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Creating a Prosurvival Phenotype through a Histone Deacetylase Inhibitor in a Lethal Two-Hit Model

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Abstract

Objectives—Hemorrhagic shock (HS) can initiate an exaggerated systemic inflammatory response and multiple organ failure, especially if followed by a subsequent inflammatory insult (“second hit”). We have recently shown that histone deacetylase inhibitors can improve survival in rodent models of HS or septic shock, individually. In the present study, we examined whether valproic acid (VPA), a histone deacetylase inhibitor, could prolong survival in a rodent “two-hit” model: HS followed by septic shock from cecal ligation and puncture (CLP).

Methods—Male Sprague-Dawley rats (250–300 g) were subjected to sublethal HS (40% blood loss) and then randomly divided into two groups (n = 7/group): VPA and control. The VPA group was treated intraperitoneally with VPA (300 mg/kg in normal saline [NS], volume = 750 μ L/kg). The control group was injected with 750 μ L/kg NS. After 24 h, all rats received CLP followed immediately by injection of the same dose of VPA (VPA group) or NS (vehicle group). Survival was monitored for 10 days. In a parallel study, serum and peritoneal irrigation fluid from VPA- or vehicle-treated rats were collected 3, 6, and 24 h after CLP, and enzyme-linked immunosorbent assay was performed to analyze myeloperoxidase activity and determine tumor necrosis factor α and interleukin 6 concentrations. Hematoxylin-eosin staining of lungs at 24-h time point was performed to investigate the grade of acute lung injury.

Results—Rats treated with VPA (300 mg/kg) showed significantly higher survival rates (85.7%) compared with the control (14.3%). Moreover, VPA significantly suppressed myeloperoxidase activity (marker of neutrophil-mediated oxidative damage) and inhibited levels of proinflammatory cytokine tumor necrosis factor α and interleukin 6 in the serum and peritoneal cavity. Meanwhile, the severity of acute lung injury was significantly reduced in VPA-treated animals.

Conclusions—We have demonstrated that VPA treatment improves survival and attenuates inflammation in a rodent two-hit model.

Keywords

Hemorrhage; sepsis; shock; resuscitation; valproic acid; acetylation; survival; cytokine; acute lung injury

Introduction

Hemorrhagic shock (HS) is a major cause of morbidity and mortality among trauma patients. Sepsis or septic shock (SS) is a leading cause of mortality in intensive care units (1). Even when patients survive the acute episode of blood loss, they often exhibit a systemic inflammatory response syndrome, which is often further complicated by the subsequent development of infections (2). Despite advances in supportive treatments, the mortality and morbidity remain high (3, 4) with a substantial burden on health care; thus, it is essential not only to elucidate the pathogenesis but also to develop more effective treatments (5). The classic view regarding the pathogenesis of sepsis proposes a hyper-inflammatory response with excessive production of cytokines (e.g., tumor necrosis factor α [TNF- α]) and a robust activation of the immune system. Inflammation is a double-edged sword; under optimal circumstances, it is a self-limiting and protective response of the body in which cytokine production is well controlled, and leukocytes are activated to eliminate the harmful pathogens. However, an exaggerated or dysregulated response can lead to immune-mediated cell damage and organ injury (1). It has also been shown that trauma (in the absence of any infectious agents) can stimulate a systemic inflammatory response very similar to sepsis (6).

Protein acetylation as a posttranslational modification controls many cellular processes, and increasing evidence has implicated protein acetylation in immunological pathways. Two opposing enzyme families, histone deacetylases (HDACs) and histone acetyl transferases, control the balance of acetylation in a cell and can be therapeutically targeted by a class of drugs called HDAC inhibitors (HDACIs) (7). Histone deacetylases play a key role in “acetylation-homeostasis” involving histone and nonhistone proteins and in regulating multiple fundamental cellular activities. Histone deacetylases remove acetyl groups from lysine residues of target proteins, which alter their functions. Inhibitors of HDAC restore acetylation, which in turn rapidly modulates the function of various inflammatory and immunologic pathways (8). At the molecular level, it has been reported that hemorrhage and sepsis lead to an imbalance in acetylation of proteins and that HDACI treatment can restore balance and improve outcomes (7, 9, 10).

Recently, our laboratory has demonstrated that HDACIs can significantly improve survival in rodent models of HS or SS, individually (10, 11). However, it still remains unknown whether HDACI treatment can change the outcomes in the more complex “two-hit” insult, i.e., HS complicated by SS. In the present study, we investigated whether valproic acid (VPA), one of the HDACIs, could prolong survival in a rodent model of HS followed by cecal ligation and puncture (CLP).

Materials and Methods

All the research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals. The study adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, and was approved by the Institutional Animal Care and Use Committee.

Animal preparation

Male Sprague-Dawley rats (250–300 g) were housed in their cages, with free access to food and water, and kept at room temperature before the experiment.

Surgical procedure

According to our published protocol (11), VPA (Calbiochem, San Diego, Calif) solution was prepared fresh by dissolving it in filtered distilled water to create a 400-mg/mL solution. Anesthesia was induced with 5% isoflurane (Abbott Laboratories, North Chicago, Ill) mixed with air in an induction chamber. Rats were then fitted with a nose cone scavenging system and allowed to breathe spontaneously. Anesthesia was maintained by delivering 0.7% to 1.2% isoflurane via the nose cone using a veterinary multichannel anesthesia delivery system and vaporizer (Kent Scientific Corporation, Torrington, Ct). After injecting 0.2 mL of 0.5% bupivacaine (AstraZeneca, Wilmington, Del) for local anesthesia, an incision was made over the right femoral vessels; the femoral artery and vein were dissected, and both vessels were cannulated with polyethylene 50 (PE50) catheters (Clay Adams, Sparks, Md). The venous cannula was used for hemorrhage, whereas the arterial catheter was connected to the Ponemah Physiology Platform (Gould Instrument Systems, Valley View, Ohio) for continuous hemodynamic monitoring in the first hit of HS.

HS protocol

Estimated total blood volume was calculated based on methods by Fukudome et al. (11) (estimated total blood volume [in mL] = weight [in g] $\times$ 0.06 [in mL/g] + 0.77). Venous blood was withdrawn at a steady rate using infusion and withdrawal syringe pumps (Kent Scientific Corporation).

To induce HS, baseline arterial blood samples were obtained, and anesthetized rats were hemorrhaged 40% of their total blood volume over 10 min. The animals were then randomly assigned to receive treatment immediately with either (a) VPA (300 mg/kg; volume = 750 μ L/kg, intraperitoneally [i.p.]; n = 7) or (b) vehicle control (750 μ L/kg of 0.9% saline, i.p.; n = 7). After 1 h of observation, postshock arterial blood samples were drawn. Cannulas were then removed, vessels were ligated, skin was closed, and animals were recovered from anesthesia and returned to their cages for close observation.

SS protocol

Twenty-four hours later, inhaled isoflurane 5% was used to induce anesthesia and then decreased to 2% for maintenance during the procedure. CLP was performed according to the model established by Rittirsch et al. (12). The abdomen was opened via a 2-cm midline

incision, and cecum was carefully exposed and isolated. Next, the cecum was ligated at the designated position (75%) with 3-0 black braided silk nonabsorbable suture and perforated by a single through-and-through puncture midway between the ligation and the tip of the cecum in a mesenteric-to-antimesenteric direction using an 18-gauge needle. After removing the needle, a droplet of feces was extruded from both holes to ensure patent perforations. After returning the cecum to the abdominal cavity, it was closed in two layers (fascia and skin). The sham animals underwent identical laparotomy and cecal manipulation without ligation and puncture. The second dose of VPA or 0.9% normal saline (NS) was given via i.p injection; animals recovered from anesthesia and transferred to their cages for observation.

Experimental design

This study included two experiments. The first one is a survival experiment. All the animals (VPA treatment and vehicle control groups) were subjected to the two-hit insult (see *Hemorrhagic shock protocol* and *Septic shock protocol*), and survival was monitored for 10 days.

In the second experiment, rats were also subjected to the two-hit model and killed at 3, 6, and 24 h after CLP (n = 3 rats per group and per time point). At the time the rats were killed, the abdominal cavity was opened and washed with 2 mL NS, and this peritoneal fluid was collected for analysis. Cardiac puncture was performed to collect blood, and serum was prepared for the measurement of circulating cytokines. Lungs were harvested from these rats to analyze protein activity or expression. A rapid tracheal infusion method for routine lung fixation was used to preserve the left lung for histological evaluation as previously described (13). As a control, tissue samples were also obtained from sham animals that were anesthetized and instrumented without hemorrhage or CLP. All animals at the end of the study were killed by pentobarbital overdose (100 mg/kg, intravenously [i.v.]) for tissue harvest.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity in peritoneal irrigation fluid was determined using the Myeloperoxidase Assay Kit (Cell Sciences Inc, Canton, Mass) according to the manufacturer's instructions. The peritoneal cavity was irrigated with 2 mL NS, and the fluid was saved for analysis. Tissue samples were then obtained. The samples were centrifuged at 3,000g at 4°C for 10 min, and supernatants were analyzed for MPO levels.

Cytokine measurements

TNF α and interleukin 6 (IL-6) in serum and peritoneal irrigation fluid were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems Inc, Minneapolis, Minn). The concentrations of cytokines were measured by optical densitometry at 450 nm in a SpectramaxPlus 384-microplate reader (Molecular Devices, Sunnyvale, Calif). All of the analyses were performed in triplicates.

Acute lung injury scoring

The acute lung injury (ALI) scoring was performed by a board-certified pathologist blinded to the treatment assignment of the samples. The method for objective quantification of the injury has been described previously (14). In brief, ALI was classified into four categories based on the severity of alveolar congestion and hemorrhage, infiltration of neutrophils in the air spaces or vessel walls, and the thickness of alveolar wall. The severity of each category was graded from 0 (minimal) to 4 (maximal), and the total score was calculated by adding the scores in each of these categories. In each animal, three separate lung sections were graded to generate the mean score.

Statistical analysis

Survival rates were compared by Kaplan-Meier log-rank test. Continuous data were summarized as means $\pm$ standard deviation (SD). Student *t* test was used to compare the differences between two groups. Differences between three or more groups were assessed using one-way analysis of variance followed by Bonferroni post hoc testing for multiple comparisons. Mann-Whitney *U* test was used for nonparametric data, and $P < 0.05$ was considered to be statistically significant. Data were analyzed using SPSS for Windows (version 15.0; SPSS Inc, Chicago, Ill).

Results

VPA improves survival in a rat two-hit model of HS and SS

As shown in Figure 1, 85.7% of rats in the control group died within 6 days, with most of the deaths within the first 24 h. However, VPA-treated animals displayed a significantly higher long-term survival rate (85.7% of rats survived >10 days). The sham-operated animals (no two-hit, no treatment) all survived (data not shown). These results indicate that VPA treatment significantly improves survival in this two-hit model ($P < 0.05$).

VPA decreases CLP-induced MPO activity

As shown in Figure 2, MPO activity was very low in the sham group, whereas CLP resulted in a significant ($P < 0.05$) increase in the MPO activity. In contrast, VPA treatment was associated with a significant attenuation in the MPO rise.

VPA suppresses production of proinflammatory cytokines

CLP in the vehicle group was associated with a significant elevation in the levels of TNF- α in peritoneal fluid at 3 h and in the peritoneal fluid and blood at 3 and 6 h (Fig. 3). IL 6 was slower to rise, and an increase in its levels was not noted in the peritoneal fluid and blood until 24 h after CLP (Fig. 4). Treatment with VPA significantly attenuated all of these changes (Figs. 3 and 4).

VPA attenuates two-hit-induced ALI

The sham group showed normal lung histology. In contrast, the lung tissues of the vehicle control group were significantly damaged, with interstitial edema, hemorrhage, thickening of the alveolar wall, and infiltration of inflammatory cells. These histology changes were

prevented with the VPA treatment (Fig. 5). Objective ALI scoring, performed by a pathologist blinded to the treatment, revealed that the two-hit model increased the score, whereas VPA treatment significantly reduced it ($P < 0.05$).

Discussion

In the present study, we have demonstrated that administration of VPA significantly improves survival in a rodent model of HS followed by CLP-induced SS. Further analysis shows that VPA inhibits activity of MPO in peritoneal exudate, attenuates the increased levels of TNF- α and IL-6 proteins in the peritoneal irrigation fluid and blood, and alleviates the ALI caused by the two-hit.

VPA, a well-known antiepileptic drug, has also been used to treat several other neurological disorders and more recently as an antineoplastic drug (15). As additional properties of this molecule are discovered, its therapeutic applications are likely to expand even further (7, 16). We now know that VPA (in high doses) can act as an HDACI to regulate key cellular mechanisms by rapidly increasing the acetylation of nuclear and nonnuclear proteins. Not only can it modulate the transcription of many important genes, but it can also rapidly alter the functions of many preexisting proteins. These proteins can influence several important pathways such as cell cycle, survival, differentiation, DNA repair, and apoptosis (7, 16). In HS models, we have demonstrated that VPA treatment improves survival and modulates numerous critical pathways (e.g., Akt [also known as protein kinase B] survival pathway members, heat shock proteins, early/immediate kinases) through acetylation (16–19). The cumulative result of all these changes is attenuation of inflammation and creation of a “prosurvival” phenotype. In addition to modulating the inflammatory cascades, VPA may also be able to more directly attenuate the oxidative process. For example, we have discovered that VPA decreases 8-isoprostane expression in pulmonary tissue in a rodent model of intestinal ischemia reperfusion (13). 8-Isoprostane is a prostaglandin-like compound formed *in vivo* from the free radical-catalyzed per-oxidation of arachidonate independent of the cyclooxygenase. In addition to being an important marker of oxidant stress, a number of reports have shown that 8-isoprostane also possesses potent biological activity and mediates many aspects of oxidative process. Thus, it is conceivable that inhibition of 8-isoprostane is a key mechanism for the prevention of ALI.

Sepsis with multiple system organ failure is a common cause of morbidity and mortality in severely injured patients. In recent years, the concept of two-hit has gained popularity as an explanation for the development of multiple system organ failure. The first hit (hemorrhage/tissue injury) is believed to result in priming of the inflammatory system, which responds in an exaggerated fashion in response to the second insult (sepsis) to cause immune mediated organ injury. Cecal ligation and puncture causes severe intra-abdominal infection, which creates a robust model of polymicrobial sepsis (12, 20, 21). We selected CLP as the second hit in our experiments as it is widely used as a standard insult in sepsis research (12, 20, 21). The outcome after CLP is closely associated with several factors such as the length of the cecum ligated, the size of the needle, and the number of punctures. In the current study, we used the Rittirsch protocol (12) and obtained consistent results with majority of the vehicle-treated animals dying in the first 24 h.

The release of proinflammatory and anti-inflammatory cytokines induces systemic inflammatory alterations. An overproduction of proinflammatory cytokines has been found to be associated with worse organ dysfunction and a higher mortality (22). Recently, HDACIs have emerged as potent prosurvival and anti-inflammatory drugs, offering new lines of therapeutic intervention for HS and SS. We and other groups have found that HDACIs such as VPA, suberoylanilide hydroxamic acid, and trichostatin A prevent hemorrhage-associated lethality in rat and swine models of HS, suppress expression of proinflammatory cytokines, and improve survival in a mouse model of SS (10, 11, 15, 23).

In the present study, we found that 24 h is a critical time point. Most of the death occurs around 24 h after CLP. We confirmed it in our nonsurvival experiment and did not harvest lung tissues from the vehicle control groups for pathological examination if the rats could not survive for 24 h. We observed severe pneumoedema and diffuse suppurative peritonitis from rats in the vehicle control group, which corresponds with our findings shown in Figures 3, 4, and 5. In contrast, there was no obvious infiltration detected from lung tissues and peritoneal cavity of VPA-treated rats. Our data suggest that animals possibly died of SS and acute respiratory failure.

The dose of VPA we used in this study is relatively high. At the high dose of 300 mg/kg, we have recently demonstrated that administration of VPA enhances nuclear histone acetylation and improves survival before and after lethal hemorrhage in rats and swine. In addition to HDACI activity, the survival advantage of the high dose of VPA has been found (a) because of stimulation of the transcription of survival gene bcl-2 and protection of cells after severe insult through the β -catenin survival pathway in a rodent model of HS (24) and (b) because of better tolerance of shock by cells and prevention of the Akt survival pathway in cells in a swine model of HS (25). Moreover, VPA (300 mg/kg) can attenuate ALI through the significant decrease in lung tissue concentration of cytokine-induced neutrophil chemoattractant, MPO, and 8-isoprostane in a rodent model of intestinal ischemia reperfusion (13). Preclinical data in hemorrhage models have been promising enough that the Food and Drug Administration has approved our application to begin a phase 1, single-ascending-dose, double-blind, placebo-controlled study to evaluate the safety and tolerability of VPA in healthy volunteers or trauma patients.

We have to point out that i.p. injection of VPA in the present study is different from i.v. injection of VPA in our previous investigation (16). They are all sublethal models with 40% of total blood loss. There is dissimilarity that VPA is slowly absorbed in present study, while the drug is circulating in the blood quickly in the previous model. However, the VPA treatment in both studies works well, although the drug administration route is different. In the current study, the rats were bled for 10 min, the model was less lethal, and VPA was given i.p. immediately after 10-min blood loss. In the previous study, the rats were kept under unresuscitated shock for 30 min after the 40% blood loss, and the model was more severe, and the i.v. VPA administration was also postponed to 30 min after the shock compared with the present model. Therefore, the slowly absorbed VPA after i.p. injection can still prolong animal life in a mild hemorrhagic model.

Work by Iskander et al. (26) has shown that there is very little lung damage in the CLP model of sepsis. We also observed the same result in a rat model of CLP-induced sepsis (data not shown). However, a single hit of CLP is different from the two-hit model—HS plus CLP-induced sepsis. Given that HS can induce pulmonary inflammation and ALI, it is understandably easy to interpret our results that the second hit of CLP-induced sepsis aggravates the already existing ALI caused by the first hit of HS.

Our study has some limitations. Blood and tissue sampling time points were limited. Similarly, for logistical reasons, we focused our attention on selected cytokines and pathways, and it is highly likely that many more pathways are altered. Currently we are exploring a number of these pathways, using chromatin immunoprecipitation, genomic DNA microarray, and high-throughput proteomic techniques.

In summary, our study presents the first direct evidence for creating a prosurvival phenotype through VPA treatment in a two-hit model. We have shown that VPA treatment improves survival, suppresses MPO activity, inhibits proinflammatory cytokine TNF- α and IL-6, and attenuates ALI in a rat model of HS followed by CLP-induced SS.

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Fig. 1. Effect of VPA on survival in a rat two-hit model of HS and SS

Male Sprague-Dawley rats (250–300 g) were subjected to sublethal HS and then randomized into two groups ($n = 7/\text{group}$): VPA and vehicle control. The VPA group was treated with VPA (300 mg/kg in NS, volume = 750 $\mu\text{L}/\text{kg}$, i.p.). The control group was injected with 750 $\mu\text{L}/\text{kg}$ NS vehicle. After 24 h, all rats received CLP followed immediately by injection of the same dose of VPA (VPA group) or NS (vehicle group). Survival was monitored for 10 days. The Kaplan-Meier curve illustrates survival over the 10-day observation period. Treatment with VPA 300 mg/kg significantly improved survival compared with NS vehicle control (*85.7% vs. 14.3% survival; $P < 0.05$).

Fig. 2. Valproic acid (VPA) decreases CLP-induced MPO activity

MPO activity in peritoneal irrigation fluid was analyzed by ELISA from rats treated with or without VPA at 3 h after CLP. All analyses were performed in triplicate. Values represent the means $\pm$ SD ($n = 3$). Two-hit significantly increases MPO activity, and VPA treatment can attenuate MPO activity. *Value significantly differs from the vehicle control group ($P < 0.05$). #Value significantly differs from the sham control group ($P < 0.05$).

Fig. 3. Valproic acid (VPA) treatment decreases level of TNF- α in serum and peritoneal irrigation fluid

The levels of TNF- α (pg/mL) were analyzed in peritoneal irrigation fluid and serum of rats treated with or without VPA by ELISA (described in MATERIALS AND METHODS). All analyses were performed in triplicate. Values represent means $\pm$ SD ($n = 3$). *Value significantly ($P < 0.05$) differs from the vehicle control group. #Value significantly ($P < 0.05$) differs from the sham group.

Fig. 4. Valproic acid (VPA) treatment decreases level of IL-6 in serum and peritoneal irrigation fluid

The levels of IL-6 (in pg/mL) were analyzed in peritoneal irrigation fluid and serum of rats treated with or without VPA by ELISA (described in MATERIALS AND METHODS). All analyses were performed in triplicate. Values represent means $\pm$ SD ($n = 3$). *Value significantly ($P < 0.05$) differs from the vehicle control group. #Value significantly ($P < 0.05$) differs from the sham group.

Fig. 5. Valproic acid attenuates two-hit-induced ALI

Representative slides of lung sections ($n = 3$) from animal groups of the sham, vehicle control, and VPA treatment. The pathologic changes were examined by hematoxylin-eosin staining and light microscopy in the lung tissues of rat treated with or without VPA at 24 h after CLP. The VPA group had less alveolar congestion, neutrophil infiltration, and alveolar wall thickness. Acute lung injury (ALI) was scored as described in MATERIALS AND METHODS and expressed as mean values $\pm$ SD. *Value significantly differs from vehicle control group ($P < 0.05$). #Value significantly differs from sham group ($P < 0.05$). Original magnification $\times 40$.