Published in final edited form as:

Brain Res. 2009 May 26; 1272: 71–80. doi:10.1016/j.brainres.2009.03.034.

Sumo-2/3-ylation following *in vitro* modeled ischemia is reduced in delayed ischemic tolerance

Liam T. Loftus, Rosaria Gala, Tao Yang, Veronica J. Jessick, Michelle D. Ashley, Andrea Ordonez, Simon J. Thompson, Roger P. Simon, and Robert Meller

Robert S. Dow Neurobiology Laboratories, Legacy Clinical Research and Technology Center, 1225 NE 2nd Avenue, Portland, OR 97232, USA.

Abstract

Several recent studies suggest that sumo-2/3 modification of proteins occurs following harmful ischemia, however, sumo-2/3-ylation may also be associated with hibernation-mediated neuroprotection. Here we investigate the sumoylation of proteins following ischemia and ischemic tolerance using our established *in vitro* model of ischemia (oxygen and glucose deprivation; OGD). Following harmful ischemia (120 min OGD), we observed a significant increase in the sumo-2/3-ylation of high molecular weight proteins (>85 kDa), but not sumo-1-ylation of proteins. Sumo-2/3-ylation following 120 min OGD was reduced when cultures were preconditioned with non-harmful 30 min OGD 24 hours earlier (delayed ischemic tolerance). However, we observed no change in sumo-2/3-ylation in a model of rapid ischemic tolerance. The effects of preconditioning on sumo-2/3-ylation following harmful ischemia was blocked by the protein synthesis inhibitor cycloheximide (1.0 μ M), a known inhibitor of delayed ischemic tolerance. In addition, we observed a reduction in sumo-2/3-ylation using hypothermia (4°C 30 min) as the preconditioning stimuli to induce delayed ischemic tolerance. Further studies show sumo-2/3-ylation occurs during the ischemic insult and that preconditioning does not change expression of the sumo E1- and E2-ligases (UBA2 and Ubc9) or the sumo specific isopeptidases (SenP1–3). While sumo-2/3-ylation is enhanced under conditions of cell stress, it is not yet clear whether this is a cause or consequence of harmful ischemia-induced cell damage.

Keywords

hypothermia; ischemia; OGD; neurons; preconditioning; sumo; tolerance cycloheximide

2. Introduction

Sumo (small ubiquitin-related modifier) proteins act by covalent attachment to lysine residues on larger proteins [22]. The best-characterized members of the sumo family are sumo-1, 2 and 3. Sumo-1 shares 44% sequence identity with sumo-2 and sumo-3, which share 86% identity with each other [40]. Sumo-4 has recently been identified, but little is known of its biological

© 2009 Elsevier B.V. All rights reserved.

Author for correspondence:- Robert Meller, Robert S. Dow Neurobiology Laboratories, Legacy Clinical Research and Technology Center, 1225 NE 2nd Avenue, Portland, OR 97232, USA. rmeller@downeurobiology.org, Telephone +1 503 4132581, Fax +1 503 4135465.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

function [2,34]. Sumo-1, 2 and 3, unlike ubiquitin, do not directly target acceptor proteins for degradation. While their wide variety of functions makes it difficult to ascribe generalized roles to these modifier proteins, numerous examples exist of sumo conjugation altering the activity and subcellular localization of transcription factors [15,24,32,41].

The process of sumo conjugation is reversible, highly dynamic and involves four different classes of enzymes. The first of these is the E1 activating enzyme AOS1-UBA2, which facilitates the formation of a thioester bond between the sumo and the catalytic cystine residue of UBA2 [18,33]. Next, the sumo is transferred to the E2 conjugating enzyme UBC9. UBC9 transfers sumo to its target protein, a process generally facilitated by a diverse class of sumo E3 ligases (approximately 10 enzymes) [17]. Sumo is removed from target proteins by isopeptidases known as SENPs, which vary widely in their specificity for sumo-1, 2 or 3 [30]: reviewed in [8].

Ischemic tolerance is the phenomenon whereby a sublethal insult (preconditioning) protects the brain from a subsequent, normally harmful, ischemic event. Two mechanisms of tolerance have been identified in the brain: rapid and delayed ischemic tolerance (for a review see [6, 9]). Rapid ischemic tolerance occurs within 1 hour of the preconditioning ischemia, is protein synthesis-independent, and is mediated by protein kinases and the ubiquitin-proteasome system [25]. In contrast, delayed ischemic tolerance develops 24 hours to 72 hours following the preconditioning stimuli [16,37]. Protein kinases regulate delayed ischemic tolerance [11,26, 35], but in addition, delayed ischemic tolerance is blocked by the protein synthesis inhibitor cycloheximide in both *in vivo* and *in vitro* models, suggesting that delayed tolerance requires *de-novo* protein synthesis [1,26].

A number of recent publications have suggested that the sumo conjugation of target proteins plays a role in the cellular response to ischemia. It was first shown in hibernating arctic ground squirrels that protein sumo-1-ylation and sumo-2/3-ylation of high molecular weight proteins occurs in the torpor state [20]. During mammalian hibernation, the oxygen and glucose supply to the brain is reduced to otherwise lethal levels, but no cellular damage occurs [4,39]. Hence a neuroprotective role of protein sumoylation was suggested. Transient focal and global ischemia result in the increase in sumo-2/3-ylation which may suggest that sumoylation plays a role in ischemia-induced cell damage [5,42,43]. The authors raised several interesting questions including (a) what is the temporal profile of these sumoylation changes, (b) does preconditioning activate sumoylation, and (c) does preconditioning affect the sumoylation induced by ischemia [43]? Hence we decided to investigate protein sumoylation in our established ischemic tolerance paradigm using cultured cortical neurons and oxygen/glucose deprivation (OGD). We studied the effect of both ischemic and hypothermic preconditioning on sumo-2/3-ylation events and the effects of these preconditioning stimuli on the sumo-2/3-ylation induced by subsequent harmful ischemia.

3. Results

In vitro modeled ischemia and tolerance

The *in vitro* modeled ischemia used in the present study is derived from that first presented by Goldberg and Choi [10]. Ischemia was modeled *in vitro* by subjecting 10–14 day old primary cortical neurons to either 30 or 120 minutes oxygen and glucose deprivation (OGD). When grown under these conditions, cell death is 5–10% as determined by propidium iodide exclusion assay [25]. Exposure of cultures to 120 minute OGD increases cell death (approximately 50–55% [25]), while 30 minute OGD did not (Figure 2b, lower panel). In addition, we observed that both 30 minute OGD and 120 minute OGD results in dramatic reductions in intracellular ATP levels (78% and 98% reductions, respectively; Supplementary Data Figure 2), consistent with the findings of other groups [14]. Delayed ischemic tolerance was induced using our

established model: Preconditioning cell cultures with 30 minute OGD protects against the harmful effects of subsequent 120 minute OGD 24 hours later [25,26,38].

Harmful, but not preconditioning ischemia increases high molecular weight sumo-2/3-ylation but not sumo-1-ylation

Primary cortical neuronal cultures were subjected to either 30 or 120 minutes OGD and harvested at 1, 4, 8 and 24 hours after re-oxygenation. Sumo-2/3 and Sumo-1 conjugation was monitored by immunoblotting of cell lysates. A significant increase in the levels of sumo-2/3 immunoreactive high molecular weight species (85–300 kDa) was observed after 120 minute OGD. The intensity of the 85+ region peaked during the first hour after the end of harmful 120 minute OGD (~350% of control) and was not significantly greater than control by 4 hours (Figure 1a and 1b). Subjecting cells to harmful 120 minute OGD resulted in a gradual reduction in sumo-1 immunoreactive high molecular weight species (85+ kDa) that was significant at 24 hours (Figure 1c and 1d). In contrast, sumo-2/3 conjugation did not increase at any time point after 30 minute OGD (Figure 1e and 1f), i.e., ischemic preconditioning does not activate sumo-2/3-ylation.

Preconditioning OGD reduces sumo-2/3-ylation after delayed harmful OGD

We used our established model of delayed ischemic tolerance whereby cells are preconditioned with 30 min OGD, 24 hours before being subject to harmful 120 min OGD. In the present study, 120 minutes OGD caused delayed lactate dehydrogenase (LDH) release associated with cell death (~300% of control), while 30 minute OGD did not, consistent with previous results [26]. Preconditioning cell cultures with 30 min OGD 24 hours prior to 120 minute OGD resulted in a 51% reduction in LDH release compared to 120 minute OGD treated cells (Figure 2c, lower panel). This is consistent with our previous ischemic tolerance studies [26].

We determined the effect of preconditioning ischemia on the sumoylation response to harmful ischemia. Sumo2/3-ylation was enhanced one hour following harmful ischemia (300% of control). Subjecting tolerant cells to 120 minute OGD resulted in a 43% reduction in sumo-2/3-ylation compared to subjecting non-tolerant cells to 120 minute OGD (Figure 2a and 2c). In contrast, preconditioning the cells with 30 minutes OGD 24 hours prior to harmful 120 minute OGD treatment did not block the reduction in sumo-1-ylation (Supplementary Data 2). Indeed, sumo-1 immunoreactivity of the 85+ region appeared lower in tolerant samples than in non-preconditioned samples, although there was not a significant difference between the groups (Supplementary Data 2). Since we did not observe a causal role of sumo-1 in ischemic tolerance, we focused our studies on sumo-2/3-ylation events. Therefore, ischemic preconditioning reduced both the sumo-2/3-ylation and cell death caused by subsequent harmful OGD.

Hypothermic preconditioning reduces sumo-2/3-ylation after delayed harmful OGD

We also investigated the effects of an additional preconditioning event, hypothermia. Previous studies show that hyperthermia enhances sumoylation of proteins [20]. Hypothermic preconditioning was induced by incubating cells at 4 °C for 30 min. This preconditioning event reduced LDH release by 62% compared to non-preconditioned 120 minute OGD treated cells (Figure 2d, lower panel).

Using this additional preconditioning model, we investigated protein sumo-2/3-ylation following harmful ischemia. Preconditioning with 30 minute hypothermia prior to 120 minute OGD resulted in a 54% reduction in sumo-2/3-ylation compared to 120 minute OGD treated cells (Figure 2b and 2d). Therefore, hypothermic preconditioning reduces both protein sumo-2/3-ylation and cell death caused by subsequent harmful OGD. This suggests that multiple preconditioning agents reprogram the sumo-2/3-ylation response to harmful ischemia.

Sumo-2/3-ylation is not blocked in the context of rapid ischemic tolerance

Previously published studies from this laboratory have shown that ischemic preconditioning one hour before harmful OGD reduces the cell death normally caused by harmful OGD, a phenomenon known as rapid ischemic tolerance [25]. Rapid ischemic tolerance was induced by preconditioning cell cultures with 30 minute OGD, one hour prior to harmful 120 minute OGD. This did not result in a reduction in sumo-2/3-ylation compared to 120 minute OGD treated cells (data not shown), indicating that sumo-2/3-ylation is not blocked by the induction of rapid ischemic tolerance. In addition, the sumo-2/3-ylation response to harmful ischemia was not reduced when hypothermia was used in a rapid ischemic tolerance paradigm (not shown).

Delayed ischemic tolerance against sumo-2/3-ylation is reduced by the protein synthesis inhibitor cycloheximide

We focused our experiments to study the effect of preconditioning ischemia on protein sumo-2/3-ylation, due to a lack of correlation seen with sumo-1-ylation levels and tolerance. Delayed ischemic tolerance is blocked by protein synthesis inhibitors, such as cycloheximide [1,26], which suggests that new protein synthesis is required for delayed ischemic tolerance. Cycloheximide (1.0 μ M) blocks protein biosynthesis *in vitro*, but does not induce neuroprotection at this concentration [7,31]. Preconditioned cultures were subjected to 30 minute OGD and then maintained in media with or without 1 μ M cycloheximide for 24 hours prior to 120 minute OGD. Control and 120 minute OGD cultures were also maintained in media with or without cycloheximide for 24 hours prior to treatment. Without cycloheximide, preconditioning with 30 minute OGD prior to 120 minute OGD resulted in a 45% reduction in sumo-2/3-ylation compared to 120 minute OGD treated cells (Figure 3a and 3b). However, cycloheximide completely prevents the reduction in sumo-2/3-ylation normally afforded by delayed ischemic tolerance (Figure 3a and 3b). These data indicate that the mechanism by which protein sumo-2/3-ylation is blocked in ischemic tolerance requires new protein synthesis.

Neither sumo conjugation enzymes UBA2 and UBC9, nor the SUMO specific isopeptidases SenP1–3 are changed following preconditioning ischemia

Our studies of protein sumoylation suggest that sumo-2/3-ylation occurs during the harmful ischemic challenge (Figure 4a and 4b). Sumo-2/3-ylation was observed from lysates prepared from cells subjected to 60 minutes OGD and harvested immediately without reoxygenation. Following 60 min OGD, ATP levels in the cells are depleted (Supplementary figure 2a). As such these data suggest that an effect of preconditioning ischemia would be evident when the cells are tolerant, because the sumoylation event during harmful ischemia is immediate. Hence, we investigated sumo conjugation and isopeptidase enzyme levels in tolerant cells 24 hours following preconditioning ischemia.

The process of sumo conjugation/de-conjugation has numerous potential regulation points. The enzymes responsible for the first two steps in the conjugation process are the E1- and E2-ligases, UBA2 and Ubc9. We conducted experiments to determine whether the blockage of sumoylation observed after ischemic preconditioning correlated with a reduction in the overall levels of these proteins. Cultures were exposed to 30 minutes preconditioning ischemia and harvested 24 hours later. As can be seen in Figure 4c and 4e, no reduction in the overall levels of UBA2 or Ubc9 is evident after preconditioning.

As a second point of regulation, if sumoylation levels are lower in tolerant cells, this may be due to more active de-conjugation. We investigated the expression of the sumo specific isopeptidases SenP1, SenP2 and SenP3 in tolerant cells 24 hours following preconditioning. As can be seen in Figure 4d and 4f, preconditioning did not significantly increase the expression

of SenP1, SenP2 or SenP3. Taken together, these data show that preconditioning cells with 30 min ischemia does not change the expression of sumo conjugation or de-conjugation associated proteins.

4. Discussion

Protein sumo-2/3-ylation induced by harmful ischemia is blocked in ischemic tolerance

The experiments in this study were conducted to determine whether sumoylation of proteins is a relevant mechanism of ischemic tolerance. We show that sumo-2/3-ylation of proteins change following harmful ischemia and that this effect is blocked in tolerant cells, which were subjected to preconditioning ischemia 24 hours earlier. Several recent publications have noted high molecular weight sumoylation, four in the context of ischemia [5,20,42,43] and four not directly related to ischemia [3,21,23,36]. Specifically, an increase in sumo2/3-ylation of high molecular weight proteins was observed (>85 kDa) in both focal and global models of ischemia in rodents [5,42,43]. In contrast, a recent study in arctic ground squirrels show that protein sumo-1- and sumo-2/3-ylation is increased in the torpor phase of hibernation, a natural ischemia tolerant state [20]. We did not find any evidence for sumo-1-ylation responses that correlated with preconditioning-induced neuroprotection. This is surprising in the context of the studies of Lee et. Al. [20], which also show that that sumo-1-ylation is associated with ischemic tolerance in neuroblastoma cells. Hence, our study would appear to be consistent with studies that show increased sumo2/3-ylation following harmful ischemia [5,42]. However, these studies did not clearly define whether the sumoylation events are a cause or consequence of the ischemia.

Our data suggest that protein sumo-2/3-ylation following harmful ischemia is a transient event. Indeed, sumoylation was detected in samples subject to as little as 60 minutes OGD, which correlated with a depletion of cellular ATP levels in our model. Following harmful ischemia (120 min OGD) sumo-2/3 conjugation dropped rapidly and was not significantly greater than control at 4 hours. Yang et. al. speculate that the abundance of free sumo-2/3 under resting conditions could relate to an important role in the cellular response to ischemic stress, i.e. the cell maintains a readily available pool of free sumo-2/3 for conjugation [43]. The rapid and transient nature of the conjugation that we observed during injurious ischemia is consistent with this idea, while further suggesting that sumo-2/3-ylation plays a role in the acute cellular response to harmful ischemia.

It is still not clear from these studies whether protein sumo-2/3-ylation is a protective response activated following exposure of the cells to harmful ischemia, or whether the conjugation of the proteins is a mechanism whereby cell death proceeds. Indeed, multiple biochemical pathways have been shown to be activated following harmful ischemia. The rapid nature of the sumoylation event during harmful ischemia is puzzling. It has been shown that reactive oxygen species induce protein sumoylation [3], but we observed an inability of anti-oxidant agents to block protein sumoylation (data not shown). A key barrier to this determination is that the identities of the sumoylated species are, as yet, unknown. Investigations into the nature of these sumoylated proteins are currently underway in this laboratory. However, in the absence of small molecule inhibitors, which selectively block the process of sumo-2/3-ylation, it is difficult to directly test whether blocking protein sumoylation would be beneficial or detrimental to ischemia induced brain injury (see foot note).

Preconditioning ischemia does not affect levels of sumo conjugating or de-conjugating enzymes

The process of sumo conjugation is reversible and highly dynamic. To date, it is unclear how these complex processes are altered in the context of ischemia, preconditioning and tolerance.

Our experiments did not reveal any change in levels of these enzymes at the time point when they would have directly influenced protein sumoylation in tolerant cells. However, a limitation of these experiments is that we only measured total protein levels, rather than enzyme activity. It has been reported that the increase in sumoylation seen during hibernation (a natural tolerant state) in ground squirrels correlates with increased expression levels of Ubc9, while levels of SenP-1 did not change [20]. However, as part of a study involving transient global ischemia, no change in the mRNA levels of Ubc9 occurred after ischemia, but a drop in Ubc9 protein levels was observed [43]. The authors speculate that sumo de-conjugation may be the point of control of sumoylation following ischemia, i.e. harmful ischemia reduces SENP activity, leading to increased stability of sumoylated proteins, although data from our experiments suggest no change in isopeptidase expression levels. Hence it is not yet clear how the preconditioning stimuli reduces protein sumoylation in response to harmful ischemia.

There are some technical differences between our study and other studies in ischemia. We have not used heat treatment of our sample or an alkylating agent to reduce isopeptidase activity in our samples [5,20,42,43]. When experiments were performed with heat treated samples, we find that the magnitude of change of protein sumoylation was very small (see Supplementary Figure1). As such, we did not use heat treatment or NEN to block isopeptidases in our samples. This raises an interesting question regarding the nature of the sumoylation event. First, it is unclear whether the proteins are modified by sumo-2 or sumo-3, as the antibody recognizes both species. Second, by not blocking the isopeptidases, this suggests that the protein sumoylation we are measuring may be isopeptidase-resistant. Whether this represents protein mono-sumoylation or perhaps a poly-sumoylation event [12,13] is not yet clear, but is currently under investigation.

The role of sumoylation in ischemia and ischemic tolerance

The present work adds to the growing body of literature linking sumoylation events and the cellular response to ischemia. The covalent attachment of sumo to multiple cellular targets leads to a wide variety of effects, often mediated by distinct cellular pathways operating within distinct timeframes. Immunocytochemical studies indicate that sumo-2/3 immunoreactive species move to the nucleus following harmful ischemia [42]. Given that numerous examples exist of sumo conjugation altering the activity and localization of transcription factors [15, 24,32,41], it is plausible to suggest that the sumo-2/3-ylation induced by harmful ischemia regulates a pattern of gene expression and that this pattern of gene expression is changed in the tolerant state. This hypothesis would be consistent with the differences in gene expression between harmful ischemia and ischemic tolerance [38]. However, the identities of the sumo-2/3-ylated species have not been established and it is therefore unknown whether they are directly altering transcription or gene expression patterns.

Conclusion

The present study indicates that multiple preconditioning paradigms, which result in delayed ischemic tolerance, blocks the sumo-2/3-ylation event following harmful ischemia. The effect of preconditioning requires the synthesis of new proteins. The mechanism by which blockade of sumo-2/3-ylation occurs in ischemic tolerance remains to be determined, but does not involve regulation of E1- and-E2 ligase, or isopeptidase protein levels. The methods developed in the present study for manipulating the sumo-2/3-ylation response to ischemia represent a platform for understanding the cellular pathways linking ischemia to sumo conjugation. However, whether sumoylation is beneficial or detrimental to cell outcome following harmful ischemia is still not clear. The identification of sumoylation targets and regulatory mechanisms as well as specific sumo inhibitors will help reveal the role of sumo in ischemia.

Footnote

Since the preparation of this manuscript, it was reported that sumoylation was observed following preconditioning ischemia in mouse and rat embryonic cultures (5–7 DIV) [19]. In addition, overexpression of sumo-1 and sumo-2 resulted in reduced cell death following an ischemic challenge. However, methodological differences exist between this manuscript and the Lee study, which may account for the differences in interpretation of the sumoylation response of the cells to ischemia.

4. Experimental Procedure

Cortical Cell Culture

Sprague-Dawley rat pups were used to prepare cortical neuronal cultures as previously described [27]. Briefly, cortices were dissected from 10–12 rat pups (P1–2) and enzymatically dissociated with papain (Worthington Biochemicals, Lakewood, NJ). Cells were plated at a density of 3.5×10^6 cells/3.5 cm culture dish (Primara; BD Biosciences, San Jose, CA) in Neurobasal-A/B27 medium (Invitrogen) for 10–14 days.

Ischemic Challenges

Oxygen and glucose deprivation (OGD) was performed by washing the cells three times with phosphate-buffered saline (NaCl (1.37 mM), KCl (2.7 mM), Na_2HPO_4 (10 mM), KH_2PO_4 (1.7 mM), pH 7.4) supplemented with 0.5 mM CaCl_2 , 1.0 mM MgCl_2 and placing culture dishes in an anaerobic chamber for 30 or 120 minute (Forma Scientific, Marjetta, OH) (85% N_2 , 5% H_2 , 10% CO_2 ; 35 °C) as previously described [26,29]. Anaerobic conditions in the chamber were monitored using Gaspack anaerobic indicator strips (BD Biosciences, San Jose, CA). OGD was terminated by removing cells from the anoxia chamber, replenishing with Neurobasal-A medium, and replacing them in the normoxic incubator. Cultured cells were subjected to the following treatments: (1) Control cells had their Neurobasal-A/B27 medium removed and replaced by Neurobasal-A medium without B27 supplement at the same time that OGD treatment ended for test cells; (2) Some cells were subjected to either 30 or 120 minute OGD and allowed to recover in Neurobasal-A medium for 1, 4, 8 or 24 hours; (3) Some cells were preconditioned with 30 minute OGD (preconditioning ischemia) and allowed to recover for 24 hours in Neurobasal-A medium prior to 120 minute OGD (harmful ischemia); (4) Some cells were preconditioned with 30 minute hypothermia (4°C Neurobasal-A medium was added directly prior to the culture being placed in a 4°C environment in air) and then recovered for 24 hours in Neurobasal-A medium prior to 120 minute OGD; (5) Some cells were subjected to 120 minute hypothermia and allowed to recover in Neurobasal-A medium for 1 hour; (6) Some cells were preconditioned with 30 minute OGD (preconditioning ischemia) and allowed to recover for 24 hours in Neurobasal A medium supplemented with 1 μM cycloheximide prior to 120 minute OGD (harmful ischemia). Each experimental dataset was gathered using “sister” cultures. Cultures were harvested for immunoblotting as described below, at the timepoints indicated in the results section. Cell death analysis by LDH assay (Roche Applied Science, Indianapolis, IN) was performed 24 hours following the final OGD challenge.

Immunoblotting

Immunoblotting was performed as previously described [28]. Briefly, tissue samples were lysed in a nondenaturing buffer containing 0.5% Nonidet P-40, the protease inhibitors phenylmethylsulfonyl fluoride (100 $\mu\text{g}/\text{ml}$), aprotinin (1 $\mu\text{g}/\text{ml}$), leupeptin (1 $\mu\text{g}/\text{ml}$) and pepstatin (1 $\mu\text{g}/\text{ml}$) prior to storage at -80°C .

Many sumo conjugates are highly sensitive to isopeptidase degradation under native conditions [5]. Heat treatment directly after cell lysis or use of the inhibitor N-ethylmaleimide to inactivate these enzymes is standard practice when working with sumoylated proteins [5]. In order to

determine appropriate treatment for the lysates, we performed preliminary experiments using heat treatment of cell lysates (95°C for 5 minutes directly after lysis) or not heat-treating cell lysates on sumo-2/3 conjugation (see Supplementary Figure 1). Heat treatment enhanced the stability of all sumo conjugates. However, the difference between treatment groups was substantially reduced when heat-treated samples were analyzed (supplementary figure one). Therefore, all data except that in Supplementary Data Figure 1 are for non-heat-treated samples.

Protein concentration was determined by Bradford reagent spectrophotometrically at A₅₉₅. Protein samples (50 µg) were denatured in a gel-loading buffer at 100 °C for 5 minutes and then loaded onto 8% SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes at 30 V overnight and incubated with primary antibodies at room temperature for 2 hours. The sumo-2/3 antibody used was a gift from J. Hallenbeck, NINDS/NIH Bethesda, MD, USA. UBA2 antibody was provided by Lifespan Biosciences, Seattle, WA, USA. Ubc9 antibody was provided by Santa Cruz Biotechnology, Santa Cruz, CA, USA. Membranes were incubated with anti-rabbit or anti-goat IgG conjugated to horseradish peroxidase (Cell Signaling, Danvers, MA or Santa Cruz Biochemical, Santa Cruz CA) followed by chemiluminescence detection (Upstate, Charlottesville, VA) and digitally imaged using Kodak Image Station 2000RT, (Kodak, Rochester, NY). Alpha-tubulin reprobes were conducted to confirm equal protein loading. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, St. Louis, MO.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) or two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test or Dunnett's test for multiple comparisons to a single control group. All analyses were performed using Graphpad Prism v. 4.0 statistical software. Statistical significance was accepted at P<0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health/ NINDS Grants NS050669 and NS054023 (R.M.) and NS024728 (R.P.S.). We thank Dr. John Hallenbeck (NINDS/ NIH Bethesda, MD, USA) for the gift of sumo-1 and sumo-2/3 antibodies, and for advice and input. We wish to thank Kristin McCarthy for her assistance with preparing the manuscript.

References

1. Barone FC, White RF, Spera PA, Ellison J, Currie RW, Wang X, Feuerstein GZ. Ischemic preconditioning and brain tolerance: temporal histological and functional outcomes, protein synthesis requirement, and interleukin-1 receptor antagonist and early gene expression. *Stroke* 1998;29:1937–1950. [PubMed: 9731622]discussion 1950-1931
2. Bohren KM, Nadkarni V, Song JH, Gabbay KH, Owerbach D. A M55V polymorphism in a novel SUMO gene (SUMO-4) differentially activates heat shock transcription factors and is associated with susceptibility to type I diabetes ,mellitus. *J Biol Chem* 2004;279:27233–27238. [PubMed: 15123604]
3. Bossis G, Melchior F. Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. *Mol Cell* 2006;21:349–357. [PubMed: 16455490]
4. Carey HV, Andrews MT, Martin SL. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiol Rev* 2003;83:1153–1181. [PubMed: 14506303]
5. Cimarosti H, Lindberg C, Bomholt SF, Ronn LC, Henley JM. Increased protein SUMOylation following focal cerebral ischemia. *Neuropharmacology* 2008;54:280–289. [PubMed: 17991493]

6. Dirnagl U, Simon RP, Hallenbeck JM. Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci* 2003;26:248–254. [PubMed: 12744841]
7. Furukawa K, Estus S, Fu W, Mark RJ, Mattson MP. Neuroprotective action of cycloheximide involves induction of bcl-2 and antioxidant pathways. *J Cell Biol* 1997;136:1137–1149. [PubMed: 9060477]
8. Geiss-Friedlander R, Melchior F. Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol* 2007;8:947–956. [PubMed: 18000527]
9. Gidday JM. Cerebral preconditioning and ischaemic tolerance. *Nat Rev Neurosci* 2006;7:437–448. [PubMed: 16715053]
10. Goldberg MP, Choi DW. Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. *J Neurosci* 1993;13:3510–3524. [PubMed: 8101871]
11. Gonzalez-Zulueta M, Feldman AB, Klesse LJ, Kalb RG, Dillman JF, Parada LF, Dawson TM, Dawson VL. Requirement for nitric oxide activation of p21(ras)/extracellular regulated kinase in neuronal ischemic preconditioning. *Proc Natl Acad Sci U S A* 2000;97:436–441. [PubMed: 10618436]
12. Green LM, Wagner KJ, Campbell HA, Addison K, Roberts SG. Dynamic interaction between WT1 and BASP1 in transcriptional regulation during differentiation. *Nucleic Acids Res* 2009;37:431–440. [PubMed: 19050011]
13. Hay RT. SUMO: a history of modification. *Mol Cell* 2005;18:1–12. [PubMed: 15808504]
14. Iijima T, Mishima T, Tohyama M, Akagawa K, Iwao Y. Mitochondrial membrane potential and intracellular ATP content after transient experimental ischemia in the cultured hippocampal neuron. *Neurochem Int* 2003;43:263–269. [PubMed: 12689606]
15. Kishi A, Nakamura T, Nishio Y, Maegawa H, Kashiwagi A. Sumoylation of Pdx1 is associated with its nuclear localization and insulin gene activation. *Am J Physiol Endocrinol Metab* 2003;284:E830–E840. [PubMed: 12488243]
16. Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, et al. 'Ischemic tolerance' phenomenon found in the brain. *Brain Res* 1990;528:21–24. [PubMed: 2245337]
17. Knipscheer P, Dijk WJvan, Olsen JV, Mann M, Sixma TK. Noncovalent interaction between Ubc9 and SUMO promotes SUMO chain formation. *Embo J* 2007;26:2797–2807. [PubMed: 17491593]
18. Lee GW, Melchior F, Matunis MJ, Mahajan R, Tian Q, Anderson P. Modification of Ran GTPase-activating protein by the small ubiquitin-related modifier SUMO-1 requires Ubc9 an E2-type ubiquitin-conjugating enzyme homologue. *J Biol Chem* 1998;273:6503–6507. [PubMed: 9497385]
19. Lee YJ, Castri P, Bembry J, Maric D, Auh S, Hallenbeck JM. SUMOylation participates in induction of ischemic tolerance. *J Neurochem*. 2009
20. Lee YJ, Miyake S, Wakita H, McMullen DC, Azuma Y, Auh S, Hallenbeck JM. Protein SUMOylation is massively increased in hibernation torpor and is critical for the cytoprotection provided by ischemic preconditioning and hypothermia in SHSY5Y cells. *J Cereb Blood Flow Metab* 2007;27:950–962. [PubMed: 16955077]
21. Li T, Santockyte R, Shen RF, Tekle E, Wang G, Yang DC, Chock PB. Expression of SUMO-2/3 induced senescence through p53- and pRB-mediated pathways. *J Biol Chem* 2006;281:36221–36227. [PubMed: 17012228]
22. Mahajan R, Gerace L, Melchior F. Molecular characterization of the SUMO-1 modification of RanGAP1 and its role in nuclear envelope association. *J Cell Biol* 1998;140:259–270. [PubMed: 9442102]
23. Manza LL, Codreanu SG, Stamer SL, Smith DL, Wells KS, Roberts RL, Liebler DC. Global shifts in protein sumoylation in response to electrophile and oxidative stress. *Chem Res Toxicol* 2004;17:1706–1715. [PubMed: 15606148]
24. Mauri F, McNamee LM, Lunardi A, Chiacchiera F, Del Sal G, Brodsky MH, Collavin L. Modification of Drosophila p53 by SUMO modulates its transactivation and pro-apoptotic functions. *J Biol Chem* 2008;283:20848–20856. [PubMed: 18492669]
25. Meller R, Cameron JA, Torrey DJ, Clayton CE, Ordonez AN, Henshall DC, Minami M, Schindler CK, Saugstad JA, Simon RP. Rapid degradation of Bim by the ubiquitin-proteasome pathway

- mediates short-term ischemic tolerance in cultured neurons. *J Biol Chem* 2006;281:7429–7436. [PubMed: 16431916]
26. Meller R, Minami M, Cameron JA, Impey S, Chen D, Lan JQ, Henshall DC, Simon RP. CREB-mediated Bcl-2 protein expression after ischemic preconditioning. *J Cereb Blood Flow Metab* 2005;25:234–246. [PubMed: 15647742]
 27. Meller R, Schindler CK, Chu XP, Xiong ZG, Cameron JA, Simon RP, Henshall DC. Seizure-like activity leads to the release of BAD from 14-3-3 protein and cell death in hippocampal neurons in vitro. *Cell Death Differ* 2003;10:539–547. [PubMed: 12728252]
 28. Meller R, Skradski SL, Simon RP, Henshall DC. Expression proteolysis and activation of caspases 6 and 7 during rat C6 glioma cell apoptosis. *Neurosci Lett* 2002;324:33–36. [PubMed: 11983288]
 29. Meller R, Stevens SL, Minami M, Cameron JA, King S, Rosenzweig H, Doyle K, Lessov NS, Simon RP, Stenzel-Poore MP. Neuroprotection by osteopontin in stroke. *J Cereb Blood Flow Metab* 2005;25:217–225. [PubMed: 15678124]
 30. Mikolajczyk J, Drag M, Bekes M, Cao JT, Ronai Z, Salvesen GS. Small ubiquitin-related modifier (SUMO)-specific proteases: profiling the specificities and activities of human SENPs. *J Biol Chem* 2007;282:26217–26224. [PubMed: 17591783]
 31. Neve KA, Molinoff PB. Turnover of beta 1- and beta 2-adrenergic receptors after down-regulation or irreversible blockade. *Mol Pharmacol* 1986;30:104–111. [PubMed: 3016496]
 32. Oishi Y, Manabe I, Tobe K, Ohsugi M, Kubota T, Fujiu K, Maemura K, Kubota N, Kadowaki T, Nagai R. SUMOylation of Kruppel-like transcription factor 5 acts as a molecular switch in transcriptional programs of lipid metabolism involving PPAR-delta. *Nat Med* 2008;14:656–666. [PubMed: 18500350]
 33. Okuma T, Honda R, Ichikawa G, Tsumagari N, Yasuda H. In vitro SUMO-1 modification requires two enzymatic steps, E1 and E2. *Biochem Biophys Res Commun* 1999;254:693–698. [PubMed: 9920803]
 34. Owerbach D, McKay EM, Yeh ET, Gabbay KH, Bohren KM. A proline-90 residue unique to SUMO-4 prevents maturation and sumoylation. *Biochem Biophys Res Commun* 2005;337:517–520. [PubMed: 16198310]
 35. Raval AP, Dave KR, DeFazio RA, Perez-Pinzon MA. epsilonPKC phosphorylates the mitochondrial K(+) (ATP) channel during induction of ischemic preconditioning in the rat hippocampus. *Brain Res* 2007;1184:345–353. [PubMed: 17988655]
 36. Rizos H, Woodruff S, Kefford RF. p14ARF interacts with the SUMO-conjugating enzyme Ubc9 and promotes the sumoylation of its binding partners. *Cell Cycle* 2005;4:597–603. [PubMed: 15876874]
 37. Simon RP, Niuro M, Gwinn R. Prior ischemic stress protects against experimental stroke. *Neurosci Lett* 1993;163:135–137. [PubMed: 8309618]
 38. Stenzel-Poore MP, Stevens SL, Xiong Z, Lessov NS, Harrington CA, Mori M, Meller R, Rosenzweig HL, Tobar E, Shaw TE, Chu X, Simon RP. Effect of ischaemic preconditioning on genomic response to cerebral ischaemia: similarity to neuroprotective strategies in hibernation and hypoxia-tolerant states. *Lancet* 2003;362:1028–1037. [PubMed: 14522533]
 39. Storey KB. Mammalian hibernation. Transcriptional and translational controls. *Adv Exp Med Biol* 2003;543:21–38. [PubMed: 14713112]
 40. Su HL, Li SS. Molecular features of human ubiquitin-like SUMO genes and their encoded proteins. *Gene* 2002;296:65–73. [PubMed: 12383504]
 41. Tsuruzoe S, Ishihara K, Uchimura Y, Watanabe S, Sekita Y, Aoto T, Saitoh H, Yuasa Y, Niwa H, Kawasuji M, Baba H, Nakao M. Inhibition of DNA binding of Sox2 by the SUMO conjugation. *Biochem Biophys Res Commun* 2006;351:920–926. [PubMed: 17097055]
 42. Yang W, Sheng H, Warner DS, Paschen W. Transient focal cerebral ischemia induces a dramatic activation of small ubiquitin-like modifier conjugation. *J Cereb Blood Flow Metab* 2008;28:892–896. [PubMed: 18167501]
 43. Yang W, Sheng H, Warner DS, Paschen W. Transient global cerebral ischemia induces a massive increase in protein sumoylation. *J Cereb Blood Flow Metab* 2008;28:269–279. [PubMed: 17565359]

Figure 1. 120 minute OGD causes a significant increase in high molecular weight sumo-2/3 conjugation

A. Representative sumo-2/3 immunoblot from 30 minute OGD/hypothermia experiment. Control cultures (Con) were harvested 24 hours after treatment. Hypothermia cultures (H) were harvested 1 hour after treatment, while 30 minute OGD cultures were harvested at 1, 4, 8 and 24 hours. **B.** Representative sumo-2/3 immunoblot from 120 minute OGD/hypothermia experiment. Control cultures (Con) were harvested 24 hours after treatment. Hypothermia cultures (H) were harvested 1 hour after treatment, while 120 minute OGD cultures were harvested at 1, 4, 8 and 24 hours. **C.** Quantification of sumo-2/3 intensities of the 85+ kDa region from 30 and 120 minute OGD experiments. Normalization was carried out against the

intensity of controls, the error bars are standard deviation, *** $P < 0.001$, two-way ANOVA with *post-hoc* Bonferroni compared to control. **D.** Representative sumo-1 immunoblot from 120 minute OGD/hypothermia experiment. Control cultures (Con) were harvested 24 hours after treatment. Hypothermia cultures (H) were harvested 1 hour after treatment, while 120 minute OGD cultures were harvested at 1, 4, 8 and 24 hours. **E.** Quantification of sumo-1 intensities of the 85+ kDa region from 120 minute OGD experiments (* denotes $P < 0.05$). All data are the result of 4 to 5 independent experiments.

Figure 2. Ischemic preconditioning and hyperthermic preconditioning reduces sumo-2/3-ylation and cell death after harmful ischemia

A. Representative sumo-2/3 immunoblot from ischemic preconditioning experiment. Control cultures and cultures subjected to 30 or 120 minute OGD were harvested 1 hour after treatment. Preconditioned cultures were subjected to 30 minute OGD 24 hours prior to 120 minute OGD and harvested 1 hour later. **B.** Representative sumo-2/3 immunoblot from hyperthermic preconditioning experiment. (The dotted lines are indicative of the fact that this image is a rearranged composite created from a single immunoblot, for reasons of clarity and correspondence with Figure 2A. Any adjustments to brightness or contrast were performed on the entire image prior to clipping.) Control cultures and cultures subjected to 120 minute OGD

or 30 minute hypothermia were harvested 1 hour after treatment. Preconditioned cultures were subjected to 30 minute hypothermia 24 hours prior to 120 minute OGD and harvested 1 hour later. **C.** Upper panel, quantification of sumo-2/3 intensities of the 85+ kDa region after preconditioning experiments. All cultures harvested 1 hour after treatments. Lower panel, LDH release 24 hours after preconditioning experiments. Normalization was carried out against control values, the error bars are standard deviation. ** $P < 0.01$, *** $P < 0.001$ compared to control, # $P < 0.05$, ## $P < 0.01$ compared to 120 minute OGD, one-way ANOVA with *post hoc* Bonferroni's test. **D.** Upper panel, quantification of sumo-2/3 intensities of the 85+ kDa region after preconditioning experiments. All cultures harvested 1 hour after treatments. Lower panel, LDH release 24 hours after preconditioning experiments. Normalization was carried out against control values, the error bars are standard deviation. ** $P < 0.01$, *** $P < 0.001$ compared to control, ## $P < 0.01$, ### $P < 0.001$ compared to 120 minute OGD, one-way ANOVA with *post-hoc* Bonferroni's test. All data are the result of 4 to 5 independent experiments.

Figure 3. Protein synthesis inhibition by cycloheximide prevents the reduction of sumo-2/3-ylation by ischemic preconditioning

A. Representative sumo-2/3 immunoblot from cycloheximide/ischemic preconditioning experiment. Cycloheximide group: Control cultures and cultures subjected to 120 minute OGD were maintained in media containing 1 μ M cycloheximide for 24 hours prior to treatment. Preconditioned cultures were subjected to 30 minute OGD and maintained in media with 1 μ M cycloheximide for 24 hours prior to 120 minute OGD. All cultures were harvested 1 hour after treatment. No cycloheximide group: Identical procedures, but without cycloheximide. **B.** Quantification of sumo-2/3 intensities of the 85+ kDa region after cycloheximide/preconditioning experiments. All cultures harvested 1 hour after treatments. Normalization

was carried out against control values, the error bars are standard deviation. * $P < 0.05$, *** $P < 0.001$ compared to control, # $P < 0.05$ compared to 120 without cycloheximide, ++ $P < 0.01$ compared to 30, 120 without cycloheximide, oneway ANOVA with *post-hoc* Bonferroni's test. All data are the result of 4 to 5 independent experiments.

Figure 4. Rapid changes in protein sumoylation following ischemia and preconditioning does not reduce the levels of sumo conjugating and de-conjugating enzymes

A. Representative sumo-2/3 immunoblot from OGD/recovery time course experiment (non-heat-treated tissue). Control cultures were harvested 60 minutes after treatment. OGD cultures were harvested after 30, 60, 90, or 120 minute OGD. Recovery cultures were replenished with Neurobasal A medium at the end of 120 minute OGD and harvested 30 or 60 minutes later. **B.** Quantification of sumo-2/3 intensities of the 85+ kDa region from OGD/recovery time course experiments. Normalization was carried out against the intensity of controls, the error bars are standard deviation, * $P < 0.05$, ** $P < 0.01$, one-way ANOVA *post hoc* with Dunnett's test compared to control. All data are the result of 4 to 5 independent experiments. **C.**

Representative Ubc9 and UBA2 immunoblots from ischemic preconditioning experiment. Control cultures and cultures subjected to 30 minute preconditioning OGD were harvested 24 hours after treatment. Alpha tubulin is shown as a loading control. **D.** Representative SenP1, SenP2 and SenP3 immunoblots from ischemic preconditioning experiment. Control cultures and cultures subjected to 30 minute preconditioning OGD were harvested 24 hours after treatment. **E.** Quantification of Ubc9 levels and UBA2 levels from ischemic preconditioning experiment. Normalization was carried out against the intensity of controls, the error bars are standard deviation, data were tested for significance using two-way ANOVA with *post hoc* Bonferroni's test compared to control. All data are the result of 3 or 4 independent experiments. **F.** Quantification of SenP1, SenP2 and SenP3 levels from ischemic preconditioning

experiment. Normalization was carried out against the intensity of controls, the error bars are standard deviation. Data were tested for significance using two-way ANOVA with *post hoc* Bonferroni's test compared to control. All data are the result of 3 independent experiments.