

Toll-like Receptor 4 Mediates Histone Subunit H3-induced Endothelial Dysfunction in Human Lung Endothelium

Pratap Karki¹, Chenou Zhang¹, Yue Li¹, Kamolrip Promnares², Konstantin G. Birukov², Anna A. Birukova¹

¹Division of Pulmonary and Critical Care Medicine, Department of Medicine, ²Department of Anesthesiology, University of Maryland School of Medicine, Baltimore, MD 21201

INTRODUCTION

- Severe trauma and associated multiple organ failure is one of the leading causes of death. In trauma and sepsis patients, cell damage in the injured tissues leads to extracellular release of DNA-binding proteins histones known as danger-associated molecular pattern (DAMP) which trigger host immune response and vascular dysfunction.
- Elevated histone plasma levels serves as a marker of poor outcomes in severely injured patients and trauma non-survivors. Also, patients undergoing trauma-associated lung injury show much higher levels of circulating plasma histones in the range of 10-230 µg/ml, as compared to median 2 µg/ml in healthy donors.
- Patients with 50 µg/ml or greater histone levels develop respiratory failure and similar doses of histones cause endothelial permeability and cell death.
- Endothelial dysfunction is a major pathological hallmark of severe lung injuries such as acute respiratory distress syndrome (ARDS) and ARDS patients present a much higher levels of extracellular histones of 50-96 µg/ml compared to only 1-3 µg/ml in healthy controls.
- Studies have now established that high levels of H3 or H4 histone subunits are responsible for endothelial damage and ensuing lung injuries, but precise mechanisms involved in histone-induced endothelial dysfunction-derived pathologies remains to be elucidated.
- To expand our understanding of the role of histones in endothelial barrier disruption and inflammation, this study examined the role of various signaling pathways including toll-like receptors (TLRs), RAGE, and NLRP3.

MATERIALS AND METHODS

Cell culture: Human pulmonary artery endothelial cells (HPAECs) at passages 5-7 were cultured using the complete growth media obtained from Lonza. Cells were exposed to purified recombinant histone H3 or other subtypes in the complete growth media supplemented with 2% FBS.

Endothelial barrier function. Endothelial permeability was measured by monitoring transendothelial electrical resistance (TER) using an electric cell-substrate impedance sensing system, ECIS Z (Applied Biophysics). Endothelial permeability to macromolecules was determined by utilizing express permeability testing (XPerT) assay developed by our group.

Immunofluorescence microscopy was employed to visualize endothelial junction assembly by staining cells with VE-cadherin.

qPCR. The mRNA expression analysis of selected endothelial inflammation marker genes was carried out by quantitative real time PCR. The fold changes in mRNA transcripts were calculated using GAPDH as an internal control and non-stimulated cells as baseline control.

Protein expression. Western blot was performed to determine the levels of various proteins associated with endothelial inflammation. Sub-cellular fractionations and biotinylation assays were carried out and respective protein samples were run on western blot.

ELISA. The secretory levels of ICAM-1 and other pro-inflammatory cytokines such as IL-6 and IL-8 was determined by ELISA using the commercially available kits (R & D systems).

SUMMARY AND CONCLUSION

- The first comprehensive analysis of effects of histone subunits on lung endothelial function revealed contrasting differences between the histone isotypes. While H1, H2A, and H2B did not affect EC barrier function and inflammation, H3 and H4 subunits caused pronounced endothelial permeability and inflammatory responses.
- Histone H3-induced endothelial dysfunction was neither affected by inhibitors of RAGE, NLRP3 inflammasome signaling, nor it involved Rho-mediated contractile mechanisms of endothelial barrier disruption. Rather, histone-induced endothelial permeability was associated with breakdown of cell junctions assembly.
- TLR4 inhibitor CLI-095 significantly attenuated histone H3-induced endothelial permeability and completely blocked inflammatory signaling cascades. TLR1/2 inhibitor CU-CPT22 had no barrier protective or anti-inflammatory effects against H3.
- These results establish that TLR4 is a critical mediator of histone H3-induced endothelial dysfunction and suggest the therapeutic potential of TLR4 inhibitors in mitigating histone-caused cellular injuries.

References:

1. Abrams, S.T., N. Zhang, J. Manson, T. Liu, C. Dart, F. Baluwa, S.S. Wang, K. Brohi, A. Kipar, W. Yu, G. Wang, and C.H. Toh, *Circulating histones are mediators of trauma-associated lung injury*. *Am J Respir Crit Care Med*. 2013; **187**(2): p. 160-9.
2. Silk, E., H. Zhao, H. Weng, and D. Ma, *The role of extracellular histone in organ injury*. *Cell Death Dis*. 2017; **8**(5): p. e2812.
3. Lv, X., T. Wen, J. Song, D. Xie, L. Wu, X. Jiang, P. Jiang, and Z. Wen, *Extracellular histones are clinically relevant mediators in the pathogenesis of acute respiratory distress syndrome*. *Respir Res*. 2017; **18**(1): p. 165.

Funding : RO1 HL155051, RO1 HL152761 (AAB)

RESULTS

Histones H3 and H4 induce endothelial permeability

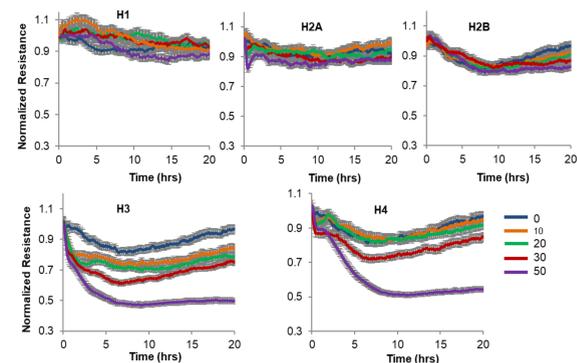


Figure 1. Only histone subunits H3 and H4 induce endothelial permeability. HPAECs were stimulated with indicated doses of histone subtypes and endothelial permeability was determined by monitoring TER over indicated time.

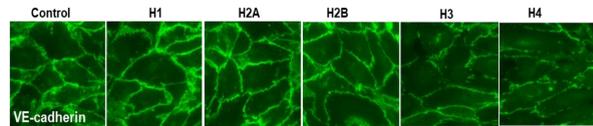


Figure 2. Only histone subunits H3 and H4 cause endothelial barrier disruption. HPAECs were stimulated with 50 µg/ml of indicated histone subtypes for 6 h and immunofluorescence staining with VE-cadherin was performed to visualize adherens junctions.

Histones H3 and H4 induce endothelial inflammation

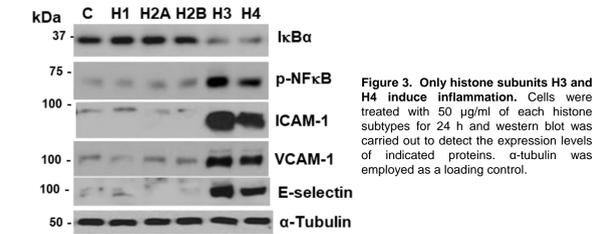


Figure 3. Only histone subunits H3 and H4 induce inflammation. Cells were treated with 50 µg/ml of each histone subtypes for 24 h and western blot was carried out to detect the expression levels of indicated proteins. α-tubulin was employed as a loading control.

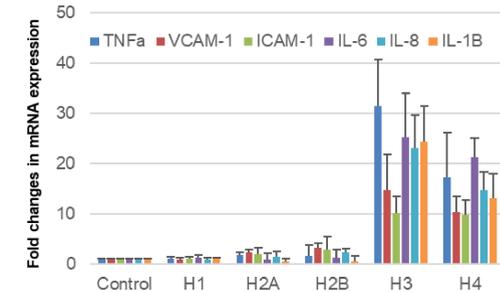


Figure 4. Only histone subunits H3 and H4 induce inflammation. Cells were treated with 50 µg/ml of each histone subtypes for 3 h and qPCR was carried out to detect the mRNA expression levels of indicated inflammatory marker genes.

Histone H3 induces inflammation in dose- and time-dependent fashions

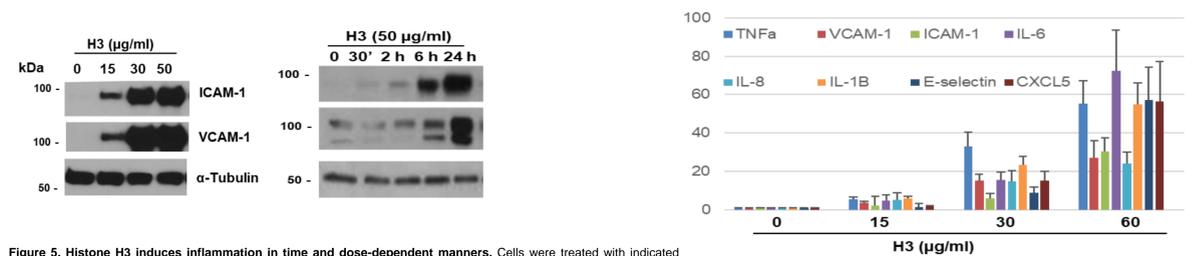


Figure 5. Histone H3 induces inflammation in time and dose-dependent manners. Cells were treated with indicated concentration of histone H3 for specified time periods. Western blot and RT-PCR were carried out to determine the protein and mRNA expression levels of indicated endothelial inflammation marker molecules.

TLR4 inhibition rescues Histones H3-induced endothelial permeability and inflammation

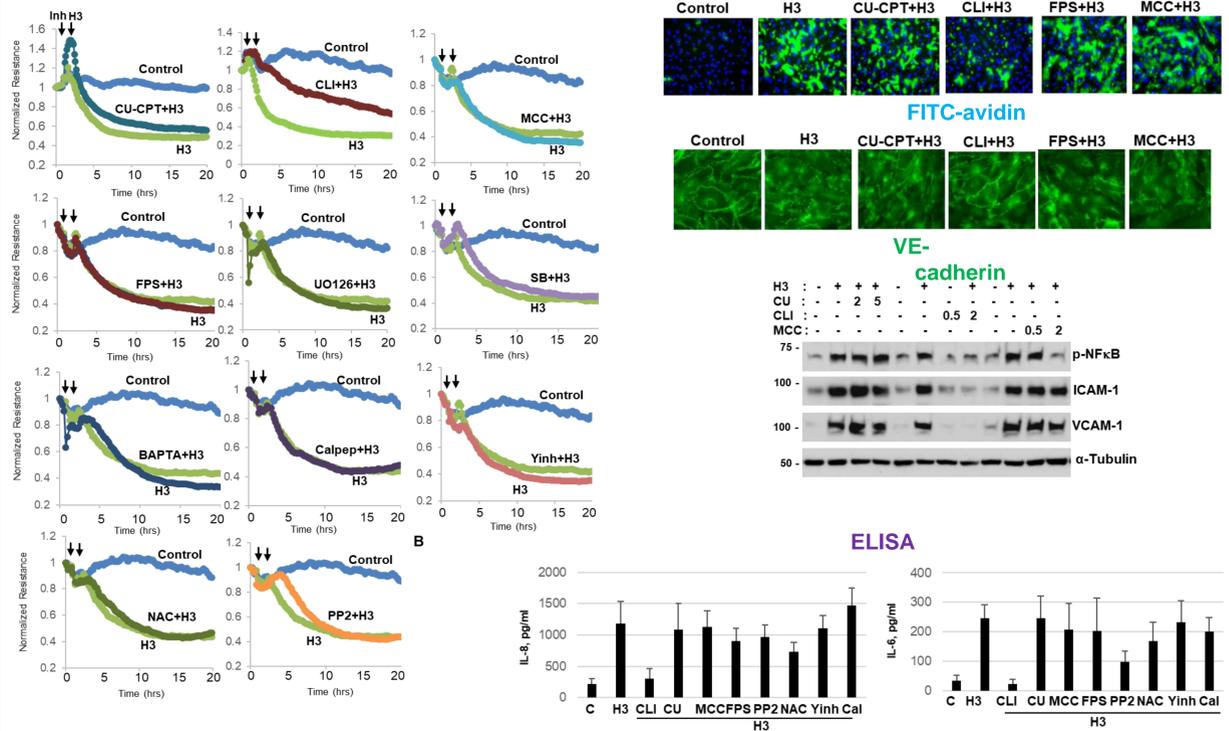


Figure 8. TLR4 inhibition rescues histone H3-induced endothelial permeability and inflammatory responses. HPAECs were pre-treated with CU-CPT (TLR1/2 inhibitor, 5 µM), or CLI-095 (TLR4 inhibitor, 1 µM), or MCC350 (NLRP3 inflammasome inhibitor, 2 µM), or FPS ZMI (RAGE antagonist, 3 µM), or UO126 (MEK inhibitor, 5 µM), or SB203580 (p38 MAPK inhibitor, 10 µM), or BAPTA-AM (Ca²⁺ chelator, 1 µM), or Calpeptin (Calpain inhibitor, 1 µM), or Y27632 (Rho kinase inhibitor, 1.5 µM), or NAC (anti-oxidant, 1 mM), or PP2 (Src inhibitor, 10 µM) for 30 min, followed by addition of 50 µg/ml of histone. ECIS, XPerT, and VE-cadherin immunostaining was done to analyze endothelial permeability. Similarly, western blot, ELISA, and RT-PCR were carried out to check the expression of proteins, secretory levels, and mRNA transcripts of indicated pro-inflammatory mediators.