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ACTIVATION OF THE XBP1S/O-GLCNACYLATION PATHWAY IMPROVES FUNCTIONAL OUTCOME AFTER CARDIAC ARREST AND RESUSCITATION IN YOUNG AND AGED MICE

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ABSTRACT—After cardiac arrest (CA) and resuscitation, the unfolded protein response (UPR) is activated in various organs including the brain. However, the role of the UPR in CA outcome remains largely unknown. One UPR branch involves spliced X-box-binding protein-1 (XBP1s). Notably, XBP1s, a transcriptional factor, can upregulate expression of specific enzymes related to glucose metabolism, and subsequently boost O-linked β-N-acetylglucosamine modification (O-GlcNAcylation). The current study is focused on effects of the XBP1 UPR branch and its downstream O-GlcNAcylation on CA outcome. Using both loss-of-function and gain-of-function mouse genetic tools, we provide the first evidence that activation of the XBP1 UPR branch in the post-CA brain is neuroprotective. Specifically, neuron-specific *Xbp1* knockout mice had worse CA outcome, while mice with neuron-specific expression of *Xbp1s* in the brain had better CA outcome. Since it has been shown that the protective role of the XBP1s signaling pathway under ischemic conditions is mediated by increasing O-GlcNAcylation, we then treated young mice with glucosamine, and found that functional deficits were mitigated on day 3 post CA. Finally, after confirming that glucosamine can boost O-GlcNAcylation in the aged brain, we subjected aged mice to 8 min CA, and then treated them with glucosamine. We found that glucosamine-treated aged mice performed significantly better in behavioral tests. Together, our data indicate that the XBP1s/O-GlcNAc pathway is a promising target for CA therapy.

KEYWORDS—Aging, brain ischemia, ER stress, glucosamine, knockout, neuroprotection, UPR

INTRODUCTION

Cardiac arrest (CA) and resuscitation results in brain ischemia/reperfusion (I/R) injury, which is considered a key contributor to the high incidence of post-CA neurologic deficits and mortality (1). Currently, therapeutic hypothermia is the only available treatment option for CA patients after resuscitation. Despite great efforts, no pharmacologic intervention has yet been shown to improve neurologic outcome for CA patients. CA-induced damage in the brain is closely related to selective death of vulnerable neurons. Thus, it is reasonable to believe that enhancing neuronal resistance to I/R injury would improve neurologic outcome in CA survivors. To this end, boosting endogenous pro-survival pathways may be a promising strategy because the primary purpose of these pathways is to promote recovery of pivotal cellular functions impaired by stress and to also maintain cellular homeostasis (2–4). The unfolded protein response (UPR) is a pro-survival pathway that is activated when function of the endoplasmic reticulum (ER) is impaired and ER stress occurs (3). The main purpose of the UPR is to restore ER homeostasis, and promote cell survival. It has been well established that brain I/R insult caused by transient ischemic stroke or CA/resuscitation leads to ER stress and subsequent activation of the UPR (5–8). Moreover, mounting evidence indicates that ER stress plays a key role in the progression of brain I/R injury (5–8). Therefore, a better understanding of the role of the UPR in CA pathophysiology would be expected to inform development of new therapeutic strategies for CA patients.

The UPR has three key response branches, but the role of these individual UPR branches in CA outcome is largely undefined. One branch is mediated by an ER stress sensor—inositol-requiring enzyme-1 (IRE1). Activated IRE1 can splice X-box binding protein-1 (*Xbp1)* mRNA (9), which leads to translation of a new 54-kDa XBP1s protein. XBP1s is a transcriptional factor that controls expression of many genes related to protein homeostasis (10). Notably, XBP1s couples the UPR with the hexosamine biosynthetic pathway (HBP) by upregulating expression of major HBP enzymes (11). HBP produces uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), which is the substrate for O-GlcNAc modification (O-GlcNAcylation), a post-translational modification that acts as a potent protective pathway under many stress conditions (11–13). For example, after heart ischemia and focal brain ischemia (i.e., ischemic stroke), this XBP1s/HBP/O-GlcNAcylation axis is activated, which has been shown to be beneficial for recovery of functional outcome (8, 11).

Notably, ischemia-induced activation of O-GlcNAcylation is impaired in the aged brain (6, 8, 14). We have provided the first evidence that pharmacologic treatment to reverse this impairment significantly improves ischemic stroke outcome in aged mice (8). Thus, this axis appears to be an excellent representative of unique pro-survival pathways that are critical to maintaining cellular homeostasis and promoting cell survival

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under ischemic stress conditions, but that, during aging, become functionally compromised. Targeting such pathways may hold great promise for achieving therapeutic efficacy in ischemia- and aging-related diseases such as CA. Indeed, most CA patients are elderly, and clinical evidence shows that CA outcomes worsen with advancing age (15). Our previous study has demonstrated that the XBP1s/HBP/O-GlcNAc axis functions in the brain, and is activated after ischemic stroke or CA (6, 8). Here, our goal was to clarify the effect of this axis on functional outcome after CA in young and aged mice.

MATERIALS AND METHODS

Animal experiments were approved by the Duke University Animal Care and Use Committee. All studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals and ARRIVE guidelines. Animals were randomized into groups using an online tool, and all evaluations were performed in a blinded fashion.

Animals

C57Bl/6 mice were obtained from The Jackson Laboratory (Maine, USA). Two XBP1-specific genetically modified mouse lines (both on C57Bl/6 background) were used, one with *Xbp1* deletion and the other with *Xbp1s* overexpression in forebrain neurons as previously described (8). Briefly, *Xbp1*$^{f/f}$ mice and Emx1$^{Cre/Cre}$ mice (JAX stock #005628) were mated to generate *Xbp1*$^{f/f}$;Emx1-Cre (Xbp1-cKO) mice. TRE-XBP1s mice and Camk2a-tTA mice (Tet-off system; JAX stock #007004) were mated to generate double transgenic TRE-XBP1s;Camk2a-tTA (XBP1s-TG) mice. Of note, expression of transgene *Xbp1s* in XBP1s-TG mice is suppressed by adding doxycycline to the drinking water. Simply changing to regular drinking water activates *Xbp1s* expression in these mice. Information for genotyping primers has been provided previously (8).

Animal surgery

Young male mice (3–4 mo) and aged male mice (20–22 mo) were used. CA/CPR surgery was performed as previously described (6, 16), with minor modifications. Briefly, following anesthesia induction and endotracheal intubation, mice were maintained on 1.5% to 1.7% isoflurane with a body temperature of 37.0°C ± 0.2°C (rectal probe) before CA induction. The electrocardiogram (ECG) and pericranial temperature were continuously monitored during the whole procedure. Peripheral blood flow on left paw was also monitored by a laser Doppler blood flow meter (Moor Instruments). After CA induction by injecting a bolus of potassium chloride *via* the jugular vein, the ventilator and body temperature control system were immediately turned off, allowing the body to cool down, and the pericranial temperature was maintained at 38.3°C ± 0.2°C (young mice) or 38.0°C ± 0.2°C (aged mice) using a coil with circulating warm water. CA duration was 8.5 min (young mice) or 8 min (aged mice). Resuscitation was initiated by injecting a bolus of epinephrine (100 μL; 32 μg/mL) followed by continuous infusion of 150 μL of epinephrine (25 μL/min) and CPR with chest compression. Return of spontaneous circulation (ROSC), as indicated by a stable ECG sinus rhythm, was usually achieved after 4 min of CPR. If not, the resuscitation effort was terminated, and mice were excluded from analysis. Once mice regained spontaneous breathing, they were removed from the surgical bench, and placed into a warm chamber to recover for 2 h. Of note, for the experiment using XBP1s-TG mice after surgery, each mouse was dosed with doxycycline (60 mg/kg) via intraperitoneal injection daily for 3 consecutive days starting 2 h post ROSC.

Glucosamine administration

Glucosamine (150 mg/mL; Sigma-Aldrich, St. Louis, Mo) was dissolved in saline (vehicle). Each mouse was dosed 200 mg/kg glucosamine *via* intravenous injection at 1 h after ROSC, and 300 mg/kg glucosamine *via* intraperitoneal injection on day 1 after CA/CPR. This dosage was based on previous studies (17, 18).

Behavioral tests

All tests were conducted by experimenters who were blinded to genotypes and treatments, using our standard protocols (6). Sham groups have been evaluated in our previous studies (16, 19), and thus were not included in these tests.

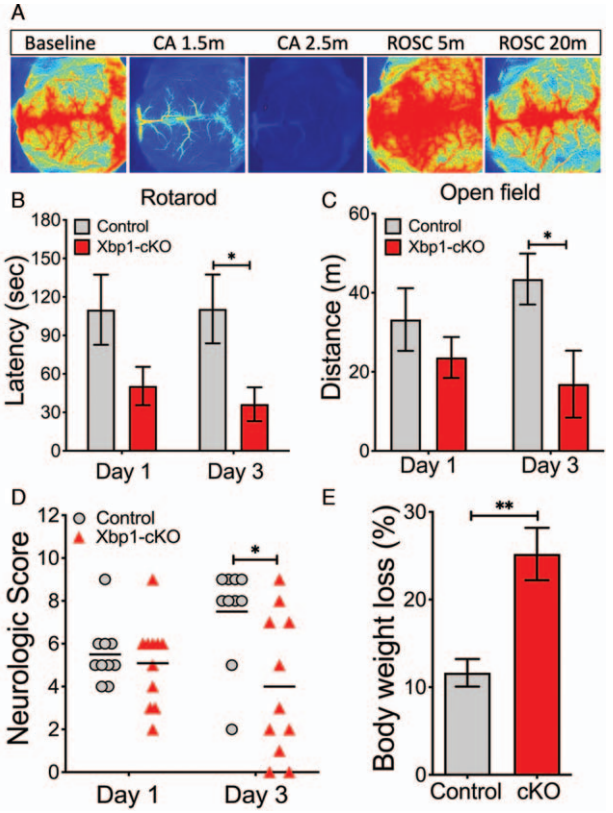

FIG. 1. **CA outcome was worse in young mice with *Xbp1* deletion in forebrain neurons.** A, Representative laser speckle contrast images showing cortical cerebral blood flow changes in our CA/CPR model. B to E, Young Xbp1-cKO (cKO) and *Xbp1*$^{f/f}$ littermates (control) were subjected to 8.5 min CA. Rotarod (B), spontaneous locomotor activity (traveled distance during a 10-min test period in an open field test (C), and neurologic score (D) were evaluated on days 1 and 3 after CA. Body weight loss was evaluated on day 3 after CA (E). Data are presented as mean ± SEM or median (n = 10–11/group). * $P < 0.05$; ** $P < 0.01$. CA indicates cardiac arrest.

Neurologic score

A nine-point scoring system was used to evaluate neurologic deficits. A total neurologic score was computed on the basis of performance (9 points = normal, and 0 points = severe injury).

Rotarod

A rotating device with an accelerating rod (4 rpm–40 rpm; Med Associates Inc, St. Albans, Vt) was used. Mice received rotarod training for 3 days prior to CA/CPR surgery to ensure a similar baseline. Latency to fall from the rotarod was calculated by averaging the data from three trials.

Open field

After mice were transferred to the test room, they were allowed to stay in the cage for 1 h prior to the test. To start the test, each mouse was placed in the center of an open field test box (50 × 50 × 50 cm, CleverSys Inc, Reston, Va). Spontaneous locomotion was video-recorded for 10 min, and then analyzed using the automatic tracking system TopScan (CleverSys).

Laser speckle flowmetry

Laser speckle imaging of the brain was performed using the full-field laser perfusion imager RFLS III, according to the manufacturer's instructions (RWD Life Science Co, Kent, Del). A midline scalp incision was made to expose the

skull for laser speckle imaging, and the imager was positioned directly above the skull. The following imaging parameters were used: display rate (25 Hz), time constant (1 s), camera exposure time (10 ms), camera frame rate (37.59), laser intensity (60 mA), and resolution (2,048 × 2,048). After images were taken, the signal intensities of cerebral blood flow (CBF) in the region of interest (marked with a rectangle) were calculated at each time point by the RWD Laser Speckle Imaging System software, and presented as percentages of pre-CA baselines.

Western blotting

Western blot analysis was performed as previously described (6). Brain samples were homogenized by sonication using 2% SDS lysis buffer. The following primary antibodies were used: anti-O-GlcNAc (677902; BioLegend), and anti-β-actin (A3854; Sigma).

Statistical analysis

All data analyses were performed using Prism 8 (GraphPad Software). The primary outcome was neurologic score, which was used to determine the group size for each experiment based on our previous studies or pilot experiments. Statistical significance was assessed by unpaired Student *t* test or Mann–Whitney *U* test. For more than two groups, one-way or two-way ANOVA with *post hoc* Holm-Sidak correction for multiple comparisons was used. Survival curve was analyzed with Log-rank (Mantel-Cox) test. Data are presented as mean ± SEM or the median (neurologic score). The level of significance was set at $P < 0.05$.

RESULTS

CA outcome was worse in young mice with neuron-specific deletion of Xbp1 in the brain.

The XBP1 UPR pathway is activated after CA/CPR (6), but its effect on CA outcome is completely unknown. To address this critical question, we first used neuron-specific *Xbp1* knockout mice, i.e., Xbp1-cKO (8). Our mouse CA/CPR model has been well characterized in previous studies (6, 16, 19, 20). Using laser speckle contrast imaging (LSCI), we further showed here that in our model, the cortical CBF decreased dramatically, and reached a zero level during CA (Fig. 1A). Consistent with a previous report, we also observed a short period of apparent hyperemia after ROSC before returning to baseline (21). This finding confirmed a brain I/R insult in our CA/CPR model.

We then subjected Xbp1-cKO and littermate control mice to CA/CPR, and examined short-term outcome during the first 3 days after CA/CPR (Fig. 1). Compared with control mice, Xbp1-cKO mice exhibited a trend toward greater functional deficits as early as day 1 after CA/CPR, based on the rotarod and open field tests (Fig. 1, B and C). On day 3, these knockout mice performed significantly worse on all three functional tests, and had lost more body weight, compared with control mice (Fig. 1, B–E). These data suggest that activation of the XBP1 UPR branch is neuroprotective in CA.

CA outcome was improved in young mice with neuron-specific expression of Xbp1s in the brain

To further test the notion that the XBP1 UPR branch is neuroprotective in CA, we set out to directly assess CA outcome in young mice with neuron-specific overexpression of *Xbp1s* in the brain. For this purpose, we used XBP1s-TG transgenic mice in which the Camk2a promoter-controlled expression of the X*bp1s* transgene can be induced by removing doxycycline from the drinking water (8). Both XBP1s-TG and littermate control mice were maintained with drinking water containing doxycycline. Expression of the *Xbp1s* transgene in the XBP1s-TG mice was induced by changing to regular drinking water 6 days before surgery, as described in our previous study (8). Mice were then subjected to 8.5 min CA followed by CPR. On day 3 after CA/CPR, no clear difference in loss of body weight was observed; however, neurologic scores were significantly better in XBP1s-TG versus control mice (Fig. 2). Collectively, therefore, our data indicate that activation of the XBP1 UPR pathway in the brain is an endogenous neuroprotective response in CA.

Short-term CA outcome in young mice was improved after post-CA treatment with glucosamine

Studies have shown that deletion of *Xbp1* suppresses, while overexpression of *Xbp1 s* boosts, O-GlcNAcylation, and XBP1-mediated protective effects in heart ischemia and ischemic stroke are mediated *via* the XBP1s/HBP/O-GlcNAc axis (8, 11). Based on these findings, CA outcome would be expected to improve if O-GlcNAcylation is increased. Indeed, post-CA treatment with thiamet-G, a small molecule that increases global O-GlcNAcylation by inhibiting removal of GlcNAc from existing O-GlcNAcylated target proteins, is protective in mice after CA/CPR (6). However, we do not yet know whether increasing O-GlcNAcylation by directly boosting this axis pharmacologically also provides beneficial effects on CA outcome. This is a critical question because these two manipulations may target different sets of O-GlcNAcylated proteins, which in turn could lead to distinct effects. Answering this question may also expand the pharmacologic options for targeting this axis in CA. To this end, we decided to use glucosamine because glucosamine can readily enter the cells and be used by the HBP to boost UDP-GlcNAc levels, and consequently increase O-GlcNAcylation. Indeed, it has been shown that glucosamine treatment increases O-GlcNAcylation in the mouse brain (22).

Therefore, we subjected young mice to CA/CPR, and administered the first dose of glucosamine or vehicle at 1 h after ROSC and second dose on day 1 after CA. On day 1 post CA, no difference in performance on the rotarod was observed between the two groups; however, on day 3, the glucosamine-treated mice performed significantly better on the rotarod (Fig. 3A).

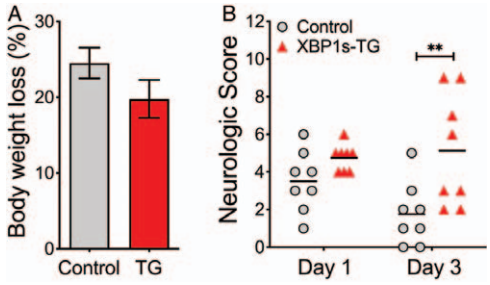

FIG. 2. **CA outcome was improved in young mice with neuron-specific overexpression of *Xbp1s*.** Young XBP1s-TG (TG) and littermate control mice were subjected to 8.5 min CA followed by resuscitation. Body weight loss was evaluated on day 3 after CA (A). Neurologic scoring was performed on days 1 and 3 after CA (B). A total of six mice (four control mice and two TG mice) died on day 2 or day 3, and were excluded from data analysis. Data are presented as mean ± SEM or median (n = 8/group). $^{**}P < 0.01$. CA indicates cardiac arrest.

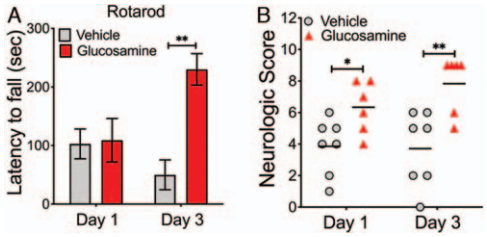

Fig. 3. **Short-term CA outcome was improved in young mice after post-CA treatment with glucosamine.** Young C57Bl/6 mice were subjected to 8.5 min CA followed by resuscitation. After 1 h and 1 day of reperfusion, mice were treated with saline (vehicle) or glucosamine. Rotarod performance (A) and neurologic score (B) were evaluated on days 1 and 3 after CA. Data are presented as mean ± SEM or median (n = 6–7/group). $^{*}P < 0.05$; $^{**}P < 0.01$. CA indicates cardiac arrest.

Further, the glucosamine-treated mice consistently exhibited better scores on the neurologic assessment during the 3-day observational period (Fig. 3B).

Long-term CA outcome in aged mice was improved after post-CA treatment with glucosamine

Since many CA patients are elderly, it would be clinically relevant to examine the effects of glucosamine treatment on functional outcome in aged mice over a relatively long period (in our case, 7 days) after CA/CPR. To reduce the high mortality that was observed in aged mice during our pilot studies, we modified our standard procedure. Specifically, the CA duration was shortened from 8.5 min to 8 min, and the cranial temperature during CA was maintained at 38.0°C ± 0.2°C. We also performed the first comparison of cortical CBF changes in young versus aged brains during the CA/CPR procedure using the LSCI technique (Fig. 4). Interestingly, we noted clear differences in CBF recovery after ROSC between young and aged brains. Compared with aged brains, the cortical CBF in young brains appeared to recover faster, while after the hyperemia phase, it dropped slower. Of note, young and aged mice were subjected to different CA durations (young: 8.5 min vs. aged: 8 min). Even with a shorter duration, CBF changes appeared to be unfavorable during the early ROSC phase in aged mice. The significance of this finding remains to be clarified with regard to its role in the observed worse CA outcome in aged versus young mice. Eventually, no significant difference in the extent of CBF recovery was observed between the two groups (Fig. 4).

To examine the effects of glucosamine in aged animals, we first demonstrated that O-GlcNAcylation can be increased in the aged brain after glucosamine treatment (Fig. 5A). Then, we subjected aged mice to 8 min CA followed by CPR. Glucosamine or vehicle was administered according to the same protocol used in the experiment with young mice. On day 1 after CA, all mice survived, and no obvious difference in neurologic scores was observed between the two groups (Fig. 5B). On the following days, some of these mice, especially from the vehicle group, died, as expected. Notably, on days 3 and 7 after CA/CPR, mice from the glucosamine group had significantly better neurologic scores (Fig. 5B). The open field test also indicated beneficial effects of glucosamine in CA mice, as glucosamine-treated mice traveled longer in the open

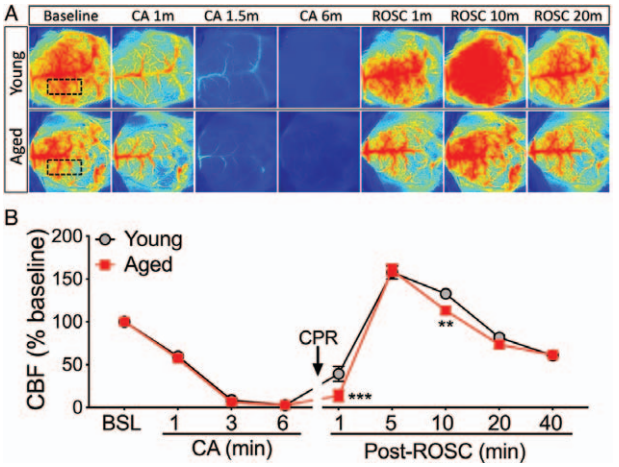

Fig. 4. **Age affected dynamics of cerebral blood flow (CBF) recovery after CA/CPR.** Young and aged mice were subjected to 8.5 min or 8 min CA, respectively. During the entire procedure (except the CPR step), brain cortical CBF was monitored using laser speckle contrast imaging (LSCI). A, Representative LSCI images. The regions for quantification were marked with dashed rectangles. B, Quantitative CBF measurement of the cortical areas by LSCI at the indicated time points over the course of the CA/CPR procedure. Baseline was set at 100% for each mouse. CBF change at each time point was calculated by comparing to baseline. Data are presented as mean ± SEM (n = 4/group). $^{**}P < 0.01$; $^{***}P < 0.001$. CA indicates cardiac arrest; CBF, cerebral blood flow.

field test compared with vehicle-treated mice on day 7 after CA/CPR (Fig. 5C). Moreover, body weight recovered significantly better in CA mice after glucosamine treatment (Fig. 5D). Finally, the overall survival rates over 7 days after CA/CPR were 50.0% (vehicle) versus 88.9% (glucosamine) ($P = 0.104$; Log-rank/Mantel-Cox; Fig. 5E). Taken together, our findings indicate that glucosamine treatment improves CA outcome in both young and aged mice.

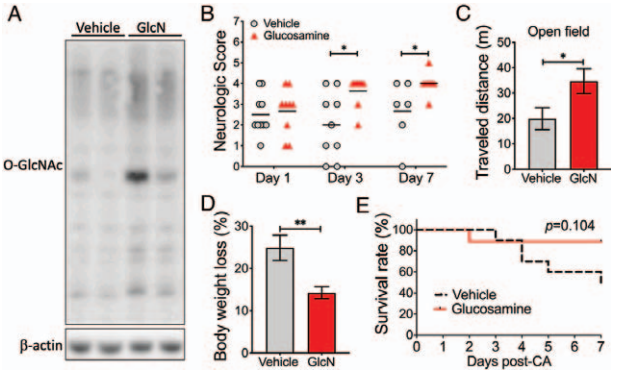

Fig. 5. **Long-term CA outcome was improved in aged mice after post-CA treatment with glucosamine.** A, Glucosamine-induced increase in O-GlcNAcylation in aged brains. Aged C57Bl/6 mice were dosed with glucosamine (GlcN) or vehicle, and 3 h later, brain cortex samples were collected for Western blotting. B to E, Long-term CA outcome. Aged mice (n = 9–11/group) were subjected to 8 min CA. Glucosamine was dosed at 1 h after ROSC, and then on day 1 after CA. CA outcome evaluations included neurologic score (B), spontaneous locomotor activity (open field test; C), and body weight loss on post-CA day 7 (D). E, Survival rates over 7 days of observation. Of note, one mouse from the vehicle group appeared to have hemangioma, and was excluded from analysis. Data are presented as mean ± SEM or median. $^{*}P < 0.05$; $^{**}P < 0.01$. CA indicates cardiac arrest; ROSC, return of spontaneous circulation.

DISCUSSION

Although it has long been established that CA/CPR activates the UPR, its effect on CA outcome remains largely unknown. In the current study, we focused on the XBP1s/HBP/O-GlcNAc axis in CA. We first demonstrated that the XBP1 UPR branch is a neuroprotective pathway in CA using both loss-of-function and gain-of-function mouse genetic tools. Then, we provided evidence that glucosamine treatment increased O-GlcNAcylation in aged mouse brains and importantly, post-CA treatment with glucosamine significantly improved CA functional outcome in both young and aged mice.

I/R insult is a well-known pathologic condition under which ER homeostasis is disturbed (i.e., ER stress) and the UPR is activated (3, 4). In addition to the IRE1/XBP1s branch, the UPR has two other branches: the PERK (protein kinase RNA-like ER kinase) branch and the ATF6 (activating transcription factor 6) branch. In the context of CA outcome, only the PERK branch has been studied to some extent. Activation of the PERK branch increases phosphorylation of eukaryotic initiation factor-2α, leading to suppression of global protein synthesis. In 1971, Kleihues and Hossmann reported that protein synthesis is severely inhibited after brain ischemia (23). Consistent with this observation, it was later found that after CA/CPR, the PERK branch is activated in various organs including the brain, kidney, and spinal cord (6, 24). Using mice with global deletion of *Perk*, except in pancreatic beta cells, Owen et al. (25) showed that these PERK knockout mice died shortly after CA/CPR, suggesting that deletion of *Perk* is detrimental in CA. However, it has yet to be established whether such a devastating effect on CA outcome is primarily due to deficiency of *Perk* in the brain. In this regard, future studies may benefit from using neuron-specific *Perk* knockout mice, as we did in our recent study (5).

Activation of the ATF6 UPR branch after CA/CPR has not yet been verified. Kumar et al. (26) did not observe the activated form of ATF6 (i.e., cleaved ATF6) in the rat brain up to 4 h of reperfusion after CA. Using GRP78 expression as an alternative indicator of ATF6 branch activation, studies have found that GRP78 levels are slightly increased in the brain, spinal cord, and kidney after CA/CPR (6, 27), which may suggest modest activation of the ATF6 branch. Of note, however, activation of the XBP1 branch can also upregulate GRP78 (28). Thus, the role of the ATF6 branch in CA warrants further research, especially considering the potent protective effects reported under various ischemic conditions (7).

The IRE1/XBP1 UPR branch is activated in the brain as well as other organs after CA/CPR (6, 29). In the current study, we provide the first evidence that activation of the XBP1 UPR branch in the post-CA brain is neuroprotective, as knockout mice with neuron-specific deletion of *Xbp1* in the brain exhibited worse CA outcome, while transgenic mice with neuron-specific expression of *Xbp1s* in the brain showed improved CA outcome. Previous studies indicate that under ischemic conditions, protective effects of the XBP1 branch are largely mediated *via* the XBP1s/HBP/O-GlcNAc axis (8, 11). Indeed, activation of this axis by either overexpressing *Xbp1s* or by directly boosting O-GlcNAcylation exerts beneficial effects after heart or brain ischemia. For example, overexpression of *Xbp1s* exerts marked protection in the heart, reducing the infarct area by nearly 50% after I/R (11). Many studies on myocardial ischemia and ischemic stroke have shown that pharmacologically boosting O-GlcNAcylation before or during ischemia renders the heart or brain more tolerant to I/R injury (8, 22, 30, 31). Inhibition of O-GlcNAc modification, however, has the opposite effect (32).

O-GlcNAcylation is a post-translational modification that adds GlcNAc to target proteins and thus, modulates their function or subcellular location. The sugar donor in this modification is UDP-GlcNAc, which can be generated *via* the HBP, a glucose metabolism pathway. Notably, the concentration of UDP-GlcNAc positively regulates O-GlcNAc transferase activity, and eventually, the level of O-GlcNAc-modified proteins (33). Thus, one way to elevate O-GlcNAcylation is to increase UDP-GlcNAc. Here, we used glucosamine to boost the HBP flux and consequently, increase O-GlcNAcylation. Interestingly, glucosamine has shown protective effects in many ischemic diseases. For example, glucosamine can increase O-GlcNAcylation in the heart, and improve the tolerance of the heart to I/R by either pretreatment before ischemia or post-treatment during reperfusion (30, 34, 35). Using an I/R retinal injury model, Chen et al. (36) showed that glucosamine mitigates I/R-induced retinal thinning, and preserves retinal functions. Moreover, glucosamine treatment improves outcome after ischemic stroke (17, 22), including long-term recovery of motor function (22). Consistent with these previous studies, we showed here that glucosamine treatment after CA/CPR significantly improved CA functional outcome in young mice. More critically, we found that glucosamine can increase O-GlcNAcylation in aged brains, and that post-CA treatment with glucosamine improved long-term CA functional recovery in aged mice. Although it is conceivable that this improvement is due to collectively beneficial effects of glucosamine on various organs after CA/CPR, our data on neuron-specific *Xbp1* knockout or *Xbp1s* transgenic mice suggest that the effect of glucosamine on the brain plays a key role. Taken together, our data indicate that the XBP1/HBP/O-GlcNAc axis is a promising target for CA therapy.

Potential mechanisms underlying the beneficial effects provided by O-GlcNAcylation are multifactorial. These include restoration of protein homeostasis (proteostasis), and attenuation of oxidative stress, calcium-mediated pathologic processes, and mitochondrial calcium overload, and its anti-inflammatory effects (35, 37). For example, increasing O-GlcNAcylation pharmacologically or genetically preserves mitochondrial membrane potential stress and prevents cytochrome C release after hypoxia or H_2O_2-induced oxidative (32, 38, 39). Further, activation of the HBP and increasing O-GlcNAcylation protects against Ca^{2+} overload and inhibits ischemia-induced activation of Ca^{2+}-mediated proteases (30, 34). Using an ischemic stroke model, it has been shown that thiamet-G treatment to boost O-GlcNAcylation significantly inhibits induction of several pro-inflammatory cytokines in the post-ischemic brain (40). Since these

pathologic processes have been implicated in I/R injury, it is likely that all these mechanisms synergistically contribute to the improved outcome offered by increasing O-GlcNAcylation after CA/CPR.

Evaluating a therapeutic CA treatment in aged animals is important because many CA patients are elderly. However, current experimental CA studies have been almost exclusively performed on young animals. The reasons for this situation are multifold, but include the high cost of aged animals as well as the technical difficulties in subjecting aged animals to CA/CPR surgery while ensuring that these CA mice not only have detectable neurologic deficits, but also have an acceptable mortality rate. These obstacles must be overcome, however, because one lesson we have learned from the disappointing progress in translating experimental stroke therapeutics from bench to bedside is that the use of aged animals in preclinical stroke studies is critical (2, 4, 41). This is likely also the case for experimental CA research.

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