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Regulation of the Activity and Expression of Aryl Hydrocarbon Receptor by Ethanol in Mouse Hepatic Stellate Cells

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Abstract

Background—During the course of alcohol-induced liver damage, hepatic stellate cells are transformed into proliferative, fibrogenic, and contractile myofibroblasts. Aryl hydrocarbon receptor (AhR) is a transcription factor that controls the expression of genes involved in the metabolism of xenobiotics, inflammation, cell proliferation, and death.

Methods—Immortal mouse hepatic stellate cells (MHSCs) were isolated from transgenic mice that expressed a thermolabile SV40 tumor antigen. Quantitative real-time reverse transcription polymerase chain reaction assays, Western blot analysis, promoter activity assays, and chromatin immunoprecipitation analyses were performed for studying the effect of ethanol (EtOH) on AhR expression and transcriptional activity.

Results—Treatment of MHSCs with 50 to 200 mM EtOH for 6 hours induced AhR nuclear translocation, enhanced the promoter activity of cytochrome P450 (CYP) 1A1, increased the amount of AhR bound to the promoter of CYP1A1 and 1B1, and up-regulated the mRNA expression of these AhR target genes in a dose-dependent manner. In contrast, EtOH exposure down-regulated AhR mRNA and protein expression. Similarly, benzo(a)pyrene (BaP) at 10 nM reduced AhR and increased CYP1A1 and 1B1 mRNAs. Pretreatment of MHSCs with 50 mM EtOH for 7 days diminished the capacity of MHSCs to express CYP1A1 and 1B1 induced by a 200 mM EtOH challenge, or by 10 nM BaP. However, the up-regulatory effect of EtOH on solute carrier family 16, member 6 (SLC16a6) was unaffected by EtOH pretreatment. Similar to EtOH, dimethyl sulfoxide (DMSO) at concentrations of 50 to 100 mM down-regulated AhR and up-regulated CYP1A1 mRNA expression in a dose-dependent manner.

Conclusions—These data, for the first time, demonstrate that EtOH activates MHSC AhR and down-regulates its expression. Chronic EtOH pretreatment lowers the availability of AhR, and specifically diminishes the inducibility of CYP genes. The effect on AhR appears to not be an EtOH-specific response, as DMSO alone (and possibly other organic solvents) was also able to activate AhR.

Keywords

Ethanol; Mouse Hepatic Stellate Cells; Aryl Hydrocarbon Receptor; Cytochrome P450 Protein

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Activation of hepatic stellate cells has been suggested as the central event in alcoholic fibrosis and cirrhosis (Ahrendt et al., 2000). In the normal liver, stellate cells remain in a quiescent state. When liver is damaged by alcohol and/or other hepatotoxic agents, the quiescent stellate cells are transformed into proliferative, fibrogenic, and contractile myofibroblasts (Maher, 1997). These dramatic phenotypic changes involve global reprogramming of gene expression. Currently, a number of genes, whose encoded proteins control cell proliferation and differentiation, have been suggested to either promote or inhibit hepatic stellate cell activation and liver fibrosis induced by various stimuli, such as viral infection and heavy long-term alcohol consumption (Mann and Marra, 2010).

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor and was initially recognized as a regulator of the expression of xenobiotic-metabolizing enzymes (Fujii-Kuriyama and Mimura, 2005). Recent studies suggest that AhR also regulates the expression of genes involved in cell death, fibrogenesis, proliferation, and differentiation. For example, it has been shown that treatment of animals with exogenous AhR ligands induces liver hyperplasia (Nebert and Karp, 2008), while AhR-null knockout mice are more susceptible to spontaneous apoptosis and fibrosis in mouse liver (Schmidt et al., 1996). However, the mechanisms underlying the pathological effects induced by activation and deletion of AhR have not been fully defined.

There are indications that exposure to ethanol (EtOH) might affect the expression and activity of AhR and its target genes. For example, EtOH stimulates the production of arachidonic acid metabolites in hepatocytes (Enomoto et al., 2000; Nanji et al., 1993) and tryptophan metabolites of the kynurenine pathway (Badawy et al., 2009). These compounds are able to bind AhR and thus induce AhR target gene expression. The molecular processes by which AhR regulates gene expression have been clearly described (Fujii-Kuriyama and Mimura, 2005). In the absence of ligands, AhR exists predominantly in the cytosolic compartment in association with a chaperone complex (Hsp90/XAP2/p23). Upon binding to ligands, AhR translocates to the nucleus and forms a heterodimer with AhR nuclear translocator already present in the nucleus (Hankinson, 1995). This heterodimer binds to consensus regulatory sequences (xenobiotic response elements [XREs]) located upstream in the promoter of the target genes. Although hundreds of genes throughout the genome contain XREs in their promoter regions, the regulatory effect of AhR on these genes varies with ligands, cell types, and the cell's oxidative status. For example, different AhR ligands have been reported to selectively induce the expression of different genes, and therefore either provoke (Marlowe and Puga, 2005; Park et al., 2005) or suppress (Marlowe et al., 2004; Paajarvi et al., 2005; Pang et al., 2008) cell death and growth. More recently, we reported that the gene expression profile induced by the AhR ligand benzo(a)pyrene (BaP) varied greatly in cells with or without overexpression of the hydrogen peroxide scavenger catalase (Wang et al., 2009).

In this article, we studied the impact of EtOH on the expression and activity of AhR in mouse hepatic stellate cells (MHSCs). Our data demonstrate that exposure of MHSCs to EtOH induces AhR translocation to the nucleus and up-regulates its target genes cytochrome P450 (CYP) 1A1 and 1B1. In addition, we observed that EtOH down-regulated AhR expression. Chronic exposure to 50 mM EtOH kept AhR expression at a low level, but diminished the inducibility of AhR target gene CYP1A1 and 1B1 in response to the AhR ligand BaP, as well as in response to an acute higher dose EtOH challenge. These findings contribute to our understanding of the mechanism underlying the synergistic toxicity induced by EtOH and polycyclic aromatic hydrocarbons (PAHs).

MATERIALS AND METHODS

Materials

Antibodies against AhR (sc-8089), albumin (sc-50536), EMR1 (sc-25830), CD31 (sc-1506), β-actin (sc-4778), heterogeneous nuclear ribonucleoprotein (hnRNPU) (sc-25374), nonspecific goat IgG (sc-2028), salmon sperm DNA/protein A-agarose, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody against desmin (ab-5200) was purchased from Abcam Inc. (Cambridge, MA). Lipofectamine 2000 kit, platinum Taq DNA polymerase, and tissue culture reagents, unless otherwise indicated, were obtained from Invitrogen (Carlsbad, CA). Luciferase assay kits and pGL2 luciferase vector were purchased from Promega (Madison, WI). M-PER mammalian protein extraction reagent and BCA protein assay kits were purchased from Pierce (Rockford, IL). QIAquick PCR purification kit, RNeasy Plus Mini kits, and primers for polymerase chain reaction (PCR) were purchased from Qiagen (Valencia, CA). EtOH (E7023), dimethyl sulfoxide (DMSO) (D-5879), and BaP (B1760) were purchased from Sigma-Aldrich (St. Louis, MO). For the treatment of cells, 1, 2.9, 5.8, or 11.6 μl of EtOH was added into 1 ml culture medium to make final concentrations of 17, 50, 100, and 200 mM, respectively; 0.5, 1, 3.5, or 7 μl of DMSO was added into 1 ml culture medium to make final concentrations of 7, 14, 50, and 100 mM, respectively. For the treatment of cells with BaP, a 20 μM BaP stock solution was prepared using a 100× diluted DMSO solution (140 mM); 1 μl of this stock solution was added into 2 ml of culture medium to make final concentrations of BaP and DMSO of 10 nM and 70 μM, respectively.

Isolating and culturing MHSCs

H-2K^b-tsA58 transgenic mice were generated by Jat and colleagues (1991). These mice express a thermolabile SV40 tumor antigen (tsA58) driven by a mouse major histocompatibility complex *H-2K*^{b} promoter (Jat et al., 1991). MHSCs were isolated from *H-2K*^{b}-tsA58 mice by in situ collagenase perfusion and Nycodenz gradient centrifugation (Kawada et al., 1996). Briefly, mice at 4 months of age were anesthetized with ketamine hydrochloride (100 mg/ml) and xylazine hydrochloride (20 mg/ml) at 0.8 μl/g body weight. Mouse livers were perfused through the portal vein with a perfusion solution containing 137 mM NaCl, 5.4 mM KCl, 0.6 mM NaH_2PO_4, 0.8 mM Na_2HPO_4, 10 mM HEPES, 4.2 mM $NaHCO_3$, 5.5 mM glucose, 3.8 mM $CaCl_2$, and 180 mg/L collagenase (pH 7.4) at 37 °C at a flow rate of 7 ml/min for 10 min. The liver was then excised and incubated in the perfusion solution supplemented with 400 mg/l pronase E and 20 mg/l DNase I at 37°C for 20 min with gentle stirring. The mixture was then filtered through a mesh (pore size: 150 mm) and centrifuged at 450 × *g* for 7 min. The supernatant enriched with stellate, Kupffer, and endothelial cells was overlaid with a triple-layered density cushion (Gey's balanced salt solution/8.2% Nycodenz/17% Nycodenz) and centrifugated at 1,400 × *g* for 20 min (Kawada et al., 1996). Gey's balanced salt solution contains 120 mM NaCl, 5 mM KCl, 0.84 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 1.85 mM $MgCl_2$, 1.53 mM $CaCl_2$, 27 mM $NaHCO_3$, and 5.5 mM glucose. Stellate cells in the upper white layer were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) and cultured in 100-mm dishes at a density of 1×10^3 cells per dish. Because of the *H-2K*^{b}-tsA58 transgene, cells obtained from these mice continuously divide at 34°C (Jat et al., 1991). After a 5-day culture period, cell colonies were picked up and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 34°C in a humidified atmosphere of air and 5% CO_2. The mRNAs and proteins of desmin, albumin, platelet/endothelial cell adhesion molecule (CD31), and epidermal growth factor receptor–like module containing mucin-like, hormone receptor-like 1 (EMR1) were measured with quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot analyses. Desmin (Riccalton-Banks

et al., 2003), albumin (Riccalton-Banks et al., 2003), CD31 (Li et al., 2010), and EMR1 (Taylor et al., 2005) are markers for stellate cells, hepatocytes, endothelial cells, and macrophages (Kupffer cells), respectively. Cells that expressed desmin were passaged at 34°C and transferred to 37°C before EtOH treatment.

Quantitative Real-Time RT-PCR Assay

In acute studies, MHSCs grown to confluence in 6-well plates were made quiescent in serum-free DMEM for 12 hours, and then treated with EtOH, BaP, or DMSO at concentrations indicated in all Figure legends for 6 hours. In chronic studies, MHSCs were incubated with 50 mM EtOH or culture medium alone for 7 days. For long-term EtOH treatment, loss of EtOH during the incubation period was a general concern. In our preliminary studies, we monitored the disappearance of EtOH under the culture conditions by measuring the EtOH concentrations in the culture medium using a Shimadazu 14A gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD) connected with a Agilent J&W GC column (Agilent Technologies, Santa Clara, CA). We observed that incubation of cell-free medium containing 50 mM EtOH at 37°C for 6 and 24 hours reduced the concentration of EtOH about 10 and 36%, respectively. In the presence of cells, the concentration of EtOH in the medium was reduced about 14 and 50% within 6 and 24 hours, respectively. In chronic studies, therefore, the medium with or without EtOH was changed every 24 hours. After 7 days, the medium was replaced with fresh medium supplemented with 200 mM EtOH or 10 nM BaP, as indicated in the legends of Figs 5, 6 and 7. Cells were harvested after 6 hours of culture. Total RNA was extracted using RNeasy Plus Mini kits (Qiagen) and subjected to quantitative real-time RT-PCR using the iCycler system (Bio-Rad, Hercules, CA) as previously described (Lin et al., 2011). These specific primers were used for amplification: AhR forward: 5′-ACTTCACACCTATTGGTTGT-3′, reverse: 5′-ATGCCACTTTCTCCAGTCTT-3′; CYP1A1 forward: 5′-CTAGACACAGTGATTGGCAG-3′, reverse 5′-GATGGTGAAGGGGACGAAGG-3′; CYP1B1 forward: 5′-CAACCGCAACTTCAGCAACT-3′, reverse 5′-GAAAGGGTGTCCTGGCTGGC-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward: 5′-GAGCCAAAAGGGTCATCATC-3′, reverse: 5′-TAAGCAGTTGGTGGTGCAGG-3′. The expression levels of target mRNAs were normalized to GAPDH mRNA.

Western Blot Analysis

Quiescent MHSCs in serum-free DMEM were treated with EtOH at the concentrations and times noted in the text. For whole-cell protein extraction, cells were lysed in M-PER (Thermo Scientific, Rockford, IL). For nuclear extracts, nuclei were isolated using NE-PER (Nuclear and Cytoplasmic Extraction Reagent) according to the manufacturer's protocol (Thermo Scientific). Samples containing either 5 μg (for the detection of β-actin and nuclear marker hnRNPU) or 40 μg of protein (for the detection of other proteins of interest) were resolved on 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels. Proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membrane was blocked with 3% fat-free milk in TBS-T (2.5 mM Tris, 15 mM NaCl, 0.1% Tween 20; pH 7.6), and then subsequently incubated with primary and secondary antibodies, as previously described (Lin et al., 2011). Immunoreactive bands were visualized using ECL plus chemiluminescence reagent (GE Healthcare Healthcare–Amersham, Piscataway, NJ) and analyzed with a GS-700 Imaging Densitometer (Bio-Rad).

Recombinant Plasmid Construction and Cell Transfection

Computer analysis of the CYP1A1 promoter showed 6 XREs in a 1-kb length fragment upstream from the transcription start site (TSS) of the mouse CYP1A1 gene. Two XREs located 987 and 57 nucleotides, respectively, upstream of the TSS are required for

constitutive and ligand-induced CYP1A1 expression (Jones et al., 1990; Neuhold et al., 1989). In this study, a 1,353-bp CYP1A1 promoter fragment was amplified by PCR from wild-type C57BL/6J mouse genomic DNA using Platinum *Taq* DNA polymerase (Invitrogen). The forward primer (5′-ATCCGCTCGAGTGGACACACGCTGTTCAGGC-3′) starts at position 1,264-bp upstream of the CYP1A1 TSS. The reverse primer (5′-ATCCCAAGCTTAGCACTCACCTTGGGCTGTA-3′) starts at position 89-bp downstream of the CYP1A1 TSS. The PCR products were cloned into the *Xho*I/*Hind*III sites of the pGL2 luciferase vector (Promega).

The recombinant plasmid constructs were transfected into MHSCs using a Lipofectamine 2000 kit (Invitrogen) according to the manufacturer's protocol. Briefly, MHSCs grown in 6-well plates to approximately 60 to 70% confluence were incubated with 2 ml/well serum-free DMEM containing 10 μl of Lipofectamine 2000, and 4 μg of a pGL2 plasmid encoding the CYP1A1 promoter fragment. At 6 hours posttransfection, the cells were replenished with fresh medium containing 10% FBS and then cultured for an additional 24 hours. The cells were then grown in serum-free DMEM for 4 hours, followed by incubation for 6 hours with 100 or 200 mM EtOH or culture medium alone prior to reporter gene analysis.

Luciferase Assays

For the luciferase assays, transfected cells were lysed with 100 μl of reporter lysis buffer provided by a luciferase assay kit (Promega). Lysate (10 μl) was incubated in a 96-well plate at room temperature for 2 min with 100 μl of luciferase assay reagent (Promega). Luminescence was measured using the BL10000 LumiCount (Packard BioScience, Meriden, CT). The protein level in the lysate was determined using a BCA protein assay kit (Pierce). The luciferase activity was expressed as the luminescence intensity relative to the protein level.

Chromatin Immunoprecipitation

Quiescent MHSCs were treated with culture medium alone, 10 nM BaP, or 100 or 200 mM EtOH for 6 hours. AhR binding to the CYP1A1 and 1B1 promoter region was determined by chromatin immunoprecipitation (ChIP) as previously described (Tang et al., 2010). Briefly, cells were cross-linked with 1% formaldehyde at room temperature and then lysed in 500 μl of cell lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% Nonidet P-40, 1 mM PMSF, and protease inhibitor cocktail, pH 8.0). Nuclei were isolated and homogenized in 300 μl of nuclear lysis buffer (50 mM Tris–HCl, 1% SDS, 10 mM EDTA, and protease inhibitor cocktail, pH 8.1). The resulting nuclear lysate was sonicated until the cross-linked chromatin was sheared to an average length of 0.3~1.0 kb. Supernatant (5 μl) was used as an input control. The remaining lysate was diluted 10-fold with ChIP dilution buffer (16.7 mM Tris–HCl, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and protease inhibitor cocktail, pH 8.1) and precleaned with salmon sperm DNA/protein A-agarose (Santa Cruz). The precleaned samples were incubated with AhR antibody or nonspecific IgG as a control, followed by salmon sperm DNA/protein A-agarose. Bound protein–DNA complexes were eluted with a solution containing 0.1 M $NaHCO_3$ and 1% SDS. Following reversion of the protein–DNA cross-links, DNA fragments in the eluate and input controls were purified using a QIAquick PCR purification kit (Qiagen), and then subjected to quantitative real-time PCR using an iCycler system (Bio-Rad). A 172- and 273-bp DNA fragments, respectively, containing the CYP1A1 and 1B1 XRE sequences were amplified. The forward primer (5′-GTATGGCTTCTTGCCTATCT-3′) for CYP1A1 starts at position 156-bp upstream of its TSS, while the reverse primer (5′-GCTCCAAGAACTACCACCTT-3′) starts at position 16-bp downstream of its TSS. The forward primer (5′-GGGAAACAAGCTGAGTGAGT-3′) for CYP1B1 starts at position 11101-bp upstream of

its TSS, while the reverse primer (5′-AAGTTTCTGCTGTCGCCTCC-3′) starts at position 829-bp upstream of the CYP1B1 TSS. The amount of AhR bound to the CYP1A1 and 1B1 promoter region was expressed as a ratio of the PCR products amplified from the immunoprecipitated samples to the input controls, which was calculated with the following formula: (PCR product from the AhR antibody immunoprecipitant – PCR product from the nonspecific IgG immunoprecipitant) / PCR product amplified from the input controls.

Statistical Analysis

Data are reported as the mean ± the standard error of the mean. Differences between MHSCs with or without EtOH and/or BaP treatments were analyzed by 1-way or multiple factor analyses of variance and the Student's unpaired *t*-test. Differences were considered statistically significant when p was <0.05. Statistix software (Statistix, Tallahassee, FL) was used for statistical analyses.

RESULTS

Identification of MHSCs

Liver is composed of multiple types of cells, including hepatocytes, stellate cells, endothelial cells, and Kupffer cells. We isolated mouse liver cells using density gradient centrifuge followed by colony screening. The typical images of PCR product electrophoresis and Western blots in Fig. 1 show that the different cell colonies isolated from the mouse liver selectively expressed the cell-specific markers albumin, desmin, EMR1, or CD31. Desmin is a marker gene of stellate cells, while albumin, EMR1, and CD31 are marker genes for hepatocytes, Kupffer cells (macrophages), and endothelial cells, respectively (Li et al., 2010; Riccalton-Banks et al., 2003; Taylor et al., 2005). Cells that expressed desmin (MHSCs) were used in this report.

EtOH-Induced AhR Nuclear Translocation and Reduced its Expression

Binding of ligands to AhR has been shown to induce AhR translocation to the nucleus (Hankinson, 1995; Wang et al., 2009) and reduce its expression (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Wang et al., 2009). Thus, this study examined the effect of EtOH on AhR expression in whole-cell lysates as well as nuclear fractions of MHSCs. Data in Fig. 2 show dose- and time-related changes in AhR expression after cells were treated with EtOH. Specifically, addition of 50, 100, and 200 mM EtOH into the culture medium for 6 hours resulted in about a 27, 50, and 81% reduction in whole-cell lysate AhR mRNA, respectively (Fig. 2*A*). Because blood EtOH concentration could reach 100 mM (Lindblad and Olsson, 1976) or higher (O'Neill et al., 1984) in alcoholic subjects (Lindblad and Olsson, 1976), we performed subsequent experiments using 100 mM EtOH. Based on a basic formula suggested by the National Highway Traffic Safety Administration for the general publics to estimate blood alcohol concentrations (Hustad and Carey, 2005), consumption of ~6 and 12 drinks within 1 hour would elevate blood alcohol concentration to 50 and 100 mM, respectively, for an individual with body weight of 100 lb, in which 1 drink is defined as 37.5 ml of 40%distilled spirits or equivalent.

Incubation of MHSCs with 100 mM EtOH for 1, 3, and 6 hours reduced the AhR protein in the whole-cell lysates by about 9, 45, and 68%, respectively, as compared to untreated control cells (Fig. 2*B*,*C*). In contrast, the same dose and time periods of EtOH treatment elevated AhR protein levels in MHSC nuclei (Fig. 2*D*). For example, treatment of MHSCs with 100 mM EtOH for 1, 3, and 6 hours increased nuclear AhR protein by about 23, 67, and 111%, respectively. These data clearly indicate that exposure of MHSCs to EtOH-induced AhR translocation to nuclei and reduced AhR whole-cell expression at the mRNA and protein level.

Similar to EtOH, the typical AhR ligand BaP reduced the levels of AhR protein (Fig. 2*E,F*) and mRNA (Fig. 6*A*). Although the mechanism by which ligands down-regulate AhR mRNA expression remains unknown, ligand-initiated degradation is widely accepted as an important mechanism underlying the regulation of AhR (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000). It is believed that nuclear AhR, after execution of its transcriptional activity, is exported back to the cytosol and undergoes proteasomedependent degradation (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000).

EtOH Induces CYP1A1 and 1B1 Expression

Although AhR binds to XREs in hundreds of genes throughout the genome, CYP1 family genes are among the most highly induced and are central to the oxidative metabolism of many AhR ligands (Nebert and Karp, 2008). Thus, this study examined the effect of EtOH on the expression of MHSC CYP1A1 and 1B1. Data in Fig. 3 show that the mRNA level of CYP1B1 was about 86-fold higher than that of CYP1A1 in control MHSCs. This result is consistent with our previous observation that the basal expression level of CYP1B1 is higher than that of CYP1A1 in mouse aortic endothelial cells (Wang et al., 2009). However, EtOH exposure induced both CYP1A1 and 1B1 expression in a dose-dependent manner. Specifically, EtOH at 17 mM, a concentration commonly used as a solvent in cell culture studies, did not significantly alter the expression of AhR and CYP1A1 and 1B1 mRNAs (Fig. 3). In contrast, incubation of MHSCs with 100 and 200 mM EtOH for 6 hours increased CYP1A1 mRNA level by 2.1- and 3.9-fold, and increased CYP1B1 mRNA level by 3.7- and 4.7-fold (Fig. 3).

EtOH Enhances AhR Binding to the CYP1A1 and 1B1 Promoter Region and Increases CYP1A1 Promoter Activity

Having demonstrated the up-regulatory role of EtOH on AhR nuclear translocation and CYP gene expression, we then examined the binding of AhR to the promoter region of CYP1A1 and 1B1 using ChIP analysis. The PCR primers used for CYP1A1 amplified a 172-bp fragment containing 1 XRE spanning nucleotides −57 to −61 upstream of the TSS of CYP1A1. The primers for CYP1B1 amplified a 273-bp fragment containing 4 XREs, respectively, spanning nucleotides −854 to −858, −873 to −877, −960 to −964, and −1,015 to −1,019 bp, upstream of the TSS of CYP1B1. Under control conditions, the DNA-binding activity of AhR was barely detectable. After cells were treated with EtOH, the binding of AhR to CYP1A1 and 1B1 promoter region was significantly increased. Specifically, the amount of AhR bound to the CYP1A1 promoter region was about 1.5- and 3.3-fold higher in MHSCs, respectively, treated with 100 and 200 mM EtOH than in untreated control cells (Fig. 4*A*). Similarly, exposure of MHSCs to 100 and 200 mM EtOH, respectively, resulted in about 2.2- and 3.3-fold increase in AhR binding to the CYP1B1 promoter region, as compared to the amount in untreated control cells (Fig. 4*A*). The data in Fig. 4*A* also showed that treatment of MHSCs with 10 nM BaP increased the level of AhR bound to the CYP1A1 and 1B1 promoter regions by ~5-fold.

In this report, we also examined the transcriptional activity of the CYP1A1 promoter using a luciferase assay. The CYP1A1 promoter region studied in this report included 6 XREs; 2 of them have been shown to be required for efficient basal and induced CYP1A1 expression (Jones et al., 1990; Neuhold et al., 1989). Incubation of MHSCs with 100 and 200 mM EtOH increased the CYP1A1 promoter activity by about 29 and 52% (Fig. 4*B*). These data suggest that EtOH induced the binding of AhR to its target genes and enhanced their promoter activity.

Chronic EtOH Exposure Persistently Suppresses AhR Expression and Diminishes the Inducibility of CYP1A1 and 1B1

To study the effect of chronic EtOH exposure on AhR, CYP1A1, and CYP1B1 expressions, MHSCs were preincubated with or without 50 mM EtOH for 1 week. The medium was then changed to one with or without 200 mM freshly prepared EtOH and the incubations continued for an additional 6 hours. Chronic 50 mM EtOH exposure kept AhR at very low expression levels seen earlier when cells were treated with the same concentration of EtOH for only 6 hours (Fig. 2*A*–*C* vs. Fig. 5*A*–*C*). For example, the AhR mRNA and protein levels in the MHSCs incubated with 50 mM EtOH for a week were about 23 and 39% lower, respectively, than those in control cells lacking EtOH. Addition of 200 mM EtOH to the cultures for an additional 6 hours resulted in a further reduction in AhR mRNA and protein expression (Fig. 5*A*–*C*).

The data in Fig. 5*D,E* also indicate that chronic EtOH exposure reduced the inducibility of CYP1A1 and 1B1 in MHSCs following the exposure to a challenge dose of 200 mM alcohol. For example, after 1-week preincubation of MHSCs with 50 mM EtOH, treatment of MHSCs with 200 mM EtOH for 6 hours elevated CYP1A1 and 1B1 mRNA levels only by 19 and 21%, respectively, as compared to the cells without EtOH treatment. In contrast, the same dose of EtOH increased CYP1A1 and 1B1 mRNA levels by about 3.9- and 4.7-fold, respectively, in cells without EtOH preincubation (Fig. 3*A,B*).

The data in Fig. 6 illustrate that chronic EtOH exposure significantly decreased AhR mRNA and diminished the inducibility of CYP1A1 and 1B1 in response to BaP. For example, when BaP was added for 6 hours to control cells that had not been exposed to 50 mM EtOH, there was a robust increase in CYP1A1 and 1B1 mRNAs (about 12- and 3.9-fold, respectively). In contrast, the same dose of BaP resulted in only about 6.5- and 2.7-fold increase in cells that had been preincubated for 7 days with EtOH (Fig. 6*B,C*).

Chronic EtOH Exposure Does not Affect the Expression of Solute Carrier Family 16, Member 6

A microarray analysis from our laboratory showed that EtOH exposure resulted in a robust increase in solute carrier family 16, member 6 (SLC16a6) expression in MHSCs. To determine whether chronic EtOH exposure also reduces the inducibility of other genes besides the CYP genes, we examined the expression of SLC16a6. The data in Fig. 7 show that acute 6-hour EtOH exposure induced SLC16a6 expression in a dose-dependent manner. Incubation of MHSCs with 100 and 200 mM EtOH for 6 hours elevated the SLC16a6 level by about 2.3- and 6.7-fold, respectively. Chronic 1-week EtOH exposure, however, did not reduce the subsequent inducibility of SLC16a6. Specifically, 1 week of 50 mM EtOH treatment elevated the SLC16a6 level by about 3-fold. Treatment of EtOH pretreated MHSCs with a 200 mM EtOH challenge for 6 hours elevated the SLC16a6 level by about 9.7-fold, which is significantly higher than that induced by 200 mM EtOH in cells without EtOH preincubation. These observations suggest that the reduction in the inducibility of CYP genes is a gene-specific event, and not a common effect of EtOH on the genome.

DMSO Altered AhR and CYP1A1 Expression

Besides being used for making alcoholic beverages, EtOH is commonly used as a solvent to dissolve water-insoluble reagents in laboratories. To determine whether other organic solvents besides EtOH also regulate the expression of AhR and its target genes, we study the effect of DMSO at low (7 and 14 mM) and high (50 and 100) concentrations on the levels of AhR and CYP1A1 mRNAs. The DMSO concentrations in cell culture medium are usually lower than 14 mM (0.1%). As the data in Fig. 8 showed, DMSO at 7 and 14 mM did not significantly alter the expression of AhR and CYP1A1. In contrast, the addition of 50 and

100 mM DMSO into the culture medium for 6 hours resulted in about 18 and 45% reduction in AhR mRNA, respectively (Fig. 8). The same concentrations of DMSO induced a 2.2- and 3.7-fold increase in the CYP1A1 mRNA level (Fig. 8). These observations suggest that the inductive effect on AhR is not a specific action of EtOH, that is, DMSO and possibly other organic solvents at high concentrations are also able to activate AhR, and therefore alter the expression of AhR and its target genes. Low concentrations of DMSO, thus, should be used for dissolving water-insoluble compounds in cell culture studies.

DISCUSSION

The present study demonstrates that exposure of MHSCs to EtOH activates AhR, induces AhR translocation to the nucleus and binding to the promoter region of target genes, and up-regulates mRNA expression of these genes. Together with the ligand-dependent characteristics of AhR, these findings suggest that EtOH stimulates MHSCs to generate chemical compounds that can function as AhR ligand(s). Various classes of endogenous AhR ligands have been identified, including (i) tryptophan metabolites of the kynurenine pathway, (ii) tetrapyrroles such as bilirubin and biliverdin, and (iii) arachidonic acid metabolites such as several prostaglandins, thromboxanes, leukotrienes, and lipoxins (Nebert and Karp, 2008). EtOH has been shown to increase the generation of some of these compounds. For example, it has been reported that alcohol consumption increases the generation of thromboxane B2, leukotriene B4 (Nanji et al., 1993), and prostaglandin E2 (Enomoto et al., 2000). In addition, it has been suggested that alcohol increases tryptophan 2,3-dioxygenase activity, the first and rate-limiting enzyme of the kynurenine pathway of tryptophan degradation (thus elevating the generation of kynurenine) (Badawy et al., 2009). Further studies are required to identify those EtOH-induced compounds that are able to activate AhR in MHSCs.

Another important finding is that EtOH exposure induced a time-related decline in AhR mRNA and protein level in MHSCs. Ligand-induced down-regulation of AhR protein has been observed in a variety of cultured cell lines, including Hepa-1 (mouse hepatocytes), NIH-3T3 (mouse embryo fibroblasts), A7 (rat smooth muscle cells), C2C12 (mouse skeletal muscle myoblasts) (Pollenz, 1996), and mouse endothelial cells (Wang et al., 2009), as well as in rat liver, spleen, thymus, and lung (Pollenz et al., 1998). Ligand-initiated degradation is widely accepted as an important mechanism underlying the regulation of AhR (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000). It is believed that nuclear AhR, after execution of its transcriptional activity, is exported back to the cytosol and undergoes proteasome-dependent degradation (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000). In addition to a decreased AhR protein level, we observed a significant reduction in AhR mRNA levels in MHSCs following EtOH exposure. These findings indicate that EtOH-induced down-regulation of AhR can occur at the transcriptional level, in addition to the proteasome-dependent protein degradation. Ligand-induced down-regulation of AhR is important for avoiding overexpression of its target genes. However, persistent underexpression of AhR would reduce its availability, and therefore diminish the inducibility of its target genes in response to ligands. Indeed, data from the present report demonstrated that long-term EtOH treatment diminished the capability of MHSCs to express AhR target genes CYP1A1 and 1B1 in response to EtOH and the toxicant BaP.

Even though the physiological significance of an EtOH-induced reduction in AhR expression has not been studied, existing data suggest that underexpression of AhR might facilitate liver fibrosis. For example, the livers of AhR-null mice show areas of fibrosis and scattered foci of apoptosis in the parenchyma (Schmidt et al., 1996). The hepatic level of transforming growth factor-β (TGF-β) was found to be elevated in AhR-null mice, particularly in those areas coincident with fibrosis. The TGF-β gene family is known to

promote cell growth and differentiation (Breitkopf et al., 2005) and reports indicate that EtOH elevates TGF-β expression in the liver (Maher, 1997). Further studies are required to determine whether persistent inhibition of AhR expression is a mechanism by which chronic EtOH consumption induces liver fibrosis and cirrhosis.

We do believe, however, that EtOH-induced underexpression of AhR affects the capability of cells to detoxify xenobiotics. Thus far, it has been reported that knockout of AhR enhances (Shimizu et al., 2000), inhibits (Sagredo et al., 2006), or does not alter (Kondraganti et al., 2003) the detoxification of PAH compounds, such as BaP. Although the mechanism underlying these conflicting findings has not been defined, it is known that the toxicity of these compounds is determined mainly by the balance between phase I and phase II xenobiotic-metabolizing enzymes. Certain imbalances in the expression of these enzymes could result in a toxic effect (Korashy and El-Kadi, 2006). Many of these xenobiotic-metabolizing enzymes are regulated directly or indirectly by AhR (Nebert, 1994; Nebert et al., 1993). It has not been studied whether EtOH exposure alters the balance of phase I and phase II enzymes. However, EtOH has been shown to aggravate the toxicity induced by exogenous AhR ligands. For example, it has been reported that the combined treatment of cells with EtOH and BaP induces more BaP-DNA adducts than BaP treatment alone (Barnes et al., 2000) and that alcohol consumption and cigarette smoking show synergistic effects on the induction of p53 mutations (Ahrendt et al., 2000), cancer development, and nonmalignant chronic respiratory illness (Klatsky et al., 1981). Cigarette smoke contains PAH compounds, such as BaP (Zhang et al., 1998). On the other hand, smoking also aggravates alcohol-induced toxicity. Alcoholics who also smoke cigarettes experience higher risk of cirrhosis than those who do not smoke (Klatsky and Armstrong, 1992). Currently, it remains unknown whether underexpression of AhR is a mechanism responsible for the synergistic toxicity induced by EtOH and PAHs.

In summary, this report is the first that demonstrates the regulatory role of EtOH on AhR expression in MHSCs. We show that acute EtOH exposure activated AhR and induced its target gene expression. Long-term EtOH exposure sustained AhR expression at a low level and diminished the inducibility of AhR target genes in response to ligands. In a separate study, we observed that EtOH is also able to down-regulate AhR, and up-regulate CYP gene expression in hepatocytes and endothelial cells obtained from the mouse liver (data not shown). These findings provide the ground-work for further study of the role of AhR in the hepatic toxicity induced by EtOH and PAH compounds such as BaP.

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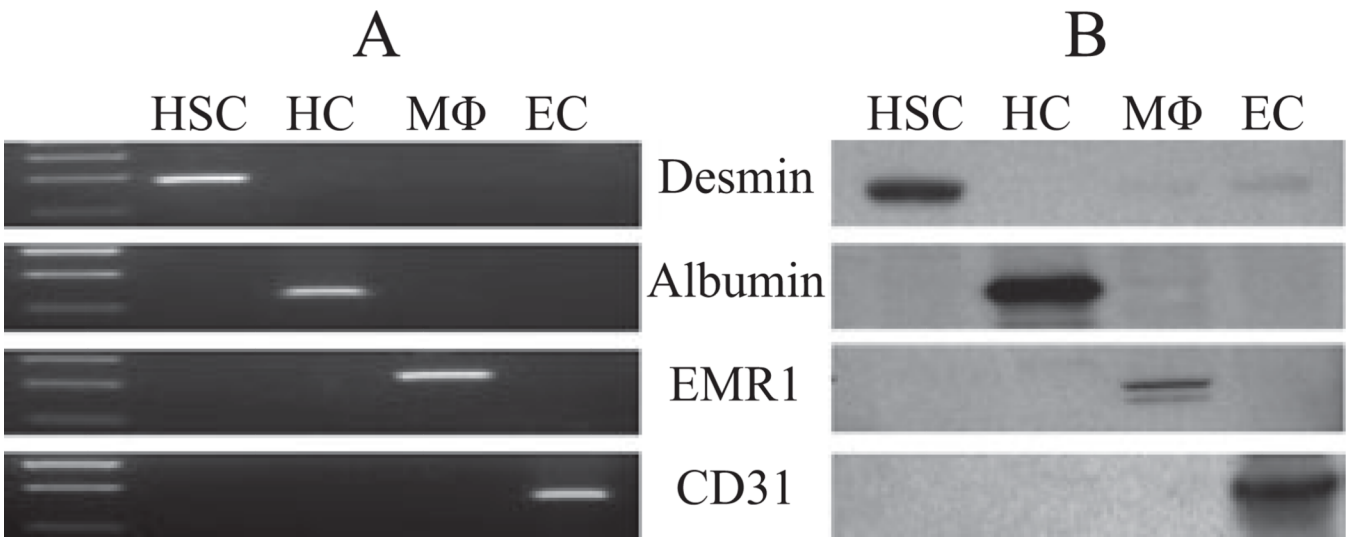

Fig. 1.

Characterization of mouse hepatic stellate cells (MHSCs). Cell colonies were obtained from the livers of *H-2K*^{b}-tsA58 transgenic mice as described in Materials and Methods. (**A**) The mRNAs of MHSC marker desmin, hepatocyte (HC) marker albumin, macrophage (MΦ) marker EMR1, and endothelial cell (EC) marker CD31 were detected by reverse transcription polymerase chain reaction. (**B**) Desmin, albumin, EMR1, and CD31 protein were detected by Western blot analysis.

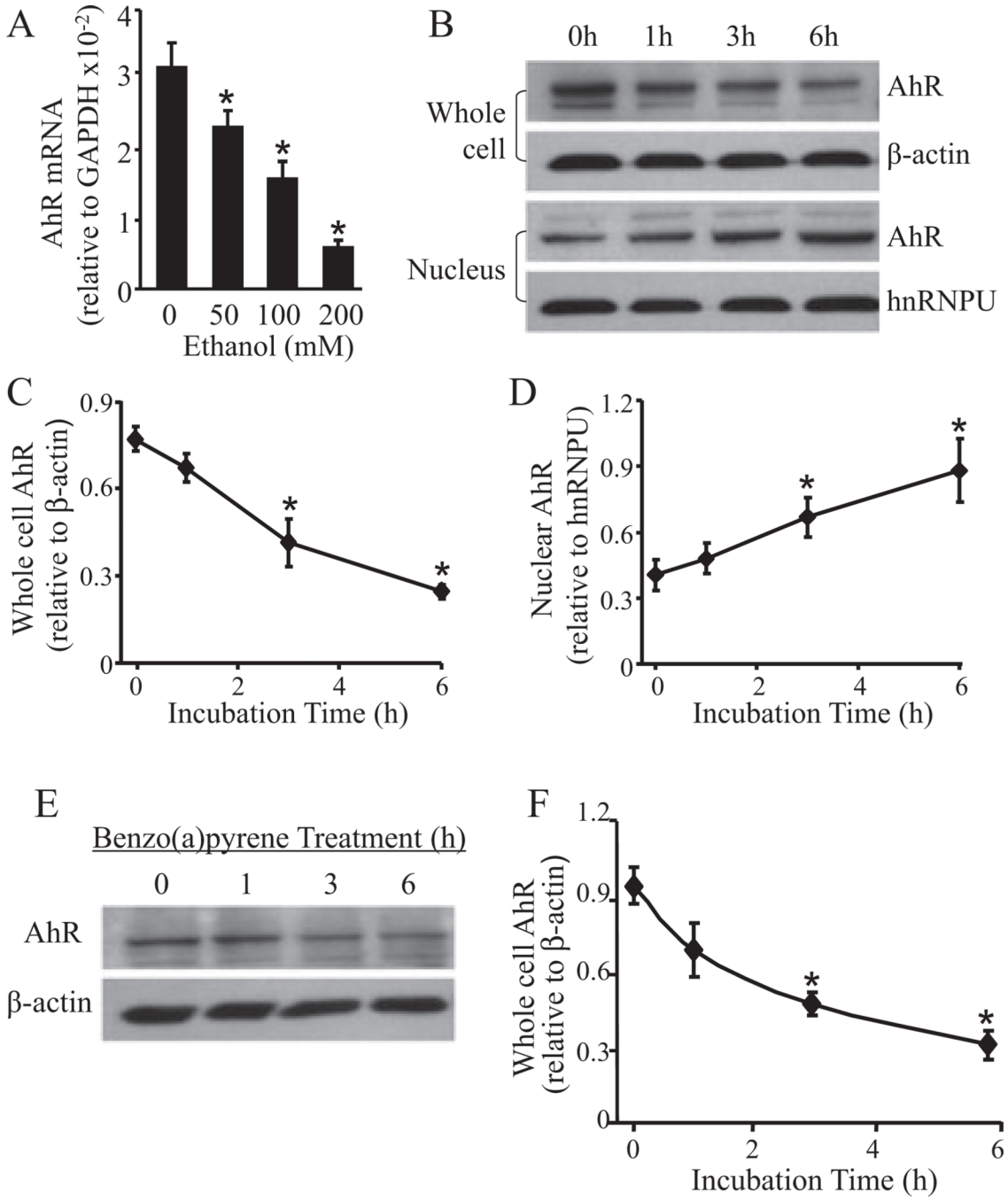

Fig. 2.

The effect of acute ethanol (EtOH) and benzo(a)pyrene (BaP) exposure on aryl hydrocarbon receptor (AhR) expression. (**A**) Mouse hepatic stellate cells (MHSCs) were incubated with 0, 50, 100, or 200 mM of EtOH for 6 hours. (**B–D**) MHSCs were treated with 100 mM EtOH for 0, 1, 3, or 6 hours. (**E–F**) MHSCs were incubated with 10 nM BaP or equal amount of vehicle dimethyl sulfoxide for 0, 1, 3, and 6 hours. The levels of AhR protein in the whole-cell lysates and nuclear extracts were measured by Western blot analysis and were expressed as a ratio of their immunoblot intensity relative to β-actin or to heterogeneous nuclear ribonucleoprotein (hnRNPU), respectively. The level of AhR mRNA was

determined by quantitative real-time reverse transcription polymerase chain reaction and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Values represent the mean ± SEM of 5 independent experiments. *$p < 0.05$ versus cells without EtOH or BaP treatment.

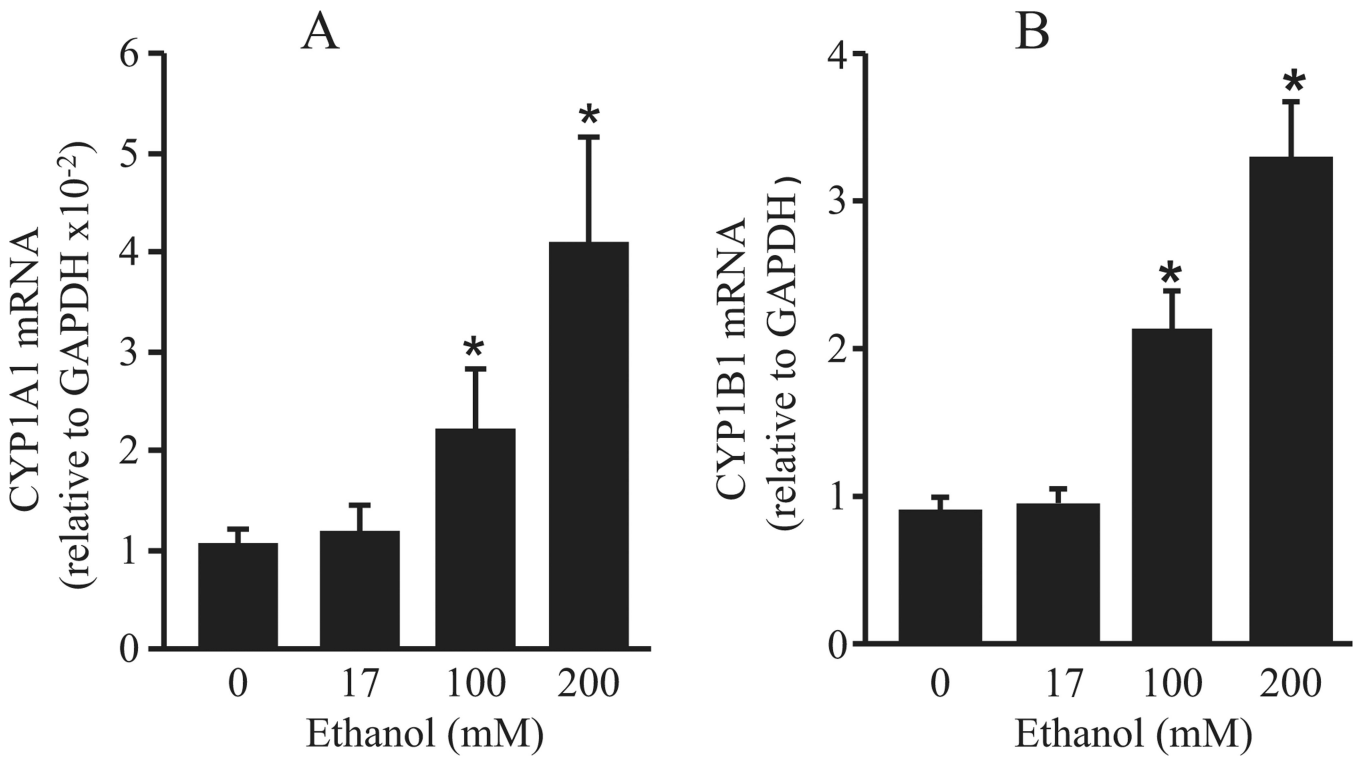

Fig. 3.

Up-regulation of cytochrome P450 (CYP) 1A1 and 1B1 expression by acute ethanol (EtOH) exposure. Mouse hepatic stellate cells (MHSCs) were treated with 0, 17, 100, or 200 mM of EtOH for 6 hours. The CYP1A1 and 1B1 mRNA levels were determined by quantitative real-time reverse transcription polymerase chain reaction, and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Values represent the mean ± SEM of 5 independent experiments. *$p < 0.05$ versus cells without EtOH treatment.

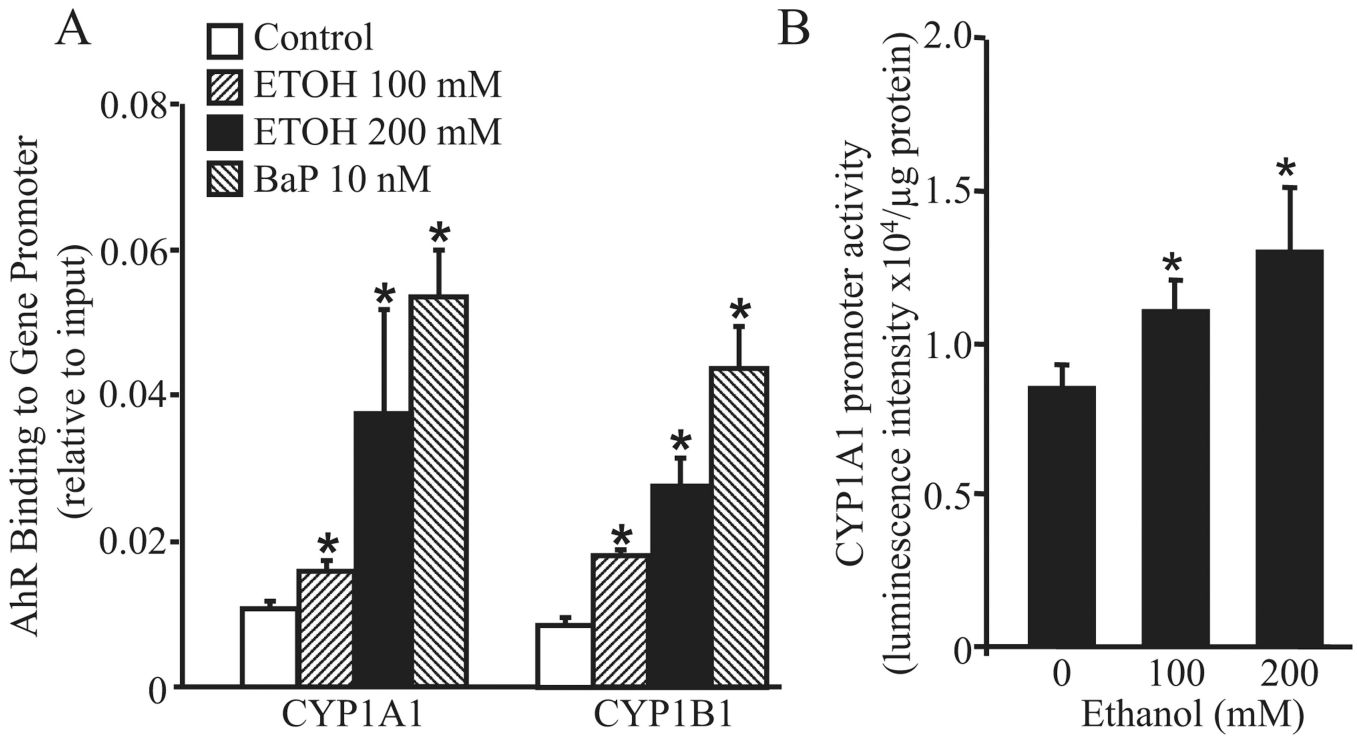

Fig. 4.

Enhancement of aryl hydrocarbon receptor (AhR) binding to the cytochrome P450 (CYP) 1A1 and 1B1 promoters and augmentation of CYP1A1 promoter activity by acute ethanol (EtOH) exposure. (**A**) Mouse hepatic stellate cells (MHSCs) were treated with 0, 100, or 200 mM of EtOH or 10 nM benzo(a)pyrene (BaP) for 6 hours. AhR binding to the CYP1A1 or 1B1 promoter was assessed by chromatin immunoprecipitation analysis. Genomic DNA bound to AhR was recovered from the immunoprecipitant and quantified by real-time reverse transcription polymerase chain reaction (RT-PCR) using primer pairs specific for the CYP1A1- and 1B1-xenobiotic response element regions. The DNA-binding activity of AhR was expressed as the ratio of the PCR product from the immunoprecipitant to that from the input control. (**B**) The CYP1A1 promoter activity was determined by luciferase assays. MHSCs were transfected with a CYP1A1 promoter-luciferase reporter plasmid, and then treated with EtOH at the indicated concentrations for 6 hours. Luciferase activity was measured using a luminescence assay and expressed relative to the protein level. Values represent the mean ± SEM of 5 independent experiments. *$p < 0.05$ versus cells without EtOH treatment.

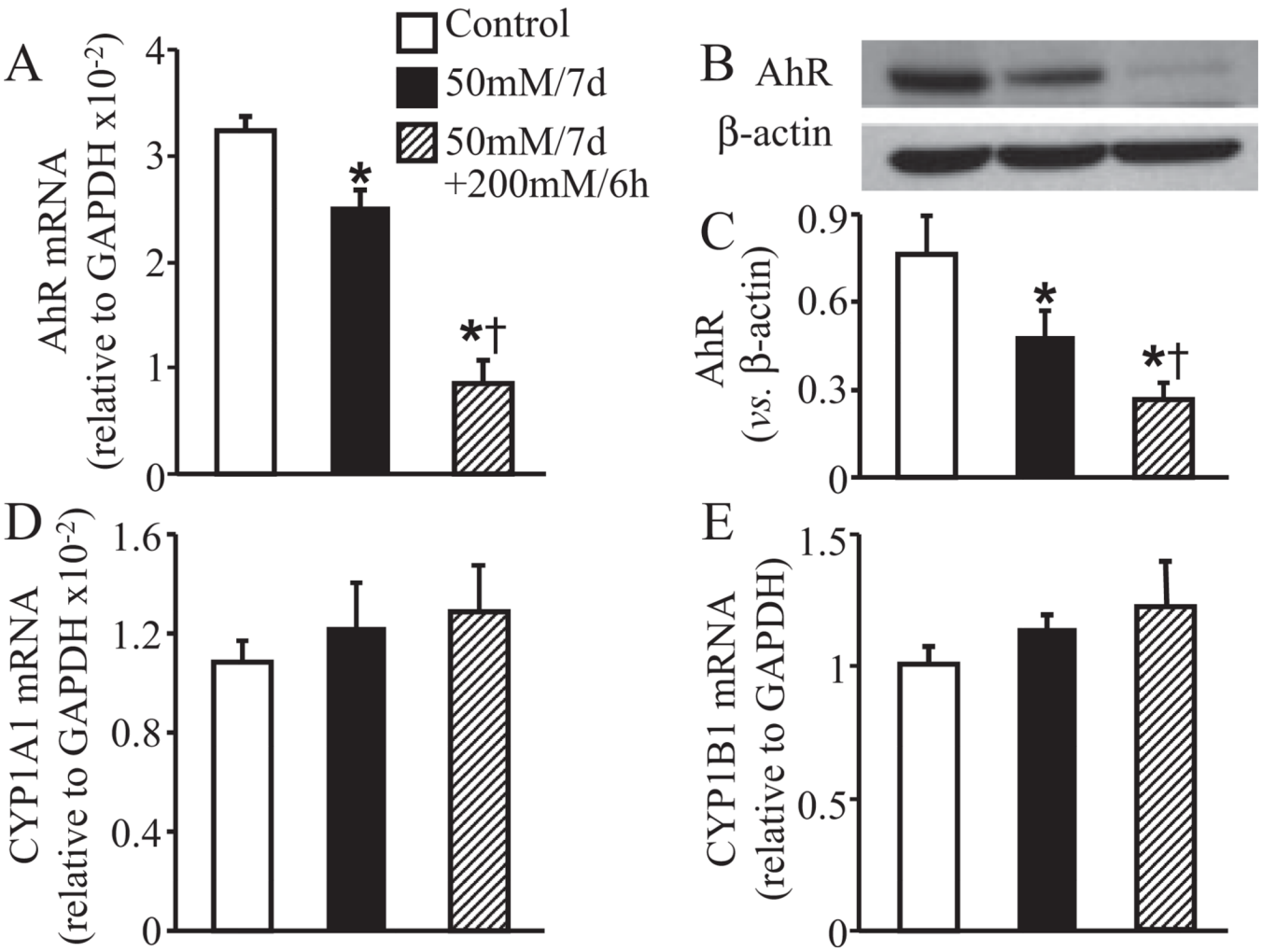

Fig. 5.

The effect of chronic ethanol (EtOH) exposure on aryl hydrocarbon receptor (AhR), cytochrome P450 (CYP) 1A1, and CYPB1 expression. Mouse hepatic stellate cells (MHSCs) were incubated with culture medium alone (control), 50 mM EtOH for 7 days (50 mM/7 d), or 50 mM EtOH for 7 days followed by 200 mM EtOH treatment for another 6 hours (50 mM/7 d + 200 mM/6 h). The level of AhR protein was determined by Western blot analysis and expressed relative to β-actin. (**A–B**) The level of AhR mRNA was determined by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and AhR protein by Western blot analysis and expressed relative to β-actin. (**C–D**) CYP1A1 and 1B1 mRNAs were determined by RT-PCR, normalized to GAPDH. Values represent the mean ± SEM of 5 independent experiments. *$p < 0.05$ versus cells without EtOH treatment (control), $^{\dagger}p < 0.05$ versus cells treated with 50 mM EtOH alone for 7 days.

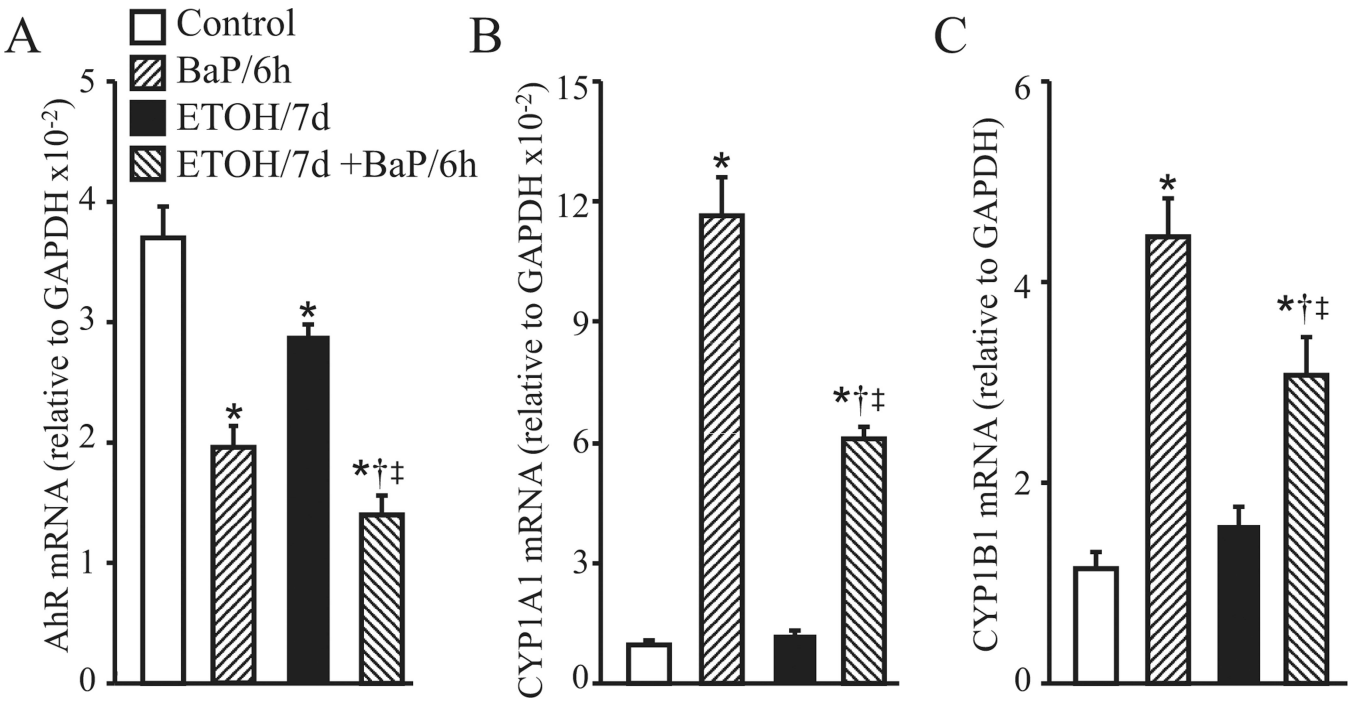

Fig. 6.

The effect of chronic ethanol (EtOH) exposure on benzo (a)pyrene (BaP)-induced changes in expression of aryl hydrocarbon receptor (AhR), cytochrome P450 (CYP) 1A1, and 1B1. Mouse hepatic stellate cells were incubated with culture medium alone (control), 10 nM BaP for 6 hours (BaP/6 h), 50 mM EtOH for 7 days (EtOH/7 d), or 50 mM EtOH for 7 days followed by 10 nM BaP treatment for another 6 hours (EtOH/7 d + BaP/6 h). For cells that were not treated with BaP, an equal amount of vehicle dimethyl sulfoxide was added to the culture medium. The levels of AhR, CYP1A1, and CYP1B1 mRNAs were determined by quantitative real-time reverse transcription polymerase chain reaction and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Values represent the mean ± SEM of 5 independent experiments. *$p < 0.05$ versus cells without EtOH treatment (control), †$p < 0.05$ versus cells treated with 50 mM EtOH alone for 7 days, ‡$p < 0.05$ versus cells treated with BaP but without EtOH.

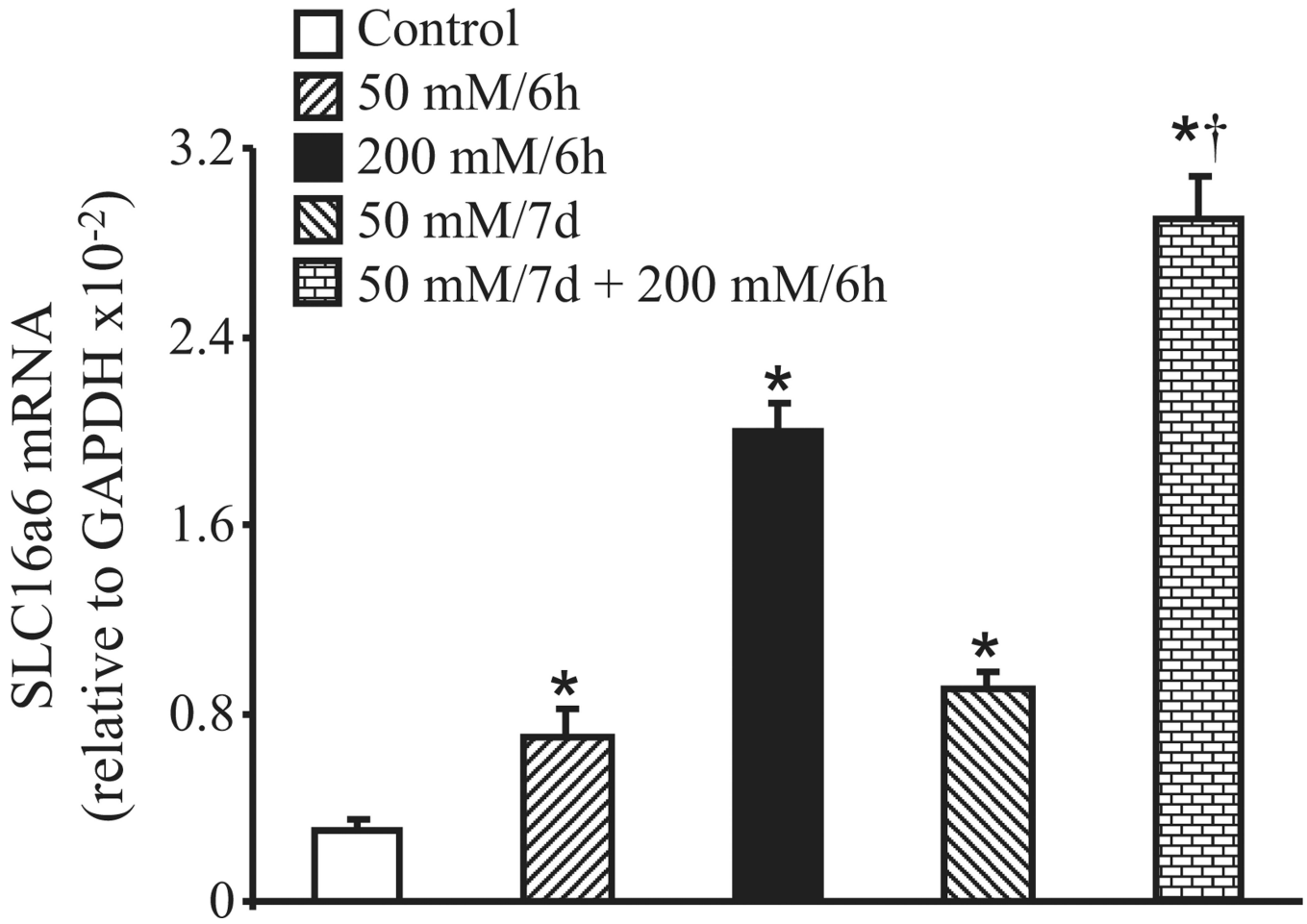

Fig. 7.

The effect of acute and chronic ethanol (EtOH) exposure on the expression of solute carrier family 16, member 6 (SLC16a6). Mouse hepatic stellate cells were incubated with culture medium alone (control), 50 mM EtOH for 6 hours (50 mM/6 h), 200 mM EtOH for 6 hours (200 mM/6 hours), 50 mM EtOH for 7 days (50 mM/7 d), or 50 mM EtOH for 7 days followed by 200 mM EtOH treatment for another 6 hours (50 mM/7 d + 200 mM/6 h). The mRNA level of SLC16a6 was determined by quantitative real-time reverse transcription polymerase chain reaction and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Values represent the mean ± SEM of 5 independent experiments. *$p <$ 0.05 versus control cells, †$p < 0.05$ versus cells treated only with 50 mM EtOH for 7 days and versus cells treated only with 200 mM EtOH for 6 hours.

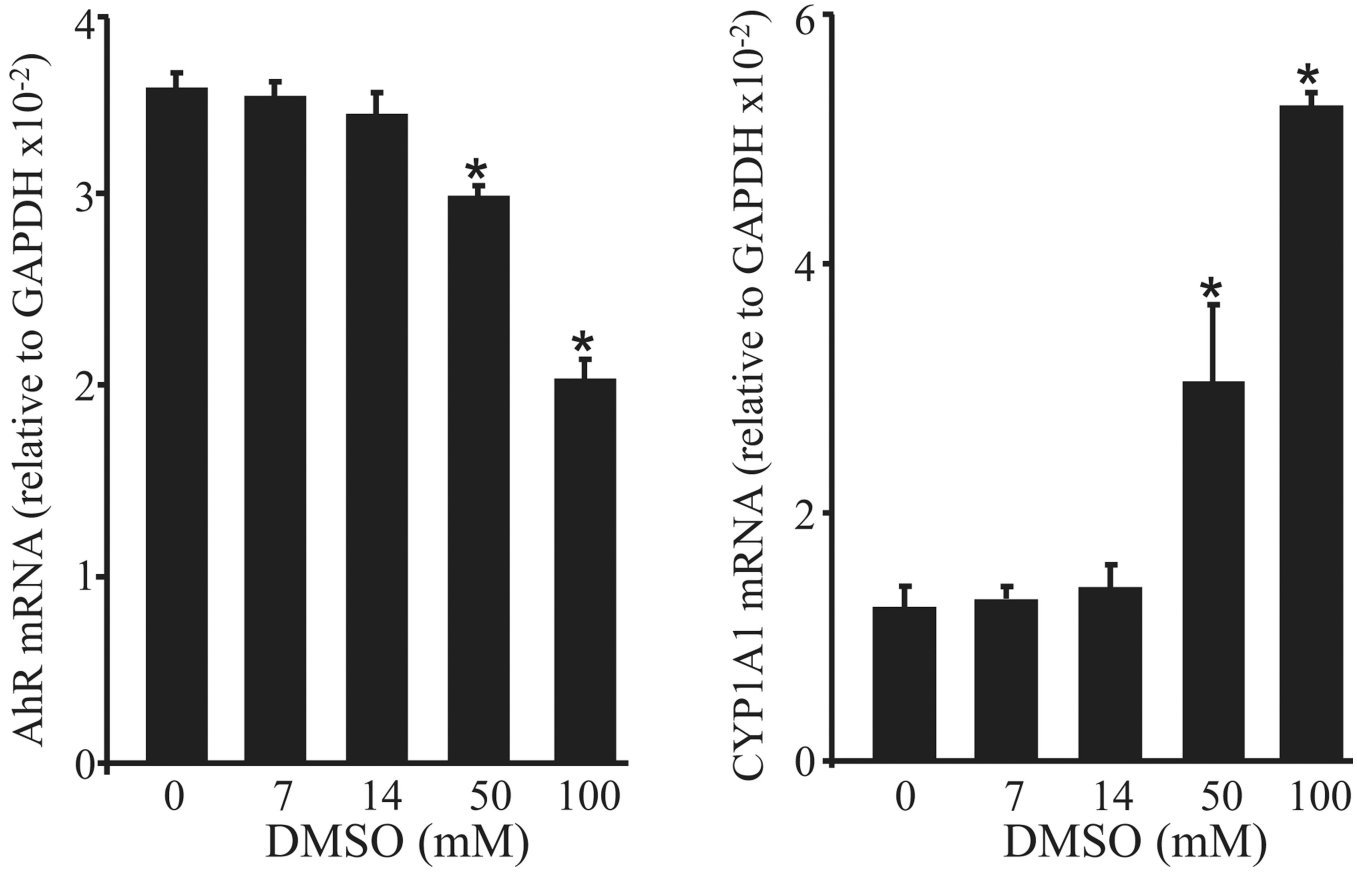

Fig. 8.

The effect of dimethyl sulfoxide (DMSO) on aryl hydrocarbon receptor (AhR) and cytochrome P450 (CYP) 1A1 expression. Mouse hepatic stellate cells were treated with 0, 7, 14, 50, 100, or 200 mM DMSO for 6 hours. The AhR and CYP1A1 mRNA levels were determined by quantitative real-time reverse transcription polymerase chain reaction and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Values represent the mean ± SEM of 5 independent experiments. *$p < 0.05$ versus cells without DMSO treatment.