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HYPOTHERMIA AND VALPROIC ACID ACTIVATE PRO-SURVIVAL PATHWAYS AFTER HEMORRHAGE

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Abstract

Background—Therapeutic hypothermia (Hypo) and valproic acid (VPA, a histone deacetylase inhibitor) have independently been shown to be protective in models of trauma and hemorrhagic shock (HS), but require logistically challenging doses to be effective. Theoretically, combined treatment may further enhance effectiveness, allowing us to use lower doses of each modality. The aim of this study was to determine whether a combination of mild hypothermia and VPA treatments would offer better cytoprotection compared to individual treatments in a hemorrhage model.

Materials and methods—Male Sprague-Dawley rats were subjected to 40% volume-controlled hemorrhage, kept in shock for 30 minutes, and assigned to one of the following treatment groups: normothermia (36–37°C), Hypo (30±2°C), normothermia+VPA (300mg/kg), and Hypo+VPA (n=5/group). After three hours of observation, the animals were sacrificed, liver tissue was harvested and subjected to whole cell lysis, and levels of key proteins in the pro-survival Akt pathway were measured using Western Blot.

Results—Activation of the pro-apoptotic protein cleaved-caspase-3 was significantly lower in the combined treatment group relative to normothermia (P<0.05). Levels of the pro-survival Bcl-2 was significantly higher in the combined treatment group relative to sham, normothermia, and

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Disclosure

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normothermia+VPA groups ($P<0.005$). The downstream pro-survival protein phospho-GSK-3 β was significantly higher in the sham, Hypo, and combined treatment groups compared to normothermia groups with or without VPA ($P<0.05$). Levels of the pro-survival β -catenin were significantly higher in the combined treatment group relative to normothermia ($P<0.01$).

Conclusions—This is the first in-vivo study to demonstrate that combined treatment with VPA and hypothermia offers better cytoprotection than these treatments given independently.

Keywords

hemorrhagic shock; resuscitation; hypothermia; apoptosis

1. Introduction

Hemorrhage is a leading cause of morbidity and mortality in civilian and combat trauma (1,2). Conventional treatment strategies for hemorrhagic shock focus on correcting blood loss by administering fluids or blood components. While fluid resuscitation restores tissue perfusion, specific anti-inflammatory or pro-survival benefits are highly dependent upon the choice of the fluids (3–5). Standard crystalloid resuscitation is perhaps the least effective fluid not only due to rapid extravasation out of the vascular system, but also by disrupting endothelial and coagulation functions (6,7). Recent research has focused on novel strategies to maintain cellular viability during shock. For example, treatment with high doses of valproic acid (VPA, a histone deacetylase inhibitor), have been shown to improve survival by activating innate cellular survival mechanisms (8). Similarly, hypothermia decreases tissue metabolism and oxygen consumption, thereby making the body more resistant to oxygen deprivation during shock. Besides decreasing the metabolic demands of the tissues, other non-metabolic pathways are likely to be involved as well (8–10). For example, both hypothermia and VPA upregulate the pro-survival phosphoinositol 3-kinase (PI3K)/Akt pathway (11), which decreases apoptosis by phosphorylating Akt and GSK-3 β , ultimately inhibiting the activation of the pro-apoptotic enzyme Caspase-3 (Figure 1). While these strategies have both shown to be protective in models of trauma and hemorrhagic shock, profound hypothermia and high doses of VPA each present unique challenges in the clinical setting. Induction of profound hypothermia requires specialized instrumentation to reduce core temperatures below 15°C. Moreover, in rodent models VPA must be given in a dose that is six-fold higher than is clinically approved, which may have potential side effects. Effective doses of VPA in patients with hemorrhagic shock remains unknown, and we are currently trying to fill this gap through a Phase I dose escalation trial (ClinicalTrials.gov Identifier: NCT01951560). In theory, combined therapy with both hypothermia and VPA may yield better outcomes, which would potentially allow us to use lower doses of each, with a better overall safety profile.

However, there is limited literature available about the interplay between VPA and hypothermia, and the possible benefits of combined therapy. We recently investigated the effects of combined VPA and mild hypothermia (32°C) treatment using an in vitro model of neuronal cells. We showed that combined treatment improves survival and decreases cell death after chemically-induced hypoxia in mouse HT22 hippocampal cells (8). As an extension of our previous work, the current study aims to obtain proof-of-concept in vivo,

by testing VPA and hypothermia in a rodent model of hemorrhagic shock. We hypothesize that combined therapy with VPA and mild hypothermia will upregulate the PI3/Akt pro-survival pathway compared to individual treatments alone.

2. Methods

Guidelines in the Animal Welfare Act and other federal statutes were followed for all experiments. The institutional animal care and use committee approved this study, and all procedures complied with the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (1996).

2.1 Animal Preparation and Monitoring

25 male Sprague-Dawley rats (225–300 grams; Charles River Breeding Labs, Wilmington, MA) were sedated with 4% isoflurane and maintained at 1% to 3% isoflurane for the duration of the experiment. 0.3 ml of 0.25% Sensorcaine (APP Pharmaceuticals, Schamburg, IL) was administered subcutaneously into the right leg, and the right femoral artery and vein were dissected and cannulated with polyethylene 50 (PE50) catheters (Clay Adams, Sparks, MD). The arterial catheter was used for hemorrhage and hemodynamic monitoring using the Ponemah Physiology Platform (Gould Instrument Systems, Valley View, OH). The venous cannula was used for VPA administration.

2.2 Treatment groups

Animals were divided into the following treatment groups: normothermic hemorrhage (NH), hypothermic hemorrhage (HH), normothermic hemorrhage with VPA resuscitation (NH+VPA), and hypothermic hemorrhage with VPA resuscitation (HH+VPA). We included normal rats (no hemorrhage or anesthesia) as a control group (n=5/group). During cannulation, NH and NH+VPA animals were kept at 37°C using a heating pad, and HH and HH+VPA animals were kept at room temperature to induce mild hypothermia. By the end of cannulation, the hypothermic animals reached a body temperature of around 32 °C and after hemorrhage temperature was maintained between 29 and 31 °C. Normothermic animals remained at a temperature of 36–38 °C for the duration of the experiment. Core temperature was monitored using an indwelling rectal thermometer.

2.3 Hemorrhage and resuscitation protocol

Rats were subjected to a controlled hemorrhage of 40% of total blood volume over 10 minutes. The 40% hemorrhage volume was based on the following formula for estimated total blood volume: $\text{weight (g)} \times 0.06 \text{ (mL/g)} + 0.77 \text{ (12)}$. All animals were subsequently left in shock for 30 minutes. NH+VPA and HH+VPA animals were then resuscitated with a single dose of VPA over a 10-minute period. Based on a previous dose-optimization study in rodents (13), a single dose of 300 mg/kg was used, which is substantially higher than the approximately 20–60 mg/kg dose for approved for human use. Fresh VPA stock solution was prepared each day using 200 mg VPA powder (Calbiochem, San Diego, CA) mixed with 335 μL sterile water. The resuscitation dose for each animal was based on the following calculation: $\text{body weight (g)} \times 0.3 \text{ (mg VPA/weight (g))} / 0.4 \text{ (mg VPA/}\mu\text{L VPA stock solution)}$. Normal saline was added to the VPA solution to make a total volume of 250 μL .

After the 30 minute shock period and subsequent resuscitation, animals were monitored for 3 hours.

2.4 Tissue sample collection

Arterial blood gas was analyzed at baseline, post-hemorrhage, post-shock, and before sacrifice. At the end of the three-hour observation period, all animals were killed by an isoflurane overdose and exsanguination. Livers were harvested, flash-frozen in liquid nitrogen, and stored at -80°C . 50 mg of tissue was homogenized in 1 ml of RIPA whole cell lysis buffer with the addition of phosphatase and protease inhibitors (HaltTM, Thermo Scientific, Waltham, MA). Tissue lysates were centrifuged at 10000 rpm for 15 min at 4°C , and the supernatant was used for western blot analysis.

2.5 Western blotting

After balancing of the samples based on protein amount, Western blotting was performed to investigate the expression of caspase-3, cleaved caspase-3, Bcl-2, phospho-GSK-3 β , β -catenin, and histone 3 acetylated at lysine 9 (ACH3K9). Equal quantities of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels and subsequently transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in 0.05% tris-phosphate buffered saline-Tween 20 (TBS-T) containing 5% milk and then incubated in primary antibody diluted in TBS-T containing 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) overnight at 4°C . Primary antibodies for caspase-3, cleaved caspase-3, Bcl-2, and phospho-GSK-3 β were purchased from Cell Signaling Technologies (Danvers, MA), β -catenin from Invitrogen (Camarillo, CA), ACH3K9 from Millipore (Temecula, CA), and β -actin from Sigma-Aldrich. Each primary antibody was detected using IRDye 680RD conjugated secondary antibodies (LI-COR Bioscience, Lincoln, NE) at 1:5000 in TBS-T with 5% milk at room temperature for one hour. The signals were visualized using Odyssey CLx Infrared Imaging System (LI-COR) and protein density was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

2.6 Statistical analysis

Data represent mean $\pm$ standard error of the mean. One-way analysis of variance with Bonferroni post-hoc testing was used to test for differences between the groups. Statistical analyses were performed using IBM SPSS Statistics version 20.0 (IBM, New York USA). A P-value of <0.05 was considered as statistically significant.

3. Results

3.1 Hemodynamic data

The mean arterial pressure (MAP) data are shown in Figure 2. Baseline MAP started around 100 mmHg for all groups, which dropped to around 50 mmHg after volume-controlled hemorrhage. The MAP in the NH group was higher during the hemorrhage period compared to the hypothermia groups (64.00 ± 1.15 mmHg in NH vs. 37.50 ± 3.72 mmHg in HH ($p=0.02$) and 34.54 ± 4.31 mmHg in HVPa ($p=0.01$)). Post-hemorrhage MAP in the NH

+VPA group was 45.19 ± 6.70 mmHg. From the post-shock period until the end of the experiment, MAP increased from around 60 mmHg to 75 mmHg in all groups.

3.2 Physiologic parameters

Serial arterial blood gasses at baseline, post-hemorrhage, post-shock, and before harvesting (end) time points are shown in Table 1. Induction of hypothermia at baseline affected several parameters. For example, the pH of HH group was lower relative to the normothermia groups, and pO₂ was lower in the NH+VPA group compared to hypothermia treatment. At the end of the experiment, we found higher pCO₂ levels and lower pH values in the hypothermia groups. Lactate was higher in VPA groups.

3.3 Protein expression

3.3.1 Acetylation of histone protein—The acetylation of histone 3 at lysine 9 (ACH3K9) was measured to determine whether the dose of VPA was effective and high enough to alter histone acetylation. Three hours after administration of VPA, both the NH+VPA and HH+VPA groups had significantly higher levels of protein acetylation relative to NH, HH, and sham groups ($P<0.01$, Figure 3). There was no difference in acetylation between the two VPA groups.

3.3.2 Akt pathway—Expression of the pro-apoptotic protein cleaved caspase-3 increased due to hemorrhagic shock, but combined treatment with HH+VPA significantly attenuated its expression ($P<0.05$, Figure 4). The pro-survival protein Bcl-2 was significantly higher in the combined HH+VPA group compared to sham, NH, and NH+VPA groups ($P<0.005$, Figure 4). Levels of phospho-GSK-3 β were significantly higher in both the HH and HH+VPA groups relative to NH and VPA-only treatment ($P<0.05$, Figure 5). The expression of the pro-survival protein β -catenin showed a significant increase due to combined HH+VPA treatment ($P<0.01$, Figure 6). β -catenin expression in the NH+VPA group also increased, but this did not reach statistical significance.

4. Discussion

This study investigated whether combined treatment with VPA and mild hypothermia could yield superior benefits in a rodent model of hemorrhagic shock. We have shown that combined treatment with VPA and hypothermia is superior compared to when these treatments are given alone. Our evidence suggests that this is, at least partly, due to modulation of the Phosphoinositide 3-Kinase (PI3K)-Akt/PKB pathway (Figure 1), a pathway that is known to enhance cell survival in many cell types including cardiomyocytes, endothelial cells, and hepatocytes (11,14,15). Our data shows that hemorrhagic shock increased the levels of cleaved caspase-3 (pro-apoptotic) and that combined treatment with HH+VPA significantly attenuated its expression (Figure 4). We also found an upregulation of several pro-survival proteins such as Bcl-2 and β -catenin. Combined treatment with HH+VPA was the only treatment that significantly increased β -catenin expression relative to the hemorrhage-only group. Bcl-2 expression was significantly greater in the HH+VPA group relative to both the NH and NH+VPA groups, suggesting that the addition of mild hypothermia to VPA treatment confers superior benefits (Figures 4 and 6). Levels of

phospho-GSK-3 β were significantly higher in both the HH and HH+VPA groups (Figure 5). Our data suggest that the combined treatment provided cytoprotection independent of the hemodynamic status or tissue perfusion (e.g. lactic acidosis). These data provide in-vivo validation of our previous findings, which showed that combined treatment of VPA and mild hypothermia improved survival and decreased cell death after chemically-induced hypoxia in mouse HT22 hippocampal cells (8).

Hypothermia is a well known and effective strategy for cellular preservation during ischemia and reperfusion, and the effects of hypothermia on improving long-term survival has been described in multiple animal models of complex injuries and lethal hemorrhage (16). Historically, hypothermia has been well known for its ability to decrease tissue metabolism and oxygen consumption (17,18). However, our lab has also shown that it not only decreases tissue oxygen consumption, but also directly alters the expression profiles of key genes. For example, we demonstrated that profound hypothermia improved survival in a rodent model of lethal low-flow state, while creating a pro-survival and anti-inflammatory profile at the level of gene transcription (19). The role of hypothermia alone on the activation of the PI3K-Akt pathway has also been previously described in the literature. For example, induction of hypothermia has been shown to preserve the Akt signaling pathway in cardiomyocytes, resulting in a decrease in cardiac apoptosis after 40% hemorrhage in rats (11). The current study is a logical extension of this previous work, as it shows that the combination of hypothermia with VPA may be even more protective.

VPA is a commonly prescribed antiepileptic drug that acts as a histone deacetylase inhibitor (HDACI) in large doses. It has been shown to be protective in models of trauma and hemorrhagic shock (20–22). Using high-throughput techniques, our lab has demonstrated that VPA alters gene expression via epigenetic modulation (9). VPA treatment down regulated early transcription of genes that modulate apoptosis, cell death, and necrosis in a large animal model of traumatic brain injury and hemorrhagic shock (9). Several possible mechanisms may explain the beneficial effect of VPA on the PI3K-Akt pathway and cell survival signaling during hemorrhagic shock. For example, VPA induces phosphorylation of Akt by inhibiting TRB3 and PTEN. P-Akt then stimulates the transcription of numerous downstream cell survival pathways, one of which is via phosphorylation of GSK-3 β . Upon phosphorylation, GSK-3 β becomes inactivated, which in turn inhibits β -catenin degradation. In addition, VPA acetylates β -catenin which translocates to the nucleus to initiate the transcription of survival genes. Phosphorylation of Akt may therefore represent a key mechanism through which VPA attenuates organ damage and promotes survival in hemorrhagic shock.

Although effective, profound hypothermia and high doses of VPA each may have limitations in the clinical setting. Induction of profound hypothermia (core temperature 10–15°C) requires specialized instrumentation and is therefore technically very challenging (23). Profound hypothermia may also produce adverse side effects that are proportional to the depth of hypothermia, such as inducing coagulopathy and impairing the immune response. In contrast, mild hypothermia as used in this study (32°C) is relatively easy to induce via passive cooling. VPA has been clinically used since 1978 for the treatment of seizures and mood disorders. However, the HDAC inhibiting dose of VPA (250–300 mg/kg)

is six-fold higher than is clinically approved, which may cause potential side effects. For example, chronic VPA use has been associated with mitochondrial dysfunction and subsequent hepatotoxicity and pancreatic dysfunction (24,25). In this study and our previous large animal models, we noticed that a single large dose of VPA was associated with transient elevation in lactate levels (20,26). Whether elevated lactate is a direct pharmacodynamic effect of VPA remains unclear. We are currently conducting a Phase 1 dose-escalation trial of VPA where we hope to further elucidate this phenomenon. Yet, we feel that the ability of VPA to activate pro-survival pathways while attenuating apoptosis and inflammation at the cellular level far outweighs these potential risks when resuscitating a severely injured patient in the hospital or on the battlefield.

This study has several limitations. The animals were subjected to a sub lethal hemorrhage and the observation period was short. This was done to avoid a “survivor bias” during analysis and to ensure that tissues from all the animals were available for comparisons. The next step would be to validate these findings in a more lethal model of hemorrhage with survival as an endpoint.

The relatively small sample size of five animals per group, while adequate to reveal differences in protein expression, may limit further elucidation of the mechanisms of VPA and hypothermia treatment at the cellular level. For example, β -catenin levels increased in the NH+VPA group, but this did not reach statistical significance. Had a larger sample size been used, it is possible that this difference could have reached statistical significance.

As the first in vivo demonstration of the benefits of combined HH+VPA treatment, our findings represent a proof of concept study. Differences in animal physiology as well as response to trauma require further validation in large animal models and human studies. Our lab is currently addressing these issues by examining the efficacy of VPA treatment in a porcine survival model of combined traumatic brain injury and hemorrhagic shock. In addition, we are conducting a Phase 1 dose escalation trial of VPA in healthy human subjects and trauma patients.

One advantage of combined treatment is the ability to use a lower dose of VPA. Another strategy is the targeting of specific HDAC isoforms. At least eighteen different HDACs in three classes have been reported in humans. VPA is a class I HDACI which acts as a pan-HDAC inhibitor. Yet, different HDAC isoforms may play unique roles during shock. We have previously demonstrated that isoform-specific HDACI are effective in treating lethal sepsis (27–29). Unpublished data from our lab has revealed that several isoform-specific HDACI may be equally or more effective relative to pan-HDACI treatment in in vitro and in vivo models of shock. These promising agents may permit the targeted therapy of specific HDAC that are known to play significant roles in injury. Moreover, these isoform-specific HDACI exhibit greater potency compared to VPA, allowing us to use lower doses to achieve therapeutic efficacy. Ongoing studies in our lab are examining combined therapy using isoform-specific HDACI with mild hypothermia, which may potentially yield greater pro-survival benefits than the results of this study, with even better safety profiles.

5. Conclusions

In summary, this is the first in vivo study to demonstrate that combined treatment with VPA and mild hypothermia offers superior cytoprotection than these treatments given independently, in a rodent model of hemorrhagic shock.

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Figure 1. Schematic diagram of the phosphoinositol 3-kinase (PI3K)/Akt survival pathway. Adapted from Shuja et al. (6).

Figure 2.

Mean Arterial Pressure at selected time points. Data are presented as mean $\pm$ standard error of the mean. * $P < 0.05$ between NH, HH, and HH+VPA at the post-hemorrhage (PH) time point. BL, baseline; PS, post-shock; PS1, one hour post-shock; PS2, two hours post-shock; End, time of sacrifice at three hours post-shock.

Figure 3. Western blot of acetylated histone 3 at lysine 9 (ACH3K9) in liver tissue following hemorrhagic shock and resuscitation treatments. Protein band density was quantified as mean $\pm$ standard error of the mean. * $P < 0.05$ compared to Sham, NH, and HH. Representative protein bands from western blots are shown below the densitometric data.

Figure 4.

Western blots of proteins in liver tissue following hemorrhagic shock and resuscitation treatments. (A) Cleaved-caspase-3 and pro-caspase-3, with * $P < 0.05$ compared to NH. (B) Bcl-2, with * $P < 0.005$ compared to Sham, NH, and NH+VPA. Protein band density was quantified as mean $\pm$ standard error of the mean. Representative protein bands from western blots are shown below the densitometric data.

Figure 5.

Western blot of phospho-GSK-3β in liver tissue following hemorrhagic shock and resuscitation treatments. Protein band density was quantified as mean $\pm$ standard error of the mean. *P < 0.05 compared to NH and NH+VPA. Representative protein bands from western blots are shown below the densitometric data.

Figure 6. Western blot of pro-survival β -catenin in liver tissue following hemorrhagic shock and resuscitation treatments. Protein band density was quantified as mean $\pm$ standard error of the mean. * $P < 0.01$ compared to NH. Representative protein bands from western blots are shown below the densitometric data.

Table 1

Selected intraoperative and postoperative arterial blood gas parameters.

Parameter	Group	BL	PH	PS	End
pH	NH	7.42 ± 0.02	7.440 ± 0.041	7.432 ± 0.010	7.456 ± 0.050
	HH	7.34 ± 0.04	7.381 ± 0.040	7.273 ± 0.063	7.299 ± 0.032
	NH+VPA	7.42 ± 0.02	7.422 ± 0.051	7.455 ± 0.030	7.448 ± 0.013
	HH+VPA	7.37 ± 0.04	7.392 ± 0.035	7.329 ± 0.032	7.192 ± 0.063
PO ₂ (mmHg)	NH	98.7 ± 8.8	131.5 ± 37.5	120.3 ± 20.7	99.0 ± 40.5
	HH	115.3 ± 11.8	152.3 ± 18.4	119.0 ± 20.4	117.6 ± 18.3
	NH+VPA	96.9 ± 10.9	134.3 ± 28.1	125.1 ± 33.2	103.4 ± 10.3
	HH+VPA	120.5 ± 8.6	163.6 ± 22.3	146.3 ± 21.8	86.8 ± 49.5
PCO ₂ (mmHg)	NH	34.5 ± 4.6	30.3 ± 4.7	30.9 ± 3.2	31.6 ± 5.2
	HH	39.6 ± 3.9	37.3 ± 4.8	50.2 ± 7.9	53.0 ± 6.5
	NH+VPA	34.2 ± 4.7	29.7 ± 2.8	26.1 ± 4.3	31.9 ± 2.2
	HH+VPA	38.2 ± 4.6	34.6 ± 4.0	40.5 ± 5.5	70.1 ± 14.3
Lactate (mmol/L)	NH	1.38 ± 0.54	3.40 ± 0.69	3.50 ± 1.85	1.55 ± 1.08
	HH	1.20 ± 0.28	1.88 ± 0.41	2.00 ± 0.86	0.66 ± 0.23
	NH+VPA	1.48 ± 0.35	4.10 ± 0.70	4.48 ± 0.93	2.13 ± 1.27
	HH+VPA	1.46 ± 0.47	2.26 ± 0.65	2.92 ± 0.66	3.30 ± 1.08
Hemoglobin (g/dL)	NH	9.92 ± 1.30	9.28 ± 0.33	8.67 ± 1.00	8.45 ± 0.57
	HH	11.56 ± 0.86	10.36 ± 1.39	9.86 ± 0.92	9.30 ± 0.86
	NH+VPA	10.82 ± 1.58	9.25 ± 0.97	8.45 ± 0.88	9.40 ± 1.04
	HH+VPA	11.46 ± 1.36	10.36 ± 0.92	9.56 ± 0.74	8.26 ± 2.10

Data shown as mean values ± standard error of the mean.

* P < 0.05. Upper and lower ends of each bracket indicate the two groups in the comparison.

BL, Baseline; PH, post-hemorrhage; PS, post-shock; VPA, valproic acid. NH, normothermia hemorrhage; HH, hypothermia hemorrhage.