

Chronic Ethanol Exposure Increases the Binding of HuR to the TNF α 3'-Untranslated Region in Macrophages*

Received for publication, May 1, 2003, and in revised form, June 27, 2003
Published, JBC Papers in Press, July 21, 2003, DOI 10.1074/jbc.M304566200

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Tumor necrosis factor α (TNF α) expression is a key mediator of ethanol-induced liver disease. Increased lipopolysaccharide (LPS)-stimulated TNF α expression in macrophages after chronic ethanol feeding is associated with a stabilization of TNF α mRNA (Kishore, R., McMullen, M. R., and Nagy, L. E. (2001) *J. Biol. Chem.* 276, 41930–41937). Here we show that the 3'-UTR of murine TNF α mRNA was sufficient to mediate increased LPS-stimulated expression of a luciferase reporter in RAW 264.7 macrophages after chronic ethanol exposure. Further, we show that HuR, a nuclear/cytoplasmic shuttling protein, which binds to TNF α mRNA, is required for increased expression of TNF α after chronic ethanol. In Kupffer cells, HuR was primarily localized to the nucleus and then translocated to the cytosol in response to LPS in both pair- and ethanol-fed rats. After chronic ethanol feeding, HuR quantity in the cytosol was greater, both at baseline and in response to LPS, compared with pair-fed controls. Using RNA gel shift assays, we found that LPS treatment increased HuR binding to the 65-nucleotide A + U-rich element of the TNF α 3'-UTR by 2-fold over baseline in Kupffer cells from pair-fed rats. After chronic ethanol feeding, HuR binding to the TNF α A + U-rich element was increased by more than 5-fold at baseline and in response to LPS, compared with pair-fed controls. Down-regulation of HuR expression by RNA interference prevented the chronic ethanol-induced increase in expression of luciferase reporters containing the TNF α 3'-UTR. Taken together, these data demonstrate that increased binding of HuR to the TNF α 3'-UTR contributes to increased LPS-stimulated TNF α expression in macrophages after chronic ethanol exposure.

Tumor necrosis factor α (TNF α)¹ is one of the principal mediators of the inflammatory response in mammals, transducing differential signals that regulate cellular activation and proliferation, cytotoxicity, and apoptosis (1, 2). TNF α has been implicated in the pathogenesis of a wide variety of inflammatory diseases (2–5), including the progression of alcoholic liver

disease (6, 7). Circulating TNF α is increased in the blood of alcoholics and in animals chronically exposed to ethanol (8, 9). Treatment of rats with antibodies to TNF α prevents liver damage resulting from chronic ethanol exposure (6). Similarly, transgenic mice lacking the TNF α receptor I gene are resistant to chronic ethanol-induced liver damage (10). Although the importance of TNF α to the progression of alcoholic liver disease is clear, the mechanism(s) by which ethanol increases TNF α production are not well understood. One contributing factor to enhanced TNF α production is an increased exposure to LPS after ethanol consumption. LPS levels are increased in the blood of alcoholics (11, 12) and rats exposed to ethanol via gastric infusion (13). Moreover, long term ethanol exposure also increases the sensitivity of macrophages to LPS activation. For example, long term ethanol consumption results in an increased susceptibility to endotoxin-induced liver injury (14). LPS-stimulated TNF α production is increased in hepatic macrophages (Kupffer cells) after chronic ethanol feeding (15, 16), as well as after *in vitro* exposure of RAW 264.7 macrophages to ethanol during culture (17, 18).

Enhanced secretion of TNF α by macrophages after chronic ethanol exposure is associated with increased accumulation of TNF α mRNA. This increase is associated with a chronic ethanol-induced stabilization of TNF α mRNA (17). Modulation of mRNA stability is an important mechanism in the regulation of TNF α biosynthesis (19, 20). Stabilization of mRNAs contributes to the strong and rapid induction of genes in the inflammatory process. The TNF α mRNA, like other short-lived mRNAs, contains A + U-rich elements (ARE) in its 3'-untranslated region (UTR) that function as destabilizing elements, as demonstrated in transgenic mice in which the TNF α -ARE is deleted (21), as well as in various *in vitro* systems (19, 22). In addition to the destabilizing activity of the TNF α 3'-UTR, the ARE elements in the 3'-UTR allow for stabilization of the TNF α mRNA in response to activation (23, 24).

Stability of the TNF α mRNA is controlled by *trans*-acting factors that bind to the TNF α mRNA. A large number of mRNA-binding proteins regulate both stabilization and destabilization (25). Of these, several proteins that bind to the TNF α mRNA, specifically to its 3'-UTR, have been identified; tristetraprolin (26) and HuR (23) act to regulate mRNA stability, whereas TIA-1 and TIAR act to regulate translational efficiency (27, 28). Tristetraprolin is a zinc-finger protein induced by LPS in macrophages that acts to destabilize TNF α mRNA (26, 29). In contrast, HuR, a member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins, primarily acts to stabilize the TNF α mRNA (23, 30). Here we report that the TNF α 3'-UTR is sufficient to convey the effect of chronic ethanol on increasing LPS-stimulated luciferase reporter activity. Further, we show that chronic ethanol exposure enhances the LPS-dependent translocation of HuR from the nucleus to the cytosol, as well as increases the binding of HuR

* This work was supported by National Institutes of Health Grant AA 11975 (to L. E. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: TNF α , tumor necrosis factor α ; ARE, A + U-rich elements; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LPS, lipopolysaccharide; LUC, luciferase; PBS, phosphate-buffered saline; siRNA, small inhibitory RNA; siHuR, small inhibitory HuR; UTR, untranslated region.

to the ARE in TNF α 3'-UTR, likely contributing to chronic ethanol-induced stabilization of TNF α mRNA.

EXPERIMENTAL PROCEDURES

Materials—LPS from *Escherichia coli* serotype 026:B6 (tissue culture tested) was purchased from Sigma; all experiments were carried out with a single lot of LPS (lot number 111K401). Antibodies to HuR were a gift from H. Furneaux or from Molecular Probes, Eugene OR. Anti-mouse IgG-peroxidase (Fab fragments) were purchased from Chemicon (Temecula, CA). All cell culture reagents were from Invitrogen. Low endotoxin plasmid preparation kits were from Qiagen (Valencia, CA). Luciferase reporter constructs have been described previously: pTNF α -LUC (containing -615+1 nucleotide TNF α promoter in a luciferase reporter) was from N. Mackman, University of California, San Diego, CA (31); pTNF α -5'-UTR-LUC (containing -993+110 of the TNF α promoter and 5'-UTR in pGL2 vector) and pTNF α -5'-UTR-LUC-3'-UTR (containing -993+100 of the TNF α promoter and 5'-UTR, as well as the 833-base TNF α 3'-UTR in pGL2 vector) luciferase reporters were from D. Joyce, University of Western Australia (32, 33); and SV40-LUC-3'-UTR luciferase reporter (containing the TNF α 3'-UTR in pGL2 vector) and the SV40-LUC-3'-UTR-ARE deletion were from V. Kruys (34). Plasmids used for synthesis of RNA probes, including 65-nucleotide TNF α ARE (1281-1350 in the 3'-UTR and the full-length TNF α 3'-UTR 1110-1627), were from P. Blackshear, National Institute of Environmental Health Sciences, National Institutes of Health.

Animals and Chronic Ethanol Feeding Protocol—Adult male Wistar rats weighing 150 g were purchased from Harlan Sprague-Dawley and allowed free access to the Lieber DeCarli ethanol diet (ethanol-fed; Dyets, Bethlehem, PA) or pair-fed a control diet, which isocalorically substitutes maltose-dextrin for ethanol as described previously (17). Procedures involving animals were approved by the Institutional Animal Care Board at Case Western Reserve University.

Kupffer Cell Isolation and Culture—Kupffer cells were isolated as described previously (15) except that CMRL medium was used to isolate and culture Kupffer cells. Briefly, livers were perfused with 0.05% collagenase, and the resulting suspension of liver cells was treated with 0.02% Pronase for 15 min at 12 °C. The resulting cell suspension was centrifuged three times at 50 \times g for 2 min, and the supernatant was collected after each centrifugation. The pooled supernatant was then centrifuged at 500 \times g for 7 min to collect non-parenchymal cells. Kupffer cells were then purified by centrifugal elutriation (15). The yield and purity of isolated Kupffer cells did not differ between pair-fed and ethanol-fed rats (15). Isolated Kupffer cells were suspended in CMRL with 10% fetal bovine serum and penicillin-streptomycin at a concentration of 2 \times 10⁶ cells/ml and plated onto 6-well plates (2 ml/well). After 2 h, non-adherent cells were removed by aspiration, and fresh media were supplied. Assays were carried out after 24 h in culture.

Culture of RAW 264.7 Macrophages and Luciferase Assays—The RAW 264.7 macrophage-like cell line was cultured routinely in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37 °C and 5% CO₂ (18). For luciferase reporter assays, RAW 264.7 macrophages were grown in 6-well plates to 60–70% confluency and then transiently transfected with control and expression vectors using Superfect transfection reagent (Qiagen), according to the manufacturer's instructions. Cells were co-transfected with pTK-RL (Promega), an expression vector for Renilla luciferase under the control of the thymidine kinase promoter, as a control for transfection efficiency. Transfected cells were sub-cultured and seeded at 10.2 \times 10⁴/cm² in 96-well plates. After 2 h, medium was changed to DMEM plus 10% FBS with or without 25 mM ethanol. Control and ethanol-treated plates were wrapped with parafilm to prevent evaporation of ethanol; parafilm wrapping had no effect on the pH value of the cell culture media over the 48 h in culture (data not shown). The concentration of ethanol in media was measured by enzymatic assay (Sigma) and was within 80% of the starting concentration after 24 h (data not shown). After 48 h, media were removed, and cells were stimulated or not with LPS in DMEM/FBS. Cells were then extracted in lysis buffer after 4 h of stimulation with LPS, and luciferase activities were measured using the DUAL luciferase assay system (Promega).

Subcellular Fractionation and Western Blot Analysis—Nuclear and cytosolic extracts were prepared (23) from Kupffer cells isolated from ethanol- and pair-fed rats after stimulation with 0 or 100 ng/ml LPS for up to 3 h. Nuclear and cytosolic samples were separated by 10% SDS-PAGE and probed with antibodies to HuR by Western blot. Bound

antibody was detected by enhanced chemiluminescence (Amersham Biosciences). Immunoreactive protein quantity was assessed by scanning densitometry.

Preparation of RNA Transcripts—Labeled RNA probes for RNA gel shift assays were prepared by *in vitro* transcription. The plasmids containing mTNF α 1281-1350 and mTNF α 110-1627 in Bluescript or pTRI-actin-mouse (Ambion), used as a non-relevant RNA control 300 nt in length, were linearized with *Xba*I to generate the DNA template for *in vitro* transcription. Template (1 μ g) was incubated in the presence of 0.5 mM unlabeled ATP, CTP, and GTP and 32.5 μ M UTP (containing 50 μ Ci of 800 Ci/mmol [α -³²P]UTP) and 30 units of T7 RNA polymerase at 37 °C for 10 min. Template DNA was removed, and the reaction was stopped by incubating the mixture with DNase I for 15 min at 37 °C and adding 1 μ l of 0.5 M EDTA, respectively. Free nucleotide was then removed by gel filtration using a Micro Bio-Spin P-30 column (Bio-Rad). The fragment was then gel-purified on a 5% denaturing polyacrylamide gel and eluted overnight at 37 °C in 0.5 M sodium acetate, 1 mM EDTA, and 0.2% SDS.

RNA Gel Shift Assays—RNA gel shift assays were carried out as described by Dean *et al.* (23). 10 μ g of cytosolic extracts from Kupffer cells were incubated at room temperature for 30 min in 10 mM HEPES (pH 7.6), 3 mM MgCl₂, 40 mM KCl, 1 mM dithiothreitol, 5% glycerol, and 20 fmol of labeled RNA probe. For antibody supershift experiments, 50 units/ml RNase T1 was included in the incubation buffer, and 4 μ g of anti-HuR was added to the reaction mixture and incubated for 30 min at room temperature. Heparin sulfate (5 mg/ml) was then added for 10 min at room temperature. Samples separated on 4 or 4–20% gradient acrylamide gels in 0.5 \times Tris borate-EDTA at 200 V at 4 °C. Gels were then fixed, dried, and analyzed by phosphorimaging.

Immunohistochemistry—Isolated Kupffer cells were cultured overnight in LabTek chamber slides and then stimulated or not with 100 ng/ml LPS for 0–120 min. Cells were fixed in freshly prepared 4% paraformaldehyde for 20 min at room temperature. Slides were then quenched in 25 mM glycine in PBS and permeabilized with 0.1% Triton X-100 for 3 min. Slides were then blocked for 1 h in 2% bovine serum albumin, 5% fish gelatin in PBS and then incubated with antibody to HuR (20 μ g/ml) in blocking solution overnight at 4 °C. Slides were washed three times for 15 min in PBS and then incubated for 1 h with AlexaFluor 488-conjugated anti-mouse IgG (1:300) (Molecular Probes, Eugene, OR) for 1 h in blocking buffer. Slides were finally washed three times for 15 min in PBS and then mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Samples were blinded, and cells were examined with a Bio-Rad confocal microscope under a \times 60 lens. Multiple cells from at least four separate preparations were examined. Nonspecific binding was assessed in cells incubated in the absence of primary antibody (data not shown).

RNA Interference—RAW 264.7 macrophages were plated in 6-well plates and after 48 h, when cells were about 70% confluent, transfected with siRNA (2 μ g) using the TransMessenger transfection reagent (Qiagen) according to the manufacturer's instructions. siRNA directed against HuR (siRNA HuR) was targeted to positions 163–183 relative to the start codon of HuR. This sequence was based on previously published reports (35). Sense and antisense oligonucleotides were synthesized and annealed by Qiagen. 2 μ g of siRNA HuR or control (non-silencing; Qiagen), along with 1.2 μ g of pTNF α -5'-UTR-LUC-3'-UTR or LUC-3'-UTR (luciferase reporters) and 0.1 μ g of pTK-RL (Renilla reporter), were diluted in 6.6 μ l of Enhancer R (ratio of RNA/DNA to Enhancer R is 1:2) and 85 μ l of Buffer EC-R (Qiagen). 8 μ l of Transfection reagent was added after 5 min at room temperature. RAW 264.7 macrophages were washed twice with PBS and then the RNA/DNA transfection mixture, diluted to 800 μ l of DMEM with FBS, added dropwise to the cells. Cells were incubated with the transfection mixture for 3 h and then media were changed to DMEM with FBS. Cells were subcultured to 96-well plates (for luciferase assays), LabTek chamber slides (for immunohistochemistry), or 24-well plates (for Western blotting) at a density of 0.4 \times 10⁶ cells/ml, allowed to attach, and then treated with or without 25 mM ethanol for 48 h. Transfection efficiencies were determined by transfecting RAW 264.7 macrophages with a fluorescein-labeled control (non-silencing) siRNA (Qiagen), followed by immunohistochemistry after 2 h in culture and ranged from 35–45% efficiency.

Statistical Analysis—Because of the limited number of Kupffer cells available from each animal, data from several feeding trials are presented in this paper; each trial consisted of six rats per feeding group. Values reported are means \pm S.E. Data were analyzed by Student's *t* test or general linear models procedure (SAS, Carey, IN) using the least square means test to determine differences.

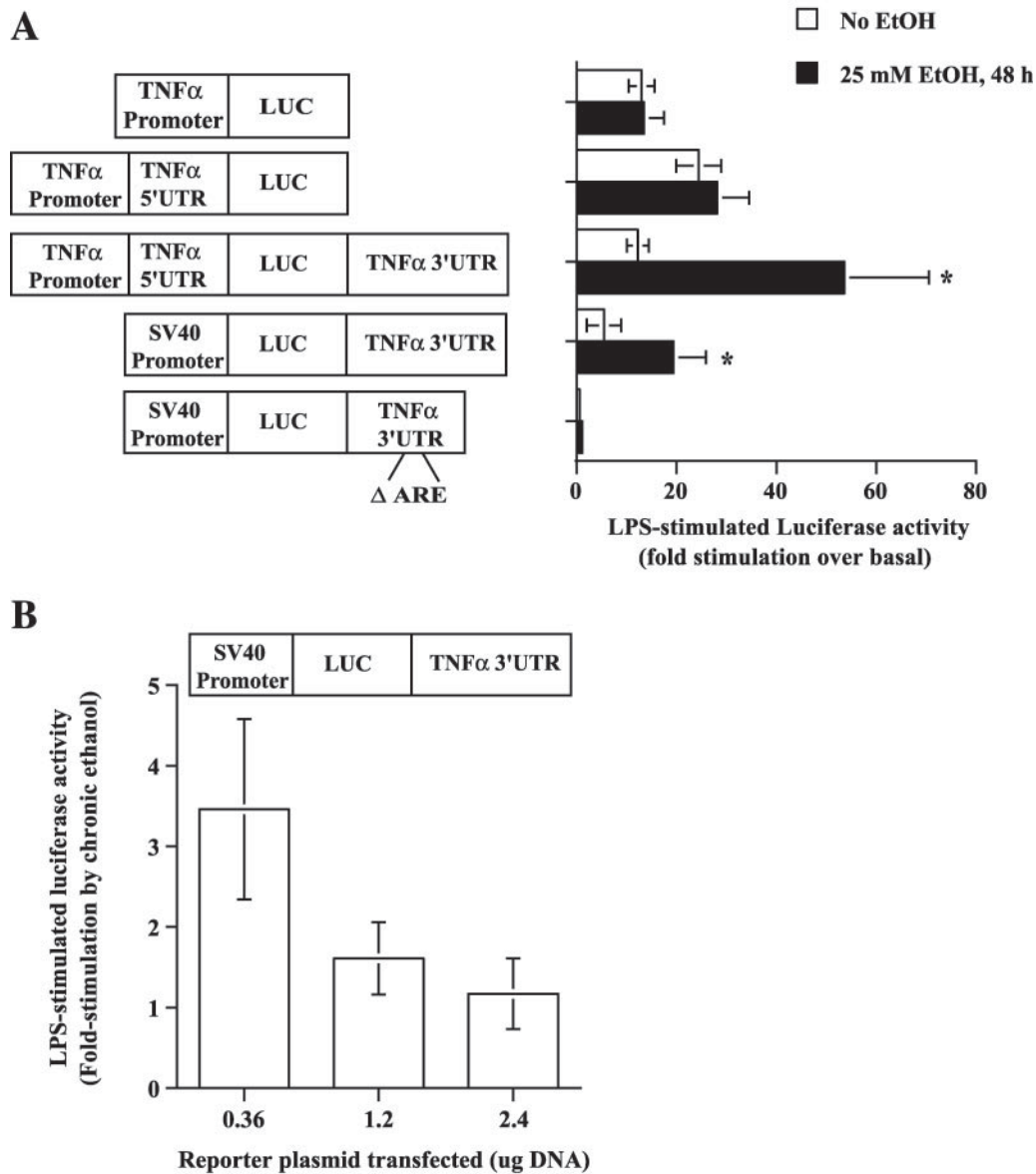


FIG. 1. Sequences in the TNF α 3'-UTR are sufficient to increase LPS stimulation of a heterologous luciferase reporter after chronic ethanol exposure. A, RAW 264.7 macrophages were transfected with luciferase reporter constructs as illustrated, along with a plasmid expressing Renilla luciferase for control for transfection efficiency. Cells were then subcultured and treated with or without 25 mM ethanol for 48 h and then stimulated or not with 100 ng/ml LPS. Luciferase and Renilla luciferase activity were measured after 4 h, and luciferase activity is expressed as -fold stimulation by LPS over basal. Values represent means \pm S.E., $n = 5$; *, $p < 0.05$ compared with cells not treated with ethanol. B, RAW 264.7 macrophages were transfected with increasing concentrations of a luciferase reporter containing the TNF α -3'-UTR under the control of the SV40 promoter (SV40-LUC-TNF α -3'-UTR). Cells were then cultured with or without 25 mM ethanol and stimulated or not with 100 ng/ml LPS as in A. Luciferase activity is expressed as the -fold stimulation of LPS-stimulated luciferase activity in cells treated with chronic ethanol compared with control cells. Values represent means \pm S.E., $n = 4-9$.

RESULTS

Chronic exposure to ethanol, either *in vivo* during ethanol feeding or *in vitro* upon exposure to ethanol in culture, increases LPS-stimulated TNF α mRNA accumulation in isolated Kupffer cells and RAW 264.7 macrophages, respectively (17). Increased LPS-stimulated TNF α mRNA accumulation after chronic ethanol is associated with a stabilization of TNF α mRNA (17). Here we have asked whether sequences in the 5'-UTR and 3'-UTR of the TNF α mRNA are sufficient to increase LPS-stimulated expression of a heterologous luciferase reporter after chronic ethanol exposure. RAW 264.7 macrophages were transfected with constructs containing the TNF α promoter, 5'-UTR, and/or 3'-UTR and then cultured with or without 25 mM ethanol for 48 h and stimulated or not with 100 ng/ml LPS. LPS-stimulated luciferase expression driven by the

TNF α promoter alone was not affected by chronic ethanol exposure (Fig. 1) (18), consistent with data demonstrating that chronic ethanol exposure has no net effect on LPS-stimulated transcription of TNF α in macrophages (18). Similarly, inclusion of the TNF α 5'-UTR did not convey a chronic ethanol-induced increase in LPS-stimulated luciferase activity (Fig. 1). Inclusion of the TNF α 3'-UTR in this reporter construct decreased LPS-stimulated activity in control cells, consistent with mRNA instability elements within the TNF α 3'-UTR (21). In contrast, after chronic ethanol exposure, LPS-stimulated luciferase activity was 4.4-fold higher when the TNF α 3'-UTR was included in the reporter construct compared with controls (Fig. 1).

To test whether the 3'-UTR alone was sufficient to convey increased LPS-stimulated luciferase activity after chronic eth-

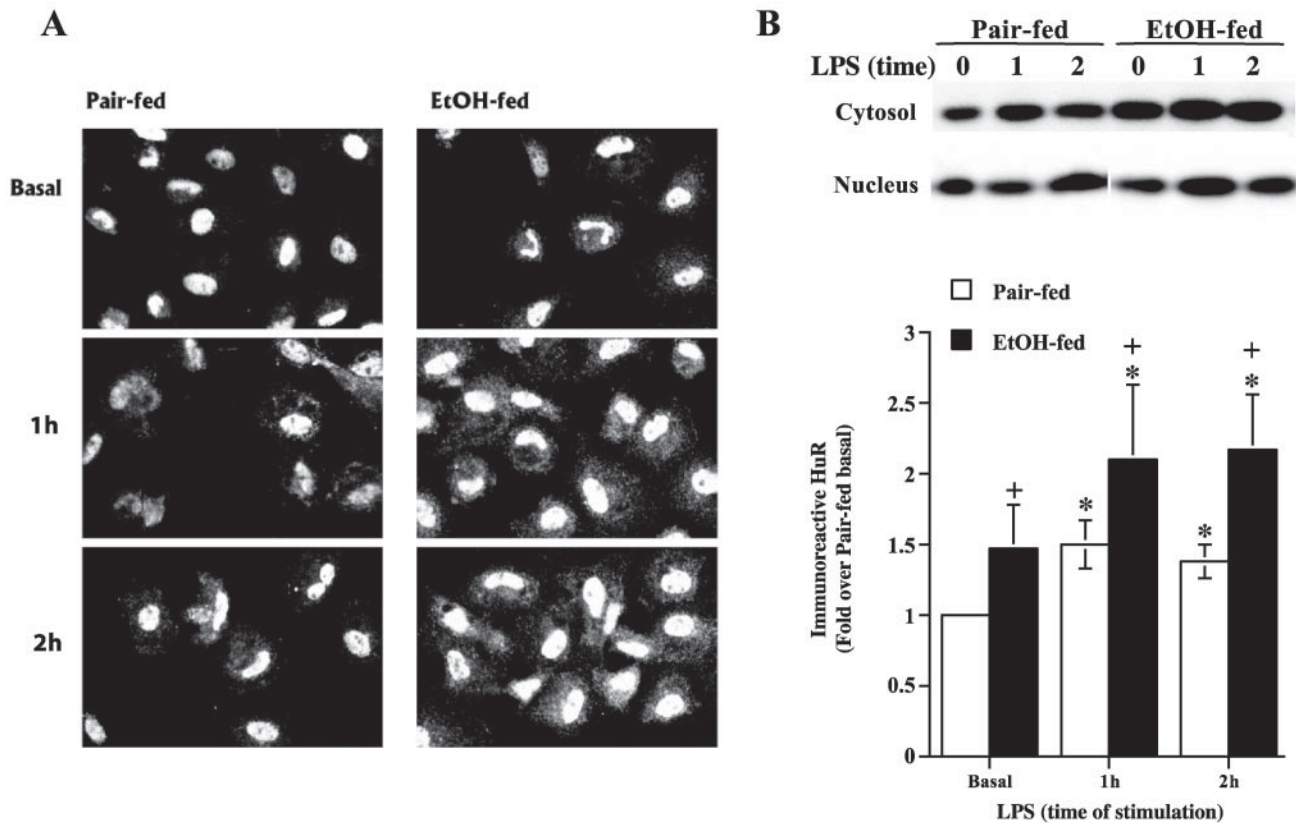


FIG. 2. Chronic ethanol feeding disrupts the subcellular localization of HuR. A, Kupffer cells isolated from pair- and ethanol-fed rats were cultured on LabTek chamber slides and then stimulated or not with 100 ng/ml LPS for 0–2 h. Cells were then fixed and processed for confocal immunohistochemistry for HuR. Pictures are representative of five separate experiments. B, Kupffer cells isolated from pair- and ethanol-fed rats were cultured for 24 h and then stimulated or not with 100 ng/ml LPS for 0–2 h. Cytosolic and nuclear fractions were then isolated. Proteins (cytosol (100 μ g) and nuclear (10 μ g)) were then separated by SDS-PAGE and probed for HuR by Western blot analysis. Values represent immunoreactive HuR in cytosol, means \pm S.E., $n = 5$; *, $p < 0.05$ compared with basal; +, $p < 0.05$ compared with pair-fed.

anol exposure, RAW 264.7 macrophages were transfected with a luciferase reporter containing the TNF α 3'-UTR under the control of the SV40 promoter (SV40-LUC-TNF α 3'-UTR). Luciferase reporter constructs under the control of the SV40 promoter (SV40-LUC, pGL2), but without the TNF α 3'-UTR, did not respond to LPS stimulation (data not shown). Insertion of the TNF α 3'-UTR (SV40-LUC-TNF α 3'-UTR) conveyed LPS-stimulated luciferase activity (Fig. 1) (33). Moreover, including the TNF α 3'-UTR increased LPS-stimulated luciferase activity after chronic exposure to ethanol by 3.5-fold compared with control cells (Fig. 1). LPS stimulation of luciferase activity, as well as increased expression after chronic ethanol, were lost when the class II ARE was deleted from TNF α 3'-UTR, indicating a critical role for this ARE element in mediating responses to both LPS and chronic ethanol.

We have reported previously that overexpression of a luciferase reporter construct containing the TNF α 3'-UTR under the control of the SV40 promoter was not sufficient to convey chronic ethanol-induced stabilization to luciferase mRNA (17). To reconcile this previous result with the current data suggesting an essential role of the 3'-UTR in mediating the chronic effects of ethanol, we hypothesized that constitutive high levels of overexpression of the 3'-UTR might titrate out specific proteins required to mediate LPS and/or chronic ethanol-induced regulation of luciferase expression. To test this hypothesis, RAW 264.7 macrophages were transfected with increasing quantities of the SV40-LUC-TNF α 3'-UTR reporter. At low levels of expression, stimulation with LPS increased luciferase activity, and this response was enhanced after chronic ethanol by 3.5-fold (Fig. 1B). However, at higher level of expression of

the SV40-LUC-TNF α 3'-UTR reporter, basal luciferase activity increased. As basal expression of luciferase increased, the ability of LPS to stimulate luciferase activity over baseline was diminished from 5.6- and 10.8-fold over basal when 0.36 μ g of DNA was transfected for control and ethanol-treated, respectively, to 3.5- and 2.9-fold when 4.8 μ g of the SV40-LUC-3'-UTR was transfected in control and ethanol-treated cells, respectively. Furthermore, as the expression level of the reporter increased, the ability of chronic ethanol to enhance LPS-stimulated luciferase activity was diminished (Fig. 1B). This saturation in the enhancing effect of chronic ethanol on LPS-stimulated luciferase activity is consistent with our previous observations that chronic ethanol only enhances LPS-stimulated TNF α mRNA accumulation and peptide accumulation at sub-maximal LPS concentrations (15, 36).

Because the TNF α 3'-UTR is sufficient to mediate increased LPS stimulation after chronic ethanol, we next investigated the role of the mRNA-binding protein, HuR, in mediating the effects of chronic ethanol. HuR is a ubiquitously expressed nuclear/cytoplasmic shuttling protein that stabilizes a number of short-lived mRNAs, including TNF α (23, 25). HuR primarily resides in the nucleus in resting cells but exits to the cytoplasm in response to a number of stimuli, including exposure to LPS (25). We first investigated whether chronic ethanol feeding disrupts the intracellular localization of HuR in isolated Kupffer cells from pair- and ethanol-fed rats. Isolated Kupffer cells were stimulated or not with 100 ng/ml LPS for 0–2 h. In Kupffer cells from both pair- and ethanol-fed rats, HuR was primarily localized to the nucleus at baseline (Fig. 2A). In response to LPS, HuR moved from the nucleus to punctate

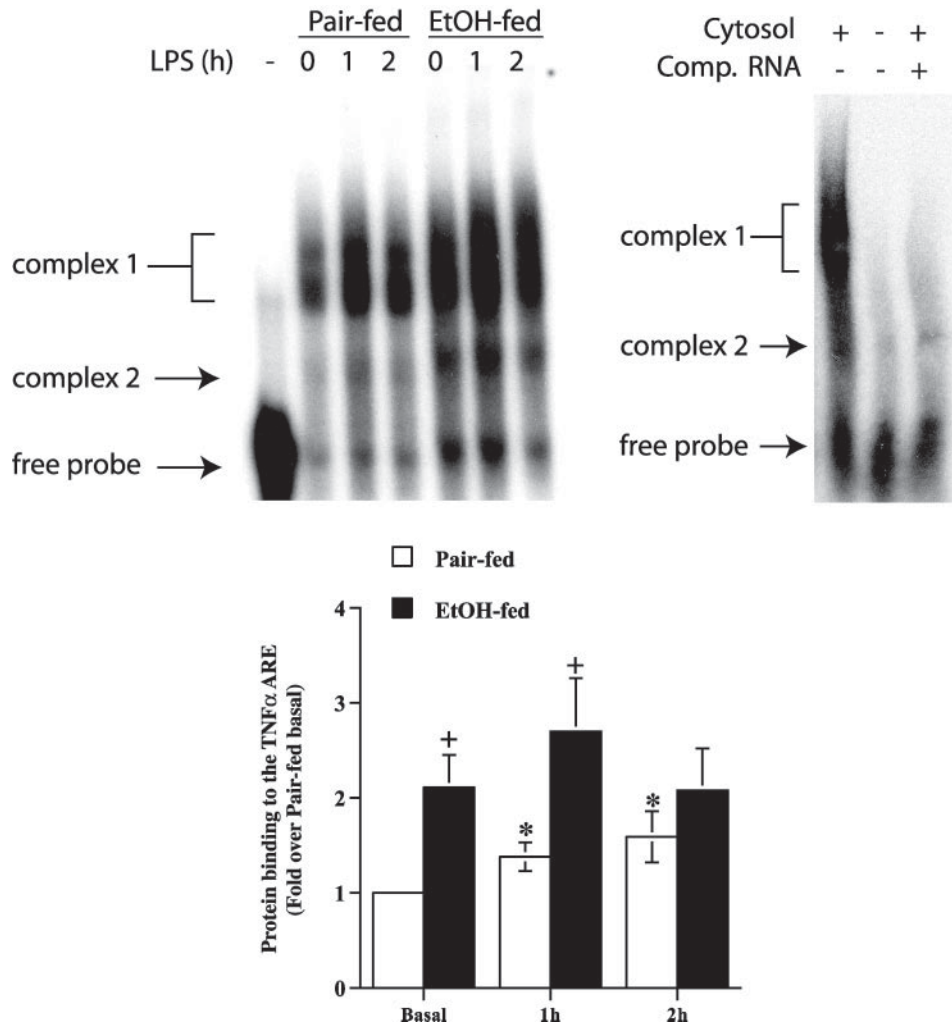


FIG. 3. **Chronic ethanol increases the binding of cytosolic proteins from Kupffer cells to the TNF α 3'-UTR-ARE.** Kupffer cells isolated from pair- and ethanol-fed rats were treated or not with 100 ng/ml LPS for 0–2 h. Cytosolic extracts were then prepared and incubated with 20 fmol of 32 P-labeled RNA probe for the 65-nucleotide TNF α 3'-UTR-ARE. Some extracts were pre-incubated with unlabeled probe (200 \times excess unlabeled RNA *in vitro* transcribed without the addition of [32 P]UTP) or without cytosol to demonstrate the specificity of the protein binding. Protein-RNA complexes were then resolved by electrophoresis on 4% acrylamide gels. Bands were quantified by phosphorimaging, and relative quantities of complex 1 are shown as means \pm S.E., $n = 4$; *, $p < 0.05$ compared with basal; +, $p < 0.05$ compared with pair-fed.

regions of the cell (Fig. 2A). This typical cytosolic/punctate distribution of HuR has been suggested to indicate a localization of HuR to polyribosomes (37) or to stress granules (38). After chronic ethanol feeding, HuR was already present to some extent in the cytosol at baseline. LPS further increased HuR content in the cytosol compared with baseline after ethanol feeding; HuR quantity in the cytosol after LPS treatment also remained higher after ethanol feeding compared with controls (Fig. 2A). Cytosolic and nuclear extracts from Kupffer cells were probed for HuR protein by Western blot. LPS stimulation increased the quantity of HuR in cytosolic fractions in Kupffer cells isolated from both pair- and ethanol-fed rats (Fig. 2B). However, after chronic ethanol feeding, the localization of HuR to the cytosol was higher at both baseline and after LPS treatment (Fig. 2B). On average, there were no detectable changes in HuR content in the nucleus either in response to LPS or chronic ethanol, most likely because of the high concentration of HuR in the nucleus relative to the cytosol.

To determine whether these chronic ethanol-induced shifts in intracellular distribution of HuR were associated with increased TNF α mRNA binding activity, we carried out RNA gel shift assays. Using 20 fmol of 32 P-labeled TNF α 3'-UTR-ARE (65 nucleotides in length from positions 1281 to 1350) (23), multiple complexes formed between the cytosolic extracts and

the labeled probe (Fig. 3). The complexes were competed from binding to the 32 P-labeled TNF α ARE probe by inclusion of an excess quantity of unlabeled 65-nucleotide probe and was not observed when the 32 P-labeled probe was incubated in the absence of cytosolic extracts (Fig. 3). LPS treatment increased the formation of complex 1 over 0–2 h of stimulation (Fig. 3) but had no significant effect on the formation of complex 2 (data not shown). In Kupffer cells isolated from rats fed ethanol, the quantity of complex 1 formed both at baseline and, in response to LPS, was higher than in pair-fed controls (Fig. 3).

HuR binds to the TNF α 3'-UTR ARE in response to stimulation with LPS in RAW 264.7 macrophages (23). Here we asked whether increased quantity of HuR in the cytosol (Fig. 2, A and B) after chronic ethanol resulted in an increase in the binding of HuR to the TNF α 3'-UTR. To increase the resolution of the HuR supershift in this assay, we used 4–20% gradient gels to better resolve high molecular weight components (Fig. 4A). The larger complex 1 was well resolved on these gels (Fig. 4A), whereas the lower molecular weight complex and free probe were not well resolved (data not shown). Inclusion of 100-fold molar excess of unlabeled 65-nucleotide probe in the reaction mixture prevented formation of labeled complexes (Fig. 4C), and no protein complexes were formed with a non-specific actin RNA probe (Fig. 4D). As in Fig. 3, an increase in

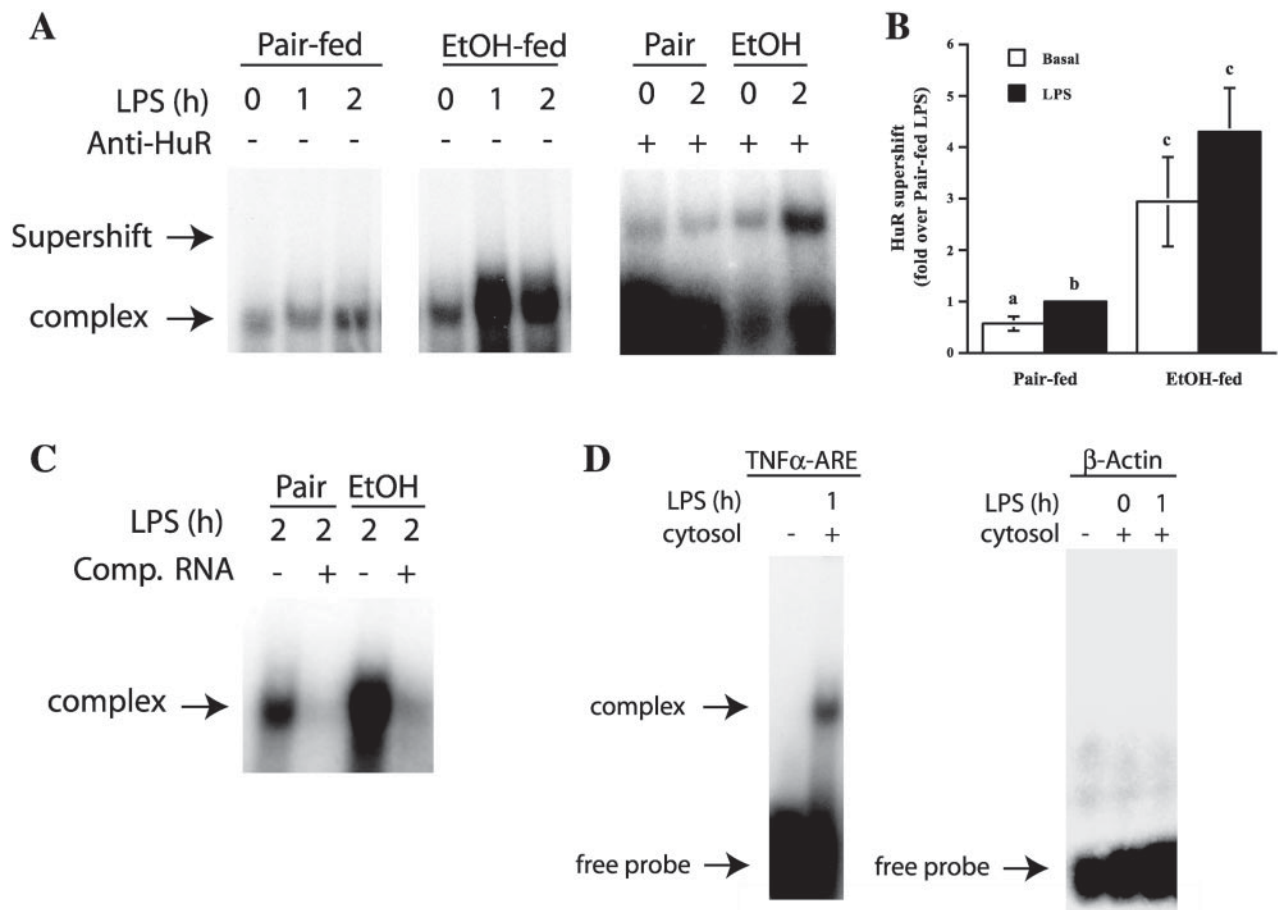


FIG. 4. HuR binding to TNF α 3'-UTR ARE is increased after chronic ethanol feeding. *A*, Kupffer cells isolated from pair- and ethanol-fed rats were treated or not with 100 ng/ml LPS for 2 h. Cytosolic extracts were prepared and then incubated with 20 fmol of the *in vitro* transcribed 32 P-labeled RNA probe for the 65-nucleotide TNF α 3'-UTR-ARE. Samples were then incubated or not with anti-HuR monoclonal antibody (4 μ g/reaction) as described under "Experimental Procedures" and separated on a 4–20% gradient acrylamide gel. *B*, supershifts were quantified by phosphorimaging, and the relative quantities of supershifted complex are shown as means \pm S.E., $n = 10$; values with different letters are significantly different; $p < 0.05$. *C*, Kupffer cells isolated from pair-fed rats were treated or not with LPS for 1 h. Cytosolic extracts were prepared and incubated with 20 fmol of 32 P-labeled RNA probe for the 65-nucleotide TNF α 3'-UTR-ARE. Extracts were pre-incubated with or without excess unlabeled probe (100 \times excess unlabeled RNA *in vitro* transcribed without the addition of 32 P-UTP) and separated on a 4–20% gradient acrylamide gel. *D*, Kupffer cells isolated from ethanol-fed rats were treated or not with LPS for 1 h. Cytosolic extracts were prepared and incubated with 20 fmol of 32 P-labeled RNA probe for the 65-nucleotide TNF α 3'-UTR-ARE or 20 fmol of 32 P-labeled actin RNA and separated on a 4–20% gradient acrylamide gel.

the formation of a protein-RNA complex after chronic ethanol feeding was observed (Fig. 4A). Inclusion of antibodies specific for HuR resulted in a supershifting of this complex, demonstrating that HuR is a component of this complex (Fig. 4A). However, antibody to HuR did not completely supershift the RNA-protein complex, even at higher concentrations of antibody (data not shown), suggesting that additional proteins also form complexes with the TNF α -ARE. In Kupffer cells from pair-fed rats, HuR was present to some extent in the complex at baseline and increased 2-fold in response to LPS. In contrast, after chronic ethanol feeding, HuR binding to the TNF α 3'-UTR ARE was higher at both baseline and in response to LPS compared with pair-fed controls (Fig. 4, A and B).

If this chronic ethanol-induced increase in the mRNA binding activity of HuR was required to mediate the chronic effects of ethanol on TNF α expression, we hypothesized that knocking down HuR expression using siRNA (39) should prevent the chronic effects of ethanol. RAW 264.7 macrophages were transfected with an siRNA designed to interfere with HuR expression (35), along with the pTNF α -5'-UTR-LUC-3'-UTR or SV40-LUC-TNF α 3'-UTR luciferase reporter plasmids. After 48 h in culture, the expression of HuR protein was decreased by 40–50% as assessed by Western blot (Fig. 5A). After culture with or

without ethanol for 48 h, cells were stimulated or not with 100 ng/ml LPS for 4 h, and luciferase activity was measured. In cells transfected with non-silencing (control) siRNA, LPS stimulation of luciferase activity was increased after chronic exposure to ethanol (Fig. 5B). In cells transfected with siHuR RNA, LPS stimulation was decreased moderately in control cells (Fig. 5B), consistent with the increased binding of HuR to the TNF α mRNA after LPS stimulation (Fig. 4). However, transfection with siHuR RNA completely eliminated the increase in LPS-stimulated luciferase activity observed after chronic ethanol exposure compared with control cells (Fig. 5B).

DISCUSSION

HuR, a widely expressed member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins, selectively binds to AU-rich elements in the 3'-UTR of a number of short-lived mRNAs (25), including the TNF α mRNA (23). HuR is a nuclear/cytoplasmic shuttling protein that is postulated to bind to specific mRNAs in the nucleus, shuttling with them to the cytoplasm, providing protection from RNA degradation machinery (25). Cytoplasmic localization of HuR is associated with conditions of cellular stress including heat shock (37), UV irradiation (40), and amino acid starvation (41), as well as stim-

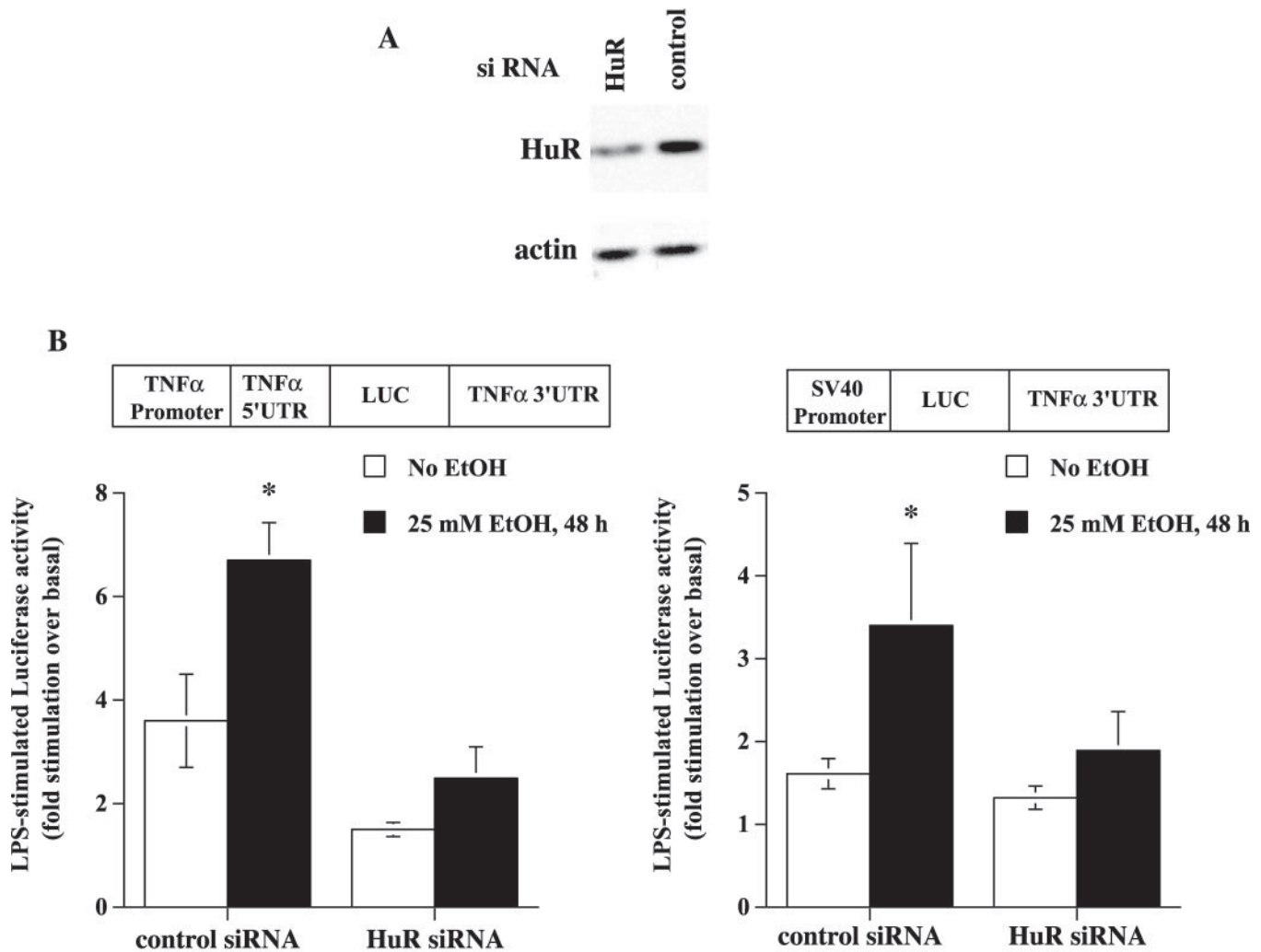


FIG. 5. Knock-down of HuR with siHuR RNA prevents chronic ethanol-induced increases in LPS-stimulated luciferase activity in a heterologous reporter containing the TNF α 3'-UTR. *A*, RAW 264.7 macrophages were transfected with siHuR RNA or a non-silencing (control) siRNA, and expression of HuR was measured by Western blot after 48 h. 20 μ g of total cell lysates were separated by SDS-PAGE and probed for HuR or actin by Western blotting. The figure is representative of three experiments. *B*, RAW 264.7 macrophages were transfected with the pTNF α -5'-UTR-LUC-3'-UTR or SV40-LUC-TNF α 3'-UTR reporter constructs, as well as a Renilla luciferase reporter under the control of the thymidine kinase promoter and siHuR RNA or non-silencing (control) siRNA, as described under "Experimental Procedures." After transfection, cells were replated in 96-well plates and cultured with or without 25 mM ethanol for 48 h. Cells were then stimulated or not with 100 ng/ml LPS for 4 h, and activity of luciferase and Renilla luciferase was measured. Values are expressed as -fold stimulation by LPS over basal, means \pm S.E., $n = 4$; *, $p < 0.05$ compared with cells not cultured with ethanol.

ulation of macrophages with LPS (Fig. 3) (23). Here we have demonstrated that the TNF α 3'-UTR is sufficient to convey increased LPS-stimulated responses to a heterologous reporter construct after chronic ethanol exposure. We have also found that chronic ethanol exposure increases the activity of HuR. Not only did chronic ethanol feeding increase the localization of HuR to the cytosol of Kupffer cells (Fig. 2), *in vitro* binding of HuR to the TNF α 65 nucleotide ARE was also increased after chronic ethanol exposure. When HuR expression was decreased in RAW 264.7 macrophages using an siRNA directed against HuR, chronic ethanol exposure no longer increased LPS-stimulated expression of luciferase reporters containing the TNF α 3'-UTR. Taken together, these data demonstrate a critical role for HuR in mediating the chronic effects of ethanol on LPS-stimulated TNF α production.

The TNF α 3'-UTR is involved in the regulation of the rate of translation of the TNF α mRNA, as well as TNF α mRNA stability (42, 43). Several lines of evidence indicate that chronic ethanol acts primarily to enhance TNF α mRNA stability rather than to increase translation. First, there is a very good correlation between the time course of chronic ethanol-induced in-

creases in LPS-stimulated accumulation of TNF α mRNA and secreted peptide in RAW 264.7 macrophages (18). Second, we have found that the half-life of TNF α mRNA after LPS stimulation is increased after chronic ethanol exposure to >100 min, compared with <40 min in control cells (17). Our current data demonstrating an essential role for HuR in mediating the chronic effects of ethanol in macrophages is consistent with a chronic ethanol-induced stabilization of TNF α mRNA in macrophages.

Regulation of HuR expression has been shown to modulate the stability of a number of mRNAs containing 3'-UTR-AREs, including TNF α mRNA. For example, transient overexpression of HuR in HeLa cells stabilizes rabbit β -globin mRNA containing 44 nucleotides of the TNF α 3'-UTR ARE (23), as well as AREs from granulocyte-macrophage colony-stimulating factor and *c-fos* (44). Further, expression of antisense HuR destabilizes p21 mRNA (40), as well as cyclins A and B1 (45). Stabilization of the mRNA for soluble guanylyl cyclase α -1 subunit involves HuR (35). Knock-down of HuR expression, by transfection with an siRNA targeted against HuR, decreases the α -1 subunit mRNA and protein expression (35). Further, the level

of HuR expression in human colon cancer cells is positively correlated with expression of cyclooxygenase-2 (46), another message that is stabilized by HuR binding (47). Here we show that knock-down of HuR expression using siRNA eliminated the chronic effects of ethanol on LPS-stimulated luciferase activity in reporter constructs containing the TNF α 3'-UTR, demonstrating an essential role for HuR in mediating the chronic effects of ethanol on LPS-stimulated TNF α production.

Regulation of the subcellular localization of HuR plays an important role in the cellular response to several types of cellular stress, including amino acid starvation (41) and heat shock (37), as well as to activation/stimulation, such as in response to activation of macrophages with LPS (23). HuR export from the nucleus utilizes two independent nuclear export pathways (48–50). In non-stimulated cells, HuR export from the nucleus is dependent on its endogenous shuttling domain, termed HNS (HuR nucleocytoplasmic shuttling) domain (44). In contrast, HuR export is dependent on CRM1 (chromosomal region maintenance protein 1) nuclear export machinery after heat shock (49). However, it is not clear whether the CRM1 pathway is involved in HuR export from the nucleus in response to other stressors such as UV irradiation or LPS. Here we show LPS stimulation results in the rapid translocation of HuR from the nucleus to the cytoplasm (Fig. 2). After chronic ethanol exposure, there was increased accumulation of HuR in the cytosol, both in non-stimulated cells and in response to LPS, compared with controls (Fig. 2). This increased cytosolic distribution of HuR, even in non-stimulated cells, suggests that chronic ethanol exposure may impact on the pathways maintaining HuR sequestration to the nucleus. Ethanol effects the subcellular distribution of other nuclear/cytoplasmic shuttling proteins, including cAMP-dependent protein kinase (51), the delta and epsilon forms of protein kinase C (52), and RACK 1 (receptor for activated protein kinase 1) (53). The mechanism for abnormal nuclear localization of these signaling proteins in response to ethanol is not known. Taken together, this disruption in the cytoplasmic/nuclear localization of a number of signaling proteins, as well as HuR, suggests that ethanol may impair the regulation of proteins involved in nuclear export, such as the CRM1 pathway.

Interestingly, our data also suggest that there may be an activation of HuR binding activity after chronic ethanol exposure. Although the HuR protein quantity in the cytosol increased by 50% after chronic ethanol exposure compared with controls (Fig. 2B), binding of HuR to the TNF α 3'-UTR ARE increased by 5.5-fold (Fig. 4). Mechanisms for the regulation of HuR RNA binding activity are not well understood. LPS stimulation induces the methylation of HuR by CARM1 (coactivator-associated arginine methyltransferase 1) (54); however, it is not known whether methylation of HuR impacts on its ability to stabilize labile mRNAs and/or its relative distribution between the nucleus and cytosol (54). Regulation of HuR activity by phosphorylation/dephosphorylation has been suggested, because inhibitors of protein phosphatase 2A modulate HuR activity (48). Further, cAMP-dependent protein kinase is required for stabilization of the Na⁺-coupled glucose cotransporter (SLGT1) (55), and cAMP-dependent protein kinase regulates the binding of HuR to the SLGT1 3'-UTR (56). In a screen of a number of protein kinases known to be involved in the regulation of unstable mRNAs, such as the mitogen-activated protein kinase family members, extracellular signal-regulated kinase, p38, and c-Jun N-terminal kinase, as well as protein kinases C and A, none were found to affect the cytoplasmic localization of HuR (57). Of all the kinases tested, only AMP-activated protein kinase was involved in the regulation of HuR localization. Activation of AMP-activated protein kinase

decreased HuR in the cytosol, whereas inhibition of this stress-activated kinase increased HuR in the cytosol and stabilized a number of short-lived mRNAs (57). Chronic ethanol exposure disrupts the activity of a number of cell signaling pathways in macrophages, including cAMP-dependent protein kinase (15), as well as the mitogen-activated protein kinases, extracellular signal-regulated kinase 1/2 and p38 (16); however, no data are available on the effect of ethanol on AMP-activated protein kinase. The elucidation of the specific signaling pathways targeted by chronic ethanol leading to increased HuR RNA binding activity will require further investigation.

In summary, we have found that chronic ethanol exposure increases the RNA binding activity of HuR and that HuR is required to mediate the chronic effects of ethanol on LPS-stimulated TNF α production. These data thus add chronic ethanol exposure to the growing list of cellular stresses, including heat shock (37), UV irradiation (40), and amino acid starvation (41), that regulate the intracellular distribution and RNA binding activity of HuR. Considering the essential role of TNF α in mediating the chronic effects of ethanol, it is likely that disruption of the subcellular localization of HuR and its RNA binding activity by chronic ethanol in macrophages may contribute to the progression of alcoholic liver injury. Moreover, because HuR is involved in the stabilization of a number of short-lived mRNAs, chronic ethanol-induced changes in HuR function may also contribute to the increased production of other cytokines and inflammatory mediators during chronic ethanol exposure.

Acknowledgments—We are thankful to Ibrahim Yaman for assistance in developing the RNA gel shift assays. We are also thankful to Drs. V. Krays, N. Mackman, and D. Joyce for kindly providing luciferase reporter constructs.

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