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Inhibition of the Kupffer Cell Inflammatory Response by Acute Ethanol: NF-κB Activation and Subsequent Cytokine Production

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Suppression of host defense mechanisms plays a critical role in the response to infectious agents in patients with alcohol-induced liver disease. Kupffer cells, the resident hepatic macrophage population, are an essential component of host defense mechanisms against infection. Thus, regulation of the Kupffer cell inflammatory response by ethanol may be a key component of that immunosuppression. The aim of this study is to test the hypothesis that ethanol directly and specifically inhibits Kupffer cell activation. Kupffer cells were incubated in 100 mM ethanol for 90 minutes, whereupon cells were washed and incubated without ethanol for LPS activation. Such treatments lead to inhibition of LPS-mediated NF-κB activation. Consistent with these data, steady state levels of TNF-α and TNF-α secretion were depressed throughout a range of LPS concentrations. The inhibition induced by ethanol was time dependent and completely reversible. These data show that the suppressive effects of ethanol affect the the earliest steps of the LPS signal transduction cascade as it is currently understood. © 1996 Academic Press, Inc.

Suppression of immune and host defense mechanisms plays a critical role in the response to infectious agents in patients with alcohol-induced liver disease [1,2]. Following the ingestion of ethanol, significant alterations occur in reticuloendothelial system (RES) function which results in increased host susceptibility to infection [1,3,4].

Kupffer cells comprise the largest resident macrophage population within the RES and this tissue-fixed macrophage population is a key component of the inflammatory response and elaborates many mediators that initiate, perpetuate and modulate this response [5]. Several studies have shown that Kupffer cell function is affected by alcohol in both experimental animals and man. Clearance of microaggregated albumin and endotoxin is depressed in rats following both acute and chronic administration of alcohol [6-8]. Alcoholics with no evidence of liver disease have also shown depressed RES function that returns to normal several days following alcohol withdrawal [9]. Furthermore, RES depression in patients with alcoholic liver disease has been shown by reduced clearance of microaggregated albumin [10], and the lack of a concentration gradient of immune complexes between portal and hepatic veins [11].

The potential role of endotoxin in hepatic injury due to long term ethanol exposure has been demonstrated experimentally [12], consistent with classic studies of chronic liver injury [13]. Similarly pharmacological elimination of Kupffer cells is protective in chronically ethanol-fed rats [14]. Other studies with chronically ethanol-fed rats have suggested that Kupffer cells may be directly activated by ethanol [15,16]. Inflammation within the liver is a complication of both chronic [14] and acute ethanol exposure [17,18]. The observations of enhanced response to endotoxemia in livers of ethanol-fed animals is consistent with such an inflammatory expansion [19]. These inflammatory cells could complicate subsequent *in vitro* studies of

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acute ethanol's effect on Kupffer cell function. We have shown that the resident hepatic macrophage population isolated from acutely injured livers is phenotypically and functionally distinct from normal Kupffer cells [20,21]. Thus cells isolated from animals fed ethanol may not accurately reflect the response of the Kupffer cell population to infection in the face of acute ethanol intoxication.

The goal of the present study was to identify the molecular mechanism for ethanol-mediated immunosuppression in a pure population of Kupffer cells. *In vivo* studies have shown that a single acute dose of ethanol is associated with significant mortality and decreased Kupffer cell function during polymicrobial sepsis [22]. To minimize the potential effects of ethanol metabolites or *in vitro* evaporation [23], Kupffer cells were pulsed with ethanol and then washed and stimulated with physiologically relevant dosages of LPS in the absence of ethanol. Understanding the effect of ethanol on the key molecular regulatory pathways for the inflammatory response may point to potential therapeutic approaches to restoring immune function diminished by ethanol.

MATERIAL AND METHODS

Animals. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250-300 g were given food and water *ad libitum*, and were quarantined for at least two days before cell isolations. All animals were handled according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* All procedures were approved by the Animal Care Committee of Saint Louis University Health Sciences Center.

Isolation of Kupffer cells. Kupffer cells were isolated by collagenase-pronase perfusion and purified by centrifugal elutriation [24]. All buffers for this procedure were formulated in pyrogen-free water (Abbott Labs, Chicago, IL). Both viability, determined by trypan blue exclusion, and purity, determined by endogenous peroxidase staining, exceeded 90%. Kupffer cells were routinely cultured overnight in DMEM (high glucose) containing 25 mM HEPES and 10% heat-inactivated fetal calf serum.

Experimental design. To determine the effect of ethanol on the LPS-mediated TNF-α secretion, 8×10^5 Kupffer cells were plated in 24 well dishes. The effect of ethanol on steady state levels of TNF-α mRNA was determined from 5×10^6 cells plated in 35 cm^2 dishes. The effect of ethanol on activation of the transcription factor NF-κB was determined in nuclear extracts prepared from 8×10^6 Kupffer cells plated in either 35 or 60 cm^2 tissue culture dishes. After overnight culture, Kupffer cells were washed twice with serum-free DMEM. Cells were incubated in DMEM, containing 100 mM ethanol for 90 minutes, the plates were washed twice and stimulated with *E. coli* 011:B4 LPS (2.5, 5, 10, or 50 ng/ml) for an additional 6 hr. After LPS exposure, the medium overlying the Kupffer cells was harvested and analyzed for TNF-α by ELISA. The effect of ethanol treatment on LPS-mediated TNF-α mRNA levels was determined in cells pretreated for 90 minutes with 100 mM ethanol, washed twice, and stimulated for 3 hrs with 2.5, 5.0, 7.5 or 10 ng/ml LPS. Steady state levels of TNF-α mRNA were determined by Northern blot analysis. The effect of ethanol treatment on LPS-mediated NF-κB activation was determined in Kupffer cells pretreated for 90 minutes with 100 mM ethanol, washed twice, followed by 1 hr of stimulation with 5 or 10 ng/ml LPS. Nuclear NF-κB binding activity was measured by electrophoretic mobility shift assay.

TNF-α ELISA. Antigenic TNF-α concentrations were measured in experimental supernatants by sandwich ELISA exactly as previously described [20].

RNA Isolation and Northern Blot Analysis. RNA was isolated from approximately 5×10^6 cells with RNASTAT 60 (Tel-Test B., Friendswood, TX) following the manufacturer's instructions. Total RNA from 5×10^6 cells was separated by electrophoresis in a 1% agarose-formaldehyde gel and transferred to a nylon membrane by capillary transfer. Total RNA in each lane was determined by ethidium bromide staining. Expression of mRNA for γ-actin (ATCC, Rockville, MD) [25] and TNF-α (Chiron Corporation, Emeryville, CA) [26] was performed by random priming of the specific cDNA followed by hybridization using standard techniques [27]. Northern blot analysis of γ-actin mRNA reflected loading variability observed with ethidium bromide staining for total RNA. Phosphorimaging was performed and TNF-α gene expression was adjusted based on the steady state levels of the γ-actin mRNA.

Preparation of nuclear extracts. The medium overlying the cells was removed and replaced with ice-cold PBS. Kupffer cells were harvested by scraping followed by centrifugation (1000×g). Nuclear extracts were prepared using the procedure of Schreiber [28]. The protein concentration of the nuclear extracts was determined with the Bradford protein assay [29].

Electrophoretic mobility shift assay for NF-κB. Binding reactions were established in 25 μl of binding buffer using 3 μg of nuclear extract protein per reaction. In the binding reaction, nuclear extracts were incubated for 15 min at 20°C with 0.1 ng (10,000 cpm) of ^{32}P-end labeled double-stranded NF-κB consensus oligonucleotide. For competition

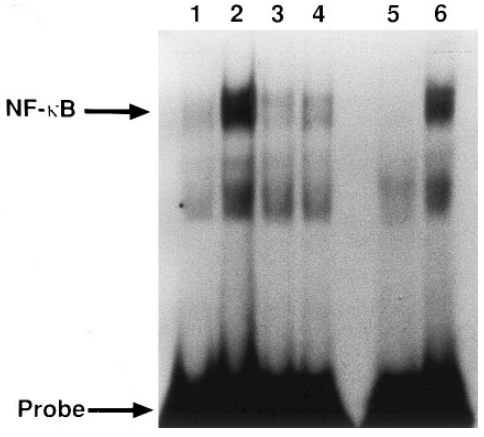

FIG. 1. Effect of ethanol on LPS-mediated NF-κB activation. Kupffer cells were incubated with 100 mM ethanol for 90 minutes, washed, and stimulated with 5 or 10 ng/ml of *Eschericia coli* 011B4 LPS. After 60 minutes of LPS exposure, nuclear extracts were prepared and NF-κB activation was determined by electrophoretic mobility shift assay. An autoradiograph representative of three independent studies is shown.

studies, a 50-fold excess of unlabeled NF-κB oligonucleotide or an unrelated oligonucleotide (AP-1) was incubated with nuclear extracts for 5 min before the addition of labeled NF-κB oligonucleotide. Samples were electrophoresed through a 4% polyacrylamide gel at a constant voltage of 155 V, and the gel was dried before autoradiography.

Statistical analysis. Values are expressed as the mean$\pm$SE. Data were analyzed by ANOVA and differences between means was assessed using the Newman-Keuls multiple comparison test. Significance is defined as p-values of less than 0.05.

RESULTS

Due to the essential role of NF-κB activation in regulation of the inflammatory response, we examined the effect of acute ethanol on LPS-mediated NF-κB activation. Cells were pretreated for 90 minutes with 100 mM ethanol, washed and stimulated for 60 minutes with 5 or 10 ng/ml LPS. Nuclear extracts were prepared and NF-κB activation was determined by electrophoretic mobility shift assay. The identity of the shifted band as NF-κB was made by cold competition. A representative gel shift of three independent studies is shown in Figure 1. Lane 1 contains a nuclear extract from control untreated cells while lane 2 is nuclear extracts from cells stimulated with 10 ng/ml LPS for 60 minutes. LPS stimulation leads to an increase in nuclear NF-κB binding activity. An excess of unlabeled NF-κB oligonucleotide (lane 5) blocked binding while an equal amount of unlabeled AP-1 oligonucleotide had no effect (lane 6). Nuclear extracts in lanes 3 and 4 were prepared from Kupffer cells treated with 100 mM ethanol for 90 minutes and washed prior to 60 minutes of stimulation with either 5 or 10 ng/ml LPS. Ethanol pretreatment resulted in marked suppression of LPS-mediated NF-κB activation.

To determine whether ethanol-induced decrease in NF-κB activation lead to a significant biological effect, we measured the effect of ethanol pretreatment on steady state levels of TNF-α mRNA. Kupffer cells were exposed to 100 mM ethanol for 90 minutes, washed, and stimulated (in the absence of ethanol) with LPS (2.5-10 ng/ml) for three hours. Total RNA was isolated and steady state mRNA levels of TNF-α and γ-actin (as a housekeeping gene) were determined by northern blot analysis. Figure 2 shows a representative autoradiograph of 3 independent studies. Phosphorimaging of all blots showed that the induction of TNF-α mRNA levels by LPS was dose dependent in control cells, and steady state TNF-α mRNA

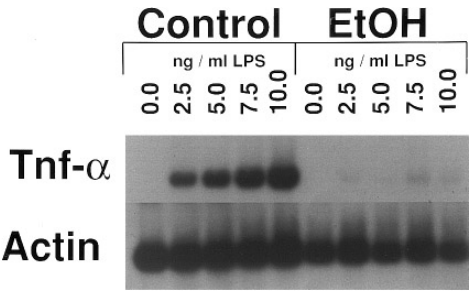

FIG. 2. Effect of ethanol on LPS-mediated TNF-α mRNA induction. Kupffer cells were incubated with 100 mM ethanol for 90 minutes, washed, and stimulated with various concentrations of LPS. After 3 hours of LPS exposure, total RNA was harvested and analyzed for steady state levels of TNF-α and γ-actin mRNA by Northern Blot Analysis. An autoradiograph representative of three independent studies is shown.

levels were at least ten fold less in ethanol treated cells (not shown). Consistent with these data, TNF-α secretion was similarly suppressed (Figure 3). Cells were exposed to 100 mM ethanol for 90 minutes, washed, and stimulated (in the absence of ethanol) with LPS at 2.5-50 ng/ml for six hours. Secretion of TNF-α by the cells into the medium was determined by specific sandwich ELISA. The response of control Kupffer cells to LPS was linear and dose dependent while the exposure to ethanol lead to significant suppression of TNF-α secretion at 5, 10, and 50 ng/ml LPS. ($p<0.001$, LPS vs ETOH treated samples). LPS at a dose of 2.5 ng/ml produced only modest TNF-α secretion in control cells, and this was not significantly suppressed by ethanol treatment. TNF-α secretion by unstimulated cells was not detectable (data not shown).

The effect of varying the duration of ethanol pretreatment on subsequent LPS-mediated TNF-α secretion is shown in Figure 4. Exposure times as short as 15 minutes lead to significant suppression, in the absence of ethanol, of TNF-α secretion induced by LPS ($p<0.01$ vs LPS alone). Longer (90 or 120 minutes) pretreatments had a significantly greater effect than 15 minutes of pretreatment ($p<0.05$). These data raised a possibility of toxicity with longer periods of ethanol treatment. To address this question, cells were exposed to 100 mM ethanol for 90 minutes, washed, and recultured overnight. After overnight recovery, cells were stimulated with 100 ng/ml LPS and TNF-α secretion was determined by ELISA. There was no

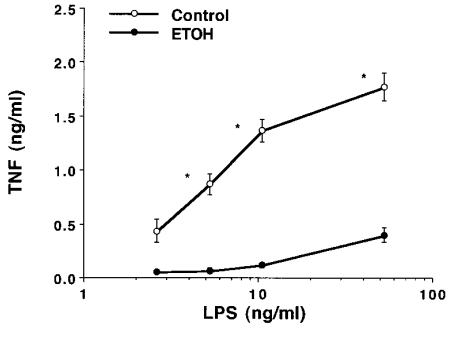

FIG. 3. Effect of ethanol on LPS-mediated TNF-α secretion by Kupffer cells. Kupffer cells were incubated with 100 mM ethanol for 90 minutes, washed, and stimulated with various concentrations of LPS. After 6 hours of LPS exposure, the medium overlying the Kupffer cells was harvested and analyzed for TNF-α by ELISA. (Mean $\pm$ SE, n=4 separate isolations, 16 determinations at each point, *$p<0.001$)

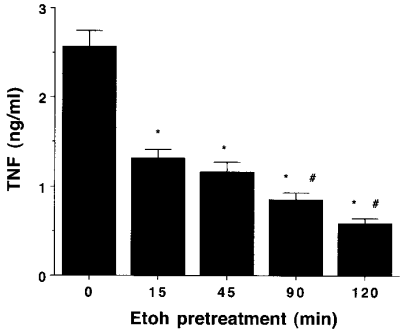

FIG. 4. Effect of varying ethanol pretreatment time on LPS-mediated TNF-α secretion by Kupffer cells. Kupffer cells were incubated with 100 mM ethanol for 15–120 minutes, washed, and stimulated with 50 ng/ml LPS. After 6 hours of LPS exposure, the medium overlying the Kupffer cells was harvested and analyzed for TNF-α by ELISA. (Mean±SE, n=3 separate isolations, 20 determinations at each point, *$p<0.01$ vs LPS alone, #$p<0.05$ vs 15′ ethanol)

significant difference in LPS responsiveness between control cells (3.0 ± 0.157 ng/ml TNF-α) and ethanol exposed cells (2.6 ± 0.191 ng/ml TNF-α, p=0.14, n=4 independent isolations, 30 determinations at each point). Thus ethanol pretreatment lead to an acute, reversible LPS-hyporesponsiveness which acts at the earliest steps of the inflammatory cascade, as it is currently understood.

DISCUSSION

Health problems involving the effects of alcohol on the liver affect many residents of the United States. The acute and chronic use of ethanol has been associated with a greater rate of infections [30-32]. These studies have reported both defects in cytokine secretion and increased intracellular survival of microbial pathogens suggesting direct and specific effects of ethanol on mechanisms of host defence. A key component of host defence are the Kupffer cells of the liver. *In vivo* acute ethanol suppresses Kupffer cell function and increases mortality during polymicrobial sepsis [22]. *In vitro* studies, where ethanol was present throughout the protocol, show suppression of TNF-α gene induction or secretion [33-35]. In contrast to previous investigations, the present study treated Kupffer cells with ethanol for a relatively short time whereupon the cultures were exposed to LPS in the absence of ethanol. This protocol was used to test if ethanol exposure has prolonged effect on the molecular components of the subsequent inflammatory response. This experimental design does not allow strict comparison to animal feeding studies designed to determine the pathogenesis of alcohol induced liver injury.

Data from this study show for the first time that ethanol induces suppression of LPS-stimulated NF-κB activation that is associated with induction of TNF-α. They show that ethanol pretreatment for as short as 15 minutes result in a LPS hyporesponsiveness which is long lasting in the absence of ethanol. Furthermore, these results suggest that the site of the ethanol-induced suppression occurs in the LPS-induced signaling pathway proximal to NF-κB activation. While these studies are consistent with other studies in diverse monocytic populations they are the first to demonstrate that ethanol exerts an immunosuppressive effect at the earliest steps of the inflammatory cascade which persists beyond the period of ethanol exposure.

We chose to analyze the effect of ethanol on LPS-mediated NF-κB activation as a first step towards dissecting the underlying molecular mechanism for ethanol mediated immunosuppression. NF-κB is a pluripotent transcription factor whose activation is re-

quired to induce transcription of cytokine, chemokine and growth factor genes [reviewed in 36]. In quiescent cells, NF-κB is maintained in the cytoplasm bound to its inhibitor I-κB. Inducers of NF-κB activation, which include LPS, cytokines and oxidants, lead to degradation of I-κB and translocation of NF-κB to the nucleus where it interacts with specific binding sites upstream of its target genes. The observation of enhanced intracellular microbial survival during ethanol exposure [30,32] may be associated with suppression of reactive oxygen species and oxidative stress associated with NF-κB activation [36]. The present study has shown that acute ethanol exposure dramatically inhibits LPS-induced NF-κB activation as determined by electrophoretic mobility shift assay.

We have shown that isolated Kupffer cells exposed to ethanol for 90 minutes fully recover following overnight culture. We estimate that the recovery period may be between 3 and 6 hours post-ethanol. Three hours was the endpoint for northern blot studies which showed a complete suppression of TNF-α mRNA while 6 hours was the endpoint for ELISA studies in which the magnitude of the TNF-α response was associated with how long the cells were treated with ethanol. Defined studies of rapid responses to LPS stimulation, such as NF-κB activation, will allow delineation of the phenomenon of recovery and the allow molecular dissection of associated signal transduction events modulated by exposure to ethanol. Further analysis of the molecular mechanism for ethanol-mediated immunosuppression may point to potential immunomodulatory therapies to augment host defences affected by ethanol.

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