Ethanol-induced HO-1 and NQO1 Are Differentially Regulated by HIF-1α and Nrf2 to Attenuate Inflammatory Cytokine Expression

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Oxidative stress plays an important role in alcohol-induced inflammation and liver injury. Relatively less is known about how Kupffer cells respond to oxidative stress-induced expression of heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase (NQO1) to blunt inflammation and liver injury. We showed that Kupffer cells from ethanol-fed rats and ethanol-treated rat Kupffer cells and THP-1 cells displayed increased mRNA expression of HO-1, NQO1, and hypoxia-inducible factor-1α (HIF-1α). Our studies showed that silencing with HIF-1α and JNK-1 siRNAs attenuated ethanol-mediated mRNA expression of HO-1, but not NQO1, whereas Nrf2 siRNA attenuated the mRNA expression of both HO-1 and NQO1. Additionally, JunD but not JunB formed an activator protein-1 (AP-1) oligomeric complex to augment HO-1 promoter activity. Ethanol-induced HO-1 transcription involved antioxidant response elements, hypoxia-response elements, and an AP-1 binding motif in its promoter, as demonstrated by mutation analysis of the promoter, EMSA, and ChIP. Furthermore, livers of ethanol-fed c-Junα/β mice showed reduced levels of mRNA for HO-1 but not of NQO1 compared with ethanol-fed control rats, supporting the role of c-Jun or the AP-1 transcriptional complex in ethanol-induced HO-1 expression. Additionally, attenuation of HO-1 levels in ethanol-fed c-Junα/β mice led to increased proinflammatory cytokine expression in the liver. These results for the first time show that ethanol regulates HO-1 and NQO1 transcription by different signaling pathways. Additionally, up-regulation of HO-1 protects the liver from excessive formation of inflammatory cytokines. These studies provide novel therapeutic targets to ameliorate alcohol-induced inflammation and liver injury.

Alcoholism is the third leading cause of preventable mortality in the United States behind cigarette smoking and obesity and contributes to about 100,000 deaths annually (1, 2). Histopathological features of alcoholic liver disease include fatty liver (steatosis) followed by liver inflammation (steatohepatitis), cirrhosis, and hepatocellular carcinoma (3–5). It has been hypothesized that reactive oxygen species (ROS)3 generated by ethanol metabolism via CYP2E1 in hepatocytes contributes to liver injury. However, no differences in liver injury and ROS formation are observed in CYP2E1−/− mice compared with wild-type mice upon ethanol feeding (6). However, studies show ethanol increases gut permeability to Gram-negative bacteria endotoxins, which activate Kupffer cells to generate ROS and the potent inflammatory mediator TNF-α, causing liver injury (5). It has been shown that oxidants derived from NADPH oxidase in Kupffer cells contribute to cytotoxic TNF-α production. This is demonstrated in studies wherein ethanol-fed p47phox knock-out mice, lacking a critical NADPH oxidase subunit, show reduced ROS and TNF-α production in Kupffer cells and result in reduced liver injury (7).

To counteract these oxidative stress insults, higher animals have evolved defense mechanisms, including antioxidant proteins and phase II detoxifying enzymes (8, 9). Induction of phase II enzymes, e.g. heme oxygenase-1 (HO-1) and NADPH-quinone oxidoreductase-1 (NQO1), renders cells more resistant to the potential subsequent challenges of greater stress. The importance of HO-1 expression in mediating antioxidant and anti-inflammatory effects has been well characterized both in vitro and in vivo (10–14) and corroborated in HO-1 knock-out mice (15, 16) and patients with HO-1 deficiency (17).

The antioxidant response element (ARE), a cis-acting element, has been shown to be involved in the transcriptional regulation of redox-regulated gene products, including HO-1 (18). Further studies demonstrate that nuclear factor erythroid 2-related factor (Nrf2) binds to ARE sites in the promoter of cytoprotective phase II genes to regulate their expression (19–21). Nrf2 is bound to its inhibitor Keap1 in the cytosol of the cell. Under oxidative stress conditions, Nrf2 dissociates from Keap1

3 The abbreviations used are: ROS, reactive oxygen species; HO-1, heme oxygenase-1; NQO1, NAD(P)H-quinone oxidoreductase-1; KC, Kupffer cell; pKC, rat KC; eKC, ethanol-fed KC; c-KC, isocaloric-fed control KC; HIF-1α, hypoxia-inducible factor-1α; Nrf2, nuclear factor erythroid 2-related factor; AP-1, activator protein-1; ARE, antioxidant response element; HRE, hypoxia response element; DPI, diphenyliodonium; TBP, TATA binding protein; Dn, dominant negative; qRT, quantitative real-time; PI, phosphatidylinositol; MAP, mitogen-activated protein.
Ethanol-induced HO-1 and NQO1 Are Differentially Regulated

and translocates to the nucleus wherein it oligomerizes with the proteins Maf, Jun, Fos, ATF4, and/or CBP (cAMP-response element-binding protein (CREB)-binding protein) and induces transcription by binding to the ARE sites of target genes (21, 22). The role of Nrf2 in up-regulating the expression of phase II enzymes has been corroborated in vivo using Nrf2 knock-out mice (23).

HO-1 expression has been shown to be induced by a variety of stimuli, such as LPS, cytokines, cigarette smoke, oxidative stress (17, 19, 24), and antioxidant photochemicals (25). The ARE core sequence is present in mouse and human HO-1 promoters (26) as well as other phase II enzymes, which have an AP-1 or AP-1 like motif (8). Recent studies have shown that JunB binding to the AP-1 site activates, whereas JunD represses human HO-1 gene expression (27). LPS and cytokine-mediated induction of HO-1 has been shown to involve AP-1 transcription factors (28). However, relatively less is known of the molecular mechanism by which ethanol-mediated oxidative stress insult is counteracted in liver cells.

In this report Kupffer cells (KCs) derived from ethanol-fed rats and ethanol treatment of rat Kupffer cells and human THP-1 monocytic cells showed increased mRNA expression of HIF-1α, HO-1, and NQO1. Our studies showed that ethanol-mediated up-regulation of HO-1 involved activation of JNK-1, HIF-1α, and Nrf2, whereas NQO1 expression utilized only Nrf2. Furthermore, the AREs, hypoxia-response elements (HREs), and an AP-1 binding motif in the promoter of human HO-1 were essential for ethanol induced HO-1 transcription. Additionally, JunD augmented, whereas JunB repressed ethanol-induced HO-1 promoter activity. Furthermore, livers of ethanol-fed c-Jun^fl/fl mice showed attenuated mRNA levels of HO-1, but not NQO1, supporting the role of c-Jun in ethanol-induced HO-1 expression. Our in vitro and in vivo results provide evidence that ethanol-induced oxidative stress differentially regulated the expression of HO-1 and NQO1 in liver cells. Moreover, our studies for the first time demonstrated that attenuated levels of HO-1 in vitro and in vivo led to increased expression of proinflammatory cytokines, indicating that liver HO-1 levels affect the inflammatory state of the liver. These studies also showed that oxidative stress-induced signaling that leads to the expression of phase II enzymes, namely HO-1 and NQO1, occurred through divergent pathways.

EXPERIMENTAL PROCEDURES

Animal Experiments—The experimental protocol and use of animals was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California for this study. Studies of chronic alcohol administration in male Wistar rats involved implantation of gastrostomy catheters as previously described (29). These rats were fed a high-fat diet (35% calories as corn oil) via intragastric infusion supplemented with an increasing dose of ethanol (9–16 g/kg per day) or isocaloric dextrose solutions (30) and weekly enteral lipopolysaccharide (5 mg/kg) over 9 weeks (ALPD Animal Core, University of Southern California).

Because the c-Jun-null mutation results in embryonic lethality, we utilized c-Jun floxed mice (c-Jun^fl/fl) (31), which were kindly provided by Dr. Bruce D. Carter (Vanderbilt University Medical School, Nashville, TN). Briefly, knock-out of c-Jun in immune cells and sinusoidal endothelial cells was accomplished by crossing heterogeneous c-Jun^fl/fl mice with Mx-1-Cre mice (The Jackson Laboratory, Bar Harbor, ME) to generate c-Jun^fl/fl,Mx-1-Cre(+) mice, which were then crossed with c-Jun^fl/fl mice to generate c-Jun^fl/fl,Mx-1-Cre(−) mice. Methods used for genotyping were described previously (37). Mice were bred to homozygosity, as measured by PCR analysis of tail DNA. Poly(I:C) was injected to induce the knock-out of c-Jun by recombination. In c-Jun^fl/fl,Mx-1-Cre mice, liver Cre expression was augmented by poly(I:C) (GE Healthcare). Briefly, 300 µl of poly(I:C) solution (1 mg/ml in PBS) was injected intraperitoneal 3 times at 48-h intervals. These mice were then used for ethanol challenge studies, where they were fed in pairs with the Lieber-DeCarli control liquid diet or ethanol (36% of total calories) containing liquid diet (32). Both control and ethanol diet also contained 18% of calories from protein and 8% from fat; for the ethanol diet, a portion of the carbohydrates was isocalorically replaced with ethanol (33). Liver samples were obtained from c-Jun^fl/fl mice and wild-type mice that were either fed control or ethanol diet.

Isolation and Culture of Kupffer Cells and THP-1 Monocytic Cells—Kupffer cells were isolated from normal male Wistar rats, alcohol-fed (E-rKCs) and pair-fed control rats (C-rKCs) by the Non-Parenchymal Liver Cell Core of Southern California Research Center for Alcoholic Liver and Pancreatic Diseases and Cirrhosis at the University of Southern California. Briefly, livers were digested in situ with Pronase and collagenase. The digests were subjected to arabinogalactan density gradient sedimentation followed by isolation and purification of KCs as previously described (30). Normal KCs were cultured in low glucose, DMEM supplemented with 5% FBS for 2 days before initiation of experimental protocols. KCs isolated from alcohol-fed and pair-fed rats were cultured for 3 h in serum-free medium before treatment. THP-1 cells were maintained in culture (34) and kept overnight in serum-free medium before treatment.

Reagents—DPI and GF109203X were purchased from Sigma. LY294002, SB203580, SP600125, and R59949 were obtained from Tocris (Ellisville, MO). Primary antibodies against HIF-1α, β-actin, phospho-JNK-1 and JNK, HO-1 and secondary antibodies conjugated to HRP were obtained from Santa Cruz, CA. Protein phosphatase 1 was purchased from Biomol International Inc. (Plymouth Meeting, PA). These pharmacological inhibitors were utilized at the following concentrations, as deemed optimal from the literature: DPI (10 µM), GF109203X (25 µM), LY294002 (15 µM), SB203580 (1 µM), SP600125 (100 nM), R59949 (30 µM), and protein phosphatase 1 (10 µM). Primary antibodies against HIF-1α, β-actin, phospho-JNK-1 and JNK, HO-1, and secondary antibodies conjugated to HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HIF-1α siRNA was synthesized at the University of Southern California Comprehensive Cancer Center Microchemical core facility as described previously (35). siRNAs for p47phox, p38 MAPK, JNK-1, JNK-2, Src-1, Nrf2, p65 (a subunit of NF-kB), c-Jun, JunD, JunB, and control siRNA were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The dominant negative (Dn) PI3K expression plasmid was kindly provided by Dr. Debbie Johnson (University of Southern California, Los Angeles, CA). The targets of the inhibitors and siRNA mentioned are described in

35360 JOURNAL OF BIOLOGICAL CHEMISTRY
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supplemental Table I. The human −9.2-kb HO-1-pGL3, −4.5-kb HO-1-pGL3, and mutant construct of the −4.5-kb HO-1-pGL3 promoter with a deletion of the ARE enhancer region E1 and the JunB expression plasmid were generously provided by Dr. Anupam Agarwal (University of Alabama, Birmingham, AL) (27, 36). Mutant constructs of the HO-1-luc promoter were generated using wild-type −4.5-kb HO-1 construct as a template with the QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) using the primers shown in Table 1. Mutations were confirmed by sequencing. c-Jun, c-Fos, and JunD expression plasmids were kindly provided by Dr. Michael Karin (University of California, San Diego, CA). All siRNAs and overexpression plasmids used in this study were shown to knock down or augment both the mRNA (supplemental Fig. S2) and protein (supplemental Fig. S3) expression of their corresponding genes. The oligonucleotides used for electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) analysis were obtained from Integrated DNA Technologies (Coralville, IA). All other reagents were obtained from Sigma unless otherwise indicated.

Isolation of RNA and qRT-PCR—Total RNA was extracted from KCs, THP-1, and liver samples using TRIzol reagent (Invitrogen). Cells in culture were treated with 100 mM ethanol, unless otherwise indicated, for the indicated time periods. Cells were preincubated for 30 min with pharmacological inhibitors before ethanol treatment. Using specific mRNA primers shown in Table 1, mRNA expression levels of genes were determined and quantified. Real-time quantitative PCR of mRNA templates was performed using the iScript SYBR Green One-Step RT-PCR Kit (Bio-Rad) and the ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). Briefly, PCR amplification of RNA (100 ng) was performed under the following conditions: cDNA synthesis at 50 °C for 10 min, iScript reverse transcriptase inactivation at 95 °C for 5 min, and PCR cycling and detection for 40 cycles at 95 °C for 10 s followed by 60 °C for 30 s. Values are expressed as relative mRNA expression that has been normalized to housekeeping GAPDH mRNA.

Preparation of Cytosolic and Nuclear Extracts and Western Blot Analysis—Cultured THP-1 and KCs in serum-free medium were treated with ethanol (100 mM) for the indicated time periods. Cytosolic and nuclear extract proteins were obtained as previously described (37) and were subjected to SDS-gel electrophoresis followed by transfer to nitrocellulose membranes. Membranes were probed with a HIF-1α antibody (1:250), HO-1 antibody (1:250), or a phosphorylated JNK-1 antibody (p-JNK-1) (1:250) as described. Blots were also probed with primary antibodies for p47phox (1:500), p38 MAPK (1:500), Src-1 (1:250), Nrf2 (1:100), c-Jun (1:200), c-Fos (1:200), JunD (1:200), and JunB (1:200) followed by incubation with anti-rabbit or anti-goat IgG secondary antibodies. These membranes were stripped and re-probed with β-actin (1:2500), TATA-binding protein (TBP; 1:2000), GAPDH (1:2000), or unphosphorylated JNK antibodies (1:250) to monitor protein loading. For Western blots of THP-1 or KC nuclear extracts, either β-actin or TBP was used as a loading control, as neither protein expression changed in response to treatment and both were found to be expressed in nuclear extracts (supplemental Fig. S4). Antibodies to HO-1 and TBP were obtained from Abcam Inc. (Cambridge, MA), whereas the remaining antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The protein bands were detected using Immobilon Western reagents (Millipore Corp., Billerica, MA) or LumiGlo reagents (Thermo Scientific, Rockford, IL) and exposed in a Bio-Rad Chemidoc XRS/HQ.

THP-1 Transient Transfections with Promoter Constructs and siRNA—Cultured THP-1 (1 × 10⁶ cells) were suspended in 100 μl of Nucleofector Solution (Nucleofector kit V, Amoxa Biosystems, Cologne, Germany) with either 50 nm siRNA constructs or 0.5 μg of luciferase reporter plasmids and 0.5 μg of a pCMV-β-galactoside vector followed by nucleofection in accordance with the manufacturer’s instructions using the V-001 program (Amaza Nucleofector II). The cells were kept overnight in complete RPMI 1640 medium containing 10% FBS. After 24 h post-transfection, the cells were harvested and incubated in serum-free medium for 3 h and then treated with ethanol for the indicated time periods. The cells were collected, lysed, and analyzed for luciferase activity using a luminometer (Lumat LB 950, Berthold, Badwildbad, Germany) and β-galactosidase activity using kits (Promega, Madison, WI) as described previously (37). For transfection efficiency, luciferase values were normalized to β-galactosidase values. Data are expressed relative to promoter-less pGL3 vector activity.

Transient Transfections of Kupffer Cells with siRNAs—Cultured rat Kupffer cells (1 × 10⁶ cells) were suspended in 100 μl of Lipofectamine 2000 plus reagent (Invitrogen) and 50 nm siRNA constructs followed by nucleofection in accordance with the manufacturer’s instructions using the V-001 program (Amaza Nucleofector II). The cells were kept overnight in complete low glucose-DMEM supplemented with 5% FBS. After 24 h post-transfection, the cells were harvested and incubated in serum-free medium for 3 h and then treated with ethanol for 12 h. The cells were collected, lysed, and analyzed for mRNA expression using qRT-PCR.

EMSA for Nrf2, AP-1 Complex, and HIF-1α—The double-stranded consensus oligonucleotides (shown in Table 1) were biotin-labeled using a light shift chemiluminescent EMSA kit (Pierce) as previously described (37). The probes corresponded to ARE, AP-1, and HRE flanking the respective ARE (−3936 to −3927), AP-1 (−913 to −907), and HRE (−42 to −39) sites of the wild-type HO-1 promoter and HO-1 promoter with mutations in these sites. Briefly, the DNA binding reaction consisted of 10 μg of nuclear extract, 5% glycerol, 5 mM MgCl₂, 50 ng/μl poly(dI-dC), 0.05% Nonidet P-40, and 0.5 ng of biotinylated duplex DNA, which was incubated for 20 min at room temperature. The samples were then subjected to gel electrophoresis and transferred to a Hybond-N⁺ nylon membrane (Amersham Biosciences). Band detection was performed using streptavidin–HRP/chemiluminescence kit (Pierce). DNA–protein interaction specificity was established using a 50-fold excess of unlabeled probe or use of a mutant probe.

ChIP Assay—THP-1 (1 × 10⁶ cells) was cultured in serum-free medium and treated with ethanol (100 mM) for the indicated time period with or without pharmacological inhibitors. Chromatin immunoprecipitation analysis (37) was performed using a HIF-1α antibody. Immunoprecipitated DNA was PCR-
amplified using primers for the HO-1 promoter region (−862 to −666 bp) (Table 1), yielding a 350-bp product. PCR products were subjected to 2% agarose gel electrophoresis, visualized by staining with ethidium bromide, and quantitated by densitometric analysis (37). All values were normalized to input DNA.

Enzyme-linked Immunosorbent Assay (ELISA) for TNF-α and IL-1β Release—THP-1 (1.5 × 10^6 cells/ml) was transfected with siRNAs for either Nrf2 or c-Jun and then cultured in serum-free medium before ethanol (100 mM) treatment for 24 h. Supernatants from these cells were collected and assayed for TNF-α and IL-1β release using specific DuoSet Enzyme-Linked Immunosorbent Assays (R&D Systems, Minneapolis, MI) for TNF-α and IL-1β, respectively. All assays were performed in accordance with manufacturer’s instructions.

Statistical Analysis—Data are presented as the means ± S.D. Student’s t test was utilized to determine the significance of difference between untreated and alcohol-treated samples. The significance of difference in mean values between multiple groups was conducted with parametric one-way analysis of variance followed by a Tukey-Kramer using the Instat 2 software program (GraphPad, San Diego, CA). Values of p < 0.05 were considered statistically significant.

RESULTS

Ethanol Augments HO-1 and HIF-1α mRNA Expression in Rat Kupffer Cells (rKCs) in Vivo and in Vitro—KCs isolated from ethanol-fed rats (E-rKCs) compared with KCs from isocaloric fed control rats (C-rKCs) showed an ∼2.5-fold increase in HO-1 mRNA expression as determined by qRT-PCR. Additionally, there was ∼1.7-fold increase in HIF-1α mRNA expression in E-rKCs compared with C-rKCs (Fig. 1A). We have previously shown that ethanol-mediated expression of RANTES (regulated upon activation normal T cell expressed and secreted) and endothelin-1 was dose-dependent (25–100 mM) with optimal expression at 100 mM ethanol (38, 39); thus, we utilized this dose of ethanol for studies described herein. Although this concentration of alcohol (0.46%) may seem physiologically high, studies (40) show that the metabolism of ethanol increases to accommodate increased alcohol intake in chronic alcoholics. Treatment of C-rKCs with ethanol (100 mM) showed 3- and 7-fold increase in HO-1 mRNA expression at 12 and 24 h, respectively. Moreover, HIF-1α mRNA levels increased 2- and 6-fold at 12 and 24 h, respectively.

Ethanol Augments HO-1 and NQO1 Are Differentially Regulated

Ethanol augments HO-1 mRNA expression in rKCs. A, shown is ethanol-induced mRNA expression of HO-1 and HIF-1α in rKCs isolated from ethanol-fed rats (E-rKCs) as determined by qRT-PCR. The data represent the fold increase after ethanol feeding compared with isocaloric fed control in KCs. B, shown is ethanol-induced mRNA expression of HO-1 and HIF-1α in ethanol-treated rKCs. The data of ethanol-treated rKCs represent a fold increase in mRNA expression after treatment with ethanol (100 mM) for the indicated time period compared with no ethanol treatment. All mRNA expression levels were normalized to GAPDH mRNA levels, and the data shown represent three independent experiments (mean ± S.D.). p values are denoted as: **, p < 0.01; and ***, p < 0.001.

Ethanol Increases mRNA Expression of HO-1 in THP-1 Cells via Activation of Src-1 Kinase, PI 3-Kinase, p38 MAP Kinase, and JNK-1—Because ethanol in vivo and in vitro induced HO-1 mRNA expression in rKCs, we examined whether a human monocytic cell line (THP-1) exhibited a similar response to ethanol as was seen in rKCs. We utilized THP-1 as a model system for elucidating ethanol-induced cell signaling pathways for ease of culture and transfection efficiency. There was an increase in HO-1 mRNA expression at 4, 8, and 24 h, with maximal and sustained increase of 5-fold after 8 h (Fig. 2A). Moreover, there was a dose-dependent increase in HO-1 mRNA level in response to 25–100 mM ethanol treatment for 8 h, which was maximal at 100 mM (data not shown). Additionally, ethanol-induced HO-1 mRNA levels were attenuated by 4-methylpyrazole (alcohol dehydrogenase inhibitor) and cyanamide (aldehyde dehydrogenase inhibitor) to the extent of 99 ± 4.1 and 95 ± 2.5%, respectively (supplemental Fig. S1). Acetaldehyde (1.0 mM)-induced HO-1 expression was attenuated by cyanamide but not with 4-methylpyrazole (supplemental Fig. S1). The viability of THP-1 cells was unaffected by ethanol (100 mM) treatment for 8 h. As shown in Fig. 2B, transfection of THP-1 cells with siRNAs for p47phox (NADPH oxidase subunit), p38 MAP kinase, Src-1 kinase, and N-terminal Jun kinase (JNK-1) resulted in 80–100% (p < 0.001) attenuation of ethanol induced HO-1 mRNA expression. Transfection with Dn PI 3-kinase also reduced >90% ethanol-induced HO-1 mRNA expression. However, transfection of THP-1 cells with JNK-2 siRNA or mock siRNA did not affect HO-1 mRNA expression (Fig. 2B). Taken together, these results suggested that ethanol metabolism was required for HO-1 expression. Moreover, these studies showed the involvement of Src-1 kinase, NADPH oxidase, PI 3-kinase, p38 MAP kinase, and JNK-1 in ethanol induced HO-1 mRNA expression.

Ethanol-induced HIF-1α mRNA and Protein Expression—Previous studies (41) show that hypoxia causes an increase in HIF-1α protein levels without affecting HIF-1α mRNA levels. We observed that ethanol (100 mM) treatment of THP-1 cells led to a time-dependent increase in HIF-1α mRNA expression at 4, 8, and 24 h, with a maximal increase of 3-fold at 8 h (Fig. 2C). As shown in Fig. 2D, Dn PI3K attenuated HIF-1α mRNA expression, whereas p47phox siRNA did not affect HIF-1α mRNA levels. Because siRNA for p47phox inhibited HO-1 mRNA expression (Fig. 2B), we examined whether ROS
affected HIF-1α protein levels. As shown in Fig. 2E, ethanol induced a time-dependent (2–4 h) increase in HIF-1α protein levels in nuclear extracts of THP-1 cells, which was maximal (2.3-fold) at 2 h. Furthermore, ethanol-induced HIF-1α protein levels were reduced below basal level by PI 3-kinase inhibitor (LY294002) but were unaffected by NADPH oxidase inhibitor (DPI) (Fig. 2F). As shown, the levels of β-actin protein remained constant in response to ethanol treatment of THP-1 cells. These results indicated that ethanol-mediated PI 3-kinase activation led to increase in both mRNA and protein levels of HIF-1α, whereas the NADPH oxidase pathway was not involved in HIF-1α expression.

**Ethanol-mediated Nrf2 mRNA Expression Involves Src-1 Kinase, NADPH Oxidase, PI 3-Kinase, and p38 MAP Kinase**—Previous studies (10) show the importance of transcription factor Nrf2 in regulating ARE-dependent transcription of HO-1 and NQO1 genes. LPS-induced NQO1 expression requires the transcriptional activation of Nrf2 (10). Thus, we determined whether ethanol augmented mRNA expression of Nrf2 in THP-1 monocytic cells. Ethanol treatment of THP-1 cells resulted in an ~5-fold increase in Nrf2 mRNA expression (Fig. 3A). Transfection of THP-1 cells with siRNAs for p47phox, p38 MAP kinase, Nrf-2, and Src-1 kinase resulted in complete abrogation of ethanol-induced Nrf2 mRNA expression. However, transfection of THP-1 cells with either JNK-1 or JNK-2 siRNAs did not affect ethanol-mediated Nrf2 mRNA expression (Fig. 3A). Taken together, these results showed that ethanol induced signaling leading to Nrf2 mRNA expression involved Src-1 kinase, NADPH oxidase, PI 3-kinase, and p38 MAP kinase but not JNK-1 and JNK-2.

**Ethanol-induced NQO1 Gene Expression Involves Src-1 Kinase, NADPH Oxidase, PI 3-Kinase, and p38 MAP Kinase but Not JNK-1/2**—Ethanol treatment of THP-1 cells for 8 h resulted in an ~4-fold increase in NQO1 mRNA expression (Fig. 3B). Transfection of THP-1 cells with siRNAs for p47phox and p38...
MAP kinase and Dn PI 3-kinase expression plasmid completely abrogated ethanol-induced NQO1 expression to either basal or below basal levels (Fig. 3B). Transfection of THP-1 with siRNAs for Nrf2 or Src-1 reduced /H1101175% of ethanol-mediated NQO1 expression, whereas JNK-1 siRNA and JNK-2 siRNA had no effect (Fig. 3B). Taken together, these results showed that ethanol-mediated cell signaling leading to NQO1 expression involved Src-1 kinase, NADPH oxidase, PI 3-kinase, and p38 MAP kinase. However, JNK-1 and JNK-2 were not involved in NQO1 expression, as observed for Nrf2 expression.

Ethanol-mediated HO-1 Expression Involves Both HIF-1α and Nrf2 Transcription Factors in Human Monocytic Cells—To determine whether HIF-1α and Nrf2 played a role in ethanol-mediated HO-1 expression, THP-1 cells were transfected with siRNAs for HIF-1α and Nrf2. As shown in Fig. 4A, transfection with HIF-1α siRNA completely reduced HO-1 expression, whereas control mock siRNA had no effect. Moreover, transfection of THP-1 cells with siRNA for Nrf2 attenuated HO-1 expression by ~60%. These data indicate the involvement of Nrf2 and HIF-1α in ethanol-mediated HO-1 expression in human THP-1 monocytic cells.

Differential Roles of HIF-1α and Nrf2 in Ethanol-induced Expression of HO-1 and NQO1 in rKCs—Because ethanol-mediated HO-1 expression in THP-1 cells showed the involvement of Nrf2 and HIF-1α, we determined whether the same transcription factors were involved in ethanol-mediated activation of phase II enzymes, namely HO-1 and NQO1, in rat Kupffer cells. Treatment of C-rKCs with ethanol led to an increase in the mRNA expression of HIF-1α, Nrf2, HO-1, and NQO1 (Fig. 4B). Transfection with HIF-1α siRNA attenuated HIF-1α and HO-1 mRNA expression, whereas expression of Nrf2 and NQO1 was not affected (Fig. 4B). Additionally, transfection of rKCs with Nrf2 siRNA followed by ethanol treatment resulted in ~80% reduction of Nrf2, HO-1, and NQO1 mRNA expression (Fig. 4B), whereas expression of HIF-1α remained unchanged. Taken together, these results indi-
cated the involvement of both Nrf2 and HIF-1α in ethanol-mediated HO-1 gene expression. However, Nrf2, but not HIF-1α, was involved in NQO1 gene expression.

Differential Role of JNK-1 in Ethanol-mediated HO-1 and NQO1 mRNA Expression in rKCs—As shown in Fig. 4B, transfection of rKCs with JNK-1 siRNA followed by ethanol treatment resulted in complete attenuation of HO-1 mRNA expression, compared with ethanol-treated rKCs. However, mRNA expression of HIF-1α, Nrf2, and NQO1 were not affected. These results indicated that JNK-1 was involved in ethanol-mediated HO-1 expression. However, JNK-1 did not play a role in ethanol-mediated gene expression of HIF-1α, Nrf2, and NQO1 in rKCs.

Ethanol-mediated HO-1 Protein Expression—As shown in Fig. 4C, ethanol treatment of rKCs resulted in an increased HO-1 protein expression in the nuclear extracts of these cells. Inhibitors of NADPH oxidase (DPI), PI 3-kinase (LY294002), and p38 MAP kinase (SB203580) reduced ethanol-induced HO-1 protein expression below the basal level (Fig. 4C). These results indicated that ethanol-mediated HO-1 protein expression involved activation of NADPH oxidase, PI 3-kinase, and p38 MAP kinase.

Role of ARE in Ethanol-mediated Activation of HO-1 Promoter Activity—The 5′-flanking region of the HO-1 gene contains potential NF-κB, C/EBP, NF-1L6, AP-1, AP-2, ARE, and GATA motifs (12). An in silico analysis of the human HO-1 promoter element (−4500 bp to −1 bp) (GenBankTM accession number AF145047) revealed the presence of two inverted hypoxia response element motifs (RGCAC) at −862 to −859 bp and −669 to −666 and one HRE (RCGTG) at −42 to −39 bp relative to the transcriptional start site, as shown in schematics of Fig. 5A. Furthermore, AP-1 binding sites were found at −1884 to −1878 bp, −1003 to −986 bp, and −913 to −907 bp (Fig. 5A).

Because ethanol-mediated HO-1 expression was attenuated by HIF-1α siRNA and Nrf2 siRNA, we determined whether this occurred via HREs and AREs present in the promoter region of HO-1. The full-length human HO-1 promoter (−15 kb) contains two ARE sites in the enhancer regions at −4.3 and −9 kb, designated as E1 and E2, respectively (36). As shown in Fig. 5B, both −9.2 kb and its serially deleted −4.5-kb HO-1 promoter construct showed an 8-fold induction of promoter activity in response to ethanol, indicating that the −4.5-kb region was sufficient for promoter activity. Deletion of ARE site (E1) in the −4.5-kb region resulted in 68 ± 4% reduction in promoter activity. Furthermore, pharmacological inhibitors of p38 MAP kinase (SB203580), protein kinase C (GF109203X), HIF-1α (R59949), PI 3-kinase (LY294002), NADPH oxidase (DPI), Src kinase (PPI), and JNK (SP600125), which attenuated ethanol-induced HO-1 mRNA expression, also reduced HO-1 promoter activity (Fig. 5C). Because pharmacological inhibitors cannot be nonspecific, we utilized a targeted gene knockdown approach. Transfection of THP-1 cells with siRNAs for p47phox, p38 MAP kinase, Src-1 kinase, and the p65 subunit of NF-κB and dominant negative PI 3-kinase resulted in 61–88 ± 4–6% inhibition of ethanol-induced HO-1 promoter activity (Fig. 5D). However, JNK-1 siRNA, but not JNK-2 siRNA, completely reduced HO-1 promoter activity. Furthermore, transcription with siRNAs for c-Jun, Nrf2, and HIF-1α also completely abrogated ethanol-mediated HO-1 promoter activity (Fig. 5D). Transfection with mock siRNA did not reduce ethanol-mediated HO-1 promoter luciferase activity (Fig. 5D).

To substantiate the role of the HREs and AP-1 in HO-1 promoter activity, we generated mutants of the HRE and AP-1 sites (Table 1) of the HO-1 promoter. Ethanol-induced HO-1 promoter activity was reduced 80–90% upon mutation of either the proximal HRE or AP-1 sites in the promoter region of HO-1 (Fig. 5E). However, mutation of the AP-1 sites designated AP-1_1, AP-1_2, and AP-1_3, as illustrated in the schematics of Fig. 5A, did not show reduced HO-1 promoter activity (Fig. 5E). These results showed that the proximal HRE-1 site (−42 to −39 bp) and proximal AP-1 site (−913 to −907 bp) in the HO-1 promoter were effective in mediating ethanol-mediated HO-1 transcription.

Ethanol-induced Activation of JNK Involves Upstream Activation of Protein Kinase C, Src-1 Kinase, and p38 MAP Kinase—Because ethanol-induced HO-1 transcription involved JNK-1, we examined upstream signaling events involved in its activation. Ethanol caused a time-dependent (5–60 min) change in JNK-1 phosphorylation, with maximal phosphorylation (about 2.2-fold) at 10 min (Fig. 6A, upper panel). The levels of JNK-1 or JNK-2 protein did not change in response to ethanol treatment of THP-1 (Fig. 6A, lower panel). Preincubation of THP-1 cells with inhibitors of protein kinase C (GF109203X), Src-1 kinase (protein phosphatase 1), and p38 MAP kinase (SB203580) resulted in an −45% reduced JNK-1 phosphorylation at 10 min after ethanol treatment (Fig. 6B). However, inhibitor of JNK (SP600125) reduced the phosphorylation of JNK-1 by −70% (Fig. 6B). These results showed that ethanol-mediated JNK activation involved upstream activation of protein kinase C, Src-1 kinase, and p38 MAP kinase.

Ethanol-induced HO-1 Promoter Activity Involves AP-1 Complex Binding Proteins—Because HO-1 promoter activity was attenuated by c-Jun siRNA and the HO-1 promoter contains an AP-1 motif, we determined the role of candidate Jun proteins in the AP-1 complex that regulated ethanol-mediated human HO-1 transcription. Co-transfection studies were performed using c-Fos, c-Jun, JunB, and JunD expression plasmids and the −4.5-kb HO-1 luciferase promoter construct. As shown in Fig. 6C, ethanol treatment of THP-1 increased HO-1 promoter activity by −5-fold compared with untreated cells. Overexpression of c-Fos did not change promoter activity; however, c-Jun caused an −8-fold increase in HO-1 promoter activity (Fig. 6C). Also c-Jun together with c-Fos augmented HO-1 promoter activity by −8.5-fold. JunB alone repressed, whereas JunD enhanced HO-1 promoter activity over and above that seen with ethanol treatment (Fig. 6C). Additionally, JunB co-transfected with c-Fos and c-Jun did not affect HO-1 promoter activity compared with cells transfected with c-Jun.
Ethanol-induced HO-1 and NQO1 Are Differentially Regulated

A

| ARE | AP-1 | AP-1 | AP-1 | HRE | HRE | HRE |
|-----|------|------|------|-----|-----|-----|
| (E-1) | (AP-1) | (AP-1) | (AP-1) | (HRE-3) | (HRE-2) | (HRE-1) |

AF145047 (Human HO-1 -4.5kb)

B

**Mean RLU (fold-change)**

| Ethanol (8h) | + | + | + | + | + |
|-------------|---|---|---|---|---|
| -9.2kb-HO-1:wt | - | - | - | + | - |
| -4.5kb-HO-1:wt | - | - | + | - | - |
| -4.5kb-HO-1ΔE1 | - | - | - | + | - |

C

**Mean RLU (fold-change)**

| Ethanol (8h) | - | + | + | + | + | + | + | + | + | + | + | + | + |
|-------------|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SB203580 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| GF109203X | - | - | - | - | - | - | - | - | - | - | - | - | - |
| RS9949 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| LY294002 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| DPI | - | - | - | - | - | - | - | - | - | - | - | - | - |
| PP1 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| SP600125 | - | - | - | - | - | - | - | - | - | - | - | - | - |

D

**Mean RLU (fold-change)**

| Ethanol (8h) | - | + | + | + | + | + | + | + | + | + | + | + | + |
|-------------|---|---|---|---|---|---|---|---|---|---|---|---|---|
| -4.5kb-HO-1:wt | - | - | - | - | - | - | - | - | - | - | - | - | - |
| p47 siRNA | - | - | - | - | - | - | - | - | - | - | - | - | - |
| D/n PI-3 kinase | - | - | - | - | - | - | - | - | - | - | - | - | - |
| p38 siRNA | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Src-1 siRNA | - | - | - | - | - | - | - | - | - | - | - | - | - |
| p65 siRNA | - | - | - | - | - | - | - | - | - | - | - | - | - |
| JNK1 siRNA | - | - | - | - | - | - | - | - | - | - | - | - | - |
| JNK2 siRNA | - | - | - | - | - | - | - | - | - | - | - | - | - |
| c-Jun siRNA | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Nrf2 siRNA | - | - | - | - | - | - | - | - | - | - | - | - | - |
| HIF-1α siRNA | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Mock siRNA | - | - | - | - | - | - | - | - | - | - | - | - | - |

E

**Mean RLU (fold-change)**

| Ethanol (8h) | - | + | + | + | + | + |
|-------------|---|---|---|---|---|---|
| -4.5kb-HO-1:wt | - | - | - | - | - | - |
| -4.5kb-HO-1:AP-1:M1 | - | - | - | - | - | - |
| -4.5kb-HO-1:AP-1:M1 | - | - | - | - | - | - |
| -4.5kb-HO-1:AP-1:M2 | - | - | - | - | - | - |
| -4.5kb-HO-1:AP-1:M3 | - | - | - | - | - | - |
and c-Fos (Fig. 6C). In contrast, JunD augmented c-Fos and c-Jun mediated HO-1 promoter activity to ~12-fold (Fig. 6C).

To further validate the opposing roles of JunD and JunB in regulating HO-1 transcription, THP-1 cells were co-transfected with HO-1 promoter constructs along with siRNAs for c-Jun, JunD, or JunB. As shown in Fig. 6D, ethanol-mediated HO-1 promoter activity was attenuated with siRNAs for c-Jun and JunD but enhanced with JunB siRNA. Taken together, these results suggested that c-Jun/c-Fos and JunD formed an active AP-1 transcriptional complex that interacted with the AP-1 site of the HO-1 promoter, augmenting its activity. However, JunB was observed to have no effect on c-Fos/c-Jun-mediated HO-1 promoter activity (Fig. 6C), indicating that JunD and JunB had opposite roles in regulating ethanol-mediated HO-1 transcription in THP-1 cells.

Ethanol Increases Nuclear Protein Binding to HRE, ARE, and AP-1 Consensus DNA Sequence in HO-1 Promoter as Determined by EMSA—Nuclear extracts from ethanol-treated THP-1 cells showed a severalfold increased binding to an oligonucleotide encompassing the HRE consensus sequence (Table 1) of the HO-1 promoter region (Fig. 6E, lane 2). The protein-DNA complex formation was attenuated completely by HIF-1α siRNA (Fig. 6E, lane 3). The protein-DNA complex formation was also completely eliminated by a 50-fold excess of nonbiotinylated oligonucleotide probe (Fig. 6E, lane 4). Additionally, oligonucleotides with mutations in the HRE consensus sequence of the HO-1 promoter (Table 1) did not show formation of the DNA–protein complex (Fig. 6E, lane 5). These results showed that ethanol increased binding of HIF-1α protein from the nuclear extracts to HRE binding sites in the HO-1 promoter.

The role of Nrf2 and AP-1 protein complex in the binding to ARE and AP-1 sites, respectively, in HO-1 promoter was determined by EMSA. Ethanol augmented the binding of nuclear extract protein from ethanol-treated THP-1 cells to biotinylated oligonucleotide probe (Fig. 6F, lane 2) corresponding to the ARE region in the HO-1 promoter (Table 1). Furthermore, Nrf2 siRNA attenuated protein-DNA complex formation (Fig. 6F, lane 3). The protein-DNA complex formation was inhibited completely by a 50-fold excess of nonbiotinylated oligonucleotide probe (Fig. 6F, lane 4). Additionally, the oligonucleotide probe with a mutation in ARE-1 site of HO-1 promoter (Table 1) did not show DNA–protein complex formation (Fig. 6F, lane 5). These results showed that ethanol augmented binding of Nrf2 protein in nuclear extracts to ARE binding sites in the HO-1 promoter.

Ethanol also increased binding to an oligonucleotide probe encompassing AP-1 consensus sequence (Table 1) in the human HO-1 promoter (Fig. 6F, lane 7). The protein-DNA complex formation was completely attenuated by c-Jun siRNA (Fig. 6F, lane 8) and by a 50-fold excess of nonbiotinylated oligonucleotide probe (Fig. 6F, lane 9). Additionally, oligonucleotide with a mutation in the AP-1 consensus sequence (Table 1) of the HO-1 promoter did not exhibit DNA–protein complex formation (Fig. 6F, lane 10). These results showed that nuclear extracts derived from ethanol-treated THP-1 cells exhibited increased binding of c-Jun/AP-1 protein complex to AP-1 binding site of the human HO-1 promoter.

Ethanol Augments HIF-1α Binding to the HRE Site in the HO-1 Promoter Region in the Chromatin—The binding of HIF-1α to the HO-1 promoter in the chromatin of THP-1 cells was supported by ChIP analysis (Fig. 6G). Chromatin samples from ethanol-treated THP-1 were immunoprecipitated with HIF-1α antibody, which showed an ~2.2-fold increase in the expected PCR product size of 350 bp, corresponding to the HO-1 promoter region (~862 to ~666 bp) containing the HRE-2 and HRE-1 sites utilizing the primers listed in Table 1. As shown in Fig. 6G, pretreatment of THP-1 with SP600125 (JNK kinase inhibitor) had no effect, and R59949 (HIF-1α inhibitor) attenuated an ethanol-induced increase in the expected PCR product by ~125%, as determined by densitometry. The amplification of input DNA before immunoprecipitation was equal in all samples (Fig. 6G, middle panel). Immunoprecipitation of chromatin samples with rabbit IgG as a control did not show any amplification of the expected products (lower panel). Taken together, these data indicated that ethanol increased HIF-1α binding to the HO-1 promoter to up-regulate the expression of HO-1 in vivo.

Ethanol-induced HO-1 Protein Expression Involves HIF-1α, Nrf2, and c-Jun—Because ethanol enhanced HO-1 protein expression in ethanol-treated rKCs, and HIF-1α and Nrf2 and c-Jun were found to be involved in regulating HO-1 mRNA expression, we determined whether these transcription factors affected HO-1 protein expression. As shown in Fig. 6H, ethanol-induced HO-1 protein levels (~1.7-fold) in nuclear extracts of THP-1 cells were reduced below the basal levels (>100%) when cells were transfected with siRNAs for HIF-1α, Nrf2, and c-Jun, indicating the role of these factors in regulating both HO-1 mRNA and protein expression.

Differential Expression of HO-1 and NQO1 mRNA in the Livers of Ethanol-fed c-Junfl/fl Mice—Because in vitro studies showed involvement of JNK and c-Jun in ethanol-mediated expression of HO-1, we determined whether c-Jun played a role in its expression in vivo. Because the c-Jun-null mutation is embryonic lethal, we used c-Jun floxed mice (c-Junfl/fl) (31) for these studies. mRNA was isolated from the livers of isocaloric fed control and c-Junfl/fl mice, ethanol-fed wild-type, and ethanol-fed c-Junfl/fl mice followed by measurement of HO-1 and NQO1 mRNA levels.

FIGURE 5. Identification of functional cis-acting elements in ethanol-induced HO-1 promoter activation. A, schematics of human HO-1 promoter (~3936/−1 bp, relative to the transcription start site), indicating the location of HRE-1 to −3, proximal and distal AP-1 binding sites, and AREs. B, shown is ethanol-induced −9.2kb-HO-1-luc and −4.5kb-HO-1-luc human promoter activity in THP-1 cells. Where indicated, the ARE enhancer region (E1) of the −4.5-kb HO-1 promoter was deleted (E1). C, shown is the effect of pharmacological inhibitors on HO-1 promoter activity in response to ethanol treatment. Where indicated, cells were preincubated with the indicated inhibitors for 30 min before ethanol treatment for 8 h. Relative luciferase units (RLU). D, THP-1 cells were transiently transfected with indicated siRNAs or Dn FL 3-kinase expression plasmid followed by ethanol treatment for 8 h. E, shown is the effect of mutations of AP-1 (AP-1_1, AP-1_2, and AP-1_3) and HRE (HRE-1) binding sites on −4.5-kb HO-1 promoter activity in response to ethanol treatment. Luciferase assay data are expressed as fold change and have been normalized relative to the change in luciferase activity of the untreated controls and to transfection efficiency with β-galactosidase. The data shown represent three independent experiments in duplicate (means ± S.D.), p values are denoted as ***, p < 0.001; **, p < 0.01, and ns (not significant).
### TABLE 1
Oligonucleotide primers used in this study

| Organism/Gene | Method | Forward sequence | Reverse sequence |
|---------------|--------|------------------|------------------|
| R, GAPDH     | PCR    | ttcaatggcacagtcaaggc | tcaccccatttgatgttagcg |
| R, HIF-1α    | PCR    | cgagctgcctctcgggctga | cccagccgctggagctag |
| R, HIF-1β    | PCR    | cctacgctgccgcatcacg | gctcggcgggtatatgct |
| R, HO-1      | PCR    | tggctctctgtaattctg | tgggccaggcggaaccttaag |
| R, Nrf2      | PCR    | gccagctgaactccttagac | gattcgtgcacagcagca |
| R, HO-1      | PCR    | ttgtctctctggaatggaagg | ctctaccgaccattctg |
| R, NQO1      | PCR    | cattctgaaaggctggtttga | ctagctttgatctggttgtcag |
| R, p47phox   | PCR    | gttccgcctggccca | cataacccgccgagc |
| R, p38 MAPK  | PCR    | cctgataagagggctcagttga | cctgctgagagctaatggccacttgcc |
| H, GAPDH     | PCR    | aacctgccaagtacgatgacatc | gtagcccaggatgcccttg |
| H, HIF-1α    | PCR    | ttgatcgcctctcgggctga | cccagccgctggagctag |
| H, Nrf2      | PCR    | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, HO-1      | PCR    | ttgtctctctggaatggaagg | ctctaccgaccattctg |
| H, NQO1      | PCR    | cattctgaaaggctggtttga | ctagctttgatctggttgtcag |
| H, p47phox   | PCR    | gttccgcctggccca | cataacccgccgagc |
| H, p38 MAPK  | PCR    | cctgataagagggctcagttga | cctgctgagagctaatggccacttgcc |
| H, p65       | PCR    | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, Src-1     | PCR    | aacctgccaagtacgatgacatc | gtagcccaggatgcccttg |
| H, JNK-1     | PCR    | cctgataagagggctcagttga | cctgctgagagctaatggccacttgcc |
| H, JNK-2     | PCR    | ttgatcgcctctcgggctga | cccagccgctggagctag |
| H, c-Jun     | PCR    | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, c-Fos     | PCR    | aacctgccaagtacgatgacatc | gtagcccaggatgcccttg |
| H, JunB      | PCR    | ttgatcgcctctcgggctga | cccagccgctggagctag |
| H, JunD      | PCR    | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, HO-1-HRE-1| SDM    | gttccgcctggccca | cataacccgccgagc |
| H, HO-1-AP-1 | SDM    | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, HO-1-AP-2 | SDM    | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, HO-1-AP-3 | SDM    | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, HO-1 [ARE consensus oligo] | EMSA   | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, HO-1 mutARE consensus oligo | EMSA   | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, HO-1 [HRE consensus oligo] | EMSA   | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, HO-1 mutHRE consensus oligo | EMSA   | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, HO-1 WTAP-1 consensus oligo | EMSA   | gccagctgaactccttagac | gattcgtgcacagcagca |
| H, HO-1 ChIP  | ChIP   | gccagctgaactccttagac | gattcgtgcacagcagca |
Ethanol-induced HO-1 and NQO1 Are Differentially Regulated

NQO1 mRNA expression by qRT-PCR. Ethanol feeding to control mice resulted in ~3- and ~2-fold increased mRNA levels of HO-1 and NQO1, respectively (Fig. 7A, lane 3) compared with isocaloric-fed control mice (Fig. 7A, lane 1). However, there was complete attenuation of the ethanol-induced increase of HO-1 mRNA levels in the livers of ethanol-fed c-JunF/F mice (Fig. 7A, lane 4) compared with ethanol-fed wild-type mice (Fig. 7A, lane 3). The mRNA levels of NQO1 remained the same in the livers of ethanol-fed c-JunF/F mice (Fig. 7A, lane 4) as in ethanol-fed wild-type mice (Fig. 7A, lane 3). Taken together, these results showed that c-Jun or AP-1 complex contributed to ethanol-mediated HO-1 expression, but not to NQO1 expression, in vivo.

Silencing with siRNA for Either Nrf2 or c-Jun Augments Release of Inflammatory Mediators from Ethanol-treated THP-1 Cells—Because we observed that silencing with Nrf2 siRNA attenuated ethanol-induced HO-1 and NQO1 expression, whereas JNK-1 siRNA only abrogated HO-1 expression, we determined whether the expression of these proteins conferred protection against formation of proinflammatory cytokines. Silencing with Nrf2 siRNA, which attenuated ethanol-induced HO-1 and NQO1 mRNA expression, augmented the release of TNF-α by 1.4-fold and IL-1β by 1.5-fold from ethanol-treated THP-1 cells (Fig. 7B, lane 3) compared with control untreated THP-1 cells (Fig. 7B, lane 1). Similarly, silencing with c-Jun siRNA, which reduced expression of HO-1 only, augmented the release of TNF-α by ~1.5- and IL-1β by ~1.5-fold from ethanol-treated THP-1 cells (Fig. 7B, lane 4) compared with control cells (Fig. 7B, lane 1). However, the levels of TNF-α and IL-1β (Fig. 7B) in THP-1 cells transfected with control (mock) siRNA (Fig. 7B, lane 5) were similar to that of ethanol-treated THP-1 cells (Fig. 7B, lane 2). These results showed that reduced expression of both HO-1 and NQO1 or HO-1 alone augmented ethanol-mediated expression of proinflammatory cytokines in THP-1 monocytic cells.

HO-1 mRNA Levels and Proinflammatory Cytokines Are Expressed in a Reciprocal Manner in Liver Tissue of Ethanol-fed Mice—Because we observed an inverse correlation between HO-1 levels and expression of inflammatory cytokines upon treatment of monocytic cells in culture with ethanol, we determined whether a similar correlation existed in the livers of ethanol-fed mice. As shown in Fig. 7C, liver tissue of ethanol-fed mice showed a 4-fold increase in HO-1 mRNA expression and a severalfold decrease in TNF-α and IL-1β mRNA expression (lane 3) compared with control mice fed an isocaloric diet (lane 1). However, the livers of isocaloric fed c-JunF/F mice showed a 5-fold reduced mRNA expression of HO-1, a 15-fold increase in TNF-α, and a 6-fold increase in IL-1β mRNA expression (Fig. 7C, lane 2) compared with livers from isocaloric-fed control animals (Fig. 7C, lane 1). Similarly, livers derived from ethanol-fed c-JunF/F mice showed reduced mRNA expression of HO-1, an ~17-fold increase in TNF-α, and an ~11-fold increase in IL-1β mRNA expression (Fig. 7C, lane 4) compared with livers from ethanol-fed wild-type animals (Fig. 7C, lane 3). Taken together, these results showed that a decrease in HO-1 mRNA expression resulted in an increased expression of inflammatory cytokines in the liver and vice versa, indicating a reciprocal relationship in the expression levels of HO-1 to inflammatory status of the liver.

DISCUSSION

Acute and chronic alcohol exposure leads to enhanced ROS generation and a concomitant reduction of antioxidant levels, culminating in oxidative stress. To compensate for these oxidative stress insults, higher animals have evolved physiological defense mechanisms, including antioxidant proteins and phase II detoxifying enzymes (8, 9). Induction of phase II enzymes such as HO-1 renders cells more resistant to potential subsequent challenges of greater stress. Moreover, studies have shown that silencing of HO-1 and NQO1 results in increased expression of inflammatory cytokines in vitro (10), whereas induction of these enzymes protects against excess proinflammatory responses (10, 12).

In the present study we delineated the molecular mechanism by which ethanol modulated the expression of phase II enzymes, namely HO-1 and NQO1. Our studies showed that Kupffer cells from ethanol-fed rats and acute treatment of rKCs with ethanol resulted in a severalfold increase in the mRNA expression of HO-1, NQO1, and HIF-1α. Although human monocytic THP-1 cells have some unique characteristics as a cancer cell line, treatment of these cells also showed similar increase in mRNA expression of the same genes; thus, we utilized THP-1 cells for ease of culture and transfection as a model system.
system for delineating cell signaling pathways as previously described (55). We showed that ethanol-induced HO-1 expression in rKCs and THP-1 involved activation of Src-1, p38 MAP kinase, and JNK-1, but not JNK-2, as demonstrated by the use of pharmacological inhibitors and knockdown of these kinase genes with siRNAs. Moreover, transfection with Dn PI3K and p47phox siRNA (a subunit of NADPH oxidase) attenuated ethanol-mediated HO-1 expression, indicating the involvement of PI 3-kinase and NADPH oxidase in HO-1 gene expression as illustrated in the schematics of Fig. 7D. Previous studies show the involvement of PI 3-kinase/Akt, p38 MAP kinase and JNK in HO-1 expression in response to diverse stimuli (42). Our studies showed that selective activation of JNK-1 by ethanol, among ubiquitously expressed JNK-1 and JNK-2, up-regulated the expression of HO-1 but not NQO1. Previous studies (43) have identified 13 MAP kinase kinases (MKKs) that regulate JNKS, but how these MKKs differentially regulate JNKS is not completely understood. Studies utilizing JNK-1 and JNK-2 knock-out mice reveal differential roles of JNK isoforms in steatohepatitis; that is, knockdown of JNK-1 augments steatosis and hepatitis (44). In contrast, knockdown of JNK-2 attenuates hepatocyte cell death (44, 45). Differential roles of JNKS have been observed in inflammation and insulin resistance wherein disruption of the JNK-1 gene prevents development of insulin resistance in obese and diabetic mice (46). JNK-1 also has been shown to determine the oncogenic or tumor suppressor activity of the integrin-related kinase in human rhabdomyosarcoma (47). Differential roles of JNKS in regulating the central tran-

FIGURE 7. Role of HO-1 and NQO1 in attenuating expression of proinflammatory cytokines in vitro and in vivo. A, mRNA from liver tissue of control isocaloric fed wild-type, ethanol-fed wild-type, isocaloric fed c-Junfl/fl, and ethanol-fed c-Junfl/fl mice were analyzed for the expression of HO-1 and NQO1. The data represent livers from six rats in each category, and the assay was performed in duplicate (mean ± S.D.). B, TNF-α and IL-1β protein release from THP-1 treated with ethanol is shown. Where indicated, cells were transfected with indicated siRNA or control mock-siRNA constructs. The data represent four independent experiments, and the ELISA assay was performed in duplicate (mean ± S.D.). C, mRNA levels of HO-1 show inverse correlation with TNF-α and IL-1β mRNA levels in liver tissue. mRNA isolated from control isocaloric-fed wild-type, ethanol-fed wild-type, isocaloric-fed c-Junfl/fl, and ethanol-fed c-Junfl/fl mice were analyzed by qRT-PCR. The data represent livers from six mice in each category, and the assay was performed in duplicate (mean ± S.D.). p values are denoted as: ***, p < 0.001 and ns, not significant. D, shown is an illustration of proposed ethanol-induced cell signaling pathways involved in regulating Nrf2 and HO-1 transcription.
Ethanol-induced HO-1 and NQO1 Are Differentially Regulated

scription initiation factor TBP have been identified, wherein JNK-1 augments, whereas JNK-2 decreases TBP expression (48). JNK-1 has been shown to increase c-Jun activity and stability, whereas JNK-2 binds to c-Jun and targets it for degradation (49). Thus, we suggest that ethanol-mediated JNK-1 activation via phosphorylation likely leads to activation of c-Jun and formation of an AP-1 transcriptional complex to affect the expression of HO-1.

By utilizing a siRNA knockdown approach, we demonstrated the involvement of the Nrf2 transcription factor in ethanol-mediated expression of both HO-1 and NQO1 in Kupffer cells and THP-1. Furthermore, our studies showed that ethanol-induced Nrf2 mRNA expression and cell signaling involved Src-1 kinase, NADPH oxidase, p38 MAP kinase, and PI 3-kinase, as illustrated in Fig. 7D. Previous studies show that oxidant stress and antioxidant phytochemicals increase Nrf2 protein translocation into the nucleus for the purpose of gene induction through stress-responsive elements or AREs in the promoters of cytoprotective genes, including HO-1 (12, 21, 50, 51). Multiple ARE sites in the enhancer regions of the full-length (15 kb) HO-1 promoter have been identified (51). Because ARE sites have an AP-1 or AP-1 like binding motif in the promoter of these cytoprotective battery of genes (8, 18), it has been proposed that members of the AP-1 protein family may act on AREs (20), and such a concept was subsequently challenged (52). Our studies showed that mutation of the AP-1 binding site other than that in an ARE site in the HO-1 promoter resulted in substantial reduction of ethanol-induced HO-1 promoter activity. Furthermore, we showed that c-Jun and JunD formed an active AP-1 transcriptional complex with other members of the AP-1 family to interact with the AP-1 site in the HO-1 promoter to augment its activity in response to ethanol challenge. However, JunB was observed to repress c-Fos/c-Jun-mediated HO-1 promoter activity. Because JunB and JunD are present endogenously, we selectively silenced JunD and JunB with siRNAs and observed that attenuation of JunD repressed HO-1 promoter activity, whereas a decrease of JunB increased HO-1 promoter activity. These results show that JunD and JunB have opposing roles in regulating ethanol-mediated HO-1 transcription. Previous studies in HK-2 cells, an immortalized human renal epithelial cell line, show that hemin-induced HO-1 promoter activity is augmented by c-Jun and JunB but repressed by JunD (27). These results and data from our studies implicate differential roles of JunD and JunB in regulating HO-1 gene expression and are context-sensitive to the nature of stimuli and cell type. Additionally, our studies showed that the c-Jun, a component of the AP-1 complex, was involved in regulating ethanol-mediated HO-1 expression in vivo, as livers derived from ethanol-fed c-Jun<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> mice showed attenuated levels of HO-1 mRNA compared with wild-type mice fed ethanol for the same duration. It is possible that c-Jun and Nrf2 interact with common ARE sites to regulate HO-1 induction. Because the ARE site of interest is located ∼3.9 kb upstream of the proximal HRE site, chromatin folding may allow bound HIF-1α at the HRE to interact with components of the ARE.

Because ethanol-induced HO-1 expression was attenuated by HIF-1α siRNA in vitro, we performed an in silico analysis of the promoter region of HO-1 and identified several possible HREs that were located at −42 to −39 bp and two inverted HREs (RGCAC) at −862 to −859 bp and −669 to −666 bp in human HO-1 promoter. Utilizing a gene knockdown approach and mutation of consensus HIF-1α DNA binding sites, our studies demonstrated that proximal HREs in the HO-1 promoter were involved in ethanol-mediated HO-1 transcription, as was further substantiated by EMSA and ChIP. Previous studies show that hypoxia-induced HO-1 expression in rat tissues occurs via HIF-1α activation (53). Our studies showed that the AP-1 complex played an important role in ethanol-mediated HO-1 expression, as demonstrated by EMSA, ChIP, and silencing with c-Jun siRNA. Overall, our results suggest that ethanol-mediated HO-1 expression in THP-1 and Kupffer cells involved AREs, HREs, and AP-1 binding motifs in the HO-1 promoter. The ethanol-induced cell signaling pathways involved in regulating HO-1 transcription are illustrated in Fig. 7D.

Furthermore, our studies showed that ethanol-mediated up-regulation of HO-1 and NQO1 in THP-1 cells led to significantly reduced levels of inflammatory cytokines, such as TNF-α and IL-1β. In contrast, attenuation of HO-1 expression in monocytic cells with either Nrf2 siRNA or c-Jun siRNA resulted in an increased expression of TNF-α and IL-1β. These results in vitro showed that ethanol-induced expression of phase II enzymes, i.e. HO-1 and NQO1, led to attenuated expression of proinflammatory cytokines. Our studies corroborate previous findings wherein LPS and prostaglandin J<sub>2</sub>-induced expression of HO-1 in monocytic cells showed an anti-inflammatory effect (10, 54). Moreover, our in vivo studies showed that increased mRNA levels of HO-1 in the livers of ethanol-fed mice correlated with a concomitant decrease in TNF-α and IL-1β mRNA expression. Conversely, livers of ethanol-fed c-Jun<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> mice or control c-Jun<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> mice, compared with control or ethanol-fed wild-type mice showed lower levels of HO-1 and exaggerated levels of cytokines. These studies showed for the first time that ethanol-induced up-regulation of HO-1 in the liver leads to reduced expression of inflammatory cytokines in the liver.

In conclusion, our studies show that ethanol-induced oxidant stress leads to up-regulation of HO-1 and NQO1. Although both of these enzymes are phase II-detoxifying enzymes, they are regulated by common and divergent pathways. The ethanol-mediated expression of HO-1 and NQO1 involves common Nrf2 binding to AREs in their promoter to up-regulate their expression. The signal transduction pathways for induction of the HO-1 gene deviates; ethanol-induced expression of HO-1 involves activation of JNK-1, HIF-1α, and an AP-1 transcriptional complex composed of c-Jun, c-Fos, and JunD but not JunB. However, a subset of these transcription factors, namely HIF-1α and AP-1, do not participate in ethanol-induced NQO1 expression. We show that ethanol-induced expression of HO-1 or HO-1 and NQO1 in monocytic cells contributes to reduced expression of proinflammatory cytokines in vitro. Furthermore, we show that reduced expression of HO-1 in the liver correlates with increased inflammatory state as seen in ethanol-fed c-Jun<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> mice. The ethanol-induced oxidative stress leading to up-regulation of HO-1 and a concomitant decrease in expression of inflammatory cytokines in the liver in vivo suggests a reciprocal relationship between expres-
Ethanol-induced HO-1 and NQO1 Are Differentially Regulated

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