Chronic Ethanol Exposure Potentiates Lipopolysaccharide Liver Injury Despite Inhibiting Jun N-terminal Kinase and Caspase 3 Activation*

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Although ethanol is known to sensitize hepatocytes to tumor necrosis factor (TNFα) lethality, the mechanisms involved remain controversial. Recently, others have shown that adding TNFα to cultures of ethanol-pre-treated hepatocytes provokes the mitochondrial permeability transition, cytochrome c release, procaspase 3 activation, and apoptosis. Although this demonstrates that ethanol can sensitize hepatocytes to TNF-mediated apoptosis, the hepatic inflammation and ballooning hepatocyte degeneration that typify alcohol-induced liver injury suggest that other mechanisms might predominate in vivo. To evaluate this possibility, acute responses to lipopolysaccharide (LPS), a potent inducer of TNFα, were compared in mice that had been fed either an ethanol-containing or control diet for 5 weeks. Despite enhanced induction of cytokines such as interleukin (IL)-10, IL-15, and IL-6 that protect hepatocytes from apoptosis, ethanol-fed mice exhibited a 4–5-fold increase in serum alanine aminotransferase after LPS, confirming increased liver injury. Six h post-LPS histology also differed notably in the two groups, with control livers demonstrating only scattered apoptotic hepatocytes, whereas ethanol-exposed livers had large foci of ballooned hepatocytes, inflammation, and scattered hemorrhage. No caspase 3 activity was noted during the initial 6 h after LPS in ethanol-fed mice, but this tripled by 1.5 h after LPS in controls. Procaspase 8 cleavage and activity of the apoptosis-associated kinase, Jun N-terminal kinase, were also greater in controls. In contrast, ethanol exposure did not inhibit activation of cytotoxic mitogen-activated protein kinases and Akt or attenuate induction of the anti-apoptotic factors NF-κB and inducible nitric oxide synthase. Consistent with these responses, neither cytochrome c release, an early apoptotic response, nor hepatic oligonucleosomal DNA fragmentation, the ultimate consequence of apoptosis, was increased by ethanol. Thus, ethanol exacerbates TNF-related hepatotoxicity in vivo without enhancing caspase 3-dependent apoptosis.

Although ethyl alcohol has been recognized as a significant hepatotoxin for centuries, the mechanisms involved in alcohol-induced liver disease remain uncertain. It is known that chronic alcohol ingestion potentiates liver injury inflicted by many other toxins (1), viral hepatitis (2, 3), and hepatic hypoxia-reoxygenation (4, 5) or ischemia-reperfusion (6) and yet fails to cause serious liver damage in most healthy adults (7–9). These clinical observations suggest that ethanol exposure enhances hepatic vulnerability to a secondary inflammatory or oxidative stress, such that serious liver injury is most likely to occur when proinflammatory/pro-oxidant factors are superimposed onto a background of ethanol use (10). Because tumor necrosis factor-α (TNFα) and cytokines that potentiate TNF activity are the common, proximal mediators of inflammatory liver injury (11–13), several laboratories have been evaluating the possibility that ethanol may sensitize hepatocytes to TNF-related toxicity (14–18). The evidence supporting this concept is growing. For example, animal studies demonstrate that ethanol enhances liver injury caused by lipopolysaccharide (LPS) (14), a process that requires TNFα (19). Moreover, disruption of the gene that encodes the TNF type 1 receptor (TNFR-1) completely protects mice from the liver injury that results from chronic intragastric infusions of ethanol (17). The latter finding demonstrates that TNFα is required for ethanol-induced liver disease in mice. Assuming that similar mechanisms operate in humans who habitually consume alcohol-containing beverages, then it is important to delineate the cellular mechanisms that mediate ethanol-related sensitization to TNF lethality.

Healthy hepatocytes are typically resistant to TNFα toxicity unless they have been pretreated with agents that inhibit protein synthesis. Under such circumstances, TNFα induces the mitochondrial permeability transition, cytochrome c release, caspase 3 activation, and eventually causes death by apoptosis (20). Recently, Hoek and colleague (18) showed that ethanol mimics these effects of protein synthesis inhibitors. That is, when HepG2 cells or primary rat hepatocytes that have been pretreated with ethanol are exposed to TNFα in vitro, mitochondrial permeability transition, cytochrome c release, caspase 3 activation, and apoptosis ensue. Because treatment of these cultures with mitochondrial permeability transition inhibitors rescues them from TNF lethality, these authors (18) concluded that ethanol sensitizes the liver to injury by potentiating TNF-induced apoptosis.

The purpose of the present study is to determine whether similar mechanisms operate in vivo. To accomplish this, ethanol and pair-fed mice were given a single intraperitoneal injection of LPS, and subsequent liver damage as well as antecedent activation of mechanisms that promote and inhibit apoptosis were compared. Surprisingly, although ethanol exposure mark-

The abbreviations used are: TNF, tumor necrosis factor; LPS, lipopolysaccharide; TNFR, TNF receptor; PF, pair-fed; JNK, Jun N-terminal kinase; EMSA, electrophoretic mobility shift assay; IL, interleukin; iNOS, inducible nitric oxide synthase; Hsp-70, heat shock protein-70; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; Akt, protein kinase B; IKK, inhibitor κB kinase.
EDY ENHANCED LPS-INDUCED LIVER INJURY UNDER THESE EXPERIMENTAL CONDITIONS, IT ACTUALLY INHIBITED CASPASE 3 ACTIVATION. MOREOVER, THE INCREASED LIVER DAMAGE IN THE ETHANOL-FED GROUP WAS NOT ASSOCIATED WITH INCREASED CYTOKINE RELEASE OR NUCLEAR OLIGONUCLEOSOMAL DNA FRAGMENTATION. TAKEN TOGETHER, THESE FINDINGS DEMONSTRATE A DISCORDANCE BETWEEN THE PREVIOUSLY REPORTED IN VITRO MECHANISMS FOR ETHANOL HEPATOTOXICITY AND THOSE THAT OPERATE TO SENSITIZE ETHANOL-EXPOSED LIVERS TO TNF TOXICITY IN VIVO. IN VIVO, ETHANOL-RELATED POTENTIATION OF TNF-MEDIATED LIVER INJURY DOES NOT REQUIRE CASPASE 3 ACTIVATION, AND HEPATOCYTE DEATH APPEARS TO RESULT MORE FROM LYsis RATHER THAN FROM INCREASED, CLASSICAL APOPTOSIS.

MATERIALS AND METHODS
ANIMAL STUDIES—EIGHT-WEEK-OLD MALE C57BL6-6 MICE WERE PURCHASED FROM THE JACOBSON LABORATORY (BAR HARBOR, ME). MICE WERE Housed IN INDIVIDUAL, STAINLESS STEEL, WIRE CAGES IN A TEMPERATURE-CONTROLLED ANIMAL FACILITY WITH A 12-H LIGHT-DARK CYCLE. AFTER A 1-WEEK EQUILIBRATION PERIOD, THE MICE WERE DIVIDED INTO TWO GROUPS. HALF (N = 15) WERE FED ETHANOL-CONTAINING LIQUID DIETS AND HALF (N = 15) WERE FED SIMILAR CONTROL DIETS IN WHICH ETHANOL WAS SUBSTITUTED ISOCALORICALLY WITH DEXTRAN MALTOSE. BOTH DIETS WERE PURCHASED FROM BIOSERV, INC. (FRENCHTOWN, NJ). THE ETHANOL-FED GROUP WAS INTRODUCED TO THE ETHANOL DIET GRADUALLY, STARTING WITH 5% ETHERAL LIVER. AT ANY TIME, 3 TO 4 DAYS UNTIL THE MICE WERE CONSUMING DIETS CONTAINING 4% (V/V) ETHANOL. THE TOTAL DURATION OF ETHANOL FEEDING WAS 5 WEEKS. DURING THE ENTIRE FEEDING PERIOD, THE CONTROL MICE WERE PAIR-FED AN ISOCALORICALLY IDENTICAL VOLUME OF ETHANOL-FREE DIET DAILY. ALL MICE WERE WEIGHED AT THE BEGINNING OF THE STUDY AND WEEKLY THEREAFTER. THE ETHANOL-FED AND PAIR-FED (PF) CONTROL MICE GAINED WEIGHT SIMILARLY DURING THIS PERIOD. HOWEVER, THREE ETHANOL-FED MICE DIED DURING THE 5-WEEK FEEDING PERIOD. AT THE END OF THE STUDY, ALL SURVIVING MICE RECEIVED A SINGLE INTRAPERITONEAL INJECTION OF 10 µG OF LPS (SIGMA), AND ANIMALS WERE KILLED AT 0.5, 1.5, AND 6 H POST-LPS. AT THE TIME OF SACRIFICE, A SMALL SEGMENT OF EACH LIVER WAS FIXED IN BUFFERED FORMALIN FOR SUBSEQUENT EVALUATION OF HISTOLOGY. THE REMAINING LIVER TISSUES WERE FREEZE-CLAMPED IN LIQUID NITROGEN AND STORED AT −80 °C UNTIL ANALYSIS.

LIVER HISTOLOGY—FORMALIN-FIXED TISSUES WERE EMBEDDED IN PARAFFIN, SECTIONS, AND STAINED WITH HEMATOXILIN AND EOSIN. CODED SECTIONS WERE EVALUATED BY LIGHT MICROSCOPY AT 200–400x MAGNIFICATION FOR EVIDENCE OF FAT ACCUMULATION, HEPATOCYTE APOPTOSIS, INFLAMMATION, AND HEMORRHAGE BY TWO INDEPENDENT REVIEWERS. EACH REVIEWER ASSESSED A MINIMUM OF 10 MICROSCOPIC FIELDS/TISSUE SECTION. PAT ACCUMULATION WAS SCORED AS FOLLOWS: 0 (NONE), 1 (<30% OF HEPATOCYTES HAVE MICRO- OR MACROVESICULAR FAT), 2 (30–60% OF HEPATOCYTES ARE FATTY), 3+ (>60% OF HEPATOCYTES ARE FATTY) ON SECTIONS REVIEWED AT 200X. SECTIONS WERE EXAMINED UNDER 400X MAGNIFICATION FOR THE PRESENCE OF APOTOTIC HEPATOCYTES, INFLAMMATORY FOI, AND HEMORRHAGIC FOI. LIVERS WERE CONSIDERED INJURED IF ANY OF THESE LESIONS WERE DETECTED. INJURY WAS SCORED AS 0 (NONE), 1+ (RARE FOCAL APOPTOSIS, INFLAMMATION, OR HEMORRHAGE), 2+ (FOCAL APOPTOSIS, INFLAMMATION, AND/OR HEMORRHAGE IN 25–50% OF THE FIELDS), 3+ (FOCAL APOPTOSIS, INFLAMMATION, AND/OR HEMORRHAGE IN >50% OF THE FIELDS).

SERUM AMINOTRANSFERASE ACTIVITY—THE ACTIVITY OF THE LIVER-ASSOCIATED AMINOTRANSFERASE, ALANINE AMINOTRANSFERASE, WAS MEASURED IN SERUM SAMPLES OBTAINED 6 H AFTER LPS ADMINISTRATION. ASSAYS WERE PERFORMED IN THE CLINICAL CHEMISTRY LABORATORY OF THE JOHNS HOPKINS HOSPITAL USING A MULTICHANNEL AUTOANALYZER.

DNA ISOLATION AND FRAGMENTATION ASSAYS—DNA WAS ISOLATED FROM SAMPLES OF SNAP-FROZEN LIVER THAT HAD BEEN STORED AT −80 °C, AND OLIGONUCLEOSOMAL DNA FRAGMENTATION WAS ASSESSSED USING REAGENTS FROM TRENGEN (GAITHERSBURG, MD) ACCORDING TO THE MANUFACTURER’S INSTRUCTIONS. BRIEFLY, 100 MG OF FROZEN LIVER WAS GRINDED INTO A POWDER UNDER LIQUID NITROGEN AND SUSPENDED IN BUFFER AND INCUBATED AT 50 °C OVERNIGHT. THE DNA pellets were precipitated, and after lyse and extraction, 1–2 µG OF DNA WERE SEPARATED ON 1.5% AGAROSE GELS, AND DNA FRAGMENTS WERE VISUALIZED BY ETIUMID BRIDING STAINING.

LIVER PROTEIN ISOLATION—LIVER TISSUE FROM EACH MOUSE WAS USED TO OBTAIN WHOLE LIVER PROTEIN AND NUCLEAR PROTEIN. WHOLE LIVER HOMOGENATE WAS PREPARED BY HOMOGENIZING FROZEN TISSUE IN HOMOGENIZATION BUFFER (50 M M HEPES, PH 7.5, 150 M NaCl, 1.5 M MgCl2, 10% GLYCEROL, 5 M EGTA, 1% TRITON X-100) ON ICE FOR 10 MIN FOLLOWED BY CENTRIFUGATION IN A MICROCENTRIFUGE FOR 15 MIN. NUCLEAR PROTEINS WERE PREPARED FROM THE SAME TISSUES ACCORDING TO THE PROTOCOL OF LAVERY AND SCHILBER (13), AS WE HAVE DESCRIBED (22). PROTEINS WERE ISOLATED FROM ETHANOL-FED AND PAIR-FED MOUSE LIVER TISSUES CONCURRENTLY, AND THE PROTEIN CONCENTRATION IN EACH EXTRACT WAS DETERMINED WITH DYE-BINDING ASSAYS USING REAGENTS FROM FERGIE.

IMMUNOBLOT ANALYSIS—TO EVALUATE VARIATIONS IN THE EXPRESSION OF SPECIFIC PROTEINS, IMMUNOBLOT ANALYSIS WERE PERFORMED. LIVER PROTEINS IN LAEMMLI SAMPLE BUFFER WERE SEPARATED BY ELECTROPHORESIS ON POLYACRYLAMIDE GELS AND TRANSFERRED TO A NITROCELLOUS FILTER. PROTEINS WERE HEAT TREATED WITH 5% LOW FAT MILK TO BLOCK SPECIFIC BINDING, MEMBRANES WERE EXPOSED OVERNIGHT TO SPECIFIC ANTI-SERA AT 4 °C. IMMUNOBLOTTING WAS PERFORMED AT 37 °C, AND IMMUNOBLOTTING WAS PERFORMED AT 37 °C. IMMUNOBLOTTING WAS PERFORMED AT 37 °C.

RESULTS
INCREASED LPS-INDUCED LIVER DAMAGE IN ETHANOL-FED MICE—HISTOLOGICALLY, CHRONIC CONSUMPTION OF ETHANOL IS KNOWN TO INCREASE VULNERABILITY TO SUBSEQUENT LPS-INDUCED HEPATOTOXICITY (14). THE PRESENT STUDY REPRODUCES THOSE RESULTS. A SINGLE INTRAPERITONEAL INJECTION OF LPS (10 µG/MOUSE) CAUSES SIGNIFICANTLY GREATER ACUTE MORTALITY IN ETHANOL-FED MICE (2 DEATHS/12 MICE, 17% MORTALITY) THAN PF CONTROLS (0 DEATHS/DURING THE
Chronic Ethanol Exposure Potentiates LPS Liver Injury

Hematoxylin and eosin-stained liver sections were prepared from mice that were sacrificed either before (0) or at various time points (0.5, 1.5, or 6 h) after LPS treatment. Sections from three mice/group were evaluated at each time point, and the severity of fat accumulation, apoptosis, inflammation, and hemorrhage were assessed as described under “Materials and Methods.” Injury was diagnosed when any apoptosis, inflammation, and/or hemorrhage was identified. Mean scores (±S.D.) are shown.

| Post-LPS (h) | Fat Injury |
|--------------|------------|
|              | PF         | EthOH     | PF         | EthOH     |
| 0            | 0 (0.2)    | 1 (0.2)   | 0 (0)      | 1 (0.2)   |
| 0.5          | 1 (0.9)    | 2 (0.7)   | 1 (0.9)    | 2 (0.1)   |
| 1.5          | 1 (0.8)    | 3 (0.3)   | 1 (0.7)    | 2 (0.3)   |
| 6            | 1 (1.2)    | 3 (0.5)   | 1 (1.0)    | 3 (0.1)   |

Initial 6 h after LPS). Liver damage is also more extensive in the ethanol-fed mice than in the PF mice that survived for 6 h post-LPS (Table I). In the PF group, most liver lobules contain normal appearing hepatocytes with only occasional hepatocytes having histologic features of apoptosis (i.e. condensed nuclear chromatin with reduced cytoplasmic volume) without associated inflammatory cell infiltrates or hemorrhage (Fig. 1a). In contrast, small-droplet fat accumulation is prominent in most hepatocytes in ethanol-fed mice, and the livers have large areas of ballooned, hypereosinophilic hepatocytes that are typically associated with focal inflammatory cell infiltrates and/or hemorrhage (Fig. 1b). These foci of injury occur predominantly in acinar zone 3 (i.e. near terminal hepatic venules). This histologic evidence of more severe liver damage in the ethanol-fed group is associated with significantly greater increases in serum alanine aminotransferase values in the ethanol-fed mice compared with the pair-fed controls following LPS treatment (Fig. 1c). Hence, ethanol- and pair-fed groups differ significantly with regards to both the histologic patterns of injury and the magnitude of alanine aminotransferase released into the blood after LPS injection.

Decreased Activation of Procaspase 3 in Ethanol-fed Mice—In cultured hepatocytes that have been exposed to ethanol, in vitro treatment with TNFα is known to cause death by mechanisms that involve procaspase 3 (18). Procaspase 3 is a distal, downstream target of apoptotic signaling that is initiated by TNFR-1 activation (28). The cleavage of procaspase 3 releases smaller 17- and 11-kDa peptides that function as terminal effector caspases during hepatic apoptosis (29). Because ethanol-induced vulnerability to TNF-killing is caspase 3-dependent in vitro (18), immunoblot analysis was done to determine whether prior ethanol exposure also accentuates procaspase 3 cleavage in vivo (Fig. 2). Interestingly, the hepatic content of procaspase 3 is somewhat reduced in ethanol-fed mice compared with PF controls both before and after LPS treatment. Following LPS treatment, PF mice exhibit a transient increase in the 11-kDa activated form of caspase 3 relative to the amount of procaspase 3, consistent with their histologic evidence of increased hepatocyte apoptosis during this time period. In contrast, neither increases in the absolute content of 11-kDa activated caspase 3 nor increases in the ratio of activated caspase 3 to procaspase 3 are observed in the livers of ethanol-fed mice after LPS exposure. This finding is surprising, given the previously published in vitro data (18) and evidence that these ethanol-fed mice develop significantly more liver damage than PF controls when challenged acutely with LPS. Indeed, because caspase 3 functions as the executioner caspase during hepatocyte apoptosis (29), the inhibited activation of procaspase 3 in the ethanol-fed group suggests that increases in apoptotic activity might not cause the ethanol-related exacerbation of LPS-liver injury. To further evaluate this possibility, the hepatic content of procaspase 3 was compared in ethanol- and pair-fed mice. During TNF-induced hepatocyte apoptosis, cleavage of procaspase 8 activates this enzyme, which in turn activates Bid and leads to mitochondrial release of cytochrome c (30). At every time point evaluated, the levels of procaspase 8 are greater in ethanol-fed mice than in controls (Fig. 3a). These differences are not due to generalized, ethanol-related increases in hepatic protein content, because the levels of caspase 3 (Fig. 2) and Hsp-70 (Fig. 3a) are consistently reduced in ethanol-exposed livers compared with controls. Moreover, steady state levels of caspase 8 mRNA are similar in the two groups (data not shown). Although these findings suggest that ethanol inhibits LPS-induced cleavage of caspase 8, further studies must be done to determine whether caspase 8 activation is truly inhibited by ethanol, when agents that recognize activated forms of murine caspase 8 become available. Nevertheless, the combination of increased procaspase 8 content with reduced hepatic accumulation of caspase 3 cleavage products after LPS exposure suggests that classical apoptosis might even be inhibited by prior ethanol exposure. To further evaluate this possibility, cytochrome c release (an event which precedes procaspase 3 activation) and nuclear oligonucleosomal DNA fragmentation (an event that follows procaspase 3 activation) (31) were compared in ethanol- and pair-fed mice at various time points following LPS injection. No ethanol-related increases in cytosolic cytochrome c content (as assessed by immunoblot analysis) or oligonucleosomal DNA fragmentation (evaluated by agarose gel electrophoresis) were observed. Thus, although ethanol exposure clearly potentiates LPS-induced hepatocyte lethality, the mechanisms that mediate this response in vivo appear to differ from those that operate when ethanol-exposed hepatocytes are treated with TNFα under in vitro conditions.

Inhibited Activation of JNK in Ethanol-fed Mice—The interaction of TNFα with TNFR-1 can initiate a complex signaling cascade that eventually activates procaspase 3 to cause apoptosis. Recent reports suggest that Jun N-terminal kinase (JNK), the terminal enzyme in the TNF-regulated, stress-activated kinase cascade, is required for procaspase 3 activation (32, 33). This finding complements other evidence demonstrating that JNK activation is necessary for apoptosis in many cells types (34–36). TNFα is a potent activator of JNK in hepatocytes (23, 37). Therefore, similar to procaspase 3, JNK is expected to be activated after LPS treatment in pair-fed mice, but this response might be inhibited in ethanol-fed mice, which fail to activate procaspase 3 following LPS exposure. To evaluate this possibility, JNK activity was compared in ethanol- and pair-fed mice (Fig. 3b). In pair-fed mice, JNK activity increases transiently, peaking at almost 8-fold above base-line values by 1.5 h post-LPS exposure. However, in ethanol-fed mice, LPS-related increases in JNK activity are inhibited. Thus, in ethanol-fed mice, JNK activity barely rises above base-line levels at any time after LPS treatment. The failure of ethanol-fed mice to activate JNK following LPS cannot be explained by ethanol-related increases in Hsp-70, because the expression of this JNK inhibitor is actually reduced in the livers of ethanol-fed mice (Fig. 3a). Nevertheless, given evidence that JNK promotes mitochondrial release of cytochrome c in other cells (32), JNK inhibition is likely to contribute to the inhibited procaspase 3 activation that was also observed in the ethanol-fed mice following LPS exposure.

Differential Induction of Other TNF-regulated Kinases in Ethanol-fed Mice—To determine whether other TNF-regulated kinases are similarly inhibited in the ethanol-fed group,
changes in the phosphorylation status of the MAPKs, Erk-1 and Erk-2, were assessed by immunoblot (Fig. 4). In PF mice, LPS treatment induces a transient 3–4-fold increase in phospho-MAPKs (Erk-1 and Erk-2) within 0.5–1.5 h. Ethanol-fed mice have even greater MAPK activation after LPS, exhibiting a biphasic pattern of MAPK induction that leads to a 6–8-fold increase in phospho-MAPKs at 0.5 and 6 h post-LPS. Thus, the induction of mitogen-activated kinases is not inhibited by chronic ethanol exposure. Protein kinase B/AKT is another TNF-regulated kinase (39). Similar to MAPK, AKT promotes cellular viability (40–43). Because ethanol-related inhibition of
JNK, an apoptosis-associated kinase, and stimulation of MAPKs cannot easily be used to explain why chronic ethanol exposure potentiates LPS hepatotoxicity. AKT activity was compared in ethanol- and pair-fed mice to determine whether ethanol might promote liver damage by inhibiting this survival factor. As shown in Fig. 5, although LPS induction of AKT is somewhat delayed in the ethanol-fed mice, the livers of ethanol- and pair-fed mice have a similar content of phospho-AKT by 6 h after LPS. Thus, the activation levels of two different cytoprotective kinase cascades, MAPK and AKT, are preserved after chronic ethanol exposure. In contrast, prior ethanol treatment suppresses the acute induction of JNK, a stress-related kinase that promotes apoptosis (32). Ordinarily, this pattern of kinase activation protects cells from TNF-mediated apoptosis (41). Hence, other mechanisms must be responsible for ethanol-related increases in LPS-related liver damage.

**Induction of NF-κB and iNOS, an NF-κB Target Gene, in Ethanol-fed Mice**—One of the targets of the MAPK and AKT cascades is IKK, the kinase complex that phosphorylates IkBa, the cytosolic binding protein for NF-κB (44). Phosphorylation of IkBa promotes its degradation by the 26 S proteasome. This releases NF-κB subunits, which translocate to nuclei to regulate the expression of various survival genes including iNOS (45, 46). NF-κB functions as a critical viability factor for hepatocytes, because interventions that prevent the activation of this transcription factor following TNFα exposure markedly enhance hepatocyte apoptosis (47). Because LPS acutely increases MAPK and AKT activity in both PF- and ethanol-fed mice, increases in NF-κB DNA binding activity were expected in both groups. EMSA confirms this prediction, in general. However, subtle differences in the pattern of NF-κB induction occur in the two groups following LPS (Fig. 6a). In PF mice, significant increases in NF-κB binding activity occur from 0.5 to 1.5 h after LPS. Supershift analysis demonstrates that these DNA-binding complexes contain large amounts of NF-κB p65. The kinetics of NF-κB activation by LPS are similar in ethanol-fed mice. However, total NF-κB binding activity is consistently less in the ethanol-fed group than in the PF group at all time points evaluated. Moreover, this ethanol-related decrease in total NF-κB binding activity appears to reflect a selective inhibition of NF-κB p65 binding activity (Fig. 6b). Consistent with the presumably normal activation of the IKK complex in the ethanol-fed group, subsequent experiments demonstrated that NF-κB p65 subunits accumulate similarly within liver nuclei of ethanol- and pair-fed mice (Fig. 6c). These observations suggest that nuclear extracts from ethanol-exposed livers might contain a factor that selectively inhibits the DNA binding activity of NF-κB p65. In other systems, nuclear IkBa or Bel-3 have been identified as NF-κB p65-binding proteins, which inhibit its DNA binding activity (48). However, immunoblot analysis does not demonstrate increased IkBa or Bel-3 accumulation in the hepatic nuclear extracts from ethanol-fed mice (data not shown). Hence, the mechanism that attenuates LPS induction of NF-κB p65 DNA binding activity after chronic ethanol exposure remains unknown. Moreover, because the regulation of NF-κB p65 transcriptional activity is complex, it is not clear that slight decreases in p65 binding activity will lead to reduced transcription of NF-κB-regulated survival genes. Indeed, the latter might even be enhanced by reduced p65 function, given the evidence that homodimers of NF-κB p50 promote tolerance to LPS toxicity (49).

To evaluate the latter possibility, the induction of iNOS was compared in ethanol- and pair-fed mice. iNOS is one of several TNF-regulated genes that are known to protect hepatocytes from LPS toxicity (50–52). Moreover, LPS-mediated transcriptional activation of the murine iNOS gene requires TNF-initiated signals that enhance NF-κB activity (53–55). LPS increases iNOS mRNA levels similarly in ethanol-and pair-fed mice.
mice (Fig. 7a). Consistent with this finding, the induction of iNOS protein occurs normally in the livers of ethanol-fed mice following LPS exposure (Fig. 7b). Hence, the slight shift in the relative abundance of p65 and p50 in NF-kB complexes that we observed in ethanol-fed mice appears to have little adverse consequence in this model because the activation of iNOS, a key NF-kB-regulated survival gene, proceeds normally. Moreover, this normal post-LPS pattern of iNOS induction complements the data that demonstrate that ethanol exposure does not prevent the activation of other mechanisms that normally limit LPS-related hepatocyte apoptosis.

**Enhanced Hepatic Induction of TNFα-inhibitory Cytokines in Ethanol-fed Mice**—Interventions that inhibit TNF activity have been shown to prevent liver damage from LPS, demonstrating that TNFα is a proximal mediator of LPS-induced hepatotoxicity (56). Studies of mice with targeted disruption of the gene for TNFR-1 also demonstrate that TNFα signaling is required for ethanol to cause liver damage (17). Given the key roles of TNFα and TNFR-1 in LPS and ethanol-related liver damage, RNAse protection analysis was done to determine whether our findings might be explained by altered hepatic expression of TNFα and/or TNFR-1. The results demonstrate similar steady state expression of TNFα and TNFR-1 transcripts in the livers of ethanol- and pair-fed mice (data not shown). However, LPS induction of other cytokines, such as IL-10, IL-15, and IL-6, that negatively modulate TNFα activity (12) differed somewhat in ethanol-fed and control mice (Fig. 8). Transcripts for these cytokines could not be demonstrated by ribonuclease protection analysis of total liver RNA obtained from pair-fed mice before LPS treatment, whereas faint expression of IL-10 and IL-15 were noted in one of the two samples from the ethanol-fed group. Following LPS challenge, IL-10, IL-15, and IL-6 were up-regulated rapidly in both groups, but the overall induction pattern for these TNFα inhibitors tended to be greater in ethanol-fed mice than in controls. Ethanol-related increases in IL-15 expression from 0 to 0.5 h after LPS exposure might be particularly relevant because this cytokine is known to inhibit TNF-initiated apoptosis by activating mechanisms that interfere with the recruitment of adaptor molecules to TNFR-1, blocking the propagation of death receptor signals (57). Thus, ethanol exposure alters the cytokine microenvironment, which might explain why TNF-mediated apoptosis is thwarted in vivo but potentiated under in vitro conditions where protective cytokines such as IL-15 are absent.

**DISCUSSION**

Decades of research have culminated in relatively recent insights that are widely believed to explain paradoxical inter- and intra-individual differences in the vulnerability to alcohol-induced liver damage. Namely, several lines of evidence sug-
gest that serious alcohol-related liver damage requires the introduction of an inflammatory or oxidant stress to ethanol-exposed livers that have become partially depleted of various survival factors that normally protect hepatocyte viability (58). Thus, moment-to-moment differences in the balance between cytotoxic and cytoprotective factors dictate the severity of alcohol-related liver damage at any given point in time. Several laboratories have exploited this general concept to develop cell culture and animal models that can be studied to characterize molecular targets for future therapeutic interventions. Work from many different groups identifies TNFα as a critical mediator of alcohol-related liver damage (14–18). Recent studies of hepatocytes that were exposed to ethanol and then treated with TNFα in vitro demonstrated an enhanced induction of several events that are involved in apopotic cell death (18), raising the exciting possibility that agents that prevent TNF-dependent activation of caspase 3 might be effective as treatments for alcoholic liver disease. However, although there is evidence of increased hepatocyte apoptosis in some patients and experimental animals with alcohol-related liver damage (59–62), the typical histologic features of alcohol-induced steatohepatitis include hepatocyte swelling and inflammation (63). Classically, such features have been attributed to necrotic processes (64). Therefore, it is important to confirm that enhanced vulnerability to TNF-mediated apoptosis actually occurs during in vivo situations in which ethanol is known to potentiate TNF-related liver damage.

The results of our study demonstrate that ethanol-related potentiation of TNF-mediated liver injury does not require the activation of procaspase 3. Moreover, we find that increased liver damage in ethanol-fed mice occurs despite robust, acute induction of various survival responses, including MAPKs, AKT, NF-κB, and iNOS, which usually protect hepatocytes from apoptosis following LPS exposure (30, 47, 55, 65). Indeed, in this in vitro model of alcohol-related liver damage, there is compelling evidence that prior ethanol exposure inhibits classical apoptotic signaling because it prevents the activation of both procaspase 8 and JNK. The former normally functions as an initiator caspase for death receptor-induced apoptosis, and the latter is necessary for stress-induced procaspase 3 activation and subsequent apoptosis in many cell types (32–36, 66). Consistent with this concept, we were unable to demonstrate increased cytochrome c release or nuclear oligonucleosomal DNA fragmentation in LPS-treated ethanol-fed mice, although these animals developed worse liver damage than the PF controls. Taken together, these results suggest that enhanced apoptosis might not be the major mechanism for alcohol-related liver damage.

This conclusion is supported by earlier work from Coler et al. (67), which showed that hepatocytes from ethanol-fed rats are deficient in mitochondrial glutathione and, after incubation with TNFα in vitro, produce large amounts of hydrogen peroxide and undergo necrosis. Evidence that ethanol potentiates TNF-mediated hepatocyte necrosis is not inconsistent with other reports showing that ethanol potentiates TNF-induced hepatocyte apoptosis (18), because seemingly discordant effects of ethanol on apoptosis can be reconciled by model-dependent differences in factors that modulate cellular responses to TNFα. For example, other cytokines are powerful regulators of TNFα bioactivity (12). These factors are produced primarily by hepatic macrophages (68) and thus are not abundant in hepatocyte cultures. Our ribonuclease protection analyses of liver RNA demonstrate that in vivo exposure to ethanol amplifies basal and LPS-related induction of several cytokines that inhibit apoptosis. At least one of these (i.e., IL-15) inhibits the recruitment of adaptor molecules to the cytosolic domains of Fas and TNFR-1, blocking the propagation of apoptotic signals that are normally initiated by these receptors (57). Therefore, in intact mice, IL-15 and other cytokines may abrogate apoptotic responses that proceed unchecked in isolated hepatocytes cultured without these factors. Additional experiments are needed to evaluate this possibility directly. Chronic ethanol exposure also modifies the availability of intracellular factors that regulate apoptosis. For example, chronic consumption of ethanol inhibits hepatic mitochondrial respiration and ATP synthesis in patients and experimental animals (69, 70). This influences the zonal distribution of alcohol-related liver injury, which typically clusters in the most hypoxic parts of liver lobules around terminal hepatic venules, where we observed the most severe injury after LPS. However, hepatocytes in more well oxygenated areas of the liver usually are less ATP-depleted and survive (4, 5). Indeed, moderate reductions in cellular ATP content might even be somewhat protective (71) because the final stages of caspase 3 activation do not occur when ATP levels dip below a certain threshold (72). However, any situation that causes extreme ATP depletion impairs the functioning of ion transporters that regulate membrane permeability, and eventually this leads to organelle and cell lysis, i.e., necrosis (5, 73, 74). The consequent release of intracellular contents into the hepatic microenvironment activates liver macrophages and helps to recruit inflammatory cells to the damaged liver (75–77), resulting in the typical histological features of alcohol-related steatohepatitis (10). Because the liver perfusion protocols and other techniques required for hepatocyte purification and culture impose considerable hypoxic stress (78–83), it is unlikely that ethanol-exposed hepatocytes with marginal ATP stores can survive this process. Thus, only the healthiest liver cells with relatively normal ATP contents are likely to be cultured from ethanol-treated animals. This methodological obstacle is not overcome by exposing primary hepatocytes from ethanol-naive animals to ethanol in vitro because brief periods of ethanol treatment are probably not sufficient to reproduce the full spectrum of ethanol-related mitochondrial toxicity that inhibits hepatic ATP synthesis during chronic in vivo exposure to ethanol (84). Even efforts to substitute hepatocyte cell lines for primary hepatocytes are potentially problematic because, like other neoplastic cells, HepG2 cells have induced nonmitochondrial pathways for ATP synthesis (38) and thus might not experience ATP depletion despite ethanol-related mitochondrial toxicity. The fact that cells with relatively normal ATP stores are over-represented in the previously mentioned in vitro models introduces a bias that favors the apoptotic (rather than necrotic) death pathway.

Thus, because in vitro hepatocyte culture systems do not fully reproduce the extra- or intracellular microenvironment of...
hepatocytes in intact livers, it is somewhat risky to extrapolate the resultant in vitro data to more clinically relevant in vivo models of chronic ethanol exposure (69, 70). This, in turn, has potentially important therapeutic implications that merit future evaluation because the in vitro data suggest that pharmacologic inhibition of caspase 3 may prove to be unnecessary (because it occurs naturally) in subjects who habitually consume alcohol. If so, then this intervention will not protect alcohol abusers from alcohol-related liver injury, and alternative therapeutic strategies will need to be developed for this important cause of chronic liver disease.

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