ERK1/2 and Egr-1 contribute to increased TNF-α production in rat Kupffer cells after chronic ethanol feeding

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Kishore, Raj, Jeanette R. Hill, Megan R. McMullen, Julia Frenkel, and Laura E. Nagy. ERK1/2 and Egr-1 contribute to increased TNF-α production in rat Kupffer cells after chronic ethanol feeding. Am J Physiol Gastrointest Liver Physiol 282: G6–G15, 2002. First published September 21, 2001; 10.1152/ajpgi.00328.2001.—Activation of Kupffer cells by lipopolysaccharide (LPS) is a critical step in the pathogenesis of alcoholic liver disease. Kupffer cells isolated from rats fed ethanol in their diet for 4 wk accumulated 4.3-fold more tumor necrosis factor (TNF)-α in response to LPS compared with pair-fed rats. In contrast, LPS-stimulated interleukin (IL)-1 accumulation was 50% lower after ethanol feeding. LPS-stimulated TNF-α mRNA accumulation was twofold higher after ethanol feeding, whereas IL-1β mRNA accumulation was blunted. To understand the mechanisms for this differential response, we investigated the effects of ethanol on LPS-dependent signal transduction. Chronic ethanol feeding increased LPS-stimulated extracellular receptor-activated kinases 1/2 (ERK1/2) activation. Activation of ERK1/2 was required for maximal increases in TNF-α and IL-1β mRNA and was associated with increased binding of early growth response-1 (Egr-1) to the TNF-α promoter after ethanol feeding. In contrast, ethanol feeding completely abrogated activation of nuclear factor-κB DNA-binding activity by LPS and had no effect on AP-1 binding. Together, these data suggest that enhanced activation of ERK1/2 and Egr-1 contributes to increased TNF-α production after chronic ethanol feeding.

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ETHANOL DISRUPTS HEPATIC FUNCTION with the eventual appearance of alcoholic liver disease (18). Kupffer cells, the resident macrophages in the liver, are critical to the onset of ethanol-induced liver injury. Ablation of Kupffer cells prevents the development of fatty liver and inflammation, early events in the progression of ethanol-induced liver damage, in rats chronically exposed to ethanol via intragastric feeding (1). Endotoxin (or lipopolysaccharide (LPS)), a component of the cell wall of gram-negative bacteria, is an important inflammatory mediator. Its concentration is increased in the blood of alcoholics (5, 9) and rats exposed to ethanol via gastric infusion (26). Lipopolysaccharide (LPS) exposure also appears to play a role in the development of liver injury in rats after long-term ethanol exposure; when ethanol-infused rats are treated with antibiotics, endotoxin concentrations are decreased and the development of fatty liver and inflammation reduced (2).

Despite the clear role of LPS and Kupffer cells in the development of alcoholic liver disease, very little is known about the effects of chronic ethanol exposure on Kupffer cell responses to LPS. Because exposure to LPS is increased during ethanol feeding, Kupffer cell responses to LPS might be desensitized, similar to the phenomenon of endotoxin tolerance described both in vivo and in vitro on repeated exposure to LPS (31). However, both in vivo and in vitro data suggest that chronic ethanol sensitizes Kupffer cells to at least some LPS-mediated responses. For example, long-term ethanol consumption increases the susceptibility of rats to endotoxin-induced liver injury (14, 21). Moreover, Kupffer cells isolated from rats fed ethanol in their diet for 4 wk accumulate up to fivefold more tumor necrosis factor (TNF)-α peptide in response to low doses of LPS or phagocytosis of latex beads (3). In contrast, LPS stimulation of nitric oxide production by Kupffer cells was decreased after 9–13 wk of ethanol feeding (24). Together, these data suggest that chronic ethanol feeding may differentially regulate LPS-dependent responses.

Production of inflammatory cytokines is a highly regulated process; regulation has been reported at the levels of transcription, translation, and secretion (27, 43). Transcriptional activation of TNF-α and interleukin (IL) 1β by LPS involves activation of several transcription factors. Increased TNF-α expression in response to LPS requires the activation of a distinct set of transcription factors binding to at least two regions of the TNF-α promoter, which include nuclear factor-κB (NF-κB), early growth response-1 (Egr-1), and AP-1 binding sites (40, 48). Whereas the exact array of transcription factors interacting with the TNF-α promoter is, to some extent, cell and species specific (22), recruitment of NF-κB and Egr-1 protein, as well as increased c-jun binding to a CRE/AP-1 site, appear to be required for full activation of TNF-α expression in most types of macrophages (40, 48). LPS-induced in-
creases in IL-1β mRNA are primarily mediated by activation of NF-κB and AP-1 (43), but in some cell types, the LPS response also involves activation of extracellular receptor-activated kinases 1/2 (ERK1/2) (22, 32). Activation of each of these nuclear transcription factors is mediated by specific LPS-mediated signaling cascades. LPS binds to a cell surface receptor, CD14, which, via interactions with the toll-like receptor 4 (28), stimulates a complex array of signal-transduction cascades (35, 41). Stimulation of macrophages with LPS activates tyrosine kinases, protein kinase C, NF-κB, as well as members of the mitogen-activated protein kinase family including ERK1/2, p38, and c-Jun NH2-terminal kinase (JNK) (35).

Ethanol disrupts a number of hormone- and neurotransmitter-dependent signaling pathways (8), including many of the same signaling pathways activated by LPS in macrophages. However, few investigations have addressed the specific effects of ethanol on LPS-mediated signal transduction. Therefore, we hypothesized that chronic ethanol feeding may have specific effects on individual LPS-activated signaling cascades, thus contributing to the differential regulation of LPS-dependent responses. Here, we show that chronic ethanol feeding differentially regulates the expression of NF-κB and IL-1β in Kupffer cells and that these differential responses are associated with impaired LPS activation of NF-κB countered by enhanced activation of ERK1/2 and Egr-1.

MATERIALS AND METHODS

Adult male Wistar rats weighing 150 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). Lieber DeCarli ethanol diet was prepared as previously described (45) or purchased from Dyets (Bethlehem, PA). Cell culture reagents were from Gibco (Grand Island, NY). Antibodies were from the following sources: active ERK1/2 (Promega, Madison, WI), ERK1/2 (Upstate Biotechnology, Lake Placid, NY), CD-14 (Zymed, South San Francisco, CA), and Egr-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-rabbit and anti-mouse IgG-peroxidase were purchased from Boehringer Mannheim (Indianapolis, IN). The Egr-1 binding site in the murine TNF-α promoter was synthesized by IDT Technologies (Coralville, IA), and the AP-1 and NF-κB consensus binding site oligonucleotides were purchased from Santa Cruz Biotechnology. PD-98059 was from Calbiochem (La Jolla, CA). LPS from E. coli serotype 026:B6 was purchased from Sigma (St. Louis, MO).

Chronic ethanol feeding protocol. Rats were acclimatized for 3 days after arrival and provided with free access to Purina rat chow and water. All rats were then allowed free access to liquid diet (19) without ethanol for 2 days and then randomly assigned to the ethanol- or pair-fed groups. The ethanol-fed group was allowed free access to liquid diet with 17% of calories as ethanol for 2 days and then provided with diet containing 35% of the calories from ethanol for 4 wk. Controls were pair fed a liquid diet that was identical to the ethanol diet except that maltose dextrins were isocalorically substituted for ethanol. Pair-fed rats were given the same amount of food as their ethanol pair consumed in the preceding 24 h. Serum ethanol concentrations measured 3 h after the end of the dark cycle were routinely 35–40 mM in the ethanol-fed rats (10). 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 previously described (3) except that Connaught’s Medical Research Laboratory (CMRL) media 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 g for 2 min, and the supernatant was collected after each centrifugation. The pooled supernatant was then centrifuged at 500 g for 7 min to collect nonparenchymal cells. Kupffer cells were then purified by centrifugal elutriation (3).

Isolated Kupffer cells were suspended in CMRL with 10% fetal bovine serum (FBS) and penicillin streptomycin at a concentration of 2 × 10⁶ cells/ml and plated onto 96 (0.2 ml/well), 6 (3 ml/well), or 100-well (5 ml/well) culture plates. Nonadherent cells were removed by aspiration after 2 h, and fresh media were supplied. Assays were initiated after 20–24 h in culture. The number of Kupffer cells isolated from pair-fed rats [26 ± 3 × 10⁶] did not differ from ethanol-fed rats [33 ± 4 × 10⁶ cells, n = 19]. Similarly, the number of cells per well or protein concentration per well at 24 h after culture did not differ between pair- and ethanol-fed rats [10.5 ± 0.5 × 10⁴ cells/well (in a 96-well plate)] in pair-fed compared with 9.0 ± 0.4 ± 10⁴ cells/well in ethanol-fed rats (n = 19) and 4.5 ± 0.7 μg protein/well in pair-fed compared with 3.9 ± 0.4 μg protein/well in ethanol-fed rats (n = 16).

Accumulation of TNF-α and IL-1. After culture for 20 h, cell culture media was removed and replaced with fresh media with or without 10% FBS. Kupffer cells were then stimulated with 0–1,000 ng/ml LPS. In some experiments, cells were preincubated with PD-98059 or vehicle (DMSO) for 2 h before LPS stimulation. After 4 and 24 h stimulation with LPS, cell culture media were removed and stored at −20°C for assay of TNF-α (4 h) or IL-1 (24 h). LPS-stimulated TNF-α accumulation was maximum at 4 h, whereas IL-1 was maximal at 24 h (data not shown). TNF-α and IL-1 accumulated in the extracellular media were measured in bioassays as previously described (3, 13).

Western blot analysis of CD14. Isolated Kupffer cells were homogenized in 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 250 mM sucrose with protease inhibitors (Complete Roche Molecular Biochemicals, Indianapolis, IN), 1 mg/ml bacitracin, and 1 mg/ml benzamidine. A plasma membrane-enriched fraction was prepared by first centrifuging homogenates at 200 g for 15 min to remove nuclei and unbroken cells. The resulting supernatant was then centrifuged at 18,000 g for 30 min. The resulting pellet was resuspended in homogenizing buffer, and protein concentration was measured and diluted to 1 mg protein/ml in SDS sample buffer. Samples were separated by SDS-PAGE and probed by Western blotting with an antibody specific for CD14 (1:500 dilution). Bound antibody was detected by chemiluminescence. Immunoreactive protein quantity was assessed by scanning densitometry.

Ribonuclease protection assay for TNF-α and IL-1. After 20 h in culture, Kupffer cell media were removed and replaced with fresh media containing 10% FBS. Cells were then stimulated with 0 or 100 ng/ml LPS for 1 h. In some experiments, cells were preincubated with PD-98059 or vehicle (DMSO) for 2 h before LPS stimulation. Total RNA was isolated by the TRIzol method (GIBCO). Rat cytokine multiscribe DNA templates (Pharmingen, San Diego, CA) were used to synthesize in vitro transcribed antisense riboprobes, and ribonuclease protection assays were carried out following manufacturer’s instructions. Samples were run on 5% sequencing gels, dried, and autoradiographed.
Fig. 1. Differential activation of tumor necrosis factor (TNF-α) and interleukin (IL) 1 accumulation by lipopolysaccharide (LPS) in Kupffer cells after chronic ethanol feeding. Kupffer cells were isolated from ethanol- and pair-fed rats and cultured for 24 h. Cells were then stimulated with 0–1,000 ng/ml LPS in Connaught’s Medical Research Laboratory (CMRL) medium with 10% fetal bovine serum. After 4 (TNF-α) or 24 h (IL-1), supernatants were harvested. TNF-α and IL-1 concentrations were measured by bioassay. Concentrations after treatment with LPS were normalized to the concentration measured in cells not treated with LPS. Quantity of TNF-α in the absence of LPS was 1.2 ± 0.3 in pair-fed rats and 4.4 ± 2.0 ng/10⁶ cells in ethanol-fed rats (P = 0.17); IL-1 was 926 ± 165 in pair-fed rats and 1,931 ± 338 ng/10⁶ cells in ethanol-fed rats (P < 0.05). Values represent means ± SE; n = 6 for TNF-α and 7 for IL-1. *P < 0.05 compared with pair-fed rats.

**Activation of ERK1/2.** After 24 h in culture, cells were treated with or without 100 ng/ml LPS in DMEM/10% FBS. At the end of each treatment, cells were moved to ice, washed with 2 ml ice-cold PBS buffer, and then lysed in lysis buffer [20 mM Tris-HCl, pH 8, 1% Triton X-100, 100 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and protease inhibitors (Complete)] for 30 min at 4°C. Lysates were centrifuged at 14,000 g for 15 min at 4°C. Proteins were separated by SDS-PAGE, transferred to polyvinylidene fluoride, and probed using anti-active ERK1/2 antibodies. Membranes were then stripped and reprobed with anti-ERK1/2 antibodies.

**Electrophoretic mobility shift assays.** Nuclei were isolated, and proteins were extracted in 0.4 M NaCl (33). Nuclei were centrifuged at 14,000 g for 15 min after extraction, and the supernatants were used for electrophoretic mobility shift assays. An oligonucleotide corresponding to the Egr-1 binding site in the promoter region of the TNF-α gene (5'-AACCTCTGCCCCCG ATGGAG-3') was used to measure the DNA-binding activity of Egr-1. Oligonucleotides for the consensus NF-kB or AP-1 binding sites were used to assess NF-kB and AP-1 DNA-binding activity. Double-stranded oligonucleotides were end-labeled with [γ-32P]ATP. 32P-labeled oligonucleotides (20,000–50,000 cpm) were used in each binding reaction (20 μl), which contained 0.2 pmol of DNA probes, 3–5 μg of nuclear extracts, and binding buffer [5% glycerol, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 25 ng/ml of poly(dI-dC)]. After being incubated on ice for 20 min, the mixtures were loaded onto 6% nondenaturing polyacrylamide gels prerun with a buffer [20 mM Tris base (pH 8.3), 20 mM boric acid, and 0.5 mM EDTA] with 1% glycerol and 4 mM MgCl₂ at 120 V for 30 min. The gels were run at 120 V, dried, and autoradiographed.

**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 five or six rats per feeding group. Values reported are means ± SE. Data were analyzed by Student’s t-test or general linear models procedure (SAS, Carey, IN) blocking for trial effects if data from more than one trial were used. Data were log transformed if needed to obtain a normal distribution.

**RESULTS**

Kupffer cells isolated from rats fed ethanol for 4 wk accumulated up to 4.3-fold higher concentrations of TNF-α in their media in response to challenge with LPS, with no difference in TNF-α accumulation between pair- and ethanol-fed rats among cells not treated with LPS (Fig. 1) (3). In contrast, basal accumulation of IL-1 in the extracellular media of Kupffer cells from ethanol-fed rats was 2.1-fold greater than pair-fed rats (see legend of Fig. 1). LPS stimulation further increased IL-1 accumulation above basal in cells from both pair- and ethanol-fed rats; however, Kupffer cells from ethanol-fed rats were less sensitive to LPS stimulation of IL-1 accumulation compared with pair-fed rats (Fig. 1). Maximal stimulation of IL-1 production by LPS was 4.3-fold over basal in pair-fed rats but only 2.1-fold over basal in ethanol-fed rats (Fig. 1).

Activation of macrophages by LPS requires the binding of LPS with CD14, a glycosylphosphatidylinositol-
anchored membrane protein. The interaction of LPS with its cell surface receptor is facilitated via the interaction of LPS with LPS-binding protein (LBP) (41). LBP is a 60-kDa acute-phase protein present in normal serum; LPS-LBP complexes are more potent activators of TNF-α production than LPS alone (12, 38). Intragastric infusion of ethanol as well as long-term ethanol feeding have been reported to increase the expression of CD14 mRNA in liver of rats (15, 34). CD14 protein expression in isolated Kupffer cells has also been reported to increase after long-term ethanol feeding (16). We measured the concentration of CD14 in Kupffer cells and found that immunoreactive CD14 protein was increased by 50% after ethanol feeding (Fig. 2). To assess the involvement of CD14 in the differential effects of ethanol feeding, we investigated the effects of serum, as a source of LBP, on LPS-dependent TNF-α and IL-1 accumulation. In the absence of serum, LPS-stimulated TNF-α accumulation was reduced compared with stimulation in the presence of serum in Kupffer cells from both pair- and ethanol-fed rats (Fig. 3). However, even in the absence of serum, LPS-stimulated TNF-α accumulation was greater in Kupffer cells from ethanol-fed compared with pair-fed rats (Fig. 3), suggesting that increased TNF-α accumulation after ethanol feeding was not completely due to increased CD14 expression. In contrast, whereas serum potentiated LPS-stimulated IL-1 production in pair-fed rats, there was no serum stimulation of IL-1 after ethanol feeding (Fig. 3). Thus, despite an increase in the quantity of the CD14 receptor for LPS, serum stimulation of LPS-dependent IL-1 accumulation was impaired after ethanol feeding.

We next investigated whether these differential effects of LPS on TNF-α and IL-1 production were associated with differences in mRNA levels. Because detection of IL-1 in the media of Kupffer cells requires a 24-h treatment with LPS compared with only 4 h for TNF-α, we were concerned that the differential responses of TNF-α and IL-1 to LPS after ethanol feeding might be due to the differences in time in culture and/or time of LPS treatment. Therefore, we measured the effects on chronic ethanol feeding on LPS-stimulation of TNF-α and IL-1 mRNA after 60 min stimulation with 100 ng/ml LPS. In nonstimulated cells, mRNA levels for TNF-α and IL-1 were undetectable (Fig. 4). LPS stimulation of TNF-α mRNA accumulation was twofold greater in Kupffer cells from ethanol-fed compared with pair-fed rats (Fig. 3).
with pair-fed rats (Fig. 4). LPS primarily increased expression of IL-1β rather than IL-1α in Kupffer cells (data not shown). Although LPS stimulated IL-1β mRNA accumulation in both pair- and ethanol-fed rats, the response was blunted after ethanol feeding to ~70% of the response in pair-fed rats (Fig. 4).

LPS-dependent increases in TNF-α and IL-1β mRNA are regulated by both transcriptional and post-transcriptional mechanisms (27, 43). Activation of NF-κB is a critical mediator of LPS-stimulated cytokine mRNA expression. Whereas chronic exposure to ethanol decreases NF-κB activation in liver in response to partial hepatectomy (47), no data are available on the ability of LPS to activate NF-κB in Kupffer cells after chronic ethanol exposure. Treatment of Kupffer cells with 100 ng/ml LPS for 60 min increased the binding of nuclear proteins to an oligonucleotide for the consensus sequence for NF-κB binding by fivefold in pair-fed rats. However, challenge with LPS failed to increase NF-κB binding after ethanol feeding (Fig. 5). Thus it is unlikely that NF-κB activity contributes to enhanced LPS-stimulated TNF-α production. Indeed, these data suggest that other compensatory mechanisms must be involved in the Kupffer cell response to chronic ethanol: first to maintain LPS-stimulated increases in TNF-α and IL-1β mRNA, but also to mediate the enhanced TNF-α response after chronic ethanol feeding.

AP-1 is a second critical mediator of LPS-dependent increases in TNF-α and IL-1β expression in macrophages (40, 43). Chronic ethanol increases the expression of c-jun and c-fos in liver (42), as well as increases...
in response to LPS after ethanol feeding was associated with ERK1/2 (L. Shi and L. E. Nagy, unpublished data). Therefore, we investigated the effects of chronic ethanol on ERK1/2 activation. Chronic ethanol feeding increased phosphorylation of ERK1/2 compared with pair-fed rats (Fig. 7). In RAW264.7 macrophages, LPS-stimulated ERK1/2 activation leads to increased expression of the transcription factor Egr-1 in the nucleus, as well as increased Egr-1 binding to the TNF-α promoter (L. Shi and L. E. Nagy, unpublished data). Therefore, we asked whether the enhanced phosphorylation of ERK1/2 in response to LPS after ethanol feeding was associated with increased Egr-1 quantity and DNA-binding activity in Kupffer cells. Stimulation with LPS led to a rapid increase in the expression of Egr-1 protein in Kupffer cells (Fig. 8A). After 30 min exposure to 100 ng/ml LPS, the quantity of Egr-1 was threefold greater in Kupffer cells from ethanol-fed compared with pair-fed rats (Fig. 8A). Consistent with the higher expression of Egr-1 after ethanol feeding, the DNA-binding activity of Egr-1 was also increased twofold after ethanol feeding compared with the LPS-dependent response in pair-fed rats after stimulation with LPS for 60 min (Fig. 8B).

**DISCUSSION**

Current models for the pathogenesis of alcoholic liver disease implicate activation of Kupffer cells as an important initial step in the development of alcoholic liver injury. The activation of Kupffer cells, which serves to protect the liver from injury, becomes dysregulated in alcoholic liver disease. This leads to an excessive inflammatory response that is associated with fibrosis. The dysregulation of Kupffer cell activation is likely to result from an altered response to the lipopolysaccharide (LPS) produced by Gram-negative bacteria. LPS binding to the LPS receptor complex, also known as the Toll-like receptor-4 (TLR-4) complex, results in activation of the MAPK kinases p38, ERK1/2, and JNK. These MAPK kinases activate transcriptional factors such as c-Jun and C/EBP, which are necessary for the production of cytokines such as TNF-α and IL-1β. Therefore, modulation of the MAPK kinases could be important in the treatment of alcoholic liver disease.

Because ERK1/2 activation was essential for maximal LPS-induced increases in TNF-α and IL-1β mRNA, we next investigated the effects of chronic ethanol on activation of ERK1/2. Chronic ethanol feeding increased phosphorylation of ERK1/2 compared with pair-fed rats (Fig. 7). In RAW264.7 macrophages, LPS-stimulated ERK1/2 activation leads to increased expression of the transcription factor Egr-1 in the nucleus, as well as increased Egr-1 binding to the TNF-α promoter (L. Shi and L. E. Nagy, unpublished data). Therefore, we asked whether the enhanced phosphorylation of ERK1/2 in response to LPS after ethanol feeding was associated with increased Egr-1 quantity and DNA-binding activity in Kupffer cells. Stimulation with LPS led to a rapid increase in the expression of Egr-1 protein in Kupffer cells (Fig. 8A). After 30 min exposure to 100 ng/ml LPS, the quantity of Egr-1 was threefold greater in Kupffer cells from ethanol-fed compared with pair-fed rats (Fig. 8A). Consistent with the higher expression of Egr-1 after ethanol feeding, the DNA-binding activity of Egr-1 was also increased twofold after ethanol feeding compared with the LPS-dependent response in pair-fed rats after stimulation with LPS for 60 min (Fig. 8B).

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essential element in the progression of liver damage (37). However, the mechanisms of Kupffer cell activation during ethanol exposure are not completely understood. Here, we report that chronic ethanol feeding differentially affects inflammatory cytokine production by Kupffer cells in response to challenge with LPS. Whereas LPS-dependent TNF-α production is increased after ethanol feeding, IL-1 accumulation in response to LPS is blunted. Increased LPS-stimulated TNF-α accumulation was associated with an increase in TNF-α mRNA. In contrast, ethanol feeding decreased LPS-stimulated accumulation of IL-1β mRNA. To understand the mechanisms for this differential response, we assessed the effects of chronic ethanol feeding on LPS-dependent signal-transduction pathways involved in increasing TNF-α and IL-1β expression. We demonstrated for the first time that ethanol feeding enhances LPS-dependent activation of ERK1/2. Activation of ERK1/2 was required for maximal increases in TNF-α and IL-1β mRNA accumulation and was associated with an increase in the DNA-binding activity of the transcription factor Egr-1 to the TNF-α promoter. In contrast, activation of NF-κB DNA-binding activity was completely abrogated after chronic ethanol feeding, likely contributing to the decreased ability of LPS to increase IL-1β expression.

Egr-1, a member of the immediate-early gene family, is a zinc finger transcription factor thought to play a role in mediating cellular responses to environmental stress, such as ischemia, mechanical injury, and ionizing radiation (46). Egr-1 binding to the TNF-α promoter is required for full activation of TNF-α transcription (48). Egr-1 is rapidly induced on LPS treatment in murine peritoneal macrophages (L. Shi and L. E. Nagy, unpublished data). Furthermore, LPS increases Egr-1 binding to the TNF-α promoter in macrophages (40, 48). Here, we show that nuclear Egr-1 protein quantity and binding to the Egr-1 binding site on the TNF-α promoter is increased in Kupffer cells exposed to LPS (Fig. 7). Moreover, chronic ethanol feeding potentiated both LPS-stimulated Egr-1 nuclear protein accumulation and DNA-binding activity (Fig. 7).

One of the earliest events in the Kupffer cell response to LPS is activation of the nuclear transcription factor NF-κB (4), with increased DNA-binding activity observed within 30 min of LPS exposure (39). Chronic exposure of rodents to ethanol via gastric infusion increases baseline NF-κB DNA-binding activity in liver assessed in vivo (17, 25). However, chronic ethanol feeding ameliorates the increase in NF-κB binding induced by partial hepatectomy (47). In vivo activation of hepatic NF-κB binding is likely the net result of a complex array of hormonal and cytokine-mediated events, probably involving multiple cell types within the liver (25). Thus the contrasting effects of ethanol on in vivo NF-κB activation suggest that chronic ethanol may have a differential impact on the ability of specific stimuli to activate NF-κB. LPS-activated NF-κB binding in alveolar macrophages (11) and RAW264.7 macrophages (L. Shi and L. E. Nagy, unpublished data) is decreased by chronic exposure to ethanol, both in vivo and in culture, respectively. Here, we report that chronic ethanol feeding completely abrogates the ability of LPS to increase NF-κB DNA-binding activity in Kupffer cells. Because NF-κB is a key mediator/integrator of inflammatory responses, the consistent decrease in LPS-mediated activation of NF-κB in sev...
eral different types of macrophages [alveolar (11), RAW264.7 macrophages (L. Shi and L. E. Nagy, unpublished data), and Kupffer cells (Fig. 5)] suggests that impaired NF-κB function may be an important contributor to abnormal immune responses (for review, see Ref. 36) observed after chronic ethanol exposure.

LPS also increases the DNA binding of another transcription factor, AP-1, but with different kinetics from the activation of NF-κB in Kupffer cells (41). Whereas chronic ethanol feeding or gastric infusion has been reported to upregulate expression of AP-1 family members in liver (42, 49) and to increase DNA-binding activity of AP-1 (20, 49), no information is available as to the effects of chronic ethanol on LPS-dependent stimulation of AP-1 DNA-binding activity in macrophages. Here, we found that chronic ethanol feeding increased basal AP-1 DNA-binding activity but had no effect on the ability of LPS to activate AP-1 DNA-binding activity.

Recent evidence has demonstrated that LPS-dependent activation of ERK1/2 phosphorylation mediates increased Egr-1 expression and DNA-binding activity and, consequently, is required for maximal increases in TNF-α expression in macrophages (L. Shi and L. E. Nagy, unpublished data) (40). Here, we have shown that inhibition of ERK1/2 phosphorylation by pre-treatment with PD-98059, an inhibitor of ERK1/2 activation, decreased the accumulation of both TNF-α mRNA and peptide (Fig. 6). Similarly, inhibition of ERK1/2 activation decreased LPS-stimulated IL-1β mRNA. Chronic ethanol feeding was associated with an increase in LPS and phorbol 12-myristate 13-acetate-stimulated ERK1/2 phosphorylation (Fig. 7). This enhanced activation is similar to that observed after chronic exposure of RAW264.7 macrophages to ethanol during culture (L. Shi and L. E. Nagy, unpublished data), indicating that it is likely a direct response to ethanol rather than an indirect/systemic response to ethanol exposure in vivo. Long-term culture of PC12 cells or hepatocytes with ethanol also enhances growth factor-induced activation of ERK1/2 (29, 30, 44). However, chronic ethanol feeding to rats decreases ERK1/2 activation in hepatocytes in response to stimulation with growth factors (6). Importantly, ethanol-induced increases in ERK1/2 activation are associated with changes in cellular function. For example, in PC12 cells, ethanol-mediated increases in ERK1/2 activation are associated with increased nerve growth factor-stimulated neurite outgrowth (30). Here, we report that in Kupffer cells, chronic ethanol-induced increases in ERK1/2 activation contributed to LPS-induced TNF-α and IL-1β mRNA accumulation.

Chronic ethanol feeding caused complex changes in activation of transcription factors involved in the regulation of TNF-α and IL-1β expression. Measurements of the DNA-binding activity of transcription factors are indicative of the potential of these transacting factors to activate transcription rather than actual measures of transcriptional activity. Therefore, whereas our results suggest that NF-κB-dependent transcriptional activation is decreased after chronic ethanol feeding and that Egr-1-dependent activation of transcription is increased, further investigations examining the functional activity of these factors will need to be carried out to directly assess activation of transcription. On the basis of the continued ability of LPS to increase TNF-α and IL-1β mRNA despite a loss of NF-κB binding activity after ethanol feeding, it will also be critical to investigate the combined interactions of these key transcription factors. Deletion analysis of the TNF-α promoter has shown that a number of transcription factor binding sites, including NF-κB, AP-1, and Egr-1, is required for full activation of LPS-stimulated TNF-α production (40, 48). However, while elimination of any single site, such the NF-κB binding site, decreases stimulation by LPS, responses to LPS are not completely prevented (48). Thus the increase in Egr-1 DNA-binding activity after ethanol feeding indicates that, under some conditions, enhanced Egr-1 activity can, at least partially, compensate for decreased NF-κB activity. The enhanced inhibitory effect of PD-98059 on accumulation of both LPS-stimulated TNF-α and IL-1β mRNA in ethanol-fed compared with pair-fed rats (Fig. 6) is consistent with the hypothesis that ERK1/2 activity activation plays a greater role in LPS-dependent responses after ethanol feeding.

Whereas it is clear from many studies that LPS stimulates transcription of TNF-α and IL-1β, our data on the steady-state mRNA levels cannot distinguish between an effect of ethanol on transcription and/or mRNA stability. Studies are currently under way to distinguish these possibilities. The complex responses of Kupffer cells to LPS stimulation reported here suggest that chronic ethanol feeding likely impacts on cytokine production at multiple levels of regulation. Regulation at multiple sites will likely contribute to the differential effects of ethanol on TNF-α and IL-1 production. For example, the increase in baseline accumulation of IL-1 after chronic ethanol, despite undetectable levels of IL-1 mRNA in Kupffer cells not treated with LPS, suggests a posttranslational site of regulation.

Kupffer cell responses to in vivo exposure to ethanol likely result from both direct effects of ethanol on signal-transduction pathways as well as indirect/systemic responses to ethanol. When RAW264.7 macrophages are cultured with ethanol, LPS-dependent ERK1/2 activation is increased (L. Shi and L. E. Nagy, unpublished data), suggesting that this reponse is a direct effect of ethanol. Therefore, the parallel increase in LPS-stimulated ERK1/2 observed in Kupffer cells after ethanol feeding may also be a direct effect of ethanol on this signal-transduction pathway. In contrast, it is also possible that some of the complex responses of Kupffer cells to ethanol feeding are the result of systemic effects of ethanol in vivo. For example, we would expect that increased endotoxin exposure reported during ethanol exposure in vivo, with endotoxin concentrations ranging from 50 to 100 pg/ml (5, 9, 26), would result in the development of endotoxin tolerance, observed both in vivo and in vitro on prolonged exposure to LPS (31). However, Jarvalainen et
al. (15) reported that the characteristic changes in liver function observed after chronic ethanol feeding were distinct from those observed after long-term exposure to endotoxin. Endotoxin tolerance is a complex response, but it is characterized by a decrease in NF-κB and AP-1 DNA-binding activity as well as decreased ERK1/2 activation on restimulation with LPS in mouse macrophages (23). Decreased LPS-stimulated NF-κB DNA-binding activity reported here after ethanol feeding (Fig. 5) is thus characteristic of endotoxin tolerance. However, the sustained ability of LPS to activate AP-1 (Fig. 5) and enhanced stimulation of ERK1/2 (Fig. 7) after ethanol feeding contrasts with typical endotoxin tolerance. Together, these data suggest that some components of the chronic ethanol-induced dysregulation of LPS-stimulated cytokine production in Kupffer cells may result from prolonged exposure to endotoxin in vivo. However, other chronic ethanol-induced defects in LPS-dependent signaling are not typically associated with tolerance to endotoxin. In particular, our data suggest that the specific increase in the ability of LPS to activate ERK1/2 after ethanol exposure may be an important therapeutic target in decreasing the inflammatory component of alcoholic liver disease.

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REFERENCES


