High Mobility Group Box 1/Toll-like Receptor Danger Signaling Increases Brain Neuroimmune Activation in Alcohol Dependence

Fulton T. Crews, Liya Qin, Donna Sheedy, Ryan P. Vetreno, and Jian Zou

Background: Innate immune gene expression is regulated in part through high mobility group box 1 (HMGB1), an endogenous proinflammatory cytokine, that activates multiple members of the interleukin-1/Toll-like receptor (TLR) family associated with danger signaling. We investigated expression of HMGB1, TLR2, TLR3, and TLR4 in chronic ethanol-treated mouse brain, postmortem human alcoholic brain, and rat brain slice culture to test the hypothesis that neuroimmune activation in alcoholic brain involves ethanol activation of HMGB1/TLR danger signaling.

Methods: Protein levels were assessed using Western blot, enzyme-linked immunosorbent assay, and immunohistochemical immunoreactivity (+IR), and messenger RNA (mRNA) levels were measured by real time polymerase chain reaction in ethanol-treated mice (5 g/kg/day, intragastric, 10 days + 24 hours), rat brain slice culture, and postmortem human alcoholic brain.

Results: Ethanol treatment of mice increased brain mRNA and +IR protein expression of HMGB1, TLR2, TLR3, and TLR4. Postmortem human alcoholic brain also showed increased HMGB1, TLR2, TLR3, and TLR4 +IR cells that correlated with lifetime alcohol consumption, as well as each other. Ethanol treatment of brain slice culture released HMGB1 into the media and induced the proinflammatory cytokine, interleukin-1 beta (IL-1β). Neutralizing antibodies to HMGB1 and small inhibitory mRNA to HMGB1 or TLR4 blunted ethanol induction of IL-1β.

Conclusions: Ethanol-induced HMGB1/TLR signaling contributes to induction of the proinflammatory cytokine, IL-1β. Increased expression of HMGB1, TLR2, TLR3, and TLR4 in alcoholic brain and in mice treated with ethanol suggests that chronic alcohol-induced brain neuroimmune activation occurs through HMGB1/TLR signaling.

Key Words: Alcoholism, cerebral cortex, cytokines, ethanol, interleukin-1, Toll-like receptors

Neuroinflammation is implicated in the etiology of many brain diseases. Recent discoveries indicate that endogenous danger-associated molecular pattern agonists and Toll-like receptors (TLRs) contribute to neuroinflammation (1–3). High mobility group box 1 (HMGB1) is an endogenous danger signaling cytokine that acts on multiple interleukin-1(IL-1)/TLRs (2,4,5). The family of IL-1/TLRs signal through kinases that activate the proinflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) increasing expression of cytokines, oxidases, and other genes associated with innate immune responses (6). Although there are multiple TLRs in the brains of mice and humans, Toll-like receptor 2 (TLR2), Toll-like receptor 3 (TLR3), and Toll-like receptor 4 (TLR4) are the most commonly studied. HMGB1/TLR danger signaling is associated with persistent chronic inflammatory diseases (7), and our studies find that once induced, neuroimmune activation in brain persists for long periods. For example, ethanol (8) and endotoxin (9) treatment of mice increases proinflammatory gene expression in brain that persists for weeks to months after treatment. Thus, we hypothesized that HMGB1/TLR danger signaling might contribute to ethanol-induced neuroinflammation and the neurobiology of alcoholism.

We have previously found that ethanol increases expression of brain proinflammatory genes through activation of the transcription factor, NF-κB (6,10). Chronic ethanol-induced expression of brain proinflammatory cytokines and oxidases are linked to neurodegeneration (8,11) and inhibition of neurogenesis (12). In postmortem human alcoholic brain, we found increased expression of the proinflammatory chemokine, monocyte chemoattractant protein-1 (MCP-1), and markers of microglial activation (13) and increased expression of interleukin-1 beta (IL-1β) and inflammasome proteins (14), as well as increased expression of proinflammatory nicotinamide adenine dinucleotide phosphate oxidase (NOX) (15), indicating significant neuroimmune activation in alcoholic human brain. Further, transgenic mice lacking TLR4 are protected against ethanol-induced proinflammatory gene induction, glial activation, and neurotoxicity (16). HMGB1/TLR danger signals may also impact responses to ethanol since knockdown of amygdala TLR4 with small inhibitory RNA (siRNA) blunts ethanol-dependent rat operant responding for ethanol (17) and acute behavioral responses to ethanol are altered in transgenic mice lacking TLR2 and/or TLR4 (18). Studies in mice have found that neuroimmune activation increases ethanol drinking (19), whereas transgenic mice lacking innate immune genes drink less than their wild-type control mice (20). These studies suggest that the actions of ethanol in brain include neuroimmune activation consistent with danger signaling through TLR4 and perhaps other TLRs. Thus, alcoholic human brain shows neuroimmune activation and ethanol-induced neuroimmune activation is linked to drinking behavior, alcohol responses, TLR upregulation, and neurodegeneration.

From the Bowles Center for Alcohol Studies (FTC, LQ, RPV, JZ), Department of Pharmacology and Psychiatry, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; and Discipline of Pathology (DS), The University of Sydney, Sydney, New South Wales, Australia.

Address correspondence to Fulton T. Crews, Ph.D., The University of North Carolina at Chapel Hill, Bowles Center for Alcohol Studies, The School of Medicine, CB #7178, 1021 Thurston-Bowles Building, Chapel Hill, NC 27599-7178; E-mail: ftcrews@med.unc.edu.

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Table 1. Patient Characteristics of Human Postmortem Brains

Group	Age at Death	Sex	Postmortem Interval	Clinical Cause of Death	Lifetime Ethanol (gm)
Control	60	Male	28	Ischemic heart disease	0
Control	62	Male	46	Ischemic heart disease	5000
Control	50	Male	30	Coronary artery disease	5500
Control	50	Male	40	Hemopericardium	9000
Control	43	Male	66	Aspiration pneumonia	13,000
Control	46	Male	29	Acute myocardial infarction	17,300
Control	24	Male	43	Undetermined (but consistent with idiopathic cardiac arrhythmia)	22,000
Control	48	Male	24	Ischemic heart disease	59,000
Control	44	Male	50	Ischemic heart disease	69,000
Control	53	Male	16	Dilated cardiomyopathy	102,000
Alcoholic	43.5	Male	43.5	Carbon monoxide intoxication/alcohol intoxication	506,000
Alcoholic	44	Male	15	Ischemic heart disease	639,000
Alcoholic	42	Male	41	Combined bromoxynil and alcohol toxicity	1,052,000
Alcoholic	49	Male	44	Ischemic heart disease	1,181,000
Alcoholic	45	Male	7.5	Drowning	1,271,000
Alcoholic	49	Male	16	Coronary artery thrombosis	1,278,000
Alcoholic	51	Male	27	Gastrointestinal hemorrhage	1,863,000
Alcoholic	50	Male	17	Ischemic heart disease	1,958,000
Alcoholic	61	Male	23.5	Atherosclerotic cardiovascular disease	3,158,000
Alcoholic	61	Male	59	Myocarditis	5,811,000

Brains were collected by the New South Wales Tissue Resource Center brain donor program at the University of Sydney. Alcohol consumption rates varied with time, such that the reporting of alcohol consumption also varied. The alcohol consumption rate was recorded in grams of ethanol/day and calculated from the reported number of standard drinks consumed per day. A range of maximum and minimum consumption rates was obtained from hospital records and questionnaires to family members. A mean of the maximum and minimum rates was used for classification (alcoholics consumed >80 g/day, control subjects consumed little or no alcohol). The age of commencement of drinking was assumed to be 25 years of age in all cases, except where other ages were recorded. The 25-year age minimum allowed for increasing consumption at earlier ages until a regular drinking pattern was established. Any period(s) of known abstinence was subtracted from the potential drinking years. Only recorded periods of abstinence greater than 6 months were considered in our calculations. The duration of alcohol consumption was calculated for each case. Lifetime alcohol consumption (expressed in kilograms of 100% alcohol) was calculated by multiplying the duration and the mean consumption rate.

We report here that ethanol treatment of mice or rat brain sections in vitro increases expression of HMGB1/TLRs. Postmortem human alcoholic brain was found to have significantly more HMGB1, TLR2, TLR3, and TLR4, which correlated with lifetime alcohol consumption. Furthermore, ethanol was found to release HMGB1 from brain slice culture, activating proinflammatory IL-1β synthesis through TLR4. These findings support the hypothesis that ethanol activation of HMGB1/TLR danger signaling contributes to neuroimmune activation in alcoholic brain and to the neurobiology of alcoholism.

Methods and Materials

Animals

Eight-week-old male (20–22 g) C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). After at least 1 week of acclimation to the animal colony, mice were divided into control and ethanol groups. Mice were treated with water (control) or ethanol (5 g/kg, intragastric, 25% ethanol wt/vol), with volumes matched, daily for 10 days and sacrificed 24 hours later. The average blood ethanol concentration at 1 hour after the first ethanol treatment and the last ethanol treatment was 291 ± 16 mg/dL (wt/vol, $n = 10$) and 301 ± 19 mg/dL (wt/vol, $n = 10$), respectively. The blood ethanol concentration is high modeling alcoholic binge drinking (21). Brain slice culture was prepared from postnatal day 7 pups from timed pregnant Sprague-Dawley rats.

All protocols and procedures in this study were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the National Institute of Health Regulations for the Care and Use of Animals in research. Details on reagents and reagent sources and

Table 2. Real-Time Polymerase Chain Reaction Primers

	Primer Pairs
Mouse TLR2	5′-TGC TTT CCT GCT GAA GAT TT-3′
	5′-TGT ACC GCA ACA GCT TCA GG-3′
Mouse TLR3	5′-TTG TCT TCT GCA CGA ACC TG-3′
	5′-GGC AAC GCA AGG ATT TTA TT-3′
Mouse TLR4	5′- ACC TGG CTG GTT TAC ACG TC-3′
	5′-GTG CCA GAG ACA TTG CAG AA-3′
Mouse HMGB1	5′-CCA TTG GTG ATG TTG CAA AG-3′
	5′-CTT TTT CGC TGC ATC AGG TT-3′
Mouse β-actin	5′-GTA TGA CTC CAC TCA CGG CAA A-3′
	5′-GGT CTC GCT CCT GGA AGA TG-3′
Rat HMGB1	5′-ATGGGCAAAGGAGATCCTA-3′
	5′-ATTCTCATCATCTCTTCT-3′
Rat IL-1β	5′- GAAACAGCAATGGTCGGGAC-3′
	5′-AAGACACGGGTTCCATGGTG-3′
Rat β-actin	5′-CTACAATGAGCTGCGTGTGGC-3′
	5′-CAGGTCCAGACGCAGGATGGC-3′

HMGB1, high mobility group box 1; IL-1β, interleukin-1 beta; TLR2, Toll-like receptor 2; TLR3, Toll-like receptor 3; TLR4, Toll-like receptor 4.