Involvement of TLR4/Type I IL-1 Receptor Signaling in the Induction of Inflammatory Mediators and Cell Death Induced by Ethanol in Cultured Astrocytes

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Involvement of TLR4/Type I IL-1 Receptor Signaling in the Induction of Inflammatory Mediators and Cell Death Induced by Ethanol in Cultured Astrocytes

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Activated astroglial cells are implicated in neuropathogenesis of many infectious and inflammatory diseases of the brain. A number of inflammatory mediators and cytokines have been proposed to play a key role in glial cell-related brain damage. Cytokine production seems to be initiated by signaling through TLR4/type I IL-1R (IL-1RI) in response to their ligands, LPS and IL-1β, playing vital roles in innate host defense against infections, inflammation, injury, and stress. We have shown that glial cells are stimulated by ethanol, up-regulating cytokines and inflammatory mediators associated with TLR4 and IL-1RI signaling pathways in brain, suggesting that ethanol may contribute to brain damage via inflammation. We explore the possibility that ethanol, in the absence of LPS or IL-1β, triggers signaling pathways and inflammatory mediators through TLR4 and/or IL-1RI activation in astrocytes. We show in this study that ethanol, at physiologically relevant concentrations, is capable of inducing rapid phosphorylation within 10 min of IL-1R-associated kinase, ERK1/2, stress-activated protein kinase/JNK, and p38 MAPK in astrocytes. Then an activation of NF-κB and AP-1 occurs after 30 min of ethanol treatment along with an up-regulation of inducible NO synthase and cyclooxygenase-2 expression. Finally, we note an increase in cell death after 3 h of treatment. Furthermore, by using either anti-TLR4- or anti-IL-1RI-neutralizing Abs, before and during ethanol treatment, we inhibit ethanol-induced signaling events, including NF-κB and AP-1 activation, inducible NO synthase, and cyclooxygenase-2 up-regulation and astrocyte death. In summary, these findings indicate that both TLR4 and IL-1RI activation occur upon ethanol treatment, and suggest that signaling through these receptors mediates ethanol-induced inflammatory events in astrocytes and brain. The Journal of Immunology, 2005, 175: 6893–6899.

The CNS is particularly prone to the harmful effects of ethanol, and its chronic abuse and acute intoxication can lead to brain damage and, in some cases, neurodegeneration (1, 2). Although the mechanisms by which ethanol can cause brain damage are not fully understood, some recent studies suggest the participation of an inflammatory process (3, 4), and indeed, neuroinflammation has been involved in the pathogenesis of several neurodegenerative disorders, such as Alzheimer’s and Parkinson’s diseases (5, 6). In addition, ethanol is known to have immunomodulatory effects, causing suppression of the general immune system and the defense against microbial and viral infections (7).

In the CNS, the interaction among various cell types, neurons, astrocytes, microglia and oligodendrocytes, is important in the immune and inflammatory responses. Among these cells, astrocytes are known to play a pivotal role in CNS immunity as immunocompetent cells by secreting cytokines and inflammatory mediators (8, 9). IL-1β is highly produced by glial cells under conditions of damage, stress, and disease, and it is able to strongly activate astrocytes to produce additional cytokines and growth factors, promoting inflammatory activity in the brain (10).

The ability of IL-1β and LPS to influence astrocyte function not only depends on the expression of the appropriate receptors, but also on the activation of specific intracellular signaling pathways. Thus, the proinflammatory cytokine IL-1β mediates its effects on immunity and inflammation by interacting with the type I IL-1R (IL-1RI), a receptor expressed on astrocytes, particularly after injury, suggesting a specificity association with the inflammatory responses (11). TLR4 is a member of the IL-1R/TLR superfamily that is required for LPS responsiveness and is involved in the host defense against Gram-negative bacteria (12). Recent findings indicate that TLR4 is expressed in primary astrocytes, providing a mechanistic link between bacterial challenge and immune responses in the inflammatory CNS disorders (13). The IL-1R/TLR superfamily is a recently defined group of receptors that shares a cytoplasmic Toll/IL-1R domain and participates in host responses to injury and infection (12, 14).

After ligand binding, TLRs/IL-1Rs dimerize and undergo the conformational change required for the recruitment of downstream signaling molecules, including the adaptor molecule MyD88, IL-1R-associated kinase (IRAK), TNFR-associated factor 6 and NF-κB-inducing kinase (14, 15). The recruitment of these molecules triggers the stimulation of downstream kinases, including MAPKs such as ERK1/2, p38 MAPK, and stress-activated protein kinase (SAPK)/JNK, as well as activation of the transcriptional factors NF-κB and AP-1 (14, 15). The activation of these transcriptional factors leads to the induction of genes encoding cytokines and...
inflammatory mediators, such as inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) (16, 17). Cell damage and apoptosis occur concomitantly as a result of the stimulation of signaling pathways and inflammatory mediators associated with TLR4/IL-1RI, which, in turn, induces a highly inflammatory response in the CNS under conditions of damage, disease, or stress (18).

Our recent studies demonstrate that chronic ethanol intake enhances inflammatory mediators (IL-1β, COX-2, and iNOS) in brain, activating stress and MAPK pathways as well as transcriptional factors (NF-κB and AP-1) associated with activation of TLR4 and IL-1RI. Notably, these effects were associated with an increase in apoptotic cell death, suggesting the involvement of inflammatory damage in ethanol-induced brain injury. Our results also suggest the participation of astrocytes in these inflammatory events, because ethanol exposure stimulates astrocytes to produce IL-1β by activating the same inflammatory mediators and signaling events as those observed in the cerebral cortex of ethanol-fed animals (4).

The above findings raise the question of whether ethanol could interact with TLR4 and IL-1RI, triggering signaling transduction pathways in a similar manner as LPS and IL-1β. We show in this study that ethanol rapidly stimulates the phosphorylation of IRAK, ERK1/2, SAPK/JNK, and p38 MAPK, followed by the activation of NF-κB and AP-1, and also the transcriptional up-regulation of iNOS and COX-2. We also show that these events are associated with an increase in cell death. Finally, we are able to demonstrate that blocking TLR4 and IL-1RI with neutralizing Abs abolishes most of the effects of ethanol on the inflammatory signaling events and prevents cell death. The findings suggest that TLR4 and IL-1RI are targets of ethanol-induced inflammatory mediators in both brain and astrocytes.

Materials and Methods

**Abs and reagents**

Anti-phospho-ERK1/2 (anti-p-ERK1/2), anti-ERK1/2, anti-p38 kinase (anti-p38), anti-INK, anti-IgB-α, anti-iNOS, HRP-conjugated goat anti-mouse and anti-rabbit IgG (Santa Cruz Biotechnology), anti-p38, anti-p-INK (Cell Signaling Technologies), anti-COX-2 (Cayman Chemicals), anti-α-tubulin (Sigma-Aldrich), and anti-IRAK (Transduction Laboratories), an Ab that detects an ~100-kDa band corresponding to the phosphorylated form of IRAK (19) as we previously demonstrated (20). The blocking Abs used were anti-IL-1RI (BD Pharmingen) and anti-TLR4 (Santa Cruz Biotechnology).

**Culture of astrocytes and experimental protocol**

Primary cultures of rat cortical astrocytes were prepared from the cerebral cortex of 21-day-old rat fetuses, as previously described (21). Cells were plated on 30-cm² plates in DMEM containing 20% FBS, supplemented with l-glutamine (1%), HEPES (10 mM), fungizone (1%), and antibiotics (1%). Cultures were grown in a humidified atmosphere of 5% CO₂/95% air at 37°C. After 1 wk of culture, the FBS content was reduced to 10%, and the medium was changed twice a week. Cells were grown to confluence and used after 12 days in culture. The purity of astrocytes was verified by competition assays with a 100-fold excess of the unlabeled oligonucleotide. All reactions were run on 5% polyacrylamide gel. The gel was dried, and NF-κB and AP-1 binding was visualized by competition assays with a 100-fold excess of the unlabeled oligonucleotide. Cell death was measured using a nucleosomal DNA ELISA (Cell Death Detection ELISA kit; Roche), which quantified histone-associated DNA fragments. After ethanol treatment, cells were lysed and centrifuged. The supernatant was used to quantitate apoptotic cell death following the manufacturer’s instructions.

**Statistical analysis**

Results are reported as the mean ± SD. Data were analyzed using one- and two-way ANOVA.

**Results**

Ethanol induces rapid activation of TLR4/IL-1RI signaling pathway in astrocytes

Several lines of evidence indicate that stimulation of TLR4 and IL-1RI by their ligands, LPS and IL-1β, respectively, can trigger MAPK and transcription factor activation, leading to the production of inflammatory mediators, such as IL-1β, TNF-α, iNOS, and COX-2, over a short period of time (23, 24). To determine whether acute ethanol treatment is able to activate signaling pathways and inflammatory mediators associated with TLR4 and IL-1RI, we first analyzed the phosphorylation of IRAK, ERK1/2, p38 MAPK, and SAPK/JNK in ethanol-treated astrocytes over different time periods (10 and 30 min, 1, 3, 12, and 24 h). Ethanol treatment significantly stimulated the activity of IRAK, ERK1/2, p38 MAPK, and SAPK/JNK in a time-dependent manner, as shown in Fig. 1. The phosphorylation peak occurred very rapidly in all these kinases,
within 10 min of ethanol treatment. This fast response was similar to that observed when astrocytes were stimulated with LPS or IL-1β (data not shown).

We then went on to test whether other ethanol concentrations or other types of alcohols could also activate the IRAK/MAPK pathway. Astrocytes were incubated for 10 min with different ethanol concentrations (2, 8, 10, 20, 40, 50, 100, and 200 mM), and IRAK and ERK1/2 phosphorylations were analyzed by Western blot. Interestingly, 10 mM ethanol caused a similar activation of IRAK and ERK1/2 as 50 mM ethanol (Fig. 2A).

We also examined the potential effect of short-chain n-alcohols on the IRAK/ERK pathway stimulation. Fig. 2B shows that although a 10-mM concentration of methanol, propanol, or butanol at 10 min slightly increased the IRAK activity, ethanol had the greatest effect on both IRAK and ERK1/2 phosphorylation. These results indicate that although several alcohols are able to stimulate IRAK phosphorylation, the IRAK/ERK pathway is mainly activated by ethanol. No significant changes in the total levels of ERK1/2, p38, and JNK1 were noted in the above experiments.

**Ethanol up-regulates the levels of COX-2 and iNOS and causes cell death in astrocytes**

Stimulation of TLR4 and IL-1RI can trigger MAPK and NF-κB activation, leading to the production of inflammatory mediators, such as IL-1β, COX-2, and iNOS (23, 24). Therefore, we examined whether ethanol-induced activation in the IRAK/MAPK pathway could lead to the stimulation of NF-κB, triggering the induction of COX-2 and iNOS. Experiments performed with 50 mM ethanol treatment indicated that the maximal activation of NF-κB occurred after a 30-min ethanol treatment (21). Notably, although iNOS and COX-2 expression was detectable as early as 10 min after ethanol exposure, maximum levels of expression were observed at 30 min and 24 h of ethanol treatment (Fig. 3A).

To determine whether an activation of the TLR4/IL-1RI signaling response is associated with cell damage, apoptotic cell death
was assessed at different times of ethanol treatment, and histone-associated oligonucleosomal DNA fragments were quantified. Interestingly, a significant increase in apoptosis was noted in astrocytes treated with 50 mM ethanol for 3 and 24 h (Fig. 3B).

Collectively, these results suggest that ethanol induces a number of rapid response genes involved in the inflammatory response, including kinases and inflammatory mediators that could produce cell damage in astrocytes.

Blocking TLR4 and IL-1RI prevents the activation of inflammatory pathways and cell death induced by ethanol in astrocytes

The above results support the idea that ethanol induces the same inflammatory events as those associated with the LPS and IL-1β responses, suggesting a potential activation of TLR4 and/or IL-1RI by ethanol. To study this possibility more directly, astrocytes were preincubated with anti-IL-1RI- or anti-TLR4-blocking Abs for 30 min before and during 50 mM ethanol treatment. Activation of IRAK/MAPKs, iNOS and COX-2 expressions, and cell death were analyzed at different ethanol treatment times. Blocking TLR4 or IL-1RI abolished the ethanol-induced activation of IRAK/MAPKs (Fig. 4) as well as the up-regulation of iNOS and COX-2 expression (Fig. 5A) and ethanol-induced cell death (Fig. 5B). The blocking Abs also decreased the minor basal IRAK, p-ERK, and iNOS expression and basal cell death, suggesting that these basal activities/expression/cell death are triggered by traces of toxins that may be present despite all the precautions to prevent toxin presence in the cultures. In any case, the findings support the hypothesis that the up-regulation of inflammatory mediators and cell death caused by ethanol treatment are mediated by TLR4 and IL-1RI, and that ethanol might directly or indirectly activate these receptors.
Anti-TLR4- and anti-IL-1RI-blocking Abs abolish the ethanol-induced activation of NF-κB and AP-1

Activation of NF-κB and AP-1 transcription factors is known to be involved in the inflammatory response (15, 25, 26). NF-κB and AP-1 activation occurs in brains of chronic ethanol-fed animals (4), and we wondered whether ethanol-induced activation of NF-κB and AP-1 was regulated via TLR4 and IL-1RI. To answer this question, astrocytes were treated with blocking Abs against TLR4 and IL-1RI before and during ethanol stimulation. NF-κB and AP-1 were then analyzed by EMSA. Fig. 6 shows how 30 min of ethanol treatment significantly increased the DNA-binding activity of NF-κB and AP-1. Notably, preincubating the cells for 30 min with either anti-IL-1RI- or anti-TLR4-blocking Abs prevented the ethanol-induced activation of NF-κB and AP-1, suggesting an involvement of TLR4 and IL-1RI in the activation of these transcriptional factors.

Discussion

Growing evidence indicates the role that inflammation plays as a potential pathogenic factor in many CNS diseases, including neurodegenerative diseases (5, 6). The hallmark of the neuroinflammation is the activation of glial cells and the production of cytokines and inflammatory mediators that trigger neural damage (6). Alcohol abuse can result in brain damage and neurodegeneration (1, 2), and there is evidence to indicate that ethanol not only stimulates glial cells, but may also induce a proinflammatory response in the brain (3, 21). Indeed, our recent findings show that chronic ethanol intake up-regulates inflammatory mediators in both brain and astroglial cells, activating signaling events associated with inflammation (4). Notably, the intracellular signal transduction events induced by ethanol were the same as those associated with the TLR4 and IL-1RI signaling responses. Therefore, a question we addressed in this report is whether ethanol-induced inflammatory processes in the brain and astrocytes are mediated by the activation of TLR4 and IL-1RI. We show for the first time in this study that ethanol directly or indirectly interacts with these receptors, activating signal transduction pathways in a similar manner as LPS and IL-1β. These findings suggest that TLR4 and IL-1RI are targets of ethanol-induced inflammatory mediators in both brain and astrocytes.

The Toll/IL-1R superfamily, a group of structurally homologous proteins, plays a vital role in the innate host defense against infections, inflammation, injury, and stress (12, 13). Activation of TLR4 and IL-1RI leads to receptor dimerization and rapid recruitment of different proteins, such as MyD88, IRAK, and TNFR-associated factor 6, which triggers the stimulation of various intracellular signaling pathways, including the MAPK and SAPK/JNK pathways, and the downstream activation of NF-κB and AP-1 transcription factors. The present findings demonstrate that ethanol at physiologically relevant concentrations (10 or 50 mM) is able to stimulate astrocytes, inducing a rapid activation of the TLR4/IL-1RI signal transduction pathways in a similar manner as when cells are stimulated with LPS and IL-1β (27–30). Thus, key indications of the activation of these receptors upon ethanol stimulation include the rapid phosphorylation (within 10 min) of IRAK, ERK1/2, SAPK/JNK, and p-38 MAPK; the subsequent downstream activation of the transcription factors NF-κB and AP-1; and the up-regulation of iNOS and COX-2 noted after 30-min ethanol treatment. Notably, we found a time lag between the maximal COX-2 and iNOS expression and the increase in cell death observed at 3 h of ethanol treatment. Consistent with the hypothesis that ethanol mediates inflammatory events by activating TLR4/IL-1RI, we show that by blocking the activation of these receptors with neutralizing Abs, both ethanol-induced inflammatory mediators and cell death in astrocytes are abolished. Our results also demonstrate that although other short-chain n-alcohols slightly increase IRAK activity, they fail to affect ERK phosphorylation, suggesting that only ethanol is able to activate the IRAK/MAPK pathways, as occurs with LPS or IL-1β stimulation (27, 29, 30). These effects are clearly not mediated by the triggering by ethanol of a short-term increase in IL-1 release, because no detectable levels of this cytokine, measured in culture supernatants using an immunoassay (20), were found before 20–30 min of incubation. At 20–30 min, the level was <23 pg/ml, far below the level (5.3 ng/ml) causing a half-maximal effect (31).

Immunomodulatory effects of ethanol are complex and depend on dose, ethanol exposure duration (acute vs chronic), cell type,
and the absence or the presence of other additional stimuli, such as LPS. Ethanol is known to increase susceptibility to infections, in part by suppressing macrophage function. Acute ethanol treatment impairs the TLR-mediated macrophage inflammatory response, thus affecting both MAPK activation and cytokine production (32, 33). Ethanol also inhibits LPS-induced IL-1β and NF-κB activation in microglial cells (34). In contrast to these negative effects, activation of the LPS signaling response in Kupffer cells (35) and hepatocytes (20) appears to be involved in early alcohol-induced liver injury (36), and increased levels of proinflammatory cytokines are normally found in the serum of alcoholic patients with liver damage (37). Consistent with these data, we have previously shown that chronic ethanol intake up-regulates serum, liver, and brain levels of proinflammatory cytokines (IL-1β and TNF-α), activating IRAK/MAPK/NF-κB pathways in both the liver and the brain of rats (4, 20). These apparently opposite effects of ethanol on the TLR4/IL-1RI response would be explained by the current data, which demonstrate that although low ethanol concentrations, such as those found in alcoholics (10–50 mM; ~0.05–0.2 g/dl), stimulate the TLR4/IL-1RI signaling response, high concentrations (>100 mM; 0.4 g/dl) either inhibit the receptor-mediated response or have no effect. Furthermore, when ethanol was used along with other stimuli (e.g., LPS), we usually observed an inhibitory effect, rather than an additive or synergistic effect, in both hepatocytes (20) and astrocytes (data not shown). In agreement with these findings, other studies have shown that ethanol suppresses both cytokine-induced iNOS expression (38, 39) and LPS-induced NO production (40) in glial cells.

The mechanism by which ethanol either activates or inhibits TLR4/IL-1RI signaling is presently uncertain. As with most effects of ethanol on membrane protein functions, the mechanism might be indirect, through the interactions of ethanol with membrane lipids (41), either facilitating or disrupting the recruitment of TLR4/IL-1RI into lipid rafts. The presence of TLRs molecules within lipid rafts and their subsequent clustering in response to LPS are crucial for signaling (42, 43), playing important roles in the innate immune response (43–45); indeed, disruption of lipid rafts leads to an inhibition of TLR internalization and signaling (43). High concentrations of ethanol appear to interfere with lipid raft clustering, leading to the suppression of TLR4 signaling demonstrated in murine macrophages (46). The present observations on the activation by low concentrations of ethanol of TLR4/IL-1RI signaling might result from the opposite effect: at low concentrations, ethanol might promote receptor recruitment into lipid rafts and dimerization. Our previous finding of facilitation of ceramide signaling by low concentrations of ethanol (47) would agree with this effect. The interference with ethanol-induced activation by the blocking Abs used in this study would result from hampering by the Abs of receptor dimerization, possibly because of steric constraints in the receptor-Ab complex. Furthermore, because these receptors, particularly IL-1RI, signaling requires an appropriate assembly of the receptor with cell adhesion complexes and actin cytoskeleton organization (48), low ethanol concentrations might also facilitate translocation of IL-1RI to focal adhesions, favoring activation of this receptor. In contrast, high ethanol concentrations, by disrupting actin organization and cell adhesion molecules (49)

FIGURE 6. Acute ethanol treatment activates DNA binding of NF-κB (A) and AP-1 (B) via TLR4/IL-1RI in astrocytes. Active NF-κB and AP-1 were assayed in nuclear protein extracts from control and ethanol-exposed (50 mM) astrocytes for 30 min, with or without IL-1RI- and TLR4-blocking Ab incubation (10 μg/ml) for 30 min before and during ethanol treatment, by EMSA with consensus oligonucleotides labeled with [γ-32P]ATP, as detailed in Materials and Methods. Densitometric values of the NF-κB and AP-1 bands from four different experiments are shown in the bar graph. A representative autoradiography of EMSA is shown, * , p < 0.03 (vs untreated astrocytes); #, p < 0.02 (vs ethanol-treated cells).
could inhibit IL-1RI recruitment and signaling activation. Low concentrations (10 mM) of other straight short-chain alcohols also facilitate the recruitment and activation of the TLR4/IL-1RI, as demonstrated by IRAK phosphorylation. In summary, although we cannot exclude a possible direct effect of ethanol on these receptors, as demonstrated with some neurotransmitter receptors (50), indirect mechanisms, such as alterations in lipid raft or cell matrix changes, can explain the effects of ethanol on the TLR4/IL-1RI response.

In conclusion, the present data indicate that activation of TLR4/IL-1RI and their signaling responses occur upon ethanol treatment. Although additional studies are required to understand the mechanisms by which ethanol activates these receptors, our findings suggest that TLR4 and IL-1RI are targets of ethanol-induced inflammatory damage in both astrocytes and brain.

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Disclosures
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