TRANSLATIONAL PHYSIOLOGY

A novel pharmacological strategy by PTEN inhibition for improving metabolic resuscitation and survival after mouse cardiac arrest

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SUDDEN CARDIAC ARREST (SCA) affects 350,000 people each year in the United States with an overall survival rate of about 7% (35). Significant mortality is due to a post-SCA syndrome with severe cardiac and neurologic dysfunction that is related to impaired metabolism and systemic inflammation (2, 19, 27). Failure of metabolic recovery results in cardiac stunning, poor tissue perfusion, and brain injury (27, 28, 49). Serum hyperglycemia and elevated lactate reflect a failed energetic recovery of critical organs (3, 43). Associated with this metabolic dysfunction, an intense inflammation described as a sepsis-like syndrome occurs with systemic pro-inflammatory cytokine release (2).

Cooling after SCA can improve both heart function and neurologically intact survival (25, 49). Our previous studies have shown that intra-ischemic cooling in heart cells exposed to ischemia/reperfusion (I/R) and in mice cooled during cardiopulmonary resuscitation (CPR) is highly protective and appears mediated via Akt, a pro-survival kinase that has both metabolic and anti-inflammatory effects (5, 38). Furthermore, although intra-CPR cooling may maximally protect heart and brain function it is difficult to achieve clinically (25). Possible mechanisms of cooling protection include inhibition of phosphatase and tensin homolog deleted on chromosomal 10 (PTEN), a ubiquitously expressed and constitutively active phosphatase and tensin homolog deleted on chromosomal 10 (PTEN), a ubiquitously expressed and constitutively active phosphatase that converts PIP3 to PIP2 and negatively regulates Akt activity (20).

In support of a critical role for PTEN in I/R injury and protection, recent work by us demonstrated that VO-OHpic (VO), a vanadyl small molecule compound that demonstrates potent inhibition of PTEN (24, 36), induces cooling-like cardioprotection with an almost fourfold reduction in cell death and significant increase of p-Akt (48). To extend these cell studies further, we studied whether VO could induce cooling-like protection with improved recovery and survival in an established mouse model of SCA.
METHODS

Mouse sudden cardiac arrest model. All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago and University of Chicago. The procedures have been previously described (1, 5, 10). In brief, adult female C57BL/6 (Taconic, Germantown, NY) mice were randomized into three groups [sham, normal saline (NS), and VO] and anesthetized with 100 mg/kg of ketamine and 10 mg/kg xylazine. Mice were intubated, ventilated, and cannulated. A pressure-volume catheter (Millar Instruments, Houston, TX) was inserted into the left ventricle for hemodynamic measurements. Following 20 min stabilization, asystolic cardiac arrest was then induced with mean arterial pressures (MAP) > 80 mmHg and a partial pressure of end-tidal CO2 (PETCO2) > 35 mmHg by intravenous administration of 0.08 mg/g potassium chloride solution. After 8 min of arrest, CPR was attempted with chest compressions, mechanical ventilation, and scheduled fluid administration. A time course of SCA was documented by ECG. Figure 1 illustrated a case for how induction and recovery of all the animals received either NS or VO in this study. Successfully resuscitated mice were monitored hemodynamically on mechanical ventilation for up to 2 h. VO (Sigma, St. Louis, MO) or vehicle NS was administered 30 min before SCA intraperitonally at 10 μg/kg body wt. This protocol was based upon work by others showing that similar vanadium compound treatment 30 min before ischemia in models of heart and brain injury is protective and within 30 min of intraperitoneal administration can increase tissue p-Akt concentrations (22, 50). Sham mice underwent instrumentation but not arrest.

Survival studies. Mice with successful return of spontaneous circulation (ROSC) were used for the survival study. After 2 h hemodynamic monitoring, mice were disconnected from the ventilator. Once the mouse was determined to breathe on its own, it was extubated and vascular access sites were surgically repaired. Mice were then monitored for up to 72 h.

Cardiac function. During the 2-h hemodynamic monitoring period, the cardiac function was acquired and analyzed using PowerLab Chart (ADInstruments, Colorado Springs, CO) and Millar PVAN 3.0 software (Millar Instruments, Houston, TX) as previously described (5).

Parameters, such as heart rate and left ventricular performance indexes including left ventricular maximum pressure (LVPmax) and maximum rate of change in the left ventricular pressure (dP/dmax) were assessed using Millar Pressure-Volume Conductance System. MAP was also measured in a separate set of mice using a pressure catheter. In addition, CPR quality parameters were recorded, such as chest compression rate (CC), partial pressure end-tidal CO2 (PETCO2), and time to achieve ROSC.

Neurological evaluation. Neurological function was evaluated at 6, 24, and 72 h following resuscitation (R6h, R24h, and R72h) using an established scoring system that includes level of consciousness, corneal reflex, respiration, righting reflex, coordination, and movement/activity (Table 1). The neurological score was assigned according to the prior studies (1, 11). The scores range from 0 (death) to 12 (normal neurological function).

Western blot analysis. The tissues were collected at 30 min and 2 h post-ROSC (R30 and R2h) from three groups of mice (sham, NS, and VO; n = 5 in each group) and lysed for protein analysis by Western blot analysis as previously described (23). The protein phosphorylation and expression were detected with antibodies against phosphorylation of Akt Thr308, p-Akt Ser473, and GSK3β Ser9 (Cell Signaling Technology, Danvers, MA), pyruvate dehydrogenase (PDH) E1α subunit (p Ser293; Novus Biologicals, Littleton, CO), phospholamban (p-PLB Thr17), sarco(endo)plasmic reticulum Ca2+ -ATPase (SERCA2α; Santa Cruz Biotechnology, Dallas, TX), α-tubulin (Neo-Markers, Fremont, CA), and β-actin (Sigma-Aldrich, St. Louis, MO). Quantitative results were obtained via densitometry (NIH ImageJ version 1.42; National Institutes of Health, Bethesda, MD).

Assessment of energetic recovery by 31P NMR. Two hours after the in vivo arrest and resuscitation protocol, mouse hearts were excised, retrograde perfused, and positioned in a 14.1 T vertical-bore NMR magnet (89 mm) (Bruker Biospin, Billerica MA) for spectroscopic assessment of metabolic and energetic recovery. 31P NMR spectra provided the ratio of phosphocreatine to ATP (PCr:ATP) as an index of the energetic state, as previously described (18, 39). Energetics was assessed for three groups: sham (n = 4), NS (n = 5), and VO (n = 5).

Measurement of plasma lactate and glucose. Plasma collected from mice (sham, NS, and VO; n = 5 in each group) at R30 and R4h was...
used to measure L-lactate using a colorimetric assay kit (Abcam, Cambridge, MA) and glucose using a colorimetric glucose assay kit (Sigma).

Cytokine measurement. The expression of multiple cytokines were measured in the plasma samples collected from mice (sham, NS, and VO; n = 5 in each group) at R30 and R4h using Bio-Plex cytokine assay kit (Bio-Rad, Hercules, CA) and Pre-B cell colony enhancing factor (PBCEF) ELISA kit (MBL International, Woburn, MA). The plasma samples were used as a 1:4 dilution, and the assay was performed in accordance with manufacture protocols. Suspended bead array was used for assessing the levels of the multiple cytokines (IL-6, KC, TNF-α, IL-1β, INFγ, and IL-10). Data from the reactions were acquired using a flow cytometry system (X Map-100; Luminex, Austin, TX) and accompanying software (Bio-Plex Manager software; Bio-Rad). The values reported represent median reporter fluorescence intensity of at least 50 beads. All samples were read in duplicate.

Statistic analysis. All statistical analysis was performed using OriginPro 8.5 (OriginLab, Northampton, MA). Results are expressed as means ± SE. For comparison among the different treatment groups, one-way ANOVA were used with post hoc examination by Tukey’s test. Kaplan-Meier survival analysis was performed using log-rank (Mantel-Cox) testing. T-test was used where applied. A value of P < 0.05 was considered statistically significant.

RESULTS

PTEN inhibitor VO improved neurologically intact survival. Twenty mice were randomized into NS and VO group (n = 10 each group). As depicted in Table 2, the parameters at both baseline and resuscitation were similar in both groups. Nine out of 10 mice in NS group with successful ROSC compared with 10 out of 10 mice in VO group achieved ROSC. Among the mice with successful ROSC, more mice (4/9) in NS group died within 4 h (R4h) compared with the VO group (0/10) (Fig. 2A). Long-term 72 h survival was significantly higher in the VO group compared with the NS group (50% vs. 10%; P < 0.05; Fig. 2A). As seen in Fig. 2B, only two mice survived to 6 h in the NS group compared with 7 in the VO group. Neurological function continued to improve at R24h and R72h. However, only one mouse in the NS group survived to R72h (10%) with a deteriorated neurological score in comparison with 5 mice in the VO group with close to normal neurological scores.

The cardiac function and hemodynamic parameters were assessed at baseline, R30, and R2h. When compared with NS, VO demonstrated a reduced deterioration in LVPmax by R2h (Fig. 3A), dp/dtmax (Fig. 3B), MAP (Fig. 3C), and PETCO2 (Fig. 3D) as early as R30. No difference was observed on recorded ECG between NS and VO group (Fig. 1).

VO increased the phosphorylation of Akt and GSK3β in heart and brain. Heart and brain samples were collected from three groups including sham, NS, and VO at R30 to study the effect of early signaling of Akt on cardiac function and survival. When compared with sham, cardiac p-Akt was decreased at R30 in the NS group and increased by VO at p-Akt Thr308 (Fig. 4, A and B), p-Akt Ser473 (Fig. 4, A and C). Similar results were observed for the Akt-related target p-GSK3β Ser9 (Fig. 4, A and D). In parallel studies, VO treatment also increased both p-Akt and p-GSK3β in brain tissue (Fig. 4, E–H).

VO increased cardiac phospholamban phosphorylation. Heart tissue collected at R30 was used to measure PLB phosphorylation, an additional Akt target and related to contractile function and SERCA2α expression by Western blot. P-PLB was over fourfold higher in VO group compared with NS group (Fig. 5, A and B). The SERCA2α expression was not changed by VO treatment (Fig. 5, A and C).

VO treatment enhanced cardiac energetic recovery (PCR:ATP). The 31P NMR spectrum in Fig. 6A illustrates the relative levels of phosphocreatine (PCr) and ATP in an isolated, perfused shammart. The PCR:ATP ratio serves as an index of the intracellular bioenergetic state (15, 18, 34). The ratio for the sham heart. The PCr:ATP ratio was 1.7 (Fig. 6B), in agreement with previously published data (29). The ratio was significantly reduced in the NS group (1.5). The reduced PCr recovery relative to ATP

Table 1. Neurological function scoring system

<table>
<thead>
<tr>
<th>Neurological Function Scoring system</th>
<th>Level of consciousness</th>
<th>Corneal reflex</th>
<th>Righting flex</th>
<th>Coordination</th>
<th>Movement/activity</th>
<th>Total possible score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1. No reaction to pinching of tail</td>
<td>1. No blinking</td>
<td>1. No turning attempts</td>
<td>1. No movement</td>
<td>1. No spontaneous movement</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

Table 2. Characteristics at baseline and CPR period in NS and VO groups

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>NS</th>
<th>VO</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
<td>29.1</td>
<td>28.4</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td></td>
<td>24.4</td>
<td>312.2</td>
</tr>
<tr>
<td>Left ventricular maximum pressure, mmHg</td>
<td>88.7 ± 3.1</td>
<td>86.0 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>Maximum rate of change in the left ventricular pressure, mmHg/ms</td>
<td>6319.1 ± 337.6</td>
<td>6018.7 ± 234.8</td>
<td></td>
</tr>
<tr>
<td>Stroke volume, μl</td>
<td></td>
<td>13.5</td>
<td>13.3</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td></td>
<td>4.3</td>
<td>4.1</td>
</tr>
<tr>
<td>PETCO2, mmHg</td>
<td></td>
<td>37.7</td>
<td>37.2</td>
</tr>
<tr>
<td>CPR Chest compression rate, beats/min</td>
<td>344.2 ± 4.1</td>
<td>356.1 ± 8.3</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>171.0 ± 1.1</td>
<td>16.6 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>PETCO2, mmHg</td>
<td></td>
<td>21.7</td>
<td>22.6</td>
</tr>
<tr>
<td>ROSC, n (%)</td>
<td></td>
<td>9 (90)</td>
<td>10 (100)</td>
</tr>
<tr>
<td>Time to ROSC, s</td>
<td></td>
<td>145.3</td>
<td>125.6</td>
</tr>
</tbody>
</table>

CPR, cardiopulmonary resuscitation; NS, normal saline; VO, VO-OHpic; ROSC, return of spontaneous circulation.
Fig. 2. Effect of VO on neurological function and survival after sudden cardiac arrest (SCA). A: Kaplan-Meier 72 h survival plot of NS and VO group (n = 10 in each group, 50% NS vs. 10% VO; #P < 0.05). B: Assessment of neurological function score (0 representing death of the animal and 12 reflecting a full neurological recovery) at 6 h (R6h; left), 24 h (R24h; middle), and 72 h (R72h; right).

Fig. 3. Effect of VO on cardiac function and hemodynamic performance after SCA. A: left ventricular maximum pressure (LVPmax) at 30 min and 2 h post-ROSC (R30 and R2h, respectively) post-SCA in NS and VO groups was measured by Millar Pressure-Volume Conductance System (n = 10 each group; #P < 0.05). B: maximum rate of change in the left ventricular pressure (dP/dtmax) was assessed at R30 and R2h in NS and VO groups by Millar Pressure-Volume Conductance System (n = 10 each group; #P < 0.05 at R30 and *P < 0.01 at R2h). C: mean arterial pressures (MAP) was evaluated at R30 and R2h post-SCA in NS and VO groups by a pressure catheter (n = 10 each group; #P < 0.05). D: P ETCO2 was measured at R30 and R2h in NS and VO groups by Millar Pressure-Volume Conductance System (n = 10 each group, *P < 0.01). Data presented are means ± SE.
content is indicative of a low intracellular phosphorylation potential and impaired mitochondrial recovery. In the VO group, the ratio of PCr:ATP (1.8) was significantly greater than the NS and not significantly different from the shams. Intracellular organic phosphate was not discernible in 31P NMR spectra at 2 h, thereby precluding evaluation of intracellular pH based on the resonant position of Pi.

Active PDH. Pyruvate dehydrogenase (PDH) is a rate-limiting enzyme of pyruvate dehydrogenase complex. P-PDH at R30 in NS- and VO-treated heart tissues were analyzed and demonstrated a decrease by VO (Fig. 6C), suggesting improved pyruvate oxidation and glucose utilization.

VO attenuated plasma lactate and glucose and modulated mediators of inflammation. VO attenuated plasma lactate levels at both R30 and R2h compared with NS (Fig. 7A). Plasma glucose was also reduced by VO at R4h (Fig. 7B). A panel of cytokines including IL-6, KC, TNF-α, INF-γ, IL-1β, and IL-10 were noticed to elevate significantly, consistent with the cytokine storm reported after human SCA (2). VO treatment most affected IL-1β and IL-10. IL-1β, a pro-inflammatory cytokine, was elevated almost twofold at R4h and was attenuated by VO. In contrast, IL-10, an anti-inflammatory cytokine, was marked increased by VO (Fig. 8, A and B). In addition, we assessed PBEF, an adipokine/pro-inflammatory cytokine that has been recently associated with decompensated heart failure (37), diabetes type II (7), and hemorrhagic shock (4). When compared with sham, plasma PBEF levels were rapidly increased as early as R30 and continued to increase upwards of 20-fold by R4h. VO attenuated PBEF levels at both time points (Fig. 8C). Other cytokines were not affected by VO treatment (Fig. 8, D–G).

**DISCUSSION**

The present study builds upon prior laboratory work suggesting that cooling protection after SCA may be mediated by PTEN inhibition and enhanced Akt signaling, and in heart cells can be reproduced without cooling using PTEN inhibition strategies (48). The present study intended to study the effect
of early signaling of Akt on cardiac function and survival given the fact that in human studies, early recovery of MAP within hours of resuscitation correlates with improved survival and better neurological outcomes due to better tissue perfusion (9, 33). Although a few studies have reported vanadium small molecule PTEN inhibitor protection in acute myocardial infarction and acute stroke models, the present study for the first time extends this prior work to the systemic I/R injury of sudden cardiac arrest. Optimal VO administration given before mouse SCA significantly increased Akt activation in both heart and brain as measured by p-Akt and phosphorylation of two Akt targets GSK3β and phospholamban (Figs. 4 and 5). When compared with NS, VO increased overall metabolic recovery after SCA as measured by decreased plasma lactate and glucose concentrations (Fig. 7). VO also decreased systemic inflammation as measured by increased anti-inflammatory cytokine plasma concentrations (i.e., IL-10) along with reduced pro-inflammatory cytokines (i.e., IL-1β and PBEF) within 4 h after CPR (Fig. 8). VO reduced cardiac performance deterioration as measured by LVPmax and dP/dtmax within 2 h after CPR (Fig. 3) and improved heart energetics assessed by PCr:ATP and glucose oxidation measured by p-PDH (Fig. 6). In addition to early cardiovascular recovery, VO results in improved 72 h neurologically intact survival (Fig. 1).

PTEN inhibition and optimal Akt activation after SCA. Previous studies show that cooling protection against I/R in both heart and brain is mediated by Akt signaling (5, 38, 46, 47). Intra-CPR cooling appears highly protective in animal models against the injury of sudden cardiac arrest (1, 44) and its protection abrogated by partial Akt deletion (5). Given that intra-CPR cooling is technically difficult to achieve clinically (25, 26), pharmacologic approaches that duplicate mechanisms of cooling protection could provide new strategies for SCA treatment.

Inhibition of PTEN has been shown to reduce I/R injury. Cardiomyocyte-specific conditional PTEN deletion limited myocardial infarction size after exposure to hypoxia (30). Its inhibitor VO was also shown to improve LVSP and reduce infarction size following myocardial infarction (50). In addition, similar small molecule inhibitors of PTEN have been reported to reduce acute stroke injury (22) and improved left ventricular function postinfarction (17). The current study extends this prior work to the disease of SCA. The present work demonstrates the importance of traditional roles of Akt related to improved glucose utilization and new roles of Akt associated with improved energetics and decreased plasma lactate and glucose concentrations. These changes are relevant clinically to improved human SCA survival (Figs. 5 and 6) (3, 43). It also identifies additional targets that may relate to SCA recovery, such as enhanced phosphorylation of phospholamban (Fig. 5) that is phosphorylated by Akt at its Thr17 site (41) and increases cardiac contractility.

Energetic recovery with VO treatment. The additional novelty of the current study is that the ischemic insult and resuscitation were performed, in vivo, 2 h before exciting the mouse heart to access energetic recovery. This adds a far greater level of physiological sophistication by integrating whole body effects of SCA on cardiac recovery. Figure 6, A and B, shows that the energetic recovery (PCr:ATP) from SCA is significantly improved with VO treatment compared with nontreated NS group. Relative to the sham group, the treated group reveals significantly improved with VO treatment compared with nontreated NS group. Relative to the sham group, the treated group reveals significantly improved with VO treatment compared with nontreated NS group. Relative to the sham group, the treated group reveals significantly improved with VO treatment compared with nontreated NS group. 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Because baseline measures of PCr and ATP were not measured before the SCA event, in vivo, PCr and ATP signal for the isolated-perfused sham hearts are used as a relative index of baseline content for all isolated-perfused hearts. The NS group shows both a reduced ATP and PCr pool relative to shams. The PCr pool for the VO treated hearts is modestly greater than the sham group, while ATP was slightly reduced in two of the five hearts (nonsignificant). Therefore, the overshoot is accounted for by both the modest increased in PCr or loss in ATP (nonsignificant).

In addition, VO decreased PDH phosphorylation, demonstrating a significant increase in the amount of PDH in the active form (Fig. 6C). This would be consistent with increased activity through the enzyme and suggests increased pyruvate oxidation and glucose utilization. These consequences may be the results of increased glucose uptake by Glut 4 and upregulated PDH activity by GSK3β and FOXO-1 that was regulated by Akt activation (12, 13, 16, 21, 45) to improve glycolysis and mitochondrial oxidation.

Metabolic recovery and inflammation after SCA. Consistent with prior studies, pro-inflammatory (IL-1β) and anti-inflammatory cytokines (IL-10) are elevated after mouse SCA. VO reduces IL-1β by 4h while increasing IL-10 (Fig. 5). This is consistent with reports of Akt-enhancement of IL-10 production and inhibition of proinflammatory cytokine secretion in several immune cells (8, 14, 40). The current study extends this prior work further by demonstrating that increases in plasma concentrations of the pro-inflammatory cytokine PBEF are associated with poor SCA outcome in the mouse and are significantly reduced by VO. Of note, PBEF/Nampt is a critical salvage enzyme related to NAD⁺ synthesis that may be released into blood during tissue energetic stress (4). VO treatment reduced the release and retained PBEF/Nampt in the cells that could subsequently increase NAD⁺ content along with improved glucose oxidation leading to improved SCA survival. The trend reported here after mouse SCA on PBEF/Nampt is similar to that reported in clinical studies associated with acute heart failure and in a mouse hemorrhagic shock model (4, 37).

Fig. 7. Plasma lactate and glucose were measured by assay kits. A: plasma lactate level at R30 (#P < 0.05) and within 4 h (R4h; *P < 0.01). B: plasma glucose level at R4h (*P < 0.01). Data presented are means ± SE of 5 mice.

Fig. 8. Plasma inflammatory cytokines were assessed by Bio-Plex cytokine assay kit at R30 and R4h. A: IL-1β at R4h (*P < 0.01) was attenuated by VO (#P < 0.05). B: IL-10 at R4h (#P < 0.05) was further increased by VO (*P < 0.01). C: pre-B cell colony enhancing factor (PBEF) was elevated as early as R30 (#P < 0.01) and continued to increase by R4h (*P < 0.01). VO attenuated it at both R30 (*P < 0.01) and R4h (#P < 0.05). D–G: VO had no effect on IL-6, murine IL-8 (KC), TNF-α, and INFγ (P = not significant). Data presented are means ± SE of 5 mice.
Reduced plasma glucose by VO demonstrated in this study is supported by the previous report that PBef/Nampt increased insulin secretion by pancreatic β-cells (32). This report suggests a possible link between PTEN/PBef and SCA in which hyperglycemia and insulin resistant are often seen. Furthermore, upregulated plasma cytokines associated with enhanced metabolic recovery observed in the present study suggests that improved glycolysis and glucose oxidation provides energy, such as ATP, required for immune cells, including neutrophils, to augment the immune responses (6). Of note, the observation that resuscitation within 4h did not decrease the plasma concentration of IL-1β and PBef, which were reduced by VO indicating regulation of these two cytokines are related to PTEn inhibition by VO. In contrast, IL-10 was increased by both reperfusion and VO, suggesting that a consequence of both reduced organ damage and PTEn effect may be involved.

I/R injury, like SCA, causes accumulation of osmotic metabolites and (such as lactate and ions) due to impaired glucose oxidation resulting in drop of pH, acidosis and increase lactate concentrations in blood, ultimately leading to dysregulation of mechanical contraction and ionic homeostasis imbalance.

The current study has limitations. We used pretreatment of VO (before SCA) to ensure optimal Akt activation in heart and brain within minutes after CPR as reported. From a translational perspective, further work is needed to confirm the protective effect during CPR that can similarly optimize Akt activity to improve SCA survival. The present work provided insights into possible mechanisms of PTEN inhibition in mediating the metabolic recovery and resuscitation after SCA.

Conclusions

This is one of the first reports to show that PTEN inhibition can mimic critical aspects of intra-CPR cooling protection after mouse SCA by enhancing heart and brain Akt signaling and increasing neurologically intact survival. Additional work is justified to further test agents capable of optimally enhancing tissue Akt during CPR.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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