Published in final edited form as:

J Trauma Acute Care Surg. 2018 February ; 84(2): 386–392. doi:10.1097/TA.0000000000001753.

Tubastatin A Prevents Hemorrhage-Induced Endothelial Barrier Dysfunction

Peter J. Bruhn, MD^{a,b}, Vahagn C. Nikolian, MD^a, Ihab Halaweish, MD^a, Zhigang Chang, MD^a, Martin Sillesen, MD PhD^{a,b}, Baoling Liu, MD^a, Yongqing Li, MD PhD^a, and Hasan B. Alam, MD, FACS^a

^aDepartment of Surgery, University of Michigan, Ann Arbor, Michigan

^bDepartment of Surgical Gastroenterology, Rigshospitalet, University of Copenhagen Hospital, Copenhagen, Denmark

Abstract

Background—Microvascular hyperpermeability resulting from endothelial barrier dysfunction (EBD) is associated with worse clinical outcomes in trauma-induced hemorrhagic shock. We have previously shown that treatment with Tubastatin A (TubA), a histone deacetylase 6 inhibitor, improves outcomes in animal models of shock. In this study, we investigate whether TubA treatment may prevent trauma-related EBD.

Methods—Wistar-Kyoto rats subjected to 40% hemorrhage were treated with TubA or vehicle control. Acute lung injury (ALI) was assessed histologically from tissues harvested 6 hours post-hemorrhage. In vitro, human umbilical vein endothelial cells (HUVECs) were cultured in EGM BulletKit medium. Medium was exchanged for glucose-free Dulbecco's Modified Eagle Medium (0.5% fetal bovine serum) with or without TubA, and cells were placed in an anoxic chamber (5% CO₂, 95% N₂, 20–48 hours). Expression of acetylated tubulin and hypoxia-inducible factor 1 α (HIF-1 α) were measured by Western blot. Soluble Interleukin Adhesion Molecule-1 concentration within the medium, a marker of endothelial integrity, was determined using enzyme-linked immunosorbent assay. Monolayers were assessed for permeability via transwell assays using fluorescein isothiocyanate-labeled albumin.

Corresponding Author: Hasan Alam, MD, FACS, Norman Thompson Professor of Surgery, Head of General Surgery, University of Michigan Health System, 2920 Taubman Center/5331, University of Michigan Hospital, 1500 E. Medical Center Drive, Ann Arbor, MI 48109-5331, alamh@med.umich.edu.

Conflicts of Interest

The authors have no conflicts to declare.

Meeting presentation: This study was presented at the 2016 annual Clinical Congress of the American College of Surgeons, October 16-20, 2016, Washington, D.C.

Author Contributions

Study conception and design: PJB, VCN, IH, HBA

Acquisition of data: PJB, ZC

Analysis and interpretation of data: PJB, VCN, ZC, BL, YL

Drafting of manuscript: PJB

Critical revision: VCN, MS, HBA

Results—Rats treated with TubA had significantly reduced ALI relative to vehicle control. In vitro, TubA significantly attenuated anoxia-induced hyperpermeability, HIF-1 α expression, and glycocalyx shedding.

Conclusions—Our findings demonstrate that TubA prevents hemorrhage-induced ALI in rats. Additionally, we have shown that TubA prevents anoxia-induced EBD in vitro. Taken together, these results suggest that TubA could attenuate microvascular hyperpermeability related to hemorrhagic shock.

Keywords

Endothelial barrier dysfunction; histone deacetylase inhibitors; acute lung injury; endothelial hyperpermeability; injury

Background

Trauma-related injuries are the leading cause of death in individuals younger than 46 years of age(1). Endothelial barrier dysfunction (EBD) might play a role in trauma-related morbidity and mortality(2). In intact blood vessels, endothelial cells and the endothelial glycocalyx layer (EGL) provide a semipermeable barrier, through which fluid and solutes can move in a regulatable manner(3, 4). EBD-related endothelial hyperpermeability is known to cause hypovolemic shock, organ edema and poor tissue perfusion, leading to numerous adverse events, including acute lung injury (ALI), abdominal compartment syndrome and multiple-organ dysfunction syndrome (MODS)(5–8). Trauma patients who survive the initial hemorrhagic shock (HS) often develop MODS, which contributes to trauma-related morbidity and mortality(9, 10). Tissue hypoxia with anaerobic metabolism is considered a major contributor to the development of post-HS MODS(11).

Several pathophysiological mechanisms work in concert to cause EBD. Increased paracellular permeability is a central event in EBD, and can be caused by numerous physiological conditions(12–14). Specifically, stress-induced dysfunction of endothelial tight junctions (ETJ) complexes and cytoskeletal contraction causes paracellular gap formation, a key feature of EBD(15–17). ETJ complex function and endothelial cell contraction is modulated by multiple up-stream effectors, which in turn are modulated through a number of physiological response mechanisms(16). One of these effectors is hypoxia-inducible factor 1 α (HIF-1 α), which plays a role in EBD under hypoxic conditions, through cytoskeletal contraction and compromised ETJ integrity(16, 17). HIF-1 α also exacerbates EBD through increased expression of myosin light chain kinase protein, vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase(18–20).

Restoring perfusion to hypoxic tissue through fluid replacement is still the main focus of resuscitation therapies for HS, but this strategy is often unsuccessful because of impaired mitochondrial function(11, 21). Our group has previously investigated the role of pharmacological resuscitation with histone deacetylase (HDAC) inhibitors in maintaining cellular viability and function during shock(22). Shock can decrease an HDAC-mediated decrease in cellular acetylation, which affects the functions of numerous regulatory proteins

and genes(22, 23). In fact, pharmacological inhibition of HDACs has been shown to improve survival in rat and swine models of lethal HS(24–27).

To date, 18 HDACs have been identified(9). HDAC6 is a mainly cytoplasmic enzyme which regulates pathways in many biological processes, including cytoskeletal rearrangement and cell-cell interaction(28). Our group has previously shown that HDAC6 inhibition with Tubastatin A (TubA) leads to improved survival in a rat model of HS(24). In this study, we sought to determine whether HS-induced acute lung injury and EBD could be prevented by HDAC6 inhibition.

Methods

Sub-lethal HS Protocol and Lung Tissue Harvest

Male Wistar-Kyoto rats (227-311g) were purchased from Charles River Breeding Laboratories, Inc. (Chicago, IL). Animals were allowed food and water ad libitum.

TubA (70mg/kg, Calbiochem, San Diego, CA) solution was prepared with dimethyl sulfoxide (DMSO, 1μL per gram bodyweight, Sigma-Aldrich, St. Louis, MO) as solvent. Inhalation anesthesia was induced with 4% isoflurane (Abbott Laboratories, North Chicago, IL) mixed with air in an induction chamber. Between 0.8% and 1.5% isoflurane was delivered via nose cone, using a veterinary multichannel anesthesia delivery and vaporizer system (Kent Scientific Corporation, Torrington, CT). Body temperature was controlled using an automated heating pad. Local anesthesia was achieved by injection with bupivacaine (0.2mL of 0.25%, APP Pharmaceuticals, LLC. Schaumburg, IL). Femoral artery cannulation with polyethylene 50 catheter (Clay Adams, Sparks, MD) was performed using a micro cut-down technique, and was used for blood withdrawal and hemodynamic monitoring (Ponemah Physiology Platform, Gould Instrument Systems, Valley View, OH).

To eliminate any survival bias in tissue sample collection, a sublethal HS protocol was designed. Total blood volume was calculated (estimated total blood volume [mL] = weight [g] × 0.06 [mL/g] + 0.77 mL)(29), and 40% volume was drawn over 10 minutes, followed by 30 minutes of unresuscitated shock. Next, rats were given either TubA (70 mg/kg, treatment group, n=6) or vehicle (DMSO, 1μL/g, vehicle group=4). No additional fluid resuscitation was administered. Rats were euthanized 6 hours post-HS, lung tissues were harvested, washed with cold saline, frozen in liquid nitrogen, and stored at –80°C. For microscopy, the tissue was paraffin fixed. Vehicle and treatment group tissues were compared with lung tissues from normal rats (control group, n=3).

Acute Lung Injury Assessment

An independent pathologist, who was blinded to the study groups, assessed ALI through scoring of lung histologic injury. Hematoxylin-eosin stained slides of lung tissues were scored from 0-4 to quantify increasing severity of leukocyte infiltration, hemorrhage and congestion, as previously published to generate composite ALI scores(30, 31).

Cell culture and treatments

Tubastatin A (Calbiochem, San Diego, CA) was diluted in phosphate buffered saline (PBS) at a stock concentration of 500 μ M, aliquoted and frozen for later experiments. Human umbilical vein endothelial cells (HUVEC) (CC-2519, Lonza, Basel, Switzerland) were grown in EGM BulletKit medium (Lonza, Basel) in a humidified atmosphere of 95% air and 5% CO₂. Cells were used for experiments from passage 3-8. After cells had reached 70% confluency, medium was exchanged with glucose-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.5% fetal bovine serum (FBS), with and without 15 μ M TubA. Cells subjected to normoxic conditions were placed in a humidified chamber with 95% air and 5% CO₂. For anoxic treatment, HUVECs were placed in a MIC-101 modular incubator chamber (Billups-Rothenburg, Del Mar, CA, USA), which was flushed with 5% CO₂ and 95% N₂ for 15 minutes, and maintained in a 37°C environment for 24-48 hours. The anoxic conditions for final testing were selected after a series of pilot experiments. Our goal was to stress the cells without a major impact on their actual viability, as proposed by Lee et al(32).

The study groups were: (1) normoxia, (2) normoxia plus 15 μ M TubA, (3) anoxia, and (4) anoxia plus 15 μ M TubA.

Monolayer Permeability Assay

HUVECs were seeded onto Polyester Membrane Transwell-Clear Inserts (0.4 μ m pore, 6.5 mm diameter; Corning Inc., Corning, NY), and exposed to the treatment once they were at 100% confluency (3 days after seeding) for 30 hours. Next, the medium in all inserts was replaced with DMEM with 0.5% FBS and 10mg/ml fluorescein isothiocyanate conjugated (FITC) albumin (66 kDa) (Sigma-Aldrich, St Louis, MO). Medium in the bottom well was replaced with DMEM with 0.5% FBS. After 1 hour, samples were carefully removed in triplicate from the bottom well and read in a fluorometer (FluorStar Optima, BMG Labtech, excitation 485 nm, emission 520 nm). All experiments were repeated six times.

Western Blot Analysis

HUVECs were harvested after 24 hours incubation and were prepared using a whole cell lysis buffer kit (BD Biosciences, Bedford, MA). Equal amounts of samples were added to each lane after determination of protein concentration using the bicinchoninic acid method. Proteins were detected using antibodies against acetylated α -tubulin (Lys40, #3971, Cell Signaling Technology Inc., Danvers, MA), and HIF-1 α (HIF-1 α , #3716, Cell Signaling Technologies Inc., Danvers, MA) and incubated with corresponding fluorescent conjugated secondary antibodies (Alexa Fluor Dyes, Molecular Probes; Life Technologies, Carlsbad, CA). Signals were obtained using Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). The results were quantified using Image Studio Lite software (LI-COR Biosciences, Lincoln, NE). Ac-tubulin experiments were repeated five times; HIF-1 α experiments were repeated four times.

Glycocalyx Shedding Assay

After 48 hours of treatment, cell-culture supernatants were removed, centrifuged at 1000 $\times$ g for 15 minutes and stored at -80°C. Samples were thawed immediately before analysis and

assayed in triplicate for concentrations for human sICAM-1 using a quantitative enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (Quantikine, R&D Systems Inc., Minneapolis, MN). Absorbance was set to 450 nm with wavelength correction set to 540 nm and 570 nm. Experiments were repeated three times for groups (1), (2) and (4) and five times for group (3).

Statistical analysis and data presentation

Data in figures are presented as group means $\pm$ standard deviation (SD). Continuous variables were tested for normal distribution using the Shapiro-Wilk test. Continuous variables between three or more groups were compared using the Kruskal-Wallis H test, and intergroup comparisons were conducted using the Dunn's multiple comparisons test. Statistical analysis was performed using IBM SPSS Statistics (version 19 for iOS, IBM Corporation, Armonk, NY). Figures were created using GraphPad Prism (version 6 for iOS, GraphPad Software, San Diego, CA).

Results

Acute Lung Injury

Compared to vehicle control, treatment with TubA significantly attenuated ALI (mean ALI 3.25 and 1, respectively; $p=0.032$) (figure 1). There was no significant difference in ALI between normoxic control and treatment groups (1.33 and 1, respectively; $p=0.786$). Hemorrhagic shock caused an increase in ALI that was not statistically significant (1.33 and 3.25, respectively; $p=0.400$).

Monolayer Permeability Assay

A key feature of EBD is paracellular hyperpermeability. We therefore thought to assess monolayer permeability using a transwell assay of HUVEC monolayers after 30 hours normoxia or anoxia, with or without TubA. Anoxia caused a statistically significant mean permeability increase of 68.8% when compared to normoxic controls ($p=0.004$) (figure 2). Treatment with TubA and anoxia caused a mean permeability increase of 5%, which was significantly less than anoxia alone ($p=0.004$). There was no significant difference in permeability between normoxic control and anoxia plus TubA groups ($p=0.240$).

Tubulin acetylation

We analyzed the concentrations of acetylated α -tubulin, a marker for HDAC6 inhibition (figure 3). After 24 hours, treatment with TubA caused a 92-fold mean increase of acetylated α -tubulin levels in normoxic cells, compared with non-treated normoxic cells ($p=0.008$). TubA caused a 23-fold mean increase of acetylated α -tubulin levels in anoxic cells, compared with non-treated anoxic cells ($p=0.008$). There was a 68-fold mean increase in acetylated α -tubulin levels in treated anoxic cells, compared with non-treated normoxic cells ($p=0.008$). There was a non-significant 3-fold mean increase in non-treated anoxic cells, compared with non-treated normoxic cells ($p=0.548$).

Hypoxia-Inducible Factor 1 α

As HIF-1 α has been shown to play an important role in EBD, we assessed expression using Western blot (figure 3). After 30 hours of anoxia treatment, there was a 188% mean increase in HIF-1 α expression in non-treated cells, compared with non-treated normoxic cells ($p=0.029$). Treatment with TubA caused a 56% decrease in HIF-1 α expression, compared with non-treated normoxic cells, which was a significantly lower expression than in non-treated anoxic cells ($p=0.029$). The 56% decrease in HIF-1 α expression found in TubA-treated anoxic cells, compared with non-treated normoxic cells, was statistically significant ($p=0.029$).

Glycocalyx Shedding

A marker for glycocalyx shedding, sICAM-1, was quantified in cell culture supernatants (figure 4). 48 hours of anoxic insult induced a significant 175% mean rise in sICAM-1 concentration, compared to non-treated normoxic cells ($p=0.036$). Treatment with TubA and anoxia caused a 6% decrease in sICAM-1 concentration, which was significantly less than anoxia alone ($p=0.036$). The 6% decrease found in TubA-treated anoxic cells, compared with non-treated normoxic cells, was not statistically significant ($p=0.700$).

Discussion

Hemorrhagic shock is a major cause of death in the immediate post-traumatic hours(1, 33). The current treatment for HS focus on replacing the lost blood, but this often fails to correct the cellular disturbances(11, 21). For the Military, the logistical challenges of the battlefield make large volume resuscitation impractical, and increasingly efforts are being focused on pharmacological adjuncts that can make resuscitation more effective, or even eliminate the need for pre-hospital fluid administration(22). Our group has investigated pharmacological treatment with HDAC inhibitors as a promising treatment to maintain life under austere circumstances (“bridge” therapy during evacuation to higher levels of care). We have shown improved survival and significantly attenuated organ injury following treatment with non-selective HDACI in animal models of HS, polytrauma, sepsis and traumatic brain injury (TBI)(22). In fact, we have just finished a federally funded phase 1 clinical trial ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study?term=NCT01951560) Identifier:NCT01951560) to test valproic acid treatment (a non-selective HDACI), and are ready to start a phase 2 trial in trauma patients. Although effective, there are concerns related to the large doses of non-selective HDACI that are needed to achieve a biological effect(22). Recently, more potent isoform specific HDACI have become available, and they offer the potential for using lower doses, and theoretically a better safety profile. However, isoform-specific inhibition of HDAC6 with Tubastatin A in trauma is a largely unstudied concept. It has previously been reported that HS-induced tissue damage occurs not only due to lack of tissue perfusion, but also due to impaired mitochondrial function(11, 21), and ineffective oxidative phosphorylation(34). Our group has previously shown that HDAC6-inhibition with TubA protects cells against HS-induced mitochondrial dysfunction(24). The underlying mechanisms, however, remain poorly defined.

In this study, we have shown that hemorrhage causes ALI in rats, which has previously been linked with EBD as a causative mechanism(35–37). We have also shown that treatment with

Tubastatin A prevents hemorrhage-induced ALI, which offers a potential explanation for the beneficial outcomes reported previously(24). To further elucidate the mechanisms by which trauma-induced EBD is ameliorated, we designed an in-vitro model using HUVECs. We found that exposure of HUVECs to anoxia and hemorrhage-mimetic medium caused a significant increase in HIF-1 α expression, with a consequential increase in monolayer permeability and glycocalyx shedding. Exposure of the anoxic cells to Tubastatin A prevented these effects. Taken together, these findings suggest that HDAC6 inhibition exerts a protective effect by maintaining the integrity of the vascular endothelium, with attenuation of downstream effects such as ALI.

As previously mentioned, HIF-1 α is known as an important cellular mediator of hypoxia-induced EBD(17, 38). HIF-1 α exerts this effect, at least in part, through pathways that compromise ETJ integrity(17, 39). HIF-1 α -induced cytoskeletal contraction along with decreased ETJ complex integrity leads to formation of paracellular gaps and consequential endothelial monolayer hyperpermeability(16, 17, 40).

The vascular endothelium is covered by a thin layer of glycoproteins and proteoglycans, the endothelial glycocalyx (EGL)(4, 41). The EGL plays an important role as a regulatable barrier to transport of proteins, fluids and inflammatory cells, as well as in coagulation and as a mechano-transducer of shear and pressure stress to the vascular wall(4, 41, 42). Upon stimulation with proinflammatory cytokines, reactive oxygen species, complement activation products or endotoxin, the endothelium becomes activated and changes phenotype(43). During endothelial activation, as in the setting of hemorrhagic shock, the EGL is shed, the endothelium becomes procoagulant, upregulates cellular adhesion molecules, and vascular permeability is greatly enhanced(43–47). Soluble ICAM-1 has been shown to be a marker of this physiological mechanism in vitro(48).

Our study was designed as a proof-of-concept study, and therefore holds certain inherent limitations. For the in vitro experiments, we used cultured HUVECs due to their reliability and widely applied use as a laboratory model for the human endothelium in general. Cultured HUVECs form a homogenous and nonexchanging barrier. In an experimental setting, this provides for good reproducibility, but poor abstraction. Studies of cultured cells also do not allow for influences of factors such as paracrine functions of local auxiliary cells, and in vitro studies have shown that permeability of cultured monolayers is far greater than in vivo(49). We addressed this issue by conducting in vivo as well as in vitro experiments, which showed consistent results. This consistency between microscopic and macroscopic levels mirror a previous study by Ramirez et al which showed that ischemia-reperfusion of in vivo micro-cannulated rat mesenteric venules indeed causes EBD(50). Finally, we only tested the HIF-1 α pathway as a mediator of EBD. Although HIF-1 α is an important mediator of EBD, other pathways are certainly relevant and should be analyzed in the setting of HDAC6-inhibition.

In conclusion, our study demonstrates that HDAC6 inhibition with TubA prevents hemorrhage-induced ALI in rats. Additionally, we have shown that TubA prevents anoxia-induced EBD in vitro. We showed this through demonstration of ALI prevention in a rat model of sublethal HS, and prevention of endothelial monolayer hyperpermeability in an

vitro model of HS. Taken together, these findings may relate to our previously reported findings that HDAC6 inhibition improves survival in a lethal model of HS in rats. Our data suggests that HIF-1 α plays a central role in mediating trauma-induced EBD, and inhibition of HIF-1 α prevents the detrimental effects of hemorrhage. Our results indicate HDAC6 should be further studied as a promising target for inhibition in future posttraumatic pharmacological resuscitation therapies.

Acknowledgments

Source of Funding:

This research was funded by a grant from the National Institute of Health, 2 R01-GM084127 awarded to HBA.

References

1. Rhee P, Joseph B, Pandit V, Aziz H, Vercruysse G, Kulvatunyou N, Friese RS. Increasing trauma deaths in the United States. *Ann Surg*. 2014 Jul; 260(1):13–21. [PubMed: 24651132]
2. Zikria BA, Bascom JU. Multiple organ failure syndrome. *JAMA*. 1994 Jul 20; 272(3):202–3.
3. Stevens T, Garcia JG, Shasby DM, Bhattacharya J, Malik AB. Mechanisms regulating endothelial cell barrier function. *Am J Physiol Lung Cell Mol Physiol*. 2000 Sep; 279(3):L419–22. [PubMed: 10956614]
4. Pries AR, Secomb TW, Jacobs H, Sperandio M, Osterloh K, Gaehtgens P. Microvascular blood flow resistance: role of endothelial surface layer. *Am J Physiol*. 1997 Nov; 273(5 Pt 2):H2272–9. [PubMed: 9374763]
5. Gulati A. Vascular Endothelium and Hypovolemic Shock. *Curr Vasc Pharmacol*. 2016; 14(2):187–95. [PubMed: 26638794]
6. Kasa A, Csontos C, Verin AD. Cytoskeletal mechanisms regulating vascular endothelial barrier function in response to acute lung injury. *Tissue Barriers*. 2015; 3(1-2):e974448. [PubMed: 25838980]
7. Duchesne JC, Kaplan LJ, Balogh ZJ, Malbrain ML. Role of permissive hypotension, hypertonic resuscitation and the global increased permeability syndrome in patients with severe hemorrhage: adjuncts to damage control resuscitation to prevent intra-abdominal hypertension. *Anaesthesiol Intensive Ther*. 2015; 47(2):143–55. [PubMed: 25293626]
8. Bhatia M, Moochhala S. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. *J Pathol*. 2004 Feb; 202(2):145–56. [PubMed: 14743496]
9. Li Y, Alam HB. Creating a pro-survival and anti-inflammatory phenotype by modulation of acetylation in models of hemorrhagic and septic shock. *Adv Exp Med Biol*. 2012; 710:107–33. [PubMed: 22127890]
10. Sauaia A, Moore EE, Johnson JL, Chin TL, Banerjee A, Sperry JL, Maier RV, Burlew CC. Temporal trends of postinjury multiple-organ failure: still resource intensive, morbid, and lethal. *J Trauma Acute Care Surg*. 2014 Mar; 76(3):582–92. discussion 92-3. [PubMed: 24553523]
11. Crouser ED. Mitochondrial dysfunction in septic shock and multiple organ dysfunction syndrome. *Mitochondrion*. 2004 Sep; 4(5-6):729–41. [PubMed: 16120428]
12. Campos SB, Ashworth SL, Wean S, Hosford M, Sandoval RM, Hallett MA, Atkinson SJ, Molitoris BA. Cytokine-induced F-actin reorganization in endothelial cells involves RhoA activation. *Am J Physiol Renal Physiol*. 2009 Mar; 296(3):F487–95. [PubMed: 19144696]
13. Zheng HZ, Zhao KS, Zhou BY, Huang QB. Role of Rho kinase and actin filament in the increased vascular permeability of skin venules in rats after scalding. *Burns*. 2003 Dec; 29(8):820–7. [PubMed: 14636758]
14. Wolfson RK, Chiang ET, Garcia JG. HMGB1 induces human lung endothelial cell cytoskeletal rearrangement and barrier disruption. *Microvasc Res*. 2011 Mar; 81(2):189–97. [PubMed: 21146549]

15. Chu ZG, Zhang JP, Song HP, Hu JY, Zhang Q, Xiang F, Huang YS. p38 MAP kinase mediates burn serum-induced endothelial barrier dysfunction: involvement of F-actin rearrangement and L-caldesmon phosphorylation. *Shock*. 2010 Sep; 34(3):222–8. [PubMed: 20160665]
16. Rigor RR, Shen Q, Pivetti CD, Wu MH, Yuan SY. Myosin light chain kinase signaling in endothelial barrier dysfunction. *Med Res Rev*. 2013 Sep; 33(5):911–33. [PubMed: 22886693]
17. Wang P, Qi H, Sun C, He W, Chen G, Li L, Wang F. Overexpression of hypoxia-inducible factor-1alpha exacerbates endothelial barrier dysfunction induced by hypoxia. *Cell Physiol Biochem*. 2013; 32(4):859–70. [PubMed: 24081113]
18. Chong W, Li Y, Liu B, Liu Z, Zhao T, Wonsey DR, Chen C, Velmahos GC, deMoya MA, King DR, et al. Anti-inflammatory properties of histone deacetylase inhibitors: a mechanistic study. *J Trauma Acute Care Surg*. 2012 Feb; 72(2):347–53. discussion 53–4. [PubMed: 22327976]
19. Kannan KB, Colorado I, Reino D, Palange D, Lu Q, Qin X, Abungu B, Watkins A, Caputo FJ, Xu DZ, et al. Hypoxia-inducible factor plays a gut-injurious role in intestinal ischemia reperfusion injury. *Am J Physiol Gastrointest Liver Physiol*. 2011 May; 300(5):G853–61. [PubMed: 21183660]
20. Yeh WL, Lu DY, Lin CJ, Liou HC, Fu WM. Inhibition of hypoxia-induced increase of blood-brain barrier permeability by YC-1 through the antagonism of HIF-1alpha accumulation and VEGF expression. *Mol Pharmacol*. 2007 Aug; 72(2):440–9. [PubMed: 17513385]
21. Fink MP. Cytopathic hypoxia. Mitochondrial dysfunction as mechanism contributing to organ dysfunction in sepsis. *Crit Care Clin*. 2001 Jan; 17(1):219–37. [PubMed: 11219231]
22. Halaweish I, Nikolian V, Georgoff P, Li Y, Alam HB. Creating a “Prosurvival Phenotype” Through Histone Deacetylase Inhibition: Past, Present, and Future. *Shock*. 2015 Aug; 44(Suppl 1):6–16. [PubMed: 25565645]
23. Lin T, Chen H, Koustova E, Sailhamer EA, Li Y, Shults C, Liu B, Rhee P, Kirkpatrick J, Alam HB. Histone deacetylase as therapeutic target in a rodent model of hemorrhagic shock: effect of different resuscitation strategies on lung and liver. *Surgery*. 2007 Jun; 141(6):784–94. [PubMed: 17560255]
24. Chang Z, Li Y, He W, Liu B, Halaweish I, Bambakidis T, Liang Y, Alam HB. Selective inhibition of histone deacetylase 6 promotes survival in a rat model of hemorrhagic shock. *J Trauma Acute Care Surg*. 2015 Dec; 79(6):905–10. [PubMed: 26680133]
25. Shults C, Sailhamer EA, Li Y, Liu B, Tabbara M, Butt MU, Shuja F, Demoya M, Velmahos G, Alam HB. Surviving blood loss without fluid resuscitation. *J Trauma*. 2008 Mar; 64(3):629–38. discussion 38–40. [PubMed: 18332802]
26. Li Y, Liu B, Sailhamer EA, Yuan Z, Shults C, Velmahos GC, deMoya M, Shuja F, Butt MU, Alam HB. Cell protective mechanism of valproic acid in lethal hemorrhagic shock. *Surgery*. 2008 Aug; 144(2):217–24. [PubMed: 18656628]
27. Alam HB, Shuja F, Butt MU, Duggan M, Li Y, Zacharias N, Fukudome EY, Liu B, Demoya M, Velmahos GC. Surviving blood loss without blood transfusion in a swine poly-trauma model. *Surgery*. 2009 Aug; 146(2):325–33. [PubMed: 19628092]
28. Valenzuela-Fernandez A, Cabrero JR, Serrador JM, Sanchez-Madrid F. HDAC6: a key regulator of cytoskeleton, cell migration and cell-cell interactions. *Trends Cell Biol*. 2008 Jun; 18(6):291–7. [PubMed: 18472263]
29. Lee HB, Blaufox MD. Blood volume in the rat. *J Nucl Med*. 1985 Jan; 26(1):72–6. [PubMed: 3965655]
30. Chen HY, Li L, Fu ZJ. Histone deacetylase inhibitors trichostatin A and suberoylanilide hydroxamic acid attenuate ventilator-induced lung injury. *Pharmacazie*. 2014 Jan; 69(1):55–9. [PubMed: 24601225]
31. Ji MH, Li GM, Jia M, Zhu SH, Gao DP, Fan YX, Wu J, Yang JJ. Valproic acid attenuates lipopolysaccharide-induced acute lung injury in mice. *Inflammation*. 2013 Dec; 36(6):1453–9. [PubMed: 23846716]
32. Lee C, Cheng W, Chang M, Su Y, Chen C, Hsieh F. Hypoxia-induced apoptosis in endothelial cells and embryonic stem cells. *Apoptosis*. 2005 Aug; 10(4):887–94. [PubMed: 16133878]

33. Demetriades D, Murray J, Charalambides K, Alo K, Velmahos G, Rhee P, Chan L. Trauma fatalities: time and location of hospital deaths. *J Am Coll Surg*. 2004 Jan; 198(1):20–6. [PubMed: 14698307]
34. Levy B. Lactate and shock state: the metabolic view. *Curr Opin Crit Care*. 2006 Aug; 12(4):315–21. [PubMed: 16810041]
35. Evers LH, Bhavsar D, Mailander P. The biology of burn injury. *Exp Dermatol*. 2010 Sep; 19(9): 777–83. [PubMed: 20629737]
36. Deitch EA. Multiple organ failure. Pathophysiology and potential future therapy. *Ann Surg*. 1992 Aug; 216(2):117–34. [PubMed: 1503516]
37. Demling RH. The burn edema process: current concepts. *J Burn Care Rehabil*. 2005 May-Jun; 26(3):207–27. [PubMed: 15879742]
38. Qi H, Wang P, Liu C, Li M, Wang S, Huang Y, Wang F. Involvement of HIF-1 α in MLCK-dependent endothelial barrier dysfunction in hypoxia. *Cell Physiol Biochem*. 2011; 27(3-4):251–62. [PubMed: 21471714]
39. Morin-Brureau M, Lebrun A, Rousset MC, Fagni L, Bockaert J, de Bock F, Lerner-Natoli M. Epileptiform activity induces vascular remodeling and zonula occludens 1 downregulation in organotypic hippocampal cultures: role of VEGF signaling pathways. *J Neurosci*. 2011 Jul 20; 31(29):10677–88. [PubMed: 21775611]
40. Wang W, Dentler WL, Borchardt RT. VEGF increases BMEC monolayer permeability by affecting occludin expression and tight junction assembly. *Am J Physiol Heart Circ Physiol*. 2001 Jan; 280(1):H434–40. [PubMed: 11123261]
41. Tarbell BM, Fu JM. Mechano-sensing and transduction by endothelial surface glycocalyx: composition, structure, and function. *Wiley Interdiscip Rev Syst Biol Med*. 2013 May-Jun; 5(3): 381–90. [PubMed: 23401243]
42. Weinbaum S, Tarbell JM, Damiano ER. The structure and function of the endothelial glycocalyx layer. *Annu Rev Biomed Eng*. 2007; 9:121–67. [PubMed: 17373886]
43. Tsiotou AG, Sakorafas GH, Anagnostopoulos G, Bramis J. Septic shock; current pathogenetic concepts from a clinical perspective. *Med Sci Monit*. 2005 Mar; 11(3):RA76–85. [PubMed: 15735579]
44. Peng Z, Pati S, Potter D, Brown R, Holcomb JB, Grill R, Wataha K, Park PW, Xue H, Kozar RA. Fresh frozen plasma lessens pulmonary endothelial inflammation and hyperpermeability after hemorrhagic shock and is associated with loss of syndecan 1. *Shock*. 2013 Sep; 40(3):195–202. [PubMed: 23807246]
45. Ostrowski SR, Berg RM, Windelov NA, Meyer MA, Plovsing RR, Moller K, Johansson PI. Coagulopathy, catecholamines, and biomarkers of endothelial damage in experimental human endotoxemia and in patients with severe sepsis: a prospective study. *J Crit Care*. 2013 Oct; 28(5): 586–96. [PubMed: 23731819]
46. Johansson PI, Stensballe J, Rasmussen LS, Ostrowski SR. A high admission syndecan-1 level, a marker of endothelial glycocalyx degradation, is associated with inflammation, protein C depletion, fibrinolysis, and increased mortality in trauma patients. *Ann Surg*. 2011 Aug; 254(2): 194–200. [PubMed: 21772125]
47. Torres LN, Sondeen JL, Ji L, Dubick MA, Torres Filho I. Evaluation of resuscitation fluids on endothelial glycocalyx, venular blood flow, and coagulation function after hemorrhagic shock in rats. *J Trauma Acute Care Surg*. 2013 Nov; 75(5):759–66. [PubMed: 24158192]
48. Videm V, Albrigtsen M. Soluble ICAM-1 and VCAM-1 as markers of endothelial activation. *Scand J Immunol*. 2008 May; 67(5):523–31. [PubMed: 18363595]
49. Kvietys PR, Granger DN. Endothelial cell monolayers as a tool for studying microvascular pathophysiology. *Am J Physiol*. 1997 Dec; 273(6 Pt 1):G1189–99. [PubMed: 9435543]
50. Ramirez R, Chong T, Curran B, Victorino GP. Role of endothelin-1 and cyclic nucleotides in ischemia/reperfusion-mediated microvascular leak. *J Trauma*. 2006 Mar; 60(3):515–20. discussion 20-22. [PubMed: 16531848]

Figure 1. TubA attenuates acute lung injury

Treatment with TubA significantly decreased acute lung injury (ALI) compared with vehicle control ($p=0.032$). Data presented as group means $\pm$ SD. * $p < 0.05$

Figure 2. TubA prevents anoxia-induced hyperpermeability in vitro

Endothelial monolayer permeability is presented as relative fluorescence units of fluorescein isothiocyanate conjugated (FITC) albumin flux across human umbilical vein endothelial cell monolayers. Monolayers grown on transwell inserts were placed in anoxia for 30 hours.

Treatment with TubA significantly prevented anoxia-induced hyperpermeability ($p=0.004$).

Data presented as group means $\pm$ SD. * $p < 0.05$

Figure 3. TubA increases acetylation of tubulin and prevents HIF-1α expression

(A) Western blot assays of hypoxia inducible factor 1α (HIF-1α), acetylated tubulin (ac-tubulin) and tubulin show that (B) treatment with TubA increases acetylation of tubulin in normoxia ($p=0.008$) as well as in anoxia ($p=0.008$), and (C) anoxia increases HIF-1α expression ($p=0.029$), which is prevented by TubA ($p=0.029$). Data expressed as group means $\pm$ SD. * < 0.05 . NC: normoxic control, NT: normoxia+TubA, AC: anoxic control, AT: anoxia+TubA.

Figure 4. TubA prevents anoxia-induced glycocalyx shedding

Concentration of sICAM-1 in cell culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA). Anoxia significantly increased sICAM-1 concentration ($p=0.036$), an effect that was prevented by treatment with TubA ($p=0.036$). Data expressed as group means $\pm$ SD. * < 0.05