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RESEARCH ARTICLE

Bidirectional Regulation of NAD(P)H Quinone Dehydrogenase 1 Expression in Mouse Hepatic Stellate Cells- Acute versus Long-Term Ethanol Exposure

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ABSTRACT

Background: The expression of NAD(P)H quinone dehydrogenase 1 (NQO1) is controlled hierarchically by aryl hydrocarbon receptor (AhR) and nuclear factor erythroid 2-related factor-2 (Nrf2). This study examined whether ethanol regulates NQO1 expression via the AhR-Nrf2 pathway.

Methods: Immortal mouse hepatic stellate cells (MHSCs) were treated with ethanol and/or an AhR ligand and benzo(a)pyrene (BaP). Real-time quantitative reverse transcription polymerase chain reaction assays, western blot analysis, and promoter activity assays were performed to determine the expression and promoter activity of NQO1.

Results: Treatment of MHSCs with 25–100 mM ethanol for 6 h elevated NQO1 mRNA and protein in a dose-dependent manner. Exposure of MHSCs to 50 mM ethanol for 6 h reduced whole-cell AhR protein level but increased the following: nuclear AhR protein, whole-cell and nuclear Nrf2 protein, and NQO1 promoter activity. Mutation of the Nrf2 binding site in the NQO1 promoter region inhibited the basal and ethanol-induced NQO1 promoter activity, while mutation of the AhR binding site did not affect the NQO1 promoter activity. Knocking down AhR suppressed ethanol-induced Nrf2 and NQO1 expression, while knocking down Nrf2 inhibited both basal and ethanol-induced NQO1 expression. Exposing MHSCs to 50 mM ethanol for 72 h reduced the following: whole cell AhR, Nrf2 and NQO1 protein levels, Nrf2 and NQO1 mRNA levels, and nuclear AhR and Nrf2 protein levels. Treating MHSCs with 10 nM BaP for 6 h increased Nrf2 and NQO1 mRNA and protein levels. Pretreatment of MHSCs with 50 mM ethanol for 72 h diminished the capacity of MHSCs to express NQO1 and Nrf2 upon induction by BaP.

Conclusions: Our findings suggest that ethanol upregulates NQO1 expression by activating the AhR-Nrf2 pathway. Long-term ethanol exposure sustains low level of AhR protein and diminishes the inducibility of its target genes Nrf2 and NQO1 by BaP. These findings will contribute to understanding the synergistic toxicity of ethanol and polycyclic aromatic hydrocarbon compounds, such as BaP.

Keywords: ethanol, benzo(a)pyrene, aryl hydrocarbon receptor, nuclear factor erythroid 2-related factor-2, NAD(P)H quinone dehydrogenase 1.

INTRODUCTION

Chronic use of tobacco cigarettes [1-3] or alcohol [4-6] has been shown to influence both the incidence and outcome of several human diseases, including cancer and diabetes as well as a number of neurological, cardiovascular, pulmonary and hepatic diseases. Combined tobacco and alcohol use have been reported to result in higher morbidity and mortality for some cancers, coronary heart disease, and stroke [7-9] than either of them alone. While previous studies have explored additional risks, such as oxidative stress and inflammation, in animal models to compare the combination of alcohol use and cigarette smoke exposure to either alone, these studies have not defined the mechanism underlying the synergistic toxicity of alcohol drinking and cigarette smoking.

The pathogenesis of cigarette smoking has been attributed at least in part to the cytotoxicity of polycyclic aromatic hydrocarbons (PAHs), a group encompassing over 100 different chemicals. Besides cigarette smoke, PAHs also exist in industrial effluents, vehicle exhausts, charred foods, and oil well fires [1, 10]. We are exposed to PAHs on a daily basis, mainly via ingestion of contaminated foods and/or inhalation of polluted air. Some individuals, like cigarette smokers, are exposed to high levels of such pollutants.

Mammalian cells detoxify PAHs using xenobiotic-metabolizing enzymes (XMEs). Specifically, phase I XMEs such as cytochrome P450 (CYP) 1A1 and 1B1 oxidize PAHs to generate reactive intermediates. Phase II XMEs glutathione S-transferases (GSTs), UDP glucuronosyl-transferases (UGTs), and sulfotransferases (SULTs) then convert these PAH intermediates to water soluble conjugates [11, 12]. NAD(P)H quinone dehydrogenase 1 (NQO1) reduces reactive oxygen species (ROS) generation by inhibiting the PAH quinone redox cycles [13]. The expression of CYP1A1 and 1B1 is controlled by aryl hydrocarbon receptor (AhR), while the expression of NQO1 and several GSTs and UGTs is controlled by AhR and nuclear factor-E2-related factor-2 (Nrf2) [14]. AhR is a ligand-activated transcription factor; its ligands include PAHs. Upon ligand binding, AhR translocates to the nucleus and binds to consensus regulatory sequences called xenobiotic response elements (XREs) in the promoters of target genes [9] and upregulate their expression. Nrf2 is an AhR target gene. PAHs, such as benzo(a)pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), have been shown to enhance AhR binding to the Nrf2 promoter and upregulate Nrf2 expression [15]. Nrf2 then upregulates the expression of its target genes by interacting with the antioxidant response element (ARE) in their promoter region. It has been documented that the promoter of some phase II XMEs, such as UGT1A9 and UGT1A1, contains both the XRE and ARE; hence, they can be activated by both AhR and Nrf2 [14, 16]. On the other hand, the promoter of some phase II XMEs, such as NQO1, GSTp1 and UGT1A6, can be activated by Nrf2 but not directly by AhR. Rather, the expression of these genes is controlled hierarchically by AhR and Nrf2, *i.e.,* the ligand-activated AhR upregulates Nrf2 expression, which in turn enhances the expression of its target genes [14-16].

The pathogenesis of ethanol results mainly from its metabolic intermediate acetaldehyde that has been reported to cause mitochondrial damage, oxidative stress, apoptosis, and DNA damage, to name a few. Humans metabolize ethanol via two steps of oxidation. First, alcohol dehydrogenase or cytochrome P450 2E1 (Cyp2E1) oxidize ethanol to acetaldehyde. Next, aldehyde dehydrogenase oxidizes acetaldehyde to acetate [4, 17]. In addition, ethanol has been suggested to stimulate cells to generate biologically active compounds. For instance, ethanol may modulate the metabolism of arachidonic acids and tryptophan, and boosts the generation of thromboxane B2, leukotriene B4 [18], prostaglandin E2 [19] and kynurenine [20]. These compounds have been considered as AhR ligands. Indeed, Zhang, et al. reported that treating mouse hepatic stellate cells (MHSCs) with ethanol induced AhR translocation to the nucleus and its binding to the promoter region of phase I enzyme gens CYP1A1 and 1B1 to upregulate their mRNA expression [21]. More recently, Dong et al. reported that feeding mice with an alcohol-containing liquid diet activated AhR in liver cells [22]. The purpose of this report was to study the effect of acute and long-term ethanol exposure on the expression of the phase II enzyme NQO1, as well as the regulatory role of the AhR-Nrf2 pathway in ethanol-induced changes in NQO1 expression in MHSCs.

MATERIALS AND METHODS

Materials

Antibodies against AhR, NQO1, Nrf2, β-actin, and histone H3 as well as horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Nrf2 antibody was purchased from R&D Systems (Minneapolis, MN). 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). Galacto-Star™ ß-Galactosidase Reporter Gene Assay System for Mammalian Cells was obtained from Applied Biosysytems (Foster City, CA). The 8-isoprostanes enzyme immunoassay (EIA) kit was purchased from Cayman Chemicals (Ann Arbor, MI).

Ethanol (E7023) and benzo(a)pyrene (BaP) (B1760) were purchased from Sigma-Aldrich (St Louis, MO). For treatment of cells, 14.5, 29, or 58 µl of ethanol were added to 10 ml of culture medium to obtain final ethanol concentrations of 25, 50, and 100 mM, respectively. For treatment of cells with BaP, a 20 µM BaP stock solution was prepared using a 100x-diluted DMSO solution (140 mM); 1 µl of this stock solution was added to 2 ml of culture medium to obtain final concentrations of BaP and DMSO of 10 nM and 70 µM, respectively.

The immortal mouse hepatic stellate cells (MHSCs) were developed previously [21, 23]. These cells express a thermolabile SV40 tumor antigen and grow at 34°C to confluency.

Real-time quantitative RT-PCR assay

Confluent MHSCs were treated with ethanol at 37°C for 6 h or 72 h at concentrations indicated in figure legends. We previously observed that incubation of 50 mM ethanol in cell-free medium or with cells at 37°C for 24 h reduced the concentration of ethanol about 36 and 50%, respectively [21]. Therefore, media with or without ethanol were changed every 24 h.

In experiments using BaP, 10 nM BaP was added to cells that had been cultured with and without 50 mM ethanol for 72 h. Total RNA was extracted using RNeasy Plus Mini kits and subjected to real-time quantitative RT PCR using the iCycler system (Bio-Rad, Hercules, CA) as previously described [24]. The primers used for amplification were as follows: AhR forward: 5'-ACTTCACACCTATTGGTTGT-3', reverse: 5'-ATGCCACTTTCTCCAGTCTT-3'; NQO1 forward: 5'-TTCTGTGGCTTCCAGGTCTT-3', reverse: 5'-AGGCTGCTTGGAGCAAAATA-3'; Nrf2 forward: 5'-CCTCGCTGGAAAAAGAAGTG-3', reverse: 5'-GGAGAGGATGCTGCTGAAAG-3'; and GAPDH forward: 5'-GAGCCAAAAGGGTCATCATC-3', reverse: 5'-TAAGCAGTTGGTGGTGCAGG-3'. The expression levels of mRNAs of interest were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level.

Western blot analysis

MHSCs were treated with ethanol and BaP at the concentrations and times noted in the text. For whole cell protein extraction, cells were lysed in M-PER. For nuclear extract preparation, nuclei were isolated using the Nuclear and Cytoplasmic Extraction Reagent (NE-PER) according to manufacturer's protocol (Thermo Scientific). Samples containing 10–40 µg of protein were resolved on 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels. Proteins were transferred to a polyvinylidene fluoride (PVDF) 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 incubated with primary and secondary antibodies, as previously described [24]. 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).

Small interfering RNA (siRNA) knockdown

MHSCs grown in six-well plates to approximately 60–70% confluency were transfected with control siRNA or siRNA targeting AhR or Nrf2 (Santa Cruz Biotechnology) using the GenMute siRNA & DNA Transfection Reagent (SignaGen Laboratories, Ijamsville, MD) according to manufacturer's instructions. After 6 h of transfection, the cells were given fresh media and cultured for an additional 24 h. These transfection procedures were repeated once more to enhance transfection efficiency. Finally, the transfected cells were treated with ethanol or vehicle medium for 6 h and harvested for measurements of protein and mRNA levels.

Reporter gene assay

The promoter activity of NQO1 was determined by a dual luminescence-based reporter gene assay for luciferase and β-galactosidase, as described previously [24]. Briefly, a 1101-bp NQO1 promoter fragment was prepared by PCR from mouse genomic DNA and cloned into the pGL2 luciferase vector. Mutations in the binding sites for Nrf2 (ARE) or AhR (XRE) within the NQO1 promoter were generated by site-directed mutagenesis of the wild-type pGL2-NQO1-promoter plasmid [24]. The recombinant NQO1 promoter-luciferase constructs and a β-galactosidase expression plasmid, pCMV-SPORT-βgal, were co-transfected into MHSCs using the Lipofectamine 2000 kit following manufacturer's instructions. The transfected cells were treated with media

containing ethanol or vehicle for 6 h and then lysed with lysis buffer. The lysates were incubated with luciferase assay reagent or galactosidase reaction buffer provided by the luciferase assay kit and the galactosidase assay kit, respectively. Luminescence was measured using the BL10000 LumiCount (Packard BioScience, Meriden, CT). Luciferase activity was expressed as a ratio of luminosity of the luciferase assay to that of the β-galactosidase assay.

Measurement of plasma 8-isoprostanes

The concentration of 8-isoprostanes was determined using an enzyme immunoassay (EIA) kit as described previously [25]. Briefly, MHSCs were lysed with NET lysis buffer (100mM NaCl, 1mM sodium EDTA, 10mM Tris, 0.5% Triton X-100: pH 7.4) supplemented with 0.01% butylated hydroxytoluene. The lysates were mixed with a lipid solvent [containing chloroform, isopropranol and NP-40 at a ratio of 7:11:0.1 (v/v)] at three times the volume of the lysis buffer. The lipid phase was separated by centrifugation and evaporated under vacuum. The dried pellet was resuspended in ethanol: 10% KH_2PO_4 (3:1, v/v). Ethanol was evaporated by vacuum centrifugation. The 8-isoprostanes in the remaining supernatant were purified with an affinity column and assayed following manufacturer's protocol. Protein levels in the lysates were determined using a BCA protein assay kit (Thermo Scientific). Concentrations of 8-isoprostanes were expressed as ng/mg cell proteins.

Statistical analysis

GraphPad was used for statistical analysis. Data were reported as mean ± SEM. Data distribution was examined using the Shapiro-Wilk normality test. Differences among groups were analyzed using Student's unpaired *t*-test (for two groups) and one-way analysis of variance (ANOVA, for more than two groups), followed by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of ethanol on NQO1 expression

A basic formula proposed by the National Highway Traffic Safety Administration for the general public to estimate blood-alcohol concentrations [26] estimates the consumption of approximately six and 12 drinks within 1 h to elevate blood alcohol concentration to 50 and 100 mM, respectively, for an individual with body weight of 100 lb. In this formula, one drink is defined as 37.5 ml of 40% distilled spirits or equivalent. Blood ethanol concentration in alcoholics has been reported to reach 100 mM [27] or higher [28]. We previously observed that ethanol at 17 mM, a concentration commonly used as a solvent in cell culture studies, did not significantly alter the expression of AhR target genes CYP1A1 and 1B1 [21]. In this study, we examined the concentration- and dose-related effects of ethanol on NQO1 expression in MHSCs. We found ethanol induce NQO1 expression in a dose-dependent manner (Fig. 1A–1C). Specifically, the presence of 25, 50, and 100 mM ethanol in the culture media for 6 h resulted in ~54%, 134%, and 198% increase in NQO1 mRNA levels, respectively (Fig. 1A). The same ethanol doses and incubation period increased NQO1 protein levels by ~31%, 110%, and 214%, respectively (Fig. 1B–C).

We also observed the inducibility of NQO1 by ethanol to be inversely correlated with incubation time (Fig. 1D–1F). Specifically, treating MHSCs with 50 mM ethanol for 6 h, 24 h, and 72 h resulted in ~135% and 48% increase as well as ~36% decrease in NQO1 mRNA level, respectively, compared to control (Fig. 1D–1F). Treating the cells with the same dose of ethanol for these three time periods caused NQO1 protein level to increase by ~91% and 15%, and to decrease by 29%, respectively (Fig. 1D–1F). The mRNA and protein levels of NQO1 in MHSCs treated with 50 mM ethanol for 24 and 72 h were significantly lower than those in cells treated with ethanol for 6 h (Fig. 1D–1F). Similarly, the NQO1 mRNA and protein levels in cells treated with ethanol for 72 h were significantly lower than those in cells treated with ethanol for 24 h (Fig. 1D–1F).

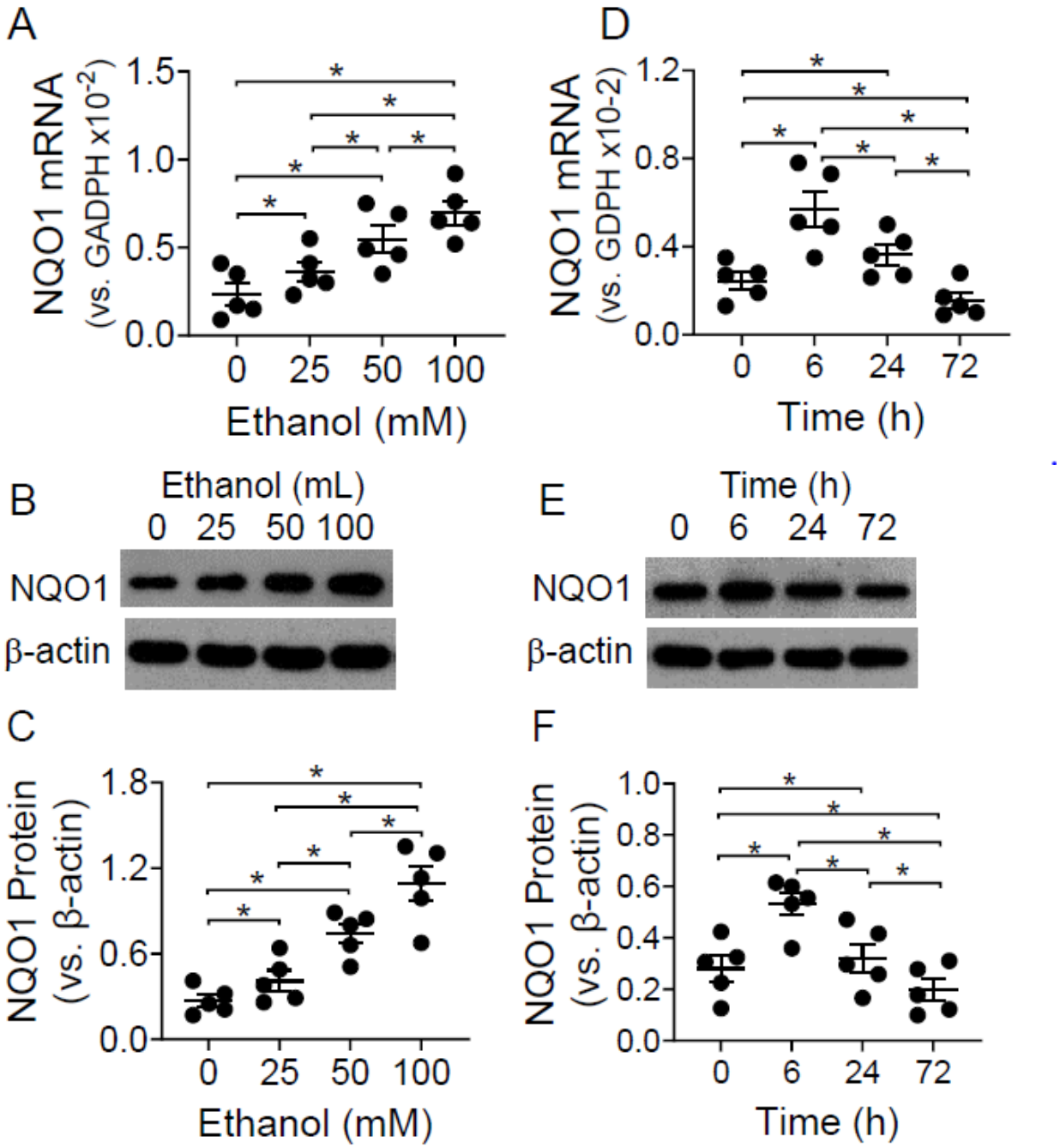

Fig. 1. Dose- and time- and dependent effect of ethanol on NQO1 expression. Mouse hepatic stellate cells were treated with 0, 25, 50 or 100 mM of ethanol for 6 h or treated with 50 mM of ethanol for 0, 6, 24 or 72 h. The NQO1 protein level was determined by western blot analysis, and expressed relative to β-actin (**A-D**). The NQO1 mRNA level was determined by quantitative real-time RT-PCR, and expressed relative to GAPDH mRNA (**E-F**). Values represent the mean ± SEM of four independent experiments. * $P<0.05$ vs. cells without ethanol treatment.

Effect of AhR and Nrf2 knockdown on NQO1 expressions

The transcription of NQO1 induced by AhR ligand BaP is hierarchically controlled by AhR and Nrf2 [24]. To validate the involvement of AhR and Nrf2 in ethanol-induced NQO1 expression, we transfected MHSCs with AhR siRNA or Nrf2 siRNA. We confirmed the knockdown efficiency by western blot analysis. As shown in Fig. 2A–B, the protein level of AhR and Nrf2 in AhR siRNA- or Nrf2 siRNA-transfected MHSCs were ~44% and ~46% lower, respectively, than those in control siRNA-transfected cells. While Nrf2 knockdown also reduced the protein level of NQO1, AhR knockdown did not

significantly alter the protein levels of Nrf2 and NQO1. Specifically, the Nrf2 and NQO1 protein levels in AhR siRNA- and control siRNA-transfected MHSCs were comparable, whereas the NQO1 protein level was ~61% lower in cells transfected with Nrf2 siRNA than in those transfected with control siRNA. Taken together, these observations suggest that the basal expression of NQO1 is regulated by Nrf2 but not by AhR.

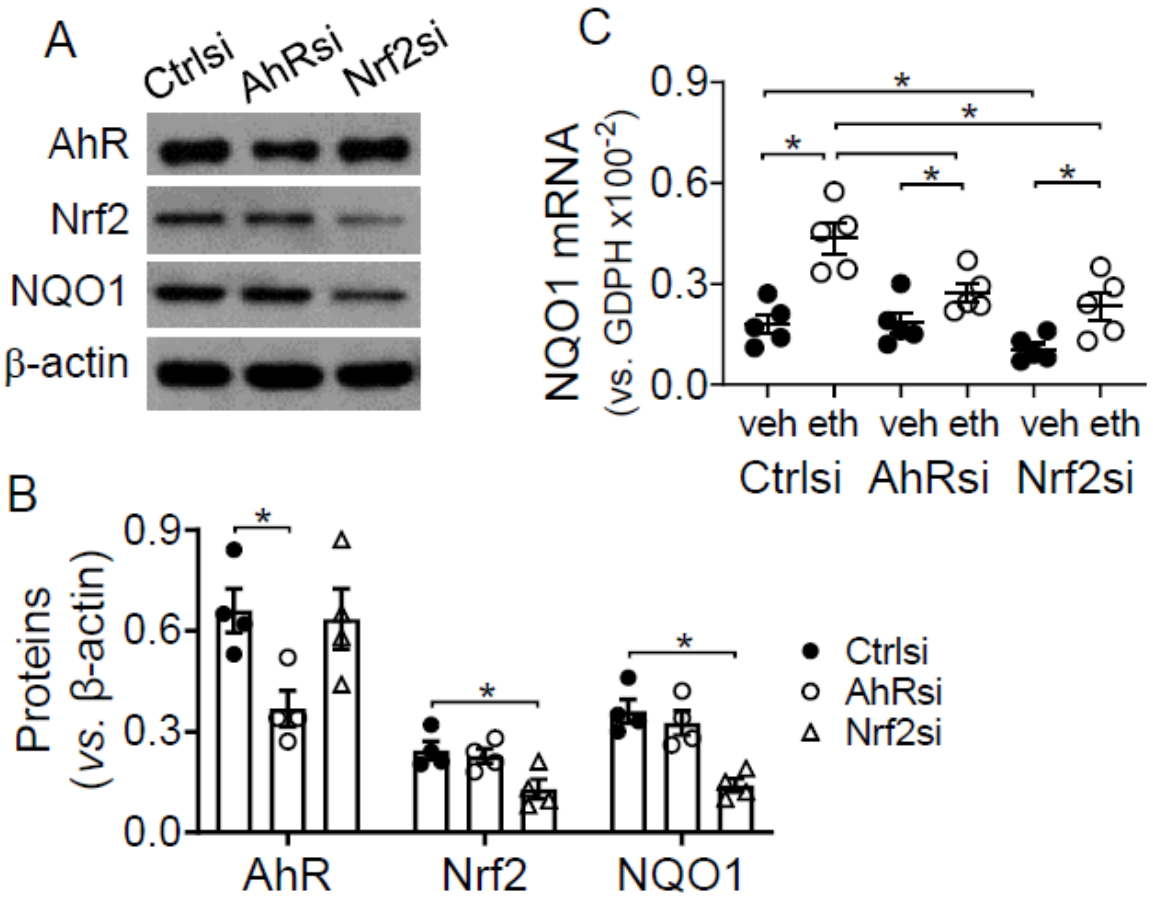

Fig. 2. Elimination of ethanol-induced NQO1 promoter activity by mutation of ARE but not by mutation of XRE in the NQO1 promoter. The NQO1 promoter-luciferase reporter plasmids were constructed and cotransfected into MHSCs with a *β-galactosidase* expression plasmid. **A.** Schematic diagram depicting wild-type, ARE-mutated, and XRE-mutated NQO1 promoter-luciferase constructs. **B.** Transfected MHSCs were treated with 50 mM ethanol or vehicle medium for 6 h. Luciferase activity was measured using a luminescence assay and expressed relative to the luminosity of the β-galactosidase assay. Values represent the mean ± SEM of four independent experiments. * $P<0.05$ *vs.* cells transfected with same plasmid and treated with vehicle medium; † $P<0.01$ *vs.* cells transfected with wild-type promoter and treated with vehicle medium and ethanol, respectively.

Exposure of control siRNA-transfected cells to 50 mM ethanol for 6 h increased NQO1 mRNA level by 142%. This was comparable to the increase induced by ethanol in cells that were not transfected with siRNAs (Fig. 2C versus Fig. 1A), indicating that the presence of siRNA itself did not alter the effect of ethanol on NQO1 mRNA expression. In contrast, transfection with AhR-specific siRNA significantly inhibited the ethanol-induced increase in NQO1 mRNA level, in spite of not altering its basal level. We found the NQO1 mRNA level to be ~37% lower in AhR siRNA-transfected cells than in control siRNA-transfected cells upon treatment with 50 mM ethanol for 6 h ($P<0.05$). On the other hand, transfection with Nrf2-specific siRNA inhibited NQO1 mRNA expression with and without ethanol treatment. The NQO1 mRNA levels in Nrf2 siRNA-transfected MHSCs under the basal and ethanol treatment conditions are ~41% and 46% lower, respectively, than in control siRNA-transfected cells under the same conditions.

Effect of ethanol on NQO1 promoter activity

Having demonstrated the regulatory role of AhR and Nrf2 in ethanol-induced NQO1 expression,

we then examined the transcriptional activity of the NQO1 promoter using a luciferase assay. To do this, we use a 1-kb mouse NQO1 promoter fragment consisting of an XRE and an ARE (Fig. 3A) [24]. The ARE is located ~426-441 nucleotides upstream of the transcription start site (TSS), while the XRE is located ~379-386 nucleotides upstream of the TSS. We transfected MHSCs with the promoter-luciferase constructs containing the wild-type (P1), mutated ARE (P2), or mutated XRE (P3) NQO1 promoter sequences (Fig. 3A). Treatment of P1-transfected cells with 50 mM ethanol increased luciferase promoter activity by 73% (Fig. 3B). Mutations in the ARE (P2) reduced the NQO1 promoter activity by 51% in the absence of ethanol, and ethanol exposure did not induce transcriptional activity in cells transfected with P2 (Fig. 3B). We observed the luciferase activity to be ~66% lower in P2-transfected MHSCs than in P1-transfected cells upon ethanol exposure. Collectively, these results indicate that the NQO1-ARE is essential for the basal and ethanol-induced expression of NQO1 in MHSCs. In contrast, mutations in the XRE did not significantly alter NQO1 promoter activity. In fact, the XRE-mutated NQO1 promoter was as active as the wild-type promoter in MHSCs with or without ethanol treatment (Fig. 3B). Namely, the basal luciferase activities of cells transfected with either P3 or P1 were comparable. Treatment of P3-transfected cells with 50 mM ethanol increased promoter activity by 81%. These observations suggest that AhR does not activate NQO1 promoter directly.

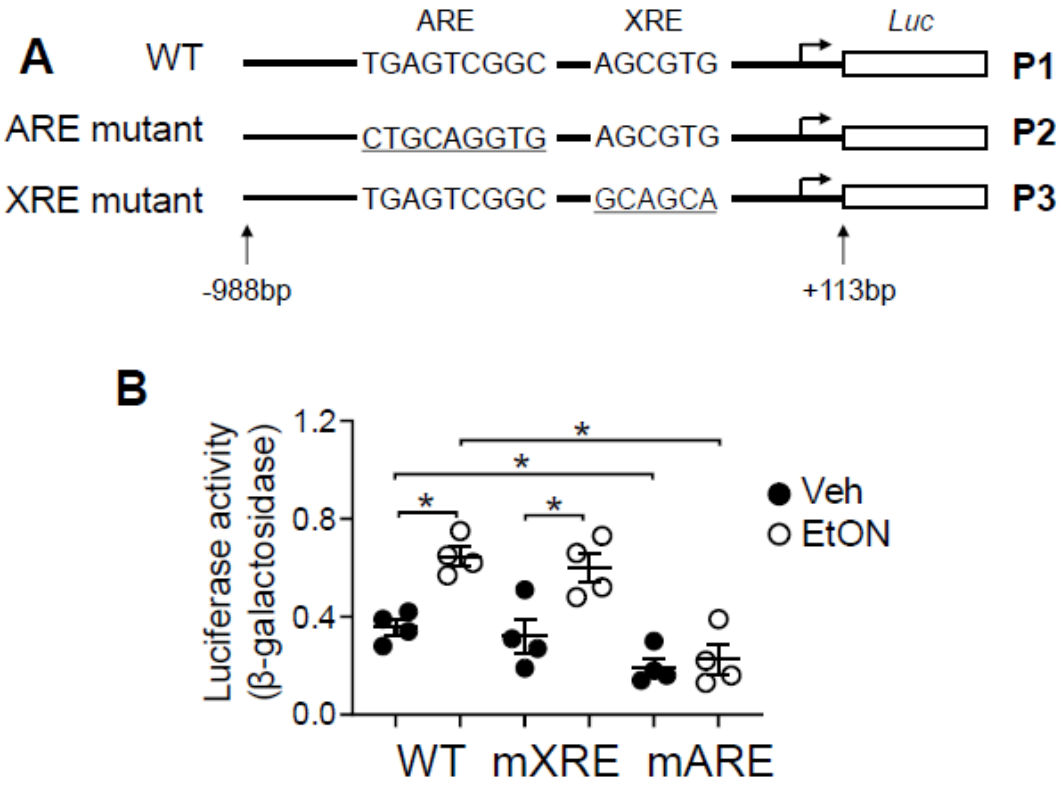

Fig. 3. Inhibition of ethanol-induced NQO1 expression by siRNA knockdown of AhR and Nrf2. **A.** MHSCs were transfected with control scrambled siRNA (ctrlsi), AhR siRNA (AhRsi) or Nrf2 siRNA (Nrfsi). The protein levels of AhR, Nrf2 and NQO1 were determined by western blot analysis, and expressed relative to β-actin. **B.** MHSCs transfected with ctrlsi, AhRsi, Nrfsi or both AhRsi and Nrfsi were treated with ethanol or vehicle medium for 6 h. The mRNA levels of NQO1 were determined by quantitative real-time RT-PCR, and expressed relative to GAPDH mRNA. Values represent the mean ± SEM of five independent experiments. * $P<0.05$ *vs.* the same genotype cells with same siRNA transfection and without BaP treatment; # $P<0.05$ *vs.* the same genotype cells transfected with control siRNA and treated with or without BaP; † $P<0.05$ *vs.* BaP-treated wild-type cells with same siRNA transfection.

Time-dependent effect of ethanol on whole-cell and nuclear AhR and Nrf2 proteins

We examined the time-dependent effect of ethanol on AhR expression. Incubation of MHSCs with 50 mM ethanol for 6, 24 and 72 h reduced the AhR protein in the whole-cell lysates by about 34, 58 and 79%, respectively, as compared to ethanol-untreated control cells (Fig. 4A and 4B). In contrast, addition of 50 mM ethanol to the culture for the same time periods induced a bidirectional change

in nuclear AhR protein level (Fig. 4 D). Specifically, treatment of MHSCs with 50 mM ethanol for 6 h resulted in an ~86% increase in nuclear AhR protein as compared to that in ethanol-untreated control cells; while exposure of cells to the same concentration of ethanol for 24 and 72 h increased nuclear AhR protein level only 41 and 8%, respectively. These data are consistent with our previous finding showing that ethanol induces AhR translocation to nuclei and downregulates AhR expression, and that long-term ethanol exposure keeps AhR at very low expression level and reduces its availability to translocate to the nucleus.

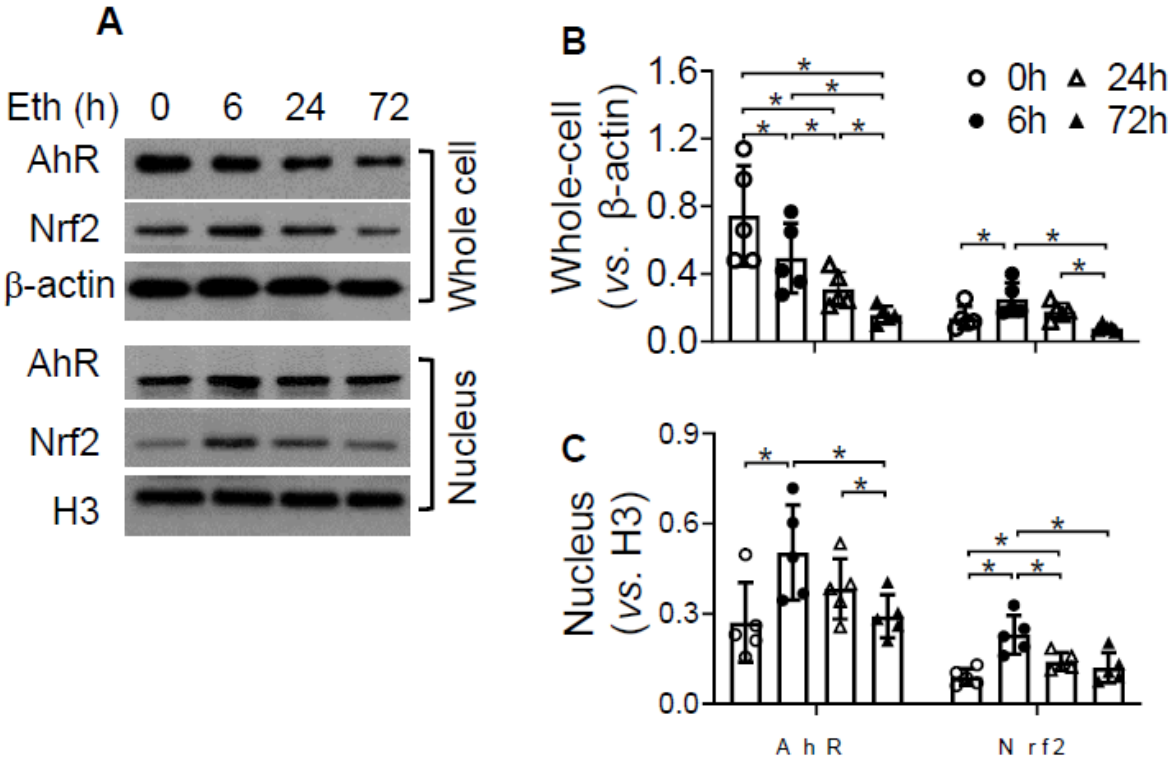

Fig. 4. Time-dependent effect of ethanol exposure on whole-cell and nuclear AhR and Nrf2. Mouse hepatic stellate cells were incubated with 50 mM of ethanol for 0, 6 24 and 72 h. The protein levels of AhR and Nrf2 in the whole-cell lysates (**A-B**) and nuclear extracts (**C-D**) were measured by western blot analysis, and were expressed as a ratio of their immunoblot intensity relative to β-actin or to nuclear marker histone H3 (H3), respectively. Values represent the mean ± SEM of five independent experiments. * *P* <0.05 *vs.* cells without ethanol or BaP treatment.

Exposure of MHCS to ethanol also induced bidirectional changes in Nrf2 protein level. Specifically, the Nrf2 protein level in the whole cell lysate of the MHSCs incubated with 50 mM ethanol for 6, 24 and 72 h were ~79 and 28% higher, and ~42% lower, respectively, than those in control cells lacking ethanol treatment. The differences of the Nrf2 protein levels between the cells treated with ethanol for 6 h and those treated for 24 or 72 h are statistically significant (P<0.05). Reciprocal change in nuclear Nrf2 protein level was observed in ethanol-treated cells. The nuclear level of Nrf2 protein level in the MHSCs incubated with 50 mM ethanol for 6 h was ~158% higher compared to those in control cells lacking ethanol, while exposure of cells to the same concentration of ethanol for 24 and 72 h increased nuclear Nrf2 protein level only ~58 and 33%, respectively. The Nrf2 protein level in the MHSCs treated with ethanol for 6 h was significantly higher than that in the cells treated for 24 or 72 h (P<0.05).

Long-term ethanol exposure diminishes the inducibility of Nrf2 and NQO1 by BaP

Mouse hepatic stellate cells were pre-incubated with 50 mM ethanol or medium alone as a control for 72 h, and then treated with vehicle solution or 1 μM of AhR ligand BaP for 6 h. As the data in Fig. 6 show, long-term ethanol exposure reduced the mRNA levels of Nrf2 and NQO1 by ~36% and ~49%, respectively; and the protein levels of Nrf2 and NQO1 by ~35% and 36%, respectively. Data in Fig. 6 also show that BaP exposure upregulated Nrf2 and NQO1 expression in both ethanol-pretreated and untreated MHSCs. Specifically, when 1 μM BaP was added for 6 h to cells that had not been exposed to ethanol, there was a significant increase in NQO1 mRNA and

protein (~83% and 75%, respectively) and Nrf2 mRNA and proteins (~65% and 79%, respectively). The same dose of BaP resulted in ~72% and 73% increase, respectively, in NQO1 mRNA and protein; and ~65% and~69% increase, respectively, in Nrf2 mRNA and proteins in cells that had been preincubated with 50 mM ethanol for 72 h (Fig. 6). However, the mRNA and protein levels of NQO1and Nrf2 were significantly lower in cells treated with both ethanol and BaP than in those treated with BaP alone (Fig. 6).

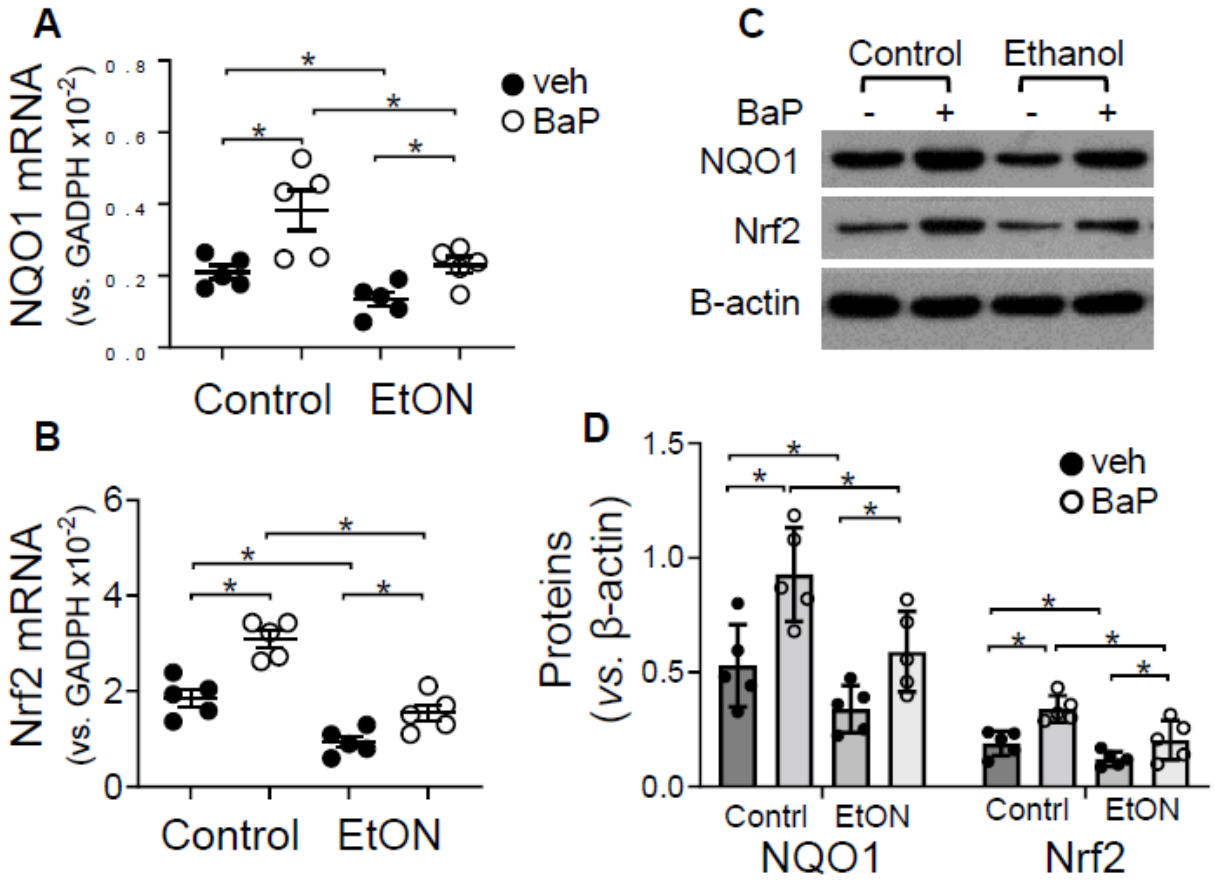

Fig. 5. Effect of long-term ethanol exposure on benzo(a)pyrene-induced changes in expression of NQO1 and Nrf2. Mouse hepatic stellate cells were incubated with culture medium alone (control) or 50 mM ethanol for 72 h, followed by vehicle medium or 10 nM BaP treatments for another 6 h. **A-B:** The mRNA level of Nrf2 and NQO1 was determined by quantitative real-time RT-PCR and normalized to GAPDH mRNA. **C-D:** The protein level of Nrf2 and NQO1 was determined by western blot analysis, and expressed relative to β-actin. Values represent the mean ± SEM of five independent experiments. * *P* <0.05 *vs.* cells without ethanol treatment (control), † *P* <0.05 *vs.* cells treated with 50 mM ethanol alone for 7 days, and ‡ P<0.05 *vs.* cells treated with BaP but without ethanol.

8-Isoprostanes are prostaglandin-like compounds produced non-enzymatically by free radical-catalyzed peroxidation of arachidonoyl lipids and are believed to be good indicators of oxidative stress [29]. The present study examined the time-dependent effect of ethanol on cellular 8-isoprostane level. As seen in Fig. 6, exposure of MHSCs to 50 mM ethanol for 6, 24 and 72 h elevated 8-isoprostane level by ~33, 69 and 82%, respectively, as compared to ethanol-untreated control cells. The isoprostane level was significantly higher in all the three groups of ethanol-treated MHSCs than in ethanol-untreated cells, and was significantly higher in cells treated with ethanol for 24 and 72 h than in those that were treated with ethanol for 6 h. This suggests that ethanol exposure induced a time-associated increase in cellular oxidative stress, reflected by elevated lipid peroxidation.

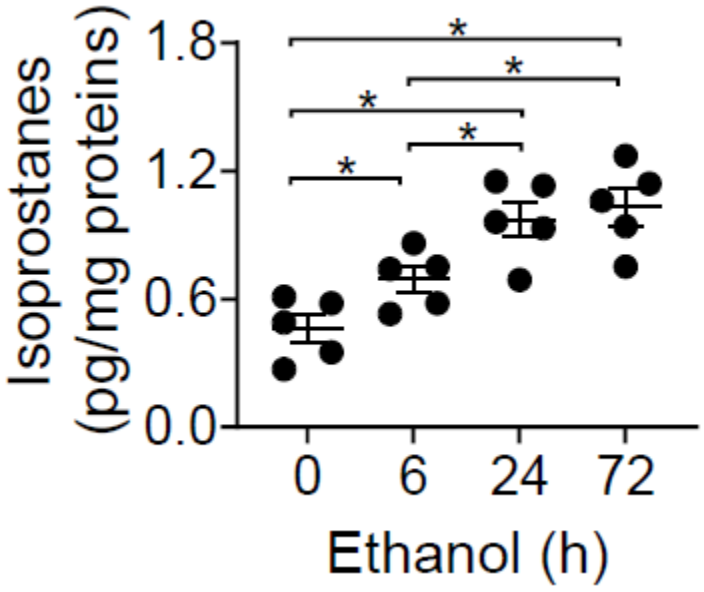

Fig. 6. Plasma 8-isoprostanes in normotensive controls and hypertensive patients. Concentrations were measured using an enzyme immunoassay as described in the Materials and Methods. Values represent the mean ± SEM. * represents a statistically significant difference from normotensive controls.

Ethanol exposure increases lipid peroxidation

DISCUSSION

The current report demonstrated the bidirectional effect of ethanol on NQO1 expression in MHSCs, *i.e.,* short-term ethanol exposure increases and long-term ethanol exposure decreases the protein and mRNA levels of NQO1. Our study also revealed the causative role of the transcription factor Nrf2 in ethanol-induced NQO1 expression. Specifically, we observed that short-term ethanol exposure elevated the protein level of Nrf2 in the whole-cell lysate and nuclear extract. Knockdown of Nrf2 significantly diminished ethanol-induced NQO1 expression. Mutation of the ARE, the consensus regulatory sequence for Nrf2 binding, in the NQO1 promoter region suppressed the basal and ethanol-induced activities of the NQO1 promoter. These are consistent with our previous findings showing that knockdown of Nrf2 repressed BaP-induced NQO1 expression, and mutation of the ARE in the NQO1 promoter diminished its basal and BaP-induced activities [24]. Taken together, it is highly likely that Nrf2 is an essential transcription factor for modulation of NQO1 expression, under both the unstressed and stressed conditions.

It has been well documented that, under the unstressed conditions, Nrf2 is sequestrated in the cytosolic compartment by Kelch-like-ECH-associated protein 1 (keap1), where Nrf2 is ubiquitinated and degraded in proteasomes [30]. In the cells exposed to electrophilic and oxidative stresses, Nrf2 escapes from keap1's sequestration and the subsequent ubiquitin-mediated proteasomal degradation, resulting in greater translocation of Nrf2 to the nucleus and increased expression of its target genes [30]. It has been known that ethanol can increase cellular oxidative stress via multiple means [4, 17]. Specifically, the Cyp2E1-catalyzed oxidation of ethanol to acetaldehyde converts NADPH to NADP+ and generates ROS. The aldehyde dehydrogenase- catalyzed oxidation of acetaldehyde to acetate also generates ROS. In addition, the reactive intermediate of ethanol, acetaldehyde, could induce damage to the mitochondria and activate membrane NADPH oxidases, and thus increasing ROS production. Indeed, increase in oxidative stress has been suggested as a mechanism of increased Nrf2 protein in mice with acute ethanol exposure [31]. Data from the current study showed that exposure of MHSCs to ethanol increased 8-isoprostane levels, suggesting that oxidative stress is, at least partially, responsible to the increased Nrf2 protein in the cells with short-term ethanol treatment.

Our data also show that short-term ethanol exposure increased the mRNA level of Nrf2 in MHECs, suggesting that ethanol not only enhances the stability of Nrf2 protein but also upregulates Nrf2 expression at the transcriptional level. Several transcription factors, such as AhR, activating transcription factor and nuclear factor-κB, have been reported to regulate the transcription of Nrf2 [32]. We previously reported that short-term ethanol

elevates nuclear AhR and upregulates the mRNA expression of its target genes CYP1A1 and 1B1 in MHSCs [21]. The current report confirmed the nuclear AhR-elevating effect of short-term ethanol exposure. In addition, we observed that knockdown of AhR reduced the mRNA and protein level of Nrf2, suggesting that activation of AhR is accountable for ethanol-induced Nrf2 expression. In addition, our data revealed that knockdown of AhR reduced the ethanol-induced NQO1 expression; however, mutation of the XRE did not alter the promoter activity of NQO1. Together with the regulatory role of AhR on Nrf2 and the role of Nrf2 on NQO1, our data suggest that ethanol-induced NQO1 expression is controlled hierarchically by AhR and Nrf2.

Activation of the AhR-Nrf2 pathway is also a mechanism by which BaP, a typical AhR ligand, induces NQO1 expression [24]. It has been well documented that the ligand-associated AhR translocates into the nucleus, where it forms a heterodimer with the AHR nuclear translocator [33]. This heterodimer binds to XREs of target genes, inducing transcription [33]. Besides numerous xenobiotics, a number of endogenously generated compounds also can function as AhR ligands. They include, but are not limited to, tryptophan metabolites, tetrapyrroles, 7-ketocholesterol, and arachidonic acid metabolites [34]. There are indicators that ethanol might modulate the metabolism of arachidonic acids and tryptophan, and boosts the generation of compounds that can bind AhR and trigger its transcriptional activity [18-20]. Further studies are required to identify those ethanol-induced compounds that are able to activate AhR and upregulates the expression of Nrf2 and NQO1.

We, in the present study and a previous report, constantly observed that exposure of cells to ethanol, whether short- or long-term, significantly reduced the mRNA and protein levels of AhR. Downregulation of AhR also has been observed in cells treated with its ligands, such as such as 2,3,7,8-tetrachlorodibenzo-*p*-dion [35] or BaP [36]. It is highly likely that the AhR ligands induced by ethanol are able to inhibit AhR expression. Ligand-induced downregulation of AhR is important for avoiding over-expression of its target genes. However, under-expression of AhR would reduce the amount translocated to the nucleus in response to endogenous and/or exogenous ligands, and therefore diminish the expression of its target genes. Indeed, our data show that long-term ethanol exposure reduced nuclear AhR protein level and NQO1 and Nrf2 expression. In addition, long-term ethanol exposure diminished the capability of MHSCs to increase nuclear AhR protein in response to BaP, and reduced BaP-induced Nrf2 and NQO1 expression. Reduction in NQO1 expression might be of importance to ethanol-induced cytotoxicity and the synergistic effect of ethanol and PAHs. Through its catalytic activity in reduction of quinone to hydroquinone, NQO1 has been implied in a number of physiological activities [37], such as reducing the toxicities of exogenous quinone compounds and endogenously generated quinones; enhancing the antioxidant properties of ubiquinone and vitamin E; and igniting the cofactor activity of vitamin K in biosynthesis of blood-clotting protein prothrombin and bone-building osteocalcin. In addition, NQO1 have been documented to protect antitumor protein p53 from degradation and bioactivates some antitumor agents [37].

Besides modulating NQO1 expression, the AhR-Nrf2 pathway also regulates the expression of a number of GSTs and UGTs [14-16]. Ethanol-induced inhibition of the AhR-Nrf2 pathway might downregulate these phase II XMEs, and therefore slow down the clearance of the reactive PAH intermediates. Furthermore, Nrf2 regulates the expression of a number of enzymes/proteins involved in antioxidant defense and NADPH regeneration [37]. Downregulation of the AhR-Nrf2 pathway might also lessen these cytoprotective molecules, and therefore contribute to the pathologic process induced by ethanol and PAHs. Ethanol has been reported to aggravate the toxicity induced by PAHs. For example, combined treatment of cells with ethanol and BaP has been shown to induce more BaP-DNA adducts than BaP treatment alone [38], and that alcohol consumption and cigarette smoking show synergistic effects on the induction of p53 mutations [39], cancer development, and nonmalignant chronic respiratory illness [8]. 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 [9]. Currently, it remains unknown whether downregulation of the AhR-Nrf2 pathway is a mechanism responsible for the synergistic toxicity induced by alcohol and cigarette.

CONCLUSION

The current report brings to light the bidirectional effect of ethanol on Nrf2 and NQO1 expression and the causal role of AhR in this ethanol-induced episode. Specifically, our data demonstrated that short-term ethanol exposure upregulates the expression of Nrf2 and its target gene NQO1, and that activation of AhR is accountable for the increased expression of Nrf2 and the subsequent expression of NQO1. On the other hand, ethanol inhibits AhR expression. Long-

term ethanol exposure sustains AhR at a low level, and therefore reduces the inductility of Nrf2 and NQO1 in response to endogenous and exogeneous ligands. Besides NQO1, the AhR-Nrf2 pathway has been shown to regulate hundreds of genes throughout the genome. Results from the current study provide a groundwork for further exploring the genes that contribute to the synergistic toxicity of ethanol and PAH compounds.

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STATEMENT OF ETHICS

This research did not involve human subjects or animal models.

CONFLICTS OF INTEREST

The authors declare that no conflicts of interest exist.

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AUTHOR CONTRIBUTIONS

Zhongmao Guo and Hong Yang designed the experiments, analyzed the data, wrote the manuscript, and acquired funding for the experiments. Ningya Zhang performed the experiments and was involved in data analysis.

DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are reported in the paper.

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