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J Neurochem. Author manuscript; available in PMC 2013 November 01.

Published in final edited form as:
J Neurochem. 2012 November ; 123(3): 349–359. doi:10.1111/j.1471-4159.2012.07916.x.

Moderate hypothermia induces marked increase in levels and nuclear accumulation of SUMO2/3-conjugated proteins in neurons

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Abstract

Deep hypothermia protects the brain from ischemic damage and is therefore used during major cardiovascular surgeries requiring cardiopulmonary bypass and a period of circulatory arrest. Here, we demonstrated that small ubiquitin-like modifier (SUMO1-3) conjugation is markedly activated in the brain during deep to moderate hypothermia. Animals were subjected to normothermic (37°C) or deep to moderate (18°C, 24°C, 30°C) hypothermic cardiopulmonary bypass, and the effects of hypothermia on SUMO conjugation were evaluated by Western blot and immunohistochemistry. Exposure to moderate 30°C hypothermia was sufficient to markedly increased levels and nuclear accumulation of SUMO2/3-conjugated proteins in these cells. Deep hypothermia induced nuclear translocation of the SUMO conjugating enzyme Ubc9, suggesting that the increase in nuclear levels of SUMO2/3-conjugated proteins observed in brains of hypothermic animals is an active process. Exposure of primary neuronal cultures to deep hypothermia induced only a moderate rise in levels of SUMO2/3-conjugated proteins. This suggests that neurons *in vivo* have a higher capacity than neurons *in vitro* to activate this endogenous potentially neuroprotective pathway upon exposure to hypothermia. Identifying proteins that are SUMO2/3 conjugated during hypothermia could help to design new strategies for preventive and therapeutic interventions to make neurons more resistant to a transient interruption of blood supply.

Keywords

cardiopulmonary bypass; hypothermia; neuronal cell cultures; oxygen/glucose deprivation; stress response pathways; SUMO2/3 conjugation; Ubc9

Introduction

Small ubiquitin-like modifier (SUMO1-3) is a group of proteins that conjugate to lysine residues of target proteins, and thereby modulate various processes that play important roles in key cellular functions under normal and pathological conditions. These functions include gene expression and genome integrity, proteasomal degradation of proteins, protein quality control, and DNA damage repair (Hay 2005; Heun 2007; Prudden *et al.* 2007; Bergink and

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The authors declare no conflicts of interest.

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Jentsch 2009; Geoffroy and Hay 2009; Tatham *et al.* 2011). In the intact brain, levels of SUMO2/3-conjugated proteins are very low, but are massively increased after transient cerebral ischemia and during deep hypothermia (Lee *et al.* 2007; Cimarosti and Henley 2008; Yang *et al.* 2008b,c; Cimarosti *et al.* 2008). The post-ischemic activation of SUMO2/3 conjugation is believed to be an endogenous neuroprotective stress response, since neurons are extremely sensitive to even a very short period of ischemia-like conditions when SUMO2/3 expression is silenced (Datwyler *et al.* 2011).

Brain damage caused by transient cerebral ischemia is of significant clinical relevance, as it is a complication of major cardiovascular surgeries in pediatric and adult patients. To protect organs from ischemic damage, major cardiovascular operations requiring a period of circulatory arrest are usually performed during deep hypothermia. Depending on the clinical center where these procedures are performed, patients are exposed to various degrees of deep or moderate hypothermia, ranging from 16°C to 30°C (Jacobs *et al.* 2001; Arnaoutakis *et al.* 2007; Khaladj *et al.* 2009; Kamiya *et al.* 2007; Camboni *et al.* 2008; Apaydin *et al.* 2009; Numata *et al.* 2009; Takami *et al.* 2009; Elefteriades 2010).

Using a rat model of deep hypothermic cardiopulmonary bypass (CPB), we have demonstrated that deep hypothermia at 18°C massively activates the SUMO conjugation pathway (Yang *et al.* 2009). In the present study, we investigated whether more moderate hypothermia is sufficient to activate SUMO conjugation. Because the potential of manipulating the SUMO conjugation pathway for preventive and therapeutic purposes is of tremendous clinical interest, we evaluated the effects of hypothermia on SUMO conjugation using an animal model of CPB and exposed animals to normothermic (37°C) or hypothermic (30°C, 24°C, or 18°C) CPB.

Here we demonstrated that deep hypothermia triggered a shift in immunoreactivity of the SUMO conjugating enzyme Ubc9 from the cytoplasm to the nucleus of neurons. Furthermore, moderate hypothermia at 30°C was sufficient to induce a marked rise in levels and nuclear accumulation of SUMO2/3-conjugated proteins in neurons. Since various pathways modulated by SUMO conjugation are nuclear processes pivotal for cells to recover from stress, as discussed above, and since mounting evidence suggests that activation of SUMO conjugation increases the tolerance of cells to stress conditions (Lee *et al.* 2007,2009; Yang *et al.* 2009; Datwyler *et al.* 2011; Lee *et al.* 2011), it is conceivable to conclude that SUMO conjugation plays a role in the organ protection that hypothermia provides.

Materials and methods

Animal experiments

This study was approved by the Duke University Animal Care and Use Committee, and complies with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Normothermic (37°C) or moderate to deep hypothermic (30°C, 24°C or 18°C) cardiopulmonary bypass (CPB) experiments were performed on 12- to 14-week-old male Sprague-Dawley rats (Charles River, Boston; n = 5/group) anesthetized with isoflurane and mechanically ventilated, as previously described (Yang *et al.* 2009; Jungwirth *et al.* 2006). In short, the tail artery was cannulated for aortic inflow, and a cannula was advanced through the right external jugular vein into the right atrium for venous return. Mean arterial blood pressure (MAP) was monitored and baseline physiologic measurements including MAP, temperature (pericranial and rectal), blood gases and glucose levels were recorded. Animals were cooled over a period of 30 minutes to a rectal temperature of 30°C, 24°C or 18°C by using a heat exchanger and topical cooling. Animals subjected to normothermic (37°C) CPB served as controls. At the end of the experiments, immediately

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after the animals had reached the target temperature or after 60 minutes exposure to the target temperature, rats were decapitated. Brains used for Western blot analysis of SUMO conjugation were quickly removed, immediately frozen and stored at −80°C. Brains used for immunohistochemistry were fixed with buffered 4% paraformaldehyde solution.

Primary neuronal cell cultures

Primary neuronal cell cultures were prepared from the cortex of embryonic rat brains at gestation day 18, essentially as described elsewhere (Paschen *et al.* 1996) with some modifications. In short, the entire cortex was dissected and dissociated with trypsin. Cells were plated at a density of 100,000 cells/cm^2 in Neurobasal medium (Invitrogen) supplemented with B27, glutamax I, 5% fetal bovine serum, and 1 μg/mL gentamicin. After 3 days in culture, cytosine-β-D-arabino-furanoside was added to a final concentration of 5 μM. Cells were then fed twice a week with serum-free Neurobasal/B27 medium for an additional 7–9 days. When used for OGD experiments, cultures exhibited about 95%–98% pure neuronal cells.

Experiments on primary neuronal cell cultures

For induction of hypothermia, the Neurobasal/B27 medium was replaced with Neurobasal medium pre-cooled to 16°C or 4°C and cultures were placed in a 16°C or 4°C environment. Cultures were exposed to oxygen/glucose deprivation (OGD, a model of ischemia) by using an anoxic chamber (Forma Scientific Anaerobic System). Glucose-free and L-aspartic acid-, L-glutamic acid-, and sodium pyruvate-free Neurobasal medium (Gibco) was equilibrated overnight in the anoxic chamber with the anoxic gas mixture (85% N_2, 10% H_2, 5% CO_2). Cultures were transferred to the anoxic chamber and washed 3 times with the anoxic medium. After 30 min OGD exposure, the anoxic medium was replaced with Neurobasal/B27 medium and cells were transferred back to the incubator set at 37°C with a gas mixture of 95% air/5% CO_2 for an additional 24 hours.

To quantify the extent of OGD-induced cell death, cells were stained with Hoechst 33342 and propidium iodide (PI) to visualize the nuclei of all cells and dead cells, respectively. Six fields were randomly selected in each well, and experiments were repeated three times. The extent of cell death was based on the ratio of PI-positive cells to total number of cells.

Immunofluorescence staining was used to identify morphological damage following deep hypothermia. Cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes. After blocking with PBS supplemented with 5% goat serum, 2% BSA, and 0.1% Triton-X100, cells were incubated with anti-MAP2 (1:500, Thermo Scientific) at 4°C overnight, followed by incubation with Alexa Fluor 594-conjugated goat anti-chicken IgG (Invitrogen, 1:400) at room temperature. Nuclei were stained with Hoechst 33342 (3 μg/mL). To evaluate the extent of deep hypothermia-induced cell death, neurons were washed once with PBS and incubated with PI (1 μg/mL) at room temperature for 15 minutes, followed by a brief wash with PBS. Cells were then fixed with 4% paraformaldehyde and stained with Hoechst 33342.

Western blot analysis

To avoid the risk of de-SUMOylation of proteins during sample preparation, brains and tissue specimens remained frozen as long as endogenous SUMO-specific proteases were potentially active. Frozen brains were processed in a cryostat set at −16°C, and samples were excised from the cerebral cortex, hippocampus and striatum. Proteins were extracted using lysis buffer composed of β-glycerophosphate (50 mmol/L; pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 0.5 mmol/L Na_3VO_4, 1% Triton X-100, and 2% sodium dodecyl sulfate (SDS) to block de-SUMOylation by inhibiting SUMO-specific proteases (Malakhov *et al.*

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2004). Frozen tissue specimens were weighed and homogenized by a short sonication for 10 seconds, immediately followed by heat inactivation at 99°C for 10 minutes and centrifugation for an additional 10 minutes. Protein concentration of extracts was measured using the BCA assay (Thermo Scientific).

Western blotting was performed using SDS-PAGE 4%–15% gels (Bio-Rad). After electrophoresis, proteins were transferred to PVDF membranes (Bio-Rad); membranes were blocked using Tris/HCl-buffered salt solution supplemented with 0.1% Tween 20 and 5% skim milk powder, and incubated with anti-SUMO1 (by courtesy of Dr. John M. Hallenbeck) or anti-SUMO2/3 polyclonal antibody (Covance) overnight at 4°C. After extensive washing, membranes were incubated with anti-rabbit horseradish peroxidase conjugates (Santa Cruz Biotechnology) for 1 hour at room temperature. Proteins were then visualized using the enhanced chemiluminescence analysis system (GE Healthcare). A monoclonal antibody against β-actin (Sigma) was used as loading control. Changes in levels of SUMO-conjugated proteins following hypothermia were evaluated by image analysis (ImageJ). The higher-molecular-weight area in each lane was cropped and analyzed.

Microscopy

Brains were fixed with paraformaldehyde and embedded in paraffin. After deparaffinization, heat-mediated antigen retrieval was performed for sections in sodium citrate buffer for 2.5 minutes. After blocking at room temperature for 1 hour, sections were incubated with rabbit anti-SUMO2/3 antibody (Covance, 1:2000) or mouse anti-Ubc9 antibody (BD Bioscience, 1:100), and chicken anti-microtubule-associated protein 2 (MAP2) antibody (Thermo Scientific, 1:500) at 4°C overnight. Sections were then incubated with Alexa Fluor 594-conjugated goat anti-chicken IgG (Invitrogen, 1:400), Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, 1:500), and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, 1:500) at room temperature for 1 hour. Images were captured on a Leica SP5 confocal microscope (Leica Microsystems) using a 40X/1.25–0.75 Plan APO oil objective.

Statistical Analysis

Data are presented as means ± SD with 5 independent brain samples per group and 3 independent cell culture samples per group for Western blot analysis, and 3 independent experiments for analysis of the extent of cell death following OGD. Statistically significant differences between groups were evaluated by analysis of variance (ANOVA), followed by Fisher's protected least-significant difference (PLSD) test. A probability of 95% was taken to indicate significant differences between groups.

Results

Recently, using the same animal model as in the present study, we demonstrated that exposing rats to one hour of 18°C deep hypothermic CPB markedly activates SUMO2/3 conjugation, and to a lesser extent, SUMO1 conjugation (Yang *et al.* 2009). This effect was observed in the hypothermic CPB animals but not in the normothermic CPB group, indicating that the surgical procedure per se does not cause the activation (Yang *et al.* 2009). Here, we exposed animals to CPB at varying temperatures ranging from 18°C to 30°C, ie, the full range of deep to moderate hypothermia used clinically for major cardiovascular surgeries, as discussed above. Specifically, rats were subjected to 37°C, 18°C, 24°C or 30°C CPB for 1 hour, and changes in levels of SUMO-conjugated proteins were analyzed by Western blot. A moderate increase in SUMO1 conjugation and a marked increase in SUMO2/3 conjugation occurred in the hypothermic CPB animals (Figure S1; Figure 1A,B). Levels of SUMO1-conjugated proteins rose 2.7 ± 0.6-fold, 3.8 ± 1.0-fold, and 4.1 ± 0.7-fold in the cortex, 3.5 ± 0.7-fold, 4.2 ± 0.8-fold, and 3.8 ± 0.7-fold in the hippocampus, and 2.8 ±

0.8-fold, 3.3 ± 0.4-fold, and 2.7 ± 0.4-fold in the striatum of animals exposed to 30°C, 24°C, or 18°C CPB, respectively, compared to normothermic 37°C CPB (Figure S1). Changes in levels of SUMO2/3-conjugated proteins were more pronounced, increasing 11.1 ± 3.1-fold, 17.8 ± 5.5-fold, and 17.1 ± 6.3-fold in the cortex, 5.7 ± 3.2-fold, 8.0± 1.5-fold, and 5.6 ± 2.8-fold in the hippocampus, and 10.8 ± 3.0-fold, 15.4 ± 3.3-fold and 13.0 ± 4.5-fold in the striatum of animals exposed to 30°C, 24°C, or 18°C, respectively, compared to normothermic 37°C CPB (Figure 1). These data indicate that SUMO2/3 conjugation was activated even after moderate hypothermia of 30°C.

In the first set of experiments, CPB animals were cooled down to the target temperature over a period of 30 minutes, and brains were processed 1 hour after animals had reached the target temperature. To determine whether SUMO2/3 conjugation is activated after a very short exposure to deep hypothermia, CPB animals were cooled down to 18°C, and brains were sampled immediately when animals reached the target temperature, ie, 30 minutes after starting the cooling process. Levels of SUMO2/3-conjugated proteins rose 9.5 ± 1.8-fold, 5.7 ± 1.2-fold, and 7.0 ± 1.0-fold in the cortex, hippocampus and striatum, respectively (Figure 1C).

A large portion of SUMO conjugation targets are nuclear proteins involved in various pathways that play key roles in the recovery of stressed cells. Thus, for the activation of SUMO2/3 conjugation to be of key functional significance, we would expect it to occur preferentially in nuclei of neurons. To investigate the relevance of hypothermia in this process, CPB animals were cooled down to 18°C, 24°C, or 30°C, brains were sampled when animals had reached the target temperature, and changes in SUMO2/3 conjugation were evaluated by immunohistochemistry. Since activation of SUMO2/3 conjugation in the brain is predominantly a neuronal stress response (Yang *et al.* 2008b), sections were co-stained for the neuron-specific cytoplasmic marker MAP2. In normothermic brains, SUMO2/3 immunoreactivity was mainly confined to the cytoplasm of neurons, both in the cortex and the hippocampal CA1 subfield (Figure 2A,B). However, in hypothermic animals, strong SUMO2/3 immunoreactivity was present in nuclei and markedly less in the cytoplasm of neurons.

To determine whether the nuclear SUMO2/3 immunoreactivity found in hypothermic animals represented free SUMO2/3, conjugated SUMO2/3 or both, we separated brain extracts into cytosolic and nuclear fractions. In nuclear fractions isolated from both 37°C normothermic and 18°C hypothermic animals, we did not find free SUMO2/3 (Figure S2). This suggests that nuclear SUMO2/3 immunoreactivity found in neurons of hypothermic animals (Figure 2A+B) represented SUMO2/3-conjugated proteins and not free SUMO2/3.

To evaluate the mechanisms underlying the observed increase in nuclear SUMO2/3 immunoreactivity in hypothermic animals, we stained brain sections of 37°C normothermic and 18°C hypothermic animals for Ubc9, the only SUMO conjugating enzyme identified so far, and MAP2 as neuronal cytoplasm marker. In normothermic brains, Ubc9 immunoreactivity was mainly confined to the cytoplasm of neurons, both in the cortex and hippocampal CA1 subfield (Figure 3A,B), similar to the pattern described by Lee et al. (2011). However, in 18°C hypothermic animals, Ubc9 immunoreactivity was also present in the nuclei and, clearly less, in the cytoplasm of neurons. This suggests that Ubc9 nuclear translocation contributes to the marked increase in levels of SUMO2/3-conjugated proteins found in the nuclei of neurons of hypothermic animals.

In search of a cell culture model for investigating the mechanisms underlying activation of SUMO2/3 conjugation under deep hypothermic conditions, we used primary neuronal cultures. Human neuroblastoma SHSY5Y cells and primary cortical neurons pre-exposed to

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4°C deep hypothermia are more tolerant to transient OGD (Lee *et al.* 2007; Loftus *et al.* 2009). We exposed primary neuronal cell cultures, prepared from cortices of embryonic rat brains, to deep hypothermia. Since activation of SUMO2/3 conjugation is a response of cells to various kinds of stresses (Yang *et al.* 2008a), we used MAP2 immunostaining to identify hypothermia-induced morphological changes as an indicator of stressed cells following hypothermia exposure (Figure 4). Cells exposed to 4°C, but not 16°C, showed marked dendrite degeneration including beading formation and fragmentation (Figure 4A). We therefore focused on 4°C deep hypothermia for further analyses. Next, cells were exposed to 4°C for 5, 15 or 30 minutes (Figure 4B). We observed degenerative changes in cells exposed to prolonged 4°C hypothermia (Figure 4B, 30 min).

To elucidate the effects of deep hypothermia on SUMO2/3 conjugation, neuronal cultures were exposed to 4°C deep hypothermia with or without normothermic recovery (Figure 5). A moderate rise in levels of SUMO2/3-conjugated proteins occurred during a short exposure to 4°C deep hypothermia (Figure 5A). However, the change in the pattern of SUMO2/3 conjugation was only transient, as it was almost normalized after only 5 minutes of exposure. Activation of SUMOylation was rapidly reversed when cells were transferred back to 37°C normothermic conditions (Figure 5B).

In the last set of experiments, cells were subjected to 30 minutes OGD and 24 hours of recovery with or without prior deep hypothermia exposure. Hoechst/PI staining indicated that transient OGD induced severe cell death that was partially suppressed in cultures exposed to 4°C deep hypothermia for 5 minutes, but not for 15 minutes, 2 hours prior to normothermic OGD (Figure 6A). When the interval between hypothermia and OGD was extended to 24 hours, this protective effect vanished (Figure 6B). We quantified the extent of cell death following OGD by counting Hoechst- and PI-positive cells. Cell death was suppressed by about 25% after a short 5-minute period of deep hypothermia 2 hours prior to OGD (Figure 6C).

Discussion

The potential of deep hypothermia to protect organs from damage induced by transient ischemia is well established. However, the mechanisms underlying organ protection by deep hypothermia and strategies to maximize its efficacy still need to be established. Understanding these mechanisms would be a pivotal step toward designing therapeutic strategies to activate these processes and thus induce a state of tolerance to transient ischemia without risking the adverse effects associated with deep hypothermia. The most promising strategy would be to activate endogenous neuroprotective pathways before performing surgical procedures that require a period of circulatory arrest, and thus increase the resistance of neurons to a transient interruption of blood supply. The small ubiquitin-like modifier (SUMO) conjugation pathway could be such a pathway, which, when activated, protects neurons from ischemic insults.

A massive activation of SUMO conjugation induced by deep hypothermia was first reported by John Hallenbeck and his colleagues (Lee *et al.* 2007). Using hibernating squirrels as an experimental model to identify endogenous neuroprotective stress response pathways, they found massive activation of SUMO conjugation during the state of hibernation torpor (Lee *et al.* 2007). During torpor, when the body temperature of animals is sharply reduced to about 5°C, blood flow, energy consumption, and protein synthesis are lowered to otherwise lethal levels (Carey *et al.* 2003; Frerichs and Hallenbeck 1998; Frerichs *et al.* 1994, 1998). The investigators therefore hypothesized that SUMO conjugation plays a role in tolerance to the severe ischemia associated with hibernation torpor. This hypothesis was substantiated by results of experiments on cell cultures exposed to transient oxygen/glucose deprivation

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(OGD, ischemia-like conditions) (Lee *et al.* 2007,2009; Datwyler *et al.* 2011). Furthermore, using transgenic mice overexpressing exogenous Ubc9, the only SUMO conjugation enzyme identified to date, a recent study showed that increased SUMO conjugation was associated with an increased tolerance to transient focal cerebral ischemia (Lee *et al.* 2011).

The most prominent finding of the present study is that exposure to moderate 30°C hypothermia in rats was sufficient to markedly modify the pattern and the subcellular localization of SUMO2/3 in neurons, and that hypothermia triggered nuclear translocation of the SUMO conjugating enzyme Ubc9 (Figures 1–3). In brains of animals exposed to normothermic CPB, most of the SUMO2/3 immunoreactivity was detectable as free SUMO (Figure 1, band at about 18 kDa), and strong SUMO2/3 immunoreactivity was present in the cytoplasm but not in the nuclei of neurons (Figure 2; 37°C). In animals exposed to hypothermic CPB, however, levels of SUMO2/3-conjugated proteins increased markedly (Figure 1), and strong SUMO2/3 immunoreactivity was present in nuclei but not in the cytoplasm of neurons (Figure 2). This suggests a massive nuclear accumulation of SUMO2/3-conjugated proteins in neurons of hypothermic animals.

It is well established that the speed of chemical and biochemical reactions is temperature-dependent, a decrease in temperature retarding the reactive process. One plausible explanation for the neuroprotective effects of hypothermia, therefore, is that it protects cells from ischemic damage by retarding the rate of energy depletion during ischemia. Hypothermia does indeed depress the tricarboxylic acid flux (Kaibara *et al.* 1999) and thus preserve cerebral energy metabolism during ischemia (Laptook *et al.* 1995; Williams *et al.* 1997; Yager *et al.* 1996), resulting in delayed anoxic depolarization (Kaminogo *et al.* 1999). These observations imply that hypothermia-induced neuroprotection is a passive process whereby the rate of glucose metabolism is lowered, consequently delaying the time to terminal depolarization. This would shorten the period of terminal depolarization during transient ischemia and thus mitigate all pathological processes triggered during the state of energy depletion and manifested after recovery from ischemia.

On the other hand, transient hypothermia induces rapid and delayed forms of tolerance to ischemic injury, suggesting that it can trigger an active process that protects cells from damage caused by ischemia (Nishio *et al.* 2000; Yunoki *et al.* 2002,2003). Given that SUMO2/3 conjugation protects neurons from ischemia-like conditions (Datwyler *et al.* 2001), that hypothermia provides neuroprotection, and that hypothermia triggers nuclear translocation of Ubc9 and activation of nuclear SUMO2/3 conjugation (Figures 2,3), we conclude that SUMO contributes the neuroprotective process induced by hypothermia.

In search of a simplified experimental model to investigate, in future studies, the mechanisms underlying the deep hypothermia-induced rise in levels of SUMO2/3-conjugated proteins and to screen for drugs that activate this process in the absence of hypothermia, we cultured primary neurons from embryonic rat brains. Exposure to deep hypothermia induced only a short-lasting very moderate increase in levels of SUMO2/3-conjugated proteins (Figure 6). This suggests that neurons in culture have a much lower capacity for activating SUMO2/3 conjugation than neurons in the intact brain. This notion is supported by our recent observation that transient OGD induced only a moderate rise in levels of SUMO2/3-conjugated proteins in primary neuronal cultures (Datwyler *et al.* 2011). However, SUMO2/3 conjugation was dramatically increased in post-OGD B35 neuroblastoma cells (Yang *et al.* 2012) and also in neurons *in vivo* subjected to a transient interruption in blood supply (Yang *et al.* 2008a,b). This indicates that the capacity of the SUMO conjugation machinery to respond to stressful conditions is much lower in primary neurons than in established cell lines in culture, and much less in cultured neurons than in neurons in the intact brain.

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Various factors may contribute to this obvious discrepancy, including the developmental state of neurons and the microenvironment. The expression levels of SUMO2/3-conjugated proteins and the SUMOylation machinery are temporally regulated in the developing brain (Loriol *et al.* 2012), which may explain why primary neurons in culture isolated from embryonic brains react to hypothermic or metabolic stress differently than neurons in the adult brain. Furthermore, neurons are post-mitotic non-dividing cells that survive *in vivo* for the entire lifespan of the animal, but only for a limited time when they are isolated from brains and kept in culture. It would be interesting to determine whether the viability of neurons in culture is limited by inactivity of the SUMOylation machinery.

The microenvironment may also contribute to the different response of *in vivo* vs. *in vitro* neurons to both deep hypothermia and transient ischemia/OGD. In the intact brain, neurons are physically and functionally tightly associated with glial cells, and this mutual interaction is important for physiologic function. *In vitro*, culture conditions are usually chosen that suppress growth of glial cells, resulting in very pure neuronal cultures. Furthermore, neurons are surrounded by a very small extracellular space *in vivo*, while neurons in culture are bathed in an ocean of medium. Elucidating the mechanisms underlying the limited ability of cultured neurons to respond to stress conditions by massively activating SUMO2/3 conjugation may help to better understand the role of SUMO conjugation in protecting cells from damage.

Many of the SUMO2/3 conjugation target proteins identified so far are transcription factors and other nuclear proteins that modify gene expression and play key roles in DNA damage repair. We therefore expect the massive nuclear accumulation of SUMO2/3-conjugated proteins in neurons of hypothermic animals to contribute to the neuroprotective effects of hypothermia. Deep hypothermia does indeed modify gene expression and downregulates expression of several genes that are associated with the pathological process triggered by transient ischemia (Yang *et al.* 2009). These include chemokines, intracellular adhesion molecule-1, interferon regulatory factor-1, and interleukin-1β and interleukin-6 (Yang *et al.* 2009).

Recently, an increasing number of SUMOylation target proteins have been identified in neurons that are not nuclear but rather cytosolic or cell membrane proteins (Martin *et al.* 2007b; Wilkinson *et al.* 2010). These include the mRNA-binding protein La (van Niekerk *et al.* 2007), the kainate receptor subunit GluR6 (Martin *et al.* 2007a), and the potassium channel Kv1.5 (Benson *et al.* 2007). This suggests a role for SUMO conjugation in modulating axonal mRNA trafficking, excitability of neurons, and ion fluxes (Wilkinson et al. 2010). Whether SUMOylation of cytosolic or cell membrane proteins is activated during hypothermia and contributes to the protective effect must be determined in future studies. We observed that during hypothermia most of the SUMO2/3 immunoreactivity is present in the nuclei of neurons (Figure 2). This supports the notion that nuclear processes mediated by SUMO2/3 conjugation are activated during hypothermia.

The rise in levels of SUMO2/3-conjugated proteins in neurons in the intact brain exposed to deep hypothermia or transient ischemia is much more dramatic than in cultured neurons exposed to deep hypothermia or transient ischemia-like conditions, as discussed above and demonstrated recently (Datwyler *et al.* 2011). *In vivo* studies are therefore needed to verify whether the activation of SUMO conjugation during hypothermia or after ischemia is indeed a protective stress response, and to establish strategies to manipulate the SUMO conjugation pathway for preventive and therapeutic purposes. Transient hypothermia/ischemia experiments on SUMO knockout animals will provide important information on the role of individual SUMO paralogues in protecting neurons from ischemic damage. SUMO transgenic animals may help us to identify proteins that are SUMO2/3-conjugated in

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neurons of hypothermic brains. Such investigation will be integral to unraveling the mechanisms by which SUMO conjugation imparts neuroprotection during hypothermia. This will be pivotal to identify drugs that activate these processes in normothermic animals, thus avoiding the potentially adverse effects associated with deep hypothermia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by RO1 grant HL095552 from the National Institutes of Health, and by funds from the Department of Anesthesiology, Duke University Medical Center. The authors thank Kathy Gage, Research Development Associate, for her excellent editorial contributions in the preparation of this manuscript and Pei Miao for her excellent technical assistance. We are grateful to Dr. John M. Hallenbeck, National Institutes of Health, for providing SUMO1 antibody.

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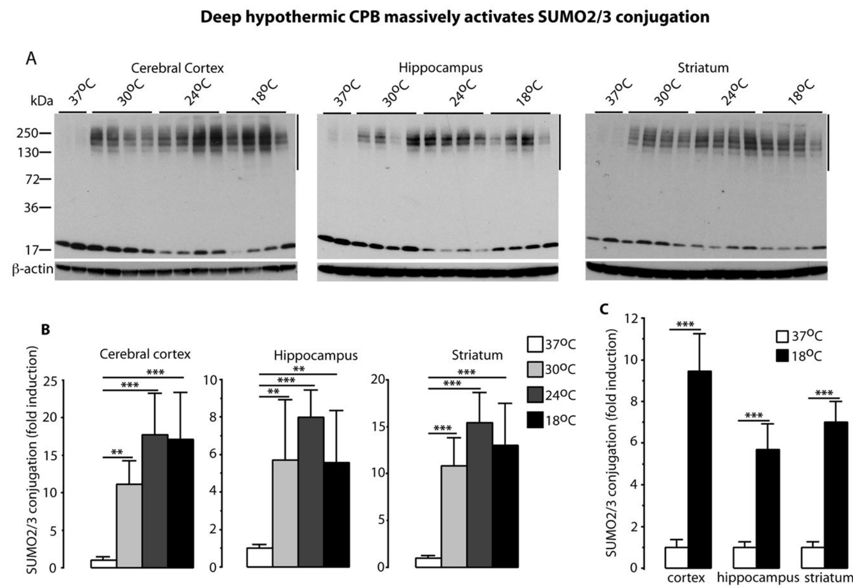

Figure 1.

Marked activation of SUMO2/3 conjugation during moderate to deep hypothermia. Western blot analysis depicts the pattern and summary of SUMO2/3 conjugation in the brains of animals subjected to normothermic (37°C) or 1 hour of moderate to deep hypothermic (30°C, 24°C, or 18°C) cardiopulmonary bypass (CPB; A,B), or 0 minutes 18°C deep hypothermic CPB (C). Monoclonal antibody against β-actin was used as loading control. The high-molecular-weight area in each lane, as indicated in A, was cropped and analyzed. Quantification data are presented as means ± SD (n=5/group). ** P<0.01; *** P<0.001 (ANOVA, followed by Fishers PLSD test).

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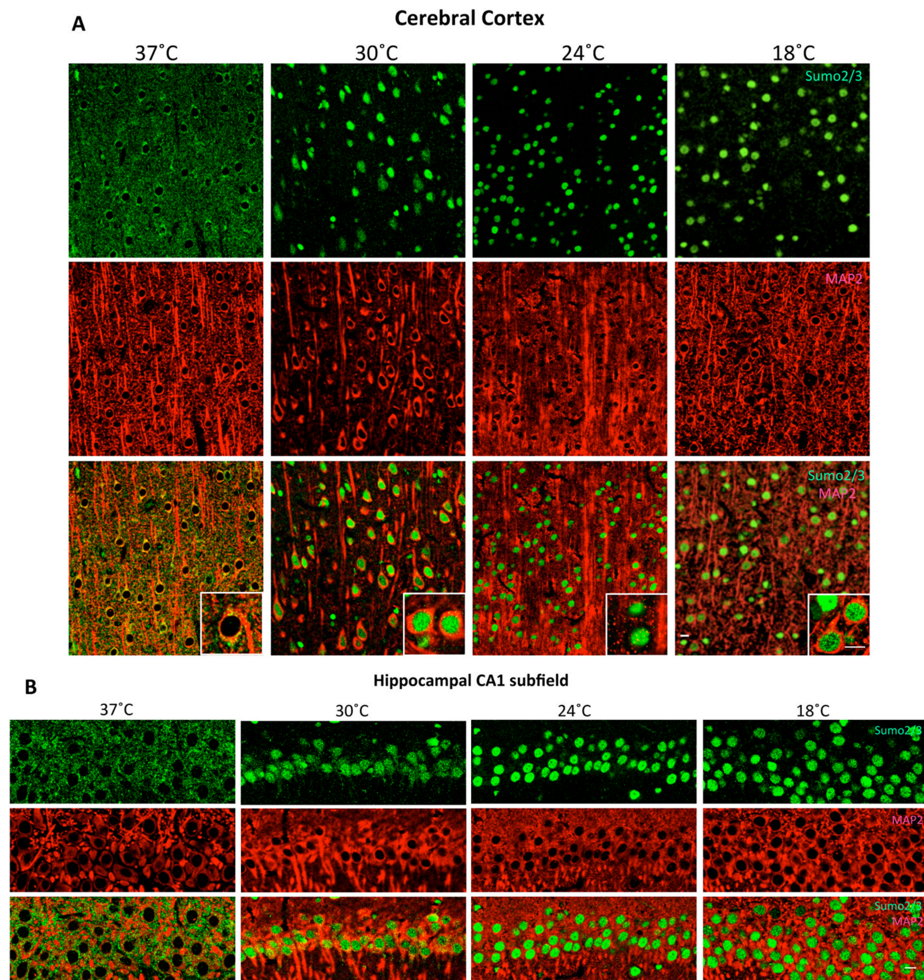

Figure 2.

Massive nuclear accumulation of SUMO2/3-conjugated proteins in cortical and hippocampal neurons exposed to even moderate hypothermia. Representative immunofluorescence staining shows the pattern of SUMO2/3 conjugation in the cortex (A) and hippocampus (B) of animals subjected to normothermic (37°C) or moderate to deep hypothermic (30°C, 24°C, or 18°C) cardiopulmonary bypass (CPB). MAP2 staining (red) was used to identify neurons. Scale bar: 10 μm.

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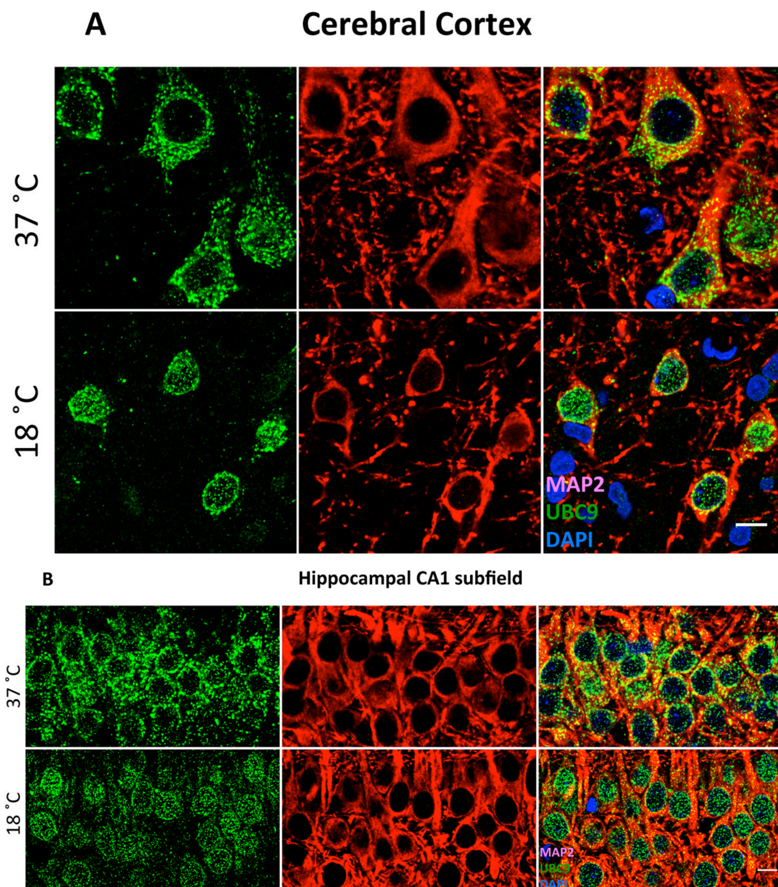

Figure 3.
Nuclear translocation of Ubc9 immunoreactivity in cortical and hippocampal neurons of animals exposed to 18°C hypothermia. Representative immunofluorescence staining shows the pattern of Ubc9 in the cortex (A) and hippocampus (B) of animals subjected to normothermic (37°C) or hypothermic (18°C) cardiopulmonary bypass. MAP2 staining (red) was used as a cytoplasmic marker of neurons. Scale bar: 10 μm.

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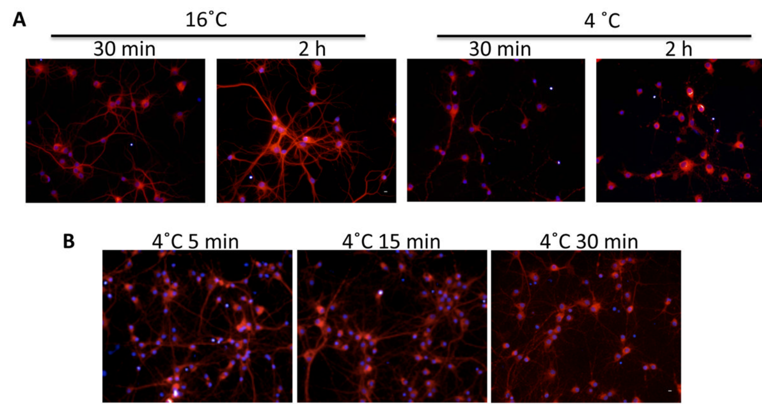

Figure 4.

Morphological damage, indicative of cellular stress, illustrated by dendritic degeneration following prolonged exposure of primary neuronal cell cultures to 4°C deep hypothermia. Primary neuronal cultures were prepared from cortices of embryonic rat brains. Cultures were exposed to 16°C or 4°C hypothermia for 30 minutes or 2 hours (A), or to 5, 15 or 30 minutes 4°C hypothermia (B). MAP2 (red), and DAPI (blue) staining was used to identify neurons and nuclei, respectively.

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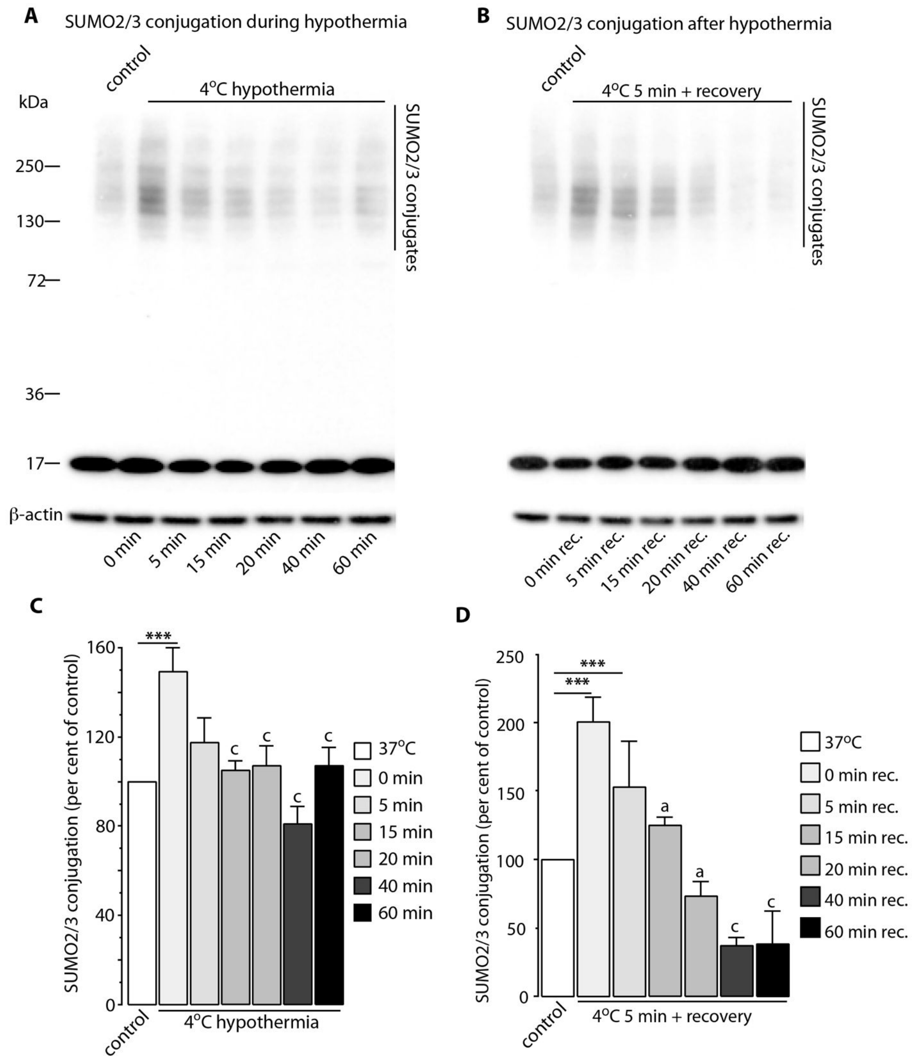

Figure 5.

Small transient rise in levels of SUMO2/3-conjugated proteins in primary neurons exposed to 4°C hypothermia. Western blot analysis depicts the pattern (A,B) and summary (C,D) of SUMO2/3 conjugation in primary neuronal cultures exposed to 4°C hypothermia without (A,C) or with (B,D) normothermic recovery. Monoclonal antibody against β-actin was used as loading control. The high-molecular-weight area in each lane, as indicated in A,B, was cropped and analyzed. Quantification data are presented as means ± SD (n=3/group). *** $P<0.001$; a,c $P<0.05$, $P<0.001$ vs. 0 minutes (0 min, C) or 0 minutes recovery (0 min rec., D), respectively (ANOVA, followed by Fishers PLSD test).

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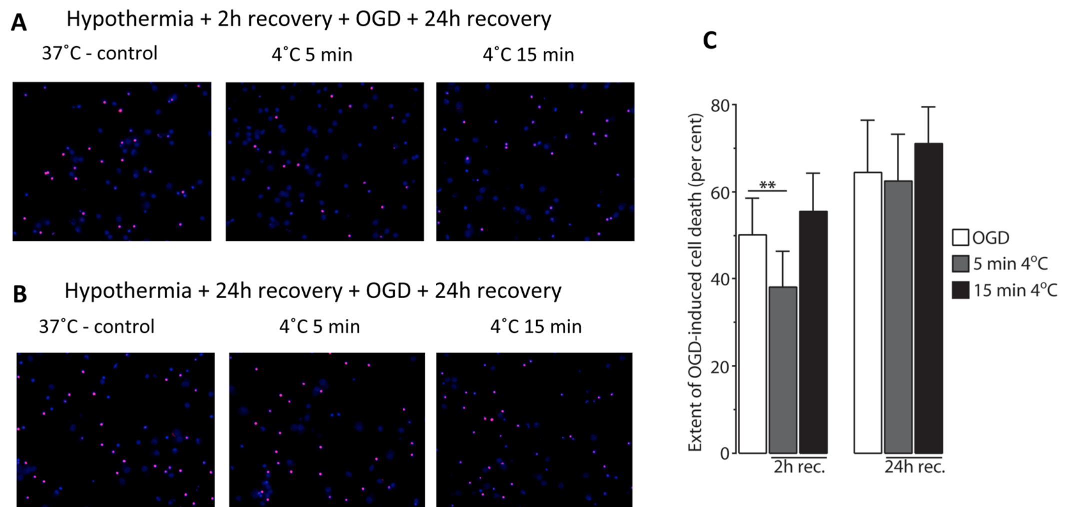

Figure 6.

Acute but not delayed tolerance to OGD following a short period of 4°C hypothermia. Primary neurons were exposed to 5 or 15 minutes 4°C hypothermia (37°C, control) 2 hours (A) or 24 hours (B) prior to 30 minutes normothermic transient OGD and 24 hours recovery. Representative immunofluorescence staining depicts the pattern of nuclei (Hoechst staining) and damaged cells (PI staining), respectively. Quantification data (C) are presented as means ± SD (n=3/group). ** P<0.01 hypothermia vs. normothermia (ANOVA, followed by Fishers PLSD test).