

Aging Is Associated With Impaired Activation of Protein Homeostasis-Related Pathways After Cardiac Arrest in Mice

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Background—The mechanisms underlying worse outcome at advanced age after cardiac arrest (CA) and resuscitation are not well understood. Because protein homeostasis (proteostasis) is essential for cellular and organismal health, but is impaired after CA, we investigated the effects of age on proteostasis-related prosurvival pathways activated after CA.

Methods and Results—Young (2–3 months old) and aged (21–22 months old) male C57Bl/6 mice were subjected to CA and cardiopulmonary resuscitation (CPR). Functional outcome and organ damage were evaluated by assessing neurologic deficits, histological features, and creatinine level. CA/CPR-related changes in small ubiquitin-like modifier conjugation, ubiquitination, and the unfolded protein response were analyzed by measuring mRNA and protein levels in the brain, kidney, and spinal cord. Thiamet-G was used to increase O-linked β -N-acetylglucosamine modification. After CA/CPR, aged mice had trended lower survival rates, more severe tissue damage in the brain and kidney, and poorer recovery of neurologic function compared with young mice. Furthermore, small ubiquitin-like modifier conjugation, ubiquitination, unfolded protein response, and O-linked β -N-acetylglucosamine modification were activated after CA/CPR in young mice, but their activation was impaired in aged mice. Finally, pharmacologically increasing O-linked β -N-acetylglucosamine modification after CA improved outcome.

Conclusions—Results suggest that impaired activation of prosurvival pathways contributes to worse outcome after CA/CPR in aged mice because restoration of proteostasis is critical to the survival of cells stressed by ischemia. Therefore, a pharmacologic intervention that targets aging-related impairment of proteostasis-related pathways after CA/CPR may represent a promising therapeutic strategy. (*J Am Heart Assoc.* 2018;7:e009634. DOI: 10.1161/JAHA.118.009634.)

Key Words: brain • cardiac arrest • ER stress • ischemia/reperfusion injury/neuroprotection • kidney • O-GlcNAc • proteostasis

Aging plays a major role in outcomes after cardiac arrest (CA), as it does after myocardial ischemia and stroke.^{1,2} The mechanisms that drive CA-, myocardial ischemia-, and stroke-induced cell death have been extensively investigated in animals, but pharmacologic interventions that improve outcome in animals have largely failed in clinical trials.^{3–5} This translational roadblock has been widely discussed; however, the potential role of aging has not received much attention.² Indeed, most experimental studies have been conducted in

young animals,⁶ whereas it is most often elderly patients who experience these potentially devastating events. Furthermore, we know that aging has a profound effect on how cells respond to the severe form of stress associated with ischemia and reperfusion (I/R).⁷

When challenged by I/R stress, cells activate many pathways that are believed to result in cellular dysfunction and death. To date, these pathologic pathways have been the predominant targets of pharmacologic interventions.

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Clinical Perspective

What Is New?

- Various proteostasis-related pathways, such as small ubiquitin-like modifier conjugation, ubiquitination, the unfolded protein response, and O-linked β -N-acetylglucosamine modification, are rapidly activated in the brain, spinal cord, and kidney after cardiac arrest (CA) and cardiopulmonary resuscitation.
- CA/cardiopulmonary resuscitation–induced activation of these proteostasis-related pathways is largely impaired in aged mice.
- Posttreatment with thiamet-G to pharmacologically increase O-linked β -N-acetylglucosamine modification improves CA outcome in young mice.

What Are the Clinical Implications?

- CA/cardiopulmonary resuscitation causes whole-body ischemia/reperfusion insult, which perturbs cellular proteostasis in various organs, and cells subsequently activate prosurvival pathways to restore proteostasis and promote cell survival.
- This study provided evidence that the capacity of cells to activate these proteostasis-related pathways and, thus, restore cellular functions after CA/cardiopulmonary resuscitation is impaired at advanced age.
- Our data suggest that pharmacologic interventions that rescue aging-related impairment of proteostasis-related pathways may hold great promise for improving outcomes in elderly patients with CA.

However, cells also activate many prosurvival pathways to restore cellular functions impaired by I/R stress. When prosurvival pathways dominate, outcomes improve. Experimental ischemic stroke studies have shown that the capacity of cells to activate prosurvival pathways markedly declines with increasing age.⁸ Notably, I/R stress-induced activation of prosurvival pathways that maintain and restore cellular protein homeostasis (proteostasis) is particularly impaired at advanced age.^{8,9} On resuscitation after CA, all organs are challenged by I/R stress, and, thus, we hypothesized that aging is associated with impairment of proteostasis-related pathways activated after CA and resuscitation. Herein, we focused on small ubiquitin-like modifier (SUMO) conjugation (SUMOylation), ubiquitination, and the unfolded protein response (UPR) and its associated O-linked β -N-acetylglucosamine modification (O-GlcNAcylation).

The ubiquitin conjugation pathway plays a key role in degrading unneeded cellular proteins, and SUMOylation is a major component of the system that controls the quality of newly synthesized proteins.^{10–12} UPR is a highly conserved pathway activated in stress conditions that impair

proteostasis and result in accumulation of unfolded/misfolded proteins in the endoplasmic reticulum (ER).⁹ The primary purpose of UPR is to restore proteostasis impaired by stress, and its activation is controlled by 3 stress sensor proteins: ATF6 (activating transcription factor 6), IRE1 (inositol-requiring enzyme 1), and PERK (protein kinase RNA-line ER kinase). Two different approaches are frequently used to study whether UPR is activated in a pathological state: evaluating activation of the ER stress sensor proteins ATF6, IRE1, and PERK; or analyzing downstream processes induced by activated stress sensor proteins. Herein, we decided to use the second approach, because we wanted to clarify the effect of age on activation of UPR-dependent proteostasis-related pathways after CA. In this study, we performed the first systematic analysis of these proteostasis-related pathways in young and aged mice after CA and resuscitation, and also provided evidence that pharmacologically increasing O-GlcNAcylation, a proteostasis-related pathway, improved CA outcome.

Methods

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure. The request should be addressed to the corresponding author. The protocol for all experiments conducted in this study was approved by the Institutional Animal Care and Use Committee at Duke University (Durham, NC).

Animals

Young (2–3 months old) and aged (21–22 months old) male C57Bl/6 mice were obtained from Jackson Laboratory and the National Institute on Aging. Mice (3–5/cage) were kept in a temperature- and humidity-controlled room and a light/dark cycle of 14:10 hours. In this study, a total of 59 mice (42 young and 17 aged mice) were used. In the thiamet-G experiment, young mice (n=7/group) were randomly assigned to saline or thiamet-G groups by software.

Cardiac Arrest and Cardiopulmonary Resuscitation

CA/cardiopulmonary resuscitation (CPR) surgery was performed as described previously, with minor modifications.¹³ Briefly, anesthesia was induced with 5% isoflurane. After endotracheal intubation, mice were maintained on 1.5% to 1.7% isoflurane before CA induction. Body temperature was measured with a rectal temperature probe and was maintained at $36.5 \pm 0.5^\circ\text{C}$ during the whole procedure. An ECG

was continuously recorded. After withdrawal of 0.3 mL blood from the jugular vein, 30 μ L of 0.5 mol/L KCl was infused to induce asystole, which was verified by ECG and an absence of spontaneous respiration. On CA onset, lung ventilation ceased, and the blood was reinfused 3 minutes later. At 8 minutes after CA, delivered oxygen was adjusted to 100%. At 8.5 minutes after CA, mechanical ventilation was resumed, and a bolus of epinephrine (100 μ L of 32 μ g/mL) was given, followed by continuous infusion (20 μ L/min). Chest compression was performed using a single finger at \approx 300 strokes/min until return of spontaneous circulation, defined as appearance of stable ECG sinus rhythms. If return of spontaneous circulation could not be achieved within 3 minutes, resuscitation was abandoned, and the animal was excluded from the study. When spontaneous respiration was adequate, mice were returned to their home cages. For the thiamet-G experiment, to facilitate detection of its effect on neurologic functions, we slightly modified the CA/CPR procedure by maintaining the pericranial temperature at $38.5\pm 0.2^\circ\text{C}$ while allowing body temperature to decrease during CA. As a first exploratory experiment, only male mice were used. Thiamet-G (30 mg/kg) was administered by intravenous injection at 30 minutes after return of spontaneous circulation.

Behavioral Tests

All evaluations were performed by observers who were blinded to group assignment, except for the experiments using aged mice. For these experiments, because of obvious differences in body weight and appearance between young and aged mice, the behavioral tests could not be performed in a blinded manner. All tests were conducted in the light phase.

Neurologic score

A 9-point scoring system was used to evaluate neurologic deficits.¹³ A total neurologic score was computed, on the basis of performance (9 points=normal; and 0 points=severe injury).

Rotarod test

Mice were placed on an accelerating rotating rod. Latency to fall from the rod was recorded, and recovery of performance was calculated as a percentage change in post-CA from pre-CA latency.⁷

Spontaneous locomotor activity

Mice were placed in a SmartCage (AfaSci, Redwood City, CA) or an open field (50 \times 50 \times 50 cm; CleverSys), and allowed to move freely. Recording of locomotor activity was started 20 seconds later for a period of 10 minutes. Active time and distance traveled were calculated for the 10-minute period of the test.¹³

Histological Features

Mice were anesthetized and transcardially perfused with saline, followed by 4% paraformaldehyde. Frozen brain sections (20 μ m thick) were used for Fluoro-Jade C staining. Briefly, the slices were incubated in 0.06% potassium permanganate for 10 minutes, and then immersed in Fluoro-Jade C staining solution (Chemicon, Temecula, CA) for 30 minutes. Fluoro-Jade C–positive cells/mm² of brain cortex were counted (4 fields of interest per mouse). Paraffin-embedded kidney and brain sections (5 μ m thick) were used for hematoxylin-eosin staining. The investigators, who were blinded to experimental conditions, assigned a score to each section on the basis of the degree of kidney injury and counted dead neurons in the hippocampal cornu ammonis 1 (CA1) subfield of each brain section (around bregma -2.06 mm).¹⁴

Creatinine Measurement

Creatinine concentrations in serum samples were determined using a colorimetric assay kit (Cayman, Ann Arbor, MI), according to the manufacturer's protocol. Briefly, creatinine standards and serum samples were mixed with reaction buffer and color reagent on a 96-well plate. Absorbance (490 nm) was measured at 1 and 7 minutes of incubation in a microplate reader (Tecan). These 2 measurements were then used to calculate the creatinine concentration of each sample.

Quantitative Reverse Transcription–Polymerase Chain Reaction

Using quantitative reverse transcription–polymerase chain reaction (PCR), levels in mRNAs were analyzed, as described.⁷ In short, total RNA was extracted from brain cortex and kidney samples using TRIzol reagent (Invitrogen), and used to generate cDNA samples. PCRs were performed using a Lightcycler 2.0 (Roche, Mannheim, Germany). All primers used in this study are listed in Table 1.

Western Blotting

Tissue samples were processed for Western blotting using our standard method, as described.⁷ Briefly, tissue samples (brain cortex, spinal cord, and kidney) were dissected on ice and snap frozen in liquid nitrogen as quickly as possible to prevent deconjugation of SUMOylated and ubiquitinated proteins. Tissue samples were homogenized by sonication using lysis buffer supplemented with 2% SDS. Quantification of signal intensities was performed using ImageJ (NIH, Bethesda, MD). The primary antibodies are listed in Table 2.

Table 1. List of Primer Sequences

Gene		Primer Sequences (5'→3')
<i>chop</i>	Forward	GAATAACAGCCGGAACCTGA
	Reverse	CGTTTCCTGGGGATGAGATA
<i>xbp1s</i>	Forward	GGTCTGCTGAGTCCGCAGCAGG
	Reverse	GAAAGGGAGGCTGGTAAGGAAC
<i>grp78</i>	Forward	CGTATGTGGCCTTCACTCCT
	Reverse	TTTCTTCTGGGGCAAATGTC
<i>gfat1</i>	Forward	TAAGGAGATCCAGCGGTGTC
	Reverse	CAGCTGTCTCGCCTGATTGA
<i>ogt</i>	Forward	ACTGTGTTGCGAGTGACCTG
	Reverse	ATTTGGGTCAAGGGTGACAG
<i>oga</i>	Forward	GCCTGGATTACTGCTTCGTC
	Reverse	ACACCTCCTGCTTTCATGG
<i>β-Actin</i>	Forward	TAGGCACCAGGGTGTGATG
	Reverse	GGGGTGTGAAGGTCTCAAA

chop indicates C/EBP homologous protein; *gfat1*, glutamine fructose-6-phosphate amidotransferase 1; *grp78*, glucose-regulated protein 78; *oga*, O-GlcNAcase; *ogt*, O-GlcNAc transferase; *xbp1s*, spliced X-box binding protein 1.

Statistical Analysis

The primary functional outcome for the CA experiments was neurologic score, which was used to determine group size, on the basis of our previous studies. Data were analyzed using Prism 6 software (GraphPad, LaJolla, CA). Statistical analysis was assessed by Mann-Whitney *U* test for neurologic scores and kidney injury scores, 1-way ANOVA for creatinine levels, Mantel-Cox log-rank test for survival rates, and unpaired Student *t* test for all other data. Data are presented as

mean±SEM, percentage, or median. The level of significance was set at $P<0.05$.

Results

CA Outcome in Young and Aged Mice

We compared CA outcome in young (24.10 ± 0.43 g) versus aged (31.69 ± 1.03 g) mice using our recently established CA/CPR model.¹³ After 8.5 minutes CA, 3 mice (1 in 8 young and 2 in 9 aged mice) were not successfully resuscitated and, therefore, excluded. During the 3-day observation, 40% of aged mice died, whereas all young mice survived (Figure 1). Thus, functional recovery was evaluated only at 24 hours after surgery, when all experimental mice were still alive (Figure 1). In all behavioral tests (neurologic score, rotarod, and spontaneous locomotor activity), aged mice exhibited significantly worse performance compared with young mice (Figure 1A–C). On day 3 after CA, survived mice were analyzed for brain and kidney damage. Of note, the sample size was small. Nevertheless, aged brains appeared to have more degenerative neurons, indicated by Fluoro-Jade C staining (Figure 2A). To evaluate kidney injury, we performed histologic analysis and measured serum creatinine. A trend toward more severe kidney tubular injury was exhibited in aged mice versus young mice (Figure 2B and 2C). Consistent with this finding, serum creatinine levels were significantly higher in aged versus young mice after CA/CPR (Figure 2D). Together, these data indicate that both brain and kidney sustained more severe damage in aged versus young mice in our CA/CPR model, which agrees with previous reports.^{15,16}

Activation of Proteostasis-Related Pathways in Young Mice After CA/CPR

This study is the first to systematically characterize activation of proteostasis-related pathways after CA/CPR. Herein, we performed a time-course analysis of post-CA/CPR activation of UPR, SUMOylation, ubiquitination, and O-GlcNAcylation in the central nervous system (brain and spinal cord) and a periphery organ (kidney), using quantitative reverse transcription-PCR and Western blotting (Figures 3 through 5). For the UPR pathway, we analyzed mRNA levels of spliced *Xbp1* (*xbp1s*), *grp78*, and *chop*, and protein levels of phosphorylated eIF2 α (eukaryotic translation initiation factor 2 subunit α), GRP78 (glucose-regulated protein 78), and PDI (protein disulfide isomerase). For SUMOylation, we measured levels of both SUMO1- and SUMO2/3-conjugated proteins. For O-GlcNAcylation, we measured levels of global O-GlcNAcylation and analyzed expression of GFAT1 (glutamine fructose-6-phosphate amidotransferase 1), OGT (O-GlcNAc transferase),

Table 2. List of Primary Antibodies

Antibody (Clone/Reference No.)	Species	Manufacturer
p-eIF2 α (9721)	Rabbit	Cell Signaling Technology
GRP78 (610978)	Mouse	BD Biosciences
PDI (SC-20132)	Rabbit	Santa Cruz Biotechnology
GAPDH (D16H11)	Rabbit	Cell Signaling Technology
O-GlcNAc (CTD110.6)	Mouse	Covance
OGT (O6264)	Rabbit	Sigma
OGA (SAB4200267)	Rabbit	Sigma
SUMO1 (21C7)	Mouse	Hybridoma Bank
SUMO2/3 (custom service)	Rabbit	Covance
Ubiquitin (3936)	Mouse	Cell Signaling Technology

GRP78 indicates glucose-regulated protein 78; OGA, O-GlcNAcase; O-GlcNAc, O-linked β -N-acetylglucosamine; OGT, O-GlcNAc transferase; PDI, protein disulfide isomerase; p-eIF2 α , phosphorylated eukaryotic translation initiation factor 2 subunit α ; SUMO, small ubiquitin-like modifier.

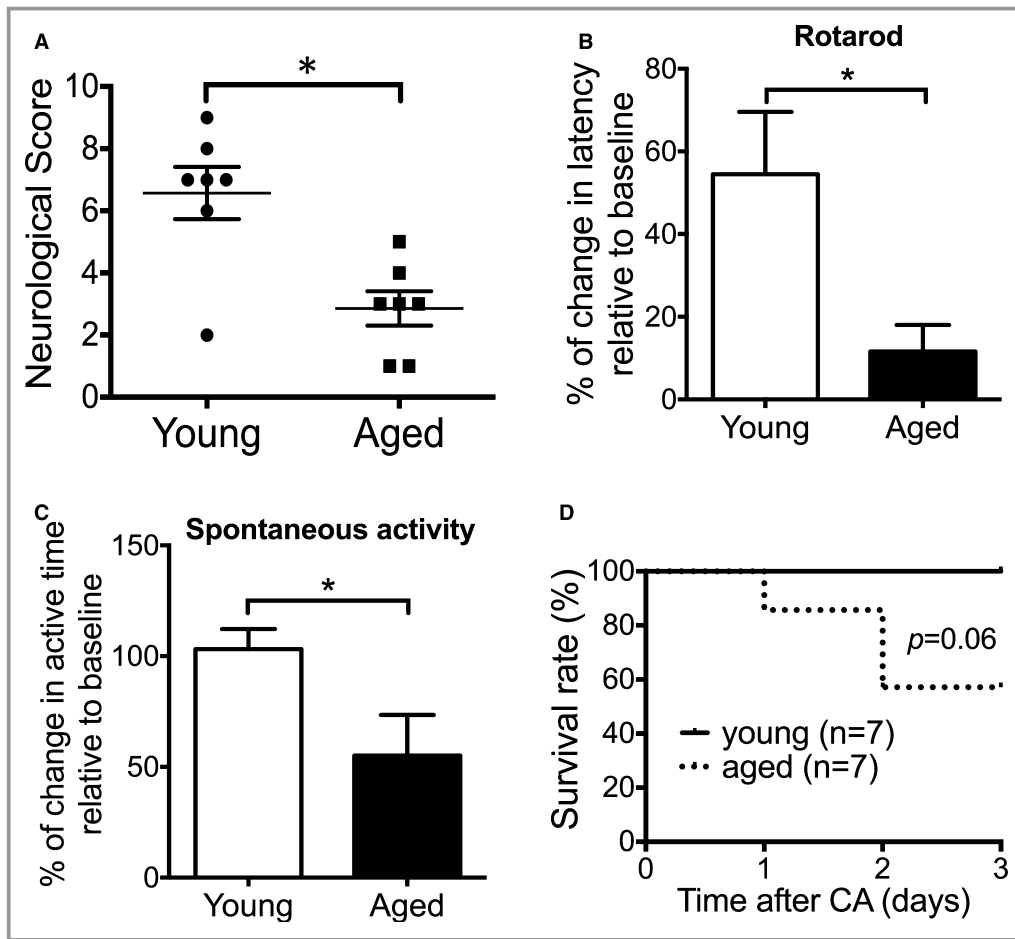


Figure 1. Functional recovery and survival rates after cardiac arrest and cardiopulmonary resuscitation (CA/CPR) in young and aged mice. A through C, Aged mice exhibited worse functional recovery after CA/CPR compared with young mice. Assessments of neurologic score (A), rotarod (B), and spontaneous locomotor activity (10-minute period; C) were performed on day 1 after CA/CPR. D, Survival rates during the first 3 days after CA/CPR. n=7 per group. * $P < 0.05$.

and OGA (O-GlcNAcylase). GFAT1 is an enzyme that controls the rate of UDP-GlcNAc generation (the substrate for O-GlcNAcylation). OGT adds O-GlcNAc to target proteins, and OGA removes O-GlcNAc from target proteins.

The levels of *xbp1s* mRNA and eIF2 α were significantly increased in the brain at 1 hour reperfusion after CA, indicating activation of the IRE1 and PERK UPR branches, respectively, whereas levels of *gpr78* mRNA and GPR78 protein (the ATF6 UPR branch) were only modestly increased at later reperfusion times (Figure 3A and 3C). Global SUMOylation, particularly SUMO2/3 conjugation, and ubiquitination were significantly increased in the brain after CA/CPR (Figure 3B), similar to our previous findings using a forebrain ischemia model.⁷ In contrast to SUMOylation, ubiquitination did not decrease, even at 24 hours' reperfusion (Figure 3B). As expected, O-GlcNAcylation was activated in the brain after CA/CPR, and peaked at ≈ 3 hours' reperfusion (Figure 3D).

With respect to OGA, OGT, and GFAT1 expression, we found only a modest decrease in OGA protein levels at 1 hour reperfusion (Figure 3D). Finally, our data showed that the spinal cord responded to CA/CPR in a manner that largely resembled the response in the brain, as indicated by activation of UPR, SUMOylation, ubiquitination, and O-GlcNAcylation (Figure 4).

In the kidney, the patterns of CA/CPR-induced activation of the pathways under investigation were similar to those in the brain; however, there were some noteworthy differences (Figure 5). In the kidney, expression of *chop*, a UPR-related proapoptosis gene, was markedly increased early after reperfusion (Figure 5A); ubiquitination was only modestly activated (Figure 5B). Activation of phosphorylation of eIF2 α was less pronounced, whereas O-GlcNAcylation of proteins was more induced in kidney versus brain at 1 or 3 hours' reperfusion (Figure 5C and 5D).

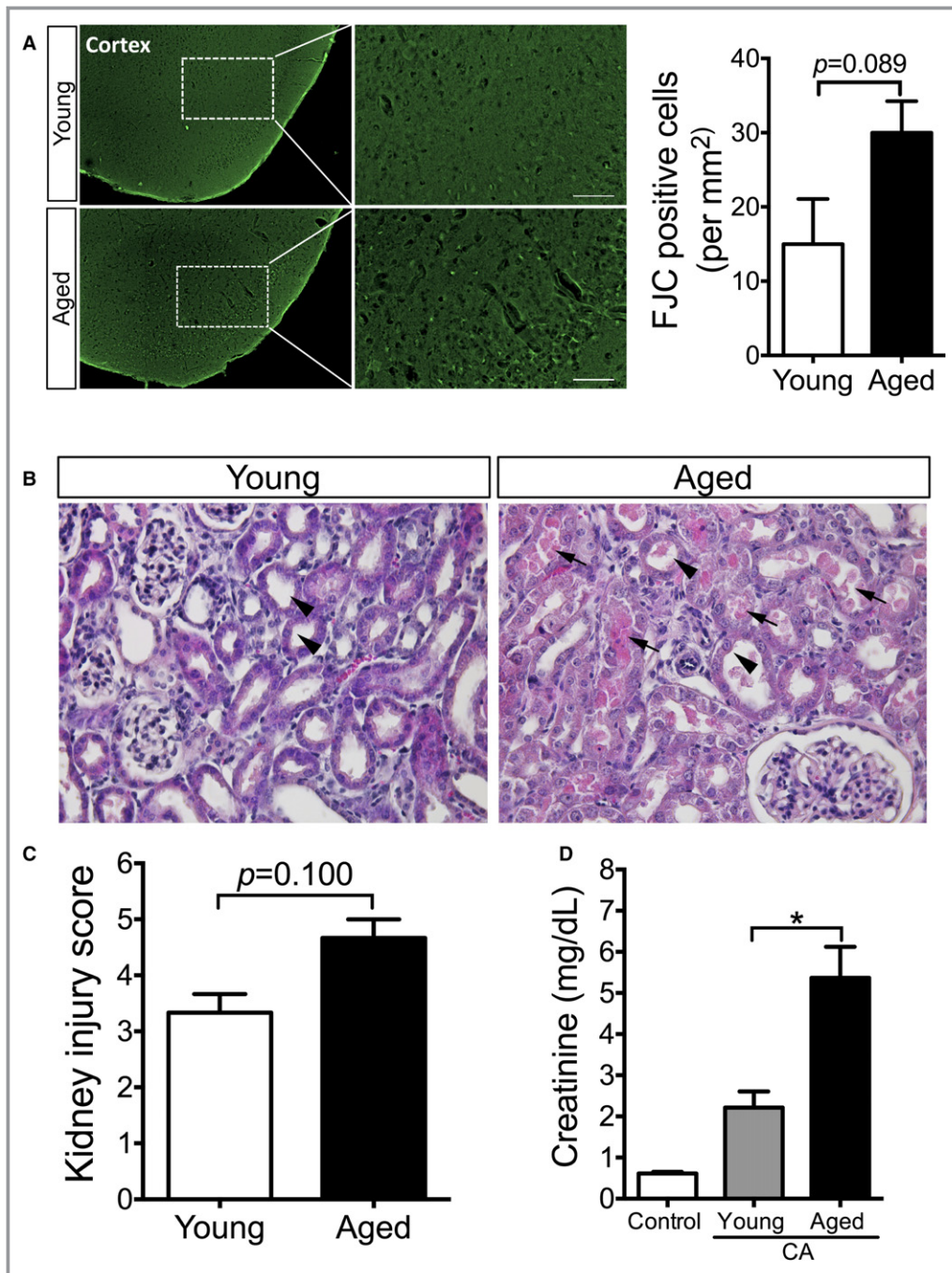


Figure 2. Effect of age on tissue injury in the brain and kidney after cardiac arrest and cardiopulmonary resuscitation (CA/CPR). Mice were subjected to CA/CPR, and after 3 days, blood and tissue samples were collected and analyzed (n=3–4). A, Fluoro-Jade C (FJC)-positive cells in the brain cortex of young and aged mice after CA/CPR. Quantitative data were shown as in the bar graph. B, Representative images of hematoxylin and eosin (H&E) staining of kidney sections from young and aged mice after CA/CPR. Arrows and arrowheads depict cast formation and loss of brush borders, respectively. C, Kidney injury scores were determined using H&E-stained kidney sections. D, Serum creatinine levels in young and aged mice after CA/CPR. * $P < 0.05$.

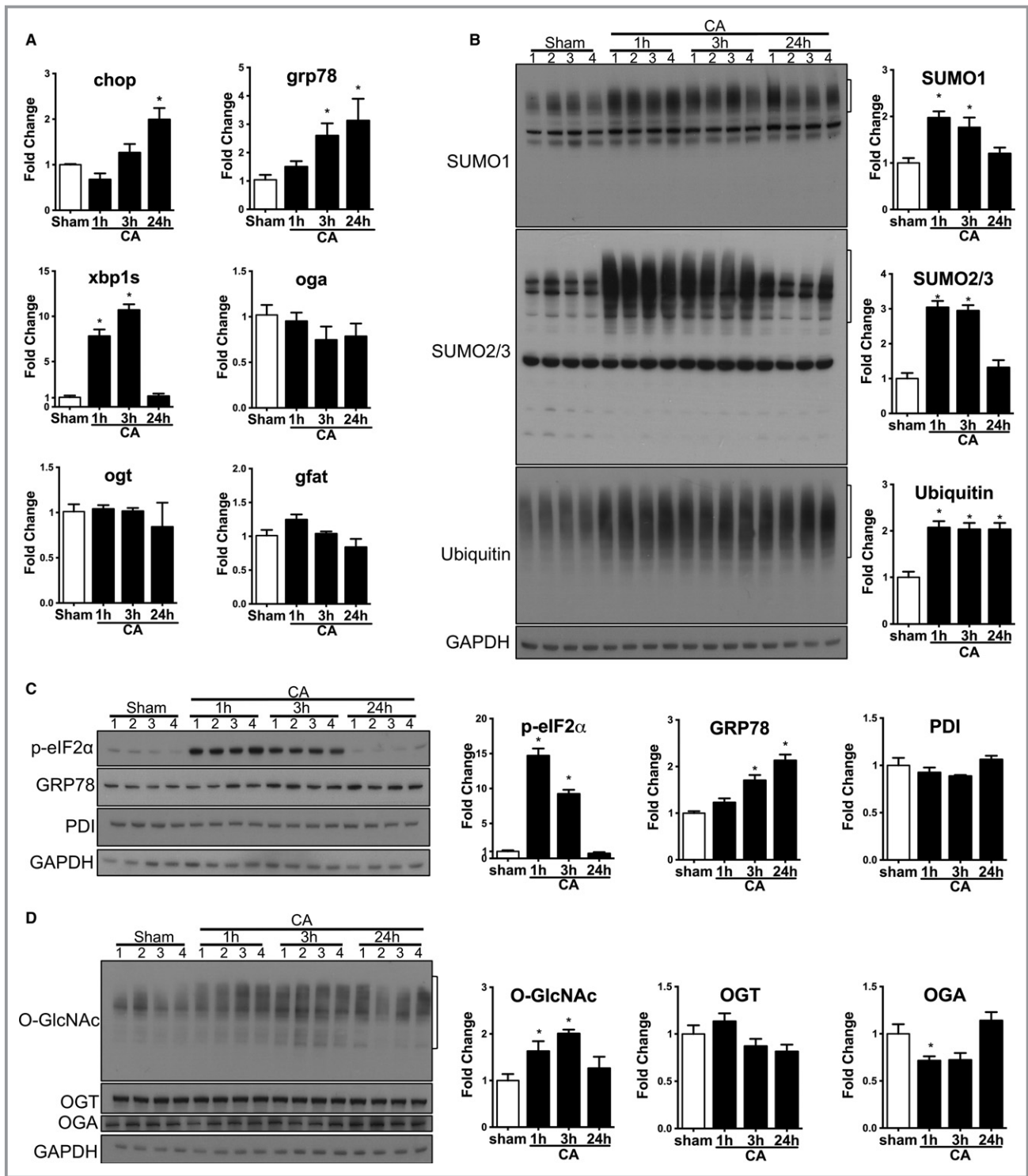


Figure 3. Activation of proteostasis-related pathways in the brain after cardiac arrest and cardiopulmonary resuscitation (CA/CPR). Young mice were subjected to CA/CPR, and cortex samples were collected at 1, 3, or 24 hours of reperfusion. Sham-operated mice served as controls. A, Quantitative reverse transcription–polymerase chain reaction (PCR) analysis. B, Small ubiquitin-like modifier (SUMO) conjugation and ubiquitination pathways. C, Unfolded protein response pathway. D, O-linked β-N-acetylglucosamine (O-GlcNAc) modification. The high-molecular-weight regions, marked by brackets, were used for quantification. All individual data were normalized for GAPDH. For calculation of fold change, the mean values of sham groups were set to 1.0. Data are presented as mean±SEM (n=4 per group). chop indicates C/EBP homologous protein; gfat, glutamine fructose-6-phosphate amidotransferase; GRP78, glucose-regulated protein 78; OGA, O-GlcNAcase; OGT, O-GlcNAc transferase; PDI, protein disulfide isomerase; p-eIF2α, phosphorylated eukaryotic translation initiation factor 2 subunit α; xbp1s, spliced X-box binding protein 1. *P<0.05, vs respective sham group.

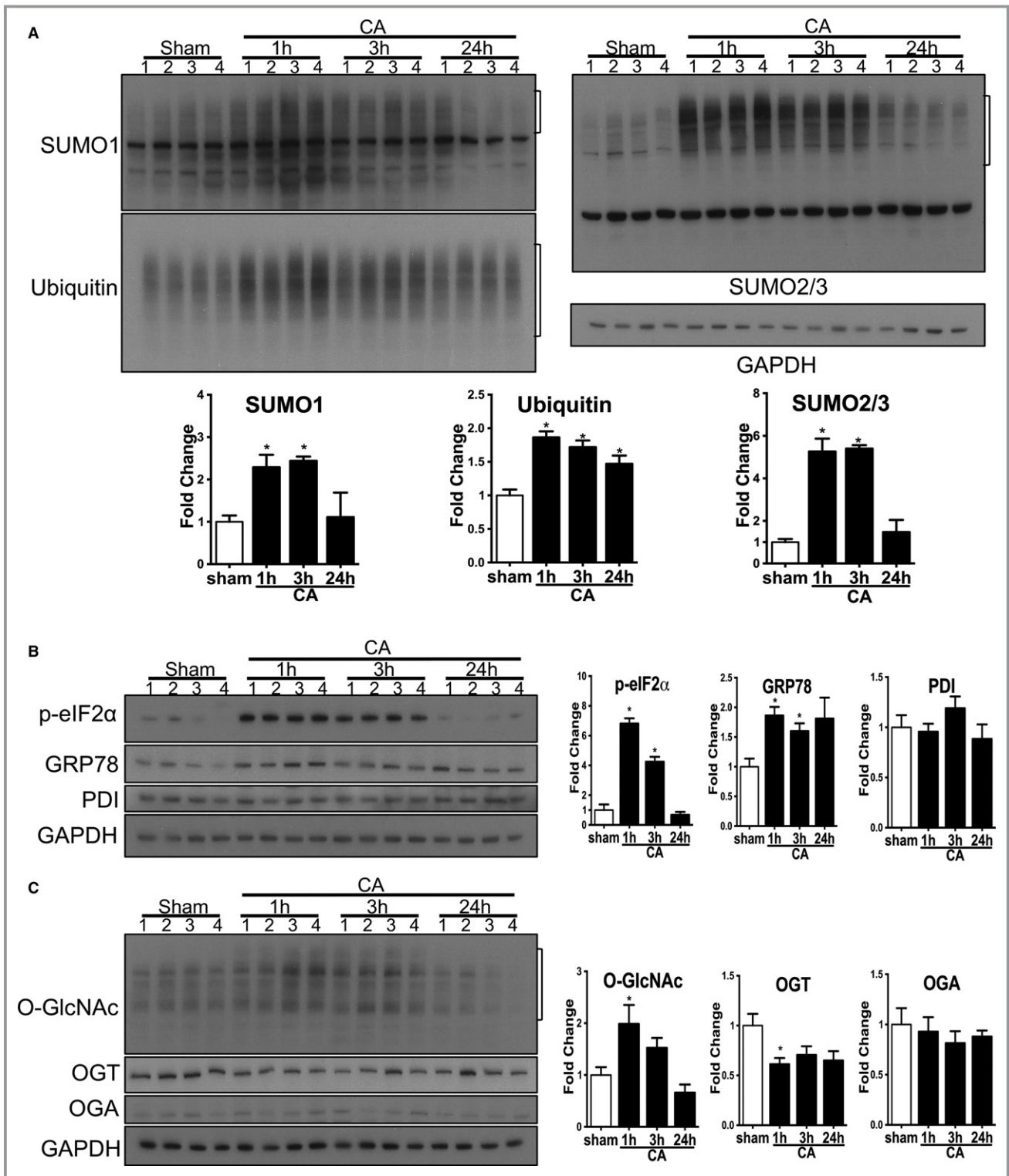


Figure 4. Activation of proteostasis-related pathways in the spinal cord after cardiac arrest and cardiopulmonary resuscitation (CA/CPR). Young mice were subjected to CA/CPR, and spinal cord samples were collected at 1, 3, or 24 hours of reperfusion. Sham-operated mice served as controls. A, Small ubiquitin-like modifier (SUMO) conjugation and ubiquitination pathways. B, Unfolded protein response pathway. C, O-linked β -N-acetylglucosamine (O-GlcNAc) modification pathway. The high-molecular-weight regions, marked by brackets, were used for quantification. All individual data were normalized to GAPDH. For calculation of fold change, the mean values of sham groups were set to 1.0. Data are presented as mean \pm SEM (n=4 per group). GRP78 indicates glucose-regulated protein 78; OGA, O-GlcNAcase; OGT, O-GlcNAc transferase; PDI, protein disulfide isomerase; p-eIF2 α , phosphorylated eukaryotic translation initiation factor 2 subunit α . *P<0.05, vs respective sham group.

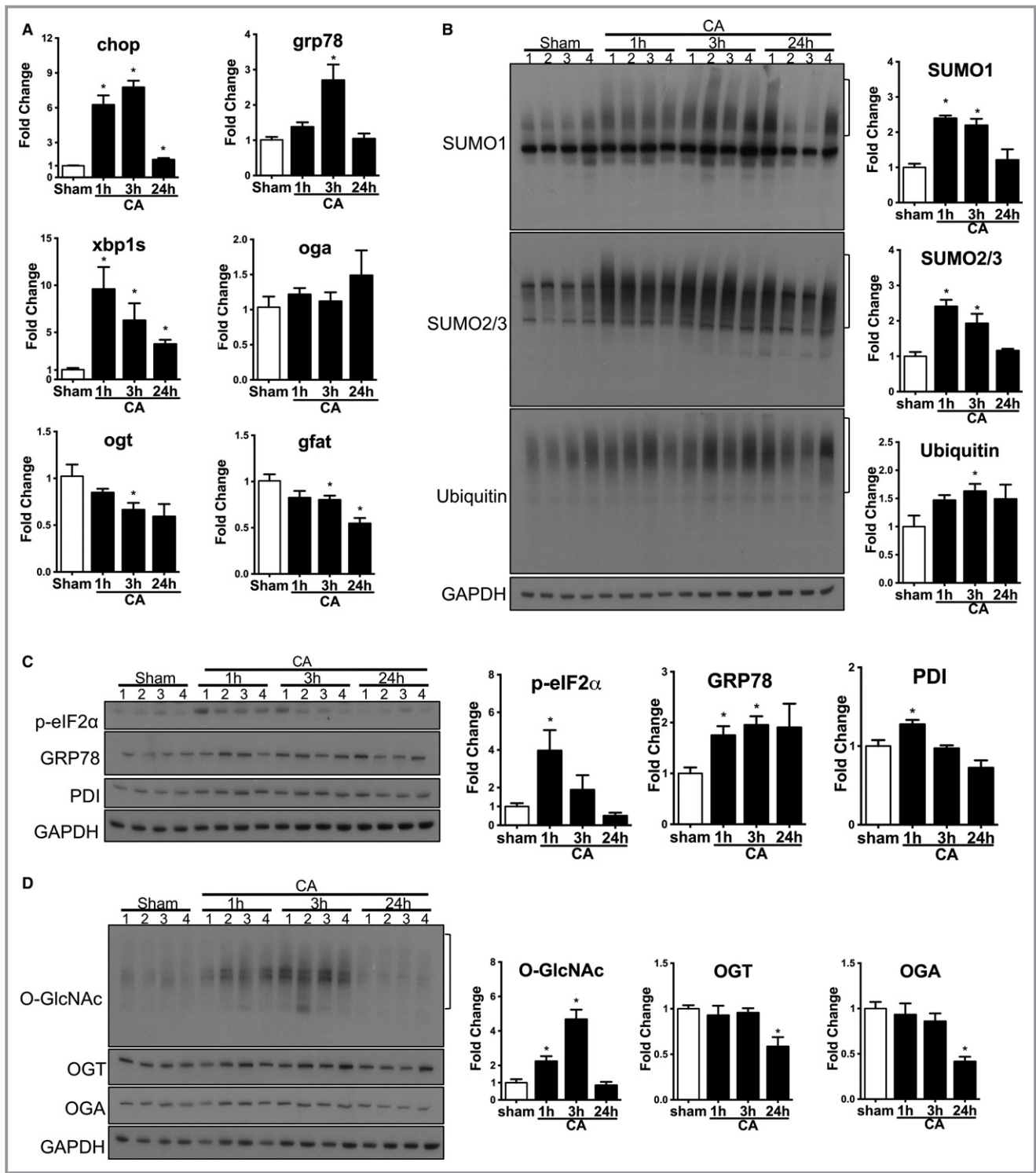


Figure 5. Activation of proteostasis-related stress response pathways in the kidney after cardiac arrest and cardiopulmonary resuscitation (CA/CPR). Young mice were subjected to CA/CPR, and kidney samples were collected at 1, 3, or 24 hours of reperfusion. Sham-operated mice served as controls. A, Quantitative reverse transcription–polymerase chain reaction (PCR) analysis. B, Small ubiquitin-like modifier (SUMO) conjugation and ubiquitination pathways. C, Unfolded protein response pathway. D, O-linked β -N-acetylglucosamine (O-GlcNAc) modification. The high-molecular-weight regions, marked by brackets, were used for quantification. All individual data were normalized to GAPDH. For calculation of fold change, the mean values of sham groups were set to 1.0. Data are presented as mean \pm SEM (n=4 per group). chop indicates C/EBP homologous protein; gfat, glutamine fructose-6-phosphate amidotransferase; GRP78, glucose-regulated protein 78; OGA, O-GlcNAcase; OGT, O-GlcNAc transferase; PDI, protein disulfide isomerase; p-eIF2 α , phosphorylated eukaryotic translation initiation factor 2 subunit α ; xbps1, spliced X-box binding protein 1. *P<0.05, vs respective sham group.

Differential Activation of Proteostasis-Related Pathways in Young and Aged Mice After CA/CPR

We previously reported that activation of O-GlcNAcylation is impaired in the aged brain in both ischemic stroke and forebrain ischemia models, a finding that is thought to have important implications in understanding worse outcome in aged brain after ischemia.^{7,8} Therefore, we decided to systematically compare activation of proteostasis-related pathways in young versus aged mice. Because O-GlcNAcylation was maximally activated at 3 hours after CA/CPR in young mice (Figures 3 and 5), we used this time point for the following analyses. We first compared post-CA/CPR mRNA levels of genes associated with UPR (*chop*, *xbp1s*, and *grp78*) and O-GlcNAcylation (*ogt*, *oga*, and *gfat1*) in the brain and kidney of young versus aged mice. Notably, we did not find any significant differences in CA/CPR-induced changes in mRNA levels of these genes in young versus aged animals (Table 3).

However, in the brain, CA/CPR-induced increases in SUMOylation were significantly higher in young versus aged animals (Figure 6A). Notably, GRP78, a major component of the activating transcription factor 6 UPR branch, was not increased in aged brains, but was increased ≈ 2 -fold in young brains (Figure 6B). In a previous study, we found that activation of O-GlcNAcylation is severely impaired in aged compared with young brains at 1 hour reperfusion after forebrain ischemia.⁷ Herein, this impairment was still evident at 3 hours' reperfusion after CA/CPR (Figure 6C). In the spinal cord, the overall activation of these pathways was greater in young versus aged mice after CA/CPR, except ubiquitination and phosphorylation of eIF2 α (Figure 7).

In the kidney, differences in baseline and CA/CPR-induced activation of these pathways in young versus aged mice were similar to those found in the spinal cord (Figure 8). Interestingly, however, aged mice seemed to exhibit higher baseline levels of SUMOylation and O-GlcNAcylation in the kidney, suggesting that at advanced age, this organ is already in a

state of stress. Because the present study focused on effects of aging on CA/CPR-induced activation of prosurvival pathways associated with proteostasis, we did not pursue this potentially important finding.

Increased O-GlcNAcylation Improves CA Outcome in Young Mice

Our data indicated that CA/CPR-induced activation of proteostasis-related pathways was impaired in aged mice, and this impairment may contribute to worse CA outcome. To investigate the link between activation of proteostasis-related pathways and CA outcome, we decided to boost these pathways pharmacologically. As a first step, we chose the O-GlcNAcylation pathway, because its activation was impaired in both brain and kidney after CA/CPR in aged mice (Figures 6 and 8), and thiamet-G, a potent pharmacologic tool to increase O-GlcNAcylation, is available. Thiamet-G is a highly specific OGA inhibitor with excellent in vivo efficacy. To evaluate the effects of increased O-GlcNAcylation on CA outcome, mice were subjected to 8.5 minutes CA. At 30 minutes after resuscitation, mice were treated with saline (control) or thiamet-G. Four mice that experienced unsuccessful return of spontaneous circulation and one outlier, as determined by statistical analysis from the control group, were excluded. As shown in Figure 9, compared with controls, thiamet-G-treated mice showed a trended improvement in the rotarod test (Figure 9A), and exhibited significantly better performance on neurologic scores and the open field test on day 3 after CA (Figure 9B and 9C). Moreover, there were fewer dead CA1 neurons in the thiamet-G-treated mice (Figure 9D).

Discussion

Proteostasis is the state of equilibrium required for optimal protein function in cells, and is achieved by quality control of

Table 3. Summary of Quantitative Reverse Transcription–PCR Results

Gene	Brain			Kidney		
	Young	Aged	Young vs Aged (P Value)	Young	Aged	Young vs Aged (P Value)
<i>chop</i>	1.26 \pm 0.19	1.54 \pm 0.30	0.47	7.76 \pm 0.58	8.02 \pm 0.73	0.79
<i>grp78</i>	2.49 \pm 0.41	2.36 \pm 0.36	0.82	2.67 \pm 0.44	2.62 \pm 0.25	0.92
<i>xbp1s</i>	10.15 \pm 0.59	10.45 \pm 0.81	0.81	6.04 \pm 1.74	7.57 \pm 2.52	0.63
<i>oga</i>	0.73 \pm 0.14	1.06 \pm 0.20	0.23	1.12 \pm 0.13	0.77 \pm 0.17	0.15
<i>ogt</i>	1.01 \pm 0.03	1.04 \pm 0.11	0.77	0.66 \pm 0.07	0.64 \pm 0.09	0.85
<i>gfat1</i>	1.03 \pm 0.03	1.14 \pm 0.17	0.54	0.80 \pm 0.05	0.84 \pm 0.12	0.79

Data are presented as mean \pm SEM (n=4–5 per group). Data represent fold changes of cardiac arrest vs sham of the respective groups. Statistical analysis was performed using Student *t* test. *chop* indicates C/EBP homologous protein; *gfat1*, glutamine fructose-6-phosphate amidotransferase 1; *grp78*, glucose-regulated protein 78; *oga*, O-GlcNAcase; *ogt*, O-GlcNAc transferase; PCR, polymerase chain reaction; *xbp1s*, spliced X-box binding protein 1.

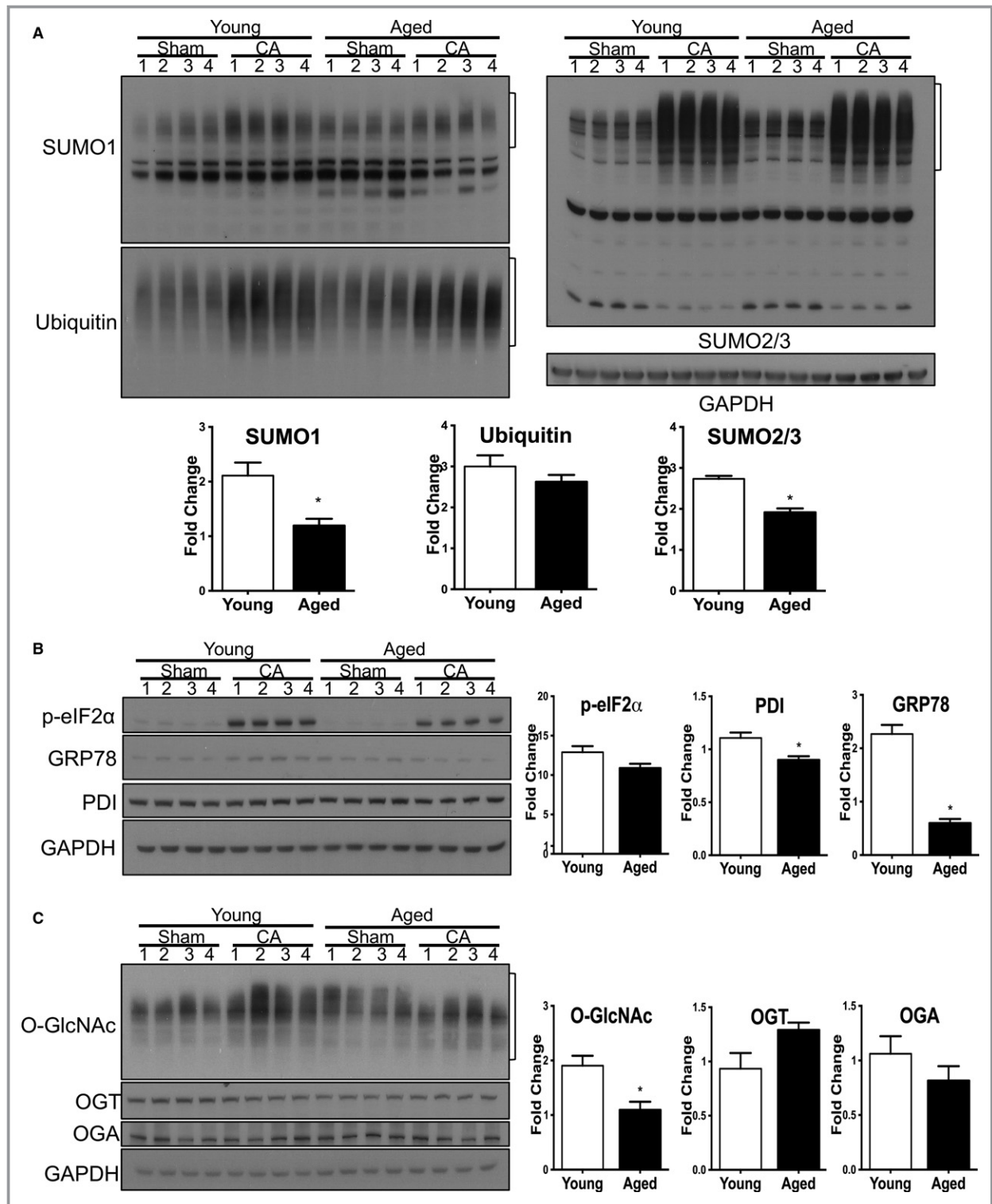


Figure 6. Effects of age on activation of proteostasis-related pathways in the brain after cardiac arrest and cardiopulmonary resuscitation (CA/CPR). Young and aged mice were subjected to CA/CPR, and after 3 hours of reperfusion, cortical samples were collected for Western blot analysis. Sham-operated mice served as controls. A, Small ubiquitin-like modifier (SUMO) conjugation and ubiquitination pathways. B, Unfolded protein response pathway. C, O-linked β -N-acetylglucosamine (O-GlcNAc) modification. The high-molecular-weight regions, marked by brackets, were used for quantification. All individual data were normalized to GAPDH. For calculation of fold change, the mean values of sham groups were set to 1.0. Data are presented as mean \pm SEM ($n=4$ per group). GRP78 indicates glucose-regulated protein 78; OGA, O-GlcNAcase; OGT, O-GlcNAc transferase; PDI, protein disulfide isomerase; p-eIF2 α , phosphorylated eukaryotic translation initiation factor 2 subunit α . * $P<0.05$.

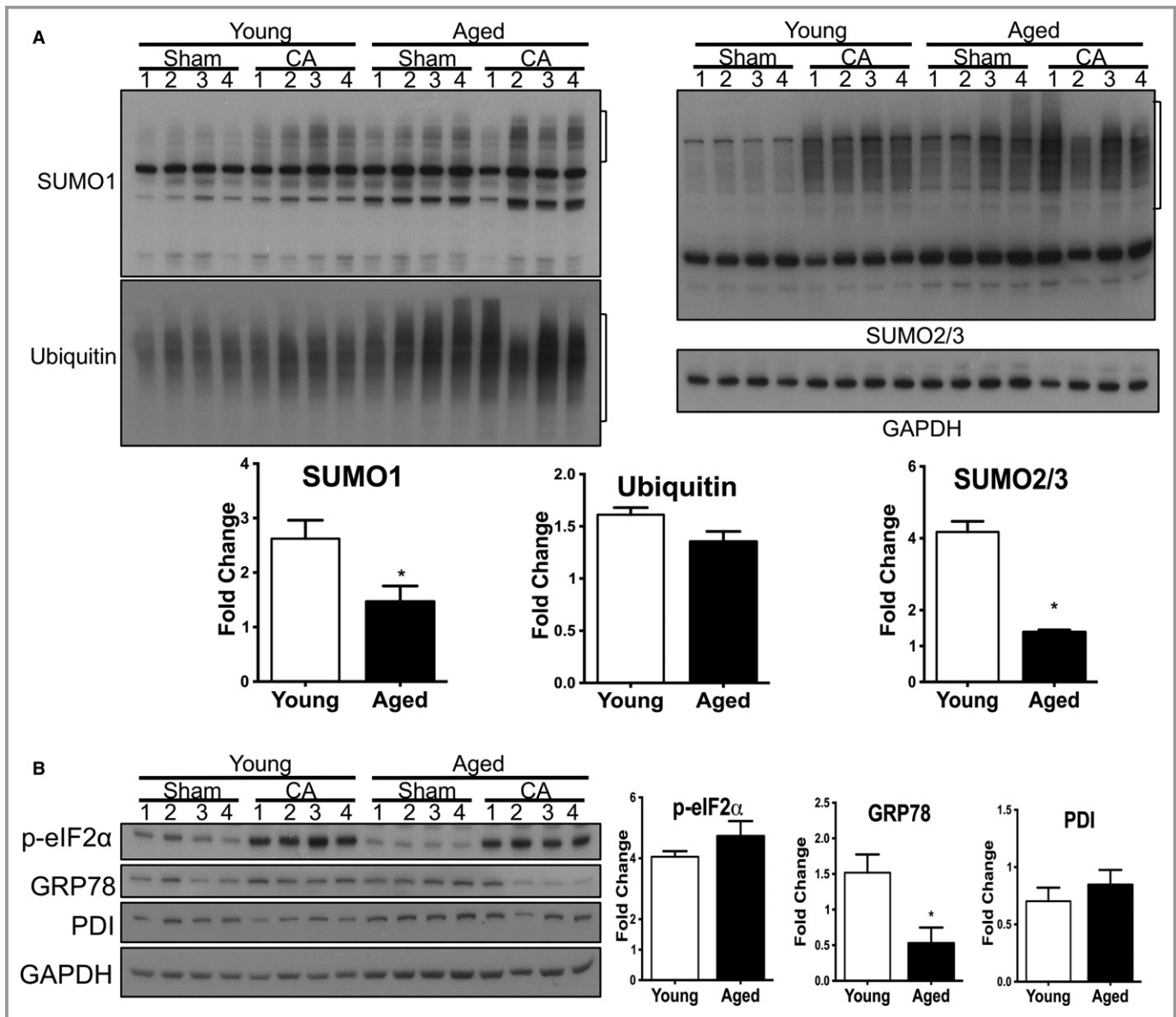


Figure 7. Effects of age on proteostasis-related pathways in the spinal cord after cardiac arrest and cardiopulmonary resuscitation (CA/CPR). Young and aged mice were subjected to CA/CPR, and after 3 hours of reperfusion, spinal cord samples were collected for Western blot analysis. Sham-operated mice served as controls. A, Small ubiquitin-like modifier (SUMO) conjugation and ubiquitination pathways. The high-molecular-weight regions, marked by brackets, were used for quantification. B, Unfolded protein response pathway. All individual data were normalized to GAPDH. For calculation of fold change, the mean values of sham groups were set to 1.0. Data are presented as mean \pm SEM (n=4 per group). GRP78 indicates glucose-regulated protein 78; PDI, protein disulfide isomerase; p-eIF2 α , phosphorylated eukaryotic translation initiation factor 2 subunit α . * P <0.05.

newly synthesized proteins, processes that help to maintain protein function under stress conditions, and degradation of misfolded/unfolded or otherwise nonfunctional proteins. Proteostasis is critical to cell survival; however, the capacity to maintain and restore proteostasis declines with increasing age.¹⁷ Thus, it is reasonable to propose that at advanced age, cells are more sensitive to a severe form of stress that impairs proteostasis. Indeed, such findings have been recently reported after myocardial ischemia and stroke.^{8,18} For CA, however, the role of aging-related decline in the capacity of

cells to restore proteostasis impaired by I/R stress has not yet been considered a potential contributor to worse outcome in elderly individuals.

Thus, in this study, we compared activation of proteostasis-related pathways, including SUMOylation, ubiquitination, and the UPR, and outcomes after CA/CPR in young versus aged mice. Previously, we and others have provided evidence that these pathways are activated in brains of young mice subjected to ischemic stress.^{19–26} Because CA results in whole-body ischemia, we expected these

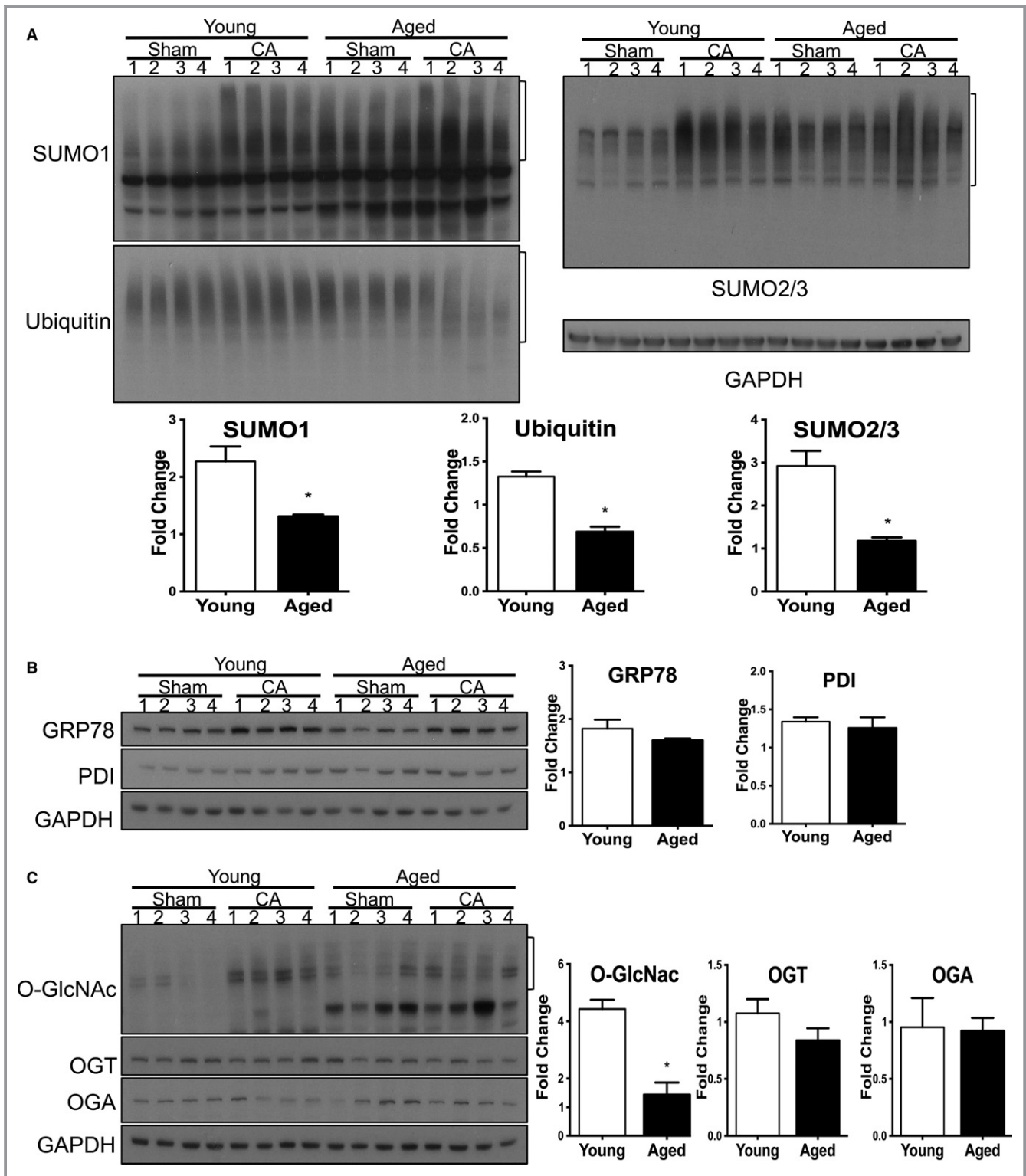


Figure 8. Effects of age on activation of proteostasis-related pathways in the kidney after cardiac arrest and cardiopulmonary resuscitation (CA/CPR). Young and aged mice were subjected to CA/CPR, and after 3 hours of reperfusion, kidney samples were collected for Western blot analysis of proteostasis-related pathways. Sham-operated mice served as controls. A, Small ubiquitin-like modifier (SUMO) conjugation and ubiquitination pathways. B, Unfolded protein response pathway. C, O-linked β -N-acetylglucosamine (O-GlcNAc) modification. The high-molecular-weight regions, marked by brackets, were used for quantification. All individual data were normalized to GAPDH. For calculation of fold change, the mean values of sham groups were set to 1.0. Data are presented as mean \pm SEM (n=4 per group). GRP78 indicates glucose-regulated protein 78; OGA, O-GlcNAcase; OGT, O-GlcNAc transferase; PDI, protein disulfide isomerase. * P <0.05.

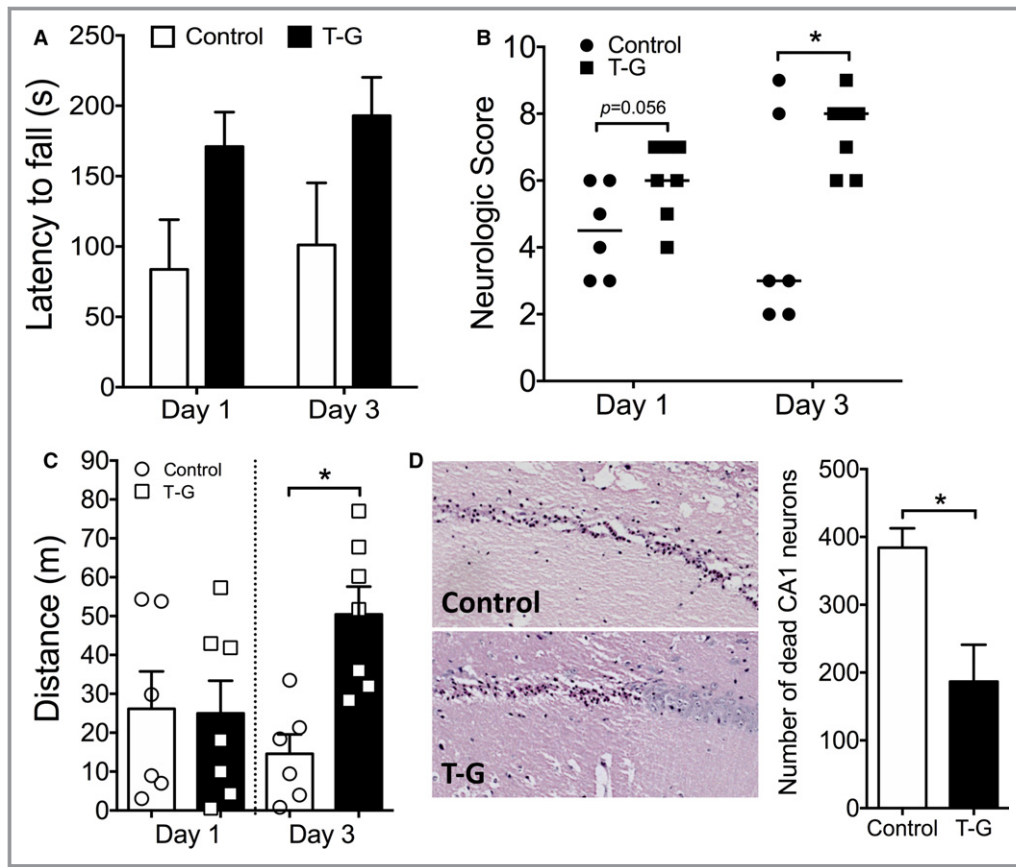


Figure 9. Posttreatment with thiamet-G improves cardiac arrest (CA) outcome. Mice were subjected to 8.5 minutes CA, followed by resuscitation. After 30 minutes of reperfusion, mice were treated with saline (control) or thiamet-G (T-G). Rotarod (A), neurologic score (B), and spontaneous locomotor activity (traveled distance during a 10-minute test period; C) were evaluated on days 1 and 3 after CA. Data are presented as mean \pm SEM or median (n=6–7 per group). D, Representative hematoxylin/eosin staining of the hippocampal cornu ammonis 1 (CA1) subfield on day 3 after CA and numbers of dead CA1 neurons. Data are presented as mean \pm SEM (n=5–6 per group). * P <0.05.

conserved stress response pathways to be activated in all organs investigated after CA/CPR. Indeed, our data showed that these pathways were activated in the brain, spinal cord, and kidney of young mice subjected to CA/CPR, and that most of the observed changes were evident early after onset of reperfusion. We also found, however, that activation of these pathways was largely impaired in aged mice. Therefore, considering that these pathways play vital roles in restoring proteostasis impaired by stress, and that proteostasis is critical to cell survival, it is plausible to suggest that the aging-related decline in the capacity of cells to activate such pathways contributes to the worse outcome observed in elderly individuals after CA/CPR.

Cells have developed different lines of defense to cope with a severe form of stress by activating prosurvival pathways, including regulation of gene expression and posttranslational protein modifications. Transcription is activated shortly after ischemia when energy metabolism recovers. Our observation that aging did not modify CA/CPR-

induced changes in mRNA levels of *chop*, *grp78*, and *xbp1s* suggests that transcription (*chop* and *grp78*) and posttranscriptional processing (*xbp1s*) of stress genes are not impaired at advanced age. However, we observed that post-CA/CPR activation of SUMOylation, ubiquitination, and O-GlcNAcylation is reduced in the organs of aged mice compared with young mice. These findings suggest that aging is associated with a decline in the capacity of cells to activate these posttranslational modifications, particularly SUMOylation and O-GlcNAcylation. Rapid activation of both modifications has been reported in various postischemic organs.^{6,8,27–30} Notably, in the present study, these were the only posttranslational modifications that showed impaired activation in both brain and kidney of aged mice.

A large body of evidence suggests that SUMOylation is an endogenous prosurvival pathway, and that increasing global SUMOylation protects the brain against ischemic insult.^{31–33} For example, transgenic mice that exhibit elevated basal levels of global SUMOylation are more resistant to ischemia-

induced brain damage,³² whereas transgenic mice in which SUMO expression is significantly silenced show worse functional outcome after global cerebral ischemia.³¹ Currently, only a limited number of small molecules that may be capable of increasing SUMOylation in vivo are available for testing in disease models related to I/R injury.³³ In an effort to address this deficit, we recently established a platform designed to effectively identify and characterize new compounds that target the SUMO system.³⁴

Ischemia-induced O-GlcNAcylation, which was evident in all post-CA/CPR organs that we studied herein, occurs downstream of the IRE1 UPR branch.^{8,29} More important, this activation was severely impaired in both brain and kidney of aged mice. This inability to activate O-GlcNAcylation after CA/CPR may have a major impact on outcome, as is suggested in current literature. O-GlcNAcylation regulates the function of many proteins, and protects cells in a variety of stress environments.³⁵ In the heart, activation of O-GlcNAcylation protects against the calcium paradox and ischemic damage, and also blocks ischemic stress-induced mitochondrial calcium overload and loss of membrane potential.³⁶ Recently, we reported that aging is associated with impaired activation of O-GlcNAcylation in the stroke penumbra and worse outcome, and that pharmacologic activation of O-GlcNAcylation improves stroke outcome.⁸ Considering that increased O-GlcNAcylation is neuroprotective,⁸ cardioprotective,²⁹ and renoprotective,³⁰ pharmacologic boosting of O-GlcNAcylation is expected to protect a variety of organs exposed to I/R injury, which would be particularly beneficial in CA/CPR that causes whole-body I/R injury. Indeed, our data showed that posttreatment with thiamet-G improved functional outcome and reduced CA1 neuronal death after CA/CPR (Figure 9). On the basis of these results, it is reasonable to speculate that thiamet-G treatment could also improve CA outcome in aged mice by increasing O-GlcNAcylation. Notably, we have reported previously that thiamet-G can increase O-GlcNAcylation in aged brains.⁸

Recently, we proposed that the aging-related decline in the body's self-healing capacity may contribute to the disparity in the observed success of pharmacologic interventions in young animals versus failure in elderly patients after brain ischemia/stroke.² This likely is also the case in preclinical CA research. Therefore, experiments on aged animals should be included in the repertoire of preclinical CA studies before moving forward to clinical trials. Finally, our findings suggest that proteostasis-related pathways play an important role in CA outcome. Our observation that activation of these pathways is impaired in aged animals therefore implies that insufficient restoration of proteostasis contributes to the worse CA outcome at advanced age. Because levels of O-GlcNAcylation affect outcomes after experimental myocardial ischemia and stroke,^{8,29} and pharmacologically boosting O-GlcNAcylation improves ischemic stroke

outcome even in aged mice,⁸ future studies to determine whether this promising pharmacologic intervention can improve CA outcome in aged animals are warranted.

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Disclosures

None.

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