SUMOYLATION OF LYS590 OF THE f-LOOP OF NCX3 BY SUMO1 PARTICIPATES IN BRAIN NEUROPROTECTION INDUCED BY ISCHEMIC PRECONDITIONING

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NEUROPROTECTIVE EFFECT OF NCX3 SUMOYLATION

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Abstract
The small ubiquitin-like modifier (SUMO), a ubiquitin-like protein involved in post-translational protein modifications, is activated by several conditions, such as heat stress, hypoxia, and hibernation. Recent evidence indicates that sumoylation confers neuroprotection against stressful stimuli by regulating the function and the fate of proteins involved in stress signalling pathways. Sumoylation enzymes and substrates are expressed not only in the cytoplasmic and nuclear compartments, but also at the plasma membrane level. Among the numerous plasma membrane proteins controlling ionic homeostasis during cerebral ischemia, NCX3, one of the three Sodium/Calcium exchangers expressed in the CNS, exerts a protective role during ischemic preconditioning. In this study we evaluated whether NCX3 is a target for sumoylation and whether this post-translational modification participates in ischemic preconditioning-induced neuroprotection. To test these hypotheses, we analyzed (1) SUMO1 conjugation pattern after ischemic preconditioning; (2) the effect of SUMO1 knockdown on the ischemic damage after transient middle artery occlusion (tMCAO) and ischemic preconditioning; (3) the possible interaction between SUMO1 and NCX3; and (4) the molecular determinants of NCX3 sequence responsible for sumoylation.

We found that (1) SUMO1 knockdown worsened ischemic damage and reduced the protective effect of ischemic preconditioning; (2) SUMO1 bound to NCX3 at the lysine residue 590 of the f-loop, and its silencing increased NCX3 degradation; and (3) NCX3 sumoylation took part to SUMO1 protective role during ischemic preconditioning. Thus, our results demonstrate that NCX3 sumoylation by SUMO1 confers additional neuroprotection in ischemic preconditioning.

Finally, this study suggests that NCX3 sumoylation might be a new potential target to enhance ischemic preconditioning-induced neuroprotection.
Introduction

Small Ubiquitin-like Modifier (SUMO) conjugation is an enzymatic process that is biochemically analogous to, but functionally distinct from, the ubiquitination process. It involves the covalent attachment of SUMO to substrate proteins, however, unlike ubiquitination, sumoylation does not lead to degradation of the target substrate but may modulate intracellular protein localization, activity, and stability (Pichler and Melchior, 2002; Gill, 2003; Seeler and Dejean, 2003; Hay, 2005; Mukhopadhyay and Dasso, 2007; Zhao, 2007). So far, four SUMO proteins have been identified, SUMO1–4. SUMO 1-3 are present in the brain, whereas SUMO4 is mainly localized in the kidney (Bohren et al., 2004). Interestingly, sumoylation is activated after different stress conditions, including anoxia, hypothermia, and hypoxia (Yang et al., 2008c; Yang et al., 2008a, b; Lee et al., 2009). Changes in brain sumoylation pattern have also been reported after focal cerebral ischemia (Cimarosti and Henley, 2008), where it may represent a protective response. Furthermore, SUMO-1 knockdown decreases cell resistance to oxygen–glucose deprivation (OGD), whereas SUMO-1 overexpression renders neurons less susceptible to OGD-induced cell damage (Lee et al., 2009). Intriguingly, sumoylation is strongly activated in the brain of hibernating animals during the torpor phase, thus shielding neurons from damage induced by reduced blood flow and substrate deprivation (Lee et al., 2009).

To date, no information is available regarding a potential neuroprotective role of protein sumoylation during endogenous neuroprotective events like ischemic preconditioning in vivo. Ischemic preconditioning, a phenomenon whereby a subliminal injurious stimulus applied before a longer harmful ischemia produces neuroprotection (Kirino, 2002; Dirnagl et al., 2003; Gidday, 2006), shares several trasductional pathways with hibernation, a condition in which sumoylation is activated. Several proteins involved in the neuroprotection mediated by ischemic preconditioning, have been proposed as sumoylation substrates (Rajan et al., 2005). Recently, convincing evidence has revealed that sumoylation enzymes and substrates are also present at the plasma membrane level, wherein they regulate plasma membrane protein expression. This finding led us to hypothesize that sumoylation might influence ionic homeostasis by regulating the stability and the expression of those transmembrane proteins involved in brain ischemia, specifically NCX3.

The reason for investigating NCX3 as a possible target for SUMO-mediated neuroprotective effect is that NCX3 plays a relevant neuroprotective role in cerebral ischemia (Pignataro et al., 2004; Molinaro et al., 2008), since both NCX3
knockdown and NCX3 knockout exacerbate the ischemic damage induced by permanent and transient middle cerebral artery occlusion (pMCAO and tMCAO) (Pignataro et al., 2004; Molinaro et al., 2008). More intriguingly, we have also identified NCX3 as a new additional effector of ischemic preconditioning (Pignataro et al., 2012). Given these premises, the aim of the present study was to evaluate whether NCX3 is a target for sumoylation and whether this post-translational modification participates in promoting ischemic preconditioning-induced neuroprotection. For these aims, we analyzed: (1) SUMO1 conjugation pattern after ischemic preconditioning, (2) the effect of SUMO1 knockdown on ischemic damage after tMCAO and ischemic preconditioning, (3) the possible interaction between SUMO1 and NCX3, and, finally, (4) the molecular determinants of NCX3 sequence responsible for sumoylation.
METHODS

Experimental Groups
Male Sprague–Dawley rats (n=110; 250-300 g; Charles River) were housed under diurnal lighting conditions (12 h darkness/light). Experiments were performed according to the international guidelines for animal research and approved by the Animal Care Committee of "Federico II" University of Naples (Naples, Italy).

Focal Ischemia
Transient focal ischemia was induced, as previously described (Pignataro et al., 2008), by suture occlusion of the middle cerebral artery (MCA) in male rats anesthetized with 1.5 % sevofluorane, 70% N₂O, and 28.5% O₂. In brief, a 2-O surgical monofilament nylon suture (Doccol, CA, USA) was inserted from the external carotid artery into the internal carotid artery and advanced into the circle of Willis until it reached the branching point of the MCA, thereby occluding the MCA (Longa et al., 1989). Achievement of ischemia was confirmed by monitoring regional cerebral blood flow in the area of the right MCA. Cerebral blood flow (CBF) was monitored through a disposable microtip fiber optic probe (diameter 0.5mm) connected through a Master Probe to a laser Doppler computerized main unit (PF5001; Perimed, Sweden) and analyzed using PSW Perisoft 2.5 (Pignataro et al., 2008). Animals not showing a cerebral blood flow reduction of at least 70%, as well as those dying after ischemia induction (5%), were excluded from the study. Rectal temperature was maintained at 37±0.5°C with a thermostatically controlled heating pad and lamp. All surgical procedures were performed under an operating stereomicroscope. After 100 min MCA occlusion, rats were re-anesthetized and the filament was withdrawn to restore blood flow. Animals were randomized into the different experimental groups in a blind manner.

Preconditioning Experimental Protocol
Ischemic preconditioning was induced as previously described (Pignataro et al., 2008). In brief, preconditioning was achieved by subjecting rats to 30 min of MCAO, followed by 72 h of reperfusion. At the end of the reperfusion period, the MCA was re-occluded for 100 min. Animals were then recovered for 24 h. The success of the experimental procedures was confirmed by measuring CBF in all the experimental steps.

Evaluation of the Infarct Volume
For the analysis of ischemic damage, rats were sacrificed 24h after tMCAO or preconditioning+tMCAO. The ischemic volume was evaluated with 2,3,5-triphenyl tetrazolium chloride (TTC) staining. In particular, the brains were cut into 1-mm coronal slices with a vibratome (Campden Instrument, 752M) and the sections were incubated in 2% TTC for 20 min and in 10% formalin overnight. The infarction area was calculated with image analysis software (Image-Pro Plus) (Bederson et al., 1986). The total infarct volume was expressed as percentage of the volume of the hemisphere ipsilateral to the lesion in order to correct brain edema. Ischemic damage was evaluated in a blind manner.

**Intracerebroventricular administration of siRNA**

In rats positioned on a stereotaxic frame, a 23-g stainless steel guide cannula was implanted into the right lateral ventricle using the stereotaxic coordinates from the bregma: 0.4mm caudal, 2mm lateral and 2mm below the dura (Franklin and Paxinos, 1997; Pignataro et al., 2004).

Two different siRNAs were tested (Quiagen siRNA-A #SI01910034 and siRNA-B #SI01910027). siRNAs (10μl, 2μM or 10μM) and siCTL were icv administered twice a day starting from 72 hours before preconditioning or tMCAO induction. Since their effectiveness was similar (about 50% reduction), all the experiments were performed with siRNA A (10μl, 10μM), which was administered twice a day, starting from 72 hours before preconditioning or tMCAO induction.

**Western Blotting Analysis**

Cortical samples were harvested from ischemic brains of rats subjected to 100 min of MCAO, from brains of preconditioned rats, and from rats subjected to preconditioning+tMCAO. Ipsilateral cortex and striatum were obtained at four different reperfusion time intervals after the last occlusion, at 3, 5, 24, and 72 hours. The same sample groups were obtained from brains of sham-operated animals.

Rat brain samples were first homogenized with an 18-gauge needle in a lysis buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 50 mM EDTA) containing a protease inhibitor cocktail, 1 mM PMSF, and 10 mM NEM. Then, they were sonicated for 10 sec, heated at 99 °C for 10 min, and centrifuged for 15 min at 14,000 rpm. Protein concentration was estimated using the Bradford reagent (Bio-Rad Laboratories, Segrate, Milan, Italy). Twenty micrograms or 50 μg of proteins were mixed with a Laemmli sample buffer. Next, the samples were separated on 8% sodium dodecyl sulfate polyacrylamide gel...
electrophoresis, and transferred onto Amersham™ Hybond™-ECL nitrocellulose membranes (GE Healthcare, Milan, Italy). The non-specific binding sites were blocked with an incubation of 5% non-fat dry milk in Tris buffered saline (TBS) 0.1% Tween 20 (Sigma-Aldrich, Milan, Italy) for 2 hours at room temperature. After that, blots were probed with SUMO-1 (1:1000) antibody (kindly given by Hallenbeck J.M. from National Institute of Neurological Disorders and Stroke (NINDS) and National Institutes of Health (NIH), Bethesda, Maryland, USA), with NCX3 antibody (kindly given by Philipson K. from David Geffen School of Medicine at UCLA, Los Angeles, CA), and with β-actin (1:1000, Sigma-Aldrich, Milan, Italy) overnight at 4 °C. Finally, detection was achieved using a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:2000; Cell Signaling; 60min at room temperature in 5% non-fat milk) and an enhanced luminescence kit (GE Healthcare, Milan, Italy) (Cuomo et al., 2008).

Generation and Stable Expression of Wild-Type and Mutant NCX3<sup>FLAG</sup> cDNAs

Murine NCX3 cDNA with a 3XFLAG epitope at the C-terminus (NCX3<sup>FLAG</sup>) was cloned in pReceiver-M13 expressing vector by Genecopoeia. Site-directed and deletion mutants of NCX3<sup>FLAG</sup> were generated by means of QuikChange strategy (Stratagene, La Jolla, CA). In particular, the amino acid regions 292-710 and 528-676 were deleted from NCX3<sup>FLAG</sup> obtaining a mutant lacking the entire f loop, NCX3<sup>Δf</sup> (NCX3<sup>Δ292-710</sup>), and another lacking a portion of f loop, NCX3<sup>Δ528-676</sup>, respectively. Two site-directed mutants were obtained by substituting lysine 456 with glutamate (NCX3K456E), and phenylalanine 589 and lysine 590 with leucine and glutamate, respectively (NCX3FK589LE). All mutant exchangers obtained were verified by sequencing both DNA strands (Primm, Milan, Italy). To stably express wild-type, site-directed and deletion mutants of NCX3<sup>FLAG</sup>, pReceiver-M13 plasmids carrying cDNAs were transfected into wild-type BHK cells by Lipofectamine 2000 (Life Technologies, San Giuliano Milanese, Italy) protocol. Stable cell lines were selected by G418 resistance and by a Ca<sup>2+</sup>-killing procedure (Molinaro et al., 2013).

Immunoprecipitation assay

BHK cells expressing NCX3 wild type and mutant forms, and brain tissue from control rats and rats exposed to tMCAO or preconditioning followed by tMCAO were homogenized in lysis buffer containing (mM) 50 mM Hepes, pH 7.4, 100 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 % NP40 with protease inhibitor cocktail, 1 mM PMSF, and 20 mM NEM. One milligram of
lysate was precleared using protein A/G plus (Santa Cruz) for 1 h at 4°C with constant rotation and centrifuged for 2 min at 8000 rpm. Precleared lysates from BHK cells were immunoprecipitated with anti-SUMO1 antibody (1:100; monoclonal anti-SUMO1 antibody, Qiagen). In particular, lysates were incubated overnight with anti-SUMO1; then protein A/G plus was added to the lysates for 2 h at 4°C with constant rotation. Finally, immunoprecipitates were washed 4 times, and the total cell lysates or immunoprecipitates were resolved by SDS-PAGE gel and transferred to a nitrocellulose membrane. Immunoblot analysis was performed using an anti-flag antibody. As for the lysates from brain tissue, 1 mg of precleared lysate was immunoprecipitated with an anti-NCX3 antibody (1:100 polyclonal anti-NCX3 antibody, Swant) using the same experimental procedure described above. Finally, total lysates or immunoprecipitates were resolved by SDS-PAGE gel and transferred to a nitrocellulose membrane. Immunoblot analysis was performed using anti-SUMO1 and anti-NCX3 antibodies.

**Primary cortical neurons and Immunocytochemistry**

Mixed cultures of cortical neurons from 2-4-day old Wistar rat pups were prepared by modifying a previously described method (Abramov et al., 2007; Scorziello et al., 2013). In brief, the tissue was minced, trypsinized (0.1% for 15 min at 37°C), triturated, plated on poly-D-lysine-coated coverslips, and cultured in Neurobasal medium (Life Technologies, San Giuliano Milanese, Italy) supplemented with B-27 (Life Technologies, San Giuliano Milanese, Italy) and 2 mM L-glutamine. The cells were plated at a concentration of 1.8x10^6 on 25-mm glass coverslips. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air, fed twice a week, and maintained for a minimum of 10 days before experimental use.

For the immunocytochemistry experiments, cortical neurons were rinsed twice in cold 0.01 M phosphate buffered saline (PBS) at pH 7.4 and fixed at room temperature in 4% (w/v) paraformaldehyde (Sigma-Aldrich, Milan, Italy) for 20 minutes. Following three washes in PBS, cells were blocked in PBS containing 10% FBS and the following antibodies: anti-NCX3 rabbit (kindly supplied by Dr. Philipson, dilution 1:4000), anti-SUMO monoclonal mouse (dilution 1:1000, Santa Cruz). Neurons were then incubated overnight at 4°C. Next, slides were washed in PBS, incubated with anti-rabbit cy2 antibody (dilution 1:200, Jackson Immunoresearch Laboratories) and anti-mouse cy3 antibody (dilution 1:200, Jackson Immunoresearch Laboratories) for 1 hour at room temperature (25°C) under dark conditions, and washed again with PBS. Finally, they were mounted onto Slow
fade™ antifade (Invitrogen-Molecular Probes) and images were observed using a Zeiss LSM510 META/laser scanning confocal microscope. Single images were taken with an optical thickness of 0.7 µm with a size of 1024×1024 pixels.

**Tissue processing, immunostaining and confocal immunofluorescence**

For the histological examination, animals were transcardially perfused under deep anesthesia with saline solution containing 0.01 ml heparin, followed by 60 ml of 4% paraformaldehyde in saline solution. The brains were removed and post-fixed overnight at +4 °C and cryoprotected in 30% sucrose in 0.1 M phosphate buffer (PB) with sodium azide 0.02% for 48 h at 4 °C. The brains were sectioned and frozen on a sliding cryostat at 40 µm thickness. The sections were then incubated with PB triton x 0.3% blocking solution (0.5%milk, 10%FBS, 1%BSA) for 2 hours. Rabbit anti-NCX3 antibodies (kindly provided by Dr. Philipson, dilution 1:4000) and mouse anti-SUMO monoclonal antibodies (dilution 1:500, Santa Cruz) were used as primary antibodies. The sections were then incubated with the corresponding fluorescent-labelled secondary antibodies (Alexa 488/Alexa 594-conjugated antimouse/antirabbit IgGs). Nuclei were counterstained with Hoechst. Images were observed with a Zeiss LSM510 META/laser scanning confocal microscope. Single images were taken with an optical thickness of 0.7 µm with a size of 1024×1024 pixels. In double-labelled sections, the pattern of immunoreactivity for both antigens was identical to that seen in single-stained material. In control groups primary antisera were replaced with normal serum. A possible cross-reactivity between IgGs in double immunolabelling experiments was assessed by processing sections through the same immunocytochemical sequence except that the primary antisera were replaced either with normal serum or with only one primary antibody; the full complement of the secondary antibodies was maintained. In addition, the secondary antibodies used in this study are highly pre-adsorbed to the IgGs of numerous species. Tissue labelling without primary antibodies was also tested to exclude autofluorescence. No specific staining was observed under these control conditions, thus confirming the specificity of the immunosignals.

**Statistic Analysis**

Values are expressed as means ± S.E.M. Statistical analysis was performed with 2-Way ANOVA, followed by Newman-Keuls test. Statistical significance was accepted at the 95% confidence level (p < 0.05).
RESULTS

SUMO1 conjugation increases in the temporoparietal cortex after ischemia, preconditioning, and preconditioning+ischemia

The role played by SUMO1 conjugation during cerebral ischemia and ischemic preconditioning was assessed by evaluating the sumoylation pattern in the ipsilesional temporoparietal cortex and striatum of rats subjected to ischemia, preconditioning, and preconditioning+ischemia and later sacrificed at different reperfusion time intervals: 3, 5, 24, and 72 hours. In the ipsilesional temporoparietal cortex, SUMO1 protein conjugation significantly increased at 5 and 24 hours after tMCAO, at 3, 5, 24, and 72 hours after preconditioning, and at 5 hours after preconditioning+tMCAO (Fig. 1A, 1B and 1C). However, in the striatum no changes were observed under all the experimental conditions (Fig.2A, 2B and 2C). The results obtained by Western blot were confirmed by confocal immunofluorescent experiments. In particular, 5 hours after tMCAO, preconditioning, or preconditioning+tMCAO, SUMO1 immunolabelling increased in the temporoparietal cortex (Fig. 3), whereas no variations were assessed in the striatum (Fig. 4).

SUMO1 colocalizes with NCX3 in primary cortical neurons, in temporoparietal cortex, and in striatum of ischemic or preconditioned rats and immunoprecipitates with the antiporter isoform in NCX3 stably transfected BHK cells.

To identify new targets of SUMO1 that might contribute to the neuroprotective effect of sumoylation during ischemic conditions, we verified whether NCX3 might be a potential candidate for the sumoylation process. Immunohistochemical analysis performed on rat brain slices after tMCAO, preconditioning, and preconditioning+tMCAO showed colocalization between NCX3 and SUMO1 signals in the temporoparietal cortex and striatum (Fig. 3 and 4). To further confirm the colocalization between NCX3 and SUMO1, we performed an immunocytochemical analysis in primary rat cortical neurons. We found that these two proteins colocalized in neuronal cell bodies (Fig. 5). Then, by using the SUMO plot analysis program (http://www.abgent.com/sumoplot), we analyzed the probability of NCX3 sumoylation. NCX3 sequence contains nine sequences that might be recognized by the SUMO conjugation enzyme; however one of these regions is localized extracellularly (Fig. 6A) according to the newly proposed NCX3 topology (Ren and Philipson, 2013). To assess the interaction between SUMO1 and NCX3, we performed
immunoprecipitation experiments on wild-type BHK cells and on BHK cells stably transfected with NCX3^{FLAG} (NCX3^{F}) and we found immunoprecipitation between NCX3 and SUMO1 (Fig. 6B). Furthermore, to better determine a possible sumylation site, we performed experiments on an NCX3^{FLAG} mutant lacking the f-loop (NCX3^{FΔf}), since this region contained most of the lysine residues with the highest probability of SUMO1 binding. Wild-type and stably transfected BHK cell lysates were immunoprecipitated with SUMO1 antibody and then immunoblotted with an anti-flag antibody. Results showed the presence of an immunoprecipitate containing NCX3^{F} and SUMO1 in NCX3^{F}-transfected BHK cells. By contrast, no immunoprecipitate was observed in NCX3^{FΔf}-transfected BHK cells, thus restricting the putative sumylation site in the f-loop of the antiporter (Fig. 6B).

The substitution of lysine456, a residue with high probability of conjugation, with glutamate (NCX3K456E) did not prevent co-immunoprecipitation between NCX3 and SUMO1 (Fig. 6C). To restrict the analysis of the putative sumoylation sites, we removed the Ca^{2+} binding domain 2 (CBD2), a subregion of the f-loop 528-676 (NCX3^{FΔ528-676}) that contains three of the seven sequences present in the f-loop. This NCX3 deletion mutant showed no immunoprecipitation with SUMO1, thus suggesting that the lysine responsible for sumoylation was present in this region. To determine the single amino acid residue involved in SUMO1 attachment, we performed immunoprecipitation on a site-directed mutant obtained by substituting phenylalanine589 and lysine590 with leucine and glutamate, respectively (NCX3FK589LE). In this case as well, no immunoprecipitation was found, thus demonstrating that lysine590 is required for sumoylation (Fig. 6C).

The biochemical interaction between SUMO1 and NCX3 was also confirmed in vivo. Indeed, results from immunoprecipitation experiments performed on rat cortex from ischemic and preconditioned animals showed that NCX3 immunoprecipitated with SUMO1 (Fig. 7A).

**SUMO1 knockdown downregulates NCX3 protein levels after preconditioning+tMCAO and reduces the neuroprotective effect elicited by ischemic preconditioning in rats.**

The effectiveness of siRNAs against SUMO1 was previously evaluated in non-ischemic rats intracerebroventricularly injected with the silencing agents. Western Blot analysis revealed a 50% reduction of SUMO1 both in cortex and in striatum (Fig. 8A). Two different
siRNAs were tested as indicated in materials and method section, and, since their efficacy was equivalent, all the experiments were performed only with siRNA-A.

To correlate the neuroprotection induced by preconditioning+tMCAO with the interaction between NCX3 and SUMO1, we verified whether SUMO1 knockdown was able to modulate NCX3 expression after preconditioning+tMCAO. As shown in Fig. 7, in the temporoparietal cortex of rats subjected to preconditioning+tMCAO and previously treated with siRNA targeting SUMO1, NCX3 protein levels significantly decreased both 24 and 72 hours after tMCAO (Fig. 7B) compared to control. By contrast, in the temporoparietal cortex of rats receiving siRNA control and exposed to the same protocol of preconditioning+tMCAO, NCX3 expression significantly increased 72 hours after ischemia (Fig. 7B), confirming our previous results (Pignataro et al., 2012). Finally, to evaluate the effect of SUMO1 silencing on the protection induced by ischemic preconditioning, rats were intracerebroventricularly injected with siRNA-A targeting SUMO1 before preconditioning, tMCAO, or preconditioning followed by tMCAO. SUMO1 knockdown induced a significant increase in ischemic volume after tMCAO (Fig. 8B) and, more interestingly, it partially prevented the neuroprotective effect induced by ischemic preconditioning (Fig. 8C) (% infarct volume: 50.9 ±3 in rats treated with siRNA control and subjected to tMCAO vs 63±1.3 in rats treated with siRNA-A SUMO1 and subjected to tMCAO; 15.3±1.65 in rats treated with siRNA control and subjected to preconditioning+tMCAO vs 39.1±9 in rats treated with siRNA-A SUMO1 and subjected to preconditioning+tMCAO).
DISCUSSION

The results of the present paper demonstrate that SUMO1 is involved in the neuroprotective mechanisms elicited by in vivo ischemic preconditioning, and, more interestingly, propose for the first time NCX3 as a new putative effector of SUMO1 neuroprotective action.

Several proteins are known to participate in the pathophysiology of brain ischemia and in ischemic preconditioning-induced neuroprotection. Although some of these proteins have been proposed as sumoylation substrates (Rajan et al., 2005), so far, no correlation has yet been found between them and SUMO levels.

Recent evidence indicates that the sumoylation enzymes and substrates are present not only in the nuclear compartment but also at the plasma membrane level (Rajan et al., 2005). In particular, sumoylation emerged also as a mechanism able to regulate plasmamembrane proteins involved in ionic homeostasis regulation (Silveirinha et al., 2013). In the present paper we hypothesized that a new substrate for sumoylation process may be represented by the isoform 3 of the plasmamembrane sodium calcium exchanger. NCX3 has been recognized as a key player in the evolution of the ischemic brain damage (Molinaro et al., 2008) and, more interestingly, as a new additional effector of ischemic preconditioning (Pignataro et al., 2012; Sisalli et al., 2014). Indeed, we found that, SUMO1 conjugation pattern significantly increased after ischemia in the ipsilateral temporoparietal cortex, whereas no changes were observed in the striatum. The observation that this increase occurred in a brain area that is far less damaged by ischemic injury than the striatum supports our hypothesis that sumoylation constitutes a cell protective response to stress associated to ischemic injury. More important, evidence showing that SUMO1 conjugation pattern also increased in the ipsilateral temporoparietal cortex during ischemic preconditioning, where a reduction of the damage occurred, thus suggesting a role of SUMO1 in ischemic preconditioning-induced neuroprotection.

In further support of a possible interaction between NCX3 and SUMO1, confocal analysis revealed that NCX3 and SUMO1 colocalized in the same neurons of the temporoparietal cortex under physiological conditions, after preconditioning, tMCAO and preconditioning followed by tMCAO. More interestingly, SUMO1 and NCX3 signals increased in the same neurons after preconditioning and preconditioning followed by tMCAO. Colocalization results were further reinforced by the co-immunoprecipitation study, showing a direct binding between NCX3 and SUMO1 in BHK cells stably expressing NCX3.
To investigate the molecular determinants involved in NCX3 sumoylation we identified, by using a bioinformatic approach, nine putative consensus sequences of the NCX3 protein that could be recognized by SUMO conjugating enzymes. Eight sequences were localized in the cytosolic side, seven of which in the f-loop region, which plays a key regulatory function in the antiporter activity. Interestingly, the removal of the whole f-loop prevented the binding between NCX3 and SUMO1, thus indicating that this region contains the molecular determinants responsible for sumoylation. Similarly, when we removed the CBD2 domain, corresponding to the subregion 528-676 of the f-loop, immunoprecipitation between NCX3 and SUMO1 was also hampered. This finding thus indicated that the putative sumoylation site is indeed localized in the CBD2 domain containing the three identified putative binding sites. Moreover, since the FKND sequence, present at the level of 589-592 aa, showed the highest score for sumoylation, we hypothesized that lysine590 was the sumoylation site for NCX3. Indeed, the substitution of phenylalanine589 and lysine590 with leucine and glutamate, respectively, rendering this sequence unrecognizable for the sumoylation enzymes, prevented NCX3 and SUMO1 co-immunoprecipitation.

Results showing that downregulation of NCX3 protein levels occurred in SUMO1-silenced rats exposed to preconditioning+tMCAO suggested that this post-translational modification may increase NCX3 stability, thus preventing NCX3 from the degradation following tMCAO (Pignataro et al., 2004; Pignataro et al., 2012).

According to previous studies, suggesting a neuroprotective role for SUMO1, we found that SUMO1 silencing significantly increased the ischemic damage induced in rats by tMCAO. More relevant, our findings demonstrated that SUMO1 silencing partially reverted the neuroprotection mediated by ischemic preconditioning.

The involvement of SUMO1 in preconditioning was also supported by previous in vitro observations, showing that preconditioned neurons maintained elevated SUMO-1 conjugation levels and SUMO-1-silenced neurons showed a reduced survival rate after oxygen and glucose deprivation and an attenuated protective response to preconditioning (Lee et al., 2009). Similarly, in vivo studies have shown that transgenic mice overexpressing SUMO conjugating enzyme, Ubc9, are more resistant than wild type mice to brain ischemia (Lee et al., 2011). Moreover, it has been demonstrated that a massive conjugation of SUMO1 is highly activated in the torpor phase of hibernating ground squirrels (Lee et al., 2009), which are naturally tolerant to oligemic conditions. Indeed,
hibernation torpor has been shown to provide neuroprotection against a superimposed lethal ischemic insult (Frerichs and Hallenbeck, 1998).

NCX3 downregulation during ischemic preconditioning in rats silenced for SUMO1 along with the observation that SUMO1 silencing significantly increased the ischemic damage and partially reverted the neuroprotection exerted by ischemic preconditioning support the hypothesis that sumoylation of NCX3 may play a key role in the neuroprotection during ischemic preconditioning. In fact, sumoylation of NCX3 might be considered a protective mechanism to preserve NCX3 from degradation during ischemic conditions.

Collectively, our results show that SUMO1 plays a fundamental role in the neuroprotection elicited by ischemic preconditioning and that its protective role might be, at least in part, mediated by NCX3 conjugation. Finally, this post-translational process may represent a potential pharmacological target to enhance the beneficial effect of ischemic preconditioning.
REFERENCES


Figure 1: Time-course of SUMO1 conjugation pattern following tMCAO, preconditioning, and tMCAO+preconditioning in the ipsilateral temporoparietal cortex

Western Blot analysis of SUMO1 conjugation pattern after tMCAO, preconditioning, and tMCAO+preconditioning in ipsilateral temporoparietal cortex are represented. Data were normalized on the basis of actin levels and expressed as percentage of sham-operated controls, taken as 100%. Values represent means±SEM (n=6). *p<0.05, compared to control animals. Representative blots are on the left.

Figure 2: Time-course of SUMO1 conjugation pattern following tMCAO, preconditioning, and tMCAO+preconditioning in striatum

Western Blot analysis of SUMO1 conjugation pattern after tMCAO, preconditioning, and tMCAO+preconditioning stimulus in striatum (B) are represented. Data were normalized on the basis of actin levels and expressed as percentage of sham-operated controls, taken as 100%. Values represent means±SEM (n=6). *p<0.05, compared to control animals. Representative blots are on the left.

Figure 3: SUMO1 and NCX3 expression following tMCAO, preconditioning, and tMCAO+preconditioning in ipsilateral temporoparietal cortex by confocal analysis

Confocal microscopic images displaying SUMO1 (A-M) (green), NCX3 (B-N) (red), HOECHST (C-O) (blue), and Merge (D-P) (yellow) in the temporoparietal cortex region of rats exposed to tMCAO, preconditioning, or preconditioning+tMCAO after 5 hours of reperfusion. A representative brain slice cartoon indicating the area of interest at the top of the Figure. Scale bars in A-P: 50 μm

Figure 4: SUMO1 and NCX3 expression following tMCAO, preconditioning, and tMCAO+preconditioning in striatum by confocal analysis

Confocal microscopic images displaying SUMO1 (A-M) (green), NCX3 (B-N) (red), HOECHST (C-O) (blue), and Merge (D-P) (yellow) in striatum of rats exposed to tMCAO, preconditioning, or preconditioning+tMCAO after 5 hours of reperfusion. A representative brain slice cartoon indicating the area of interest is at the top of the Figure. Scale bars in A-P: 50 μm
Figure 6: SUMO1-NCX3 colocalization in cortical neurons
Confocal microscopic images displaying SUMO1 (green), NCX3 (red), HOECHST (blue),
and merge (yellow) in primary cortical neurons. Scale bars: 10 μm

Figure 7: SUMO1 and NCX3 interaction in BHK cells transfected with NCX3 mutants
(A) Schematic representation of NCX3 mutants used for the immunoprecipitation assay.
(B) Immunoprecipitation experiments on WT BHK cells and in BHK cells transiently
transfected with NCX3 flag (NCX3^F), or NCX3^F lacking f-loop (NCX3 Δf). Cell lysates were
immunoprecipitated with SUMO1 and immunoblotted for anti-flag antibody. Both total
extracts and immunoprecipitates were divided into two parts and each part was run in 8 or
12% gel. (C) Immunoprecipitation experiments on WT BHK cells and in BHK cells
transiently transfected with NCX3^F, NCX3^F Δ528–676, NCX3^F K456E, or NCX3^F FK489LE.
Cell lysates were immunoprecipitated with SUMO1 and immunoblotted for anti-flag
antibody.

Figure 7: SUMO1 and NCX3 interaction in brain tissue and effect of SUMO1 silencing
on NCX3 expression after preconditioning+ischemia
(A) Immunoprecipitation experiments for the analysis of NCX3-SUMO1 interaction.
Brain tissue from control and ischemic rats was immunoprecipitated for NCX3 and then
blotted for SUMO1. The same filter was then incubated with NCX3 antibody, to confirm the
results. (B) Western Blot analysis of NCX3 after preconditioning+tMCAO in cortex of rats
previously treated with siRNA-A targeting SUMO1 and in rats treated with siRNA control
and exposed to preconditioning+tMCAo. The data were normalized on the basis of actin
levels and expressed as percentage of sham-operated controls, taken as 100%. Values
represent means±SEM (n=6). *p< 0.05, compared to control animals.

Figure 8: Effect of siRNA targeting SUMO1 on ischemic damage induced in male
rats by tMCAO and by preconditioning+tMCAO
(A) Representative blots and densitometric analysis of SUMO1 protein levels after icv
infusion of siRNA control, siRNA-A, or siRNA-B in rat cortex and striatum. Data were
normalized on the basis of β-actin levels and represented as % of SUMO1 expression in
control animals. Values represent means±SEM (n=3). *p<0.05 versus SUMO1 levels in
controls. (B) Effect of siRNA control or siRNA-A on infarct volume induced by tMCAO.
Each column represents the mean±SEM of the percentage of the infarct volume. siRNA-A
(10μl, 10μM) or siCTL were icv administered twice a day starting from 72 hours before or
tMCAO induction. n=5-8 animals in each group. *p<0.05 versus siRNA control treated group. (C) Effect of siRNA control or siRNA-A on neuroprotection mediated by preconditioning+tMCAO. Each column represents the mean±SEM of the percentage of the infarct volume. siRNA-A (10μl, 20μM) or siCTL were icv administered twice a day starting from 72 hours before preconditioning induction. n=5-8 animals in each group. *p<0.05 versus siRNA control treated group.
Figure 7

A

IP: NCX3
IB: SUMO1

105 KDa

CTL tMCAO Prec+ tMCAO

IP: NCX3
IB: NCX3

105 KDa

CTL tMCAO Prec+ tMCAO

B

NCX3

β-Actin

NCX3/βActin (% of Control)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NCX3/βActin</th>
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</thead>
<tbody>
<tr>
<td>Sham + siRNA CTL</td>
<td>80%</td>
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<tr>
<td>siRNA CTL + Prec + tMCAO 72h</td>
<td>120%</td>
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<tr>
<td>siRNA SUMO1 + Prec + tMCAO 24h</td>
<td>*</td>
</tr>
<tr>
<td>siRNA SUMO1 + Prec + tMCAO 72h</td>
<td>*</td>
</tr>
<tr>
<td>Sham + siRNA SUMO1</td>
<td>120%</td>
</tr>
</tbody>
</table>
Figure 8

- **A**: SUMO Pattern
  - siCTL
  - siRNA-A 2μM
  - siRNA-A 10μM
  - siRNA-B 2μM
  - siRNA-B 10μM

- **B**: SUMO1/Actin (% of Control)
  - siCTL
  - siRNA-A 2μM
  - siRNA-A 10μM
  - siRNA-B 2μM
  - siRNA-B 10μM

- **C**: % Ischemic Damage
  - tMCAO
  - tMCAO + siRNA CTL
  - tMCAO + siRNA A-SUMO1