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Expression and function of striatal enriched protein tyrosine phosphatase is profoundly altered in cerebral ischemia

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Abstract

Striatal enriched protein tyrosine phosphatase (STEP) acts in the central nervous system to dephosphorylate a number of important proteins involved in synaptic function including ERK and NMDA receptor subunits. These proteins are also linked to stroke, in which cerebral ischemia triggers a complex cascade of events. Here we demonstrate that STEP is regulated at both the transcriptional and the post-transcriptional levels in rat models of cerebral ischemia and that its regulation may play a role in the outcome of ischemic insults. After transient middle cerebral artery occlusion, there are profound decreases in the levels of STEP mRNA, whilst in global ischemia STEP mRNA is selectively down-regulated in areas susceptible to ischemic damage. In a neuroprotective preconditioning paradigm, and in regions of the brain that are relatively resistant to ischemic damage, STEP mRNA levels are increased. Furthermore, there is a significant processing of STEP after ischemia to generate a novel species, STEP₃₃, resulting in a redistribution of STEP from membrane-bound to soluble compartments. Concomitant with the cleavage of mature forms of STEP, there are changes in the phosphorylation state of ERK. We show that the cleavage of STEP leads to a catalytically active form, but this cleaved form no longer binds to and dephosphorylates its substrate pERK. Therefore, in response to ischemic insults, there are profound reductions in both the amount and the activity of STEP, its localization, as well as the activity of one of its key substrates, pERK. These changes in STEP may reflect a critical role in the outcomes of ischemic brain injury.

Keywords

calpain; ERK; MCAO; NMDA receptor; STEP; rat

Introduction

Ischemic insults are initiated by an interruption in cerebral blood flow that limits oxygenation of brain tissue. This leads to cell death in the ischemic core as well as subsequent peripheral injury in the penumbra as reperfusion occurs. A variety of mechanisms are initiated to counteract the initial insult and protect against further damage. These include changes in gene expression (Tang *et al.*, 2002; Rickhag *et al.*, 2006), modulation of intracellular signaling

cascades (Lopez-Neblina & Toledo-Pereyra, 2006) and induction of inflammatory responses (Wang *et al.*, 2007). A better understanding of these mechanisms could lead to improved therapeutic interventions (Weinberger, 2006; Mehta *et al.*, 2007).

One of the earliest causes of neuronal death in the ischemic brain is secondary to the release of the excitatory neurotransmitter glutamate (Choi & Rothman, 1990). Glutamate stimulates large increases in Ca^{2+} flux through NMDA receptors, leading to the generation of nitric oxide and excitotoxic free radicals (Iadecola, 1997). Furthermore, tyrosine phosphorylation of NMDA receptor subunits markedly alters following ischemic insults, leading to further excitotoxicity (Matsumoto *et al.*, 2002).

The mitogen-activated protein kinase (MAPK) pathways are activated following cerebral ischemia (Nozaki *et al.*, 2001). These pathways include the extracellular signal-regulated kinase 1 and 2 (ERK), p38, and c-jun N-terminal kinase (JNK). ERK activation occurs in response to growth factors, activation of glutamate receptors and oxidative stress, all of which occur following ischemia (Won *et al.*, 2002; Saito *et al.*, 2005). Increases in ERK activity have been shown to be neuroprotective (Irving *et al.*, 2000; Namura *et al.*, 2001), although inhibition of ERK activity has also been shown to provide neuroprotection (Runden *et al.*, 1998; Namura *et al.*, 2001). These conflicting results may be due to a complex spatiotemporal profile in ERK signaling that affects neuronal survival.

A modulator of both NMDA receptors and ERK is striatal enriched protein tyrosine phosphatase (STEP). This phosphatase is expressed widely throughout the central nervous system (CNS) (Lombroso *et al.*, 1993; Boulanger *et al.*, 1995) and plays key roles in normal synaptic function and pathological states (reviewed in Braithwaite *et al.*, 2006b). STEP dephosphorylates a regulatory tyrosine residue on the NR2B subunit of NMDA receptors, resulting in their endocytosis (Pelkey *et al.*, 2002; Snyder *et al.*, 2005; Braithwaite *et al.*, 2006a). STEP also binds to, and dephosphorylates, ERK, therefore modulating the duration of ERK signaling (Pulido *et al.*, 1998; Paul *et al.*, 2003). Given its role in the regulation of key substrates implicated in cerebral ischemia, STEP is potentially an important molecule to study. Intriguingly, an earlier study demonstrated that in a model of perinatal ischemic-hypoxic injury, STEP is processed from its normally expressed form, STEP₆₁, to a lower molecular-weight isoform, STEP₃₃ (Gurd *et al.*, 1999). This isoform of STEP is produced through a calpain-mediated cleavage (Nguyen *et al.*, 1999), although it remains unclear whether the cleavage of STEP results in a change in function. Calpain acts to cleave a variety of proteins, in addition to STEP, and calpain inhibitors are being investigated for their neuroprotective effects in cerebral ischemia (Ray, 2006).

In the present study, we explored how ischemic damage leads to changes in STEP. The results demonstrate that STEP mRNA is significantly down-regulated following ischemia, while at the same time pre-existing STEP protein is rapidly cleaved. We have determined that cleavage of STEP following cerebral ischemia results in an isoform that is no longer able to bind to and dephosphorylate ERK. Therefore, STEP is regulated in multiple ways following ischemic injury, profoundly regulating its levels, activity and functional properties.

Materials and methods

Animal preparation and experimental groups

All experimental procedures were approved by AGY's Animal Care and Use Committee and were performed in line with guidelines of the National Institute of Health using male Wistar rats. For surgical procedures animals were anesthetized using 3% isoflurane in a mixture of air and oxygen (70 : 30) Transient middle cerebral artery occlusion (tMCAO) was performed as described previously (Memezawa *et al.*, 1992; Zhao *et al.*, 1994) with some minor

modifications. Briefly, a small incision was made in the common carotid artery and a nylon monofilament was inserted into the internal carotid artery through the common carotid artery. An occlusion time of 2 h was used in all rats subjected to MCAO and the monofilament was removed at the end of this period. The body temperature of rats subjected to MCAO was maintained at 37 ± 1 °C for 6 h after occlusion. All animals were tested for neurological status according to the neurological examination grading system described previously (Bederson *et al.*, 1986). Only rats that consistently circled toward the paretic side after the 2 h of MCAO were included in this study. Global ischemia with (PC) or without (NC) preconditioning was performed as previously described (Shamloo & Wieloch, 1999). Male Wistar rats were used in the two-vessel occlusion model of global cerebral ischemia. Ischemia was induced by withdrawal of blood through a venous catheter to a blood pressure of 50 mmHg followed by clamping of the common carotid arteries with clips. Ischemia was terminated after 10 or 3 min by removing the clips and increasing the blood pressure to over 100 mmHg. In the preconditioning protocol, the second ischemic event was induced in a similar manner at 2 days following the 3-min preconditioning ischemia. Sham operated animals were treated similarly to those subjected to ischemia, except for the occlusion of the carotid arteries and the induction of hypotension.

***In situ* hybridization**

Animals were killed using 5% isoflurane in a mixture of air and oxygen (70 : 30) and the brains were collected at indicated reperfusion times, embedded in OCT embedding medium and frozen on dry ice. Rat brain coronal sections of 15 µm thickness were cut and prehybridized by fixation in 4% paraformaldehyde followed by processing in 0.1 mol/L triethylamine (TEA), 0.25% acetic anhydride/TEA, 100% chloroform, and alcohol dehydration. A ribonucleotide probe was generated using a clone of rat STEP₆₁ as template for *in vitro* transcription and labeled with [α -³³P]UTP (Perkin Elmer Life, Boston, MA, USA). The sections were then probed with 200 µL hybridization cocktail containing 10⁵ c.p.m. for 18 h at 55 °C, followed by an RNase A treatment and stringent wash in standard saline citrate buffer. Finally, the sections were dehydrated and exposed to phosphorimager screens (Cyclone, Packard Instruments, Meridan, CT, USA) for 5–7 days at room temperature before image processing. The specificity of *in situ* hybridization experiments was confirmed using labeled sense probe as well as competition studies by using unlabeled probe at 100 times the concentration of the labeled probe (data not shown).

Protein biochemistry

Brains were homogenized in RIPA buffer [0.1% sodium dodecyl sulphate (SDS) 1% NP40, 0.5% sodium deoxycholate; in phosphate-buffered saline (PBS)] and sonicated to extract proteins. Protein concentrations were determined by the BCA method (Pierce Biotechnology, Rockford, IL, USA). Lysates were prepared in SDS-loading buffer and proteins separated by SDS-polyacrylamide gel electrophoresis on Tris-glycine gels (Invitrogen, Carlsbad, CA, USA). Proteins were transferred to nitrocellulose membranes and blots probed with either anti-STEP (Novus Biologicals, Littleton, CO, USA), antiphospho-ERK or anti-ERK (Invitrogen) antibodies overnight at 4 °C. Membranes were washed and incubated with appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature, followed by development using enhanced chemiluminescence techniques (Amersham). Western blots were quantified using ImageJ and statistical analyses were performed using *t*-tests.

Subcellular fractionation

Homogenized and sonicated brain lysates were used for subcellular fractionation. The nuclear fraction (P1) was removed by centrifugation at 1000g. A crude synaptosomal fraction (P2) was

prepared by centrifugation at 10 000g and centrifugation of the supernatant (S2) at 100000g produced microsomal fraction (P3); the remaining supernatant (S3) consisting of mainly cytoplasmic proteins was retained.

Calpain cleavage of STEP *in vitro*

The bacterial expression construct and purification of GST-STEP₄₆ fusion protein used in this study have been previously described (Lombroso *et al.*, 1993); GST-STEP₄₆ with its kinase interaction motif deleted [GST-STEP₄₆ (Δ KIM)] was generated by the same methods. To isolate STEP₃₃ protein from GST-STEP₄₆ fusion protein, 20 μ g of GST-STEP₄₆ was digested with 5 units of active calpain I (Calbiochem, San Diego, CA, USA) in digestion buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM CaCl₂) at 30 °C for 30 min. After digestion, GST-STEP₄₆ N-terminal fragment was removed by affinity purification using glutathione-sepharose beads (Amersham Pharmacia) and the remaining C-terminal STEP₃₃ fragment was checked for purity on Western blots.

Phosphatase assays

Phosphatase activity of GST-STEP₄₆ and STEP₃₃ was assayed in the presence of 50 mM imidazole buffer, pH 7.0, and 2.5 mM para-nitro phenyl phosphate (Sigma, St Louis, MO, USA) at 30 °C for 30 min, as previously described (Paul *et al.*, 2000). The reaction was terminated by the addition of 0.2 N NaOH and optical density measured at 405 nm. Results are expressed as molar concentration of p-nitrophenolate ions produced in the reaction per minute.

Dephosphorylation of phospho-ERK was performed by incubating increasing amounts of GST-STEP₄₆ or STEP₃₃ proteins with 10 ng of phospho-ERK (pERK; Calbiochem) in assay buffer (25 mM HEPES, pH 7.3, 5 mM EDTA, 10 mM DTT) at 30 °C for 30 min. The reaction was terminated by adding SDS buffer and samples were run on SDS-PAGE. The resolved proteins were transferred to nitrocellulose membrane and immunoblotted with anti-pERK antibody (Cell Signaling, Boston, MA, USA).

Immunoprecipitation

Rats were killed by decapitation and their brains were rapidly removed and placed into ice-cold homogenization buffer. Differential centrifugation was performed as previously described. For each immunoprecipitation, 10 μ g of GST-STEP₄₆ or STEP₃₃ protein was incubated with anti-STEP antibody (Novus Biologicals) and protein G-agarose beads (Upstate) in RIPA buffer for 2 h at 4 °C. S2 homogenates (100 μ g) were precleared with protein G beads, and incubated at 4 °C overnight with the protein-antibody complex. For GST pull-down assays, 10 μ g of GST-STEP₄₆ or GST-STEP₄₆ N-terminal fragments were conjugated to glutathione-sepharose beads (Amersham) and incubated with S2 homogenates in RIPA buffer overnight at 4 °C. GST protein was used as a negative control. The precipitates were washed thoroughly with RIPA buffer and resuspended in 2 $\times$ SDS sample buffer and subjected to SDS-PAGE. For immunoblots assay, the separated proteins were transferred to PVDF membranes and incubated with the indicated antibodies. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody and immunoreactivity was visualized by chemiluminescence (Pierce) as previously described.

Results

STEP mRNA is down-regulated following transient MCAO

Cerebral ischemia triggers a complex range of pathophysiological responses that are indicative of both cell death and initiation of survival and recovery mechanisms. Many genes are differentially expressed following tMCAO with a marked temporal profile (Rickhag *et al.*,

2006). We first investigated the changes in STEP mRNA after a 2 h occlusion of the middle cerebral artery followed by varying periods of reperfusion. In control animals, in agreement with previous studies (Lombroso *et al.*, 1993), or animals that have undergone a sham surgery, STEP is highly expressed in the striatum and is also present in the cortex (Fig. 1a and b). Immediately prior to reperfusion and after 2 h of MCAO, STEP mRNA expression levels were unchanged (Fig. 1c), but following reperfusion there was a time-dependent decrease in levels of STEP mRNA in the ischemic hemisphere in striatum and later in the cortical region in both the ischemic core and the penumbra (Fig. 1 d–i). After 24–48 h of reperfusion, STEP mRNA was virtually undetectable in the ischemic regions. It is important to note that the down-regulation of STEP mRNA in both regions precedes cell death as no histological sign of injury is detectable (Garcia *et al.*, 1995; Rojas *et al.*, 2006).

STEP mRNA expression in global cerebral ischemia

We further analysed STEP mRNA expression in a model of global ischemia. A brief period of ischemia (3 min) induced minimal injury to the hippocampus, while 10 min of ischemia led to selective and irreversible damage to cornu ammon 1/2 (CA1 / CA2) layers of the hippocampus. Exposure to 3 min of ischemia 2 days prior to a second ischemic episode (10 min) actually protected the hippocampus against ischemic neuronal injury, a phenomenon known as preconditioning (Shamloo *et al.*, 1999). By *in situ* hybridization we observed that STEP message was highly expressed in the hippocampus, particularly in the CA2 region in sham operated animals (Fig. 2a, e and i), consistent with previous data on STEP protein expression (Boulanger *et al.*, 1995). Animals were subjected to 3 or 10 min of global ischemia with and without preconditioning followed by 12, 18 and 24 h of reperfusion. Exposure to 10 min of ischemia induced a significant and persistent down-regulation of STEP mRNA in the cortex, CA1/CA2 layers of the hippocampus as well as subcortical areas at all time points after reperfusion (Fig. 2b–d). By contrast, in the dentate gyrus, a hippocampal region which is resistant to ischemic injury, 10 min of ischemia resulted in up-regulation of STEP mRNA (Fig. 2b–d). This indicates that STEP mRNA is down-regulated in vulnerable (cortex, CA1 and CA2) and up-regulated in resistant (dentate gyrus) regions of the brain in response to damaging periods of global cerebral ischemia. By contrast, a single 3 min of ischemia, which does not result in damage, induced up-regulation of STEP mRNA throughout the brain (Fig. 2e–h). This up-regulation correlates with the tolerance developed in the brain tissue against injury by this short episode of ischemia. Finally in the preconditioning model, in which a 3-min ischemic challenge was given 2 days before 10 min of ischemia, STEP mRNA was not down-regulated to the same extent in cortex or CA2 cell layer of the hippocampus (Fig. 2i–l). In addition, no up-regulation of STEP mRNA was detected in dentate gyrus. This result suggests that when the brain is protected by preconditioning against ischemic injury STEP mRNA is not regulated in the same way as after 10 min of detrimental ischemia. Our results suggest a strong correlation between down-regulation of STEP mRNA and neuronal injury, and an up-regulation of STEP mRNA in regions that are resistant to ischemic neuronal injury.

Cleavage of STEP after tMCAO

Previously it has been demonstrated that in a perinatal model of hypoxia–ischemia, STEP is processed into a 33-kDa isoform (STEP₃₃) (Gurd *et al.*, 1999). This isoform was produced by calpain-mediated cleavage (Nguyen *et al.*, 1999). Therefore, we performed experiments to confirm that cerebral ischemia in adult rats can result in post-translational effects on STEP as well as affecting its mRNA levels. Lysates were prepared from the ischemic (ipsilateral) and contralateral hemispheres of animals that had undergone 2 h of tMCAO followed by varying periods of reperfusion. We found that within 45 min following tMCAO, STEP₃₃ was detected in the ischemic side of the brain and increased over time, being still present at high levels 48 h after reperfusion commenced (Fig. 3a–d). With the appearance of STEP₃₃ there was a

significant loss of STEP₆₁ (Fig. 3b) and a trend towards reduced levels of STEP₄₆ (Fig. 3c), suggesting that STEP₃₃ is produced by cleavage of these higher molecular-weight species.

STEP cleavage results in a redistribution to soluble compartments

STEP₆₁ contains hydrophobic sequences within its N-terminal domain that target it to membraneous compartments, and previous reports have demonstrated that STEP₆₁ is localized, in part, to the endoplasmic reticulum (Boulanger *et al.*, 1995). In contrast, STEP₄₆ does not have putative membrane spanning domains and is cytoplasmic (Lombroso *et al.*, 1993), and STEP₃₃, although its sequence is not known, has also been found to be cytoplasmic (Gurd *et al.*, 1999). We therefore studied the redistribution of STEP in the tMCAO model using subcellular fractionation. Brains were taken from animals that had undergone tMCAO immediately after the ischemic insult or after 6 h of reperfusion. Centrifugation was performed to remove nuclei, and further rounds of centrifugation were performed to separate large membraneous, primarily synaptosomal, components (P2 fraction) and small membraneous, microsomal compartments (P3 fraction), leaving the cytoplasmic supernatant (S3 fraction). The majority of STEP₆₁ is found in membrane-bound compartments, particularly the microsomal P3 fractions, with a reduction in STEP₆₁ levels particularly evident after 6 h of reperfusion in this compartment (Fig. 4). It is also notable that there is a small downward shift in the STEP₆₁ band in the ipsilateral side of the brain, observed immediately after the period of MCAO. STEP₄₆ and STEP₃₃ are found predominantly in the cytoplasmic fraction (Fig. 4) indicating that these are soluble forms. Therefore, tMCAO leads to a redistribution of STEP from membrane-bound compartments to the cytoplasm.

ERK phosphorylation is altered after MCAO

We next wanted to determine the consequences that STEP cleavage might have on the activity of ERK. To this end, samples were analysed for their levels of ERK phosphorylation after tMCAO. pERK significantly increased 45 min after reperfusion, but then returned to baseline levels by 12 h after the ischemic challenge (Fig. 5a and c), whilst total levels of ERK were unchanged (Fig. 5b). These experiments showed that subsequent to tMCAO there are significant changes in the levels of ERK phosphorylation, but this does not correlate closely with the temporal profile of STEP cleavage (Fig. 3).

STEP₃₃ phosphatase activity

The formation of STEP₃₃ could lead to direct changes in its activity and also a redistribution of STEP activity away from, or towards, its substrates. To test first whether STEP₃₃ can regulate pERK activity, we isolated STEP₃₃ from calpain-cleaved, recombinant, GST-STEP₄₆ protein *in vitro*. After digestion, glutathione-sepharose beads were added to remove the N-terminal of GST-STEP₄₆ (GST-STEP₄₆ N-ter), leaving behind the C-terminal fragment (STEP₃₃). After pulldown of GST-STEP₄₆ N-ter, we tested the purity of the samples by immunoblotting with anti-STEP or anti-GST antibodies, and found that calpain digestion appeared to be complete and separation of each fragment was effective (Fig. 6a). Using pNPP as a substrate, we next tested for phosphatase activity of STEP₃₃ and compared its activity with that of uncleaved GST-STEP₄₆. STEP₃₃ was as active as uncleaved STEP₄₆ (Fig. 6b). Therefore, the cleavage of STEP does not affect its enzymatic activity towards the small molecule substrate pNPP.

STEP₃₃ binding and activity towards pERK

As STEP₄₆ and STEP₆₁ are known to bind to ERK through a kinase interaction motif (KIM) (Pulido *et al.*, 1998), we next assessed whether STEP₃₃ could still bind to ERK after cleavage. Immunoprecipitations were performed using GST-STEP₄₆ or calpain-digested STEP₃₃. Phosphorylated ERK from rat brain homogenates co-immunoprecipitated with GST-STEP₄₆, but not with STEP₃₃ (Fig. 6c). We also performed GST pulldown assays of pERK with GST-

STEP₄₆ and the N-terminal fragment remaining after cleavage, GST-STEP₄₆ N-ter (Fig. 6d). We observed that the N-terminal cleavage product was also unable to bind to pERK (Fig. 6d). Therefore, cleavage of GST-STEP₄₆ results in the elimination of any STEP species that has the ability to bind to pERK.

As we had demonstrated that STEP₃₃ retained catalytic activity (Fig. 6b), yet was unable to bind to pERK (Fig. 6c), we next tested whether STEP₃₃ could dephosphorylate pERK. GST-STEP₄₆ demonstrated a dose-dependent dephosphorylation of purified pERK (Fig. 6e, top panels). However, STEP₃₃ was unable to dephosphorylate pERK (Fig. 6e, bottom panels). Previous studies have shown that the intact KIM domain is essential for STEP-pERK interactions (Pulido *et al.*, 1998). We therefore tested whether the calpain cleavage site(s) might lie within the KIM domain and result in its destruction. To test this hypothesis, we compared the digestion pattern between GST-STEP₄₆ and a construct in which the KIM domain had been deleted, GST-STEP₄₆ (Δ KIM). As shown above, GST-STEP₄₆ was digested by calpain to generate STEP₃₃ (Fig. 6f, top panel); however, GST-STEP₄₆ (Δ KIM) was not cleaved by calpain *in vitro* (Fig. 6f, bottom panel). Therefore, calpain-mediated cleavage is likely to occur within the KIM domain, generating STEP₃₃ that is no longer able to bind to and dephosphorylate pERK.

Discussion

The results presented here demonstrate that there are significant, rapid and long-lasting changes in STEP at multiple levels in response to cerebral ischemia. We have demonstrated that, at the transcriptional level, the mRNA message for STEP is down-regulated after tMCAO. The down-regulation initiates in the ischemic core and extends over time into the penumbra region in a pattern that follows ischemic damage to tissue. Similarly, in a model of global ischemia, the mRNA for STEP is down-regulated throughout most of the brain; however, in the dentate gyrus, an area that is relatively spared from ischemic damage (Kirino, 1982; Pulsinelli *et al.*, 1982; Yamashima *et al.*, 2007), STEP message appears to be up-regulated. With a sublethal preconditioning paradigm (3 min of ischemia alone) in which there is no resultant ischemic damage, STEP message appears to be up-regulated throughout the brain. When the previously damaging 10 min of ischemia is preceded by a preconditioning paradigm, the dramatic changes in hippocampal STEP mRNA levels are no longer observed. These findings, across different models, suggest that reductions in STEP message levels correlate with the progression of ischemic cell death, while higher expression levels of STEP mRNA are associated with regions that are protected from ischemic injury.

We also found that there are profound changes in STEP protein in the brain. Within 45 min of reperfusion after tMCAO, a short isoform of STEP is detected, STEP₃₃, with a concomitant reduction in the higher molecular-weight STEP₆₁ and STEP₄₆ isoforms. These changes in STEP lead to a reduction in membrane-localized STEP and its redistribution to soluble compartments. Mechanistically, it has been proposed that STEP₃₃ is formed by calpain-mediated cleavage of STEP₆₁ (Nguyen *et al.*, 1999). We also observed a downward shift in the band corresponding to STEP₆₁ subsequent to ischemia, potentially due to a change in the phosphorylation state of STEP₆₁, making it a substrate for calpain cleavage. Previous studies have also found rapid changes in STEP levels. For example, fear conditioning training in rats leads to the *de novo* expression of the STEP₄₆ isoform in the lateral amygdala (Paul *et al.*, 2007). Therefore, it appears that STEP is dynamically regulated at the protein level to respond to changes in neuronal input, stress, injury or behavioral training.

STEP has been found to play a regulatory role in normal synaptic function, plasticity and learning (Pelkey *et al.*, 2002; Paul *et al.*, 2007) as well as pathological states including Alzheimer's disease (Snyder *et al.*, 2005) and epilepsy (Choi *et al.*, 2007). These findings fit

with the central importance of STEP's known substrates, NMDA receptors (Pelkey *et al.*, 2002), ERK (Pulido *et al.*, 1998) and non-receptor tyrosine kinases (Nguyen *et al.*, 2002). These studies have shown that STEP can act to dephosphorylate its substrates and limit their activity. In our studies we have demonstrated that cleavage of STEP takes place subsequent to tMCAO and reperfusion, occurring within the KIM domain, rendering STEP unable to bind to one of its important substrates, pERK, and therefore unable to dephosphorylate it, although STEP₃₃ does retain intrinsic phosphatase activity. Further work is necessary to define the exact site of cleavage and the implications it has on STEP's subcellular localization and roles with respect to other substrates. The normal function of STEP in relation to ERK appears to be in regulating the duration of ERK signaling (Paul *et al.*, 2003) and acting as a key intermediary between glutamatergic and dopaminergic inputs to regulate these patterns of ERK phosphorylation (Valjent *et al.*, 2005). A critical serine residue in the KIM domain of STEP (S49 in STEP₄₆, S221 in STEP₆₁) is phosphorylated by protein kinase A (Paul *et al.*, 2000), sterically blocking the ability of STEP to bind to its substrates. Conversely, NMDA receptor-mediated activation of calcineurin leads to the dephosphorylation of this residue and the subsequent activation of STEP (Paul *et al.*, 2003). Therefore, normal signaling mechanisms exist in the brain to control STEP's activity with respect to its substrate ERK.

Our findings suggest that cerebral ischemia leads to a rapid change in STEP that circumvents this fine control of its normal function. It is known that subsequent to ischemic damage, there is a massive release of glutamate (Choi & Rothman, 1990). This could potentially act through NMDA receptors to activate STEP, allowing it to dephosphorylate ERK in the absence of any other control mechanism. By cleavage of STEP in the KIM domain, the deactivation of ERK is prevented even in the presence of excessive glutamate and therefore potentially acting to retain its signaling ability. ERK phosphorylation is important in the responses to ischemia, with activation being neuroprotective (Shamloo & Wieloch, 1999; Shamloo *et al.*, 1999), while extreme and prolonged ERK phosphorylation can lead to apoptosis (Nozaki *et al.*, 2001). Our finding that subsequent to ischemia there is a transient increase in ERK phosphorylation that returns to basal levels indicates that cerebral ischemia initiates a transient ERK signaling that could be an attempt at neuroprotection. The processing of STEP to produce forms that are unable to dephosphorylate ERK may facilitate ERK activation, even in the presence of large amounts of glutamate that would otherwise counteract this response. The reduced levels of active STEP and the decrease in STEP mRNA suggest that the later reductions in ERK phosphorylation observed are mediated by the kinases that regulate ERK activity, or by other phosphatases within the ischemic region. The relationship between STEP₃₃ and its other potential substrates has not yet been studied directly. It will prove interesting to investigate the roles of STEP₃₃ with respect to NMDA receptors, activation of which has been linked to damage in stroke (Simon *et al.*, 1984). Previously, it has been demonstrated that STEP dephosphorylates NMDA receptors resulting in their removal from synaptic membranes and reduced NMDA receptor function (Pelkey *et al.*, 2002; Snyder *et al.*, 2005; Braithwaite *et al.*, 2006a). It is notable that focal and global ischemic episodes induce an up-regulation of NMDA receptor tyrosine phosphorylation during reperfusion (Shamloo *et al.*, 1999; Matsumoto *et al.*, 2002; Takagi *et al.*, 2003) that may also be a result of the decrease in the ability of STEP to interact with its substrates. Therefore, STEP cleavage subsequent to tMCAO, and down-regulation of STEP message during the reperfusion period, may be a multifaceted attempt for the CNS to counteract the consequences of excessive glutamate release and so minimize damage to the brain.

Our results suggest that STEP is regulated at the levels of transcription, localization and function following cerebral ischemia. These changes are apparently induced to limit neuronal damage and can clearly impact the outcome of injury, indicative of a central role of STEP in CNS function and pathology.

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Abbreviations

CNS, central nervous system; JNK, c-jun N-terminal kinase; KIM, kinase interaction motif; MAPK, mitogen-activated protein kinase; (p)ERK, (phosphorylated) extracellular signal-regulated kinase 1 and 2; STEP, striatal enriched protein tyrosine phosphatase; (t)MCAO, (transient) middle cerebral artery occlusion.

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Fig. 1.

STEP mRNA expression is dramatically reduced in the ischemic hemisphere of the brain during the reperfusion period after MCAO. (a) *In situ* hybridization shows that in control animals STEP is highly expressed in the striatum, but also present in the cortex. (b) In sham operated animals 24 h after surgery, STEP levels remain unaffected. (c–i) Animals that have undergone 2 h of transient MCAO with varying periods of reperfusion show a time-dependent decrease in STEP mRNA levels initially in the ischemic core region, and later extending into the penumbra. Data are representative of at least three animals at each timepoint

Fig. 2.

STEP mRNA levels are altered in a model of global ischemia. (a,e,i) In control brains from sham operated animals, STEP message is observed throughout the brain, with notably high levels in the CA2 and subiculum regions of the hippocampus. (b–d) Animals that have undergone 10 min of global ischemia (10' ischemia) followed by varying periods of recovery show a time-dependent decrease in STEP mRNA in the CA1, CA2 and subiculum cell layers of the hippocampus with an increase in the dentate gyrus. (e–h) A short, 3-min carotid artery occlusion (3' ischemia) results in increased STEP mRNA levels throughout the brain. (i–l) When the 10-min global ischemia is preceded by a 3-min preconditioning episode, STEP mRNA was not down-regulated to the same extent in subiculum and CA2 regions as in non-conditioned ischemia during 12–24 h of reperfusion. In addition no up-regulation of STEP was detected in dentate gyrus. Images are representative of at least four animals at each timepoint.

Fig. 3.

STEP is cleaved after transient MCAO. (a) Western blot of brain lysates prepared from the ipsilateral (i) or contralateral (c) side of the brain demonstrates that STEP is rapidly processed from its major isoforms, STEP₆₁ and STEP₄₆, to generate STEP₃₃ on the ischemic side of the brain. Results are representative of three animals at each time point. (b) Quantification of STEP₆₁ levels shows that there are significant reductions in the ischemic hemisphere (●) compared with the contralateral (■) 45 min, 6 h and 48 h after MCAO (* $P < 0.05$). (c) Quantification of levels of STEP₄₆. (d) Quantification of STEP₃₃ shows significantly increased levels in the ischemic hemisphere compared with the contralateral after 3, 12 and 48 h (* $P < 0.05$) and at 24 h (** $P < 0.01$)

Fig. 4.

Transient MCAO leads to redistribution of STEP from membrane-bound to soluble compartments. Subcellular fractionation was performed on ipsilateral (ips) or contralateral (con) hemispheres of brains taken from animals that had undergone 2 h of tMCAO. Brains were taken either immediately (0 h) or after 6 h of reperfusion (6 h). Results are representative of three animals at each time point.

Fig. 5.

Timecourse of ERK phosphorylation changes after transient MCAO. Brains were isolated from animals that had undergone 2 h of MCAO and varying periods of reperfusion, and were dissected into the ipsilateral (i) and contralateral (c) hemispheres. (a) Western blots were probed for phosphorylated ERK (representative of three independent experiments.) (b) Samples were reprobed for total levels of ERK. (c) Quantification of ERK phosphorylation demonstrates significant increases in the ischemic (●) relative to the contralateral (■) hemisphere 45 min and 3 h after MCAO (** $P < 0.005$) and at 6 h (* $P < 0.05$) before returning to basal levels after 12 h.

Fig. 6.

Calpain-mediated cleavage of STEP results in the formation of STEP₃₃, which is catalytically active, yet unable to bind to and dephosphorylate pERK. (a) Recombinant GST-STEP₄₆ protein was digested with active calpain I *in vitro*. After digestion, the N-terminal fragment was affinity purified (designated as GST-STEP₄₆ N-ter), leaving a pure preparation of the C-terminal fragment (STEP₃₃). The efficiency of separation of the two fragments was tested with anti-GST and anti-STEP antibodies. (b) Phosphatase activity of GST-STEP₄₆ and STEP₃₃ was compared using pNPP. GST alone was used as a negative control. (c) Immunoprecipitation experiments were performed with either GST-STEP₄₆ (10 μg) or STEP₃₃ (10 μg) added to rat whole brain homogenates (100 μg, S2 fractions) and immunoprecipitated with anti-STEP

antibody. Co-precipitated pERK was detected by immunoblot using anti-pERK (upper panel). The same blot was stripped and reprobed with anti-STEP antibody (lower panel). Protein G-agarose beads alone were used as a negative control (lane 2). (d) GST pull-down experiments were performed with 10 μ g of the indicated GST fusion proteins added to 100 μ g of S2 fractions and glutathione-sepharose beads. Co-precipitated pERK was detected by anti-pERK (upper panel). The same blot was stripped and analysed with anti-GST antibody (lower panel). (e) Increasing amounts of GST-STEP₄₆ (upper panels) or STEP₃₃ (bottom panels) were tested for phosphatase activity using pERK as substrate. Samples were resolved by SDS-PAGE, followed by immunoblot assay using anti-pERK antibody (upper panels). Levels of STEP were confirmed with anti-STEP antibody (lower panels). (f) Full-length GST-STEP₄₆ (100 ng) or GST-STEP₄₆ (Δ KIM; 100 ng) were incubated with calpain I. The digested material was processed by SDS-PAGE and immunoblotted with anti-STEP antibody.