Mechanisms Promoting ERK-Dependent Neuronal Oxidative Toxicity

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MECHANISMS PROMOTING ERK-DEPENDENT NEURONAL OXIDATIVE TOXICITY

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Glutamate-induced oxidative toxicity in HT22 cells and primary immature cortical cultures provides an excellent model system for studying oxidative stress-dependent neurodegeneration. Glutamate treatment leads to cysteine and subsequent glutathione depletion, followed by the steady accumulation of reactive oxygen species (ROS). This form of cell death depends upon the persistent activation, via phosphorylation, of extracellular signal regulated kinase (ERK) kinase-1/2 (ERK-1/2) that occurs during oxidative stress. However the mechanisms responsible for this chronic activation of ERK during oxidative stress have not been well characterized. In this thesis, I demonstrate that ERK activation is dependent upon the tonic activity of the phosphatidylinositol-3-kinase (PI3K)-Akt pathway and the subsequent activation of MEK. Furthermore, the persistent ERK activation that leads to cellular toxicity can be driven by the oxidative-dependent inactivation of ERK-phosphatases. Thus the balance of activating kinase activity and inactivating phosphatase activity dynamically regulates ERK-dependent signaling and is a major determinant of neuronal cell responses to oxidative stress. The overexpression of a negative regulator of the ERK MAPK pathway, the ERK-specific phosphatase MKP3, led to protection of both HT22 cells and primary immature cortical cultures from oxidative toxicity. Furthermore, a catalytically inactive form of MKP3 (MKP3 C293S) was shown to physically restrict activate ERK to the cytoplasm. Because overexpression of MKP3 C293S was also shown to be neuroprotective, translocation of active ERK to the nucleus,

but not ERK activation alone, must be required for glutamate-induced oxidative toxicity. Collectively, these results clearly place ERK activation as a necessary event that leads to neuronal cell death during oxidative stress and have revealed some unique mechanisms by which ROS accumulation drives ERK activation.

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1. PREFACE

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2. INTRODUCTION

2.1. Oxidative stress and neurodegenerative disease

2.1.1. Physiological sources of ROS

Reactive oxygen species (ROS) are a family of oxygen containing molecules that include superoxide anion (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2). Superoxide anions can interact with nitrogen containing molecules such as nitric oxide (NO^-) to form peroxynitrite ($ONOO^-$), a prototype member of the family of reactive nitrogen species (RNS). Collectively, ROS and RNS molecules vary in both their stability and ability to oxidize proteins, lipids, and nucleic acids in the cell, but the accumulation of these molecules in general is detrimental to normal cellular physiological function and often leads to cellular demise.

As the primary site of oxygen consumption in the cell, mitochondria represent an important source of intracellular ROS production. Oxidative phosphorylation, the process by which energy is captured via the step-wise reduction of O_2 to H_2O, occurs in the mitochondria of eukaryotic cells. Though by far most O_2 becomes fully reduced to H_2O during this process, partial reduction of oxygen leads to the formation of unstable oxygen radicals that quickly interact with molecular oxygen or water to form ROS. Cells that have greater energy requirements and a higher metabolic rate, such as the cardiomyocytes found in cardiac tissue and the neurons within the nervous system, generally consume more oxygen and produce a greater amount ROS due to increased demands on mitochondrial oxidative phosphorylation. Thus, these cells may be at increased risk for the accumulation of damaging ROS relative to most other cells in the body.

However, not all ROS production in the cell leads to cellular damage. ROS production by NADPH oxidases and myeloperoxidases in discrete compartments within phagocytic

neutrophils is part of an important host defense mechanism that allows for microbiocidal action in the phagolysosome. Congenital defects in this system can have devastating compromising effects on immune function, underscoring the importance of ROS production to normal function (Cotran et al., 1999). Furthermore, it is becoming clear that low levels of ROS can act as important signaling molecules in nearly all cells in the body via oxidation-dependent and reversible changes in cellular protein function. The role of localized ROS production and participation in normal, physiological signaling events is an emerging field that is only beginning to be studied and understood. The role of ROS in both normal and maladaptive signaling events will be discussed in more detail below.

2.1.2. ROS Defense Systems

To combat the deleterious effects of unnatural accumulation of ROS, several ROS defense systems have evolved to both passively and actively scavenge these molecules at different sites both within and outside of the cell. Lipid-based scavengers of ROS are important pools of reducing agents that can quench both ROS and lipid peroxides that are themselves self-propagating mediators of further oxidative damage. These molecules include Coenzyme Q in the inner leaflet of the mitochondrial membrane and vitamin E in the plasma membrane. Both CoQ and vitamin E have a passive mechanism of action by which electrons are transferred from ROS and lipid peroxides to sites on the molecule that are easily oxidized. For CoQ, two electrons can be accepted in the formation of the stable, oxidized quinone form, while vitamin E can accept one electron in the formation of a resonance-stablized oxidized state. Ascorbic acid (vitamin C), glutathione, and thioredoxin are major soluble ROS scavenging molecules that exist in the cytoplasm of the cell. Ascorbic acid can accept two electrons in the conversion from the

reduced, enol tautomeric form to the fully oxidized ketone form. Interestingly, ascorbic acid is important for restoring vitamin E to its reduced form in vivo. Glutathione and thioredoxin can specifically reverse the oxidation of thiol residues on proteins and collectively represent an important pool of soluble reducing agents. Glutathione is a modified tripeptide (Glu-Cys-Gly) with the capacity to disrupt oxidized thiols on target proteins by direct nucleophilic reaction between the reduced cysteine of glutathione and the oxidized thiol in the target protein molecule. Thioredoxin is a ubiquitously expressed and highly conserved protein that utilizes two vicinal cysteine residues in its active site to transfer electrons from oxidized thiols / disulphides within target proteins (Holmgren, 1995). While molecules such as CoQ, vitamin E, and vitamin C are important in preventing ROS accumulation, their action is necessarily stoichiometric and therefore can be easily overwhelmed by the presence of particularly high loads of ROS, oxidized lipids, and other oxidized molecules. However, gluthathione and thioredoxin can be restored to their reduced forms by reductases, and this provides a mechanism by which these anti-oxidants can scavenge larger pools of ROS.

Several enzymatic systems with differing substrate specificities have evolved to actively quench and deactivate even larger pools of ROS. The ubiquitous enzyme catalase, which specifically converts H_2O_2 to H_2O and O_2, is a prototype for this class of enzymes. It is interesting to note that catalase has one of the most rapid rates of catalysis ever identified (Stryer, 1995), further underscoring the importance of ROS scavenging and breakdown to the maintenance of normal cellular function. Superoxide dismutase (SOD) has several isoforms that specifically convert superoxide radicals to hydrogen peroxide and molecular oxygen. Within the cell, both manganese and copper/iron containing SOD isoforms function to scavenge superoxide radicals, while extracellular SOD functions in the connective tissue outside of the cell (Fattman

et al., 2003). Other ROS scavenging enzymes, such as thioredoxin- and glutathione-reductases, act indirectly by restoring the cellular pool of soluble scavengers to functional, reduced states. Examples of several ROS scavenging enzymatic and non-enzymatic reactions are shown in Figure 1 below.

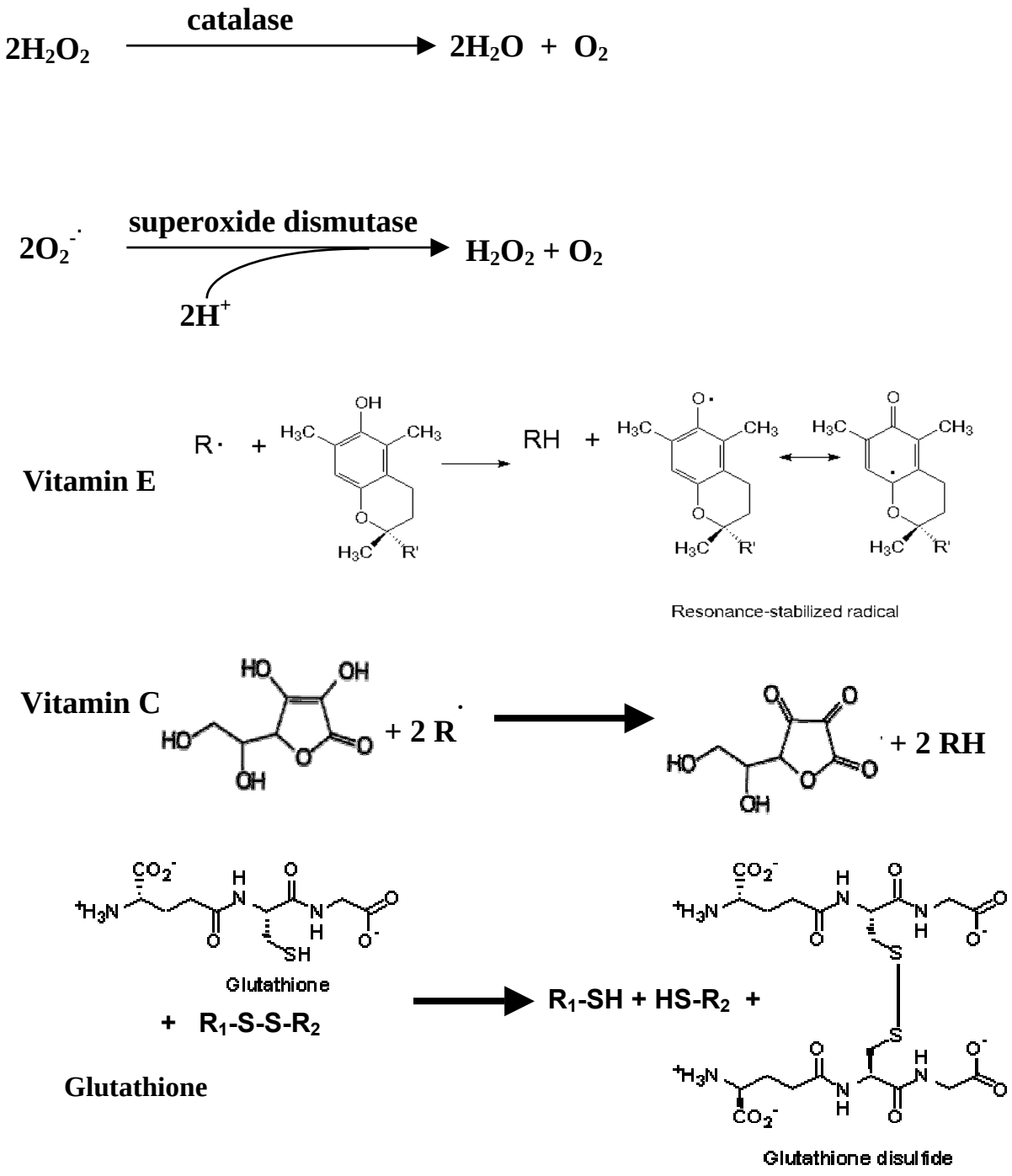


Figure 1 Prototypical reactive oxygen species (ROS) scavenging enzymes and small molecules

2.1.3. Role of ROS in neurodegenerative diseases

The accumulation of ROS resulting from impairments in normal pro-oxidant / anti-oxidant homeostasis, generally known as oxidative stress, has been implicated in the pathophysiology of a vast array of neurodegenerative disorders. These diseases include stroke, Parkinson's Disease, Alzheimer's Disease, and Amyotrophic Lateral Sclerosis, among others, which collectively account for the most prevalent forms of neurodegenerative disorders in humans. Furthermore, these diseases often involve the degeneration of specific subsets of neurons and do not appear to be general processes that affect the nervous system as a whole. Because most of these diseases occur over years or decades, yet oxidative stress can lead to neuronal demise in hours or days (e.g. during ischemia/reperfusion-induced neuronal degeneration), it is likely that these neurodegenerative disorders occur via the gradual, but selective, effect of a steady loss of neurons. Eventually, disease symptoms are manifested when remaining neurons fail to compensate for this steady loss. However, whether these disorders occur via a mechanism of gradual, selective loss of single neurons or a steady decline in functioning of all neurons within a specific neural system remains a subject of great debate.

The underlying cause of oxidative stress in chronic neurodegenerative disease is similarly controversial, and explanations ranging from purely environmental to purely genetic have been postulated. While the literature investigating the link between oxidative stress and neurodegenerative disease is quite extensive and is beyond the scope of this discussion, several excellent reviews have been written on this topic (Simpson et al., 2003; Barnham et al., 2004; Milton, 2004). However, highlighting a few examples of these links would be illustrative in underscoring the pivotal role of oxidative stress in neurodegeneration. For example, idiopathic Parkinson's Disease (PD) has been associated with the exposure to certain pesticides that can

inhibit specific enzymatic complexes that underlie oxidative phosphorylation in the mitochondria (Liou et al., 1997; Greenamyre and Hastings, 2004), while a familial form of the same disease can be manifested by mutations in the gene encoding the protein parkin (Kitada et al., 1998). Recently, deficits in complex I function have been demonstrated in leukocytes from Parkinson's patients with this parkin mutation, implying that parkin functions in part to regulate oxidative phosphorylation in mitochondria (Muftuoglu et al., 2004). Complex I inhibition could be an important causal link between parkin mutations and the development of oxidative stress in the development of PD. Most recently, mutations in the gene encoding the mitochondrial protein PTEN-induced kinase 1 (PINK1) have been associated with familial PD (Valente et al., 2004). This defect reveals yet another link between mitochondrial dysfunction and the development of PD. Other familial forms of neurodegenerative disorders are also intimately linked with the regulation of ROS production. For example, mutations in the Cu/Zn SOD1 isoform are associated with the development of the familial form of ALS (Rosen et al., 1993), and these mutations inhibit the dimerization of SOD1 necessary for full enzymatic activity (Deng et al., 1993). Furthermore, increased sensitivity to ROS/RNS may underlie the phenotype of familial forms of Parkinson's disease, which have been associated with mutations in the genes encoding the protein α-synuclein (Polymeropoulos et al., 1997). Oxidation of α-synuclein is necessary for the dimerization and aggregation of the protein (Norris et al., 2003), and the two major α-synuclein mutants, A30P and A53T, exhibit increased sensitivity to oxidation-dependent aggregation (Krishnan et al., 2003). Thus critical mutations in specific genes may drive the development of neurodegenerative disorders either by direct impairment of oxidative phosphorylation and anti-oxidant systems or by increasing the sensitivity of proteins to the detrimental effects of ROS/RNS. However, the familial forms of these neurodegenerative

disorders represent the minority of cases. Which mechanisms operate in any one affected individual is difficult to predict or determine, and it is likely that the sporadic forms of many of these diseases represent the cumulative effect of multiple maladaptive disruptions in several independent pathways.

The role of ROS in the pathology of acute neuronal injury, such as that seen during stroke, is well established. Periods of ischemia followed by reperfusion can cause a robust increase in ROS in selective regions of the brain, such as the hippocampus, and lead to an apoptotic neuronal cell death that occurs over a short period of time (i.e. hours to days) (Kirino, 1982; McCulloch and Dewar, 2001). This form of delayed toxicity typifies the neuronal cell death that is observed in the penumbral region of tissue surrounding the ischemic, necrotic core (Kametsu et al., 2003). Similarly, acute neuronal death due to potent neurotoxins can occur via the production of ROS. For example, MDMA (also known as the street drug Esctasy) administration in mice induces striatal neuronal cell death that is dependent upon free radical formation in affected neurons (Camarero et al., 2002). Furthermore, the household biocide, methylisothiozolinone, causes acute toxicity in cultures of cortical neurons via the cellular production of ROS (Du et al., 2002). Thus, ROS production and neuronal toxicity are intimately linked across a range of neurodegenerative disorders both chronic and acute.

2.2. Mitogen-activated protein kinases (MAPKs)

2.2.1. MAPK members and general signaling pathway module

The mitogen-activated protein kinase (MAPK) family is involved in the signaling of numerous stimuli and stresses that impinge upon the cell and regulates such important cellular

responses as adhesion, growth, proliferation, survival, and programmed cell death. These molecules are highly conserved across a wide range of organisms – from yeast to humans – and play a key role in both normal physiology and disease. MAPK members have traditionally been categorized into three primary divisions that include the extracellular signal-regulated kinase (ERK), the c-jun N-terminal kinases/stress activated protein kinase (JNK/SAPK), and the p38 kinase families (Garrington and Johnson, 1999). However, several new members of the MAPK family have been identified, such as ERK5 (Zhou et al., 1995) and ERK1b (Yung et al., 2000).

MAPKs operate within a canonical three-member signaling module that includes upstream MAPK kinases (MEKs or MKKs) and MEK/MKK kinases (MKKKs) (Robinson and Cobb, 1997). Signals from nearly every ligand/receptor interaction are transduced via the activation of some MAPK cascade module, and the mechanisms by which receptors and various cellular stressors are linked to this activation have been the focus of innumerable studies. In brief, both tyrosine kinase and G-coupled receptor activation can lead to the recruitment of the small GTPase, Ras, which can in turn drive the activation of the ERK signaling module through Ras-dependent activation of the various MKKK isoforms Raf1, A-Raf, and B-Raf (Robinson and Cobb, 1997).

All MAPK members are activated by dual-phosphorylation on a conserved activation loop on both a theonine and a tyrosine residue of the sequence T-X-Y (Bellon et al., 1999). Phosphorylation at both sites induces a conformational shift in the protein that leads to enzymatic activation, while removal of either phosphate or both leads to enzymatic inactivation (Canagarajah et al., 1997). MAPKs themselves are effector kinases directed toward serine and threonine residues on target proteins both in the cytoplasm and in the nucleus. Many of these targets are kinases themselves, and are generally referred to as MAPK-activated protein kinases

or MAPKAPKs. ERK1 (44 kDa) and ERK2 (42 kDa) are two closely associated isoforms of ERK that appear to be regulated in parallel in most systems. Functional distinctions between the two have been difficult to identify pharmacologically due to the lack of specific ERK1 or ERK2 inhibitors (e.g. MEK1/2 inhibitors such as PD98059 and U0126 reduce ERK1 and 2 phosphorylation levels equally). However, ERK1 and ERK2 knockout mice exhibit drastically differing phenotypes. ERK1-deficient animals are viable, fertile, and grow to normal sizes. However, ERK1 plays a specific role in the development of thymocytes, as mature thymocytes were reduced by half in the ERK1-deficient animals (Pages et al., 1999). Furthermore, ERK1-deficient animals exhibit enhanced synaptic plasticity and learning in striatal-mediated tasks (Mazzucchelli et al., 2002), but not in hippocampal- or amydgala-dependent tasks (Selcher et al., 2001). Conversely, ERK2-deficient animals are embryonic lethal, indicating that ERK2 is critical for overall cellular function and embryonic development and that ERK1 cannot compensate for the loss of ERK2 (Mazzucchelli et al., 2002). The determination of role of specific JNK isoforms in normal functioning has been similarly constrained by lack of specific inhibitors. The generation of specific JNK isoform knockouts and double knockouts has revealed that the loss of single isoforms can impact development. While JNK1, JNK2, and JNK3 single knockouts are overtly viable, JNK1 knockouts display malformations of the anterior commisure and a generalized disruption in microtubule assembly in neurons (Chang et al., 2003). Interestingly, JNK1 knockouts feature decreased adiposity and greater insulin sensitivity than normal mice (Himosumi et al., 2002). JNK1/JNK2 double knockouts display alterations in brain development due to both increased and decreased apoptosis in various subregions of the neural tube but are ultimately embryonically lethal (Kuan et al., 1999). Finally, hippocampal neurons in JNK3 knockout mice are resistant to apoptosis during excitotoxicity (Yang et al., 1997). Thus,

specific isoforms within each MAPK family may indeed have distinct and possibly opposing functions, and these subtleties in function are not typically dissociable in the majority of studies that use inhibitors that reduce the function of all isoforms equally. This caveat must be held in mind when interpreting results using inhibitors of upstream kinases that regulate these MAPK family members.

For ERK1/2, a generalized phosphorylation consensus sequence in target proteins is Pro-X-Ser/Thr-Pro (Davis, 1993). While this consensus sequence confers some specificity to ERK1/2 action, ERK1/2-dependent phosphorylation of target proteins is also dependent on a complex set of interactions that involve the physical association of ERK1/2 with scaffolding proteins and other ERK1/2 binding proteins in various compartments of the cell. Thus, the physical restriction of ERK1/2 and other MAPKs to cytosolic scaffolds may restrict MAPK functioning to cytoplasmic targets in various cell types and in varying conditions (Morrison and Davis, 2003). ERK1/2-dependent phosphorylation of nuclear targets is largely responsible for the effects of ERK1/2 activation on gene transcription. Although the mechanism remains to be fully delineated, nuclear translocation of active ERK1/2 is an important event in ERK-dependent signal transduction. This occurs in part by dimerization of two activated ERK molecules and the subsequent uptake of this complex into the nucleus (Khokhlatchev et al., 1998). However, monomeric ERK can passively diffuse through the nuclear pore, as dimerization-deficient ERK mutants enter the nucleus following mitogenic stimulation, while the same ERK mutants fused to β-gal do not (Adachi et al., 1999). An intriguing mechanism by which ERK is transported out of the nucleus was revealed by both general inhibitors of nuclear export and by microinjection of ERK into the nucleus (Adachi et al., 2000). Coinjection of MEK1/2, but not a mutant MEK1/2 that was unable to physically interact with ERK1/2, allowed for nuclear export of ERK.

Furthermore, introduction of a peptide corresponding to the ERK1/2 binding site blocked the ability of MEK1/2 to initiate nuclear export (Adachi et al., 2000). These data represent compelling evidence for an active role of MEK1/2 in the export of ERK1/2 to the cytoplasm, but the functional consequences of this mechanism have not been investigated. The dynamics governing the shuttling of both inactive and active ERK into and out of the nucleus are still not fully understood and remain a subject of debate.

The list of identified nuclear targets of ERK1/2 has remained fairly small. Most identified targets are transcription factors, such as CREB, Elk-1, and the AP-1 complex, underscoring the importance of active ERK1/2 translocation to the nucleus in the mediation of signaling events that lead to changes in gene transcription. Furthermore, ERK may affect transcription indirectly via the activation of cytoplasmic targets, such as p90 ribosomal S6 kinase (RSK), that translocate into the nucleus and phosphorylate transcription factors that include the estrogen receptor, c-Fos, and CREB (Frodin and Gammeltoft, 1999). However, some nuclear targets for ERK1/2 are regulators of ERK1/2 activity themselves. For example, ERK1/2-dependent phosphorylation of the phosphatase MKP1 leads to stabilization of the protein and facilitates ERK1/2 dephoshorylation (Brondello et al., 1999). This finding has led to an increasing focus on the nucleus as an important site of ERK1/2 signal termination (Volmat et al., 2001; Pouyssegur and Lenormand, 2003).

2.2.2. Importance of both temporal and spatial pattern of ERK activation in physiological outcome and regulation

MAPK activation represents a point of convergence and integration in the information processing of the cell. For example, hormones and other ligands bind to a wide variety of

receptors and can lead to ERK1/2 activation, yet cellular responses to those different factors can vary from proliferation to differentiation. Thus, the information contained in the MAPK module must be more complex than a simple "on/off" switch. Information coding in the ERK1/2 pathway, and MAPK pathways in general, is the result of a complex interplay of both temporal and spatial patterning, as well as cross-talk with other signaling modules. One well characterized example of the dependence on the temporal pattern of ERK activation in the determination of biological outcome is the diametrically opposed effect of EGF and NGF in PC12 cells. In this system, EGF stimulates proliferation and causes a brief activation of ERK, while NGF induces differentiation and is characterized by a more prolonged, sustained pattern of ERK activation (Marshall, 1995). As mentioned above, the association of ERK1/2 with different anchors can alter the subcellular localization of active ERK, and ERK translocation is often critical for effects on gene transcription. For example, β-arrestin plays an important role in the signal transduction of several G-protein coupled receptors (GPCRs) by recruiting and maintaining ERK1/2 in the cytoplasm. Thus, receptors with higher affinities for β-arrestin lead to an increase in cytoplasmic ERK1/2 signaling, and a decrease in ERK1/2 nuclear translocation, upon activation when compared to receptors that operate independently of β-arrestin (Tohgoet et al., 2003). Recently, the identification of PEA-15 as novel, soluble, and cytoplasmic ERK1/2 binding protein has shed light on the complexities of ERK1/2 signaling. PEA-15 expression can block ERK translocation via direct protein-protein interactions without affecting the activity of ERK (Formstecher et al., 2001). Thus a complex interaction of signal intensity, signal duration, and signal localization within the cell, truly a three-dimensional integration, determines the specific information coded within the activation of a MAPK module.

2.2.3. Role of MAPKs during cellular stress

MAPKs are activated by numerous stressors, including oxidative stress, in a wide range of tissues including the heart, lungs, skeletal muscle, liver, kidney, and brain. Early reports linked such stressors as heat and osmotic shock, UV irradiation, and exposure to DNA-damaging agents or inflammatory cytokines with the activation of either JNK or p38 MAPKs (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997). Furthermore, early studies tended to associate ERK1/2 activation with cellular survival and p38 / JNK activation with cell death in a variety of model systems, including neurotrophin rescue of serum-starved PC12 cells (Xia et al., 1995) and H_2O_2 treatment of HeLa cells (Wang et al., 1998). This led to a fairly simplistic, but quite influential, view that the balance of activation between p38 / JNK and ERK regulated cell death in a directly opposing manner. It is now clear that the regulation of cell death by MAPKs is far more complex. More recent studies have demonstrated an important cytoprotective role of both p38 (Lee et al., 2003) and JNK activation (Andreka et al., 2001) in a variety of model systems, and conversely, and increasing number of studies have demonstrated ERK-dependent cell death (Stanciu et al., 2000; Chu et al., 2004). Thus the view that various classes of MAPKs have specific, predetermined roles in regulating cell death is quickly waning. The outcome of MAPK recruitment during cellular stress is more likely due to the complex interaction of MAPK members with scaffolds and the temporal and spatial pattern of activation, as discussed above, and can likely shift the information coded within a specific pathway between such diametric cell responses as cellular survival or death.

2.2.4. ERK signaling during neuronal stress – Neurotoxicity or Neuroprotection?

As would be predicted from the discussion above, ERK1/2-dependent signaling during stress-induced neurodegeneration has not consistently been shown to function solely as a pro-survival or a pro-cell death response. For example, the activation of ERK1/2 has been shown to underlie the protective effect of growth factor rescue of serum-starved neuronal cultures (Xia et al., 1995) and in an in vivo rat model of hypoxia-ischemia (Han and Holtzman, 2000). In several other models of toxicity, ERK1/2 activation has been shown to be necessary for neuronal survival, such as in the BDNF-mediated rescue of camptothecin-treated cortical neuronal cultures (Hetman et al., 1999).

However, an increasing number of studies have now demonstrated that ERK1/2 activation during neuronal stress is necessary for neurotoxicity. Glutamate-induced oxidative toxicity (Stanciu et. al., 2000; Levinthal and DeFranco, 2004), zinc toxicity (Seo et al., 2001), Fe / amyloid β toxicity (Kuperstein and Yavin, 2002), focal ischemia (Alessandrini et al., 1999; Noshita et al., 2002), traumatic brain injury (Mori et al., 2002), seizure-induced neurotoxicity (Murray et al., 1998), 6-hydroxydopamine toxicity (Kulich and Chu, 2001), methylisothiazolinone and Zn toxicity (Du et al., 2002), and okadaic-acid treatment of hippocampal slice cultures (Runden et al., 1998) have all been shown to display ERK-dependent neuronal cell death. However, it is interesting to note that the role of ERK1/2 recuitment can be different even within the same cell type, as forced activation of ERK1/2 protects HT22 cells from serum withdrawal, but not from glutamate-induced oxidative toxicity (Rossler et al., 2004).

The divergent roles of ERK1/2 in various neurotoxicity models are likely due to the heterogeneity in the temporal and spatial pattern of ERK1/2 activation and the manner in which this pattern alters the transcription of specific target genes. An emerging pattern appears to

associate transient ERK1/2 activation with neuroprotection (i.e. in growth factor rescue of neurons) and sustained, prolonged ERK1/2 activation with neurotoxicity. However, this assertion may be overly simplistic, and more work will be needed to firmly establish a given temporal and spatial profile of ERK activation with such specific and divergent functional outcomes. With the advent of gene array and interference RNA technology, studies of the differential patterns of gene expression that occur with such divergent profiles of ERK1/2 activation, combined with studies of the functional role of identified genes, is likely to contribute greatly to our understanding of role of ERK1/2 signaling during neurotoxicity.

2.2.5. Cross-talk with other pathways

The MAPK modules are integrated with other signaling pathways and engage in "cross-talk". In neurons, the activation of several pathways can be linked to the activation of ERK1/2. For example, in PC12 and hippocampal neurons, Ca^{2+} and PKA-dependent CRE-mediated gene transcription was shown to require ERK1/2 activation, and ERK1/2 translocation was dependent upon PKA activity (Impey et al., 1998). Furthermore, ERK1/2 activity can be dependent upon PKC activation as demonstrated in phorbol ester-induced dendritic spine formation in cultured hippocampal neurons (Goldin and Segal, 2003).

Phosphatidylinositol 3-kinase (PI3K) is an enzyme that phosphorylates the D3 position of various lipid inositols to form PI3, PI3,4, and PI3,4,5. PI3K activation is generally linked to both tyrosine kinase and G-protein coupled receptors, and the PI3K-dependent production of PI3,4,5 is required by the 3-phosphoinositide dependent kinase 1 (PDK1) to phosphorylate and activation of another important kinase, Akt. Thus in most instances, PI3K activity results in Akt phosphorylation, and these kinases comprise a well-described signaling module.

The PI3K-Akt and Raf-MEK-ERK pathways are functionally coupled in many instances, and across many cell types. Early reports found that PI3K can act as an intermediate in G-protein-associated signaling upstream of Ras activation and that PI3K activity was thus necessary for the eventual recruitment of the Raf-MEK-ERK pathway (Hawes et al., 1996; Lopez-Ilasaca et al., 1997). In neurons, there is much evidence to support significant crosstalk between the PI3K and ERK pathways in other non-G-protein mediated signaling events. For example, in striatal cultures, ERK signaling downstream of AMPA and NMDA receptor activation was shown to depend upon PI3K activity (Perkinton et al., 1999, 2002). PI3K was also demonstrated to be required for the NGF-induced sustained activation of ERK during PC12 differentiation (York et al., 2000). Furthermore, PI3K activation was shown to occur upstream of ERK activation in vivo in a rat model of associative learning (Lin et al., 2001). Finally, PI3K activity has been shown to be required for the recruitment of ERK in the absence of direct ligand-receptor interactions. For example, ERK1/2 activation due to hydrogen peroxide-induced oxidative stress in primary cortical neuronal cultures can be PI3K dependent (Crosswaite et al., 2002). Thus there appears to exist a mechanism for functional coupling of the PI3K and ERK pathways in neurons, with the potential for PI3K to act as a required upstream activator for the transduction of a variety of signals.

2.3. Phosphatases

2.3.1. Major classes of phosphatases

Phosphatases are enzymes that hydrolyze phosphorylated protein, lipid, and small molecule substrates and act in opposition to the multitude of kinases in the cell. Phosphatases

that are directed against protein substrates have received considerable attention due to their potential role in the regulation of cellular signaling events. Most protein phosphatases can be classified into one of three general groups according to their specificity of action. These groups are the Ser/Thr-, Tyr-, and dual-Ser/Thr and Tyr-directed phosphatases and are also referred to as protein phosphatases (PPs), protein tyrosine phosphatases (PTPs), or dual-specificity phosphatases (DUSPs or DSPs), respectively. Historically, the PPs were among the earliest to be identified (1970's), followed by PTPs (1980's), and finally, DSPs (1993 to the present), and thus the understanding of the regulation of these classes of enzymes is greatest for the PPs, while the mechanisms governing PTP and DSP regulation are only beginning to become clear.

2.3.2. Substrate specificity and role as important regulators of cellular signaling

PPs, PTPs, and DSPs act upon a wide variety of proteins targets that include structural elements, metabolic enzymes, transcription factors and general transcriptional machinery, receptors, ion channels, adapter proteins, and signaling molecules. However, specific phosphatases act on restricted subsets of targets, and the mechanisms governing this specificity of action are active areas of study. Most PTPs share a common modular structure in which regulatory and binding domains are localized in the N-terminal region, while the conserved catalytic domain is located in the C-terminal region. A targeted, physical interaction of these phosphatases with a substrate protein appears to lead to specificity for many PTPs, which display great heterogeneity in amino acid sequences in the N-terminal region. For example, the PTP SHP-2 contains an SH2 domain that specifically targets the phosphatase to phospho-tyrosine moieties on receptor tyrosine kinase molecules (Neel et al., 2003), thus linking the recruitment of these phosphatases to the auto-phosphorylation reactions that occur at receptor tyrosine kinases

when bound to ligand. For other PTPs of the membrane-bound, receptor class (i.e. the receptor protein tyrosine phosphatase α [RPTPα]), extracellular fibronectin-type and immunoglobulin domains are involved in the regulation of intracellular phosphatase domains (Denu and Dixon, 1998). For example, RPTPα can act as a transducer of mechanical force and extracellular matrix (ECM) contact by stabilizing α_v/β_3 integrin-cytoskeleton interactions (von Wichart et al., 2003), and this interaction leads to RPTPα-dependent dephosphorylation of phospho-tyrosine residues on Src kinase. However, RPTPα does not appear to directly bind ECM proteins but acts in cis with other membrane receptors (von Wichart et al., 2003). To date, the endogenous ligands for RPTPs have not been well characterized, and only one endogenous ligand has been identified for RPTPβ, the cytokine pleiotrophin (Meng et al., 2000). Recently, an intriguing study identified the *Helicobacter Pylori* vacuolating toxin VacA as a potent xeno-ligand for RPTPβ (also known as PTP-zeta) (Fujikawa et al., 2003). In this study, RPTPβ-deficient animals were shown to be insensitive to the formation of VacA-dependent gastric ulcers during *H. Pylori* infection, while gastric pleiotrophin administration in wild type animals caused ulceration. These findings clearly establish aberrant RPTP-β activation as a causative factor in the development of gastric ulceration, highlighting the importance of phosphatase dysregulation in pathology.

Within the PTPs and DSPs, an important domain referred to as the kinase-interaction motif (KIM) appears to confer great specificity to these enzymes and targets them to specific kinases, such as ERK1/2 and other MAPKs (Tanoue et al., 2002). Interestingly, KIM domains are found within multiple members of MAPK modules, including upstream activators (e.g. MEKs) as well as downstream effectors (e.g. MAPKAPKs), and appear to contribute to the affinity of these molecules for specific MAPK members (Tanoue and Nishida, 2003). The KIM domains of multiple MKPs have been identified, and due to the specificity of the protein-protein

interactions at this site, disruption of KIM-MAPK interactions are an intriguing site for therapeutic development using small peptide inhibitors. Table 1 shows the sequence of known KIM domains in several MEK/MKKs, MAPKAPKs, MAPK-directed phosphatases, and transcription factors, along with the specific MAPKs that interact with that site (adapted from Tanoue et al., 2000; Tanoue and Nishida, 2003). Note that all of these sites contain several basic residues that are necessary for binding (indicated in bold).

Class of Docking Protein	**Specific Protein**	**Kinase Docking Site**	**Specific MAPK Target**
MAPKK	MEK1	MP**KKK**PTPIQLNPNP	ERK
	MEK2	MLA**RRK**PVLPALTINP	ERK
	MKK3	**K**G**K**S**KRKK**DL**R**I	p38
	MKK6	S**K**G**KKR**NPGL**K**IP	p38
	SEK1/MKK4	QG**KRK**AL**K**LNF	JNK, p38
	MKK7	EA**RRR**IDLNLDISP	JNK
	MEK5	L**KK**SSAEL**RK**IL	ERK5
MAPKAPK	RSK1	SSILAQ**RR**V**RK**LPSTTL	ERK
	RSK2	**R**STLAQ**RR**GI**KK**ITSTAL	ERK
	RSK3	SSNLAQ**RR**GM**KR**LTST**R**L	ERK
	MNK1	**K**S**R**LA**RRR**ALAQAG**R**S**R**D	ERK p38
	MNK2	QS**K**LAQ**RR**Q**R**ASLSATPV	ERK
	MSK1	**K**APLA**KRRK**M**KK**TSTSTE	ERK, p38
	MAPKAPK2	NPLLL**KRRKK**A**R**ALEAAA	p38
	MAPKAPK3	N**R**LLN**KRRKK**QAGSSSAS	p38

	PRAK	NNPIL**RKRK**LLGT**KPK**DS	p38
	RSKB	NAPLA**KKRK**Q**K**L**R**SATAS	p38
PTP	EC-PTP	GLQE**RR**GSNVSLTLDM	ERK
	He-PTP	**R**LQE**RR**GSNVALMLDV	ERK
	STEP	GLQE**RR**GSNVSLTLDM	ERK
MKP	MKP1	**R**FSTIV**RRR**A**K**GA**K**GAG	ERK, p38, JNK
	MKP2	**R**CNTIV**RRR**A**K**GSVLE	ERK, p38, JNK
	MKP3	PGIML**RR**L**RK**GNLPI**R**	ERK
	MKP4	LPALLL**RR**L**RR**GSLSV**R**	ERK
	MKP5	CAD**K**IS**RRR**LQQG**K**TIV	p38, JNK
	Pyst2/MKP-X	PGLML**RR**L**RK**GNLPI**R**	ERK
	PAC1	PWNALL**RRR**A**R**A**R**GPP	ERK, p38, JNK
	B23	LNSVVL**RR**A**R**GGAVSA	ERK
	M3/6 / hVH5	S**K**LV**KRR**LQQG**K**VTI	p38, JNK
Transcription factor	MEF2A	NS**RK**PDL**R**VVIPPSS**K**	p38
	SAP-1	**R**S**KK**P**K**GLGLAPTLVIT	ERK, p38
	Elk-1	**K**G**RK**P**R**DLELPLSPSLL	ERK, p38

Table 1 MAPK-docking sites on MAPK interacting molecules

2.3.3. Mechanisms of catalysis

Two distinct mechanisms of catalysis are employed by the PPs and the PTP/DSPs. Dependence upon metal centers typify the PPs in general, and this metal center activates a water molecule in the active site to promote a one-step phosphate hydrolysis reaction. The PPs can be divided into PPP and PPM subclasses depending upon their dependence for various specific metals in the catalytic center. PPPs such as PP1, PP2A, and PP2B (calcineurin), are dependent upon a di-nuclear ion center that is composed of Mn, Fe and/or Zn in various combinations (Nishito et al., 1999), while PPMs, such as the PP2C-like phosphatases, are dependent on Mg and/or Mn (Zhou et al., 2002). The PTPs and DSPs both share a canonical $HC(X)_5R$ motif in their active sites, and the cysteine residue is absolutely critical for enzymatic function. Catalysis is dependent upon the formation of a stable phosphoryl-intermediate that is stabilized by surrounding residues, including an important, conserved arginine. Another conserved asparatic acid functions as a general acid/base to complete the hydrolysis reaction (Denu and Dixon, 1998). The binding pockets of PTPs have been shown to be ~9Å, while those of the DSPs tend to be shallow at ~6Å. This difference is thought to contribute to the differential specificity for tyrosine and serine/threonine phosphates – the shorter serine/threonine residues may not gain access to the deeper binding pocket of the PTPs and are therefore spared from hydrolysis (Denu and Dixon, 1998).

For many PTPs, physical interaction via the KIM domain of the phosphatase and the target protein is required for conformational changes in the phosphatase that significantly increase enzymatic activity. This mechanism appears to be fairly generalized across the MKPs (Farooq et al., 2003; Farooq and Zhou, 2004), but has been most studied with MKP3. For example, ERK2 binding to MKP3 via the KIM domain on MKP3 (Nichols et al., 2000) stabilizes

the active conformation of the active site cysteine (Farooq et al., 2001) and results in an approximately 100-fold increase in enzymatic activity (Fjeld et al., 2000). Interestingly, MKP3 appears to first dephosphorylate the phospho-threonine residue within dually phosphorylated T-X-Y motif on ERK2, dissociate, and subsequently reassociate with monophosphorylated ERK2-phoshotyrosine (Zhao and Zhang, 2001). Importantly, MKP3 has been found to engage in intramolecular dephosphorylation of ERK with a binding stoichiometry of 1:1 (Kim et al., 2003). Thus ERK-binding dependent increases in MKP3 activity result in the dephosphorylation of the same bound ERK molecule, and not a freely soluble secondary ERK molecule. These considerations have important implications for the negative regulation of ERK given that active ERK1/2 forms dimers in solution, which may be resistant to inactivation by MKP3. Actve ERK1/2 dimers translocate into the nucleus, and ERK inactivation may be driven by dimer dissociation and subsequent binding to MKPs.

2.3.4. MAPK-directed phosphatases

While protein phosphatases are directed against a vast array of targets, an increasing number of studies have focused on the regulation and physiological role in both normal and abnormal signaling events of a subset of phosphatases targeted to various MAPK members. These phosphatases, some of which are targeted only to specific MAPK family members, act as important negative regulators and can function in a negative feedback loop to limit MAPK activation. In a variety of cell types, ERK1/2 activity is regulated by several phosphatases including PP2A (Zhou et al., 2002), PTP-SL / He-PTP (Pulido et al., 1998; Nika et al., 2004), PTP-ER (Karim and Rubin, 1999), STEP (Pulido et al., 1998), and the cdc25s (Vogt et al., 2003), among numerous others. An emerging group of specific-MAPK phosphatases, or MKPs,

are DSPs that remove both phosphatases from the T-X-Y activation motif in the P-loops of several MAPK members. MKPs show differing specificities for the MAPKs, but some are exquisitely specific for only ERK1/2, such as B23, MKP3, and MKP-X (Mustelin et al., 2002). Table 2 lists some of the known MAPK phosphatases and their substrate specificities (adapted from Mustelin et al., 2002) that have activity against ERK1/2 and shows the various MAPK specificities of other identified MKPs.

While the expression patterns for the various MKPs have not been fully completely delineated, MKPs clearly have been shown to play important physiological roles in a wide variety of organ systems. Well described roles for MKPs are found within the hematopoetic and immune systems. For example, the PTP member He-PTP has specificity for ERK1/2, and is involved in signaling events in hematopoietic cells (Gronda et al., 2001), and a key component of the T-cell antigen receptor (TCR) signaling complex, ZAP-70, phosphorylates and activates the phosphatase VHR to downregulate ERK1/2 in response to ligand binding at the TCR (Alonso et al., 2003). In neurons, STEP is another PTP that plays an important role in NMDA receptor signaling (Paul et al., 2003). Furthermore, PTP-ER has been shown to be necessary for photoreceptor development via its regulation of ERK (Rintelen et al., 2003). Interestingly, both MKP1 and MKP3 expression is potently increased in the hippocampi of rats following global ischemia (Kawahara et al., 2004). Clearly, more studies of the tissue specific expression pattern of MAPK phosphatases will be needed to elucidate their role in both normal functioning and pathology.

MKP	Substrate	Subcellular Localization
MKP1	ERK, JNK, p38	Nucleus
MKP2	ERK, JNK, p38	Nucleus
MKP3	ERK	Cytoplasm
MKP4	ERK>JNK=p38	Cytoplasm>Nucleus
MKP5	JNK, p38α and β	Cytoplasm=Nucleus
MKP6	ERK, JNK	Cytoplasm
MKP7	JNK, p38α and β	Cytoplasm
MKP-X	ERK	Cytoplasm
PAC1	ERK, p38	Nucleus
B23	ERK	Nucleus
VHR	ERK, JNK	Cytoplasm
M3/6	JNK, p38	Cytoplasm=Nucleus
PTP		
He-PTP	ERK	Cytoplasm
EC-PTP	ERK	Cytoplasm
STEP	ERK	Cytoplasm

Table 2 MAPK substrate specificities and subcellular localization of various MKPs

2.4. Oxidation Mediated Inhibition of Phosphatases

2.4.1. Sensitivity of PTPs and DSPs to redox inhibiton and mechanisms of inhibition

Cellular redox conditions can have a profound effect on signal transduction pathways, and over the past several years, a growing body of work has identified a unique sensitivity of PTP and DSP function to changes in redox conditions. This linkage between ROS and signaling has led to a novel view of ROS as potential signaling molecules themselves in a mechanism now referred to as oxidative signaling. Thus oxidative conditions may lead to phosphatase inhibition and alter the steady state level of phosphorylation on important signaling molecules, such as kinases and transcription factors.

PTPs and DSPs share significant sequence homology in their catalytic sites, possessing the canonical $HC(X)_5R$ motif metioned above. Early studies of the effects of oxidation on phosphatase function identified the catalytic cysteine within the active site as being particularly susceptible to oxidation with a measured pK_a of 5.5 (Denu and Tanner, 1998). Thus, at physiological pH, the catalytic cysteine within these phosphatases is approximately 1000-fold more deprotonated than free cysteine (pK_a = 8.5) and ~300 times more deprotonated than the cysteine residues found in most proteins (pK_a ~8). This uniquely enables PTPs and DSPs to function effectively as redox sensors in the cell.

The mechanisms that govern redox modification of phosphatase function are varied. Oxidation of the catalytic cysteine is believed to progress through a step-wise series of oxidation reactions that begin with the interaction of H_2O_2 or other oxidants with the thiolate anion (i.e. $R\text{-}S^-$) to yield a potentially stable sulfenic acid (i.e. –SOH) intermediate. This modification is reversible by thiol reducing agents (Denu and Tanner, 1998). Further oxidation of the sulfenic

acid can yield sulphinic acid (i.e. $–SO_2H$) and sulphonic (i.e. $–SO_3H$) forms that are irreversibly oxidized. However, the sulfenic acid intermediate represents a point of divergence in redox-dependent phosphatase inhibition, as several different phosphatases display a variety of mechanisms of reversible oxidation. For example, several phosphatases can form a stable intramolecular disulphide between vicinal cysteines within the active site. This mechanism has been demonstrated in LMW-PTP (Chiarugi et al., 2001) and Cdc25C (Savitsky and Finkel, 2002). Oxidation dependent changes in the catalytic cysteine may also lead to siginificant conformational changes that affect function. The oxidation of the catalytic cysteine in RPTPα leads to a novel intramolecular interaction between the C-terminus and the spacer region to stabilize RPTPα dimers, resulting in enzymatic inactivation (Blanchetot et al., 2002). Finally, an intriguing stable-intermediate form of oxidized PTP1B was crystallized by two groups independently. In this protein, oxidation of the catalytic cysteine results in the formation of a stable sulphenyl-amide between the cysteine and the main chain nitrogen of the adjacent residue (Salmeen et al., 2003; van Montfort et al., 2003). It is difficult to predict which mechanisms predominate in a given phosphatase, but it appears that many phosphatases have evolved mechanisms to preserve function by enabling the formation of stable, oxidized intermediate forms that protect the enzyme from irreversible oxidation.

Much less is known about the mechanisms that govern oxidative inhibition of ser/thr protein phosphatases such as PP1, PP2A, PP2B. Recently, PP2A has been shown to be sensitive to H_2O_2 in both aging fibroblasts (Kim et al., 2003) and brain tissue (Foley et al., 2004). These studies both find that PP2A activity is inhibited by oxidative stress in a thiol-reducant reversible manner, implying that the oxidation of cysteine residues within the protein, or associated subunits, leads to inhibition. Studies using the bacteriophage λ protein phosphatase, a general

model for all protein phosphatase metalloenzymes, have shown that multiple combinations of metal species can exist in the metal center and are susceptible to subtle alterations in redox conditions (Reiter and Rusnak, 2004). Interestingly, PP1C-α was found to be sensitive to oxidation in vitro due to both thiol-oxidation and the oxidation of the metal center (Kim et al., 2003), while PP2B was similarly inhibited by oxidation of two vicinal cysteines as well as by identified redox changes (i.e. Fe^{2+}-Zn^{2+} oxidized to Fe^{3+}-Zn^{2+}) in the metal center (Bogumil et al., 2000; Namgaladze et al., 2002). Thus, both alterations in metal chemistry and thiol-oxidation are likely mechanisms by which oxidative stress may impact the activity of the PPs.

2.4.2. ROS mediated inhibition of phosphatases in vivo

ROS-mediated inhibition of phosphatase function may act within normal signaling events or can be involved in cellular pathology. The transduction of the growth receptors has been a model system for the study of localized ROS production and transient, reversible phosphatases inhibition. In Rat-1 cells, PDGF-treatment and PDGF receptor activation is associated with the brief production of ROS and a coincident inhibition of specific phosphatases, including SHP-2 (Meng et al., 2002). Phosphorylated ERK levels increased in a ROS-dependent manner, strongly suggesting that the inactivation of specific phosphatases plays an important and active role in this process. ROS-dependent inactivation of phosphatases has also been demonstrated in several model systems, including cell adhesion in several T-cell (Laakko and Juliano, 2003) and fibroblast cell lines (Chiarugi et al., 2003), ligand-induced Rho-GTPase dependent changes in actin cytoskeleton (Nimnual et al., 2003), and EGF-receptor activation in a breast cancer cell line (Reynolds et al., 2003). While innumerable studies have investigated ROS-dependent toxicity, abnormal recruitment of signaling pathways via ROS-dependent phosphatase inhibition has

remained unexplored. This mechanism could be an important feature of MAPK activation that occurs during oxidative stress, and phosphatases could very well be the redox sensors that initiate aberrant signaling in this context.

2.5. THE MODEL SYSTEM: Glutamate-induced oxidative toxicity

2.5.1. The X_c^- glutamate / cystine antiporter

Extracellular glutamate acts on neurons via several pathways that include binding to ionotropic receptors (i.e. kainate, AMPA, and NMDA receptors), metabotropic receptors (mGluRs), high affinity glutamate uptake transporters, and a chloride-dependent amino acid antiporter (X_c^-). The X_c^- system is ubiquitously expressed in mammalian cells and is involved in the exchange of intracellular glutamate for extracellular cystine, the oxidized, disulphide form of cysteine (Sato et al., 1999). Since cystine is rapidly reduced to free cysteine in the cytoplasm, and intracellular glutamate concentrations are much larger than outside the cell, there is a considerable concentration gradient that drives this exchange. Intracellular cysteine levels are dependent upon the activity of the X_c^- exchanger, and free cysteine is the rate-limiting precursor for glutathione synthesis. Therefore, the activity of the X_c^- exchanger directly controls glutathione levels in the cell, and inhibition of the exchanger, by increasing extracellular glutamate, leads to the depletion of intracellular glutathione. While both neurons and astrocytes express and utilize the X_c^- system, there are no reported differences in the efficiency of this system between these two cell types (Allen et al., 2002).

2.5.2. Glutamate-induced oxidative toxicity in HT22 and primary immature cortical cultures

Glutamate-induced oxidative toxicity is a useful model system for studying the effects of oxidative stress on cell death that relies upon the inhibition of the X_c^- system. Several cellular systems have been shown to be sensitive to the effects of extracellular glutamate via this mechanism, including HT22 cells (Behl et al., 1995; Li et al., 1997), primary immature cortical cultures (Murphy et al., 1990), mature enriched cortical neuronal cultures (Schubert and Piasecki, 2001), PC12 cells (Froissard et al., 1997), and C6 glioma cells (Mawatari et al., 1996). However, the analysis in some of these systems is confounded by the presence of ionotropic receptors and a potential for excitotoxic cell death. HT22 is a mouse hippocampal-derived cell line, immortalized with a temperature-sensitive SV-40 T antigen, which does not express ionotropic glutamate receptors. Thus, this cell line is uniquely suited for studying the effects of glutamate-mediated X_c^- inhibition and subsequent glutathione depletion. Similarly, immature primary cultures of embryonic cortical cells (DIV 1-4) show a specific sensitivity to glutamate-induced oxidative toxicity (Murphy et al., 1990). This is due to a delay in the expression of functional ionotropic glutamate receptors until DIV 7 or later, when greater than 80% of cultured neurons display inward currents in the presence of glutamate and other agonists, such as NMDA (Murphy and Baraban, 1990).

2.5.3. Signaling events during glutathione depletion in HT22 and primary immature cortical cultures

Glutamate-induced oxidative toxicity is characterized by a gradual depletion of intracellular glutathione levels that occurs over several hours. In HT22 cells, glutathione

depletion leads to the activation of 12-lipoxygenase (12-LOX) upstream of a massive influx of Ca^{2+} and subsequent ROS accumulation (Li et al., 1997). Similarly, toxicity in primary cortical cultures is also dependent upon the activation of 12-LOX and rises in intracellular Ca^{2+} (Li et al., 1997; Stanciu et al., 2000). The characteristics of the Ca^{2+} channels that are involved in Ca^{2+} influx have not been identified.

HT22 cells have remained a standard neuronal cell line for the study of glutamate-induced oxidative toxicity, but the signaling events that underlie toxicity in these cells remains somewhat controversial. Several studies have demonstrated that glutamate-induced oxidative toxicity is dependent upon the activity of the phospholipase C (PLC) isoform phosphatidylcholine-specific PLC (PS-PLC) and protein kinase C (PKC) (Li et al., 1998; Maher, 2001). However, while one study concluded that multiple MAPK members are involved in toxicity and that ERK may play a protective role (Maher, 2001), we have found that the development of glutamate-induced oxidative toxicity depends specifically upon the persistent activation of ERK1/2. This finding was perhaps the first reported finding of a neurotoxic role for ERK1/2 (Stanciu et al., 2000), and has been replicated in both HT22 cells and in immature primary cortical cultures (Satoh et al., 2000; Stanciu et al., 2000; Stanciu and DeFranco, 2002; Levinthal and DeFranco, 2004). Thus, MEK inhibitors such as U0126 protect both HT22 cells and primary cortical neurons from oxidative toxicity. The exact targets of ERK1/2 activation that are required for oxidative toxicity have not been identified.

3. THESIS GOALS

The work presented here was initiated with two broad research goals. Because glutamate-induced oxidative toxicity in both HT22 and immature primary cortical cultures

previously had been shown to be dependent upon the recruitment of ERK, I focused on studying factors that determined ERK activation in these cells during oxidative toxicity. Early studies with inhibitors revealed a unique coupling of the PI3K pathway to eventual ERK activation in primary immature cortical cultures and protection of neurons from oxidative toxicity via PI3K inhibition (Chapter 1). However, I also focused on the regulation of endogenous phosphatases during oxidative stress and the impact of overexpression of a specific ERK-phosphatase, MKP3, on neuronal oxidative toxicity.

First, I sought to determine whether oxidative stress altered the activity of phosphatases that are targeted to the ERK MAPK pathway, the biochemical nature of those phosphatases, and the potential mechanisms of redox modification. Due to the wealth of information implicating oxidative stress-induced phosphatase inhibition, I hypothesized that the persistent ERK activation observed during oxidative toxicity was due to the specific inhibition of ERK-directed phosphatases and a loss of negative feedback on the ERK MAPK signaling pathway. The results from these studies are presented in Chapter 2.

Second, I sought to manipulate ERK activity and subcellular localization to specifically determine whether ERK translocation to the nucleus was indeed required for neuronal oxidative toxicity. The ERK-specific phosphatase MKP3 previously had been shown to be a useful tool to inhibit the activity (MKP3 WT overexpression) and to force the cytosolic localization (MKP3 C293S overexpression) of ERK in a landmark study that characterized this approach in fibroblasts (Brunet et al., 1999). I hypothesized that MKP3 WT overexpression would be neuroprotective in both HT22 cells and primary immature cortical neurons by reducing the levels of phosphorylated, active ERK. Furthermore, studies in our laboratory suggested that ERK nuclear translocation was associated with oxidative toxicity in HT22 cells. Thus, I hypothesized

that the physical restriction of active ERK to the cytoplasm would directly impact the development of glutamate-induced oxidative toxicity by denying active ERK access to downstream nuclear targets. The data from these genetic manipulation studies is presented in Chapter 2.

4. CHAPTER 1: Transient Phosphatidylinositol-3 Kinase Inhibition Protects Immature Primary Cortical Neurons from Oxidative Toxicity via Suppression of Extracellular-Signal Regulated Kinase Activation

4.1. SUMMARY

Oxidative stress has been shown to underlie a diverse range of neuropathological conditions. Glutamate-induced oxidative toxicity is a well-described model of oxidative stress-induced neurodegeneration that relies upon the ability of extracellular glutamate to inhibit a glutamate / cystine antiporter, which results in a depletion of intracellular cysteine and the blockade of continued glutathione synthesis. Glutathione depletion leads to a gradual, toxic accumulation of reactive oxygen species (ROS). We have previously determined that glutamate-induced oxidative toxicity is accompanied by a robust increase in activation of the mitogen-activated protein kinase (MAPK) member extracellular-signal regulated kinase (ERK) and that this activation is essential for neuronal cell death. This study demonstrates that delayed ERK activation is dependent upon the activity of phosphoinositol-3 kinase (PI3K) and that transient, but not sustained, PI3K inhibition leads to significant protection of neurons from oxidative stress-induced neurodegeneration. Furthermore, we show that transient PI3K inhibition prevents the delayed activation of MEK-1, a direct activator of ERK, during oxidative stress. Thus, this study is the first to demonstrate a novel level of cross-talk between the PI3K and ERK pathways in cultured immature cortical neuronal cultures that contributes to the unfolding of a cell death program. The PI3K pathway, therefore, may serve opposing roles during the progression of oxidative stress in neurons, acting at distinct kinetic phases to either promote or limit a slowly developing program of cell death.

4.2. INTRODUCTION

Neuronal cell death due to oxidative stress links such diverse conditions as Parkinson's Disease, Alzheimer's Disease, Amyotrophic Lateral Sclerosis, and stroke (Coyle and Puttfarcken, 1993; Mattson et al., 2001). Glutamate-induced oxidative toxicity has become an excellent paradigm for studying the effects of oxidative stress in primary immature neuronal culture (Murphy et al., 1990; Ratan et al., 1994; Li et al., 1997; Stanciu et al., 2000). In this model, inhibition of a glutamate / cystine antiporter deprives cells of essential precursors for glutathione synthesis. An increased load of ROS results from this glutathione depletion (Murphy et al., 1990; Li et al., 1997) and activates intracellular signaling events that engage an apoptotic-like cell death program (Ratan et al., 1994).

The MAPK family member ERK is activated by a vast array of stimuli that impinge upon the cell and acts on a diverse range of cellular targets (Davis, 1993; Garrington and Johnson, 1999; Grewel et al., 1999). In neurons, ERK can function to either support cell survival or promote cell death. ERK was originally implicated in neuronal cell survival in differentiated PC12 cells that required neurotrophic factor support (Xia et al., 1995), and many subsequent studies have confirmed its contribution to neuronal cell survival in other systems (Bonni et al., 1999; Gonzalez-Zulueta et al., 2000; Han and Holtzman, 2000; Zhu et al., 2000). However, in many other models of neuronal cell death, ERK activation has been found to be associated with cell death (Murray et al., 1998; Alessandrini et al., 1999; Stanciu et al., 2000; Namura et al., 2001; Du et al., 2002; Mori et al., 2002; Noshita et al., 2002; Stanciu and DeFranco, 2002). The mechanisms that underlie such diametric effects of ERK are unclear but could be based on differences in both the temporal and spatial pattern of ERK activation induced by the various treatments (Stanciu and DeFranco, 2002).

The PI3K-Akt pathway has been found to consistently serve a pro-survival function in neurons exposed to various apoptosis-inducing stimuli (Datta et al., 1999). These effects are thought to occur via Akt-mediated inactivation of Bad (Datta et al., 1997), caspase-9 (Cardone et al., 1998), or members of the Forkhead transcription factor family (Bonni et al., 1999), among others. In neurons, there is much evidence to support significant crosstalk between PI3K and ERK (Perkinton et al., 1999; York et al., 2000; Lin et al., 2001; Perkinton et al., 2002; Crossthwaite et al., 2002), with the potential for PI3K to act as a required upstream activator of ERK.

We have previously demonstrated in immature primary cortical neurons that activation of ERK is necessary for glutamate-induced oxidative toxicity (Stanciu et al., 2000). We now demonstrate that this ERK activation is PI3K-dependent, and that transient, but not sustained, PI3K inhibition leads to significant protection of neurons. Thus, the PI3K pathway may serve opposing roles during the progression of oxidative stress in neurons, acting at distinct kinetic phases to either promote or limit a slowly developing program of cell death.

4.3. EXPERIMENTAL PROCEDURES

Primary Cortical Cultures Cortices from embryonic day 17 Spague-Dawley rat fetuses (Hilltop Lab Animals, Scottdale, PA) were dissected and manually dissociated by repeated trituration using fire-polished glass pipettes 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+} (Invitrogen, Carlsbad, CA) followed by passage through a 40 μm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) to remove clumped cells. Cells were counted and plated on 50 μg/ml poly-D-lysine treated culture plates at a density of ~2.1 x 10^4

cells / cm^2. Cell viability was routinely greater than 80% as assessed by uptake of trypan blue dye upon plating. Cultures were maintained for 3-4 days in media (DMEM (Invitrogen), 10% fetal calf serum (Hyclone, Logan, UT), 10% Ham's F12 Nutrient Supplement (Invitrogen), 1.9 mM glutamine, 24 mM Hepes Buffer, and 4.5 mg/mL glucose) at 37°C and 5% CO_2. All experiments were performed on DIV3-4 day old cultures to avoid the confounding effect of functional ionotropic glutamate receptor expression, which is not present in immature cultures, but which begins soon after this time period (Mizuta et al., 1998). At this time, these cultures contain approximately 20% $GFAP^+$ glial elements. Unless otherwise stated, all chemicals and reagents used were purchased from Sigma Chemical Corporation, St. Louis, MO.

Cell Line Culture HT22 cells, a hippocampal cell line that is particularly sensitive to glutamate-induced oxidative toxicity (Davis and Maher, 1994), were maintained in DMEM supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA), 100 Units Penicillin, and 100 µg/mL Streptomycin at 37ºC and 5% CO_2.

Cell Viability Eighteen hours following the initiation of all treatments, cultures were incubated for 10 minutes with 6.25 µg/mL propidium iodide (PI) to visualize dead or dying PI-positive cells. Cells were observed under an inverted fluorescence microscope equipped with phase contrast optics (Nikon Eclipse TE200), and PI-labeled and unlabeled cells were counted by a non-blinded observer. The percentage of labeled cells in each field was then calculated (approximately 200 cells per field at 400x). Three random fields were counted for each condition in at least three separate cultures.

Western Blot Analysis Cells were treated as described, scraped and collected into phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4), pelleted at 2-3 x 10^3 rpm for 5 minutes, and lysed in Lysis Buffer (50mM Tris-Cl, pH 7.5, 2mM EDTA, 100 mM NaCl, 1% NP-40, 100μM $NaVO_4$, 100 μM NaF, 2mM DTT) supplemented with 5μL protease inhibitor cocktail per milliliter of lysis buffer. Total protein concentrations were determined using the Bio-Rad™ Kit. Equivalent amounts of total protein (either 20 or 30 μg) were separated by SDS-PAGE on 10% polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were blocked with 5% dry milk in PBS / 0.1% v/v Tween-20 (PBST). Membranes were incubated with primary antibodies (anti-phospho ERK, anti-total ERK, anti-phospho Akt, and anti-total Akt, all from Cell Signaling, Beverly, MA) overnight at 4ºC with 3% dry milk or 5% BSA, washed 3x 10 minutes with PBST, and then exposed to the appropriate horseradish-peroxidase (HRP)-conjugated secondary antibody for one hour at room temperature. Membranes were again washed 3x 10 minutes with PBST, and immunoreactive bands detected by enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ) using standard x-ray film (Kodak, Rochester, NY). Several different exposure times were used for each blot to ensure linearity of band intensities.

MEK Kinase Activity Assay MEK kinase activity was determined via the ability of immunoprecipitated MEK to phosphorylate purified, unphosphorylated GST-ERK2 protein using ^{32}P-labelled ATP. In brief, 400 μg of total lysate protein from primary immature cortical cultures were pre-cleared with 50 μl of a 100 mg/mL stock of protein-A Sepharose beads (Amersham Biosciences) for 1 hour at 4ºC on a rotating shaker. The supernatants were then

immunocomplexed with a non-specific rabbit IgG antibody (FITC-conjugated rabbit anti-sheep) or a rabbit polyclonal antibody directed against the N-terminal region of MEK-1 (anti-MEK1 NT; Upstate, Lake Placid, NY) overnight at 4ºC on a rotating shaker. The immunocomplexes were absorbed to 80 µL of the 100 mg/mL stock of Protein-A Sepharose beads for 2 hours at 4ºC on a rotating shaker, and subsequently washed twice with ice-cold Lysis Buffer (see above). The pelleted beads were resuspended in 50 µl Kinase Buffer (Lysis Buffer + 50mM $MgCl_2$ and 100 µM ATP) supplemented with 10 µCi ^{32}P-labelled ATP (Amersham) and 0.250 µg non-phosphorylated GST-ERK2 (Upstate) per reaction. The kinase reaction was maintained at 37 ºC for 25 minutes and terminated by pelleting the Sepharose beads, and adding 10 µL 6x Laemmli Buffer to the individual supernatant fractions, which were then placed on ice. After boiling for 10 minutes, 20 µl of each of the samples was loaded onto a 10 % polyacrylamide gel and electrophoresed at 150 V for 1 hour. Autoradiographic exposures of the gels were performed for 4-12 hours at -80ºC.

Indirect Immunofluorescence Cortical cells were plated directly onto 0.05 mg/mL poly-lysine coated 12-well tissue culture plates. Cells were treated with or without 5 µM cytosine-arabinoside (Ara-C) at DIV2. On DIV4, cells were washed twice with PBS, fixed in 4% paraformaldehyde for 18 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, and blocked for 1 hour with PBS supplemented with 10% goat serum (Invitrogen) at room temperature. The fixed cells were incubated with anti-GFAP (1:500) in PBS with 10% goat serum overnight at 4˚C and washed three times with PBS before incubation with a fluorescent-labelled secondary antibody (1:1000 anti-rabbit IgG conjugated to Alexa Fluor 488; Molecular Probes, Eugene, OR) and the nuclear stain DAPI (1:1000). Images were viewed with a Zeiss

Axiophot inverted fluorescence microscope, and the number of glial cells was counted in three random fields and calculated as a percentage of total cells.

Detection of intracellular ROS Intracellular ROS was measured as previously described (Hsieh et al., 1998). Primary cortical cells were treated as described, and the dye DCFH-DA (50 μM) (Molecular Probes, Eugene, OR) was added for 1 hour to establish a stable intracellular level of the probe. DCFH-DA is taken up by cells, where it is converted by esterases to DCFH, and is, in turn, oxidized to DCF in the presence of ROS. The extent of increases in the DCF fluorescence signal is therefore correlated with increases in intracellular oxidative stress. After one hour, cells were washed with HBSS, scraped from their plates, and measured for DCF fluorescence intensity (excitation 475, emission 525) in a fluorometer (Wallac Victor^2, Perkin Elmer, Wellesley, MA). Cell counts from each sample were used to normalize the DCF signal intensity. A 500 μM dose of H_2O_2 (2 hours) was used as a positive control for the detection of oxidative stress. Data were expressed as the fold-change in DCF signal intensity compared to control cells within each independent experiment.

Statistics One-way ANOVA was performed with Bonferroni's post-hoc correction for multiple comparisons. P values less than 0.05 were taken to be significant.

4.4. RESULTS

4.4.1. LY294002 Protects Primary Mixed Cortical Cultures from Glutamate-Induced Oxidative Toxicity

Primary immature cortical neuronal cultures (DIV 3-4) undergo an apoptotic cell death within 24 hours of exposure to 5 mM glutamate (Ratan et al., 1994). This glutamate-induced oxidative toxicity has been used extensively as a model for oxidative stress in neurons (Murphy et al., 1990; Ratan et al., 1994; Li et al., 1997; Stanciu et al., 2000). We have previously determined that glutamate-induced oxidative toxicity can be abrogated in cortical cultures by administration of the MEK inhibitor U0126 (10 μM) (Stanciu et al., 2000). Mixed cultures of immature cortical cells (DIV3) were either left untreated, treated with 5 mM glutamate, or treated with 5 mM glutamate administered with 10 μM U0126 for 24 hours. As shown in Figures 2A and 2B, U0126 protects these cells from glutamate-induced oxidative toxicity. Treatment with U0126 alone did not affect cell viability or morphology (data not shown). To examine the role of PI3K-Akt in glutamate-induced oxidative toxicity, we used various doses of the specific PI3K inhibitor, LY294002 (K_i = 1.4 μM). Because the PI3K-Akt signaling pathway has been associated so reliably with neuroprotection in a wide variety of neurotoxicity models, we anticipated that the administration of LY294002 would not be able to prevent glutamate-induced oxidative toxicity. Surprisingly, LY294002 conferred significant neuroprotection against glutamate-induced oxidative toxicity at doses ranging from 10 μM to 40μM (Figure 2B, 20 μM and 40 μM doses shown).

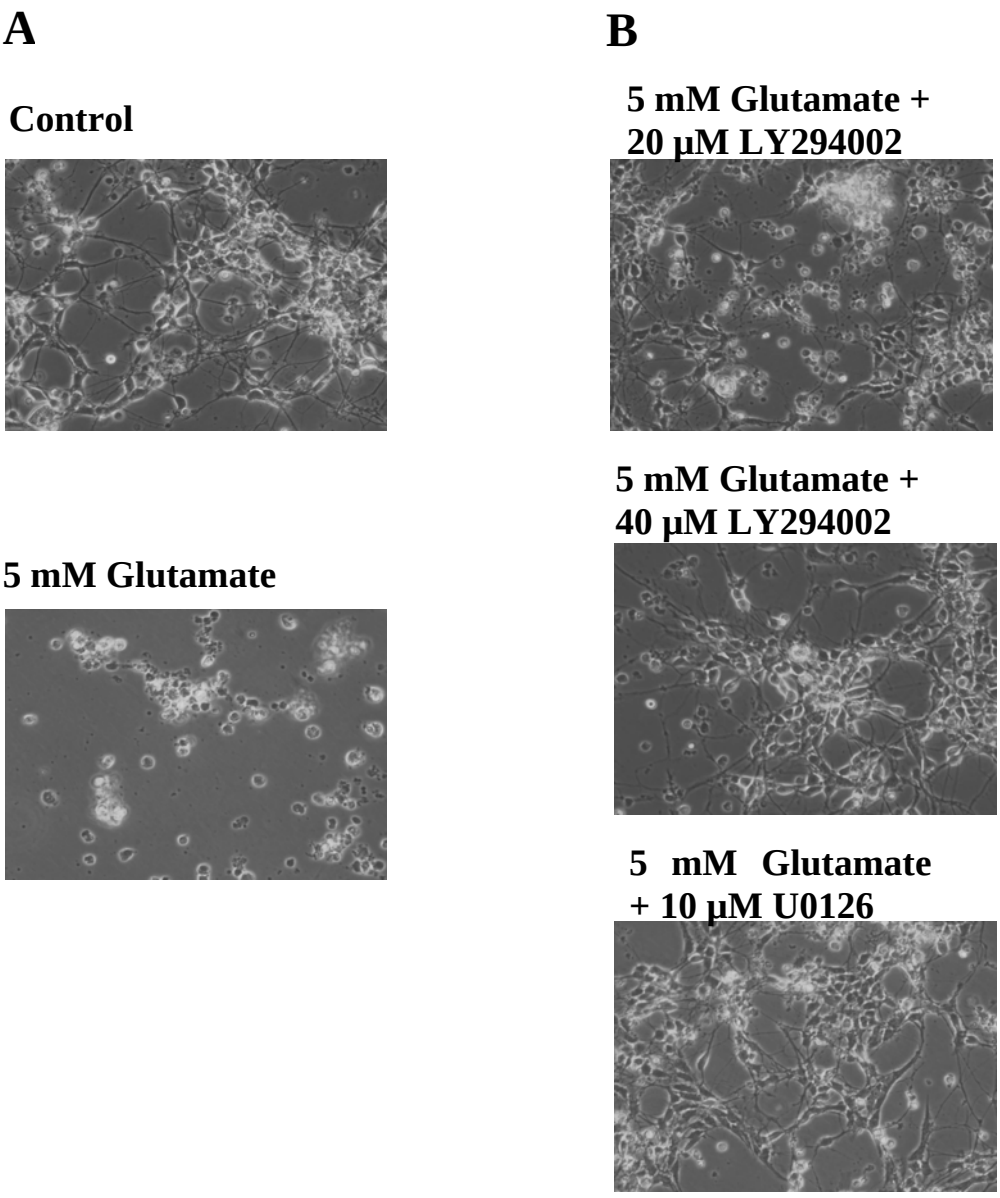


Figure 2 Representative phase-contrast images (400x magnification) demonstrating glutamate-induced oxidative toxicity in primary cortical neurons and neuroprotection via PI3K or MEK-1 inhibition. A, Immature primary cortical cultures (DIV3) were either left untreated or treated with 5 mM glutamate for 24 hours. B, Cultures were treated with glutamate plus either 20 µM LY294002, 40 µM LY294002, or 10 µM U0126. Both compounds led to significant neuroprotection from glutamate-induced oxidative toxicity, as assessed 24 hours after the initiation of glutamate treatment.

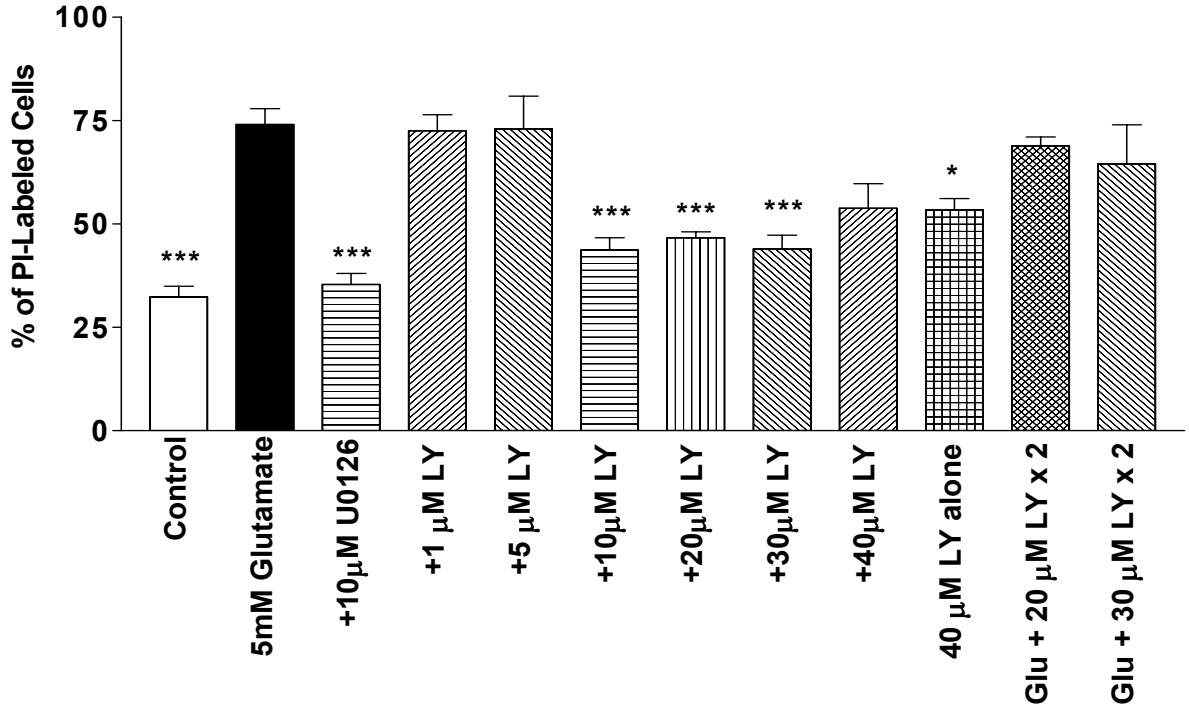
To quantify the ability of these inhibitors to abrogate glutamate-induced oxidative toxicity, we employed the PI-uptake assay, which utilizes the property of this fluorescent dye to be taken up selectively by dying or dead cells, but not by viable cells. PI uptake has been demonstrated in cells ungoing either necrosis or apoptosis and thus does not identify a specific cell death mechanism. Nonetheless, it is a sensitive measure of membrane integrity and is a marker of cell health. PI labeled and unlabeled cells were counted in three fields per experimental condition in at least three separate cultures 18 hours after the initial exposure to glutamate. At this time point, cells destined to die have begun to retract their processes, have shrunken cell bodies, and display condensed nuclei. The vast majority of cells treated with glutamate display all of these features within 20-24 hours and become detached from the plate soon after this time. Figure 3 demonstrates the dose dependency for the neuroprotective effects of LY294002. Doses of LY294002 ranging from 1 μM to 5 μM did not abrogate glutamate-induced oxidative toxicity, whereas higher doses (e.g. 10-30 μM) significantly protected cells when compared to glutamate treated cultures ($p < 0.001$). The administration of 20 μM alone did not lead to cell death (Figure 5C), but 40 μM LY294002 alone exhibited some toxicity (Figure 3A). Wortmannin, which inhibits PI3K through a different mechanism than LY294002 but is much less stable in aqueous solution (Walker et al., 2000), also protected against glutamate-induced oxidative toxicity and was not toxic when administered alone (Figure 3C).

To examine whether a subthreshold 0.5 μM U0126 dose (i.e., a dose that did not by itself protect neurons from glutamate-induced oxidative toxicity; Figure 3B) could potentiate the neuroprotective effect of LY294002, 0.5 μM U0126 and increasing doses of LY294002 (1 μM – 40 μM) were co-administered to cortical cultures with 5 mM glutamate. In the presence of this subthreshold dose of U0126, the minimum dose of LY294002 that was found to be significantly

neuroprotective was 5 μM, half the dose than that found to be effective in the absense of U0126 (Figure 3B). These results suggest that some crosstalk between the PI3K-Akt and MEK-ERK pathways may be operating in these neurons undergoing oxidative stress.

The manner in which LY294002 was administered was critical for its neuroprotective effect. For example, one bolus of inhibitor (30 μM) given at the initiation of glutamate treatment led to protection from toxicity (Figure 3A). However, administering one 20 μM dose of LY294002 at the initiation of glutamate treatment and a subsequent 20 μM dose 6 hours later completely abolished the neuroprotective effect of the drug (Figure 3A). This phenomenon was also observed for an analagous 30 μM - 30 μM LY294002 dosing schedule (Figure 3A). The administration of two 20 μM doses of LY294002 six hours apart by itself was not toxic to the cells (Figure 5C). Furthermore, delaying the administration of LY294002 by 6 hours after the addition of glutamate abolished any protective effect of the compound (Figure 3C). This evidence would suggest that some critical PI3K-dependent event occurs within 6 hours, but not later, and that this event is necessary for glutamate-induced oxidative toxicity.

A


% of PI-Labeled Cells
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75
50
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Control
5mM Glutamate
+10μM U0126
+1 μM LY
+5 μM LY
+10μM LY
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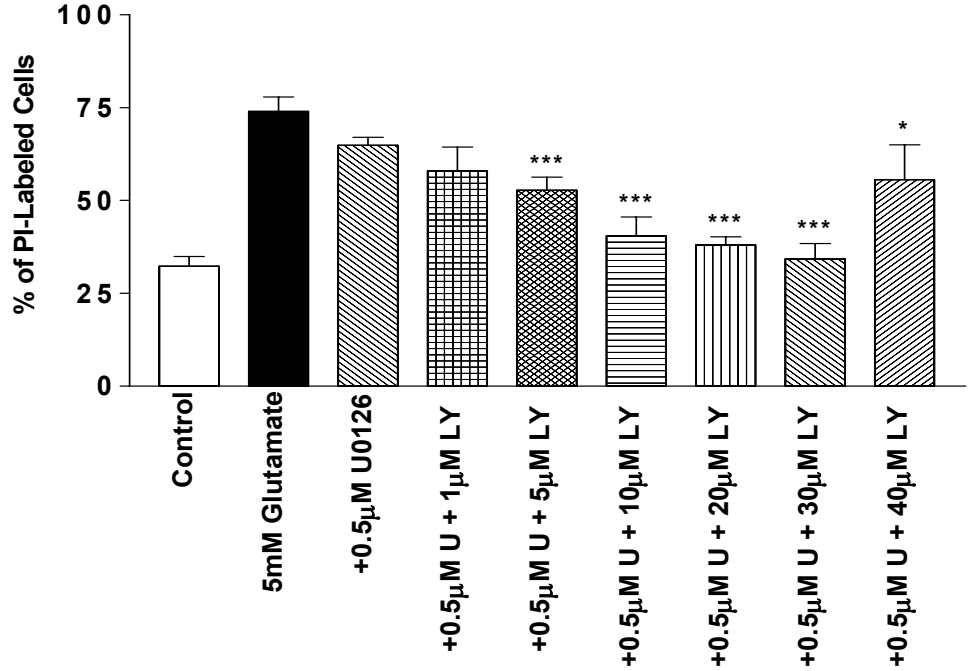
+30μM LY
+40μM LY
40 μM LY alone
Glu + 20 μM LY x 2
Glu + 30 μM LY x 2

B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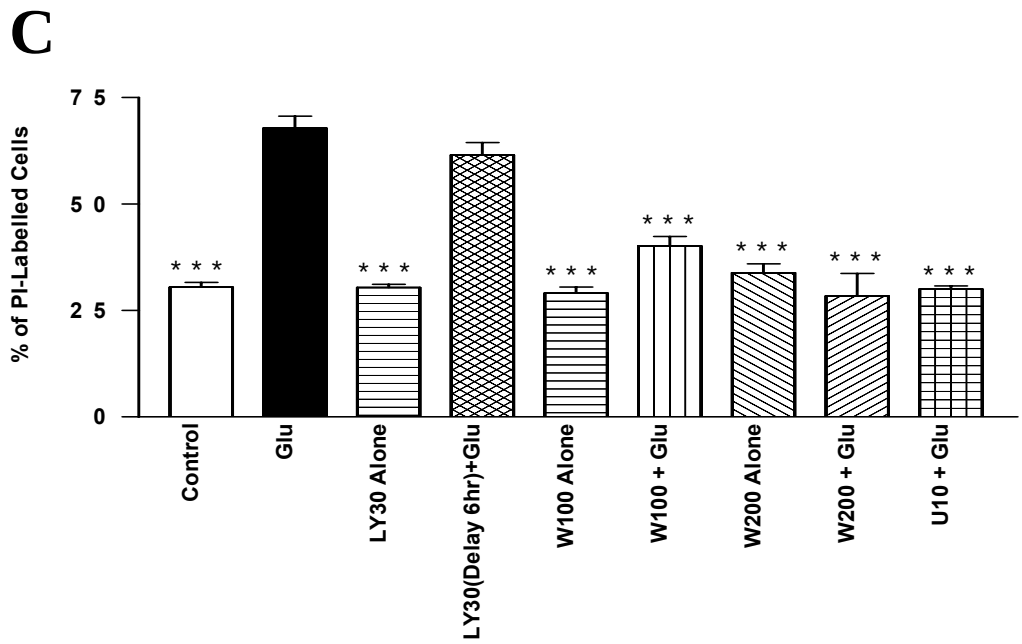


Figure 3 The neuroprotective effect of LY294002 is dose-dependent, and concurrent inhibition of both MEK and PI3K can potentiate neuroprotection from glutamate-induced oxidative toxicity. *A*, Primary immature cortical cultures (DIV 3) were treated with 5 mM glutamate and increasing single doses of LY294002 (1 μM to 40 μM), two doses (20 μM or 30 μM) of LY294002 each administered six hours apart (i.e. one with the initial treatment of glutamate and another six hours later), 10 μM U0126, or 40 μM LY294002 alone. Eighteen hours after initial treatment, cells were incubated for 10 minutes with propidium iodide (PI) to visualize dead or dying PI-positive cells using an inverted fluorescence microscope equipped with phase-contrast optics. Both PI-positive and PI-negative cells were counted, and the percentage of PI-positive cells was calculated for three random fields in at least three separate cultures. LY294002 exihibited significant protection at doses of 10 μM or higher, while giving two separate doses of LY294002 abrogated its protective effect. All comparisons were made relative to glutamate treatment alone. *B*, Subthreshold doses of U0126 can potentiate the protective effect of LY294002. A subthreshold dose of U0126 (0.5 μM) alone, or with increasing doses of LY294002, was administered at the initiation of 5 mM glutamate treatment. While 0.5 μM U0126 did not significantly protect cultures from glutamate toxicity, the lowest significantly neuroprotective dose of LY294002 was 50% lower (i.e. 5 μM vs. 10 μM) in the presense of 0.5 μM U0126. All comparisons were made relative to glutamate treatment alone. *C*, Wortmannin Protects Primary Cortical Cultures from Oxidative Toxicity. Either a 100nM or 200nM dose of wortmannin was given alone or concurrently with glutamate, and toxicity was measured using PI. Both doses significantly protect neurons from cell death, and neither is toxic alone. Interestingly, a 6 hour delay in the administration of LY294002 (30 μM) abolishes its ability to protect neurons from oxidative toxicity. All comparisons were made relative to glutamate treatment alone. * = $p<0.05$ and *** = $p<0.001$; U=U0126, LY=LY294002, W=Wortmannin.

4.4.2. Both LY294002 and Wortmannin Only Transiently Inhibit PI3K in Mixed Cortical Cultures

To confirm that LY294002 and wortmannin indeed inhibited the PI3K-Akt pathway in our cultures, we administered the drugs for various amounts of time and monitored levels of phosphorylated Akt as an output measure of PI3K activity. As shown in Figure 4A, in primary immature mixed cortical cultures, a single LY294002 treatment (20 μM) only transiently inhibited Akt phosphorylation. This dose had conferred significant neuroprotection from glutamate-induced oxidative toxicity (Figure 1B, Figure 3A). This was similarly observed in wortmannin-treated cultures (Figure 4B). Consistently, phosphorylated Akt returned to baseline levels within about 6 hours after the initial administration of both drugs. Thus, LY294002 and wortmannin administration to mixed primary cortical cultures both inhibit PI3K transiently and have no long term effect (i.e., beyond 6 hours) on the PI3K-Akt pathway. Figure 3 shows representative Western blots from at least 3 independent experiments.

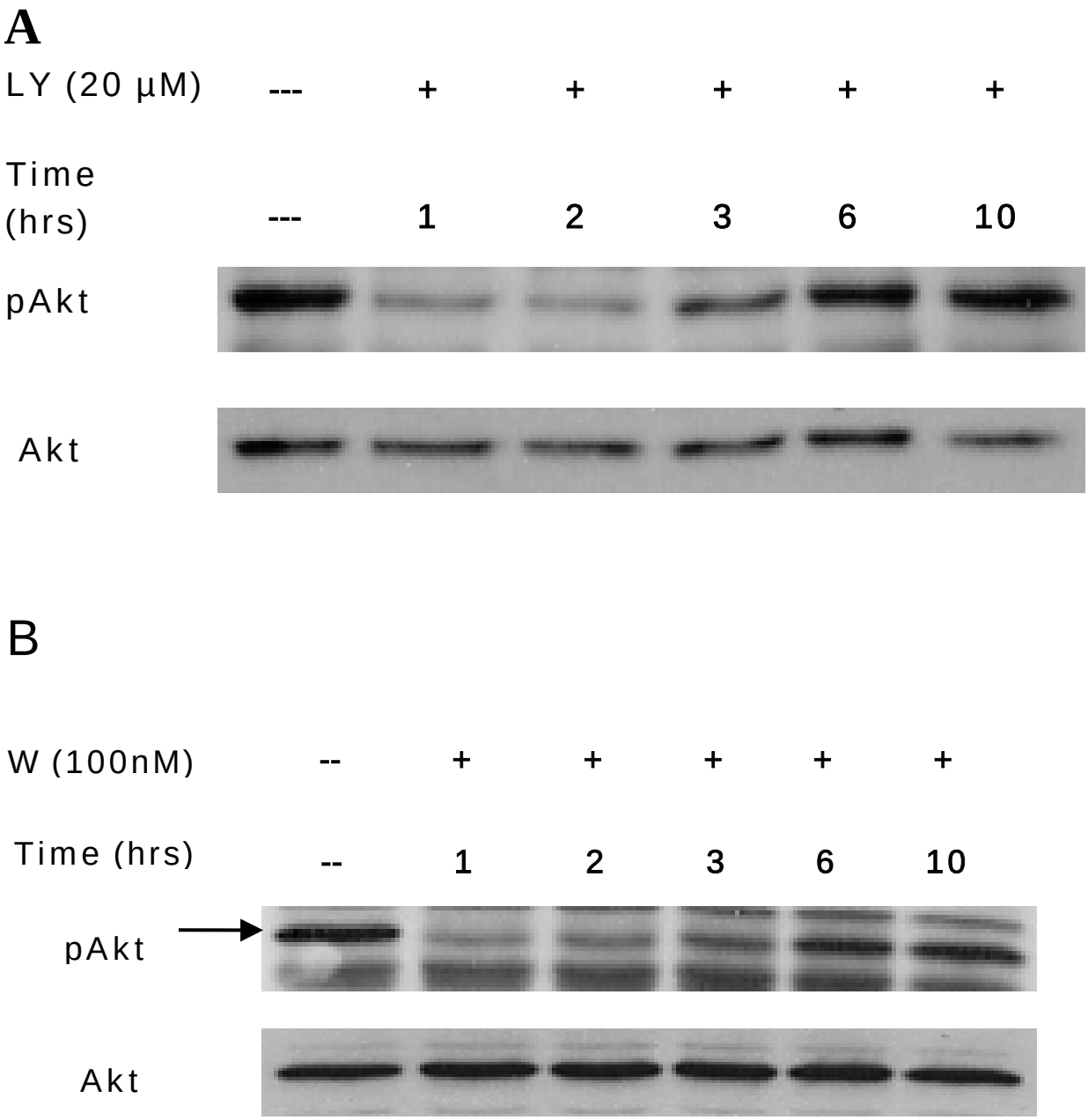
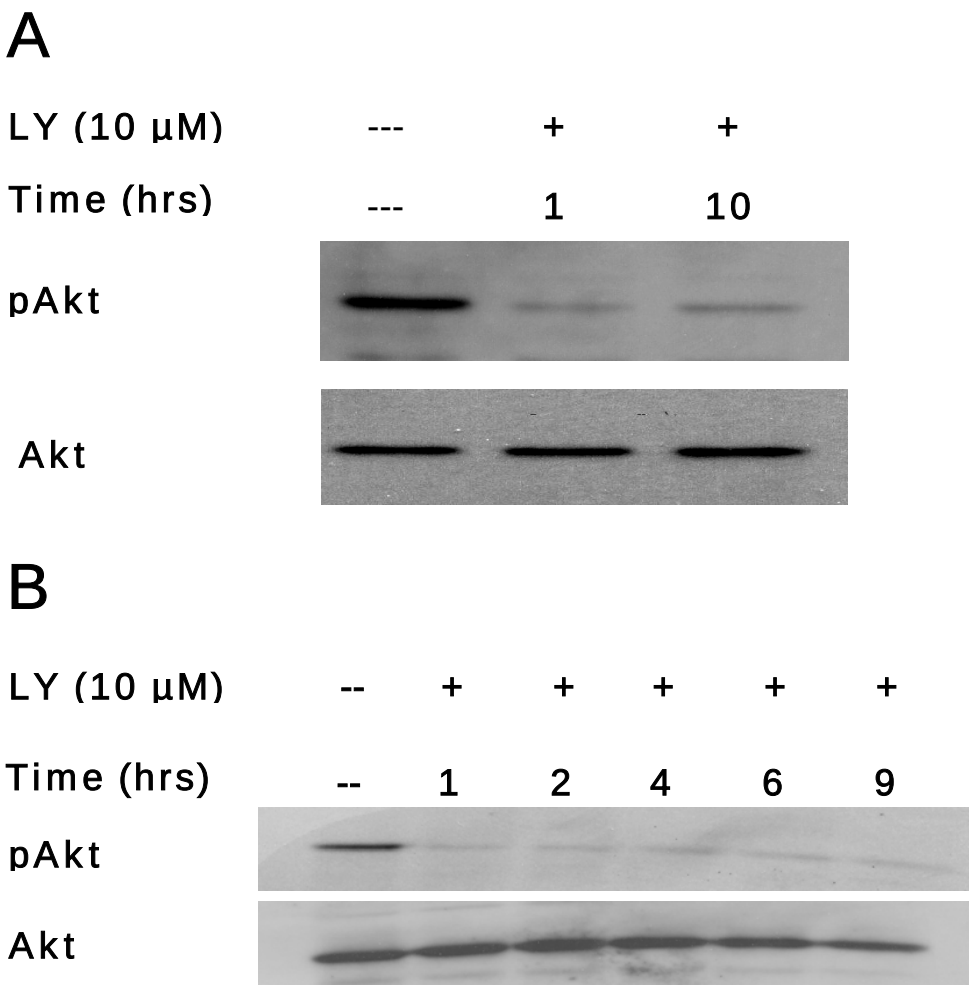


Figure 4 Both LY294002 and wortmannin only transiently down-regulate phosphorylated Akt in primary immature cortical cultures. Representative Western Blot (n=3) performed using whole cell lysate protein (20 µg per lane) from cultures treated for 1, 2, 3, 6, or 10 hours with LY294002 (20µM) (*A*), wortmannin (100nM) (*B*) or from untreated control cultures. Blots were probed with anti-phospho-Akt specific antibodies, stripped, and reprobed with anti-total Akt antibodies. LY = LY294002, W=Wortmannin.

4.4.3. LY294002 Is Sequestered or Metabolized by Glial Cells in Mixed Cortical Cultures

Because LY294002 is known to be stable in aqueous solution, the apparent loss after 6 hours of its efficacy to inhibit PI3K action on one of its downstream targets (i.e., Akt) would be consistent with metabolism or sequestration of the compound. To further explore this phenomenon of transient PI3K inhibition in our culture system, we asked whether the presence of glial elements in the system was responsible for this apparent LY294002 inactivation. Our cultures typically contain about 20-25% GFAP-positive cells at DIV 3-4, as determined by immmunocytochemical staining (20.3% +/- 7.2 SEM). The anti-mitotic agent cytosine arabinoside (Ara-C) was added to the mixed cultures, and LY294002 administered 2 days later, after the vast majority of glial cells were eliminated. The efficacy of Ara-C in eliminating GFAP-positive cells was verified by immunocytochemistry (i.e., fewer than 1% of cells were GFAP-positive). As shown in Figure 5A, a single administration of 20 μM LY294002 to these enriched neuronal cultures is very effective in inhibiting PI3K activity for a prolonged period of time (i.e., 10 hours). This implies that the transient inhibition of PI3K activity in mixed cultures given a single dose of LY294002 may be due to glial metabolism or sequestration of the drug. To further test this hypothesis, we administered a lower dose of LY294002 (10 μM) to the hippocampal cell line, HT22. These cultures are devoid of glial cells, and therefore we expected LY294002 to have a prolonged effect in inhibiting PI3K. Indeed, as shown in Figure 5B, a single dose of LY294002 (10 μM) was sufficient to inhibit PI3K for extended periods of time. Furthermore, low doses of LY294002 are toxic to HT22 cells (data not shown), which confirms that prolonged maintenance of PI3K activity plays an important role in neuronal cell survival.

To further confirm that the maintenance of PI3K activity is important to the survival of primary cortical neurons, we measured the viability of cells in Ara-C-treated, neuron-enriched cultures following treatment with LY294002. As shown in Figure 5C, 20μM LY294002, administered in one single dose, or two doses six hours apart, to mixed cortical cultures shows no toxic effect. However, in neuron-enriched cultures, single doses of LY294002 ranging from 10 μM to 30 μM showed significant neurotoxicity (Figure 5C, $p < 0.001$). Thus prolonged PI3K inhibition alone is sufficient to undermine cell survival in our cultures.



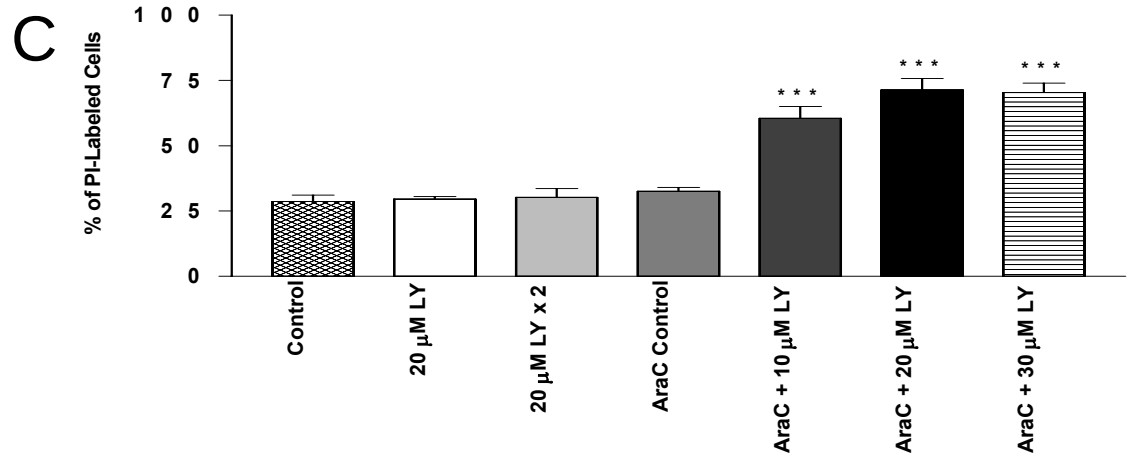
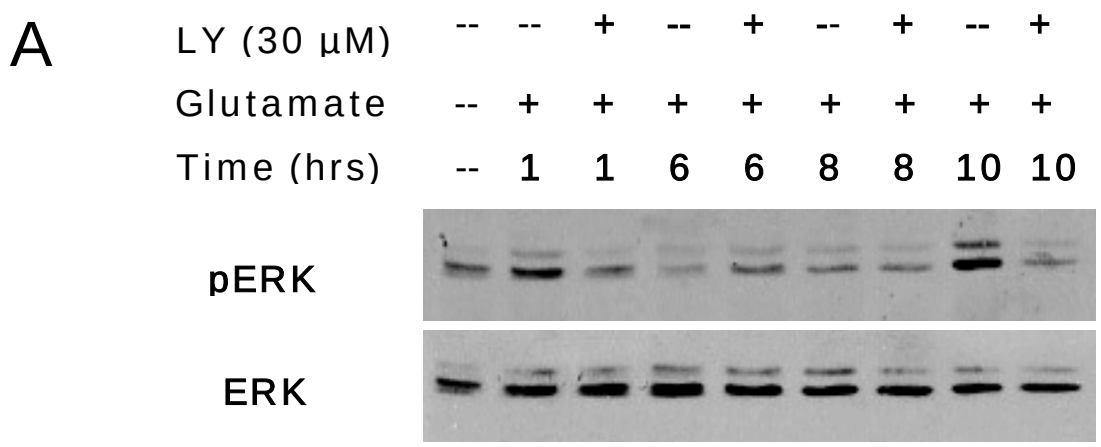


Figure 5 LY294002 is metabolized or sequestered by glial cells in mixed cortical cultures. Western Blots were performed using whole cell lysate protein (20 μg per lane) from neuron-enriched cultures and HT22 cells (a hippocampal cell line). Blots were probed with anti-phospho-Akt specific antibodies, stripped, and reprobed with anti-total Akt antibodies. *A*, The transient inhibition of PI3K (as assessed by phosphorylated Akt) observed in mixed cortical cultures is not seen in neuron-enriched cultures. Neuron-enriched cultures were obtained by treating mixed cultures (DIV 2) with 5 μM cytosine-β-D-arabinofuranoside (Ara-C) for two days. These cultures were then treated with a 20 μM dose of LY294002 for either one hour or ten hours, and whole cell lysate protein was collected for Western Blot analysis. *B*, LY294002-induced PI3K inhibition is prolonged in HT22 cells. HT22 cells were treated with 10 μM LY294002 for 1, 2, 4, 6, or 9 hours, and whole cell lysates were collected for Western Blot analysis. *C*, The toxicity of various doses of LY294002 in mixed- and neuron-enriched cortical cultures was assessed by PI-uptake (see Figure 2) eighteen hours after treatment. Neither 20 μM LY294002 administered in one single dose or in two repeated doses six hours apart were toxic to mixed-cortical cultures. However, single doses of LY294002 (10 μM – 30 μM) were significantly toxic to neuron-enriched cultures. *** = $p < 0.001$; LY=LY294002; Ara-C = neuron-enriched cultures.

4.4.4. Transient, Early PI3K Inhibition Prevents the Development of a Delayed ERK Activation

We have previously determined that glutamate-induced oxidative toxicity is associated with a delayed chronic activation of ERK1/2 and that this activation is necessary for neuronal cell death (Stanciu et al., 2000) (Figures 2 and 3A). In cortical cultures, we typically observe this delayed activation 10 hours or more after the initiation of glutamate treatment, which correlates with the times of increasing oxidative stress in these cells (Li et al., 1997) (Figure 8). We therefore asked whether LY294002 or wortmannin, both of which protect these cultures from glutamate-induced oxidative toxicity (Figures 2 and 3), interfere with the development of this delayed ERK1/2 activation. As shown in Figure 6A, 30 μM LY294002, a dose that significantly protected cortical neurons, is effective in preventing the delayed activation of ERK1/2. Furthermore, treatment with either 100 nM or 200 nM wortmannin showed attenuation of the ERK activation measured 10 hours after glutamate administration (Figure 6B). Interestingly, a 6 hour delay in the administration of LY294002 after glutamate, a protocol that failed to protect cortical neurons from oxidative toxicity (Figure 3C), abolished the ability of the compound to prevent the later ERK activation seen during oxidative toxicity (data not shown). Thus, only an early, transient inhibition of PI3K activity in immature cortical neurons can inhibit the delayed activation of ERK that accompanies glutamate-induced oxidative stress.



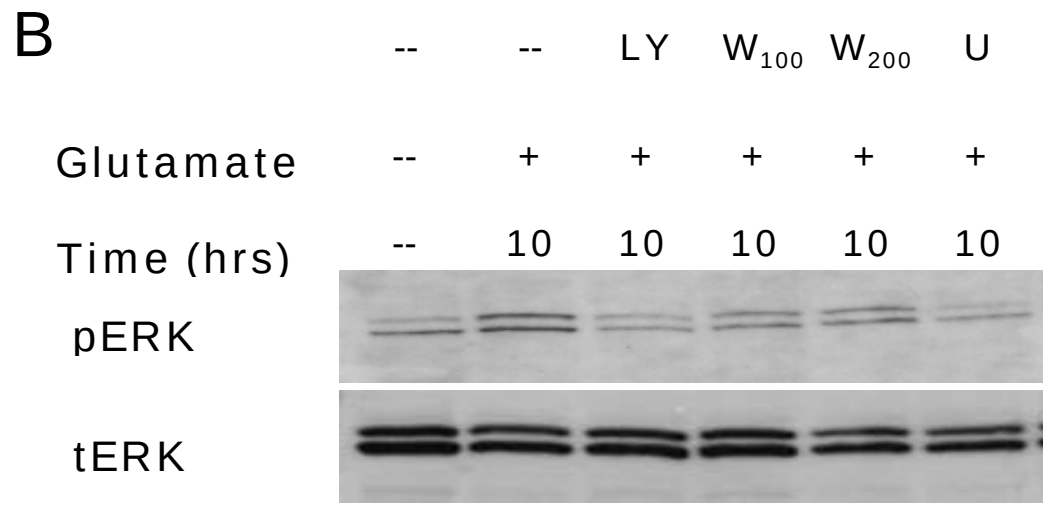


Figure 6 PI3K inhibition prevents the delayed activation of ERK in glutamate-treated immature primary cortical cultures. *A*, 30 μM LY294002 prevents the delayed activation of ERK due to 5 mM glutamate exposure. Whole cell lysates from cultures treated with glutamate and with or without 30 μM LY294002 for varying amounts of time were compared to control lysates. ERK activation is typically observed 10 hours or later after glutamate treatment in this system. *B*, Both 100 nM and 200 nM doses of wortmannin attenuate ERK activation at 10 hours. Blots were probed with anti-phospho ERK antibodies, stripped, and reprobed with anti-total ERK antibodies (20 μg of total protein were loaded per lane). A representative blot (n=4) is shown. LY=LY294002, W=Wortmannin.

4.4.5. PI3K Inhibition Prevents the Delayed Activation of MEK-1

PI3K has been found to exert either a stimulatory or an inhibitory effect on ERK activation depending, in part, upon the identity and strength of the applied extracellular stimulus (Duckworth and Cantley, 1997; Wennstrom and Downward, 1999; Moelling et al., 2002). In cases in which PI3K is necessary for ERK activation, B-Raf appears to be a major conversion point for these two pathways, especially in neurons (York et al., 2000). Because MEK-1 is the major target of all Raf isoforms, we used an immunoprecipitation (IP)-kinase assay to monitor changes in MEK-1 activity in extracts prepared from primary neurons treated with glutamate in the presence or absence of LY294002. In this way, we could determine whether PI3K acted at the level of ERK or on upstream kinases, such as Raf / MEK-1, during the development of the PI3K-dependent, delayed ERK activation. Previous studies have implicated PI3K activity in MEK-independent ERK activation. Interestingly, in one study of PDGF stimulated fibroblasts, ERK activation was shown to follow a biphasic pattern with a brief rise and fall in activity followed by sustained activity (Grammer and Blenis, 1997). The early rise in ERK activity was MEK-dependent, but the delayed, sustained activation was PI3K dependent and MEK-independent (Grammer and Blenis, 1997). This would imply that PI3K dependent signaling events can potentially lead to direct ERK activation.

As is shown in Figure 7, glutamate-induced oxidative toxicity in primary immature cortical cells leads to the delayed recruitment of active MEK-1 at 10 hours, which is consistent with the observed onset of ERK activation. This result is not surprising given that the MEK-1 inhibitor U0126 was previously shown to prevent the development of ERK activation at this time point (Stanciu et al., 2000). However, administration of LY294002 at the initiation of glutamate treatment prevented the recruitment of MEK-1 activity at 10 hours, showing that the late

recruitment of MEK-1 is dependent upon early, but not late PI3K activity (Figure 7). Thus, PI3K activity must affect the ERK pathway at the level of MEK-1 or upstream of MEK-1 during glutamate-induced oxidative toxicity, formally eliminating the possibility that PI3K activity influences ERK phosphorylation independent of MEK-1.

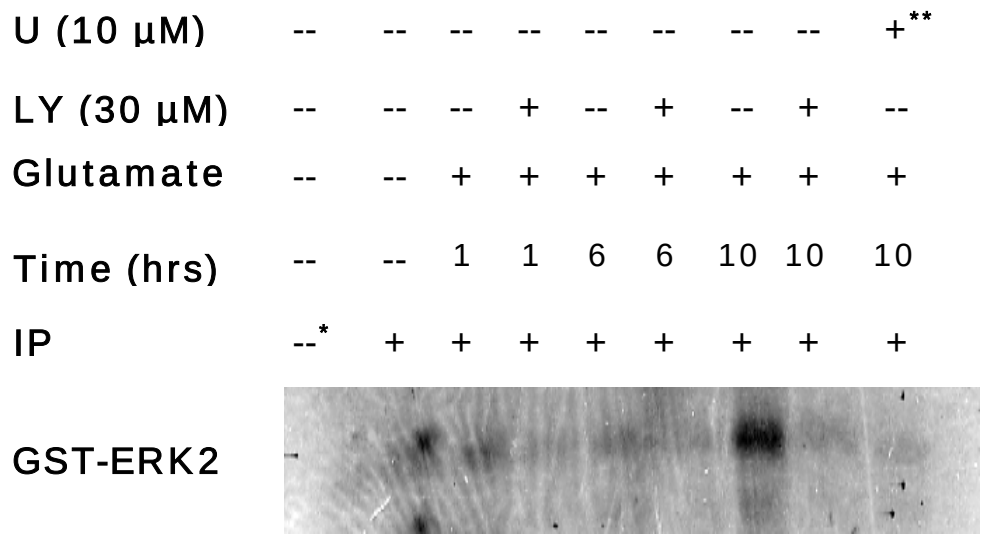


Figure 7 PI3K inhibition prevents the delayed recruitment of MEK-1 activity in glutamate-treated immature primary cortical cultures. MEK-1 immunocomplexes were isolated using Protein-A Sepharose beads, and MEK-1 activity was determined via the incorporation of ^{32}P into purified, unphosphorylated GST-ERK2 protein (see Methods). MEK-1 activity was strongly induced at 10 hours, and early LY294002 administration abrogated this induction. Image shown is representative of 3 independent experiments. * = negative control: control lysates were immunocomplexed with a non-specific FITC conjugated rabbit anti-sheep antibody; ** = negative control: the 10 hour glutamate sample was processed as specified, except 10 µM U0126 was added into the second Lysis Buffer wash and during the kinase reaction.

4.4.6. Neither PI3K Inhibition nor MEK Inhibition Prevents Oxidative Stress in Primary Mixed Cortical Cultures

To test the hypothesis that the protective effects of PI3K inhibition or MEK inhibition may be due to the prevention of oxidative stress in our cultures, we measured ROS production using the dye DCF. As can be seen in Figure 8, a significant increase in DCF fluorescence intensity is seen 10 hours, but not 4 hours, after the administration of glutamate. A high dose of H_2O_2 was used as a positive control in the assay. Interestingly, neither LY294002 (30μM) nor U0126 (10μM) significantly affected the extent of oxidative stress measured 10 hours after glutamate administration. This implies that the loss of ERK activation 10 hours after glutamate treatment (via PI3K inhibition or direct MEK inhibition) can uncouple cells from oxidative toxicity despite the ongoing presence of oxidative stress. Further experiments will be needed to determine if cells eventually recover anti-oxidant control systems and reduce oxidative stress in the presence of U0126 or LY294002. This may explain the longevity of our cultures in during ERK inhibition in the long term. Furthermore, the mechanism by which continued oxidative stress during MEK-inhibition fails to lead to the activation of other pathways is currently unknown. It is possible that ERK activity is needed for the recruitment of these potential alternative signaling events.

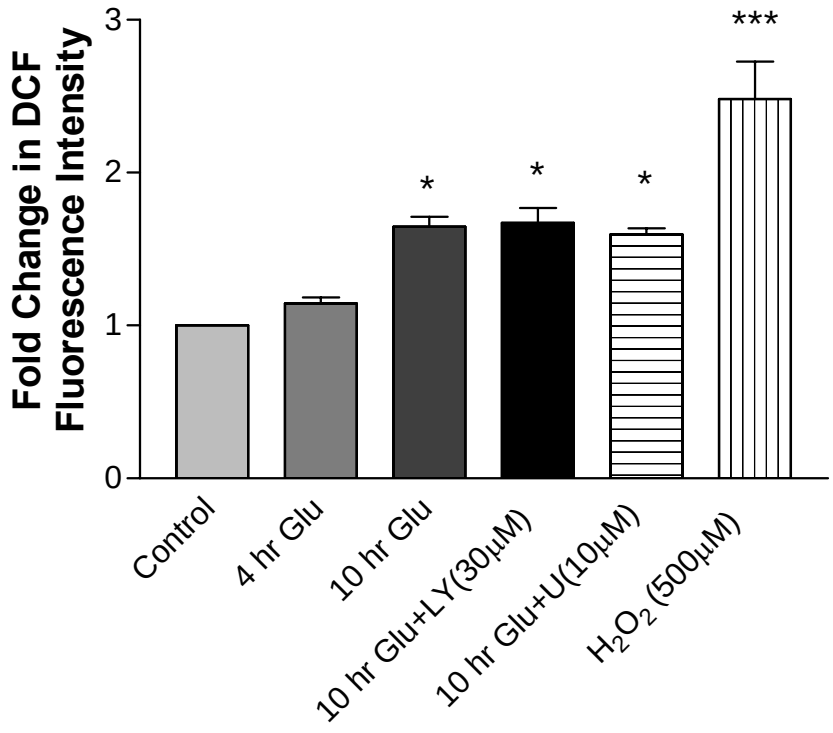


Figure 8 Neither PI3K nor MEK inhibition prevents the development of glutamate-induced oxidative stress in immature primary cortical cultures. Cells were treated with glutamate for either 4 hours or 10 hours, or 10 hours with glutamate with either LY294002 (30 μM) or U0126 (10 μM), or with 500 μM H_2O_2 for 2 hours, or left untreated. One hour prior to analysis, cells were loaded with 50 μM DCFH-DA. Cells were washed in HBSS, collected, and tested for the intensity of DCF fluorescence (Excitation 475, Emission 525). DCF intensities were adjusted to cell counts, and the signal was compared to untreated cells (fold-change). Data presented represents the average of 4 independent experiments. * = $p < 0.05$; *** = $p < 0.001$

4.5. DISCUSSION

The PI3K-Akt pathway is well recognized for its ability to mediate neuronal protection from a wide range of toxic insults and conditions (Brunet et al., 2001). In many cases, inhibition of PI3K (e.g., wortmannin, LY294002) has been instrumental in establishing the neuroprotective action of this pathway (Zheng et al., 2002). However, we report here that transient inhibition of the PI3K-Akt pathway can protect primary neurons from oxidative toxicity. Thus, PI3K activity may be required for some initial steps of a cell death program that unfolds when neurons are subjected to oxidative stress. To our knowledge, this is the first demonstrated example of PI3K activity associated with cell death**.** Importantly, a transient "window" of PI3K inhibition in mixed cortical cultures exposed to LY294002 or wortmannin enabled us to distinguish between the short-term and long-term requirements for PI3K activity in this neurotoxicity model. Transient PI3K inhibition is apparently insufficient to undermine the long-term pro-survival function of this pathway. Since neuron-enriched cultures of the same age treated with LY294002 do not exhibit a transient inhibition of PI3K activity, it seems likely that glial cells in our cultures (~20-25%) are responsible for the metabolism, inactivation, or sequestration of LY294002. Although we have not detected appreciable inactivation of LY294002 in enriched, mature astrocyte cultures (data not shown), there are other glial cell types in our mixed cultures that could exhibit this activity.

Our results are consistent with the wealth of data supporting a neuroprotective role for the PI3K pathway, including in an established cell culture model of oxidative toxicity (i.e. HT22 cells). We are able in our mixed cultures to generate prolonged inhibition of PI3K by simply administering LY294002 in two boluses 6 hours apart. In the case in which PI3K activity was inhibited for prolonged periods of time (i.e., up to 12 hours), the neuroprotective effects of

LY294002 were abrogated. Furthermore, in neuron-enriched cultures, a single bolus of LY294002 was toxic due to prolonged inhibition of the PI3K-Akt pathway (Figure 5C). This finding is consonant with recent data showing that a single 20 μM dose of LY294002 is toxic to neuron-enriched primary hippocampal cultures (Luo et al., 2003).

Transient inhibition of the PI3K-Akt pathway does not affect neuronal survival in other models in which prolonged inhibition would be predicted to lead to cellular demise. For example, infusion of the PI3K inhibitor wortmannin, an unstable compound in aqueous solution, into the amygdala of rats impaired their ability to learn in a fear-conditioning paradigm soon after the administration of the drug, but did not affect their ability to be effectively re-trained on the same paradigm several days later (Lin et al., 2001). This implies that the effects of wortmannin in vivo are reversible and do not result in neuronal cell death, at least not in the amygdala. Similarly, LY294002 has been used in vivo in models of transient focal cerebral ischemia- or seizure-induced neuronal toxicity, and in these studies LY294002 alone did not lead to increased neuronal cell death in the hippocampus or the caudate / putamen (Noshita et al., 2001; Henshall et al., 2002), presumably due to the surrounding glia. Our demonstration of transient PI3K inhibition by LY294002 in mixed neuronal / glial cultures provides strong support for the notion that metabolism, inactivation, or sequestration of LY294002 can have functional consequences for neuronal cell responses to a cell death-inducing stimulus. This finding would be of great interest in the development of therapeutic uses of these compounds in vivo, where surrounding glial elements would likely alter the pharmacodynamics of these drugs in the central nervous system.

We have previously determined that glutamate-induced oxidative toxicity is associated with the development of a delayed activation of ERK1/2, and that the MEK inhibitor prevents

this activation and is significantly neuroprotective (Stanciu et al., 2000; Stanciu et al., 2002). Interestingly, the ability of transient PI3K inhibition to protect these cultures is associated with an abrogation of delayed MEK-1 activation (Figure 7) and ERK1/2 phosphorylation (Figure 6). This further establishes ERK1/2 activation in this model as a requisite event for eventual cellular demise. In addition, because of the temporal disparity between the period of PI3K inhibition and the regulation of ERK1/2 activity, we can place PI3K activation upstream of the course of events that lead to MEK-1 and ERK1/2 activation. Furthermore, the fact that neither PI3K nor MEK inhibition blocks oxidative stress implies that somehow PI3K inhibition can uncouple ERK activation from oxidative stress and that oxidative stress is not dependent upon ERK activation in this model (Figure 8).

Cross-talk between the PI3K-Akt and Raf-MEK-ERK pathways has been demonstrated in an increasingly diverse set of circumstances and in different cell types. Although initially this link was reported to be involved in the signaling of G-protein coupled receptors (Hawes et al., 1996; Lopez-Ilasaca et al., 1997), PI3K activity can be necessary for the recruitment of the ERK pathway in signaling events associated with other types of receptors as well. For example, PI3K activity is required for in vitro ERK activation in AMPA and NMDA receptor-mediated signaling events in striatal cultures (Perkinton et al., 1999; Perkinton et al., 2002) and for NGF-induced sustained activation of ERK during PC12 differentiation (York et al., 2000). Furthermore, PI3K activation was shown to occur upstream of ERK activation in a rat model of associative learning (Lin et al., 2001). Of great interest is the recent finding that PI3K positively contributes to the activation of ERK1/2 in cortical cultures exposed to oxidative stress induced by hydrogen peroxide (Crossthwaite et al., 2002). Thus, there appears to exist a mechanism for

functional coupling of the PI3K and ERK pathways in neurons, with the potential for PI3K to act as a required upstream activator.

Contradictory effects of PI3K on the ERK pathway have been noted and were initially attributed to cell-type specific signaling events that reflected a wide variety of direct and indirect interactions between the two pathways (Hawes et al., 1996; Lopez-Ilasaca et al., 1997; Zimmermann and Moelling, 1999). However, recent reports showing opposing effects of PI3K on ERK activation within a single cell type in response to one signal reveal the importance of signal intensity in dictating the eventual outcome of PI3K/ERK cross-talk (Duckworth and Cantley, 1997; Wennstrom and Downward 1999; Moelling et al., 2002). Furthermore, EGF-induced ERK activation was found to be PI3K-dependent under conditions under which PI3K activity remained near basal levels (Wennstrom and Downward, 1999). This previously described "permissive" effect of the PI3K pathway on ERK activation (Duckworth and Cantley, 1997; Sutor et al., 1999; Wennstrom and Downward, 1999; Crossthwaite et al., 2002) appears to operate in our neuronal cultures, given that increased stimulation of PI3K activity in response to glutamate treatment is minimal (data not shown). However, our neuronal cultures exhibit a substantial baseline level of PI3K activity (Figures 4A, 4B and 5A). Interestingly, our results are unique in that the coupling between PI3K and ERK is restricted to a distinct kinetic phase during the progression of an oxidative-stress induced cell death pathway. Thus, inhibition of basal PI3K activity (as assessed by levels of phosphorylated Akt) at early times following the initiation of glutamate treatment (i.e., within 4-6 hours) impacts ERK activation that is only apparent following an additional 4-6 hours.

We consider the observed coupling in our primary neuron cultures to reflect a permissive effect of PI3K on ERK activation. Glutamate-induced oxidative toxicity in immature neurons

and in HT22 cells is driven by a delayed rise in intracellular ROS and Ca^{2+} (Tan et al., 1998). The rapid burst and accumulation of ROS and Ca^{2+} that ultimately results from glutamate treatment are likely to be proximal to the terminal execution phases of the cell death program that operates in these cells (Tan et al., 1998). PI3K has been found to influence plasma membrane Ca^{2+} channel activity through activated Akt and thereby contribute to neuronal cell survival in response to neuroprotective factors such as IGF-1 (Blair et al., 1999). Furthermore, a VDCC-dependent form of long-term potentiation at hippocampal CA1 region synapses was found to require PI3K activity, implying that PI3K activity can directly regulate calcium entry into neurons (Sanna et al., 2002). In addition, PI3K has been demonstrated to be required for the activation of voltage-independent Ca^{2+} channels in a CHO cell system (Kawanabe et al., 2002) and VDCCs in vascular smooth muscle cells (Seki et al., 1999), non-selective cation channels (NSCCs) (Kawanabe et al., 2003), and for Ca^{2+} release from intracellular SOCCs (Kawanabe et al., 2003). Furthermore, there is increasing evidence showing that PI3K activity is not only necessary for increases in intracellular Ca^{2+} but that this increase in Ca^{2+} is necessary for ERK activation (Kansra et al., 2001). Interestingly, PI3K-dependent increases in intracellular Ca^{2+} have been shown to be dissociated from increases in phosphorylated Akt and may represent a point of divergence between action of PI3K on the Akt versus the ERK pathway (Kansra et al., 2001; Kawanabe et al., 2003). This is consistent with our findings that Akt activation (above baseline) is minimal during the development of glutamate-induced oxidative toxicity (data not shown). Thus, it is tempting to speculate that the permissive effects of PI3K on ERK-dependent cell death in oxidatively stressed primary neurons might be due to effects of basal PI3K activity on either plasma membrane Ca^{2+} channels, or intracellular store-operated channels. ROS generated by the mitochondria contribute to Ca^{2+} influx and accumulation in glutamate-treated

HT22 cells and primary cortical neurons (Li et al., 1997; Tan et al., 1998), and a Ca^{2+} channel response to ROS that requires some direct or indirect priming action of the PI3K pathway may very well be the link between the PI3K and ERK pathways in our system. Previous studies have demonstrated that HT22 cells and primary cortical neurons are protected from oxidative toxicity by blocking plasma membrane Ca^{2+} channels (i.e., with $CoCl_2$) or by inhibiting Ca^{2+}-uptake into the mitochondria (i.e., with ruthenium red) (Tan et al., 1998). Thus PI3K inhibition may protect neurons from oxidative toxicity by altering the activity of Ca^{2+} channels and transporters.

The identification of multiple intracellular signaling pathways that operate to promote or limit neuronal cell death and the delineation of various levels of cross-talk between these pathways both present unique challenges to the development of pharmacological neuroprotective therapies. Our results suggest that depending upon the nature and identity of the coupled signal transduction pathways, a temporal window may exist that allows for the transient inhibition of a specific signal transduction pathway. Thus, rapid metabolism or sequestration of drugs targeted to specific signaling pathways may prove beneficial particularly in cases where unique coupling between signal transduction pathways occurs at distinct kinetic phases of a progressing cell death program.

5. CHAPTER 2: Reversible oxidation of ERK-directed protein phosphatases drives oxidative toxicity in neurons

5.1. SUMMARY

Oxidative stress links diverse neuropathological conditions that include stroke, Parkinson's Disease, and Alzheimer's Disease, and has been modeled in vitro with various paradigms that lead to neuronal cell death following the increased accumulation of reactive oxygen species (ROS). For example, immortalized neurons and immature primary cortical neurons undergo cell death in response to depletion of the anti-oxidant glutathione, which can be elicited by administration of glutamate at high concentrations. We have previously demonstrated that this glutamate-induced oxidative toxicity requires activation of the mitogen-activated protein kinase member ERK1/2, but the mechanisms by which this activation takes place in oxidatively stressed neurons are still not fully known. In this study, we demonstrate that during oxidative stress, ERK-directed phosphatases of the serine/theonine- and tyrosine-directed classes are selectively and reversibly inhibited via a mechanism that is dependent upon the oxidation of cysteine thiols.

Furthermore, the impact of ERK-directed phosphtatases on ERK1/2 activation and oxidative toxicity in neurons was tested in a neuronal cell line and in primary cortical cultures. Overexpression of the highly ERK-specific phosphatase MKP3 and its catalytic mutant MKP3 C293S were neuroprotective in transiently transfected HT22 cells and primary neurons. The neuroprotective effect of the MKP3 C293S mutant, which enhances ERK1/2 phosphorylation but blocks its nuclear translocation, demonstrates the necessity for active ERK1/2 nuclear localization for oxidative toxicity in neurons. Together, these data implicate the inhibition of

endogenous ERK-directed phosphatases as a mechanism that leads to aberrant ERK1/2 activation and nuclear accumulation during oxidative toxicity in neurons.

5.2. INTRODUCTION

Oxidative stress is a common feature of a diverse range of neuropathological conditions, including stroke, Parkinson's Disease, Alzheimer's Disease, and Amyotrophic Lateral Sclerosis (Coyle and Puttfarken, 1993; Mattson et al., 2001). Glutamate-induced oxidative toxicity provides an excellent model for studying the effects of oxidative stress in immortalized neurons and in primary neuronal cultures (Murphy et al., 1990; Ratan et al., 1994; Li et al., 1997). In this model, inhibition of a glutamate/cystine antiporter, known as system x_c^-, leads to decreased accumulation of intracellular free cysteine, a necessary precursor of glutathione, and to eventual glutathione depletion. As a result, reactive oxygen species (ROS) accumulate and activate cellular signaling events that contribute to neuronal cell death. The extent to which system x_c^- is involved in adult neurons in vivo is not fully known, but studies have demonstrated that this amino acid antiporter is widely expressed in the adult nervous system. However, the system is expressed primarily in the periventricular areas of the adult mouse brain and in the meninges rather than in major nuclei or the cortex (Sato et al., 2002). Thus in the adult brain, this system may function more to maintain the redox state in the CSF (i.e., by regulating the cysteine/cystine ratio) than to maintain neuronal levels. However, in cultured immature primary cortical neurons, this system is clearly expressed and is fully functional (Murphy et al., 1990). We have previously shown that oxidative toxicity causes a delayed, sustained activation of ERK1/2 that is necessary for neuronal cell death (Stanciu et al., 2000; Stanciu and DeFranco, 2002; Levinthal

and DeFranco, 2004), but the mechanisms by which oxidative stress drives ERK1/2 activation have not been fully elucidated.

The regulation of ERK1/2 phosphorylation and activation reflects a subtle balance between ERK-directed kinase and phosphatase activity. A diverse range of phosphatases directed against phospho-serine/theonine, phospho-tyrosine, or both have been identified as negative regulators of ERK1/2. PP2A (Silverstein et al., 2002; Kim et al., 2003[2]) and STEP (Paul et al., 2003), among numerous others, can function as ERK-directed phosphatases in neurons. Dynamic changes in ERK-directed phosphatase activity can result from several mechanisms, including ERK-dependent upregulation of phosphatase expression (Bhalla et al., 2002), phosphorylation-dependent increases in phosphatase stability (Brondello et al., 1999), and protein-protein interaction–dependent activation of phosphatase activity (Camps et al., 1998). These events collectively have been shown to function in a negative feedback loop that terminates ERK signaling (Bhalla et al., 2002).

Recently, the role of oxidative stress in the regulation of phosphatase activity has received much attention (Meng et al., 2002; Leslie et al., 2003; Persson et al., 2004). Several phosphatases have been shown to be redox sensitive and can be either reversibly or irreversibly inhibited, depending upon the degree and mechanism of oxidation (Meng et al., 2002; Tonks, 2003). Oxidative phosphatase inhibition can impact various cellular signaling pathways and accounts for a mechanism now referred to as oxidative signaling. The extent to which oxidative inhibition of phosphatases plays a role in driving signaling events during neurotoxicity remains relatively unexplored.

We sought to characterize the effect of glutamate-induced oxidative toxicity on ERK-directed phosphatases in primary neuronal cultures. In this study, we show that endogenous

ERK-directed phosphatase activity is specifically and reversibly inhibited during oxidative stress consistent with a mechanism involving the oxidation of cysteine thiols. This inhibition of ERK-directed phosphatases correlates with an increase in phosphorylated ERK levels. We show that this aggregate phosphatase activity is likely comprised of PP2A and a vanadate-sensitive component. The impact of ERK-directed phosphatase activity on oxidative toxicity was revealed by the neuroprotective effects of overexpressed MKP3 and its catalytic mutant, MKP3 C293S, in both HT22 cells and primary cortical neurons. Collectively, these data implicate oxidative inhibition of ERK-directed phosphatases in neuronal oxidative toxicity induced by glutathione depletion.

5.3. EXPERIMENTAL PROCEDURES

Plasmids The expression plasmids for MKP3 WT and MKP3 C293S, as well as the parent plasmid (pSG5), were kind gifts from Dr. Steven Keyse and Dr. Anne Brunet (Groom et al., 1996; Dowd et al., 1998; Brunet et al., 1999). The mitochondria-targeted eYFP expression plasmid was a gift from Dr. Ian Reynolds, and the expression plasmid for the Elk-1/GAL4 fusion protein, the luciferase reporter, and the constitutive renilla plasmid (all from Strategene, La Jolla, CA) were purchased or donated by Dr. Elias Aizenman.

Primary Cortical Cultures Cortices from embryonic day 17 Spague-Dawley rat fetuses (Hilltop Lab Animals, Scottdale, PA) were dissected and manually dissociated by repeated trituration using fire-polished glass pipettes 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+} (Invitrogen, Carlsbad, CA) followed by passage through a 40 μm cell

strainer (Becton Dickinson Labware, Franklin Lakes, NJ) to remove clumped cells. Cells were counted and plated on 50 μg/ml poly-D-lysine treated culture plates at a density of ~2.1 x 10^4 cells / cm^2. Cell viability was routinely greater than 80% as assessed by uptake of trypan blue dye upon plating. Cultures were maintained for 3-4 days in media (DMEM (Invitrogen), 10% FCS (Hyclone, Logan, UT), 10% Ham's F12 Nutrient Supplement (Invitrogen), 1.9 mM glutamine, 24 mM Hepes Buffer, and 4.5 mg/mL glucose) at 37°C and 5% CO_2. At this time, these mixed cortical cultures are predominately neuronal, with approximately 20% $GFAP^+$ staining cells (Murphy et al., 1990). Unless otherwise stated, all chemicals and reagents used were purchased from Sigma Chemical Corporation, St. Louis, MO.

Cell Line Culture HT22 cells, a hippocampal cell line that is sensitive to glutamate-induced oxidative toxicity (Li et al., 1997), were maintained in DMEM supplemented with 10% FCS (Atlanta Biologicals, Norcross, GA), 100 Units Penicillin, and 100 μg/mL Streptomycin at 37ºC and 5% CO_2.

Transfections in Primary Cortical Neuronal Cultures A number of transfection reagents and protocols that were tested proved toxic to our primary immature cortical cultures. However, limited toxicity was observed when the 25 kD polyamine polymer poly(ethyleneimine) (PEI) was used to form DNA-complexes and to transfect primary neurons (Guerra-Crespo et al., 2003; Horbinski, et al., 2001). In brief, 2 μL of a 100 mM PEI stock solution was added to 250 μL 150 mM NaCl, while 6.5 μg of DNA was added to 250 μL 150 mM NaCl. Two μg of a mitochondria-targeted enhanced YFP (mt-eYFP) plasmid DNA and 4.5 μg of MKP3 plasmids were used per condition. After 5 min, the two solutions were mixed. PEI/DNA complexes were

allowed to form for 10 min, and then diluted into 10 mL MEM (Invitrogen). Conditioned media was removed from the primary neuronal cultures and replaced with MEM. One mL of transfection solution was added to each 35 mm well and left to incubate at 37ºC for 1 hr. The transfection media was then removed and replaced with conditioned media for the remainder of the experiment. The transfection efficiency with this method varied between 0.1 – 0.5 %. Glutamate treatment was initiated 24 hrs following transfection.

Transfections in HT22 cells The cationic lipid reagent Lipofectamine 2000 (Invitrogen) was used to transiently transfect HT22 cells. For toxicity experiments, 2 μg of DNA was used per well of a 12-well tissue culture plate, in a DNA (μg) / Lipofectamine 2000 (μL) ratio of 1.5. Typically, 1 μg of mt-eYFP plasmid DNA and 1 μg of MKP3 plasmid DNA were used for these transfections. For the Elk-1-dependent luciferase expression experiments, ~8 μg total plasmid DNA was used for each 60 mm plate (3 μg pSG5, MKP3 WT, or MKP3 C293S, 4 μg Luciferase reporter plasmid [pFR-Luc], 0.12 μg Elk-1/GAL4 fusion expression plasmid [pFC-Elk-1/GAL4], or the GAL4 DNA binding domain expression plasmid [pFC-GAL4 DBD], and 1.2 μg constitutive Renilla expression plasmid [PRK-tk Renilla]), with a DNA/Lipofectamine 2000 ratio of 1.5. DNA and Lipofectamine 2000 were diluted in OptiMEM (Invitrogen) for 5 min and then combined to allow DNA complexes to form for 20 min. The transfection solution was added to HT22 cells in serum-free DMEM without antibiotics for 5 hr, after which cells were returned to normal serum and antibiotic containing media (see above). Cells were harvested as described beginning 16 to 18 hrs following transfection, and luciferase/renilla activity was measured (see below).

Elk-1/GAL4 Fusion Protein-Dependent Luciferase Expression Components of the PathDetect in Vivo Signal Transduction Pathway trans-Reporting System (Stratagene, La Jolla, CA) were used in order to monitor Elk-1-dependent gene expression in HT22 cells. Cells were transfected with an expression plasmid coding for an Elk-1/GAL4 fusion protein, a reporter plasmid containing the luciferase gene under a synthetic promoter containing 5 tandem GAL4 binding sites, and a MKP3 expression plasmid. In this system, luciferase activity is a measure of the extent of Elk-1 activation. HT22 cells were co-transfected with the Elk-1 fusion plasmid (pFA2-Elk-1) or its negative control containing only GAL4 (pFC2-DBD), the luciferase reporter plasmid (pFR-Luc), a constitutive expression plasmid coding for Renilla under control of the CMV promoter (PRK-tk Renilla), and either the pSG5 empty vector, MKP3 WT, or MKP3 C293S plasmids. Sixteen hr after transfection, HT22 cells were left untreated or treated with glutamate for 7.5 hr. Cells were then harvested and lysed in Lysis Buffer (see below). Both luciferase activity and renilla activity were measured with the Dual-Glo Luciferase Assay System (Promega, Madison, WI) using a luminometer (Wallac Victor^3, Perkin Elmer Biosciences, Boston, MA). All luciferase signals were normalized to renilla within each sample.

Viability Measurements in Transfected Cells Primary cortical neuronal cultures (DIV3) display cell body shrinkage, process retraction, and nuclear condensation at 18-20 hr following treatment with 5 mM glutamate. After 24 hr, cells begin to detach from the tissue culture plate. To assess cell viability in transfected neurons (i.e. mt-YFP positive cells; see Methods), we treated cells with the DNA dye propidium iodide (PI), which is excluded from healthy, intact cells and only gains access into cells with a compromised plasma membrane. Eighteen hours following the initiation of all treatments, cultures were incubated for 10 min in media containing a final

concentration of 6.25 μg/mL PI. Cells were observed under an inverted fluorescence microscope equipped with phase contrast optics (Nikon Eclipse TE200), and mt-eYFP positive, PI-labeled and unlabeled cells were counted. The percentage of dually-labeled cells in each well was then calculated as a percentage of all transfected cells counted (approximately 50 cells per well). Multiple wells were counted for each condition in at least three separate tranfected cultures (total cell population at least 250 per condition).

HT22 cells become PI-positive beginning 6-7 hr following 5 mM glutamate treatment and begin to detach from the culture plate after 9 hr. We thus assessed toxicity in transfected, mt-eYFP-marker positive cells at 7 hr using the PI staining method as described above. Cells were observed under an inverted fluorescence microscope equipped with phase contrast optics (Nikon Eclipse TE200), and mt-eYFP positive, PI-labeled and unlabeled cells were counted. The percentage of dually-labeled cells in each field was then calculated as a percentage of all transfected cells counted (approximately 60 cells per field at 100x), and multiple fields were counted for each condition in at least three separate transfected cultures (total cell population at least 500 per condition).

Indirect Immunofluorescence HT22 cells were plated on 12-well tissue culture plates in normal media (see above), and then transfected with either the pSG5 parent plasmid, MKP3 WT, or MKP3 C293S expression plasmids for 16-18 hr. Cells were then washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4), fixed with -20º methanol for 5 min, washed with PBS, permeabilized with PBS containing 0.1% Triton X-100 for 10 min, and then blocked for 1 hr with PBS supplemented with 3% BSA at room temperature. The fixed cells were then incubated with an anti-c-myc antibody (1:500;

Cell Signaling, Beverly, MA) or an anti-GAL4 DBD antibody (1:500; Sigma) in PBS with 3% BSA for 1 hr at room temperature, washed three times with PBS before incubation with a fluorescent-labelled secondary antibody (1:1000 anti-mouse IgG conjugated to Alexa Fluor 488; Molecular Probes, Eugene, OR) and the nuclear stain Hoescht 33258 (1 μg/ml). After washing stained cells twice with PBS, images were viewed with a Zeiss Axiophot inverted fluoroscence microscope coupled to a cooled CCD camera (Leica DC 300F, Bannockburn, IL).

ERK-directed Phosphatase Activity Assay We modified a non-radioactive method for determining ERK-directed phosphatase activity in whole cell lysates (Laakko and Juliano, 2003). This method relies on detecting dephosphorylation of a purified, dually phosphorylated His_6-tagged ERK upon incubation with whole cell lysate. Thus, alterations in ERK-directed phosphatase activity within the lysate can be monitored by measuring changes in the phosphorylation state of the isolated substrate, as assessed by Western blotting with a phospho-specific ERK1/2 antibody. In brief, 150 μg of whole cell lysate (see below) lacking DTT, Na_3VO_4, or NaF, was diluted into a total volume of 250 μL in phosphatase assay buffer (10 mM $MgCl_2$, 10 mM Hepes, pH 7.5, and 10 μM of the MEK inhibitor U0126). Recombinant, phosphorylated His_6-ERK2 (Biomol, Plymouth Meeting, PA) was added to each sample at 30 ng/sample, and the reactions were maintained at 37 ºC for 40 min. As a positive control for phosphatase activity, 1200 Units of the dual-specificity phosphatase Lambda Protein Phosphastase (λ-PPase, New England BioLabs, Beverly, MA) was included in a separate reaction. Treatment with 50 mM DTT or phosphatase inhibitors was accomplished during a 30-min incubation on ice, prior to the addition of purified ERK. Where DTT and inhibitors were used, the samples were pre-incubated for 30 min on ice with DTT, followed by a 15 min

incubation with inhibitors. After 40 min at 37ºC, the reactions were stopped by the addition of 250 µL wash buffer (8 M Urea, pH 8.6 containing 10 mM imidizole), and 30 µL of Ni^{2+}-conjugated, magnetic beads (Qiagen, Valencia, CA) were added to each reaction. After 90 min of rocking at 4ºC, the samples were washed twice with wash buffer and once in 300 mM NaCl, 25 mM Tris, pH 7.5. The beads were then suspended in NaCl/Tris and Laemmli buffer, boiled for 5 min, loaded onto a 10% polyacrylamide gel, transferred to polyvinylidine fluoride (PVDF) membranes (Millipore, Bedford, MA), and subjected to Western Blotting to detect phosphorylated ERK and total ERK (see below).

JNK-directed Phosphatase Activity Assay This method was performed exactly as described above, except that each sample was incubated with 30 ng of purified, dually-phosphorylated His_6-tagged JNK-1 protein (Upstate, Waltham, MA), and Western blots were probed with anti-phospho JNK and total-JNK antibodies.

Western Blot Analysis Cells were treated as described, scraped and collected into PBS, pelleted at 3000 rpm for 5 min, and resuspended in Lysis Buffer (50mM Tris-Cl, pH 7.5, 2mM EDTA, 100 mM NaCl, 1% NP-40, 100µM Na_3VO4, 100 µM NaF, 2mM DTT) supplemented with 5µL protease inhibitor cocktail (Sigma) per mL of lysis buffer. Total extract protein concentrations were determined using the Bio-Rad™ reagent. Equivalent amounts of total protein (either 15 or 20 µg) were separated by SDS-PAGE on 10% polyacrylamide gels and then transferred to PVDF membranes (Millipore, Bedford, MA). 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 JNK, anti-total JNK, or anti-c-myc, all from Cell

Signaling) overnight at 4ºC with 3% dry milk or 5% BSA, washed 3x 10 min with PBST, and then exposed to the appropriate horseradish-peroxidase (HRP)-conjugated secondary antibody for one hour at room temperature. Membranes were again washed 3x 10 min with PBST, and immunoreactive bands detected by enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ) using standard x-ray film (Kodak, Rochester, NY). Several different exposure times were used for each blot to ensure linearity of band intensities. Densitometry was performed using a Personal Densitometer SI (Molecular Dynamics, Amersham Biosciences) linked to the ImageQuant 5.2 software (Molecular Dynamics).

Statistics Comparisons of multiple mean values were accomplished by ANOVA with either Tukey's or Bonferroni's post-hoc tests for significance. Comparisons of two means were performed using a paired t-test. P values less than 0.05 were taken to be significant, and all data was analyzed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA).

5.4. RESULTS

5.4.1. ERK-Directed Phosphatase Activity is Specifically Inhibited during Glutamate-Induced Oxidative Toxicity in Primary Immature Cortical Cultures

We have previously reported that in primary immature cortical cultures, ERK1/2 phosphorylation is elevated during oxidative stress generated by glutamate-induced glutathione depletion (Stanciu et al., 2000; Levinthal and DeFranco, 2004). Given the sensitivity of protein phosphatases to cellular redox state (Meng et al., 2002), we set out to examine whether ERK-

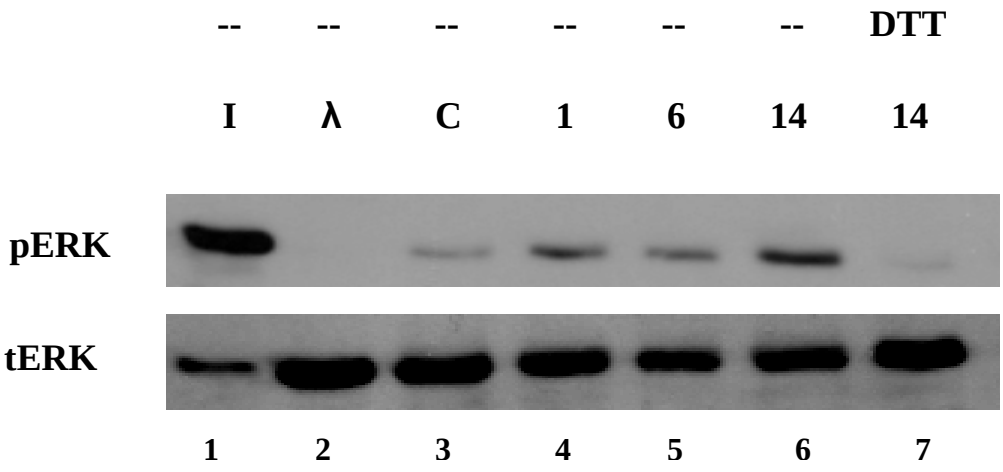
directed phosphatase activity was altered during glutamate-induced oxidative toxicity. Toward this end, we adapted a novel in vitro phosphatase assay that uses whole cell lysates to dephosphorylate purified dually-phosphorylated His_6-tagged ERK-2 (see Methods). This assay revealed the robust ERK-phosphatase activity of exogenously applied lambda protein phosphatase, a non-specific dual-specificity phosphatase that we employed as a positive control. Furthermore, the method proved to be quite sensitive in detecting changes in phosphatase activity, as the application of well-characterized phosphatase inhibitors were effective in inhibiting phosphatase activity in preparations of whole cell lysate (see below). Though not as active as purified λ-phosphatase, whole cell lysates prepared from primary cortical neurons possess robust ERK-directed phosphatase activity (Figure 9A, Lane 1). When extracts were prepared from cultures treated with glutamate for 14 hours, a decrease in ERK-directed phosphatase activity was revealed (Figure 9A, Lane 6). Quantification of several independent experiments revealed a significant decrease in ERK-phosphatase activity in cultures treated for 14 hours with glutamate, as reflected in a 5-fold increase ($p < 0.01$) in the normalized intensity of the phosphorylated ERK2 band when compared to untreated cultures (Figure 9B). Shorter treatments with glutamate (i.e., 1 and 6 hours) did not significantly affect ERK-directed phosphatase activity in the primary neuron culture extracts (Fig 9A, lanes 4 and 5). Thus, the observed inhibition of ERK-directed phosphatase activity in these extracts coincided with times of maximal oxidative stress in which glutathione levels are depleted and ERK phosphorylation is increased (Li et al., 1997; Levinthal and DeFranco, 2004). Interestingly, inhibition of ERK-directed phosphatase activity could be completely reversed by treatment of the extracts with 50 mM DTT (Figure 9A, Lane 7), implying that phosphatase function is impaired in oxidatively stressed cells due to cysteine oxidation. The restoration of ERK-phosphatase activity in

oxidatively stressed cells with DTT consistently yielded a level of activity greater than that found in untreated cells (e.g., compare lanes 3 and 7, Figure 9A). Indeed, DTT treatment of lysates from primary cortical neuronal cultures not exposed to glutamate led to increased ERK-directed phosphatase activity (data not shown). Thus, a small pool of ERK-phosphatases may be normally under tonic, reversible oxidative inhibition in primary neurons, despite intracellular conditions that are generally reducing. Because oxidative toxicity induced by glutamate in these primary cultures depends upon the depletion of glutathione, we sought to reverse the effects of thiol oxidation-mediated phosphatase inhibition by re-introducing reduced glutathione into whole cell lysates from oxidatively stressed cells. However, glutathione addition to these extracts prepared from oxidatively stressed cells did not increase ERK-phosphatase activity (data not shown). Others have observed this phenomenon, as DTT, but not glutathione, can reverse oxidation-dependent inhibition of the phosphatase cdc25 (Sohn and Rudolph, 2003). It is unclear whether this reflects an inability of reduced glutathione to gain access to the oxidized cysteine moiety in the ERK-directed phosphatases or cofactors due to steric interference, or effects of competing oxidized substrates in the extracts. Future studies will employ a mixture of reduced glutathione, NADPH, and glutathione reductase to allow for a sustainable pool of reduced glutathione. This approach may demonstrate that glutathione can indeed reverse oxidation-dependent phosphatase inhibition in the whole cell lysates from oxidatively stressed cells.

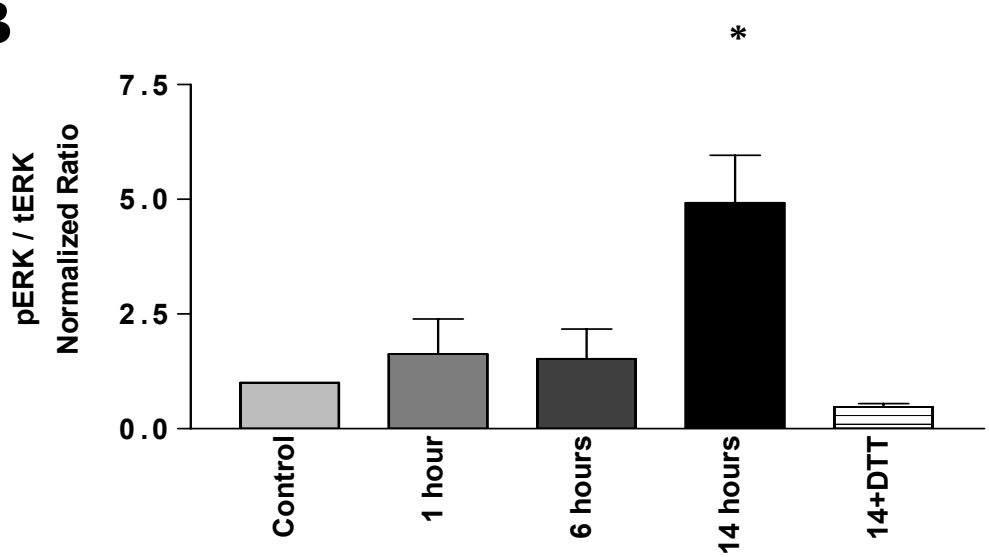
To determine the substrate specificity of phosphatase activity affected by oxidative stress, we assayed the activity of JNK-directed phosphatases using purified His_6-tagged dually phosphorylated JNK1 protein in the same primary neuron lysates used to monitor ERK dephosphorylation. As shown in Figure 9C, JNK1-directed phosphatase activity is not

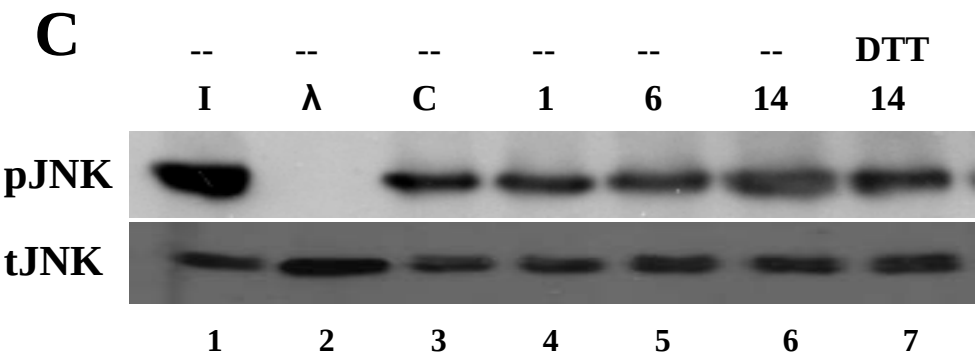
significantly altered during glutamate-induced oxidative toxicity. Furthermore, the addition of 50 mM DTT did not enhance JNK-phosphatase activity in the lysates, unlike the restoration of ERK-directed phosphatase activity by DTT in the same lysates. These extracts do contain some active JNK-phosphatases, as phosphorylated JNK levels were reduced upon incubation with extracts as compared to input (compare lanes 1 and 3, Figure 9C). To examine the phosphorylation state of endogenous JNK1 (p46) and JNK2/3 (p54) in the primary neurons, we performed Western Blot analysis using a phospho-specific JNK antibody. As can be seen in Figure 9D, JNK phosphorylation does not change during glutamate-induced oxidative toxicity, even during times of increased oxidative stress (i.e. 12-14 hours after the addition of glutamate). This finding is consistent with the lack of regulation of JNK-phosphatase activity by oxidative stress. Thus oxidative stress in immature cortical neuron cultures specifically inhibits the activity of a subset of phosphatases that act upon specific MAPK members. Furthermore, the selectivity of oxidative inhibition of MAPK-directed phosphatases revealed in the in vitro assay correlate with the specificity of MAPK activation in oxidatively stressed neurons.

A


-- -- -- -- -- -- DTT
I λ C 1 6 14 14
pERK
tERK
1 2 3 4 5 6 7

B


*
pERK / tERK
Normalized Ratio
7.5
5.0
2.5
0.0
Control
1 hour
6 hours
14 hours
14+DTT



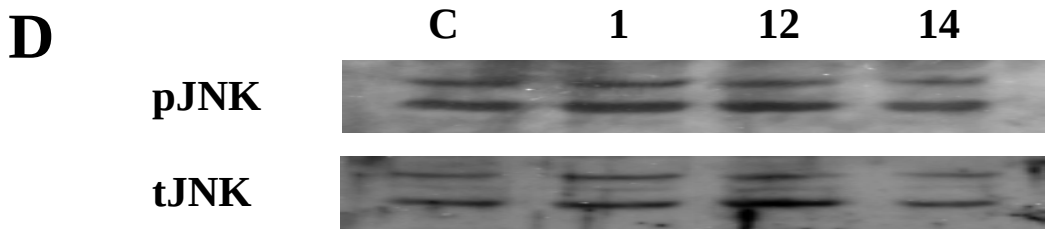


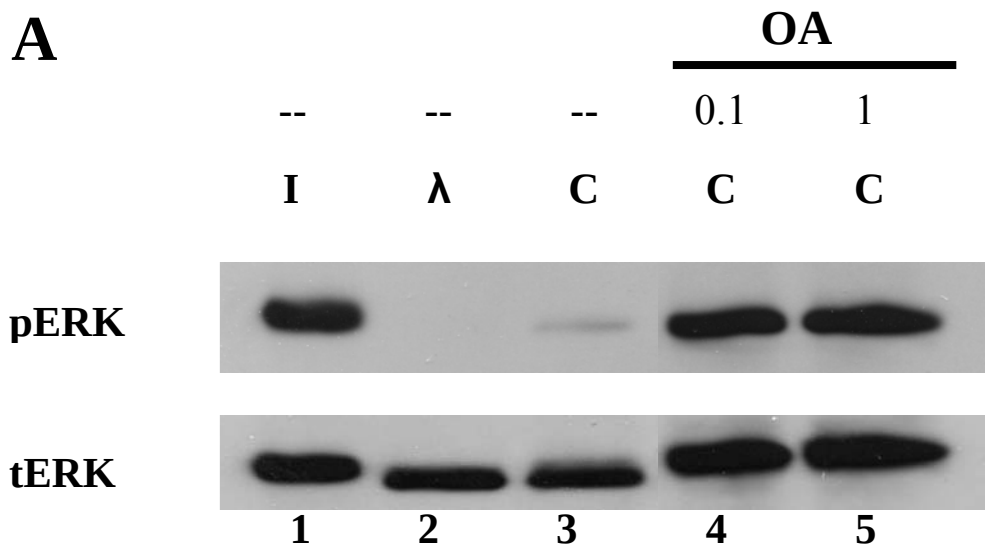
Figure 9 ERK-directed phosphatase activity is specifically inhibited during glutamate-induced oxidative toxicity in primary immature cortical cultures. Primary immature cortical cultures were left untreated or treated with glutamate for 1, 6, or 14 hours. Whole cell lysates from these samples were utilized in an in vitro ERK-directed phosphatase activity assay (see Methods). ERK-directed phosphatase activity is significantly reduced during oxidative stress (i.e. 14 hours following glutamate treatment), and this inhibition is completely reversible with 50 mM DTT (A). The results of between four and eight independent experiments were quantified, revealing a significant increase in ERK-directed phosphatase inhibition during oxidative stress and a significant restoration of this activity with DTT (B). The effects of oxidative toxicity on MAPK-directed phosphatases are specific to ERK, as JNK-directed phosphatase activity is not altered by glutamate treatment (n=3) (C). Furthermore, endogenous JNK phosphorylation is not altered by glutamate treatment (n=3) (D). * = $p < 0.05$; I = phosphatase assay input; λ = lambda phage protein phosphatase (positive control).

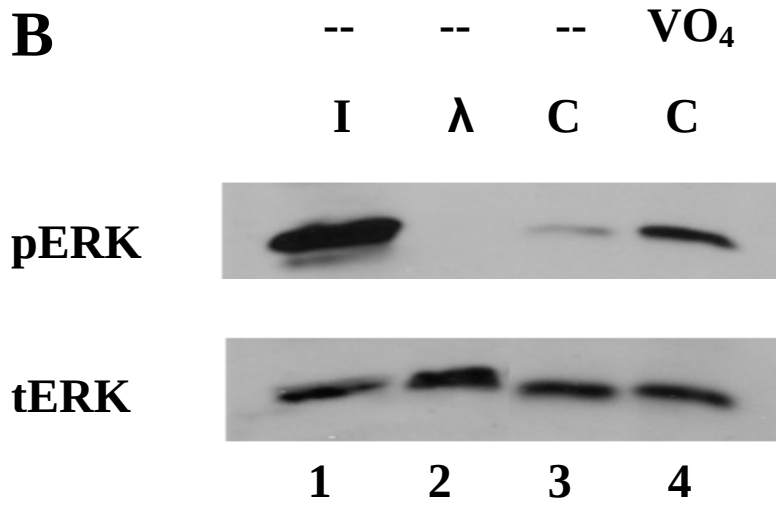
5.4.2. ERK-Directed Phosphatase Activity Can be Inhibited by Okadaic Acid and Orthovanadate

In order to ascertain the contribution of tyrosine/dual-specificity versus serine/threonine-directed phosphatases to ERK dephosphorylation in primary neuron cultures, we utilized two inhibitors: okadaic acid (OA) and sodium orthovanadate. OA is a well characterized serine/threonine directed protein phosphatase inhibitor with a high degree of selectivity at low concentrations for PP2A. Furthermore, a major component of ERK-phosphatase activity in neurons is contributed by PP2A (Foley et al., 2004). As shown in Figure 10A, treatment with either 0.1 μM or 1.0 μM OA led to near complete inhibition of ERK-directed phosphatase activity in lysates from untreated control cultures (compare lanes 4 and 5 to lane 3, Figure 10A). Thus, our in vitro assay confirms the expected contribution of PP2A to ERK-dephosphorylation in neurons.

Sodium orthovanadate is a potent inhibitor of tyrosine- and dual-specificity phosphatases that does not affect serine/threonine-directed phosphatases. Currently, there are few compounds available that inhibit specific tyrosine- or dual-specificity phosphatases, yet orthovanadate remains a useful tool to delineate the contribution of these classes of phosphatases to the regulation of protein phosphorylation. To examine the role of tyrosine- and dual-specificity phosphatases to the observed phosphatase activity in untreated neuronal cultures, we treated lysates from these cells with 1 mM Na_3VO_4. As is shown in Figure 10B and 10C, orthovanadate significantly inhibited ERK-directed phosphatase activity in lysates from untreated cultures. Importantly, the degree of inhibition observed with orthovanadate was less than that observed with OA, further confirming the predominant role of PP2A as an ERK-directed phosphatase

activity in neurons. Nonetheless, tyrosine- and/or dual-specificity phosphatases appear to operate in primary neurons as ERK-directed phosphatases.





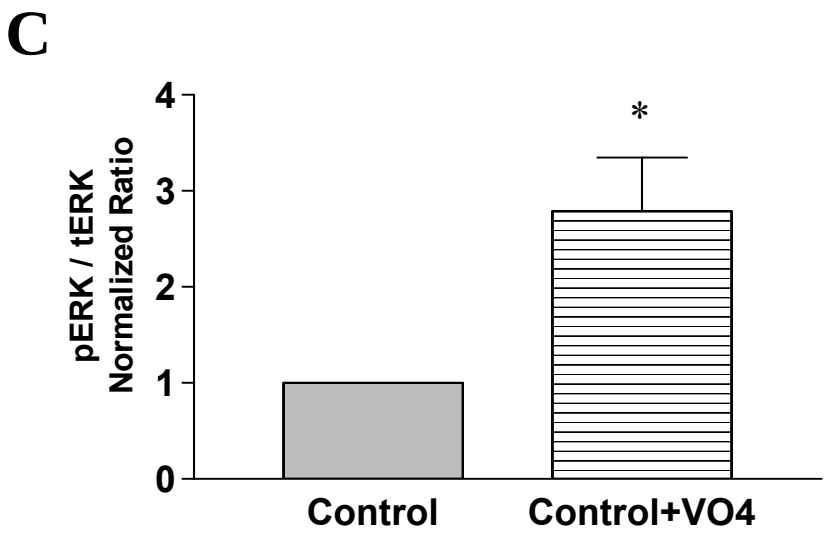


Figure 10 ERK-directed phosphatase activity is inhibited by okadaic acid and orthovanadate. The nature and identity of the components of the basal ERK-directed phosphatase activity were probed using the phosphatase inhibitors okadaic acid, an inhibitor of PP2A, and sodium orthovanadate, a general inhibitor of tyrosine-directed and dual specificity phosphatases. Whole cell lysates from untreated primary immature cortical neurons were incubated with vehicle, 0.1 μM or 1 μM okadaic acid, or 1 mM orthovanadate for 30 minutes prior to use in the in vitro ERK-directed phosphatase activity assay. Both doses of okadaic acid were sufficient to inhibit activity (A). Because orthovanadate led to less inhibition of ERK-directed phosphatase activity than okadaic acid (B,C), it is likely that tyrosine-directed or dual-specificity phosphatases that contribute to this activity, while PP2A is the major component. All experiments were conducted at least 4 times. * = $p < 0.05$

5.4.3. The Reversibly Oxidized Pool of ERK-Directed Phosphatase Activity Can be Inhibited by Okadaic Acid and Orthovanadate

Having established parameters for pharmacologic inhibition of ERK-directed phosphatases in vitro using lysates from control cultures, we next sought to reveal the nature of phosphatase activity recovered by DTT treatment of extracts from oxidatively stressed cells. As shown in Figure 11, 0.1 μM Okadaic acid and 1 mM Na_3VO_4 were both capable of blocking ERK-phosphatase activity restored by DTT in lysates from oxidatively-stressed cells (compare lanes 6-8 with lane 5). Thus, the reversibly inhibited pool of ERK-directed phosphatases that we have identified in oxidatively stressed neuronal cultures is likely composed of PP2A and vanadate-sensitive activities.

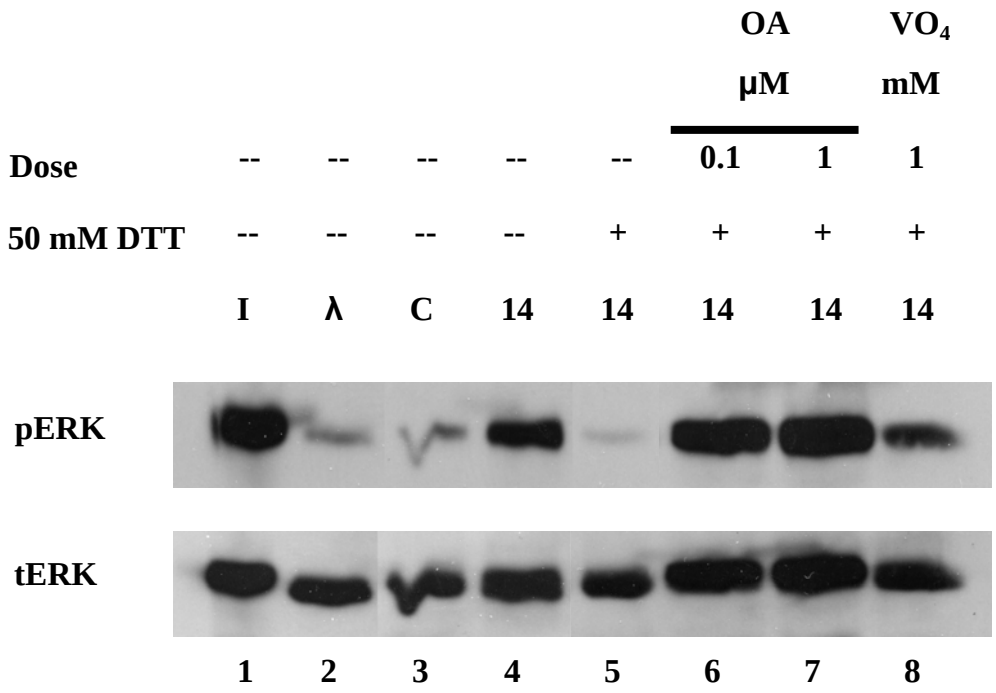


Figure 11 The reversibly oxidized pool of ERK-directed phosphatase activity is inhibited by okadaic acid and orthovanadate. Whole cell lysates were prepared from primary immature cortical neurons that had been left untreated or treated with glutamate for 14 hours. The lysates from glutamate treated cells were incubated with DTT alone (45 minutes), or DTT (30 minutes) followed by a 15-min incubation with 0.1 μM or 1 μM okadaic acid, or 1 mM sodium orthovanadate, prior to use in the in vitro ERK-directed phosphatase activity assay. The reversible pool of oxidized ERK-directed phosphatases is inhibited by okadaic acid and orthovanadate. Results shown are representative of four independent experiments.

5.4.4. Overexpression of MKP3 WT and MKP3 C293S significantly alter ERK Phosphorylation and Translocation in HT22 Cells

If reversible oxidation of ERK-directed phosphatases such as PP2A or some necessary cofactors accounts in part for persistent ERK activation in oxidatively stressed neurons, overexpression of these phosphatases could overcome this inhibition and reduce ERK phosphorylation. Although PP2A exists as a multimeric complex in vivo, expression of the catalytic subunit alone (i.e. C subunit) can be sufficient for activity (Chung and Brautigan, 1999). However, manipulation of PP2A activity in vivo upon catalytic subunit overexpression is complicated by the auto-inactivation of PP2A activity that results from C subunit overexpression (Chung and Brautigan, 1999; Baharians and Schonthal, 1998). Futhermore, PP2A has a wide range of substrates other than ERK1/2, making it difficult to ascribe biological effects of PP2A activity manipulation to effects on a particular substrate (i.e. ERK1/2). We therefore decided to assess the effects of MKP3 overexpression on glutamate toxicity in HT22 cells. MKP3 is a dual-specificity phosphatase with a high degree of selectivity toward ERK1/2 that is predominantly localized in the cytoplasm (Muda et al., 1996). The MKP3 point mutant, MKP3 C293S, was also tested, because it acts as a dominant negative to block dephosphorylation of ERK by endogenous MKP3 as well as to prevent the translocation of phosphorylated ERK to the nucleus (Brunet et al., 1999).

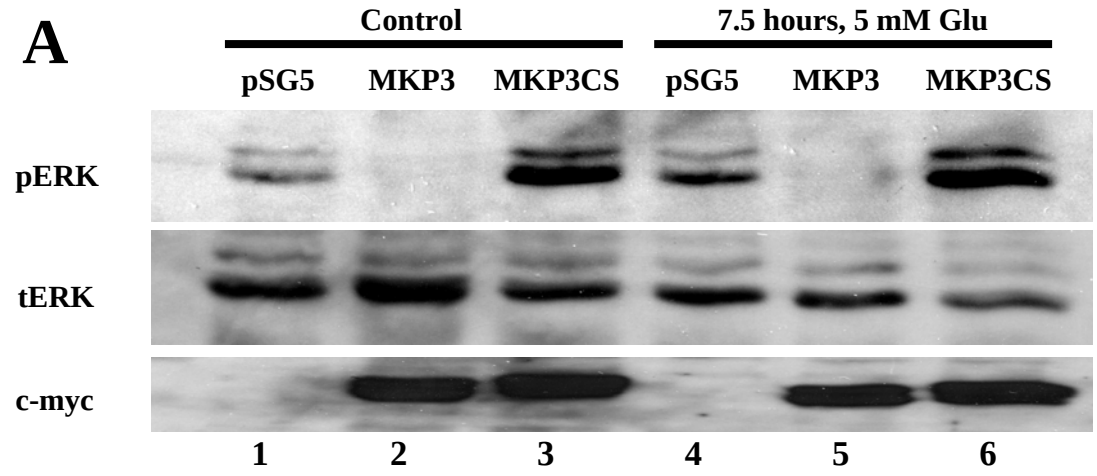
As expected from previous analyses of MKP3 compartmentalization, MKP3 WT and MKP3 C293S are clearly localized in the cytoplasm of transfected HT22 cells (Figures 12B-D). In HT22 cells that overexpress transfected MKP3 WT, phospho-ERK levels were dramatically reduced (Figure 12A). The high degree of transfection efficiency that we obtain with the HT22 cells (i.e., ~80% or higher) allowed for such significant reductions of ERK phosphorylation to be

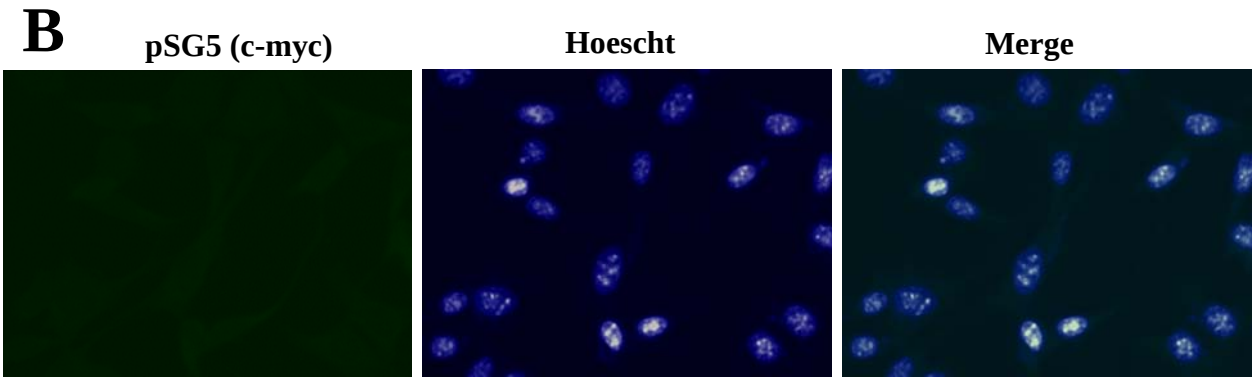
detected in extracts prepared from the total pool of cells. Overexpression of the MKP3 C293S mutant in the HT22 actually led to an enhancement of ERK phosphorylation which may be explained by the dominant negative effect of this mutant on basal MKP3 activity. Given the ability of MKP3 overexpression to affect ERK phosphorylation, we examined their effects on glutamate induced oxidative toxicity, which we have shown previously to be ERK-dependent (Stanciu et al., 2000; Levinthal and DeFranco, 2004). In agreement with earlier studies from our group, glutamate treatment increased phospho-ERK levels in HT22 cells transfected with an empty plasmid (Figure 12A, lanes 1 and 4). As observed in untreated HT22 cells, overexpression of MKP3 dramatically reduced ERK phosphorylation levels in oxidatively stressed cultures (Figure 12A, lanes 2 and 5). Furthermore, MKP3 C293S overexpression was as effective as in untreated cultures in increasing phospho-ERK to levels even higher than those attained with glutamate treatment alone (Figure 12A, lanes 3 and 6). Thus, MKP3 overexpression can be used to manipulate ERK phosphorylation in HT22 cells either under basal or oxidative stress conditions.

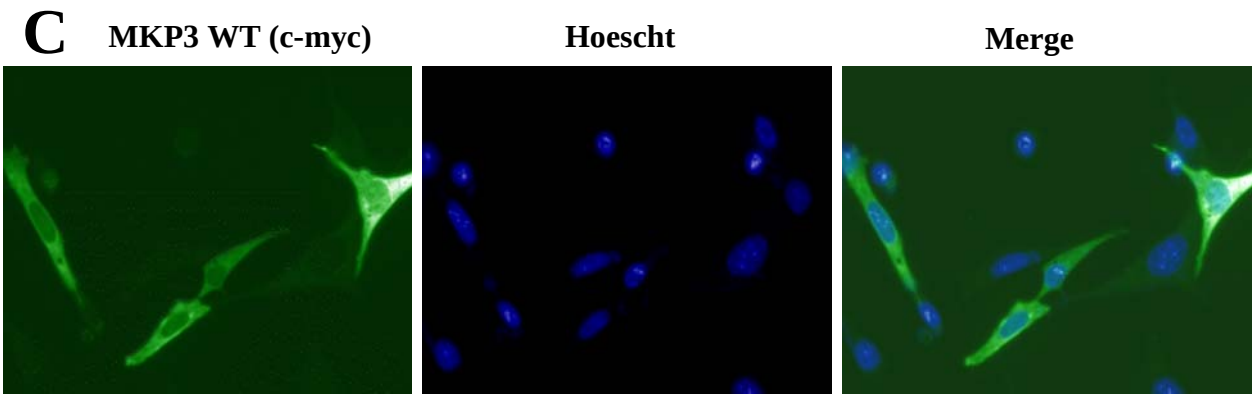
MKP3 C293S acts not only as a dominant negative to sequester ERK away from endogenous MKP3, but restricts ERK to the cytoplasm via its selective, high-affinity interaction with ERK via its N-terminal domain (Brunet et al., 1999; Tanoue and Nishida, 2003). As we have previously proposed a role for nuclear-localized ERK in glutamate-induced oxidative toxicity (Stanciu and DeFranco, 2002), we sought to establish a sensitive, functional test for detecting active ERK in the nuclei of MKP3 transfected cells. Therefore, HT22 cells were transfected with MKP3 expression plasmids along with an expression vector for an Elk-1/GAL4 DBD fusion protein and a luciferase reporter gene under the control of a promoter containing 5 tandem GAL4 DNA binding sites. Given that Elk-1 is an established nuclear target of ERK,

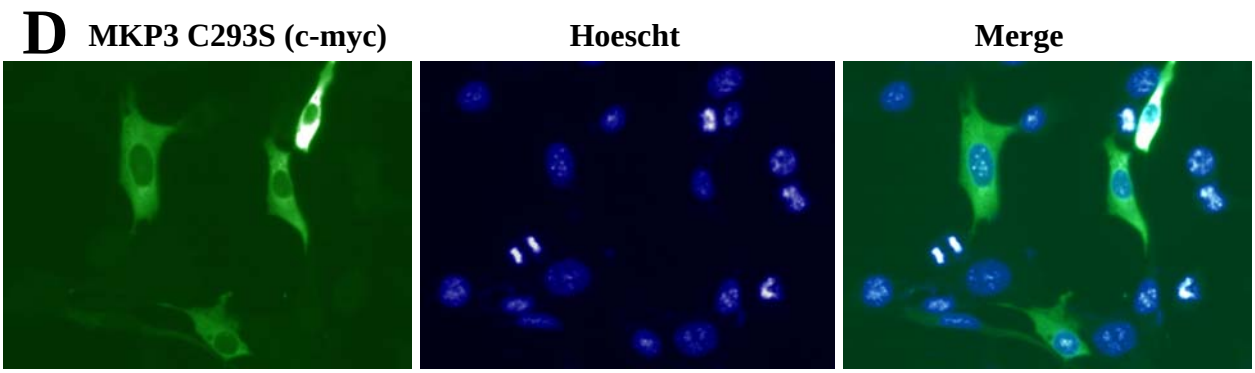
enhanced transactivation activity resulting from its phosphorylation by nuclear localized ERK is easily monitored through the activity of the luciferase reporter in this system.

As confirmation of the sensitivity of this system to the presence of activated ERK in the nucleus, luciferase activity was induced nearly two-fold upon glutamate treatment of HT22 cells transfected with an empty vector (Figure 12E). Treatment with the MEK inhibitor, U0126, blocked the glutamate-induced increase in luciferase activity. As shown in Figure 12E, MKP3 WT overexpression reduced luciferase expression below baseline in both untreated and glutamate-treated cells, as would be expected from the dramatic reduction in ERK phosphorylation (see Figure 12A). Interestingly, MKP3 C293S overexpression, which led to a significant increase in total ERK phosphorylation, led to reduction in luciferase activity in both untreated and glutamate-treated cells (Figure 12E). This result again confirms the sensitivity of the system to detect the activity of phosphorylated ERK within the nucleus and establishes the ability of MKP3 C293S to restrict nuclear translocation of active ERK in a neuronal cell line, consistent with previous work in fibroblast cell lines (Brunet et al., 1999). Collectively, these data show that glutamate-induced oxidative toxicity in HT22 cells is indeed accompanied by a functional increase in ERK signaling in the nucleus.


A
Control
7.5 hours, 5 mM Glu
pSG5
MKP3
MKP3CS
pSG5
MKP3
MKP3CS
pERK
tERK
c-myc
1
2
3
4
5
6


B
pSG5 (c-myc)
Hoescht
Merge


C
MKP3 WT (c-myc)
Hoescht
Merge


D
MKP3 C293S (c-myc)
Hoescht
Merge

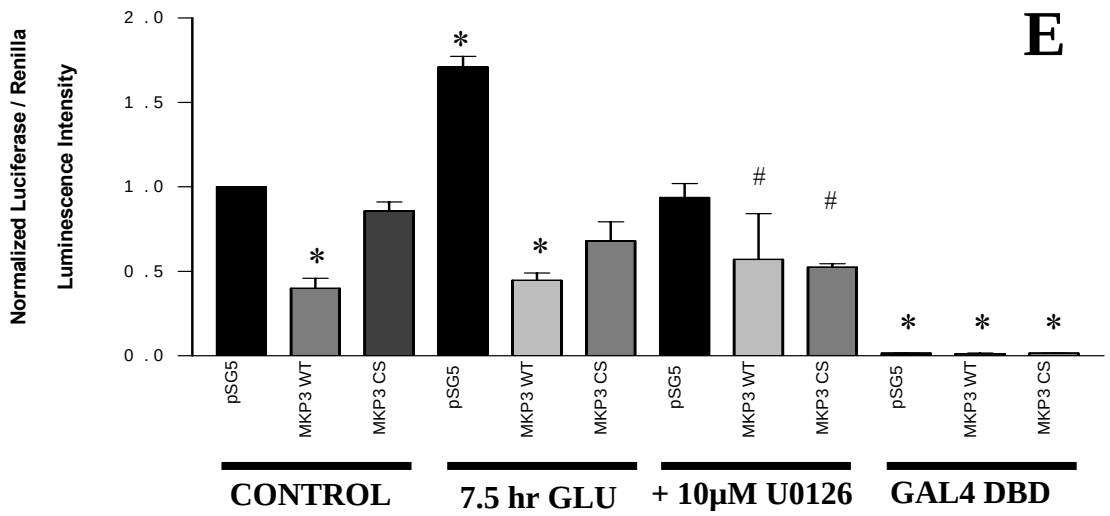


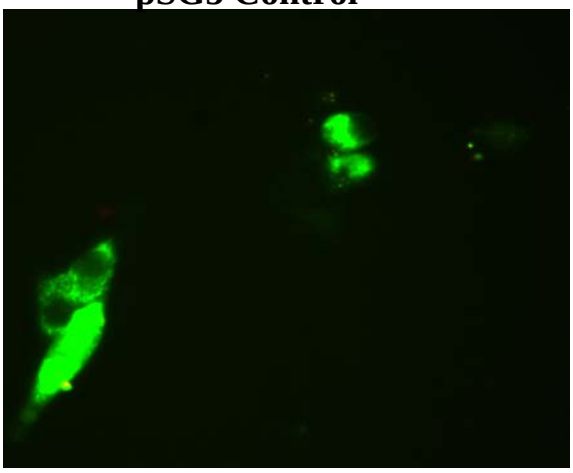
Figure 12 Overexpression of MKP3 WT and MKP3 C293S significantly alter ERK phosphorylation and translocation in HT22 Cells. HT22 cells transfected with either empty plasmid (pSG5), MKP3 WT, or MKP3 C293S were left untreated or treated with 5 mM glutamate for 7.5 hours, and whole cell lysates were prepared. Western blots from these cells demonstrate glutamate-dependent ERK activation in empty vector transfected cells, while MKP3 WT abrogated, and MKP3 C293S markedly accentuated, ERK activation independently of glutamate treatment (A). Immunocytochemistry against the myc epitope of the transfected myc-tagged MKP3WT and MKP3 C293S demonstrate a lack of staining in empty plasmid transfected HT22 cells (B) and a clearly cytoplamsic pattern of staining in cells transfected with the MKP3 WT (C) and MKP3 C293S (D) expression plasmids. HT22 cells were transfected with an Elk-1/GAL4 fusion expression plasmid, a Luciferase reporter plasmid, a Renilla expression plasmid, and either an empty plasmid, or either MKP3 WT or MKP3 C293S expression plasmids. Glutamate treated, empty plasmids transfected cells demonstrated a significant increase in luciferase expression, whereas both MKP3 WT or MKP3 C293S as well as U0126 abrogated this increase (E). * = $p < 0.001$ compared to pSG5 control; # = $p < 0.05$ compared to pSG5 control.

5.4.5. Overexpression of MKP3 WT and MKP3 C293S Protects both HT22 Cells and Primary Immature Cortical Neurons from Glutamate-Inducted Oxidative Toxicity

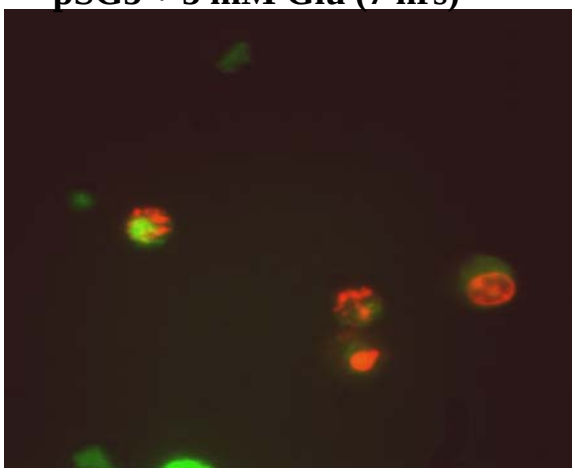
Given that MKP3 WT and MKP3 C293S overexpression was effective in manipulating both ERK activation and translocation, we sought to determine their impact on glutamate-induced oxidative-toxicity in both HT22 and primary immature cortical cell cultures. A single-cell, toxicity assay was devised that relied on the dual transfection of a mitochondrial-targeted eYFP expression plasmid (mt-eYFP) along with either an empty plasmid (pSG5), or either MKP3 WT or MKP3 C293S expression plasmids. After 6-7 hours of glutamate treatment, HT22 cells with a compromised plasma membrane become permeable to the PI, a DNA dye. Toxicity can be assessed in transfected HT22 cells by determining the percentage of dually-labelled, $eYFP^+/PI^+$ cells in untreated and glutamate-treated groups. As can be seen in Figure 13A, following transfection with the pSG5 empty plasmid, a significant proportion of HT22 cells were dually-labelled upon glutamate treatment. Co-transfection with either MKP3 WT (Figure 13B) or MKP3 C293S (Figure 13C) significantly blocked oxidative toxicity as reflected by a decrease in the percentage of dually-labelled cells compared to cultures transfected with empty vector. These results are quantified and summarized in Figure 13D.

A

pSG5 Control

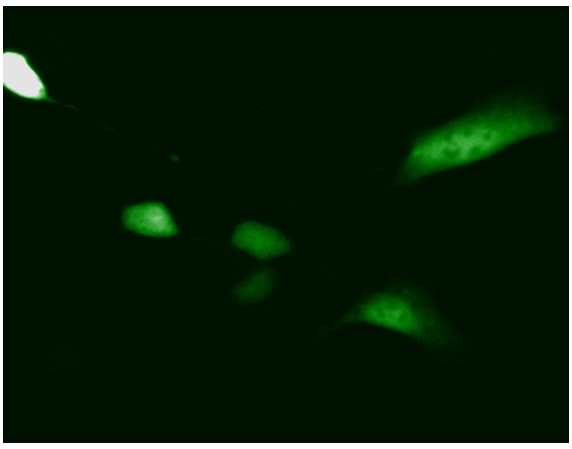


pSG5 + 5 mM Glu (7 hrs)

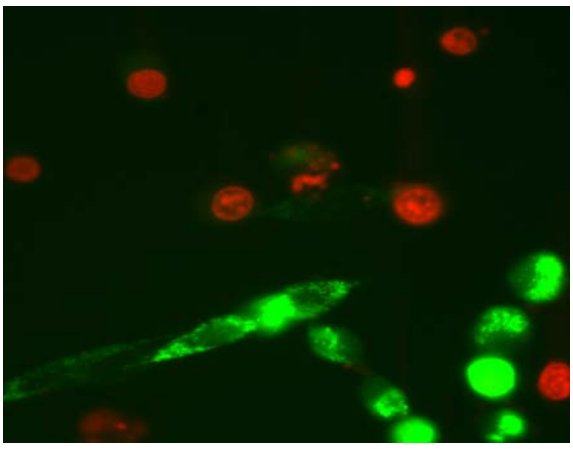


B

MKP3 WT Control

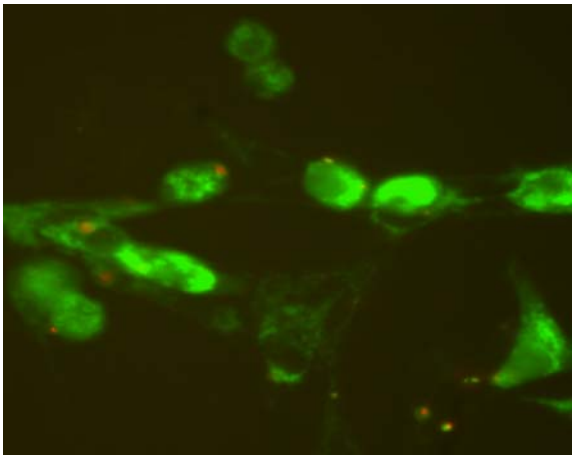


MKP3 WT + 5 mM Glu (7 hrs)

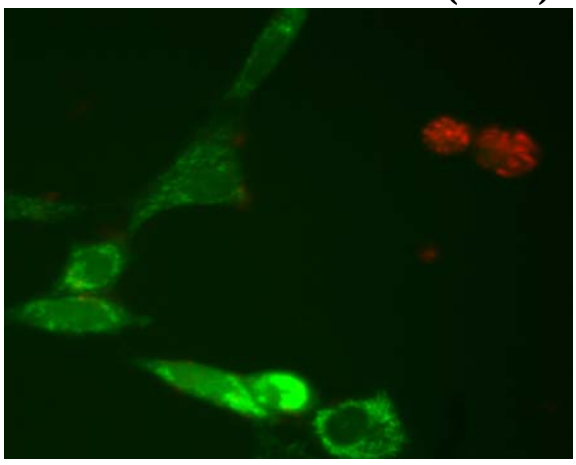


C

MKP3 C293S Control



MKP3 C293S + 5 mM Glu (7 hrs)



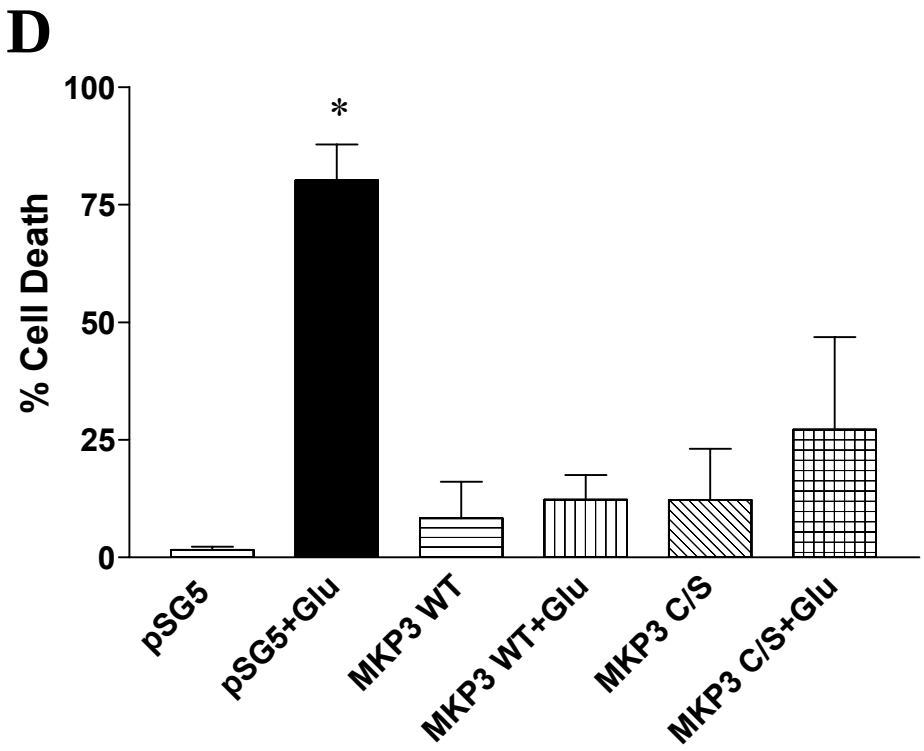
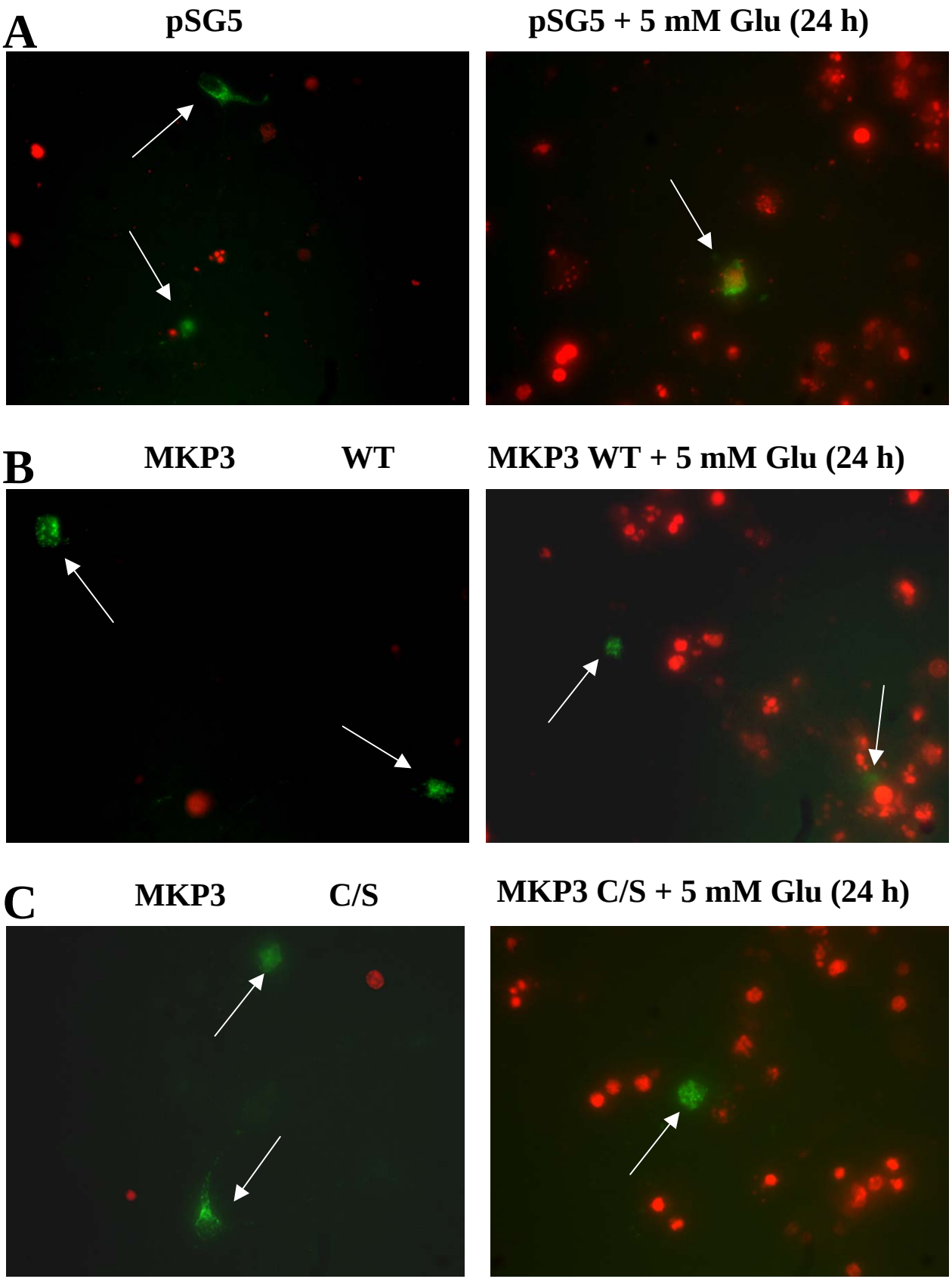


Figure 13 Overexpression of both MKP3 WT and MKP C293S protects HT22 cells from glutamate-induced oxidative toxicity. HT22 were co-tranfected with a mitochondrial-targeted eYFP expression plasmid and either an empty plasmid (pSG5), or either MKP3 WT or MKP3 C293S expression plasmids. Toxicity was measured as the percentage of propidium iodide (PI) positive cells within the transfected population in untreated and 5 mM glutamate-treated cells. Photomicrographs of untreated and treated empty plasmid (A), MKP3 WT (B), and MKP3 C293S (C) transfected HT22 cells are shown. A summary of counts from three random fields from at least three independent transfections is shown (D). * = $p < 0.01$ when compared to pSG5 control.

This finding was extended to primary immature cortical neuronal cultures. These cultures were transfected with both the mt-eYFP marker and either pSG5, MKP3 WT, or MKP3 C293S expression plasmids. We confirmed that the mt-eYFP transfection marker was localized in neurons by immunocytochemical staining of transfected cultures with an antibody to the neuronal marker NeuN (data not shown). Cultures were treated with glutamate, and PI-uptake in transfected cells was determined 18-20 hours later (at a time in which cells become PI labeled, but before a significant portion have detached from the culture dish). An example of a dually-labelled cell in glutamate-treated cultures transfected with empty vector is seen in Figure 14A. However, in cultures transfected with MKP3 WT (Figure 14B) or MKP3 C293S (Figure 14C), transfected cells were significantly spared from glutamate-induced oxidative toxicity as reflected by a reduced proportion of dually-labelled cells within the transfected population. These results are quantified and summarized in Figure 14D.

The results of the transfection experiments in HT22 cells and primary immature cortical neurons confirm that ERK activation is necessary for cell death during glutamate-induced oxidative toxicity. Furthermore, the fact that MKP3 C293S significantly protected both HT22 cells and primary neurons from cell death implies that activation of ERK1/2 alone is not sufficient to induce toxicity in response to glutathione depletion. Active ERK1/2 must be capable of translocating to the nucleus in order to trigger cell death in oxidatively stressed neurons.


A
pSG5
pSG5 + 5 mM Glu (24 h)
B
MKP3
WT
MKP3 WT + 5 mM Glu (24 h)
C
MKP3
C/S
MKP3 C/S + 5 mM Glu (24 h)

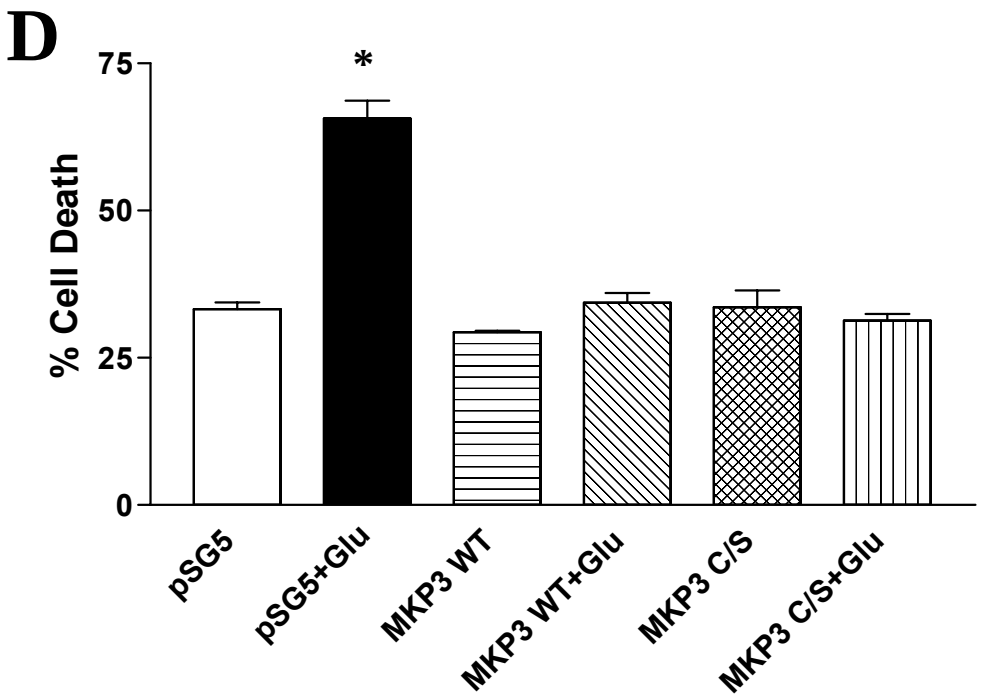


Figure 14 Overexpression of both MKP3 WT and MKP C293S protects primary immature cortical HT22 cells from glutamate-induced oxidative toxicity. Primary immature cortical cells were co-transfected with a mitochondrial-targeted eYFP expression plasmid and either empty plasmid (pSG5), MKP3 WT, or MKP3 C293S expression plasmid. Toxicity was measured as the percentage of propidium iodide (PI) positive cells within the transfected population in untreated and 5 mM glutamate treated cells. Photomicrographs of untreated and treated empty plasmid (A), MKP3 WT (B), and MKP3 C293S (C) transfected HT22 cells are shown. A summary of counts from three random fields from at least three independent transfections is shown (D). * = $p < 0.001$ when compared to untreated pSG5.

5.5. DISCUSSION

Although thiol-oxidation and inactivation of phosphatases has been shown in numerous cellular systems to be a feature of normal cellular signaling events (Meng et al., 2002) and to occur during ROS accumulation in neuronal cell lines (O'Loghlen et al., 2003), the role of this mechanism in oxidative stress-induced neurotoxicity has not been assessed previously. In this report, ERK-directed phosphatase activity was found to be specifically inhibited during times of oxidative stress, while phosphatases regulating other MAPKs, such as JNK1, were not affected. This inhibition of ERK-directed phosphatase activity is coincident with the temporal pattern of ERK activation that occurs during glutamate-induced oxidative toxicity – only at times in which inhibition of ERK-directed phosphatase activity is detected do we observe a concurrent increase in phosphorylated ERK1/2. These findings of substrate specificity and temporal congruence strongly indicate that oxidative inhibition of ERK-directed phosphatase activity is a mechanism that drives ERK activation and propels neurons toward a cell-death pathway. Based upon inhibitor studies, PP2A and vanadate-sensitive phosphatases appear to be the likely phosphatases that mediate this effect.

Many studies have demonstrated p38 and JNK activation during oxidative stress in numerous cell types, including primary cortical neuronal cultures (McLaughlin et al., 2001; Crossthwaite et al., 2002). We do not observe JNK or p38 activation in either HT22 cells or primary cortical neuronal cultures during glutatamte-induced oxidative toxicity. A potential reason for this disparity could be attributed to differences in the complement of MAPK-directed phosphatases that are expressed in various cellular systems or to the specificity to which certain phosphatases, even within one cell type, may be inhibited by oxidative stress. We have shown that JNK-directed phosphatases are not inhibited by oxidative stress in primary cortical cultures,

and that this correlates with the lack of increased JNK phosphorylation during oxidative stress. Thus JNK-directed MAPK phosphatases, such as M3/6, may not be as sensitive to oxidation as ERK-directed phosphatases, such as MKP3 or STEP. Further work clearly needs to be done to determine the differential sensitivities of various phosphatases to oxidation, as this has a direct implication for potential mechanisms of oxidative signaling.

A number of mechanisms could account for oxidation-dependent phosphatase inhibition. Protein-tyrosine phosphatases exhibit sensitivity toward oxidation of the catalytic cysteine (Sohn and Rudolph, 2003), and inactivation can occur by both reversible and irreversible mechanisms. Because both tyrosine-directed and dual-specificity phosphatases share a canonical $HC(X)_5R$ motif in their active sites, it is likely that both classes undergo similar mechanisms of inhibition. Conversion of the active-site cysteine to a metastable sulfenic acid (Cys-SOH) (Meng et al., 2002) or the involvement of this cysteine in the formation of an intramolecular disulfide bond (Sohn and Rudolf, 2003) are both reversible with thiol-reducing agents. A novel mechanism of reversible oxidative inhibition of PTP-1B was recently identified that involves the formation of a sulphenyl-amide stable intermediate resistant to further oxidation (van Montfort et al., 2003; Salmeen et al., 2003). However, the extent to which this mechanism operates in other phosphatases is currently unknown. Progression of these reversible states to sulfinic ($Cys\text{-}SO_2H$) or sulfonic ($Cys\text{-}SO_3H$) acid forms leads to irreversible phosphatase inhibition. Since we observe total reversibility of oxidative phosphatase inhibition with the thiol-reductant DTT, oxidation of ERK-directed phosphatases to sulfinic and sulfonic forms are unlikely to occur in the oxidatively stressed neuronal cultures that we analyzed. Furthermore, the fact that activity is recoverable with DTT rules out the possibility that decreased ERK-directed phosphatase activity during oxidative stress reflects decreased phosphatase expression (Vasudevan et al., 2004) or

increased phosphatase degradation (Lin et al., 2003). The restoration of ERK-directed phosphatase activity with DTT is likely to represent a direct effect on the phosphatases themselves. However, it is formally possible that DTT is reversing the thiol-oxidation of cofactors necessary for ERK-directed phosphatase function. The identification of both the sites of oxidation and the mechanism of inhibition of specific ERK-directed phosphatases during oxidative stress will be the subject of future studies.

Although redox modification of cysteine residues plays an important role in protein phosphatase inhibition, other mechanisms are possible, such as nitrosylation or glutathionylation of residues within the protein, or oxidation of Fe-Zn metal clusters that are critical for the function of Ser/Thr-directed phosphatases. In fact, oxidative-inhibition of Ser/Thr-directed phosphatases is not well understood and may reflect effects on oxidant-sensitive regulatory subunits. Nonetheless, oxidative-stress induced inhibition of phosphatases is likely to represent a common mechanistic thread that drives cellular signaling events during neuronal cell death in a diverse range of conditions, such as in stroke, Alzheimer's Disease, or Parkinson's Disease. Indeed, PP2A activity has been found to be reduced in the cortices of Alzheimer's patients as compared to control patients (Gong et al., 1995), and ERK has been found to be activated in these tissues (Pei et al., 2002). Tau is a well-described ERK1/2 target (Pei et al., 2003), and the hyperphosphorylation of tau protein and the development of neurofibrillary tangles in Alzheimer's Disease pathology could reflect aberrant ERK1/2 activity (Pei et al., 2003). Interestingly, mice expressing a dominant negative form of PP2A in neurons display features of Alzheimer's pathology (Kins et al., 2003). Thus, oxidative stress-mediated PP2A inhibition in Alzheimer's disease may account for enhanced ERK1/2 activity and subsequent tau hyperphosphorylation and neurofibrillary tangle formation.

Because the endogenous pool of PP2A and vanadate-sensitive phosphatases is inactivated during oxidative toxicity in primary neurons, we sought to overexpress one of these phosphatases to attempt to overcome this inhibition and impact ERK activation. The choice of MKP3 as a tool to manipulate ERK activity in our system was made for several reasons. MKP3 is localized in the cytoplasm and physically interacts with ERK1/2 at a kinase-interacting motif (KIM) domain in its N-terminus in a highly specific manner. Brunet et al. elegantly showed that the overexpression of MKP3 WT could extinguish ERK1/2 signaling, whereas overexpression of the catalytic mutant, MKP3 C293S, could abolish ERK1/2 signaling specifically in the nucleus by acting to trap activated ERK1/2 in the cytoplasm via its physical interaction with MKP3 (Brunet et al., 1999).

Shuttling of phospho-ERK into and out of the nucleus is a dynamic process, and standard, static immunocytochemical fluorescence imaging techniques may not be sufficient to detect small shifts in the equilibrium of the subcellular distribution of phospho-ERK. However, these small changes in the distribution of phospho-ERK could have important biological consequences. We therefore employed a sensitive, quantifiable, and functional assay to assess nuclear residency of active ERK during glutamate-induced oxidative toxicity. In this assay, luciferase expression was controlled by the activation of an Elk-1-GAL4 fusion by active-ERK in the nucleus. We found that Elk-1-Gal4 directed luciferase expression increases with oxidative stress in HT22 cells and that both MKP3 WT and MKP3 C293S block this increase. Thus, glutamate-induced oxidative stress leads to an increase in active ERK function within the nucleus. Even though it is predominantly localized within the cytoplasm, overexpressed wild-type MKP3 can deplete the total cellular pool of active ERK most likely by acting upon dynamically shuttling ERK during its transit through the cytoplasm. Overexpression of the

MKP3 C293S mutant exerts a dominant negative effect to block endogenous MKP3 and elevate ERK phosphorylation. Elevated phospho-ERK in this case is not accessible to the Elk-Gal4 fusion in the nucleus due to its sequestration with the cytoplasm by the MKP3 C293S mutant (Brunet et al., 1999).

Despite the likelihood that multiple phosphatases from a diversity of classes are responsible for the regulation of ERK phosphorylation, MKP3 overexpression was sufficient to manipulate ERK activity in oxidatively stressed neurons and revealed consequences of ERK activation and localization on toxicity. Thus, inhibition of ERK activation was brought about by MKP3 overexpression and led to significant protection from oxidative toxicity in both HT22 cells and immature cortical neuronal cultures. Obviously, the overexpression was sufficient to overcome the oxidation-induced inhibition of endogenous ERK-directed phosphatases in the primary neurons. Elevation of ERK activation brought about by MKP3 C293S overexpression also was protective in both contexts presumably based upon the sequestering of active ERK in the cytoplasm. Thus, we hypothesize that ERK-directed toxicity in oxidatively stressed neurons requires some translocation of active ERK to the nucleus. We are currently in pursuit of specific targets of ERK that may be mobilized, even in response to the transient nuclear accumulation of active ERK, to trigger neuronal cell death in response to oxidative stress.

ERK activation occurs downstream of ROS production in primary immature cortical cultures undergoing glutamate-induced oxidative toxicity. We have previously monitored ROS in primary immature cortical cultures, and ERK inhibition via U0126 administration did not alter the rate or extent of ROS production in these cells during glutamate-induced oxidative toxicity (Levinthal and DeFranco, 2004). We observe that ERK-directed phosphatases are still inhibited in glutamate-induced oxidative toxicity in the presence of U0126 (data not shown), a treatment

that protects these cultures from cell death. This indicates that the ERK-directed phosphatase inhibition is not sufficient for toxicity in of itself, but must act through specific activation of ERK. Furthermore, it also implies that there is a requirement for MEK activity to provide a forward drive for ERK activation during oxidative stress (Levinthal and DeFranco, 2004) in primary immature cortical cultures.

The results reported here represent the first demonstration of the critical toxic role of ERK1/2 activity in the nucleus using a molecular approach rather than relying upon inhibitor studies, which can not resolve the distinction between ERK1/2 activation as a whole versus the translocation of active ERK1/2. Thus, the spatio-temporal pattern of ERK activation during glutamate-induced oxidative toxicity is critical for determining the cellular response to this insult. Nuclear targets of ERK must be mobilized to bring about oxidative toxicity in neurons. We also have implicated an intriguing mechanism by which oxidative stress may be coupled to ERK activation. Specific oxidation of ERK-directed phosphatases during glutamate-induced oxidative toxicity could drive ERK activation, and nuclear accumulation, leading to neuronal cell death. We are now fully engaged in determining the specific phosphatases that are involved in mediating this effect and the mechanism by which they are inhibited by oxidative stress.

6. CONCLUDING REMARKS

The recruitment and activation of cellular signaling pathways were once thought to be primarily involved in the integration and transduction of signals emanating from hormones, neurotransmitters, growth factors, cell-cell and cell-extracellular matrix contact, among numerous other factors, that impinged upon the cell from the surrounding milieu. Recently, cellular stressors such as irradiation, ischemia, heat shock, and oxidative stress have been shown to activate cell signaling pathways in the absence of clearly defined ligand-receptor interactions. The mechanisms by which cellular stressors lead to the activation of signaling events and eventual stress responses are just beginning to be identified, but clearly these responses are highly individualized across specific cell types and for specific stressors. The data presented here provide insight into the mechanisms by which a specific cellular signaling pathway, the ERK-MAPK pathway, is recruited during oxidative stress-induced neuronal cell death. This ERK activation was shown to be dependent upon the tonic activity of the PI3K-Akt pathway and subsequent PI3K-dependent MEK activation. Furthermore, the persistent ERK activation that leads to cellular toxicity can be driven by the oxidation-dependent inactivation of ERK-phosphatases. The balance of activating kinase activity and inactivating phosphatase activity dynamically regulates ERK-dependent signaling and is a major determinant of neuronal cell responses to oxidative stress. Thus, the reduction of ERK signaling by the administration of inhibitors (Chapter 1) and the overexpression of ERK-specific phosphatases (Chapter 2) can overcome glutamate-induced oxidative toxicity in both HT22 cells and in primary immature cortical cultures. These results clearly place ERK activation as a necessary event that leads to neuronal cell death during oxidative stress. A summary of the important cellular events during glutamate-induced oxidative toxicity is shown in the model diagram below (Figure 15).

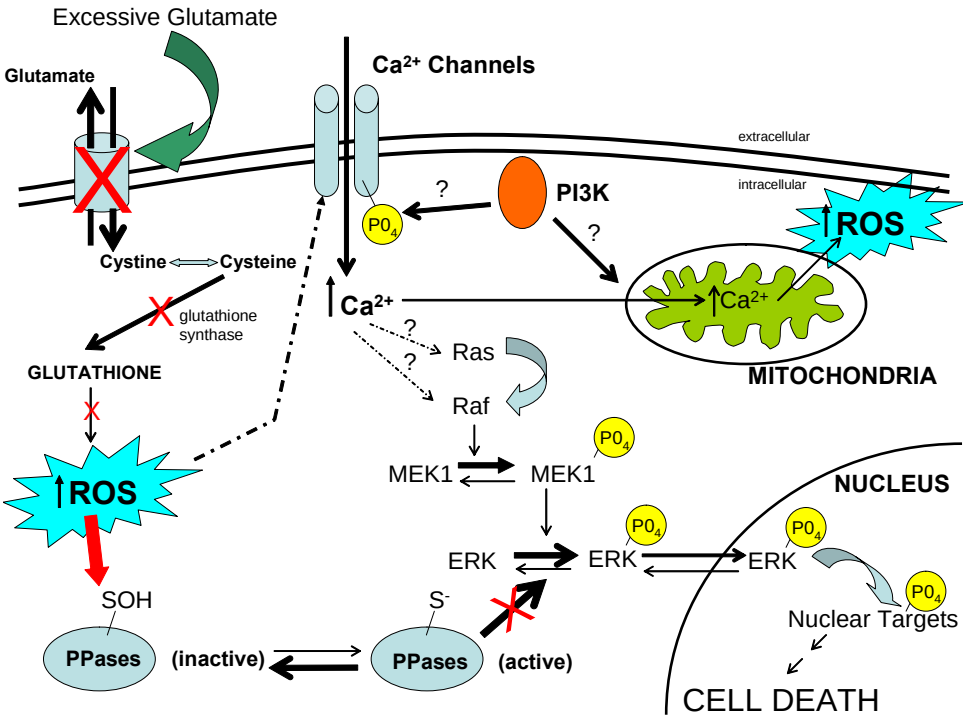


Figure 15 Schematic summary diagram.

6.1. PI3K as a Mediator of Neuronal Cell Death

ERK activation during glutamate-induced oxidative toxicity in primary cortical cultures is PI3K-dependent during its initial phase, but not for the delayed, prolonged ERK activation that occurs at times of increasing oxidative stress. Cross-talk between the PI3K-Akt and ERK signaling pathways has been demonstrated in many studies, but typically "cross-talk" implies a direct action of one pathway on another (i.e., via direct phosphorylation). Because the loss of PI3K-Akt signaling several hours preceding, but not during, the accumulation of ROS leads to

the abrogation of ERK signaling, it is unlikely that PI3K activity is directly involved in the activation of ERK. Interestingly, we did not observe phosphorylation of Akt, the primary downstream target of PI3K, upon glutamate administration, and thus the link between PI3K activity and ERK activation is not likely via an Akt-dependent mechanism. A potential link between these two signaling pathways could be via calcium channels that are under the tonic control of PI3K. As discussed in Chapter 1, several calcium channels in both neurons and non-neuronal cells have been identified as PI3K-sensitive and are relatively impermeable to calcium flux in the presence of the PI3K inhibitors LY294002 or wortmannin. Because calcium influx into the cell via plasma membrane Ca^{2+} channels is an important feature of glutamate-induced oxidative toxicity (Tan et al., 1998), it is possible that PI3K inhibition renders these channels inactive, breaking the mechanistic link between oxidative stress, calcium influx, MEK activation, and eventual ERK activation. Although MEK inhibitors have been shown to block rises in intracellular calcium levels in HT22 exposed to glutamate-induced oxidative toxicity (implying that MEK functions upstream of calcium entry in these cells), the relationship between calcium and ERK in primary neurons has not been investigated. Future studies will be needed to determine whether calcium entry in primary immature cortical neurons exposed to glutamate-induced oxidative toxicity (Li et al., 1997) functions upstream of MEK and subsequent ERK activation, the nature of those calcium channels (i.e. L-type versus N-type), and the PI3K-senstivity of those calcium channels.

The identification of PI3K as a positive contributer to oxidative stress-induced neuronal cell death is quite surprising given that this pathway has been universally ascribed a role in cell survival across a vast array of cell types and cellular stressors. The data described here are the first to demonstrate that PI3K activity is necessary for some forms of cell death. Interestingly,

since the publication of the material in Chapter 1 (Levinthal and DeFranco, 2004), two research groups have identified a similar mechanism of PI3K dependent cell death. One study has made use of a model of interferon (IFN)-induced cell death in multiple myeloma cells. In this case, although IFN did not activate Akt above basal levels, PI3K inhibition with LY294002 protected against toxicity by preventing the recruitment and activation of caspases (Thyrell et al., 2004). Furthermore, NO-induced apoptosis was found to be PI3K-dependent in cultured mouse astrocytes (Yung et al., 2004). Additionally, this study revealed the lack of Akt activation during toxicity, and LY294002 was not toxic to astrocytes when administered alone (Yung et al., 2004). All of these results are completely consistent with my findings in glutamate-treated primary immature cortical cells and underscore the potential role for PI3K to act independently of Akt to contribute to a cell death process.

6.2. Oxidation Dependent Inhibition of ERK-directed Phosphatases

The identification of mechanisms by which oxidative stress conditions are linked to cellular signaling events has received an increasing amount of attention and is truly an area of intense investigation. As mentioned above, oxidative stress is a cellular condition that appears to lack the specificity of traditionally described ligand-receptor or protein-protein interactions that initiate signaling events. However, oxidative stress conditions do specifically activate subsets of signaling pathways, and this phenomenon is now referred to as oxidative signaling given that cellular oxidants are now becoming viewed as bonefide second messengers. The identification of several protein phosphatases that are uniquely sensitive to reversible oxidative inhibition has led to a conceptual model in which protein phosphatases act as redox sensors for the cell and

function proximally to initiate signaling. The oxidation of specific phosphatases could be a generalized mechanism by which oxidative stress is linked to the activation of specific signaling cascades. The development of strategies that disrupt these mechanisms during pathological conditions are likely to lead to novel therapeutic approaches to several disorders that depend upon the production and accumulation of ROS and in which aberrant signaling events have been shown to occur, such as ischemia/reperfusion injury following a stroke, Parkinson's Disease, Alzheimer's Disease, and Amyotrophic Lateral Sclerosis.

Most of the data supporting a role for oxidation-dependent phosphatase inhibition has come from studies of growth factor ligand-receptor interactions in fibroblast cell lines. In these models, the recruitment and activation of signaling complexes at the plasma membrane in response to ligand binding is accompanied by the localized production of ROS that inactivate specific phosphatases. These inactivated phosphatases, such as SHP-2, are necessary for the amplification of signaling downstream of activated receptors (e.g., PDGF receptors) and treatment with antioxidants can abrogate signaling (Meng et al., 2002). Although oxidative conditions do not universally inhibit all phosphatases, the mechanisms governing the oxidative sensitivity of one phosphatase versus another are currently unknown. Furthermore, the extent to which oxidation-dependent inhibition of phosphatases operates as a generalized mechanism in growth factor signaling in all cell types is being actively pursued by several research groups. Localized production of ROS may function in normal signaling events as important second messengers, and this discovery is likely to revolutionize the field of cellular signaling much in the same way that it was changed following the identification of nitric oxide and carbon monoxide as gaseous signaling molecules. The development of new tools to measure oxidation-dependent modifications on target proteins is likely to drive an explosion in future research. A

recent proof-of-principle study used antibodies raised against the oxidized form of the active site cysteine residue within the phosphatase receptor-like PTPα (RPTP). Oxidation-dependent modifications on this phosphatase and others, including SHP-2, were detected during exposure to hydrogen peroxide and during normal PDGF signaling (Persson et al., 2004). This approach is quite exciting, as the future of this field of research is likely to employ these reagents much in the same way phospho-specific antibodies are used now to detect post-translational phosphorylation modifications on target proteins.

The data presented in Chapter 2 are the first to implicate oxidation-dependent phosphatase inhibition as an important event during the unfolding of oxidative-stress dependent neuronal cell death. Several groups have identified ROS-dependent MAPK member activation in both neuronal and non-neuronal cells, but the role of potentially reversible, inactivated phosphatases acting as the causal link between ROS and MAPK activation had not been explored. I have found that ERK-directed phosphatase activity is inhibited by oxidation during times of oxidative stress that correlate with the upregulation of ERK activity. This occurs specifically for the ERK MAPK pathway, as JNK-directed phosphatase activity is not inhibited and JNK activation is not observed. Furthermore, inhibition of ERK-directed phosphatase activity was completely reversible by thiol-reducing agents such as DTT. Although this is clear evidence that oxidation-dependent inhibition of ERK-phosphatase activity occurs through the oxidation of cysteine thiols, it is not clear whether this represents a direct effect on the phosphatases themselves, or whether thiol-oxidation alters one or more intermediate phosphatase regulatory proteins. This issue may be more relevant for serine/threonine-directed protein phosphatases, which in many cases interact with regulatory subunits in vivo, than for the PTPs/DSPs, which do not typically interact with subunits other than their specific protein targets.

Further studies monitoring the activity of isolated phosphatases from oxidatively stressed cells (i.e., via immunoprecipitation and phosphatase activity assays) will be necessary to determine the direct versus indirect nature of oxidation-dependent phosphatase inhibition.

I used inhibitors to determine the nature of the oxidation-sensitive phosphatases in primary immature cortical cultures and found that the main component of ERK-directed phosphatase activity was inhibited by okadaic acid, while a smaller component was inhibited by orthovanadate. This implicates PP2A as a primary oxidation-sensitive ERK-directed phosphatase in cultured neurons, while the remaining phosphatases are likely PTPs or DSPs. However, as mentioned above, future studies will require direct measurements of PP2A activity to prove that this phosphatase becomes directly inhibited during oxidative stress and that this inhibition can be reversed by thiol-reducing agents. These studies can be accomplished by immunoprecipitating PP2A and determining phosphatase activity directly by measuring the extent of cleavage of well-characterized, phosphorylated substrates such as pNPP or Malachite Green. Although orthovanadate lacks resolution as an inhibitor, there are several vanadate-sensitive attractive candidate PTPs/DSPs that operate within neurons. STEP, MKP3, and MKP1, are expressed in neurons and are active against phosphorylated ERK. The oxidation-dependent inhibition of these targets will need to be tested, as they could also contribute to persistent ERK activation during oxidative stress.

While glutamate-induced oxidative toxicity in primary immature cortical cultures is a convenient model system for the study of oxidative stress-induced neurodegeneration, the study of animal models of oxidative stress-dependent neuronal toxicity may be more directly relevant to human pathology. The middle cerebral artery occlusion (MCAO)-reperfusion model in rodents is an excellent paradigm that models a stroke in vivo, and a significant proportion of

strokes in humans develop subsequent to transient blockages in the MCA or its branches. In the MCAO model, the middle cerebral artery, which in rats supplies oxygen and nutrients to most of the cortex, hippocampus, and striatum, is blocked either from within the vessel lumen or by constriction from outside of the vessel, and neuronal damage is restricted to the ipsilateral side. Other models of more global neuronal damage, such as the four-vessel occlusion model, are more relevant for modeling the brain damage that occurs in conditions where oxygenated blood flow is impaired systemically, such as during a heart attack. Several aspects of the MCAO focal ischemia-reperfusion model have been characterized, including the regional pattern and time course of neuronal degeneration, histological and biochemical markers of necrosis and apoptosis, as well as the dependence upon the activation of specific signaling pathways. After a brief decrease immediately after MCAO, ROS steadily accumulate above baseline over long occlusive periods (i.e., 3 hours or more) and show a robust increase in formation and accumulation in the minutes following reperfusion (Peters et al., 1998). Whereas the initial accumulation of ROS during ischemia has been associated with mitochondrial dysfunction, the cellular source of the burst of ROS during reperfusion is not well characterized (Becker, 2004). However, because the production and accumulation of ROS is a necessary feature of neurodegeneration in the MCAO model and ERK is specifically activated and associated with neuronal cell death in this context (McCulloch and Dewar, 2001; Noshita et al., 2002), oxidation-dependent inhibition of ERK-directed phosphatases could drive ERK activation and neuronal toxicity during MCAO/reperfusion. Indeed, transgenic mice that overexpress Cu/Zn SOD1 show resistance to MCAO-induced neurodegeneration specifically by preventing the activation of ERK via decreases in ROS production (Noshita et al., 2002). Future studies in our group will investigate the potential role of oxidation dependent ERK-phosphatase inhibition in the activation of ERK in

several disease models, including the MCAO model, that have been shown to involve oxidative toxicity. These studies may suggest novel approaches to therapies that target the potential maladaptive interaction of phosphatases and ERK during oxidative stress.

6.3. Subcellular Localization of ERK during Oxidative stress

In Chapter 2, I conclusively demonstrate that ERK translocation to the nucleus is necessary for neuronal oxidative toxicity in both HT22 cells and primary immature cortical cultures. I used two important molecular tools to manipulate ERK in transfected cells. Overexpression of the cytosolic, ERK-specific phosphatase MKP3 and a catalytically inactive mutant MKP3 C293S can impact ERK activation and translocation, respectively. These two forms of MKP3 were first used in an influential and elegant study of ERK nuclear translocation and cell cycle dysregulation in fibroblasts (Brunet et al., 1999), but they have not been previously used in models of neuronal toxicity. This molecular approach is superior to the traditional use of chemical inhibitors because these inhibitors may impact other pathways and, more importantly, they fail to dissociate ERK activation from ERK translocation to the nucleus. MKP3 is a specific ERK phosphatase that resides in the cytoplasm. MKP3 C293S overexpression leads to increased levels of phosphorylated ERK, yet prevents its translocation to the nucleus by physically binding to active ERK in the cytoplasm. The use of MKP3 WT and MKP3 C293S will likely provide insights into the necessity of ERK activity and nuclear translocation both in normal neuronal functioning and in various models of neuronal toxicity. Furthermore, MKP3 WT and MKP3 C293S could be useful tools to resolve long-standing controversies surrounding ERK localization in neurons. For example, although current

immunohistochemical or cellular fractionation techniques can be spatially precise in identifying ERK localization at a given moment in time, they are hampered by an inability to monitor complex events that involve rapid shuttling between cellular compartments over time. Thus, studies using these techniques cannot give an accurate representation of the nuclear residency time for ERK and other proteins that rapidly shuttle between the nucleus and the cytoplasm, nor can they definitively demonstrate the necessity of ERK translocation to the nucleus in the studied paradigm. Several studies have implicated the nuclear localization of ERK as a requisite event in the development of long term potentiation at hipppocampal synapses. For example, cAMP-mediated long term potentiation is associated with nuclear translocation of phospho-ERK in hippocampal slice cultures (Patterson et al., 2001), and ERK-dependent CREB phosphorylation (Impey et al., 1998; Davis et al., 2000). However, the absolute necessity of nuclear localized ERK for the development of long lasting LTP is not known and has been difficult to establish because MEK inhibitors, which do disrupt LTP formation (Davis et al., 2000), globally suppress ERK phosphorylation in all cellular compartments. Thus, the expression of MKP3 C293S in hippocampal neurons could physically restrict ERK to the cytoplasm and would definitively demonstrate the requirement of ERK nuclear translocation in the development of LTP. Similarly, ERK translocation during Aβ and Fe-induced neuronal toxicity in primary cortical cultures has been implicated as a necessary feature of this mode of neuronal cell death by immunocytochemical methods and the application of MEK inhibitors (Kuperstein and Yavin, 2002). Such data is only associative, and the expression of MKP3 C293S in these cells would again definitely demonstrate the necessity of ERK translocation in Aβ/Fe neuronal toxicity. The use of molecular tools, such as MKP3 WT and MKP3 C293S, to independently manipulate both

the subcellular localization and activity of ERK in neurons is an exciting development that will greatly refine and enhance studies that focus on this pathway in neurons and in other cell types.

The use of genetically altered mice has been somewhat instructive in examining the functioning of ERK isoforms during higher order processes and behaviors. As described earlier, although ERK2 knockout (KO) mice show an embryonically lethal phenotype, some forms of learning are enhanced in ERK1 KO mice (Mazzucchelli et al., 2002). However, these studies made use of global knockouts of ERK1 or ERK2, and the interpretation of the results are complicated by the same issues that plague most KO studies – the potential compensation for function by other pathways, the diffuse nature of the genetic manipulation, and developmental effects, among others. Temporally controlled downregulation of the ERK1/2 pathway in a subset of cells could be achieved by placing MKP3 WT under a tetracycline regulatable, tissue specific promoter. However, this system would still rely on alterations in gene expression, and tight temporal control would be difficult to achieve. These issues are significantly resolved by the use of high affinity tamoxifen-binding estrogen receptor (ER) mutant fusion proteins. Many enzymes, when physically tethered to ER within a chimeric fusion protein, are inactive in the absence of tamoxifen but become responsive in the presence of this drug. One of the first uses of this technology was in the construction of temporally and spatially inducible KO mice in which the chimeric CRE-recombinase/ER fusion was expressed under tissue specific promoters, and floxed genes containing Lox-P CRE-targeting sites were specifically disrupted in those tissues upon tamoxifen treatement (Li et al., 2000). Furthermore, the activity of other enzyme-ER chimeras has been shown to be tamoxifen dependent in vivo. For example, tamoxifen has been shown to drive Raf-1-ER fusion protein-dependent MEK and ERK activation in stably-transfected cell lines (Le Gall et al., 2000). Placing the expression of other chimeric enzymes

under tissue specific promoters may thus lead to unparalleled temporal and spatial control over enzymatic functioning in vivo.

Particularly intriguing is the possibility of constructing tamoxifen-inducible ER-MKP3 WT or ER-MKP3 C293S fusion proteins. These constructs would add a dimension of temporal control such that MKP3 activity or MKP3 C293S action would be unleashed in the presence of tamoxifen. The ability to restrict ERK activity directly or to restrict ERK localization to the cytoplasm in a temporally controlled manner would be an impressive tool to probe multiple systems. If these constructs operate as predicted in vitro, then we would expect tamoxifen treatment to protect transfected HT22 cells and primary neuronal cultures from oxidative toxicity. If this experimental paradigm were successful in cultured cells, we would proceed to engineer transgenic animal expressing each of these constructs under tissue specific promoters, such as the calmodulin II (CamII) kinase promoter that specifically targets the neurons of the forebrain. Thus these animals would be sensitive to tamoxifen and would respond by downregulating ERK activity or ERK translocation specifically in neurons. Major studies that have relied on MEK inhibitors to determine the ERK-dependence of such important and diverse processes as learning and memory and neuronal cell death after ischemia / reperfusion could be replicated in these transgenic animals with a specificity never achieved before.

In summary, the work presented in this thesis has definitively established that the regulation of ERK activation and translocation is a major determinant of cell fate during glutamate-induced oxidative toxicity in both HT22 cells and primary immature cortical cultures. ERK activation is dependent upon tonic PI3K activity in cortical cultures, and sustained activation is likely driven by the oxidation-mediated inhibition of ERK-directed phosphatases. Manipulations of ERK activity and nuclear translocation using MKP3 show that it is the

translocation of active ERK to the nucleus, not ERK activation per se, that is necessary for toxicity. Collectively, these data have clarified the role of ERK during glutamate-induced oxidative toxicity and have identified a novel mechanism that drives ERK-dependent neuronal cell death. Future studies will be directed toward manipulating active ERK in the nuclear compartment by expressing nuclear, ERK-directed phosphatases that can act either to diminish ERK activity (i.e., through the WT form of the phosphatase) or lead to sequestration of active ERK in the nucleus (i.e., through the catalytically inactive form of the phosphatase). Good candidate phosphatases for manipulating ERK in this manner would be MKP1 and B23.

Exploration of the mechanisms governing the redox sensitivity and reversible inactivation of phosphatase function is an intriguing new horizon in the field of cellular signaling research. Indeed, redox-dependent alterations in protein functioning may be revealed as a ubiquitous mechanism by which purposefully and locally produced ROS/RNS act as signaling molecules themselves during a host of normal cellular processes. Thus, thiol-oxidation and other redox dependent changes could enter the pantheon of post-translational protein modifications that include phosphorylation, glycosylation, and ubiquitination, among numerous others. The identification of oxidation-inactivated phosphatases as uniquely sensitive redox sensors has bridged the mechanistic link between ROS production and the activation of specific cellular signaling pathways. It is likely that future studies of disease will reveal this mechanistic link to be involved in a host of disorders that involve oxidative stress, and future therapies may target this mechanism.

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