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Different mechanisms account for ERK activation in distinct brain regions following global ischemia and reperfusion

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Abstract

Oxidative stress after cerebral ischemia and reperfusion activates extracellular signal-regulated kinases (ERK) in brain. However, the mechanism of this activation has not been elucidated. We have previously reported that in an in vitro model of oxidative stress in immature cortical neuronal cultures, the inhibition of ERK phosphatase activity contributes to ERK1/2 activation and subsequent neuronal toxicity. This study examined whether ERK activation was associated with altered activity of ERK phosphatases in a rat cardiac arrest model. Rats in experimental groups were subjected to asphyxial cardiac arrest for 8 min and then resuscitated for 30 min. Significant ERK activation was detected in both cortex and hippocampus following ischemia/reperfusion by immunoblotting. ERK phosphatase activity was reversibly inhibited in cerebral cortex but not affected in hippocampus following ischemia/reperfusion. MEK1/2 was activated in both cerebral cortex and hippocampus following ischemia/reperfusion. Using a specific inhibitor of protein phosphatase 2A (PP2A), okadaic acid (OA), we have identified PP2A to be the major ERK phosphatase that is responsible for regulating ERK activation in ischemic brain tissues. Orthovanadate inhibited ERK phosphatase activity in brain tissues, suggesting that tyrosine phosphatases and dual specificity phosphatases may also contribute to the ERK phosphatase activity in brain tissues. Together, these data implicate ERK phosphatase in the regulation of ERK activation in distinct brain regions following global ischemia.

Keywords

Ischemia/reperfusion; ERK; Phosphatase; Oxidative stress

Oxidative stress is generated in many tissues including brain following ischemia and reperfusion. Increased levels of oxidized lipids, proteins and nucleic acids are observed after reperfusion with oxygenated blood but do not accompany the initial ischemic event. The depletion of antioxidants observed soon after reperfusion (i.e. 10 min) contributes to the accumulation of damaging reactive oxygen species (ROS) (Katz et al., 1998). In reperfusion

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that occurs in brain tissue following global ischemia, antioxidants recover within 120 min. Nonetheless, this transient reperfusion-induced oxidation appears to contribute to neuronal injury that occurs following both transient global and focal ischemia.

Cerebral ischemia and reperfusion produces multiple changes in cellular signaling. Specifically, kinases in the mitogen activated protein kinases (MAPK) family are activated during reperfusion after both focal (Alessandrini et al., 1999) and global ischemia (Hu et al., 2000; Hicks et al., 2000b). Members of the MAPK family that are activated during reperfusion include the extracellular-signal regulated kinase (ERK) and N-terminal Jun-kinase (JNK). The activation of MAPKs after ischemia is multiphasic, including both an early onset, rapid increase during the first 30–60 min of reperfusion, and a later onset, more protracted, increase between 12 and 24 hrs after reperfusion (Hicks et al., 2000b). In a transient focal ischemia model, ERK activation was found 5 min following ischemia/reperfusion and persists for 24 hrs (Alessandrini et al., 1999; Namura et al., 2001). After global ischemia/reperfusion, ERK activation has also been noted both at 30 min and persisting to 24 hrs (Hu et al., 2000; Hicks et al., 2000b; D'Cruz et al., 2002).

ERK1/2 activation is a well established response in many cells to stimulation by mitogens such as neurotrophins and results in neuronal proliferation and differentiation in vitro (Pearson et al., 2001). Increased ERK activation is also associated with cell survival in some in vivo systems. After global ischemia ERK was activated at 30 min of reperfusion in surviving dentate gyrus (DG) cells, but not in vulnerable hippocampal CA1 neurons (Hu et al., 2000). We previously reported that in a rat asphyxial cardiac arrest model, a regimen of hypothermia that decreases hippocampal neuronal death increases ERK activation in the hippocampus (Hicks et al., 2000b).

In contrast, a growing body of studies has revealed a role of ERK in neuronal death. For example, pharmacological inhibition of ERK1/2 activation using U0126, a specific MEK1/2 inhibitor, reduced neuronal injury that results from focal ischemia (Alessandrini et al., 1999; Namura et al., 2001). ERK1/2 activation has been found to be necessary for neuronal toxicity in vitro in response to various cell death inducing stimuli such as glutamate (Stanciu et al., 2000; Levinthal and DeFranco 2004), zinc (Seo et al., 2001), Fe/Amyloid beta (Kuperstein and Yavin, 2002), 6-hydroxydopamine (Kulich and Chu, 2001) and methylisothiazolinone (Du et al., 2002).

ERK activity is regulated by its phosphorylation state, which is the result of the balanced action of both ERK kinases and ERK-directed protein phosphatases. The immediate upstream kinase of ERK1/2 is MEK1/2. MEK1/2 is also activated by phosphorylation utilizing kinases in the Raf family including A-Raf, B-Raf and Raf-1 (Pearson et al., 2001). Protein phosphatases that can affect ERK phosphorylation include tyrosine phosphatases, serine/threonine phosphatases, and dual-specificity phosphatases (Keyse, 2000). PP2A (Silverstein et al., 2002; Kim et al., 2003) and MKP3 (Camps et al., 1998) are two major ERK phosphatases.

We have previously shown that ERK activation following oxidative stress in immature primary neuronal cultures is driven by the oxidative inhibition of ERK phosphatases (Levinthal and DeFranco, 2005). The relative contribution of upstream kinases and phosphatase activity to ERK activation after ischemia and reperfusion is unknown. This study tested whether ERK activation was associated with altered activity of ERK phosphatases in an in vivo ischemia/reperfusion model. We have employed a rat asphyxial-induced cardiac arrest model to induce global ischemia (Hicks et al., 2000a). This model mimics brain damage that occurs during the conditions when blood flow to the entire brain is impaired.

EXPERIMENTAL PROCEDURES

Subjects and experimental design

Animal protocols were approved by the University of Pittsburgh Animal Care and Use Committee. Male Sprague-Dawley rats (n=18) weighing 300–350 g were housed individually with food and water ad libitum. Rats were randomly assigned to three treatment groups: sham (n=6), asphyxial cardiac arrest without resuscitation (n=6) and asphyxial cardiac arrest followed by 30 min of reperfusion (n=6). Sham rats received anesthesia and surgery but were not subjected to asphyxia.

During procedures, temperature was monitored by computer and regulated via computer-driven relays connected to a 100-W heating lamp and a cooling fan (Colbourne et al., 1996; Hicks et al., 2000a). At least 3 days prior to the procedure, a 5 mm, 20-gauge stainless steel guide-cannula was stereotactically placed over the parietal cortex to allow placement of a battery-operated, wireless temperature probe (XH-FM-BP, MiniMitter, Sun River, OR, U.S.A.). All rats were maintained at 37°C during procedures and until sacrifice.

In order to induce asphyxia, rats were anesthetized with halothane, orotracheally intubated and mechanically ventilated as described previously (Hicks et al., 2000a & 2000b). Ventilation was titrated to blood gases monitored via femoral arterial catheters. Rats were chemically paralyzed with vecuronium (2 mg/kg), halothane was discontinued, and the fraction of inspired oxygen was reduced to 0.21 (room air) for 2 min. Mechanical ventilation was discontinued at end-expiration for 8 min, resulting in bradyasystolic circulatory arrest. For rats undergoing resuscitation, ventilation resumed with 100% oxygen, chest compressions were delivered at 200/min, and intravenous epinephrine (0.005 mg/kg) and bicarbonate (1 mEq/kg) were injected. These interventions reliably restored pulses in 30–60 sec. Rats were supported with mechanical ventilation until sacrifice.

Sham rats were sacrificed by decapitation after surgery and vecuronium administration, but prior to asphyxia. Asphyxia rats were sacrificed by decapitation after 8 min of asphyxial cardiac arrest. Resuscitated rats were sacrificed at 30 min after restoration of circulation by restarting halothane and then decapitation. Brains were dissected into chilled phosphate buffered saline (PBS) and cooled for 1 min. The cerebral cortex and hippocampus were dissected onto a cold metal stage and then frozen at –70°C.

ERK-directed phosphatase activity assay

Frozen tissues were solubilized by sonication for 5 sec on ice in 0.5ml of lysis buffer (50mM Tris-Cl, pH7.5, 2mM EDTA, 100mM NaCl, 1% Nonidet P-40, supplemented with protease inhibitor (Protease inhibitor cocktail, Sigma). The solubilized tissues were then centrifuged at 13,000 rpm for 5 min at 4°C and supernatants were collected for further analysis.

We have modified a nonradioactive method for determining ERK-directed phosphatase activity in tissue extracts (Laakko T and Juliano RL., 2003). This method relies on detecting dephosphorylation of a purified, dual-phosphorylated, His₆-tagged ERK upon incubation with the tissue extracts (Levinthal and DeFranco, 2005).

The alterations of ERK phosphatase activity within the tissue extracts can be monitored by measuring changes in the phosphorylation state of the isolated phosphorylated ERK substrate, as shown by Western blotting with a phospho-specific ERK1/2 antibody. Briefly, 150µg of tissue extracts were diluted into a total volume of 250µl in phosphatase assay buffer (10mM MgCl₂, 10mM Hepes, pH7.5 and 10µM of the MEK inhibitor, U0126). Recombinant dual phosphorylated His₆-ERK2 (Biomol, Plymouth Meeting, PA) was added to each sample (30ng/sample), and the reactions were maintained at 37°C for 15 min where indicated. 50mM DTT

or 10nM okadaic acid (OA) or 1mM Na₃VO₄ was added to the sample for 30 min on ice, prior to the addition of purified pERK. Following a 15 min incubation at 37°C, the reactions were stopped by the addition of 250ul of wash buffer (8M urea, pH8.6, containing 10mM imidazole). 30ul of Ni²⁺-conjugated, magnetic beads (Qiagen, Valencia, CA) was then added to each reaction. After 90 min of rocking at 4°C, the samples were washed twice with wash buffer followed by one wash in 300mM NaCl, 25mM Tris, pH7.5. The beads were then suspended in Laemmli sample buffer, boiled for 5 min, loaded onto a 10% polyacrylamide gel, transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) and subjected to Western blotting to detect phosphorylated ERK and total ERK.

JNK-directed phosphatase activity assay

This method was performed exactly the same as described for ERK phosphatase assay, except that the samples were incubated with 30ng of purified, dual-phosphorylated His₆-tagged JNK3 protein (Upstate, Waltham, MA), and immunoblot with anti-phospho-JNK and total JNK antibodies (from Cell Signaling).

Immunoblot analysis

The tissue extracts were obtained as described above. Protein concentrations of the extracts were determined using the Bio-rad reagent. Equivalent amount of total protein (20–30ug) were separated by SDS-PAGE on 10% polyacrylamide gels and then transferred to polyvinylidene membranes (Millipore). Membranes were blocked with 5% dry milk in PBS/0.1% (v/v) Tween 20 (PBST). Membranes were then incubated with primary antibodies (anti-phospho-ERK, anti-total ERK, anti-phospho-MEK1, anti-total MEK1, all from Cell Signaling) overnight at 4°C with 2% BSA in PBST. The membranes were then washed three times with PBST (10 min each time), incubated with the appropriate horseradish, peroxidase-conjugated secondary antibody for 40 min at room temperature and followed by three time washes with PBST. Immunoreactive bands were then revealed by enhanced chemiluminescence (ECL, Amersham Biosciences) using standard x-ray film (Eastern Kodak Co., Rochester, NY). Densitometry was performed using a Personal Densitometer SI (Amersham Biosciences) linked to the ImageQuant 5.2 software (Amersham Biosciences).

Statistical Analysis

Comparison of two means was performed using a paired *t* test. Comparison of multiple mean values was performed by analysis of variance with Bonferroni's *post hoc* tests for significance. *p* value of <0.05 were considered to be significant. All data were analyzed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA).

RESULTS

ERK phosphatase activity is inhibited in ischemic/reperfusion cerebral cortex following cardiac arrest

Rats in experimental groups were subjected to asphyxia for 8 min and then resuscitated for 30 min while rats in sham group were only subjected to identical anesthesia and surgery without asphyxia. Rats in I/NR group were subjected to 8 min of asphyxia but not resuscitation. Baseline physiological variables were similar between all groups.

We previously reported that increased activation of ERK1/2 occurs within 12 h of reperfusion following asphyxial-induced cardiac arrest and persists for at least 24 h post-ischemia/reperfusion (Hicks et al., 2000b). In middle cerebral artery-occlusion (MCAO) focal ischemia models, ERK1/2 was activated as early as 5 min following ischemia/reperfusion (Alesandrinni et al., 1999; Namura et al., 2001). It has been shown that the early post-ischemic period is

associated with a large increase in oxidation (Katz et al., 1998). To investigate the effect of ischemia-induced oxidative stress on ERK1/2 activation, we examined if ERK1/2 activation was associated with early post-ischemic period in brain tissues following cardiac arrest. As cerebral cortex is one of the major sites of damage following cardiac arrest, extracts were prepared from adult male rat cerebral cortex isolated 30 min following global ischemia/reperfusion or control groups. Western blot analysis was performed to detect phospho-ERK1/2 and total ERK1/2. We found that ERK1/2 was dramatically activated in cortex following ischemia/reperfusion but not in sham animals or the ischemia/non-reperfusion animals (Fig 1A). To reveal if the inhibition of ERK-directed phosphatases by ischemia-induced oxidative stress contributes to the post-ischemic ERK1/2 hyperphosphorylation, ERK phosphatase activity was measured in extracts prepared from adult male rat cerebral cortex isolated 30 min following global ischemia/reperfusion or control groups using His₆-tagged phospho-ERK2 as a substrate. Compared with the robust ERK phosphatase activity in extracts of sham and ischemia/no reperfusion animals, ERK2-directed phosphatase activity in extracts from animals subjected to ischemia and a 30 min reperfusion decreased significantly (Fig 1B, C). The effects of ischemia/reperfusion on ERK2-directed phosphatases are selective as the phosphatases in cortical extracts that act on the MAPK family member JNK are not affected (Fig 1D).

DTT can reverse the inhibition of ERK phosphatase activity in cerebral cortex following ischemia/reperfusion

The selective inhibition of ERK1/2-directed phosphatase activity that occurs in oxidatively stressed primary neuronal cultures can be reversed upon the addition of DTT to neuronal extracts (Levinthal and DeFranco, 2005). We therefore added 50mM DTT to extracts of the cerebral cortex prepared following 30 min post-ischemia to examine whether the inhibition of ERK1/2 phosphatase activity following ischemia/reperfusion is reversible. The inhibition of ERK-directed phosphatase activity could be reversed by the addition of 50mM DTT to extracts prepared from ischemic cortical tissues (Fig 2A, B). Together with Fig 1, these results suggest that analogous to *in vitro* models of neuronal oxidative toxicity, the activation of ERK1/2 in cerebral cortex 30 min following global ischemia/reperfusion is driven in part by reversible inhibition of some ERK1/2-directed phosphatases.

ERK phosphatase activity is not changed in ischemic/reperfusion hippocampus following cardiac arrest

In addition to the cortex, global ischemia can affect multiple brain regions including the hippocampus (Katz et al., 1998) with histological signs of injury in the rat asphyxial model being more pronounced in hippocampus rather than cortex (Liachenko 1998; Hickey 2000). Furthermore, after reperfusion, cortex and hippocampus blood flow are similarly depressed when assessed using MRI (Liachenko 2001; Xu 2002). In that study, perfusion was related to the duration of resuscitation more than the duration of ischemia. We therefore examined whether activation of ERK1/2 in hippocampus is also associated with phosphatase inhibition. As observed in cortex, ERK1/2 is significantly activated in hippocampus 30 min following ischemia/reperfusion (Fig 3A). Interestingly, unlike the cortex, ERK phosphatase activity in hippocampus was not altered within 30 min of ischemia/reperfusion (Fig 3B, C). Thus, the robust activation of ERK1/2 in hippocampus at early times of reperfusion following global ischemia is not due to effects on ERK phosphatase activity, at least as measured in crude extracts.

pMEK is activated in both ischemic/reperfusion cerebral cortex and hippocampus

The activation state of ERK1/2 reflects the balance of its upstream activating kinase and inhibiting phosphatase. MEK1/2 is the upstream activating kinase of ERK1/2 and the activation state of MEK1/2 is reflected by its phosphorylation. Therefore, we examined whether MEK1/2

was activated in brain regions following ischemia/reperfusion. The level of activated MEK1/2 is increased in both cerebral cortex and hippocampus following ischemia/reperfusion when compared with control groups (Fig 4). Together with previous results on ERK phosphatase activity, these results strongly suggest that different mechanisms account for ERK activation in distinct brain regions following ischemia/reperfusion.

PP2A is the predominant ERK phosphatase in rat brain

Since the antibody used to detect His-tagged phospho-ERK2 in the phosphatase assay recognizes dually-phosphorylated ERK1/2, the reduction in signal upon incubation with brain extracts could reflect protein phosphatase action on either the ERK2 phosphotyrosine or phosphothreonine residue. A major component of ERK phosphatase activity in neurons is contributed by PP2A, a serine/threonine protein phosphatase (Millward et al., 1999; Virshup 2000). To determine the contribution of PP2A to ERK dephosphorylation in rat brain tissues, a specific inhibitor, okadaic acid (OA), was utilized. OA is a well-characterized serine/threonine-directed protein phosphatase inhibitor with a high degree of selectivity at low concentrations for PP2A. The addition of OA at the concentration that selectively blocks PP2A (10nM), inhibited the robust ERK-directed phosphatase activity in extracts prepared from normal cerebral cortex (Fig 5A, 5B) and hippocampus (Fig 5D, 5E) in rats, suggesting that PP2A is likely to be the predominant ERK2-directed phosphatase in these brain regions. Furthermore, these results show that both hippocampus and cortex contain active PP2A, although it is differentially responsive to ischemia/reperfusion injury in these tissues (see Fig 1C, 3C). Western blot using an antibody against PP2A C-subunit showed that the level of PP2A did not change following ischemia/reperfusion in both cerebral cortex and hippocampus (Fig 5C, 5F).

Tyrosine and dual specificity phosphatases also contribute to ERK phosphatase activity in rat brain

Sodium orthovanadate is a potent inhibitor of tyrosine and dual specificity phosphatases and does not affect serine/threonine phosphatases. To examine the role of tyrosine and dual specificity phosphatases in the observed ERK phosphatase activity in rat brain, we treated lysates from the cortex of sham animals with 1mM Na₃VO₄. Orthovanadate dramatically inhibited ERK phosphatase activity in extracts from cortex in sham animals (Fig 6A&B). Moreover, the extent of inhibition observed with orthovanadate was less than that observed with OA, confirming the predominant role of PP2A in ERK-directed phosphatase activity in rat brain (Fig 6C). However, tyrosine and/or dual specificity phosphatases seem to play some roles in dephosphorylating ERK in rat brain.

DISCUSSION

ERK activation occurs in a variety of brain regions in response to ischemia/reperfusion but the mechanism of this activation has not been thoroughly analyzed. In this report, we report regional differences in the response of the ERK signaling pathway to ischemia/reperfusion and a differential sensitivity of ERK phosphatases to oxidative stress that accompanies reperfusion. Specifically, the reversible inhibition of ERK phosphatases by ischemia/reperfusion in a cardiac arrest model contributes to early ERK activation in the rat cerebral cortex but not in hippocampus. Further examination of the profile of MEK1/2 activation has revealed the activation of MEK1/2 in both cerebral cortex and hippocampus following ischemia/reperfusion. These data suggest that different mechanisms could account for ERK activation in distinct brain regions following ischemia/reperfusion. In cerebral cortex, ischemia/reperfusion-induced oxidative stress results in both the activation of MEK1/2 and the inhibition of ERK phosphatases, leading to the ultimate hyperphosphorylation of ERK. In hippocampus, ERK activation following ischemia/reperfusion is achieved mainly through the activation of

MEK1/2 but not with detectable inhibition of ERK phosphatases. PP2A appears to be the major ERK phosphatase that is responsible for regulating ERK activation in ischemic brain tissues. Our results must still be interpreted with caution since ERK phosphatase measures were made in crude tissue homogenates. Once the mechanism responsible for phosphatase inhibition in ischemic tissue is revealed, molecular probes may be devised that would allow the definitive assessment of phosphatase inhibition *in vivo*.

Our study has revealed a region-specific pattern of the inhibition of ERK phosphatase activity. ERK phosphatase activity was inhibited in the ischemic cerebral cortex but not in the ischemic hippocampus. Several possibilities could account for the region specificity in ERK phosphatase activity we have observed. In our study, we assume that the same levels of ischemia/reperfusion affect all brain regions. However, changes in regional cerebral blood flow before, during, and after asphyxia-induced cardiac arrest were not monitored by laser-Doppler flowmetry. Therefore, recirculation failure for the various brain regions may account for the difference in observed inhibition of ERK phosphatase activity in different brain regions. Moreover, even if different brain regions are subject to the same constant impact of ischemia/reperfusion, the extent of oxidative stress, i.e., the level of ROS generated, may be different. Difference in the level of ROS in specific brain regions following ischemia/reperfusion is very likely to explain the specificity of ERK phosphatase inhibition. This will be addressed by measuring ROS levels in extracts of different brain regions following ischemia/reperfusion. By taking extracts from distinct brain regions, we assume that the complement of cell types in the extracts is the same (e.g., glia, neurons). It is also possible that the hippocampus has more glia cells in the extract and this explains the difference in measured phosphatase activity. However, this possibility is excluded since the proportion of glial cells has been analyzed by the Western blot analysis of their specific marker, GFAP, in both cortex and hippocampus. Same levels of GFAP have been found in both brain regions, suggesting that the difference in glial cell components could not account for the difference in phosphatase activity we observed. However, we did not measure the component of microglial cells in these two brain regions and this could be analyzed by using a specific marker of microglial cells.

Impairment of PP2A activity has been associated with an enhanced activation of ERK and hyperphosphorylation of tau, a specific ERK1/2 target, in Alzheimer's disease (Gong et al., 1995; Zhao et al., 2003). Transgenic mice expressing a mutant PP2A catalytic unit exhibited activation of ERK and JNK pathways as well as the phosphorylation of endogenous tau, similar to the key pathological features in Alzheimer's disease (Kins et al, 2001 & 2003). Although PP2A is the major phosphatase contributing to ERK dephosphorylation in cortical and hippocampal extracts, it appears that ischemia/reperfusion injury selectively affects this phosphatase in cortex but not hippocampus. Since both hippocampus and cortical tissues are subjected to oxidative stress at early times after reperfusion, PP2A may be differentially sensitive to oxidative inhibition in different brain regions. This implies that the response of individual signaling molecules to oxidative stress within individual neurons may be subjected to multiple levels of regulation that are not reflected strictly in the levels of oxidants. In addition to ERK1/2, JNK has also been shown to be a substrate of PP2A (Shanley et al., 2001). However, direct or indirect effects of ischemia/reperfusion do not appear to impact JNK phosphatases suggesting that, unlike dominant negative PP2A C subunit effects, the reversible inhibition of PP2A activity that occurs upon ischemia/reperfusion injury applies to select substrates.

Several studies on the impact of ischemia on protein phosphatases have focused on the eukaryotic initiation factor 2 alpha (eIF2 α) and its phosphatases. Inhibition of eIF2 α is an established mechanism for inhibiting protein synthesis following ischemia (DeGracia et al., 2002). Phosphorylation of eIF2 α during ischemic reperfusion (30 min) inactivates eIF2 α and increased eIF2 α P levels during reperfusion have been observed in various experimental models of transient global ischemia in the rat (DeGracia et al., 1999; de la Vega et al., 2001) and a few

studies of transient focal cerebral ischemia (Althausen et al., 2001; Mengesdorf et al., 2002). In a rat cardiac arrest model, eIF2 α phosphorylation increased rapidly and significantly in the brains following ischemia/reperfusion while there was no change in the eIF2 α phosphatase activity (DeGracia et al., 1999). In contrast to that study, eIF2 α phosphatase activity significantly decreased at 10–15 min of ischemia/reperfusion in a rat transient forebrain ischemia model (Martin de la Vega et al., 2001) while no activation of any eIF2 α kinase during ischemia/reperfusion was found. Experiments using a specific inhibitor of PP1 and PP2A to determine the contribution of PP1 and PP2A in brain extracts suggested that PP1 might be the major ischemia-inactivated eIF2 α phosphatase (Martin de la Vega et al., 2001).

In another model of transient forebrain ischemia in rat, calcineurin activity was selectively decreased in various brain regions examined, as the activity of PP2A did not change (Morioka et al., 2001). In various assays of protein phosphatase activity, different substrates and in vitro assay conditions have been utilized, which could obscure substrate-specific or oxidant-sensitive effects on these enzymes. The regional inhibition of ERK phosphatases in cerebral cortex extracts prepared after ischemia/reperfusion was reversed by an in vitro incubation with a thiol-reducing agent (DTT). Since both hippocampus and cortical tissues are subjected to oxidative stress at early times after reperfusion, select ERK phosphatases (e.g. PP2A) must be differentially sensitive to oxidative inhibition in different brain regions. This implies that the response of individual signaling molecules to oxidative stress within individual neurons may be subjected to multiple levels of regulation that are not reflected strictly in the levels of oxidants.

We have previously reported that in an in vitro model of oxidative stress in immature cortical neuronal cultures, the inhibition of ERK2 phosphatase activity contributes to ERK1/2 activation and subsequent neuronal toxicity (Levinthal and DeFranco, 2005). In this report, we establish for the first time a role for phosphatase inhibition in ERK activation following global ischemia. Furthermore, our data provide insights into the different regulating mechanisms on ERK activation in distinct brain regions following global ischemia/reperfusion. Considering the important association between ERK activation and neuronal survival in global ischemia, a better understanding of the mechanisms regulating ERK activation following ischemia/reperfusion could lead to new therapeutic strategies that target specific targets that regulate selective responses to oxidative stress.

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Abbreviations

ERK	Extracellular-signal regulated kinase
ROS	Reactive oxygen species
MAPK	mitogen activated protein kinases
JNK	N-terminal Jun-kinase
OA	okadaic acid
MCAO	middle cerebral artery-occlusion
PBS	phosphate buffered saline

eIF2 α

eukaryotic initiation factor 2 alpha

Figure 1. Inhibition of ERK-directed phosphatase activity in reperfused cerebral cortex

A. Endogenous pERK1/2 and ERK1/2 levels were measured by Western blot in corresponding extracts from adult male rat cortical tissue isolated 30 min following ischemia/reperfusion (I), control sham operations (C) or ischemia/without reperfusion (I/NR).

B. Extracts prepared from adult male rat cerebral cortex isolated 30 min following global ischemia/reperfusion (I), control sham operations (C) or ischemia/without reperfusion (I/NR) were incubated with His₆-tagged phospho-Erk-2. Western blot analysis was then performed of affinity purified His₆-tagged Erk-2 to reveal phospho-Erk-2 (pERK2) or total Erk-2 (ERK2) levels. IN: phosphatase assay input; C: control sham; I: ischemia/reperfusion; I/NR: ischemia without reperfusion.

C. Statistical analysis of two independent ERK2 phosphatase assays (n=6 in each group).
p<0.001.

D. Extracts prepared as described in B were incubated with His₆-tagged phospho-JNK3. Western blot analysis was then performed of affinity purified His₆-tagged JNK3 to reveal phospho-JNK3 or total JNK3 levels. IN: phosphatase assay input; C: control sham; I: ischemia/reperfusion; I/NR: ischemia without reperfusion.

Figure 2. Recovery of ERK2-directed protein phosphatase activity in ischemic/reperfusion cerebral cortex

A. Ischemic/reperfusion extracts from cerebral cortex shown in Fig 1 were incubated with 50 mM DTT where indicated prior to assaying for p-Erk-2 phosphatase activity.

B. The statistical analysis of two independent ERK2 phosphatase assays (n=6 in each group). $p < 0.05$.

Figure 3. ERK phosphatase activity in hippocampal extracts is unaffected by ischemia/reperfusion

A. Endogenous pERK1/2 and ERK1/2 levels were measured by Western blot in corresponding extracts from adult male rat hippocampal tissue isolated 30 min following ischemia/reperfusion (I), control sham operations (C) or ischemia/without reperfusion (I/NR).

B. Extracts prepared from adult male rat hippocampus isolated 30 min following ischemia/reperfusion (I), control sham operations (C) or ischemia/without reperfusion (I/NR) were incubated with His₆-tagged phospho-Erk-2. Western blot analysis was then performed of affinity purified His₆-tagged Erk-2 to reveal total Erk-2 (ERK2) or phospho-Erk-2 (pERK2) levels. IN: phosphatase assay input. C: control sham; I: ischemia/reperfusion; I/NR: ischemia without reperfusion.

C. Statistical analysis of two independent phosphatase assays (n=6 in each group). There is no significant difference between the groups.

Figure 4. MEK is activated in both ischemic cortex and hippocampus

Endogenous pMEK1/2 and MEK1/2 levels were measured by Western blot in corresponding extracts from adult male rat in cortical (CTX: A) or hippocampal (HPC: C) extracts isolated 30 min following ischemia/reperfusion (I), control sham operations (C) or ischemia/without reperfusion (I/NR). Statistical analysis of two independent Western blot analysis in B and D (n=6 in each group). $P < 0.001$.

Fig 5. PP2A is the predominant ERK2-directed phosphatase in rat cerebral cortex and hippocampus

A & D. Extracts prepared from control sham operated adult male rats in corresponding tissues (CTX & HPC) were incubated with 10nM OA where indicated prior to assaying for p-Erk-2 phosphatase activity. Input phospho-Erk-2 (IN) as well as phospho-Erk-2 digested with purified phosphatase (lambda) were included.

B & E. The statistical analysis of two independent phosphatase assays in corresponding tissues (n=6 in each group). ***: $p < 0.001$; **: $p < 0.01$.

C & F. Western blot analysis of PP2A C-subunits in corresponding ischemia/reperfusion tissues (I), control sham (C) or ischemic without reperfusion (I/NR).

Figure 6. Tyrosine phosphatases also contribute to ERK-directed phosphatase activity in rat cerebral cortex

A. Extracts prepared from control sham operated adult male rat in cerebral cortex were incubated with 1mM VO₄ where indicated prior to assaying for ERK2 phosphatase activity. Input phospho-ERK2 (IN) was included.

B. Statistical analysis of two independent ERK2 phosphatase assays (n=6 in each group). P<0.001.

C. Statistical analysis of two independent ERK2 phosphatase assays between OA inhibition and VO₄ inhibition experiment. The effect of OA inhibition of ERK phosphatase activity was statistically significant than that of VO₄. p<0.001.