Extracellular Histones Enhance LPS-induced Cytokine Production

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\section*{Abstract}

Over the last twenty years, the debilitating clinical condition known as sepsis has tripled in frequency to become the tenth leading cause of death in the United States. Extracellular histones were previously reported to mediate cell damage and organ dysfunction during hyper-inflammatory response, a characteristic feature of sepsis. Lipopolysaccharide (LPS) is an endotoxin found in Gram-negative bacteria, which account for most sepsis cases.

In this study we evaluate the histone-to-LPS relationship and investigate the molecular mechanisms responsible for histone-mediated hyper-inflammation. Cytokine levels as a result of histone plus LPS stimulation were compared via ELISA assays. The impact of histone classes (H1, H2A, H2B, H3, H4) and kinase inhibitors (SB202190, U0126, LY294002) on cytokine levels were also tested. Western blotting and Coomassie blue staining were used to detect possible protein receptors. Histone plus LPS stimulation synergistically yielded cytokine levels over 1000-fold greater than separate histone or LPS stimulation, respectively. Cells given H2A and H2B on average contained cytokine (TNF-\(\alpha\), IL-8) levels over 1000-fold higher when compared to LPS treatment. Treatments with kinase inhibitors indicate that ERK1/2 and p38 are associated with histone signaling in cytokine production. Two target proteins were indicated with MW 37 kD and \(\sim 90\) kD, which will require further investigation. The roles of ERK1/2 and p38 in inflammatory signaling also need further study to determine the molecular mechanisms responsible for histone-mediated hyper-inflammatory responses in septic patients and ultimately to develop therapeutic interventions for sepsis.

\section*{Introduction}

Sepsis, a typical example of systemic inflammatory response syndrome (SIRS) caused by infection, is a major challenge in the intensive care unit (ICU), where it is one of the leading causes of death\textsuperscript{1}. Sepsis is increasingly prevalent with an overall 2.3-fold increase in cases from 2000 to 2007\textsuperscript{2}. Early warning signs are often nonspecific and inconspicuous, exacerbating late diagnosed treatment\textsuperscript{3}. As the number of sepsis hospitalizations increases, mortality rates remain high, far exceeding those of common medical conditions such as myocardial infarction and cardiovascular accident\textsuperscript{4}. Despite advanced medical care, sepsis leads to roughly 225,000 annual deaths among 750,000 patients in the US alone\textsuperscript{1}.

While sepsis results from various infections, such as bacterial infection and fungal infection, Gram-negative bacterial infection is the most common cause of sepsis, and outbreaks of Escherichia coli (E. coli), the most common Gram-negative bacterium, are currently a major issue among global health concerns, causing roughly 73,480 illnesses, 2,168 hospitalizations, and 61 deaths annually in the United States alone\textsuperscript{5,6}. Lipopolysaccharide (LPS), an endotoxin found on the surface of Gram-negative bacteria such as E. coli, is known to activate the innate immune system and elicits strong immune responses in humans via Toll-like receptor (TLR) signaling pathways\textsuperscript{7}. Consequently, LPS challenge is currently widely implemented in research investigating the hyper-inflammatory response exhibited in sepsis\textsuperscript{8}. In addition, previous studies have reported marked increase of histone levels during LPS-induced septic shock\textsuperscript{9}.

During an innate immune response, neutrophils can release histones, which serve to kill microbes caught in neutrophil extracellular traps (NETs)\textsuperscript{10}. However histones found in NETs, especially H3 and H4, are also cytotoxic to tissue\textsuperscript{11}. Xu et al. (2011) revealed that antibodies used to block extracellular histones could rescue animals from LPS-mediated death. Thus histones released in response to bacterial challenge mediate endothelial dysfunction, organ failure, and death during sepsis\textsuperscript{12}. Therefore, during massive cellular activation triggered by either infectious pathogens or non-infectious stimuli, extracellular histones are released, inducing a cytokine storm and amplifying inflammation through inflammatory receptors such as TLR4 in SIRS13.

Binding of LPS to TLR4 on the surface of monocytes triggers the recruitment of adaptor molecules, such as MyD88 and activates various kinases, including IRAK-4 and TRAF-614. Mitogen-activated protein kinase (MAPK) pathways are then activated, leading to the activation of transcription factors, including NF-\(\alpha\)B15. MAPKs have been implicated as key regulators of the production of pro-inflammatory cytokines\textsuperscript{16}. Extracellular signal-regulated kinases (ERKs) and p38K are two major classes of MAPKs that play important roles in transducing inflammation\textsuperscript{17}. Schabbauer et al. (2004)\textsuperscript{15} reported that the phosphatidyl-inositol-3 kinase (PI3K) pathway suppresses LPS signaling, effectively regulating LPS-induced inflammatory response. Both MAPK and PI3K signaling pathways may be blocked via available specific inhibitors, making them key molecular targets for therapeutic intervention.

With the recent finding of histones mediating cell death in sepsis, a unique connection between histones and LPS seems likely\textsuperscript{9}. We, therefore, hypothesized that histones actively interact with LPS to facilitate cytokine production. More importantly, despite numerous investigations performed studying the roles MAPK and PI3K pathways play in sepsis, the molecular mechanisms responsible for histone-mediated hyper-inflammation in septic patients remain unclear\textsuperscript{11}. Herein, through ELISA and Western blot analysis, we examine the specific effects of histones on LPS-
induced pro-inflammatory cytokine production in human HL-60 and THP-1 cells, the roles of signaling receptors other than TLR2 or TLR4 in contributing to histone-mediated hyper-inflammatory responses, and possible protein receptors that transduce signals.

Materials and Methods

Human HL-60 (ATCC#: CCL-240) and THP-1 inflammatory cells (ATCC#: TIB-202) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and L-glutamine (Invitrogen Corporation, Camarillo, CA). Both HL-60 and THP-1 cell lines (1×106 cells/ml) were treated with histones (50 μg/ml), LPS (1 μg/ml), or both (Sigma-Aldrich Corporation, St. Louis, MO). All cells were incubated for 1 hour, 2 hours, 4 hours, and 24 hours. TNF-α and IL-8 levels of conditioned media were measured with respective human cytokine ELISA kits according to manufacturer's instructions (Invitrogen Corporation, Camarillo, CA). HL-60 and THP-1 cells were treated with histones, LPS, LPS plus H1, H2A, H2B, H3, H4 (10 μg/ml) (New England Biolabs, Ipswich, MA) separately or with histones (50 μg/ml) for indicated periods of time. Cells were pretreated with or without p38 kinase inhibitor SB202190, ERK1/2 inhibitor U0126, or PI3K pathway inhibitor LY294002 (10 μM). TNF-α and IL-8 levels of conditioned media were determined via respective ELISA assays using the manufacturer's protocol. Cross-linking procedure was performed in accordance with manufacturer's protocol (Pierce Biotechnology, Rockford, IL). Trifunctional cross-linker Sulfo-SBED (45 mg/ml in DMSO) was added to histones (10 mg/ml in Phosphate Buffered Saline (PBS)) at room temperature (RT) and incubated without light for 30 minutes. Buffer exchange was performed via spin column with PBS. Cells were centrifuged at 500 × g for 1 minute and washed with cold PBS twice. Cells were suspended in 1 ml cold PBS, mixed with Sulfo-SBED-histones (50 μg/ml), and incubated on ice for 10 minutes. Samples were centrifuged and washed with cold PBS twice, treated with UV light (365 nm) 5 cm away for 15 minutes at RT, and washed twice afterwards. The cell pellet was then lysed with cold PBS containing 1X protease inhibitor cocktail and 0.5% NP-40 and incubated on ice for 5 minutes. Nuclei were spun down at 1500 × g for 5 minutes at 4°C. The resulting supernatant was collected and centrifuged at 10000 × g for 5 minutes at 4°C. The supernatant was mixed with Avidin-Affi-Gel 10 resin and rocked for 2 hours at 4°C. Samples were centrifuged and washed four times with cold PBS containing 0.1% TX-100 at 500 × g for 1 minute. 1X SDS sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 100 mM 2-mercaptoethanol, 0.01% w/v bromophenol blue) was added to the samples and then heated at 100°C for 5 minutes (Pierce Biotechnology, Rockford, IL). Electrophoresis was performed as specified by the manufacturer (Bio-Rad, Hercules, CA). The above eluate was loaded into a Mini-PROTEIN TGX any kD SDS-PAGE gel (Bio-Rad, Hercules, CA) (10 μl/lane), and a current of 200 V was applied to the apparatus for approximately 26 minutes. The gel was stained with Coomassie Blue for 1 hour at RT and washed 3 times with TTBS for every 5 minutes. The membrane was incubated with Streptavidin-HRP (1 μg/ml in TTBS) for 30 minutes and washed 3 more times. Afterwards, the membrane was incubated with Immobilon Chemiluminescent HRP Substrate (Millipore, Billerica, MA) for 1 minute, drained of excess solution, wrapped in plastic wrap, and exposed to X-ray film. Gel staining procedure was performed according to manufacturer's instructions (Pierce Biotechnology, Rockford, IL). After electrolysis, the gel was washed twice with deionized (DI) water for 15 minutes each. GelCode Blue Stain Reagent (Pierce Biotechnology, Rockford, IL) was added to the gel and shaken for 1 hour. The gel was destained with DI water for 30 minutes each. Any protein bands that were considered sufficient were exposed to the Molecular Biology-Proteomics Facility at the University of Oklahoma Health Sciences Center for in-gel tryptic digestion and LC/MS/MS identification. Results were expressed as means ± SD of experiments performed in duplicate. Student t test and one-way ANOVA with Tukey post-tests were used for two-group and multiple-group comparisons, respectively, with differences considered statistically significant at P < 0.05.

Results

In order to determine if extracellular histones and LPS can induce enhanced cytokine production in cell culture, TNF-α and IL-8 cytokine levels in neutrophilic HL-60 and monocyctic THP-1 cell conditioned medium after histones and LPS stimulation were determined throughout a 24 hr period after challenge with triplicate samples taken at 1, 2, 4, and 24 hr. Histone-plus-LPS stimulation was found to increase cytokine levels in a manner that was not additive but rather synergistic (Fig. 1).

Both HL-60 and THP-1 cells had a significant increase in TNF-α production that peaked 4 hr after challenge. At 4 hr for HL-60 cells, TNF-α levels were >200% higher in the combined treatment than with only histones and >300% higher than with only LPS (Fig 1A). In addition TNF-α concentrations in THP-1 cells under combined stimulation were greater than those with only histones by a staggering >1600% and those with LPS by >200% (Fig 1B). A similar trend was observed when examining IL-8 concentrations. For HL-60 cells, those treated with both histones and LPS produced >600% more IL-8 than those treated with histones and >250% more than those treated with LPS alone (Fig 1C). Moreover, THP-1 cells under combined stimulation had IL-8 cytokine production >100% greater than those under LPS stimulation alone; THP-1 cells with only histones produced insignificant amounts of IL-8 (Fig. 1D). These findings indicated that histones and LPS together disproportionately boost cytokine release.

To determine the role of individual histones, cytokine levels in HL-60 and THP-1 cell conditioned medium given different classes of histones plus LPS were measured in triplicate (Fig. 2). In HL-60 cells treated with H2A and LPS, TNF-α cytokine concentrations were the highest, while those of cells treated with H4 and LPS were the lowest (Fig. 2A). However, in the case of TNF-α concentrations in THP-1 cells, histone H2B yielded the highest cytokine production, while H4 still yielded the lowest (Fig 2B). IL-8 levels were highest in HL-60 cells under H2A stimulation and lowest with H4 treatment (Fig. 2C). Nonetheless in THP-1 cells, H2B most effectively stimulated IL-8 cytokine
Figure 1. Histones enhance LPS-induced cytokine production in HL-60 and THP-1 cells. TNF-α or IL-8 levels in the conditioned medium of HL-60 (A and C) or THP-1 cells (B and D) after histones, LPS, or histones-plus-LPS stimulation were determined at the indicated time points. Experiments were performed in triplicate. Student t test: *P < 0.05 compared to LPS treatment.

ANOVA: (A) P = 8.27×10^{-5}. (B) P = 2.85×10^{-11}. (C) P = 7.32×10^{-7}. (D) P = 3.46×10^{-5}.

production, and, once again, H4 yielded lowest cytokine production with a concentration of 0.15 ng/ml (Fig. 2D). Overall, H2A and H2B treatments on average yielded cytokine levels >1800% and >1100% higher when compared to LPS challenge, respectively; histones H2A and H2B were most effective in increasing cytokine production, while H4 contributed the least.

To determine the potential roles of various signaling pathways, kinase inhibitors were used and the resulting cytokine levels were measured; SB202190, U0126, and LY294002 are specific inhibitors for p38, ERK1/2, and PI3K pathways respectively (Fig. 3). TNF-α concentrations in histone and LPS stimulated HL-60 cell conditioned medium were lowest when the cells were pretreated with U0126 and highest when pretreated with LY294002 inhibitor (Fig. 3A). THP-1 cells pretreated with U0126 also had lower levels of TNF-α as opposed to those pretreated with SB202190 and LY294002 (Fig. 3B). Furthermore, HL-60 cells pretreated with U0126 yielded IL-8 levels of 0.22 ng/ml, while those pretreated with SB202190 and LY294002 yielded concentrations of 0.27 ng/ml and 1.09 ng/ml respectively (Fig. 3C). THP-1 cells had significantly decreased IL-8 cytokine production when pretreated with U0126 when compared to the IL-8 levels of cells pretreated with SB202190 or LY294002 (Fig. 3D).

Overall, the induction of cytokines in response to histones and LPS is likely mediated by ERK1/2 or p38 and less likely impacted by PI3K signaling. To identify proteins that interact with histones, cell surface proteins on HL-60 or THP-1 cells were cross-linked with histones, labeled with biotin, and subsequently separated by SDS-PAGE. Western blots were then used to determine the approximate molecular weight (MW) of the biotin-labeled proteins.

As indicated in Fig. 4, there are two distinct proteins of interest: one with MW ~90 kD and the other with MW 37 kD present in both HL-60 and THP-1 cell samples. Meanwhile another SDS-PAGE gel was stained with Coomassie blue in order to obtain desired protein bands of ~90 kD and 37 kD. These protein bands were excised from the gel and then underwent in-gel tryptic digestion and LC/MS/MS...
Discussion
Through ELISA cytokine assays, a strong relationship for histone-facilitated cytokine production in LPS-induced hyper-inflammation was clearly indicated (Fig. 1). HL-60 cells after histone-plus-LPS stimulation had significantly greater TNF-α and IL-8 cytokine production at 2 hr and 4 hr after challenge (Fig. 1A, 1B). Similarly, THP-1 cells had dramatic increases in TNF-α and IL-8 production when given histone-plus-LPS challenge as opposed to either treatment alone (Fig. 1C, 1D). Overall, histone-plus-LPS stimulation synergistically yielded cytokine levels up to >1600% and >300% greater than separate histone and LPS stimulation, respectively. These results indicate that the effect of histone-plus-LPS stimulation did not yield an additive relationship, but rather a unique connection that disproportionately promoted cytokine production.

After discovering the intimate relationship between extracellular histones and LPS, histones H1, H2A, H2B, H3, and H4 were individually compared to determine the potency of each histone type regarding cytokine production (Fig. 2). ELISA tests indicated that cells given H2A on average produced cytokine levels >1800% higher when compared to LPS treatment. Similarly, H2B increased cytokine
Figure 3. Kinase inhibitor effect on histone and LPS-induced cytokine production in HL-60 and THP-1 cells. TNF-α or IL-8 levels in the conditioned medium of HL-60 (A and C) or THP-1 cells (B and D) after histones, LPS, or histones-plus-LPS stimulation in the absence or presence of indicated kinase inhibitor were determined 4 hr after challenge. Experiments were performed in triplicate. Student t test: *P < 0.05 compared to LPS treatment. ANOVA: (A) P = 0.0374; (B) P = 6.54×10^-3; (C) P = 0.0131; (D) P = 6.15×10^-3. SB=SB202190, U=U0126, LY=LY294002.

production >1100%. Histone H4 played a considerably less active role in promoting cytokine production, inducing >100% boost in cytokine concentrations. Hence histone H2A and H2B play major roles in facilitating LPS-induced cytokine production, while histone H4 was significantly less potent.

In determining the signaling pathways responsible for histone and LPS effect, p38K, ERK1/2, and PI3K inhibitors were given to the cells and subsequent cytokine production was measured (Fig. 3). U0126 was clearly most effective in suppressing TNF-α and IL-8 production by lowering the average cytokine concentration by nearly 90%. This indicates that the corresponding pathway, ERK1/2, is likely involved in histone-mediated LPS-induced hyper-inflammation. It is also significant to note that SB202190 was relatively effective in blocking cytokine production, indicating that the role of p38 pathway is important as well. However, as the LY294002 kinase inhibitor had minimal effect on cytokine concentrations, the PI3K signaling mechanism is unlikely to be involved in this signaling pathway.

In an attempt to discover signaling transduction receptors for histones, biotin-labeled histones were cross-linked with cell surface proteins on HL-60 or THP-1 cells using tri-functional cross-linker, Sulfo-SBED. A biotin group was subsequently conjugated on the cell surface, marking the proteins that interacted with histones. Western blots were used to determine the molecular weights of target proteins. The most abundant biotin-labeled proteins were present at MWs of 37 kD and ~90 kD. MPO, an 89 kD protein found in HL-60 cell
sample, was the only protein band able to be identified, indicating a possible role for MPO in histone-mediated inflammatory signaling in neutrophils. Coincidentally, the MWs of the remaining target protein bands (37 kD and ~90 kD) roughly match with those of scavenger receptor class E and scavenger receptor class A, respectively. However, LC/MS/MS of additional samples is needed for more conclusive information.

This study identifies a novel relationship between extracellular histones and LPS to induce hyper-inflammation in human HL-60 and THP-1 cell lines. In addition histones H2A and H2B were specifically shown to be effective stimuli of cytokine production, while H4 displayed the least involvement. ERK1/2 and p38 signaling were primarily involved in this process, and candidate receptor proteins for histones were found with MW 37 kD and ~90 kD.

This study exposes the interaction between histones and LPS – a relationship that must be fully understood to effectively treat sepsis. From this connection, future works can determine the specific signaling that occurs between extracellular histones and LPS; such signals can then be blocked, effectively reducing inflammation from substantial cytokine production.

Histone H2A and H2B were found to be the primary contributors of hyper-inflammation in this cell type. Thus additional studies should be conducted to target the specific receptors and to investigate possible treatments that involve the blockage of these histones, thereby significantly reducing inflammatory response.

ERK1/2 was discovered to play a very important role in the apparent synergism between extracellular histones and LPS. Since SB202190 and U0126 appear to have relatively similar inhibition effects, the p38 kinase pathway seems to be involved in histone-mediated hyper-inflammatory signaling as well. However, future studies are necessary to elucidate the roles of ERK1/2 and p38 in regards to the relationship between histones and LPS.

Candidate histone receptors were uncovered at MW 37 kD and ~90 kD via cross-linking with histone on cell surface, and MPO found in the HL-60 cell sample was identified as one of such proteins. MPO is an enzyme known to mediate the activation of neutrophils as well as p38 MAPK through interaction with integrins. However the question of how histones interact with MPO to enhance LPS-induced inflammatory response is still unresolved and should be subjected to future investigations. The MWs of class E scavenger receptors and class A scavenger receptors correlate with those of the remaining proteins, so it is possible that the scavenger receptors are the other proteins of interest that were unable to be identified. Furthermore, scavenger receptors have been shown to play roles in the signaling of inflammation. However, from additional LC/MS/MS studies, more information will be provided regarding the remaining proteins of interest.

Sepsis is caused by both Gram-positive and Gram–negative bacteria; therefore lipopeptides may be used in place of LPS in following studies. Consequently, the relationships between histones, Gram-positive bacteria, and inflammatory response would be clarified, and many answers would be provided to yet another common cause of sepsis.

Figure 4. Western blotting of biotin-labeled proteins interacted with histones. 37 kD and ~90 kD cell surface biotin-labeled proteins from HL-60 and THP-1 cells were indicated.

Figure 5. Coomassie blue staining of the same samples separated by SDS-PAGE shown in Fig. 4. With exception to MPO, no obvious target bands could be obtained for tryptic digestion and LC/MS/MS analysis.

References
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Acknowledgements
We thank Dr. Charles Esmon for generously sponsoring this study at the Oklahoma Medical Research Foundation and Xiaomei Zhang for assistance.