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Increased protein SUMOylation following focal cerebral ischemia

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Abstract

Stroke is a major cause of death and disability, which involves excessive glutamate receptor activation leading to excitotoxic cell death. We recently reported that SUMOylation can regulate kainate receptor (KAR) function. Here we investigated changes in protein SUMOylation and levels of KAR and AMPA receptor subunits in two different animal stroke models: a rat model of focal ischemia with reperfusion and a mouse model without reperfusion. In rats, transient middle cerebral artery occlusion (MCAO) resulted in a striatal and cortical infarct. A dramatic increase in SUMOylation by both SUMO-1 and SUMO-2/3 was observed at 6 h and 24 h in the striatal infarct area and by SUMO-2/3 at 24 h in the hippocampus, which was not directly subjected to ischemia. In mice, permanent MCAO resulted in a selective cortical infarct. No changes in SUMOylation occurred at 6 h but there was increased SUMO-1 conjugation in the cortical infarct and non-ischemic hippocampus at 24 h after MCAO. Interestingly, SUMOylation by SUMO-2/3 occurred only outside the infarct area. In both rat and mouse levels of KARs were only decreased in the infarct regions whereas AMPARs were decreased in the infarct and in other brain areas. These results suggest that posttranslational modification by SUMO and down-regulation of AMPARs and KARs may play important roles in the pathophysiological response to ischemia.

Keywords: SUMO; Ischemia; Stroke; Kainate receptor; AMPA receptor; Posttranslational modification

1. Introduction

Cerebral ischemia has profound effects on multiple cellular processes including posttranslational modification of proteins (Dorval and Fraser, 2007; Durukan and Tatlisumak, 2007). One such posttranslational modification is SUMOylation that occurs when *S*mall *U*biquitin like *MO*difier (SUMO), a 97-residue protein, covalently binds to lysine residues on target proteins. Protein SUMOylation has been most extensively studied in the nucleus where it can alter the subcellular localization, protein partnering, and DNA-binding and/or transactivation functions of transcription factors (Hilgarth et al., 2004).

There are four SUMO paralogues (SUMO-1–4) in vertebrates. Of these SUMO-4 is mainly localized to the kidney (Bohren et al., 2004) while the other forms are present in brain. SUMO-2 and SUMO-3 differ by only four N-terminal amino acids and no difference in their functional roles has yet been reported (Hay, 2005). Together SUMO-2/3 shares only ~50% sequence identity to SUMO-1.

It is becoming increasingly apparent that protein SUMOylation is involved in diverse cellular processes including transcriptional regulation, nuclear transport, maintenance of genome integrity, cell signalling, plasma membrane depolarization and signal transduction (Johnson, 2004; Wilson and Rosas-Acosta, 2005; Kerscher et al., 2006; Scheschonka et al., 2007). The functional consequences of SUMO attachment vary greatly from substrate to substrate, and in many cases are not well understood. SUMO-1 and SUMO-2/3 also differ in their conjugation dynamics and responses to cellular stress. Under resting conditions, very little SUMO-1 is present in an unconjugated form, yet there is a large free pool of SUMO-2/3. In response to cellular stresses, such as oxidative

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stress, osmotic stress or heat shock, an increase in SUMO-2/3 conjugation occurs suggesting that SUMO-2/3 could act as a cellular SUMO reserve to allow an efficient response to stress (Saitoh and Hinchey, 2000).

Protein SUMOylation is dramatically increased in the brain of ground squirrels during hibernation and it has been proposed that this may provide a mechanism to protect cells from otherwise lethally low levels of oxygen and glucose due to reduced blood flow (Lee et al., 2007). Furthermore, recently it has been reported that transient global cerebral ischemia induces a marked increase in protein SUMOylation in the hippocampus and cerebral cortex by SUMO-2/3 in mice (Yang et al., in press).

There are multiple SUMOylated proteins at synapses (Martin et al., 2007) consistent with bioinformatics analysis data indicating that a diverse range of synaptic proteins are potential SUMO targets (Yang et al., 2006). More specifically, the KAR subunit GluR6 is a SUMO target. GluR6 exhibits low levels of SUMOylation under resting conditions but is rapidly SUMOylated in response to agonist activation. Reducing GluR6 SUMOylation using the specific de-SUMOylation enzyme SENP-1 prevented the agonist-evoked KAR endocytosis. KAR-mediated excitatory postsynaptic currents (EPSCs) in hippocampal slices are decreased by SUMOylation and enhanced by de-SUMOylation (Martin et al., 2007). This is of interest because GluR6 has been implicated in neuronal cell death following ischemic insult via a JNK activation pathway (Pei et al., 2006; Zhang et al., 2006) and KARs are also involved in ischemia-induced white matter injury (Tekkok et al., 2007). Therefore, we formulated the hypothesis that one role of increased SUMOylation could be to down-regulate the surface expression of KARs, which in turn, would reduce excitotoxicity and subsequent cell death.

Here we have investigated changes in the pattern of protein SUMOylation and in levels of AMPAR and kainate receptor subunits in MCAO-induced focal ischemia in rat and mouse, which is a model for human stroke. In mice permanent middle cerebral artery occlusion results in a cortical infarct whereas in rat transient MCAO results in a predominantly striatal infarct. At 6 h and 24 h post-MCAO, ischemic tissue from cortex and striatum was dissected and processed to analyse protein SUMOylation. In addition, non-ischemic hippocampal tissue was analysed from both MCAO models. Thus we studied protein SUMOylation in brain tissue from the infarcted area as well as outside the ischemic regions from two different species with or without reperfusion. Our data show that ischemia evokes dramatic changes in the pattern of protein SUMOylation and causes decreases in both AMPAR and KAR levels.

2. Methods

Animal care and all experimental procedures were conducted in accordance with Danish animal protection legislation and the experimental protocols were approved by the Danish National Committee for Ethics in Animal Research.

2.1. Transient middle cerebral artery occlusion in rats

Transient middle cerebral artery occlusion (tMCAO) was induced in Wistar rats weighing 240 ± 15 g essentially as described previously (Kelsen et al., 2006). Rats were treated with 5% Xylocain gel (AstraZeneca, Denmark) locally and Rimadyl 5 mg/kg s.c. 30 min pre-surgery and subsequently anaesthetized with 2–3% isoflurane (Baxter, Denmark) in a gas mixture of nitrous oxide (0.7 l/min) and oxygen (0.3 l/min). During surgery, isoflurane was decreased to 1–2% and the rats were placed on a heating pad (CMA/150, Carnegie Medicin AM, Sweden) to provide a core body temperature of 37 ± 0.5 °C. The right common carotid artery (CCA) was isolated through a small midline incision in the neck region. The right occipital artery (OA) and the pterygopalatine artery (PA) were permanently ligated to assure that the filament was not trapped in wrong side branches. The superior thyroid artery (STA) was coagulated and transected to mobilize the external carotid artery (ECA). The ECA was ligated where it branches into the lingual (LA) and the maxillary artery (MA). Distal to the ligature the LA and MA were coagulated and cut. A small arteriotomy was made in the ECA stump and a filament with a rounded tip was introduced and manoeuvred into the internal carotid artery (ICA) and advanced 22 mm beyond the carotid bifurcation. During the entire ischemic challenge, the right CCA was clamped to diminish blood flow. The intraluminal filament blocked the right MCA origin for 60 min. After withdrawal of the filament, the ECA stump was ligated and the CCA clamp removed. Reperfusion of the ICA was observed before wound closure. At 6 h or 24 h after surgery the rats were decapitated and the brains were quickly removed, relevant brain regions dissected and frozen on liquid nitrogen.

2.2. Permanent middle cerebral artery occlusion in mice

Focal ischemia was induced on outbreed Naval Marine Research Institute (NMRI) mice by permanent occlusion of the right middle cerebral artery (MCA) (Moller et al., 1995). Mice weighing 30 g were anaesthetized with 2% isoflurane (Baxter, Denmark) in a gas mixture of nitrous oxide (0.7 l/min) and oxygen (0.3 l/min) and placed on a heating pad (CMA/150, Carnegie Medicin AM, Sweden) to provide a core body temperature of 37 ± 0.5 °C. With the mice lying at the left side a small skin incision was made between the right orbita and the external ear opening. The underlying temporalis muscle was loosened from the skull and reflected forwards. Using a dental drill a craniectomy was made over the proximal part of the MCA. Without penetrating the dura mater the exposed MCA was occluded with a bipolar electrocoagulator with forceps (MK20 Beta-Surge, Beta Ltd, USA). After occlusion the temporal muscle was repositioned and the skin incision closed with 3/0 silk sutures. The wound was treated with 5% Xylocain gel (AstraZeneca, Denmark) and the mice were returned to their cages. At 6 h or 24 h after surgery the mice were decapitated and the brains were quickly removed, relevant brain regions dissected and frozen on liquid nitrogen.

2.3. Western blots and densitometry

Frozen brain regions prepared at NeuroSearch were added to 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% mammalian protease inhibitor and 20 mM NEM, and homogenized on ice. The homogenates were sonicated for 10–15 s at 4 °C and the protein concentration determined. Samples were boiled for 5 min at 95 °C with 5% β-mercaptoethanol and 2% glycerol and subjected to SDS–PAGE (8% or 10%) loaded at 15–60 μg protein/lane. Western blot analyses were performed using the following antibodies: rabbit polyclonal anti-SUMO-1 (1:1000) and rabbit polyclonal anti-SUMO-2/3 antibodies (1:2000) kindly provided by Dr. M. Dasso (NIH, USA), rabbit polyclonal anti-SUMO-1 antibody (1:1000) purchased from Cell Signaling, anti-SUMO-2/3 antibody (1:250) purchased from Zymed, rabbit polyclonal anti-GluR2/3 antibody (1:1000) purchased from Chemicon, rabbit monoclonal anti-GluR6/7 antibody (1:1000) purchased from Upstate, and mouse monoclonal anti-β-actin antibody (1:10,000) purchased from Sigma Chemicals. In the figures, representative blots obtained by using the anti-SUMO-1 from Dr. M. Dasso and the anti-SUMO-2/3 from Zymed are shown. Similar results were obtained with the other SUMO-1 and SUMO-2/3 antibodies tested. The band intensity was quantified by densitometry using ImageJ (NIH). For SUMO conjugate band analysis the entire lane in the range 37–250 kDa was sampled. For each structure (cortex, striatum and hippocampus) from each hemisphere (contralateral and ipsilateral), MCAO groups (6 h and 24 h) were compared to control groups not exposed to MCAO on the same