Regulation of protein phosphatases in oxidatively stressed neurons

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Submitted to the Graduate Faculty of

Arts and Science in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2006

UNIVERSITY OF PITTSBURGH

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Oxidative stress induced by glutathione depletion in the mouse HT22 neuronal cell line and embryonic rat immature cortical neurons causes a delayed, sustained activation of extracellular signal-regulated kinases-1/2 (ERK1/2), which results in cell death. Previous studies from our lab have shown that this sustained activation of ERK1/2 is mediated primarily by a selective, reversible inhibition of ERK1/2-directed phosphatases. However, the mechanisms underlying the inhibition remain unclear.

Results from this thesis demonstrate that the inhibition of ERK1/2 phosphatases in HT22 cells and immature neurons is a consequence of oxidative stress induced by glutathione depletion as phosphatase activity is restored in cells treated with the antioxidant BHA. This agent leads to reduced ERK1/2 activation and neuroprotection. However, we also show that an increase in free intracellular Zn^{2+} that accompanies glutathione depletion-induced oxidative stress in HT22 cells and immature neurons contributes to selective inhibition of ERK1/2 phosphatase activity and consequently ERK1/2 activation and cell death. ERK1/2 also functions to maintain elevated levels of Zn^{2+}. Thus the elevation of intracellular Zn^{2+} within neurons subjected to oxidative stress can trigger a robust positive feedback loop operating through activated ERK1/2 that rapidly sets into motion a Zn^{2+} -dependent pathway of cell death.

Previous data from our lab have suggested that PP2A, a Serine/Threonine protein phosphatase, is the predominant ERK1/2 phosphatase in primary neurons. I have confirmed this

result by using specific peptide inhibitor of PP2A. Furthermore, I have revealed for the first time the reversible cysteine oxidation in the catalytic subunit of PP2A, suggesting that PP2A may be the phosphatase that is susceptible to oxidation and inactivation following glutamate treatment in HT22 cells and primary neurons.

ERK1/2 activation contributes to neuronal death following focal ischemia/reperfusion. In the third part of my thesis, I investigated the mechanisms responsible for ERK1/2 activation following ischemia/reperfusion. I have demonstrated that the selective inhibition of ERK1/2 phosphatases contributes to ERK1/2 activation following ischemia/reperfusion in both focal and global ischemia models. Altogether, these results implicate the inhibition of ERK1/2 phosphatases as an important mechanism for ERK1/2 activation in oxidatively stressed neurons.

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Dedicated to my parents

Pui Man Ng & Yuen Lung Ho

PREFACE

The work presented here could have never been accomplished without the help, support and encouragement of many people.

First of all, I would like to express my deep gratitude to my supervisor, Dr Donald DeFranco. Being a great scientist and an outstanding mentor, Don has taught me to think beyond the individual experiment and focus on the whole picture of the project. More importantly, Don has taught me the attitude towards life which he applies also to science: be optimistic, and not to be afraid to take risks. Don, I appreciate deeply your encouragement and guidance which helped me go through all the frustrations and hard times. Thank you.

I also want to thank all the members of my committee for providing me with valuable suggestions and guidance over the years. They are: Dr. Teresa Hastings, Dr. Michael Cascio, Dr. Yu Jiang, Dr. Stephen Meriney, Dr. Edda Thiels and Dr. Eric Klann. Thanks also go to many of my collaborators. Without their great help, I could not have finished the work in this thesis. Dr Megan Knoch and Dr Elias Aizenman helped me with zinc imaging experiment. Dr Pier Mastroberardino and Dr Tim Greenamyre assisted me with the thiol affinity chromatography experiment. Focal ischemia samples were provided by Dr Feng Zhang and Dr Jun Chen and global ischemia samples were provided by Rick Logue and Dr Cliff Callaway.

Thank you to all the members of the DeFranco Lab, past and present. They are David Levinthal, Xinjia Wang, Yue Luo, John Caltagarone, Marjet Heizer, Gary Kohanbash, Natalia

Kubicki, Marcia Lewis, Terry McGuire, Haibing Jiang, Kyunsoo Park, Jennifer Guerrero-Santoro, Anne Stetler, Bassem Dekelbab, and Meral Guzey. You all have made my time in the lab more enjoyable and fun. I would like to specifically thank Dr David Levinthal for his great help in my project and excellent training for culturing primary neurons.

Finally, I would like to thank my family, my husband, Yikpan Ng, my parents, Puiman Ng and Yuenlung Ho, and my brother, Kwan Ho. Although they are thousands of miles away from me, their unconditional love, encouragement and support has sustained me over the past five years and made me overcome all difficulties placed before me. Without their support and understanding, I could never have come so far. Thank you all for your love.

1.0 INTRODUCTION

1.1 OXIDATIVE STRESS IN NEURONS

1.1.1 ROS and oxidative stress

Oxidative stress is the result of the accumulation of reactive oxygen species (ROS) and is brought about by a disruption of the physiological balance between normal oxidant production and the action of various anti-oxidant defense systems. ROS includes a family of chemically reactive oxygen containing molecules, such as superoxide (O_2^-), hydroxyl radical (OH-) and hydrogen peroxide (H_2O_2). As the major sites of energy generation in the cell, mitochondria are the most important sources of ROS generation during the process of oxidative phosphorylation (Jezek and Hlavata, 2005). Unstable oxygen radicals are generated as the by-products of oxidative phosphorylation due to the incomplete reduction of oxygen in mitochondria.

ROS react with and modify most macromolecules, such as proteins, lipids, and nucleic acids in the cell. Therefore, the accumulation of ROS will cause damage to those molecules and subsequently lead to the dysfunction of normal cellular activities. However, not all ROS are harmful. ROS generated by NADPH oxidase complex represents an important defense system in phagocytes to intruding bacteria (Nordberg and Arner, 2001). Moreover, ROS have been found to regulate a variety of signal transduction pathways as well as the activity of transcription

factors such as nuclear factor – kappa B (Kabe et al., 2005) and activator protein-1 (AP-1) (Nordberg and Arner, 2001). For example, H_2O_2 could activate tyrosine kinases such as Lck or Src and then tyrosine phosphorylate the inhibitor of NF-kB (IkB). The phosphorylation of IkB then releases NF-kB from its association and activate NF-kB (Kabe et al., 2005). Therefore, it is clear that ROS act as important regulators of signaling pathways in normal cellular functions as well as under stress conditions.

1.1.2 ROS defense systems

Several antioxidant ROS defense systems exist to protect cells from harmful effects of excessive ROS accumulation. The ROS defense systems can be widely divided into two groups: enzymatic and non-enzymatic. Superoxide dismutases (SODs) and catalase are the most important enzymes that scavenge specific ROS species (Nordberg and Arner, 2001). SOD specifically converts superoxide radicals to hydrogen peroxide and molecular oxygen. Several isoforms of SOD have been identified. Manganese SOD (Mn-SOD) is located in mitochondria and essential for normal cellular function. Specifically, Mn-SOD knockout mice die neonatally or develop severe neurodegenerative diseases (Li et al., 1995b; Melov et al., 1998). Cytosolic copper and zinc-containing SOD (Cu/Zn SOD) and extracellular SOD also function together to scavenge superoxide radicals inside and outside of cells (Maier and Chan, 2002). Catalase is located in peroxisomes and specifically catalyzes the conversion of hydrogen peroxide to water and molecular oxygen.

Non-enzymatic ROS defense systems include a network of low molecular weight antioxidants, such as ascorbic acid (vitamin C), glutathione (GSH) and thioredoxin. Glutathione and thioredoxin function as thiol-reducing agents in the cytoplasm. However, due to the high

concentration of GSH, the GSH system constitutes the predominant redox buffer in cells (Jezek and Hlavata, 2005). GSH is a tripeptide (Glu-Cys-Gly) with a free thiol group that functions to reverse oxidized thiols in target molecules. Once oxidized, two GSH molecules form glutathione disulfide (GSSH). GSSH is then recycled to GSH by glutathione reductase (GR). Therefore, the ratio of GSH/GSSH reflects the status and extent of oxidative stress. Ascorbic acid can reduce peroxide and superoxide as well as convert alpha-tocopherol (vitamin E) to its reduced form (Buettner, 1993). By reducing alpha-tocopherol radicals, ascorbic acid prevents lipid peroxidation in plasma lipoproteins such as LDL (Sies et al., 1992). This may account for the proposed mechanism of vitamin C in preventing atherosclerotic plaque formation (Reaven and Witztum, 1996).

1.1.3 ROS and chronic neurodegenerative diseases

Due to its high consumption of oxygen as well as relative deficiency in antioxidant defense systems (low level of catalase activity and moderate SOD activity), the central nervous system (CNS) is particularly sensitive to ROS-induced damage (Rego and Oliveira, 2003). Oxidative stress is implicated in the pathogenesis of a wide range of chronic neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Alzheimer's disease (Simonian and Coyle, 1996; Mattson et al., 2001). These neurodegenerative diseases are characterized by progressive degeneration of specific neuronal populations and have familial and sporadic forms. Oxidative stress may play a role in some familial forms of these diseases. Mutations in the Cu/Zn SOD (SOD1) gene have been identified in a subset of familial ALS (Rosen et al., 1993). These mutations affect the structure of SOD and its activity (Deng et al., 1993). A familial form of Parkinson's disease has been associated with mutations in the gene for

protein alpha-synuclein (Polymeropoulos et al., 1997). Selective and extensive alpha-synuclein nitration has been found in synuclein aggregates in sporadic Parkinson's disease (Giasson et al., 2000). In vitro exposure to oxidative and nitrative species enhances stable formation of alpha-synuclein polymers, which is necessary for their accumulation within aggregations (Souza et al., 2000). Two major alpha-synuclein mutants, A30P and A53T, have an increased propensity to dimerization following oxidative exposure (Krishnan et al., 2003). Moreover, an increase in the expression of alpha-synuclein can itself induce oxidative stress (Hsu et al., 2000). Parkin is another protein whose mutations have been revealed to be responsible for a familial form of Parkinson's disease (Kitada et al., 1998). Overexpression of wild type parkin protects dopaminergic neuroblastoma cells from dopamine-induced toxicity by decreasing ROS generation and attenuating protein oxidation, while mutant parkin abrogates these effects (Jiang et al., 2004b). Moreover, this protective effect of wild type parkin is achieved through the limit of ROS production by suppressing the expression of monoamine oxidase, which produces large amounts of ROS by oxidizing dopamine (Jiang et al., 2006).

Apart from alpha-synuclein and parkin, the identification of mutations in DJ1 (Bonifati et al., 2003) and phosphatase and tensin homologue (PTEN)-induced kinase 1 (PINK1) (Valente et al., 2004) provides more evidences for a relation between the pathogenesis of Parkinson's disease and oxidative stress. DJ1 has been found to exhibit antioxidant properties (Canet-Aviles et al., 2004). By upregulating glutathione synthesis, overexpression of DJ1 protects cultured dopaminergic cells from oxidative stress (Zhou and Freed, 2005). DJ1 also protects cells from A53T alpha-synuclein-induced toxicity (Zhou and Freed, 2005). DJ1 knockout mice have an increased level of ROS generation and exhibit increased sensitivity to the exposure of an exogenous oxidative stress insult (Kim et al., 2005). Amyloid beta-peptide (1-42), which is

central to the pathogenesis of Alzheimer's disease, has been found to induce oxidative stress, including ROS generation, protein oxidation, and lipid peroxidation (Butterfield et al., 2001; Butterfield, 2002).

Although many genetic mutations are associated with the pathogenesis of neurodegenerative diseases, the majority of those diseases are still sporadic with no definitive known causes. The popular view is that the environment is the most influencing factor in disease etiology. Epidemiologic studies have suggested that environmental toxins that inhibit mitochondrial complex I contribute to the pathogenesis of Parkinson's disease (Sherer et al., 2002a). 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) inhibits complex I and reproduces Parkinson's disease like symptoms in rats (Dawson and Dawson, 2003). The best animal model of Parkinson's disease has been established by Greenamyre's lab (Betarbet et al., 2000). Rats chronically exposed to a commonly used pesticide, rotenone, develop Parkinson's disease-like symptoms, including selective dopaminergic neuron degeneration and the formation alpha-synuclein rich inclusions (Betarbet et al., 2000). Rotenone is a highly selective complex I inhibitor. Inhibition of complex I by MPTP and rotenone has been shown to cause oxidative damage by increasing ROS generation or decreasing GSH levels (Cassarino et al., 1997; Votyakova and Reynolds, 2001; Sherer et al., 2002b). Therefore, deficits in mitochondrial complex I function establish a consistent connection between the pathogenesis of Parkinson's disease and oxidative stress.

1.1.4 ROS and ischemia

The role of oxidative stress in the pathogenesis of acute neuronal injury, such as stroke, is well established. Reperfusion following ischemia causes a significant increase in ROS generation in

the perfusion territory of the occluded blood vessel, resulting in delayed neuronal cell death in that area (McCulloch and Dewar, 2001; Schaller and Graf, 2004). A series of complex neurochemical events occurs following ischemia/reperfusion, such as increased ROS generation, decreased GSH levels and antioxidant enzyme activities, and the impairment of mitochondrial functions. Various cellular events, such as the activation of the mitogen-activated regulated kinases (MAPK) signaling pathway, and initiation of protein synthesis machinery, such as eukaryotic initiation factors (eIFs), also occur (Schaller and Graf, 2004). Both apoptosis and necrosis are found in ischemia-induced neuronal death. While most cell death is necrotic in the ischemia core, apoptosis is more obvious in the penumbra zone, particularly the inner border regions surrounding the ischemia core (Li et al., 1995a). Delayed cell death is another characteristic of ischemia cell death (Lipton, 1999; Schaller and Graf, 2004). The delay varies from hours (McGee-Russell et al., 1970) to days or even weeks (Kirino, 1982; Du et al., 1996), depending on the extent of the insult and the brain regions affected. Animal models to study ischemia include global ischemia, which models cardiac arrest, and focal ischemia, which models stroke in human diseases. Global ischemia occurs when there is no blood flow to all or most of the brain and is most commonly produced by vessel occlusions (Lipton, 1999; Traystman, 2003). Focal ischemia is defined by a reduction in the cerebral blood flow to a distinct and specific brain area. Middle cerebral artery occlusion (MCAO) represents the most common model of focal ischemia (Lipton, 1999; Traystman, 2003).

Due to the critical role of oxidative stress in the pathogenesis of ischemia, a variety of pharmacological and genetic manipulations to reduce oxidative damage have been shown to attenuate ischemia damage (Chan, 2001; McCulloch and Dewar, 2001; Margaill et al., 2005). SOD1 transgenic mice exhibit attenuated neuronal death in global and focal ischemia models

(Chan et al., 1998; Noshita et al., 2002). Various antioxidant strategies have been evaluated for their efficacy in attenuating ischemia, including the inhibition of free radical generation, scavenging of free radicals, and enhancement of free radical degradation (Margaill et al., 2005). Dehydroascorbic acid (DHA), a blood-brain-barrier (BBB)-permeable oxidized form of ascorbic acid, has been shown to significantly reduce infarct volume following ischemia (Huang et al., 2001). Nitrone radical-trapping compounds, such as NXY059, have been reported to have significant neuroprotective effects following transient focal ischemia in rats (Kuroda et al., 1999). Clinical trials have been performed or are being performed on four antioxidants for their efficacy in attenuating ischemia (Margaill et al., 2005). Ischemia-induced oxidative stress has been shown to activate various cellular signaling pathways, including the well-established extracelluar-regulated kinase (ERK) pathway. A recent report has revealed that pharmacological inhibition of the extracelluar-regulated kinase (ERK) pathways reduces the extent of ischemia damage (Namura et al., 2001). This study provides some insights into the new therapeutic targets of ischemia and demonstrates the effectiveness of targeting intracellular signaling events in attenuating ischemia.

1.2 MAPK SIGNALING PATHWAYS

1.2.1 MAPK signaling cascades

The MAPKs family is a group of signaling modules involved in cellular responses to various stimuli and impacts diverse responses, such as adhesion, cell differentiation and proliferation,

synaptic plasticity, survival, and apoptosis. MAPK members include ERKs, Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPK kinase families (Pearson et al., 2001). Other new members of ERK family include ERK3 (Boulton et al., 1991; Gonzalez et al., 1992), ERK5 (Lee et al., 1995; Zhou et al., 1995), and ERK7 (Abe et al., 1999). The MAPKs are activated by dual-phosphorylation of a conserved Threonine-X-Tyrosine (T-X-Y) motif by upstream MAPK kinases (MEKs or MKKs), which are in turn, activated by their upstream kinases, MEK/MKK kinases (MEKKs or MKKKs) (Pearson et al., 2001).

Different isoforms exist in each MAPK family. ERK1 (44kDa) and ERK2 (42kDa) are two closely related isoforms of ERK. In the activation loop of ERK1 and ERK2, threonine and tyrosine are separated by a glutamate residue which forms a T-E-Y motif (Payne et al., 1991). The ERK signaling pathway is an excellent example that illustrates how an outside stimulus is relayed and translated into cellular signals that induce expression of specific proteins for distinct cellular events. Activation of receptor tyrosine kinases or G protein-coupled receptors by outside signals leads to the formation of a multiprotein complex that includes monomeric G proteins such as Ras. Ras then activates various MEKK isoforms, including Raf-1, A-Raf, and B-Raf, which in turn, activate MEK1 and MEK2 for the activation of ERK (Pearson et al., 2001). Apart from phosphorylating cytosolic and membrane-bound targets, active ERK also translocates to the nucleus to phosphorylate various targets. Phosphorylation of nuclear targets by ERK1/2 is a prerequisite for the effects of ERK-dependent gene transcription and subsequent cellular response (Brunet et al., 1999). The known nuclear targets of ERK1/2 are predominantly transcription factors such as CREB, ELK-1, c-Fos, and the AP-1 family. This highlights the importance of the nuclear translocation of ERK in regulating gene transcription. ERK cytoplasmic targets include cytosolic phospholipase A2 and protein kinases, such as pp90

ribosomal S6 kinases (RSKs). Once activated, RSKs translocate into the nucleus and phosphorylate downstream targets, such as CREB, CBP and c-Fos.

1.2.2 The regulation of MAPK signaling cascades

A variety of extracellular stimuli can induce ERK activation but the cellular responses resulting from ERK activation vary from cell proliferation to cell differentiation and even to cell death. Given the ubiquitous expression and numerous substrates of ERKs, there must exist a precise and complex machinery operating to ensure the conduction and conversion of specific signals to specific cellular responses. The fidelity and spatio-temporal regulation of ERK pathways have been the subjects of many studies. MAPK pathways not only share a high homology in their module organization but also a high similarity in their protein primary sequences (Pouyssegur et al., 2002). Furthermore, all MAPKs share similar phosphorylation consensus sequences in their substrates: Ser/Thr-Pro (S/T-P) (Pouyssegur et al., 2002). Therefore, it is very important to prevent the inappropriate cross-talk between different MAPK pathways to ensure appropriate cellular response. One mechanism to ensure this precise regulation of MAPK signaling is by the action of scaffolding proteins that interact with and bring together components of each MAPK module (Morrison and Davis, 2003). The formation of these multi-enzyme complexes facilitates the rapid and precise transmission of signals through the individual cascade.

Kinase suppressor of Ras (KSR) was identified as a positive regulator of Ras/MAPK signaling (Kornfeld et al., 1995). KSR deletion severely reduces ERK activation in both *C. elegans* and *Drosophila*, demonstrating the requirement of KSR for Ras-dependent ERK activation in these models (Anselmo et al., 2002; Ohmachi et al., 2002). β-arrestins have

important roles in G-protein-coupled-receptor (GPCR) signaling. Apart from interacting with GPCR and targeting it for endocytosis, β-arrestins can also mediate the interaction of GPCR with other signaling pathways, including ERK pathways (Morrison and Davis, 2003). It has been shown that β-arrestins can mediate GPCR signaling by the retention of active ERK in the cytoplasm, which results in a decrease in ERK nuclear translocation as well as subsequent transcriptional activities (Tohgo et al., 2002). Therefore, the affinity between GPCR/β-arrestins could determine the mechanisms of ERK activation and the subsequent biological outcomes (Tohgo et al., 2003).

Specific protein interactions and subsequent enzyme reactions between MAPKs and their activators, substrates, and regulators depend on specific docking sites on both MAPKs and their interacting proteins. Almost all members of MAPK interacting proteins in MAPK modules, such as its activator MAPK kinases (MEKs), its downstream substrates (MAPKAPKS), as well as its regulator MAPK phosphatases (MKPs), have a MAPK-docking site (Tanoue and Nishida, 2002). Characterized by a cluster of positively charged amino acids, the docking sites of MAPK-interacting molecules are necessary for the binding of these proteins to MAPK since mutations generated in these sequences result in the disruption of the interactions between MAPK interacting proteins and MAPKs (Tanoue and Nishida, 2002). The docking site on ERK2 contains a group of negatively charged amino acids and serves as a common docking (CD) site for MEK1, MKP3 (a specific MKP for ERK), and Mnk1 (a substrate specifically activated by ERK and p38) (Tanoue et al., 2000). Similar to those docking sites on MAPK-interacting proteins, mutations in this common docking site on ERK markedly decreases the efficiency of this enzyme (Tanoue et al., 2000). A corresponding common docking site is also found in p38 and JNK, suggesting that a common mechanism of increasing protein-protein interaction is

conserved in all MAPK pathways (Tanoue et al., 2000). The identification of these docking sites has provided new insights into the regulation of MAPK pathways. For example, injection of a peptide corresponding to the ERK1/2 binding site of MEK1/2 into the nucleus disrupts the association between ERK1/2 and MEK1/2, and therefore, inhibits the nuclear export of ERK1/2 by MEK1/2 (Fukuda et al., 1996; Adachi et al., 2000). Similarly, the introduction of a peptide corresponding to the docking site of ERK1/2 on p90RSK to the nucleus disrupts the association between ERK1/2 and their specific phosphatases and, therefore, increases ERK phosphorylation and activation in the nucleus (Volmat et al., 2001).

Because the substrates of ERK1/2 distribute in various cellular compartments, the subcellular localization of active ERK1/2 is very critical for its ability to gain access to its potential targets and determines the specific cellular responses and biological outcomes it will elicit. As mentioned above, cytoplasmic sequestration of active ERK1/2 by β-arrestins blocks the nuclear translocation of ERK1/2 as well as ERK-dependent transcriptional activities (Tohgo et al., 2002). Enforced cytoplasmic retention of active ERK1/2 inhibits ERK1/2–mediated nuclear gene transcription and growth factor-induced DNA replication (Brunet et al., 1999). PEA-15, a cytoplasmic protein, has been shown to anchor active ERK1/2 in the cytoplasm and block ERK1/2-dependent gene transcription and cell proliferation (Formstecher et al., 2001). Deletion of PEA-15 restores the nuclear translocation of ERK1/2 and ERK-dependent cFos transcription as well as cell proliferation (Formstecher et al., 2001). These studies highlight the critical role of ERK1/2 nuclear translocation in mediating gene transcription and proper cellular responses.

It is now known that ERK shuttles between the nucleus and the cytoplasm. However, the mechanisms governing ERK nuclear import and export have remained largely unknown.

Monomeric ERK2 can enter into the nucleus even when active nucleocytoplasmic trafficking is blocked (Adachi et al., 1999), which suggests that passive diffusion is a mechanism for ERK nuclear entry. However, when ERK2 is fused to beta-gal, a high molecular weight molecule, it can still enter into the nucleus (Adachi et al., 1999), suggesting that active nuclear transport is also responsible for translocating ERK into the nucleus. It has been reported that phosphorylation and homodimerization of ERK1/2 is necessary for its nuclear translocation (Khokhlatchev et al., 1998). Dimerization motif mutants of ERK2, when fused to beta-gal, are unable to enter the nucleus (Adachi et al., 1999), further demonstrating that dimerization of ERK is prerequisite to its active nuclear transport. Recently, evidence has been presented that ERK can pass through the nuclear pore by direct binding with nuclear pore proteins (Matsubayashi et al., 2001), which suggests that ERK may not use a classical nuclear import transporter for its nuclear import.

The mechanism responsible for ERK nuclear export is also not clear. Leptomycin B (LMB) blocks active nuclear export by interfering with the binding between proteins containing a nuclear export signal (NES) and their nuclear export receptor, exportin 1 (Ossareh-Nazari et al., 1997). It has been found that LMB traps ERK and MEK in the nucleus (Adachi et al., 2000; Volmat et al., 2001). MEK contains a nuclear export sequence (NES) and may play a critical role in nuclear export of ERK. Co-injection of ERK1/2 into the nucleus with wild-type MEK1/2, but not the NES-disrupted MEK1/2, triggers nuclear export of ERK1/2 (Adachi et al., 2000). Moreover, inhibiting the binding between ERK1/2 and MEK1/2 by introducing a peptide corresponding to the docking site of ERK1/2 on MEK1/2 blocks the nuclear export of ERK1/2 (Adachi et al., 2000).

The integration of spatial and temporal regulation of ERK activation in mediating ERK-dependent specific biological outcomes is best illustrated in the classic example of the responses

of PC12 cells to EGF and NGF stimulation (Marshall, 1995). In this system, EGF induces transient cytoplasmic ERK activation and causes cell proliferation. In contrast, NGF stimulates a more prolonged and sustained nuclear ERK activation and triggers cell differentiation (Marshall, 1995). The importance of regulating the duration of ERK activation in achieving different cellular responses is also demonstrated in many other systems, including hepatocytes, neuroepithelioma cell lines, and fibroblasts (Colucci-D'Amato et al., 2003). Recently, our lab has shown that the duration of ERK activation can determine the divergent effects of ERK to promote cell survival or cell death even within a single cell type (Luo and DeFranco, 2006).

In summary, the regulation of MAPK activation is a very complex process, involving the integration of signal recognition, conveyance to appropriate pathways, localization and duration of the signals, cross-talk with other signaling pathways, and finally, signal relay to specific downstream effectors. The integration of all these factors determines the specificity of the signal transduction pathway in mediating specific biological outcomes.

1.2.3 Functions of MAPK

Compared with numerous *in vitro* studies on the physiological roles of MAPK pathways, gene-targeting studies provide important information on the roles of these pathways *in vivo*. In the past 10 years, mice genetically deleted for various MAPK members have been generated, allowing the examination of physiological roles of specific isoforms in each MAPK family (Pearson et al., 2001; Kuida and Boucher, 2004). ERK1 knockout mice are fertile and normal in many aspects. However, defects in thymocyte maturation have been found, suggesting that ERK1 may play a role in the development of normal thymocytes (Pages et al., 1999). Interestingly, ERK1-deficient mice have been shown to exhibit enhanced synaptic plasticity in the striatum and increased

performance in striatum-mediated learning tasks (Mazzucchelli et al., 2002). In contrast, ERK2-deficient mice exhibit abnormal placenta development and fail to induce the mesoderm, causing embryonic lethality (Hatano et al., 2003; Yao et al., 2003). This suggests an indispensable role of ERK2 in embryonic development. Although individual JNK1, JNK2, and JNK3 knockout mice are normal and viable, JNK1/JNK2 double knockout mice have embryonic lethality due to an abnormality in neural tube closure (Kuan et al., 1999; Sabapathy et al., 1999). JNK1/JNK2 may play an important role in regulating apoptosis, since fibroblasts from JNK1/JNK2 double knockout mice are resistant to stress-induced apoptosis (Tournier et al., 2000). Compared with JNK1 and JNK2, JNK3 is specifically expressed in the nervous system. JNK3 knockout mice exhibit a normal phenotype without embryonic lethality. However, deletion of JNK3 in mice causes resistance to excitotoxicity-induced apoptosis in the hippocampus (Yang et al., 1997), suggesting a role of JNK3 in mediating neuronal apoptosis.

MAPK pathways are activated in response to a variety of stress signals, including heat shock, UV-radiation, inflammatory cytokines, DNA-damaging agents, and oxidative stress (Robinson and Cobb, 1997). Conventional views tend to associate the activation of JNK or/and p38 signaling pathways with cell death while correlating the activation of the ERK pathway with cell survival. For example, growth factor withdrawal-induced apoptosis in PC12 cells requires the activation of JNK and p38 pathways, whereas ERK activation is necessary for growth factor rescue of serum-starved neuronal cultures (Xia et al., 1995). However, this may represent an oversimplistic picture of the roles of MAPK family members under stress. Although JNK and p38 pathways are mainly involved with cell death, many recent studies have revealed a role for ERK1/2 in contributing to cell death (Colucci-D'Amato et al., 2003; Chu et al., 2004). These studies suggest that MAPK pathways may have multiples roles in response to stress. The final

cellular response may depend on the spatial and temporal pattern of the activation of each MAPK pathway, the specificity of the stimuli and cell type, and more importantly, the interactions and cross-talk between each MAPK pathway.

1.2.4 Role of ERK activation in stressed neurons

As mentioned above, ERK activation has been considered predominantly as transducing pro-survival signals in stressed neurons in a variety of models (Hetman and Gozdz, 2004). For example, ERK1/2 activation is necessary for NGF-mediated pro-survival signaling in PC12 cells (Xia et al., 1995) and responsible for the neuroprotective effect of BDNF-mediated rescue of trophic factor-deprived neuronal cultures (Bonni et al., 1999). Furthermore, ERK1/2 activation also has been shown to be neuroprotective in a variety of neuronal damage models, including BDNF-mediated neuroprotection against DNA damage in cortical neurons (Hetman et al., 1999), estrogen-mediated neuroprotection against glutamate excitoxocity in neuronal cultures (Singer et al., 1999), as well as *in vitro* (Gonzalez-Zulueta et al., 2000) and *in vivo* (Han and Holtzman, 2000) models of hypoxia-ischemia.

However, ERK1/2 activation has also been found to contribute to neuronal cell death in a variety of *in vitro* and *in vivo* neurotoxicity models (Chu et al., 2004). For example, ERK1/2 activation has been shown to be necessary for glutamate-induced oxidative toxicity (Stanciu et al., 2000; Levinthal and DeFranco, 2004; Choi et al., 2006), zinc toxicity (Seo et al., 2001), methylisothiazolinone and Zn toxicity (Du et al., 2002), 6-hydroxydopamine toxicity (Kulich and Chu, 2001), amyloid β toxicity (Kuperstein and Yavin, 2002), tau-induced neurotoxicity (Amadoro et al., 2006), and focal ischemia (Alessandrini et al., 1999; Namura et al., 2001; Noshita et al., 2002). The opposing roles of ERK1/2 activation may depend on the spatial and

temporal pattern of ERK activation. Interestingly, our lab has recently shown that ERK1/2 activation has dual roles in promoting cell survival and/or cell death even within the same cell type (Luo and DeFranco, 2006), which highlights the importance of the kinetics of ERK1/2 activation in mediating divergent functions in neuronal survival or neuronal death.

1.3 PROTEIN PHOSPHATASES AND OXIDATIVE STRESS

1.3.1 Classification and catalytic mechanism of protein phosphatases

Protein phosphorylation is regulated by two types of enzymes: protein kinases, which catalyze the covalent attachment of a phosphate group to an amino acid, and protein phosphatases, which catalyze the removal of the phosphate. Due to their important roles in regulating reversible phosphorylation of proteins in nearly every aspect of cellular signaling, protein phosphatases have been extensively studied. Most of the protein phosphatases fall into one of the three following categories in terms of their substrate specificity: protein serine/threonine phosphatase (PSTP), protein tyrosine phosphatase (PTP), and dual-specificity phosphatase (DSP), which can dephosphorylate both serine/threonine and tyrosine residues.

Although all three types of protein phosphatases catalyze the same reaction of hydrolysis of phosphate monoesters, their structural organization and mechanisms for catalysis are distinct. PSTPs are metalloenzymes that contain two metal ions within their catalytic center. Depending on the identity of the metal ions, PSTPs can be divided into PPPs such as PP1, PP2A, PP2B (calcineurin) and PPMs such as PP2C. Although the specificity of the metal ions is still slightly controversial, it is generally accepted that PPPs contain various combination of Fe, Mn and/or Zn

dinuclear ion centers while PPMs contain Mn in their catalytic centers (Barford et al., 1998). Most PSTPs are holoenzymes that consist of multiple subunits, including catalytic subunits and regulatory subunits. Although catalytic subunits dictate the catalytic ability of the phosphatases, the regulatory subunits determine the substrate specificity as well as the subcellular trafficking of the enzymes. Therefore, the different combinations of catalytic subunits and regulatory subunits confer diverse functions of PSTPs. PTPs are composed of a single polypeptide chain and depend on structural variations in catalytic and regulatory domains built in the same peptide chain for the diversity of their functions.

The catalytic mechanisms employed by PSTPs and PTPs/DSPs also are different. PSTPs depend on the metal center to activate its surrounding water molecule to attack directly the phosphoryl group of the substrate. PTPs/DSPs are characterized by a PTP signature motif $HC(X)_5R$ in their active sites, which contains cysteine (Cys) and arginine (Arg) residues that are essential for enzyme catalysis. PTPs/DSPs employ a two-step strategy for phosphate monoester hydrolysis (Fig 1). The first step involves a nucleophilic attack of the active site cysteine on the phosphate ester of the bound substrate. This step leads to the formation of a phosphocysteine intermediate between the substrate and the enzyme (Barford et al., 1998; Denu and Dixon, 1998). Arginine is important in substrate recognition and transient state stabilization. In the second step, the phosphoenzyme intermediate is hydrolyzed by the attack of a water molecule, which is activated by a conserved aspartic acid (Asp) in the phosphatase. This step results in the release of inorganic phosphate and restores the enzyme.

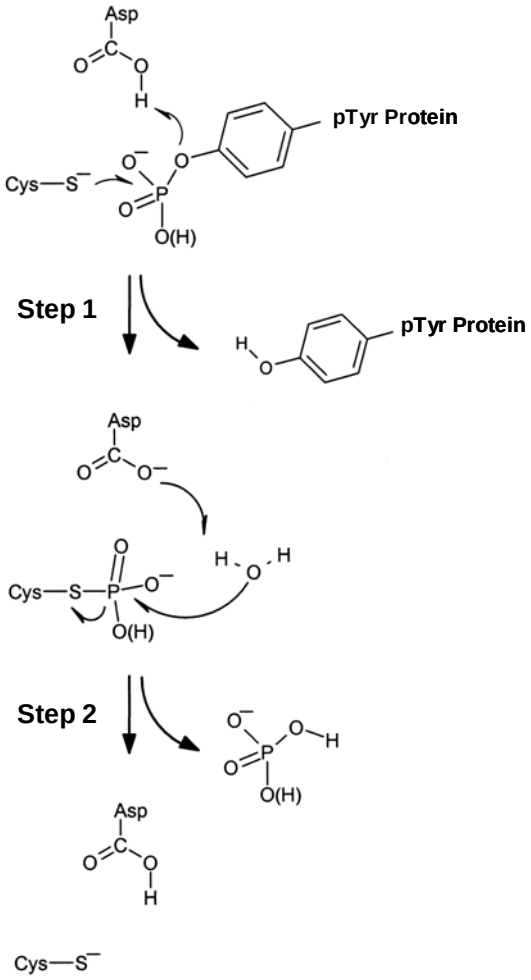


Figure 1.1. Reaction mechanism catalyzed by PTPs.

Adapted from (Kolmodin and Aqvist, 2001).

1.3.2 MAPK phosphatases

The magnitude and duration of the activation of MAPK pathways determine the effects of MAPK-mediated biological outcomes. The phosphorylation and activation of MAPK pathways reflects a balance between their upstream kinase activators and downstream phosphatase inactivators. MAPK-directed phosphatases serves as negative regulators of MAPK activation and

therefore play critical roles in regulating the spatio-temporal pattern of MAPK activation, which, in turn, determines the diverse cellular responses mediated by MAPK activation.

A variety of protein phosphatases can inactivate MAPKs. Among them, MKPs (MAPK phosphatases) are a group of DSPs that specifically dephosphorylate tyrosine and threonine in the P-loop of MAPK members. MKP members are distinct in their substrate (MAPKs) specificity, subcellular localization, as well as tissue distribution. For example, while most other MKPs act on all MAPK members with different affinities, MKP3, MKP-X and B23 are only specific for ERK1/2 dephosphorylation. MKP3 exists exclusively in the cytosol, whereas MKP1 and MKP2 are only expressed in the nucleus. Apart from MKPs, several PTPs, such as He-PTP and STEP, and PSTPs, such as PP2A, are also able to inactivate MAPK members. Table 1 lists some MAPK phosphatases in terms of their substrate specificity, subcellular localization, and tissue distribution {Adapted from (Haneda et al., 1999; Camps et al., 2000; Keyse, 2000; Saxena and Mustelin, 2000; Farooq and Zhou, 2004)}.

Although MAPK phosphatases have been shown to play important roles in various tissues and cell types, the roles of MAPK phosphatases in regulating neuronal function and development is still not well established. Recently, it has been shown that MKP1 and MKP3 are differentially activated in the acute induction period and chronic maintenance period following methamphetamine administration (Takaki et al., 2001). In the early induction period, MKP1 and MKP3 are activated in all brain regions, while during the chronic maintenance period, MKPs are only activated in restricted brain regions. This suggests a role for MKPs in behavioral sensitization induced by methamphetamine administration. Moreover, a striatal-enriched tyrosine phosphatase (STEP) has been shown to play important roles in regulating ERK activation in NMDA- and dopamine-mediated signaling in neurons (Paul et al., 2003; Valjent et al., 2005),

providing new insights into the roles of MAPK phosphatases in a variety of physiological and pathological neurological conditions, including long-term synaptic plasticity and drug abuse.

Table 1. Characterization of MAPK phosphatases

Name	**MAPK specificity**	**Subcellular localization**	**Tissue Distribution**
DSPs			
MKP1	JNK=P38>ERK	Nucleus	Ubiquitous
MKP2	ERK=JNK=p38	Nucleus	Ubiquitous
MKP3	ERK	Cytoplasm	Ubiquitous
MKP4	ERK>JNK=P38	Cytoplasm>nucleus	Ubiquitous
MKP5	JNK=P38>ERK	Cytoplasm>nucleus	Ubiquitous
MKP6	ERK=JNK>p38	Cytoplasm	Ubiquitous
MKP-X	ERK	Not determined	Ubiquitous
B23	ERK	Nucleus	Ubiquitous
PAC-1	ERK=p38	Nucleus	Ubiquitous
VHR	ERK>JNK=P38	Cytoplasm	Ubiquitous
M3/6	JNK>p38>ERK	Nucleus and cytoplasm	Ubiquitous
PTPs			
He-PTP	ERK=P38	Cytoplasm	Lymphoid-specific
STEP	ERK	Cytoplasm	Neuronal specific
PSTPs			
PP2A	ERK>P38=JNK	Cytoplasm	Ubiquitous
PP2C	p38,JNK	Cytoplasm	Ubiquitous

1.3.3 PP2A

PP2A is the major Ser/Thr protein phosphatase in eukaryotic cells. As a holoenzyme complex, the core enzyme of PP2A consists of a 36kDa catalytic subunit (C subunit) and a 65kDa structure subunit, A subunit (PR65). The core enzyme forms complexes with a variety of regulatory subunits (B subunits) that regulate the substrate specificity and subcellular localization of the PP2A holoenzyme. Four different families of B subunits have been identified, including B family (PR55), B'family (PR61), B" family (PR72) and B"' family (PR93/PR110) (Janssens and Goris, 2001). Multiple isoforms exist in each B subunit family, giving rise to a variety of holoenzyme combinations. Different holoenzymes dephosphorylate distinct substrates in specific cellular compartments, and this may explain the diversity of biological outcomes brought about by PP2A. It has been shown that overexpression of PP2A Bγ subunit in a stably transfected PC6-3 neuronal cell line promotes cell differentiation (Strack, 2002), while in the same cell line, overexpression of neuronal-specific PP2A Bβ2 subunit promotes apoptosis induced by serum withdrawl (Dagda et al., 2003). The apoptosis induced by the latter is actually caused by the targeting to PP2A to mitochondria by PP2A Bβ2 subunits and probably is through the dephosphorylation of various mitochondrial pro- and anti-apoptotic proteins, such as proteins of the Bcl-2 family (Dagda et al., 2003).

Substrates of PP2A are varied, ranging from protein kinases mediating cell growth to transcriptions factors regulating apoptosis. PP2A is the major PSTP that regulates the activation of protein kinases and signaling cascades in eukaryotic cells, in particular the ERK/MAPK cascade. Kinases in the ERK signaling modules, including Raf (Abraham et al., 2000; Kubicek et al., 2002; Ory et al., 2003), MEK (Westermarck et al., 2001; Yu et al., 2004), and ERK1/2

(Alessi et al., 1995; Zhou et al., 2002), are all substrates of PP2A. PP2A can both positively and negatively regulate ERK activation, depending on the entry points into the ERK pathway. PP2A dephosphorylates and inactivates MEK and ERK1/2 *in vitro* (Anderson et al., 1990; Gomez and Cohen, 1991). Transient expression of SV40 small T antigen (which inhibits PP2A by displacing the PR55-B subunit) activates the MEK1-ERK1/2 pathway (Sontag et al., 1993). Recently, it has been revealed that PP2A can positively regulate the ERK pathway by activating Raf-1 and subsequent MEK/ERK pathways through the dephosphorylation of the inhibitory Ser 259 in Raf-1 (Abraham et al., 2000; Kubicek et al., 2002; Ory et al., 2003). Impairment of PP2A has been implicated in a variety of oxidative stress-induced neurodegenerative diseases. Reduced 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 exhibit activation of ERK and JNK pathways as well as the phosphorylation of endogeneous tau, similar to the key pathological features in Alzheimer's disease (Kins et al., 2001; Kins et al., 2003). These results suggest that oxidative stress-induced PP2A inhibition in AD may be responsible for the enhanced ERK1/2 activation and subsequent tau hyperphosphorylation and neurofibrillary tangle formation.

1.3.4 Inhibition of protein phosphatases by oxidation

Over the past several years, it has been realized that ROS play an important role in regulating signal pathways. This has been referred to as *redox signaling*. By modifying the structure and function of specific proteins, ROS regulate signaling pathways involved in a variety of cellular responses. Recently, various protein phosphatases have been identified as targets of ROS, thus

establishing a critical link between oxidative inhibition of phosphatases and ROS-mediated signaling pathways.

Most results on the redox regulation of protein phosphatases are based on the study of redox regulation of PTPs (den Hertog et al., 2005; Tonks, 2005). PTPs and DSPs share a signature motif $HC(X)_5R$ in their active catalytic site. As mentioned above, the cysteine residue is critical for the formation of a cysteine-phospho intermediate for the catalytic function of PTPs. Distinct from cysteines in other proteins with a pKa ~8.5, the low pKa (~4.5) of cysteines in the signature motif of PTPs and DSPs renders them more vulnerable to oxidation. Oxidation of the catalytic cysteine residue then inactivates the phosphatase.

ROS transiently inactivate PTPs, leading to an imbalance between the activity of protein tyrosine kinases (PTKs) and PTPs and a subsequent enhancement of tyrosine phosphorylation of potential targets. Therefore, reversibility of the oxidative inhibition of PTPs is very important for regulating redox signaling. The oxidation of catalytic cysteines to sulfenic acid is reversible while further oxidation to sulfinic acid and sulfonic acid are all irreversible (Denu and Tanner, 1998). Another reversible oxidation mechanism is the intramolecular formation of disulfide bonds between the catalytic cysteine and its neighboring cysteine. This has been demonstrated in several non-classical PTPs, including LMW-PTP (Chiarugi et al., 2001), PTEN (Lee et al., 2002b), and Cdc25C (Savitsky and Finkel, 2002). Recently, the crystal structure of oxidized PTP1B has been revealed, suggesting a new form of reversible oxidation in PTPs (Salmeen et al., 2003; van Montfort et al., 2003). This formation has been termed sulfenylamide and is formed by the oxidized catalytic cysteine and the nitrogen backbone of the adjacent serine residue. Sulfenyladmide is not susceptible to further oxidation into sulfinic acid and sulfonic acid, establishing the basis for reversible oxidation of PTP1B. In contrast to other PTPs, receptor

protein tyrosine phosphatases (RPTPs) can be regulated negatively through the oxidation of a catalytic cysteine in a domain (D2 domain) that lacks phosphatase activity. Dimerization of RPTPα is stabilized by the oxidation of catalytic cysteines in the D2 domain, resulting in the activation of RPTPα (Blanchetot et al., 2002; van der Wijk et al., 2003). Various oxidation states of cysteines are listed in Table 2 {Adapted from (Barford, 2004)}.

Table 2. Oxidation states of cysteine

	Formulae	**Oxidation state**	**Reversibility with thiols**	**Catalytically active**
Cysteine	-SH or -S-	Reduced state	Not applicable	Yes
Sulfenic acid	-SOH	Single oxidation	Yes	No
Sulfinic acid	$-SO_2$	Double oxidation	No	No
Sulfonic acid	$-SO_3$	Triple oxidation	No	No
Disulfide	Cys-S-S-R_1	Converted from Sulfenic acid	Yes	No
Cyclic sulfonamide	Cys-S-NH-R_1	Converted from Sulfenic acid	Yes	No

The critical role of the oxidative inhibition of phosphatases in ROS-mediated signal transduction is best illustrated in the example of growth factor signaling (Aslan and Ozben, 2003). Fig 2 is a schematic diagram of the redox regulation of PTPs in a model of ligand-activation of receptor tyrosine kinase (RTK) signaling, which is the model employed by most growth factors, hormones, and cytokines. Ligand binding to RTK activates membrane NADPH oxidase, triggering the generation of ROS. By oxidizing the active cysteine site in PTP members to inactive sulfenic acid, ROS inhibits the activity of PTPs and therefore enhances tyrosine phosphorylation of RTKs. Due to the reversible oxidation property of sulfenic acid, intracellular reducing molecules, such as glutathione and thioredoxin, could reduce sulfenic acid to thiolate

anion and therefore restore the activity of PTPs. This results in the termination of tyrosine-phosphorylation-dependent signaling. Therefore, ROS generated in response to a physiological stimulus could inhibit specific PTPs that are responsible for constraining the signal pathways induced by that stimulus through the dephosphorylation of RTKs. This thus leads to the augmentation of tyrosine phosphorylation and subsequent signaling transduction.

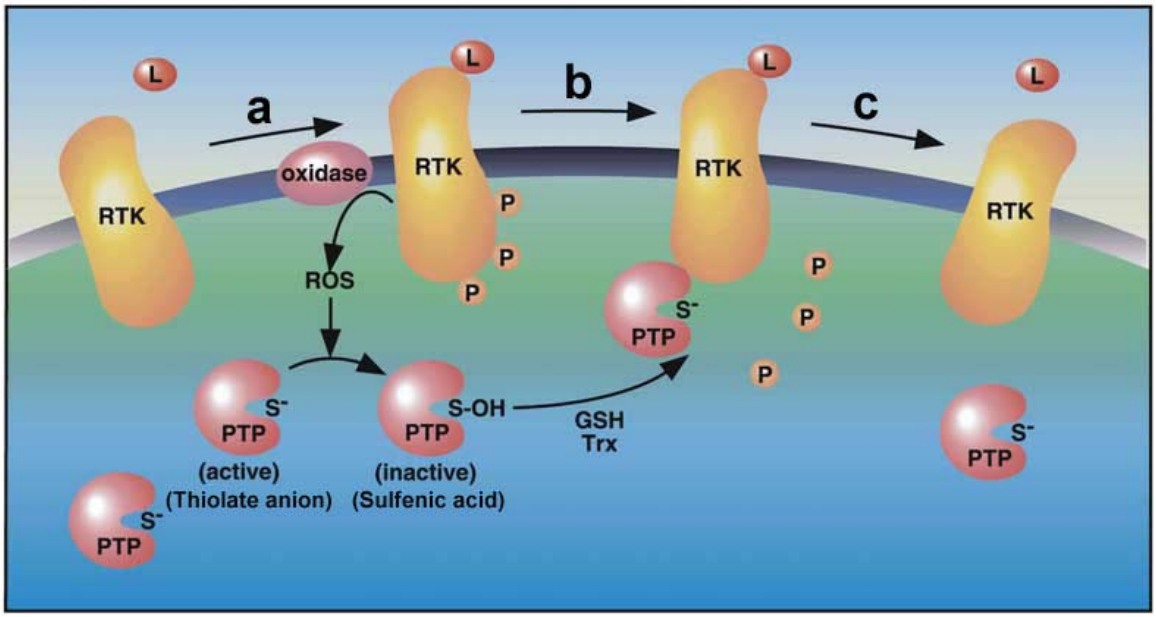


Figure 1.2 Regulation of Protein Tyrosin Phosphatase (PTP) activity by reversible oxidation. Adapted from (Tonks, 2003).

ROS are necessary for EGF signaling since antioxidants prevent the signal transduction mediated by EGF in A431 human epidermoid carcinoma cells (Bae et al., 1997). Moreover, oxidative inhibition of PTP1B contributes to EGF-mediated protein tyrosine phosphorylation. PDGF treatment induces a brief generation of ROS, which is associated with the inhibition of the tyrosine phosphatase, SHP-2, and the subsequent PDGF receptor activation in Rat-1 fibroblast cells (Meng et al., 2002). Recently, a role of oxidative inhibition of phosphatase activity that affects MAPK signaling has been demonstrated by Michael Karin's group (Kamata et al., 2005).

In this study, TNF-α treatment was found to cause ROS production and oxidative inhibition of JNK phosphatases, including MKP1, MKP3, MKP5, and MKP7, which resulted in sustained JNK activation and subsequent cell death in fibroblasts.

Compared with a well established role of oxidation in the inhibition of PTPs, the mechanism that regulates oxidative inhibition of PSTPs such as PP1, PP2A, and PP2B still remains vague. PP2B (calcineurin) has been shown to be oxidatively inhibited by H_2O_2 through the oxidation of its dinuclear metal center (Fe^{2+}-Zn^{2+}) (Wang et al., 1996) as well as the oxidation of two neighboring cysteines (Carballo et al., 1999; Bogumil et al., 2000). PP2A recently has been shown susceptible to H_2O_2 in aging fibroblasts (Kim et al., 2003) and brain tissue (Foley et al., 2004). Oxidation of cysteine residues may be responsible for oxidative inhibition of PP2A, since the inactivation of PP2A by ROS is thiol-dependent and reversible. However, the active site of PP2A does not contain an essential cysteine(Foley et al., 2004; Foley and Kintner, 2005). Thus, it is unclear which cysteine residue is oxidized and how that oxidation affects PP2A activity.

1.4 ZINC HOMEOSTASIS AND OXIDATIVE STRESS-INDUCED NEURONAL DEATH

1.4.1 Regulation of intracellular zinc homeostasis in neurons

Zinc is the second most abundant trace element in the body, particularly in the brain, which contains highest concentration of zinc. Zinc is indispensable, since deficiency in dietary zinc causes growth retardation and severe symptoms in the body, particularly in the nervous system

(O'Dell, 1993; Prasad, 1995). As a structural element or co-factor for many proteins, including transcriptional factors or even as a second messenger itself, zinc participates in almost all levels of signaling events and plays a crucial role in a wide range of physiological and pathological conditions.

In the CNS, free zinc is stored and released from presynaptic vesicles of neurons that also release glutamate (Frederickson et al., 2005; Mocchegiani et al., 2005). Relative to synaptic vesicles, free zinc ion concentrations in the cytoplasm of cultured neurons is very low, roughly femtomolar to picomolar concentrations(Frederickson et al., 2005; Mocchegiani et al., 2005). This suggests that a precise mechanism exists for the regulation of intracellular zinc homeostasis. The maintenance of intracellular zinc homeostasis is a tightly controlled process that involves uptake and export mechanisms, as well as intracellular zinc-binding proteins (Frederickson et al., 2005; Mocchegiani et al., 2005). Zinc taken up into neurons occurs by various routes, including the membrane zinc uptake transporters of the Zip family (Law et al., 2003; Seve et al., 2004) and several zinc-permeable membrane channels, including NMDA (N-methyl-D-aspartate) channels (Koh and Choi, 1994), Ca^{2+}-permeable AMPA/kainate channels (Jia et al., 2002), and voltage-gated Ca^{2+} channels (Sensi et al., 1997). The influx of zinc through these neurotransmitter receptors and channels implicates a role for zinc in modulating neurotransmission. To decrease cytosolic free zinc concentrations, zinc must be exported out of neurons or transported into intracellular synaptic vesicles by zinc transporters of the ZnTs family (Liuzzi and Cousins, 2004). Among the nine ZnT proteins that have been cloned, ZnT1 and ZnT4 are transporters mainly responsible for zinc efflux, whereas the brain and testis-specific ZnT3 regulates zinc transport into synaptic vesicles (Liuzzi and Cousins, 2004). Genetic deletion of ZnT3 in mice results in the zinc accumulation in synaptic vesicles, which demonstrates that ZnT3 is necessary

for zinc transport into synaptic vesicles (Cole et al., 1999). A novel ZnT protein, ZnT7, has been shown to promote cytoplasmic zinc transport into the Golgi apparatus (Kirschke and Huang, 2003). The variations in their regulatory control, tissue-specific expression pattern, as well as distinct target sites of the zinc transporters underlines the importance of the ZnT family in regulating intracellular zinc homeostasis. Recently, a putative mitochondrial transporter of zinc has been identified in prostate cells (Guan et al., 2003), which suggests that mitochondrial uptake of zinc may be another site for maintaining zinc homeostasis in cells.

Apart from transporters regulating influx and efflux of zinc ions, the maintenance of intracellular zinc homeostasis is a very dynamic process, involving the intracellular binding and release of zinc by zinc-binding proteins in a precisely-regulated way. The major sources of this intracellular zinc pool are metallothioneins (MTs). MTs are small proteins having a very high affinity for zinc. Each MT binds up to seven zinc ions through cysteine residues (Maret and Vallee, 1998). The equilibrium between metallated thionein (MT) and unmetallated thionein (T) is specifically regulated by the redox status in the cells. Oxidation or nitryosylation of cysteine residues reduces the zinc binding affinity of MT and therefore results in zinc release from MT, whereas reducing agents, such as GSH, favor zinc transfer and binding to thionein (Jacob et al., 1998; Jiang et al., 1998; Maret and Vallee, 1998).

Oxidative stress can also affect zinc homeostasis by influencing the nuclear-cytoplasmic distribution of MTs (Spahl et al., 2003). It has been shown that activation of cytokine-induced inducible NO synthase (iNOS) results in nuclear translocation of MT and triggers intranuclear zinc release (Spahl et al., 2003). These studies point to the role of oxidative stress as an important regulator in modulating intracellular zinc homeostasis and subsequent zinc signaling. Interestingly, several recent reports have demonstrated that PKC is another source of zinc storage

in cytoplasm. Exposure to superoxide activates PKC by dissociating and releasing zinc from its zinc-finger domain (Knapp and Klann, 2000; Korichneva et al., 2002). These results not only provide new insights into a new source of regulating zinc homeostasis but also reveal a new mechanism for regulating PKC activation by zinc.

Upon oxidative stress, zinc is released, activating a wide range of signaling pathways and influencing transcriptional regulation of various genes. A well established zinc-activated transcription factor is metal response element-binding transcriptional factor 1 (MTF-1). After binding zinc, MTF-1 is translocated from the cytoplasm to the nucleus and induces the transcriptional activation of both metallothionein and zinc transporter 1 in response to the elevation of cellular zinc (Bittel et al., 1998; Langmade et al., 2000; Smirnova et al., 2000). This may represent an important auto-regulated negative feedback mechanism to respond to excessive intracellular free zinc.

1.4.2 Oxidative stress and zinc-induced neuronal toxicity

It is now known that oxidative stress can trigger the release of intracellular stores of zinc by altering the redox state of zinc-binding proteins, such as MT. Similar to elevation of cytosolic zinc, oxidative stress can rapidly induce MT gene expression by regulating the MTF-1 transcriptional factor, which suggests that oxidative stress may lead to a rise of intracellular zinc concentrations (Dalton et al., 1996; Andrews, 2000). Several groups have shown that nitric oxide (NO) mediates intracellular zinc release by acting on the cysteine-rich zinc-binding protein, MT (Berendji et al., 1997; Cuajungco and Lees, 1998; Aravindakumar et al., 1999; St Croix et al., 2002). Overexpression of MT-III (brain specific MT form) is protective against glutamate and NO-induced neuronal death (Montoliu et al., 2000), which further supports this idea. Aizenman

et al (2000) shave found that a thiol oxidizing agent, 2, 2'-dithioldipyridine (DTDP), induces intracellular zinc release from its binding protein in cortical neurons and that this oxidative stress-induced intracellular zinc release triggers neuronal apoptosis. Compared with numerous studies that simply added exogenous zinc to induce toxicity in various cell culture models, this study was the first one showing that zinc released from intracellular stores induces toxicity in neurons. This has provided new insights into the mechanisms of zinc-induced neuronal toxicity. Sources of toxic zinc will be discussed in detail later. Moreover, this study demonstrated that intracellular zinc release is an important link and mediator between oxidative stress and neuronal injury. Given the abundant ROS generation and the role of zinc toxicity in acute brain injury and chronic neurodegenerative diseases, this study is of great significance in elucidating a potential mechanism for neuronal injury in vivo. Confirming the above findings, a more recent paper provides a link between NO and NO-triggered zinc release from intracellular stores in mediating neuronal death pathways that involve mitochondria dysfunction and MAPK activation (Bossy-Wetzel et al., 2004).

Zinc-induced neuronal toxicity has been reported to be mediated by oxidative stress and has characteristics of both necrosis and apoptosis (Kim et al., 1999a; Kim et al., 1999b; Noh et al., 1999; Seo et al., 2001). Zinc exposure induces ROS generation, which contributes to zinc-induced neuronal toxicity, since antioxidants attenuate zinc-induced toxicity (Kim et al., 1999a; Kim et al., 1999b; Noh et al., 1999; Seo et al., 2001). NADPH oxidase, a membrane free-radical generating enzyme, is activated by zinc exposure in cortical neurons and astrocytes, which suggests that NADPH is one mediator of zinc-induced oxidative stress (Noh and Koh, 2000). The most likely sources of zinc-induced ROS are mitochondria. It has been reported that zinc inhibits mitochondrial respiration (Brown et al., 2000; Bossy-Wetzel et al., 2004) and induces

mitochondrial ROS production (Sensi et al., 1999). Combined with the studies on oxidative stress-induced zinc-mediated cell death, this suggests that zinc liberated from intracellular stores may not only act as a mediator but also as an amplifier of oxidative stress signals in forming a destructive cycle to execute subsequent neuronal death.

1.4.3 Role of MAPK in zinc-induced neuronal death

Zinc participates in a wide range of cellular signaling pathways, ranging from intracellular second messenger regulation, protein kinase and phosphatase cascade regulation, as well as transcription initiation, all of which result in a variety of cellular responses, from cell proliferation and differentiation (Beyersmann and Haase, 2001) to cell death (Truong-Tran et al., 2001). For example, MAPKs have been shown to be activated by zinc in several models. In fibroblasts, zinc induces MAPK activation and protein tyrosine phosphorylation (Hansson, 1996) while the chelation of zinc decreases the activation of MAPK induced by insulin growth factor 1 (IGF-1) (Lefebvre et al., 1999). Exposure to excessive zinc induces EGF receptor phosphorylation and MAPK activation and subsequent activation of transcription factors, such as Jun and ATF-2, in human bronchial epithelial cells (Samet et al., 1998; Wu et al., 1999). In recent years, the role of MAPKs in mediating zinc-induced neuronal toxicity has been revealed. Intracelllular zinc release induced by thiol oxidizing agents, including DTDP and peroxynitrite, leads to the activation of p38 MAPK and subsequent cell death in cortical neurons (McLaughlin et al., 2001; Zhang et al., 2004). Pyrrolidine dithiocarbamate (PDTC) has been shown to induce intracellular zinc release, which in turn, activates JNK and contributes to cell death in embryonic hippcampal progenitor cells (Min et al., 2003). In PC12 cells, excessive zinc-induced sustained ERK activation contributes to zinc-induced neuronal death, which may be mediated through the

induction of an ERK downstream target, Egr-1 (Park and Koh, 1999; Seo et al., 2001). A biocide, methylisothiazolinone (MIT), induces toxicity in cortical neurons through the induction of intracellular zinc accumulation and subsequent ERK activation (Du et al., 2002). Recently, ERK-dependent cell death has been observed in peroxynitrite-induced toxicity in mature oligodendrocytes via the elevation of intracellular zinc (Zhang et al., 2006). Consistent with the roles of ERK activation in neuronal death in various models discussed earlier, ERK activation seems a prerequisite for zinc-induced neuronal death. However, the mechanisms responsible for ERK activation remain unknown. In Seo's study, a dominant negative Ras mutant was found to block zinc-induced neuronal death in PC12 cells, which suggests the role of Ras/Raf/MEK activation in zinc-induced toxicity (Seo et al., 2001).

1.4.4 Zinc and the inhibition of protein phosphatases

Zinc has been shown to modulate a various cellular signaling cascades, including the IGF-1 and EGF signaling pathways, by affecting the phosphorylation of signaling proteins. In the cases of EGFR- and IGF-1-mediated signaling, zinc can mimic the effect of EGF and insulin in the regulation of both pathways by augmenting the phosphorylation of RTKs, which in turn, activate subsequent downstream signaling cascades (Wu et al., 1999; Tang and Shay, 2001; Samet et al., 2003). Furthermore, it has been revealed that in both signaling pathways, the mechanism of zinc-mediated increased phosphorylation of RTKs lies in the inhibition of opposing PTPs (Haase and Maret, 2003; Tal et al., 2006).

Zinc inhibits PTPs at concentrations within the micromolar range (10 uM) (Brautigan et al., 1981) to nanomolar range (Maret et al., 1999; Haase and Maret, 2003). Given the fact that the concentration of cellular free zinc ions is in the nanomolar or picomolar range, zinc-mediated

inhibition of PTPs at either high or low concentrations of zinc may be mediated by distinct mechanisms. The inhibition of PTPs by high concentrations of zinc may occur under pathological conditions, where a huge influx or release of zinc is observed, such as in acute brain injury. This could result in an imbalance of phosphorylation-dependent signaling cascades and lead to the ultimate demise of neurons. On the other hand, zinc inhibition of PTPs at low concentrations of zinc may have more physiological relevance and represent the physiological role of zinc in regulating PTP activity, such as in IGF-1 mediated signaling (Haase and Maret, 2005). The mechanism of zinc-mediated inhibition of PTP activity is not known. However, it seems that the cysteine residue in the catalytic site of PTPs is not involved in an inhibitory effect of zinc (Maret et al., 1999).

As mentioned above, oxidative stress is an important regulator of PTP activity. Similar to other growth factors, insulin augments its signaling by the production of hydrogen peroxide, which provides a positive feedback by inhibiting PTP 1B (Mahadev et al., 2001). Given the fact that zinc is released from MT by oxidative stress and the inhibition of PTPs by zinc, IGF-1 signaling represents an excellent example to illustrate the interaction and crosstalk between redox and zinc signaling in response to one signal.

Much less is known about the effect of zinc on the activity of PSTPs. In one report, Zhuo and Dixon (1997) found that zinc has inhibitory effect on purified PSTPs including PP1, PP2A, and PP2B, in vitro and that this inhibition can be reversed by the addition of dithiothreitol (DTT), which is a chelator of zinc. This is the first evidence showing an inhibition of PSTP activity by zinc. Furthermore, it has been shown that zinc inhibits PSTP activity by competing with Mn^{2+} for the active site of PSTPs (Zhuo and Dixon, 1997). This is consistent with a more recent study showing that zinc inhibits calcineurin (PP2B) activity in vitro by competing with

Ni^{2+} for the active metal-binding site in calcineurin (Takahashi et al., 2003). Recently, it has been shown that zinc activates ERK in human airway epithelial cells by inhibiting ERK-directed phosphatases, which may include PSTPs, PTPs, and DSPs (Kim et al., 2006).

1.4.5 Roles of zinc toxicity in acute brain injury

Apart from various in vitro models, zinc toxicity has also been shown to contribute to acute neuronal injury in vivo, including seizures (Frederickson et al., 1989), transient forebrain global ischemia (Tonder et al., 1990; Koh et al., 1996), focal ischemia (Lee et al., 2002a), traumatic head injury (Suh et al., 2000), and hypoglycemia-induced neuronal death in vivo (Suh et al., 2004). In these models, significant zinc accumulation has been observed in selective degenerating neurons. In an elegant experiment, Koh et al (1996) have shown that intraventricular administration of a cell-impermeable zinc chelator, CaEDTA, significantly prevents zinc accumulation and reduces neuronal death in a transient global ischemia model, which suggests a critical role of zinc in mediating neuronal death in this model. Based on numerous studies, a "zinc-translocation" hypothesis has been proposed to explain the mechanism of zinc-induced neuronal death in vivo (Choi and Koh, 1998). This hypothesis proposes that following ischemia or other acute brain injury, zinc release is triggered from pre-synaptic vesicles. The presynaptic zinc then enters into postsynaptic neurons through various NMDA- or voltage-gated channels and results in neuronal injury. Chelating of extracellular zinc with membrane-impermeable agent CaEDTA reduces the entry of presynaptic zinc into postsynaptic neurons, thus preventing zinc-induced cell injury. However, in recent years, several studies have presented evidence that is inconsistent with this hypothesis. For example, zinc accumulation in degenerating neurons is still observed following kainate-induced seizures in selective vulnerable

neurons in ZnT3 knockout mice, which have no zinc in their presynpatic vesicles (Lee et al., 2000). This questions the presynaptic origin of zinc in the "zinc-translocation" hypothesis. Furthermore, CaEDTA has been shown to be able to deplete zinc from inside cells and presynaptic vesicles (Frederickson et al., 2002), arguing against the conclusions that the neuroprotective effect provided by CaEDTA in transient forebrain ischemia is solely due to the chelation of extracellular zinc released by presynaptic neurons. Therefore, a new model of excitotoxicity involving zinc release has been proposed (Frederickson et al., 2004). According to this model, presynaptic vesicle zinc release, mobilization of postsynaptic intracellular zinc, and possibly mitochondrial release of zinc are all involved in the excitotoxicity-induced zinc release events following brain injury (Frederickson et al., 2004). Although the mechanisms of zinc-mediated brain injury still remain controversial, we should bear in mind that chelation of zinc is effective in protecting neuronal death in various in vitro and in vivo models. This may provide some clues for developing therapeutic strategies in zinc-induced cell injury.

1.5 MODELS OF OXIDATIVE STRESS IN NEURONS

1.5.1 Oxidative stress models in neurons

Various *in vitro* models have been developed to analyze the molecular mechanisms of cellular responses to oxidative stress that are particularly relevant for neurodegeneration, such as amyloid β-peptide (Aβ)-induced oxidative toxicity (Akterin et al., 2005; Fonfria et al., 2005), 6-hydroxydopamine (6-OHDA)-induced toxicity (Kulich and Chu, 2001; Hanrott et al., 2006), and oxygen-glucose deprivation (OGD) reoxygenation-induced toxicity (Culmsee et al., 2005; Lim et

al., 2006) in neurons. Aβ is the major component in the plaque formation in AD and considered critical for the pathogenesis of AD, mostly probably through the generation of ROS (Butterfield, 2003). Dysregulation of the thioredoxin antioxidant system plays a role in Aβ-mediated toxicity in neuronal cultures (Akterin et al., 2005). 6-OHDA is a dopaminergic neurotoxin that generates hydrogen peroxide, superoxide, and hydroxyl radicals following its metabolism under physiological conditions (Cohen and Heikkila, 1974). 6-OHDA has been utilized in various neuronal models of PD, including B65 neuronal cell line (Kulich and Chu, 2001), primary neurons of substantia nigra (Ding et al., 2004), human dopaminergic neuronal cell line SH-SY5Y (Chen et al., 2004), and PC12 cells (Hanrott et al., 2006). OGD reoxygenation is an excellent model to mimic the oxidative stress condition following ischemia-reperfusion (Culmsee et al., 2005; Lim et al., 2006).

1.5.2 Glutamate-induced oxidative toxicity in HT22 and primary immature cortical neurons

Extracellular glutamate acts on neurons via three pathways which include the binding of glutamate to ionotropic receptor (i.e. NMDA, AMPA or kainate receptors), metabotropic receptors (mGluRs), and a cystine/glutamate antiporter (X_c^-). Ubiquitously expressed in mammalian cells, the X_c^- system is responsible for cystine uptake into the cells coupled to the efflux of glutamate (Bannai, 1986; Sato et al., 1999). The concentration of intracellular glutamate is much higher than that its extracellular concentration. In contrast, low intracellular concentrations of cystine are maintained by its rapid reduction to cysteine in cells. As cysteine is the rate-limiting precursor of glutathione synthesis, the activity of the X_c^- system regulates the

intracellular levels of glutathione. High concentrations of extracellular glutamate inhibit the exchange of the X_c^- system, thereby leading to the depletion of intracellular glutathione.

Glutamate-induced oxidative toxicity in a HT22 hippocampal cell line (Li et al., 1997a) and primary immature cortical neurons (Murphy et al., 1990) has provided a useful model for studying the effects of oxidative stress on neuronal cell death. HT22 is a mouse hippocampal-derived cell line that lacks ionotropic glutamate receptors. Excess exogenous glutamate inhibits the X_c^- system of HT22 cells, which leads to the depletion of glutathione and subsequent ROS accumulation. The ROS accumulation then in turn activates signaling cascades that eventually contribute to a novel form of cell death that has features of both apoptosis and necrosis (Li et al., 1997a, b; Sagara and Schubert, 1998; Tan et al., 1998; Tan et al., 2001). Similarly, primary immature cortical neurons (DIV 1-3) do not express functional ionotropic glutamate receptors, therefore rendering them sensitive to glutamate-induced oxidative toxicity (Murphy et al., 1990).

Depletion of glutathione in HT22 and primary immature cortical neurons leads to a series of cellular events, including 12-LOX activation followed by a significant influx of calcium and ROS accumulation, resulting in cell death (Li et al., 1997a; Stanciu et al., 2000). Distinct from classical apoptosis or necrosis, glutamate-induced oxidative toxicity shares some features of both apoptosis and necrosis. Similar to necrosis in terms of morphological criteria, such as cell swelling during cell death, glutamate-induced oxidative toxicity also has some biochemical characteristics of apoptosis, including the requirement for synthesis of new proteins (Tan et al., 2001). Therefore, the term “oxytosis” has been proposed to reflect this unique form of cell death caused by glutathione-depletion and ROS-accumulation (Tan et al., 2001).

The signaling events leading to cell death in glutamate-induced oxidative toxicity in HT22 cells and primary immature cortical neurons have been studied extensively although the

findings are somewhat controversial. PKC activation has been reported to be neuroprotective against glutamate-induced oxidative toxicity (Davis and Maher, 1994; Maher, 2001). The roles of various MAPK members have been investigated in this toxicity model and have led to the suggestion that p38 MAPK activation contributes to the toxicity while ERK1/2 activation is neuroprotective (Maher, 2001). However, numerous results obtained from our lab and several other labs have shown that sustained activation of ERK1/2 specifically contributes to the oxidative toxicity induced by glutamate in HT22 cells and primary immature cortical neurons (Satoh et al., 2000; Stanciu et al., 2000; Stanciu and DeFranco, 2002; Levinthal and DeFranco, 2004, 2005; Choi et al., 2006; Luo and DeFranco, 2006). Furthermore, it has recently been shown that reversible oxidation of ERK-directed protein phosphatases contributes to ERK activation and subsequent neuronal death (Levinthal and DeFranco, 2005). However, the mechanisms regulating the oxidation and inactivation of ERK phosphatases remain elusive. A recent study has suggested that proteasome degradation of MKP1 may contribute to ERK1/2 activation in glutamate-mediated oxidative toxicity model (Choi et al., 2006).

2.0 THESIS GOALS

This thesis was mainly focused on two specific research goals. Previous studies in our lab had shown that specific reversible inhibition of ERK-directed phosphatases contributes to the persistent ERK activation in glutamate-induced toxicity in primary immature cortical neurons (Levinthal and DeFranco, 2005). However, the mechanism underlying the oxidation and inactivation of ERK-directed phosphatase is not known. Therefore, the first specific aim of my study was to investigate the mechanism of oxidative stress-induced inhibition of ERK phosphatases in glutamate toxicity models. Considering the role of oxidative stress in triggering intracellular zinc release and the inhibition of protein phosphatases by zinc, I first sought to establish whether there exists a connection between oxidative stress-induced intracellular zinc release by glutamate and the inhibition of ERK-directed phosphatases in HT22 cells and primary immature cortical neurons. My hypothesis was that selective zinc inhibition of ERK phosphatases is responsible for ERK-dependent oxidative toxicity in neurons. The results from this part of my studies are presented in Chapter 3.

Second, I set out to determine the biochemical mechanisms underlying the oxidation and inactivation of ERK-directed phosphatases. Previous studies had shown that PP2A is the predominant ERK phosphatase in primary neurons (Levinthal and DeFranco, 2005). Therefore, I sought to examine cysteine oxidation of PP2A following oxidative stress, and the effects of PP2A oxidation on its catalytic activity. I hypothesized that the cysteine oxidation of PP2A may

contribute to the decrease of PP2A activity following oxidative stress. The results of this part are described in Chapter 4.

The second specific aim of my study focused on the inhibition of MAPK phosphatases in rodent ischemia models. ERK1/2 activation has been associated with neuronal death in the transient focal ischemia/reperfusion model (Alessandrini et al., 1999; Namura et al., 2001). In the first part of this study, I set out to investigate the inhibition of MAPK phosphatases in a focal ischemia (MCAO) model. I hypothesized that oxidative stress-induced oxidation and inactivation of ERK phosphatases contributes to ERK1/2 activation and subsequent neuronal death in this focal ischemia/reperfusion model. ERK1/2 activation also occurs following global ischemia/reperfusion, although the role of ERK1/2 activation in neuronal cell injury that results from this ischemic insult remains controversial. Therefore, I also examined the role of ERK1/2 phosphatase inhibition in mediating ERK1/2 activation following global ischemia/reperfusion in rat. The results of these studies are presented in Chapter 5.

3.0 SELECTIVE INHIBITION OF MAPK PHOSPHATASES BY ZINC ACCOUNTS FOR ERK1/2-DEPENDENT OXIDATIVE NEURONAL CELL DEATH

3.1 SUMMARY

Oxidative stress induced by glutathione depletion in the mouse HT22 neuronal cell line and embryonic rat immature cortical neurons causes a delayed, sustained activation of the mitogen-activated protein kinase (MAPK) family members, extracellular signal-regulated kinase 1/2 (ERK1/2), which is necessary for cell death. This sustained activation of ERK1/2 is mediated primarily by a selective, reversible inhibition of ERK1/2-directed phosphatases. The inhibition of ERK1/2 phosphatases in HT22 cells and immature neurons subjected to glutathione depletion is indeed the result of oxidative stress, as phosphatase activity is restored in cells treated with the antioxidant BHA. This agent leads to reduced ERK1/2 activation and neuroprotection. Furthermore, we show that an increase in free intracellular Zn^{2+} that accompanies glutathione-induced oxidative stress in HT22 cells and immature neurons contributes to selective inhibition of ERK1/2 phosphatase activity and ensuing ERK1/2 activation and cell death. ERK1/2 also functions to maintain elevated levels of Zn^{2+}. Thus, the elevation of intracellular Zn^{2+} within neurons subjected to oxidative stress can trigger a robust positive feedback loop operating through activated ERK1/2 that rapidly sets into motion a Zn^{2+} -dependent pathway of cell death.

3.2 INTRODUCTION

Oxidative stress results from the accumulation of reactive oxygen species (ROS) and is brought about by a disruption of the physiological balance between normal oxidant production and the action of various anti-oxidant defense systems. The brain is particularly sensitive to ROS accumulation, and oxidative stress is implicated in the pathogenesis of a wide range of chronic neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) (Simonian and Coyle, 1996; Mattson et al., 2001), as well as acute neuronal injury, such as stroke (McCulloch and Dewar, 2001; Cherubini et al., 2005). Various *in vitro* models have been developed to analyze the molecular mechanisms of cellular responses to oxidative stress that are particularly relevant for neurodegeneration (Akterin et al., 2005; Hanrott et al., 2006; Lim et al., 2006).

A number of cellular signaling pathways are activated by oxidative stress in neurons including the MAPKs. The MAPK family is a group of signaling modules involved in cellular responses to a wide range of stimuli that impact cell adhesion, cell differentiation and proliferation, synaptic plasticity, survival and apoptosis (Pearson et al., 2001; Thomas and Huganir, 2004). MAPK members include extracelluar-signal regulated kinase (ERK), Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAPK kinase families (Pearson et al., 2001). The MAPKs are activated by dual-phosphorylation of a conserved threonine-X-tyrosine motif by upstream MAPK kinases (MEKs or MKKs), which are in turn activated by their upstream kinases, MEK/MKK kinases (MKKKs) (Pearson et al., 2001).

Individual MAPK family members were initially considered to serve distinct roles in neuronal responses to death-inducing stresses, with activation of the JNK/SAPK and p38 pathways most often associated with neuronal cell death (Xia et al., 1995; Yang et al., 1997;

Borsello et al., 2003). In contrast, a neuroprotective role of ERK activation has been revealed in several models of neuronal toxicity, including growth factor rescue of serum-starved neuronal cultures (Xia et al., 1995), *in vivo* models of hypoxia-ischemia (Han and Holtzman, 2000), as well as the BDNF-mediated rescue of camptothecin-treated cortical neuronal cultures (Hetman et al., 1999). However, ERK1/2 activation has also been found to promote neuronal cell death in a number of *in vitro* models of neurodegeneration induced by oxidative stress (Satoh et al., 2000; Stanciu et al., 2000; Levinthal and DeFranco, 2004), zinc (Seo et al., 2001; Du et al., 2002), or 6-hydroxydopamine (Kulich and Chu, 2001). In fact, pharmacological inhibition of ERK1/2 activation *in vivo* reduces neuronal cell injury in response to transient focal ischemia (Alessandrini et al., 1999; Noshita et al., 2002). The divergent effects of ERK1/2 to either promote cell death or survival, even within a single cell type are likely to be determined by multiple factors, including the kinetics of kinase activation and/or the subcellular localization of activated kinases (Luo and DeFranco, 2006).

Glutamate-induced oxidative toxicity in the HT22 hippocampal cell line (Li et al., 1997a) and primary immature cortical cultures (Murphy et al., 1990) has provided a useful model for studying the effects of oxidative stress on neuronal cell death. Both HT22 cells and primary immature cortical neuronal cultures lack N-methyl-D-aspartate (NMDA) receptors (Murphy and Baraban, 1990). Therefore, glutamate treatment of these cells primarily inhibits a glutamate/cystine antiporter and blocks the uptake of cystine, a precursor of glutathione synthesis (Murphy et al., 1990). The resulting glutathione depletion triggers ROS accumulation, which activates signaling cascades that eventually contribute to a form of cell death that has features of both apoptosis and necrosis (Li et al., 1997a, b; Sagara and Schubert, 1998; Tan et al., 1998; Tan et al., 2001).

We have previously reported that oxidative stress in HT22 cells and immature cortical neurons causes a delayed, sustained activation of ERK1/2, which is necessary for neuronal cell death (Stanciu et al., 2000; Stanciu and DeFranco, 2002; Levinthal and DeFranco, 2004). This sustained activation of ERK1/2 is mediated primarily by a selective, reversible inhibition of ERK1/2-directed phosphatases (Levinthal and DeFranco, 2005). In this study, we examined the mechanistic basis for oxidative inhibition of ERK1/2-directed phosphatases in HT22 cells and immature cortical neuron cultures. We demonstrate that the increased intracellular accumulation of Zn^{+2} in oxidatively stressed neuronal cells is responsible for the selective inhibition of ERK-phosphatases and ensuing ERK1/2 activation and cell death. Surprisingly, ERK1/2 also functions to maintain elevated levels of Zn^{+2}. Thus, the elevation of intracellular Zn^{+2} within damaged neurons can trigger a robust positive feedback loop operating through activated ERK1/2 that rapidly sets into motion a Zn^{2+} -dependent pathway of cell death.

3.3 MATERIALS AND METHODS

Primary Cortical Cultures and Cell Lines

Primary cultures were prepared from the cortices of embryonic day 17 Spague-Dawley rat fetuses (Hilltop Lab Animals). Briefly, the cortices of embryonic day 17 Spague-Dawley rat fetuses were dissected and dissociated by repeated trituration in Hanks' balanced salt solution (5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 137 mM NaCl, 5.6 mM D-glucose, pH 7.4) without Ca^{2+} or Mg^{2+} (GIBCO). The cell suspensions then were passed through a 40-um cell strainer (BD Biosciences) to remove clumped cells. Cells then were counted and plated on 50 ug/ml poly-d-lysine coated cultures plates at a density of ~$1x10^5$

cells/cm^2. Cell viability was assessed by the uptake of trypan blue dye and was usually greater than 80%. Cultures were maintained for 2-3 days in media (Dulbecco's modified essential medium [DMEM], 10% fetal calf serum (Hyclone), 10% Ham's F12 nutrient supplement (Sigma), 1.9 mM glutamine, 24 mM Hepes buffer, and 4.5 mg/ml glucose) at 37ºC and 5% CO_2. At this time, the mixed cortical cultures are ~80% neuronal, with ~20% glial fibrillary-associated protein staining cells (Murphy et al., 1990). HT22 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units of penicillin, and 100ug/ml streptomycin at 37ºC and 5% CO_2.

Cell Viability Assay

The DNA dye propidium iodide (PI) was used to assess cell viability. PI is excluded from healthy, intact cells and can only get into cells with a compromised plasma membrane. Fourteen to 16 h after being treated with homocysteate (HCA), primary neurons were incubated for 10 min in media containing a final concentration of 6.25 ug/ml PI. Cells were observed under an inverted fluorescence microscope equipped with phase-contrast optics (Nikon Eclipse TE200). Multiple fields were counted for each condition in at least 3 independent cultures with a total cells population of at least 500 neurons per condition. Toxicity was assessed in HT22 cells after 12 h's glutamate treatment using the PI staining method described above.

ERK2- and JNK3-directed Phosphatase Activity Assay

A nonradioactive method has been modified for determining ERK2- and JNK3-directed phosphatase activity in cell lysates (Laakko and Juliano, 2003). This method is based on detecting dephosphorylation of a purified, dual-phosphorylated, His_6-tagged ERK2 or JNK3

upon incubation with the cell lysates (Levinthal and DeFranco, 2005). The alterations of ERK2 or JNK3 phosphatase activity within the cell lysates can be monitored by measuring changes in the phosphorylation state of the isolated phosphorylated ERK2 or JNK3 substrate, as shown by Western blotting with a phospho-specific ERK1/2 or JNK antibody. Briefly, 150 ug of cell lysates were diluted into a total volume of 250 ul in phosphatase assay buffer (10 mM $MgCl_2$, 10 mM Hepes, pH7.5 and 10 uM of the MEK inhibitor, U0126). Recombinant dual phosphorylated His_6-ERK2 or His_6-JNK3 (Biomol) was added to each sample (30 ng/sample), and the reactions were maintained at 37ºC for 15 min where indicated. For the *in vitro* Zn^{2+} inhibition experiment, different concentrations of $ZnCl_2$ were pre-incubated with cell lysates for 10 min at 37ºC before the addition of purified pERK2 substrate. Following a 15 min incubation at 37ºC, reactions were stopped by the addition of 250 ul of wash buffer (8M urea, pH 8.6, containing 10 mM imidazole). Thirty ul of Ni^{2+}-conjugated, magnetic beads (Qiagen) was then added to each reaction. After 90 min of rocking at 4ºC, the samples were washed twice with wash buffer followed by one wash in 300 mM NaCl, 25 mM Tris, pH 7.5. The beads were then suspended in Laemmli sample buffer, boiled for 5 min, loaded onto a 10% polyacrylamide gel, transferred to a polyvinylidine fluoride membrane (Millipore) and subjected to Western blotting to detect phosphorylated ERK1/2, total ERK1/2, phosphorylated JNK3, or total JNK3.

Western Blot Analysis

Cells were treated with HCA or glutamate, scraped, and collected in 1x PBS and then centrifuged at 7000 rpm for 3 min. The pellets were then disrupted in lysis buffer (50 mM Tris-Cl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Nonidet P-40, supplemented with protease inhibitor (Protease inhibitor cocktail, Sigma). The solubilized lysates were then centrifuged at

13,000 rpm for 5 min at 4ºC, and supernatants were collected for further analysis. Protein concentrations of the extracts were determined using the Bio-rad reagent. Equivalent amount of total protein (20-30 ug) were separated by SDS-PAGE on 10%-polyacrylamide gels and then transferred to polyvinylidine membranes. Membranes were blocked with 5% dry milk in PBS/0.1% (v/v) Tween 20 (PBST) for 1 h at room temperature. Membranes were then incubated with primary antibodies (anti-phospho-ERK1/2, anti-total ERK1/2, anti-phospho-JNK, anti-total JNK, all from Cell Signaling) overnight at 4ºC 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 washes with PBST. Immunoreactive bands were then revealed by enhanced chemiluminescence (ECL, Amersham Biosciences) using standard x-ray film (Eastman Kodak Co). Densitometry was performed using a Personal Densitometer SI (Amersham Biosciences) linked to the ImageQuant 5.2 software (Amersham Biosciences).

Intracellular Zinc Imaging

This part of work was performed by Dr Megan Knoch in Dr Elias Aizenman's lab. Neuronal cultures were incubated in a Fluozin-3 AM (5 µM, Molecular Probes)-containing solution composed of HEPES-buffered saline supplemented with 5 mg/mL BSA for approximately 30 min. Immediately following incubation, coverslips were submerged in a recording chamber (Warner Instruments) mounted on an inverted epifluorescence microscope (Nikon Eclipse TE200) equipped with a 10X and a 20X objective. MEM-HEPES-BSA was continuously perfused through the recording chamber via a gravity-driven perfusion system. Using a computer-controlled monochromator (Polychrome II, TILL Photonics) and a CCD

camera (IMAGO, TILL Photonics), images were acquired with 490 nm excitation light. Once a series of baseline fluorescence images were established, a 20 μM Tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) solution was perfused through the chamber to chelate intracellular zinc and quench the fluorescent signal. The relative fluorescence for all cells (n= 6-40) was determined by subtracting the TPEN-quenched signal from the initial fluorescence and an average value was calculated for each coverslip (n=6). Data were analyzed and plotted using Origin 6.0 (OriginLab Corporation).

Statistics

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

3.4 RESULTS

3.4.1 ERK2-directed phosphatase activity is inhibited during oxidative toxicity in HT22 cells and primary immature cortical neurons

We had previously reported that glutamate-induced oxidative stress in immature cortical neurons inhibits ERK2-directed phosphatases resulting in sustained activation of ERK1/2 (Levinthal and DeFranco, 2005). As HT22 cells exhibit analogous ERK1/2-dependent oxidative toxicity, we first set out to examine whether ERK1/2-directed phosphatases were similarly inhibited by

glutamate treatment in HT22 cells. We adopted an *in vitro* phosphatase assay described previously (Levinthal and DeFranco, 2005), which uses whole cell lysates to dephosphorylate purified, dual-phosphorylated, His_6-tagged ERK2. A nonspecific dual-specificity phosphatase, lambda protein phosphatase, was employed as a positive control for ERK2 dephosphorylation (Figure 3.1A, lane 2).

As shown in Figure 3.1A, whole cell lysates prepared from HT22 cells possess robust ERK2 phosphatase activity (Figure 3.1A, lane 3). When cell extracts were prepared from cultures treated with glutamate for 7.5 h, a decrease in ERK2-directed phosphatase activity was observed (Figure 3.1A, lane 6). Quantification of four independent experiments revealed a significant increase ($p<0.05$) in the normalized levels of phosphorylated ERK2 in HT22 cell lysates exposed to glutamate for 7.5 h when compared to untreated cultures (Figure 3.1B). When cells were exposed to shorter treatments with glutamate (i.e. 2 h and 5 h), ERK2 phosphatase activity was not significantly affected (Figure 3.1A, lane 4&5). The length of glutamate exposure that leads to inhibition of ERK2 phosphatase activity in HT22 cells is coincident with that required for maximal oxidative stress (Tan et al., 1998) and enhanced ERK1/2 phosphorylation (Stanciu et al., 2000).

To confirm the effect of glutamate on the inhibition of ERK2 phosphatase activity in primary immature cortical neurons, we used the glutamate analog homocysteate (HCA) to induce oxidative stress. HCA is a glutamate analog that has a relatively high binding affinity to glutamate/cysteine antiporter and has widely been used to induce oxidative toxicity to immature neurons (Ryu et al., 2003b; Ryu et al., 2003a). The ERK2 phosphatase assay was performed with whole cell lysates prepared from cultures treated with HCA for different times (i.e, 12 h, 14 h and 16 h). As shown in Figure 3.1C, we began to observe an inhibition of ERK2 phosphatase

activity in extracts prepared from neurons treated for 14 h with HCA. After a 16h treatment with HCA, a pronounced inhibition of ERK2 phosphatase activity was revealed (Figure 3.1C and Fig 3.1D).

3.4.2 An antioxidant that blocks oxidative toxicity reduces ERK1/2 activation and restores ERK2 phosphatase activity

The antioxidant, butylated hydroxyanisole (BHA), has previously been shown to be protective against glutamate-induced oxidative toxicity in primary immature cortical neurons (Ryu et al., 2003a). We confirmed the neuroprotective effects of BHA against glutamate toxicity in HT22 cells (data not shown). BHA has also recently been shown to prevent the oxidative stress-induced inhibition of JNK phosphatases and TNF-α induced cell death in fibroblast cell lines (Kamata et al., 2005). The effects of BHA in blocking ERK1/2 activation induced by oxidative stress has also been observed in other cell types, such as head and neck squamous carcinoma cells (Kim et al., 2006). We therefore used BHA to assess whether ERK1/2 activation and the inhibition of ERK2-directed phosphatase activity is mediated by ROS.

As shown in Figure 3.2A, a 7.5 h glutamate treatment induced significant ERK1/2 activation in HT22 cells. In primary cortical neurons, HCA activation of ERK1/2 was evident at 14 h and peaked at 16 h (Figure 3.2B), analogous to the time course of ERK1/2 activation by glutamate in immature neurons (Stanciu et al., 2000; Levinthal and DeFranco, 2004, 2005). The administration of BHA blocked ERK1/2 activation induced by oxidative stress in HT22 cells and neurons (Figures 3.2A & 3.2B).

To investigate the mechanism responsible for BHA inhibition of ERK1/2 activation, we then performed ERK2 phosphatase assays on cell lysates prepared from HT22 cells and neurons subjected to oxidative stress in the presence or absence of BHA. As shown in Figure 3.2C, BHA prevented the inhibition of ERK2 phosphatase activity following glutamate treatment in HT22 cells. Similarly, BHA abrogated the inhibition of ERK phosphatase activity in neurons subject to HCA treatment (Fig 3.2D). Thus, ROS-mediated inhibition of protein phosphatase activity is responsible, in part for the activation of ERK1/2 in oxidatively stressed neurons.

3.4.3 Selective inhibition of ERK2 phosphatase activity by Zn^{2+} in neuronal cell extracts

ERK1/2 phosphatases such as MKPs and PP2A are reversibly inactivated by thiol oxidation (Kim et al., 2003; Foley et al., 2004). Previously, we found that ERK2 phosphatase activity in extracts prepared from oxidatively stressed neurons was restored by the *in vitro* addition of DTT (Levinthal and DeFranco, 2005). This suggests that BHA-reversible oxidative stress may be acting to inhibit ERK1/2 phosphatases in HT22 cells and primary cortical neurons through direct or indirect thiol oxidation of the phosphatases. However, DTT in addition to its well-known thiol reducing properties is also an effective Zn^{2+} chelating agent (Cornell and Crivaro, 1972; Paoletti et al., 1997). Thus, restoration of ERK2 phosphatase activity in extracts prepared from oxidatively stressed neurons by DTT may not be solely due to reduction of oxidized thiols. Interestingly, Zn^{2+} is inhibitory to purified phosphatases that could act on ERK1/2, including the protein serine/threonine phosphatase (PSTP) PP2A (Zhuo and Dixon, 1997) and protein tyrosine phosphatases (PTPs) (Brautigan et al., 1981; Maret et al., 1999). As such, we investigated the effect of Zn^{2+} on ERK2 phosphatase activity.

Whole cell lysates prepared from untreated HT22 cells were pre-incubated for 10 min with different concentrations of $ZnCl_2$ before performing the ERK2 phosphatase assay. As shown in Figure 3.3A, Zn^{2+} caused a concentration-dependent inhibition of ERK2 phosphatase activity with the maximal inhibition observed at 10μM. Quantification of several independent experiments revealed a significant inhibition of ERK2 phosphatase activity by zinc at 10μM (Figure 3.3B, $p<0.01$). To determine whether the Zn^{2+} inhibition of phosphatase activity is specific to ERK phosphatases, we examined Zn^{2+} effects on JNK3 phosphatase activity. As shown in Figure 3.3C, the addition of $ZnCl_2$ to untreated HT22 cell lysates did not affect JNK3 phosphatase activity. This suggests that exogenous Zn^{2+} is selective in inhibiting ERK2-directed phosphatases in HT22 cell extracts. This is not surprising since oxidative toxicity in HT22 cells and immature neurons is not associated with an increase in JNK activation or inhibition in JNK phosphatase activity (Levinthal and DeFranco, 2005).

3.4.4 Oxidative stress triggers Zn^{2+} accumulation in HT22 cells and primary neurons

Given the inhibitory effects of exogenous Zn^{2+} on ERK1/2 phosphatase activity *in vitro*, we set out to examine whether oxidative stress-induced phosphatase inhibition in HT22 cells and immature cortical neurons is due to increased accumulation of Zn^{2+} (Aizenman et al., 2000). The impact of oxidative stress in HT22 cells and primary neurons on intracellular Zn^{2+} accumulation was assessed using FluoZin 3-AM, a fluorescent Zn^{2+} indicator dye (Minta et al., 1989; Devinney et al., 2005). Relative intracellular Zn^{2+} levels were obtained by quantitative measures of fluorescence of FluoZin-3AM, which is quenchable by the Zn^{2+} chelator, N,N,N',N'-Tetrakis-(2-pyridylmethyl)-Ethylenediamine (TPEN), within individual cells. As shown in Figure 3.4A, oxidative stress in HT22 cells and immature neurons led to a doubling of the relative TPEN-

quenchable FluoZin 3-AM fluorescence. In glutamate-treated HT22 cells, the relative intracellular Zn^{2+} levels increased from 7.8 $\pm$ 1.5 to 15.5 $\pm$ 1.7 (n=8), whereas HCA treatment of neurons led to an increase in the relative levels of intracellular Zn^{2+} from 14.6 $\pm$ 1.6 to 29.2 $\pm$ 4.2 (n=6). Thus oxidative stress in both HT22 cells and immature neurons triggers the accumulation of intracellular Zn^{2+}, similar to what we have reported in other systems (Aizenman et al., 2000; McLaughlin et al., 2001; Pal et al., 2004; Zhang et al., 2004).

In order to determine the relationship between oxidant production, intracellular Zn^{2+} accumulation, and ERK1/2 activation, neurons were exposed to HCA in the presence or absence of either the antioxidant BHA or the MEK1/2 inhibitor U0126. As shown in Figure 3.4B, BHA treatment blocked HCA-induced intracellular Zn^{2+} accumulation and exerted no significant effect on the relative intracellular Zn^{2+} levels when given alone. Interestingly, U0126 treatment also reduced TPEN-quenchable Zn^{2+} fluorescence in HCA-treated neurons and did not alter Zn^{2+} levels when given alone (Figure 3.4B). Thus, Zn^{2+} accumulation triggered by ROS may be exacerbated through a positive feedback loop driven by activated ERK1/2.

3.4.5 Chelation of intracellular Zn^{2+} blocks ERK1/2 activation and restores ERK2 phosphatase activity

Zn^{2+}-induced ERK1/2 activation has been reported to contribute to neuronal cell death (Seo et al., 2001; Du et al., 2002; Zhang et al., 2004). Given the fact that oxidative stress triggers intracellular Zn^{2+} accumulation in HT22 cells and primary neurons (Figure 3.4) and sustained ERK1/2 activation (Stanciu et al., 2000), we sought to examine whether Zn^{2+} contributes to ERK1/2 activation. To evaluate the role of Zn^{2+} accumulation in activating ERK1/2 in HT22 cells and primary neurons, we employed the high affinity Zn^{2+} chelator TPEN. HT22 cells were

treated with glutamate, in the presence or absence of 1 μM and 5 μM TPEN for 8 h. Whole cell lysates were then subject to Western blot analysis to detect phosphorylated, activated ERK1/2. As shown in Figure 3.5A, exposure of HT22 cells to glutamate induced a large increase in ERK1/2 phosphorylation that was unaffected by the addition of 1 μM TPEN (Fig 3.5A). However, the addition of 5 μM TPEN significantly blocked glutamate-induced ERK1/2 activation (Fig 3.5A). This result suggests that ERK1/2 activation is downstream of Zn^{2+} accumulation.

Similar experiments were then extended to primary neurons, which were treated with HCA, HCA plus 1 μM TPEN, and HCA plus 5 μM TPEN for 16 h. Analogous to results obtained in HT22 cells, addition of 5 μM, but not of 1 μM TPEN, significantly blocked the activation of ERK1/2 following oxidative stress in primary neurons (Figure 3.5B).

Given the fact that Zn^{2+}inhibits ERK2 phosphatase activity *in vitro* (Figures 3.3A & 3.3B), we set out to examine whether TPEN inhibition of ERK1/2 activation occurs through the reversal of Zn^{2+}-mediated inhibition of ERK1/2 phosphatases. The ERK2 phosphatase assay was performed on whole cell lysates prepared from untreated HT22 cells, or cells treated with glutamate, or glutamate plus 1 μM or 5μM TPEN. As shown in Figures 3.5C and 3.5E, ERK2 phosphatase activity was decreased following treatment with glutamate. The addition of 1 μM TPEN to HT22 cells did not reverse this inhibitory effect of glutamate (Figures 3.5C and 3.5E). However, the addition of 5 μM TPEN significantly reversed the inhibition of ERK2 phosphatase activity in HT22 cells that were treated with glutamate (Figures 3.5C and 3.5E). Similar results were obtained in primary neurons (Figures 3.5D and 3.5F). These results suggest that TPEN blocks Zn^{2+}-induced ERK1/2 activation by reversing a Zn^{2+}-mediated inhibition of ERK1/2 phosphatase activity.

As U0126 has been found to block Zn^{2+}accumulation in oxidatively-stressed cells, (Figure 3.4), we sought to examine whether U0126 could also reverse the inhibition of ERK2 phosphatase activity. The ERK2 phosphatase assay was therefore performed on cell lysates prepared from untreated HT22 cells or cells treated with glutamate or glutamate plus U0126. As shown in Figures 3.5G and 3.5H, U0126 treatment of HT22 cells reversed the inhibition of ERK2 phosphatase activity induced by oxidative stress. This result reinforces the notion that activated ERK1/2 contributes to a positive feedback loop that maintains elevated levels of intracellular $Zn^{2+}$and persistent inhibition of ERK1/2 phosphatases.

3.4.6 TPEN protects neuronal cells from oxidative toxicity

Since TPEN treatment blocks ERK1/2 activation following oxidative toxicity in HT22 cells and primary neurons (Figures 3.5A & 3.5B), we examined whether TPEN could also limit cell death. Due to the fact that Zn^{2+} is an essential metal, we limited TPEN treatment to less than 16 h in both HT22 cells and primary neurons. Prolonged exposure (i.e. 48 h) of HT22 cells and primary neuronal cultures with TPEN alone is toxic (data not shown). HT22 cells were treated for 12 h with glutamate, glutamate plus 1 μM TPEN, glutamate plus 5 μM TPEN, as well as 1 μM TPEN or 5 μM TPEN alone. A propidium iodine (PI) staining method was then used to assess cell toxicity. PI is a DNA dye that only enters cells with compromised plasma membranes. Therefore, toxicity can be assessed by determining the percentage of PI^{+} cells in different treatment groups (Levinthal and DeFranco, 2005). As shown in Figure 3.6A, a significant proportion of HT22 cells stained positive for PI upon glutamate treatment. Cells treated with glutamate plus 1 μM TPEN exhibited a similar extent of cell death. However, a 5 μM TPEN treatment significantly protected HT22 cells from glutamate-induced cell death (Figure 3.6A,

$p<0.001$). This neuroprotective effect is similar to that provided by U0126 (Figure 3.6A). A 12 h treatment with 1 μM and 5 μM TPEN alone exhibited no toxicity in HT22 cells (Figure 3.6A).

This paradigm was then extended to primary neurons. These cultures were subject to the same treatment as HT22 cells, and cell toxicity assessed after treatment for 16 h. Similar to the results obtained in HT22 cells, a 16 h treatment with HCA as well as HCA plus 1 μM TPEN induced significant cell death in primary neurons (Figure 3.6B). However, the addition of 5 μM TPEN significantly protected primary neurons from HCA-induced toxicity (Figure 3.6B). In summary, the dose of TPEN required to protect both HT22 cells and primary neurons from oxidative toxicity is coincident with that required to block ERK1/2 activation and ERK1/2 phosphatase inhibition.

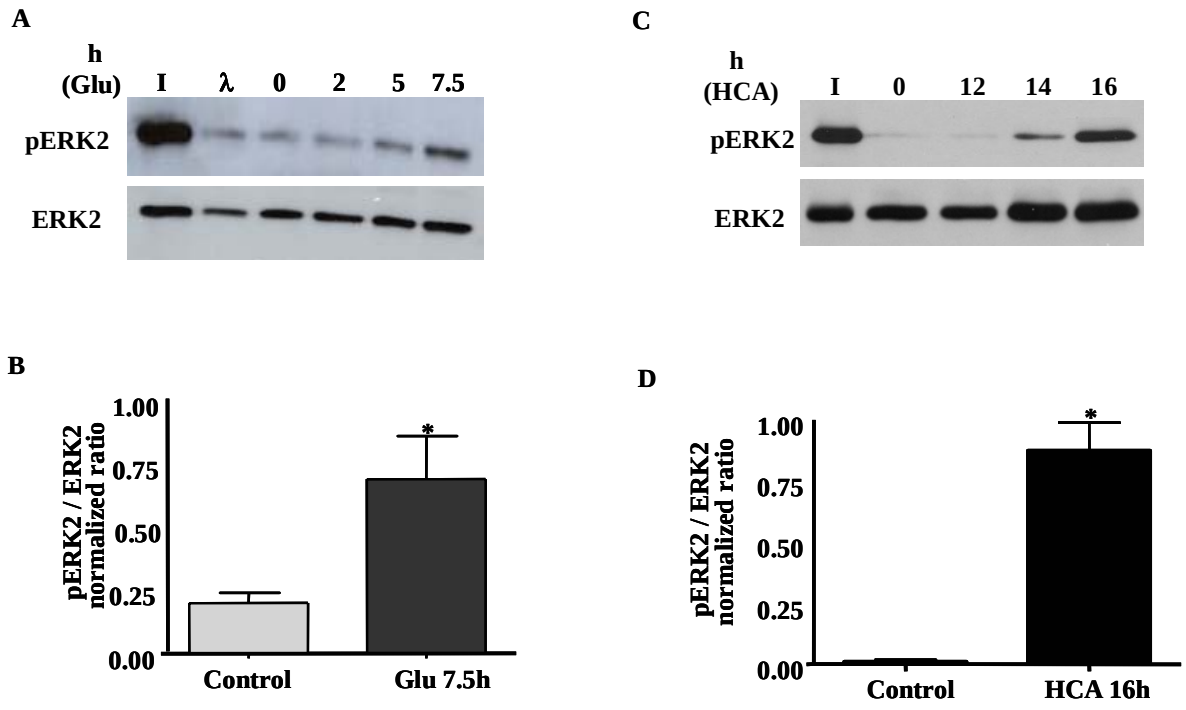


Figure 3.1 ERK2 phosphatase activity is inhibited upon oxidative toxicity in HT22 and immature primary cortical cultures.

A. HT22 cells were treated with 5mM glutamate for either 0, 2, 5, or 7.5h. Whole cell lysates were incubated with purified, phosphorylated ERK2 (pERK2) and phosphatase activity was assessed by Western blots that revealed pERK2 or total ERK2. I=phosphatase assay input; λ=lambda phage protein phosphatase (positive control).

B. Quantification of the results from 4 independent experiments, revealing a significant decrease in ERK2 phosphatase activity following glutamate-induced oxidative toxicity in HT22 cells. $P<0.05$.

C. ERK2 phosphatase activity is inhibited upon 5mM HCA treatment in primary cortical neuronal cultures. Immature primary neuronal cultures were treated with HCA for either 0, 12, 14, 16h. Whole cell lysates were incubated with purified pERK2, and phosphatase activity was assessed by Western blots that revealed either pERK2 or total ERK2. I=phosphatase assay input.

D. Quantification of the results from 3 independent experiments, revealing a significant decrease in ERK2 phosphatase activity following HCA-induced oxidative toxicity in immature primary cortical neuron cultures. $P<0.05$.

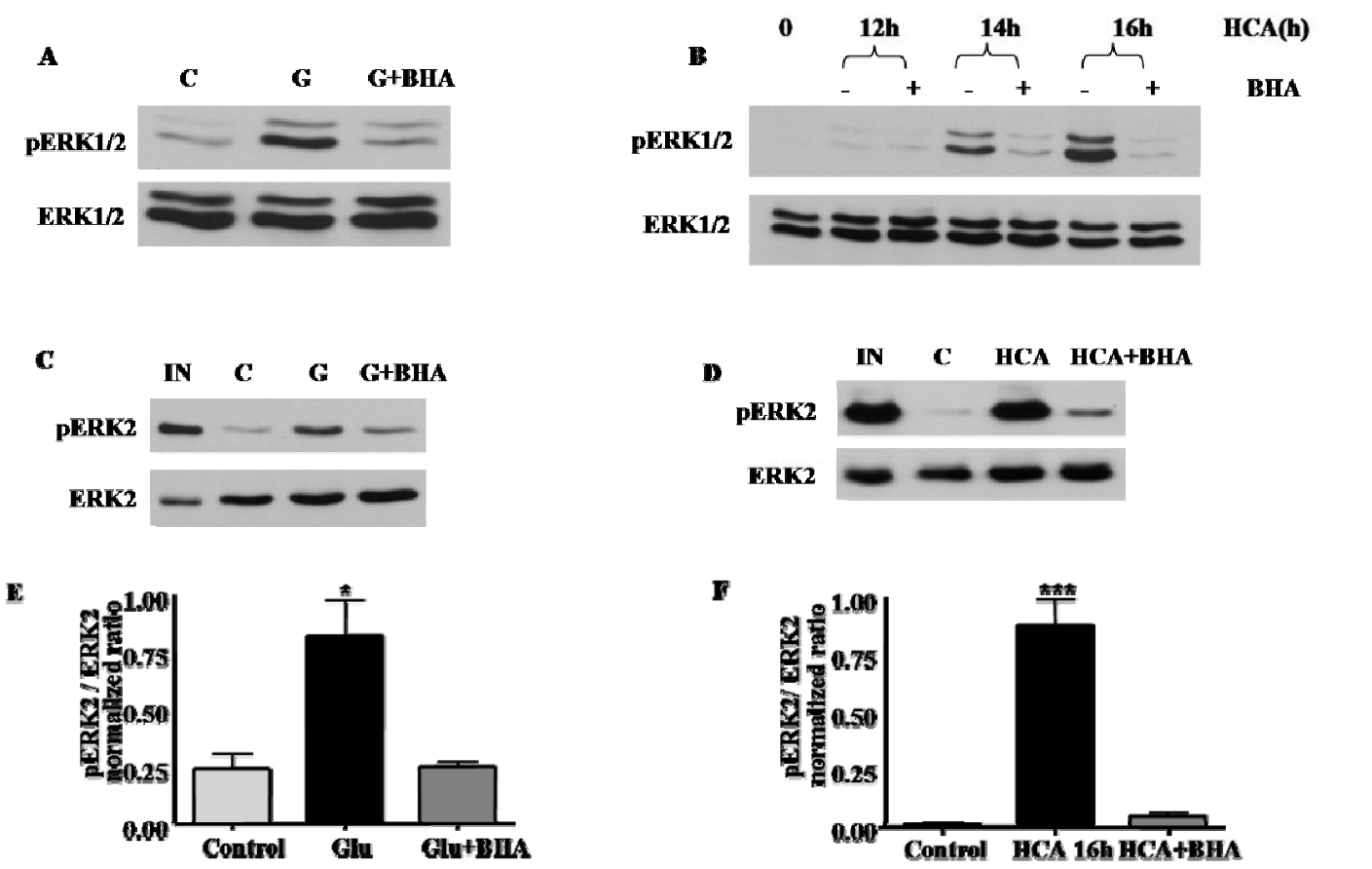


Figure 3.2 BHA blocks ERK1/2 activation and the inhibition of ERK2 phosphatase activity induced by oxidative stress in HT22 cells and immature primary cortical cultures.

A. HT22 cells were treated with glutamate or glutamate plus BHA for 7.5h. Twenty ug of total protein lysate was separated by SDS-PAGE and subjected to Western blot analysis to detect pERK1/2 and total ERK1/2 on the same blots. C=control cell lysates. The addition of BHA blocked ERK1/2 activation following glutamate-induced oxidative stress.

B. Immature primary cortical neuronal cultures were treated with HCA for either 0, 12, 14, or 16 hr in the presence or absence of BHA. Twenty ug of total protein lysate was separated by SDS-PAGE and subjected to Western blot analysis to detect pERK1/2 and total ERK1/2 on the same blots. The administration of BHA significantly blocks ERK1/2 activation following HCA treatment.

C. Whole cell lysates prepared from control untreated (C), glutamate (G)-treated, or glutamate plus BHA-treated HT22 cells were incubated with purified pERK2, and phosphatase activity was assessed by Western blot that revealed either pERK2 or total ERK2. IN=ERK2 input. The addition of BHA reverses the glutamate-induced inhibition of ERK2 phosphatase activity.

D. Whole cell extracts prepared from the control (C), HCA-treated, and HCA plus BHA-treated primary neuronal cultures were incubated with purified pERK2, and phosphatase activity was assessed by Western blot that revealed either pERK2 or total ERK2. IN=ERK2 input. The administration BHA reverses the inhibition of ERK2 phosphatase activity following HCA-induced oxidative toxicity.

E. Quantification of the results from 3 independent experiments. Co-administration of BHA abrogates the inhibition of ERK2 phosphatase activity in glutamate-treated HT22 cells. P<0.01.
F. Quantification of the results from 3 independent experiments. Co-administration of BHA abrogates the inhibition of ERK2 phosphatase activity in HCA-treated immature cortical cultures. P<0.001.

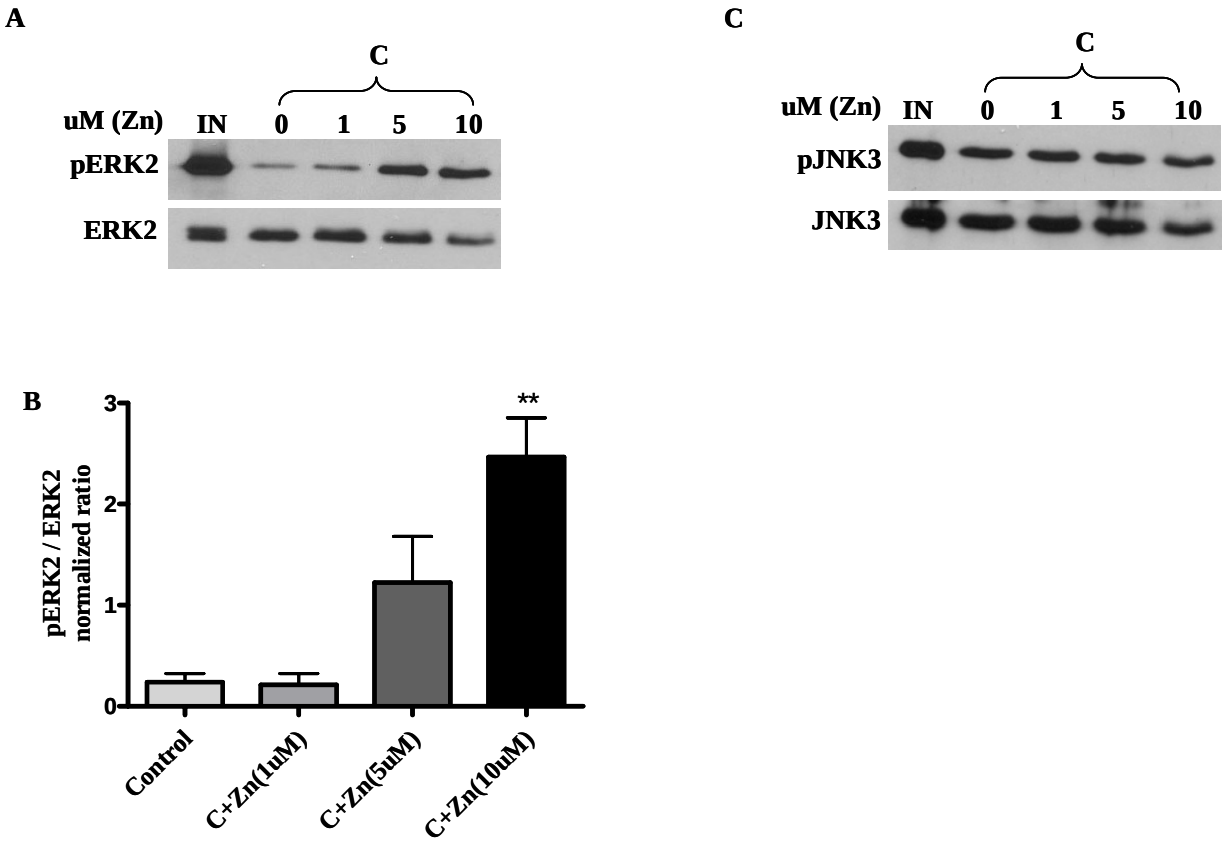


Figure 3.3 Zn^{2+} specifically inhibits ERK2 phosphatase activity in neuronal cell extracts.

A. Various concentrations of $ZnCl_2$ were pre-incubated with whole cell lysates prepared from untreated HT22 cells for 10 min at 37ºC before the addition of pERK2 substrate. ERK2 dephosphorylation was monitored by Western blot analysis as described previously.
B. Quantification of the results from 3 independent experiments. 10uM $ZnCl_2$ significantly inhibits ERK2 phosphatase activity in HT22 cells. P<0.01.
C. Various concentrations of $ZnCl_2$ were pre-incubated with whole cell lysates prepared from untreated HT22 cells for 10 min at 37ºC before the addition of pJNK3 substrate. JNK3 dephosphorylation was monitored by Western blot analysis as described previously. $ZnCl_2$ does not inhibit JNK3 phosphatase activity in HT22 cell extracts.

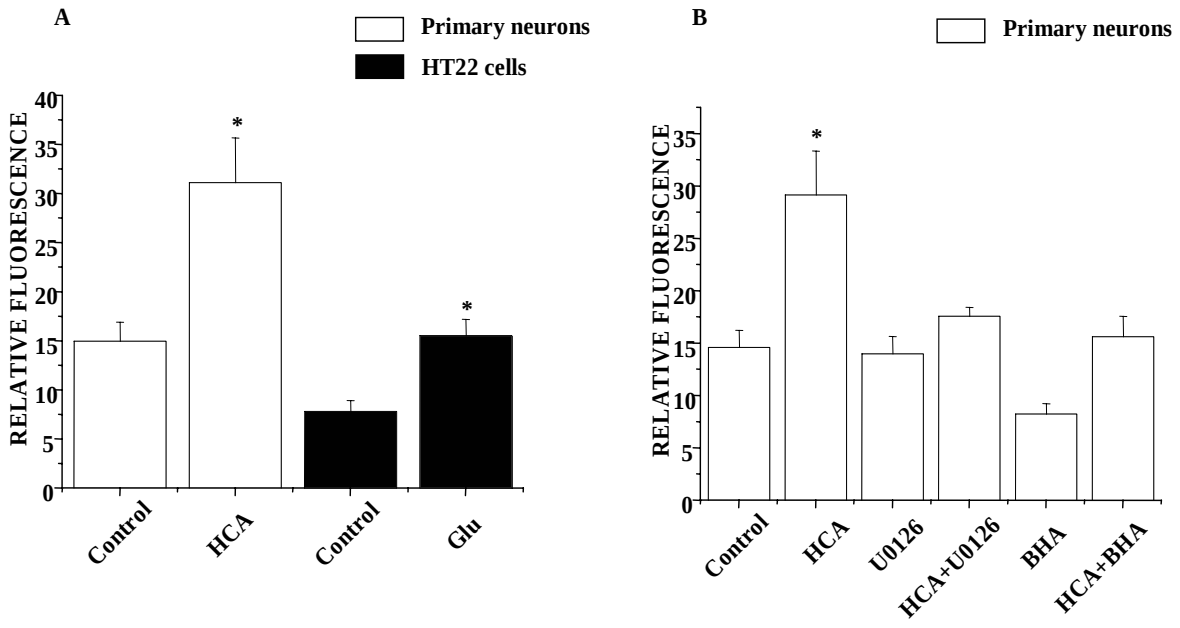


Figure 3.4 Oxidative stress triggers zinc accumulation in HT22 cells and immature primary cortical neurons.

A. HT22 cells were treated with glutamate for 8 hr and primary neuronal cultures were treated with HCA for 12 hr and loaded with FluoZin-3 for 30 min. The fluorescence imaging of intracellular zinc was then monitored by digital fluorescence microscopy and relative fluorescence was measured. There is a significant increase in zinc accumulation following oxidative stress in both HT22 cells and primary immature cortical neurons.

B. Primary immature cortical neurons were treated with HCA, HCA+U0126, HCA+BHA as well as U0126 only and BHA only and then were loaded with FluoZin-3 to measure the relative fluorescence. Co-administration of U0126 decreases the zinc accumulation in HCA-treated primary cortical neurons to basal level. Similarly, the co-administration of BHA also decreases the zinc release in HCA-treated primary cortical neurons.

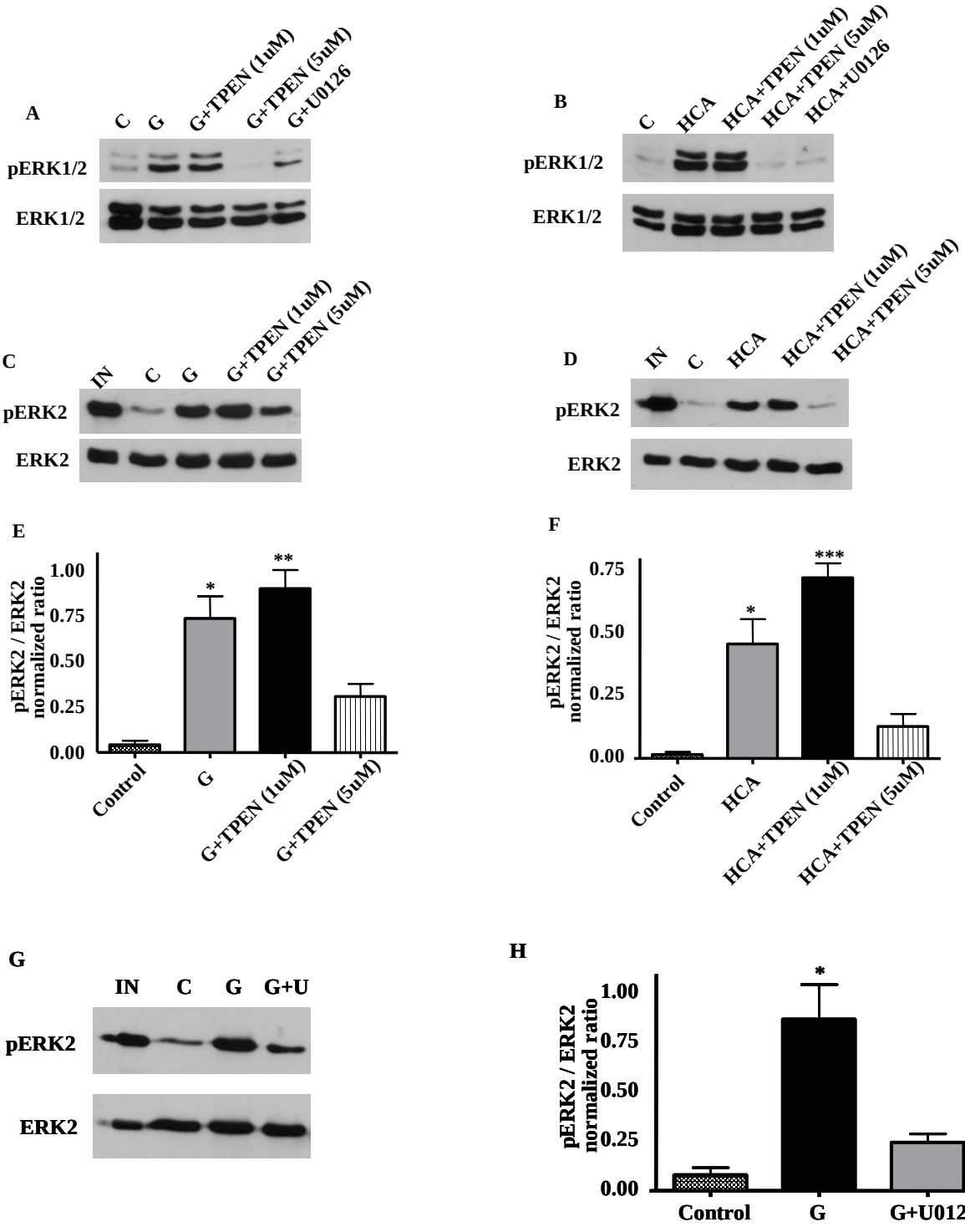

A
C
G
G+TPEN (1uM)
G+TPEN (5uM)
G+U0126
pERK1/2
ERK1/2
B
C
HCA
HCA+TPEN (1uM)
HCA+TPEN (5uM)
HCA+U0126
pERK1/2
ERK1/2
C
IN
C
G
G+TPEN (1uM)
G+TPEN (5uM)
pERK2
ERK2
D
IN
C
HCA
HCA+TPEN (1uM)
HCA+TPEN (5uM)
pERK2
ERK2
E
pERK2 / ERK2 normalized ratio
1.00
0.75
0.50
0.25
0.00
*
**
Control
G
G+TPEN (1uM)
G+TPEN (5uM)
F
pERK2 / ERK2 normalized ratio
0.75
0.50
0.25
0.00
*
***
Control
HCA
HCA+TPEN (1uM)
HCA+TPEN (5uM)
G
IN
C
G
G+U
pERK2
ERK2
H
pERK2 / ERK2 normalized ratio
1.00
0.75
0.50
0.25
0.00
*
Control
G
G+U0126

Figure 3.5. TPEN blocks ERK1/2 activation and reverses the inhibition of ERK2 phosphatase activity following oxidative stress in HT22 cells and immature cortical cultures.

A. HT22 cells were treated with either glutamate, glutamate plus 1uM TPEN, glutamate plus 5uM TPEN, or glutamate plus U0126 for 8h. Twenty ug of total protein lysate was separated by SDS-PAGE and subjected to Western blot analysis to detect pERK1/2 and total ERK1/2 on the same blots. C=control cell lysates. 5uM TPEN blocks ERK1/2 activation following glutamate treatment in HT22 cells.

B. Immature primary cortical cultures were treated with either HCA, HCA plus 1uM TPEN, HCA plus 5uM TPEN, or HCA plus U0126 for 16h. 20ug of total protein lysate was separated by SDS-PAGE and subjected to Western blot analysis to detect pERK1/2 and total ERK1/2 on the same blots. C=control cell lysates. 5uM TPEN blocks ERK activation following HCA treatment in immature primary cortical cultures.

C. An ERK2 phosphatase assay was performed on whole cell lysates prepared from either untreated HT22 cells, or cells treated with either glutamate, glutamate plus 1uM TPEN, or glutamate plus 5uM TPEN. TPEN reverses the inhibition of ERK2 phosphatase activity following glutamate treatment in HT22 cells.

D. An ERK2 phosphatase assay was performed on whole cell lysates prepared from either untreated primary immature cortical neurons, or cells treated with either HCA, HCA plus 1uM TPEN, or HCA plus 5uM TPEN. TPEN reverses the inhibition of ERK2 phosphatase activity following HCA treatment in primary immature cortical neurons.

E. Quantification of the ERK2 phosphatase activity results from 3 independent experiments in HT22 cells. $P<0.05$.

F. Quantification of the ERK2 phosphatase assays results from 3 independent experiments in primary immature cortical neurons. $P<0.05$.

G. An ERK2 phosphatase assay was performed on whole cell lysates prepared from either untreated HT22 cells, or cells treated with either glutamate, or glutamate plus 10uM U0126. The administration of U0126 reverses the inhibition of ERK2 phosphatase activity following glutamate treatment in HT22 cells.

H. Quantification of the ERK2 phosphatase assay results from 3 independent experiments in HT22 cells. $P<0.05$.

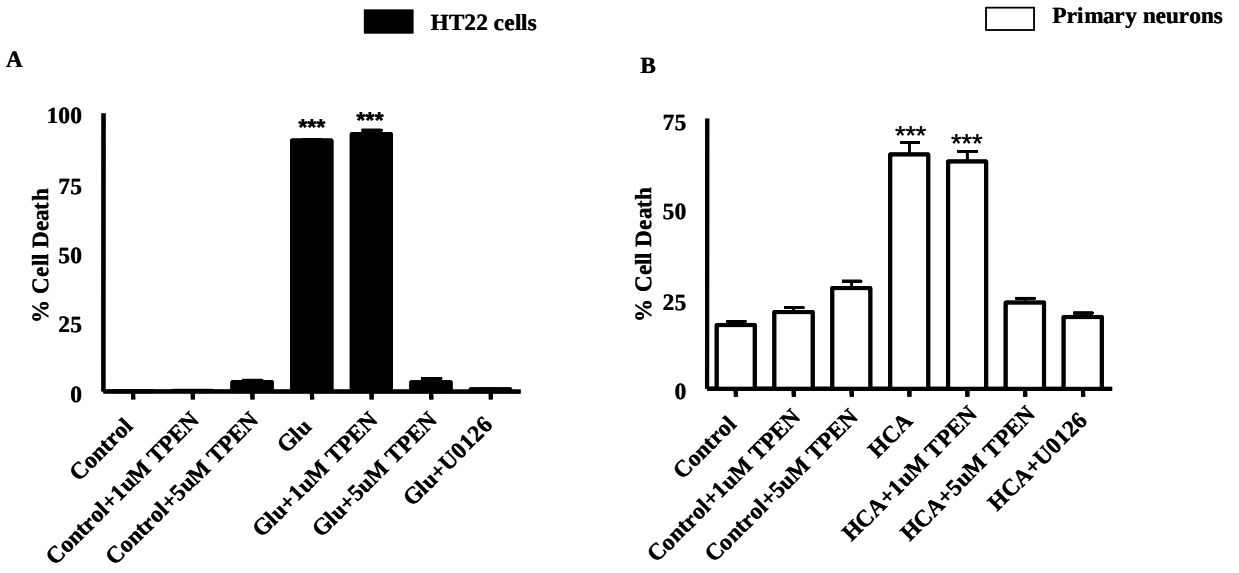


Figure 3.6. TPEN blocks oxidative toxicity in HT22 cells and immature cortical cultures.

A. HT22 cells were treated with either 1uM TPEN, 5uM TPEN, glutamate, glutamate plus 1uM TPEN, glutamate plus 5uM TPEN, or glutamate plus U0126. Toxicity was measured as the percentage of PI-positive cells after treatment for 12 h in the above groups. ***$p<0.001$. 5uM TPEN blocks glutamate-induced oxidative toxicity in HT22 cells.

B. Immature primary cortical cultures were treated with either 1uM TPEN, 5uM TPEN, HCA, HCA plus 1uM TPEN, HCA plus 5uM TPEN or HCA plus U0126. Toxicity was measured as the percentage of PI-positive cells after treatment for 16 h in the above groups. ***$p<0.001$. 5uM TPEN blocks HCA-induced oxidative toxicity in immature primary cortical cultures

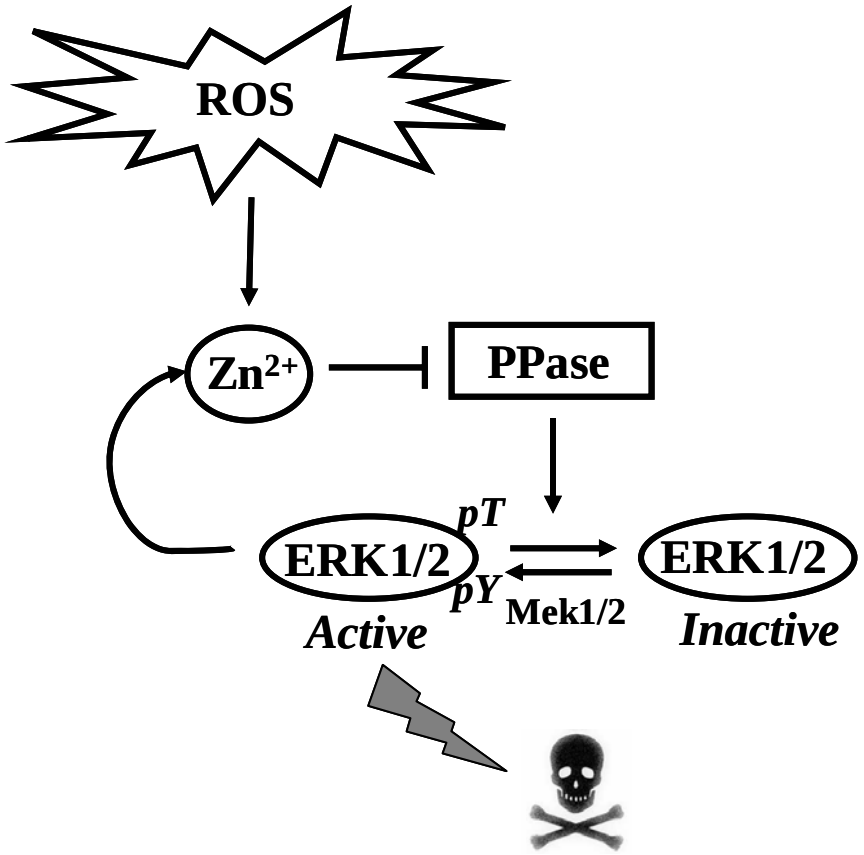


Figure 3.7 Mechanism of ERK1/2-dependent neuronal cell death triggered by ROS-mediated Zn^{2+} accumulation.
Accumulation of intracellular Zn^{2+} brought about by oxidative stress (ROS) in neurons inhibits protein phosphatases that act selectively on ERK1/2. The persistent activation of ERK1/2 (pERK1/2) functions to promote cell death in part through a positive feedback loop that maintains elevated Zn^{2+} levels.

3.5 DISCUSSION

ERK1/2 functions in many cell types to promote proliferation or survival but can also be diverted to participate in certain cell death pathways (Colucci-D'Amato et al., 2003; Chu et al., 2004). For example, chronic activation of ERK1/2 is necessary for cell death induced by oxidative stress in immature cortical neurons and HT22 cells (Satoh et al., 2000; Stanciu et al., 2000; Luo and DeFranco, 2006). In these cases, the inhibition of select protein phosphatases is primarily responsible for the persistent activation of ERK1/2 (Levinthal and DeFranco, 2005). This conclusion was confirmed in a recent report, which also revealed the minimal contribution of upstream activating kinases (i.e. MEK1/2) to prolonged ERK1/2 activation in oxidatively stressed HT22 cells and immature neurons (Choi et al., 2006).

I show here that the inhibition of ERK1/2 phosphatases in HT22 cells and immature neurons subjected to glutathione depletion is indeed the result of oxidative stress as phosphatase activity is restored in cells treated with BHA. This leads to reduced ERK1/2 activation and neuroprotection. The oxidative inhibition of protein phosphatase activity in TNF-alpha treated fibroblasts is also eliminated by BHA treatment (Kamata et al., 2005). However in TNF-α-induced oxidative toxicity and Concanavolin A-induced liver toxicity in mice, cell death is associated with the selective inhibition of JNK phosphatases (Kamata et al., 2005). Glutamate-induced oxidative stress in immature neurons does not significantly affect JNK phosphatase activity (Levinthal and DeFranco, 2005). Therefore, even though MAPK phosphatases are oxidant sensitive, they are not globally inactivated by oxidative stress in cells. Depending upon the nature of the oxidative stress imposed upon living cells, select MAPK phosphatases may be

protected from the damaging effects of reactive species due to their sequestration within subcellular compartments or multi-subunit complexes that are inaccessible to short-lived oxidants.

While several protein phosphatases are redox sensitive and inhibited by direct thiol oxidation of a catalytic-site cysteine (Meng et al., 2002; Leslie et al., 2003; Sohn and Rudolph, 2003; Tonks, 2003; Persson et al., 2004), our results suggest that MAPK phosphatase activity may also be regulated in oxidatively stressed cells by the inhibitory effects of free Zn^{2+}. The sensitivity of various MAPK phosphatases to inhibition by Zn^{2+} was established with purified phosphatase preparations suggesting that Zn^{2+} can act directly to impact the activity of these enzymes (Brautigan et al., 1981; Zhuo and Dixon, 1997). Zn^{2+} mediated inhibition of PTPs has been suggested to impact insulin/IGF (Haase and Maret, 2003) or IL-8 (Kim et al., 2006) signaling in C6 glioblastoma or BEAS-2B human airway epithelial cells, respectively. Thus, Zn^{2+}-mediated inhibition of protein phosphatase activity may not always be linked to cell death responses but could play a role in facilitating hormone signaling (Haase and Maret, 2003) or participate in activity-dependent neuronal plasticity (Klann and Thiels, 1999). However, prolonged inhibition of select MAPK phosphatases and ensuing persistent activation of specific MAPKs that drives toxicity in oxidatively stressed cells may reflect an inability to restore Zn^{2+} homeostasis (see below).

For PSTPs, Zn^{2+} may exert its inhibitory effects through the displacement of bound Mn^{+2} at the active site of their catalytic subunits (Zhuo and Dixon, 1997). Interestingly, purified PSTPs, such as PP2A, are refractory to exchange by Mn^{2+} once bound by Zn^{2+} and therefore limited recovery of their activity can be achieved by Zn^{2+} chelation *in vitro* (Zhuo and Dixon, 1997). These potent inhibitory effects of Zn^{2+} on ERK1/2 phosphatases, such as PP2A, may

explain the requirement for a 5 uM TPEN treatment to reverse the inhibition of ERK1/2 phosphatase activity in oxidatively stressed HT22 cells and immature neurons. Importantly, 5 uM TPEN was also required to block glutamate- and HCA-induced toxicity in HT22 cells and immature neurons, respectively, showing a close association between the inhibition of ERK1/2 activation, restoration of ERK1/2 phosphatase activity, and protection from oxidative stress.

The specific ERK1/2 phosphatase that is subjected to oxidative stress-induced Zn^{2+} inhibition is not known. Previous results from our group suggest that PP2A is the major ERK1/2 phosphatase in primary immature cortical neurons (Levinthal and DeFranco, 2005) and HT22 cells (data not shown), but other MAPK phosphatases in these cells may also contribute to the regulation of ERK1/2 activation. In a recent report, glutamate treatment of HT22 cells and immature neurons was found to trigger the degradation of MKP1 (Choi et al., 2006). Although MKP1 exerts a minor albeit significant effect on ERK1/2 activation and glutamate toxicity in HT22 cells (Choi et al., 2006), the loss of this enzyme cannot be solely responsible for heightened ERK1/2 activation since ERK12 phosphatase activity in extracts from oxidatively stressed neurons can be restored *in vitro* with DTT (Levinthal and DeFranco, 2005). Furthermore, the JNK family of MAPKs is the preferred substrate of MKP1, which is inconsistent with the selective effects of oxidative stress in HT22 cells and immature neurons on ERK1/2 phosphatases. PP2A, the major ERK1/2 phosphatase in HT22 cells and immature neurons (Levinthal and DeFranco, 2005), exerts minimal effects on JNK activation, and thus is a better candidate for the selective MAPK phosphatase that is affected by Zn^{2+} in oxidatively stressed neuronal cells.

Oxidative stress can trigger the release of intracellular stores of metal ions such as Zn^{2+} from redox-sensitive metal binding proteins. For example, a thiol oxidant, 2,2'-dithhildipyridine

(DTDP), has been shown to induce neuronal cell death by liberating Zn^{2+} from metal-containing proteins (Aizenman et al., 2000). Protein kinase C (PKC) delta can also release its bound Zn^{2+} when exposed to oxidants or lipid-derived signaling molecules (Knapp and Klann, 2000; Korichneva et al., 2002). Interestingly, PKC delta has recently been reported to be responsible for ERK1/2 activation and glutamate toxicity in HT22 cells and primary cortical neurons (Choi et al., 2006). Since activation of PKC delta is associated with release of its bound Zn^{2+} (Knapp and Klann, 2000; Korichneva et al., 2002), PKC delta could participate in ERK1/2-dependent toxicity through triggering the enhanced degradation of a dual-specificity protein phosphatase (DSP) acting on ERK1/2 (i.e. MKP1; (Choi et al., 2006) and/or providing free Zn^{2+} for reversible inhibition of an ERK1/2-selective PSTP (e.g., PP2A). Future work examining the impact of PKC delta on Zn^{2+} release may reveal whether such a dual regulation of ERK1/2 activation by PKC delta operates in oxidatively stressed neurons.

The decreased accumulation of free Zn^{2+} upon inhibition of ERK1/2 activation by U0126 supports the existence of a positive feedback loop that not only provides conditions for the maintenance of ERK1/2 activation (i.e., through phosphatase inhibition) but also exacerbates cellular exposure to neurotoxic levels of free Zn^{2+} (Figure 7). In this case, ROS provide the trigger that acts to promote the release of protein-bound Zn^{2+}, perhaps through thiol oxidation of specific cysteine residues that participate in Zn^{2+} binding (Aizenman et al., 2000; St Croix et al., 2002). This model would be consistent with the failure of transfected constitutively active MEK1 to induce toxicity in HT22 cells despite a robust, persistent activation of ERK1/2 within either the cytoplasmic or nuclear compartment (Luo and DeFranco, 2006). Thus, ERK1/2 may prevent the repair of oxidatively damaged Zn^{2+} binding proteins or limit their expression (Jiang

et al., 2004a). In the absence of oxidative damage to these proteins, chronic ERK1/2 activation may not impact intracellular Zn^{2+} homeostasis and promote cell death.

In summary, we have identified an important regulatory feature of protein phosphatase regulation, namely, reversible Zn^{2+} inhibition, that triggers the persistent activation of a select MAPK signaling module in neurons. Furthermore, the oxidative induction of Zn^{2+} accumulation predisposes neurons cells to a positive feedback loop driven by ERK1/2 that disrupts Zn^{2+} homeostasis and further exacerbates cellular exposure to neurotoxic levels of free Zn^{2+}.

4.0 MECHANISM OF PHOSPHATASE INHIBITION IN OXIDATIVELY STRESSED NEURONS

4.1 SUMMARY

Previous studies have shown that the reversible inhibition of ERK1/2 phosphatases contribute to ERK1/2-dependent oxidative toxicity in neurons. However, the specific ERK1/2 phosphatases and the mechanism of the oxidation and inactivation of ERK phosphatases have not been identified. This study examined the role of PP2A as the possible candidate for ERK dephosphorylation by utilizing specific inhibitors of PP2A. Using the method of thiol affinity chromatography, I have identified the thiol oxidation of cysteines in the PP2A catalytic subunit. Moreover, I have detected different patterns of migration of various PP2A regulatory subunits under reducing and non-reducing conditions, which suggests that PP2A regulatory subunits are differentially regulated by oxidative stress. However, the activity of the PP2A catalytic subunit is not affected following glutamate-induced toxicity in neurons. Altogether, these results suggest that thiol oxidation of cysteine residues in the catalytic subunit of PP2A may affect the activity of PP2A by affecting the association between regulatory subunits or associated factors with catalytic subunits, rather than by directly inactivating the total cellular pool of PP2A catalytic subunits.

4.2 INTRODUCTION

In recent years, ROS have emerged as important regulators of many signaling pathways. For example, protein phosphatases have been identified as targets of ROS, suggesting that ROS mediate signaling pathways by changing the phosphorylation status of the signaling proteins. The mechanism of ROS-mediated inhibition of protein tyrosine phosphatases has been studied extensively (den Hertog et al., 2005; Tonks, 2005). PTPs bear a signature motif $HC(X)_5R$ in their active catalytic site and these cysteine residues are critical for the formation of a cysteine-phospho intermediate involved in the catalytic function of PTPs. Due to the low pKa (~4.5) of these cysteines, they are very susceptible to oxidation, which leads to inactivation of the phosphatase. DSPs have similar signature motif as PTPs and share the same mechanism of oxidation inhibition by ROS. Compared with PTPs/DSPs, the mechanism regulating oxidative inhibition of PSTPs, such as PP1, PP2A and PP2B (calcineurin), still remains vague. Calcineurin has been shown to be oxidatively inhibited by H_2O_2 through the oxidation of its dinuclear metal center (Fe^{2+}-Zn^{2+}) (Wang et al., 1996; Namgaladze et al., 2002) as well as the oxidation of two neighboring cysteines (Carballo et al., 1999; Bogumil et al., 2000).

As the major PSTPs in eukaryotic cells, PP2A is composed of a core enzyme and various regulatory subunits. The core enzyme consists of a catalytic subunit (C subunit) and a structure subunit (A subunit) and forms complexes with a variety of regulatory subunits (B subunits) that regulate the substrate specificity and subcellular localization of the PP2A holoenzyme. Four different families of B subunits have been identified and each B subunit family contains multiple isoforms, resulting in a variety of holoenzyme combinations (Janssens and Goris, 2001). The specificity of the regulatory subunits determines the specificity of the substrates as well as the subcellular localization of PP2A. It has been shown that overexpression of PP2A Bγ subunit in

stably transfected PC6-3 neuronal cell line promotes cell differentiation (Strack, 2002), while in the same cell line, overexpression of neuronal-specific PP2A Bβ2 subunit promotes apoptosis induced by serum withdrawl (Dagda et al., 2003). The apoptosis induced by the latter is actually caused by the targeting to PP2A to mitochondria by PP2A Bβ2 subunits and mostly likely is through the dephosphorylation of various mitochondrial pro- and anti-apoptotic proteins (Dagda et al., 2003). Recently, it has been found that a PP2A associated factor α4 is required for the inhibition of apoptosis in thymocytes (Kong et al., 2004).

ERK1/2 is one of the major targets of PP2A. Different subunits of PP2A have been associated with ERK1/2-dependent apoptosis or survival (Silverstein et al., 2002; Adams et al., 2005; Van Kanegan et al., 2005), suggesting an important role of PP2A in regulating ERK1/2-mediated signaling pathways as well as cellular responses. Recently, impairment of PP2A has been involved in a variety of oxidative stress-induced neurodegenerative diseases. Reduced PP2A activity has been associated with an enhanced activation of ERK1/2 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 exhibit activation of ERK1/2 and JNK pathways as well as the phosphorylation of endogeneous tau, similar to the key pathological features in Alzheimer's disease (Kins et al., 2001; Kins et al., 2003). PP2A recently has been shown susceptible to H_2O_2 in aging fibroblasts (Kim et al., 2003) and brain tissues (Foley et al., 2004). Oxidation of cysteine residues may be responsible for oxidative inhibition of PP2A, since the inactivation of PP2A by ROS is thiol-dependent and reversible. However, the mechanism of thiol-oxidation mediated inactivation of PP2A by oxidative stress has not yet been elucidated.

We previously have shown that glutamate-induced oxidative stress reversibly inhibits ERK1/2 phosphatases activity in immature cortical neurons (Levinthal and DeFranco, 2005). However, the specific ERK1/2 phosphatase being inhibited as well as the mechanism of oxidation and inhibition of ERK1/2 phosphatase have not been identified yet. In the current study, we demonstrated that PP2A is the predominant ERK phosphatase in neurons. Moreover, cysteine thiol-oxidation of PP2A has been revealed although the association between thiol-oxidation of PP2A, and its catalytic activity remains unclear.

4.3 MATERIALS & METHODS

ERK-directed phosphatase assay

An ERK2-directed phosphatase assay was performed as described in the previous chapter. Where indicated, 2.5ug of SV40 small T antigen was incubated with control cell lysates of HT22 cells for 10 min at 37ºC before adding the pERK2 substrate. 1nM okadaic acid (OA) was incubated with control lysates from HT22 or primary neurons for 30 min on ice before adding the pERK2 substrate. 1ug of purified Inhibitor-2 (I2) was incubated with control lysates of primary neurons for 30 min on ice before adding the pERK2 substrate.

Thiol affinity chromatography

In collaboration with Drs. Pier Mastroberardino and Timothy Greenamyre, I adopted a thiol-affinity chromatography to enrich for proteins containing reversibly oxidized thiols following glutamate treatment in primary immature cortical neurons. Generally, cytosolic extracts cleared of intracellular organelles were subjected to iodoacetamide (IAA)/N-

ethylmaleimide (NEM) treatment which could react and block reduced thiols in the extracts. The modified proteins were then precipitated by cold acetone to remove the excess IAA/NEM. Tributylphosphine (TBP) was then added to modified protein samples to reduce oxidized thiols. TBP treatment could only reduce the oxidized thiols formed before IAA/NEM blocking. TBP-treated samples were then loaded to thiol-activated Sepharose 6B affinity columns (Upstate Biotechnology) for enrichment of proteins with TBP-reduced thiols. Then the proteins were eluted by the addition of 50 mM DTT. Eluted samples were then subjected to Western blot analysis using the antibody against the catalytic subunit of PP2A to detect the presence of reversibly oxidized thiols in PP2A C subunit. To show the equivalence of protein loading, same amounts of total protein were subjected to SDS-PAGE and silver staining.

Details of the procedure are as follows. Firstly, dry thiol-sepharose resin (SIGMA, St. Louis, MO, USA) was rehydrated in milliQ grade distilled water for 30 min with gently shaking at 4°C. After centrifugation for 5 min at 500g, the sedimented resin was resuspended in 10 volumes of 100mM TrisHCl, pH7.4, SDS 1% and incubated for 5 min with gently shaking at room temperature. This step was repeated for a total of three times. Cytosolic extracts cleared of intracellular organelles were then prepared from glutamate-treated or non-treated primary cortical neurons by ultracentrifugation at 100,000g for 1hr at 4ºC. The extracts were resuspended in 100mM TrisHCl, pH7.4, SDS 1% at the final concentration of 1mg/ml. Proteins were denatured heating the solution at 70°C for 5 min. N-ethylmaleimide (NEM) and iodoacetamide (IAA) were added at the final concentration of 50mM each. The mixture was incubated for 30 min at 37°C and precipitated with 5 volumes of ice cold precipitation solution (50% acetone, 25% methanol, 25% ethanol, 1 hr at -80°C). After spinning (25 min, 3200g, 4°C), the pellet was resuspended in 1 ml Tris/SDS. As a pre-clearing step, the sample was then incubated with the

thiol-sepharose medium (200 ul, about 50 mg of dry resin) for 2 hrs at room temperature. After centrifugation for 5 min with 500g at room temperature, the supernatant was saved and tributylphosphine (TBP) in dimethylformamide (DMF) (20mM final concentration) was added to reduce cysteines previously engaged in disulfides. The mixture was incubated for 15 min at room temperature. A control reaction was performed incubating proteins in DMF omitting TBP. Proteins were then precipitated as above and resuspended in 4 ml of Tris/SDS. Affinity purification was performed with incubation for 2 hrs at room temperature, with the proteins mixed with 1ml of equilibrated resin (about 125mg of dry resin). To wash the resin after incubation, the sample was spun for 5 min with 500g at room temperature. The supernatant (unbound) was saved, the sedimented resin was resuspended in 10 volumes of Tris/SDS and shaken for 3 min at room temperature and spun again. A total of 3 washes were performed. Bound proteins were eluted incubating the resin (5 min with shaking at room temperature) with Tris/SDS buffer containing 40mM TBP or 100mM DTT. After centrifugation (5 minutes, 500g, room temperature), the supernatant (eluate) was saved and the sedimented resin was incubated again with Tris/SDS buffer containing 40mM TBP or 100mM DTT. The pooled supernatants were precipitated as above and used for electrophoresis.

IP-PP2A phosphatase assays

This assay was performed following the product protocol of an IP-PP2A phosphatase assay kit (Upstate Biotechnology, Catalogue number: 17-313). Briefly, cell extracts were prepared from untreated and glutamate 8-hr treated HT22 cells in a phophatase extraction buffer containing 20mM imidazole-HCl, 2mM EDTA, 2mM EGTA, pH 7.0, 1mM benzamidine, 1mM PMSF and supplemented with protease inhibitor cocktail (Sigma). The extracts were then

sonicated for 10 sec and centrifuged at 2000g for 5 min. The supernatants were used for phosphatase activity assays. Two hundred and fifty ug of cell extracts were immunoprecipitated with commercially available PP2A C subunit antibody and protein A agarose beads for 2 hr at 4ºC. The immunocomplex was then washed three times with 1x TBS before incubating with a threonine phosphopeptide (K-R-pT-I-R-R) for 10 min at 30ºC. The activity of the PP2A C immunocomplex was then measured by dephosphorylation of this phosphopeptide with a colormetric assay using Malachite Green.

4.4 RESULTS

4.4.1 PP2A is the predominant ERK1/2 phosphatase in neuronal cells

Using okadaic acid (OA), an inhibitor of PP2A, previous results from our lab suggested that PP2A is the major ERK1/2 phosphatase in primary immature cortical neurons (Levinthal and DeFranco, 2005). However, the dosage of OA (100nM) used in those studies was too high to determine the selectivity of OA in inhibiting PP2A or PP1. Therefore, in the current study, I utilized a specific peptide inhibitor of PP2A, SV40 small T antigen, to determine the contribution of PP2A to ERK1/2 phosphatase activity in neuronal cells. Small T antigen specifically inhibits PP2A by displacing the PR55-B subunit from PP2A holoenzymes (Sontag et al., 1993). In addition, I used the pharmacological inhibitor, OA, at a low dosage of 1nM, which specifically inhibits PP2A but not PP1. To determine the contribution of PP1 to ERK1/2 phosphatase activity, a specific peptide inhibitor of PP1, Inhibitor-2 (I2) was utilized. As shown in **Fig 4.1**, ERK2 phosphatase activity in HT22 cell extracts was inhibited by the addition of 1 nM OA or

SV40 small T antigen. Similarly, we have confirmed the role of PP2A in ERK2 dephosphorylation in primary neurons (**Fig 4.2**). Treatment of extracts with the selective peptide inhibitor of PP1, Inhibitor-2 (I2), did not block ERK2 phosphatase activity (**Fig 4.2**). These data confirm that PP2A is the predominant ERK2 phosphatase in HT22 cells and primary cortical neurons.

4.4.2 Reversible thiol oxidation of PP2A C subunit following glutamate treatment in primary immature cortical neurons

Cytosolic extracts from 14-hr glutamate-treated or non-treated primary immature cortical neurons were subjected to thiol affinity chromatography (performed by Dr Pier Mastroberadino), and the eluates were analyzed for the presence of oxidized PP2A catalytic (C)-subunit by Western blots. As shown in **Figure 4.3A**, the signal of PP2A C-subunit in the eluates of the thiol-activated Sepharose 6B column was enhanced following glutamate treatment for 14 hr, which demonstrates the reversible thiol-oxidation in PP2A C subunit. **Fig 4.3B** showed the Commassie blue-staining following SDS-PAGE of 25% of eluate fractions loaded for Western blot analysis, which demonstrates the equivalence in the total loading protein.

4.4.3 PP2A activity is not changed following glutamate treatment in HT22 cells

To determine the effect of thiol-oxidation on the activity of PP2A C subunit, we performed an IP-PP2A phosphatase assay, which depends on the dephosphorylation of a threonine phosphopeptide (K-R-pT-I-R-R) to determine the activity of the immunoprecipitated PP2A complex. Cell lysates prepared from 8-hr glutamate-treated or non-treated HT22 cells were

immunoprecipitated with a commercially available PP2A C subunit antibody. The activity of the immunocomplex was then measured by the dephosphorylation of the phosphopeptide with a colormetric assay using Malachite green. As shown in **Fig 4.4A**, following glutamate treatment, there was no change in the activity of immunoprecipitated PP2A. The equivalence in the immunoprecipitated PP2A C subunit from glutamate-treated or non-treated HT22 cells was revealed by the Western blot analysis of the immunocomplex using antibody against PP2A C subunit (**Fig 4.4B**).

4.4.4 Subunits of PP2A are differentially oxidized by glutamate-induced oxidative toxicity

To further demonstrate the thiol oxidation of PP2A following glutamate-induced oxidative toxicity, SDS-PAGE was performed on either glutamate-treated or non-treated cell lysates in the absence of the reducing agent DTT. Western blot analysis was then performed using antibodies against different subunits of PP2A. As shown in **Fig 4.5**, PP2A C subunit could not be detected in SDS-PAGE in glutamate-treated cell lysates in the absence of DTT. Thus, in the absence of the reducing agent, PP2A may form into a high-molecular weight complex that can not enter into the gel. Similar results were also observed for the PP2A B**γ2** subunit (**Fig 4.5**). However, there was no difference between the migration patterns of the PP2A Bδ subunit, which suggests that different subunits of PP2A may be differentially susceptible to glutamate-induced oxidative stress.

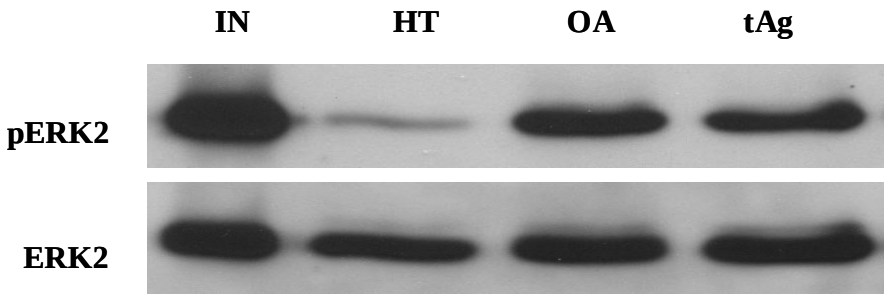


Figure 4.1 PP2A is the predominant ERK phosphatase in HT22 cell extracts
Whole cell lysates prepared from HT22 cells (HT) were incubated with purified pERK2 and phosphatase activity was evaluated by Western blot. Specific inhibitors of PP2A, 1nM okadaic acid (OA) or 2.5ug of purified small T antigen (tAg), were included with cell extracts. IN=phosphatase assay input.

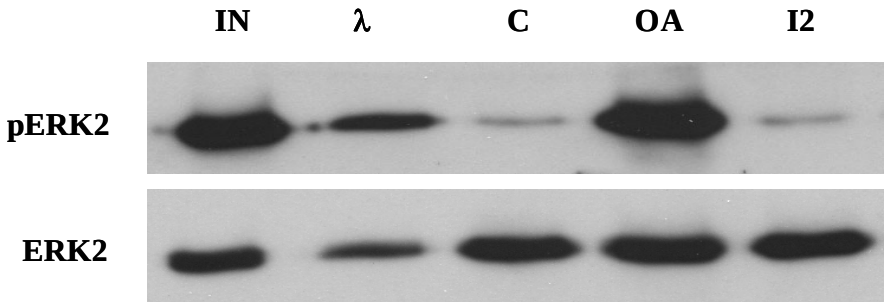


Figure 4.2 PP2A contributes to ERK-directed phosphatase activity in immature primary cortical neurons.
Whole cell lysates prepared from primary neuron culture (C) were incubated with purified pERK2 and phosphatase activity was assessed by Western blot. 1nM okadaic acid or 2 ug purified Inhibitor 2 (I2) was included with the neuronal cell extracts. IN=phosphatase asay input. λ=lambda phage protein phosphatase (positive control).

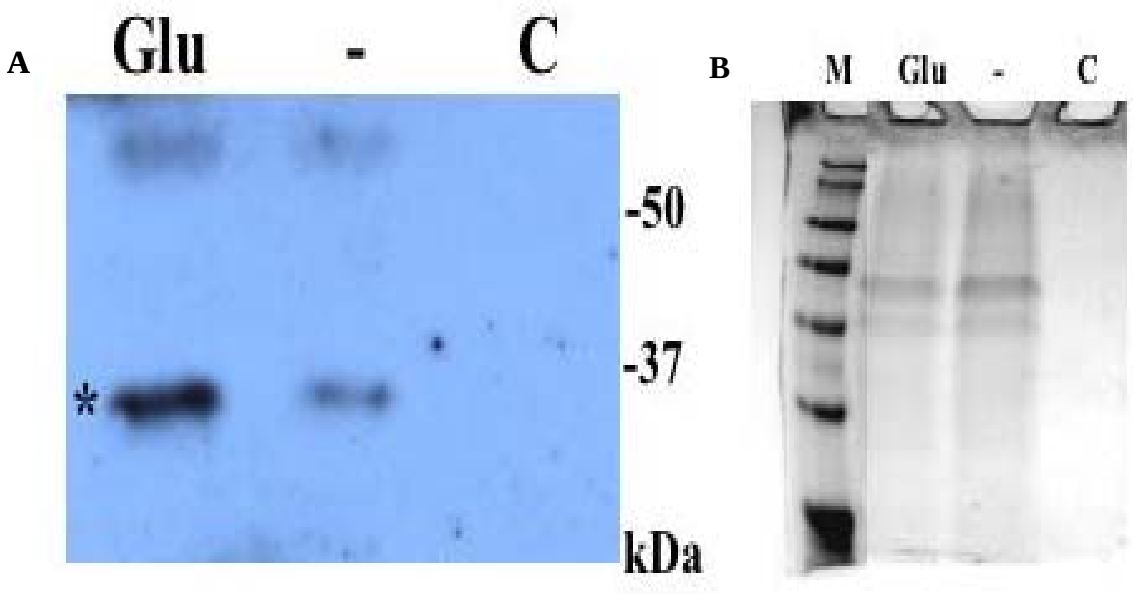


Figure 4.3 Reversible thiol-oxidation of PP2A C subunit following glutamate treatment in primary immature cortical neuronal cultures.

Primary immature cortical neurons were either untreated (-) or treated with 5mM glutamate (Glu) for 14 hr. Cytosolic extracts underwent IAA/NEM modification followed by TBP reduction of reversibly oxidized thiols. Protein samples were then subjected to thiol-affinity chromatography and eluted with 50 mM DTT.

A. Eluates from the column were detected with an antibody against the PP2A C subunit (*36 kDa) by Western blot. C stands for control lysates which were not treated with TBP before affinity chromatography. This is a result representative of 3 independent experiments.

B. Commassie blue-staining of SDS-PAGE loaded with 25% of eluate fractions for Western blot analysis

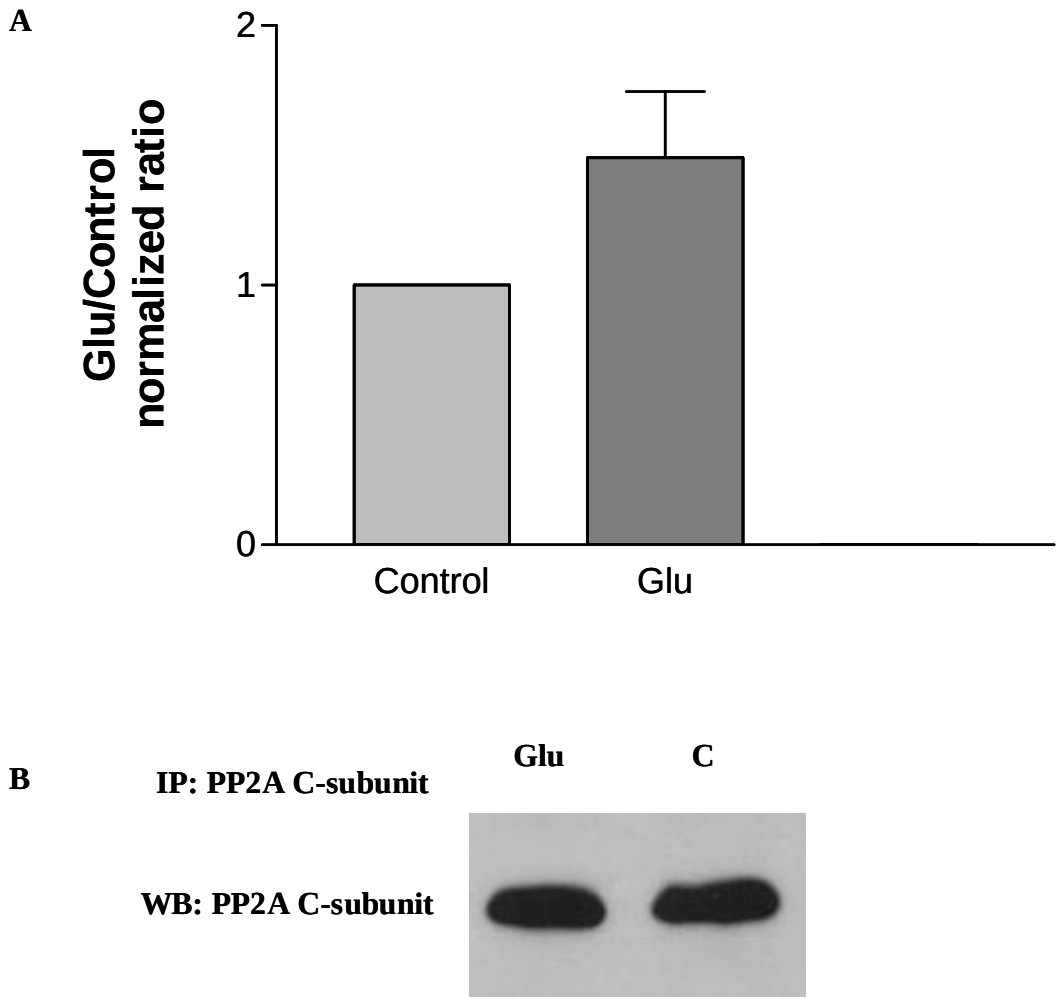


Figure 4.4 PP2A activity is not changed following glutamate treatment in HT22 cells.

A. Glutamate-treated or non-treated HT22 cell lysates were immunoprecipiated with an anti-PP2A C subunit antibody. The activity of the immunocomplex was then assessed by measuring the dephosphorylation of a phosphopeptide by the immunocomplex with a colormetric assay using Malachite Green (Upstate Biotechnology). No change in PP2A activity was detected following glutamate treatment in HT22 cells (n=3).

B. The equivalence in the immunoprecipitated PP2A C subunit from glutamate-treated or non-treated HT22 cells was revealed by the Western blot analysis of the immunocomplex using an antibody against PP2A C subunit.

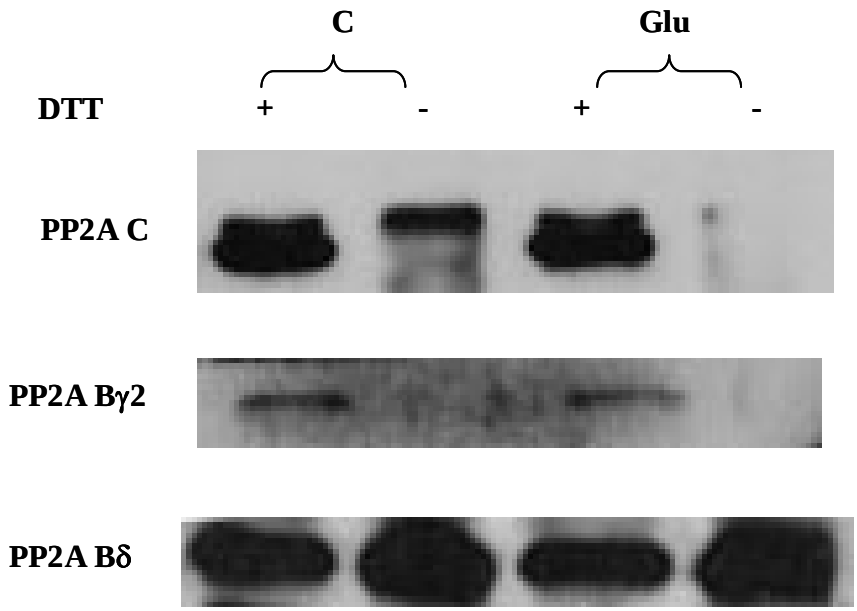


Figure 4.5 Subunits of PP2A are differentially susceptible to glutamate-induced oxidative toxicity.
SDS-PAGE were performed on either glutamate-treated (Glu) or non-treated cell lysates (C) in the presence or absence of the thiol-reducing agent DTT and Western blot analysis were performed using antibodies against different subunits of PP2A. The experiment on PP2A C subunit has been performed twice, whereas the experiment on PP2A B subunits has been performed once.

4.5 DISCUSSION

Cellular redox status has long been known as an important regulator of a variety of normal cellular signaling events. Transient and reversible thiol oxidation and inactivation of protein tyrosine phosphatases regulates the tyrosine phosphorylation-dependent signaling pathways mediated by numerous growth factors. Thiol oxidation of the sensitive catalytic cysteines in protein tyrosine phosphatases involves both reversible and irreversible mechanisms (Barford, 2004). Reversible mechanisms include the oxidation of catalytic cysteines to sulfenic acid (Meng et al., 2002), the intramolecular formation of disulfide bonds between catalytic cysteine and its neighboring cysteine (Savitsky and Finkel, 2002), as well as the formation of a novel structure, sulfenylamide, which is not susceptible to further oxidation (Salmeen et al., 2003; van Montfort et al., 2003). Further oxidation of sulfenic acid to sulfinic acid and sulfonic acid are all irreversible (Denu and Tanner, 1998).

Mechanisms of oxidative inhibition of PSTPs are not well understood. Due to the presence of the bi-nuclear metal clusters in the active site of STPPs, one mechanism responsible for oxidative inhibition of the activity of STPPs may involve the oxidation of these catalytic metal centers. Redox regulation of calcineurin has been shown to occur through the targeting of the Fe^{2+}-Zn^{2+} center at the active site of the enzyme (Namgaladze et al., 2002). Recently, cysteine oxidation and inactivation of calcineurin (Bogumil et al., 2000) and PP1 (Kim et al., 2003) have been revealed, which suggests that thiol oxidation may be another mechanism for

inhibiting these enzymes. The role of thiol oxidation in regulating the activity of PP2A remains unclear, although PP2A inactivated by H_2O_2 in Caco-2 cells (Rao and Clayton, 2002) and brain extracts (Foley et al., 2004) is reversible by the thiol reducing agent DTT. The current study shows the first direct evidence of reversible cysteine thiol oxidation in PP2A C subunit following oxidative stress. However, these modifications of PP2A C subunit were not associated with alterations in the overall PP2A activity following glutamate-induced oxidative stress.

There are six conserved cysteine residues in the catalytic subunit of PP2A although they are not considered critical for the catalysis of PP2A (Green et al., 1987). It is possible that the modification of PP2A C subunit may not change its overall activity but affect its association with various B regulatory subunits or selective substrate as well as subcellular localization. Therefore, it would be essential to identify the specific cysteine residue being thiol-oxidized and examine the effects of these modifications on the activity of PP2A. Furthermore, the antibody we used in IP-PP2A phosphatase assay detects the total PP2A pool. It is possible that only a subset of PP2A was oxidized and inactivated while the changes in total PP2A activity could not be detected. Indeed, it has been shown that only the sub-pool of PP2A associated with the rebinoblastoma protein (pRb), but not the total PP2A pool, is specifically modulated by H_2O_2 in HUVEC endothelial cells (Cicchillitti et al., 2003). Therefore, it might be useful for future studies to examine the activity of PP2A associated with ERK following glutamate-induced oxidative stress.

Since we have observed different susceptibility of PP2A B subunits to oxidative stress (Fig 4.5), it may be very possible that only a certain B subunit is sensitive to oxidation. This affects the activity of the subset of PP2A holoenzymes associated with this specific B subunit. PP2A B subunits are critical in regulating PP2A holoenzyme's activity. First, B subunits determine the distinct subcellular compartmentalizations of PP2A holoenzymes. For example,

Bα and Bγ have been found to direct PP2A to microtubules and cytoskeletal substrates (Sontag et al., 1995; Strack et al., 1998). B'α, B'β and B'ε subunits target PP2A to the cytosol, whereas B'δ and B'γ target PP2A to the nucleus (McCright et al., 1996). A recent report has shown that the Bβ2 subunit, a neuronal-specific regulatory subunit of PP2A, localizes and targets the PP2A holoenzyme to the outer membrane of mitochondria (Dagda et al., 2005). Second, B subunits determine the substrate specificity of PP2A. It has been shown that in PC6-3 neuronal cell lines, Bα and Bδ subunits target PP2A to the dephosphorylation of ERK whereas specific B' subunits regulate Akt activity (Van Kanegan et al., 2005). Third, even in the same cell type and in the regulation of the same specific target, distinct B regulatory subunit confers different effects. For example, in PC6-3 cells, overexpression of the Bγ subunit activates ERK signaling (Strack, 2002) whereas silencing of Bα and Bδ subunits disinhibits ERK activation (Van Kanegan et al., 2005). This suggests that by regulating different targets in the ERK signaling pathways, B subunits of PP2A are involved in both positive and negative regulation of the ERK cascade. Fourth, the regulation of specific targets by the same B regulatory subunits is cell-type specific. Strack's group has found that Bα and Bδ subunits negatively regulate ERK activation in PC6-3 cells (Van Kanegan et al., 2005), whereas they positively regulate ERK activation in HEK cells through the dephosphorylation of phosphor-Ser-259 inhibitory site in Raf1, which results in the subsequent MEK1 and ERK1/2 activation (Adams et al., 2005) Taken together, these studies point to the complexity of regulating PP2A holoenzyme activity by distinct B subunits. By bringing PP2A to appropriate cellular sites and dephosphorylating distinct substrates in different compartments in a cell-specific way, B subunits determine the diverse biological effects brought about by PP2A. Therefore, future studies will focus on the regulation of distinct B subunits by glutamate-induced oxidative stress. It might be useful for future studies to detect the PP2A

phosphatase activity associated with specific B subunits by immunoprecipitating the PP2A holoenzyme with specific B subunit antibodies.

DSPs bear the same signature motif $HC(X)_5R$ in their active catalytic site as PTPs. Therefore, DSPs may share the same mechanism of thiol oxidation of PTPs. A recent report has shown that proteasomal degradation of MKP1, an ERK1/2 phosphatase, contributes to glutamate-induced neuronal death in HT22 cells and primary immature cortical neurons (Choi et al., 2006). This suggests that MKP1 is another ERK1/2 phosphatase susceptible to oxidation. Indeed, previous studies have shown that a pool of ERK1/2 phosphatases are composed of PTPs and DSPs that are sensitive to orthovanadate (Levinthal and DeFranco, 2005). Moreover, a recent study has demonstrated that thiol oxidation and inhibition of MKPs, including MKP1, contribute to TNFα-mediated sustained JNK activation and subsequent cell death in fibroblast (Kamata et al., 2005). All these clues point to the possible role of MKP1 as a possible candidate of ERK phosphatase that is thiol-oxidized and inactivated in HT22 cells and primary immature cortical neurons. Further study will be focused on revealing the reversible thiol oxidation of MKP1 using thiol affinity chromatography as well as non-reducing SDS-PAGE. Moreover, the activity of MKP1 following glutamate-induced oxidative stress could be investigated using an IP-phosphatase assay.

The results presented here have confirmed that PP2A contributes to ERK-directed phosphatase activity in HT22 cells and primary cortical neurons as shown in previous studies from our lab (Levinthal and DeFranco, 2005). Moreover, we have provided the first direct evidence of reversible thiol oxidation of the cysteine residues on PP2A C subunit by using a novel thiol-affinity chromatography procedure. The feasibility of this method could be extended to the study of thiol oxidation of other possible candidates of ERK phosphatases, such as MKP1.

The thiol modification of PP2A C subunit may not directly affect its catalytic activity but more likely alters the association with regulatory subunits or specific substrates. We are now engaged in determining the effect of oxidative stress in altering the association between the PP2A C subunit and its selective substrate, ERK1/2, in our model.

5.0 INHIBITION OF MAPK PHOSPHATASES IN RODENT ISCHEMIA MODELS

5.1 SUMMARY

Oxidative stress after cerebral ischemia and reperfusion activates ERK1/2 in the 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 ERK1/2 phosphatase activity contributes to ERK1/2 activation and subsequent neuronal toxicity. The current study examined whether ERK1/2 activation was associated with altered activity of ERK1/2 phosphatases in a transient focal ischemia (middle cerebral artery occlusion – MCAO) model in mice and a global ischemia model in rat (asphyxia-induced cardiac arrest model). Consistent with previous reports, significant ERK1/2 activation was detected in ischemic cortex following MCAO. Furthermore, ERK1/2 phosphatase activity was inhibited in ischemia cortex but not affected in the contralateral non-ischemic side. This phosphatase inhibition was selective, as JNK phosphatase activity was not affected by ischemia/reperfusion. I then proceeded to examine the role of ERK1/2 phosphatase inhibition in activating ERK1/2 following ischemia/reperfusion in rat cardiac arrest model. ERK1/2 activation was revealed in both ischemic cerebral cortex and ischemic hippocampus. However, reversible inhibition of ERK1/2 phosphatase activity was only observed in the cerebral cortex but not affected in the hippocampus following ischemia/reperfusion. MEK1/2 was activated in both the cerebral cortex

and the hippocampus following ischemia/reperfusion. Using a specific inhibitor of protein phosphatase 2A (PP2A), okadaic acid (OA), I established that PP2A is the major ERK1/2 phosphatase that is responsible for regulating ERK1/2 activation in ischemic brain tissues. Orthovanadate inhibited ERK1/2 phosphatase activity in brain tissues, which suggests that PTPs and DSPs may also contribute to the ERK1/2 phosphatase activity in brain tissues. Together, these data establish a role of a specific inhibition of ERK1/2 phosphatase in ERK1/2 activation following ischemia/reperfusion. Moreover, different mechanisms for regulating ERK1/2 activation in distinct brain regions following global ischemia/reperfusion have been revealed.

5.2 INTRODUCTION

Oxidative stress is generated in many tissues including in 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 ROS (Katz et al., 1998). In reperfusion 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 MAPK family are activated during reperfusion after both focal (Alessandrini et al., 1999; Noshita et al., 2002) and global ischemia (Hicks et al., 2000b; Hu et al., 2000). Members of the MAPK family that are activated during reperfusion include ERK and

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 hours after reperfusion (Hicks et al., 2000a; Hicks et al., 2000b). In a transient focal ischemia model, ERK1/2 activation was found 5 min following ischemia/reperfusion and persists for several hours (Alessandrini et al., 1999; Namura et al., 2001; Noshita et al., 2002). After global ischemia/reperfusion, ERK1/2 activation has also been noted both at 30 min and to persist up to 24 hours (Hicks et al., 2000b; Hu et al., 2000; 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 ERK1/2 activation also is associated with cell survival in some in vivo systems. After global ischemia ERK1/2 was activated after 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 ERK1/2 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).

ERK1/2 activity is regulated by its phosphorylation state, which is the result of the balanced action of both ERK1/2 kinases and ERK1/2-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 ERK1/2 phosphorylation include PTPs, PTSPs, and DSPs (Keyse, 2000). PP2A (Silverstein et al., 2002; Kim et al., 2003) and MKP3 (Camps et al., 2000) are two major ERK phosphatases.

We have previously shown that ERK1/2 activation following oxidative stress in immature primary neuronal cultures is driven by the oxidative inhibition of ERK1/2 phosphatases (Levinthal and DeFranco, 2005). However, whether the inhibition of ERK1/2 phosphatase is associated with ERK1/2 activation following oxidative stress conditions in vivo, i.e., after ischemia/reperfusion, is not known. Moreover, the relative contribution of upstream kinases and phosphatase activity to ERK1/2 activation after ischemia/reperfusion remain elusive. Therefore, this study tested whether ERK1/2 activation was associated with altered activity of ERK1/2 phosphatases in an in vivo ischemia/reperfusion model. A mouse transient focal ischemia (middle cerebral artery occlusion – MCAO) model and a global ischemia model in rat (asphyxia-induced cardiac arrest model) have been employed for the study.

5.3 MATERIALS AND METHODS

MCAO model of focal ischemia

In collaboration with Dr Jun Chen in the Department of Neurology in the University of Pittsburgh, transient focal cerebral ischemia was produced by intraluminal occlusion of the left

middle cerebral artery (MCA) as described (Yang and Betz, 1994; Cao et al., 2002). The animals undergo left MCA occlusion for 60 min and then reperfusion for certain period of time. Control animals are sham animals with anesthesia and surgical procedure without MCAO. Changes in regional cerebral blood flow (rCBF) before, during, and after MCAO will be monitored by laser-Doppler flowmetry (Cao et al., 2002). If the laser Doppler signal declines >75% from baseline, MCAO surgery is considered successful. All the surgery procedures were performed by Dr. Feng Zhang in Dr. Jun Chen's lab.

Global ischemia model- asphyxia-induced cardiac arrest

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), or asphyxial cardiac arrest followed by 30 min of reperfusion (n=6). Sham rats received anesthesia and surgery but were not subjected to asphyxia. All the surgery procedures were performed by Rick Logue in Dr. Cliff Callaway's lab. During procedures, body temperature was monitored by computer and regulated via computer-driven relays connected to a 100-W heating lamp and a cooling fan (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 37ºC during procedures until sacrifice.

In order to induce asphyxia, rats were anesthetized with halothane, orotracheally intubated, and mechanically ventilated as described previously (Hicks et al., 2000a; Hicks et al., 2000b). Ventilation was titrated to blood gases monitored via femoral arterial catheters. Rats

were paralyzed chemically 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 within 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 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 -70ºC.

ERK1/2-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 4ºC and supernatants were collected for further analysis.

We have modified a nonradioactive method for determining ERK1/2-directed phosphatase activity in tissue extracts (Laakko and Juliano, 2003). This method relies on detecting dephosphorylation of a purified, dual-phosphorylated, His_6-tagged ERK2 upon incubation with the tissue extracts (Levinthal and DeFranco, 2005). The alterations of ERK2

phosphatase activity within the tissue extracts can be monitored by measuring changes in the phosphorylation state of the isolated pERK2 substrate, as shown by Western blotting with a phospho-specific ERK1/2 antibody. Briefly, 150ug of tissue extracts were diluted into a total volume of 250ul in phosphatase assay buffer (10mM $MgCl_2$, 10mM Hepes, pH7.5 and 10uM of the MEK inhibitor, U0126). Recombinant dual phosphorylated His_6-ERK2 (Biomol, Plymouth Meeting, PA) was added to each sample (30ng/sample), and the reactions were maintained at 37ºC for 15 min where indicated. Either 50mM DTT or 10nM okadaic acid (OA) or 1mM Na_3VO_4 was added to the sample for 30 min on ice, prior to the addition of purified pERK. Following a 15-min incubation at 37ºC, the reactions were stopped by the addition of 250ul of wash buffer (8M urea, pH8.6, containing 10mM imidazole). 30ul of Ni^{2+}-conjugated, magnetic beads (Qiagen, Valencia, CA) were then added to each reaction. After 90 min of rocking at 4ºC, 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 polyvinylidine fluoride membrane (Millipore, Bedford, MA), and subjected to Western blotting to detect phosphorylated ERK and total ERK.

JNK3-directed phosphatase activity assay

This method was performed exactly the same as described for ERK2 phosphatase assay, except that the samples were incubated with 30ng of purified, dual-phosphorylated His_6-tagged JNK3 protein (Upstate, Waltham, MA), and immunoblotted 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 amounts of total protein (20-30ug) were separated by SDS-PAGE on 10% polyacrylamide gels and then transferred to polyvinylidine 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 4ºC 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 were performed using a paired *t*-test. Comparison of multiple mean values were performed by analysis of variance with Bonferroni's *post hoc* tests for significance. A *p*-value of <0.05 was considered to be significant. All data were analyzed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA).

5.4 RESULTS

5.4.1 ERK2 phosphatase activity is specifically inhibited in ischemic/reperfusion cerebral cortex following MCAO

I have utilized a transient focal ischemia model (i.e. MCAO) in mice to evaluate the effects of oxidative stress induced by acute brain injury on MAPK phosphatases. Western blot analysis has been performed on lysates from ischemic tissues and contralateral non-ischemic tissues as well as tissues from control animal. Increased activation of ERK1/2 has been revealed in the ischemic cortex as early as 5 min and 60 min after initiation of reperfusion following MCAO (**Fig 5.1).** These data are consistent with previous reports of ERK1/2 activation during ischemia/reperfusion following transient MCAO (Alessandrini et al., 1999; Namura et al., 2001; Noshita et al., 2002). I then proceeded to examine whether inhibition of ERK1/2-directed phosphatase activity contributes to ERK1/2 hyperphosphorylation following ischemia/reperfusion. Phosphatase activity was measured in brain extracts using His_6-tagged pERK2 as a substrate. As shown in **Fig 5.2**, ERK1/2 phosphatase activity was inhibited in extracts isolated from the cerebral cortex of the ischemic hemisphere of MCAO mice. To assess if the inhibition of phosphatase activity was specific to a ERK2 phosphatase, JNK3 was used as a substrate in the *in vitro* phosphatase assays (**Fig 5.3**). There is no difference between the JNK3 phosphatase activity in the extracts from cerebral cortex in the ischemic hemisphere and the contralateral hemisphere (**Fig 5.3**). Therefore, these results suggest that selective inhibition of ERK1/2 phosphatase may contribute to the ERK1/2 activation in vulnerable brain regions

following transient focal ischemia. The result is still preliminary since one more set of samples will be needed to perform statistical analysis (n=2). Moreover, I am currently investigating the effects of longer reperfusion (3h and 6h) on the inhibition of ERK phosphatases following MCAO in mice.

5.4.2 ERK2 phosphatase activity is specifically inhibited in ischemic/reperfusion cerebral cortex in cardiac arrest model

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). 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 oxidation stress on ERK1/2 activation in global ischemia, I first examined whether ERK1/2 activation was associated with the early post-ischemic period in brain tissues following cardiac arrest. As the 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 from the cortex of control rats. Western blot analysis was performed to detect pERK1/2 and total ERK1/2. We found that ERK1/2 was dramatically activated in cortex following ischemia/reperfusion but not in the cortex of sham animals or the cortex of ischemia/non-reperfusion animals (**Fig 5.4A**). To reveal whether the inhibition of ERK-directed phosphatases by ischemia-induced oxidative stress contributes to the post-ischemic ERK1/2 hyperphosphorylation, ERK2 phosphatase activity was measured in extracts prepared from adult male rat cerebral cortex isolated 30 min following global ischemia/reperfusion or control groups using His_6-tagged pERK2 as a substrate. Compared with the robust ERK2

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 5.4B, C**). The effect of ischemia/reperfusion on ERK2-directed phosphatases is selective as the phosophatases in cortical extracts that act on the MAPK family member JNK were not affected (**Fig 5.4D**).

5.4.3 DTT can reverse the inhibition of ERK2 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). I 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 ERK2-directed phosphatase activity could be reversed by the addition of 50mM DTT to extracts prepared from ischemic cortical tissues (**Fig 5.5A, B**). Together with **Fig 5.4**, 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.

5.4.4 ERK2 phosphatase activity is not changed in hippocampus following ischemia/reperfusion hippocampus

In addition to the cortex, global ischemia can affect multiple brain regions, including the hippocampus (Katz et al., 1998). As observed in cortex, ERK1/2 is significantly activated in the hippocampus 30 min following ischemia/reperfusion (**Fig 5.6A**). I therefore examined that whether the inhibition of ERK phosphatase activity in the hippocampus contributes to ERK activation 30 min following ischemia/reperfusion. Interestingly, unlike the cortex, ERK phosphatase activity in hippocampus was not altered within 30 min of ischemia/reperfusion (**Fig 5.6B, C**). Thus, the robust activation of ERK1/2 in hippocampus at early times of reperfusion following global ischemia was not due to effects on ERK phosphatase activity, at least as measured in crude extracts.

5.4.5 pMEK is activated in cerebral cortex and hippocampus following ischemia/reperfusion

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, I 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 5.7**). Together with previous results on ERK2 phosphatase activity, these results strongly suggest that different mechanisms account for ERK1/2 activation in distinct brain regions following ischemia/reperfusion in global ischemia.

5.4.6 PP2A is the predominant ERK2 phosphatase in rat brain

A major component of ERK phosphatase activity in neurons is contributed by PP2A, a PSTP (Millward et al., 1999; Virshup, 2000). To determine the contribution of PP2A to ERK1/2 dephosphorylation in rat brain tissues, a specific inhibitor, OA, was utilized. OA is a well-characterized PSTP 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 ERK2-directed phosphatase activity in extracts prepared from normal cerebral cortex (**Fig 5.8A, 8B**) and hippocampus (**Fig 5.8D, 8E**) 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 5.4C, 5.7C**). Western blot using an antibody against the PP2A C-subunit showed that the level of PP2A did not change following ischemia/reperfusion in both cerebral cortex and hippocampus **(Fig 5.8C, 8F)**.

5.4.7 Tyrosine and dual-specificity phosphatases also contribute to ERK2 phosphatase activity in rat brain

Sodium orthovanadate is a potent inhibitor of PTPs and DSPs and does not affect PSTPs. To examine the role of PTPs and DSPs in the observed ERK2 phosphatase activity in rat brain, we treated lysates from the cortex of sham animals with 1mM Na_3VO_4. Orthovanadate dramatically inhibited ERK2 phosphatase activity in extracts from cortex in sham animals (**Fig 5.9A&B**). The extent of inhibition observed with orthovanadate was less than that observed with OA, confirming the predominant role of PP2A in ERK2-directed phosphatase activity in rat brain

(**Fig 5.9C**). However, PTPs and/or DSPs seem to play some roles in dephosphorylating ERK1/2 in rat brain.

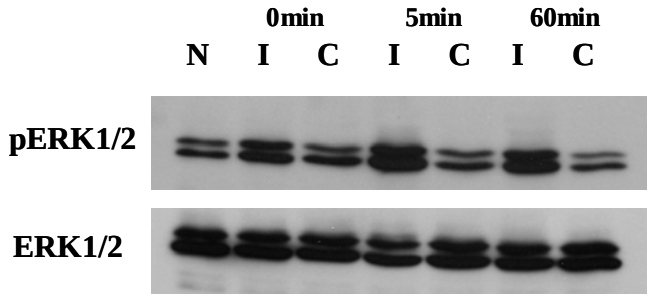


Figure 5.1 Activation of ERK1/2 in cerebral cortex following MCAO.
Western blot analysis was performed using extracts prepared at various times of reperfusion following MCAO mice from ischemic (I) and contralateral (C) non-ischemic tissue. Cerebral cortical tissue was also isolated from normal animals (N). ERK1/2 phosphorylation (pERK1/2) and total ERK1/2 levels were determined in same blot following the stripping of phospho-ERK1/2 antibody (n=2).

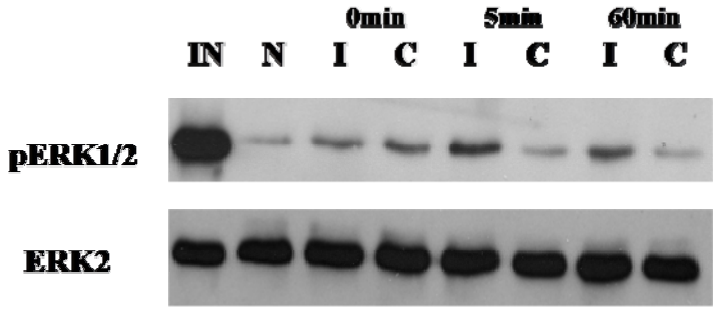


Figure 5.2 Inhibition of ERK2 phosphatase activity in cerebral cortex following MCAO.
ERK2 phosphatase assays were performed using cerebral cortical extracts prepared at various times of reperfusion following MCAO in mice from ischemic (I) and contralateral (C) non-ischemic tissue. Cerebral cortical tissue was also isolated from normal animals (N). Input (In) of purified pERK2 is included. Western blots (representative of 2 separate experiments) revealed either pERK2 or total ERK2 (n=2).

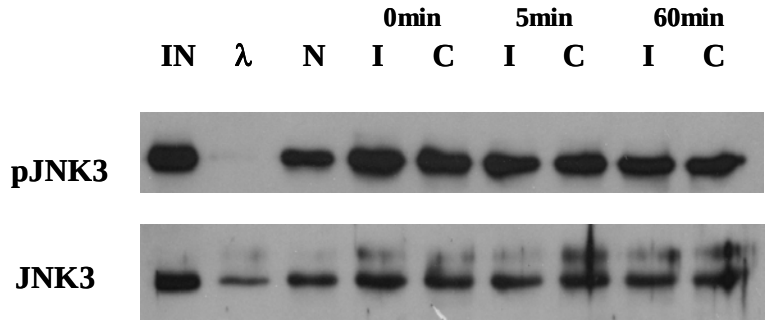


Figure 5.3 No inhibition of JNK3 phosphatase activity in cerebral cortex following MCAO.

JNK3 phosphatase assays were performed using cerebral cortical extracts prepared at various times of reperfusion following MCAO in mice from ischemic (I) and contralateral (C) non-ischemic tissue. Cerebral cortical tissue was also isolated from normal animals (N). Input (In) of purified pJNK3 is included as well as control reactions with purified l phosphatase. Western blots (representative of 2 separate experiments) revealed either pJNK3 or total JNK3 (n=2).

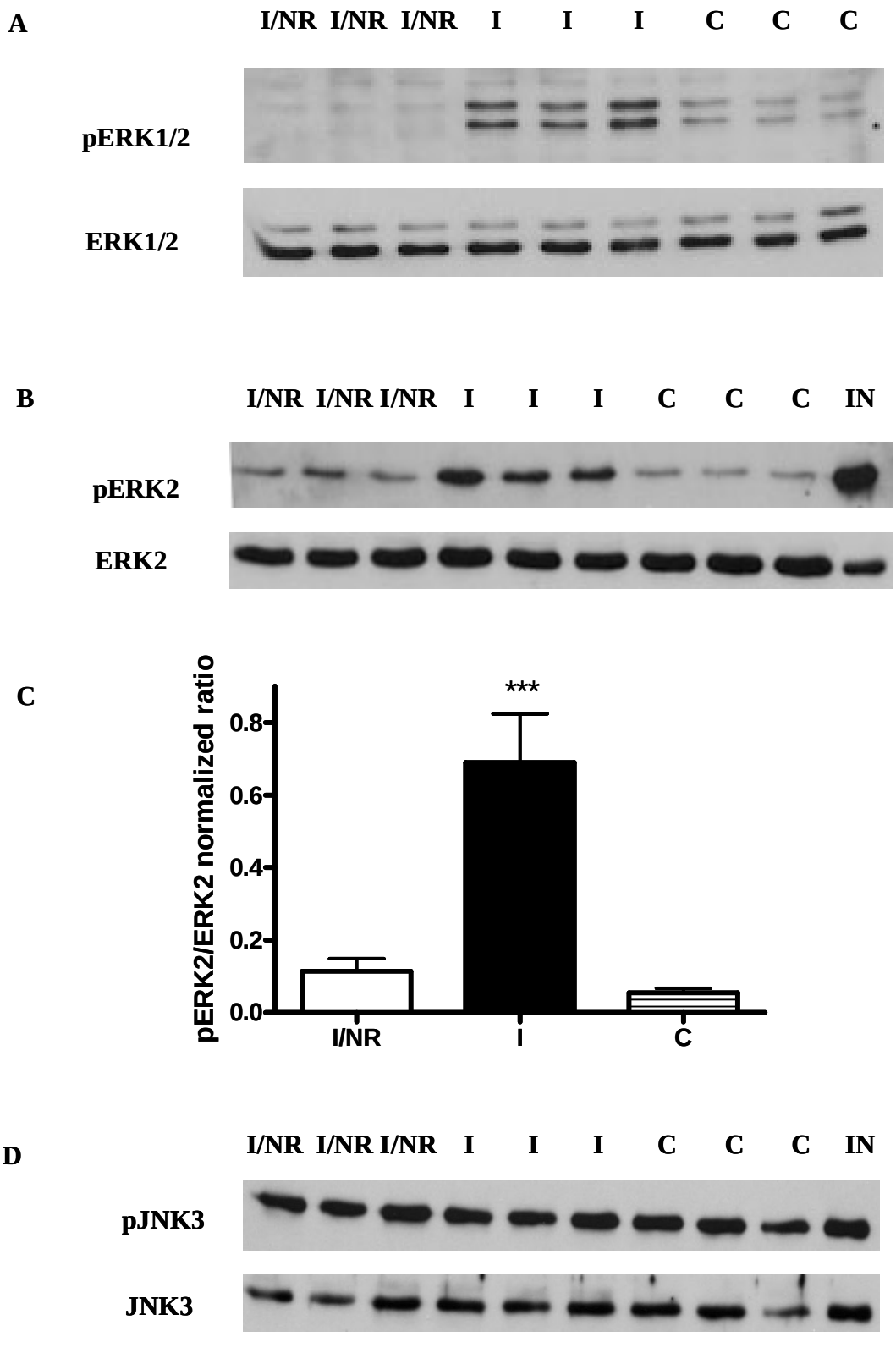

A
I/NR I/NR I/NR I I I C C C
pERK1/2
ERK1/2
B
I/NR I/NR I/NR I I I C C C IN
pERK2
ERK2
C
pERK2/ERK2 normalized ratio
0.8
0.6
0.4
0.2
0.0
***
I/NR
I
C
D
I/NR I/NR I/NR I I I C C C IN
pJNK3
JNK3

Figure 5.4 Inhibition of ERK2-directed phosphatase activity in reperfused cerebral cortex following global ischemia.

A. Endogeneous pERK1/2 and tERK levels were measured by Western blot in corresponding extracts from adult male rat cortical tissue isolated 30 min following either 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 either global ischemia/reperfusion (I), control sham operations (C), or ischemia/without reperfusion (I/NR) were incubated with His_6-tagged pERK2. Western blot analysis was then performed of affinity purified His_6-tagged pERK2 to reveal phospho-ERK2 (pERK2) or total ERK2 (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 His_6-tagged phospho-JNK3. Western blot analysis was then performed of affinity purified His_6-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.

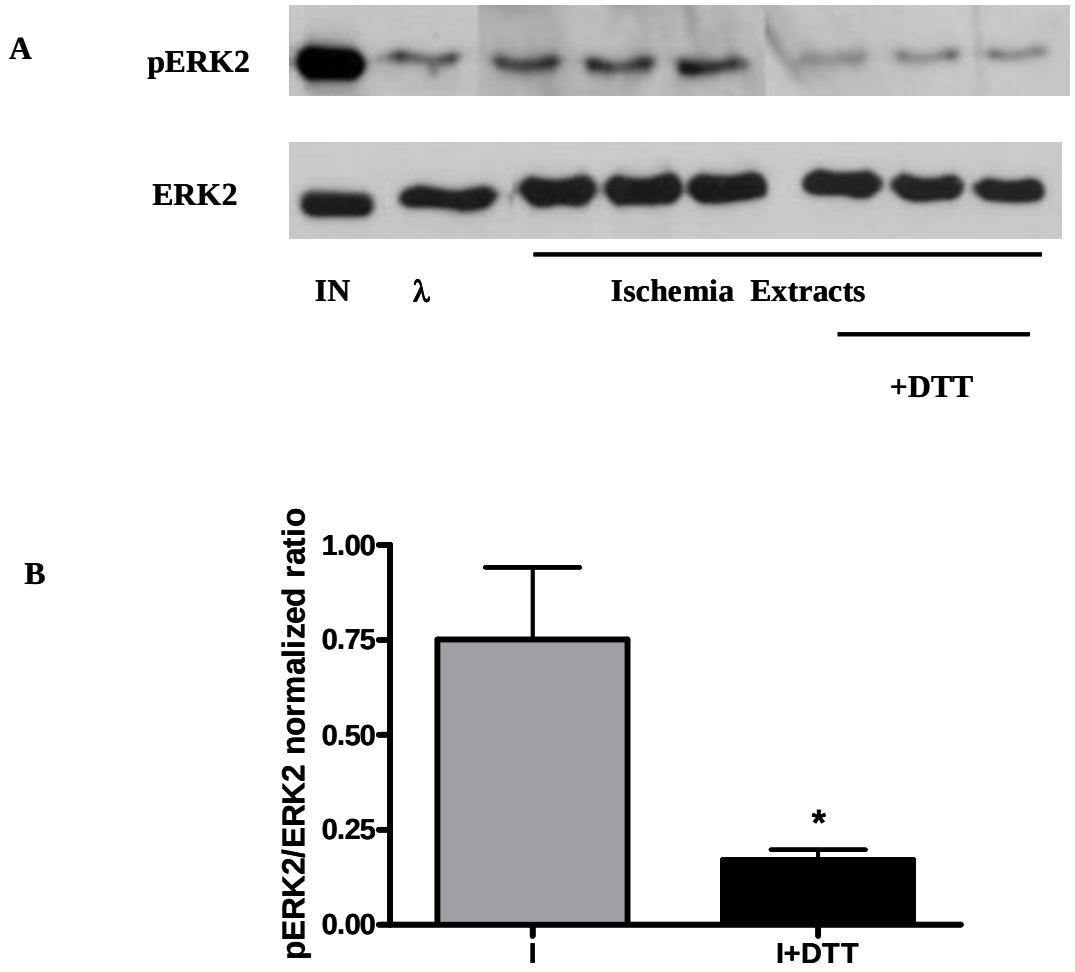


Figure 5.5 Recovery of ERK2-directed protein phosphatase activity in the cerebral cortex following ischemia/reperfusion.

A. Ischemic/reperfusion extracts from cerebral cortex shown in Fig 1 were incubated with 50 mM DTT where indicated, prior to assaying for pERK2 phosphatase activity.

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

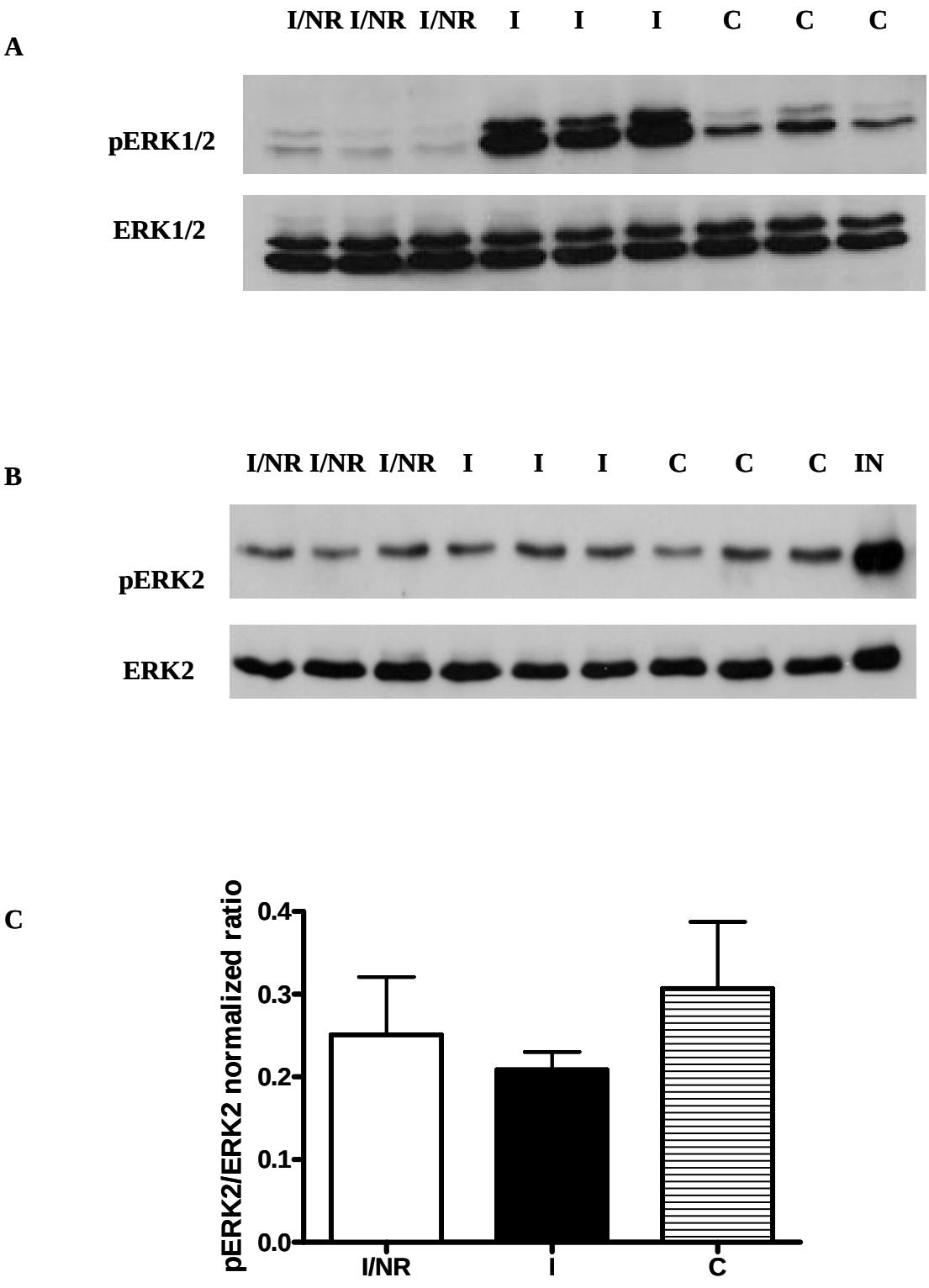
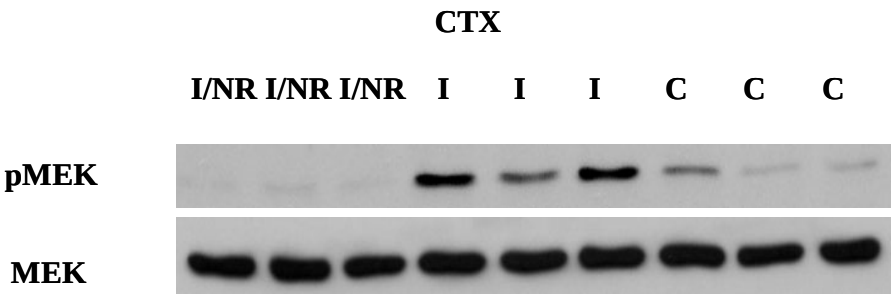


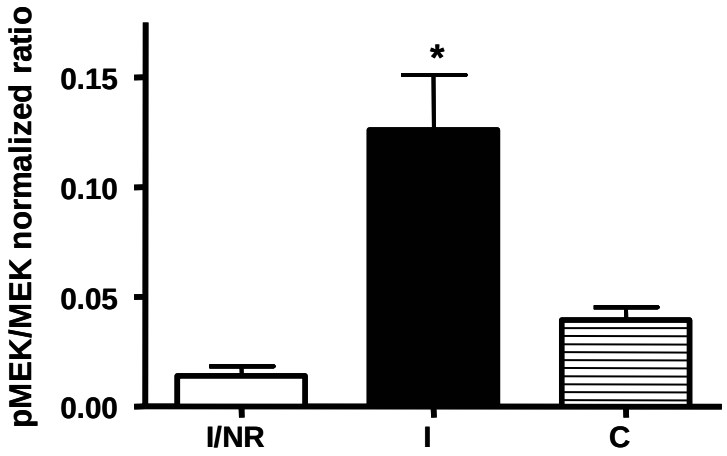
Figure 5.6 ERK2 phosphatase activity in hippocampal extracts is unaffected by ischemia/reperfusion.

A. Endogenous pERK1/2 and ERK levels were measured by Western blots in corresponding extracts from adult male rat hippocampal tissue isolated 30 min following either 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 either ischemia/reperfusion (I), control sham operations (C), or ischemia/without reperfusion (I/NR) were incubated with His6-tagged pERK2. Western blot analysis was then performed of affinity purified His6-tagged pERK2 to reveal total ERK2 (tERK) or 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.

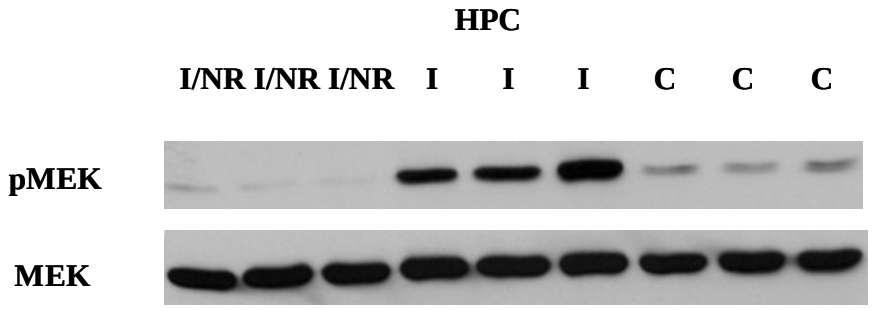
A


CTX
I/NR I/NR I/NR I I I C C C
pMEK
MEK

B


pMEK/MEK normalized ratio
0.15
0.10
0.05
0.00
*
I/NR
I
C

C


HPC
I/NR I/NR I/NR I I I C C C
pMEK
MEK

D

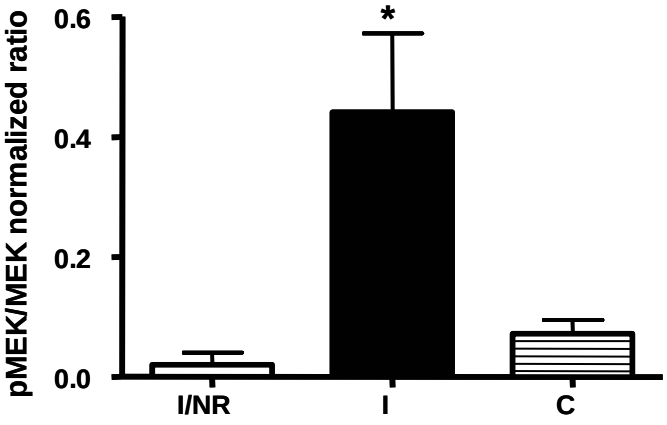
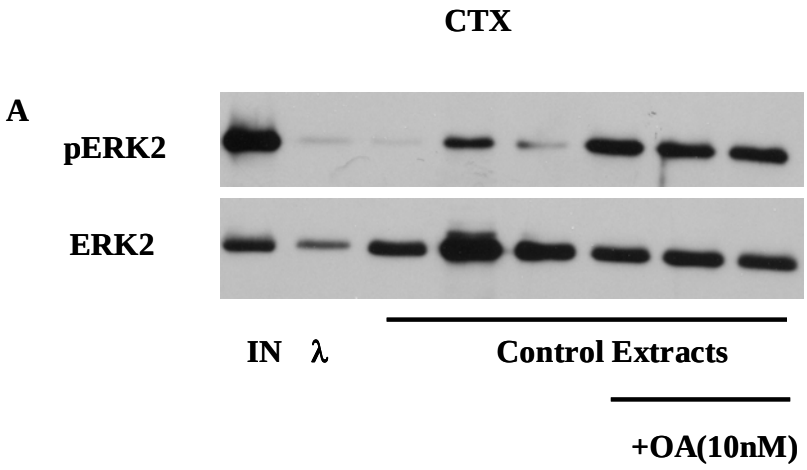
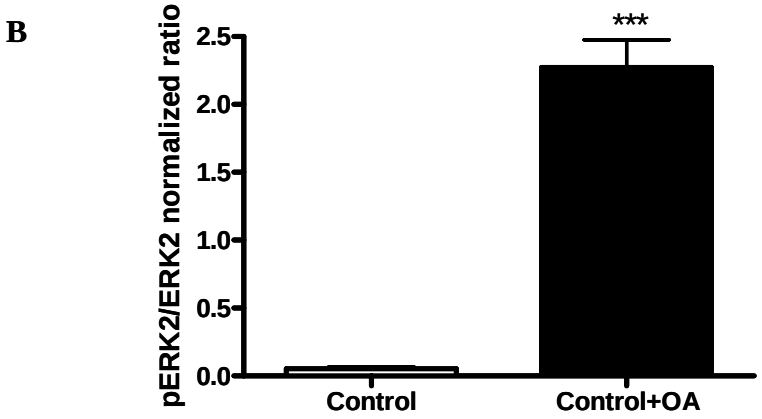
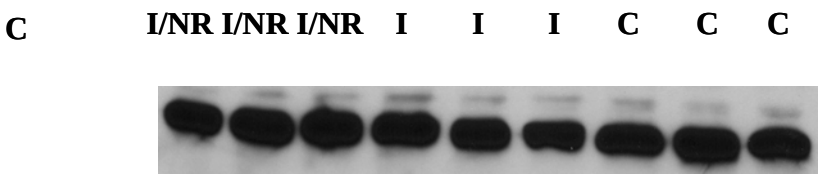

pMEK/MEK normalized ratio
0.6
0.4
0.2
0.0
*
I/NR
I
C

Figure 5.7 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 either 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). $P<0.05$.


CTX
A
pERK2
ERK2
IN
λ
Control Extracts
+OA(10nM)


B
pERK2/ERK2 normalized ratio
2.5
2.0
1.5
1.0
0.5
0.0
***
Control
Control+OA


C
I/NR I/NR I/NR I I I C C C
PP2A C-subunit

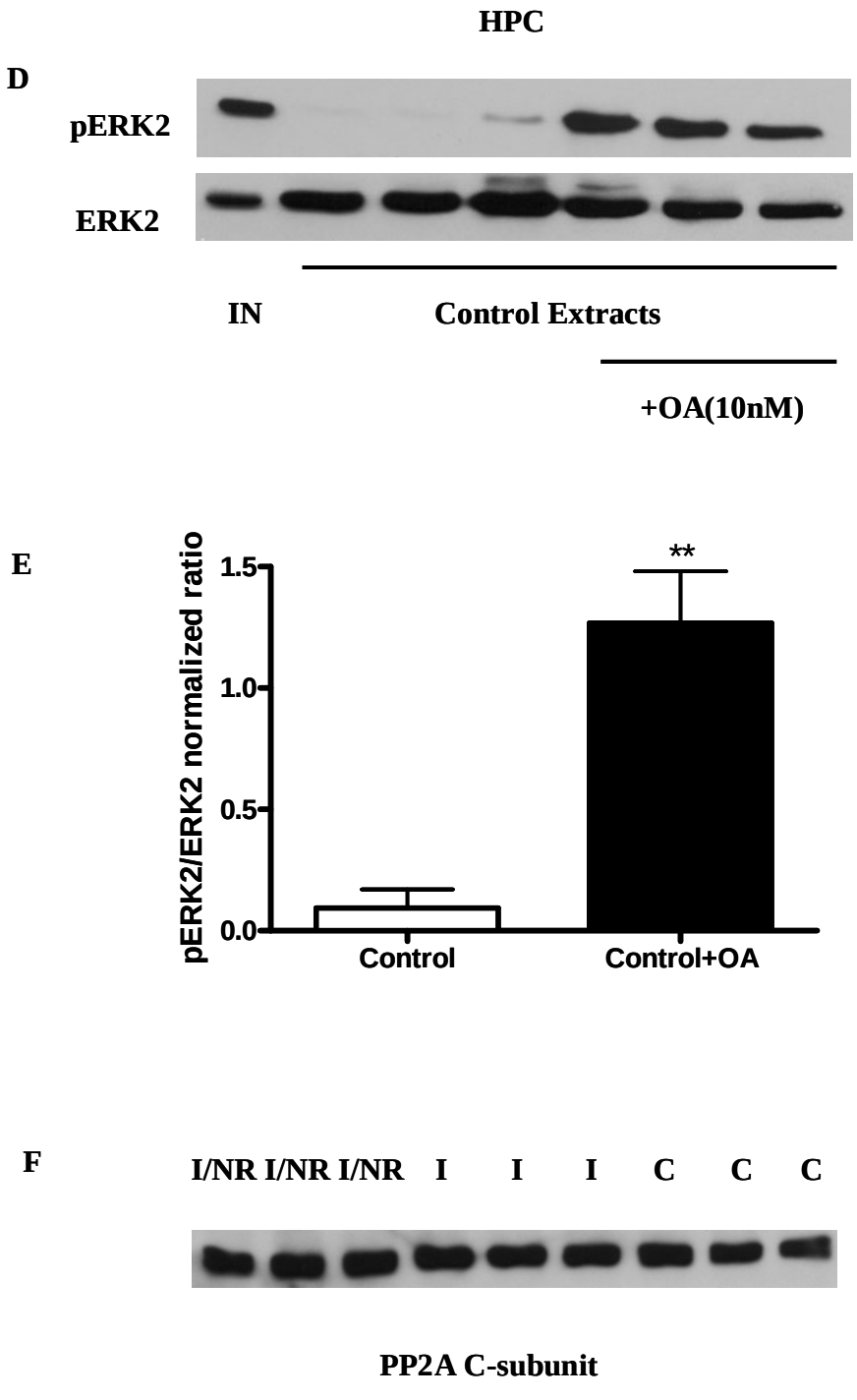
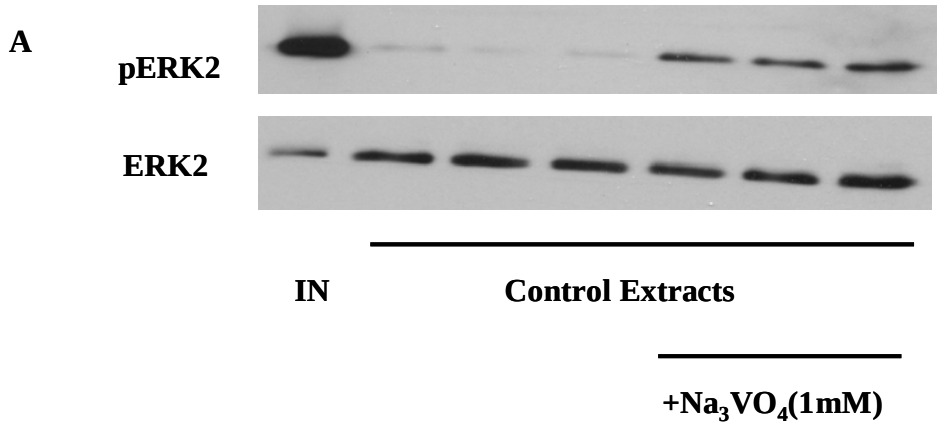
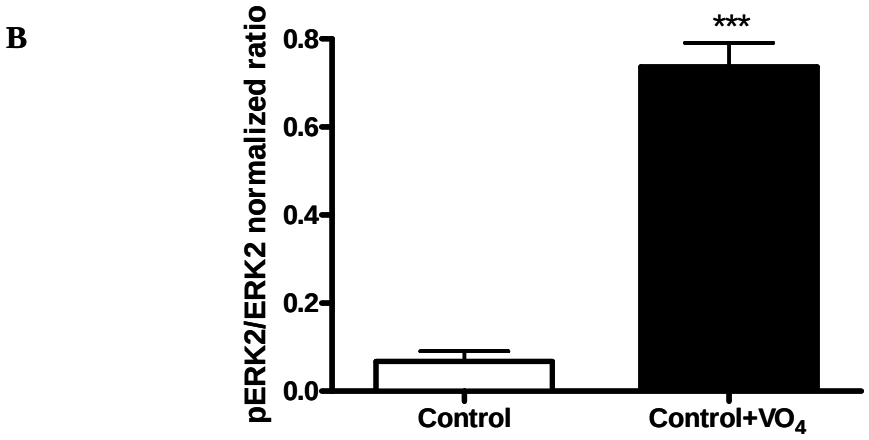


Figure 5.8 PP2A is the predominant ERK2-directed phosphatase in rat cerebral cortex and hippocampus.

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 pERK2 phosphatase activity. Input pERK2(IN) as well as pERK2 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).





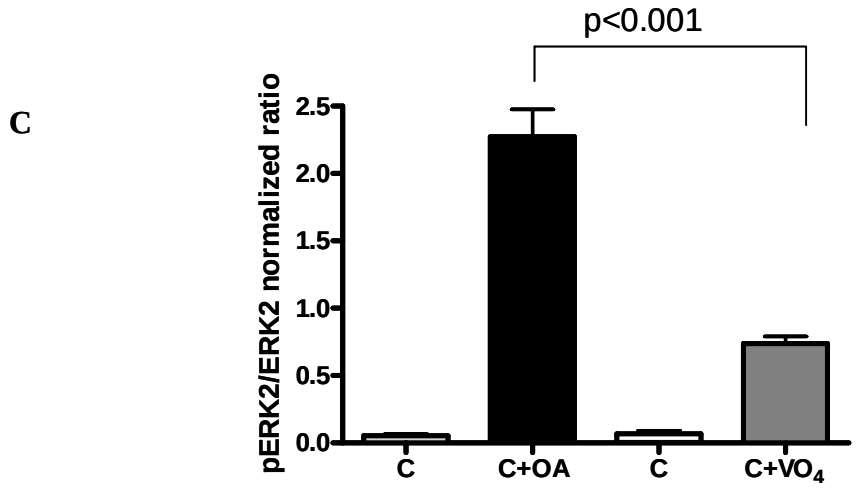


Figure 5.9 Tyrosine phosphatases also contribute to ERK-directed phosphatase activity in rat cerebral cortex.

A. Extracts prepared from control sham operated adult male rat in cerebral cortex were incubated with 1mM VO_4 where indicated prior to assaying for pERK2 phosphatase activity. Input pERK2 (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 ERK2 phosphatase activity was statistically significant than that of VO_4. $p<0.001$.

5.5 DISCUSSION

ERK1/2 activation occurs in a variety of brain regions in response to ischemia/reperfusion but the mechanism of this activation has not been analyzed thoroughly. In the preceding studies, I demonstrated that the inhibition of ERK1/2 phosphatases contributes to ERK1/2 activation following ischemia/reperfusion in both transient focal ischemia and global ischemia models. Furthermore, this inhibition of phosphatases is selective, since JNK phosphatases were not affected. I also revealed regional differences in the response of the ERK1/2 signaling pathway to ischemia/reperfusion and a differential sensitivity of ERK1/2 phosphatases to oxidative stress that accompanies reperfusion. The reversible inhibition of ERK1/2 phosphatases by ischemia/reperfusion in a cardiac arrest model contributes to early ERK1/2 activation in the rat cerebral cortex but not in the 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 strongly suggest that different mechanisms account for ERK activation in distinct brain regions following ischemia/reperfusion. In the cerebral cortex, ischemia/reperfusion-induced oxidative stress results in both the activation of MEK1/2 and the inhibition of ERK1/2 phosphatases, leading to the ultimate hyperphosphorylaton of ERK1/2. In hippocampus, ERK1/2 activation following ischemia/reperfusion is achieved solely through the activation of MEK1/2 but not with inhibition of ERK1/2 phosphatases. PP2A appears to be the major ERK phosphatase that is responsible for regulating ERK1/2 activation in ischemic brain tissues.

ERK1/2 activation has been associated with neuronal death in several transient focal ischemia models (Alessandrini et al., 1999; Namura et al., 2001; Noshita et al., 2002). ROS play a critical role in mediating ERK1/2 activation and subsequent neuronal death in this model since overexpression of SOD1 in mice attenuates ERK1/2 activation and the subsequent neuronal death following transient ischemia/reperfusion (Noshita et al., 2002). However, the mechanism of ROS-mediated ERK activation following ischemia/reperfusion remains largely unknown. Previous studies utilizing an in vitro neuronal model have shown that selectively reversible oxidation and inhibition of ERK1/2 phosphatases contributes to ERK1/2 activation and subsequent neuronal toxicity following oxidative stress (Levinthal and DeFranco, 2005). This study provided the first link between ROS-mediated dysregulation of ERK1/2 phosphatases and ERK1/2-dependent neuronal toxicity. The current study extends the above findings to in vivo models of ischemia/reperfusion, particularly in transient focal ischemia where ERK1/2 is thought to contribute to neuronal toxicity in vitro. Taken together, we have established the role of oxidative stress in the selective inhibition of ERK1/2 phosphatases both in vitro and in vivo, providing new insights into the regulation of ERK1/2 activation by ROS.

It has long been known that ROS regulate protein phosphatases, mainly based on the study of PTPs. Oxidative stress can inhibit the activity of PTPs by oxidizing and inactivating the catalytic cysteines on these phosphatases. Since both PTPs and DSPs have the same $HC(X)_5R$ motif in their catalytic sites, they may share the same mechanism of oxidative inhibition. Thiol oxidation and inactivation of PTPs/DSPs can be reversible or irreversible. Oxidation of the catalytic cysteine to sulfenic acid or the formation of intramolecular disulfide bonds or the formation of a novel reversible intermediate sulphenylamide are all reversible modifications of thiol groups (Barford, 2004). However, further oxidation of sulfenic acid to sulfinic acid and

sulfonic acid are irreversible. Compared with PTPs/DSPs, the mechanisms regulating the oxidative inhibition of PSTPs are much less known. Due to the lack of the presence of catalytic cysteines in the catalytic active sites of these enzymes, it is most likely that oxidation inhibits their activity through the oxidant-sensitive regulatory subunits or associated factors. Another important mechanism responsible for oxidative inhibition of the activity of PSTPs may lie on the oxidation of bi-nuclear metal clusters in their active site. Redox regulation of calcineurin has been shown through the targeting of Fe^{2+}-Zn^{2+} center at the active site of the enzyme (Namgaladze et al., 2002). Recently, reversible thiol-oxidation has been reported regulating the activity of PP2A, although the exact mechanism is not known (Kim et al., 2003; Foley and Kintner, 2005).

Apart from directly attacking catalytic cysteines or binuclear metal centers in protein phosphatases, oxidative stress can affect the activity of phosphatases through other signaling molecules such as zinc. Zinc is known as a key regulator of many signaling pathways, with the best example being its involvement in insulin signaling. Zinc has been found augmenting insulin signaling pathways through the inhibition of PTPs that dephosphorylate insulin receptor (Haase and Maret, 2005). It is interesting to note that insulin-stimulated ROS generation also plays a role in oxidizing and inactivating PTPs and, therefore, providing a positive feedback loop to insulin signaling. Furthermore, it should be borne in mind that oxidative stress is very important in mediating zinc release from its intracellular binding proteins. Therefore, the combination and the interactions between redox signaling and zinc signaling may consistently drive and augment the insulin-mediated signaling pathway.

Zinc release and accumulation has been associated with neuronal injury in various models of ischemia/reperfusion, although the origins of zinc remain controversial. Apart from

entering into postsynaptic neurons from presynaptic neurons, zinc has also been shown to be released from its intracellular stores by oxidative stress (Aizenman et al., 2000). ERK1/2 activation has been involved in zinc-induced neuronal toxicity in neuronal cultures (Park and Koh, 1999; Seo et al., 2001), although the mechanism of zinc-induced ERK1/2 activation is not known yet. Due to the role of zinc in inhibiting phosphatases and a recent report showing the inhibition of ERK1/2 phosphatases by zinc in human bronchial epithelial cells (Kim et al., 2006), it is tempting to hypothesize that the inhibition of ERK1/2 phosphatases by the combination of oxidative stress itself and zinc released following ischemia/reperfusion contribute together to ERK1/2 activation and subsequent neuronal toxicity in ischemia/reperfusion. To confirm the role of zinc in inhibiting ERK1/2 phosphatases in ischemia model, it will be interesting for the future study to examine the ERK1/2 phosphatases activity following the chelation of zinc by in vivo administration of either CaEDTA or TPEN. Future studies could also focus on the identification of the specific ERK1/2 phosphatases that are reversibly oxidized or inactivated in the ischemia/reperfusion model.

Impairment of PP2A activity has been associated with an enhanced activation of ERK1/2 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 ERK1/2 and JNK pathways as well as the phosphorylation of endogeneous tau, similar to the key pathological features in Alzheimer's disease (Kins et al., 2001; Kins et al., 2003). Although PP2A is the major phosphatase contributing to ERK1/2 dephosphorylation in cortical and hippocampal extracts, it appears that ischemia/reperfusion injury selectively affects this phosphatase in cortex but not in 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, ischemia/reperfusion does not appear to impact the activity of protein phosphatases to dephosphorylate JNK3 (Fig 5.3 and Fig 5.4). Unlike dominant negative PP2A C subunit-expressing transgenic mice which globally induced the hyperphosphorylation of the substrates of PP2A, including ERK1/2, JNK and tau (Kins et al., 2001; Kins et al., 2003), our results suggest that the inhibition of PP2A activity that occurs upon ischemia/reperfusion injury applies only to select substrate, i.e., ERK1/2. Given the fact that PP2A is the major ERK1/2 phosphatase in neurons and involved in the dephosphorylation of all levels of ERK1/2 signaling cascade, it is possible that ischemia/reperfusion-induced oxidative stress impact PP2A-associated ERK1/2 signaling cascade more than the JNK pathway.

It was previously reported that in an in vitro model of oxidative stress in immature cortical neuronal cultures, inhibition of ERK2 phosphatase activity contributes to ERK1/2 activation and subsequent neuronal toxicity (Levinthal and DeFranco, 2005). In this report, I have established for the first time a role for phosphatase inhibition in ERK1/2 activation following transient focal ischemia and global ischemia. Furthermore, my data provide insights into the different regulating mechanisms on ERK1/2 activation in distinct brain regions following global ischemia/reperfusion. Considering the important association between ERK1/2 activation and neuronal toxicity or neuronal survival in various ischemia models, a better understanding of the mechanisms regulating ERK1/2 activation following ischemia/reperfusion

could lead to new therapeutic strategies that target specific molecules that regulate selective responses to oxidative stress.

6.0 GENERAL DISCUSSION

As an important signaling molecule activated by various extracellular stimuli, ERK1/2 is traditionally associated with cell proliferation and differentiation as well as cell survival. In recent years, a role of ERK1/2 activation in contributing to cell death under various stresses has begun to emerge (Chu et al., 2004). However, due to the individualized responses induced by specific stimuli in different cell types, the mechanisms regulating ERK1/2 activation remains poorly understood. Previous studies in our lab have definitely established a role of ERK1/2 in contributing to neuronal cell death in response to glutamate depletion. The data presented here provide insights into the mechanism of regulating ERK activation in oxidatively-stressed neurons. Oxidation-induced inactivation of ERK1/2 phosphatases results in the hyperphosphorylation of ERK1/2 and subsequent ERK1/2-dependent neuronal cell death. Furthermore, an increase in intracellular free zinc also contributes to the inhibition of ERK phosphatases in neuronal cells following glutamate toxicity. The administration of either an antioxidant or zinc chelator restores the activity of ERK phosphatases and protects neuronal cells from oxidative stress-induced cell death. Moreover, studies examining the role of oxidative inhibition of ERK1/2 phosphatases in driving neuronal cell death have been extended to an *in vivo* model of MCAO. Specifically, I found that ERK phosphatase activity is inhibited following ischemia/reperfusion in an MCAO model, in which ERK1/2 activation contributes to neuronal cell death (Alessandrini et al., 1999). Altogether, these results establish a clear role for the

inhibition of ERK1/2 phosphatases in driving ERK1/2-dependent neuronal toxicity during oxidative stress.

6.1 OXIDATIVE INHIBITION OF ERK-DIRECTED PHOSPHATASES

In recent years, ROS have been recognized as important second messengers in mediating specific cellular signaling events. By changing the phosphorylation status of signaling proteins, several protein phosphatases have been identified as the key links between oxidative stress and regulation of specific signaling pathways. Due to the low pKa of their catalytic cysteines, PTPs are very sensitive to oxidation, which suggests that cysteine oxidation is important in regulating the activity of PTPs. Reversible or irreversible cysteine oxidation of specific PTPs then determines the distinct biological outcomes mediated by PTP-regulated signaling pathways. This has established a role for protein phosphatases as a redox sensor to mediate and regulate oxidant-induced signaling cascades.

The best example of the role of oxidative inhibition of protein phosphatases in mediating physiological responses is ligand-receptor interactions in growth factor-mediating signaling events (Droge, 2002). Growth factor-receptor binding on the plasma membrane leads to the local generation of ROS, which in turn, inactivate specific phosphatases. This leads to the augmentation of membrane receptor autophosphorylation and facilitation of ensuing signaling cascades. One intriguing problem in these models is how the specificity of the oxidative inhibition of specific phosphatase is achieved. In one single cell type (Rat-1 cells), PDGF induces the oxidation of SHP-2 (Meng et al., 2002), whereas insulin induces the oxidation of PTP1B (Meng et al., 2004). Moreover, it has been shown that a NADPH oxidase, Nox4, is co-

localized with PTP1B on intracellular membranes (Martyn et al., 2006). Nox4 has been reported to play an important role in ROS generation and oxidation inhibition of PTP1B in the insulin-mediated signaling pathway (Mahadev et al., 2004). Therefore, the local regulation of ROS generation and the proximity between ROS and phosphatases may be important in regulating the specificity of the phosphatases being affected, rather than indiscriminate inhibition of all phosphatases. A recent report revealed that distinct domains of receptor-like PTPs (RPTPα) are differentially sensitive to oxidation (Groen et al., 2005) by using a novel antibody against the oxidized form of the active site of cysteine residues. These results suggest that intrinsic difference in the susceptibility of PTP to oxidation may also determine the specificity of oxidative inhibition of distinct signaling pathways.

Our study was the first to reveal a role for oxidative inhibition of protein phosphatases in mediating neuronal death (Levinthal and DeFranco, 2005). Consistent with our study, a recent report showed that activation of MEK1 is modest in HT22 cells and primary immature cortical neurons (Choi et al., 2006), further supporting the notion that oxidative inhibition of ERK1/2-directed phosphatases is largely responsible for persistent ERK1/2 activation following glutamate-induced oxidative toxicity. Similarly, persistent JNK activation is mainly driven by the oxidative inhibition of JNK phosphatases in TNF-α treated fibroblasts and liver cells (Kamata et al., 2005). Furthermore, the administration of a broad spectrum antioxidant, BHA, restores the activity of specific MAPK phosphatases and prevents subsequent cell death in our models (results in Chapter 3) and in Kamata's study (Kamata et al., 2005). Thus, oxidative inhibition of specific protein phosphatases may represent a general mechanism for prolonged activation of protein kinases that drive cell death.

Since BHA is a broad spectrum antioxidant, it is not known which specific reactive oxygen or nitrogen species is generated following glutamate-induced oxidative toxicity and responsible for the inhibition of ERK phosphatase. Various strategies could be utilized to address this question. Specfic ROS or RNS could be detected by a variety of detection systems such as coelenterazine-enhanced chemiluminescence method to measure changes in intracellular O_2^- levels (Tarpey et al., 1999). Moreover, specific ROS generated could be characterized by various oxidant scavenging systems. Purified SOD1 or the transfection of SOD1 expression plasmid will be utilized to evaluate the role of O_2^- on glutamate-induced oxidative toxicity and the inhibition of ERK phosphatase. The role of H_2O_2 on oxidative toxicity could be determined by using its scavenger, catalase or catalase expression vectors.

Although in vivo administration of antioxidant BHA and in vitro addition of a thiol-reducing agent DTT could reverse the inhibition of ERK1/2 phosphatase activity following glutamate toxicity, the mechanism of oxidation inhibition of ERK1/2 phosphatases still remains unclear. In Chapter 4, I provided the first clear evidence of reversible cysteine thiol oxidation in PP2A C-subunit under conditions of glutamate toxicity. Moreover, more recent study in the lab has shown that the phosphatase activity of PP2A was indeed decreased following glutamate-induced oxidative toxicity by using the IP-PP2A phosphatase assay (data not shown). It still remains unclear whether the inhibition of PP2A phosphatase activity may be due to the oxidation of reactive site cysteine residue(s) in PP2A following oxidative stress. Kim et al (2003) have shown that ROS accumulation during cellular senescence in fibroblast cells inhibited PP1 activity and this might be due to the oxidation of Cys^{62} and Cys^{105} in PP1. Since there is 49% homology in the catalytic domains of PP1 and PP2A, it is very possible that the oxidation of Cys^{62} may also be critical for the impairment of PP2A phosphatase activity in our case. Future

studies will be directed to identify the specific cysteine residue(s) in PP2A which is (are) modified by oxidative stress. Since BHA restores ERK phosphatase activity and PP2A is one of the major ERK phosphatases in neurons, it would be interesting to evaluate the effect of antioxidant (BHA) on restoring the activity of PP2A following glutamate-induced oxidative toxicity. Apart from directly affecting the activity of the catalytic subunit of PP2A, thiol oxidation of PP2A C subunit may affect its association with the B regulatory subunits, which determines the substrate specificity as well as subcellular compartmentalization of the PP2A holoenzyme. Future studies will also be directed towards the examination of the association between different PP2A subpopulation and its specific substrate, ERK1/2, following glutamate-induced oxidative toxicity.

Using specific peptide and pharmacological inhibitors of PP2A, I have demonstrated that PP2A is a major ERK1/2-directed phosphatase that may be susceptible to oxidation inhibition. However, previous studies suggest that some orthovanadate-sensitive phosphatases, such as MKPs or PTPs, may also contribute to ERK1/2-directed phosphatase activity (Levinthal and DeFranco, 2005). Indeed, a recent report has demonstrated the role of MKP1 in contributing to glutamate-induced toxicity in HT22 cells and primary immature cortical neurons (Choi et al., 2006). Furthermore, oxidation inactivation of MKP1 has been demonstrated in TNF-α treated fibroblasts, and this inhibition of MKP1 contributes to persistent JNK activation that leads to apoptosis (Kamata et al., 2005). Due to the similar structure in catalytic sites as PTPs, MKPs is reversibly inhibited through the oxidation of catalytic cysteines to sulfenic acid (Kamata et al., 2005). Altogether, these results suggest that MKP1 may be another major candidate of ERK1/2 phosophatase sensitive to oxidation inhibition in our model. As I have demonstrated the feasibility of the thiol affinity chromatography to enrich for reversibly thiol-oxidized proteins

(Chapter 4), future studies will be necessary to examine the thiol oxidation of MKP1 by either thiol affinity chromatography or non-denaturing SDS-PAGE (Kamata et al., 2005). Once the thiol oxidation of MKP1 is demonstrated, the activity of thiol-oxidized MKP1 can be examined by an immunoprecipitation phosphatase assay and specific cysteine residues being oxidized could be identified by mass spectrometry.

6.2 OXIDATIVE STRESS-MEDIATED ZINC RELEASE IN ERK1/2-DEPENDENT NEURONAL DEATH

Apart from affecting the redox state of signaling proteins, oxidative stress can also alter many metabolic and signaling pathways through triggering the release of intracellular stores of metal ions such as zinc. An indispensable trace element in human body, zinc is most abundant in the brain. Zinc deficiency causes severe symptoms in nervous system and excessive zinc has been found to be toxic to neurons *in vitro* (Koh and Choi, 1994; Canzoniero et al., 1999; Seo et al., 2001). Moreover, zinc accumulation has been associated with neuronal death in both global ischemia and focal ischemia (Koh et al., 1996; Lee et al., 2002a). Although the sources of toxic zinc following neuronal injury in brain still remains controversial, a thiol oxidant, DTDP (Aizenman et al., 2000), and nitric oxide (Montoliu et al., 2000; Bossy-Wetzel et al., 2004) have been shown to trigger zinc release from the intracellular thiol-sensitive zinc-binding protein, metallothionein (MT).

Zinc has been shown to induce neuronal death through its impact on the activation of various MAPK pathways. Thiol oxidizing agents and nitric oxide mediates intracelllular zinc release, which in turn, activates p38 MAPK and leads to cell death in cortical neurons

(McLaughlin et al., 2001; Bossy-Wetzel et al., 2004; Zhang et al., 2004). Exogenous zinc induces sustained ERK1/2 activation which contributes to neuronal death in PC12 cells (Park and Koh, 1999; Seo et al., 2001). ERK1/2-dependent neuronal death has also been reported in methylisothiazolinone (MIT)-induced toxicity (Du et al., 2002) and peroxynitrite-induced toxicity in mature oligodendrocytes (Zhang et al., 2006) through the release of intracellular zinc. However, the mechanism regulating zinc phosphorylation and activation of ERK1/2 pathway still remains largely unknown. One mechanism that might be responsible for zinc-mediated protein phosphorylation involves the inhibition of protein phosphatases. For example, in EGFR and IGF-1 mediated signaling, zinc enhances the phosphorylation of receptor tyrosine kinases (RTKs) through the inhibition of PTPs (Haase and Maret, 2003; Tal et al., 2006). Zinc has been found to inhibit PTPs (Brautigan et al., 1981; Haase and Maret, 2003) and PSTPs such as PP2A (Zhuo and Dixon, 1997) and PP2B (Takahashi et al., 2003) *in vitro*. Zinc inhibition of PSTP activity involves the competition with the metal in the active sites of PSTPs. Zinc competes with Mn^{2+} in PP2A and Ni^{2+} in PP2B (Zhuo and Dixon, 1997; Takahashi et al., 2003). Recently, it has been shown that zinc activates ERK1/2 in human airway epithelial cells by inhibiting ERK1/2-directed phosphatases *in vivo* (Kim et al., 2006).

The data presented in Chapter 3 confirm the role of oxidative stress in mediating intracellular zinc release. A general antioxidant, BHA, reduces zinc accumulation in oxidatively stressed HT22 cells and primary immature cortical neurons. Furthermore, I provided the evidence that intracellular zinc-mediated inhibition of ERK1/2 phosphatases links oxidative stress and ERK1/2-dependent neuronal death in HT22 cells and immature cortical neurons. This provides important insights into the mechanism of oxidative stress-induced ERK1/2-dependent neuronal death, highlighting the importance of the inhibition of ERK1/2 phosphatases. In

summary, I propose the following: Glutamate or HCA-induced oxidative stress triggers the intracellular accumulation of zinc, which together with ROS, inactivate ERK1/2 phosphatases. This leads to ERK1/2 hyperphosphorylation and activation, resulting in neuronal death in HT22 cells and primary immature cortical neurons. Moreover, the results of U0126's reduction of zinc release and restoration of ERK1/2 phosphatase activity suggest that ERK1/2 itself can function as a positive feedback to enhance zinc release triggered by ROS.

The mechanisms of ERK1/2 itself acting to increase intracellular zinc accumulation still remain elusive. One mechanism may involve the requirement of ERK1/2 for ROS generation through the activation of NADPH oxidase as shown in a model of MIT-induced ERK-dependent neuronal death (Du et al., 2002). Although previous studies in the lab have shown that U0126 does not reduce ROS generation by measuring DCF fluorescence intensity (Stanciu et al., 2000; Levinthal and DeFranco, 2004), more sensitive detection methods may be needed in the future to re-evaluate these results. The role of ERK1/2-activated NADPH oxidase in generating ROS and subsequent zinc accumulation could be evaluated by the treatment of primary neurons with a selective NADPH oxidase inhibitor, apocynin, in the absence or presence of HCA and subsequent measurement of ROS levels and zinc accumulation. Molecular inhibitors of ERK1/2 activation such as dominant negative MEK1 could be utiltized to confirm the impact of ERK1/2 on zinc release.

Although a direct role of ROS in triggering zinc accumulation has been shown in our study (Chapter 3), it is still not known which specific ROS is responsible for intracellular zinc accumulation following oxidative toxicity. The impact of specific ROS on intracellular zinc accumulation could be determined by measuring relative intracellular zinc levels in neurons following the treatment of neurons with pharmacological or molecular oxidant scavenging

reagents in the absence or presence of HCA (or glutamate). In addition, zinc accumulation is not only a mediator of ROS-induced toxicity but also an executor itself for ROS generation. Zinc has been found to inhibit mitochondrial respiration (Brown et al., 2000; Bossy-Wetzel et al., 2004) and induces mitochondrial ROS production (Sensi et al., 1999). The effect of zinc release on ROS generation in our model has not been evaluated. This could be addressed in the future by measuring ROS and/or RNS levels in oxidatively stressed neurons treated with TPEN, a chelator of zinc.

Although we have observed the intracellular zinc accumulation following oxidative toxicity in neurons, the source of intracellular elevated zinc has not been identified yet. Extracellular zinc could be uptaken into cells through various routes including Zip family of zinc transporters and various zinc-permeable ion channels. Therefore, a cell impermeable zinc chelator, CaEDTA, will be used to evaluate the contribution of extracellular zinc in ROS-mediated zinc accumulation. Inside the cells, one major cellular source of zinc is metallothionein (MT). Measurement of intracellular zinc levels in oxidatively stressed neurons that are transfected with MT expression plasmids will enable us to determine the contribution of MTs in releasing zinc in our model of neuronal oxidative stress. Recently, PKCδ has been shown to be activated by oxidative stress in glutamate-treated HT22 cells and immature cortical neurons (Choi et al., 2006). Moreover, PKCδ activation facilitates proteasomal degradation of MKP1 and contributes to ERK1/2 activation. Interestingly, PKCδ activated by oxidative stress releases zinc from its zinc-finger domain (Knapp and Klann, 2000; Korichneva et al., 2002), which suggests that PKCδ is another source of intracellular zinc. Taken together, it is very tempting to hypothesize that PKCδ may play a role in mediating ROS-induced zinc release and subsequent ERK-dependent oxidative toxicity. Future studies will be directed to examine the effect of PKCδ

inhibition in blocking oxidative stress-induced zinc release in glutamate or HCA-treated HT22 cells or immature cortical neurons by utilizing specific pharmacological or molecular inhibitors of PKCδ.

Study from our lab has shown that ERK1/2 activation is necessary but not sufficient in mediating glutmate-induced oxidative toxicity (Luo and DeFranco, 2006). Transfection of a constitutively active MEK1 failed to induce toxicity in HT22 cells despite a robust, persistent activation of ERK1/2 within either the cytoplasmic or nuclear compartment (Luo and DeFranco, 2006). My data presented in Chapter 3 has revealed that chronically activated ERK1/2 in oxidatively stressed neurons functions as a positive feeback to maintain elevated intracellular levels of Zn^{2+} and subsequent Zn^{2+}-dependent cell death. As discussed above, one mechanism responsible for ERK1/2-regulated intracellular zinc accumulation may be due to ROS generated by ERK1/2-activated NADPH oxidase. In our model, ROS acts as the trigger to promote the release of protein-bound Zn^{2+}, perhaps through thiol oxidation of specific cysteine residues that participate in Zn^{2+} binding (Aizenman et al., 2000; St Croix et al., 2002). In this case, ERK1/2 may also block the recovery of oxidatively damaged Zn^{2+} binding proteins such as MT or limit their expression (Jiang et al., 2004a) and therefore, maintains intracellular neurotoxic levels of zinc. Thus, consistent with Luo's study (2006), in the absence of oxidative damage to these redox-sensitive proteins, chronic ERK1/2 activation alone does not promote Zn^{2+}-dependent neuronal death since it might not impact intracellular Zn^{2+} homeostasis. This hypothesis could be tested by measuring the intracellular zinc levels in HT22 cells transfected with a constitutively active MEK1 expression plasmid.

My data presented in Chapter 3 has revealed a mechanism of dual regulation of ERK phosphatase by ROS and zinc. However, it is not known if these two processes are dependent or

independent events. It is possible that selective ERK phosphatase may need to be thiol oxidized to cause the change of its conformation to bind with the inhibitory zinc. An in vitro assay has been utilized to measure zinc bound to immunoprecipitated PKCδ in H_2O_2 treated lymphocytes by using a fluorescent zinc indicator dye (TSQ) (Korichneva et al., 2002). In our case, PP2A or MKP1 could be immuoprecipitated from untreated or glutamate-treated HT22 cells and subjected to the above in vitro zinc binding assay. If oxidative stress does have an effect on the binding of zinc to PP2A or MKP1, we should observe an increase in the zinc associated with PP2A or MKP1 immunoprecipiated from glutamate-treated HT22 cells.

Fig 6.1 summarizes the mechanism of regulating ERK phosphatases by ROS and zinc in glutathione-depletion induced oxidative toxicity in HT22 cells and primary neurons.

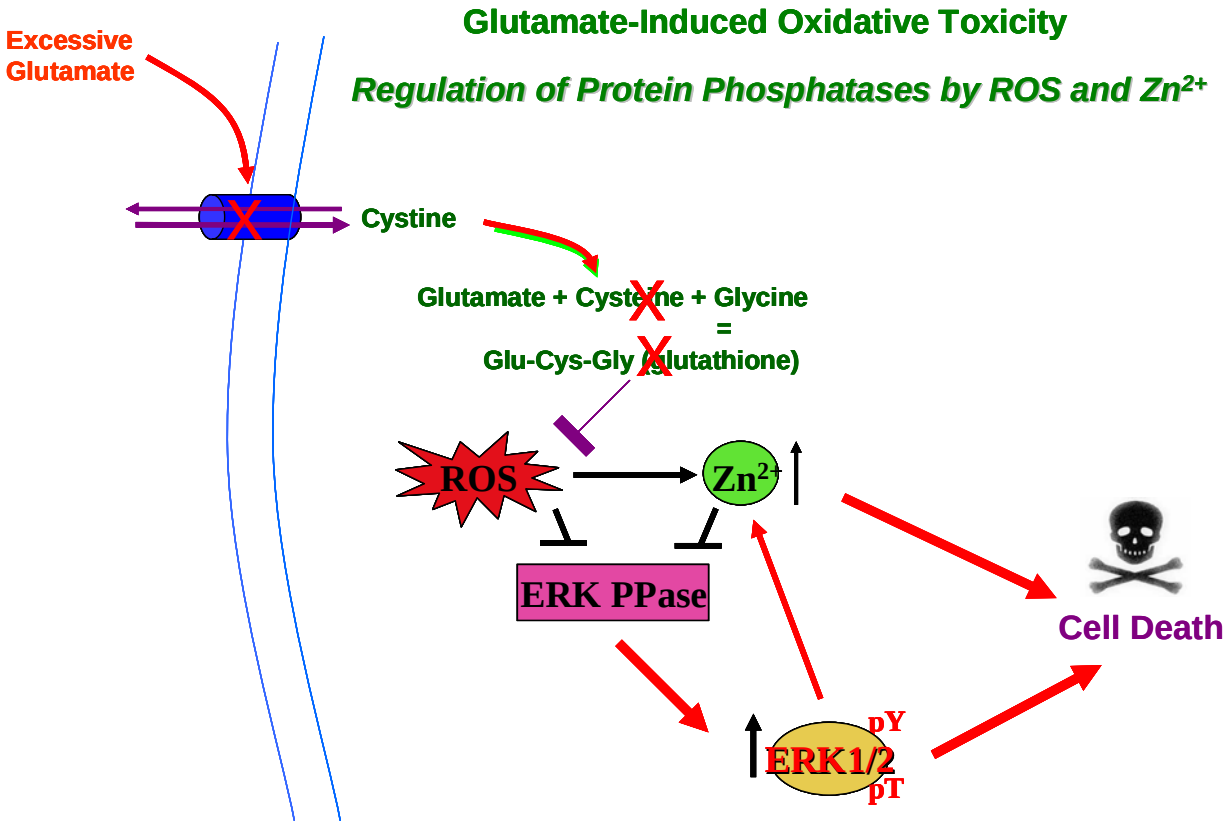


Figure 6.1 Summary of the mechanisms regulating ERK phosphatases by ROS and Zinc in glutamate-induced oxidative toxicity models.

Glutamate-induced toxicity in HT22 cells and primary immature cortical neurons provides an excellent in vitro model to study the role of oxidative stress in the pathogenesis of neurodegenerative diseases, particularly in the pathogenesis of neonatal hypoxia. This type of brain injury is often seen in neonates suffering from birth asphyxia and is a common cause for lifelong neurological disabilities. Oxidative stress is an early feature of ischemia in both adult and immature brains. Compared with adult brain, the immature brain is particularly vulnerable to oxidative stress due to its poor oxidant scavenging systems and high rate of energy consumption (Ferriero, 2004; Blomgren and Hagberg, 2006). Moreover, the vulnerability to oxidative stress is more selective for neurons than for glia cells. Therefore, oxidative stress may play a more important role in ischemia-induced neuronal death in immature brain by regulating the release of proapoptotic proteins from mitochondria (Blomgren and Hagberg, 2006). However, it should be borne in mind that oxidative toxicity and excitotoxicity all contribute to ischemia-induced neuronal death in both adult and immature brains. Thus, it would be difficult to clearly differentiate the contribution of oxidative toxicity and excitotoxicity in mediating neuronal death following ischemia in vivo although oxidative stress is known as an early triggering event in ischemia.

To make our study have more pathological relevance, we adopted several in vivo models. One in vivo model is MCAO model in rodents which mimics stroke. Although the mechanisms of neuronal death in MCAO models involve both excitotoxicity and oxidative toxicity, this model still serves as an excellent paradigm to elucidate mechanisms of oxidative stressed-induced signaling cascades and subsequent neuronal death in vivo. Scavenge of ROS generation significantly reduces neuronal death in MCAO models (Kuroda et al., 1999; Huang et al., 2001), suggesting the contribution of oxidative stress in the mechanism of neuronal death in MCAO

models. Consistent with our in vitro data, neuronal death in MCAO rodents is ERK1/2-dependent, since the pharmacological inhibition of ERK1/2 activation decreases brain injury in rodent MCAO models (Alessandrini et al., 1999). ROS such as superoxide contribute to ERK1/2-dependent neuronal death in MCAO rodents since the overexpression of SOD1 in mice reduces brain injury by preventing ERK1/2 activation induced by ROS (Noshita et al., 2002). My data presented in Chapter 5 revealed that the inhibition of ERK1/2 phosphatases contributes to ERK1/2 activation following ischemia/reperfusion in MCAO mice. This is the first demonstration of the role of oxidative inhibition of ERK1/2 phosphatases in ERK1/2 activation in an in vivo model that involves oxidative toxicity. Furthermore, the activation of MEK1 in ischemic tissues is only modest (data not shown), which suggests that the inhibition of phosphatase may play a major role in mediating ERK1/2 activation in our case. Although I found that total ERK1/2-directed phosphatase activity is inhibited, it would be important to identify the specific ERK1/2 phosphatase being affected in ischemic tissues using an IP-phosphatase assay. Given the fact that zinc accumulation in ischemic brain tissues contributes to toxicity in ischemia models (Koh et al., 1996; Lee et al., 2002a), the MCAO model could also be utilized to study the role of zinc in mediating oxidative stress-induced neuronal toxicity by using the approaches outlined in our in vitro studies. The potential role of zinc in mediating toxicity in ischemia models could be confirmed by intraventricular administration of a cell impermeable cheltor of zinc, CaEDTA, which is neuroprotective, as shown in several studies from Choi's group (Koh et al., 1996; Lee et al., 2002a). Once the neuroprotective effect of CaEDTA is confirmed in the MCAO model, we can proceed to examine the impact of zinc chelation in vivo on ERK1/2 activation and ERK1/2 phosphatase activity. Another in vivo model that will allow us to study the impact of oxidation on the inhibition of ERK phosphatase is SOD1 transgenic mice.

Transgenic mice overexpressing SOD1 exhibit resistance to MCAO-induced neuronal injury by preventing ERK1/2 activation while no effect on MEK1 activation is observed (Noshita et al., 2002). This suggests that SOD1 overexpression may prevent ERK1/2 activation by restoring ERK1/2 phosphatase activity. Thus, the analysis of ERK phosphatase activity in SOD1 transgenic mice will allow us to understand the mechanisms responsible for SOD1 effects on ERK1/2 activation following MCAO and alternatively, the mechanism of superoxide-induced ERK1/2 activation. Taken altogether, these in vivo studies will provide novel insights into the mechanisms regulating ERK1/2 phosphatases and the impact on ERK1/2-dependent neuronal death in ischemia models. The information obtained could contribute to the development of novel therapeutic strategies that target ERK1/2 phosphatases and ERK1/2 activation during oxidative stress in vivo.

Studies in another in vivo oxidative stress model, asphyxia-induced global ischemia model, have 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 level 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. Moreoever, 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.

Similar to selective inhibition of JNK phosphatase in TNF-α- induced oxidative toxicity in fibroblasts and Concanavolin A-induced liver toxicity in mice (Kamata et al., 2005), a selective inhibition of ERK phosphatase has been revealed in both in vitro and in vivo studies of our oxidative stress models. Glutamate-induced oxidative stress in immature neurons does not significantly affect JNK phosphatase activity (Levinthal and DeFranco, 2005). These results suggest that even though MAPK phosphatases are oxidant sensitive, they are not globally inactivated by oxidative stress in cells. How the specificity of the oxidative inhibition of specific phosphatase is achieved still remains vague. As discussed before, depending upon the nature of the stimulus imposed upon cells, select protein phosphatases may be subjected to different levels of oxidation. For example, in Rat-1 cells, PDGF induces the oxidation of SHP-2 (Meng et al., 2002), whereas insulin induces the oxidation of PTP1B (Meng et al., 2004). The local regulation of ROS generation and the proximity between ROS and phosphatases may be critical

in determining the specificity of the phosphatases being affected. Nox4, a NADPH oxidase which was reported playing a role in the oxidative inhibition of PTP1B in the insulin-mediated signaling pathway (Mahadev et al., 2004), has been revealed co-localizing with PTP1B on intracellular membranes (Martyn et al., 2006). Therefore, selective MAPK phosphatase may be protected from the damaging effects of reactive species by sequestering within subcellular compartments that are inaccessible to short-lived oxidants. It is also possible that selective MAPK phosphatase may form multi-units protein complexes to protect the active cysteine residue(s) from exposing to reactive species. Similarly, zinc-binding sites in selective MAPK phosphatase may also be protected by the formation of multi-units protein complexes and this may explain for the selective inhibition of ERK phosphatase but not JNK phosphatase by exogenous zinc.

In summary, the results presented in this thesis have definitively established the role of ERK1/2 phosphatases in contributing to ERK1/2 activation as well as subsequent ERK1/2-dependent neuronal death in both in vivo and in vitro oxidative stress models. I have confirmed findings from previous studies (Levinthal and DeFranco, 2005) by showing that the inhibition of ERK1/2 phosphatases in HT22 cells and primary cortical neurons subjected to glutathione depletion is indeed the result of oxidative stress as phosphatase activity was restored, leading to reduced ERK1/2 activation upon treatment with the antioxidant BHA. Moreover, I have identified a novel mechanism of protein phosphatases regulation by oxidative stress, i.e., intracellular free zinc accumulation. Oxidative stress triggers the release of intracellular zinc, which reversibly inhibits protein phosphatases. This leads to the activation of a subset of signaling molecules (i.e., ERK1/2) and subsequent ERK1/2-dependent neuronal death. Furthermore, I have also identified a positive feedback loop driven by ERK1/2 that further

exacerbates cellular exposure to neurotoxic levels of free zinc. Altogether, my study has elucidated the mechanism of protein phosphatases regulation by oxidative stress and has established a role of zinc as an important regulator of protein phosphatases. As a bridge between oxidative stress and the regulation of protein phosphatases, zinc could be the potential target of future therapeutic strategies for various neurological diseases that involve oxidative toxicity.

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