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Item Type	Journal Article
Authors	Mandrekar, Pranoti;Catalano, Donna;Szabo, Gyongyi
Citation	Int Immunol. 1999 Nov;11(11):1781-90.
Download date	2026-04-22 00:43:44
Link to Item	https://hdl.handle.net/20.500.14038/42269

Inhibition of lipopolysaccharide-mediated NF κ B activation by ethanol in human monocytes

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Keywords: alcohol, intracellular signaling, inflammatory cytokines, nuclear regulatory factors, macrophages

Abstract

Alcohol use is typically associated with impaired immunity and increased host susceptibility to infection, partially due to decreased inflammatory response. Acute ethanol exposure has been shown to down-regulate monocyte production of inflammatory cytokines. Activation of the pluripotent transcription factor NF κ B is a pivotal step in the induction of inflammatory cytokines, chemokines and growth factors. Therefore, we hypothesized that alcohol may alter NF κ B activation, thus providing a mechanism for the decreased inflammatory cytokine production by monocytes after acute alcohol treatment. We show here for the first time that alcohol inhibits lipopolysaccharide (LPS)-induced NF κ B activation in human monocytes by decreasing DNA binding of the p65/p50 heterodimer as seen in electrophoretic mobility shift and supershift assays. We also demonstrate that alcohol prevents LPS-induced nuclear translocation of p65 and to a lesser extent that of the p50 subunits. NF κ B activation is regulated via phosphorylation and proteolytic degradation of I κ B. Thus, we investigated the effect of acute ethanol treatment on I κ B in human monocytes. Alcohol did not prevent LPS-induced I κ B α degradation but decreased the levels of phospho-specific I κ B α (Ser32). Finally, for the first time we show that *de novo* protein synthesis is necessary to bring about the ethanol-mediated inhibition of LPS-induced NF κ B activation. Consequently, these results suggest that physiologically relevant concentrations of alcohol interfere with NF κ B activation and thereby may affect the regulation of NF κ B-controlled gene activation.

Introduction

The transcription factor NF κ B is comprised of homodimers and heterodimers of proteins that belong to the Rel family of nuclear regulatory factors that reside as inactive cytosolic proteins due to their interaction with inhibitory proteins called I κ Bs in most cell types including blood monocytes. NF κ B activation is induced by a wide variety of signals, such as mitogens, cytokines, bacterial lipopolysaccharide (LPS), viruses, viral proteins, double-stranded RNA and UV light (1). Treatment of cells with various inducers of NF κ B activity leads to phosphorylation and degradation of the I κ B proteins, releasing NF κ B to allow its translocation into the nucleus to activate transcription of target genes encoding for inflammatory cytokines, chemokines, growth factors and adhesion proteins (2,3). Thus, activation of the transcription factor NF κ B is a critical step in monocyte inflammatory cytokine production

after exposure to bacterial stimuli such as LPS (4–6). Among the various NF κ B/Rel dimers the p65/p50 heterodimer is the prototypical form of LPS-inducible NF κ B in monocytes (7,8).

Monocyte aberrations play an important role in impaired immunity and increased host susceptibility to infections associated with both acute and chronic alcohol use. Increased infection rate after alcohol use is secondary to abnormal inflammatory response (9–11). Inflammatory cytokines, particularly tumor necrosis factor (TNF)- α , play a pivotal role in the elimination of microorganisms by stimulating a cascade of mediators. Production of inflammatory cytokines such as TNF- α , IL-1 and IL-6 were shown to be decreased after acute moderate alcohol treatment both *in vivo* and *in vitro* in human and murine monocytic cells (11–13). Decreased TNF- α has been implicated as a mechanism for impaired anti-mycobact-

erial and anti-microbial defense in alcohol-exposed animals (10,14). Therefore, by inhibiting monocyte TNF- α production in response to bacterial and other pathogens, ethanol clearly interferes with host defense. Considering that activation of NF κ B is a pivotal molecular event in the inflammatory response to injury, we hypothesized that alcohol affects LPS-induced NF κ B activation in monocytes. Alteration of NF κ B by alcohol may provide a mechanism for decreased inflammatory cytokine production after alcohol use.

Our previous studies have shown that acute ethanol treatment of monocytes in the absence of bacterial stimulus increases nuclear NF κ B binding in monocytes (15). We also reported that the ethanol-induced increase in DNA binding activity is restricted to the p50/p50 homodimer and, in contrast to LPS stimulation, there is no increase in the p65/p50 heterodimer. Thus, the present study was designed to evaluate the effect of acute ethanol treatment on LPS-induced NF κ B activation in monocytes and to define the steps of NF κ B activation targeted by acute ethanol treatment.

Methods

Reagents

IMDM and RPMI 1640 were obtained from Gibco Life Technologies (Gaithersburg, MD) and FBS was obtained from Atlanta Biologicals (Norcross, GA). Endotoxin contamination was >8 pg/ml in the culture media and FBS. LPS (*Escherichia coli* strain 0111:B4) was obtained from Difco (Detroit, MI). NF κ B oligonucleotide was obtained from Promega (Madison, WI). Protease inhibitors such as PMSF, aprotinin, antipain and leupeptin were from Sigma (St Louis, MO).

Blood donors

Healthy normal individuals, aged 18–60, females and males, donated 120 ml peripheral blood per experiment. Blood was obtained by venipuncture and anticoagulated by heparin. Blood donors had consumed no alcohol at least 48 h prior to blood donation. Furthermore, donors had no previous alcohol abuse history and consumed less than six drinks per week.

Monocyte separation and stimulation

Monocytes from human peripheral blood were isolated by selective adherence from Ficoll-Hypaque-purified mononuclear cell preparations as previously described (12). Briefly, Ficoll-Hypaque density-separated mononuclear cells were washed in ice-cold HBSS supplemented with 3% FBS and adhered to microexudate-treated flasks at 1×10^7 cells/flask in RPMI containing 15% FBS. Non-adherent cells were removed after 2 h adherence by gentle washing with warm RPMI and then adherent cells were collected by vigorously shaking with 4 mM EDTA. This separation provides 93–95% monocyte purity. Monocytes were collected and plated in six-well plates for stimulation at 1×10^7 cells/group for nuclear and cytoplasmic extractions, 3×10^6 cells/group for bioassays and 6×10^6 cells/group for RNA extraction. Cells were stimulated with *E. coli*-derived LPS (1 μ g/ml), 25 mM ethanol, and the combination of LPS and ethanol together. The 25 mM *in vitro* ethanol concentration approximates a 0.1 g/dl blood alcohol level, which is achieved *in vivo* after a dose of moderate

drink. Monocyte viability was not affected by ethanol or LPS treatment and was 98% as reported before (12,13).

Preparation of nuclear and cytoplasmic extracts

Cells with or without 1 h stimulation at 37°C were extracted by the modified method of Dignam (16). At the end of the stimulation period, cells were washed in ice-cold PBS 3 times and scraped in cold buffer A (10 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 10 mM NaCl, 0.5 mM DTT, 0.3 M sucrose, 0.5 mM PMSF, 0.1 mM EDTA, and 1 mg/ml protease inhibitors such as aprotinin, antipain and leupeptin). Cells were then lysed using a glass Dounce homogenizer on ice with 15–20 strokes. The lysate was centrifuged at 12,000 *g* for 30 s to pellet the nuclei and the supernatant was stored at –80°C as the cytoplasmic extract. The nuclear pellet was then resuspended in ice-cold buffer B (20 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 300 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 25% glycerol). All tubes were kept on a shaker at 4°C for 30 min. The lysate was then centrifuged at 12,000 *g* for 15 min and the supernatant was stored at –80°C as the nuclear extract. Protein content was estimated in both the cytoplasmic and nuclear extract by the BioRad (Hercules, CA) Dye Reagent Assay.

Electrophoretic mobility shift assay (EMSA)

A consensus double-stranded NF κ B oligonucleotide 5'-AGTTGAGGGGACTTCCCAGGC-3' was used for EMSA. End-labeling was accomplished by treatment with T4 kinase in the presence of [³²P]ATP. Labeled oligonucleotides were purified on a polyacrylamide copolymer column (BioRad). Five micrograms of nuclear protein was added to a binding reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 20% glycerol, 20 μ g/ml BSA, 2 μ g poly(dI-dC) and 30,000 c.p.m. of ³²P-labeled NF κ B oligonucleotide. Samples were incubated at room temperature for 30 min. For supershift assays, 1 μ l of the antibody was added to the reaction after the 30 min and then further incubated for 30 min. All reactions were run on a 5% polyacrylamide gel and the dried gel was exposed to an X-ray film at –80°C overnight. Anti-human p50 antibody for supershifts was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antisera raised against the N-terminus region of human p65 was generously provided by Dr Nancy Rice (ABL-Basic Research Programs, Frederick, MD). Cold competition was done by adding a 20-fold excess of specific unlabeled double-stranded probe to the reaction mixture.

Western blots

Ten microgram aliquots of nuclear or cytoplasmic proteins were mixed with an equal volume of 2 \times sample buffer (containing 0.1% SDS and 2-mercaptoethanol) and boiled for 5 min. Proteins were separated on 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes. Non-specific binding was blocked by soaking the membranes in Tris-buffered saline/1% non-fat dried milk/0.1% Tween 20 for 2 h at room temperature. Immunoreactive proteins were detected by incubating the blots with diluted p50, p65, I κ B α and phospho-specific I κ B α antibodies at 4°C overnight. After washing the blots, in Tris-buffered saline/0.1% Tween 20, the

filters were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, Arlington Heights, IL). Blots were then developed using ECL reagents from Amersham. NF κ B p50 (SC114) affinity-purified rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology and rabbit polyclonal antibody against the C-terminal peptide of p65 (SA171) from Biomol (Plymouth Meeting, PA). I κ B α (sc-371) affinity-purified rabbit polyclonal antibody to the C-terminal of human I κ B α was obtained from Santa Cruz Biotechnology. Phospho-specific I κ B α (Ser32) antibody was obtained from New England Biolabs (Portsmouth, NH).

Detection of monocyte TNF- α and IL-1 β levels

Monocyte supernatants were assayed for TNF- α using the serum-free *in vitro* bioassay as previously described (12). IL-1 β activity in the monocytes was determined by an ELISA (Endogen, Cambridge, MA) according to the manufacturer's guidelines. Statistical significance between appropriate groups for TNF- α and IL-1 β was calculated using the Wilcoxon signed-rank non-parametric data analysis (12). Data are presented as representatives of all experiments and mean values of monokine response levels rather than statistical mean \pm SE.

TNF- α and IL-1 β RNA determination

Adherent monocytes (3×10^6 monocytes/sample) were stimulated for 1.5 h for TNF- α and 3 h for IL-1 β as indicated in each experiment, and then resuspended in 1 ml of Tri reagent (Molecular Research Center, Cincinnati, OH). Total cellular RNA was extracted based on the acid guanidinium thiocyanate-phenol-chloroform method described previously (13). Northern blots were prepared after fractionation of RNA on a 1% agarose gel containing 0.66 M formaldehyde, then transferred to nylon membrane by the capillary technique. Membranes were cross-linked under UV light, then prehybridized at 42°C for 3–4 h. The human TNF- α probe used in these experiments was a 473 bp *Eco*RI cDNA fragment and the IL-1 β probe was a 1.3 kb *Pst*I cDNA fragment (generously provided by Dr S. Clark, Genetics Institute, Cambridge, MA). The cDNA fragments were radiolabeled by the random primer method of Feinberg and Fogelstein to a sp. act. of 10^8 c.p.m./ μ g. Hybridizations were performed with 10^6 c.p.m. of denatured probe/ml hybridization buffer for 16–18 h at 42°C. Filters were washed in $2 \times$ SSPE plus 0.1% SDS twice for 30 min at 65°C. Filters were then exposed to Kodak X-Omat film at -70°C with intensifying screens to visualize by autoradiography. Filters were stripped in prehybridization solution twice for 30 min at 72°C and re-probed with radiolabeled β -actin probe (2.0 kb *Xho*I cDNA fragment).

Results

Decreased production of TNF- α and IL-1 β after acute ethanol treatment in monocytes

Both *in vivo* and *in vitro* studies have shown that acute ethanol treatment prior to bacterial stimulation has an inhibitory effect on inflammatory cytokine production including TNF- α , IL-1 β and IL-6 (11,17,18). We hypothesized that ethanol inhibits IL-1 β and TNF- α signaling pathways at the level of mRNA or

proximal to RNA induction. Figure 1 illustrates that a single dose of biologically relevant *in vitro* ethanol treatment (25 mM) given at the time of LPS stimulation resulted in a significant down-regulation of monocyte TNF- α production ($P < 0.001$, $n = 15$). Similar to the protein levels, the LPS-induced TNF- α mRNA at 2 h was substantially decreased ($58 \pm 15.1\%$; $n = 3$) by *in vitro* ethanol (25 mM) treatment given simultaneously with LPS. Figure 1 demonstrates that ethanol also down-regulates monocyte IL-1 β induction. Ethanol inhibited LPS-induced monocyte IL-1 β protein levels by $46 \pm 12\%$ ($n = 15$) with a concomitant decrease in the IL-1 β mRNA ($76.3 \pm 8.2\%$; $n = 3$), suggesting that ethanol affects early signaling elements in IL-1 β regulation. Our previous studies revealed that ethanol has no significant effect on IL-1 β mRNA stability, and, therefore, likely has a direct effect on TNF- α and IL-1 β gene activation (13).

Attenuation of LPS-induced NF κ B activation by ethanol in monocytes

Monocyte stimulation by LPS signals through the CD14 receptor and leads to activation of NF κ B, a crucial transcription factor in the production of pro-inflammatory cytokines (8). We have shown that acute ethanol treatment does not significantly decrease monocyte CD14 expression to account for decreased inflammatory cytokine induction in response to LPS (data not shown). In order to test our hypothesis that ethanol affects LPS-induced NF κ B activation in monocytes, EMSA were performed. As illustrated in Fig. 2(A), nuclear extracts from adherence-isolated normal human monocytes showed minimal NF κ B binding. There was a marked increase in nuclear NF κ B binding in monocytes stimulated for 1 h with 1 μ g/ml LPS as compared to the unstimulated cells. In contrast, when LPS was combined with *in vitro* ethanol treatment (25 mM), we found that ethanol inhibited the binding of NF κ B to the consensus DNA sequence by $74.3 \pm 7.3\%$ (five different experiments). Similar inhibition of LPS-induced NF κ B binding by ethanol was seen in monocytes at 10 min, 30 min and 2 h after stimulation (data not shown). Since NF κ B activation was maximal at 60 min this time-point was used for all future experiments. Consistent with our previous reports, alcohol alone also induced an increase in NF κ B binding (15). The specificity of NF κ B binding was confirmed by competition reactions using 20-fold excess of the oligonucleotide.

In our effort to achieve better resolution of the complexes, we used $0.25 \times$ TBE for native gel electrophoresis. This allowed for the separation of two complexes referred to as complex I and II as seen in these experiments (Fig. 2B). The upper complex (complex I) with the greater mol. wt represents the p65/p50 heterodimer while the lower mol. wt complex (complex II) represents the p50/p50 homodimer. A third complex (non-specific) also seen in all the lanes at the same intensity irrespective of the stimulation is a non-specific band. Data in Fig. 2(B) shows that ethanol had an inhibitory effect on the LPS-induced p65/p50 heterodimer induction. Consistent with our previous reports, ethanol treatment (25 mM) by itself showed a moderate increase ($29.6 \pm 3.7\%$; $n = 4$) in the levels of p50/p50 homodimer as compared to the unstimulated monocytes (15). Next, to confirm the specificity of the NF κ B complexes supershift analysis was performed using antibodies to the p65 and p50 NF κ B subunit. Data in

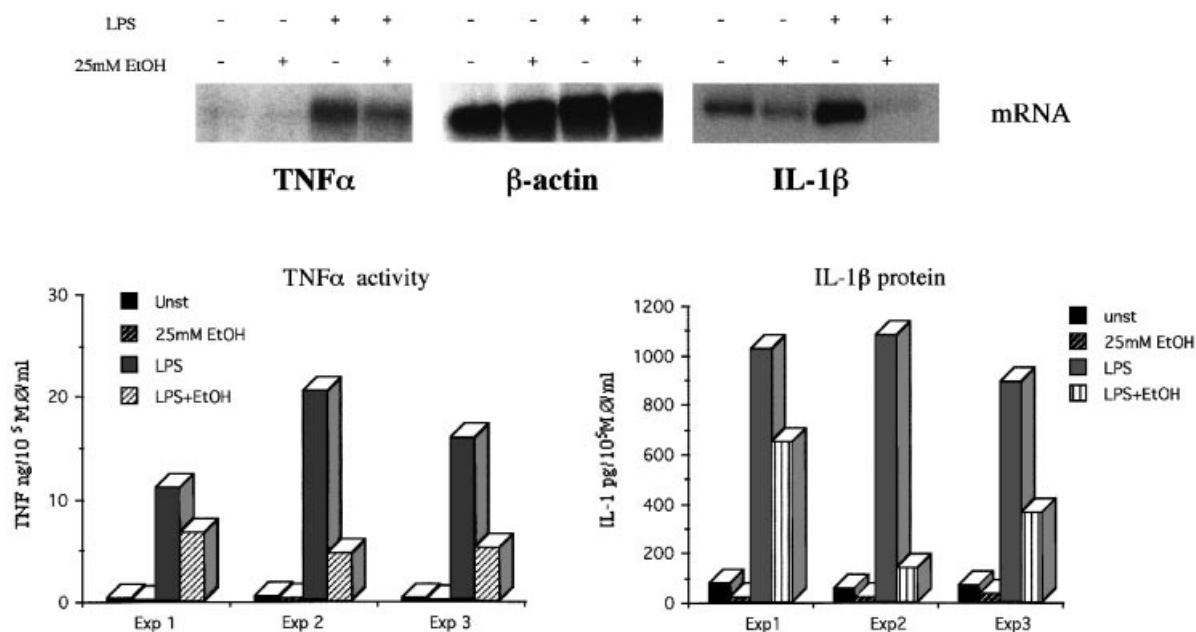


Fig. 1. Acute ethanol down-regulates TNF- α and IL-1 β mRNA and protein levels in human monocytes. Adherence-isolated monocytes (unst) were stimulated with 1 μ g/ml of LPS in the presence or absence of 25 mM ethanol for 1.5 h for TNF- α mRNA, 3 h for IL-1 β mRNA, and 16 h for TNF- α and IL-1 β protein levels. (Top panel) For mRNA analysis, total cellular RNA was extracted and Northern blot analysis was performed as described in Methods. β -Actin was used to control for loading differences. Data are representative of five separate experiments showing similar results. (Lower panel) Monocyte culture supernatants were collected for TNF- α determination by the L-M cell bioassay and IL-1 β was measured by ELISA (Endogen). Three representative experiments out of the total 15 are shown. Exp, experiment; M ϕ , monocyte

Fig. 3(A) illustrate that upon addition of the p50 antibody, NF κ B complexes are supershifted in all the unstimulated, ethanol, LPS- or LPS + ethanol- stimulated monocytes as indicated by the diminution of the bands. This suggests that both complexes I and II contain the p50 subunit. As seen in Fig. 3(B), combination of nuclear extracts with rabbit antisera against p65 of NF κ B supershifts only the p65/p50 (complex I) in the LPS-treated monocytes. There is a faint supershifted complex also seen in the LPS + ethanol-treated cells. Thus, these results confirm the previous observation that complex I contains p65 as well as p50 because antibodies to both p65 and p50 supershift this complex (19,20). Further, complex II contains p50 and not p65 because only the p50 antibody and not the p65 antibody shifts this band effectively in a supershift experiment. These results demonstrate that ethanol primarily inhibits nuclear binding of LPS-induced p65/p50 heterodimer.

Inhibitory effect of acute ethanol treatment on LPS-induced nuclear translocation of p65 and p50 subunits of NF κ B

Decreased NF κ B binding in ethanol-treated cells may be due to impaired activation of the inactive cytoplasmic NF κ B-I κ B complex or altered NF κ B nuclear translocation. Therefore, the effect of acute ethanol treatment was tested on the nuclear translocation of p65 and p50 subunits of NF κ B after bacterial stimulation in adherence-isolated normal human monocytes. Cells were stimulated with 1 μ g/ml LPS, 25 mM ethanol or their combination for 60 min. Nuclear and cytoplasmic extracts were prepared and tested for p50 and p65 protein levels by Western blotting as described in Methods. Figure 4(A) shows that adherent monocytes had a substantial amount of p50

not only in the cytoplasmic but also in the nuclear extracts. Constitutive levels of NF κ B in the nucleus of the human monocytes have been previously shown and are thought to be secondary to adherence, monocyte maturation or both (21). As reported previously, monocyte stimulation with ethanol alone increased the level of p50 in nuclear extracts (15). LPS (1 μ g/ml), a potent inducer of both inflammatory cytokines and NF κ B activation, also increased nuclear p50 levels after 60 min stimulation, suggesting that LPS brings about nuclear translocation of the p50 subunit of NF κ B. However, when LPS stimulation was combined with 25 mM ethanol for 60 min, there was a decrease in the nuclear p50 level compared to the LPS-stimulated cells, indicating that ethanol may interfere with the LPS-induced nuclear translocation of the p50 subunit of NF κ B.

Reduced nuclear p50 levels in ethanol + LPS-stimulated monocytes represent the p50/p50 homodimer or the p65/p50 heterodimer form of NF κ B. Therefore, to evaluate the effect of ethanol on the LPS-induced p65 nuclear translocation, cytoplasmic and nuclear extracts of monocyte samples stimulated for 60 min were subjected to Western blot analysis using the p65 antibody. Figure 4(B) demonstrates that ethanol stimulation alone had no significant effect on nuclear levels of p65 compared to unstimulated monocytes. In agreement with previous findings, LPS stimulation resulted in increased p65 levels in the nuclear extracts, suggesting nuclear translocation of the p65 subunit of NF κ B in blood monocytes. Consistent with the decreased p65/p50 heterodimer binding in EMSA (Fig. 3), ethanol inhibited LPS-induced p65 nuclear translocation, as indicated by low nuclear levels of p65 (Fig. 4B). Taken together, these results suggest that ethanol down-

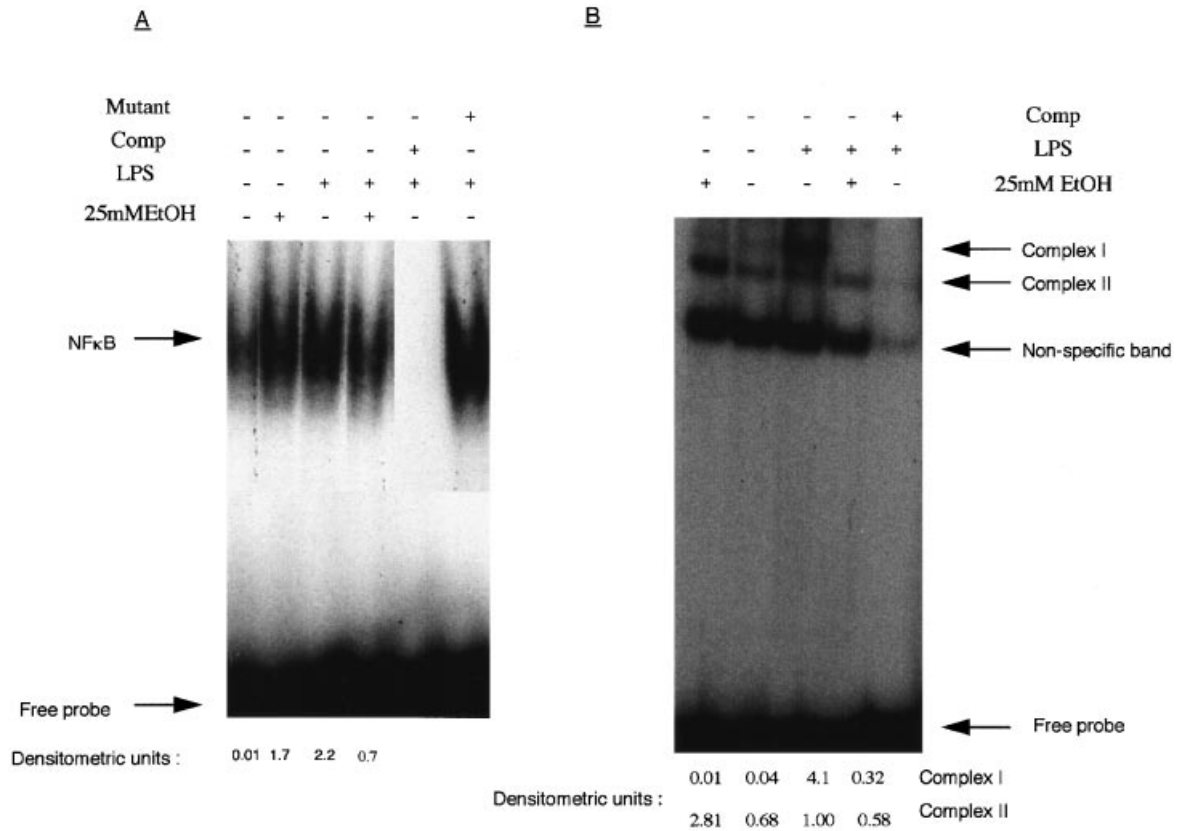


Fig. 2. Ethanol inhibits nuclear NFκB binding in peripheral blood monocytes. Adherence-isolated monocytes were stimulated with 1 μg/ml LPS in the presence or absence of 25 mM ethanol for 1 h. Nuclear extracts were then prepared and gel shift analysis performed as described in Methods. (A) Representative EMSA of three separate experiments done using [³²P]NFκB consensus oligonucleotide. A 20-fold excess of the unlabeled NFκB fragment was included as a cold competitor (comp). The extent of DNA binding was quantitated using the NIH Image program and densitometric units are indicated at the bottom of each lane. (B) EMSA showing better resolution of the NFκB complexes, achieved by using 0.25×TBE buffer in native gel electrophoresis. Corresponding densitometric units of the individual complexes are shown at the bottom of each lane. Complex I, p65/p50 heterodimer; Complex II, p50/p50 homodimer.

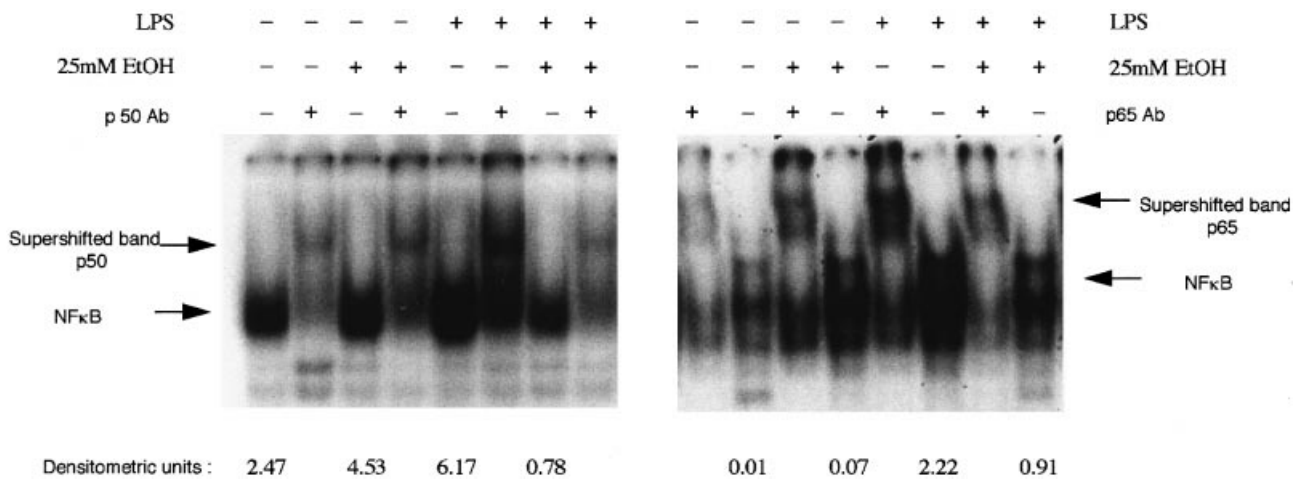


Fig. 3. Supershift analysis of NFκB in ethanol-treated monocytes. Nuclear extracts from monocytes stimulated for 1 h with 1 μg/ml LPS with or without 25 mM ethanol were subjected to gel shift analysis. All reactions were admixed with rabbit anti-p50 antibody (2 μg) (Santa Cruz Biotechnology) (A) or with anti-human-p65 antisera (generously provided by Dr Nancy Rice) (B) after addition of [³²P]NFκB oligonucleotide and incubated for 30 min. Shifted bands were detected by their retarded mobility.

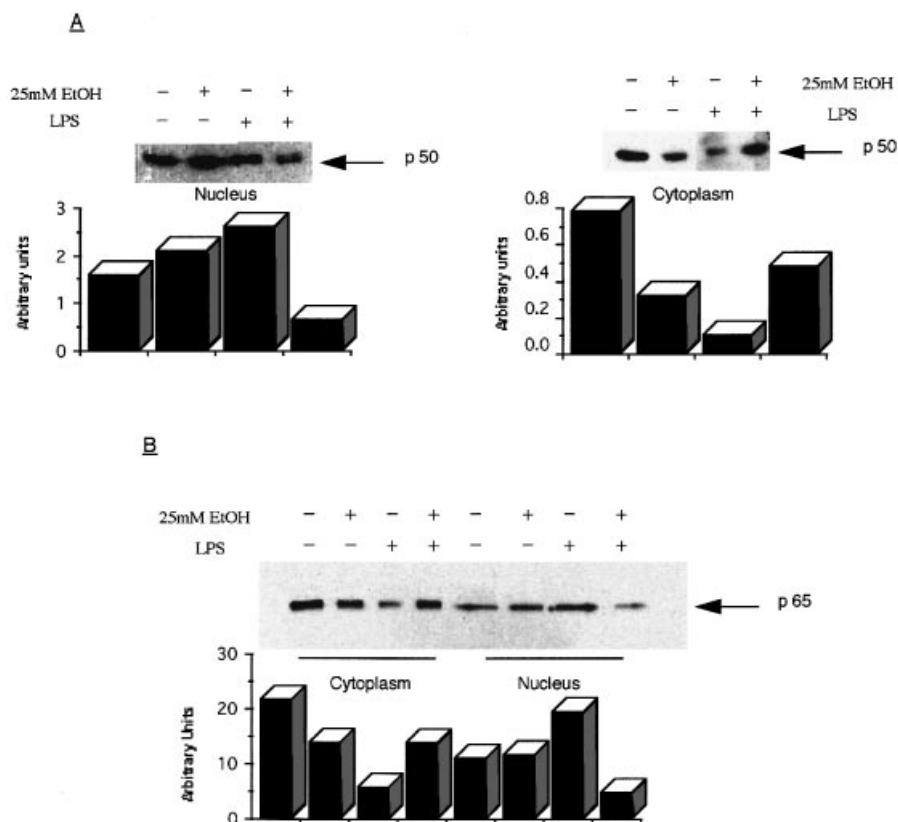


Fig. 4. Lack of translocation of NFκB subunits in ethanol-treated monocytes. Monocytes were stimulated with 1 μg/ml LPS in the presence or absence of 25 mM ethanol for 1 h. Nuclear and cytoplasmic extracts (10 μg/group) were subjected to immunoblotting using anti-p50 antibody (1:500 dilution) (A) or anti-p65 antibody (1:1000 dilution) (B) and visualized by ECL assay (Amersham). Results are representative of four experiments with different blood donors.

regulates LPS-induced translocation of p65 as well as p50 subunits of NFκB into the nucleus.

Effect of ethanol on IκBα levels

Our data so far suggest that ethanol decreases LPS-induced p65/p50 heterodimer translocation and DNA binding. The retention of NFκB in the cytosol is due to its interaction with inhibitory proteins called IκBs (22). Activation and nuclear translocation of NFκB involves phosphorylation and proteolytic cleavage of IκBα (22,23). Thus, we postulated that inhibition of LPS-induced NFκB activation by ethanol may involve modulation of IκBα levels. To test whether the ethanol-induced inhibition of NFκB translocation is due to an increase in IκBα levels in the cytoplasm, we analyzed the effect of ethanol on cytoplasmic IκBα levels and IκBα phosphorylation in LPS-induced monocytes. As expected, a substantial amount of IκBα was seen in the cytosol of unstimulated cells (Fig. 5A). The kinetics of IκBα degradation showed a rapid loss by 10 min and its complete re-accumulation by 3 h after LPS stimulation in monocytes as previously shown by others (22) (data not shown). Cytoplasmic IκBα protein levels were reduced after LPS stimulation for 1 h (Fig. 5A), consistent with the increase in p65 nuclear translocation shown in Fig. 4(B). In contrast, cells treated with ethanol alone showed no difference in the cytosolic IκBα levels as compared to

unstimulated cells suggesting that ethanol alone does not affect IκBα levels (Fig. 5A). Similar to the data in Fig. 5(A) obtained 1 h after ethanol treatment, cytoplasmic IκBα levels did not change at 10, 30 and 90 min in ethanol-treated monocytes (data not shown). More important, we found that treatment of monocytes with ethanol did not prevent LPS-induced degradation of IκBα at 1 h (Fig. 5A) or any of the other time-points tested (data not shown).

Activation of NFκB and reduction of cytoplasmic IκBα levels is preceded by IκBα phosphorylation in LPS-stimulated cells (22,24). Thus, the levels of phosphorylated IκBα in cytoplasmic extracts were evaluated utilizing the antibody specific for phospho-IκBα (Ser32) in Western blots. LPS stimulation increased monocyte phospho-IκBα expression (Fig. 5B). Data in Fig. 5(B) illustrate that only LPS-induced phospho-IκBα levels were substantially reduced by ethanol treatment. However, in monocytes treated with ethanol alone, the levels of phosphorylated IκBα did not change as compared to unstimulated monocytes (Fig. 5B). These results indicate that in human monocytes ethanol prevents LPS-induced increase in phosphorylated IκBα levels without preventing degradation of cytoplasmic IκBα levels.

Previous studies have shown that nuclear binding of NFκB causes a subsequent up-regulation of IκBα mRNA levels due to the presence of NFκB sites in the IκBα promoter which

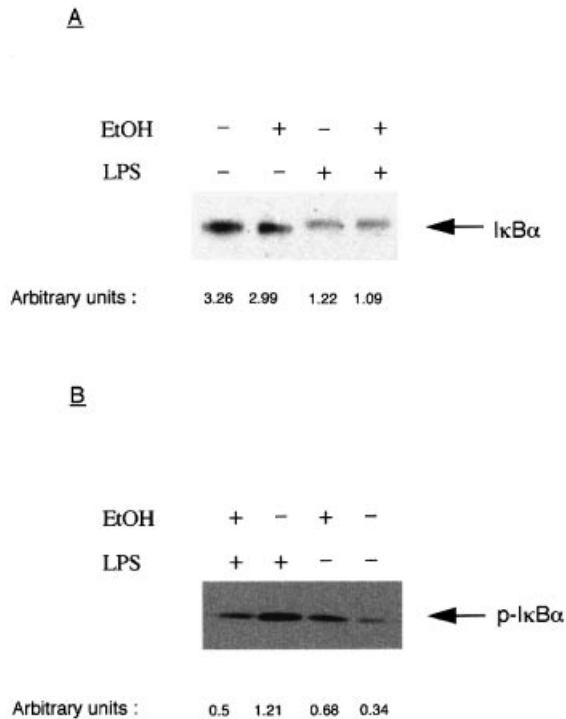


Fig. 5. Ethanol decreases LPS-induced phospho- $\text{I}\kappa\text{B}\alpha$ levels without affecting total cytoplasmic $\text{I}\kappa\text{B}\alpha$. Cytoplasmic extracts (20 $\mu\text{g}/\text{group}$) of monocytes stimulated with 1 $\mu\text{g}/\text{ml}$ LPS with or without 25 mM ethanol for 1 h were subjected to immunoblotting using the anti- $\text{I}\kappa\text{B}\alpha$ antibody (Santa Cruz Biotechnology) (A) or phospho-specific anti- $\text{I}\kappa\text{B}\alpha$ (Ser32) antibody (B) as described in Methods. Densitometric values for the individual bands are indicated below each lane.

provides a mechanism to terminate ongoing NF κ B activation (25,26). Thus, one can hypothesize that ethanol may affect the p65/p50 heterodimer translocation and DNA binding activity by increasing the re-accumulation of the $\text{I}\kappa\text{B}\alpha$ pool in the cytosol. To check whether ethanol has any effect on the re-synthesis of $\text{I}\kappa\text{B}\alpha$ protein, we performed experiments using the protein synthesis inhibitor cycloheximide (10 $\mu\text{g}/\text{ml}$) in combination with all the stimulations used in the previous experiments. Data in Fig. 6(A) demonstrate that substantial $\text{I}\kappa\text{B}\alpha$ levels were detected in unstimulated monocytes. Cycloheximide partially inhibited the recovery of $\text{I}\kappa\text{B}\alpha$ levels in LPS-stimulated monocytes at 1 h, suggesting that accumulation of $\text{I}\kappa\text{B}\alpha$ in monocytes after activation requires *de novo* protein synthesis. Monocytes pretreated with cycloheximide for 30 min and then with LPS + ethanol showed a substantial reduction in $\text{I}\kappa\text{B}\alpha$ compared to the LPS + ethanol-stimulated (no cycloheximide) cells. This indicates that ethanol affects $\text{I}\kappa\text{B}\alpha$ by a mechanism that requires *de novo* protein synthesis. Enhanced $\text{I}\kappa\text{B}\alpha$ re-synthesis, which in turn may shut off LPS-induced NF κ B (p65/p50) activation and nuclear translocation, is a likely mechanism for ethanol's effect. However, increased cytoplasmic $\text{I}\kappa\text{B}\alpha$ would be expected in such cases.

Finally, we tested whether *de novo* protein synthesis was required for inhibition of LPS-induced NF κ B nuclear binding by ethanol. We found that cycloheximide prevented the ethanol-mediated inhibition of NF κ B activation in LPS-stimulated monocytes. Consistent with previous studies showing that

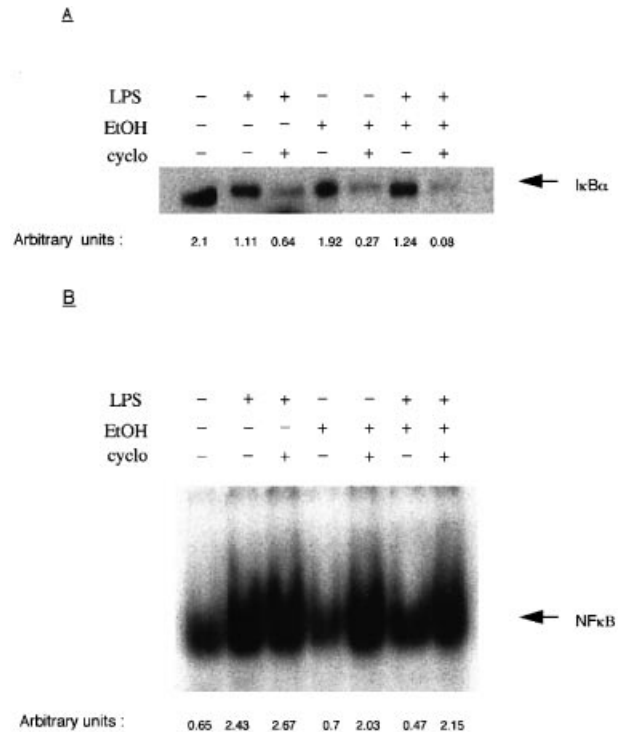


Fig. 6. Protein synthesis is required for ethanol-mediated inhibition of LPS-induced NF κ B activation. Adherence-isolated monocytes were treated with 10 $\mu\text{g}/\text{ml}$ cycloheximide (cyclo) for 30 min before the addition of 1 $\mu\text{g}/\text{ml}$ LPS in the presence or absence of 25 mM ethanol for 1 h. (A) Cytoplasmic extracts were subjected to Western blot analysis using the anti- $\text{I}\kappa\text{B}\alpha$ antibody. (B) NF κ B activation in the nuclear extracts of the same samples was determined by the gel shift analysis. Data are representative of four experiments showing similar results.

protein synthesis is not required for LPS-mediated NF κ B activation (27), there was no change in LPS-induced NF κ B in cycloheximide pre-treated monocytes (Fig. 6B). These results suggest that *de novo* protein synthesis is required for ethanol-induced inhibition of NF κ B activation. Thus, these data together suggest that ethanol-induced inhibition of LPS-induced NF κ B is a complex phenomenon and may require an association of synthetic cellular responses in human monocytes.

Discussion

The goal of this study was to define the effect of acute alcohol treatment on LPS-induced NF κ B activation and to characterize the molecular mechanisms involved in the ethanol-mediated inhibition of LPS-induced pro-inflammatory cytokines. Here, we show for the first time that ethanol affects LPS-induced NF κ B activation in human monocytes by down-regulation of its DNA binding activity. Our results also demonstrate that the LPS-induced translocation of the p65/p50 heterodimer is prevented by ethanol treatment in human monocytes. One of the significant points of this study is that since the nuclear migration and DNA binding of NF κ B has been previously associated with LPS-mediated transcriptional activation of the TNF- α and IL-1 gene in primary macrophages, alcohol may

affect TNF- α and IL-1 via NF κ B (4–6). We and others have shown that exposure of ethanol-naive cells to ethanol has an inhibitory effect on inflammatory cytokine production both *in vitro* in monocytic cells and *in vivo* in animal models (9,11,12). Furthermore, decreased induction of TNF- α , IL-1 β and IL-6 by bacterial stimuli is seen both at the protein and mRNA levels in human monocytes after acute ethanol treatment (11,13,17,28). Thus, we propose that ethanol-mediated down-regulation of LPS-induced TNF- α , IL-1 β and IL-6 may be a consequence of the decreased NF κ B DNA binding activity. In addition, we have previously reported that IL-10 can be induced *in vitro* in monocytes by acute ethanol treatment (29). We have also shown that ethanol-induced IL-10 is a late inhibitor of monocyte TNF- α production (29). Studies from other laboratories have demonstrated that IL-10 inhibits inflammatory cytokine production by affecting nuclear binding of NF κ B (30). Here, our result of decreased LPS-induced NF κ B binding by ethanol in monocytes is unlikely to be via IL-10 since increase in IL-10 production with acute ethanol treatment occurs only 10 h after stimulation (29). Our studies show that ethanol affects NF κ B as early as after 1 h, suggesting that ethanol may have a more direct and rapid effect on NF κ B binding and, hence, on TNF- α and IL-1 β expression, independent of IL-10. Furthermore, our recent reports have demonstrated that *in vitro* acute ethanol treatment of monocytes resulted in a dose-dependent attenuation of staphylococcal enterotoxin B-induced IL-8 and MCP-1 production (31). Similar to inflammatory cytokine genes, IL-8 and MCP-1 gene transcription has been shown to be regulated by NF κ B (32,33). Therefore, our data may also suggest that the ethanol-mediated NF κ B inhibition is likely to contribute to the decreased production of chemokines such as IL-8 and MCP-1.

NF κ B is a pleiotropic regulator of inflammation-induced genes. It forms a family of homo- or heterodimeric transcription factor complexes containing p50, p52 and p65 (Rel A) subunits (34). One of the most important effects of NF κ B activation in cells is the up-regulation of inflammatory cytokine, chemokine and growth factor genes. Our data demonstrate that acute ethanol exposure dramatically inhibits LPS-induced NF κ B activation in human monocytes. Studies by Fox and colleagues have shown that acute ethanol exposure of isolated rat Kupfer cells down-regulates LPS-mediated NF κ B activation *in vitro* (35). However, that study used a high, less physiologic (100 mM) dose of ethanol (equivalent to 400 mg/dl blood alcohol level) *in vitro*. Our findings of decreased NF κ B activation in human peripheral blood monocytes using a much lower dose of ethanol (25 mM; equivalent to 100 mg/dl blood alcohol level) make this data physiologically significant and relevant, suggesting that even moderate alcohol use may affect NF κ B.

A prototypical form of NF κ B is a heterodimeric complex containing the p50 and p65 subunits of the rel family of transcription factors. In our effort to delineate the effect of ethanol on hetero- and homodimers, we show that LPS significantly increases the binding of the p65/p50 heterodimer as shown earlier by other groups (7). Acute ethanol treatment of human monocytes decreases the LPS-induced p65/p50 heterodimer, as well as the p50/p50 homodimer binding activity when compared to LPS-stimulated monocytes. How-

ever, cells treated with LPS and ethanol consistently show a higher proportion of the p50/p50 homodimer as compared to the p65/p50 heterodimer. Homodimers of p50/p50 have been implicated in inhibition rather than activation of NF κ B-driven gene transcription (36). Therefore, our observations imply that ethanol may decrease the inflammatory response by affecting the NF κ B dimers in two ways. First, the decrease in cytokine gene transcription may be due to ethanol-mediated decrease in binding of the p65/p50 heterodimer to the promoter region. Second, an increased proportion of the p50/p50 homodimer in the nucleus of ethanol treated monocytes may facilitate an increase of the p50/p50 homodimer binding to the promoter over the p65/p50 heterodimer. Considering the inhibitory potential of the p50/p50 homodimer, the proportion of the stimulatory p65/p50 heterodimer versus the inhibitory p50/p50 homodimer might be important in decreasing cytokine gene activation in ethanol-exposed monocytes.

Activation of cells by specific stimuli induces the nuclear translocation of NF κ B proteins (34). Our results show that ethanol affects the nuclear translocation of p65 (RelA) as well as of p50 (NF κ B1) subunits. Thus, it appears that p65 and p50 proteins may remain sequestered in the cytoplasm. This observation may account for the lack of detectable nuclear p65/p50 heterodimers as previously shown by EMSA in the nuclear extracts of LPS + ethanol-treated cells.

The central paradigm of NF κ B activation involves the removal of I κ B proteins from a cytoplasmic complex with NF κ B. The major pathway used by a variety of stimuli to activate NF κ B involves the phosphorylation of I κ B α and its proteasome-mediated degradation. Here, ethanol has been shown to decrease the LPS-induced phosphorylation of I κ B α protein at the Ser32 residue as detected by Western blots using the site-specific (Ser32) I κ B α antibody. However, ethanol did not seem to have an effect on total I κ B α levels in LPS-induced monocytes. This apparent disparity between the decreased phosphorylation of I κ B α and unchanged total I κ B α in LPS-induced cells by ethanol remains to be further investigated. NF κ B-activating signals lead to phosphorylation possibly at one or both the Ser32 and Ser36 residues in I κ B α which, in turn, represent recognition signals are needed but not sufficient for proteolysis (37). Proteolysis of I κ B α requires additional sequences including a C-terminal PEST region (37). One possible explanation may be that a decrease in the phosphorylation at the Ser32 residue alone may not be sufficient for the I κ B α to escape degradation by the proteasome pathway. Therefore, it appears that the ethanol-mediated inhibition of LPS-induced NF κ B activation may require intracellular events which occur at a step subsequent to I κ B α degradation.

If the inhibitory effects of ethanol were restricted to a mechanism which does not require *de novo* protein synthesis, one would expect the ethanol-mediated NF κ B inhibition to occur in cells where basic cellular synthetic responses were blocked. To test this hypothesis, we determined the effect of cycloheximide on ethanol-mediated inhibition of NF κ B activation. While it is known that LPS-mediated NF κ B activation does not require protein synthesis (27), our data is the first to show that cycloheximide prevents the ethanol-mediated inhibition of LPS-induced NF κ B activation. This indicates that the inhibitory effects of ethanol require an associated cellular

synthetic event. Fox *et al.* (38) have shown that *N*-acetylcysteine-mediated inhibition of NFκB and TNF mRNA activation also requires *de novo* protein synthesis. The rapid regeneration of the cytoplasmic IκBα pool is in part transcriptionally regulated by the interaction of NFκB with DNA-binding sites located in the promoter of the IκBα gene (25,26). The newly synthesized IκBα interacts with the activated NFκB and thus decreases its activity. Our results show that blockade of *de novo* protein synthesis by cycloheximide results in a significant reduction of IκBα activity in the cytoplasm of LPS + ethanol-stimulated monocytes as compared to the LPS + ethanol (no cycloheximide)-treated cells indicating that ethanol may be affecting the regeneration of the newly synthesized IκBα pool. This in turn may be involved in the inhibition of LPS-induced NFκB activation. Hence, our data suggest that specific synthetic cellular responses are involved in the inhibition of LPS-mediated NFκB activation by ethanol.

In summary, the present data demonstrate for the first time that acute ethanol treatment prevents NFκB activation in human monocytes which is a likely mechanism in inhibition of pro-inflammatory cytokines such as TNF-α and IL-1β at the protein as well as the mRNA level. Although considerable effort has been put into the determination of the cellular mechanisms for activation, much less is known about the molecular events that regulate the termination of activation. We demonstrate that ethanol affects NFκB activation through an active mechanism requiring a cellular synthetic response. Ethanol consumption suppresses host immunological defenses and increases human susceptibility to bacterial and viral infections. Dissecting the mechanisms by which NFκB is down-regulated could provide both a theoretical understanding and clinical utility in alcohol-related infections.

Acknowledgements

This publication was made possible by grant numbers AA 08577 and AA 11576 from the NIH, and 'its contents are solely the responsibility of the authors and do not necessarily represent the official view of the NIH'.

Abbreviations

EMSA	electrophoretic mobility shift assay
LPS	lipopolysaccharide
TNF	tumor necrosis factor

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