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Valproic acid mitigates the inflammatory response and prevents acute respiratory distress syndrome in a murine model of *Escherichia coli* pneumonia at the expense of bacterial clearance

George Kasotakis, MD, MPH¹, Manuel Galvan, PhD¹, Elizabeth King, MD¹, Beda Sarkar, MD, PhD¹, Arthur Stucchi, PhD¹, Joseph P Mizgerd, ScD², Peter A Burke, MD¹, and Daniel Remick, MD³

¹Boston University School of Medicine, Department of Surgery

²Boston University School of Medicine, Departments of Medicine, Microbiology and Biochemistry, Pulmonary Center

³Boston University School of Medicine, Department of Pathology and Laboratory Medicine

Abstract

Background—Histone deacetylase inhibitors (HDACI) are members of a family of epigenetic modifying agents with broad anti-inflammatory properties. These anti-inflammatory properties may have important therapeutic implications in Acute Respiratory Distress Syndrome (ARDS). However, administration of HDACI may create an immunosuppressive environment conducive to bacterial growth. Accordingly, the aim of the current study is to investigate the effect of HDACI Valproic Acid (VPA) on host inflammatory response and bacterial burden in a murine model of *E. coli* pneumonia-induced ARDS.

Methods—ARDS was induced in male C57BL6 mice (n=24) by endotracheal instillation of 3×10^6 *E. coli*. VPA (250mg/kg) was administered 30 min after *E. coli* instillation in the intervention group. Blood samples were collected at 3 and 6 hours, and animals were sacrificed at 6 hours. Bronchoalveolar lavage (BAL) was performed and tissue specimens were harvested. Cytokine levels were measured in blood and BAL, and so was transalveolar protein transit. Cell counts, and colony forming units were quantified in BAL fluid.

Results—VPA reduced neutrophil influx into the lungs and local tissue destruction through decreased myeloperoxidase activity. It also ameliorated the pulmonary and systemic inflammatory response. This led to greater bacterial proliferation in the pulmonary parenchyma.

Author for Correspondence / Reprints: George Kasotakis, MD MPH, Assistant professor of Surgery, Boston University School of Medicine / Boston Medical Center, Section of Trauma, Acute Care Surgery & Surgical Critical Care, 840 Harrison Avenue, Dowling 2 South, Suite #2414, Boston, MA 02118, tel: (617) 414-5689, fax: (617) 414-5204, gkasot@bu.edu.

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Conclusions—Administration of VPA in a clinically relevant bacterial model of murine ARDS mitigates the host inflammatory response, essentially preventing ARDS, but creates an immunosuppressive environment that favors bacterial overgrowth.

Keywords

Acute lung injury; acute respiratory distress syndrome; angiopoietin 2; histone deacetylase inhibitors; valproic acid

Background

Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS) represent a spectrum of an inflammatory pulmonary disease, manifesting clinically with severe hypoxia and dyspnea.¹ The syndrome, which was first described in 1967, is characterized by systemic release of proinflammatory mediators and diffuse infiltration of the pulmonary parenchyma with inflammatory cells.² Neutrophil recruitment to the lungs can lead to widespread pulmonary vascular endothelial and alveolar epithelial cell damage and death, intrapulmonary hemorrhage, pulmonary edema due to disruption of the blood-alveolar barrier, and severely impaired gas exchange leading to respiratory failure.³

The syndrome is usually the result of highly inflammatory disease states that may be affecting the lungs directly (pneumonia, aspiration, pulmonary contusion, near-drowning, toxic inhalation) or indirectly (sepsis, severe multi-organ trauma, multiple transfusions, drug overdose, pancreatitis).³ More than 200,000 cases of ARDS – the definition of which was recently expanded to encompass ALI² – are diagnosed annually in the United States alone, that are associated with 3.6 million hospital days.⁴ Although mortality from ARDS appears to be improving at least in the setting of clinical trials, it remains very high, averaging above 45%, and even exceeding 60% in the most severe cases.⁵ This makes ARDS even more lethal than myocardial infarction and embolic stroke.^{6,7} Moreover, long-term disability in ARDS survivors can be significant: A longitudinal study that followed ARDS survivors over 5 years demonstrated major physical and psychological sequelae, including significant exercise limitation, poor quality of life, and increased costs associated with healthcare utilization.⁸ Despite advances in critical care and mechanical ventilation technology over the last few decades, treatment for ARDS remains largely supportive,⁹⁻¹¹ while targeted therapies have failed to improve clinically relevant outcomes, including survival in human clinical trials.¹²⁻¹⁴ The above emphasize the need to identify novel therapeutic approaches, with the aim to mitigate excessive lung inflammation and improve outcomes of this host-response disease.

ARDS represents a clinical syndrome resulting from an exaggerated immune response to noxious stimuli that is triggered from overexpression of a multitude of chemokines and activation of recruited and resident inflammatory cells.¹⁵⁻²⁰ In that regard, epigenetic modification of the harmful inflammatory pathways that become activated early in the disease course may present a unique opportunity for development of specific, targeted therapies, and may hold promise at preventing ARDS from developing and improving clinically relevant outcomes in the management of patients with or at risk for ARDS.

Preliminary studies using murine models of ARDS have mitigated the systemic and pulmonary inflammatory profile and improved survival in animals pretreated with HDACI.^{21,22} However, the studies published to date have not utilized clinically relevant models, in which animals receive treatment after the original inflammatory insult is applied, and the effect of this new anti-inflammatory therapy has not been adequately assessed on bacterial dissemination, given most cases of ARDS arise from severe infections.⁴

The aim of the current project is to determine whether Valproic Acid, one of the most widely used HDACI, ameliorates the inflammatory response in a murine model of *E. coli* pneumonia-induced ALI (one of the most common models of the disease²³), if administered shortly after the stimulus, and what its effect on pulmonary bacterial load is, once the immune response is lessened.

Methods

Mice

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were allowed to acclimate for a minimum of 3 days and maintained under specific pathogen-free conditions in a full-barrier facility until experiments, at 6-8 weeks of age. All mice were allowed food and water *ad libitum*, and all procedures were conducted after approval by Boston University's Institutional Animal Care and Use Committee, and in accordance with guidelines for ethical animal treatment.

Escherichia coli

Escherichia coli (serotype 06:K3:H1, strain 19138, American Type Culture Collection, Manassas, VA) was cultured to the desired concentration in Luria broth (LB). *E. coli* was quantified by optical density and verified by serial dilutions on LB plates.

Endotracheal Instillations and Acute Lung Injury

After anesthesia with inhaled isoflurane, mice underwent direct endotracheal instillation using the tongue pull technique. The animals were suspended by their front incisors on an angled surgery board and their tongue was retracted outward with DeBakey forceps to allow access to their larynx. Fifty microliters of sterile normal saline ($n=6$) or 3×10^6 of *E. Coli* in the same amount of saline ($n=24$, per previously published literature²¹) was instilled endotracheally by pipette. Mice were returned to their home cage once consciousness was regained. Of the 24 mice that were challenged with *E. coli*, half were randomly selected and administered 250 mg/kg of Valproic Acid (VPA) (Selleck Chemicals, Boston, MA) and the other half sterile normal saline intraperitoneally (IP) 30 minutes post endotracheal instillation (Figure 1).

Blood was collected 3 and 6 hours after *E. coli* challenge via facial vein or cardiac puncture respectively. Immediately following euthanasia at 6 hours, the animals underwent thoracotomy and had their left hilum ligated, while their trachea was surgically exposed and a tracheostomy with a 22G angiocath was performed. This time point was selected consistent with previous published methodology.^{21,23,24} Bronchoalveolar lavage (BAL) of

the right lung was performed with HBSS EDTA in a stepwise fashion until 1 cc of BAL fluid (BALF) was obtained. Cell count and differential was obtained in the BALF (Cytospin: ThermoScientific, Waltham, MA).

Histology and Confirmation of Acute Lung Injury

The left lung was fixed in 4% formalin, embedded in paraffin, sectioned, and mounted on microscopy slides. The non-lavaged lung was selected for histologic assessment to eliminate the risk of identifying alveolar wall destruction or similar pathology due to possible overdistention during lavage. The samples were then dehydrated, processed with xylene, and stained with hematoxylin and eosin. Images were examined and captured with an Olympus Upright microscope with X software version X (Olympus Corporation, Tokyo, Japan).

In a pilot experiment to be published elsewhere, using the same method and dose for *E. coli* endotracheal instillations and lung staining, but without BAL, we confirmed in animals sacrificed at the same time point, that diffuse neutrophil infiltration, alveolar damage and hemorrhage, and alveolar wall thickening with hyaline deposition and pulmonary edema were present in all 5 pulmonary lobes, as well as ratios of PaO₂/FiO₂<300 were present, thus ensuring satisfactory creation of a murine ARDS model.

Myeloperoxidase (MPO) activity assay

Assessment of MPO activity was performed as previously described,²⁵ as a measure of neutrophil activation in the pulmonary parenchyma. The absorbance was read at 450 nm and data were expressed as change in optical density (OD: the difference between the average absorbance of the three substrate wells and the three background wells for the same sample).

Cytokine Expression

The pro-inflammatory cytokines Tumor Necrosis Factor- α (TNF- α), Interleukin-6 (IL-6), CXCL1 (also known as KC), CXCL2 (also known as Macrophage Inflammatory Protein-2, MIP-2), and Angiopoietin-2 levels were determined in samples by ELISA per the manufacturers' instructions. [TNF- α and IL-6: eBioscience (San Diego, CA); MIP2, KC: R&D Systems (Minneapolis, MN); Angiopoietin 2: Abcam (Cambridge, MA)]. These cytokines were selected for measurement, as they are implicated in the pathophysiology of ARDS in both human and animals as pro-inflammatory (TNF- α , IL-6), or neutrophil-attracting chemokines (KC, MIP-2),¹⁵⁻¹⁹ or they have been recently identified as potential early biomarkers for the clinical syndrome (Angiopoietin-2).²⁶ TNF- α , IL-6, KC, and MIP-2 were measured in the BALF at the time of sacrifice (6 hours), whereas IL-6 and Angiopoietin-2 in plasma at 3 and 6 hours.

Transalveolar-capillary membrane protein transit

Albumin and Immunoglobulin M (IgM) levels were assessed in the BALF by ELISA (Bethyl Laboratories, Montgomery, TX) to assess degree of transalveolar-capillary membrane protein transit, a marker of alveolar wall destruction and alveolar-capillary membrane permeability.

Bacterial burden

Viable bacteria were quantified by colony counting,^{17,20} after serial dilutions of BALF were plated on blood agar, and colony forming units (CFU)/mL were enumerated after incubation at 37°C for 48h.

Statistics

Student's t-test or one-way analysis of variance (ANOVA) were performed to compare means between intervention groups as appropriate. Data are reported as means + standard deviations as appropriate. Statistical significance was declared at a two-sided p-value of 0.05. Statistical analyses were performed with Prism 6.0 (GraphPad Software, La Jolla, CA).

Results

Histopathology, neutrophil recruitment and activation

While lung sections from sham animals demonstrated normal pulmonary parenchymal architecture (Figure 2A), sections obtained post-mortem from the isolated left lung of the animals instilled with *E. coli* exhibited acute inflammatory histopathology consistent with ALI: Numerous neutrophils were noted in the alveolar air spaces, in the perivascular connective tissue, and the air spaces and interstitium of the conducting airways.¹⁷ Diffuse pulmonary edema was evident by fibrin deposition within the air spaces, and the expansion of the interstitial and lymphatic compartments. Focal hemorrhage with red cell extravasation into the pulmonary parenchyma suggestive of vascular endothelial disruption; alveolar wall thickening; and loss of parenchymal architectural integrity were also noted. (Figure 2B) The animals treated with VPA demonstrated improved histopathology. Neutrophil recruitment – a hallmark of acute inflammation and ALI^{27,28} – was significantly decreased: Fewer neutrophils could be seen throughout the pulmonary parenchyma after VPA administration, and the expansion of the interstitial and lymphatic compartments suggestive of pulmonary edema was mitigated. Red cell extravasation was less pronounced and architectural integrity was preserved to a greater extent. (Figure 2C).

Cytology of the BALF on Cytospin demonstrated a few resident macrophages in the sham animals (Figure 2D), with many more neutrophils having been recruited in the BALF of the *E. coli*-instilled mice. The number of the macrophages seen was similar to that of the sham animals, however, it is likely the time of sacrifice (6 hours) may have precluded more macrophages from emigrating into the lungs this early on (Figure 2E). The number of macrophages was also relatively stable in the VPA-treated mice, but the recruited neutrophils were significantly reduced (Figure 2F).

Numeric assessment of the macrophage and neutrophil counts in the BALF confirmed the above observation (Figure 3A & B), and so did neutrophil MPO assessment (Figure 3C).

Cytokine Expression

The pro-inflammatory cytokines TNF- α and IL-6 were measured in the plasma and in the BALF. Both cytokines were noted to be significantly elevated in the instilled animals' BALF

compared to the shams, and both were diminished after VPA administration, suggesting an ameliorated inflammatory response secondary to the HDACI administration. Compared to the sham animals, the IL-6 levels measured in the bloodstream at both 3 and 6 hours were elevated.²¹ TNF- α levels in plasma were below the level of detection. VPA led to a statistically significant reduction in both these cytokines at both time points. Although Angiopoietin-2 has recently been identified as a promising biomarker in predicting development of ARDS in humans,²⁶ we were not able to replicate this finding in our experiments in mice, with its levels in sham animals not being different compared to animals with ALI and those that received VPA. BALF chemokines MIP-2 and KC were increased compared to shams in the *E. coli*-instilled animals, however, VPA appeared to affect only the former. These data suggest that histone acetylation helps limit the expression of proinflammatory cytokines and neutrophil migration during *E. coli* pneumonia early on.

Transalveolar-capillary membrane protein transit

An important result of lung infection and resulting inflammation is decompartmentalization, in which the normally tight alveolar epithelial barrier is compromised and large circulating molecules from the bloodstream may extravasate into the alveolar space and locally produced proinflammatory cytokines can spill over into the systemic circulation.^{17,29,30} This pulmonary endothelial and capillary membrane injury from the local infection led to increased permeability between the sham and *E. coli*-instilled animals, and - as demonstrated earlier - the systemic IL-6 levels were elevated at both 3 and 6 hours after *E. coli* instillation compared to the *E. coli*-naïve animals. Both measurements were decreased with VPA administration. Conversely, significantly larger IgM leakage into the BALF and a trend toward greater albumin transit was noted in the *E. coli*-challenged animals. Despite the ameliorated inflammatory response in the VPA-treated animals, there was a trend toward lower plasma extravasation into the alveolar air spaces, however, this did not reach statistical significance (Figure 5). The maintenance of the alveolar-capillary barrier in the VPA-treated mice is also suggested by the fewer extravasated red cells seen in the alveolar space of the sectioned pulmonary parenchyma (Figure 1C).

Pulmonary bacterial burden

Any bacterial infection in an immune competent host leads to an inflammatory response and activation of numerous bactericidal pathways, in an attempt to curb bacterial proliferation. As might have been expected from the mitigated inflammatory response and the impaired neutrophil migration into the lungs, the VPA-treated animals' bactericidal activity was compromised, and the *E. coli* bacterial burden was exaggerated in these mice (Figure 6).

Discussion

Any bacterial infection in an immune competent host leads to an inflammatory response that includes neutrophil recruitment to the site of infection and activation of numerous bactericidal pathways, in an attempt to curb microbial proliferation. This response, in addition to bacterial killing, commonly leads to host tissue destruction, that when exaggerated, may in itself be more harmful than the original inflammatory stimulus. ALI/ARDS represents one such example, in which the diffuse pulmonary neutrophil recruitment

and alveolar epithelial membrane disruption result in intraparenchymal hemorrhage, decompartmentalization, and severely impaired gas exchange. These changes may be recognized regardless of whether the primary inflammatory insult was infectious or not. Numerous anti-inflammatory strategies have been tried in the past in an attempt to mitigate the clinical sequelae of ARDS with no clinical benefit in humans.^{12,13,31} It appears that anti-inflammatory regimens have no effect in reversing the course of disease once neutrophil recruitment and pulmonary parenchymal destruction have peaked, events that typically occur early on, before the full-blown clinical syndrome reaches its zenith. This observation allowed the focus of management of ARDS to be shifted from *treating* patients with it, to establishing strategies that *prevent* it in patients with potent enough inflammatory stimuli.³² Thankfully, recent work appears to have yielded satisfactory risk stratification tools toward identifying patients *at risk* for developing ARDS.³²⁻³⁴ An approach tailored around administering early anti-inflammatory therapies in patients with severe pneumonia or aspiration, sepsis, extensive burns, multi-organ trauma, or any other source of a major systemic inflammatory response, could pave the way toward improving ARDS-related morbidity and mortality.

In our experiment, we demonstrate that a pharmacological agent known to be a histone deacetylation inhibitor attenuated expression of IL-6, TNF- α , and MIP-2, decreased neutrophil migration to the pulmonary parenchyma, and improved decompartmentalization, overall minimizing the infection-induced lung injury and improving the histopathologic changes consistent with ALI. As a result of the impaired pro-inflammatory cytokine production and neutrophil recruitment, bacterial proliferation became uncontrolled to a greater degree in the HDACI-treated mice. One of the key mechanisms by which bacterial infections trigger an inflammatory response in the host aimed at curbing the infection is through the Toll-Like Receptor (TLR), Myeloid Differentiation Factor 88 (MyD88), Interleukin-1 Receptor Kinase (IRAK1) and NF- κ B pathway: PAMPs interface with macrophage plasma membrane TLRs and signal through MyD88 and IRAK1 to stimulate nuclear translocation of NF- κ B, subsequently turning on the transcription of proinflammatory cytokines.^{35,36} These κ B-regulated proteins include KC, MIP-2, TNF- α , IL-6 and IL-1 β and have been shown to play a pivotal role in the pathogenesis of lung injury.^{19,37-39} It is conceivable, therefore, that by timely curbing overexpression of these cytokines, histopathologic changes that lead to ALI may be minimized.¹⁷

Although histone deacetylase inhibition has been used with success in animal models with a variety of septic insults,^{40,41} the mechanisms by which it diminishes expression of pro-inflammatory mediators remain largely speculative. Histone deacetylase inhibition can decrease MyD88 and IRAK1 protein expression, likely by enhancing their degradation due to Heat Shock Protein 90 (HSP90) hyperacetylation, thereby preventing NF- κ B nuclear translocation and cytokine expression *in vivo*.⁴² This represents at least one possible mechanism by which the κ B-regulated proteins become underexpressed with histone deacetylase inhibitors. The mechanism by which valproic acid diminishes cytokine expression now becomes an important focus for further study.

One downside of Histone Deacetylase Inhibition in the presence of an acute pulmonary bacterial infection is that the downregulation of the microbicidal host immune response

impairs local bacterial clearance in the lungs. Similar findings have been observed in numerous other related settings, including but not limited to the deficiency of NF- κ B RelA, MyD88, or early response cytokines TNF and IL-1.⁴³

Due to the decreased neutrophil migration, an additional factor mitigating alveolar destruction may be the decreased MPO activity, a key lysosomal enzyme present in azurophilic granules released extracellularly during neutrophil degranulation. MPO not only is necessary for bacterial killing, it has also been increasingly implicated in the pathogenesis of ARDS^{44,45} by differentially affecting expression of claudins, molecules responsible for the maintenance of tight junctions between pneumocytes^{46,47} and indirectly upregulating MIP-1 synthesis,⁴⁴ further perpetuating neutrophil emigration to the lungs and propagating lung injury.

Unlike neutrophils, we did not identify a change in the macrophage count in the BAL, however, the samples were obtained somewhat early on, at 6 h, limiting potential migration of extra-alveolar monocytes/macrophages that typically takes place later on.^{48,49} In future experiments we plan on assessing the role of macrophages in the pathogenesis of ARDS in greater detail.

Despite the amount of attention Angiopoietin-2 has recently received, not only as a proangiogenic factor, but also as an important inflammatory mediator,⁵⁰ a surrogate marker for the degree of pulmonary endothelial inflammation,⁵¹ and a predictor of development of multiorgan failure⁵² and ARDS,²⁶ we were not able to identify an association between the severity of lung injury and its levels in our experiments. We found some levels of Angiopoietin-2 to be constitutionally expressed in the sham animals, and its levels did not change significantly in the presence of infection and significant lung injury, suggesting that in mice this molecule perhaps has non-homologous function compared to its human counterpart, or its role in ARDS may not have been reflected in this particular disease model.

Attenuation of the lung injury in bacterial pneumonias, currently the most common cause of ALI/ARDS, may have significant translational implications should it be considered for use in patients with early ARDS or at risk for it. Even though other anti-inflammatory therapies have been evaluated in human clinical trials with poor results in the past,^{12,13,31} it is likely their delayed administration during the full-blown phase of the clinical syndrome precluded any significant clinical benefits. Contrasting these findings with our preliminary results that demonstrate an ameliorated syndrome, it is possible that a therapeutic window of time exists, and if potent anti-inflammatory therapies are administered early - shortly after the original inflammatory insult and before the full-blown syndrome of ALI/ARDS develops, clinically relevant benefits may be anticipated.

There are numerous limitations to our model, including no assessment of pulmonary function, survival, and long-term outcomes, as well as the role of antibiotherapy, optimal dosing of VPA or other narrow-spectrum HDACI, or identification of specific molecular pathways that are implicated in the pathophysiology of the syndrome that may become more

specific therapeutic targets in the future. However, we aim to tackle these research questions one at a time.

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Figure 1.

Timeline of *E. coli* endotracheal instillation, VPA or vehicle intraperitoneal injection, blood collection, and bronchoalveolar fluid and tissue sampling.

Figure 2.

(From left to right) A. Lung section of sham animal; B. Inflammatory changes, including infiltration with numerous neutrophils, fibrin and hyaline membrane deposition, alveolar wall thickening and red cell extravasation, as well as loss of parenchymal architectural integrity are noted in the ARDS animals; C. Improved histopathology in the VPA-treated animals. Assessment of inflammatory cells in BALF in D. sham animals; E. ARDS animals; and F. VPA-treated mice.

Figure 3.
(Clockwise) Numeric assessment of the macrophage and neutrophil counts in the BALF, and density differential in neutrophil MPO activity. (* $p < 0.05$)

Figure 4. Decreased IL-6, TNF- α and MIP-2 levels in the BALF and IL-6 in the bloodstream with VPA administration. KC and Angiopoietin-2 levels were unchanged. (* p<0.05; ** p<0.01)

Figure 5.
Trend toward improved transalveolar-capillary membrane protein transit, as evidenced by BALF levels of IgM and albumin in the VPA-treated mice. (* $p < 0.05$)

Figure 6.

B. Bacterial burden was significantly increased in the VPA-treated mice. (** $p < 0.01$)