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Alcohol (Ethanol) Inhibits IL-8 and TNF: Role of the p38 Pathway

Saman Arbabi, Iris Garcia, Gregory J. Bauer, and Ronald V. Maier

Acute ethanol (EtOH) intoxication has been identified as a risk factor for infectious complications in trauma and burn victims. However, the mechanism of this immune dysfunction has yet to be elucidated. The monocyte/macrophage production of cytokines, in particular IL-8 and TNF-α, is critical in the regulation of the acute inflammatory response to infectious challenge. IL-8 is a potent chemo-attractant and activator of neutrophils. TNF-α, a proinflammatory cytokine, initiates expression of endothelial cell surface adhesion molecules and neutrophil migration. p38, a member of the mitogen-activated protein kinases, plays an important role in mediating intracellular signal transduction in endotoxin-induced inflammatory responses. We examined the effects of LPS and ethanol on p38 activation and the corresponding IL-8 and TNF-α production in human mononuclear cells. LPS-induced IL-8 and TNF-α production was inhibited in a similar pattern by pretreatment with either EtOH or SB202190 (1 μM), a specific inhibitor of p38 kinase. Western blot analysis, using a dual phospho-specific p38 mitogen-activated protein kinase Ab, demonstrated that EtOH pretreatment inhibited LPS-induced p38 activation. These results demonstrate that alcohol suppresses the normal host immune inflammatory response to LPS. This dysregulation appears to be mediated in part via inhibition of p38 activation. Inhibition of IL-8 and TNF-α production by acute EtOH intoxication may inhibit inflammatory focused neutrophil migration and activation and may be a mechanism explaining the increased risk of trauma- and burn-related infections. The Journal of Immunology, 1999, 162: 7441–7445.

Mitogen-activated protein kinases (MAPK) have been demonstrated to play a role in mediating intracellular signal transduction and regulating cytokine production by mononuclear cells in response to a variety of extracellular stimuli (1–3). In response to appropriate stimuli, the MAPK are activated by phosphorylation on both adjacent threonine and tyrosine residues that are separated by a single amino acid (4, 5). For extracellularly regulated kinases (ERK), the best studied of the MAPK families, this intervening amino acid is glutamate; for the p38 MAPK family, it is glycine. While ERK MAPK has been classically associated with growth- and differentiation-inducing signals, p38 MAPK is believed to be involved with inflammatory cytokines and environmental stress inducers (6). In particular, p38 plays a role in the LPS (endotoxin)-induced inflammatory response based on studies using p38 inhibitors from a class of pyridinyl imidazoles that includes SB202190 (4, 7–10).

LPS, the outer component of the Gram-negative bacterial cell wall, is a powerful activator of host mononuclear cells and prompts the synthesis and release of multiple cytokines (11). The production of these cytokines, particularly IL-8 and TNF-α, is central in the regulation of the acute inflammatory response to bacterial challenge (1–8). IL-8 or neutrophil-activating peptide is a potent chemo-attractant (12–15) as well as a crucial activator of neutrophils for an optimal immune response to bacterial foci (16–18). TNF-α, a proinflammatory cytokine critical to the initiation of multiple components of the host immuno-inflammatory response, stimulates the endothelial cell to express cell surface adhesion molecules and initiates neutrophil migration to foci of inflammation or tissue injury (19).

Several in vivo studies have demonstrated that acute alcohol intoxication inhibits neutrophil chemotaxis and suppresses the responses to various inflammatory stimuli. However, the mechanism of this action remains ill defined (20–23). Inhibition of neutrophil recruitment and migration may explain the observed increase in infection rate in trauma and burn victims with acute alcohol intoxication (24, 25). LPS-induced activation of specific signal transduction pathways, such as p38 MAPK, may be critical in the appropriate cytokine production and inflammatory cell responses to infection. In the current study we examined the effect of ethanol (EtOH) on LPS-induced p38 activation in the human mononuclear cells (HMO) and the corresponding effect on IL-8 and TNF-α production.

Materials and Methods

Cell isolation and treatment

Human blood from healthy adult volunteers was drawn into polypropylene syringes containing sodium citrate. HMO were isolated from the buffy coat layer of a Ficoll-Paque (Pharmacia, Piscataway, NJ) density centrifugation gradient. The cells were washed with normal saline and resuspended in RPMI 1640 (BioWhittaker, Walkersville, MD) with 100 μg/ml of gentamicin and 10% heat-inactivated adult bovine serum. HMO at 1 × 10⁶ cells/ml were placed in round-bottom, 12- × 75-mm polypropylene tubes and treated with or without Escherichia coli 0111:B4 LPS (Sigma, St. Louis, MO). Some cells were pretreated with either SB202190 (Calbiochem, La Jolla, CA) or absolute ethanol (McCormick Distilling, Weston, MO) at varying doses for 1 h. Ethanol doses were expressed as a percentage (weight/volume), similar to legal statutes, and values were confirmed by the Harborview Medical Center Toxicology Laboratory (Seattle, WA). After 24 h of LPS treatment, cell supernatants were harvested and frozen at −70°C for later cytokine analysis. Cell viability was confirmed by trypan blue exclusion.

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3 Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; ERK, extracellularly regulated kinase; EtOH, ethanol; HMO, human mononuclear cells.

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IL-8, TNF-α, and IL-6 determinations

IL-8, TNF-α, and IL-6 were quantitated by TiterSeive enzyme immunoassay kits from PerSeptive Biosystems (Framingham, MA), which are based on coated well, sandwich enzyme immunoassays.

Northern blots

HMO were placed in 50-ml polypropylene tubes at 15 × 10⁶ cells/tube. After 8 h of LPS stimulation, total cellular RNA was extracted using the Ultraspec RNA isolation system (Biotex Laboratories, Houston, TX). Fifteen micrograms of RNA were electrophoresed in a denaturing 1.25% agarose-formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL). IL-8 mRNA was determined by hybridizing the membrane overnight at 42°C in hybridization buffer (5X SSPE, 50% formamide, 1% SDS, and 0.1 g/ml polyethylene glycol) containing an IL-8 cDNA probe. The IL-8 probe is a 40-mer oligonucleotide containing an IL-8 cDNA probe. The IL-8 probe is a 40-mer oligonucleotide (Oncogene, Cambridge, MA) end labeled with [32P]ATP (New England Nuclear, Boston, MA). The membrane was then autoradiographed using an intensifying screen with exposure of Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 4–5 days. To assure equal loading, the membrane was stripped and rehybridized with [32P]ATP-labeled 28S ribosomal RNA cDNA probe. The autoradiograms were analyzed by densitometry using the NIH Image program.

Western blots

HMO were placed in 50-ml polypropylene tubes at 10 × 10⁶ cells/tube. After 15 min of LPS stimulation, total cellular protein was extracted by lysing the cells in 1 ml of lysis buffer (20 mM Tris, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1% SDS-PAGE, 50% formamide, 1% SDS, and 0.1 g/ml polyethylene glycol) containing an IL-8 cDNA probe. The IL-8 probe is a 40-mer oligonucleotide (Oncogene, Cambridge, MA) end labeled with [32P]ATP (New England Nuclear, Boston, MA). The membrane was then autoradiographed using an intensifying screen with exposure of Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 4–5 days. To assure equal loading, the membrane was stripped and rehybridized with [32P]ATP-labeled 28S ribosomal RNA cDNA probe. The autoradiograms were analyzed by densitometry using the NIH Image program.

Statistical analysis

Values were expressed as the mean ± SD. Data were analyzed by ANOVA, with post-hoc testing by Fisher’s least significant difference test. A p value of <0.05 was considered significant.

Results

Activation of p38 MAPK

We studied the LPS-induced p38 activation in HMO using a dual phospho-specific Ab that binds only to the activated p38. LPS activation of p38 peaked at 30 min and returned to baseline in 2 h (data not shown). Western blot analysis demonstrated that LPS, throughout the dose range of 10 pg/ml to 100 ng/ml, activated p38 in a linear fashion with respect to the log doses of LPS (Fig. 1). Western blot analysis using phospho-specific ERK Ab demonstrated no significant ERK activation in response to the same dose range of LPS (data not shown).

Alcohol pretreatment of HMO inhibited p38 activation in a dose-related manner (Fig. 2). This inhibition was demonstrated for LPS doses of both 100 pg/ml and 100 ng/ml. EtOH, 0.3 and 0.8%, inhibited p38 activation by approximately 50% and by >50%, respectively, based on OD reading of the Western blots.

IL-8 production by HMO

LPS stimulated HMO to produce up to 40 ng/ml of IL-8 compared with 1–2 ng/ml in controls (Fig. 3). However, IL-8 production, in contrast to p38 activation, which was linear with respect to log doses of LPS, reached an early plateau at the threshold LPS dose of 1 ng/ml. Increasing the LPS dose above 1 ng/ml did not significantly increase IL-8 production. IL-8 production was inhibited by SB202190 (1 μM) pretreatment, a specific p38 inhibitor, but this inhibitory effect was less efficient with increasing LPS dose beyond the threshold. Similar to the SB202190 inhibition, alcohol inhibited IL-8 production by HMO. This inhibition was also less efficient beyond the LPS dose of 1 ng/ml. Similar results were obtained using SB203580 (1 μM) pretreatment (data not shown).

At 100 pg/ml LPS, IL-8 production was inhibited by EtOH pretreatment in a dose-related manner that reached statistical significance with 0.1% EtOH (legal limit for blood alcohol level in most states) (26) and higher (Fig. 4). At 0.8% EtOH, there was 80% inhibition.

Northern blot analysis demonstrated that both SB202190 and EtOH inhibited IL-8 mRNA levels (Fig. 5). There was a low level of IL-8 mRNA in the control cells that was significantly increased by LPS stimulation. The increase in IL-8 mRNA was inhibited by pretreatment with both SB202190 and EtOH. There was a decrease...
in IL-8 mRNA with increasing pretreatment doses of EtOH. Pretreatment with EtOH at 0.3 and 0.8% inhibited the LPS-induced increase in IL-8 mRNA by approximately 60 and 90%, respectively, based on OD reading of the Northern blots. The matched 28S ribosomal RNA levels were used to demonstrate equal loading.

TNF-α production by HMO

LPS also stimulated TNF-α production by HMO (Fig. 6A). TNF production reached statistical significance at LPS doses higher than 1 ng/ml. Throughout the LPS dose range, SB202190 pretreatment inhibited TNF production by 60–70%. Similar to SB202190, 0.8% EtOH pretreatment inhibited TNF-α production by 70%. TNF production induced by 100 ng/ml of LPS was inhibited by EtOH pretreatment in a dose-related manner (Fig. 6B).

IL-6 production is not inhibited by alcohol

LPS also caused increased production of IL-6 by HMO. IL-6 production by HMO in response to LPS was not inhibited by alcohol (Fig. 7). These data demonstrate that inhibition of IL-8 and TNF-α by EtOH does not appear to be due to a global inhibition of cytokine production or diffuse nonspecific toxicity.

Discussion

Activation of the various components of the MAPK family is one of the major mechanisms used by eukaryotic cells to transduce extracellular signals into cellular response (5). p38, a member of the MAPK family, is characterized by a threonine-glycine-tyrosine sequence and is activated by dual phosphorylation of the tyrosine and threonine residues (27, 28). In this study we have confirmed the critical role of p38 activation in LPS-induced inflammatory cytokine response by HMO based on studies with the p38-specific inhibitor, SB202190. Although recently there have been some studies suggesting that SB203580, another member of the pyridinyl imidazole family, may also inhibit c-Jun N-terminal kinases and cyclo-oxygenase activity (29, 30), the inhibition demonstrated in these two studies either required much higher doses (3–10 μM for c-Jun N-terminal kinases) or was only evaluated in platelets (in

![FIGURE 3.](http://www.jimmunol.org/)

SB202190 and EtOH pretreatments inhibit LPS-induced IL-8 production in a similar pattern. HMO were pretreated with 1 μM SB202190, 0.8% EtOH, or no pretreatment for 1 h. Subsequently, HMO were stimulated with a varying dose of LPS, and the supernatants were collected after 18 h and assayed for IL-8. *, p < 0.05 compared with no pretreatment with the same LPS stimulation (n = 6).

![FIGURE 4.](http://www.jimmunol.org/)

Alcohol inhibits LPS-induced IL-8 production in a dose-dependent manner. HMO were pretreated with varying doses of EtOH (0–0.8%) for 1 h and subsequently stimulated with 100 pg/ml of LPS. The supernatants were collected after 18 h and assayed for IL-8. *, p < 0.05 compared with no pretreatment and LPS-stimulated group (n = 6).

![FIGURE 5.](http://www.jimmunol.org/)

Alcohol and SB202190 inhibit the LPS-induced increase in IL-8 mRNA. HMO were pretreated for 1 h with varying doses of EtOH, 1 μM SB202190, or no pretreatment. Subsequently, HMO were stimulated with 100 pg/ml of LPS for 8 h before cellular RNA was extracted and subjected to Northern blot analysis by hybridizing with IL-8 cDNA probe. The membrane was stripped and rehybridized with 28S ribosomal RNA probe.

![FIGURE 6.](http://www.jimmunol.org/)

A. Alcohol and SB202190 inhibit LPS-induced TNF production. HMO were pretreated with 1 μM SB202190, 0.8% EtOH, or no pretreatment for 1 h. Subsequently, HMO were stimulated with varying doses of LPS, and the supernatants were collected after 18 h and assayed for TNF-α. *, p < 0.05 compared with no pretreatment with the same LPS stimulation (n = 6). B. Alcohol inhibits LPS-induced TNF-α production in a dose-dependent manner. HMO were pretreated with varying doses of EtOH (0–0.8%) for 1 h and subsequently stimulated with 100 ng/ml of LPS. The supernatants were collected after 18 h and assayed for TNF-α. *, p < 0.05 compared with no pretreatment and the LPS-stimulated group (n = 6).
the case of cyclo-oxygenase); therefore, SB202190 is still widely accepted as a specific inhibitor of p38-MAPK in HMO (12–16).

In addition, we studied the pattern of IL-8 production by HMO and compared it to that of p38 activation. LPS induced p38 phosphorylation and activation in a dose-related manner. p38 activation occurred at low doses of LPS (<100 pg/ml) and resulted in a significant amount of IL-8 production. At lower levels of p38 activation (LPS dose <1 ng/ml), IL-8 production was attenuated with 1 μM SB202190 pretreatment, approaching control levels. However, IL-8 production, in contrast to p38 activation, which was linear with respect to log doses of LPS, reached a plateau at an LPS dose of 1 ng/ml. Increasing p38 activation with doses of LPS above 1 ng/ml did not translate into more IL-8 production. The increased levels of p38 activation, however, rendered the HMO resistant to the inhibitory effect of SB202190 pretreatment. The inability to effectively inhibit IL-8 production when the p38 level of activation was beyond the maximal response may explain the inconsistencies demonstrated between effective in vitro control of inflammatory mediators under controlled stimulatory conditions and the lack of response in clinically relevant in vivo models, where activation has exceeded the capacity of inhibitors to down-regulate the HMO and inflammatory responses.

In contrast, significant TNF-α production by HMO occurred only at higher doses of LPS (1 ng/ml and higher). Although the rate of increase in TNF-α production decreased at higher LPS doses, there was no plateau, up to 1 μg/ml of LPS (data for LPS dose >100 ng/ml not shown). The 1-μM SB202190 pretreatment continued to effectively and proportionately inhibit TNF-α production.

Similar to SB202190, EtOH pretreatment inhibited both IL-8 and TNF-α production by HMO. This inhibition was dose dependent and, in the case of IL-8, was significant at 0.1% EtOH. This observation has potential clinical significance, as approximately 30% of seriously injured patients have blood alcohol levels higher than 0.2% (25). This effect is not a global toxicity, since cells remained viable and able to exclude trypan blue over 24 h. IL-6 production by HMO was not inhibited by EtOH, and the inhibition of IL-8 production could be overcome by increasing the LPS dose.

Both EtOH and SB202190 inhibited the LPS-induced increase in IL-8 mRNA levels using Northern blot analysis. Nair et al. demonstrated a decrease in TNF-α mRNA with alcohol treatment using RT-PCR in HMO (31). To our knowledge this is the first report of inhibition of IL-8 production by alcohol pretreatment, and in the case of TNF-α our results agree with the literature (31, 32).

EtOH may inhibit LPS-induced IL-8 and TNF-α production via the p38 pathway. p38 MAPK plays a regulatory role in IL-8 and TNF-α production (33, 34). The pattern of EtOH inhibition of IL-8 and TNF-α production is very similar to that of SB202190, a p38 MAPK inhibitor. Furthermore, Western blot analysis using the dual phospho-specific p38 Ab demonstrated inhibition of LPS-induced p38 activation by EtOH pretreatment in a dose-related manner.

The mechanism of inhibition of p38 MAPK activation by alcohol is not clear. One possible mechanism may involve phospholipase D. EtOH serves as a false substrate, replacing water, in the phospholipase D-catalyzed hydrolytic cleavage of choline from phosphatidylcholine (35–37), thus producing phosphatidylethanol instead of phosphatidic acid, an important second messenger in the inflammatory response (38). Further studies are needed to investigate this potential mechanism.

In the clinical arena, acute intoxication suppresses the inflammatory response in normal subjects by inhibiting granulocyte migration (20–23). The inhibition of neutrophil migration in vivo has been demonstrated in both human and animal models. This has been implicated as a major cause of the increased risk of infection following trauma and burns (24, 25). Spagnuolo and MacGregor (22) demonstrated that acute alcohol intoxication suppresses granulocyte migration to inflammatory sites in man. To further define the mechanism of this anti-inflammatory action, granulocytes were subjected to an in vitro neutrophil migration assay. These studies demonstrated no inhibition of neutrophil chemotaxis in cells exposed to alcohol, concluding that the inhibition of neutrophil chemotaxis does not appear to be a direct effect on neutrophils.

Our studies suggest that acute ethanol intoxication may impair neutrophil chemotaxis by inhibiting IL-8 and TNF-α production at the inflammatory sites in response to stimuli such as LPS. IL-8 is a potent neutrophil chemoattractant and causes a rapid and prolonged neutrophil recruitment and accumulation at the site of injection (12–18). TNF-α also plays a key role in the neutrophil inflammatory response; specifically, TNF-α production by monocytes/macrophages at the site of inflammation activates endothelial cells to express cell surface adhesion molecules that modulate rolling, adherence, and migration of neutrophils (19). Therefore, inhibition of IL-8 and TNF-α production may explain the block of neutrophil recruitment and migration to areas of acute inflammation.

In summary, we have demonstrated that LPS induces p38 activation in HMO, which plays a key role in IL-8 and TNF-α production. Alcohol inhibits LPS-induced p38 activation. This correlates with inhibition of IL-8 and TNF-α production by HMO. This inhibition of IL-8 and TNF-α production may be a mechanism explaining the inhibition of neutrophil chemotaxis by alcohol intoxication in vivo.

References


