Histone Deacetylase Inhibitors: A Novel Strategy for Neuroprotection and Cardioprotection Following Ischemia/Reperfusion Injury

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ABSTRACT: Ischemia/reperfusion injury is a complex molecular cascade that causes deleterious cellular damage and organ dysfunction. Stroke, sudden cardiac arrest, and acute myocardial infarction are the most common causes of ischemia/reperfusion injury without effective pharmacologic therapies. Existing preclinical evidence suggests that histone deacetylase inhibitors may be an efficacious, affordable, and clinically feasible therapy that can improve neurologic and cardiac outcomes following ischemia/reperfusion injury. In this review, we discuss the pathophysiology and epigenetic modulations of ischemia/reperfusion injury and focus on the neuroprotective and cardioprotective effects of histone deacetylase inhibitors. We also summarize the protective effects of histone deacetylase inhibitors for other vital organs and highlight the key research priorities for their successful translation to the bedside.

Key Words: epigenetics ■ histone deacetylase inhibitors ■ myocardial infarction ■ stroke ■ cardiac arrest
Reperfusion exacerbates cell death through calcium overload, glutamate release, ROS formation, opening of the mitochondrial permeability transition pores, endothelial dysfunction, thrombosis, proteolysis, and activation of inflammatory pathways. Mitochondrial dysfunction also contributes significantly to reperfusion injury. After prolonged global ischemia from cardiac arrest, microthrombi formation and abnormal leukocyte adhesion in the capillaries can lead to significant secondary ischemia due to the "no-reflow" phenomenon. Hypothermic targeted temperature management is the only clinically available therapy that may attenuate reperfusion injury by upregulating prosurvival pathways after cardiac arrest. However, its efficacy is often limited by the inability to achieve target temperature within the therapeutic window, side effects, and delay in neuroprognostication.

The paucity of effective treatments for I/R injury demonstrates an urgent need for novel therapeutic approaches. Histone deacetylase inhibitors (HDACi) have emerged as a promising strategy for the treatment of I/R injury (Table). This review highlights the epigenetic modulations and protective effects of HDACi following I/R injury. We focus on the neuroprotective and cardioprotective effects of HDACi after stroke, cardiac arrest, and myocardial infarction. We also discuss the peripheral organ protective effects of HDACi. Special emphasis is placed on valproic acid (VPA), a nonspecific HDACi approved by the US Food and Drug Administration (FDA), with the most preclinical data to support its use following I/R injury.
Epigenetic modulation alters gene expression through the transcriptional regulation of 3 primary mechanisms: DNA methylation, histone modification, and 3-dimensional chromatin structural modulation. In the early studies of epigenetic modulation, I/R injury to the brain and heart were shown to cause a 40% decrease in histone H3 and H4 acetylation. The global hypoacetylation induced by I/R injury leads to chromatin condensation, which results in widespread decrease of anti-inflammatory gene transcriptions, activation of apoptosis, and increase in proinflammatory cytokines such as TNF-α (tumor necrosis factor α). These effects are mediated through histone deacetylases (HDACs) or lysine deacetylases, enzymes that alter the epigenome and gene expression by removing acetyl groups from the tails of histone and nonhistone proteins.

Histone Deacetylase Inhibitor After I/R

| In Vivo Model | Small Animal Model | Mechanistic Outcomes |
|---------------|--------------------|----------------------|
| x             |                   | Reduc...
hyperacetylation by acting directly on lysine residues to regulate fatty acid oxidation and autophagy. Over the past 2 decades, research has focused on HDACi as an innovative treatment for various diseases, given the unique organ distribution and distinct physiologic function of HDACs. In humans, there are 4 classes of HDACs with total of 18 unique isoforms. Because HDACs are overexpressed in many cancers, HDACi was first explored as a novel approach to anticancer therapy through the ability to decrease proliferating gene expression and increase cell-cycle and apoptotic gene transcriptions. To date, 20 distinct HDACi have been assessed in clinical trials for anticancer therapies.

HDACi has also been shown to be promising adjunctive therapy for trauma and sepsis resuscitation in preclinical studies. Early studies focused on nonspecific HDACi such as VPA and suberoylanilide hydroxamic acid (SAHA). Treatment with a single high-dose VPA significantly increases survival and attenuates organ dysfunction in large animal models of polytrauma. In traumatic brain injury and hemorrhagic shock, high-dose VPA administered in the early postinjury period has been shown to reduce neurologic injury, expedite recovery, and improve long-term survival.

Figure 2. Epigenetic effects of histone deacetylase (HDAC) inhibition in the brain. Chromatins are composed of negatively charged DNA wrapped around nucleosomes, each of which consists of 8 positively charged histones (2 sets of H2A, H2B, H3, and H4). HDAC inhibitors (HDACi) inhibit HDACs, resulting in the removal of positive charges on histone H3 and H4 through acetylation. The subsequent unwinding of DNA from the nucleosomes exposes genes to RNA polymerase for transcription. The relevant genes regulated by HDACi for neuroprotection after ischemia/reperfusion injury are highlighted. ARC indicates apoptosis repressor with caspase recruitment domain; BDNF, brain-derived neurotrophic factor; Foxm1, forkhead box M1; GSK3β, glycogen synthase kinase 3β; HSP70, heat shock protein 70; IL, interleukin; iNOS, inducible nitric oxide synthase; mTOR, mechanistic target of rapamycin kinase; NEUROD1, neuronal differentiation 1; PAI-1, plasminogen activator inhibitor 1; t-PA, tissue-type plasminogen activator.
neurologic outcome. In traumatic brain injury, VPA administration induces the activation of master transcription factors such as NEUROD1 (neuronal differentiation 1) and TBR1 (T-box brain transcription factor 1) to mediate the expression of downstream neurogenic and neuroplastic genes. In sepsis, VPA and SAHA treatments improve survival and attenuate organ dysfunction. The mechanisms of action of HDACi in trauma and sepsis models have been discussed extensively elsewhere.

**HDACi for Neuroprotection Following I/R Injury**

**Stroke**

Stroke is the second leading cause of death and the leading cause of long-term disability in the United States. Nearly 80% of strokes are caused by ischemia, and the remainder are caused by hemorrhage. Stroke occurs by interruption or reduction in the blood supply to the brain, resulting in the deprivation of oxygen, glucose, and electrolytes to neuronal tissues. Focal cerebral ischemia is more common in stroke, whereas global ischemia is more prevalent in systemic hypoperfusion such as cardiac arrest and hemorrhagic shock.

Small animal models of I/R injury have been used to demonstrate the neuroprotective effects of HDACi following stroke. In both transient focal and global ischemic rat models of stroke induced by middle cerebral artery occlusion (MCAO) and 4-vessel occlusion, respectively, intraperitoneal administration of VPA (300 mg/kg) significantly decreased neurologic deficit score, neuronal death, infarct size, and blood-brain barrier disruption. Similarly, pre- and postinjury treatment with 300 mg/kg of intraperitoneal VPA attenuated infarct size in mouse models of transient MCAO I/R injury. However, only pretreatment with VPA reduced infarct size after permanent MCAO in mice. Intraperitoneal VPA injections also reduced hippocampal injury in a gerbil I/R model and decreased CASP1 (caspase 1), IL-1β (interleukin 1β), and IL-18. Multiple studies have shown that the protective effects of VPA after I/R injury are dependent on the time of drug administration following insult. For example, VPA was effective when administered within the first 3 hours following neuronal I/R injury but ineffective when administered after 4 hours in mouse models, making VPA treatment following I/R injury time-sensitive but clinically feasible.

The neuroprotective effect of VPA after stroke is thought to be secondary to HDAC inhibition and up-regulation of HSP70 (heat shock protein 70). VPA increases the levels of acetylated histones H3 and H4 and HSP70. Acetylated histones H3 and H4, in turn, promote prosurvival gene expression and inhibit microglia, GSK3β (glycogen synthase kinase 3β), hippocampal pyroptosis, and cellular inflammation. Furthermore, VPA decreases hippocampal sensitivity to inducible nitric oxide synthase in rat models of focal ischemia, thereby reducing oxidative stress and ROS generation. VPA also exhibits antiapoptotic protective effects by increasing the expression of ARC (apoptosis repressor with caspase recruitment domain), ARC expression decreases CASP1, IL-1β, IL-18, NLRP1 (NLR family pyrin domain containing 1), and NLRP3, which are all part of the pyroptotic neuronal cell injury pathway. VPA’s protective effect was abolished when ARC was knocked down by small interfering RNA, further confirming ARC-mediated protection by VPA. VPA combined with hypothermia results in synergistic protective effects in hippocampal neurons in vitro. Combined treatment significantly increases cell viability and histone H3 acetylation and suppresses the release of lactate dehydrogenase compared with either treatment alone. Acetylated histone H3 increases Akt phosphorylation, resulting in the downstream inactivation of GSK3B and expression of antiapoptotic genes.

The neuroprotective effects of HDACi after stroke are not exclusive to VPA. Postinjury treatment with nonspecific HDACi such as VPA, sodium butyrate (SB), and trichostatin A (TSA) all significantly reduced neuronal injury in rat models of stroke. SB also promoted neuroprotective and neurogenic effects in rat models of neonatal postischemic brain injury, suggesting that HDACi could be useful therapy for neonatal I/R injury. The anti-inflammatory and neuroprotective effects of SB are mediated in part through increased expression of oligodendrocyte progenitor cells, which attenuates the infiltration of both microglial cells and macrophage/microcytes. SB upregulates the expression of BDNF (brain-derived neurotrophic factor) mRNA, which is believed to have neurogenic effects. Treatment of transient MCAO-induced I/R brain injury at the onset of ischemia with 50 mg/kg intraperitoneal SAHA also resulted in neuroprotection, attenuation of cerebral inflammation, and promotion of a more protective microglial phenotype. Reverse transcriptase polymerase chain reaction confirmed that SAHA significantly reduced the transcription of proinflammatory cytokine IL-1β, IL-6, and TNF-α and increased anti-inflammatory cytokines IL-10 and Arg-1 (arginase 1). These mechanisms are thought to reduce microglial activation and monocyte infiltration, resulting in a more protective M2 microglial phenotype overall.

Studies of isoform-specific HDACi for neuroprotection after stroke are limited. Tubastatin A (Tub-A), a class II specific HDAC6 inhibitor, is the most studied isoform-specific HDACi for I/R injury. Tub-A protects...
 hippocampal neurons in vitro against injuries from oxygen–glucose deprivation by modulating Akt/GSK3β signaling and inhibiting mitochondria-mediated apoptosis. Tub-A also significantly reduces neuronal death and infarct size while increasing α-tubulin and GAP43 (growth-associated protein 43) acetylation, thus protecting neurons following photothrombotic infarction in mice. Further mechanistic and translational studies are needed to validate the efficacy of isoform-specific HDACi in animal models of I/R injury.

Cardiac Arrest

More than 430,000 people experience cardiac arrest in the United States every year. Survival to hospital discharge for out-of-hospital cardiac arrest remains low at approximately 11%, and only 9% of survivors have good neurologic outcomes. Furthermore, two-thirds of out-of-hospital cardiac arrest and a quarter of in-hospital cardiac arrest survivors die from neurologic injury. Despite increased awareness in bystander cardiopulmonary resuscitation and early defibrillation, there has been minimal improvement in cardiac arrest survival and survival with good neurologic outcome during the past 5 years.

HDACi has been studied as a neuroprotective therapy following cardiac arrest. Studies of asphyxial cardiac arrest rat models have shown that intravenous high-dose VPA (300 mg/kg) significantly improves survival and neurologic outcomes when administered immediately after return of spontaneous circulation. Specifically, high-dose VPA combined with hypothermic targeted temperature resulted in significantly greater 72-hour survival. Improved survival with good neurologic outcomes and decreased seizure burden compared with normothermia or hypothermic targeted temperature management alone. As such, the addition of high-dose VPA to hypothermic targeted temperature management remains a promising therapy to improve cardiac arrest outcomes.

The mechanisms of VPA-mediated neuroprotection after cardiac arrest are likely pleiotropic. High-dose VPA induces epigenetic modulations through HDAC inhibition, antiepileptic properties, and prosurvival effects. In addition, high-dose VPA may also affect neurologic outcome through profibrinolysis. VPA has been shown to induce the release of t-PA (tissue-type plasminogen activator) in vitro and in rat models. VPA has also been shown to significantly reduce the level of PAI-1 (plasminogen activator inhibitor 1), thereby altering the t-PA/PA-1 ratio in favor of fibrinolysis. An observational study of patients with out-of-hospital cardiac arrest showed that impairment of fibrinolysis and generation of fibrin were associated with post–cardiac arrest syndrome. It is possible that VPA’s profibrinolytic effects may result in reduction of microvascular thrombi formation, thereby reducing the no-reflow phenomenon and end-organ injury. Future translational research using clinically relevant large animal models of cardiac arrest are needed before HDACi can be adopted as a neuroprotective strategy for cardiac arrest survivors.

HDACi for Cardioprotection Following I/R Injury

Cardiovascular disease is the leading cause of death globally and accounts for 1 in 3 deaths in the United States. Specifically, myocardial infarction affects >8 million people and leads to 115,000 deaths each year in the United States. Myocardial infarction causes cardiac ischemia through partial or full occlusion of blood flow to the myocardium. The brain is the organ most sensitive to oxygen and glucose deprivation. It sustains irreversible damage in <20 minutes of ischemia because of its high energetic demand and reliance on glucose. Although cardiac tissues are also highly sensitive to oxygen deprivation and can suffer irreversible damage after 20 minutes of ischemia, they do not exclusively rely on glucose as an energetic source. As such, cardiac tissue may have a longer therapeutic window than the brain, with studies demonstrating therapeutic intervention within the first 2 hours being most optimal and treatment within the first 12 hours of ischemia still associated with better outcomes.

Given the clear clinical need to improve outcomes after myocardial infarction, HDACi have also been studied for cardioprotection after I/R injury. Both nonspecific and isoform-specific HDACi have been evaluated for cardioprotection using in vitro models of I/R injury. Cultured cardiomyocytes pretreated with nonspecific HDACi SAHA demonstrated a 40% reduction in cell death after I/R injury. Further investigation suggested that SAHA treatment led to cardioprotection through the reduction of oxidative stress and induction of a protective macrophage phenotype. SAHA pretreatment of mouse cardiomyocytes in I/R injury led to fewer dysfunctional mitochondria, and this protection was lost when ATG7 (autophagy related 7) knockout myocytes were used. Moreover, 100 mg/kg of intraperitoneal SAHA administered immediately after myocardial infarction in mice increased the expression of M2 macrophages, thereby improving angiogenesis, wound healing, and left ventricle function. A similar study using isoform-specific class Ib HDAC6 inhibitor Tub-A on rat cardiomyocytes showed improved cell viability secondary to epigenetic modification. Tub-A increased the expression of acetylated histone H3, PI3K/Akt antiapoptotic pathway, and mTOR (mechanistic target of rapamycin kinase) activation.
while decreasing autophagy and cellular cytotoxicity on reoxygenation.\textsuperscript{92}

Several small and large animal studies have used the MCAO model to study the protective effects of nonspecific HDACi following myocardial infarction-induced I/R injury.\textsuperscript{80} Pretreatment with TSA, a nonspecific class I and IIb HDACi, reduced myocardial infarct size by nearly 50\%, whereas TSA treatment 12 hours following myocardial infarction had only minimal impact on infarct size, suggesting an optimal therapeutic window for TSA.\textsuperscript{83,84} SAHA pretreatment significantly reduced infarct size comparable to TSA in a rabbit model of myocardial infarction.\textsuperscript{37} High-dose VPA treatment was able to significantly reduce infarct size and preserve systolic function in a rat model of myocardial infarction. The beneficial cardiac effects of VPA are mediated through the upregulation of Foxm1 (forkhead box M1), a regulator of neonatal cardiomyocyte proliferation and repressor of proinflammatory genes.\textsuperscript{85} High-dose VPA may also promote fibrinolysis, thereby mediating a reduction of recurrent myocardial infarction in mice pretreated with intraperitoneal VPA.\textsuperscript{76}

Recent work compared the cardioprotective effects of isoform-specific HDACi such as entinostat (class I specific) and Tub-A (class IIb HDAC6 isoform-specific).\textsuperscript{86} In a rat model of global cardiac I/R injury, pretreatment with 10 mg/kg intraperitoneal entinostat protected the contractile function following I/R injury by upregulating antioxidant enzymes and decreasing ROS.\textsuperscript{86} In contrast, pretreatment with 10 mg/kg Tub-A and 1 mg/kg TSA had no significant protective effect.\textsuperscript{86} The same group verified the protective effect of entinostat in an ex vivo rat heart I/R injury model.\textsuperscript{36} Furthermore, treatment with LL-66, an isoform-specific inhibitor of HDAC1 found in cardiomyocytes, was equally protective compared with entinostat.\textsuperscript{36} Such cardioprotection was shown to be mediated by the inhibition of fatty acid oxidation through succinate dehydrogenase.\textsuperscript{36} Additionally, a meta-analysis assessing the efficacy of HDACi for organ protection in preclinical injury models of ischemia, trauma, and sepsis concluded that HDACi significantly reduced infarct size compared with untreated animals across injury models.\textsuperscript{57} These findings highlighted the heterogeneity of preclinical studies and the potential differences in the cardioprotective properties of isoform-specific HDACi following I/R injury.\textsuperscript{57,87}

### HDACi for Other Organ Protection After I/R Injury

In addition to neuroprotection and cardioprotection, HDACi have also been evaluated for protective effects of other peripheral organs after I/R injury.\textsuperscript{31,57}

Whether I/R injury is a focal primary injury or secondary injury from global ischemia, HDACi may be a protective therapy for organs including the kidneys, lungs, liver, and retinas.

#### Kidneys

VPA is the major HDACi that has been evaluated as a protective agent after renal I/R injury induced by renal artery occlusions. Multiple studies revealed that VPA attenuates renal dysfunction and expedites renal recovery following I/R injury.\textsuperscript{88–90} Although the mechanism remains unclear, a major component appears to be the regulation of inflammation and apoptosis.\textsuperscript{85,90} VPA treatment inhibits the proinflammatory response of macrophages and reduces apoptotic regulators such as TNF-\(\alpha\) after renal I/R injuries in rodent models.\textsuperscript{88–90}

#### Lungs

HDACi has been evaluated as a pulmonary protective agent in several models of I/R injury. In a rat model of acute lung injury induced by superior mesenteric artery occlusion, VPA has been shown to improve survival and mitigate lung injury through ROS reduction and anti-inflammatory effects.\textsuperscript{44} VPA treatment also provides pulmonary protection through the expression of antioxidant HMOX1 (heme oxygenase 1) after I/R injury.\textsuperscript{91} Blocking HMOX1 with zinc protoporphyrin IX mitigated the pulmonary protective effects of VPA, further supporting VPA’s antioxidant mechanisms after pulmonary I/R injury.\textsuperscript{91}

#### Liver

HDACi may also exhibit protective effects following hepatic I/R injury. Pre- and postinjury treatment with nonspecific HDACi SB has been shown to alleviate both partial and total occlusion-induced hepatic I/R damage.\textsuperscript{92–94} SB treatment at the time of injury significantly attenuated hepatic damage, which is associated with the upregulation of histone H3 acetylation and HSP70.\textsuperscript{92} Despite evidence that supports HDACi as hepatoprotective, 1 study directly contrasted with these results. VPA (300 mg/kg) and SAHA (60 mg/kg) treatments after 90-minute occlusion of left hepatic lobe failed to provide protection, and VPA treatment exacerbated I/R injury to the liver.\textsuperscript{95} However, these results might be explained by treatment dose, as VPA is known to be hepatotoxic at high doses and as rapid infusion.\textsuperscript{96,97}

#### Retina

HDACi is a potential retinal protective agent following I/R injury. In rat models of retinal I/R injury induced by increasing intraocular pressure, pretreatment with 300 mg/kg subcutaneous VPA reduced retinal
damage. Subsequent studies demonstrated that VPA attenuates retinal injury by promoting antioxidant effects, antiapoptotic pathways via histone H3 acetylation, and the upregulation of HSP70.

FUTURE TRANSLATION

Most studies of HDACi for organ protection after I/R injury have been in vitro mechanistic studies or proof-of-concept studies in small animal models. As such, the logical next step is to use clinically relevant large animal models to validate the protective effects of HDACi. Although large animal studies pose unique challenges, this step is vital before HDACi can be safely translated to treat I/R injury in clinical trials.

Among the HDACi studied, high-dose VPA has the most promising preclinical evidence supporting its use. A recent phase 1 dose-escalation trial demonstrated that healthy human participants could tolerate up to 140 mg/kg VPA as a 1-hour intravenous infusion with minimal side effects. Additional early phase clinical studies are necessary to validate VPA’s pharmacokinetics, pharmacodynamics, safety, and efficacy in patients with stroke, cardiac arrest, or myocardial infarction. In addition, dose-finding studies are also needed to determine the therapeutic window and minimal dose of HDACi required to achieve maximal protection. SAHA is another FDA-approved HDACi with unique promise as a treatment for I/R injury. It is highly potent and has the ability to induce both autophagy and mitochondrial biogenesis. Recent advancement in single-cell sequencing offers a promising strategy to investigate the cell- and disease-specific function of HDACi under different physiologic conditions. To further refine therapeutic interventions using precision medicine, high-throughput genomic and proteomic studies can help elucidate the pharmacodynamics biomarkers that can best differentiate the treatment responders from nonresponders.

Given more limited side effect profiles, isoform-specific HDACi could prove to be a superior treatment to nonspecific HDACi. However, given that these HDACi are experimental and not already FDA-approved like VPA, the timeline to translation will be significantly longer. We suspect that HDACi could even be adapted to other I/R injuries, including cardiac bypass surgery and organ transplantation, and as adjunctive therapy during extracorporeal cardiopulmonary resuscitation or aortic balloon occlusion during hemorrhagic shock.

CONCLUSIONS

I/R injury remains devastating and can cause irreversible damage to the brain, heart, and other vital organs without effective pharmacologic treatment. HDACi have shown promising protective properties in vitro and in small animal models of I/R injuries. They represent a high-impact, low cost, and clinically feasible strategy for neuroprotection and cardioprotection. Specifically, high-dose VPA improves clinical outcomes through pleiotropic effects triggered by HDAC inhibition and is the clear candidate for further clinical translation.

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