ on downregulation of PXR and P450 3A11 mRNA and ERND activity

To test the possible role of Kupffer cells in downregulation of *PXR* and *P450 3A11* expression and ERND activity during chronic alcohol intoxication, mice were administered twice weekly with $GdCl_3$ (10 mg/kg, i.v.), a selective Kupffer cell toxicant, to inactivate Kupffer cells. As further verification of $GdCl_3$ efficacy on inactivation of Kupffer cells, TNF-α, IL-1β and *IL*-6 mRNA expression was determined in liver of ethanol-treated and ethanol/$GdCl_3$-cotreated mice. *TBARS* content was measured in liver of ethanol-treated and ethanol/$GdCl_3$-cotreated mice. As shown in Fig. 8, TNF-α, IL-1β and IL-6 mRNA level significantly increased in livers of chronic ethanol-treated animals. $GdCl_3$ pretreatment blocked ethanol-mediated pro-inflammatory cytokine expression in mouse liver. In addition, $GdCl_3$ pretreatment significantly attenuated chronically ethanol-induced increase in TBARS content in mouse liver (Fig. 9).

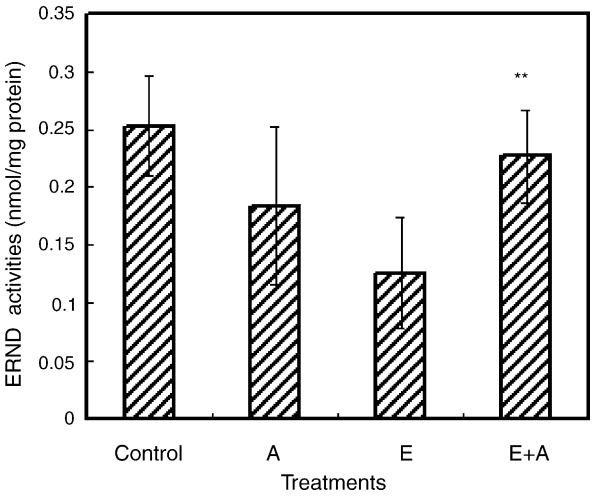

Fig. 7. Effects of antibiotics on ethanol-induced downregulation of ERND catalytic activities in mouse liver. Mice were administered with ethanol (4.0 g/(kg day), i.g.) for 5 weeks in the ethanol group. Mice were administered with ethanol (4.0 g/(kg day), i.g.) and polymyxin B (150 g/(kg day), i.g.) plus neomycin (450 g/(kg day), i.g.) in the ethanol + antibiotics group. Livers were excised and microsomes were isolated from livers at 12 h after the last ethanol treatment. ERND catalytic activities were measured as described in Section 2. Data were expressed as means ± S.E.M. of 12 mice. $^{*}P < 0.05$ as compared with the control; $^{\ddagger}P < 0.05$ as compared with the ethanol-treated group.

The effects of $GdCl_3$ on chronically ethanol-induced downregulation of the *PXR* and *P450 3A11* mRNA level are presented in Fig. 10A and B. Results showed that

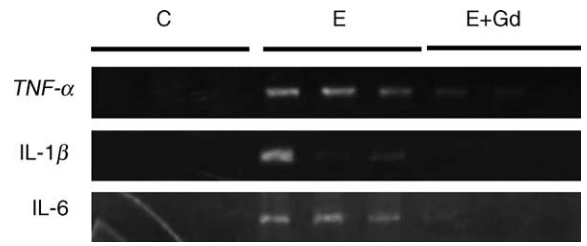

Fig. 8. The effects of $GdCl_3$ on ethanol-induced TNF-α, IL-1β and IL-6 mRNA levels in mouse liver. Mice were administered with ethanol (4.0 g/(kg day), i.g.) for 5 weeks in the ethanol group. In addition to ethanol treatment, mice were intravenously injected 10 mg/kg of $GdCl_3$ twice weekly in the ethanol + $GdCl_3$ group. Livers were excised and total RNA was extracted at 2 h after the last ethanol treatment. Total cellular RNA were extracted from three mouse livers. TNF-α, IL-1β and IL-6 mRNA levels in mouse livers were determined using RT-PCR.

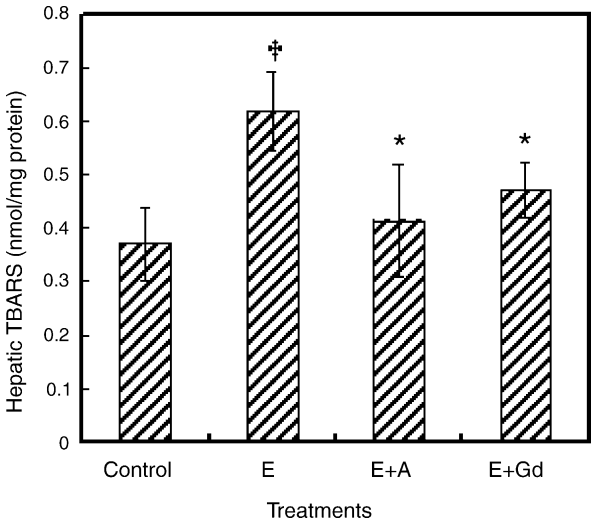

Fig. 9. The effects of antibiotics and $GdCl_3$ on ethanol-induced lipid peroxidation. Mice were administered with ethanol (4.0 g/(kg day), i.g.) for 5 weeks in the ethanol group. Mice were administered with ethanol (4.0 g/(kg day), i.g.) and polymyxin B (150 g/(kg day), i.g.) plus neomycin (450 g/(kg day), i.g.) in the ethanol + antibiotics group. In addition to ethanol treatment, mice were intravenously injected 10 mg/kg of $GdCl_3$ twice weekly in the ethanol + $GdCl_3$ group. Liver was excised and homogenized at 12 h after the last ethanol treatment. TBARS content was measured as described in Section 2. Data were expressed as means ± S.E.M. ($n = 12$). $^{\dagger}P < 0.05$ as compared with control group; $^{*}P < 0.05$ as compared with ethanol group.

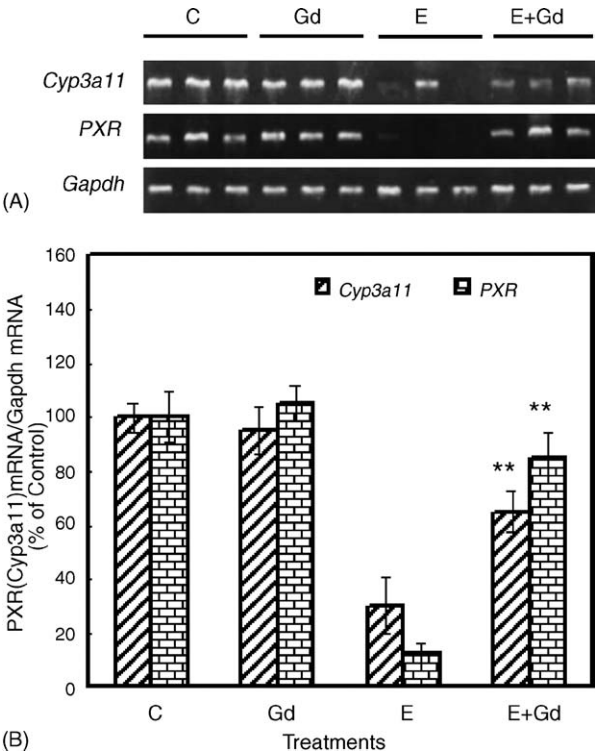

Fig. 10. The effects of $GdCl_3$ on chronically ethanol-induced downregulation of *PXR* and *P450 3A11* mRNA levels. Mice were administered with ethanol (4.0 g/(kg day), i.g.) for 5 weeks in the ethanol group. In addition to ethanol treatment, mice were intravenously injected 10 mg/kg of $GdCl_3$ twice weekly in the ethanol + $GdCl_3$ group. (A) Livers were excised and total RNA was extracted at 12 h after the last ethanol treatment. *PXR* and *P450 3A11* mRNA levels were determined using RT-PCR. (B) Quantitative analysis of *PXR* and *P450 3A11* mRNA on 12 mouse liver RNA samples at each point was performed. The *PXR* and *P450 3A11* mRNA levels were normalized to *Gapdh* mRNA level in the same samples. The *PXR* and *P450 3A11* mRNA level of control was assigned as 100%. $^{**}P < 0.01$ as compared with the control; $^{\ddagger\ddagger}P < 0.01$ as compared with the ethanol-treated group.

$GdCl_3$ pretreatment significantly attenuated chronically ethanol-induced downregulation of *PXR* and *P450 3A11* mRNA levels. $GdCl_3$ also significantly attenuated chronically ethanol-induced decrease in ERND catalytic activities in mouse liver (Fig. 11).

4. Discussion

Cytochrome P450 3A is the most important metabolic enzyme and accounts for 25–35% of the total cytochrome P450 present in adult human or rat liver, which is responsible for the oxidative metabolism of numerous clinically used drugs, including psychotropic, cardiac, analgesic, hormonal, immunosuppressant, antineoplastic and antihistaminic agents (Thummel and Wilkinson, 1998). Pregnane X receptor is a nuclear receptor that regulates *cytochrome P450 3A* (*CYP3A*) gene transcription in a ligand-dependent manner (Kliewer et al., 1998). Previous study showed that short-term ethanol exposure-induced hepatic P450 3A expression and ERND catalytic activity in rats (Roberts et al., 1995). In the present study, we investigated the effects of chronic ethanol exposure on the expressions of *PXR* and *P450 3A11* in mouse liver. Our results indicated that hepatic *PXR* and *P450 3A11* mRNA levels significantly decreased after 5-week ethanol exposure. These results are in agreement with earlier work of Rowlands et al. (2000), in which CYP3A apoprotein level and testosterone 6β-hydroxylase activities decreased in rat fed ethanol diet for 42–55 days. In mouse liver, P450 3A is the major P450 enzyme catalyzing erythromycin *N*-demethylation (Lan et al., 2000). Thus, ERND activity was used as an indicator of CYP3A expression in this study. Consistent with downregulation of *P450 3A11* mRNA, hepatic ERND catalytic activity significantly decreased during chronic alcohol intoxication.

Most animal studies with alcohol use continuous intragastric enteral feeding protocol of Tsukamoto et al.

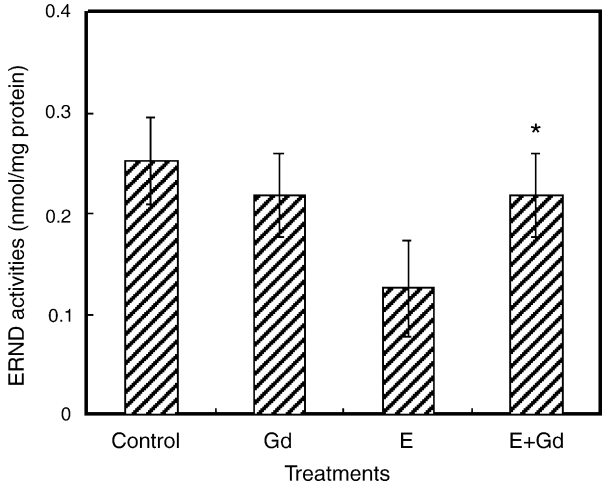

Fig. 11. Effects of $GdCl_3$ on chronically ethanol-induced downregulation of ERND catalytic activities in mouse liver. Mice were administered with ethanol (4.0 g/(kg day), i.g.) for 5 weeks in the ethanol group. In addition to ethanol treatment, mice were intravenously injected 10 mg/kg of $GdCl_3$ twice weekly in the ethanol + $GdCl_3$ group. Livers were excised and microsomes were isolated from livers at 12 h after the last ethanol treatment. ERND catalytic activities were measured as described in Section 2. Data were expressed as means ± S.E.M. of 12 mice. $^{*}P < 0.05$ as compared with the control; $^{‡}P < 0.05$ as compared with the ethanol-treated group.

(1990). However, determination of the dose of ethanol administered according this protocol depends on daily measurement of the ethanol concentration in blood or urine, which is different in the case of the mouse. In the present study, we administered mice by gavage a constant dose of ethanol every day. To ensure that controls obtain a similar calorific intake as ethanol-treated animals, we compared the body weight growth among different groups. Results showed that there were no significant differences in body weight growth among groups. To exclude the effect of ethanol-induced liver injury on *PXR* and *P450 3A11* expression and ERND activity, we also investigated the effects of ethanol on *P450 2E1* mRNA in mouse liver. In contrast to downregulation of *PXR* and *P450 3A11* expression, chronic ethanol exposure significantly increased *P450 2E1* mRNA expression in mouse liver. Our results indicate that the downregulation of *PXR* and *P450 3A11* expression in ethanol-treated mice can be attributed neither to coloric effect nor to ethanol-induced liver injury.

Endotoxemia has long been known to be associated with chronic alcohol exposure. Alcohol consumption deleteriously affects the anatomical and functional integrity of intestinal mucosa and increases intestinal permeability, thus allowing gut-derived endotoxin to escape into the blood (Yin et al., 2001; Zhou et al., 2003; Lambert et al., 2003; Uesugi et al., 2002, 2001; Enomoto et al., 1998). Indeed, the present results showed that serum endotoxin levels significantly increased after a chronic ethanol exposure. Numerous studies indicated that activation of Kupffer cells by bacterial endotoxin is mediated by CD14 and Toll-like receptor (TLR) 4 (Su, 2002). Interestingly, the present results found that a 5-week ethanol treatment also markedly increased TLR4 and CD14 mRNA levels in mouse liver. Therefore, we hypothesize that activation of Kupffer cells by gut-derived endotoxin might contribute to chronically ethanol-induced downregulation of hepatic pregnane X receptor and *P450 3A11* expression in mice.

Several studies have demonstrated that pretreatment with antibiotics to sterilize gut is an effectual remedy for ethanol-induced endotoxemia (Enomoto et al., 2000; Adachi et al., 1995). To investigate the role of gut-derived endotoxin on downregulation of hepatic *PXR* and *P450 3A11* during chronic alcohol intoxication, mice were pretreated daily with polymyxin B (150 g/(kg day)) plus neomycin (450 g/(kg day)) by gavage before ethanol administration. As expected, ethanol-evoked plasma endotoxin elevation and hepatic *CD14* and *TLR4* expressions were significantly attenuated by elimination of Gram-negative bacteria and endotoxin with antibiotics. Correspondingly, pretreatment with antibiotics reversed downregulation of hepatic *PXR* and *P450 3A11* mRNA during chronic alcohol intoxication. In addition, pretreatment with antibiotics also significantly attenuated chronically ethanol-induced decrease in ERND activity. These results suggested that gut-derived endotoxin is involved in downregulation of hepatic *PXR* and *P450 3A11* gene expression during chronic alcohol intoxication.

Our earlier study demonstrated that Kupffer cells contribute to endotoxin-induced downregulation of *PXR* and *P450 3A11* exposure in mouse liver (Xu et al., 2004). In the present study, to determine the role of Kupffer cells on chronic ethanol-induced downregulation of *PXR* and *P450 3A11* gene expression in mouse liver, mice were pretreated with $GdCl_3$ twice a week, to inactivate Kupffer cells. As expected, $GdCl_3$ pretreatment significantly inhibited chronically ethanol-induced pro-inflammatory cytokine (TNF-α, IL-1β and IL-6) mRNA expression and lipid peroxidation in mouse liver. Correspondingly, chronic ethanol-induced downregulation of hepatic *PXR* and *P450 3A11* was significantly attenuated in mice pretreated with $GdCl_3$. Furthermore, pretreatment with $GdCl_3$ also attenuated the repressive effect of chronic ethanol exposure on ERND catalytic activity in mouse liver. These results are consistent with the hypothesized role of Kupffer cells.

As noted above, ethanol-evoked plasma endotoxin elevation and hepatic *CD*14 and *TLR*-4 expressions were almost completely inhibited by elimination of Gram-negative bacteria and endotoxin with antibiotics, whereas pretreatment with antibiotics only partially reversed ethanol-induced downregulation of *PXR* and *P450 3A11*. Indeed, ethanol is a potent inducer of P450 2E1. The present study showed that a 5-week ethanol administration significantly induced *P450 2E1* mRNA expression in mouse liver. Several studies demonstrated that ethanol-evoked ROS are associated with induction of P450 2E1 (Albano et al., 1996; Navasumrit et al., 2000). Thus, our results do not exclude the involvement of P450 2E1.

In summary, the present results allow us to reach the following conclusions. First, chronic ethanol exposure significantly downregulates the expression of *PXR* and *P450 3A11* mRNA and ERND activity in mouse liver; second, activation of Kupffer cells by gut-derived endotoxin mediates, at least in part, chronic ethanol-induced downregulation of *PXR* and *P450 3A11* mRNA and ERND activity in mouse liver.

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