Analysis of Oxygen/Glucose-Deprivation-Induced Changes in SUMO3 Conjugation Using SILAC-Based Quantitative Proteomics

Wei Yang,* J. Will Thompson,* Zhengfeng Wang,*† Liangli Wang,* Huaxin Sheng,* Matthew W. Foster,* M. Arthur Moseley,* and Wulf Paschen‡,*

‡Department of Anesthesiology, Multidisciplinary Neuroprotection Laboratories, †Proteomics Core Facility, Duke Institute for Genome Science and Policy, and *Pulmonary, Allergy, and Critical Care Medicine, Duke University Medical Center, Durham, North Carolina 28810, United States

†Department of Surgery, The First Affiliate Hospital of Zhengzhou University, Zhengzhou, China

Supporting Information

ABSTRACT: Transient cerebral ischemia dramatically activates small ubiquitin-like modifier (SUMO2/3) conjugation. In cells exposed to 6 h of transient oxygen/glucose deprivation (OGD), a model of ischemia, SUMOylation increases profoundly between 0 and 30 min following re-oxygenation. To elucidate the effect of transient OGD on SUMO conjugation of target proteins, we exposed neuroblastoma B35 cells expressing HA-SUMO3 to transient OGD and used stable isotope labeling with amino acids in cell culture (SILAC) to quantify OGD-induced changes in levels of specific SUMOylated proteins. Lysates from control and OGD-treated cells were mixed equally, and HA-tagged proteins were immunoprecipitated and analyzed by 1D-SDS-PAGE—LC—MS/MS. We identified 188 putative SUMO3-conjugated proteins, including numerous transcription factors and coregulators, and PIAS2 and PIAS4 SUMO ligases, of which 22 were increased or decreased more than ±2-fold. In addition to SUMO3, the levels of protein-conjugated SUMO1 and SUMO2, as well as ubiquitin, were all increased. Importantly, protein ubiquitination induced by OGD was completely blocked by gene silencing of SUMO2/3. Collectively, these results suggest several mechanisms for OGD-modulated SUMOylation, point to a number of signaling pathways that may be targets of SUMO-based signaling and recovery from ischemic stress, and demonstrate a tightly controlled crosstalk between the SUMO and ubiquitin conjugation pathways.

KEYWORDS: cerebral ischemia, oxygen/glucose deprivation, quantitative proteomics, SILAC, small ubiquitin-like modifier, stress response, SUMOylation, ubiquitin conjugation

INTRODUCTION

Small ubiquitin-like modifier (SUMO1−3) are ubiquitin-related proteins that post-translationally modify lysine residues of target proteins in a process similar to ubiquitin conjugation, which involves SUMO-specific activating, conjugating, and ligating enzymes.1 SUMO2 and SUMO3 proteins share about 95% sequence identity. Since available antibodies cannot distinguish between SUMO2 and SUMO3, these SUMO paralogues are usually referred to as SUMO2/3. SUMOylation is a highly reversible process as SUMO-conjugated proteins are rapidly deconjugated by sentrin-specific proteases (SENP).2,3 SUMO conjugation has been shown to modulate stability, activity, and subcellular localization of proteins.1,2,4,5 A large number of SUMOylated proteins are transcription factors and other nuclear proteins involved in gene expression and genome stability.6,7 Furthermore, many SUMO target proteins have been identified in neurons that are cytosolic or cell membrane proteins.8 Therefore, any substantial change in levels of SUMO-conjugated proteins can be expected to have a major impact on the fate of cells.

SUMO conjugation is activated in various stress conditions, including hypoxia, hypo-/hyperthermia, and oxidative stress.9 The SUMOylation pathway is massively activated in hibernating animals during the torpor state when the body temperature drops to about 5 °C.10 During hibernation torpor, cerebral blood flow is reduced to below detection levels, but neurons are not damaged as they would be by an episode of transient normothermic ischemia.11 Since protein synthesis is almost completely suppressed during hibernation torpor,12 it is postulated that SUMO conjugation is a protective stress response shielding neurons from damage induced by transient ischemia.10 We and others have shown that SUMO2/3 conjugation is also sharply activated after global and focal cerebral ischemia and during deep hypothermic cardiopulmonary bypass.13–16 Further suggesting that SUMO2/3 conjugation is important for neuroprotective stress responses. After transient focal cerebral ischemia, levels of SUMO2/3-conjugated proteins are particularly high in neurons.

Received: August 27, 2011
Published: November 14, 2011
located at the border of the ischemic territory, and we have demonstrated that a short nonlethal duration of vascular occlusion is sufficient to activate this process. Furthermore, we found that SUMO2/3 conjugation protects neuronal cultures from transient oxygen/glucose deprivation (OGD)-induced damage. It is therefore of key clinical interest to identify proteins that are SUMOylated after ischemia in order to better understand the significance of this process for the fate of post-ischemic cells.

OGD is a widely used experimental approach to model the severe form of metabolic stress triggered by transient cerebral ischemia in vivo, and has been previously used to investigate the role of SUMO conjugation in ischemic cell death. Here, we sought a tractable system for investigating post-ischemic activation of SUMO conjugation of target proteins. Due to the low abundance and difficulty of enriching for endogenous SUMOylated proteins, we utilized neuroblastoma B35 cells stably expressing mouse HA-tagged SUMO3 and took advantage of a stable isotope labeling with amino acids in cell culture (SILAC) approach to quantify OGD-dependent changes in SUMO conjugation. The result is the first proteomic study to investigate the changes in SUMO conjugation in cells exposed to ischemia-like conditions.

**EXPERIMENTAL SECTION**

**Cell Culture and Transfection**

Experiments were performed on neuroblastoma B35 cells (courtesy of Dr. P. F. Maness, University of North Carolina, Chapel Hill, NC, USA). HA-tagged mouse SUMO3 expression vector was generated by cloning SUMO3 cDNA derived from mouse mRNA into pcDNA3-HA vector (Invitrogen). After verification of construct by DNA sequencing, B35 cells were stably transfected with HA-SUMO3 expression vector using Geneticin (500 μg/mL) for selection. For SILAC analyses, cells were cultured in lysine- and arginine-deficient DMEM (Pierce) containing 10% dialyzed FBS (Sigma) and 1% penicillin/streptomycin/fungizone (Invitrogen), supplemented with 10 μg/L β-glycerophosphate, 1 mM EDTA, 5 μg/mL L trypsin (Promega). Digestion was carried out overnight at 37 °C for reduction at 80 °C and finally swelled in AmBic containing 10 mM dithiothreitol (Sigma) for reduction at 80 °C for 30 min, followed by alkylation with 20 mM iodoacetamide (Sigma) at room temperature for 20 min in the dark. Gel pieces were then taken through two shrink/swell cycles alternating acetonitrile (Fisher Scientific) and AmBic and finally swelled in AmBic containing 10 ng/μL trypsin (Promega). Digestion was carried out overnight at 37 °C and was quenched, and peptides were extracted using 0.1% v/v TFA in 1:1 MeCN/water. Samples were dried and reconstituted in 10 μL 1:2:97 v/v/v TFA/MeCN/water for mass spectrometry analysis.

**Oxygen/Glucose Deprivation (OGD) and Protein Extraction**

Cultures were exposed to OGD for 6 or 8 h using an anoxic chamber (Forma Scientific Anaerobic System). Glucose-free balanced salt solution (BSS) (116 mM NaCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 5.4 mM KCl, 1 mM NaH2PO4, 14.7 mM NaHCO3, and 10 mM HEPES, pH7.4) was equilibrated over-balanced salt solution (BSS) (116 mM NaCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 5.4 mM KCl, 1 mM NaH2PO4, 14.7 mM NaHCO3, and 10 mM HEPES, pH7.4) was equilibrated over-

**Western Blotting Analysis**

Western blotting was performed using SDS-PAGE gels (Bio-Rad). Proteins were transferred to PVDF membranes (Bio-Rad), and membranes were blocked for 1 h in Tris-buffered saline solution supplemented with 0.1% Tween 20 (TBST) and 5% skim milk powder and incubated with the first antibody for 16 h at 4 °C. Membranes were then washed and incubated with goat anti-rabbit or -mouse horseradish peroxidase conjugates (Santa Cruz Biotechnology) for 1 h at room temperature. Proteins were visualized using the ECL Western blot analysis system (GE Healthcare). Antibodies used in this study include anti-SUMO2/3 polyclonal antibody (Covance), anti-HA polyclonal antibody (Cell Signaling), anti-ubiquitin monoclonal antibody (Cell Signaling), anti-TIF1β polyclonal antibody (Cell Signaling), and anti-RUNX1 polyclonal antibody (Novus Biologicals). A monoclonal antibody against β-actin (dilution 1:5000; Sigma) was used as loading control.

**Proteomic Analysis**

Five 10-cm dishes of cells were used per group. Fifteen milligrams of protein from control (light) and post-OGD (heavy) cultures were mixed, and HA-SUMO3-conjugated proteins were immunoprecipitated using monoclonal anti-HA agarose (Sigma). Beads were washed, and HA-tagged SUMO3-conjugated proteins were eluted by incubating beads with PBS buffer containing 100 μg/mL HA peptide (Sigma). Proteins were precipitated with acetone, and precipitates were dissolved in SDS loading buffer and separated approximately 1 cm on a SDS-PAGE 4–12% gel (Invitrogen). The gel was stained briefly with colloidal Coomassie (Invitrogen), and the protein-containing region was dissected into 5 adjacent slices. The excised gel slices were destained, and the proteins in the slices were reduced, alkylated, and digested with trypsin according to the “In-Gel Tryptic Digestion Protocol” available at (http://www.genome.duke.edu/cores/proteomics/sample-preparation/). Briefer, slices were destained with 1:1 MeCN/water, then dehydrated in MeCN, and swelled in 50 mM ammonium bicarbonate (AmBic) containing 10 mM dithiothreitol (Sigma) for reduction at 80 °C for 30 min, followed by alklylation with 20 mM iodoacetamide (Sigma) at room temperature for 20 min in the dark. Gel pieces were then taken through two shrink/swell cycles alternating acetonitrile (Fisher Scientific) and AmBic and finally swelled in AmBic containing 10 ng/μL trypsin (Promega). Digestion was carried out overnight at 37 °C and was quenched, and peptides were extracted using 0.1% v/v TFA in 1:1 MeCN/water. Samples were dried and reconstituted in 10 μL 1:2:97 v/v/v TFA/MeCN/water for mass spectrometry analysis.

**Analysis of Cell Death**

The extent of OGD-induced cell death was evaluated by measuring the release of LDH from cells using the LDH Cytoxicity Detection Kit (Clontech). The extent of cell death was calculated by relating LDH released from cells to total LDH activity.
SUMO Protease Treatment
To identify bona fide SUMO-conjugated proteins, HA-tagged SUMO3-conjugated proteins from the same amount of extracts of light control and heavy OGD cultures (6 h OGD with 30 min re-oxygenation) were separately immunoprecipitated using monoclonal anti-HA agarose (Sigma). Eluates from individual OGD experiment were incubated with or without SUMO protease 2 (LifeSensors) in PBS buffer pH 7.4 for 1 h at 30 °C and analyzed by Western blotting. Verifications were confirmed using biological triplicates.

SUMO2/3 Gene Silencing
B35 cells stably transfected with constructs expressing control miRNA (miR-Neg; a miRNA sequence not related to any mammalian gene) or SUMO2/3 miRNA (miR-SUMO2/3) were used as described previously.25

RESULTS
Oxygen/Glucose Deprivation Activates SUMO2/3 Conjugation
We first examined optimal conditions for investigating OGD-induced protein SUMOylation in a rat neuroblastoma cell line. B35 cells were exposed to 6 or 8 h of OGD, and the extent of OGD-induced cell death was evaluated after 22 h of recovery by measuring the release of LDH from cells/total LDH activity (fractional LDH release). In control cultures not exposed to OGD, fractional LDH release amounted to 11 ± 1% and increased to 31 ± 3% and 81 ± 15% when cells were exposed to 6 or 8 h of OGD, respectively (Figure 1A). We chose the 6 h time point for further studies because it induced only minor cell damage. To determine whether a rise in levels of SUMO2/3-conjugated proteins occurred following OGD, cells were exposed to 6 h of OGD and up to 180 min of recovery, and changes in levels of SUMOylated proteins were evaluated by Western blotting analysis (Figure 1B). As expected, levels of SUMO2/3-conjugated proteins declined during OGD, because SUMO conjugation is an energy-requiring process. During recovery from OGD, SUMO2/3 conjugation was markedly activated, as indicated by a massive increase in the smear of bands at high molecular weight and a considerable decrease in levels of free SUMO2/3 (Figure 1B, band at about 17 kDa). This is a pattern similar to that found after transient cerebral ischemia.4,15 Since activation of SUMO2/3 conjugation was most pronounced after 30 min of re-oxygenation in B35 cells, we decided to employ this time point for proteomic analysis.

Proteomic Analysis Identified Modulation of Numerous SUMO Substrates by OGD
The proteomic approach to analyze OGD-induced changes in SUMO3 conjugation is summarized in Figure 2A. A stable B35 cell line expressing HA-SUMO3 in which overexpression of HA-SUMO3 produced the similar pattern of SUMO2/3 conjugation compared to untransfected B35 cells was selected for this study (Figure 2B). B35 cells stably expressing HA-SUMO3 were grown in light or heavy Lys- and Arg-supplemented DMEM. Cells were first cultured for 8 passages, and mass spectrometry (MS) analysis revealed complete incorporation of heavy lysine and arginine and no evidence of proline conversion in the heavy cells (data not shown). Extracts were prepared from control (light) and post-OGD (heavy; 6 h OGD with 30 min re-oxygenation) cultures, which included lysis in buffer containing 1% SDS as well as heating at 95 °C to both inhibit endogenous SUMO proteases and to disrupt protein–protein interactions that might otherwise
result in the adventitious precipitation of nonsumoylated proteins. Protein concentrations were measured and adjusted to identical concentrations, and both extracts were mixed for immunoprecipitation of HA-tagged SUMO3-conjugated proteins and subsequent quantitative proteomic analysis. We confirmed the measured protein concentrations of control and post-OGD extracts by SDS-PAGE electrophoresis and Coomassie staining (Figure 2C) and examined SUMO2/3 levels in control versus OGD immunoprecipitates (Table 1 and Table S1 in Supporting Information), including runt-related transcription factor 1 (RUNX1) and transcription intermediary factor 1 (TIF1/β/TRIM28/KAP1), which were quantified by 2 and 27 SILAC pairs, respectively (Figure 3). The numbers of proteins quantified from Slice 1 (high molecular weight) to Slice 5 (low molecular weight) were 35, 81, 81, 81, and 16, respectively.

Gene ontology (GO) annotation demonstrated that a large fraction (92/174 annotated proteins) of quantified proteins were predicted to have nuclear localization, which is consistent with previous observations that sumoylation is predominantly a nuclear event.6,7 In addition to SUMO proteins themselves (see below), many quantified proteins were previously characterized SUMO substrates, including TIF1/β,26 LIG1,27 PIAS2, and PIAS4,28 and the transcription factors SOX-6 and SOX-10.29,30 There were also several potentially novel SUMO substrates, including RUNX1, atrophin-1, and matrin-3.

As expected on the basis of immunoblotting of these samples (Figure 2) the overall distribution of all quantified SILAC pairs pointed to a significant increase in SUMO-conjugated proteins after OGD (Figure 3B). As criteria for determining which of the identified proteins were most robustly regulated following OGD, we required at least 2 quantitative ratios (thus at least two peptide identifications) and an absolute fold-change ≥2. Twenty-five proteins met these criteria, but the three kinases that were apparently “downregulated” (i.e., had light Lys and Arg only) were flagged as likely contaminants. Of the remaining 22 proteins (Table 1), 18 were increased and 4 decreased with OGD treatment. As an initial validation, we confirmed the increase in high MW RUNX1 and decrease in high MW TIF1/β as a function of OGD (Figure 4) by Western blot analysis. In addition, the high MW forms of these proteins were abolished by treatment with a SUMO-specific protease, confirming SUMO conjugation.

To improve depth of coverage and, in particular, to resolve free SUMO and SUMO-conjugated proteins, we separated the SILAC-encoded immunoprecipitate by a short SDS-PAGE separation and excised five contiguous gel slices for in-gel trypsinization. 1D-LC–MS/MS analysis was performed on peptides recovered from each of these five gel slices, and putative SUMO-conjugated proteins were identified and quantified using Rosetta Elucidator (Figure 3 and Experimental Section). Overall, 939 peptides to 240 unique proteins were identified. After peptide identification and quantification, 880 of these peptides were found to belong to a total of 624 SILAC pairs. These 624 peptide ratios resulted in the quantification of 188 proteins in control versus OGD immunoprecipitates (Table 1 and Table S1 in Supporting Information), including runt-related transcription factor 1 (RUNX1) and transcription intermediary factor 1/β (TIF1/β/TRIM28/KAP1), which were quantified by 2 and 27 SILAC pairs, respectively (Figure 3). The numbers of proteins quantified from Slice 1 (high molecular weight) to Slice 5 (low molecular weight) were 35, 81, 81, 81, and 16, respectively.

Figure 1.Transient oxygen/glucose deprivation (OGD) activates SUMO2/3 conjugation and induces cell death. (A) B35 cells were exposed to 6 or 8 h of OGD and 22 h of recovery. Cell death was evaluated by measuring the release of LDH from cells and relating LDH released from cells to total LDH activity. Data are presented as means ± SD (n = 3). Statistically significant differences between groups were evaluated by ANOVA followed by Fisher’s PLSD test; ***p ≤ 0.001. (B) B35 neuroblastoma cells were exposed to 6 h of OGD and 0–180 min of recovery. Proteins were extracted as described in the Experimental Section, and OGD-induced changes in levels of SUMO2/3-conjugated proteins were evaluated by Western blotting. Free SUMO2/3 is indicated by an arrowhead.
Of the proteins that were overall greater than ±2-fold changed post-OGD, several showed a greater degree of regulation in their high than low MW forms (Table 1). For example, the E3 SUMO-protein ligase PIAS2 was increased only 1.6-fold in slice 3 but was almost 7-fold increased in the highest MW slice. Thus, even the highly approximate MW data provided by SDS-PAGE fractionation can provide important information in the quantitative analysis of stimulus-induced SUMO conjugation.

Overall, HA-SUMO3 immunoprecipitates were enriched in SUMO paralogues and SUMO (E1, E2, E3) ligases. Despite SUMO3 being present at levels presumably much higher than those of the nearly identical SUMO2 or the less homologous (~50% identical) SUMO1, both of these SUMO paralogues were also identified in high MW fractions and were increased 2.5- to 3-fold after OGD, which is consistent with previous observations.7,28 The quantity of immunoprecipitated SUMO E3 ligases PIAS2 and PIAS4 was also increased greatly after OGD. By analogy to PIAS1, which has been shown to be modified and activated by SUMO1, these modifications may further potentiate PIAS2- and PIAS4-dependent SUMOylation. On the other hand,
OGD apparently decreased SUMO3 conjugation of the SUMO E1 subunit Sae1 and SUMO E2 ligase Ubc9 (Table S1). SUMO conjugation of Ubc9 on Lys14 has been shown to differentially affect target-specific sumoylation.\textsuperscript{31}

Transcription factors (TFs) and corepressors or enhancers were among the putative SUMO substrates that were significantly modulated by OGD. The TFs RUNX1, NFAT5, SOX-6, and SOX-10 were all increased with OGD treatment, although SOX-10 and NFAT5 were identified by only one unique SILAC pair (both in two slices each; mean 5.8- and 4.5-fold increased with OGD, respectively). Of these, SUMO conjugation has been shown previously to repress the transcriptional activity of SOX-6 and SOX-10.\textsuperscript{29,30} The apparent SUMOylation of numerous corepressors was modulated by OGD, including Ngf-\textalpha binding protein 2, interferon regulatory factor 2-binding protein 1, nucleus accumbens-associated protein 1, TIF1\textbeta /TRIM28, and bromo-
main-containing protein 8 (Table 1). Additional OGD-regulated SUMO substrates that did not meet strict significance criteria, included nuclear corepressor 2, transcription enhancer factor 1, as well TIF1-α. SUMO conjugation of TIF1β is required for its transcriptional repression,26 and it is also worth noting that TIF1β itself is among TRIM proteins that possess SUMO E3 ligase activity.32

Ingenuity Pathway Analysis (IPA) was performed with proteins where SUMO3 conjugation was ≥2-fold up-regulated in any gel slice. The most significant pathway identified by IPA covers proteins playing key roles in post-translational protein modifications, gene expression, and cell cycle (IPA score 54; Figure 5). All of these proteins have predicted nuclear localization and include enzymes such as histone deacetylase 1-like protein (Hdac1l; 2.74-fold), polypyrimidine tract binding protein (PTBP1; 4.9-fold), lysine-specific histone demethylase 1A (KDM1A; 2.67-fold), and transcription elongation regulator 1 (TCERG1; 3.758-fold), as well as transcription factors, such as runt-related transcription factor 1 (RUNX1; 7.8-fold), helicase ARIP4 (RAD54L2; 6.82-fold), Ngf-A binding protein 2 (NAB2; 6.39-fold), transcription factors SOX-6 and SOX-10 (3.0- and 6.79-fold, respectively), and nuclear receptor corepressor 2 (NCOR2; 5.1-fold). The observation that the most significant pathway identified by IPA covers a large proportion of the SUMO targets found in post-OGD cells and specifically involves nuclear proteins playing key roles in gene expression strongly implies that activation of SUMOylation modulates the fate of cells exposed to ischemia-like conditions. As expected, the most significant pathway identified by IPA did not cover proteins involved in cell damage, because we used a short period of OGD that induced only minor cell death.
Ubiquitination Is Induced by OGD and Depends on SUMO Conjugation

Ubiquitin peptides were quantified in almost all of the gel slices, and although ubiquitin was increased 1.7-fold overall with OGD treatment, it was >2-fold increased in several of the high MW gel slices. Indeed, a marked increase in ubiquitin-conjugated proteins was observed in HA immunoprecipitates by immunoblotting after OGD (Figure 6A). Furthermore, SUMO protease treatment increased the mobility of ubiquitinated proteins by SDS-PAGE, indicative of a shift of these proteins to lower MW, although total ubiquitin did not appear to be substantially reduced. SUMO2/3 conjugates have been previously shown to monstrate that SUMO2/3 is required for ubiquitination in cells expressing miR-SUMO2/3 (Figure 6C). These data de-

Figure 6. Protein ubiquitination is increased after OGD and depends on SUMO expression. (A) Immunoprecipitates from control and OGD extracts were immunoblotted for ubiquitin, and SUMO protease was utilized to demonstrate SUMO conjugation as in Figure 4. (B) SUMO2/3 and (C) ubiquitin levels in B35 cells stably expressing control or SUMO2/3 miRNA were analyzed by Western blotting in untreated cells and immediately following or 30 min after 6 h of OGD. WB, Western blotting. IP, immunoprecipitation.

■ DISCUSSION

Accumulating evidence suggests that SUMO2/3 conjugation is important for neuroprotective stress responses under conditions of transient cerebral ischemia. However, the precise targets of SUMO2/3 under these conditions have not been previously identified. Here, we used a SILAC-based proteomic approach to identify SUMO3 targets and to quantify their modulation in an experimental model of transient ischemia. This study begins to address both the mechanisms by which OGD modulates protein SUMOylation and the potential functional consequences, including modulation of gene transcription, DNA repair and protein ubiquitination. While not novel, the advantages of a “GeLC” approach, which preserves protein MW information, are particular evident for the analysis of SUMO (and other ubiquitin-related) modifications, as such an analysis not only can differentiate changes in free and protein-conjugated SUMO but also may in principle allow the resolution of stimulus/stress-coupled changes in polySUMO conjugation, either through alterations to SUMO chain length or the number of SUMO-modified Lys residues in a particular protein.

Although mechanisms for OGD-regulated SUMOylation are still unknown, we can speculate that it will require post-translational modification or differential expression of components of the SUMO conjugation machinery. For example, based on the identification of PIAS2 and PIAS4 as putative targets of OGD-induced SUMOylation, we hypothesize that the increased activity or expression of these E3 ligases may be important for global increases in SUMO conjugation. It will be interesting to determine the role of these PIAS isoforms in mediating OGD-dependent SUMO conjugation and whether they exhibit unique substrate specificities. Furthermore, since Ubc9 levels have been shown to positively correlate with the degree of SUMO conjuga-

tion,10 it would be surprising if the e-

fects of OGD on Ubc9-SUMO were a consequence of reduced Ubc9 expression. Rather, SUMOylation of Ubc9 has been shown to decrease SUMO-1 conjugation via the SUMO-Ubc9 interaction,33 although more generally, OGD-mediated phosphorylation may alter the association of SUMO ligases and proteases with their targets and might in part explain the modulation of SUMO3 conjugation by OGD.

It is well established that ubiquitin conjugation is markedly activated after both transient global or focal cerebral ischemia.34,35 However, we have shown here for the first time that SUMO2/3 is required for OGD-induced protein ubiquitination. The interplay between the SUMO and ubiquitin systems is increasingly
recognized: ubiquitin is identified in SUMO2/3 immunoprecipitates,36 ubiquitin-proteasome inhibition results in accumulation of SUMO2/3 conjugates.36 SUMO itself can be ubiquitylated, and SUMO E3 ligases can modulate ubiquitin conjugation.37 Furthermore, SUMO and ubiquitin can compete for the same lysine residue, as exemplified by the NFκB inhibitor protein IκBα, which is either SUMO- or ubiquitin-conjugated at lysine K21.4 Although sumoylation can act as a signal for ubiquitination of proteins and degradation at the proteasome,37 we speculate that these modifications coordinate additional critical cellular processes post-OGD, including DNA damage repair, cell proliferation, and apoptosis.38–40 However, further investigation is required to determine the significance of SUMO-dependent ubiquitination following transient OGD.

Numerous lines of evidence suggest that the alteration of SUMO conjugation following OGD is a protective stress response that helps cells to better withstand a transient period of impaired energy metabolism. For example, in a mouse model of stroke, we found SUMO2/3 conjugation to be particularly activated in post-ischemic neurons located at the border of the ischemic territory. A short duration of vascular occlusion, which is not expected to cause major cell damage, is sufficient to activate this process,15 suggesting that SUMOylation is not an instigator of cell or tissue damage. SUMO2 and SUMO3 has been shown to promote cell survival after heat shock.28 Further work is needed to determine the significance of the OGD-modulated SUMO substrates that have been identified in the present study. Additional complementary ‘omic approaches, including analysis of transcriptome and global proteome analysis, should also provide additional clarity as to the physiological significance of SUMOylation in cellular and animal models of transient cerebral ischemia.

**CONCLUSIONS**

We demonstrate here for the first time how SUMO conjugation of target proteins is modified when cells are exposed to transient OGD, ischemia-like stress conditions, using SILAC-based quantitative proteomics analysis. Targets where the extent of SUMO conjugation was massively activated were predominantly nuclear proteins involved in gene expression. Once tools are available permitting proteomic analysis of endogenous SUMOylated proteins using brain tissue samples, the results presented here will provide an important platform to investigate in detail the role of SUMO conjugation in brains stressed by a transient interruption of blood supply. This will make it possible to design new avenues of therapeutic intervention for patients suffering from stroke or cardiac arrest followed by resuscitation. Our observation that OGD-induced activation of ubiquitin conjugation was almost completely blocked when SUMO2/3 conjugation was suppressed by silencing their expression suggests that activation of SUMO2/3 conjugation is required for activation of ubiquitin conjugation in OGD-stressed cells. Considering the various signal transduction pathways that modulate ubiquitin and SUMO conjugation and control key cellular functions, including cell proliferation, apoptosis, and DNA damage repair, the interplay between ubiquitin and SUMO conjugation may play a more prominent role than previously anticipated.

**ASSOCIATED CONTENT**

Supporting Information

All putative SUMO3 targets identified by SILAC-based quantitative proteomics including details for mass spectrometry analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

**Corresponding Author**

*Phone: 1-919-684-5576. Fax: 1-919-684-6692. E-mail: wulf.paschen@duke.edu.*

**ACKNOWLEDGMENT**

This research was supported by ROI grant HL095552 from the National Institutes of Health and by funds from the Department of Anesthesiology, Duke University Medical Center. The excellent technical support of Meredith Turner and Pei Miao is gratefully acknowledged. We also gratefully acknowledge Prof. Robert Lefkowitz for the use of the LTQ-Orbitrap mass spectrometer.

**ABBREVIATIONS**

CID, collisionally induced dissociation; HNRNP H2, heterogeneous nuclear ribonucleoprotein H2; IPA, Ingenuity Pathway Analysis; IP, immunoprecipitation; LDH, lactate dehydrogenase; miR, microRNA; MS, mass spectrometry; OGD, oxygen/glucose deprivation; PIAS, protein inhibitor of activated signal transducer and activator of transcription; PML, promyelocytic leukemia protein; RUNX1, runt-related transcription factor; SENP, sentrin-specific protease; SILAC, stable isotope labeling with amino acids in cell culture; SOX, SKY-box containing gene; SUMO, small ubiquitin-like modifier; TIF1β, transcription intermediary factor 1-β; Ubc9, ubiquitin conjugating enzyme 9 (the only SUMO conjugating enzyme identified so far); WB, Western blotting

**REFERENCES**

(3) Xu, Z.; Chan, H. Y.; Lam, W. L.; Lam, K. H.; Lam, L. S.; Ng, T. B.; Au, S. W. SUMO proteases: redox regulation and biological consequences. Antioxid. Redox Signaling 2009, 11 (6), 1453–84.


