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Moderate Alcohol Induces Stress Proteins HSF1 and hsp70 and Inhibits Proinflammatory Cytokines Resulting in Endotoxin Tolerance

Sujatha Muralidharan, Aditya Ambade, Melissa A. Fulham, Janhavée Deshpande, Donna Catalano, and Pranoti Mandrekar

Binge or moderate alcohol exposure impairs host defense and increases susceptibility to infection because of compromised innate immune responses. However, there is a lack of consensus on the molecular mechanism by which alcohol mediates this immunosuppression. In this study, we show that cellular stress proteins HSF1 and hsp70 play a mechanistic role in alcohol-mediated inhibition of the TLR4/MyD88 pathway. Alcohol exposure induced transcription factor HSF1 mRNA expression and DNA binding activity in primary human monocytes and murine macrophages. Furthermore, HSF1 target gene hsp70 mRNA and protein are upregulated by alcohol in monocytes. In vitro pre-exposure to moderate alcohol reduced subsequent LPS-induced NF-κB promoter activity and downstream TNF-α, IL-6 and IL-1β production in monocytes and macrophages, exhibiting endotoxin tolerance. Mechanistic analysis demonstrates that alcohol-induced HSF1 binds to the TNF-α promoter in macrophages at early time points, exerting transrepression and decreased TNF-α expression. Furthermore, association of hsp70 with NF-κB subunit p50 in alcohol-treated macrophages correlates with reduced NF-κB activation at later time points. Hsp70 overexpression in macrophages was sufficient to block LPS-induced NF-κB promoter activity, suggesting alcohol-mediated immunosuppression by hsp70. The direct crosstalk of hsp70 and HSF1 was further confirmed by the loss of alcohol-mediated endotoxin tolerance in hsp70- and HSF1-silenced macrophages. Our data suggest that alcohol-mediated activation of HSF1 and induction of hsp70 inhibit TLR4-MyD88 signaling and are required for alcohol-induced endotoxin tolerance. Using stress proteins as direct drug targets would be clinically relevant in alcohol abuse treatment and may serve to provide a better understanding of alcohol-mediated immunosuppression. The Journal of Immunology, 2014, 193: 000–000.

Monocytes and macrophages present the first line of host defense and are pivotal for activation of innate immune responses (1). During infection, pathogen recognition by TLRs and downstream TLR signaling play an important role in activation and function of innate immune cells. Upon stimulation with TLR4 ligand, LPS, or endotoxin, downstream MyD88-dependent signaling results in activation of NF-κB–mediated transcription of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (2). These innate immune responses are compromised by moderate or binge alcohol exposure increasing host susceptibility to infection, illustrating clinical relevance (3, 4). For example, binge alcohol–exposed macrophages and monocytes showed reduced Ag-presenting function, decreased production of proinflammatory cytokines TNF-α, IL-6, IL-8, and IL-12, and inhibition of NF-κB activation (5–9). Binge alcohol is also known to induce negative regulators of TLR4, such as IL-1 receptor associated kinase-M (IRAK-M) (10) and B cell lymphoma 3-encoded protein (Bcl-3) (11), likely contributing to endotoxin tolerance (12, 13). However, alcohol-induced cellular stress responses in innate immune cells and their contribution to immune suppression by alcohol has received no attention. In this study, we identify novel mechanisms of alcohol-mediated endotoxin tolerance, linking cellular stress pathways to innate immune signaling.

Alcohol-mediated induction of cellular stress, particularly oxidative stress by reactive oxygen species (ROS), has been previously demonstrated in heart, lung, and liver tissues (14–16). Cellular stress proteins can crosstalk with immune signaling pathways and influence immune responses (17). Among various cellular stress proteins, heat shock proteins are likely candidates for mediating alcohol-induced immune tolerance based on their widely characterized anti-inflammatory role in innate immune signaling. For instance, heat shock protein (hsp) 70 (also known as hsp72), which is induced by heat shock, inhibits LPS-induced production of cytokines such as TNF-α by macrophages (18, 19). Expression of hsp70 is regulated by its transcription factor heat shock factor 1 (HSF1) (20), which can also exert anti-inflammatory effects independent of hsp70 (21–23). These immunosuppressive roles of stress-induced HSF1 and hsp70 lend support to our hypothesis that alcohol causes endotoxin tolerance and inhibition of downstream signaling via induction of hsp70 and/or HSF1. Identifying this alcohol-mediated mechanism by which stress proteins crosstalk with inflammatory responses may provide novel mechanisms and identify therapeutic targets for restoration of normal immune functions in binge-drinking patients and trauma patients who abuse alcohol.
Previous studies from our laboratory show that moderate alcohol activates HSF1 and induces hsp70 in murine macrophages (24). The aim of this study was to delineate the mechanistic role of hsp70 and HSF1 in alcohol-induced endotoxin tolerance. Using an in vitro model of alcohol pre-exposure, we provide evidence that alcohol mediates activation of HSF1 and induction of hsp70 in human monocytes and murine macrophages. Alcohol-induced hsp70 bound to p50 subunit of NF-κB and HSF1 directly interacted with cytokine gene promoter exerting negative effects on TLR4-induced proinflammatory cytokine production. HSF1 and hsp70 were not only required, but also sufficient for alcohol-mediated induction of endotoxin tolerance. Collectively, these results uncover a crosstalk mechanism between alcohol induced stress proteins hsp70 and HSF1 and TLR4 signaling molecules resulting in endotoxin tolerance.

Materials and Methods

Human subjects and cell lines

Healthy women and men aged 18–60 years with no previous alcohol abuse history and who consumed <6 drinks/week were recruited in the study and all abstained from alcohol for 48–72 h prior to study. This study was reviewed and approved by University of Massachusetts Institutional Review Board and Department of Defense Human Research Protection Office at the Clinical Trials Unit at University of Massachusetts Medical Center. Mononuclear cells were isolated using Ficoll gradient from peripheral blood collected from the healthy human donors. Human monocytes from PBMCs were isolated by selective adherence as described previously (25).

The adherent monocytes were cultured in IMDM (Invitrogen Life Technologies) with 10% FBS (HyClone).

RAW 264.7 murine macrophages were purchased from American Type Culture Collection and maintained in DMEM (Invitrogen Life Technologies) containing 10% FBS (HyClone).

Alcohol exposures and cell stimulations

Monocytes or macrophages plated at 1 × 10⁶ cells/ml were stimulated with LPS (Sigma-Aldrich) derived from Escherichia coli (100 ng/ml) and ethanol concentrations of 25 or 50 mM as indicated in the figure legends.

For positive induction estimates a blood alcohol level of 0.1 g/dl, which is greater than the legal limit for driving in most states in the USA. Alcohol distributed in the brain and other tissues via the blood as it is metabolized and excreted. The 25 mM in vitro alcohol concentration approximates a blood alcohol level of 0.1 g/dl, which is greater than the legal limit for driving in most states in the USA. Alcohol distributed in the brain and other tissues via the blood as it is metabolized and excreted.

Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the Chromatrap ChIP assay kit according to the manufacturer’s instructions. RAW monocytes (1 × 10⁶) were treated with LPS or ethanol for indicated times, fixed with 1% of formaldehyde, and resuspended in hypotonic buffer before subjecting to chromatin shearing by sonication for 6 cycles of 10 s at 80% amplitude. Shearing efficiency was confirmed by agarose gel electrophoresis. After setting aside input chromatin, an aliquot of chromatin samples was incubated on a shaker at 4°C for 3 h with Abs against HSF1 (Enzo life sciences) or IgG control Abs as negative control in low-molarity chromatin samples was incubated on a shaker at 4°C for 3 h with Abs against HSF1 (Enzo life sciences) or IgG control Abs as negative control in low-molarity

Cell-free supernatants were collected from human monocyte or RAW macrophage cultures and analyzed for human TNF-α (Thermo Scientific), IL-6, and IL-1β (BD Biosciences) or murine TNF-α (BD Biosciences), IL-6 (BioLegend), and IL-1β (R&D Systems) according to the manufacturer’s instructions.

Nuclear lysates prepared from RAW macrophage (25 μg) were assayed for p-326 HSF1 and total HSF1 using ELISA kits from Enzo Life Sciences according to manufacturer’s instructions.

Preparation of nuclear, cytoplasmic, and whole cell extracts

Nuclear and cytoplasmic extracts from macrophages were prepared as previously described (27). At the end of the stimulation period, cells collected in ice-cold PBS were resuspended in cold hypotonic buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 10 μg/ml protease inhibitors such as aproitin, antipain, and leupeptin; Sigma-Aldrich) and incubated on ice for 20 min. Cells were then lysed with 0.6% Nonidet P-40 by vortexing for 20 s.

The lysate was centrifuged at 12,000 × g for 1 min 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 HEPES [pH 7.9], 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 20% glycerol). All tubes were kept on a shaker at 4°C for 30 min. The lysate was then centrifuged at 12,000 × g for 10 min, and the supernatant was stored at −80°C as the nuclear extract.

Monocytes/macrophages were resuspended in lysis buffer (10% glycerol, 1% Triton, 20 mM Tris [pH7.6], 150 mM NaCl, 25 mM β-glycerophosphate, 50 mM NaF, 1 mM Na-orthovanadate, 1 mM EDTA, and 1 mM DTT with protease inhibitors) and incubated for 20 min on ice. The lysates were then centrifuged at 12,000 × g for 10 min and the supernatant was stored as whole cell lysates.

Protein content was determined in the whole cell, cytoplasmic, or nuclear extracts with the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories).

Immunoblotting (Western blotting)

Heat denatured whole cell, cytoplasmic, or nuclear lysates (5–25 μg) were loaded into each well, separated on 10% SDS-polyacrylamide gel, and electroblotted onto nitrocellulose membranes. Nonspecific binding was blocked by incubation of the membranes in TBS/5% nonfat dried milk/0.1% Tween 20 or TBS/5% BSA/0.1% Tween 20 followed by the Abs indicated in each experiment. The mouse Abs against hsp70 (HSPA1A), hsp90β (HSP90AB1; constitutive form), and β-actin were purchased from StressMarq Biosciences and Abcam, respectively. The rabbit Abs against p65 and p50 were obtained from Cell Signaling Technology, and those against TBP-1 were obtained from Santa Cruz Biotechnology. The Abs were detected using HRP-conjugated anti-mouse and anti-rabbit secondary Abs (Santa Cruz Biotechnology and Abcam) and chemiluminescence assay reagents from Bio-Rad. The immunoreactive bands were quantified by densitometric analysis using an UVP System (Bio-Rad Laboratories).

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed using the Chromatrap ChIP assay kit according to the manufacturer’s instructions. RAW monocytes (1 × 10⁶) were treated with LPS or ethanol for indicated times, fixed with 1% of formaldehyde, and resuspended in hypotonic buffer before subjecting to chromatin shearing by sonication for 6 cycles of 10 s at 80% amplitude. Shearing efficiency was confirmed by agarose gel electrophoresis. After setting aside input chromatin, an aliquot of chromatin samples was incubated on a shaker at 4°C for 3 h with Abs against HSF1 (Enzo life sciences) or IgG control Abs as negative control in low-molarity

ELISA

ELISA Cell-free supernatants were collected from human monocyte or RAW macrophage cultures and analyzed for human TNF-α (Thermo Scientific), IL-6, and IL-1β (BD Biosciences) or murine TNF-α (BD Biosciences), IL-6 (BioLegend), and IL-1β (R&D Systems) according to the manufacturer’s instructions.
Transient transfection of plasmids and siRNA

The hsp70-CMV5 overexpression (pCMV5-hsp70) and 4× NF-κB tandem promoter-driven-luciferase reporter (pκBx4-luc) plasmids were gifts from Dr. R. Morimoto (Northwestern University, Chicago, IL) and Dr. N. Mackman (Scripps Research Institute, La Jolla, CA), respectively (28, 29). RAW macrophages were transfected with 2 μg plasmid at 1:3 ratio of DNA to lipofectamine in Opti-MEM for 6 h using Lipofectamine 2000 (Invitrogen). For knockdown experiments, RAW macrophages were transfected with 10 nM hsp70 siRNA (Invitrogen Stealth Library) or HSF1 siRNA (30) (synthesized by Invitrogen) or negative control in Opti-MEM for 24 h using Lipofectamine 2000 as shown previously (31). siRNA knockdown efficiency was confirmed with qPCR for hsp70 or HSF1. Twenty-four hours after transfection, cells were treated with ethanol or LPS for appropriate experiments.

 Luciferase assay

RAW macrophages were transfected with 0.5 μg pκBx4-luc alone or in combination with varying amounts of pCMV5-hsp70 using transfection reagent Lipofectamine 2000 (Invitrogen) at a 1:3 ratio of DNA to lipofectamine. Twenty-four hours after transfection, cells were treated with ethanol or LPS for indicated times and luciferase activity was assessed with Dual Luciferase reporter assay (Promega), according to the manufacturer’s instructions. Firefly and Renilla luciferase activities were determined using a Promega Glomax 96 Luminoimeter. NF-κB promoter-driven transcriptional activity, as detected by Firefly luciferase activity, was normalized with Renilla luciferase activity.

 EMSA

A double-stranded HSE (5’-GCCTCAGATGTTGCGAAGTTT-3’) consensus sequence was used for EMSA (24). End-labeling was accomplished by treatment with T4 polynucleotide kinase in the presence of [32P]-ATP (Dupont-NEN). Labeled oligonucleotide was purified on a polyacrylamide copolymer column (Bio-Rad Laboratories). Nuclear protein (5 μg) was added to a binding reaction mixture containing 20 mM HEPES (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, 200 μg/ml BSA, 2 μg polydeoxyinosinic-polydeoxyctydilic acid. Samples were incubated at room temperature for 20 min followed by incubation with 50,000 cpm [32P]-labeled HSE oligonucleotide for 10 min. For the cold competition reaction, a 20-fold excess of a specific, unlabeled, double-stranded probe was added to the reaction mixture 30 min before adding the labeled oligonucleotide. All reactions were run on a 4% polyacrylamide gel, and the dried gel was exposed to x-ray film at −80°C.

Statistical analysis

Results are presented as mean ± SD. Student t test was used to determine the statistical significance of differences between samples. The p values < 0.05 were considered to represent statistical significant differences.

Results

Pretreatment of human monocytes with a single dose of alcohol induces endotoxin tolerance and inhibits proinflammatory cytokine production

Several studies have shown that binge or short-term alcohol exposure (up to 24 h) can suppress innate immune responses (3, 4). In order to establish our in vitro model of alcohol pre-exposure followed by subsequent endotoxin stimulation, we conducted a comprehensive analysis of the effect of multiple alcohol pre-exposure time points on the expression of TLR4-induced MyD88-dependent proinflammatory cytokines TNF-α, IL-6, and IL-1β in primary human monocytes. Fig. 1 shows that treatment of monocytes with 100 ng/ml LPS for 2 h caused a significant induction of TNF-α (Fig. 1A), IL-1β (Fig. 1B), and IL-6 (Fig. 1C) mRNA expression, respectively (primers listed in Table I). However, this induction was significantly decreased in monocytes pre-exposed to 25 mM alcohol for 3–24 h before LPS stimulation. It should be noted that we used a physiologically relevant concentration of 25 mM alcohol (approximates 0.1 g/dl blood alcohol concentration), and alcohol alone did not induce any of these cytokines in monocytes. Alcohol pre-exposure and LPS treatment also did not affect cell viability (data not shown). Monocytes pretreated with low doses of LPS for 24 h prior to 100 ng/ml LPS stimulation showed inhibition of cytokine mRNA and served as a positive control for LPS and endotoxin tolerance (32). ELISA data corroborated these changes in cytokine mRNA levels. We observed inhibition of LPS-induced TNF-α (Fig. 2A), IL-1β (Fig. 2B), and IL-6 (Fig. 2C) in 3–24 h alcohol pre-exposure in human monocytes, consistent with the mRNA results. Notably, the inhibitory effect of alcohol on LPS-induced IL-1β observed even at 1 h after alcohol pre-exposure was statistically significant despite no change in IL-1β mRNA, suggesting that alcohol likely also regulates IL-1β expression at the posttranscriptional level, in addition to inhibition of mRNA transcription. Thus, our results show that pre-exposure of human monocytes to physiologically relevant concentrations of alcohol caused inhibition of LPS/TR4–stimulated proinflammatory cytokine production at the mRNA and protein levels.

Alcohol induces HSF1 expression and activation in monocytes and macrophages

The role of cellular stress proteins in TLR4 signaling and tolerance has been reported (17). We have shown previously that alcohol and LPS added together activated HSF1 in human monocytes (24). To delineate the role of stress-induced HSF1 in alcohol-mediated immune suppression, we first determined the effect of alcohol pretreatment for 2–24 h on expression and activity of HSF1 in human monocytes and RAW264.7 murine macrophages. Alcohol exposure significantly induced HSF1 mRNA in monocytes (Fig. 3A) and RAW macrophages (Fig. 3B) at 14–24 h compared with untreated cells (primers listed in Table I). Upregulation of HSF1 mRNA in RAW macrophages occurred at 50 mM alcohol concentration (Fig. 3B). This increase in HSF1 mRNA was not affected by subsequent LPS stimulation (Fig. 3C). Next, RAW macrophages were pre-exposed to alcohol followed by LPS to determine HSF1 DNA binding activity measured by EMSA. As shown in Fig. 3D, 50 mM alcohol alone for 1 h induced higher HSF1 DNA binding activity compared with untreated cells, which is consistent with our previously published results in human monocytes (24). Cells pretreated with 50 mM alcohol for 1 h prior to LPS stimulation also showed levels of HSF1 DNA-binding activity comparable to cells treated with alcohol alone but higher than LPS-stimulated macrophages, indicating that subsequent LPS stimulation did not further augment alcohol-induced HSF1 activity. Because chaperone protein, hsp90β or HSP90AB1, is known to sequester HSF1 in the cytoplasm (33), we examined cytoplasmic hsp90β and observed no significant change in alcohol-treated cells compared with untreated cells, suggesting that alcohol-induced HSF1 activation is independent of hsp90β levels (Fig. 3E). HSF1 undergoes posttranslational modifications, such as activating Ser326 phosphorylation by heat shock–induced mammalian target of rapamycin (mTOR) kinase, which affects DNA binding activity (34). To determine whether alcohol regulates HSF1 DNA binding activity via phosphorylation, we analyzed HSF1 phosphorylation at Ser326 in alcohol-treated cells using a plate-based ELISA. Heat-shocked cells showed significant induction of phospho-S326 HSF1 as expected, but we did not observe phospho-S326 HSF1 levels in the presence of alcohol alone or with alcohol pretreatment followed by LPS stimulation (Fig. 3F). This result suggests that alcohol regulates HSF1 activity independent of activating phosphorylation of S326 on HSF1 or hsp90β levels.

Alcohol-induced hsp70 expression is mediated by HSF1 in monocytes and macrophages

Stress-mediated activation of HSF1 induces target genes such as hsp70 (20). In this study, we examined the effect of alcohol on HSF1 target gene hsp70 expression. Human monocytes treated...
with 25 mM alcohol showed significant upregulation of hsp70 mRNA at 14–24 h compared with untreated cells (Fig. 4A) (primers listed in Table I). As shown in Fig. 4B, upregulation of hsp70 mRNA observed in cells pretreated with alcohol for 24 h was not augmented further by subsequent LPS stimulation, but was significantly higher than in LPS-treated cells. Heat-shocked monocytes showed significantly high expression of hsp70 mRNA and served as a positive control (Fig. 4B). Next, Fig. 4C illustrates that monocytes exposed to alcohol for 24 h exhibited significant induction of hsp70 protein as determined with immunoblotting. Pre-exposure of alcohol followed by LPS did not increase hsp70 further in monocytes compared with alcohol alone, indicating that upregulation of hsp70 is mediated solely by alcohol. Therefore, similar to heat stress, alcohol can upregulate hsp70 expression at the mRNA and protein levels in human monocytes independent of TLR4 stimulation.

Previous studies in our laboratory have shown that alcohol exposure upregulates HSF1-directed hsp70 promoter-driven transcriptional activity in macrophages (24). As illustrated in Fig. 4D, we show using ChIP analysis that binding of HSF1 to the hsp70 promoter in RAW macrophages is significantly increased upon treatment with alcohol alone for 1 h. Interestingly, 1 h of alcohol pre-exposure followed by LPS treatment for 1 h reduced HSF1 binding to the hsp70 promoter, which was still higher than in LPS-stimulated cells alone. Therefore, alcohol-mediated HSF1 activation mediates induction of hsp70 by direct binding to its promoter region in monocytes and macrophages.

**Alcohol-induced HSF1 and hsp70 mediates LPS-induced NF-κB inhibition in macrophages**

Previous studies demonstrate decreased DNA-binding activity and reduced nuclear translocation of NF-κB in cells exposed to alcohol and LPS together (27). To implicate alcohol-mediated anti-inflammatory proteins HSF1 and hsp70 in TLR4-mediated NF-κB activation, we tested whether alcohol pre-exposure affects nuclear...
been shown to be transiently inactivated via phosphorylation upon treatment with LPS alone (21). Alcohol pretreatment for 1 h prior to LPS stimulation exhibited some reduction of HSF1 occupancy of TNF-α promoter compared with LPS; however, it remained comparable to alcohol alone. This indicates alcohol-induced HSF1-mediated direct repression of TNF-α transcription could be a mechanism of alcohol-induced tolerance in monocytes and macrophages. Collectively, alcohol-induced hsp70 and HSF1 exert inhibitory effects on TLR4 signaling that result in decreased NF-κB activation.

**Overexpression of hsp70 is sufficient to induce endotoxin tolerance in macrophages**

Our results so far have shown that binge alcohol induces hsp70, which plays an important role in NF-κB inhibition and alcohol-mediated endotoxin tolerance in monocytes and macrophages. In this study, we determined whether expression of hsp70 would be sufficient to mimic alcohol-mediated tolerance and inhibition of TLR4 signaling. Transient transfection of RAW macrophages with pCMV5-hsp70 plasmid showed a significant increase in hsp70 expression, as illustrated by Western blotting (Fig. 6A). Maximal expression of hsp70 was observed at a ratio of 1:3 DNA to lipofectamine and was even higher than levels of hsp70 in heat-shocked cells (Fig. 6A). To investigate the effect of hsp70 on NF-κB activation, we transiently cotransfected RAW cells with NF-κB-promoter–driven luciferase plasmid (p(κB)2-luc) and increasing amounts of pCMV5-hsp70 while maintaining the optimal total DNA-to-lipofectamine ratio of 1:3. As illustrated in Supplemental Fig. 2, cotransfection with both plasmids did not interfere with hsp70 expression and yielded macrophages with optimal overexpression of hsp70. Fig. 6B shows that macrophages transfected with p(κB)2-luc and stimulated with LPS for 6 h significantly increased NF-κB promoter-driven luciferase activity. On the other hand, overexpression of hsp70 dose-dependently decreased NF-κB–driven luciferase activity. Therefore, hsp70 overexpression can inhibit NF-κB promoter activation downstream of TLR4 in a manner similar to macrophages exposed to alcohol (Fig. 5C).

To determine whether hsp70 is sufficient to mimic the inhibitory effect of alcohol exposure on TLR4-mediated downstream cytokine production, we examined the effect of hsp70 overexpression on LPS-stimulated proinflammatory cytokines. As shown in Fig. 6, untransfected RAW cells exhibit significant induction of TNF-α (Fig. 6C), IL-1β (Fig. 6D), and IL-6 (Fig. 6E) expression in re-

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**Table I. Sequences of primers used for qPCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>hTNFa</td>
<td>Fwd, 5′-ATCTTTTCTGAGACCCGAGATTGA-3′</td>
</tr>
<tr>
<td>hIL-6</td>
<td>Rev, 5′-CGTTACGCCCAGCCCGGAAGAA-3′</td>
</tr>
<tr>
<td>hIL-1β</td>
<td>Fwd, 5′-CAGGTCTTCCGAGATACAGGC-3′</td>
</tr>
<tr>
<td>mTNFa</td>
<td>Rev, 5′-GAGAGAACCACTCTGGTGCTTCA-3′</td>
</tr>
<tr>
<td>mIL-6</td>
<td>Fwd, 5′-ACAACCACGCGCTTCTCCTGCTT-3′</td>
</tr>
<tr>
<td>mIL-1β</td>
<td>Rev, 5′-CACGTATCCAGACATGATCA-3′</td>
</tr>
<tr>
<td>mHsp70</td>
<td>Fwd, 5′-ACTCTCAACCTGGACACACCTG-3′</td>
</tr>
<tr>
<td>mHSF1</td>
<td>Rev, 5′-GGAGGCTCTTGGGACACACAGG-3′</td>
</tr>
<tr>
<td>18S</td>
<td>Fwd, 5′-GATAACCTTGGACACCCATT-3′</td>
</tr>
<tr>
<td></td>
<td>Rev, 5′-CCCTTAAATGGGTGTAGGCA-3′</td>
</tr>
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Fwd, forward; Rev, reverse.
response to LPS, whereas overexpression of hsp70 decreased cytokine production in LPS-stimulated transfected macrophages. Taken together, our results show that proinflammatory cytokine production inversely correlated with hsp70 expression in macrophages, suggesting that hsp70 is sufficient to induce NF-κB inhibition and decrease proinflammatory cytokines demonstrated in alcohol-mediated endotoxin tolerance.

Knockdown of hsp70 and HSF1 reverses alcohol-induced endotoxin tolerance in macrophages

To determine whether HSF1 and hsp70 are required for alcohol-induced TLR4 tolerance, we used siRNA to inhibit expression of these proteins in alcohol-treated RAW macrophages. As illustrated in Fig. 7A, we achieved 80% knockdown of HSF1 in untreated and heat-shocked RAW macrophages. Pre-exposure of control transfected RAW cells to alcohol for 24 h resulted in significant inhibition of LPS-induced TNF-α production (Fig. 7B). However, silencing of HSF1 by siRNA in alcohol-exposed RAW macrophages prevented downregulation of LPS-induced TNF-α by alcohol, indicating that HSF1 plays an important role in alcohol-mediated endotoxin tolerance in macrophages. Furthermore, we wanted to ascertain the role of hsp70 in alcohol-mediated inhibition of proinflammatory cytokine production and endotoxin tolerance. We inhibited expression of hsp70 in RAW macrophages by transient transfection with siRNA and confirmed 90% knockdown in heat-shocked RAW macrophages (Fig. 7C). As observed in Fig. 7D, hsp70-deficient RAW macrophages pre-exposed to alcohol for 24 h followed by LPS stimulation did not exhibit downregulation of TNF-α production, compared with alcohol-exposed control macrophages, indicating the importance of hsp70 in alcohol-mediated endotoxin tolerance. These results show that HSF1 and hsp70 are both required for alcohol-induced endotoxin tolerance in monocytes and macrophages via mechanisms illustrated in Fig. 8.

FIGURE 2. Pre-exposure of human monocytes to alcohol inhibits TLR4-induced proinflammatory cytokine protein levels. Adherence isolated human monocytes were treated with 100 ng/ml LPS or 25 mM alcohol (Et) alone or pre-exposed to 25 mM alcohol for 1, 3, 5, 7, or 24 h followed by LPS stimulation for 18–22 h (overnight). Secreted TNF-α (A), IL-1β (B), and IL-6 (C) protein in overnight culture supernatants was analyzed using ELISA. LPS tolerance control: 10 ng/ml LPS for 24 h followed by LPS (100 ng/ml). Data summarize mean ± SD of 13 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.005. ns, not significant.
**Discussion**

Although the suppressive effect of short-term or binge alcohol exposure on innate immune activation has been documented (3, 4, 37), the underlying mechanism of alcohol-mediated endotoxin tolerance is not completely understood. In this study, we delineate novel mechanistic roles of stress proteins hsp70 and HSF1 in suppression of the TLR4/MyD88 pathway in an in vitro setting of short-term alcohol pre-exposure of human peripheral blood monocytes and murine RAW macrophages. To our knowledge, this report is the first to describe the crosstalk mechanisms between alcohol-mediated stress proteins and their relevance in suppression of TLR4/MyD88 proinflammatory cytokine responses. Previous studies have implicated kinases such as IL-1 receptor–associated kinase-M (IRAK-M) and protein kinase C (PKC) and other negative regulators including heme-oxygenase 1 (HO-1) and suppressors of cytokine signaling (SOCS) 1 and SOCS3 in alcohol-mediated anti-inflammatory effects on TLR or cytokine-stimulated cells (10, 38–40). The inhibition of TLR4 responses by alcohol has also been attributed to alcohol-induced perturbation of TLR4-CD14 association in lipid rafts (41). Recently alcohol-induced Bcl-3, an inhibitor of NF-κB signaling, has been reported to contribute to alcohol-mediated inhibition of LPS-induced TNF-α production (11). However, a lack of consensus on the mechanisms by which alcohol pre-exposure of immune cells leads to immunosuppression demands further investigation. Our group has shown previously that binge alcohol exposure induces cellular stress proteins hsp70 and HSF1 in macrophages (24). The crosstalk between stress pathways and immune signaling has been recently recognized (17, 42). Whether alcohol induced stress pathways have any effect on macrophage/monocyte inflammatory responses remains unclear. In this study, we demonstrate that alcohol-mediated endotoxin tolerance correlated with alcohol-induced increase in HSF1 activity and hsp70 expression, strongly supporting our hypothesis of a role for cellular stress proteins in alcohol-mediated endotoxin tolerance. We showed that interaction between alcohol-induced hsp70 and NF-κB subunit p50 and alcohol-

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**FIGURE 3.** Alcohol induces HSF1 expression and activation in human monocytes and RAW macrophages. (A) Human monocytes isolated by adherence were exposed to 25 mM alcohol (Et) for 2–24 h, and total RNA collected at different time points was analyzed for HSF1 mRNA using qPCR. Graph depicts mean ± SD of seven independent experiments. (B) RAW macrophages were exposed to 25 or 50 mM alcohol (Et) for 2–24 h, and total RNA collected at different time points was analyzed for HSF1 mRNA using qPCR. Data summarize the mean ± SD of three independent experiments. (C) Adherence isolated human monocytes were treated with 100 ng/ml LPS or 25 mM alcohol (Et) alone or were pre-exposed to 25 mM alcohol, followed by LPS stimulation for indicated time points. Total RNA was subjected to HSF1 mRNA determination using qPCR. Data summarize the mean ± SD of seven independent experiments. (D–F) RAW macrophages were stimulated with 100 ng/ml LPS for 30 min or 50 mM alcohol (Et) alone for 1 or 24 h, or they were pre-exposed to 50 mM alcohol for 1 or 24 h, followed by LPS stimulation for 30 min. (D) HSF1 DNA-binding activity was detected in nuclear extracts by EMSA using a 32P-labeled, double-stranded HSE oligonucleotide. A representative experiment is depicted, and the graph summarizes the mean ± SD of four independent experiments. A 20-fold excess of unlabeled oligonucleotide added to the heat-shocked sample to confirm specificity of HSF1 binding was included as competition control (Comp). (E) Expression of hsp90β in the cytoplasmic extracts was assayed with Western blotting. A representative experiment is depicted, and the graph summarizes mean ± SD of four independent experiments. (F) Phosphorylated HSF1 (phosphoserine 326) was detected in nuclear lysates by ELISA and normalized to total HSF1. The graph depicts the mean ± SD of four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.005. HS, heat-shocked control, 42°C for 45 min; ns, not significant.
mediated HSF1 binding to TNF-α promoter resulted in negative regulation of TLR4 signaling (Fig. 8).

Our results provide evidence that pre-exposure of human monocytes and macrophages to moderate physiologic concentrations of alcohol (based on National Institute of Alcohol Abuse and Alcoholism/NIH standards: 25 mM approximates 0.1 g/dl blood alcohol concentration, higher than the legal limit 0.08 g/dl, which is reached by 4–5 drinks in 2 h) inhibits proinflammatory cytokine production and nuclear translocation of NF-κB downstream of TLR4 stimulation. A comprehensive time course analysis of 1–24 h revealed a time-dependent, alcohol-mediated decrease in LPS-induced IL-6, IL-1β, and TNF-α, with maximal inhibition observed at 24 h of alcohol pretreatment in human monocytes. Our observations are in agreement with Bala et al. (11), who reported inhibition of LPS-induced TNF-α production by monocytes and macrophages after 18 h pre-exposure of binge alcohol. This anti-inflammatory effect of binge alcohol on monocyte/macrophage function is in contrast to the effects observed after long-term alcohol exposure, which sensitized macrophages to augment cytokine production in response to LPS/TLR4 signaling (10, 43, 44). We also demonstrate that moderate alcohol inhibits IL-1β and IL-6 mRNA and protein, resulting in endotoxin tolerance. Whereas alcohol exposure alone did not affect NF-κB activity, our results show an inhibitory effect of alcohol pre-exposure on subsequent LPS-induced NF-κB nuclear translocation and promoter-driven luciferase activity at the 24 h pretreatment time point in macrophages. This result was consistent with the reported decrease in NF-κB DNA-binding activity observed in 16 h alcohol-pretreated
Thus, our detailed analysis illustrates that binge alcohol-mediated endotoxin tolerance is a result of inhibition of TLR4/MyD88 dependent NF-κB activation and proinflammatory cytokine production in monocytes and macrophages.

Previous studies show that alcohol induces activation of HSF1 and induces hsp70 in monocytes and macrophages (24). In this study, we postulated that stress proteins HSF1 and hsp70 play a mechanistic role in alcohol-mediated endotoxin tolerance. HSF1 mRNA expression is constitutive in all cells, whereas its activation is regulated at the posttranslational level by cellular stress signals (20). Interestingly, our results show induction of HSF1 mRNA expression by alcohol exposure, even in cells subsequently stimulated by LPS indicating alcohol-mediated regulation of HSF1 at the transcriptional level. Transcription factor C/EBPβ has been implicated in induction of HSF1 expression in colonic epithelial cells (45), and upregulation of C/EBPβ protein in response to long-term alcohol exposure has been demonstrated in mouse liver and hepatic nuclear extracts (46, 47). Therefore, it is plausible that HSF1 mRNA induction by alcohol is regulated by C/EBPβ in monocytes/macrophages. Based on the University of California Santa Cruz Genomic Browser Database, putative binding sites for other transcription factors such as forkhead box protein O1 (FOXO1), early growth response protein 1 (EGR-1), and specificity protein 1 (SP-1) have also been reported in the HSF1 promoter region. Alcohol treatment enhances activation of SP-1 and EGR-1 (48, 49). Further investigation will be required to identify the regulatory mechanisms responsible for alcohol-induced HSF1 mRNA expression.
Alcohol exposure was also demonstrated to induce HSF1 nuclear translocation, phosphorylation, and activation in astrocytes and colonic epithelial cells (50, 51). Elevation in hsp70 mRNA and protein levels and in HSF1 protein expression was also observed in livers of mice subjected to short-term alcohol administration (52). In this study, we demonstrate that alcohol pre-exposure increased HSF1 DNA binding activity in macrophages, consistent with our previous studies of HSF1 activation in macrophages treated with alcohol and LPS together (24). These data were also corroborated by our ChIP analysis that revealed significantly higher binding of HSF1 to the promoter region of target gene hsp70 in alcohol-treated macrophages. Alcohol pre-exposure followed by LPS stimulation also induced HSF1 binding to hsp70 promoter, albeit to a lesser extent, possibly because of a concomitant increase in HSF1 binding at the TNF-α promoter observed in these alcohol-pretreated, LPS-stimulated macrophages. Alcohol-mediated activation of HSF1 may be regulated by nuclear translocation, transactivation of DNA binding activity, or posttranslational modifications. In an effort to determine the mechanism of alcohol-induced HSF1 activation, we first examined the effect of alcohol exposure on cytoplasmic levels of constitutive hsp90β (HSP90AB1), which can directly bind to and inhibit HSF1 nuclear translocation. Although we show that cytoplasmic hsp90β expression was not affected by alcohol pre-exposure, previous studies have shown that hsp90-HSF1 complexes were reduced during alcohol treatment in macrophages (24). It is likely that alcohol pre-exposure dissociates HSF1-hsp90 complexes without affecting hsp90β levels in the cytoplasm. Next, we examined the effect of alcohol on levels of phosphoserine326-HSF1 (an activating phosphorylation), which is known to be phosphorylated by mTOR kinase (34). Alcohol has been demonstrated to induce mTOR expression in murine liver and cerebral cortex, whereas other groups have shown alcohol-induced decrease in phosphorylation (activation) of mTOR in mouse myocytes and brain cells (53, 54). Based on these observations, we postulated that alcohol could modulate phosphorylation of HSF1 in alcohol-treated cells; however, we did not observe an increase in pS326-HSF1 levels in alcohol treated macrophages indicating that regulation of HSF1 phosphorylation as a mechanism of activation. It is noteworthy that alcohol-mediated induction of HSF1 and its target gene hsp70 is consistently maintained over a longer period (up to 24 h) compared with heat shock stress induction (55, 56). Alcohol exposure has been shown to enhance nuclear localization of the catalytic subunit of PKA (57), an enzyme that increases PKA activity (35). Alcohol has been shown to enhance nuclear localization of the catalytic subunit of PKA (57), enhancing PKA activity and transcription of heat shock genes (58). Future investigation will be required to determine the mechanism of alcohol-mediated HSF1 activation.
The upregulation of hsp70 mRNA at 14–24 h of alcohol exposure is consistent with the observation of alcohol-mediated endotoxin tolerance at 24 h of alcohol pretreatment. Our findings here in human monocytes support previously published results from our group showing increased hsp70 levels in RAW macrophages when treated with alcohol and LPS together (24). Importantly, alcohol-mediated upregulation of hsp70 mRNA and protein was not further altered by LPS in human monocytes, suggesting that endotoxin stimulation did not affect or enhance induction of hsp70 in alcohol-treated monocytes. Our results indicate that alcohol-induced hsp70 likely interacts with the LPS-stimulated TLR4 signaling pathway to exert its anti-inflammatory effects.

To address crosstalk mechanisms and direct effects of alcohol-induced hsp70 in macrophages, we overexpressed hsp70 and tested its effect on LPS-induced NF-κB signaling. We showed an hsp70 dose-dependent decrease in NF-κB promoter-driven luciferase activity and inhibition of expression of NF-κB–dependent proinflammatory cytokines TNF-α, IL-6, and IL-1β. These results are in agreement with previous reports of inhibition of NF-κB translocation and activation by hsp70 (59); they also corroborated our hypothesis that hsp70 expression is sufficient to mimic alcohol-induced endotoxin tolerance.

The anti-inflammatory properties of stress proteins have been reported previously (17). HSF1 has been demonstrated to exert anti-inflammatory effects by repressing transcription of TLR4 induced genes such as TNF-α (36). In this study, we show HSF1 binding to TNF-α promoter by ChIP analysis in alcohol-pretreated macrophages, indicating that alcohol-induced HSF1 could directly repress proinflammatory cytokines by binding to a specific promoter region—a plausible mechanism of alcohol-mediated endotoxin tolerance. Hsp70 has been shown to bind directly NF-κB subunit p50, resulting in decreased NF-κB activation in lymphoma cells (35). We show that the alcohol-mediated inhibition of NF-κB nuclear translocation could be attributed to the association of subunit p50 with hsp70. The interaction of hsp70 with p50 could hamper nuclear translocation and promoter transactivation by NF-κB, resulting in reduced expression of proinflammatory cytokines observed in our studies, thus providing direct links among alcohol,

**FIGURE 7.** HSF1 and hsp70 are required for alcohol-mediated TLR4 tolerance. (A and C) RAW macrophages were transfected with 10 nM siRNA targeting HSF1 (A) or hsp70 (B) or negative control (Neg ctrl) siRNA. Total RNA was subjected to HSF1 or hsp70 mRNA determination using qPCR, and percent knockdown in unstimulated and heat-shocked cells (42˚C for 45 min) was calculated for untransfected for each condition. The graph depicts mean ± SD (n = 3). (B, D) RAW macrophages were transfected with 10 nM siRNA targeting HSF1 (B) or hsp70 (D) or negative control (Neg ctrl) siRNA. Twenty-four hours after transfection, macrophages were stimulated with 100 ng/ml LPS for 2 h or 50 mM alcohol (Et) alone for 24 h, or they were pre-exposed to alcohol, followed by LPS stimulation for indicated times. TNF-α mRNA was analyzed with qPCR. The graph depicts mean ± SD of three independent experiments. ∗p < 0.05. ns, not significant.
hsp70, and endotoxin tolerance. Finally, our siRNA experiments confirm HSF1 and hsp70 as plausible intermediates in alcohol-mediated endotoxin tolerance. Our results show that knockdown of HSF1 or hsp70 prevents alcohol-mediated downregulation of TNF-α and thus endotoxin tolerance, indicating that both of these stress proteins are required for alcohol-mediated tolerance.

Collectively our studies here identify that clinically relevant alcohol pre-exposure induces stress proteins HSF1 and hsp70, which negatively regulates TLR4-mediated proinflammatory responses. It should be noted that the quantity and form of alcohol consumed also seems to be a factor in modulation of immune responses. For example, consumption of moderate amounts of red wine containing antioxidants, which would dampen cellular stress responses, did not show an immunosuppressive effect in mice and humans (60, 61). Moderate alcohol-mediated immunosuppression may also have protective effects in a tissue-specific context, such as by possibly “dampening” the inflammatory immune responses that contribute to cardiovascular disease (62, 63). In this study, we have examined the effect of moderate alcohol exposure on TLR4 responses with a specific emphasis on an alcohol pre-exposure model, which has a direct physiologic and clinical relevance. For example, accident or trauma patients are more susceptible to subsequent viral or bacterial infections because of prior consumption of alcohol that can likely compromise their innate immune responses (26). Our studies here not only test the effect of this physiologically relevant alcohol exposure on innate immune function, but also recognize novel crosstalk mechanisms affected by alcohol that reduce inflammatory responses. Whereas alcohol-induced HSF1 can exert repressive effects on cytokine production at early time points, the interaction of hsp70 and NF-κB at later time points is important in alcohol-mediated inhibition of proinflammatory cytokines. Identification of the crosstalk among HSF1, hsp70, and TLR4 signaling molecules that play a role in alcohol-induced endotoxin tolerance will provide insight into novel links between stress proteins and immune signaling pathways and will unravel novel therapeutic mechanisms for intervention in alcohol abuse patients. Consequently, the use of drugs directly targeting stress proteins hsp70 or HSF1 in innate immune cells could aid in restoration of normal immune function in alcohol-exposed individuals. The ready availability of such drugs for treatment of cancer (65, 66) adds to the appeal of these stress proteins as possible drug targets in treatment of alcohol abuse.

Disclosures

The authors have no financial conflicts of interest.

References


