Chronic Ethanol Exposure Increases the Binding of HuR to the TNFα 3’-Untranslated Region in Macrophages*

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Tumor necrosis factor α (TNFα) expression is a key mediator of ethanol-induced liver disease. Increased lipopolysaccharide (LPS)-stimulated TNFα expression in macrophages after chronic ethanol feeding is associated with a stabilization of TNFα 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 TNFα 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 TNFα mRNA, is required for increased expression of TNFα 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 TNFα 3’-UTR by 2-fold over baseline in Kupffer cells from pair-fed rats. After chronic ethanol feeding, HuR binding to the TNFα 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-increased susceptibility to endotoxin-induced liver injury. LPS-stimulated TNFα production is increased in hepatic macrophages (Kupffer cells) after chronic ethanol feeding, as well as after in vitro exposure of RAW 264.7 macrophages to ethanol during culture.

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

Stability of the TNFα mRNA is controlled by trans-acting factors that bind to the TNFα mRNA. A large number of mRNA-binding proteins regulate both stabilization and destabilization (25). Of these, several proteins that bind to the TNFα 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 TNFα 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 TNFα mRNA (23, 30). Here we report that the TNFα 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.
to the ARE in TNFα 3′-UTR, likely contributing to chronic ethanol-induced stabilization of TNFα mRNA.

EXPERIMENTAL PROCEDURES

Materials—LPS from *Escherichia coli* serotype 026:B6 (tissue culture tested) was purchased from Sigma. The 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 In-vitrogen. Low endotoxin plasmid preparation kits were from Qiagen (Valencia, CA). Luciferase reporter constructs have been described previously: pTNFα-LUC (containing −615→+1 nucleotide TNFα promoter in a luciferase reporter) was from N. Mackman, University of California, San Diego, CA (31); pTNFα-5′-UTR-LUC (containing −993→+110 of the TNFα promoter and 5′-UTR in pGLO vector) and pTNFα-5′-UTR-LUC-3′-UTR (containing −993→+110 of the TNFα promoter and 5′-UTR, as well as the 5′-3′ base TNFα 3′-UTR in pGLO vector) luciferase reporters were from D. Joyce, University of Western Australia (32, 33); and SV40-LUC-3′-UTR luciferase reporter (containing the TNFα 3′-UTR in pGLO vector) and the SV40-LUC-3′-UTR ARE deletion were from V. Kruys (34). Plasmids used for synthesis of RNA probes, including 65-nucleotide TNFα ARE (1281–1350 in the 3′-UTR and the full-length TNFα 3′-UTR 1110–1287), were from P. Blackshear, National Institutes 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 substituted maltose-dextrin for ethanol as described previously (17). Kupffer cell isolation and culture—Kupffer cells were isolated as described previously (15) except that CMRL medium was used to isolate 65-nucleotide TNFα ARE and cytosolic extracts were prepared (23) from Kupffer cells isolated from liver tested) was purchased from Sigma; all experiments were carried out according to the manufacturer’s instructions. siRNA directed against HuR (20 μg/ml) in blocking solution overnight at 4 °C. Cells were washed three times for 15 min in PBS and then incubated for 1 h with Alexa Fluor 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 ×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 Gel Shift Assays—RNA gel shift assays were carried out 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 μg of siRNA HuR or control (non-silencing; Qiagen), along with 1.2 μg of pTF-5′-UTR-LUC-3′-UTR or LUC-3′-UTR (luciferase reporters) and 0.1 μg of pTK-RL (Renilla reporter), were diluted in 6.6 μl of Enhancer R (ratio of RNA/DNA to Enhancer R is 1:2) and 85 μl of Buffer EC-R (Qiagen). 8 μl 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 μl 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 West-ERN blotting) at a density of 0.4×10⁶ cells/ml. Cells were allowed to adhere overnight, and then treated with or without 25 μM ethanol for 48 h. Transfection efficiencies were determined by transfecting RAW 264.7 macrophages with a fluorescent-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 ± S.E. Data were analyzed by Student’s t test or general linear models procedure (SAS, Carey, IN) using the least squares means test to determine differences.

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RESULTS

Chronic exposure to ethanol, either in vivo during ethanol feeding or in vitro upon exposure to ethanol in culture, increases LPS-stimulated TNFα mRNA accumulation in isolated Kupffer cells and RAW 264.7 macrophages, respectively (17). Increased LPS-stimulated TNFα mRNA accumulation after chronic ethanol is associated with a stabilization of TNFα mRNA (17). Here we have asked whether sequences in the 5′-UTR and 3′-UTR of the TNFα 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 TNFα 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 TNFα 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 TNFα in macrophages (18). Similarly, inclusion of the TNFα 5′-UTR did not convey a chronic ethanol-induced increase in LPS-stimulated luciferase activity (Fig. 1). Inclusion of the TNFα 3′-UTR in this reporter construct decreased LPS-stimulated activity in control cells, consistent with mRNA instability elements within the TNFα 3′-UTR (21). In contrast, after chronic ethanol exposure, LPS-stimulated luciferase activity was 4.4-fold higher when the TNFα 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-
ethanol exposure, RAW 264.7 macrophages were transfected with a luciferase reporter containing the TNFα 3′-UTR under the control of the SV40 promoter (SV40-LUC-TNFα 3′-UTR). Luciferase reporter constructs under the control of the SV40 promoter (SV40-LUC, pGL2), but without the TNFα 3′-UTR, did not respond to LPS stimulation (data not shown). Insertion of the TNFα 3′-UTR (SV40-LUC-TNFα 3′-UTR) conveyed LPS-stimulated luciferase activity (Fig. 1) (33). Moreover, including the TNFα 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 TNFα 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 TNFα 3′-UTR under the control of the SV40 promoter (SV40-LUC-TNFα 3′-UTR) 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-TNFα 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-TNFα 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 μg of DNA was transfected for control and ethanol-treated, respectively, to 3.5- and 2.9-fold when 4.8 μg 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 TNFα mRNA accumulation and peptide accumulation at sub-maximal LPS concentrations (15, 36).

Because the TNFα 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 TNFα (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

![Image](https://example.com/image.png)

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 μg) and nuclear (10 μg)) were then separated by SDS-PAGE and probed for HuR by Western blot analysis. Values represent immunoreactive HuR in cytosol, means ± S.E., n = 5; *, p < 0.05 compared with basal; †, p < 0.05 compared with pair-fed.

![Graph](https://example.com/graph.png)
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 TNFα mRNA binding activity, we carried out RNA gel shift assays. Using 20 fmol of 32P-labeled TNFα 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 32P-labeled TNFα ARE probe by inclusion of an excess quantity of unlabeled 65-nucleotide probe and was not observed when the 32P-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 TNFα 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. 2A and B) after chronic ethanol resulted in an increase in the binding of HuR to the TNFα 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
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 TNFα 3′-UTR 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 TNFα 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 TNFα 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 pTNFα-5′-UTR-LUC-3′-UTR or SV40-LUC-TNFα 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 TNFα 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 TNFα 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. 4.** HuR binding to TNFα 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 32P-labeled RNA probe for the 65-nucleotide TNFα 3′-UTR-ARE. Samples were then incubated or not with anti-HuR monoclonal antibody (4 μg/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 ± 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 32P-labeled RNA probe for the 65-nucleotide TNFα 3′-UTR-ARE. Extracts were pre-incubated with or without excess unlabeled probe (100× excess unlabeled RNA in vitro transcribed without the addition of 32P-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 32P-labeled RNA probe for the 65-nucleotide TNFα 3′-UTR-ARE or 20 fmol of 32P-labeled actin RNA and separated on a 4–20% gradient acrylamide gel.
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The regulation of macrophages with LPS (Fig. 3) (23). Here we have demonstrated that the TNFα 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 TNFα 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 TNFα 3′-UTR. Taken together, these data demonstrate a critical role for HuR in mediating the chronic effects of ethanol on LPS-stimulated TNFα production.

The TNFα 3′-UTR is involved in the regulation of the rate of translation of the TNFα mRNA, as well as TNFα mRNA stability (42, 43). Several lines of evidence indicate that chronic ethanol acts primarily to enhance TNFα mRNA stability rather than to increase translation. First, there is a very good correlation between the time course of chronic ethanol-induced increases in LPS-stimulated accumulation of TNFα mRNA and secreted peptide in RAW 264.7 macrophages (18). Second, we have found that the half-life of TNFα 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 TNFα mRNA in macrophages.

Regulation of HuR expression has been shown to modulate the stability of a number of mRNAs containing 3′-UTR-AREs, including TNFα mRNA. For example, transient overexpression of HuR in HeLa cells stabilizes rabbit β-globin mRNA containing 44 nucleotides of the TNFα 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 TNFα 3′-UTR, demonstrating an essential role for HuR in mediating the chronic effects of ethanol on LPS-stimulated TNFα 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 affects 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 RACK1 (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 TNFα 3′-UTR ARE is increased by 5.5-fold (Fig. 4). Mechanisms for the regulation of HuR RNA binding activity are not well understood. LPS stimulation increases 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 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 TNFα 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 TNFα 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.

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**Increased TNFα after Chronic Ethanol Involves HuR**