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Role of cofilin, an actin cytoskeletal protein, in ischemic conditions : potential therapeutic target for ischemic stroke

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Role of Cofilin, an Actin Cytoskeletal Protein, in Ischemic Conditions: Potential Therapeutic Target for Ischemic Stroke

by

Anusha Madineni

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Medicinal Chemistry

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An Abstract of

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Cerebral ischemia or stroke is a condition associated with decreased blood supply to brain leading to death of neurons. It is associated with a diverse cascade of responses involving both degenerative and regenerative mechanisms. Actin cytoskeleton changes play a key role in mediating these responses. Cofilin, an actin cytoskeletal severing protein is known to be involved in early stages of apoptotic cell death. Evidence supports its intervention in progression of disease states like Alzheimer's and ischemic kidney disease. We have hypothesized possible involvement of cofilin in cerebral ischemia and neurodegeneration. To investigate the potential actin cytoskeletal changes induced by cofilin in ischemia, various ischemic models such as oxidative stress, oxygen glucose deprivation (OGD) *in vitro* and permanent middle cerebral artery occlusion (pMCAO) *in vivo* were used. The expression profile studies by immunoblotting and fluorescence methods displayed decrease in phosphocofilin (inactive form of cofilin) levels with all models of ischemia implying stress induced cofilin activation. Furthermore, calcineurin and slingshot 1L (SSH) phosphatases were found to be the signaling mediators of the

cofilin activation pathway in ischemia elucidated by our inhibitor studies. These results strongly support cofilin role in cerebral ischemia induced actin cytoskeleton alterations leading to cell damage. We believe targeting this protein mediator has a potential for therapeutic intervention in stroke.

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Table of Contents

List of Figures

1. Introduction

1.1 Cerebral ischemia

Cerebral ischemia or Stroke is a neurodegenerative disease characterized by sudden death of cells in the brain due to inadequate blood flow. A thrombosis, an embolism or a systemic hypo-perfusion can lead to restriction of blood flow to the brain in an ischemic stroke conditions resulting in insufficient oxygen and glucose delivery to support homeostasis, consequently leading to cell death (Doyle, Simon et al. 2008). Each year in the United States approximately 700,000 individuals are afflicted with stroke and about 2 million survivors of stroke are living with disabilities. Stroke is the third leading cause of death surpassed by heart disease and cancer (Doyle, Simon et al. 2008). The economic and disability costs incurred through stroke are substantial throughout the world (Caplan 2000).

Human brain comprises 2% of body weight but requires 20% of total oxygen consumption (Stenman, Malmsjo et al. 2002). This large amount of oxygen consumption is required to generate ATP and to maintain ionic gradients across each and every neuron cell which is essential for nerve impulse transmission (Doyle, Simon et al. 2008). When the brain is deprived of adequate blood flow, the resulting ischemia is characterized by a bewildering array of interrelated physiologic and cellular responses that ultimately result in neuronal cell death due to loss of energy dependent membrane potentials. The complex cascade of events triggered in response to ischemia are excitotoxicity, acidotoxicity, ionic

imbalance, oxidative/nitrative stress, inflammation and apoptosis which can last from few minutes to days after the insult (Silbergleit, 2001). These processes can share overlapping, redundant features and subsequently lead to injury of neurons, glia and endothelial cells (Doyle, Simon et al. 2008).

The current drug therapy approved by US FDA for treatment of stroke is tissue plasminogen activator (tPA), which mainly functions to dissolve the blood clots and clear the obstruction to blood flow. This therapy is for immediate recovery of blood flow, but the neurodegeneration associated with the stroke requires attention to overcome the disabilities associated with stroke. Despite promising neuroprotective agents, various animal models of study, and various clinical testing, still there is a lack of effective methods of stroke therapy (Zeynalov, Shah et al. 2009).

Increased understanding of the brain's response to injury during the period between insult and neuronal cell death will allow us to focus on specific target oriented drug therapies for cerebral ischemia. The important pathways playing a key role in progression of events following acute brain injury are neurocytoskeletal degenerations. Research suggests disruption of the cytoskeletal proteins occurs in a variety of forms of acute brain injury including cerebral ischemia and diffuse axonal injury (Fitzpatrick, Dewar et al. 1996). Indeed cytoskeleton is the targeted structure by the cell death mediators in ischemia (Lipton 1999). Deeper insight into neuronal cytoskeleton is essential which can eventually potentiate the search for targets involved in ischemic injury.

1.2 Neuronal Cytoskeleton

This is the filamentous network that spans the cytoplasm and interconnects the cell nucleus with the extracellular matrix. It is therefore regarded as a structural link between molecules involved in cell communication and gene expression (Ramaekers and Bosman 2004). Cytoskeleton is the dynamic structure in the cell constantly adapting to the changes in the environment essentially maintaining the structural integrity (Dos Remedios, Chhabra et al. 2003). The dynamic cytoskeleton performs homeostasis functions in the cell by maintenance of cell shape, cell movement, cell replication, apoptosis, cell differentiation, and cell signaling (Fuchs and Cleveland 1998).

Neurons have elaborate cytoskeletal structures comprised of three major protein families of filamentous mesh network which together with motor proteins and their associated protein complexes form the structural elements of the cell. They are microfilaments (also called actin filaments) composed of linear polymers of protein actin, microtubules organized by polymers of α and β tubulin and neurofilaments comprising the intermediate filaments of the cell.

This filamentous network associates with hundreds of different proteins which cooperate in the organization of the complex machinery that is involved in essentially all structural and dynamic aspects of living cells (Ramaekers and Bosman 2004). The three

important protein families are constituted by filaments which have distinct mechanical, dynamic and biological role (Alberts, 2002).

1.3 Actin filaments

The migration of neurons, extension and guidance of axons including dendrites are highly dependent on the actin filament cytoskeleton (Gianluca Gallo, Actin review). Microfilaments are the two stranded helical polymers of the protein, actin. Actin is a single globular polypeptide chain which forms linear polymers by continuous assembly of its subunits. These constitute the flexible cytoskeletal structures and are considered the thinnest filaments of the cytoskeleton.

Actin is the major ubiquitous protein in all eukaryotic cells and serves various essential roles in the cell such as shape determination, cytokinesis, cell motility and cell-cell/cell-matrix interactions (Small, Rottner et al. 1999).

1.3.1 Actin structure and dynamics

Actin is considered the most abundant protein in all eukaryotes. Each actin filament is composed of two parallel protofilaments that twist around each other in a right-handed helix. All such filaments organize into a variety of linear bundles, two-dimensional networks and three-dimensional gels providing structural stability to cell.

Actin filaments in neurons are diverse in functional roles such as cell shape, motor based organelle transport, regulation of ion transport and receptor mediated

responses of the cell to external signals (Dos Remedios, Chhabra et al. 2003). It exists in two major states: globular actin (G-actin) which is the monomeric form and filamentous actin (F-actin), a polymeric form (Reisler and Egelman 2007). The dynamic equilibrium between the two forms is controlled by various factors and mediators in the cell.

Actin contains binding site for ATP or ADP nucleotides in the polypeptide chain (Fig 1.1). Each actin moiety contains Mg-ATP bound in the deep cleft, which stabilizes the molecule (Pollard and Borisy 2003). Once it is in the filament, the ATP is hydrolyzed to ADP+Pi and the ADP bound actin has higher dissociation constant than ATP-bound actin. In steady state ATP-actin associates at the barbed (growing) end and ADP-actin dissociates from the pointed (shrinking) end (Pollard and Borisy 2003).

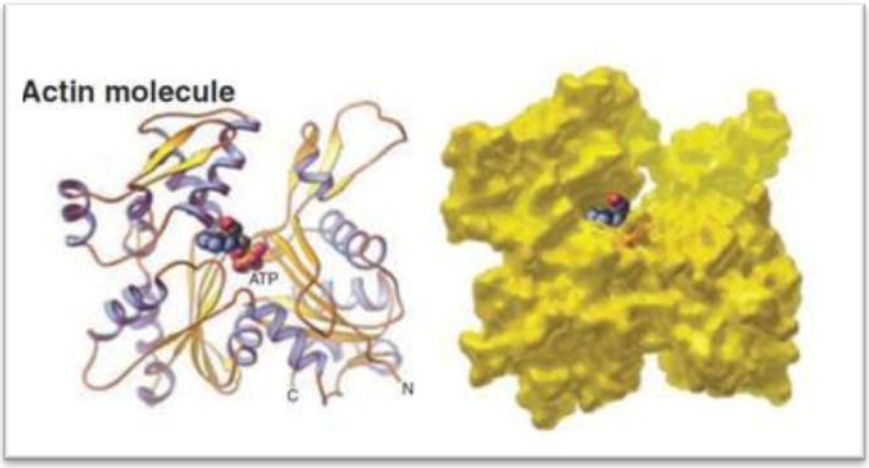

Fig 1.1 Structure of actin molecule. ATP bound state in ribbon representation and space filling model of actin PDB file 1ATN.pdb, ref (Pollard and Cooper 2009).

1.3.2 Actin binding proteins

Actin is associated with several proteins, which assist in various functions of microfilaments collectively called as actin binding proteins (ABPs). There are more than 162 distinct and separate ABPs which are diverse in their isoforms and their functions. Out of 162 ABPs, 12 ABPs are membrane associated, 9 membrane receptors/ ion transporters, 13 crosslink actin filaments and the rest participate in interaction with microtubules and intermediate filaments facilitating coordination/communication and assembly/disassembly of proteins.(Dos Remedios, Chhabra et al. 2003).

There are 7 important assembly/disassembly ABPs and among those a filament depolymerizing class of protein, cofilin, is the research interest of current study.

1.4 ADF/Cofilin Family of proteins

These are the actin depolymerizing family of proteins expressed in all eukaryotes. Members of this family are essential actin-binding proteins which have an active role in actin-filament dynamics in the cells (Bernstein and Bamburg 2010). Cofilin proteins are virtually small molecular weight (15-19KDa) and exist in various isoforms. They are ADF (also known as destrin), cofilin-1 and cofilin-2. All eukaryotes express at least one protein of this family functioning mainly in regulation of actin dynamics (Bamburg and Bernstein 2010). The central function of cofilin family is to maintain the rapid recycling of F-actin monomers associated with membrane ruffling and cytokinesis. ADF/Cofilin proteins were first extracted from embryonic chick brains by Bamburg et al. (Bamburg, Harris et al. 1980).

1.4.1 Distribution of cofilin/ADF proteins

Cofilin is diffusely distributed in the cytoplasm. In active cells, cofilin translocates to the cortical regions in which the actin cytoskeleton is highly dynamic such as ruffling of membranes of motile cells, cleavage of dividing cells and development of growth cones (Dos Remedios, Chhabra et al. 2003). Thus they are primarily concentrated in regions rich in dynamic actin structures.

Cofilin-1 (also known as n-cofilin) is the major ubiquitous form in non-muscle tissues, whereas cofilin-2 is the major form in differentiated muscle tissue. Cofilin-1 is predominantly expressed in embryonic and adult mouse cells (Hotulainen, Paunola et al. 2005) and knockdown of the cofilin-1 gene is embryonic lethal in mice (Gurniak, Perlas et al. 2005). ADF and cofilin were found to be similar in actin dynamizing activity but they vary in the ratios of their binding to actin and initiating the activity. Cofilin-1 is regarded as the most ubiquitous of the isoforms, expressed in higher levels and is crucial in development. Cofilin-1 being the abundant protein in expression in the brain, our studies were focused on cofilin-1, and mentioned as cofilin here forth.

1.4.2 Cofilin and its role in actin dynamics

Actin dynamics regulation by Cofilin is largely dependent on the concentration of cofilin both in vitro and in vivo (Bamburg and Bernstein 2008). Lower concentrations of cofilin have higher binding affinity to ADP-bound actin in the filaments, thus promoting depolymerization from the pointed end of the actin filaments (Tsai and Lee 2012). On the

other hand, higher levels of cofilin can stabilize the actin filaments and lead to nucleation of G-actins. This process can further lead to elongation and branching by profilin or Arp2/3 (Actin related protein) complex. Thus the levels of cofilin have greater influence on actin dynamics in a biphasic manner responsible for affecting cellular changes accordingly (Van Troys, Huyck et al. 2008). The depletion of cofilin-1 and/or ADF by siRNA would lead to accumulation of actin filaments and also increase the cell size (Hotulainen, Paunola et al. 2005).

Cofilin mainly functions to dynamize actin filaments by various mechanisms (Dos Remedios, Chhabra et al. 2003) such as;

Treadmilling – This is a phenomenon observed in microfilaments and microtubules, wherein one end of the filament grows in length while the other end shrinks which results in a section of filament seemingly moving across cytosol. This process is associated with constant removal of subunits at one end and constant addition at the other end of the filament. Cofilin is involved in major rate of treadmilling of actin monomers in vivo. This results in rapid accumulation of G-actin in the cytoplasm. Cofilin binding to actin filaments is restricted in regions where ATP nucleotide is converted to ADF. So, the binding is some distance away from the membrane interface where the filaments are actively growing (Dos Remedios, Chhabra et al. 2003).

Depolymerization – Cofilin is involved in rapid depolymerization of actin filaments at the pointed ends of actin filaments rather than barbed ends. Pointed ends are rich in ADP-actin facilitating cofilin binding.

Nucleation of polymerization – Cofilin not only participates in the depolymerization of actin filaments but also nucleates the filaments for polymerization (Dos Remedios, Chhabra et al. 2003). This potential of nucleating the assembly depends on the concentration of cofilin and by pH of the evironment.

Binding to ADP-actin – The binding of cofilin to actin is highly dependent on the state of the nucleotide bound to the actin. It was found that cofilin can bind to ADP-actin with greater affinity and magnitude than with ATP-actin at physiological pH ranges (Carlier, Laurent et al. 1997). Apart from its function to promote disassembly of ADP-actin monomers from filaments, it can also bind to released ADP-actin monomers and inhibit the exchange of bound nucleotide (Nishida, Muneyuki et al. 1985).

Actin severing – Actin severing activity of cofilin is in turn dependent on ratio of actin to cofilin. In vitro studies demonstrate that low cofilin to actin ratio results in persistent filament severing, but higher cofilin to actin ratio results in transient severing because higher cofilin levels saturates the filament and produces twists that facilitate severing (Bamburg and Bernstein 2010). In fact, the assembly of cofilin-ADP-actin complex can form non-dynamic actin bundles (also called rods) as an energy conserving mechanism, which is a reversible process in most cells. But in axons and dendrites of

stressed neurons, these rod like structures block transportation and cause abnormalities leading to diseases such as dementia and Alzheimer's (AD) (Minamide, Striegl et al. 2000) (Bamburg, Bernstein et al. 2010).

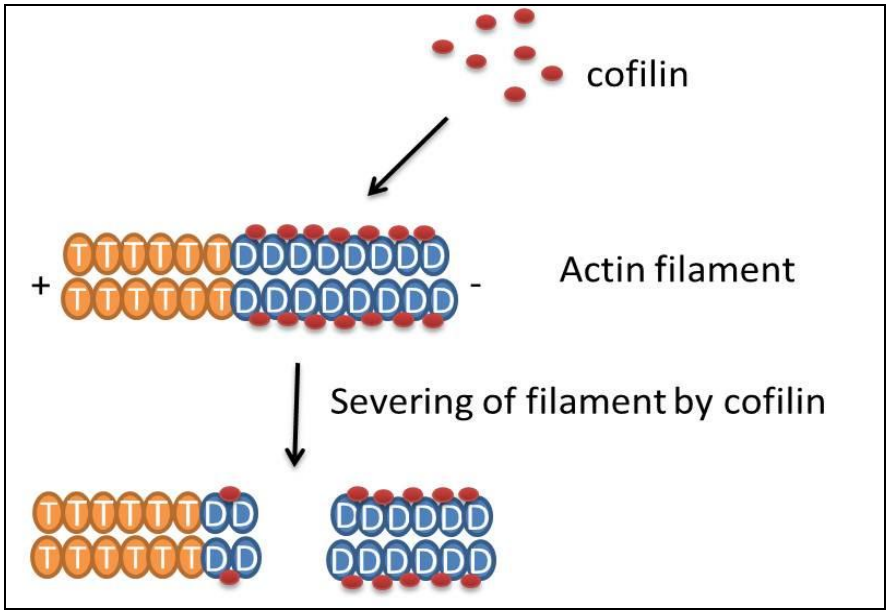

Fig 1.2 Actin filament severing by cofilin. Depicting actin filament with ADP and ATP bound in subunits. Cofilin binding to ADP-actin subunits and severing activity which is regulated by a set of phosphatases and kinases (Bamburg and Wiggan 2002).

1.4.3 Regulation of activity of cofilin

Phosphorylation

The binding of cofilin to G- and F- actin is regulated by phosphorylation of Ser-3 residue in cofilin (Morgan, Lockerbie et al. 1993). Phosphorylation of cofilin inactivates cofilin and results in sharp decline in its affinity for actin binding (Bamburg and Bernstein, 2008). It also facilitates the removal of cofilin from the bound ADP-actin

complex resulting in reincorporation of actin subunits into the filament. The cellular microfilament turnover is regulated by the cycles of phosphorylation and dephosphorylation form of cofilin (Dos Remedios, Chhabra et al. 2003).

Cofilin is phosphorylated by a family of LIM-Kinase proteins (LIMK-1 and LIMK-2) and TES kinases. LIM kinases are involved in phosphorylation of ADF/Cofilin family proteins in brain. LIMK-1 is found to be predominantly neuronal whereas LIMK-2 is wide spread. The signaling cascade for activation of LIMK-1 is by Rho family of GTPase Rac and PAK (p21 activated kinases) whereas Rho GTPase, ROCK regulates LIMK-2. Members of Rho GTPase family are the key regulatory molecules that link surface receptors to the organization molecules of cytoskeleton (Hall 1998). LIMK are the downstream mediators of Rho GTPases, p38MAPK and Protein kinase C (PKC) which indirectly direct cofilin phosphorylation (Scott and Olson 2007).

Dephosphorylation of cofilin

Cofilin is activated by two phosphatase enzymes namely chronophin (CIN) and slingshot (SSH-1, 2, 3) (Bernstein and Bamburg 2010). Chronophin is found to be highly specific for cofilin whereas SSH phosphatase is involved in dephosphorylation and also deactivation of LIMK-1. Other nonspecific phosphatases, which are involved in dephosphorylation and activation of cofilin are protein phosphatases (PP) - PP1 and PP2A. All these phosphatases are in turn regulated by different upstream mediators (Van Troys, Huyck et al. 2008).

Inhibition by Phosphatidylinositol 4,5-bisphosphate.

The membrane lipids phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP2) regulate the activity of cofilin by binding to and inhibiting the actin binding domain of cofilin (Yonezawa, Nishida et al. 1990).

Regulation by pH

Cofilin activity of assembling or disassembly of actin filaments is largely dependent on pH in vitro (Dos Remedios, Chhabra et al. 2003). In alkaline pH, cofilin can rapidly depolymerize actin filaments whereas in acidic conditions (pH < 6.8) increases the stabilization of actin filaments by cofilin.

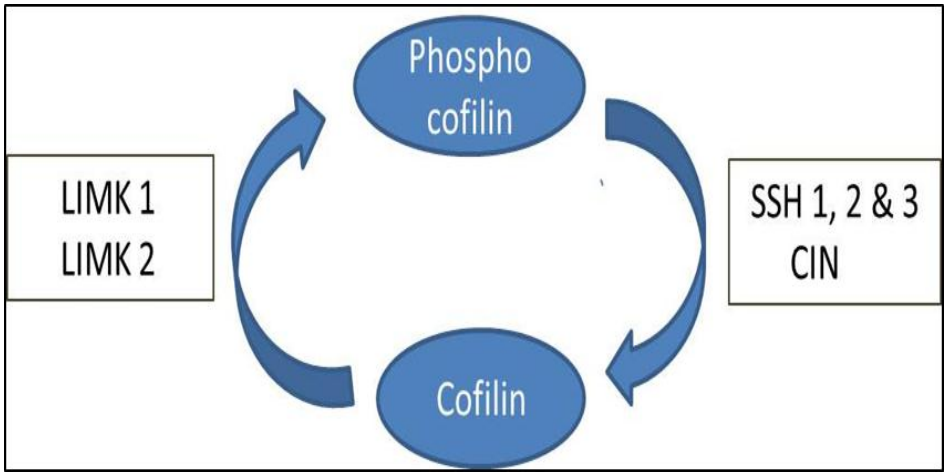

Fig 1.3: Regulation of cofilin activity. Cofilin phosphorylation is regulated by LIMK1 and LIMK2 whereas cofilin is dephosphorylated and activated by phosphatases – SSH and CIN.

1.5 Cofilin activity beyond cytoskeletal regulations – Role in pathophysiological conditions of various diseases

Cofilin is an important protein in regulation of actin cytoskeletal dynamics during steady state conditions and also various cellular functions. Hence forth also considered as homeostasis protein in the cell (Bernstein and Bamburg 2010). Apart from its role in cytoskeletal regulations, cofilin is majorly involved in certain pathophysiological states which lands cofilin as a target in major diseases.

Formation of cofilin-actin rods in stress conditions

Actin polymerization in all cells is a highly energy dependent process (Pollard 1986). In actin filament assembly process, ATP-bound actin subunits are incorporated into the filament and there is cyclic exchange between G-actin and F-actin ultimately leading to ATP hydrolysis (Pollard and Borisy 2003).

Actin is an abundant protein in the cell. Therefore, the ATP utilization and expenditure in each phase of actin polymerization and depolymerization is very high (Huang, Minamide et al. 2008). Indeed it was shown that in neurons during anoxic stress conditions, decreasing the actin turnover could attenuate ATP depletion by 50% (Bernstein and Bamburg 2003). In energy-depleted states, cells have adapted mechanisms to limit the actin turnover and restore the ATP levels. One such mechanism includes the stabilization of actin filaments by actin depolymerization factor, cofilin. Cofilin is known to rapidly reorganize with actin into rod shaped inclusion bodies in neurons as a response

to ATP depletion (Minamide, Striegl et al. 2000). Thus the cofilin-actin complexes formed organize into rod shaped bodies and attenuate cofilin-driven actin treadmilling to restore energy reserves of the cells (Huang, Minamide et al. 2008). These inclusions have discrete solubility in various cells. Persistence of these rods for longer periods can have dramatic detrimental effects on neuronal function. It results in disruption of dendritic microtubule integrity, synaptic deficits and can block intracellular trafficking leading to loss of dendritic spines (Cichon, Sun et al. 2012). Furthermore, in neurons, these rods block the vesicular transport of amyloid precursor protein (APP) and enzymes involved in processing of the β amyloid, resulting in accumulation of APP in rods progressing the pathology of Alzheimer disease (Maloney and Bamburg 2007). During ATP depletion, cofilin is activated by dephosphorylation prior to its inclusion in rod shaped bodies suggesting the importance of phosphocofilin turnover to rod formation (Minamide, Striegl et al. 2000). Overall cofilin is a mediator involved in progression of neurodegeneration states in AD and associated pathologies involving ATP depletion.

Cofilin in cancer invasion and metastasis

Multiple processes associated with reorganization of actin filaments and actin regulatory proteins - cofilin and Arp2/3 are known to be involved in metastasis and invasion of cancer cells (Tsai and Lee 2012). The cancer research group (Sidani, Mouneimne et al. 2006) showed that cofilin silencing by small interfering RNA (siRNA) suppressed cancer migration and metastasis suggesting its prominent role in cancer invasion.

ADF in renal ischemia

In vivo renal ischemia and cellular depletion of ATP levels in vitro were found to disrupt actin membrane cytoskeleton in renal proximal tubule cells (PTC) (Kellerman and Bogusky 1992). ADF, an actin depolymerizing factor of cofilin family was found to be activated by rapid dephosphorylation in the intraluminal vesicles and bleb structures of proximal tubule cells in response to ischemia in a duration dependent manner (Schwartz, Hosford et al. 1999). The structure alterations noticed in ischemia are membrane bleb formation, detachment of cells and redistribution of actin network. This resulted in loss of apical membrane potential and ion transport suggesting the irreversible damaging role of ADF apart from actin regulation.

Mitochondrial translocation and apoptosis

Mitochondria are the power-house of the cell as they generate ATP for cellular functions. During exposure of cell to Reactive oxygen species (ROS), the efficiency of mitochondria is reduced which results in decreased ATP levels and cellular dysfunction (Chen, Yoshioka et al. 2011). These conditions of ATP depletion and ATP conservation processes operating simultaneously in the cell dephosphorylate and activate cofilin (Suurna, Ashworth et al. 2006). In addition, ROS species oxidize the cofilin moieties thereby decreases its affinity for actin binding and facilitating translocation to mitochondria. In mitochondria, cofilin indirectly induces mitochondrial swelling and cytochrome c release by altering permeability transition pore as observed in phagocytes (Klamt, Zdanov et al. 2009).

In glutamate excitotoxicity mediated neuronal death, cofilin was found to translocate Bax to mitochondria (Posadas, Perez-Martinez et al. 2012). Cofilin translocation to mitochondria is indeed considered as an early step in apoptotic cascade (Chua, Volbracht et al. 2003). As translocation of cofilin to mitochondria requires dephosphorylation and activation, the overexpression of phosphorylated form (S3D) is known to inhibit staurosporine induced mitochondrial apoptosis and cytochrome c release in tumor cell line (Chua, Volbracht et al. 2003). Supporting this study, overexpression of LIMK1 in PC12 cells protected cells from serum deprived apoptosis by inactivating cofilin and inhibiting caspase 3 (Yang, Kim et al. 2004). This potential activity of cell death induced by cofilin by its translocation to mitochondria and release of cell death mediators such as cytochrome c coupled with its rod forming complexes with actin are key to explain the neurodegenerations induced by cofilin.

1.6 Hypothesis and aims

The primary goal of this study is to study the expression pattern of cofilin and also investigate the cytoskeletal alterations induced by cofilin in cerebral ischemia associated stress conditions using both in vitro and in vivo models of stroke. To achieve this goal, specific aims were established in this research study.

Aim-1: Evaluating the stress induced changes in phosphocofilin, inactive form of cofilin and total cofilin expression in differentiated neuronal and PC12 (Pheochromocytoma) cells.

This can be accomplished by employing various stress simulations such as ROS generation in cell environment (using tertiary butyl hydro peroxide, *t*-BuOOH) and an experimental ischemia situation (using oxygen glucose deprivation (OGD) conditions). The morphological changes endured during ischemia in the cell cytoskeleton and protein expression pattern will be studied using fluorescence and immunoblotting approaches.

Aim-2: Elucidating the pathways and mediators enabling the activation of cofilin and investigating their putative role in ischemic damage.

By using inhibitors which target the mediators activating cofilin and thereby studying the repercussions associated with this inhibition, the signal transduction pathways of cofilin can be elaborated. This can provide a clue on the exact cascade of responses eliciting neurocytoskeletal damage by activating cofilin in cerebral ischemia.

Aim-3: Illustrate the potential role of cofilin induced degenerations in cerebral ischemia using animal model of cerebral ischemia – pMCAO (Permanent middle cerebral artery occlusion). This is an advanced stride in understanding the cofilin involvement. Immunoblotting and fluorescence analysis of ischemic brains can possibly suggest the activation state of cofilin after stress.

2. Materials

PC12 (Pheochromocytoma) cells from ATCC (American type culture collection, Manassas, VA), NGF (Nerve growth factor, Harlan Laboratories, Indianapolis, IN), RPMI (Roswell Park Memorial Medium, Mediatech Inc, Manassas, VA), DMEM (Dulbecco modified Eagle medium, Thermo scientific, Hanover Park, IL), HBSS (Hank's Balanced Salt Solution, Thermo scientific), 0.5% Trypsin (Thermo scientific), Poly-l-lysine (Sigma chemicals, St Louis, MO), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) from Nalgene (Rochester, NY), DAPI (4'-6-Diamidino-2-phenylindole) from Santa Cruz Biotechnology(Santa Cruz, CA), Sodium orthovanadate from Sigma chemicals, FK-506 (Tacrolimus) from A.G scientific (San Diego, CA), Horse serum and fetal bovine serum (Life Technologies, Carlsbad, CA), Penicillin-streptomycin (Fischer, Hanover Park, IL), Fungazone (Fischer), tertiary butyl hydroperoxide (*t*-BUOOH; Sigma chemicals).

3. Methods

3.1 Animals

All animal protocols were approved by the University of Toledo Health science campus Institutional animal care and utilization committee. Guidelines of the National Institutes of health were followed throughout the study. Mice were procured from Charles River laboratories, Wilmington, MA. Male mice of strain C57BL/6 weighing about 20-30 gms were included in the study.

3.2 Animal studies pMCAO model of stroke induction

pMCAO (permanent middle cerebral artery occlusion) was used as a model to induce stroke in animals and study ischemia induced alterations in the protein phosphocofilin expression pattern. In the pMCAO model, the distal portion of middle cerebral artery was occluded according to a method described by (Saleem, Shah et al. 2009)

Experimental design: Mice were anesthetized using isoflurane in 25% oxygen enriched air. A 1-cm vertical incision was made between the right eye and ear. Then the temporal muscle was moved aside and the underlying temporal bone was exposed. With the aid of a surgical microscope, a 2 mm hole was drilled just over the area of the middle cerebral artery (MCA) so that it was visible through the temporal bone. After the artery is clearly visible, the main trunk of the distal MCA was occluded directly with a bipolar coagulator and the complete interruption of the blood flow at the occlusion site was

confirmed by the severance of the MCA. The successful occlusion of the MCA was confirmed by the Laser Doppler probe above the temporal ridge to confirm that the blood flow into the region is terminated. The body temperature was maintained at 37±0.5°C during and after the procedure by using a heating blanket that was attached to a temperature probe for automatic temperature regulation and placed in a temperature regulated incubator after surgery.

3.3 Perfusion procedure and isolation of Brain

After 24 hours and 7 days of ischemia, the brains of mice were isolated using the perfusion method. Mice were anesthetized with Pentobarbital sodium and transcardially perfused with physiological saline followed by 4% buffered paraformaldehyde using a peristaltic pump. The complete perfusion was ensured by checking intermittently for the hardening of the tail. Brains were then dissected from the skulls and post-fixed for 24hrs in 4% paraformaldehyde. The isolated brains were stored at -80°C.

3.4 Tissue Sectioning

The frozen tissues were sectioned using a cryostat. About 7-8µ thick sections were cut and sections were collected onto a positively charged Microslides (Superfrost Plus, VMR) which adsorbs the thin negatively charged sections readily onto the slide surface by electrostatic attraction. To prevent dehydration, sections were stored immediately at -20°C. The sections on microslides were then probed for studying the protein related changes using the immunohistochemical and TUNEL methods.

3.5 TUNEL assay for apoptosis detection

The extent of apoptosis in the infarct area was evaluated using TUNEL assay (Promega, Fitchburg, WI). Tissue sections were fixed using 4% paraformaldehyde solution in phosphate buffered solution (PBS) for 15 minutes at room temperature. The excess of paraformaldehyde was removed by rinsing the slides three times in 1X PBS solution, each wash lasting for 5-minutes. 100 µl of proteinase K solution (20µg/ml) was added to each tissue section and incubated for 20 minutes in order to enhance the permeabilization of the tissues. Sections were then washed by immersing the slides in a coplin jar containing PBS for 5 minutes. Tissue sections were refixed using 4% paraformaldehyde for 5 minutes and washed with PBS buffer.

Preparation of TUNEL reaction mixture – The reaction mixture (100 µl) was composed of 98 µl of Equilibration buffer, 1µl of Biotinylated Nucleotide mix and 1 µl of rTdT enzyme. Each tissue section was initially covered with 100 µl of equilibration buffer and incubated for about 10 minutes at room temperature. After equilibration, the excess of solution was blotted using tissue paper and then about 100 µl of rTdT reaction mixture was added. The sections were covered with plastic coverslips to ensure even distribution of the reagent. This incubation was at 37°C for 60 minutes in dark conditions inside a humidified chamber to allow for the end-labeling reaction to occur. The reaction with rTdT enzyme mixture was terminated by immersing the slides in 2X SSC (Saline sodium citrate) buffer in a coplin jar for 15 minutes and rinsed twice with PBS at room temperature. These slides were further processed for the immunohistochemical analysis.

3.6 Antigen retrieval

Heat mediated mode of antigen retrieval was performed for proteins phosphocofilin and cofilin (Abcam protocol, USA) to enhance the detection process of proteins submerged by paraformaldehyde crosslinking. Sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20) of pH 6.0 was employed. Briefly the sections were heated for 3-4 minutes in a pressure cooker containing the sodium citrate buffer and then allowed to cool for 10 minutes at room temperature. The tissue sections were then rinsed with 1X PBS to obviate the acidic effect of retrieval buffer.

3.7 Immunohistochemistry

In the immunohistochemical processing of sections, nonspecific binding sites were blocked using 3% Bovine serum albumin (BSA) (RPI, Mount Prospect, IL, USA) prepared in 1X PBS buffer for 1 hour at room temperature. About 100-150 µl of BSA was added to each section. Primary polyclonal rabbit antibody specific against proteins phosphocofilin, cofilin (Abcam, MA, USA) of dilution 1:100 was prepared in blocking buffer and incubated on sections overnight at 4°C. Para films were placed on the sections to prevent the overflow of solutions. Subsequently sections were washed thrice with PBS buffer for 10 minutes following the incubations. Donkey anti-rabbit secondary IgG antibody (1:400; Jackson ImmunoResearch, West Grove, PA, USA) which are tagged with fluorescent dyes (Texas Red) were added to the section and incubated for 1 and a half hour in dark at room temperature. Following this, three washings (each for 10 minutes) were performed in dark environment. After rinsing with PBS buffer, the excess

liquid on the slide was blotted with tissue paper. Then the slide was mounted with DAPI and immediately a coverslip was placed to allow the solution to stabilize on the section overnight. The gap between the section and coverslip was sealed and fluorescence imaging was performed. All the experiments were performed thrice using three separate batches of animals.

3.8 Protein Extraction

Protein extractions of mice brains were performed for Western blotting protocols. The brains subjected to pMCAO were collected by sacrificing mice at specific time points (6 hr, 12 hr, 1 day, 2 days, 3 days, 7 days, and 14 days after surgery). Brains were minced finely, tissue lysis and homogenization was initiated using 1X RIPA lysis buffer (20mM Tris HCL (pH 7.5), 150 mM Nacl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate). Protease and phosphatase inhibitors such as 0.1 mM sodium pyrophosphate, 50 mM sodium fluoride, 10 mM sodium vanadate, 1 mM DTT, 1 mM PMSF, 1 µl of protease inhibitor cocktail per ml of RIPA buffer were added to the lysis buffer solution. About 250 µl of lysis buffer was added to the samples, vortexed and placed on ice for 1 hour to facilitate complete homogenization of the tissue. Then the samples were subjected to centrifugation at 12000 rpm for 12 minutes at 4°C. The supernatant containing the whole cellular protein was collected into microtubes and stored at 4°C.

3.9 Protein quantification using Bradford protein assay

Bradford method of protein quantification was employed for estimating the concentration of unknown protein samples. In this assay, BSA of 1 mg/ml was used as a standard (1.46 mg/ml BSA, Bio-Rad). The serial dilutions of BSA standard solution were prepared whose concentrations were used in preparing the standard graph. Protein estimation was performed using the standard graph for each experiment.

All the samples were assayed in triplicates using a 96 well microtiter plate. Protein assay reagent (Bio-Rad Laboratories) was diluted to 1:5 ratio with distilled water and about 200 µl of this diluted protein reagent was added to the wells containing 1 µl of protein samples. Blank wells contain distilled water and protein reagent. The samples were mixed thoroughly and the absorbance was measured immediately at a wavelength of 590 nm using Synergy Hybrid plate reader (BioTek, Winooski, VT). The optical density value of each sample was subtracted from the blank values to obtain the true sample value and the values were averaged. A standard curve was plotted using optical density values of BSA versus the concentration of BSA. The unknown concentration of the protein was estimated by extrapolating the optical density values onto standard graph.

3.10 Western blotting procedures

Depending on the protein concentration values, the samples were prepared in sample buffer (25% v/v mercaptoethanol, 5% v/v glycerol, 5% w/v SDS and 0.625 M Tris chloride). Bromophenol blue was added to the sample buffer to visualize the movement of colorless protein samples during electrophoresis process. Samples were

vortexed and heated to 95°C to facilitate denaturation of proteins. 15% polyacrylamide gels were used to promote the efficient separation of smaller molecular weight proteins of interest. Equivalent amount of total nuclear protein (25 µg) was loaded onto each well and 70 V current was applied across the gel. Proteins were separated depending on molecular weight using SDS PAGE electrophoresis employing 1X Running buffer (3.03g Tris base, 14.4g glycine and 1 g SDS) as a medium in running the gel. Proteins separated on the gels were transferred onto pretreated PVDF membrane (Polyvinylidene fluoride; Bio-Rad, Hercules, CA) by applying 115 V current for 1hour across the transfer setup containing the 1X transfer buffer (3.03 g Tris base, 14.4 g glycine).

The membrane with transferred proteins was blocked with 3% BSA for 1 hour to prevent nonspecific binding. Membrane was then incubated with following primary antibodies: rabbit anti-phosphocofilin and anti-cofilin (1:1000; Abcam), rabbit anti-actin (1:2000; Sigma Aldrich, St. Louis, MO), rabbit anti-GAPDH (1:2000; Fischer, Hanover Park, IL) overnight at 4°C. Usually these solutions are packed in small polythene bags and placed on a rotary shaker to enhance the thorough distribution of the solution on the membrane.

Following the incubation, the blots were washed thrice, each wash lasting for a period of 15 minutes with 1X PBS/0.1% Tween buffer. The membrane was then incubated with HRP (Horseradish peroxidase) conjugated goat anti-rabbit secondary antibody (1:6000; Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. After the incubation, membrane was rinsed thrice with PBS-T buffer. The

protein bands were then visualized by chemiluminescence reaction produced by the interaction of HRP enzyme with the added substrate. The substrate in the form of reagents A and B (Super Signal, West Pico reagent kit) each of equal volume were added to the membrane and allowed to stand for 3 minutes facilitating the reaction. The bands were developed using autoradiography cassette (Fischer) by the method of X-ray autoradiography. Actin and GAPDH were used as loading controls in each experiment. The images were analyzed using Adobe photoshop and Image J software.

3.11 Cell Culture

PC12 (Rat Pheochromocytoma) cells were cultured in RPMI medium supplemented with 5% fetal bovine serum, 10% Horse serum, 1 mM Glutamine, 100 units/ml Penicillin-streptomycin and fungazone in a poly-l-lysine coated 6 cm petriplates. Cells were maintained at 37°C with 95% air and 5% CO2 relative humidity conditions in an incubator for 5-7days. The medium was replaced every two days with fresh medium and the cells were split at confluency using 0.5% Trypsin and 1X PBS buffer.

3.12 Differentiation of PC12 cells

PC12 cells were seeded at a population density of $0.05x10^6$ cells onto poly-l-lysine coated 6 cm petridishes. After 24 hours of plating the cells, growth medium (RPMI) was replaced with differentiating medium DMEM (Dulbecco modified Eagle medium, HyClone, Thermo scientific) supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum, 5% Horse serum and 100 ng/ml Nerve growth factor (NGF). The medium was replaced for every two days with fresh differentiating medium and the

cells were cultured until a period of 12-14 days to allow the cells to completely transform into differentiated neuronal cells.

3.13 Oxygen glucose deprivation (OGD) model of ischemia

The most important components of tissue damage in cerebral ischemia are oxygen glucose deprivation, excitotoxicity and production of harmful free radicals. An in vitro model of ischemia namely OGD (Oxygen glucose deprivation) mimics the conditions prevailing during ischemia and hence this model was used for studying ischemia-induced changes in the cells. In this model of study, the growth medium of the cells was replaced with medium lacking glucose (HBSS Phenol red medium) and placed in a chamber which was rendered anaerobic by placing a sachet containing ascorbic acid (AnaeroGenTM, OXOID, Germany) which absorbs oxygen from the surrounding atmosphere. An Anaerobic indicator (OXOID, Germany) containing resazurin sensitive to changes in oxygen levels was placed in the chamber and lid of the chamber was tightly closed. This chamber was then placed in an incubator at 37°C. Initially as there was oxygen retained in the chamber, the indicator is pink in color. The ascorbic acid in the sachet absorbs all the oxygen in the chamber and makes the environment completely anaerobic and this usually takes a period of 1 hour. The complete lack of oxygen in the chamber is indicated by change in the color of the indicator from pink to white color. The time point when the color of the indicator was white, onset time for OGD was recorded. Usually the time ranges for OGD experiments were from 1 hour to 4 hours, where there is 90% cell death occurring at 4 hours of OGD. Immediately after OGD, the cells were removed from the

chamber and subjected to experimental reagents to study the effects of OGD stress induced changes in the cells.

3.14 Treatment with Stressor (tertiary Butyl hydro peroxide)

To induce the oxidative stress conditions and study the impact of free radical production on protein changes in the cells, PC12 cells were subjected to different concentrations of t-Butyl hydro peroxide (*t*-BuOOH) for a time period of 1 hour. The concentrations used were 60 µM, 100 µM and 200 µM respectively. PC12cells were cultured and plated at a density of $0.8x10^6$ cells onto poly-l-lysine coated 6 cm plates. After 24 hrs of plating, the medium was replaced with fresh medium and the stressor was added to the medium and distributed thoroughly by shaking the petri dish gently. After the time period of treatment, the medium was removed and the cells were washed with sterile 1X PBS buffer twice, each wash lasting for 5 minutes. The cells were then harvested and the proteins were extracted using RIPA buffer for further analysis. The protein expression pattern was studied using immunoblotting procedures.

3.15 Immunocytochemistry

In these experiments PC12 cells of density $0.03x10^6$ were loaded onto poly-l-lysine coated plates containing sterile coverslips. After 24 hours of plating the cells, in order to induce differentiation of cells, the growth medium was replaced with differentiating medium. The NGF supplemented medium was added for every two days and cells were completely differentiated by a time span of 14 days. The cells thus

cultured on the coverslips in 6 well dishes were subjected to different treatments; inhibitor pretreatments (Sodium vanadate for 2 hours, FK-506 for 60 minutes, and stressor treatments i.e., *t*-BuOOH for 1 hour and oxygen glucose deprivation for 1 hour in each individual set of experiments.

Once cells were exposed to different conditions for specified time points, media was aspirated and cells were washed with sterile 1X PBS buffer. To study the morphological changes, differentiated cells were fixed with 1% para formaldehyde buffer in 1X PBS for 20 minutes at room temperature and then rinsed with 1X PBS for 5 minutes.

To increase the permeability of the cell membranes to the antibody solutions, cells were incubated in 0.3% of Triton X-100 in 1X PBS buffer for 20 minutes. Following the rinsing with 1X PBS, fixed cells were blocked with 1% BSA for 1 hour and incubated with primary antibodies – rabbit anti-phosphocofilin ,anti-cofilin (1:100; Abcam, USA) at 4°C overnight. Para films were placed on the coverslips to prevent overflow of solutions. Using PBS buffer, cells were washed three times at room temperature. Texas red labeled donkey anti-rabbit secondary IgG antibody (1:400; Jackson ImmunoResearch) was added to the cells and incubated for 1and a half hour at room temperature. This procedure was performed in dark conditions to prevent photobleaching of fluorescent dyes. After incubation, cells were washed with 1X PBS buffer and one unit of Phalloidin (Invitrogen) was added to stain F-actin in the cells for 20 minutes. After rinsing with PBS, the coverslips were carefully removed from the well and dripped slightly to remove excess

solutions. The coverslip was mounted with DAPI over the glass slide and the gap between coverslip and the slide was sealed.

3.16 MTT- Cell Viability assay

PC12 cells were seeded onto 24 well plates coated with poly-l-lysine at a seeding density of $0.4x10^6$ cells. After 24 hours of plating the cells, the cells were subjected to incubation with agents such as inhibitors (Na3VO4 for 2 hours) or FK-506 for 60 minutes. Following the treatments, the medium in the wells was replaced with glucose deficient HBSS medium and the plate was placed in OGD chamber. The anaerobic sachet, anaerobic indicator was placed in the chamber and sealed immediately. Cells were deprived of oxygen and glucose for 1 hour. After the stipulated time, cells were removed from the chamber, medium was aspirated and washed with 1X PBS buffer. To test the viability of the cells in OGD and other treatment conditions on cell viability, MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) reagent which is pale yellow tetrazole was added to the cells and incubated for 2 hours at 37°C in the incubator. Metabolically active cells react with yellow tetrazole and convert it into insoluble purple formazan. To terminate the reaction and solubilize the formazan, stop solution was added and incubated for another 1 hour. The solution in each well was taken in a microtube, centrifuged and the supernatant was collected. This assay was performed in triplicates for the sample in each well using a 96 well microtitre plate. About 100µl of distilled water was added to each well in microtiter plate and to this 100µl of the sample was added and the absorbance was measured at 570nm.

3.17 Statistical analysis

All the data were analyzed using Student's t-test and expressed as mean±SEM. A value of $p<0.05$ was considered to be statistically significant.

4. Results

4.1. Decreased expression of Phosphocofilin in PC12 Cells against t-Butyl hydro peroxide-induced oxidative stress

Oxidative stress is a predominant manifestation in cerebral ischemia generating reactive oxygen species (ROS) in the cell environment. We hypothesize the possible role of cofilin in actin cytoskeletal degenerations and associated cell death pathways by its activation by ROS. To investigate this, PC12 cells were exposed to different concentrations of t-butyl hydro peroxide (*t*-BuOOH) such as 60µM, 100µM and 200µM for 1 hour. Following the treatment, whole cell proteins were extracted and immunoblotting was performed. As cofilin is regulated by phosphorylation to an inactive form, phosphocofilin, the levels of cofilin were studied and interpreted in terms of changes in the phosphocofilin expression. As depicted in fig 4.1, Phosphocofilin expression was found to be significantly decreased with exposure to higher concentrations of *t*-BuOOH. These results imply the complete dephosphorylation of all the inactive form of cofilin to an active form, during oxidative stress conditions.

Thus, the increased cofilin levels can majorly impact actin depolymerisation and filament breakdown as a consequence of ROS generation. Actin was used as loading control in the experiments to normalize the bands and analyze the changes associated with expression pattern. Total cofilin expression was unchanged in all the treatment

groups. The ratio of phosphocofilin to total cofilin expression pattern was plotted against the treatment groups. These experiments were performed in triplicate with separate batches of PC12 cells and the cumulative result of the experiments was represented in the graph (Fig 4.1).

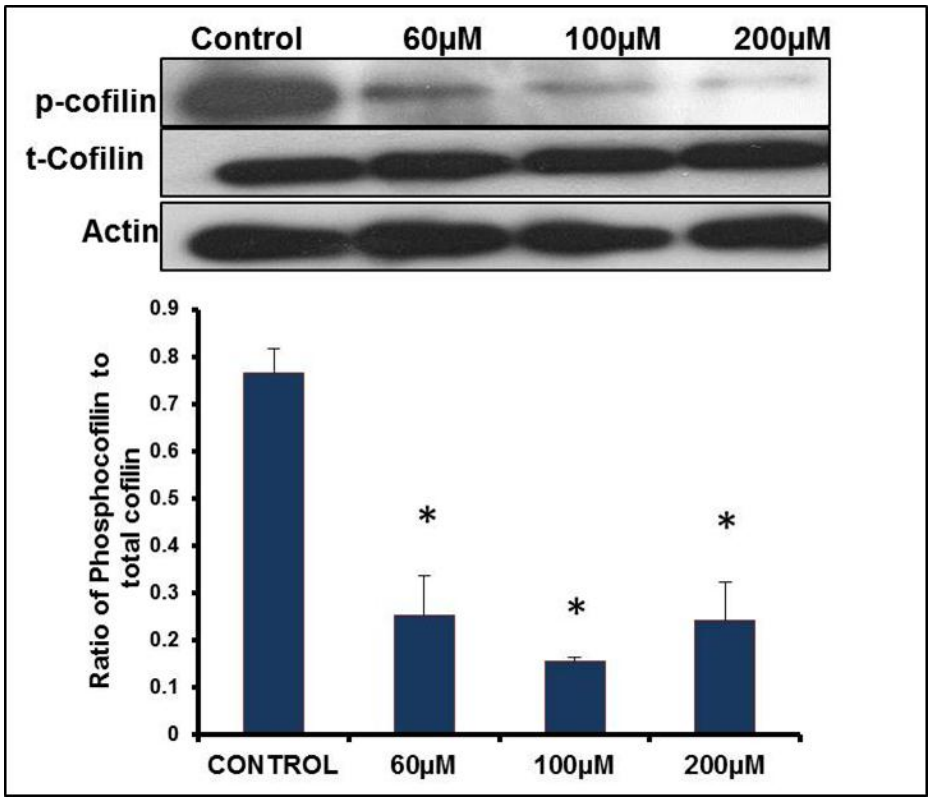

Fig 4.1: Oxidative stress induced changes in phosphocofilin expression. PC12 cells were subjected to treatment with 60µM, 100µM and 200µM concentrations of *t*-BuOOH for 1 hour and the protein expression pattern was studied using Western blotting (n=3). Phosphocofilin expression was found to be significantly decreased with increasing concentration of *t*-BuOOH. The graph represents the densitometric analysis values normalized with actin and the ratio of phosphocofilin to total cofilin levels was plotted against treatment groups. Data are expressed as mean±SEM, where the $p<0.05$ vs control.

4.2. Oxygen glucose deprivation (OGD) induced changes in phosphocofilin expression levels

To further elucidate the cofilin involvement in the ischemic stress mediated responses on actin filaments, an in vitro model of ischemia, oxygen glucose deprivation (OGD) was employed. OGD simulates the hypoxic and hypoglycemic conditions which prevail in cell during stroke. Both undifferentiated and differentiated neuronal cells were subjected to OGD for 4 hours and protein expression pattern was studied using Western blotting. We observed that phosphocofilin, the inactive form of cofilin expression was remarkably decreased when compared to control group in OGD, demonstrating cofilin hyper activation in energy depletion states which is a prevalent feature in ischemia (Fig 4.2a).

There were no discernible changes in the total cofilin levels indicating that the de-phosphorylation is an activation process in hypoglycemic and hypoxic stress conditions not a cellular degradation mechanism. In order to further delineate the time dependent changes occurring in the cofilin activation, subsequent time course experiments were conducted. The neuronal cells were exposed to different time points such as 30 minutes, 1 hour and 4 hours of OGD. It was observed that there was gradient decrease in the phosphocofilin expression with increasing periods of OGD. Phosphocofilin dephosphorylation was initially unchanged at 30 minutes of OGD, followed by a steep decline with 1 hour and almost 70% de-phosphorylation at 4 hours of OGD (Fig. 4.2 b). Together these observations confirm the cofilin activation in the neurodegeneration of ischemic stress pathways.

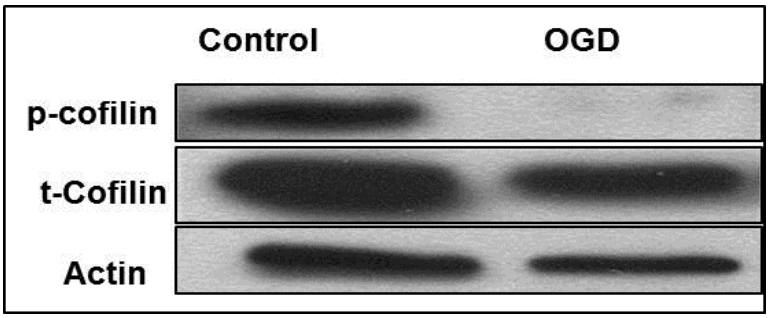

Fig 4.2a: OGD induced dephosphorylation and activation of cofilin. PC12 cells were subjected to OGD for 4 hours and the western blot analysis depicting the proteins - phosphocofilin, cofilin and actin during OGD compared with control conditions. The decreased phosphocofilin expression in OGD conditions was prominent and this indicates de-phosphorylation to a completely active form. Total cofilin levels were found to be unchanged supposedly as the changes were associated only within phosphorylated and un-phosphorylated (active) forms. Actin bands were used as loading control in these experiments.

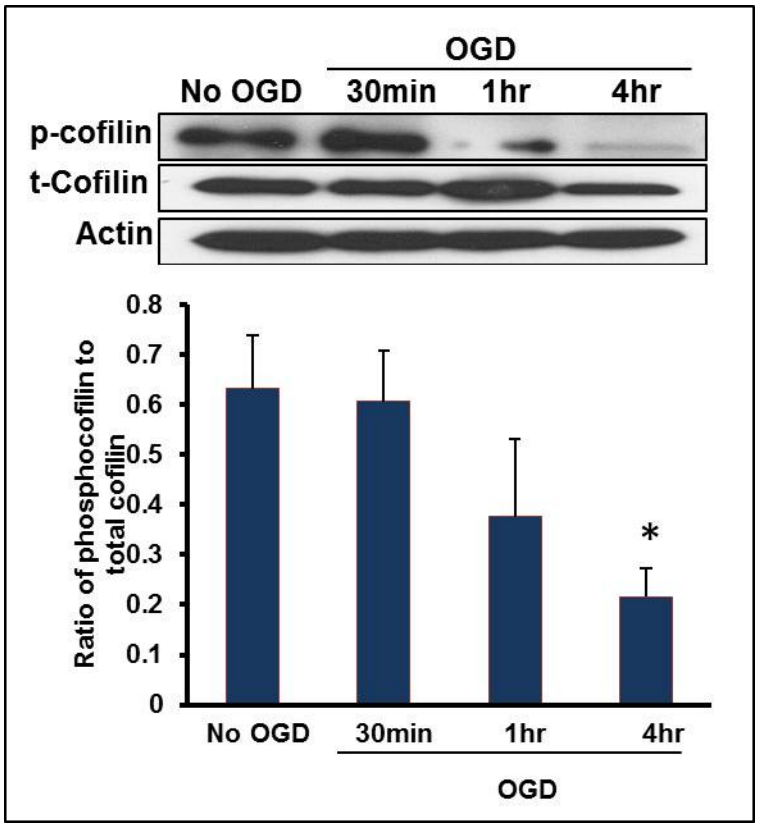

Fig 4.2b: Time-dependent phosphocofilin activation with increasing periods of OGD. Phosphocofilin, total cofilin and actin expression pattern at 30 minutes, 1 hour and 4 hours of OGD. Time course study (n=3) demonstrated the de-phosphorylation of phosphocofilin occurring with increasing periods of stress. The decline in the phosphorylated form initiated within 1 hour of OGD followed by a complete decline within 4 hours of OGD. The graphical representation shows the effect of OGD on the ratio of phosphocofilin to total cofilin levels. All the bands were normalized with actin and represented as Mean±SEM with $p<0.05$ vs. No OGD group. Experiments were performed thrice with separate batches of differentiated neuronal cells and the graph is the cumulative result of three different experiments.

4.3. Effect of nonspecific tyrosine phosphatase inhibitor (Na3VO4) on the phosphocofilin expression in oxygen glucose deprivation (OGD)

Next we sought to investigate the pathways and mediators involved in activation of cofilin during ischemic conditions. Sodium orthovanadate (Na3VO4), a nonspecific tyrosine phosphatase inhibitor was employed in the studies to target the broad range of phosphatases and thereby study the outcomes of cofilin activation inhibition. Cells were pre-incubated with 1mM Na3VO4 for 2 hours and then subjected to OGD for 1 hour duration. Western blot analysis results indicate significant restoration of phosphocofilin levels with inhibitor pretreated cells in OGD when compared to untreated cells (Fig. 4.3). So, with these findings we were able to establish the functional link between phosphatases and cofilin activation state in OGD.

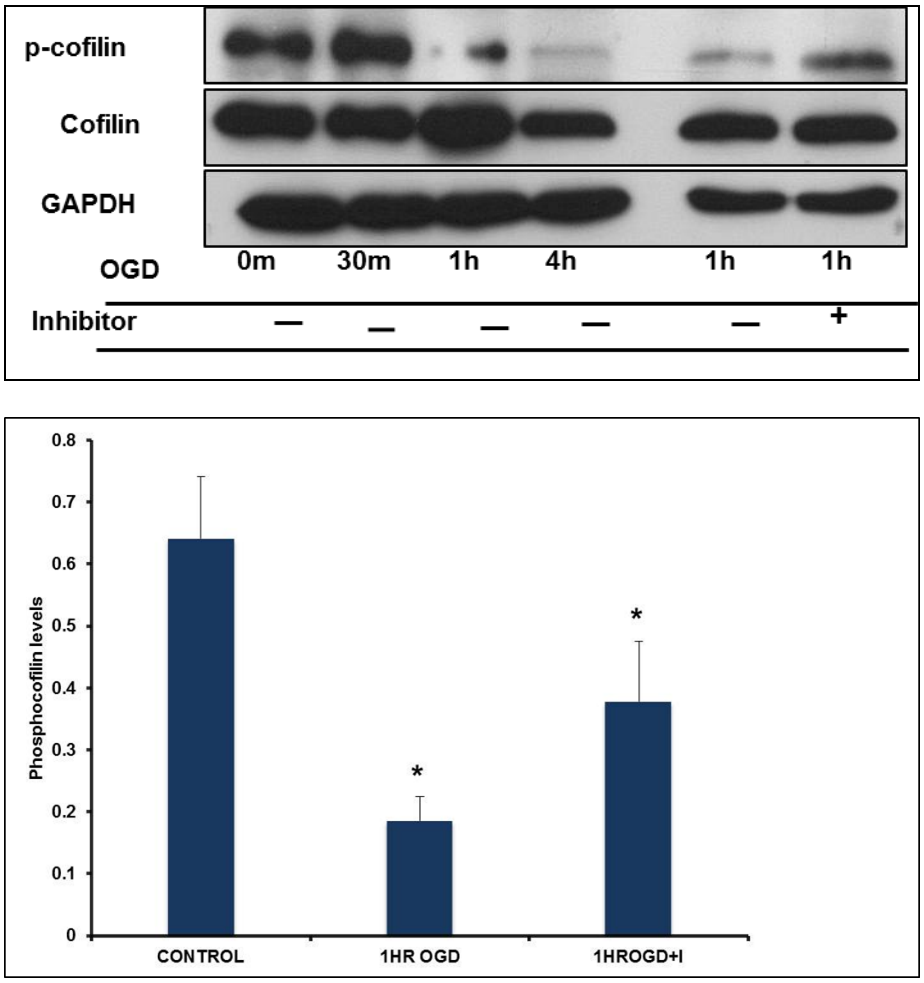

Fig 4.3: Phosphocofilin protein expression was restored in neurons pretreated with inhibitor. Western blotting data demonstrated a decrease in phosphocofilin expression with OGD and a remarkable restoration of its levels with inhibitor pretreatment. Using GAPDH as the loading control, densitometric analysis (n=3) was performed and the phosphocofilin levels with different treatment conditions were represented graphically. Data are expressed as Mean±SEM with $p<0.05$ with respect to control. These experiments were conducted in triplicates with separate batches of differentiated neuronal cells.

4.4. Inhibitor pretreatment increases Cell Viability during oxygen glucose deprivation (OGD)

We further tested the importance of restoring the levels of phosphocofilin during stress conditions using cell viability assays. To have an insight into the effect of alterations in the phosphocofilin levels on the cellular activity, these experiments were proposed. Cells were pretreated with inhibitor and subjected to OGD for a period of 1 hour and MTT assay was performed. It was observed that inhibitor pretreated neurons didn't exhibit any cell death demarcations indicating that the inhibitor by itself does not have any deleterious effect on cell viability and was comparable to control. There was about 50-60% of cell death observed in OGD, on the other hand, inhibitor pretreatment for 2 hours followed by OGD exposure showed restoration of cell viability by 27±0.5% against OGD induced stress implying the importance of phosphocofilin expression on cell viability (Fig 4.4). Ischemic stress crucially reduced the cell viability suggesting the significant role of de-phosphorylation processes taking place during stress, and in-particular activating protein like cofilin. Restoration of certain phosphorylated protein levels such as phosphocofilin during stress has a major impact on cell viability as evident from our MTT results.

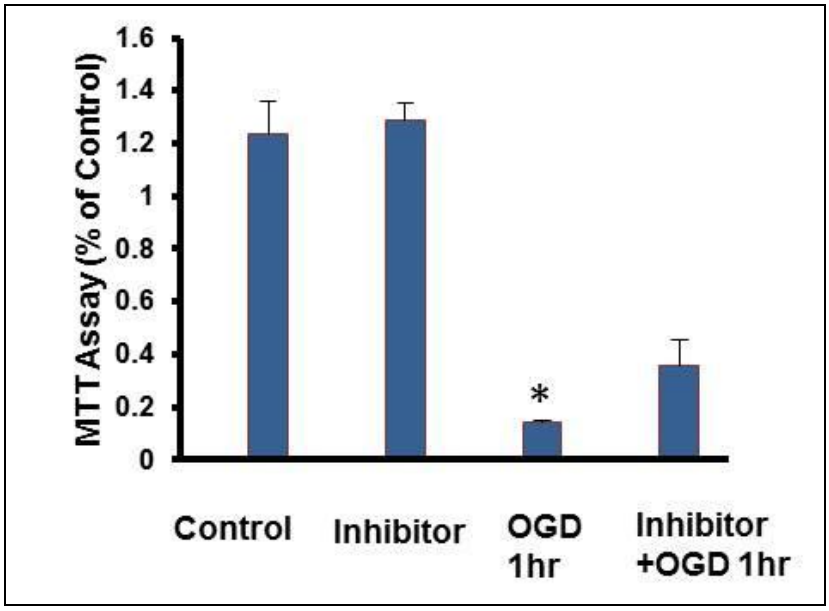

Fig 4.4: OGD stress and inhibitor pretreatment on cell survival. PC12 cells were assayed in 24 well plates for the cell viability in OGD conditions (n=3). The assay results display decreased percentage of metabolically active cells by more than 50% with ischemic stress. The inhibitor pretreatment was able to restore the cell viability when compared with OGD only. The values were expressed as percentage of control and data expressed as mean±SEM. The graph represents the cumulative data of three experiments conducted on separate batches of cells.

4.5. Immunocytochemical analysis of phosphocofilin protein expression in oxygen glucose deprivation (OGD) conditions

To visualize the expression pattern of phosphocofilin and cofilin proteins in neurons and understand the effects of OGD on the actin cytoskeleton, immunofluorescence imaging studies were performed. The inhibitor modulated effects was also confirmed by these illustrations. Differentiated neurons were pretreated with 1mM sodium vanadate for 2 hours and then subjected to OGD for an additional 1hour. Using fluorescence microscopy we observed that phosphocofilin was localized in the nucleus. Cofilin on the other hand was found co-localized with the F-actin filaments prominently and expressed in the cell periphery, cytoplasm and neurite extensions which were evident from the FITC (Fluorescein isothiocyanate) staining. Within 1 hour of OGD, the levels of phosphocofilin in the nucleus were found to be decreased but the inhibitor pretreated group displayed restored levels which was consistent with the western blotting data. Ischemic stress induced destruction of F-actin filaments was depicted from decreased FITC staining. On close observation of the morphology of the filaments, rigidity and intense staining at the filament ends was noticed suggesting the aggregation of severed actin filaments and cofilin was found to be localized with these aggregated filaments. This supports our hypothesis that cofilin enhances actin severing activity in stress conditions (Fig. 4.5a and b). These experiments were repeated twice with separate batches of differentiated cells.

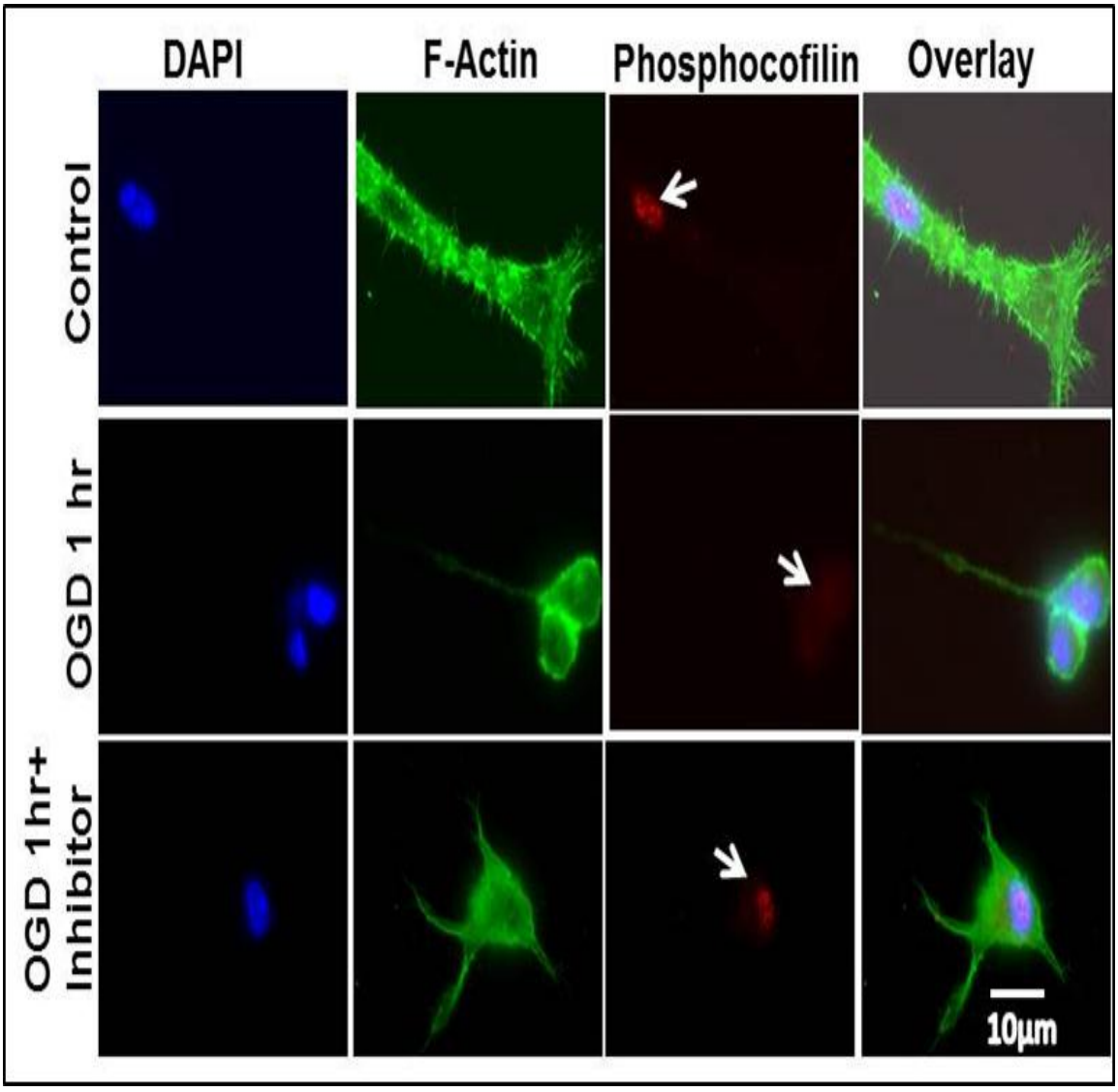

Fig4.5a: Phosphocofilin expression in OGD conditions using inhibitor assays. Nucleus is stained with DAPI (blue color), F-actin filaments stained with phalloidin (green), phosphocofilin was stained with Texas red (red). The top panel showing the control group followed by OGD and inhibitor pretreated group in middle and lower panel. The last right column of images depicts the merged overlay of all figures. The fluorescence imaging clearly illustrates that the inhibitor pretreatment restored the phosphocofilin levels in the nucleus which were decreased with OGD.

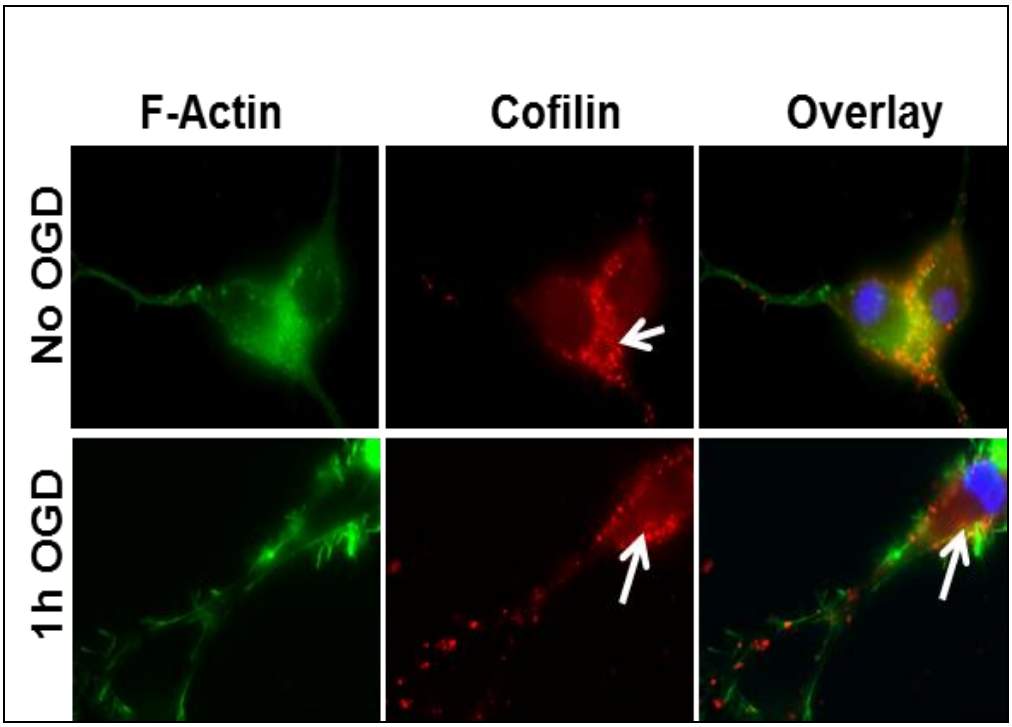

Fig 4.5b: Localization of cofilin after OGD. Cofilin (stained red) is co-localized with the F-actin filaments predominantly in the cell periphery and cytoplasm. After OGD most of the actin filaments were rigid and degraded, the areas highlighted show cofilin prevalence in aggregated actin filaments. Scale bar is 10 µm.

4.6. Calcineurin, a mediator involved in activation of SSH 1L(Slingshot phosphatase) contributes to the dephosphorylation of cofilin during OGD conditions.

These investigations broadened our scope of cofilin activation pathway by depicting the involvement of nonspecific phosphatases during ischemia. The demonstrated results stemmed our interest to delve further into the role of specific phosphatase initiating the changes in cofilin. Literature findings show that the elevated calcium levels could also impact cofilin activation furthering that responses were mediated by SSH1L via calcineurin (Wang, Shibasaki et al. 2005). Thus studies were initiated to target calcineurin and observe the consequent effects on cofilin through SSH1L phosphatase.

Calcineurin is a calcium dependent serine threonine phosphatase involved in dephosphorylation and activation of various proteins and enzymes. As calcium levels were elevated with OGD, evidenced from calcium imaging experiments, we present the hypothesis for the possible involvement of calcineurin in activation of SSH1L and cofilin during stress. Differentiated neuronal cells were pretreated with calcineurin inhibitor (FK-506) of different concentration ranges of 50nM, 100nM and 500nM for 60 minutes and subjected to OGD for 60 minutes. Proteins were extracted and probed for the expression of phosphocofilin and cofilin. The decrease in phosphocofilin levels observed with OGD were restored with 100 nM and 500 nM of calcineurin inhibitor illustrating clearly role of SSH1L phosphatase and calcineurin in the phosphocofilin dephosphorylation (Fig.4.6).

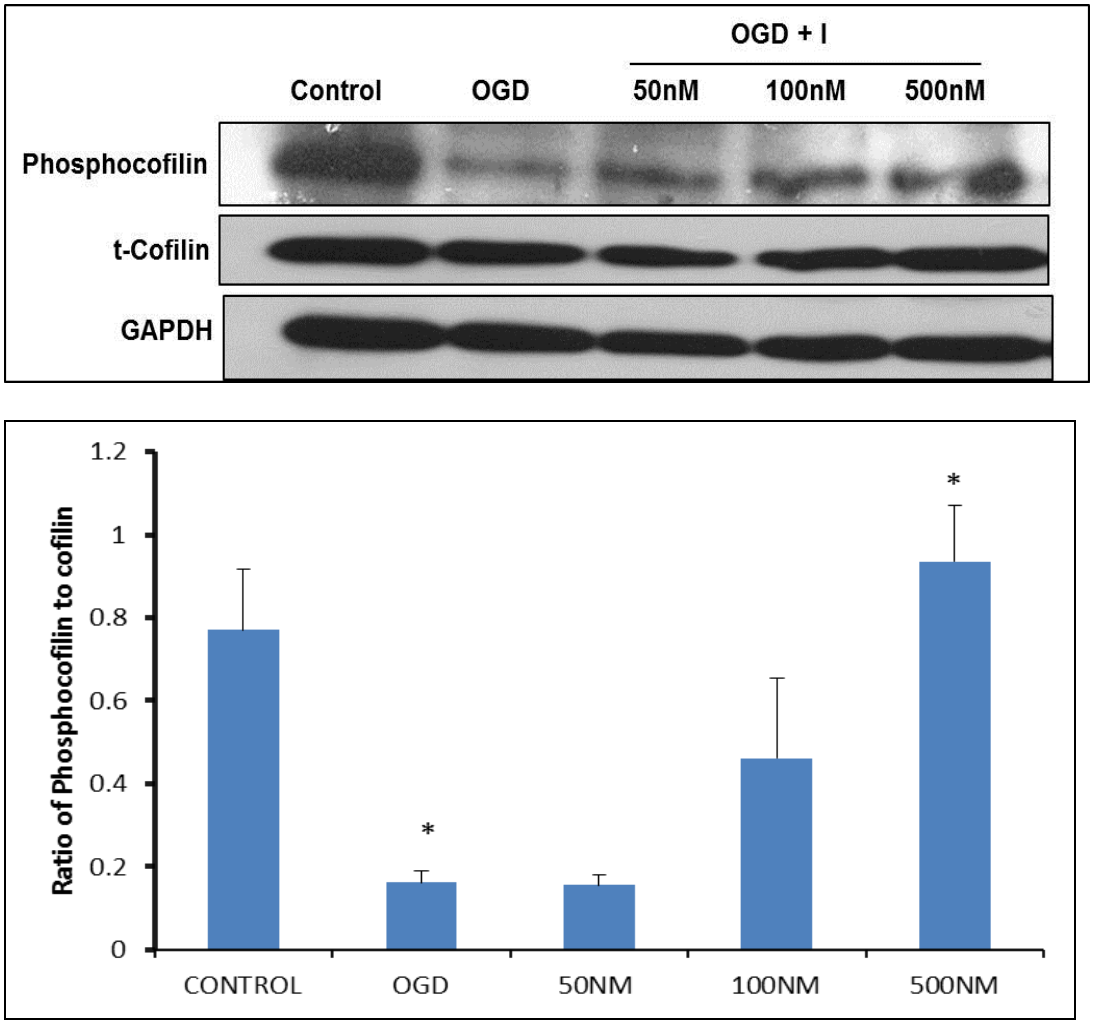

Fig 4.6: Calcineurin inhibitor studies in OGD conditions. Western blot analysis of the phosphocofilin in the differentiated neurons subjected to pretreatment with calcineurin inhibitor (FK-506) of different concentrations in OGD stress (n=3). The levels of phosphocofilin decreased in OGD stress were found to be restored with 100 nM and 500 nM concentrations of the inhibitor. The bar graph represents the cumulative result of three experiments whose densitometric values of the bands were calculated using Image J. The ratio of phosphocofilin to cofilin levels were plotted against the different treatment groups. The data was expressed as Mean±SEM.

4.7. Ischemia induced changes in phosphocofilin expression in mice subjected to permanent middle cerebral artery occlusion (pMCAO) model of ischemia.

The results from *in vitro* studies motivated us to further our understanding for the potential role of cofilin in ischemic conditions *in vivo*. Permanent middle cerebral artery occlusion (pMCAO) model of ischemia was employed as stroke model in the animal studies. Mice randomized into different groups were subjected to pMCAO model of ischemia and brains were isolated after sacrificing the mice, at time different time-points (6 hr, 12 hr, 1 day, 2 days, 3 days, 7 days and 14 days). The results illustrate that in the mice brains the acute changes and chronic changes in phosphocofilin expression were different when compared to control groups of animals. Interestingly, there was an increase in the expression during acute phase of stroke but the levels started declining at post-ischemic time points of 7 days and 14 days. These results provide the evidence of the possible mechanisms of cofilin in neuroregeneration and neurodegenerations at various stages post-ischemia (Fig. 4.7). The experiments were performed thrice with separate batches of animals for different time points of study to confirm these results.

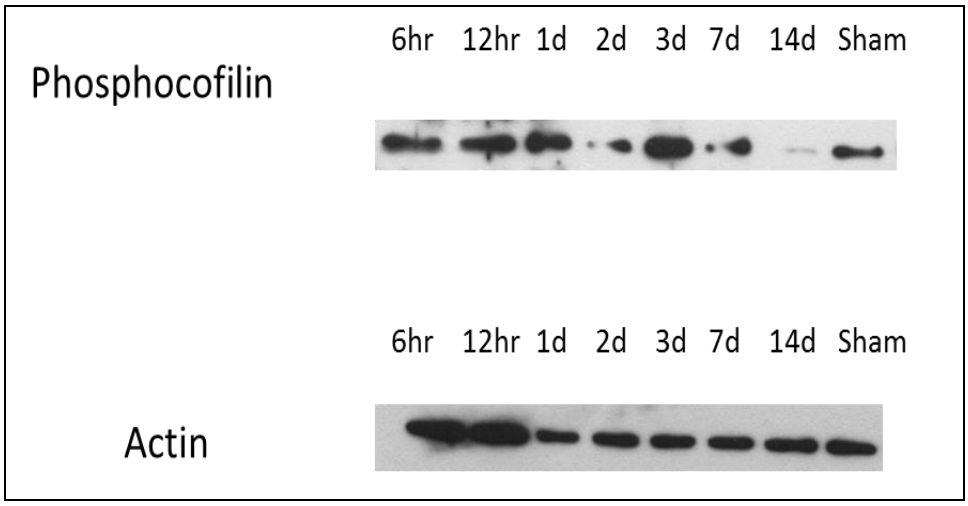

Fig 4.7: Phosphocofilin protein expression in mice subjected to pMCAO. In the permanent ischemic model of study, the significant changes in the phosphocofilin levels was observed during protracted time points of ischemia (n=3). 7days and 14days post ischemic mice brains displayed a pattern of decrease in phosphocofilin levels when compared to sham mice. Actin was the loading control in these immunoblotting experiments.

4.8. Immunohistochemical representation of protein changes in the mice brains subjected to ischemia.

We confirmed these protein changes in the brain sections of mice by immunohistochemistry and TUNEL assay method of apoptosis detection. Mice brains were collected at day 1 and 7 of post-ischemia by transcardial perfusion. TUNEL assay gave an insight into the dead regions of the ischemic brains giving scope for studying the protein expression pattern in these regions and the associated changes in the penumbra region of brain (Fig 4.9 a, b, c). Phosphocofilin levels were found to be predominant at 1day of ischemia and then decreased within 7days of ischemia. Differences in the phosphocofilin levels were observed during the protracted ischemic period. These investigations support the immunoblotting expression patterns observed previously with various post ischemic time points. Cofilin localized to the periphery of the cell and was abundantly expressed around tissues due to thorough distribution of actin filaments connecting all the tissues together (Fig 4.9 e). As a result peculiar changes in cofilin expression in control and ischemic brains were not distinguishable.

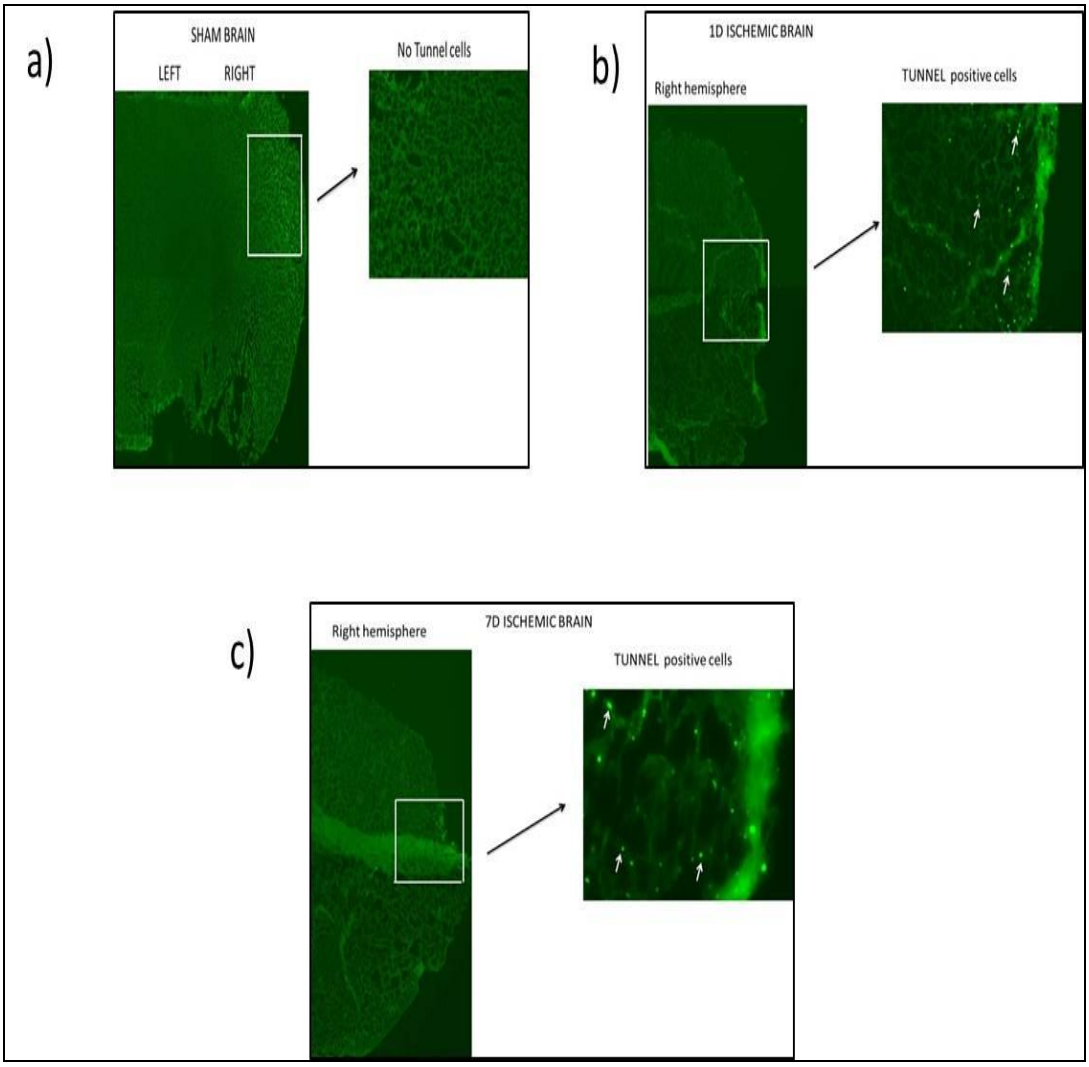

Fig 4.8a, b, and c: TUNEL assay for the control (sham) brain, 1 day and 7 day post ischemic brain showing the ischemic core regions. (a) Control brain was TUNEL negative as there was no ischemia induced in the mice. (b) The ischemic region in the 1 day and (c) 7 days post ischemic brain sections was observed to be centered in the upper right half of brain. The apoptotic (TUNEL positive cells) were the green fluorescent cells highlighted by arrows to show the cell death occurring in the ischemic portion. Scale bar 10μm for the enlarged image.

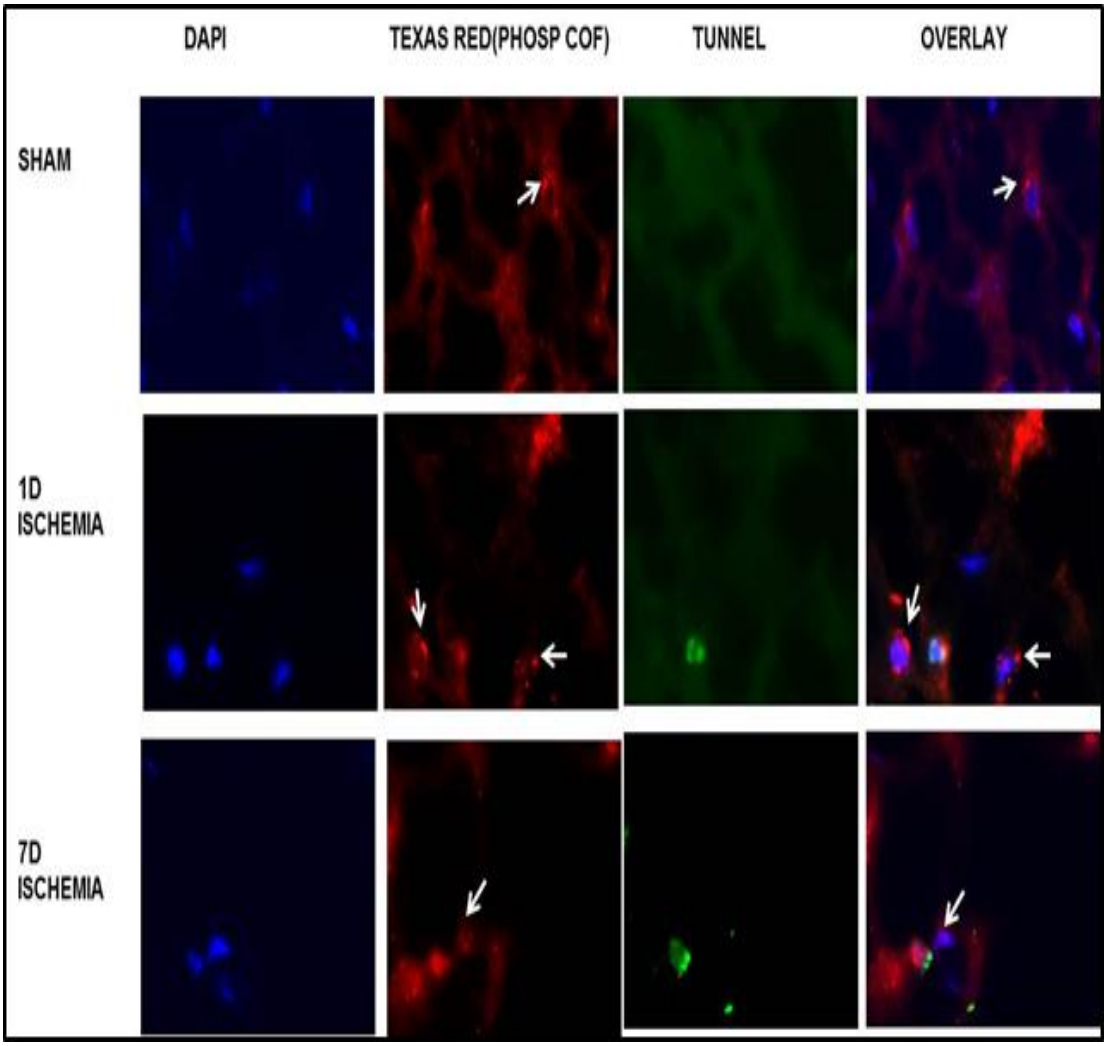

Fig 4.8d: Immunohistochemical analysis of the protein expression in mice subjected to pMCAO. The nucleus was stained with DAPI, phosphocofilin with Texas red and the TUNEL cells were stained with green. In the sham, phosphocofilin expression was localized around the nucleus. The Phosphocofilin expression pattern was found to be increased when compared to sham during 1day of ischemia following a complete decrease at 7day of ischemia. Scale bar 10µm.

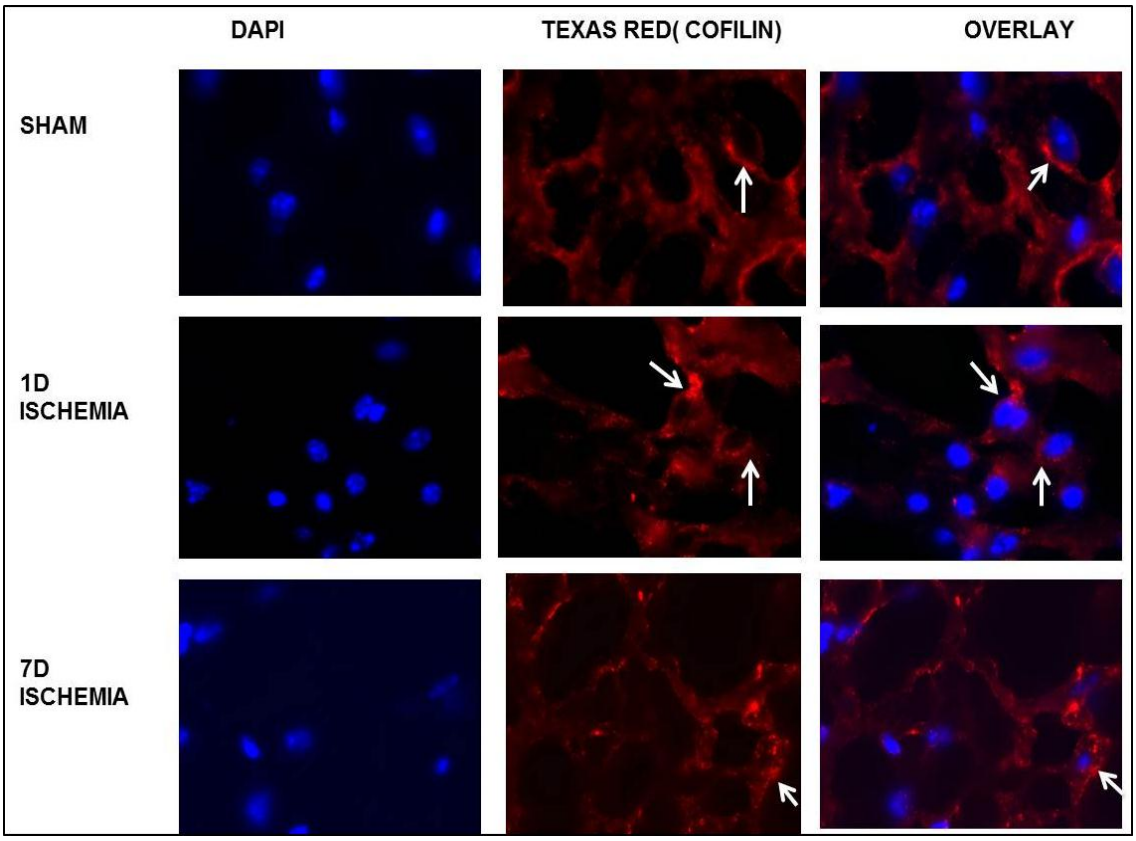

Fig 4.8e: Immunohistochemistry of cofilin expression in control and ischemic brains. The tissues abundant expression of actin and colocalized cofilin do not clearly depict the peculiar changes occuring during ischemia. Scale bar 10µm

5. Discussion

In the present study, we have demonstrated the functional role of protein cofilin in altering actin cytoskeletal associated cellular functions in cerebral ischemia using both *in vitro* and pMCAO model of ischemia. Cofilin was found to be hyperactivated by prominent dephosphorylation against the oxidative and OGD insult in a time dependent manner. Western blot analysis displayed undetectable phosphorylated cofilin after 4 hours of OGD implying complete activation of cofilin in response to OGD stress. In addition, the temporal expression study in ischemic brains displayed chronic dephosphorylation of phosphocofilin suggesting the delayed neurocytoskeletal degenerations induction period. Moreover, phosphatase inhibitor pretreatment inhibited the cofilin dephosphorylation and activation in OGD conditions. This observation was complemented by our immunofluorescence studies wherein morphological changes in the actin cytoskeleton and thereby phosphocofilin expression in OGD was demonstrated. Prominently, marked changes in actin filaments during OGD such as aggregation of actin filaments in the cell periphery and the intense staining of FITC (green) in these regions was also noted confirming the enhanced actin severing activity of cofilin. This inhibition was also found to ameliorate cell death during OGD. Our results suggest that targeting cofilin will have important role in ischemia repair and regeneration.

We have also successfully attributed the role of signaling mediators, SSH and calcineurin phosphatases, in the dephosphorylation and activation of cofilin in OGD. These results explain the cofilin predominance in ischemia conditions by dephosphorylation which were mainly mediated by SSH phosphatase upon activation by calcineurin. Together, our results implicate actin cytoskeletal degenerations in cerebral ischemia were mediated by cofilin and targeting this protein could be beneficial in ischemic regeneration and recovery.

Cerebral ischemia is a cerebrovascular disease with various pathophysiological manifestations. With very few treatments available for stroke, the essential need for identifying pathways associated with the pathology and developing new therapeutic drugs targeting neurodegenerative pathways is imperative. The ischemic core in brain where there is complete obstruction of the blood flow, predominant inflammation, excitotoxicity and necrotic cell death follows within minutes. Penumbra (region adjacent to ischemic portion of brain) acts as a buffer region due to collateral blood flow and the degree of ischemia, and the levels of reperfusion determine the fate of the cells in this region (Doyle, Simon et al. 2008). The cell death in this region is less rapid and rescuing cells in this region is critical step in neuroprotection. Cytoskeleton of neurons is the dynamic framework that has a potential to salvage the penumbra by regeneration, but the presence of certain mediators involved in the regulation outrage this dynamic nature leading to neurodegeneration. Studies have demonstrated the active involvement of MAP (Microtubule associated protein), tau and cofilin aggregations in the progression of disease states such as AD and dementia (Takahashi, Tsujioka et al. 1999). These

neurodegenerative pathologies deteriorate the disease state and thereby also regenerative strategies due to their effect on cytoskeleton.

In addition to this, cytoskeleton which forms the major neuron structure is essential for protein localizations and transport of molecules to-and-fro between dendrites and axons (Lipton 1999). Any gross changes associated with cytoskeleton can immediately trigger necrosis or apoptosis. Supporting this, a study demonstrated that activated ADF/cofilin proteins were found to disrupt actin cytoskeleton in response to ischemia in renal tubular epithelial cells (Schwartz, Hosford et al. 1999). These are consistent with our investigations in which we elucidate cofilin potential involvement in mediating neurocytoskeletal degenerations in a disease state, cerebral ischemia.

The blockade of blood flow to the brain results in upregulation of complex cascade of degenerative mechanisms and these include excitotoxicity, inflammation, oxidative stress, apoptosis and calcium overload (Won, Kim et al. 2002). Reactive oxygen radicals and OGD are the most common mechanisms of neuronal injury in stroke (Doyle, Simon et al. 2008). Combination pathologies of glutamate release, calcium influx and enhanced production of reactive metabolites was demonstrated in ischemia (Kristian and Siesjo 1998). Thus formed combinatorial mediators are responsible for targeting various structures of the cell such as mitochondria, cytoskeleton and DNA. Cytoskeletal proteins are predominant in most of the cells and this abundance makes it more susceptible target for cell death mediators (Avery 2011). Hence the perpetrators in the form of calcium, reactive oxygen species formed combined with excitotoxicity can ultimately degrade the integrity of actin filaments by indirectly modulating cytoskeletal

proteins ultimately leading to neuronal death. Our cumulative results were able to explain the potential mechanistic signaling involving cofilin, SSH and calcineurin phosphatase as one of the pathway which is possibly explored by ischemic cell death mediators to target the cytoskeleton in an ischemic situation where ROS, calcium overload and hypoxia are prevalent.

Cofilin, an important cytoskeletal protein is responsible for controlling actin dynamics in a cell. Cofilin functions beyond these regulatory activities are responsible for aberrations in cellular activity. Phosphatases were found to play a key role in activation of cofilin during ATP depletion states in endothelial cells (Huang, Minamide et al. 2008). This activation which is an energy conservatory mechanism results in bundling of actin with cofilin, eventually forming rods but potentially predisposing neuron to synaptic deficits (Cichon, Sun et al. 2012). In addition to this, cofilin hyperactivation can also affect the mitochondrial functions by altering the mitochondrial membrane permeability and facilitating the release of cell death mediators like cytochrome C (Chua, Volbracht et al. 2003). Furthermore, oxidative stress and ATP depletion states were found to oxidize cofilin and translocate it to mitochondria to induce cell death (Klamt, Zdanov et al. 2009).

In fact cofilin actin rods were formed as a consequence of ATP depletion in neurons are potential mediators of cell death (Bernstein, Chen et al. 2006). Since cerebral ischemia is a pathological condition associated with excitotoxicity, ATP depletion and oxidative stress, we choose to investigate potential cofilin involvement in ischemic stress

mediated responses on actin cytoskeleton and degenerations. The question of cytoskeletal aberrations and cofilin involvement was addressed by our current investigations. We have successfully demonstrated cofilin's active role in cerebral ischemic conditions and the cytoskeletal alterations studied by fluorescence methods confirmed the cofilin potential in altering the integrity of filaments in ischemia and thereby facilitating cell death.

In conclusion, we believe that targeting cofilin which is a subject of many adversaries and involved in mediating cell death in ischemia would be an effective strategy in treating stroke.

References

Begin Ashworth, S. L., R. M. Sandoval, et al. (2001). "Ischemic injury induces ADF relocalization to the apical domain of rat proximal tubule cells." Am J Physiol Renal Physiol 280(5): F886-894.

Ashworth, S. L., R. M. Sandoval, et al. (2001). "Ischemic injury induces ADF relocalization to the apical domain of rat proximal tubule cells." Am J Physiol Renal Physiol **280**(5): F886-894.

Bamburg, J. R. and B. W. Bernstein (2008). "ADF/cofilin." Curr Biol **18**(7): R273-275.

Bamburg, J. R. and B. W. Bernstein (2010). "Roles of ADF/cofilin in actin polymerization and beyond." F1000 Biol Rep **2**: 62.

Bamburg, J. R., B. W. Bernstein, et al. (2010). "ADF/Cofilin-actin rods in neurodegenerative diseases." Curr Alzheimer Res **7**(3): 241-250.

Bamburg, J. R., H. E. Harris, et al. (1980). "Partial purification and characterization of an actin depolymerizing factor from brain." FEBS Lett **121**(1): 178-182.

Bamburg, J. R. and O. P. Wiggan (2002). "ADF/cofilin and actin dynamics in disease." Trends Cell Biol **12**(12): 598-605.

Bernstein, B. W. and J. R. Bamburg (2003). "Actin-ATP hydrolysis is a major energy drain for neurons." Journal of Neuroscience **23**(1): 1-6.

Bernstein, B. W. and J. R. Bamburg (2010). "ADF/cofilin: a functional node in cell biology." Trends Cell Biol **20**(4): 187-195.

Caplan, L. R. (2000). "Multiple potential risks for stroke." JAMA **283**(11): 1479-1480.

Carlier, M. F., V. Laurent, et al. (1997). "Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: Implication in actin-based motility." Journal of Cell Biology **136**(6): 1307-1322.

Chen, H., H. Yoshioka, et al. (2011). "Oxidative stress in ischemic brain damage: mechanisms of cell death and potential molecular targets for neuroprotection." Antioxid Redox Signal **14**(8): 1505-1517.

Chua, B. T., C. Volbracht, et al. (2003). "Mitochondrial translocation of cofilin is an early step in apoptosis induction." Nature Cell Biology **5**(12): 1083-1089.

Cichon, J., C. Sun, et al. (2012). "Cofilin aggregation blocks intracellular trafficking and induces synaptic loss in hippocampal neurons." J Biol Chem **287**(6): 3919-3929.

Cichon, J., C. C. Sun, et al. (2012). "Cofilin Aggregation Blocks Intracellular Trafficking and Induces Synaptic Loss in Hippocampal Neurons." Journal of Biological Chemistry **287**(6): 3919-3929.

Dos Remedios, C. G., D. Chhabra, et al. (2003). "Actin binding proteins: Regulation of cytoskeletal microfilaments." Physiological Reviews **83**(2): 433-473.

Doyle, K. P., R. P. Simon, et al. (2008). "Mechanisms of ischemic brain damage." Neuropharmacology **55**(3): 310-318.

Fitzpatrick, M. O., D. Dewar, et al. (1996). "The neuronal cytoskeleton: an insight for neurosurgeons." Br J Neurosurg **10**(5): 483-487.

Fuchs, E. and D. W. Cleveland (1998). "A structural scaffolding of intermediate filaments in health and disease." Science **279**(5350): 514-519.

Hall, A. (1998). "Rho GTPases and the actin cytoskeleton." Science **279**(5350): 509-514.

Hotulainen, P., E. Paunola, et al. (2005). "Actin-depolymerizing factor and cofilin-1 play overlapping roles in promoting rapid F-actin depolymerization in mammalian nonmuscle cells." Mol Biol Cell **16**(2): 649-664.

Huang, T. Y., L. S. Minamide, et al. (2008). "Chronophin mediates an ATP-sensing mechanism for cofilin dephosphorylation and neuronal cofilin-actin rod formation." Dev Cell **15**(5): 691-703.

Keezer, S. M., S. E. Ivie, et al. (2003). "Angiogenesis inhibitors target the endothelial cell cytoskeleton through altered regulation of heat shock protein 27 and cofilin." Cancer Res **63**(19): 6405-6412.

Kellerman, P. S. and R. T. Bogusky (1992). "Microfilament Disruption Occurs Very Early in Ischemic Proximal Tubule Cell Injury." Kidney International **42**(4): 896-902.

Kim, J. S., T. Y. Huang, et al. (2009). "Reactive oxygen species regulate a slingshot-cofilin activation pathway." Mol Biol Cell **20**(11): 2650-2660.

Klamt, F., S. Zdanov, et al. (2009). "Oxidant-induced apoptosis is mediated by oxidation of the actin-regulatory protein cofilin (vol 11, pg 1241, 2009)." Nature Cell Biology **11**(11): 1387-1387.

Kristian, T. and B. K. Siesjo (1998). "Calcium in ischemic cell death." Stroke **29**(3): 705-718.

Lipton, P. (1999). "Ischemic cell death in brain neurons." Physiological Reviews **79**(4): 1431-1568.

Maloney, M. T. and J. R. Bamburg (2007). "Cofilin-mediated neurodegeneration in Alzheimer's disease and other amyloidopathies." Mol Neurobiol **35**(1): 21-44.

Minamide, L. S., A. M. Striegl, et al. (2000). "Neurodegenerative stimuli induce persistent ADF/cofilin-actin rods that disrupt distal neurite function." Nature Cell Biology **2**(9): 628-636.

Morgan, T. E., R. O. Lockerbie, et al. (1993). "Isolation and characterization of a regulated form of actin depolymerizing factor." Journal of Cell Biology **122**(3): 623-633.

Nishida, E., E. Muneyuki, et al. (1985). "An actin-depolymerizing protein (destrin) from porcine kidney. Its action on F-actin containing or lacking tropomyosin." Biochemistry **24**(23): 6624-6630.

Pollard, T. D. (1986). "Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments." Journal of Cell Biology **103**(6 Pt 2): 2747-2754.

Pollard, T. D. and G. G. Borisy (2003). "Cellular motility driven by assembly and disassembly of actin filaments." Cell **112**(4): 453-465.

Pollard, T. D. and J. A. Cooper (2009). "Actin, a central player in cell shape and movement." Science **326**(5957): 1208-1212.

Posadas, I., F. C. Perez-Martinez, et al. (2012). "Cofilin activation mediates Bax translocation to mitochondria during excitotoxic neuronal death." J Neurochem **120**(4): 515-527.

Ramaekers, F. C. and F. T. Bosman (2004). "The cytoskeleton and disease." J Pathol **204**(4): 351-354.

Reisler, E. and E. H. Egelman (2007). "Actin structure and function: what we still do not understand." J Biol Chem **282**(50): 36133-36137.

Schwartz, N., M. Hosford, et al. (1999). "Ischemia activates actin depolymerizing factor: role in proximal tubule microvillar actin alterations." Am J Physiol **276**(4 Pt 2): F544-551.

Scott, R. W. and M. F. Olson (2007). "LIM kinases: function, regulation and association with human disease." J Mol Med (Berl) **85**(6): 555-568.

Sidani, M. M., G. Mouneimne, et al. (2006). "Cofilin Knockdown Triggers Arp2/3 Relocalization and Changes the Motility Behavior of Metastatic Cancer Cells." Molecular Biology of the Cell **17**.

Singh, G., M. A. Siddiqui, et al. (2009). "Oxygen Glucose Deprivation Model of Cerebral Stroke in PC-12 Cells: Glucose as a Limiting Factor." Toxicology Mechanisms and Methods **19**(2): 154-160.

Suurna, M. V., S. L. Ashworth, et al. (2006). "Cofilin mediates ATP depletion-induced endothelial cell actin alterations." Am J Physiol Renal Physiol **290**(6): F1398-1407.

Tamura, A., D. I. Graham, et al. (1981). "Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion." J Cereb Blood Flow Metab **1**(1): 53-60.

Tsai, C.-H. and Y.-J. Lee (2012). "Focus on ADF/Cofilin: Beyond Actin Cytoskeletal Regulation." ISRN Cell Biology **2012**: 7.

Van Troys, M., L. Huyck, et al. (2008). "Ins and outs of ADF/cofilin activity and regulation." Eur J Cell Biol **87**(8-9): 649-667.

Wang, C., H. N. Nguyen, et al. (2002). "Role of intracellular calcium stores in cell death from oxygen-glucose deprivation in a neuronal cell line." J Cereb Blood Flow Metab **22**(2): 206-214.

Wang, Y., F. Shibasaki, et al. (2005). "Calcium signal-induced cofilin dephosphorylation is mediated by Slingshot via calcineurin." J Biol Chem **280**(13): 12683-12689.

Won, S. J., D. Y. Kim, et al. (2002). "Cellular and molecular pathways of ischemic neuronal death." J Biochem Mol Biol **35**(1): 67-86.

Yang, E., H. Kim, et al. (2004). "Overexpression of LIM kinase 1 renders resistance to apoptosis in PC12 cells by inhibition of caspase activation." Cell Mol Neurobiol **24**(2): 181-192.

Yonezawa, N., E. Nishida, et al. (1990). "Inhibition of the Interactions of Cofilin, Destrin, and Deoxyribonuclease-I with Actin by Phosphoinositides." Journal of Biological Chemistry **265**(15): 8382-8386.

Zeynalov, E., Z. A. Shah, et al. (2009). "Heme oxygenase 1 is associated with ischemic preconditioning-induced protection against brain ischemia." Neurobiology of Disease **35**(2): 264-269.