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Development of a novel neuroprotective strategy: Combined treatment with hypothermia and valproic acid improves survival in hypoxic hippocampal cells

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Abstract

Background—Therapeutic hypothermia and histone deacetylase inhibitors, such as valproic acid (VPA), independently have been shown to have neuroprotective properties in models of cerebral ischemic and traumatic brain injury. However, the depth of hypothermia and the dose of VPA needed to achieve the desired result are logistically challenging. It remains unknown whether these two promising strategies can be combined to yield synergistic results. We designed an experiment to answer this question by subjecting hippocampal-derived HT22 cells to severe hypoxia in vitro.

Methods—Mouse hippocampal HT22 cells were exposed to 200 μ M cobalt chloride (CoCl_2), which created hypoxic conditions in vitro. Cells were incubated for 6 or 30 hours under the following conditions: (1) Dulbecco's Modified Eagle Medium; (2) 200 μ M CoCl_2 ; (3) 200 μ M CoCl_2 plus 1 mmol/L VPA; (4) 200 μ M CoCl_2 plus 32°C hypothermia; and (5) 200 μ M CoCl_2 plus both 1 mmol/L VPA and 32°C hypothermia. Cellular viability was evaluated by (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and lactate dehydrogenase release assays at 30 hours after treatment. Levels of acetylated histone H3, hypoxia-inducible factor-1 α , phospho-GSK-3 β , β -catenin, and high-mobility group box-1 were measured by Western blotting.

Results—High levels of acetylated histone H3 were detected in the VPA-treated cells. The release of lactate dehydrogenase was greatly suppressed after the combined hypothermia + VPA treatment (0.269 ± 0.003) versus VPA (0.836 ± 0.026) or hypothermia (0.451 ± 0.005) treatments alone ($n = 3$, $P = .0001$). (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay showed that the number of viable cells was increased by 17.6% when VPA and hypothermia were used in combination ($n = 5$, $P = .0001$). Hypoxia-inducible factor-1 α and phospho-GSK-3 β expression were synergistically affected by the combination treatment, whereas high-mobility group box-1 was increased by VPA treatment, and inhibited by the hypothermia.

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Conclusion—This is the first study to demonstrate that the neuroprotective effects of VPA and hypothermia are synergistic. This novel approach can be used to develop more effective therapies for the prevention of neuronal death.

Therapeutic hypothermia is a potent protective strategy against central nervous system damage.¹ For example, it has been shown in large randomized clinical trials to improve neurologic outcomes in patients with hypoxic brain injury after cardiac arrest.^{2,3} A paucity of clinical data exists supporting the use of hypothermia in the setting of hemorrhagic shock, but preclinical evidence is very strong,⁴ and a clinical trial has been launched recently to test the feasibility of inducing hypothermia in patients with traumatic arrest ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01042015) Identifier: NCT01042015).⁵ Our team, as well as others, has shown previously that rapid induction of profound hypothermia protects neurons and astrocytes and preserves cognitive functions in large animal models of lethal hemorrhage.^{6,7} One of the advantages of therapeutic hypothermia is its ability to activate numerous pathways simultaneously during the ischemic and reperfusion window to reduce the cellular damage.^{8,9} Despite its enormous therapeutic potential, there are several logistical barriers to the application of hypothermia in the setting of lethal trauma. These include the need to decrease the core body temperature to <15°C, requirement for cardiopulmonary bypass, the very short window of time available for complex instrumentation, the need for effective heat-exchange technology or large volume of cold fluids, and the adverse effect of hypothermia on coagulopathy.¹⁰ These limitations are especially problematic in the austere prehospital environment, where most of trauma-related deaths occur.

Another promising approach is to administer a life-saving pharmacologic agent in the field that can keep injured patients alive long enough to receive definitive treatment at higher echelons of care. We have tested this concept in a number of large and small animal models and have discovered that the administration of histone deacetylase inhibitors (HDACIs) can improve survival after lethal hemorrhage, sepsis, and poly-trauma.¹¹

Some of these agents have already been in clinical use for decades (for nontrauma indications). For example, valproic acid (VPA), a commonly used antiseizure medication, has also been identified to be an HDACI with potent cell protective, anti-inflammatory, and anti-apoptotic properties.¹² In previous studies, we have shown that treatment with VPA up-regulates multiple prosurvival pathways and regulatory molecules, including phospho-GSK3 β and β -catenin,^{13–16} and protects neurons against hypoxia-induced apoptotic cell death.¹⁷ In vivo studies that use a large animal model of combined hemorrhage and traumatic brain injury have also confirmed its neuroprotective potential.¹³

On the basis of these promising data, a phase 1 dose-escalation trial recently has been initiated to test the safety of VPA ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01951560) Identifier: NCT01951560).¹⁴ A practical problem with VPA is that the dose that exerted an HDACI effect in the preclinical studies (>250 mg/kg) was 6- to 8-fold greater than the commonly used antiseizure dose.¹⁵ These large doses of VPA have potential side effects, including hepatic injury, pancreatitis, and changes in mental status.

It remains untested whether these promising strategies can be combined to enhance their effectiveness. If so, then lesser degrees of hypothermia and lower doses of VPA could be combined to create a logistically superior intervention with a better safety profile. With an improved understanding of the numerous pathways that are activated by cerebral ischemia, it seems highly unlikely that any single treatment (that affects one or more branch points of any one pathway) could alter outcomes.¹⁸ It is much more logical to combine multiple potent treatments (modulating numerous key pathways) in an effort to achieve synergistic effects.

Mild hypothermia (32°C) has the advantage of being associated with fewer and less serious side effects than deep hypothermia.¹⁹ In this study, we used cobalt chloride (CoCl₂)-treated hippocampal cells as an in vitro hypoxia model^{20–22} to test whether a combination of VPA and mild hypothermia would result in better injury protection compared with individual treatments.

MATERIALS AND METHODS

Cell culture and generation of hypoxic stress

Hippocampal cells (HT22) were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. For each assay, HT22 cells were seeded in 6- or 96-well plates according to the requirement of the experiment and treated after 24 hours of culture. To induce hypoxia, cells were treated with 200 µM of a hypoxia-inducing agent, CoCl₂ (Sigma-Aldrich, St Louis, MO). DMEM alone was used as the control.

Measurement of cytotoxicity

HT22 cells and the supernatants were harvested after 30 hours incubation with the following conditions: (1) DMEM; (2) 200 µM CoCl₂; (3) 200 µM CoCl₂ plus 1 mmol/L VPA, which is within a range that can be achieved in vivo; (4) 200 µM CoCl₂ plus 32°C hypothermia; (5) 200 µM CoCl₂ plus both 1 mmol/L VPA and 32°C hypothermia. Lactate dehydrogenase (LDH) content was determined separately for the cell extracts and corresponding media by the use of a Cytotoxicity Detection Kit (Roche, Indianapolis, IN), and the percentage of LDH released in the medium was calculated after subtracting the corresponding background value.

MTT assay

MTT (3-[3, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; M2128, Sigma Aldrich, St. Louis, MO) assay was used to assess cell viability. Thirty hours after incubation under various experimental conditions as described previously, cell medium was replaced with 20 µL of 5 mg/mL MTT dissolved in phosphate-buffered solution in each well. The plates were returned to the incubator for 3 hours, and the MTT solution was then replaced with 100 µL of DMSO to dissolve formazan. Absorbance was read by spectrometer at 590 nm.

Western blot analysis

HT22 cells were harvested after 6 or 30 hours' incubation with different treatments. Cell lysates were prepared using a whole cell lysis buffer kit (BD Biosciences, Bedford, MA) and Subcellular Proteome Extraction Kit (539791, Calbiochem, CA), according to the manufacturer's instructions. Protein concentration was determined by bicinchoninic acid method, and equal amounts of samples were loaded per lane. Proteins were detected using specific antibodies against acetylated histone H3 (Lys9, 07–352; Millipore, Billerica, MA), hypoxia-inducible factor-1 α (HIF-1 α , H6536; Sigma-Aldrich), p-GSK3 β (9336; Cell Signaling Technology, Danvers, MA), HMGB1 (6893; Cell Signaling Technology) and β -Catenin (13–8400; Invitrogen), and incubated with corresponding IRDye 680RD conjugated second antibodies (LI-COR Bioscience, Lincoln, NE). The signals were visualized using Odyssey CLx Infrared Imaging System (LI-COR), and the results were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analyses

Data are presented as group means $\pm$ standard error of mean (SEM). One-way analysis of variance was performed using Graph-Pad Prism (version 5.0 for Windows, GraphPad Software, San Diego, CA), with Tukey post-hoc analysis.

RESULTS

CoCl₂ -induced cell death in HT22 cells

Exposure of HT22 cells to 200 μ M CoCl₂ for 30 hours significantly increased the release of LDH, which is a marker of cell injury (Figs 1 and 3, $P = .0001$). MTT assay showed that the cell viability was decreased to 30% of control level (Fig 2, $P = .0001$). After exposure to CoCl₂, the cells displayed apoptotic morphology characterized by shrunken cell body, cell detachment and the condensation of the chromatin, as shown in Fig 3. We used these in vitro conditions to test the therapeutic benefits of VPA and hypothermia.

Synergetic effects of VPA and hypothermia against CoCl₂ -induced cell death

Individual treatments with VPA and hypothermia significantly reduced the LDH release (Fig 1 and 3, $P = .0001$) and improved cell survival (Figs 2 and 3, $P = .0001$) after 30 hours of hypoxic injury. However, the best cell survival (47%, $n = 6$, $P < .05$, Figs 2 and 3) and the lowest LDH levels (80% of control, $n = 3$, $P < .05$, Figs 1 and 3) were observed in the group subjected to the combined (hypothermia + VPA) treatment.

HDAC inhibition up-regulated the levels of acetylated histone 3

We examined the level of protein acetylation 6 hours after treatment with VPA and/or hypothermia. Level of acetylated histone 3 at lysine 9 significantly ($P = .0012$) decreased after exposure to CoCl₂, and was markedly elevated by the VPA treatment (Fig 4). Hypothermia, alone or in combination with VPA, restored the acetylation back to the control level (Fig 4).

VPA and hypothermic treatments synergistically suppressed CoCl₂-induced HIF-1 α expression

It is well known that CoCl₂ can induce transcriptional factor HIF-1. As shown in Fig 5, HIF-1 α expression was low in the control group, and it increased by 4.8-fold after 6-hour exposure to CoCl₂. Treatment with VPA ($3.7 \times$ control level) and hypothermia ($4.0 \times$ control level) were equally effective in blunting the HIF-1 α levels, whereas the combined treatment was associated with the lowest HIF-1 α expression ($2.0 \times$ control level, $n = 3$, $P = .0032$).

VPA and hypothermic treatments synergistically enhanced expression of phospho-GSK3 β , but not β -catenin

Six hours after exposure to CoCl₂, significant decrease in the phospho-GSK3 β ($n = 3$, $P = .001$, Fig 6) and β -catenin ($n = 3$, $P = .0117$, Fig 7) levels was detected by Western blotting. Treatment with VPA or hypothermia significantly enhanced the phospho-GSK3 β and β -catenin expression. Interestingly, VPA and hypothermia exerted a synergistic effect on the phospho-GSK3 β ($n = 3$, $P < .05$, Fig 6) but not on the β -catenin levels (Fig 7).

HMGB1 expression was enhanced by VPA and suppressed by hypothermia

Increased HMGB1 expression was observed 30 hours after exposure to CoCl₂ in the cytosolic fraction of the cellular protein. Treatment with VPA further increased the HMGB1 expression, whereas hypothermia blunted this response ($n = 3$, $P = .001$, Fig 8).

DISCUSSION

We have previously shown that treatment with VPA or hypothermia, individually, can provide sufficient neuronal protection in models of hypoxia, hemorrhagic shock and traumatic brain injury.^{6,13,17} However, the dose of VPA and the depth of hypothermia that are required to achieve this protection are logistically challenging. In the present study, we have demonstrated that these two neuroprotective treatments can be combined to yield synergistic results, which is a logical extension of our previous work and supports additional in vivo testing to identify the optimal combination (degree of hypothermia and the dose of VPA).

HT-22 cells were derived from the immortalized mouse hippocampal cell line HT-4 and are extremely suitable for in vitro testing of hypoxic stress. In this study, we exposed these HT22 cells to CoCl₂, which is widely used as a model to investigate hypoxic cellular injury.^{20,22} Dose of CoCl₂ was based on a series of pilot experiments, which were aimed to create a substantial but still reversible cellular injury. In addition to cell death, we also decided to investigate a few potential mechanisms that are known to be involved in hypoxia-induced neuronal injury.^{23,24} CoCl₂ exposure creates hypoxic conditions by causing the stabilization of HIF-1 α and preventing its degradation by the proteasome.^{25,26} We detected increased HIF-1 α levels after CoCl₂ treatment (Fig 5), which is consistent with the published literature.

Studies in animals have shown that knocking out the HIF-1 α gene or inhibiting the protein protects against hypoxic injury by preserving the integrity of the blood-brain barrier and reducing brain edema.^{27,28} As expected, we observed substantial suppression of HIF-1 α expression with VPA and hypothermia, with the most decrease in response to the combined treatment. HIF-1 α is a transcription factor that can bind with a second protein (HIF-1 β) in the nucleus to influence the promoter elements in hypoxia-responsive genes. Kim et al²⁹ have reported that HIF-1 α stability is enhanced by direct binding of oxygen-dependent degradation domain of HIF-1 α to HDAC1 and 3, which can be inhibited by VPA. Our results showed that VPA treatment greatly increased the level of acetylated histone 3 (Fig 4), which could be responsible for the observed decrease in HIF-1 α . We also noted that hypothermia decreased the HIF-1 α levels, which is consistent with a report from Tanaka et al³⁰ in which they documented a decrease in HIF-1 α protein synthesis with mild hypothermia. Our study is the first to demonstrate that combined treatment with VPA and hypothermia results in an enhancement of neuroprotective effects, but the precise underlying molecular mechanisms for this synergism still need to be identified.

In animal models of hemorrhagic shock, we have previously established that phospho-GSK-3 β and β -catenin are up-regulated when VPA¹⁶ and hypothermia³¹ are administered independently. Therefore, we decided to study these signaling cascades in the current study. GSK-3 β is a downstream protein of Akt, responsible for signaling β -catenin (a prosurvival protein), via phosphorylation, for proteosomal degradation.³² Once phosphorylated (phospho-GSK-3 β), GSK-3 β becomes inactive, resulting in decreased β -catenin degradation, which promotes cellular viability by increasing the production of anti-apoptotic proteins, such as Bcl-2.³³

Similar to our previous studies^{16,31} and other reports,³⁴ we found that the individual treatment with VPA or hypothermia greatly up regulated the phospho-GSK-3 β and β -catenin signals that had been suppressed by hypoxia (Figs 6 and 7). Interestingly, synergistic effect with the combined treatment was only seen on the phospho-GSK-3 β (Fig 6) but not on the β -catenin (Fig 7) expression. It is not surprising that the cumulative effect of two different treatments (each influencing multiple signaling pathways) on a downstream protein is different compared with what is observed after the individual treatments. We are currently studying the underlying mechanisms in more detail to better understand the cross talk between these therapies.

HMGB1 is a nuclear protein that acts as an architectural chromatin-binding factor involved in the maintenance of nucleosome structure and regulation of gene transcription.³⁵ Animal studies have demonstrated that siRNA-mediated HMGB1 knockdown or administration of anti-HMGB1 antibodies can markedly reduce brain damage after ischemic and traumatic injuries.^{36,37} On the other hand, HMGB1 has also been reported to be a substrate for histone acetylase, and HDAC inhibitors can increase the release and relocation of HMGB1 from the nucleus to the cytoplasm.^{38–40} In this study, we detected an increase in HMGB1 in the VPA treated group (Fig 8). It could be a “side effect” of VPA, although the VPA treatment exhibited an overall neuroprotective effect. Interestingly, the increase in HMGB1 by the VPA was inhibited by treatment with hypothermia. However, HMGB1 level in the

hypothermia group was no different than the CoCl₂ alone group, suggesting that different signal cascades may be involved with these conditions.

There are several limitations in this study that must be mentioned. CoCl₂ treatment was used in this study to induce the hypoxic insult. However, potential differences between CoCl₂ treatment and oxygen deprivation in a hypoxia chamber may exist. Similarly, in vitro hypoxia may differ from low flow anaerobic environment that is experienced by the cells during hemorrhagic shock. This is a proof-of-concept study, and we examined only a limited number of mechanisms. It is highly likely that many more mechanisms are influenced by hypoxia and the treatments. To address these shortcomings, we have recently completed an in vivo study using a rodent model of hemorrhagic shock, and tissue analyses are in progress.

In summary, we have shown that the combined treatment with VPA and hypothermia improves survival and decreases cell death after chemically induced hypoxia in HT22 hippocampal cells. Furthermore, our data suggest that HIF-1 α , phospho-GSK-3 β and HMGB1 are involved at the mechanistic level. Taken together with previously published results, these findings suggest that combination therapy involving a multimodal form of protection is a promising approach that warrants further investigation.

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Fig 1.

Cell injury after 30 hours of hypoxia in hippocampal HT22 cells. A substantial increase in cellular injury was detected after CoCl₂ (200 μ M) exposure. Treatment with VPA and/or hypothermia protected cells against hypoxia compared with the control group. $n = 3$, $*P < .05$ compared with CoCl₂ alone. $\#P < .05$ compared with VPA + hypothermia.

Fig 2.

Cell viability measured by MTT assay after 30 hours of hypoxia in HT22 cells. Data presented as % change compared with normoxic controls. Hypoxia caused a reduction in viability to 29.7% (SD 2.6%) in the untreated cells. Treatment with VPA and/or hypothermia significantly improved the cell survival. $n = 6$, $*P < .05$ compared with CoCl₂ alone. $\#P < .05$ compared with VPA + hypothermia.

Fig 3. HT22 cell culture after 30 hours of hypoxia. Treatment with VPA and/or hypothermia greatly reduced hypoxic injury and improved cell survival. The most significant improvement was observed in VPA + hypothermia group. Scale bar: 50 μm .

Fig 4.

Western blot for acetylated histone 3 at lysine 9 in HT22 cells after 6 hours of hypoxia.

Acetylation significantly increased after treatments with VPA and/or hypothermia. $n = 3$, * $P < .05$ compared with normoxic control; # $P < .05$ compared with CoCl_2 alone.

Fig 5.

Western blot for HIF-1 α expression after 6 hours of hypoxia. HIF-1 α was suppressed by VPA and/or hypothermia treatments. $n = 3$, * $P < .05$ compared with CoCl₂ alone. # $P < .05$ compared with VPA + hypothermia.

Fig 6.

Western blot for phospho-GSK3 β expression after 6 hours of hypoxia. Exposure to CoCl₂ reduced phospho-GSK3 β expression, whereas it was preserved after treatments with VPA and/or hypothermia. $n = 3$, * $P < .05$ compared with CoCl₂ alone. # $P < .05$ compared with VPA + hypothermia.

Fig 7.

Western blot for β -catenin expression after 6 hours of hypoxia. The level of β -catenin was reduced after exposure to CoCl₂. After treatments with VPA and/or hypothermia, the expression of β -catenin returned to normoxic control levels. No difference was detected among the VPA, hypothermia, and VPA + hypothermia groups. $n = 3$, $*P < .05$ compared with normoxic control.

Fig 8.

Western blot for HMGB1 expression after 30 hours of hypoxia. Exposure to CoCl₂ significantly induced HMGB1. The expression of HMGB1 was increased further by VPA treatment, whereas addition of hypothermia inhibited it. $n = 3$, $*P < .05$ compared with normoxic control. $\#P < .05$ compared with CoCl₂ alone.