Histone deacetylase inhibitors prevent pulmonary endothelial hyper-permeability and acute lung injury by regulating heat shock protein 90 function.

Atul D. Joshi¹, Nektarios Barabutis¹, Charalampos Birmpas¹, Christiana Dimitropoulou¹, Gagan Thangjam¹, Mary Cherian-Shaw², John Dennison² and John D. Catravas¹

¹ Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, VA 23508
² Vascular Biology Center, Georgia Regents University, Augusta, GA 30912

Running Head: HDAC-HSP90 interactions in endothelial hyper-permeability and ALI.

Corresponding Author:
Dr. John D. Catravas
Frank Reidy Research Center for Bioelectrics
Old Dominion University
Norfolk, VA 23508
jcatrava@odu.edu

Copyright © 2015 by the American Physiological Society.
ABSTRACT

Trans-endothelial hyper-permeability caused by numerous agonists is dependent on Heat shock protein 90 (Hsp90) and leads to endothelial barrier dysfunction (EBD). Inhibition of Hsp90 protects and restores trans-endothelial hyper-permeability. Hyper-acetylation of Hsp90, as by inhibitors of histone deacetylase (HDAC), suppresses its chaperone function and mimics the effects of Hsp90 inhibitors. In this study we assessed the role of HDAC in mediating LPS-induced trans-endothelial hyper-permeability and acute lung injury (ALI). We demonstrate that HDAC inhibition protects against LPS-mediated EBD. Inhibition of multiple HDAC by the general inhibitors panobinostat or trichostatin provided protection against LPS-induced trans-endothelial hyper-permeability, acetylated and suppressed Hsp90 chaperone function and attenuated RhoA activity and signaling crucial to endothelial barrier function. Treatment with the HDAC3-selective inhibitor, RGFP966, or the HDAC6-selective inhibitor tubastatin A, provided partial protection against LPS-mediated trans-endothelial hyper-permeability. Similarly, knock down of HDAC3 and HDAC6 by specific siRNAs provided significant protection against LPS-induced EBD. Furthermore, combined pharmacologic inhibition of both HDAC 3 and 6 attenuated the inflammation, capillary permeability and structural abnormalities associated with LPS-induced ALI in mice. Together these data indicate that HDAC mediate increased trans-endothelial hyper-permeability caused by LPS and that inhibition of HDAC protects against LPS-mediated EBD and ALI by suppressing Hsp90-dependent RhoA activity and signaling.

Keywords: histone deacetylases, endothelium, barrier function, RhoA, MLC, LPS, inflammation, acute lung injury

Glossary: EU – endotoxin units
INTRODUCTION

Lipopolysaccharide (LPS), a major component of the bacterial wall cell membrane, is released in the bloodstream during infection after bacteria are lysed by white blood cells (8, 44). LPS binds the TLR4 receptor complex on endothelial cells and induces multiple signaling pathways that lead to the production and release of pro-inflammatory cytokines and endothelial barrier dysfunction (EBD) (19, 45, 54). Inflammation and EBD are the primary causes of acute lung injury (ALI), and acute respiratory distress syndrome (ARDS) (3, 18, 44, 46).

The ATP-dependent molecular chaperone, Heat shock protein 90 (Hsp90), plays a central role in LPS-mediated EBD (4, 16). LPS potentiates Hsp90 chaperone function by inducing pp60Src-dependent Y300 phosphorylation of Hsp90 (9). Inhibitors of Hsp90 block LPS-induced Y300 phosphorylation in cultured human lung microvascular endothelial cells (HLMVEC) (9) and prolong survival, attenuate inflammation and reduce lung injury in a murine model of LPS-induced ALI/ARDS (15). Long-term treatment (>8h) with small molecule inhibitors of Hsp90 causes destabilization and ubiquitin-mediated degradation of numerous client proteins, such as Akt and pp60Src (10, 21, 24, 55). However, even short-term treatment (<4h) with Hsp90 inhibitors attenuates LPS-induced RhoA activation and signaling and protects against LPS-mediated EBD in HLMVEC (31).

Histone deacetylases (HDAC) remove acetyl groups from the ε-amino group of lysine residues in numerous histone and non-histone proteins such as Hsp90 (11, 61). In humans, there are 18 characterized HDAC distributed into four classes. HDAC3, a class I HDAC, is primarily a nuclear protein that shuttles to the cytoplasm when phosphorylated (53, 61), localizes to the plasma membrane and is a substrate of Src (39). Conversely, HDAC6 is primarily a cytoplasmic protein and a unique member of class II HDAC (12). HDAC6 acetylation results in nuclear translocation and regulation of gene expression (38). HDAC inhibitors exhibit anti-inflammatory properties by suppressing pro-inflammatory cytokine production (6, 22, 30, 59) and reducing disease severity.
in animal models of inflammatory and autoimmune disease (6, 43, 48). In endothelial cells, HDAC inhibitors suppress thrombin-induced EBD (49). Importantly, inhibition of HDAC induces Hsp90 acetylation at lysine 294 and suppresses its chaperone function, thereby mimicking the effect of Hsp90 inhibitor (50). Both HDAC3 and 6, interact with and deacetylate Hsp90 and inhibition of HDAC3 and 6 hyperacetylates Hsp90 (29, 35, 50) suggesting a possible role of these two HDAC in EBD.

In this study, we demonstrate that inhibition of HDAC protects against LPS-mediated EBD. Particularly, we show that selective pharmacologic or genetic inhibition of HDAC3 and/or HDAC6 provides partial protection against LPS-mediated endothelial hyper-permeability. Finally, we demonstrate that HDAC acts upstream of Hsp90 and that inhibition of HDAC acetylates Hsp90 suppressing its chaperone function. Together, our data suggests a central role for basal and induced HDAC activity in LPS-mediated endothelial hyper-permeability and EBD.
MATERIALS AND METHODS

**Antibodies and Reagents.** Antibodies against acetyl-lysine, phospho-Akt, Akt, MLC and di-phospho-MLC were purchased from Cell Signaling. Anti-Hsp90 antibodies were purchased from BD Biosciences (610418) and Enzo Life Sciences (ADI-SPA-840). Anti-beta-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO), as were antibodies against S424-HDAC3, HDAC3 and HDAC6. PY99 antibody was purchased from SantaCruz Biotechnologies (Santa Cruz, CA). The HDAC inhibitors, panobinostat (Pan), trichostatin (TSA) and the Hsp90 inhibitor, AUY-922 (S1069) were obtained from Selleck Chemicals. Selective HDAC3 inhibitor RGFP966 and selective HDAC6 inhibitor tubastatin were purchased from Selleck chemicals. HDAC3 (sc-35538), HDAC6 (sc-35544) and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligofectamine was obtained from Fisher Scientific (Pittsburg, PA).

**Cell culture.** HLMVEC were isolated and cultured in-house as described previously (14).

**HDAC activity assay.** HDAC activity was measured using fluor-de-lys HDAC activity assay kit in accordance with the manufacturer's instructions (Enzo Life Sciences). After treatment, HLMVEC were lysed and cell lysates were used to measure HDAC activity. Results were normalized to protein levels measured using the bicinchonic acid (BCA) assay reagent (Bio-rad).

**Trans-endothelial permeability.** Trans-endothelial resistance (TER) assay was performed as described previously (16). Briefly, approximately 60,000 cells were seeded on each well of a 8W10E+ ECIS array. After 24h, the media was changed and treatments began at 48h when the resistance was stable between 900-1200 ohms at a frequency of 4000Hz and the capacitance
was between 22-29 nanofarads. Each experiment was performed at least in triplicate and
repeated at least three times (n=3). Resistance was measured using the ECIS model Zθ and
normalized to each well’s value at t=0h.

RhoA activity assay. RhoA activity was determined using a Rho G-LISA™ assay kit in
accordance with the manufacturer’s instructions (Cytoskeleton Inc., Denver) using HLMVEC cell
lysates. Results were normalized to protein levels, measured by the Precision Red protein
assay reagent.

Cell fractionation. Cytoplasmic and nuclear extract were prepared using the nuclear kit from
Active Motif.

Western blotting and Immunoprecipitation. Western blot analyses and immunoprecipitation
experiments were performed as described previously (9, 31). Densitometry was performed
using Image Studio v3.1 from Licor and plotted as fold change from vehicle.

Animal experiments. Mice (C57/6; Harlan) were injected intraperitoneally with 10mg/kg each of
RGFP966 and Tubastatin. After 24h, the animal were anesthetized (ketamine/xylaxine) and
1.5mg/kg LPS was instilled through the trachea. 24h later, the animals were similarly
anesthetized and bronchoalveolar lavage (BAL) was performed with 1ml saline. BAL fluid
(BALF) was thus collected and used form measurements of cellularity and protein
concentration.
siRNA transfection: siRNAs against human HDAC3 and HDAC6 were used to knock down the expression of the respective proteins in HLMVEC. siRNA which does not lead to the degradation of any known cellular mRNA was used as control. siRNAs were diluted in Opti-MEM I Reduced Serum Medium, and Oligofectamine was diluted in an equal volume of Opti-MEM I and incubated for 30 min at room temperature. The oligomer-Lipofectamine complexes were added to cells, which were cultured in media free of antibiotics. The medium was changed 8 h after transfection. Cells were incubated at 37°C in an atmosphere of 5% CO2 and 95% air for 48 h after transfection and then assayed by Western blotting or used in ECIS experiments.

Statistical analyses. Data are presented as mean values ± SEM (standard error of mean). Comparisons among groups were performed using either paired t-test, one-way or two-way ANOVA with Bonferroni post-test, as appropriate. Differences were considered significant at p<0.05, n represents the number of experimental repeats.
RESULTS

HLMVEC grown to confluence were exposed to either PBS or LPS (0.2, 1 or 5 EU/ml) for 2h. After cell lysis, HDAC activity was measured as described in Materials and Methods. Compared to PBS treated cells, LPS-exposed cells exhibited a modest but consistent and significant increase in HDAC activity in whole cell lysates (111±3%, 122±3% and 117±2%, respectively for 0.2, 1 and 5 EU/ml LPS) indicating that LPS signaling activates cellular HDAC function. The LPS-induced HDAC activity increased as early as 1h (122±2%) and remained elevated at 2h (130±7%). Since HDAC are found in both the cytoplasm and the nucleus, we isolated the cytoplasmic and the nuclear extract and measured their HDAC activity. LPS induced HDAC activity in the cytoplasm (100±4%, 112±1% and 115±1%) as well as in nucleus (100±1%, 118±1% ad 115±1%), for 0.2, 1 and 5h, respectively.

HDAC inhibition protects against LPS-mediated decrease in trans-endothelial electrical resistance (TER). Pan-HDAC inhibitors target multiple class I and class II HDAC and have a broad effect on HDAC activity and function. Therefore, we hypothesized that treatment with pan-HDAC inhibitors would attenuate LPS-mediated endothelial hyper-permeability. ECIS arrays were used to measure TER across the endothelial cell monolayer. HLMVEC were grown on gold electrode arrays and TER values were monitored continuously until a constant value was attained, suggesting a confluent monolayer. Cells were then exposed to vehicle or the HDAC inhibitor panobinostat (Pan; 1μM) or trichostatin (TSA; 2μM) for two hours followed by PBS or LPS (1 EU/ml). These concentrations are higher than those frequently used in tumor cells, because it is well known that non-tumor, normal cells are less responsive to HDAC inhibitors (56). LPS decreased TER values suggesting an increase in monolayer permeability. Pretreatment with either Pan (Figure 1A) or TSA (Figure 1B) prevented the LPS-mediated decrease
in HLMVEC monolayer TER, suggesting that HDAC activity and function is necessary for LPS-induced endothelial hyper-permeability.

**HDAC inhibition attenuates LPS-induced Hsp90 activation and chaperone function.** Hsp90 plays a central role in LPS-mediated EBD. Therefore, we hypothesized that the HDAC effects on EBD may involve Hsp90. Confluent HLMVEC were pretreated with vehicle or the specific Hsp90 inhibitor, AUY-922, followed by exposure to LPS (1 EU/ml). At the end of treatment, cells were lysed and total cellular HDAC activity was measured, as described in Materials and Methods. LPS alone increased HDAC activity. AUY-922 did not affect neither the basal nor the LPS-induced HDAC activity (Figure 2A), suggesting that Hsp90 functions downstream of HDAC. To prove this, we then examined the effects of HDAC inhibitors on Hsp90 activation and function.

LPS induces Hsp90-Y300 phosphorylation via pp60Src to activate Hsp90 and potentiate its chaperone function. If HDACs act is upstream of Hsp90, then inhibition of HDAC function would affect LPS-induced Hsp90-Y300 phosphorylation. To test this, we pre-treated HLMVEC with panobinostat or TSA followed by exposure to LPS. Both panobinostat and TSA completely blocked LPS-induced Y300 phosphorylation of Hsp90 (Figure 2B).

Acetylation of Hsp90 suppresses its chaperone function. Therefore, we determined whether HDAC inhibition acetylates Hsp90 in HLMVEC. Treatment with panobinostat for 2h increased Hsp90 acetylation (Figure 2C). Furthermore, panobinostat completely abolished the LPS-induced and Hsp90-dependent phosphorylation of Akt, an Hsp90 client protein (Figure 2D).

Taken together, the data in Figure 2 suggest that HDAC inhibition prevents Hsp90 activation and disrupts its chaperone function.

**HDAC inhibition suppresses LPS-mediated RhoA activity and signaling.** The RhoA-ROCK pathway is an important mediator of the cytoskeletal changes associated with HLMVEC
exposure to LPS (13, 25). ROCK inhibitors block LPS-induced MLC phosphorylation and protect against LPS-induced EBD (23, 37). Recently, we reported that Hsp90 inhibition attenuates LPS-induced RhoA activation and MLC phosphorylation in HLMVEC (31). Therefore, we hypothesized that HDAC inhibition would block LPS-mediated and Hsp90-dependent RhoA activity and downstream signaling. LPS increased RhoA activity and panobinostat pre-treatment for 2h attenuated this LPS-induced RhoA activity (Figure 3A). Furthermore, LPS induces di-phosphorylation of MLC2 in HLMVEC and this induction was completely blocked by either panobinostat or TSA (Figure 3B and 3C). This data suggests that HDAC inhibition blocks RhoA activity and signaling, most likely by attenuating Hsp90 chaperone function.

**HDAC3 is involved in LPS-induced EBD.** HDAC3 and HDAC 6 have been reported to interact with Hsp90. Therefore, we hypothesized that they may be selectively involved in LPS-induced EBD. Confluent HLMVEC were exposed to vehicle or LPS (1 EU/ml) for 2h, lysed, HDAC3 was immunoprecipitated and HDAC activity was measured as described in Materials and Methods. LPS significantly increased HDAC3 activity above basal levels observed in vehicle-treated cells (Figure 4A). Immunoprecipitated HDAC3 exhibited an LPS-induced increase in S424 phosphorylation (Figure 4B) which is consistent with studies showing increased activity of S424 phosphorylated HDAC3 (62).

We then determined whether inhibition of HDAC3 blocks the LPS-mediated decrease in TER. To that end, we employed the HDAC3-selective inhibitor, RGFP966. Pretreatment with RGFP966, at a concentration similar to those reported in the literature without apparent toxic effects (57), moderately but significantly reduced total HDAC activity (Figure 4C), in a concentration-dependent manner, but nearly abolished HDAC3 activity, also in a concentration-dependent manner (Figure 4D). Importantly, pre-treatment with RGFP966 significantly
attenuated the LPS-induced decrease in TER (Figure 4E). This data suggests that HDAC3 plays a significant role in LPS-mediated EBD.

**HDAC6 inhibition attenuates the LPS-mediated EBD.** HDAC6 interacts with, and regulates Hsp90 chaperone function (7, 50). Inhibition of HDAC6 blocks endothelial hyper-permeability induced by thrombin (49). Therefore, we hypothesized that LPS modulates HDAC6 activity and that inhibition of HDAC6 may prevent the LPS-induced endothelial hyper-permeability. Confluent HLMVEC were exposed to vehicle or LPS (1 EU/ml) for 1 or 2h, lysed, HDAC6 was immunoprecipitated and HDAC activity was measured as described in Materials and Methods. LPS did not affect basal HDAC6 activity (Figure 6A). Similarly, LPS did not affect the expression of either HDAC3 or HDAC6 proteins (data not shown). We then determined whether HDAC6 is involved in the LPS-mediated decrease in TER. Pretreatment with the HDAC6-selective inhibitor, tubastatin A, for 2h and at a concentration similar to those reported in the literature without apparent toxic effects (17), moderately but significantly reduced total HDAC activity (Figure 5B), in a concentration-dependent manner, but nearly abolished HDAC6 activity, also in a concentration-dependent manner (Figure 5C). Furthermore, pre-treatment with tubastatin A blocked the LPS-induced decrease in TER (Figure 5D). This data suggests that even though HDAC6 activity is not affected by LPS, HDAC6 plays an important part in LPS-mediated EBD.

**Combined inhibition of HDAC 3 and 6 attenuates LPS-mediated EBD.** As shown in Figures 4 and 5, selective inhibition of either HDAC3 or HDAC6 provides partial but significant protection against LPS-mediated EBD. Therefore, we investigated the effect of combined inhibition of HDAC 3 and 6. HLMVEC were exposed to 5μM each of RGFP966 and tubastatin for 2h, followed by LPS (1EU/ml). This treatment essentially abolished the LPS-induced MLC2 phosphorylation (Figure 6A) and Hsp90 chaperone function, as reflected in Akt phosphorylation.
(Figure 6B). To further assess the combined role of HDAC 3 and 6 in LPS-mediated EBD, HLMVEC were transfected with siRNAs against HDAC 3 and 6. The siRNA produced >80% inhibition of HDAC3 and HDAC6 expression (Figure 7A and 7B). Subsequently, the two siRNAs were added to HLMVEC freshly plated on ECIS arrays. TER was monitored starting 24h later and for comparison purposes, data is presented normalized to the 24h values. Over the next 20h HLMVEC lacking most of HDAC3 and 6 exhibited faster proliferation (Figure 7C). At 48h post plating, LPS (1 EU/ml) was added to the arrays and, for comparison purposes, TER data was again normalized to the value just prior to LPS addition. Cells lacking HDAC3 and 6 were significantly more resistant to LPS than cells transfected with scrambled siRNA (Figure 7D).

**Combined inhibition of HDAC 3 and 6 attenuates LPS-mediated ALI.**

To investigate whether the EBD protective effects of HDAC3 and 6 inhibitors conferred similar protection against LPS-induced ALI, mice were injected intra-peritoneally with 10mg/kg each of RGFP966 and Tubastatin. After 24h, 1.5mg/kg LPS was given intratrachely to mice under anesthesia and bronchoalveolar lavage fluid (BALF) was collected 24h post-LPS instillation. Cellular infiltration into alveoli was quantified by counting number of cells in BALF. LPS induced a profound and significant increase in leukocyte infiltration compared to vehicle treatment; however, BALF from mice pre-treated with RGFP966 and Tubastatin exhibited significantly less cellular infiltration (Figure 8A). Similarly, a partial but significant attenuation of BALF protein concentration was observed in mice pre-treated with RGFP966 and Tubastatin, compared to mice treated with LPS (Figure 8B). Histological examination of lung tissue stained with H&E revealed significant protection in lungs of mice treated with RGFP966 and Tubastatin from the LPS-induced structural changes, reflected in septal thickness and cellular infiltration (Figure 8C, arrows), whereas MPO staining demonstrated the granulocytic nature of the invading cells (Figure 8D). When quantified with respect to Lung Injury index (42), the combination of HDAC3
and 6 inhibitor treatment effectively protected against LPS-induced lung pathology (Figure 8E).

Together, data in Figure 8 indicate that combined inhibition of HDAC 3 and 6 protects against LPS-mediated ALI.
DISCUSSION

Lysine acetylation is fine-tuned by the opposing actions of two families of enzymes, histone acetyltransferases (HAT) and histone deacetylases (HDAC). The HAT adds an acetyl group whereas HDAC removes acetyl groups from the ε-amino group of lysine residues (11, 61). In humans, there are 18 characterized HDAC which are distributed in four classes based on function and DNA sequence similarity (20). Despite their name, HDAC target not only histone, but also many non-histone proteins both in the nucleus and the cytoplasm and regulate a variety of functions including transcription, cytoskeletal polymerization and signaling pathways (28, 30, 51, 61).

Inhibition of HDAC activity exhibit immunosuppressive and anti-inflammatory properties by reducing cytokine production in both humans and mice (6, 22, 30, 59). HDAC inhibition also reduces disease severity in several animal models of inflammatory and autoimmune diseases (6, 43, 48). In endothelial cells, HDAC inhibitors have been shown to suppress thrombin-induced EBD (49). We now show that pharmacologic as well as genetic inhibition of HDAC protect HLMVEC from LPS-mediated EBD.

We have demonstrated that LPS activates Hsp90 chaperone function by inducing Y300 phosphorylation and that it promotes EBD in cultured HLMVEC (9, 26, 36). On the other hand, inhibition of Hsp90 prevents Y300 phosphorylation, attenuates chaperone function and protects against LPS-induced EBD in cultured HLMVEC. Furthermore, Hsp90 inhibitors prolong survival, attenuate inflammation and reduce lung injury in a murine model of LPS-induced ALI (15). Hsp90 chaperone function is also regulated by reversible acetylation at residue K294. Acetylation suppresses whereas deacetylation promotes Hsp90 chaperone function (50). HDAC inhibitors abrogate deacetylation of Hsp90 resulting in the accumulation of acetylated Hsp90. Since, we have previously shown that Hsp90 promotes EBD and ALI/ARDS (4, 16, 40), in this project we determined the role of HDAC in LPS-mediated EBD in cultured HLMVEC. We
hypothesized and demonstrated that LPS-induced HDAC activity functions upstream of Hsp90 and inhibition of HDAC would suppress Hsp90 chaperone function. We also found that inhibition of HDAC activity blocked LPS-induced Y300 phosphorylation of Hsp90 suggesting that HDAC inhibition also attenuates LPS-mediated and Src-dependent phosphorylation and activation of Hsp90 (9). Thus we propose that prevention of EBD by HDAC inhibitors is achieved by disrupting Hsp90 chaperone function. This is supported by directly demonstrating that HDAC inhibition prevents LPS-induced Akt phosphorylation (Figure 2D). Akt is an Hsp90 client protein and its activation (phosphorylation) by LPS (58) or other agonists is Hsp90-dependent.

The Rho-ROCK pathway modulates cell-cell adhesion and vascular permeability by regulating myosin-mediated contractile forces (13, 25). LPS-induced RhoA activity requires activation of Src, an Hsp90 client protein. Induction of RhoA activity phosphorylates ROCK which in turn phosphorylates MLC leading to increase in myosin-mediated contractile forces and EBD (1, 25). In HLMVEC, two ROCK inhibitors, Y27632 and GSK429286, block MLC phosphorylation and provide significant protection against LPS-mediated decrease in TER (31). Also, inhibition of Src or Hsp90 completely abolished the LPS-induced RhoA activity and MLC phosphorylation in HLMVEC (31). Since HDAC inhibition suppresses Hsp90 function we hypothesized and demonstrated that treatment with HDAC inhibitor should also attenuate RhoA activity and MLC phosphorylation. We also demonstrated that among HDACs, HDAC3 and HDAC6 are major contributors to the LPS-induced EBD and ALI.

HDAC3, a class I HDAC, is primarily a nuclear protein that regulates transcription (60). Phosphorylation at serine 424 increases HDAC3 activity and translocates HDAC3 to the cytoplasm where it interacts with and deacetylates Hsp90 and promotes its chaperone function (62). Cytoplasmic HDAC3 is a substrate of, and regulated by, Src, and has been shown to localize at the plasma membrane (39). Consistent with these observations, our data demonstrates that LPS selectively induces HDAC3 activity by inducing serine 424
phosphorylation and that selective inhibition of HDAC3 protects against LPS-induced decrease
in TER indicating that HDAC3 activity plays a crucial role in LPS-mediated EBD.

Recently, the HDAC6 specific inhibitor, tubacin, was shown to suppress thrombin-induced EBD
in human pulmonary arterial cells (49). HDAC6 is a unique member of class II because it
contains two homologous, fully functional catalytic domains. The C-terminal catalytic domain of
HDAC6 possesses α-tubulin deacetylase activity and regulates microtubule dynamics (12, 27).
HDAC6 is primarily cytoplasmic and interacts with and deacetylates Hsp90 (5, 35). The HAT,
p300, acetylates HDAC6, promotes nuclear translocation and regulates gene expression (38).
Our data demonstrates that LPS does not induce HDAC6 activity (data not shown) however,
selective pharmacologic or genetic inhibition of HDAC6 protects against the LPS-induced
decrease in TER. This suggests that basal HDAC6 activity is critical in inducing LPS-mediated
EBD and that inhibition of HDAC6 provides a possible strategy to protect against LPS-mediated
EBD.

Both HDAC3 and HDAC6 are involved in gene expression, protein function, kinase signaling
and cytoskeletal changes in endothelial cells (2, 32-34, 41, 49, 52, 63). In the cytoplasm, both
HDAC3 and HDAC6 interact with the molecular chaperone Hsp90 and regulate its chaperone
activity (47). Therefore, we speculated that combined inhibition of HDAC 3 and 6 might provide
better protection against LPS-mediated EBD. Our data demonstrates that combined
pharmacologic or genetic inhibition of HDAC 3 and 6 provides significant protection against
LPS-mediated EBD in HLMVEC and against LPS-induced ALI in mice.

Binding of LPS to the endothelial cells elicits a broad cellular response including induction of
kinase signaling pathways, production of pro-inflammatory cytokines, cytoskeletal remodeling
and loss of cell-cell adhesion. Together these effects cause an increase in trans-endothelial
permeability and lead to EBD and ALI. Hsp90 regulates the stability and activity of many
proteins in these pathways. Our present data uncovers a novel mechanism of LPS-induced endothelial hyper-permeability (Figure 9). We have recently demonstrated that src and Hsp90 interact in HLMVEC resulting in mutually augmented activities, of kinase and chaperone functions, respectively. We now demonstrate that HDAC, specifically, HDAC3 and HDAC6 are also involved. At minimum, the threesome of src, HDAC and Hsp90 promote enhancement of Hsp90 chaperone function triggered by LPS and possibly other inflammatory molecules and are thus critically important in the development of EBD and ALI. Consequently, the anti-inflammatory and endothelial barrier protective effect of HDAC and Hsp90 inhibitors could be exploited to treat vascular diseases with minimal side effects.

Grants: Supported by HL101902

Disclosures: None
REFERENCES


**Figure legends**

**Figure 1**

**HDAC inhibition protects against LPS-mediated decrease in TER.** HLMVEC were grown to confluence on ECIS arrays (8W10E+). Once a constant resistance was attained, the cells were treated with either vehicle (0.1% DMSO) or 1 μM panobinostat (Pan; panel A) or 2 μM Trichostatin (TSA; panel B) for 2h. LPS (1 EU/ml) was then added as indicated and TER values were measured using ECIS Zθ. Normalized resistance was calculated and plotted as a function of time. *: p<0.01 from vehicle, #: p<0.01 from LPS. N≥4.

**Figure 2**

**HDAC inhibition attenuates LPS-induced Hsp90 chaperone function.** A) Confluent monolayers were treated with vehicle or 2μM AUY-922 (Hsp90 inhibitor) for 4h followed by exposure to LPS (1 EU/ml) for 2h. Cells were then lysed and HDAC activity measured using the Flour-de-lys HDAC activity assay. (*: p<0.05 from Vehicle or AUY). B) Confluent monolayers were treated with vehicle, LPS (1 EU/ml), 1μM trichostatin (TSA) for 2h followed by exposure to LPS for 2h. Cells were lysed, Hsp90 was immunoprecipitated and immunoblotted for phosho-tyrosine and Hsp90. (n=3, *: p<0.05 from vehicle; #: p<0.05 from LPS.). C) HLMVEC were treated with vehicle (VEH) or 1 μM panobinostat (Pan); cells were then lysed, Hsp90 was immunoprecipitated and immunoblotted for acetyl-lysine and Hsp90. (n=3, *: p<0.05). D) HLMVEC were treated with vehicle, LPS (1 EU/ml), 1μM panobinsotat, or 1μM panobinsotat for 2h followed by exposure to LPS (1 EU/ml) for 2h. Cells were then lysed and immunoblotted for phosho-Akt (S473) and Akt. Densitometric analysis was carried out and the ratio of pAkt to Akt was plotted.
Figure 3

**HDAC inhibition suppresses LPS-induced RhoA activity and signaling.** A) Confluent HLMVEC were treated with vehicle, LPS (1 EU/ml), 1μM panobinsotat (Pan) or panobinsotat for 2h followed by exposure to LPS for 2h (Pan+LPS). Cells were then lysed and RhoA activity was measured using the G-LISA RhoA activation assay. (n=5, *: p<0.05 from Vehicle; #: p<0.05 from LPS). B) HLMVEC were grown in 6-well plates. Confluent monolayers were treated with vehicle, LPS (1EU/ml), 1μM panobinsotat or panobinsotat for 2h followed by exposure to LPS for 2h. Cells were then lysed and immunoblotted with antibodies against ppMLC2, MLC2 and β-actin. C) Cells were treated as in B except instead of panobinostat, 1μM trichostatin (TSA) was added (n=5, *: p<0.05 from Vehicle; #: p<0.05 from LPS).

Figure 4

**HDAC3 Inhibition protects from LPS-induced EBD.** A) HLMVEC were grown in 100mm dishes. When confluent, the cells were treated with either PBS or LPS (1 EU/ml) for 2h. Cells were then lysed, HDAC3 was immunoprecipitated and HDAC activity was measured using the Flour-de-lys HDAC activity assay. (n=4; *: p<0.05). B) HLMVEC were treated as above. Cells were lysed, HDAC3 was immunoprecipitated and then immunoblotted for phospho-serine 424 and HDAC3. (n=3; *: p<0.05). C,D) HLMVEC were grown in 100mm dishes. When confluent, the cells were treated with the indicated concentrations of the HDAC3 selective inhibitor, RGFP966 for 2h and total HDAC (C), as well HDAC3 activity (D) was measured, as described previously (n=4; *: P<0.05 from vehicle). E) HLMVEC were grown to confluence on ECIS arrays (8W10E+). Once a constant resistance was attained, the cells were treated with either vehicle
(0.1% DMSO) or 5μM RGFP966 (RGF) for 2h. LPS (1 EU/ml) was then added as indicated and TER values were measured using an ECIS Zθ. *: p<0.05 from vehicle; #: p<0.01 from LPS.

Figure 5

**HDAC6 inhibition protects against LPS-induced EBD.** A) HLMVEC were grown in 100mm dishes. When confluent, the cells were treated with either PBS or LPS (1 EU/ml) for 2h. Cells were then lysed, HDAC6 was immunoprecipitated and HDAC activity was measured using the Flour-de-lys HDAC activity assay. B, C) HLMVEC were grown in 100mm dishes. When confluent, the cells were treated with the indicated concentrations of the HDAC6 selective inhibitor, tubastatin for 2h and total HDAC (B), as well HDAC6 activity (C) were measured, as described previously (n=4; *: P<0.05 from vehicle). D) HLMVEC were grown to confluence on ECIS arrays (8W10E+). Once a constant resistance was attained, the cells were treated with either vehicle (0.1% DMSO) or 5 μM Tubastatin (Tub) for 2h. LPS (1 EU/ml) was then added as indicated and TER values were measured using ECIS Zθ. *: p<0.01 from vehicle; #: p<0.05 from LPS.

Figure 6

**Combined inhibition of HDAC 3 and 6 prevents MLC phosphorylation and abrogates Hsp90 chaperone function.** A) HLMVEC were grown in 6-well plates. Confluent monolayers were treated with vehicle, LPS, RT (5μM RGFP966 + 5μM Tubastatin) or RT for 2h followed by exposure to LPS (1EU/ml) for 2h. Cells were then lysed and immunoblotted with antibodies against ppMLC2 and MLC2. *: p<0.05 from vehicle; #: p<0.05 from LPS. n=4. B) Cells were
treated as in A and immunoblotted with antibodies against pAkt and Akt. *: p<0.05 from vehicle; #: p<0.05 from LPS. n=4.

Figure 7

siRNA-induced downregulation of both HDAC3 and HDAC6 protects against LPS-induced EBD. A,B) HLMVEC were transfected with either scrambled siRNA or siRNAs against both HDAC 3 and 6. 48h later transfected cells were lysed and immunoblotted with antibodies against HDAC3 (A) and HDAC6 (B). *, ***, P<0.05, P<0.001; n=4. C) HLMVEC were transfected with either scrambled siRNA or siRNAs against both HDAC 3 and 6. 24h later transfected cells were added to ECIS arrays (8W10E+) and allowed to grow to confluence. N=4. *: P<0.01 from scrambled siRNA. D) The same cells and arrays as in (C). Following 24h exposure to siRNA, TER was normalized and is presented as t=0. The cells were then treated with either vehicle (PBS) or LPS (1 EU/ml) as indicated and TER values were monitored. *: p<0.05 from vehicle; #: p<0.05 from LPS. N=4.

Figure 8

Combined pharmacologic inhibition of HDAC 3 and 6 protects against LPS-mediated ALI. Mice were injected intra-peritoneally with 10mg/kg each of RGFP966 and Tubastatin. After 24h, 1.5mg/kg LPS was given intratracheally and bronchoalveolar lavage fluid (BALF) was collected 24h later. Cellular infiltration into the alveolar spaces was quantified by counting number of cells in BALF (A). *: p<0.05 from vehicle; #: P<0.05 from LPS. N=7. Capillary permeability was estimated by measuring protein concentration in BALF using the bicinchonic acid method (B). *: p<0.05 from vehicle; #: P<0.05 from LPS; $: P<0.05 from RT. N=7. Histological evaluation of
lung samples from treated mice was also performed. Sections were stained with Eosin and Hematoxylin (C) or immunocytostained against the granulocyte marker, myeloperoxidase (MPO) (D). Arrows point to thickened septa and cellular infiltrates. Representative samples of three specimen per group. E) Estimation of Lung Injury Index as described in (42). *: p<0.05 from vehicle; $: P<0.05 from LPS; N=4.

**Figure 9**

Proposed scheme for the role of HDAC 3 and 6 in LPS-mediated EBD.
Figure 1

A

![Graph showing Normalized TER over time for different treatment groups.](image)

B

![Graph showing Normalized TER over time for different treatment groups.](image)
Figure 2

(A) Total HDAC activity (% of VEH): VEH, LPS, AUY, AUY+LPS.

(B) pY/Hsp90 (fold of VEH): VEH, LPS, TSA+LPS, Pan+LPS.

(C) acK/Hsp90 (fold of VEH): VEH, Pan.

(D) pAkt/Akt (fold of VEH): VEH, LPS, Pan, Pan+LPS.
Figure 3

A

![Graph showing RhoA activity (% of VEH) for VEH, LPS, Pan, and Pan+LPS.](image)

B

![Graph showing ppMLC2/MLC2 (fold of VEH) for VEH, LPS, Pan, and Pan+LPS.](image)

C

![Graph showing ppMLC2/MLC2 (fold of VEH) for VEH, LPS, TSA, and TSA+LPS.](image)
Figure 4

A) HDAC3 activity (% of VEH)

B) S424/HDAC3 (fold of VEH)

C) Total HDAC activity (% of VEH)

D) HDAC3 activity (% of VEH)

E) Normalized TER
Figure 5

**A**

Total HDAC activity (% of VEH)

- VEH
- 1h
- 2h

**B**

Total HDAC activity (% of VEH)

- VEH
- 0.2 μM
- 2 μM
- 5 μM

**C**

HDAC6 Activity (% of VEH)

- VEH
- 2 μM
- 5 μM
- Pan 1 μM

**D**

Normalized TER

- VEH
- LPS
- Tub
- Tub+LPS

* * *

* * 

* 

*
Figure 6

A

```
<table>
<thead>
<tr>
<th></th>
<th>ppMLC2/MLC2 (fold of VEH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEH</td>
<td>1</td>
</tr>
<tr>
<td>LPS</td>
<td>2 *</td>
</tr>
<tr>
<td>RT</td>
<td>1</td>
</tr>
<tr>
<td>RT+LPS</td>
<td>*#</td>
</tr>
</tbody>
</table>
```

B

```
<table>
<thead>
<tr>
<th></th>
<th>pAkt/Akt (fold of VEH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEH</td>
<td>1</td>
</tr>
<tr>
<td>LPS</td>
<td>1.5 *</td>
</tr>
<tr>
<td>RT</td>
<td>1</td>
</tr>
<tr>
<td>RT+LPS</td>
<td>2 *#</td>
</tr>
</tbody>
</table>
```

![ppMLC2/MLC2](image1)

![pAkt/Akt](image2)
**Figure 8**

**A**

![Bar chart showing BALF cell counts per ml of BALF](image)

- **VEH**
- **LPS**
- **RT**
- **RT+LPS**

**B**

![Bar chart showing BALF protein levels (µg/mL)](image)

- **VEH**
- **LPS**
- **RT**
- **RT+LPS**

**C:** H&E

- **VEH**
- **LPS**
- **RT**
- **RT+LPS**

**D:** MPO

- **VEH**
- **LPS**
- **RT**
- **RT+LPS**

**E**

![Bar chart showing Lung injury score](image)

- **VEH**
- **LPS**
- **RT**
- **RT+LPS**
Figure 9

Lipopolysaccharide

HDAC3

HDAC6

Src

Hsp90

pY

RhoA

ROCK

ppMLC

EC PERMEABILITY