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

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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 μM 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 μM $CoCl_2$; (3) 200 μM $CoCl_2$ plus 1 mmol/L VPA; (4) 200 μM $CoCl_2$ plus 32°C hypothermia; and (5) 200 μM $CoCl_2$ 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-1α, phospho-GSK-3β, 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-1α and phospho-GSK-3β expression were synergistically affected by the combination treatment, whereas high-mobility group box-1 was increased by VPA treatment, and inhibited by the hypothermia.*

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. (Surgery 2014;156:221-8.)*

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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 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 pro-survival pathways and regulatory molecules, including phospho-GSK3β 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 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 ($CoCl_2$)-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% CO_2. 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, $CoCl_2$ (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 $CoCl_2$; (3) 200 μM $CoCl_2$ plus 1 mmol/L VPA, which is within a range that can be achieved in vivo; (4) 200 μM $CoCl_2$ plus 32°C hypothermia; (5) 200 μM $CoCl_2$ 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-ul]-2, 5-diphenyltetrazolium bromide; M2128, Sigma Aldrich, St. Louis, MO) assay was used to assess cell