Published in final edited form as:

Resuscitation. 2011 January ; 82(1): 105–109. doi:10.1016/j.resuscitation.2010.09.469.

Histone Deacetylase Inhibitors Prevent Apoptosis following Lethal Hemorrhagic Shock in Rodent Kidney Cells

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Abstract

Background—We have previously demonstrated that treatment with histone deacetylase inhibitors (HDACI), such as valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA), can improve survival after hemorrhagic shock in animal models. Hemorrhage results in hypoacetylation of proteins which is reversed by HDACI. These agents are known to acetylate insulin receptor substrate-I (IRS-I), which in turn activates the Akt survival pathway. This study investigated whether HDACI exert their beneficial effects through the Akt survival pathway.

Methods—Wistar-Kyoto rats (N=21) underwent hemorrhage (60% blood loss) and were randomized into 3 groups; no resuscitation (NR), and treatment with VPA or SAHA. Kidneys were harvested at 1, 6, and 24 hours after HDACI treatment and analyzed for acetylated histone 3 at lysine 9 residue (Ac-H3K9), phosphorylated Akt (phospho-Akt), BAD and Bcl-2 proteins.

Results—Hemorrhaged animals were in severe shock, with mean arterial pressures of 25–30 mmHg and lactic acid 7–9 mg/ml. Only animals treated with VPA and SAHA survived to the 6- and 24- hour timepoints. Treatment with HDACI produced a biologic effect on rat kidney cells inducing acetylation of histone H3K9, which peaked after 1 hour of treatment, and was statistically significant in the VPA group ($p=0.01$) compared to NR. Phospho-Akt protein increased in the VPA group with a reciprocal decrease in the pro-apoptotic BAD protein in both groups which was statistically significant in the VPA group after 1 hour ($p=0.007$) and 24 hours ($p=0.006$) of treatment and in the SAHA group after 24 hours of treatment ($p=0.028$). Anti-apoptotic Bcl-2 protein markedly increased after 6 ($p=0.04$) and 24 hours ($p=0.014$) of VPA treatment. Bcl-2 also increased in the SAHA group, but failed to reach statistical significance.

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Conflict of interest statement

No conflicts of interest declared.

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Conclusion—Treatment with HDACI increases phosphorylation of Akt with a subsequent decrease in the pro-apoptotic BAD protein. The above mechanism facilitates the action of anti-apoptotic protein Bcl-2. HDACI protect kidney cells subjected to hemorrhagic shock in rodents through the Akt survival pathway.

Keywords

Akt; apoptosis; hemorrhage; resuscitation; survival; valproic acid; Bad; Bcl-2

INTRODUCTION

Hemorrhagic shock is a major cause of organ dysfunction, multiple organ failure, and death in trauma patients [1]. It is known to induce apoptosis in cells from diverse tissues [2]. Various pathways are known to be involved resulting in changes in expression of various apoptotic and anti-apoptotic proteins such as Bcl-2, BAX and Mcl-1 [2].

Acetylation of lysine residues on histone and non-histone proteins is a well-established cellular process, resulting in activation or inhibition of gene transcription as well as cell signaling pathways in the cytosol [3,4]. The above processes are mediated by the enzymes histone acetyl transferase (HAT) and histone deacetylase (HDAC). Histone deacetylase inhibitors (HDACI) are a group of drugs that selectively inhibit HDAC and thus induce hyperacetylation of histone and non-histone proteins [4]. VPA (2-propyl-valeric acid) is a chemical compound that is clinically used as an anticonvulsant and mood-stabilizing drug, primarily for the treatment of epilepsy, bipolar disorder and major depression. It acts as a GABA agonist, by GABA transaminase inhibition, voltage-gated sodium channel and T-type calcium channel blocker, and as an HDAC inhibitor. Suberoylanilide hydroxamic acid (SAHA), is a member of the family of compounds that act as HDACI. It is clinically approved for the treatment of cutaneous T cell lymphoma and Sezary Syndrome.

We have previously demonstrated that VPA, an HDACI enhances nuclear histone acetylation and improves survival after lethal hemorrhage in rats [5,6]. Moreover, we have shown that VPA protects cells through the β -catenin survival pathway in neuronal cells [7]. However, more than one mechanism seems to be involved: in addition to acetylation of nuclear histones to enhance gene transcription, HDAC inhibitors rapidly activate numerous other pre-formed proteins to create a “pro-survival” phenotype [8–10]. It should also be pointed out that the impact of these drugs is disease and cell type specific with markedly different effects in transformed/neoplastic, normal, and injured cells.

One possible mechanism involves the phosphoinositol 3-kinase (PI3K) and its downstream target, serine threonine kinase (AKT) survival pathway. After activation of this pathway via phosphorylation, phospho-Akt phosphorylates the pro-apoptotic protein BAD [11–13]. In the absence of phosphorylation, BAD protein binds to Bcl-2 and suppresses survival by inducing BAX homodimer formation. The phosphorylation of BAD protein by phospho-Akt promotes its association with 14-3-3 protein and allows Bcl-2 to act as an anti-apoptotic factor and promote survival.

Insulin Receptor Substrate 1 (IRS-1) is a key moderator protein of insulin action [14]. IRS-1 is an acetylated protein and treatment with HDACI can induce increases in acetylation [14]. IRS-1 has been shown to activate the Akt survival pathway [12]. In the present study we investigated the mechanism of action of HDACIs, VPA and SAHA, and specifically their involvement in the Akt survival pathway. Our hypothesis was that HDACI treatment would activate the Akt survival pathway resulting in a decrease in apoptosis.

MATERIALS AND METHODS

Materials

Primary antibodies against acetyl lysine and acetyl histone H3K9 (Ac H3K9) were purchased from Upstate (Lake Placid, NY). Primary antibodies against Bcl-2, P-Akt, and BAD were purchased from cell-signaling Technology (Danvers, MA). Anti-mouse and anti-rabbit immunoglobulin (Ig) secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ). Fetal bovine serum and phosphate-buffer-saline (PBS) were purchased from Gibco-BRL (Grand Island, NY). VPA was purchased from the Sigma Chemical Co. (St. Louis, MO) SAHA was purchased from Biomol International (Plymouth Meeting, PA).

Animal groups and tissue collection

All the animal experiments were approved by the Institutional Animal Care and Use Committee and complied with the federal regulatory requirements. Wistar-Kyoto rats (254.12 ± 2.71 g; Harlan Indianapolis, IN) were used in the model of hemorrhagic shock (60 % blood loss over 60 minutes) that has been described previously [5]. Briefly, animals were anesthetized and instrumented with 3% isoflurane, and 1% bupivacaine was injected at the operative site to achieve local anesthesia. Isoflurane (0.7%) was used to maintain anesthesia during the experiment. Animals were allowed to breathe spontaneously under a nose cone scavenging system, using a veterinary anesthesia delivery system (Kent Multi-Station Anesthesia Delivery System; Kent Scientific Corp., Torrington, CT). With aseptic technique, the femoral artery and vein were cannulated with polyethylene catheters (PE50; Clay Adams, Sparks, MD). The blood volume of each animal was estimated using the formula: [estimated blood volume (ml) = weight(g) × 0.06 ml/g + 0.77]. About 1.3 mL of blood was manually drawn at the beginning and end of hemorrhage for analysis (included in the total hemorrhage calculation). Initially, 40% of the estimated blood volume was withdrawn through the arterial catheter over 10 min (simulating the brisk bleeding that takes place after trauma), followed by another 20% blood volume *via* venous catheter over 50 min (simulating slower oozing during the transport period), using Kent Scientific adjustable pumps (Kent Scientific Corp., Torrington, CT). Blood pressure and heart rate were continuously monitored, except during arterial hemorrhage, by a Ponemah Physiology Platform (Gould Instrument Systems, Valley View, OH). Blood samples (0.3 mL) were analyzed at two time points: (1) baseline, and (2) end of hemorrhage, using a Stat Profile 2 Blood Gas and Electrolyte Analyzer (Nova Biomedical, Waltham, MA). Levels of liver transaminases, bilirubin, total protein, creatinine kinase, amylase, BUN, creatinine, and glucose, were also drawn at the same time points (1 mL) and sent to our core facility for testing. All the blood samples (total 1.3 mL) were included in the total blood loss

calculations. At the end of hemorrhage, animals were randomized into 3 groups; 1) no resuscitation (NR), 2) treatment with VPA (300 mg/kg in 0.25 ml 0.9% normal saline [NS]) and 3) treatment with SAHA (7.5 mg/kg in 0.25 mL NS) and sacrificed after 1, 6 or 24 hours. The Sham group consisted of healthy rats that were sacrificed without any hemorrhage/resuscitation. Kidneys were dissected and directly immersed into liquid nitrogen.

Subcellular protein fractionation

Fresh kidney (wet weight 50 mg) was immersed in extraction buffer and homogenized by hand using a homogenizer. Subcellular proteome extraction kit (Calbiochem, Gibbstown, NJ) was used according to instructions. Protease inhibitor cocktail was added to prevent protein degradation. The supernatants were obtained through different extraction procedures with sequential incubation and centrifugation as follows: fraction I, cytosolic protein extract; fraction II, membrane/organelle protein extract; fraction III, nucleic protein extract; and fraction IV, cytoskeletal fraction protein extract. Total protein concentration in each fraction was determined by Bratford method (Bio-Rad Laboratories, Hercules, CA).

Western Blot

Proteins (about 100 µg per lane) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked in 0.05% PBS-Tween (PBST) containing 5% milk (Bio-Rad Laboratories) and then incubated with the primary antibody at 4°C overnight. The primary antibody was detected by incubation with horseradish peroxidase-coupled second antibody (1:3,000 in PBST in 5% milk) at room temperature for 2 hours. The chemiluminescence detection was performed by using Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer LAS, Boston, MA). Films were developed using a standard photographic procedure and quantitative analysis of detected bands was carried out by densitometer scanning using VersaDoc Imaging System (Bio-Rad Laboratories Hercules, CA).

Statistical analysis

Statistical comparisons between NR and treated experimental groups were performed by Student's t-test. SPSS 12.0.p statistical package was used. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

Survival

This was a highly lethal model. Only VPA and SAHA treated animals survived to the 6- and 24-hour timepoints, whereas all the animals in the NR group died before 6 hours.

Physiologic Parameters

There were no significant differences between groups in terms of weight, blood loss, and baseline or end-hemorrhage values of lactate, hemoglobin, base deficit or other markers of

organ function. The mean arterial pressure (MAP) in animals that were subjected to hemorrhage dropped sharply after bleeding to approximately 20 mm Hg. MAP gradually improved and reached approximately 30 mm Hg at the end of hemorrhage, with no inter-group differences. The hemorrhaged animals had statistically significant hypotension compared to the Sham for the entire duration of observation. The lactate level was significantly elevated (7–9 mmol/L) after hemorrhage consistent with profound shock, and the hemoglobin dropped significantly consistent with the volume of blood loss. There were no significant differences in the post-hemorrhage lactate or hemoglobin levels between the NR and treatment groups.

Effects of VPA and SAHA on acetylation of histone H3 at K9

To assess whether VPA or SAHA affects acetylation of cells, kidney tissue from hemorrhaged rats treated with VPA and SAHA (harvested after 1, 6, and 24 hours), Sham or NR groups, was carefully dissected and fractionated. Equal amounts of protein samples were separated on SDS-PAGE and transferred to nitrocellulose membranes. We have previously shown that acetylated histone H3 at lysine 9 (H3K9) is a reliable marker for acetylation of histone protein [5–7, 9–10]. Acetylation of H3K9 was detected by Western blot with anti-acetyl histone H3K9 (Ac-H3K9) antibody. Treatment of VPA markedly increased acetylation of H3K9 at 1 ($p=0.01$), 6, and 24 hours compared to NR. The acetylation peaked at 1 hour after VPA treatment (Figure 1). These results indicate that the VPA dose was sufficient to create the expected biological effect (acetylation of histones) in the kidney cell. In the SAHA group, Ac-H3K9 was also markedly increased reaching statistical significance after 6 ($p=0.029$) and 24 ($p=0.030$) hours of treatment (Figure 1).

Effects of VPA and SAHA on Akt activation

Akt is a serine-threonine kinase that exerts its anti-apoptotic effects through multiple mechanisms including the phosphorylation of BAD protein [12–13]. The activation of Akt involves phosphorylation to phospho-Akt by various upstream signals. To test the effect of VPA and SAHA on Akt activation, kidney tissue from hemorrhaged rats treated with VPA (harvested after 1, 6, and 24 hours), SAHA (harvested after 1, 6, and 24 hours) Sham or NR, was carefully dissected and fractionated. Equal amounts of protein samples were separated on SDS-PAGE and transferred to nitrocellulose membranes. Treatment of VPA markedly increased the level of phospho-Akt after 1 hour of treatment (Figure 2). This increase was not statistically significant. In the SAHA treated group there was no increase in the levels of phospho-Akt.

Effects of VPA and SAHA on BAD levels

BAD induces apoptosis through heterodimerization with Bcl-2, neutralizing its function [12–13]. To test the effect of VPA and SAHA on BAD levels, kidney tissue from hemorrhaged rats treated with VPA (harvested after 1, 6, and 24 hours), SAHA (harvested after 1, 6, and 24 hours) sham or NR, was carefully dissected and fractionated. Equal amounts of protein samples were separated on SDS-PAGE and transferred to nitrocellulose membranes. Treatment of VPA markedly decreased the level of BAD after 1 ($p=0.007$), 6, and 24 hours ($p=0.006$). In the SAHA treatment group, the decrease was statistically significant only after 24 h of treatment ($p=0.028$) (Figure 3).

Effects of VPA and SAHA on Bcl-2 levels

Bcl-2 is a well known protein with anti-apoptotic actions. To test the effect of VPA and SAHA on Bcl-2 levels, kidney tissue from hemorrhaged rats treated with VPA (harvested after 1, 6, and 24 hours), SAHA (harvested after 1, 6, and 24 hours) Sham or NR, was carefully dissected and fractionated. Equal amounts of protein samples were separated on SDS-PAGE and transferred to nitrocellulose membranes. Treatment of VPA markedly increased the levels of Bcl-2 after 1, 6 ($p=0.04$), and 24 hours of treatment ($p=0.14$). The increase in the SAHA treatment group was not statistically significant (Figure 4).

DISCUSSION

We have shown previously that severe hemorrhage is associated with HDAC/HAT imbalance, which influences the acetylation status of cells, and that it can be corrected by treatment with pharmacologic HDAC inhibitors (HDACI) [5–8, 9]. In a rodent model of lethal hemorrhagic shock, we have recently reported that post-shock administration of HDACI, such as VPA or suberoylanilide hydroxyamic acid (SAHA), significantly improves survival even in the absence of conventional fluid resuscitation [5]. This has also been validated in a clinically realistic large animal model of poly-trauma, coagulopathy and lethal blood loss [8]. We have also demonstrated that VPA treatment prevents neuronal apoptosis through HDAC inhibition in an *in vitro* model of severe hypoxia [10]. In the present study, we have shown that in an *in vivo* rodent model of lethal hemorrhagic shock, administration of VPA and SAHA induces the cellular levels of phospho-Akt, decreases the cellular levels of BAD and increases the levels of Bcl-2. These results suggest that acetylation of cellular proteins, decreased cellular levels of BAD, and increased cellular levels of Bcl-2 are important for kidney cell survival.

IRS-I protein is a key modulator of insulin and insulin like growth factor (IGF) action [14]. It is involved in various cellular function including metabolism, growth, cell differentiation and survival [16]. Four members (IRS 1–4) have been identified that differ in tissue distribution, subcellular location, binding to insulin receptor and interaction with Src homology 2 (SH2) domains [14, 16, 17]. They are all structurally characterized by N-terminal pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domains, which are required for coupling to the activated insulin/IGF receptors, and a C-terminal region with multiple sites for tyrosine phosphorylation by the receptors. IRS proteins act thus as molecular adaptors that interact with a number of SH-2 containing proteins binding to specific phosphorylated tyrosine residues and lead to activation of various intracellular pathways including the PI3K-Akt pathway [17–19].

HDACI increase acetylation of various cellular proteins. It has been shown that IRS-I and HDAC2 interact. IRS-I is an acetylated protein, of which the acetylation is increased after treatment with HDACI [14, 16]. Acetylation of IRS-I is permissive for tyrosine phosphorylation and facilitates insulin-stimulated signal transduction [14–16]. HDACI, specifically Trichostatin A and VPA, have been shown to activate the Akt survival pathway [14, 15, 19, 20]. Multiple forms of phosphoinositide 3-kinases (PI3Ks) exist in eukaryotes, with class Ia being the most important for the production of D-3 phosphoinositides in response to growth factors [21–22]. Activated PI3K converts plasma membrane lipid

phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂] to phosphatidyl-inositol-3,4,5-trisphosphate [PI(3,4,5)P₃]. Association of serine-threonine kinase Akt and phosphoinositide-dependent kinase 1 (PDK1) with PI(3,4,5)P₃ brings these two proteins into proximity facilitating phosphorylation of Akt by PDK1 [23]. Subsequent phosphorylation of the apoptosis –inducing protein BAD promotes binding of BAD to 14-3-3 proteins and prevents BAD from binding to bcl family members Bcl-2 and Bcl-xl [23].

Limitations in our study are the small sample size which did not allow us to reach statistically significant differences in all proteins investigated, although the trend was obvious. Only one specific pathway was investigated although we believe that various pathways could potentially be involved. Apoptosis was measured by a limited number of assays as this was a proof-of-concept study, and a definitive mechanistic study would require more detailed evaluations. Finally, we did not correlate the cellular findings with long term organ function. Despite these limitations we can still draw some important conclusions. Taking into account that the kidney is an organ that is prone to ischemia, we have shown that treatment with HDACIs results in activation of the Akt pathway, a decrease in apoptosis and improved survival in rodent model of severe hemorrhage. This study is the first to report a potential anti-apoptotic effect of HDACIs on kidney cells through the Akt survival pathway. Hemorrhagic shock has devastating effects on renal function, leading to a loss of renal function. Treatment with HDACIs can potentially prevent cellular death and preserve renal function in hemorrhagic shock.

Acknowledgments

Funded by a grant from the Office of Naval Research to Dr. Alam. Additional funds provided through a generous donation by the Polsky family.

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B

Figure 1. Effect of VPA and SAHA on H3K9 levels after hemorrhagic shock

A. Protein bands quantified by densitometry were expressed as mean values $\pm$ SEM.

*= $p < 0.05$ compared to no resuscitation (NR) group. SAHA 1hr, 6hr and 24 hour represent samples obtained at 1, 6, and 24 hours after suberoylanilide hydroxamic acid treatment.

VPA 1hr, 6hr and 24 hour represent samples obtained at 1, 6, and 24 hours after valproic acid treatment. The band labeling.

B. Representative protein bands from western blots for Ac-H3K9. VPA and SAHA increase the levels of Ac-H3K9 after hemorrhagic shock. The band labeling is identical to the labels shown under the corresponding bar graphs.

Figure 2. Effect of VPA and SAHA on phospho-Akt levels after hemorrhagic shock

A. Protein bands quantified by densitometry were expressed as mean values $\pm$ SEM.

*= $p < 0.05$ compared to no resuscitation (NR) group. SAHA 1hr, 6hr and 24 hour represent samples obtained at 1, 6, and 24 hours after suberoylanilide hydroxamic acid treatment. VPA 1hr, 6hr and 24 hour represent samples obtained at 1, 6, and 24 hours after valproic acid treatment.

B. Representative protein bands from western blots for phospho-Akt. VPA increases the levels of phospho-Akt after hemorrhagic shock. The band labeling is identical to the labels shown under the corresponding bar graphs.

Figure 3. Effect of VPA and SAHA on BAD levels after hemorrhagic shock

A. Protein bands quantified by densitometry were expressed as mean values $\pm$ SEM.

*= $p < 0.05$ compared to control. NR 1Hr is the no resuscitation group at 1 hour. SAHA 1hr, 6hr and 24 hour represent samples obtained at 1, 6, and 24 hours after suberoylanilide hydroxamic acid treatment. VPA 1hr, 6hr and 24 hour represent samples obtained at 1, 6, and 24 hours after valproic acid treatment.

B. Representative protein bands from western blots for BAD. VPA and SAHA decrease the levels of BAD after hemorrhagic shock. The band labeling is identical to the labels shown under the corresponding bar graphs.

Figure 4. Effect of VPA and SAHA on Bcl-2 levels after hemorrhagic shock

A. Protein bands quantified by densitometry were expressed as mean values $\pm$ SEM.

*= $p < 0.05$ compared to control. NR 1Hr is the no resuscitation group at 1 hour. SAHA 1hr, 6hr and 24 hour represent samples obtained at 1, 6, and 24 hours after suberoylanilide hydroxamic acid treatment. VPA 1hr, 6hr and 24 hour represent samples obtained at 1, 6, and 24 hours after valproic acid treatment.

B. Representative protein bands from western blots for Bcl-2. VPA and SAHA increase the levels of Bcl-2 after hemorrhagic shock. The band labeling is identical to the labels shown under the corresponding bar graphs.