THE STUDY OF THE NEUROPHYSIOLOGY OF HIGH STRAIN RATE NERVE INJURY

A Dissertation

by

IN HONG YANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2003

Major Subject: Biomedical Engineering

THE STUDY OF THE NEUROPHYSIOLOGY OF HIGH STRAIN RATE NERVE INJURY

A Dissertation

by

IN HONG YANG

Submitted to Texas A&M University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Approved as to style and content by:

Hsin-I Wu (Co-Chair of Committee)	Theresa A. Good (Co-Chair of Committee)
William A. Hyman (Member)	Lihong Wang (Member)

William A. Hyman
(Head of Department)

December 2003

Major Subject: Biomedical Engineering

ABSTRACT

The Study of the Neurophysiology of High Strain Rate Nerve Injury.

(December 2003)

In Hong Yang, B.S.; M.S., KyungHee University

Co-Chairs of Advisory Committee: Dr. Hsin-I Wu
Dr. Theresa A. Good

The study of the mechanism of traumatic brain injury (TBI) processes at the cellular level is vital to obtain characterization of nerve cell damage after mechanical deformation. This understanding is needed to find feasible therapeutic targets for mechanically damaged neurons. To study the cellular level of TBI damage, development of a new *in vitro* cellular model of TBI might be done to simulate *in vivo* cellular TBI.

In this research, two studies were performed: (1) the design and construction of an *in vitro* cell stretching device to mechanically injure cells and (2) the characterization of the molecular and cellular level of the TBI mechanism. The cell stretching device design allows for the precise control of cell strain and duration of stretching cells such that TBI can be mimicked. Analysis of the cellular and molecular level mechanisms of TBI in the proposed *in vitro* model might help in the design of therapeutic strategies for the treatment of TBI. Our proposed mechanism of injury due to TBI is as follows: after the cell is stretched, a cellular signaling molecule is released to activate the cellular signaling pathway. The activated cell signal may activate kinases which phosphorylate proteins and initiate new protein synthesis. Newly phosphorylated and synthesized proteins may activate the apoptotic process.

Using a variety of pharmacological agents, one could block steps in the hypothesized mechanism and examine the effect of those agents on downstream cellular processes and cell apoptosis. For example, the inhibitions of calcium transport, protein synthesis, and caspases were performed to examine the initial activation of the signaling pathway and the role of both in the apoptosis process. Proteomics of TBI may help the understanding of the mechanism of TBI related protein expression. This work will contribute to the discovery of new therapeutic targets and better treatments for TBI.

DEDICATION

To my loving parents

ACKNOWLEDGEMENTS

I am thankful to many people who supported me and looked after me during my academic studies at Texas A&M University. Without them, I could not have achieved my Ph.D.

I am heartily thankful to Dr. Wu and Dr. Good. Their knowledge led me to develop a creative and independent scientific mind. Also, I am thankful for their companionship and generosity. I wish to thank my advisory committee members: Dr. Hyman, and Dr. Wang for their critical comments on my work and their time. I am also appreciative for former group members who taught me various lab skills: Dina Triyoso, and Roberto Linares and my best friends: Eddy Tay, Wanida Wattanakaroon, Sung Moon Lee, Suk Joon Yoo, Kyungnam Kang, Sungjoon Lee, Vasiliki Kazantzi, and Steven Wang who made me happy.

My family has provided great support and sacrifice for me. Also, I would like to send my love to my grandmother who passed away.

Finally, I am thankful to my puppy, Volkee who provided me with lots of fun.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Traumatic Brain Injury

Traumatic brain injury (TBI) is one of the major public health problems and the leading cause of death of individuals under 44 years old in United States. The risk of experiencing a TBI is highest among teenagers, young adults, and people older than 75 years of age. Most studies indicate that the highest rates of TBI occur between 15 to 24 years of age (CDC, 1999). The leading causes of TBI are vehicle crashes, accidental falls, bicycles accidents, pedestrian's falls, and firearms (Thurman et al., 1999). The National Institutes of Health (NIH) reported that 5 million TBI cases occur in the United States each year (CDC, 1999) among which about 100,000 people die. Nearly 750,000 people are hospitalized a year (CDC, 1999) and about 80,000 of TBI survivors live with significant disabilities (CDC, 1999). Consequences of TBI are loss memory, deficits in judgment and emotion, and other mental and physical disabilities. The cost of TBI is an estimated $56.3 billion the U.S. in 1995, (Thurman et al., 1999).

Due to the many occurrences and the significant impact of TBI on a society, a great deal of efforts has been done to prevent and to find treatments for TBI. To prevent the TBI and TBI-related disabilities, many policy and safety measures have been enacted. For instance, the increase of helmet use during recreation and sports activities, the improved use of child safety seats and seat belts, and improved trauma care surveillance systems and clinical preventive services are all examples of practices that have implanted to prevent TBI and TBI related disabilities (Pollock and Lowery, 2001).

This dissertation follows the style of Bioengineering and Biotechnology.

Because TBI does not occur in a controlled or reproducible manner, it is difficult to perform controlled laboratory experiments that mimic TBI. Studies of mechanical impact have been performed by using a dummy to characterize the forces generated upon head impact. However, the effect of mechanical displacement on nerve cells at a cellular or molecular level are difficult to study in *in vivo* models or simulation with dummies. The goal of this research is to improve the understanding of TBI at a cellular and molecular level through the development of a simple *in vitro* model of mechanical injury to neuron-like cells and the analysis of cellular and molecular level changes that occur in that model after injury .

General definition and initial mechanism of traumatic brain injury. Traumatic brain injury is also referred to as acquired brain injury. Traumatic brain injury occurs when a sudden physical impact on the brain or skull causes mechanical damage to the brain tissue. When an excessive mechanical force is applied to the brain, the brain deforms. The mechanical effects of TBI are associated with strain rate, and rate of head acceleration (Gennarelli, 1996). The strain rate to brain tissue that occurs during TBI has been estimated to vary in the range from 1 to $5s^{-1}$ (Chu et al., 1994; Ueno et al., 1995; Ommaya, 1995). In biological tissues, which are generally viscoelastic materials, some of the initial force applied to the head is absorbed, leading to a damping of the load and prolonged, oscillatory stresses within the tissue (Bradshaw et al., 2001). Bone, scalp, dermal and subcutaneous layers all play a role in absorbing and transmitting impact forces to the underlying central nervous tissue (Hubbard, 1971). Contact forces on the brain generate a mechanical stress wave and skull deformation. Deformed brain tissues undergo a variety of pathological processes that lead to the loss of function associated with TBI. The cellular events in the pathological pathways that lead to the physiological effects of TBI will be discussed later in this chapter.

Types of traumatic brain injury. TBI can be generally classified as focal brain injury or diffuse brain injury (DAI). Focal brain injury is in a restricted area of

brain while diffuse brain injury or diffuse injury describes a pattern of injury in areas of the brain that exceed the area of impact. In most cases focal injury leads to more diffuse damage. The examples of focal TBI are contusion, epidermal hematoma and subdural hematoma. Hematoma means a matter of clotted blood that forms in a tissue, organ, or body space as a result of a blood vessel rupture. Contusions are generally associated with the skull fractures. Epidermal hematoma is caused by the deformation of skull bone where dural fiber and vascular attachment are sheared. Subdural hematoma is caused by the surface artery tearing and is associated with contusion. However, there are examples of contusion such as shaken baby syndrome where contusions not associated with fracture. Diffuse axonal injury is caused by the acceleration and deceleration of tissues in the brain. The rotational acceleration is the key component of diffuse axonal injury (Gennarelli, 1996). The shearing of axons block the communication between the nerve cells. The shear and other stress associated with tissue acceleration and deceleration are what is believed to give rise to the cellular level damage to large numbers of neurons during brain injury (Margulies et al., 1990).

Primary brain and secondary brain injuries are categorized by the initial impact and subsequent injury. A primary brain injury occurs at the beginning of brain insult. Primary brain injuries are associated with the initial contact force and the subsequent inertial force. The severity of primary brain injuries depend upon numerous variables such as the intensity of initial contacting forces, degrees of tissue deformation, location of injury and skull thickness. A secondary brain injury is a result of a pathological response of brain to a primary brain injury. A secondary brain injury develops within hours or days after the primary brain injury. Blood vessel rupture, cell lysis, cell apoptosis in response to the release of high levels of excitatory neurotransmitters, and increased intracranial pressure are all associated with secondary injury.

Treatment of traumatic brain injury patient. Medical treatment of TBI is critical to prevent further damage. Improper care may cause additional brain injury and other injuries. An important step in the treatment of head injury is lowering the

intracranial pressure (ICP). Prevention of elevated ICP is the key to prevent ischemia and hematoma, both of which are associated with reduction in blood and oxygen supplies resulting in further neurodegeneration associated with TBI. ICP can be monitored by a ventricular catheter, subarachnoid bolts, and a fiber optic parenchymal monitor. The elevated cerebral fluid can be drained by only a ventricular catheter. A fiber optic paprenchymal monitor is minimally invasive device to monitor ICP. Also surgical removal of hematoma is important to prevent further brain damage. Moreover, various pharmacological agents are used to lower the elevated ICP. β-adrenoreceptor blocker and osmotic diuretics are examples of agents. To reduce the increased cellular metabolism associated with secondary injury in TBI, cooling blanket and nasogastric acetaminophen are used for hyperthermic treatment. Action is also taken to reduce glutamate and free radicals after TBI. Glutamate and free radicals contribute significantly to secondary brain injury. Glutamate is an amino acid which causes excitotoxicity via excessive introduction of calcium into cells through the NMDA receptor. Free radical can cause damage to cells similar to that caused by glutamate. Removal of glutamate, blockage of NMDA receptor, and use of free radical scavengers (antioxidants) all are used to block secondary brain injury (Triyoso and Good, 1999).

Research review of traumatic brain injury. There are several approaches that have been taken previously to try to understand the mechanism of neurodegeneration associated with injury. It is considered that direct measurement of strain is difficult or nearly impossible to perform in animal models. Therefore many theoretical models were developed to study the damage of brain on impact. However, the post traumatic symptoms and combined effect of different cells, bones, blood, and tissues are most easily researched in animal models of TBI. *In vitro* models of TBI are used to study to cellular and biochemical neurodegeneration of TBI at cellular levels. In the following section, animal, theoretical, and *in vitro* models will be individually reviewed.

Animal models of traumatic brain injury. One of the earliest and most widely used approaches to understand TBI is the use of animal models of injury. In an animal model, a defined injury can be administrated after which the cellular and tissue level damage can be examined (Gennarelli et al., 1982; Gennarelli, 1983; Tornheim et al., 1984; Nusholtz and Kaiker, 1986; Huelke and Nusholtz, 1986; Nusholtz and Ward, 1987; Martin et al., 1989). Advantages of using animal models of TBI include the ability to observe the complex interactions of tissue, blood supply, and bone. The effect on the whole animal following injury can be examined. Therapeutic strategies can be tested in the animal to examine their ability to attenuate the effect of injury. Experimental procedures to simulate the TBI in animal models include weight drops, penetrating force applications, and fluid percussions (Martin et al., 1989; Pravdenkova et al., 1996; Yakovlev et al., 1997). Pravdenkova and his coworker induced TBI in rats by dropping a 450-g weight from a height of 2 m on rat skulls (Pravdenkova et al., 1996). Brain cells and DNA were isolated after TBI and apoptosis was quantified. The maximum level of DNA fragmentation was detected at 10 h after TBI and an increase in the activity of the Ca/Mg-dependent endonuclease associated with apoptosis were detected. Conti and his coworker induced TBI in rat by lateral fluid-percussion (Conti et al., 1998). They showed that the apoptosis of injured cells differed by region and cell type. The number of apoptotic cells in the white matter was increased as early as 12 hr after injury and peaked by 1 week. An increase in apoptotic cells was observed in the hippocampus at 48 hr, whereas in the thalamus, the apoptotic response was delayed, peaking at 2 weeks after injury. Golding and coworkers induced TBI in rate by severe controlled cortical impact using cylinder which was 5 m/sec, 130-msec duration, and 3-mm deformation (Golding et al., 1998). They investigated differences in calcium levels associated with injury in their model. Using the monkeys, diffuse axonal injury was investigated by accelerating the head without mechanical impact (Gennarelli 1983). Monkey heads were accelerated without impact in one of three directions which are sagittal head motion, oblique head motion, and full lateral head motion within 10-20msec by a pneumatic shock tester. From the animal model experiments, the effects of

different types of mechanical load to the brain were characterized. However, while these studies were able to capture much of the complexity of interactions between cells and tissues during TBI, this same complexity makes it cumbersome to elucidate molecular level pathways that lead to neurodegeneration. An understanding of molecular level pathways is paramount if molecular level treatments for TBI are to be developed.

Theoretical modelings and mechanical experiments of TBI. We and others hypothesize that it is the level of shear or tensile stress the rate of strain and the extent of strain that occur in CNS tissue during TBI that are the greatest determinants of the cellular and molecular level changes that lead to neurogeneration. To this end, it is important to be able to estimate levels and rates of stress and strain during TBI. *In vivo* measurement of stress and strain during TBI is quite difficult or nearly impossible. However, over the past 25 years, many theoretical models have been developed to predict mechanical forces, stresses, and strain in the brain associated with TBI (Aldman, 1986; Takizawa et al., 1988; Ruan and Prasad, 1995; Margulies et al., 1990; Krabbel and Appel, 1995; Ommaya, 1995; Bradshaw et al., 2001; Bandak et al., 1995; Chang et al., 2000; Kleiven and von Holst, 2002). Theoretical models focused on how mechanical forces were distributed on head and neck, what the level of stress causes the deformation of tissue, and relative stress or strain within different tissues associated with different mechanical impacts. In mechanical models, brain tissues were considered as elastic materials (Ommaya, 1995) or viscoelastic materials (Lindgren, 1966). In studies of brain tissue, in order to obtain theoretical values readily, assumptions concerning mechanical properties were made, such as incompressibility, homogeneity, and linearity. To estimate mechanical damages of a skull, dummy models were frequently used for obtaining the distribution of the strain and stress on the skull during the impact (Takizawa et al., 1988; Ruan and Prasad, 1995; Bradshaw et al., 2001). In some experiments, the dummy head was filled with materials such as silicon that mimicked the viscoelastic properties of brain tissue (Bradshaw et al., 2001). Two and three-dimensional finite element approaches were also used in modeling of TBI (Takizawa et

al., 1988; Margulies et al., 1990; Krabbel and Appel, 1995; Chang et al., 2000; Pintar et al., 2001; Kleiven , 2002). Impact conditions of TBI were characterized as strain is more than 5 % and duration of strain is more than 8 ms (Margulies et al., 1990; Chu et al., 1994; Ueno et al., 1995; Bradshaw et al., 2001). The tolerance of diffuse axonal injury was characterized by using a mechanical model (Margulies et al., 1990). The threshold of diffuse axonal injury (DAI) which was empirically derived value for a critical shear strain associated with severe diffuse axonal injury in primates was 0.1 in strain. From theoretical models and mechanical experiments, effects of blood vessel rupture, the relationship between head and neck, the range and the duration of strain were characterized. Variables of TBI obtained from the theoretical models and mechanical experiments were applied to the *in vitro* and animal experiments.

***In vitro* models of traumatic brain injury.** Even though the values of stress and strain during TBI were precisely predicted from the mechanical and mathematical models, the cellular changes by mechanical impact are still not well characterized. In order to investigate the cellular response to TBI, a number of *in vitro* models of TBI have been developed. Because neurons are pivotal building block of brain, most models have focused on examining the response of neurons to specific mechanical insults. Primary neuron culture and neuron-like cell lines were used *in vitro* models of TBI. Primary neuron cultures can only be obtained in limited supply from neonatal animals. Cells are easily injured and loose their connections to each other during dissection and harvesting. To avoid some of these complications, a number of immortalized cell lines are also used (Triyoso and Good, 1999). To examine how other cells react to mechanical insult in brain, endothelial cells and astrocytes have also been used (Laplaca et al., 1997). The advantages of *in vitro* models include: precise control of strain and stress, homogeneity of cell culture, ease in characterization of the specific cellular and biochemical process; and reproducibility of results.

Stress and strain were generated in *in vitro* TBI models using following methods; axon transaction, fluid hydrodynamic shear, and biaxial strain applied to attached cells

via deformation of growth matrix. The first *in vitro* model of TBI was the transectional damage of axon of neuron to investigate the TBI (Ramon y Cajal, 1928). The most universal *in vitro* TBI models employ hydrodynamic shear stress application and stretching cells upon a membrane (LaPlaca and Thibault, 1997; Triyoso and Good, 1999). In hydrodynamiac shearing, cells were grown on a plate and shear stress was applied using rotational flow generated by a secondplate placed near the cells (LaPlaca et al., 1997; LaPlaca and Thibault, 1997; Triyoso and Good, 1999). The level of strain generated in these *in vitro* models was up to 50% and strain and stress applied to neuron *in vitro* experiments were based on the theoretical models (LaPlaca et al., 1997; Galbraith et al., 1993). Using the shear stress induced cell strain models, neurons responded to high strain rate deformation via an increase in cytosolic calcium and release of lactate dehydrogenase (LaPlaca et al., 1997). These results indicated necrotic cell death occurred in these models (LaPlaca et al., 1997; LaPlaca and Thibault, 1997). In low strain rate hydrodynamic shear models apoptosis pathways were activated. At low strain rate, pulsatile hydrodynamic shear stress led to higher levels of apoptosis than the steady hydrodynamic shear stress (Triyoso and Good, 1999). LDH release from cells was not detected.

As an alternative to hydrodynamic shear stress, tensile stress has been used to deform cells. In these models tissue or cells were grown on an elastic, homogeneous, linear, and isotropic matrix which was stretched in controlled manner (Donna and Geddes, 2001; Craig and Clark, 2001; McKinney et al., 1996). In the squid giant axon, a 20% uniaxial strain caused severe injury of axon (Galbraith et al., 1993). Astrocytes and glial cells were grown on an elastic membrane, and were stretched for 50ms (McKinney et al., 1996).

Astrocytes, glial cells, and endothelial cells were much more resistant to stretch-induced injury at the range of 50 ms and up to 170% of strain than normal state. Ellis and coworkers stretched injured astrocytes, glial cells, and endothelial cells simultaneously to detect the multi cellular damages. Intracellular calcium level was increased and glutamate concentration was sharply increased after injury. Released glutamate from the astrocyte and glial cells could activate calcium transport via NMDA receptors. mRNA expression from cells injured in a stretching device was characterized (Marciano et al., 2001). From the DNA micro array analysis of injured nerve cells, apoptotic related genes were detected.

Some investigators have used hydrostatic pressure as a means to injure neurons as an *in vitro* model of TBI. The hydrostatic pressure experiment was applied to neurons to elucidate the effect of compression at 15 atm for 10 min (Murphy and Horrocks, 1993). Results from these types of models have been largely inconclusive.

In vivo, cells undergo compression, shear stress and tensile stress during TBI. Therefore, all of the types of *in vitro* models developed could contribute to the understanding of cell response to traumatic injury. In addition, while neurons are the component of the central nervous system that receive the most attention due to their central function, many different cell types (astrocytes, glia, and endothelial cells, for example) are present in the CNS and could contribute to neurodegeneration observed after TBI.

Neuronal Cell Death

General review of neuronal cell death in neurobiochemistry and neurophysiology. The nervous system is one of most controlled and integrated systems in higher organisms. The central nervous system (CNS) is the pivotal center of entire nerve system. The key structure of the CNS is the neuron. The role of the neuron is the conduction of nerve impulses from one part of body to another. A neuron consists of a cell body, an axon, and dendrites (Figure 1-1). The cell body, also called the soma or preikaryon, contains the nucleus and is surrounded by granular cytoplasm. Dendrites are usually highly branched structures extending from the cytoplasm. Neurons normally have multiple dendrites. The axon is usually a single long and thin structure which plays a role transmitting the nerve impulse.

During TBI, neurons undergo cell death via two modes; necrosis and apoptosis. Necrosis is characterized as an abnormal cell death pathway that does not exist in normal neurophysiology. Necrosis can be triggered by extreme condition such as mechanical breakdown of cellular structures, high concentrations of glutamate, and an inflammatory response by macrophage and microglia. Necrosis can not be controlled. Apoptosis, in contrast, is a normal event during neuronal development. Apoptosis can be detected during the process of neuron differentiation. Apoptosis can be activated by specific pathways and involved a known set of proteins, caspases, that carry out the controlled dismantling of the cellIn TBI, apoptosis is considered to be the main neuronal cell death pathway.

In general, there are important chemicals associated with neurodegeneration during TBI. Those chemicals are calcium, glutamate, nitric oxide, G proteins, and phospholipase C. Calcium, the most abundant ion in the body, is an extracellular electrolyte. Calcium is not only a structural component of bones, and teeth, but also is involved in blood coagulation, excitability of nerve tissue, and neurotransmitter release. An action potential in a neuron typically leads to the opening of voltage gated calcium channels in the neuron, causing a calcium influx into the cell. The increased localized

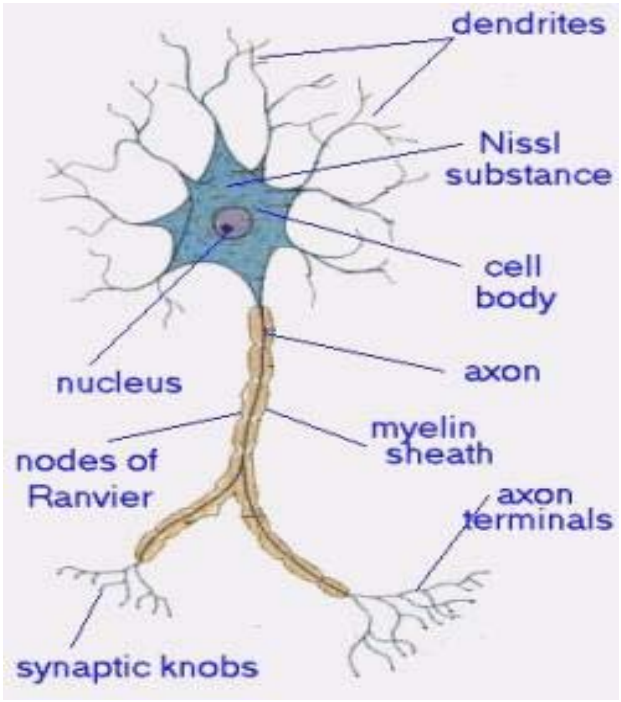

Figure 1-1. Structure of neuron.

calcium concentration triggers neurotransmitter release from the nerve terminal, either signaling another neuron or prompting some muscle action at the neuromuscular junction. On the other hand, in nerve injury, elevated intracellular calcium level is considered a key factor in cell death. Excessive intracellular calcium leads to inappropriate activation of a number of proteins, and typically leads to cell death via apoptosis.

Glutamate is one of the essential amino acids. In the central nervous system, glutamate is an excitatory neurotransmitter (it triggers the excitation of other neurons). Glutamate release from the nerve terminal (into the synapse) is triggered by intracellular calcium. Glutamate can also lead to the entry of calcium into the cell through voltage gated glutamate channels. When glutamate binds to particular ion channels (the NMDA channel, for example), calcium can enter into the cell. In neurons, excessive glutamate levels tends to lead to excitotoxicity (or cell death from over excitation of the cell). During TBI, glutamate can be released from the blood, astrocytes, other glial cells, and neurons by mechanical damage.

Another pathway of intracellular calcium increase is via G protein activation. General role of G proteins in nervous system is as a second messenger. G proteins carry signals from transmembrane receptors to intracellular effectors. One intracellular effector is phospholipase C, which when activated, can cause the release of calcium from intracellular stores. G protein signaling pathway is important in the mechanism of neuron apoptosis associated with low strain rate nerve injury. (you need references)

Nitric oxide (NO) and free radicals are considered to be neurotoxic chemicals. The role of nitric oxide in the nervous system is as a retrograde neurotransmitter, which is believed to be important in learnng and memory. However, the role of NO in the brain can change surprisingly under TBI condition. During TBI, the concentration of NO is 100 times higher than that observed under normal conditions. It reacts with the superoxide anion to form peroynitrite, which can damage cellular proteins. NO can cause energy failure and DNA damage by blocking ATP production and impairing

DNA replication (Iadecola et al., 1996). Overall apoptotic pathway of TBI are illustrated in Figure 1-2.

General neuronal cell death pathways. Necrosis: Cell death by necrosis is the abnormal pathway of cell death. It is not the normal developmental pathway of cell death in neuron. Morphology of necrosis is associated with breakdown of cell membrane and endoplasmic reticulum, cell swelling, and cell lysis (Steller, 1995; Huang and Tatro, 2002; Meloni et al., 2002). Mainly, necrosis can be caused by uncontrollable cellular reasons. Also, necrotic cell death results in an inflammatory response of microglia and macrophage. Biochemical characteristics of necrosis are rapid ATP depletion and structural change of mitochondria (Mattson et al., 1993; Steller, 1995). Mechanical damages of sub-cellular structure lead to necrotic cell death pathway. In the area of ischaemia and highly concentrated glutamate released from damaged cells, necrosis occurred frequently by extremely elevated intracellular calcium (Huang and Tatro, 2002; Berti et al., 2002; Meloni et al., 2002). Highly toxic level of glutamate was released from the neuron by TBI (Choi, 1994; Dugan and Choi, 1994). Glutamate can bind to NMDA and AMPA channels to increase the intracellular calcium level which causes the additional damages. A huge increase of calcium leads neuron to necrosis but mild increase of calcium leads to apoptosis (Ankarcrona et al., 1995). A huge increase of calcium induces the endonuclease, calpain, protease, and the production of free radical. At the period of calcium increase, mitochondria acts buffer storage of calcium. It blocks the oxidative phosphorylation and leads to release of free radicals like nitric oxide and peroxynitrite which also cause the increase of intracellular calcium. In mitochondria, condensation of the inner membrane and dissipation of metrical granules occurs during the necrosis (Trump et al., 1965). As for the appearances between necrosis and apoptosis, necrosis begins with mitochondria swelling and chromatin clusterin, while apoptosis begins with cytoplasmic condensation and separation of apoptotic bodies. The characteristics of necrosis and rate of necrosis rely upon the initial intensity of injury (Ito et al., 1975).

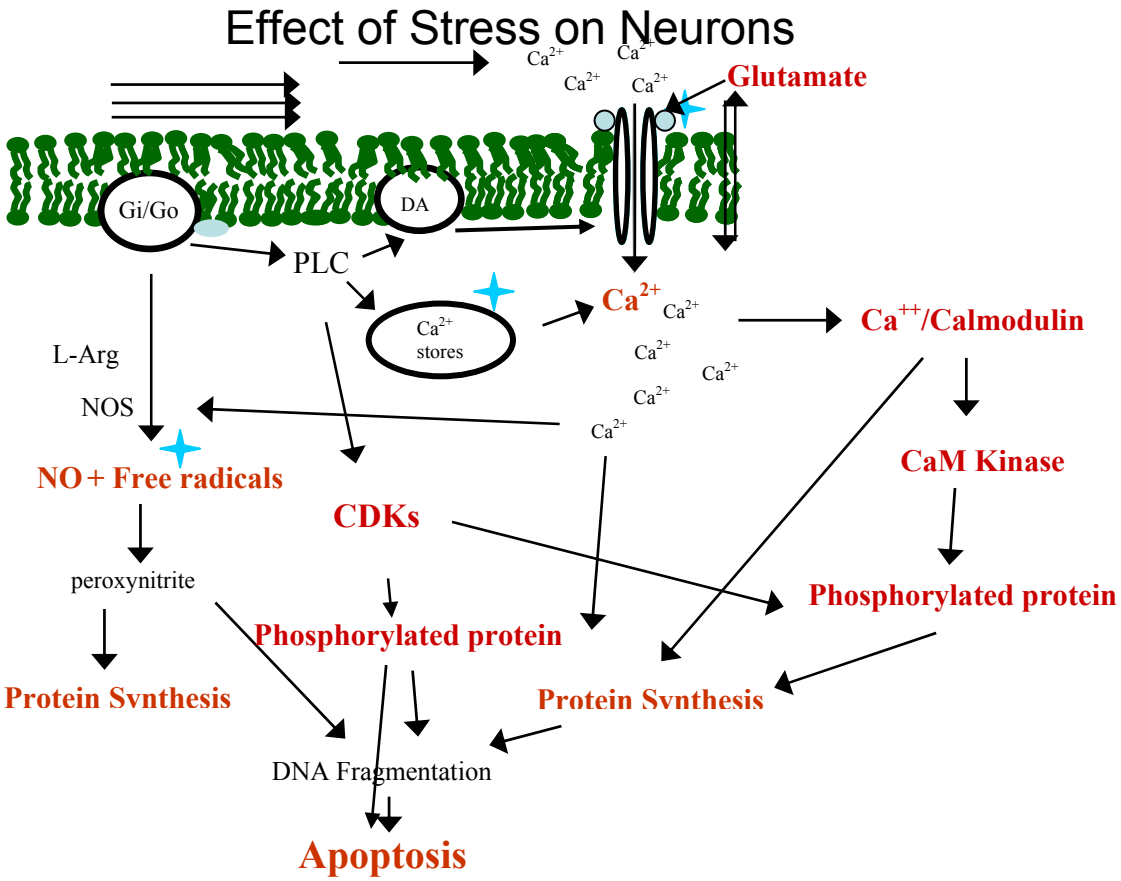

Figure 1-2. Overall process of apoptosis in a neuron as a result of mechanical insult.

Apoptosis: The meaning of apoptosis in Greek is falling down. Apoptosis is essential for normal tissue development and homeostasis, and it contributes to certain forms of neuropathological pathway. Damaged neuronal cells or not well differentiated neuronal cells could be eliminated via apoptotic pathway (Steller, 1995; Simonian et al., 1996). The morphology between apoptosis and necrosis is totally different. The morphological changes of apoptosis are chromatin condensation, cytoplasmic shrinkage, and plasma membrane blebbing (Steller, 1995). Apoptosis can be initiated in different pathways, but most common pathway is caspases related pathways. Apoptosis was first found in *C. elegans.* In development of neuron, neurons which cannot reach their target would not obtain the neurotrophic factor from the target and hence those neurons undergo apoptosis. *In vitro* neuronal culture, apoptosis can be initiated by the removal of nerve growth factors mimicking the developmental effect of neuron. In neuron development, the features of neuronal apoptosis are that apoptosis occurs only early connection period of neuronal development and it depends on the connection between neuron and target cell. The triggers of apoptosis in brain damage or TBI are broad. There are four major molecules involved in apoptotic process. These are the caspases, the adaptors, members of the tumor necrosis factor (TNF) receptor (TNF-R), and Bcl-2 proteins.

A group of cysteine proteases, so called caspases, is essential for apoptosis (Zhao et al., 1998; Woo et al., 1998). Caspases can be detected in all cells undergoing apoptosis. As a consequence, for these mice lacking caspase-3 or caspase-9 have increased numbers of neurons in the brain so caspase-3 and capases-9 are related to neuronal developmental apoptosis. A potent inhibitor of all known caspases, p35, can hamper the apoptosis in various call line (Sugimoto et al., 1994; Hay et al., 1994; Beidler et al., 1995; Datta et al., 1997). Caspases play a role in regulators of cell death initiation and degradation of cellular substrates during the end of apoptosis. At least 14 caspases have been identified in mammals (Alnemri et al 1996; Zhao et al., 1998). Caspases are synthesized as zymogens, which have very low intrinsic enzymatic activity. From the so-called initiator caspases (e.g. caspase-8 and caspase-9) start a sudden large amount of

increasing caspase activity by processing and activating so-called effector caspases (Woo et al., 1998). Some caspases, particularly effector caspases, cleave and inactivate certain vital cellular proteins, such as DNA repair enzymes, lamin, gelsolin, MDM2 (an inhibitor of p53), and protein kinase C (Thornberry and Lazebnik, 1998; Adams and Cory, 1998). There are also enzymes that can be activated directly or indirectly by caspase-mediated proteolysis. Caspase-3 can remove a negative regulatory domain from the kinase p21-activated protein kinase 2, which is deemed to trigger plasma membrane blebbing (Rudel and Bokock, 1997). The capases-activated Dnase (CAD) is normally inactivated by binding to an inhibitor, iCAD. During apoptosis, iCAD is cleaved by caspases, and this leads to release of the active endonuclease which generates the characteristic internucleosomal DNA cleavage.

Many proteins found as structural similarity with Bcl-2 which is the prototypical inhibitor of apoptosis (Adams and Cory, 1998). The Bcl-2 family consists of anti-apoptotic gene family (bcl-2, bcl-x_L bcl-w) and pro-apoptotic gene family (bax, bid, bad, bak) (Kozopas et al., 1993; Gibson et al., 1996; Lin et al., 1996). These proteins can form homodimer and heterodimer interacting with each other. Among these, the well studied protein is Bcl-2. Bcl-2 is an intracellular protein located in mitochondrial membrane and it can prevent from the apoptosis by reinstating calcium level inside of mitochondria (Chao et al., 1995). Also, Bcl-2 can inhibit the thapsigargin induced calcium level from endoplasmic reticulum (Kozopas et al., 1993). Bcl-xL is another protein which has anti-apoptotic activity. Bcl-xL can inhibit the release of cytochrom c from mitochondria, and caspases 9, activator of apoptotic cascade, can be suppressed (Kozopas et al., 1993; Lin et al., 1996). As described above, the apoptosis pathway is regulated by the caspases enzymes. Inhibition of caspases enzyme and their gene expression can protect neuron from the apoptosis (Fink et al., 1999; Piccioli et al., 2001).

TNF receptors can activate proliferation, differentiation, and cell death (Ashkenazi and Dixit, 1998; Nagata, 1997; Wallach et al., 1999). TNF receptors are activated by a group of structurally related ligands that belong to the TNF ligand family.

Members of the TNF-R family can also activate the transcriptional regulators NF-κB and Jun kinase.

In the dynamic of cell death, apoptosis varies *in vivo* and *in vitro*. In trophic factor deprivation of PC12 cell, total apoptotic events took over 1-4 days but apoptotic characteristics were detected within 6 hrs (Oberhammer et al., 1991). In case of *in vivo* axotomy and target deprivation experiments, apoptosis occurs over 7 days but within 6 hrs, apoptosis was detected morphologically.

Apoptosis and necrosis are the traditional style of cell death. Necrotic cell death pathway is quite difficult to be controlled and inhibited. However, apoptotic cell death pathway can be controlled and be inhibited by blocking the activator protein of apoptosis. Early expressed apoptotic genes can be beneficial for finding out the therapeutics and procedures of apoptosis related diseases.

The Causes of Apoptosis in TBI

Axonal injury (axotomy). The rapid mechanical displacement during TBI sheared axons at the time of injury causes them to retract and to expel a ball of axoplasm, forming a reactive axonal swelling or retraction ball, the traditional histological feature of diffuse axonal injury (Povlishock, 1992). In axonal injuries induced by TBI, the degeneration of axon is not immediate but is a delayed process for four to 24 hours depending upon the severity of injury known as secondary axotomy (Maxwell et al., 1997). In those axotomically damaged neuron, protein synthesis occurs for axonal regeneration (Fletcher and Xue, 2000; Lund et al., 2002). Transcription factors (c-jun and jun D), growth factors and receptors, cytoskeletal proteins, and cytokines are widely expressed (Maxwell et al., 1997; Castagne and Clarke, 1999; Fletcher and Xue, 2000; Castagne et al., 2001; Lund et al., 2002). Also, damages of axons can cause not only axon degeneration but also the neuronal death, which may occur via apoptosis or necrosis. Necrosis of neuron occurs when damaged axons are very close to neurons (Martin et al., 1998). In retinal ganglion cells, apoptosis occurred after axotomy but over expression of Bcl-2 inhibited apoptosis (Bonfanti et al., 1996). Some of retinal

ganglion cells died by necrosis after axotomy (Cui and Harvey, 1995). However, most of axonaly injured neuron undergo apoptosis (Povlishock et al., 1992; Lund et al., 2002). Because neurons are in axotomy states, they cannot reinstate connections with their targets. These chromatolytic neurons enter into apoptosis. Glutamate receptors and excitotoxic mechanisms are also associated with axotomized neurons (Lieberman et al., 1971; Greensmith et al., 1994; Iwasaki et al., 1995). However, it is still indistinct how axotomy causes the neuronal death. It is an important area to research for axon regeneration.

To inhibit apoptosis by axotomy and axon regeneration is one of the key factors to overcome the axonal injuries in TBI. The pathway of apoptosis induced by axotomy is similar to the pathway of apoptosis induced by damaged cell (intracellular calcium increase, caspases, free radical, glutamate). There are two possible stratagems to overcome the axotomical deaths. To inhibit the axotomical cell death requires to block the apoptosis causes. However, even though neuron was saved by the apoptosis, the connection between the neuron should be recovered by regeneration of axon and neurons (Van Ooyen and Willshaw, 1999). Recently, one report suggested that neurite outgrowth induced by bFGF, LN, or N-cadherin was strongly inhibited by a treatment with inhibitors of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), activation in embryonic chick retinal neurons (Perron and Bixby, 1999). Also, an electrical activity is important factor of neuron regeneration. Not only do neurotrophic factors need to signal neurons but neurons should be reactive to these factors by generating electrical activity of axons to elongate axons (Shen et al., 1999). Recent report suggested that to potentiate synaptic transmission by neurotrophins , presynaptic depolarization should be controlled by presynaptic cAMP levels (Boulanger and Poo, 1999).

The failure of axon regeneration could cause a malfunction of brain. Axon degradation and regeneration need precisely be controlled. To find out the therapeutic method of TBI, axotomy should be combined with the injury of cell itself.

Calcium and glutamate. Roles of calcium in nerve cells are broad and calcium is a ubiquitous intracellular signaling molecule (Sattler and Tymianski, 2000). Examples of calcium as signaling molecules are for cell growth, differentiation, exocytosis, and synaptic activity. Due to the great importance of calcium, levels of calcium are precisely controlled. Normally, the intracellular calcium concentration is about 100 nM, or 10 ~ 4 times lower than extracellular cellular calcium (Sattler and Tymianski, 2000). The excessive import of cellular calcium has been suggested to be the pathway of cell death (Choi et al., 1994; Schanne et al., 1994). If intracellular calcium level increases massively, it causes the breakdown of the plasma membrane that occurs early in necrosis. Moderate increase of intracellular calcium may mediate apoptosis. The concentration of intracellular calcium is regulated in the 50-300nM range in normal neuron. The elevated intracellular calcium level in TBI can be elevated mainly by several sources which are physical damage of cell membrane, receptor mediated glutamate toxicity, import from the voltage calcium channel, and release from the endoplasmic reticulum through phosphoinositol (Figure 1-3). Extracellular calcium levels can be increased by release of calcium from adjacent damaged cells in TBI. Through damaged cell membrane, calcium can be entered cytosol. Mostly calcium increase by damaged cell membrane causes necrosis because the membrane breakdown causes the abrupt and extreme increase of cytosolic calcium level (Steller, 1995; Huang and Tatro, 2002). Also, the concentration of intracellular calcium level can be increased by an activated NMDA receptor that was in turn activated by glutamate which was released from the damaged astrocyte or glia. About 100 μm of glutamate can cause to reach the highest calcium level that leads to necrosis in neuron and glia *in vitro* but lower than 100μm of glutamate level can cause apoptosis (Nagata, 1997). Glutamate is an essential amino acid that is the major excitatory neurotransmitter in the mammalian nervous system by increasing the intracellular calcium level. It is present at millimolar concentrations in mammalian nerve system and has an excitatory effect on the central nervous system (Vexler and Ferriero, 2001). Postsynaptic effects of glutamate are mediated by a number of cell-membrane

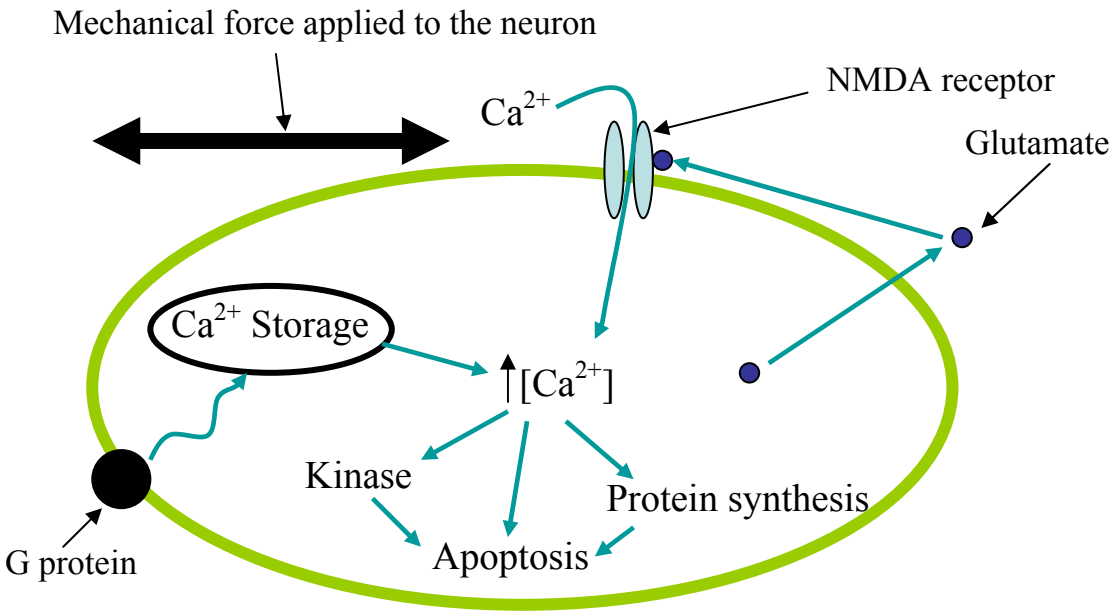

Figure 1-3. Proposed mechanism of neurodegeneration by intracellular calcium increase.

receptors (*N*-methyl-D-aspartate (NMDA), kainate, and 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate receptor). In TBI cases, intracelluar calcium increase through the NMDA receptor by glutamate has been considered as the main pathway of calcium increase (Schanne et al., 1979; Lobner and Lipton, 1993). Opening of NMDA receptor channels require glutamate and depolarization of the post synaptic neuron. NMDA receptor antagonists have placated the TBI (Graham et al., 1993; Vexler and Ferriero, 2001).

Activation of G-protein by other signals may also lead to increase of cytosolic calcium level. Activated G-protein activates phospholipase C. Phosphoilpase C produces the IP_3 from PIP_2. Released IP_3 leads release of calcium from the endoplasmic reticulum (Laplaca and Thibault, 1997). Voltage gated calcium transport is another source of intracellular calcium increase. Peripheral injections of kainate can cause degeneration of hippocampal cells (Mattson,et al., 1993, Simonian et al., 1996).

When the calcium level increases in cytoplasm, one of intracelluar calcium regulation mechanism is mitochondrial calcium buffering. Mitochondria uptake calcium to regulate the cytosolic calcium level. At a high intracellular calcium level, mitochondrial membranes would be dysfunction with swelling and would release the cytochrome c. Released cytochrom c activates Apaf-1 that in turn activates caspases. Bcl-2 family proteins, including bax, bid and bcl-xL, regulate the release of cytochrome c and other apoptogenic factors from mitochondria. Cytochrome c interacts with Apaf-1 and caspase-9 to form an apoptosome. Apoptosome can activate caspase-3. Caspases can also be activated by cell surface tumor necrosis factor (TNF) receptors via caspase-8. Also, an elevated calcium level may play a role in activation of protease, generation of free radical, and activation of new protein synthesis.

From the description given above, calcium is a vital cellular molecule in TBI. Consequently, pharmacological inhibition and restoration of increased intracellular calcium level are the primary strategy of neuroprotection in TBI. The stratagems of regulating the calcium rise in cytosol are blocking of calcium import, stimulating the calcium export, and holding the calcium by tight binder. In TBI, NMDA channels and

voltage gated calcium channels are the main ways of calcium import (Faden et al., 1989; McBurney et al., 1997; Leski et al., 1999). By blocking these channels, the apoptotic pathway can be inhibited. Calcium channel blocking agents are listed in Table 1-1.

Nitric oxide and free radicals. Nitric oxide (NO) is a free radical gas which can move through the cell membrane by diffusion. It has a short life time and unstable structure. Nitric oxide (NO) plays a key role in the pathogenesis of TBI (Beckman, and Koppenol, 1996; Triyoso and Good, 1999; Boucher et al., 1999). Nitric oxide can be synthesized by NO synthase with several calcium sources. NO synthase controlled by calmodulin is activated by an elevated intracellular calcium via NMDA channels, voltage gated channels, and release from the endoplasmic reticulum activate the neurons (Boucher et al., 1999, Fleming and Busse, 1999). Also, NO can be synthesized in astrocytes and microglia by activating the NO synthase. In TBI condition, high levels of NO react with superoxide radical (O_2^-), that forms peroxynitrite ($ONOO^-$). Peroxynitrite ($ONOO^-$) is highly reactive molecule which has a long half life comparing to NO. Peroxynitrite ($ONOO^-$) is highly reactive to OH^- radical. It travels through cell membrane and reacts with protein, membrane lipids, and nucleic acid to make oxidative forms. At low level of NO, mitochondria is the key target of cellular damage (Pellegrini-Giampietro et al., 1988; de Haan et al., 1992; Zhang et al., 2002). Nitric oxide can react with cytochrom c to inhibit the ATP production. A low level of ATP leads depolarization that slack calcium entry through the voltage gated calcium channels. It can lead cells to apoptosis.

The definition of free radical is that molecules have unpaired electrons thus it has highly reactive characteristics. Most common free radicals in TBI are superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). Superoxide can be transformed to hydrogen peroxide in aqueous solution. It reacts with metal ions and converts into hydroxyl radical. The binding between metal bound to protein and superoxide causes the protein degradation. Hydroxyl radicals react with ribose phosphate and pyrimidine

Table 1-1. Inhibitors of apoptosis.

Steps in cellular mechanism	Inhibitor	Change expected in subsequent steps
Ca^{2+} entry into cell	EGTA (Non specific calcium channel inhibitor) α-Conotoxin (voltage gated calcium channel)	G protein activity PLC activity Phosphorylation Protein synthesis Apoptosis Glutamate release
NMDA receptor	Memantine D-(-)-2-amino-5-phophonovaleric acid (AP-5) MK801	
PLC activity	Neomycin U71822	Phosphorylation Protein synthesis Apoptosis Ca^{2+} release from stores
Protein synthesis	Cycloheximide	Apoptosis Protein synthesis

residue of DNA to break and modify DNA strand and base (Wilson and Gelb, 2002). Also, it peroxidates the membrane lipids (Maas, 2001; Wilson and Gelb, 2002). Free radical can cause necrosis and apoptosis. Necrosis results from the breakdown of cell membrane lipids and proteins by free radicals. Apoptosis can be caused by NO that induces the oxidative stress to cells.

Oxidative damages in TBI are mainly caused by free radicals. To reduce oxidative stresses in TBI, free radical production should be reduced and scavenging free radicals should be activated. Scavenging agents for free radical reduction are ascorbic acid (vitamin C), α-tocopherol (vitamin E), and Glutathione. Ascorbic acid and α-tocopherol are general free radical scavengers. Glutathione can actively scavenge the singlet oxygens and singlet hydroxyl radicals, and peroxidates H_2O_2. Also, the act of inhibiting of NO synthase and scavenging NO can reduce further damages of brain (Fiebich et al., 1998; Spinnewyn et al., 1999). Activation and injection of superoxide dismutase to TBI may reduce superoxide levels (Spinnewyn et al., 1999). In Parkinson's disease, the transinfection of virus which posseses superoxide dismuatse expression system reduced the free radicals in brain (Faden et al., 1989; Barkats et al., 2002).

Possible connection of apoptosis during TBI with growth factor, heat shock proteins, DNA repair enzyme, and calpains to apoptosis. Neurotrophic factors have been reported to manage the neuronal cell death and survival (Gustafson et al., 1999; Vexler and Ferriero, 2001). In TBI, especially hypoxia and ischemia, BDNF, NGF, basic fibroblast growth factor, growth hormones are reported to have neuroprotective ability (Kirschner et al., 1996; Gustafson et al., 1999; Han et al., 2000; Vexler and Ferriero, 2001). *In vitro* studies demonstrated that the survival effects of neurotrophic factors can be mediated via activation of the phosphatidylinositol 3-kinase (PI3-kinase) pathway in granule neurons (Kirschner et al., 1996). Neuroprotection by neurotrophic factors could also be mediated via the extracellular signal-related protein kinase (ERK) pathway and activation of the phosphatidylinositol 3-kinase pathway by BDNF showed cell survival (Han et al., 2000). Heat shock protein, hemeoxydase, is limiting factor of

degradation of hemoproteins such as NOS, peroxidase, catalase and cytochrome P450 and has two isoforms, H)-1 ans HO-2 (Ferriero et al., 1990). HO-1 is related to NF-κB regulation and AP-1. High levels of HO-1 expression in TBI is considered as neuroprotection (Ferriero et al., 1990). PARP is a ubiquitous protein in repair of DNA damage. Over expression of PARP caused apoptosis in neuron and inhibition of PARP suppressed the neuronal death (Dirnagl, 1999). Calpains are calcium inducing protease. Elevated intracellular calcium level may activate the calpains and cause apoptosis. Inhibition of calpains by calpastatins that are endogenous an inhibitor of caplain showed decrease of neuronal cell death (Blomgren et al., 1999).

New protein expression and phosphorylation during TBI. Relatively, few proteins play roles in the form of primary translation product in function. Most of them are modified in one or several ways. The examples of protein modification are phosphorylation, glycosylation, fatty acid acetylation and so on. Such modifications have been shown to be essential for particular aspects of protein function. Phosphorylation is one of protein modification. In neuronal apoptosis, phosphorylation of specific protein is also considered as the one of may play a role in apoptosis associated with changes in gene expression and membrane receptor activities resulting the apoptosis and apoptosis related protein expression. One of examples of apoptotic phosphorylation is G protein activation (Laplaca and Thibault, 1997; Triyoso and Good, 1999). That is activation of specific pathway by phosphorylation the specific protein. Phosphorylation of G protein can elevate intracellular calcium via activating phospholipase C. It is considered one of sources of intracellular calcium activator in neurodegeneration. Other examples of phosphorylations of proteins are p38 MAP kinae and c-Jun N terminal kinase (Kawasaki et al., 1997; Schwarzschild et al., 1997; Yang et al., 1997). Kawasaki and coworkers found that glutamate stimulated activation of p38 and stress-activated protein kinase (SAPK, also known as c-Jun N-terminal kinase (JNK)), and the mitogen-activated protein kinase. It could activate transcription of apoptosis related genes via enhancing the transcriptional associated factor

tethering(Mattson et al., 2003). The phosphorylation of cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase whose activity is associated with postmitotic neurons (Waetzig and Herdegen, 2002). Cdk5 normally plays a role in cell cycle regulation. Waetzig and coworker showed that the deregulation of Cdk5 activity either through generating a more stable proteolytic product of p35, or by stabilizing Cdk5/p35 complex is also associated with phosphorylation of tau, a major component of neurofibrillary tangles associated with neurodegeneration. However, it is not clear that what gene's transcription is activated by what protein's phosphorylation.

The study of protein expression during TBI can give us the useful insights of possible therapeutics and treatment. Using the gene chip, Feuerstein and coworkers categorized genes after cerebral ischemia and trauma. It showed that first waves, c-fos, c-jun, and jun B, are activated within minutes of nerve injury. Hsp70 and hsp72 which are heat shock proteins were expressed as second wave within two hours. TNF-a, IL-1, Il-6, P-selec, ICAM-1, and growth factors were expressed as third wave. Forth wave is the genes related to the inflammation. Last fifth wave was genes which are associated with secondary injury and repair process of brain injury. Mattzilevich and coworker also categorized the gene expression after TBI using rat cortical impact injury. Within 3 hours after TBI, cyclin B, cyclin, GADD45, Cu Zn SOD, and neutrophil related genes were expressed. At 24 hour time point, most of gene expressions were suppressed except for cdc-2 and cyclin-B-kinase. However, apoptotic genes were not detected because whole hippocampal neurons were used for DNA micro array. From the analysis of DNA micro array, it was quite difficult to ascertain that apoptotic genes were expressed after TBI. However, the involvements of apoptotic genes and proteins in TBI have been characterized by other researchers by Northern blot (Mikawa et al., 1995; Raghupatti et al., 1996). The role of cyclin dependent kinase in TBI were considered as they delay the cell cycle to repair damages to injury or the signal of cell cycle may contribute the apoptosis (Dash et al., 2001, Park et al., 2000). Nitric oxide related genes and Cu Zn suoeroxide dismutase (SOD) genes were also expressed. Mikawa and coworkers showed that c- Jun mRNA expression was higher in an injured transgeneic

mouse which expresses Cn Zn SOD than a normal injured mouse. Lindsberg and Goodman found that heat shock proteins and cytokines were highly expressed in injured brain over 24 hours after TBI (Lindsberg et al., 1996; Goodman et al., 1990). Heat shock protein may stabilize the injured cellular structures and cytokines may be associated with inflammation response and neurotrophin release on injury. Neurotropic factors such as NGF, bFGF, and IGF were expressed in the beginning of TBI (Hashimoto et al., 1993). The release of neurotropic factors from injured neurons could reconnect the damaged synaptic connections.

As we have discussed now, many genes were involved in TBI. However, actual proteomic study has not performed until now. Though the level of mRNA expression of certain gene could be associated with the protein expression of that, there is no guarantee that mRNA expression coincide with protein expression. For example, if certain protein could be ubiqitinated and degraded by proteases, it is difficult to identify protein integrity with mRNA level. Thus, proteomic study in diseases should have been developed. The meaning of proteome is the entire protein complement in cell, or tissue. In general, proteomics research include protein activities, modifications and localization, and interactions of proteins in complexes. By studying global patterns of protein using 2 dimensional electrophoresis, it can enhance understanding of biochemical and pathological properties of TBI. In clinical and pharmacological research, proteomics can give rise to develop many possible therapeutic of TBI. Also, it can be used as one of diagnosing tool of diseases.

SH-SY5Y neuroblastoma cell line. In order to study the biochemical and pathological condition of TBI, numerous *in vitro* models have been developed and primary cell cultures and immortalized cell lines were used for their analyses. Due to the possible damage during the harvesting the primary cell cultures from the animals, a number of researchers have used immortalized cell line in neuron research.

SH-SY5Y cell were used in our research. It has a number of advantages in neuronal research. Here are: it was subcultured from human neuron; it is differentiated

by NGF-β, retinoic acid, and phorbolester 12-O-tetradecanoyl phorbol-13-acetate (TPA) easily ; differentiated SH-SY5Y cells secrete neurotransmitter; it has homogeneous cell population, biochemical property of differentiated SH-SY5Y cell is close to neuron in brain. The morphology and characteristic of SK-N-SH cell lines have been characterized (Biedler et al., 1973; Biedler et al., 1978). SH-SY5Y cells are the neurblastoma cell lines which were subcloned from SK-N-SH cell line. The doubling time of SH-SY5Y cell line is 44 hours. Differentiated SH-SY5Y cells by NGF-β show the neurite growth and cell volume increase which comes from the increased metabolism of neurotransmitter. Differentiated SH-SY5Y cells by retinoic acid show relatively long neurite. Within 30 minutes, SH-SY5Y cells show action potential (Kuramoto et al., 1981). Differentiated SH-SY5Y expresses epinephrine, dopamine-β-hydroxylase, and acetylcholinasterase which are associated with production of dopamine, acetylcholine, and gamma amino butyric acid (GABA). SH-SY5Y cells have ion channels and receptors which are ligand-gated channel, metabotropic glutamate receptor, receptors of insulin growth factor I and II etc. The calcium channels of SH-SY5Y are AMPA, kainate, G protein coupled calcium channel (not membrane calcium channel), and NMDA receptor coupled calcium channel (Naarala et al., 1990). They are featured calcium channels which are found in brain neuron. It also have been used extensively in neuronal research (Maruyama et al., 1997; Triyoso and Good, 1999; Button et al., 2003; Tang et al., 2003)

Conclusion

Numerous researches have been done in TBI. From animal models and *in vitro* models of TBI, it have been characterized that neuronal cell deaths in TBI are consequences of apoptosis and necrosis. Apoptosis and necrosis exhibit totally different cell death morphology. However, main causes of apoptosis and necrosis are connected. In TBI, apoptosis is considered pivotal pathway of neuronal death and biochemically preventable pathway. Apoptosis in neuronal death of TBI is not identical to natural apoptosis in neuron. Apoptosis can be caused by various conditions in TBI. The major pathway of apoptosis in TBI is illustrated in Figure 1-2. The prevention of apoptosis is the key component of TBI prevention, but apoptosis can be caused by various sources and apoptotic pathways also vary. Therefore, biochemical properties of cellular level of neuronal death should be analyzed and combined to find therapeutic methods of neuronal death and to block the further damages of TBI.

CHAPTER II

DEVELOPMENT OF A DEVICE FOR HIGH STRAIN RATE INJURY TO NEURONS AND NEURON-LIKE CELLS

Introduction

Traumatic brain injury results in about 100,000 deaths and 500,000 hospitalizations per year in the United States (CDC, 1999). Traumatic brain injury is caused by the displacement of brain tissue and neurons due to tissue acceleration associated with mechanical impact to the head. Mechanical insult associated with TBI causes changes in cellular events and damages cellular structures (Tornheim et al., 1984; Nusholtz and Ward, 1987; Lee and Haut, 1989). During TBI, brain tissues and cells are exposed to shear and tensile stress, and compression (Ommaya et al., 1995; Pintar et al., 2001). The predicted strain rates associated with TBI range from 1 to 5 s^{-1}, or about 10% strain in 20 to 100 ms (Chu et al., 1994; Ueno et al., 1995; Gennarell, 1996).

In order to study the changes in cells of the central nervous system during TBI at a cellular or molecular level a number of approaches have been taken, including *in vivo* animal models, *in vitro* models using hydrodynamic shear to deform cells, and *in vitro* models which rely on tensile stress applied to cells via their attachments to a growth surface (LaPlaca et al., 1997; Laplaca and Thibault, 1997; Triyoso and Good, 1999). Each of these methods has both advantages and disadvantages. Using hydrodynamic shear to deform neurons can be problematic because of difficulties in achieving strain rates that mimic the *in vivo* injuries (Chu et al., 1994; Ueno et al., 1995). However, hydrodynamic shear may be a more appropriate way to administer low strain rate injury as might occur in glaucoma or carpel tunnel syndrome (Triyoso and Good, 1999). In the case of the *in vivo* animal models, injury is applied by dropping a weight on the skull of the animal. In this model it is quite difficult to measure or to control the applied strain or stress to cells. Thus, a good *in vitro* model, in which cells are strained at an appropriate

rate and duration for TBI, and in which good control of the mechanical insult to cells is attainable, is desired. Currently, a number of *in vitro* models have been developed by several research labs that deliver appropriate magnitude of strain to cells, at a high rate. The *in vitro* model developed by Geddes and coworkers utilizes tensile stress to injure cells, but has a problem of cell spreading on a membrane and relatively long stretching time (from 50ms to 100ms) (Geddes et al., 2001). The uniaxial strain system built by Craig and coworkers also uses long times of strain which exceed those estimated during TBI(Craig and Clark, 2001). The device developed by Morrison et al can stretch cells within 20ms (Morrison et al., 1998b). However, the strain profile in the device is nonlinear, resulting in cells experiencing various levels of injury in the device. An *in vitro* TBI device was developed and validated at TAMU cellular engineering lab. The device applies a stress to cells over a period of 20 to 100ms, with a controllable strain level. The strain produced in the device is uniform and uniaxial.

Materials and Methods

General description of the high strain rate application device. The system is based on the Flex Plate system. The strain field is induced on a membrane by applying a vacuum that stretches the membrane attached on a flexible bottomed plate. Cells attached to the membrane deform with the membrane. The device is composed of four separate parts, a plate loading deck, a solenoid three way valve, a vacuum pump, and a computer (Figure 2-1). The plate loading deck has 6 planar cylinders (28mm diameter each) and one of them has an optical window that allows for viewing cells on the flexible membrane on the microscope. A flexible bottomed plate was used for cell culturing (Flexcell, Mckeesport, PA). It has 6 wells of silicon rubber membranes with an identical diameter of 35 mm. Each well of the flexible bottomed plate is centered at the

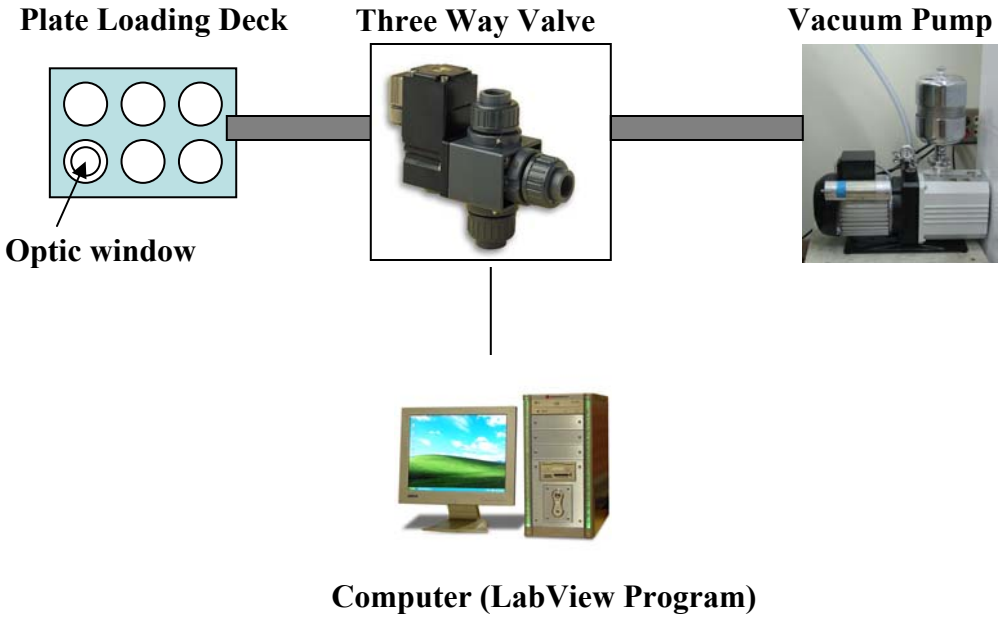

Figure 2-1. Simplified experimental set up of a high strain rate application device.

center of a planar cylinder. The solenoid three way valve (SV-900, Omega, Stamford, CT) is connected to the plate loading deck and the vacuum pump through tubings. The solenoid three way valve is controlled by the computer using LabView (National Instruments, Austin, TX). The time needed to open the solenoid three way valve is from 10 to 20 ms and the closing time is from 20 to 40ms, according to the manufacturer. In the valve closed position, the pressure between the flexible bottomed plate and the plate loading deck is approximately 1 bar. The volume of air between the flexible bottomed plate and loading deck is estimated to be less than 10 mL. The minimum pressure that vacuum pump (Trivac, Export, PA) can produce as low as $5*10^{-4}$ mbar and the pumping speed is 9.7 m^3*hr^{-1}, as stated in the manufacturer's specifications When the valve opens, air is pumped out between the flexible bottomed plate and the plate loading deck. The 10 mL of air could be theoretically removed in approximately 4 ms, if the pump were operating at maximum speed. In turn, the membrane attached to the flexible bottomed plate deforms downward, introducing an equibiaxial strain in the membrane, which is transmitted to the cells (Figure 2-2). The strain profile of the membrane was recorded with a Cannon G2 camera (Lake Success, NY) or a MTI RC300 camera (Michigan City, IN) attached to a Zeiss Axovert Microscope (Chester, VA).

High strain rate application. For a high strain rate application, membranes attached to the flexible bottomed plates were loaded on the plate loading deck. To avoid the air leaking between the deck and flexible bottomed plates, they are clamped with a plastic plate. The vacuum pump was turned on for 5 min to ensure a rapid application shown in Figure 2-2. The time of high strain rate application of 30 ms was set before opening the valve. The time of high strain rate application is the time between the signal to open and close the valve. If the valve opened slowly (20 ms) and it took an additional 4 ms to evaculate all of the air from the space between the flexible bottomed plate and loading deck, then the time need to achieve maximum strain would be approximately 24 ms. Alternatively, if the valve opened quickly and air was evacuated from the space between plate and loading deck during valve opening, then the time needed to achieve

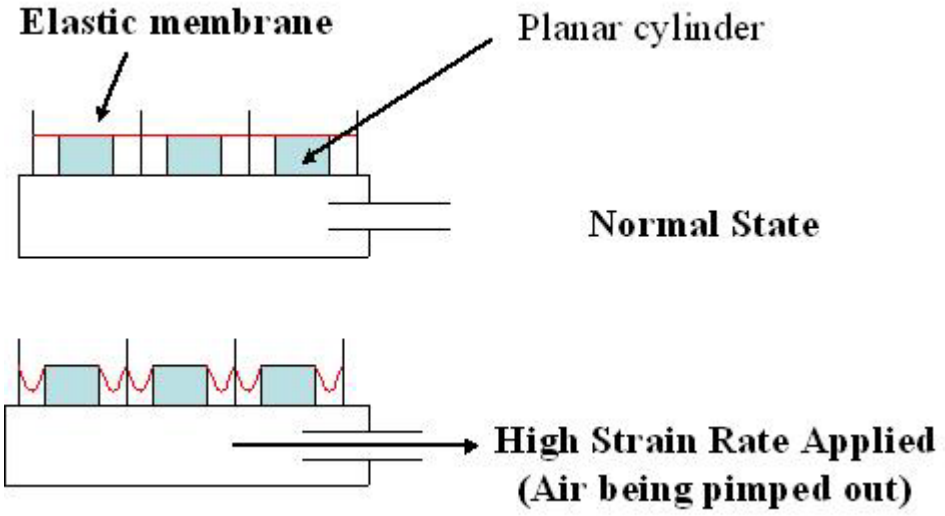

Figure 2-2. Schematic drawings of a membrane deformation device.

maximum strain would be closer to 4 ms. As an approximation, we assumed an intermediate value of 10 ms to achieve maximum strain.

Strain measurement. Three marks were placed on the membrane that defined a triangle (Figure 2-3). Using images of the membrane before and after deformation obtained using the camera attached to the microscope, triangle areas were measured. Strain was estimated as the ratio of the difference in areas after and before strain to the area after strain.

Marks used for strain measurement were placed either at the center of the flexible bottomed plate of towards the edge of the plate such that the uniformity of the strain could be estimated. For each device configuration used to generate different strain levels, strain measurements were made at least 3 times, both at the center and the edge of the strain device.

Strain rate measurement. To estimate strain rate or time needed to achieve maximum strain in the device, images of the flexible bottom plate with markings used for determination of strain were obtained as a function of time using both the CCD camera and the video camera attached to the microscope.

Results

Strain analysis of attached membrane. A theoretical analysis is formulated to verify the strain along the radial direction. The membrane that is attached to the flexible bottomed plate is circular. Thus during the deformation of the membrane, the part supported by planar cylinder experiences a uniform strain. Strain characterization of the membrane was performed. The strain profile over the membrane by changing the radius is characterized. Let the radius of membrane before the high strain application be ‘R’

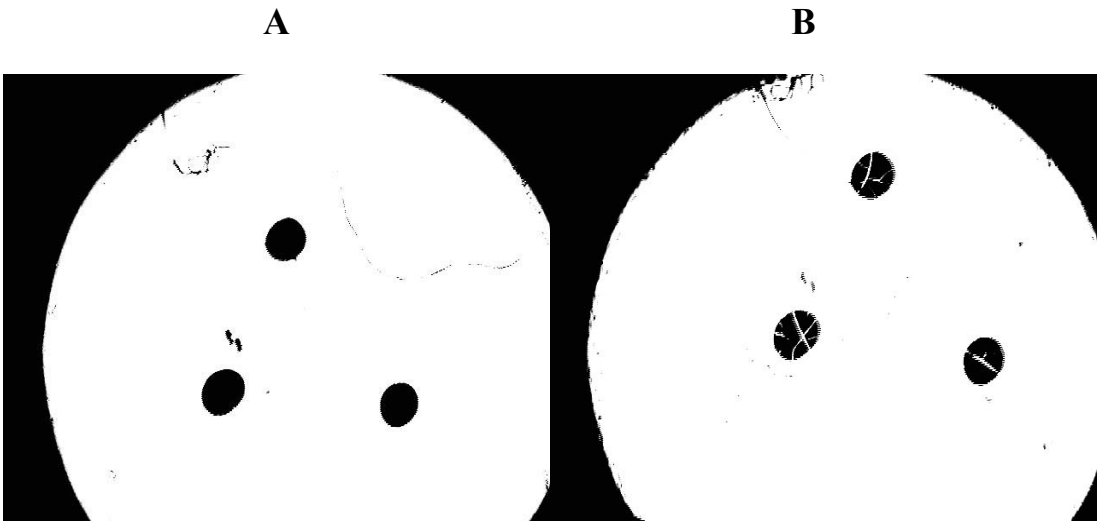

Figure 2-3. Strain analysis of a stretched membrane.

A: Normal state, B: Stretched state

and radius of membrane after the high strain rate application be 'R + Δr',

$$Area(before) = \pi R^2,$$

$$Area(after) = \pi(R + \Delta r)^2,$$

$$\varepsilon_{Area} = \frac{Area_{After} - Area_{before}}{Area_{Before}} = 2\frac{\Delta r}{R} + (\frac{\Delta r}{R})^2 \approx 2\frac{\Delta r}{R},$$

where ε_{Area} is the area strain. Thus the strain of the high strain rate application device has a near linear strain profile for small strains when the Δr^2 term is negligible.

The strain profiles of the membranes are listed in Table 2-1. The strain levels were changed by increasing the gap between the application deck and the flexible bottomed plate using a rubber plate. This enabled us to produce 3 distinct strain levels that could be used to examine the effect of strain on cellular injury. The strain profiles of the membranes were calculated by marked areas of triangles at different position on membrane to verify the homogeneity of strain of the deformed membrane. There were no statistical differences between strains at the inner and outer area of membrane (p<?). During the high strain application, most of the membrane experiences a uniform strain, except the portion that is not supported by the planar cylinder. Thus, during the cell culture and proteomic experiments (Chapter III and Chapter IV), cells located in the middle of membrane were used for analysis.

Characterization of strain rates. Strain, degree of brain tissue deformation, is one of deleterious mechanical components of TBI. Also, a strain rate (ε/time) is another key factor of TBI. From mechanical simulations of TBI, the range of strain rate of TBI was predicted from 1 to $5s^{-1}$(Chu et al., 1994; Ueno et al., 1995; Gennarell, 1996). The strain rates of our device were characterized using the CCD camera of fluorescent microscope and regular digital camera. To record the membrane deformation, the time of strain was set at 30ms. The degree of strain is controlled by adding a rubber plate to the loading deck by increasing the gap between the flexible bottomed plate and the plate loading deck. Fifteen frames per second were recorded by each CCD camera. As seen in Figure 2-4, during the high strain rate application, the membrane was stretched only at

Table 2-1. Strain profiles of membranes for different set ups.

Low: no rubber plate between the flexible bottomed plate and the plate loading deck.

Medium: Rubber plate between the flexible bottomed plate and the plate loading deck.

High: Rubber plate between the flexible bottomed plate and the plate loading deck.

Membrane Stretch	**Area Strain (ε_{area})**
Low	**0.05 ± 0.014**
Medium	**0.151 ± 0.011**
High	**0.258 ± 0.013**

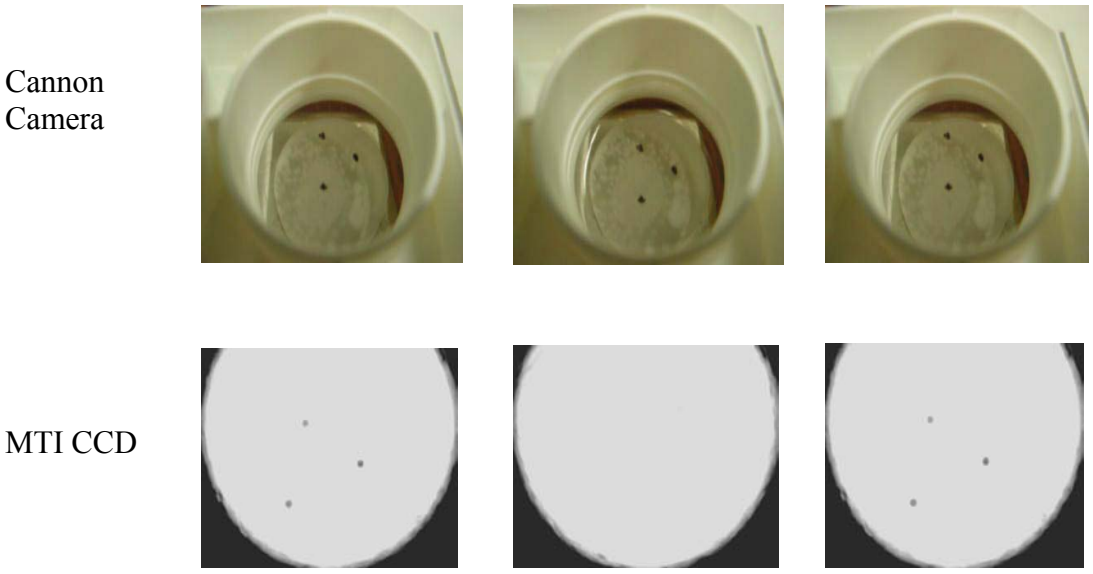

Figure 2-4. Frames captured by CCD camera during the high strain application. The time of strain was at 30ms. The time between frames is 66ms. Only middle frame shows that the membrane is being stretched.

middle frame in Cannon camera. The frames recorded by MTI CCD camera via microscope showed that only middle frame is unfocused because of dropping of membrane during stretching. From this result, the membrane stretching time is guaranteed to be less than 132ms. Even though we are not unable to claim the membrane was stretched within 10 ms to 24 ms, the lower and upper bounds of our theoretical estimates of time needed to achieve maximum strain based on pump and valve specifications, because of technical difficulty of CCD camera, repetitive experiments showed that the only one frame captured the stretched membrane state of the high strain rate application device. Theoretically, we estimate the highest strain rate achieved in the device was approximately 26 s^{-1}. Based on videomicroscopy measurements of strain as a function of time, the strain rate in the device was 2 s^{-1} or higher at the highest strain levels used. Both estimates fall within the range of strain rates expected during TBI.

Discussion

There are many techniques for mechanical stimulation of cells *in vitro*. The techniques of mechanical stimulation of cells that have been developed have in general had resulted in a physical environment *in vitro* that approximated the physical environment *in vivo.* Hydrostatic pressure and platen abutment was used for compressive loading system to study cartilage response to mechanical force (van Kampen et al., 1985,) and ligament cells response to hydrostatic pressure (Yousefian et al., 1995). The stretch system by uniaxial displacement (Vandenburgh et al., 1989) was employed to study myotube and bone cells (Murray et al., 1990). The stretch system by culture plate bending was used to research the cardiocyte growth (Decker et al., 1997). The circular culture membrane distention was applied to TBI (Ellis, et al., 1995; Morrison et al., 1998a; Geddes et al., 2001). The plane culture membrane distention was used to study mechanical force to human oral cell (Norton et al., 1995). The fluid shear system was applied to study high and slow strain rate nerve injury (Laplaca and Thibault, 1998; Triyoso and Good, 1999).

Traumatic brain injury is caused by the mechanical displacement of brain tissues. Over the past decades, many *in vitro* models of TBI have been developed to characterize the effects of mechanical forces and deformation on cells of the central nervous system (Laplaca et al., 1997; Laplaca and Thibault, 1997; Morrison et al., 1998a; Geddes et al., 2001). A number of methods have been used to injure cells that are analogous to the mechanical displacement seen in TBI including hydrodynamic shear applications to neurons, and tensile stress applied to an elastic substrate to which cells are attached (Chu et al., 1994; Ueno et al., 1995; Laplaca et al., 1997; Morrison et al., 1998a; Triyoso, e t al., 1999; Craig and Clark, 2001).

The high strain rate application device developed in the Cellular Engineering Lab at TAMU was based on an existing membrane deformation device where cells were attached to the membrane (Banes et al., 1985). The high strain rate application device has several advantages. First, the high strain rate application device has a relatively linear and homogeneous strain contour independent of cell orientations. The homogeneity and isotropy of strain enables the device produce the same mechanical deformation for the entire population of cells. The device developed by Morrison and coworkers is similar to our device, however, during the deformation, their device stretches the membrane nonlinearly resulting in nonhomogeneus and aniostropic strains over the population of cells (Figure 2-5). Differences in strain application to cells may result in different biochemical responses from cells. In addition, the flat surface for cell culture in the device developed at TAMU provides a superior surface for cell attachment, while attachment is more problematic in spherically shaped devices such as that of Morrison and co workers. Attachment is essential for transmission of the strain from the membrane to the cells (Figure 2-5). Strength of the TAMU device is the optical window below the cell culture surface, making it possible to observe cells via a microscope during the deformation. The optical window can be used to study the actual cell deformations during the high strain rate application and also to investigate, via novel detection methods, biochemical changes in cells and culture medium in real time during and after injury. The study of morphology neurodegeneration at an early stage can be

identified. Third, the strain administered in the high strain rate application device can be changed by adding rubber plates on the plate loading deck easily. To change the strain of membranes by the device developed by Morrison et al needs to replace the membrane that has different strain profile. It is quite cumbersome to change membranes to produce different strain profiles because membrane and membrane loading deck should be sterile for cell culturing. The device developed by Geddes et al was based on stretching the membrane by pressing it down with a cylinder (Figure 2-5). It may cause the cell detachment of the membrane. Also, it is quite difficult to produce different strain levels by changing the vacuum levels within 100ms. Since the high strain rate application is a fast process, it is difficult to control the deformation by using the mechanical components. The device hydrodynamical shearing of neuron developed by Laplaca and coworkers showed that the time of shear stress application was more than 200ms. It couldn't simulate the actual time of mechanical force application during TBI.

The profound understanding of neurodegeneration is required for effective treatments and new therapeutics. Precise control of the high strain rate application to neurons may enable us to more carefully study the pathological events leading to neurodegeneration. The *in vitro* high strain rate application device uses an innovative method to control the precise mechanical deformation parameters of TBI. Using our device, primary injuries of neurons can be produced and by culturing the injured neuron, secondary injuries of neuron can be studied. However, the actual strain rate was not verified because of technical difficulty. Thus, it is crucial to evaluate the real membrane deformation profile during the high strain rate application. Eventually, our device will provide a much needed method to investigate neurodegeneration caused by mechanical insults.

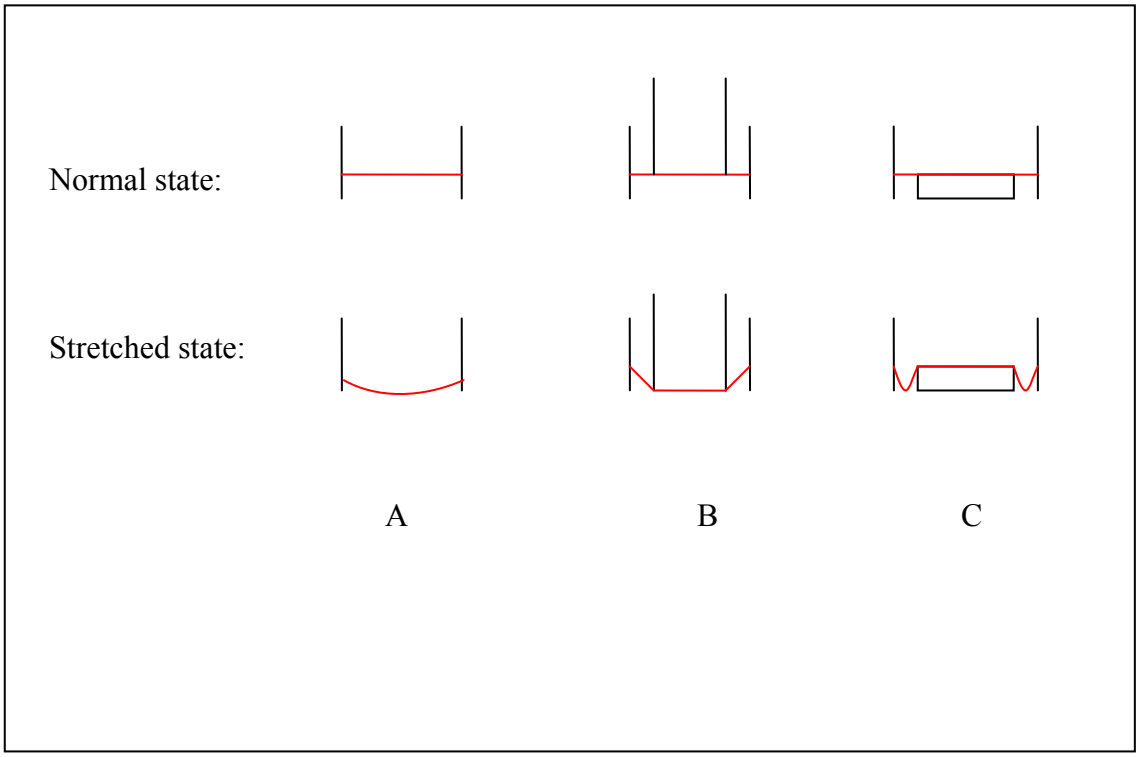

Figure 2-5. Schematic illustrations of the cells attached membrane based cell stretching devices.

A: Device developed by Morrison et al 1998a, B: Device developed by Craig and Clark, 2001, C: Device developed at TAMU cellular engineering lab.

CHAPTER III

HIGH TENSILE STRAIN RATE LEADS TO APOPTOSIS IN SH-SY5Y CELLS

Introduction

Traumatic brain injury is a leading cause of death for people under 45 years old in the United States (CDC, 1999, Thurman, 2001). The cost of TBI is an estimated $56.3 billion the U.S. in 1995, (Thurman, 2001). Traumatic brain injury is associated with mechanical displacement of brain tissue and acceleration of the head. During brain tissue damage, nerve cells may be sheared, compressed and stretched. Mechanical insults to neurons can lead to damage to the cell body and to the axon of the neuron. Numerous experiments have been carried in animal models and cell culture models to better understand the progression of TBI (Gennarelli et al., 1982; Gennarelli et al., 1983; Tornheim et al., 1984; Nusholtz and Ward, 1987; Lee and Haut, 1989). Animal models of TBI typically consist of dropping a weight from a fixed height onto the skull of the experimental animal. To mimic TBI *in vitro,* neurons have been stretched, compressed and sheared by different mechanical devices (Murphy and Horrocks, 1993; LaPlaca et al., 1997; Laplaca and Thibault, 1997; Triyoso and Good, 1999; Craig and Clark, 2001, Geddes et al., 2001). There are many advantages of *in vitro* models of TBI. In animal models, it is difficult to precisely control the mechanical insult to the tissue and it is relatively difficult to measure progression of injury. *In vitro* models, while much simpler, enable the experimentalist to more precisely control mechanical insult and isolate mechanistic steps in injury after the insult. Based on the animal models of TBI, it is believed that neurons undergo apoptosis (Dirnagl et al., 1999). Free radical generation and release of intracellular calcium may contribute to apoptosis associated with TBI (Rudel and Bokoch, 1997, Marciano et al., 2001). Loss of calcium homeostasis could result from the NMDA receptor activation by released glutamate from the damaged cells

(McBurney et al., 1997). In addition, oxidative damage caused by free radical or excess Nitric Oxide production could lead to mitochondrial dysfunction, which could contribute to apoptosis associated with TBI (Beckman and Koppenol, 1996; Triyoso and Good, 1999; Boucher et al., 1999).

In this chapter, to examine the mechanism of the neuronal cell death associated with TBI, differentiated SH-SY5Y cells were injured in the device described in Chapter III. The rate, duration, and magnitude of injury were chosen to mimic conditions occurring during TBI. The effects of high strain rate injury on neuronal death were characterized as a function of time after insult. To prevent apoptosis in injured cells different therapeutic pharmacological candidates were applied to mechanically damaged cells. The categories of therapeutic agents tested were: calcium channel blockers, NMDA receptor inhibitor, G protein inhibitor, phospholipase C (PLC) inhibitor, and protein synthesis inhibitor. All of these agents play a role in regulation of calcium homeostasis in neurons. In our experiments, only Neomycin, a nonspecific agent which blocks PLC activity as well as caspase inhibitors, had any effect on prevention of apoptosis associated with a high strain rate mechanical injury. This is in sharp contrast with result from the low strain rate injury in which the G protein, PLC pathway was very important in injury associated apoptosis. These findings may help to understand the mechanism of cell apoptosis after mechanical damage and how different forces *in vivo* contribute to injury.

Materials and Methods

Materials. Minimum essential media (MEM) Eagle's with Earl's salts and non-essential amino acids, fetal bovine serum, penicillin, streptomycin, fugizone, and trypsine/EDTA were bought from the GibcoBRL (Grand Island, NY). β–nerve growth factor, EGTA, Neomycin, U73122, ω–conotoxin, pertussis toxin, D-(-)-2-amino-5-phophsphonovaleric acid (AP5), Ryanodine, cyclohexamide, propidium iodide, and cadmium chloride were obtained from Sigma (St. Louis, MO). Nonspecific caspases inhibitor, inhibitors of caspase1, and caspase3 were obtained from R&D (Minneapolis, MN). Methanol free formaldehyde was obtained from Poly Sciences Inc. (Warrington, PA). Caspases inhibitors are small protein fragment can block the Caspases activity. TUNEL apoptosis detection kit was obtained from Promega (Madison, WI). Flexible-bottomed culture plates, BioFlex® Untreated Culture Plates, were obtained from Flexcell International (Hillsborough, NC).

Cell culture. The human neuroblastoma cell line, SH-SY5Y, was used for all experiments. SH-SY5Y cells were cultured in a humidified 5% (v/v) CO^2/air environment at 37^{o}C. The composition of medium used to grow SH-SY5Y cell were minimum essential medium with 10% fetal bovine serum, 100U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 3 mM L-glutamine. Prior to the stretch experiments, SH-SY5Y cells were grown at a density of 5,000,000 cells per 100cm^2 tissue culture flask. For experiments, SH-SY5Y cell were differentiated for 6 days by adding 30μg ml^{-1} human β-nerve growth factor to cells in the flexible bottomed plates.

Application of shear injury at a low strain rate. Differentiated SH-SY5Y cells were cultured on the flexible bottomed plate. The flexible bottomed plate was placed on the cell stretching deck and vacuum was applied. The cells were stretched for 30 ms. The strain of cells was 16.2%. Device details are described in Chapter II.

Application of low shear strain rate to cells. The shear stress apparatus was a modification of a cone and plate viscometer (Triyoso and Good, 1999). It consisted of a GlasCol stirrer and motor controller from Omega (Terre Haute, IN), and a stainless steel cone (Texas A&M Chemical Engineering machine shop, College Station, TX). The cone had a 5° angle relative to a stationary tissue culture dish containing a layer of SH-SY5Y cells. Culture medium filled the space between the cone and the layer of cells. To generate pulsatile shear stress, the rotating cone was started and stopped. In 90 minute pulsatile, the cone was rotating for 13 minutes and then stopped for two minutes, repeatedly for a period of 90 minutes. In 90 minute steady, the cone was rotating for 90 minutes without stopping. Except for the control cells, all cells were exposed to shear at 1 dynes cm^{-2}. This strain level was sufficient to cause a 5% cell strain within 10 minutes stress application (Edwards et al., 2001)

Apoptosis detection. Apoptosis was detected and measured according to the manufacturer's instructions using TdT-mediated dUTP nick and end labeling (TUNEL) assay kit from Promega (Madison, WI). For each apoptosis experiment, 1-2 million cells were used. To prepare the cell for apoptosis assay, the cell culture medium was removed from the flexible bottomed plates and trypsin/EDTA was added to detach the cells from the flexible bottomed plates. The cell suspensions were centrifuged and washed with phosphate buffer saline (PBS) two times. Cells were fixed with methanol free formaldehyde for 20 minutes. Two times of wash with PBS were done and cells were permeablized by 70 % of ethanol. After at least four hours of incubation at $-20^{o}C$, cells were washed with PBS and labeled with fluorescein-12-dUTP by terminal deoxynucleotide transferase (TdT) at $37^{o}C$ for one hour. This reaction labels broken DNA strands which are indicative of cells undergoing apoptosis. Immediately, after TdT enzyme reaction, 20mM of EDTA was added to block the further reaction. Cells were washed with PBS containing Bovine serum albumin (BSA). Afterwards, propidium iodide, which contains whole DNA, was added to cells and incubated in the dark for 30 minutes. The results of the TUNEL assay were analyzed with a FACS Caliber flow

cytometry using a 15 mW argon laser at 488nm excitation wavelength. Emission filters of 530 nm bandpass for green fluorescence (Fluorescien) and 585 nm for red fluorescence (Propidum iodide) were used. Cellquest software (Becton Dickinson) was used to determine the percentage of apoptotic cells.

Inhibition of apoptosis during high tensile strain rate induced nerve injury. Pharmacological agents were selected to block the specific steps in a hypothesized mechanism of strain induced cell apoptosis. The pharmacological agents used for inhibition of neuronal apoptosis are listed on Table 3-1. Percentage of apoptoic or TUNEL positive cells was measured 24 hours after high strain rate injury was applied to cells.

Measurement of PLC activity. PLC activity is measured using ^{3}H-Labled Phophatidylinositol as described (Fukuda et, al., 1994). To100ul substrate solution plus 50 μl Ca^{2+}/EGTA solution, 50-0 μl of water and 0-50 μl of PLC enzyme source are added to make a final volume of 200 μl. The final assay mixture contains 50 μg of PtdIns, 1%of octylglucoside, 0.1mM DTT, 1mM EGTA, and 3mM $CaCl_2$ in 50 mM HEPES buffer. After incubateing the reaction for 10 min at 37OC, the reaction is terminated by adding 1 ml of chloroform/methanol/HCl (100:100:0.6 by volume), followed by 0.3ml of 1*N* HCL containing 5mM EGTA. The aqueous and organic phases are separated by centrifugation, and the upper aqueous phase is analyzed with scintillation counter.

Data analysis. All data were shown as the mean ± the standard error of the mean of n independent determinations. Each independent determination represented a single culture well in which cells were stretched and agent was applied for a specified time. To determine if a data set was significantly different than a control or negative data set, the t-test with unequal variance was used. Criteria of $p<0.05$ and $p<0.001$ were employed.

Table 3-1. List of possible therapeutic candidates for use in high strain rate nerve injury.

Steps in cellular mechanism	Inhibitor	Amount and incubation time prior to injury
Ca^{2+} entry into cell	EGTA (chelating agent of calcium) ω-Conotoxin (voltage gated calcium channel blocker) D-(-)-2-amino-5-phophonovaleric acid (AP-5) (NMDA receptor blocker) MK801(NMDA receptor blocker)	1mM, 1.5 hours 0.4μM, 0 hours 50μM, 0 hours 50mM, 0 hours
PLC activity	Neomycin U71822	2mM, 0.25 hours 2μM, 0.25 hours
G protein signaling	Pertussis	10μg ml^{-1}, 4 hours
Protein synthesis	Cycloheximide	10ng ml^{-1}, 4 hours
Caspases Inhibition	Caspase 1 inhibitor (877 Da) Caspase 3 inhibitor(668 Da) Caspases total (inhibitors of Caspase 1 and 3)	10μM, 1 hour 20μM, 1 hour 20μM, 1 hour

Results

Application of stress to SH-SY5Y cells at a high strain rate leads to apoptosis. During TBI, neuronal cells are mechanically injured. A number of different stress act upon neurons during the TBI. To identify the specific effect of a high strain rate tensile stress on neuron-like cells, we deformed differentiated SH-SY5Y cells at high strain rate and measured the percentage of cells with fragmented DNA using the TUNEL assay. A 16% strain was applied to cells for 30ms. The strain rate was estimated to be greater than 10 s^{-1}. Before the high strain rate injury to SH-SY5Y cells, most of cells were in G1-phase as seen in Figure 3-1. In Figure 3-2, representive flow cytometer results from TUNEL stained cells exposed to high strain rate applied injury are shown. Cells in G1-phase and other phases gradually became apoptotic over 24 hours. Specifically, as seen in Figure 3-2, C, a small peak showed up between the G1 peak and the apoptosis peak. This peak could represent cells in which the cell cycle machinery was activated, suggesting that mechanical insult resulted in a certain level of cell cycle activation. From these results, we identified that apoptosis occurs over 24 hours after high strain nerve injuries. To identify the apoptosis initiation time, we the level of apoptosis after high strain rate injury application to cells at a number of times after injury. As seen in Figure 3-3, the percentage of apoptosis after high strain injury to cells was more than 7% at all times measured after injury while the percentage of apoptotic cells in the control group was less than 2.2%. This difference was significant at $p<0.05$. In the injured cells, the percentage of TUNEL positive cells increased rapidly within 30 minutes, with 90 percent of the total apoptosis fraction of injured cells reached within 1 hour. The portion of apoptotic cells after injury remained the same over a 24 hour period.

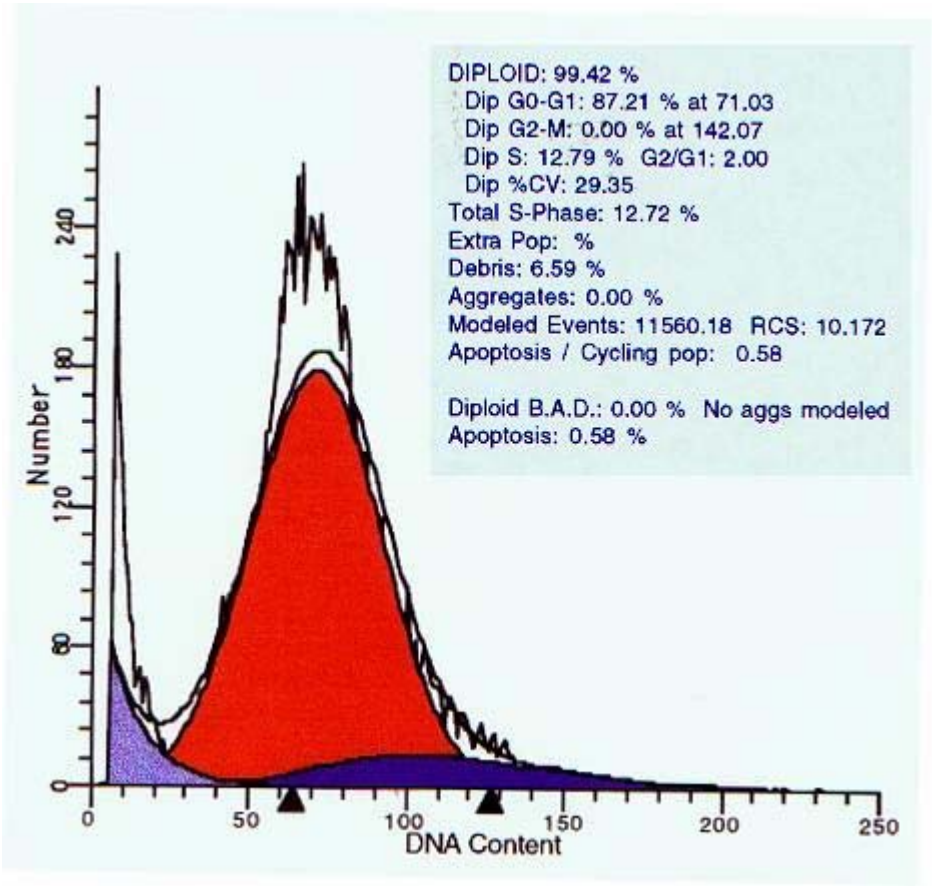

Figure 3-1. Representative flow cytometry analysis of uninjured differentiated SH-SY5Y cells stained with TUNEL agents.

Red area is G1-phase. Blue area is S-phase Sky blue area is cell debris.

Differentiation means the process by which different cell types arise to fulfill specialized function. If cells were well differentiated, cell cycle stages of differentiated cells stopped at G1/G0 phase. G1/G0 phase (Gap phase) is the starting and ending phase of cell cycle. S phase is second phase of cell cycle where cells complete the DNA synthesis. G2 phase is another gap phase after S phase. M phase is next phase of G2 where mitosis occurs. After mitosis, cell enters G1/G0 phase again.

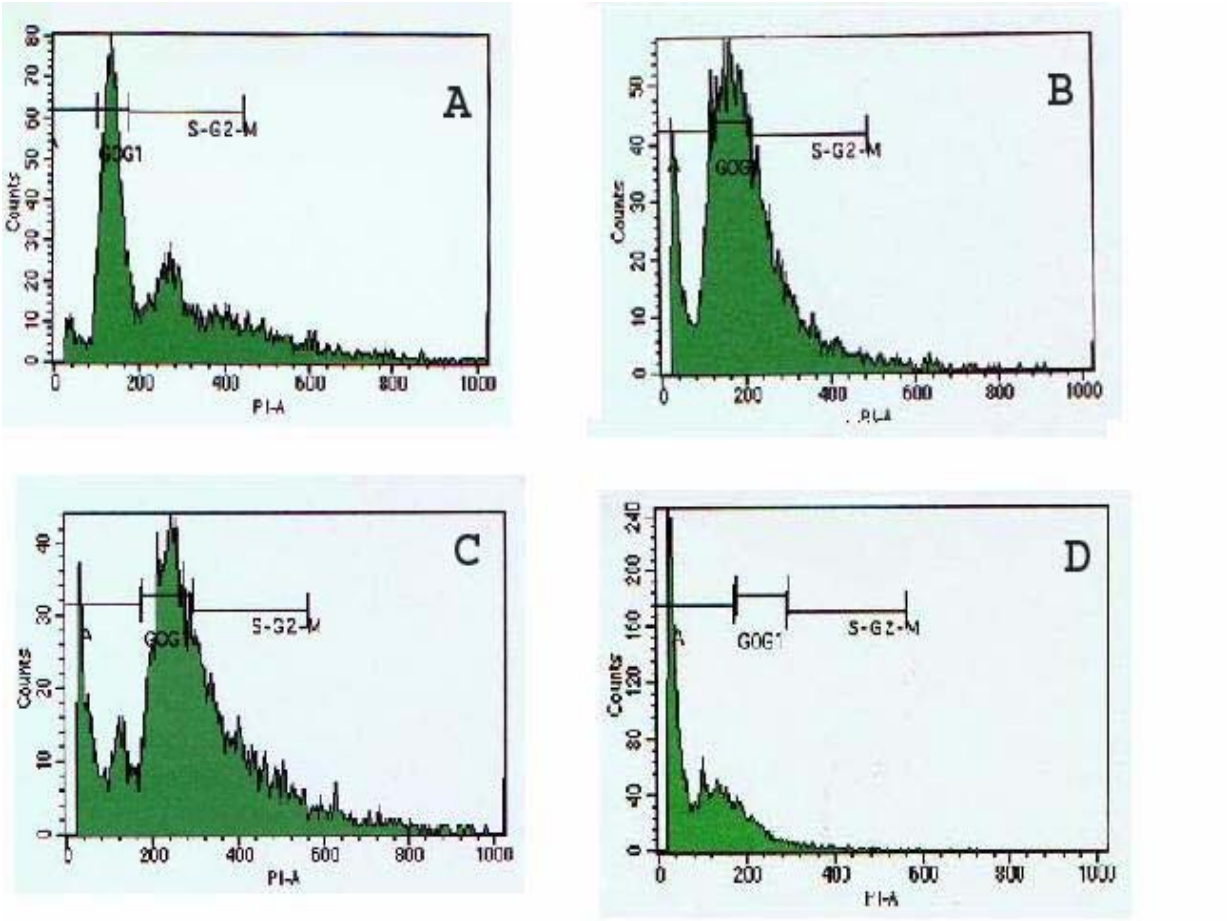

Figure 3-2. Representative flow cytometry analysis of TUNEL stained differentiated SH-SY5Y cells before and after high strain rate injury.

(A): negative control. (B): 4 hours after injury. (C): 8 hours after injury. (D): 24 hours after injury. Cells that are apoptotic A, in G0/G1, S, G2, and M phase are indicated.

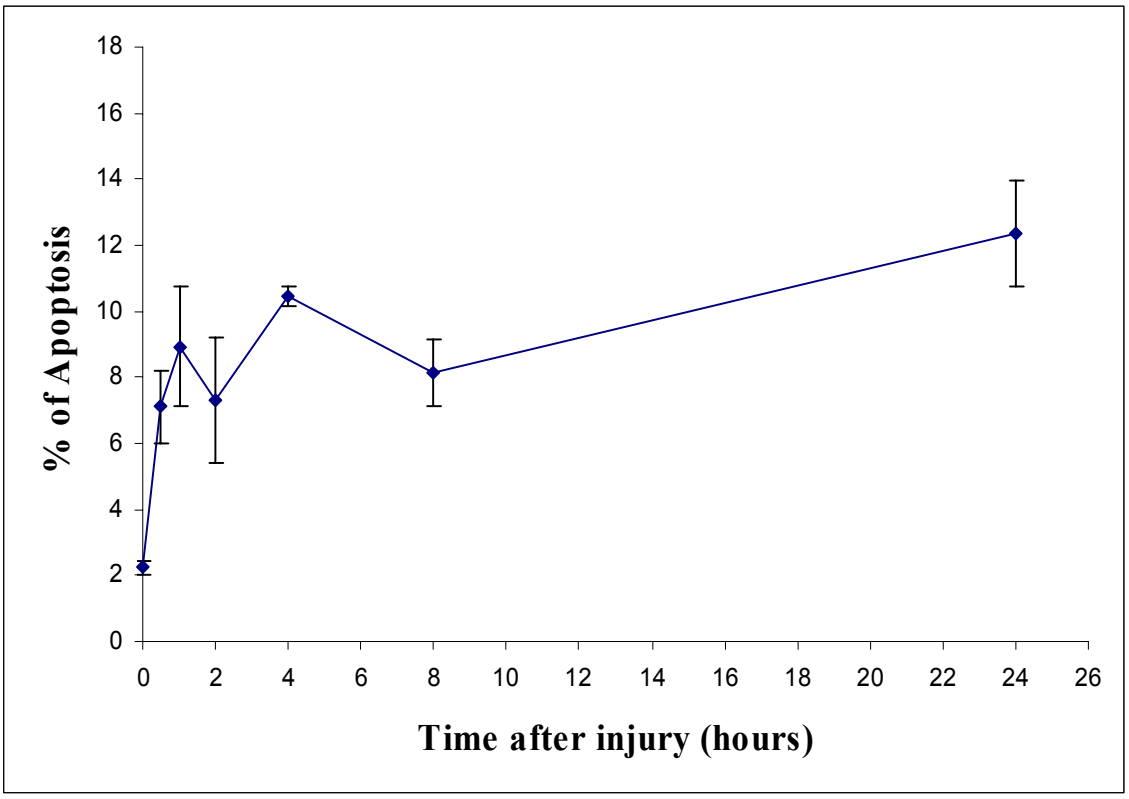

Figure 3-3. High strain rate injury leads SH-SY5Y cells to apoptosis.
TUNEL assay was performed at different times after injury. Error bars represent mean ± standard deviation and n independent measurements where n is 3. T-test showed that all data from injured cells after 0 hours are statistically different from uninjured cells at 0 hours and, p value <0.01.

Inhibition of apoptosis using caspase enzyme inhibitors. Caspases enzymes are known as the key regulators of apoptosis (Sugimoto et al., 1994; Hay et al., 1994; Beidler et al., 1995; Xue et al., 1996). At least 14 different caspases have been identified in mammals (Datta et al., 1997; Wallach et al., 1998). Caspase 1 is related to inflammation and caspase 3 is associated with chromatin degradation. To check the caspase enzyme contribution to apoptosis in cells injured at a high strain rate, caspase enzyme inhibitors were added to cells before injury. Apoptosis was measured at 24 hours after injury since apoptotic cells after injury reached a constant level after 24 hours (Figure 3-3) and in the clinical situation, doctors monitors TBI patient more than 1 day. As seen in Figure 3-4, the percentage of apoptotic cells after injury decreased in the presence of caspases inhibitors. A general caspase inhibitor was just as effective as inhibitors specific for caspase 1 and 3. These results suggest that caspase inhibitors may be able to prevent some cell damage associated with TBI.

Inhibition of G protein signaling and phospholipase C. G proteins are membrane proteins that play an important signaling role within neurons. Activation of G protein triggers phospholipases activation including phospholipase C (PLC) and protein kinase C. Activation of PLC is related to calcium release from the endoplasmic reticulum and calcium homeostasis. To examine the possible connection between G protein signaling, PLC activation and high strain rate nerve injury, pertussis toxin, U73122 and neomycin were used to block the activation of G proteins and PLC, respectively and apoptotsis was measured at 24 hours after injury. Pertussis toxin is a specific inhibitor of GTP hydrolysis and G_o/G_i activation. Neomycin is a blocker of adenylyl cyclase and PLC, and U73122 is a PLC inhibitor. As seen in Figure 3-5, Pertussis toxin exacerbated the cellular damage. However, Neomycin attenuated apoptosis of injured cells. In the case of U73122, the level of apoptosis was the same as positive controls. From these results, we observed that apoptosis of injured cells was inhibited only by Neomycin, and not by typical inhibitors of a G protein/ PLC pathway.

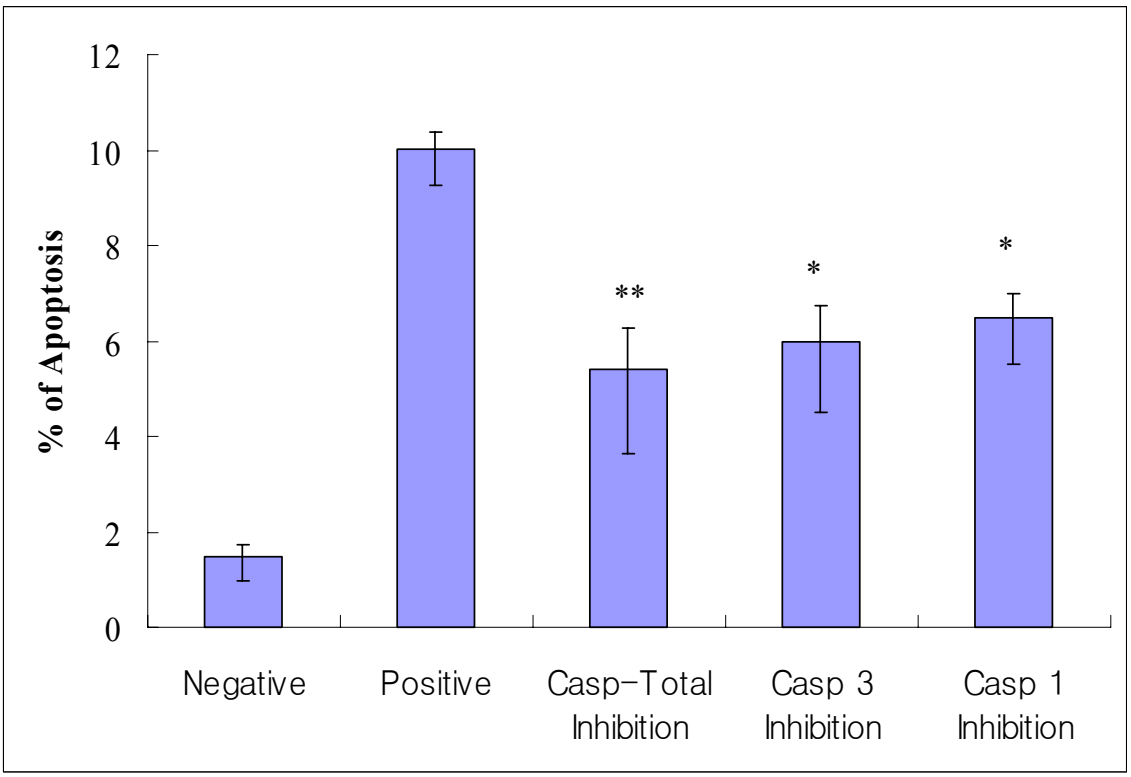

Figure 3-4. Effect of caspase inhibitors on SH-SY5Y cell apoptosis after high strain rate injury. Negative: No high strain rate applied to SH-SY5Y, Positive: High strain rate applied to SH-SY5Y cells, Casp-Total: High strain rate applied to SH-SY5Y cells with inhibitors of Caspases 1 and 3 added prior to injury, Casp 3: High strain rate applied to SH-SY5Y cells with Caspase 3 inhibitor added prior to injury, Casp 1: High strain rate applied SH-SY5Y to cells wit h Caspase 1 inhibitor added prior to injury. Error bars represent mean ± standard deviation of n independent measurements where n is 3. **: $p < 0.01$, *: $p < 0.05$ compared to positive. The treatment of Caspase inhibitors on unstrained cell had no effect.

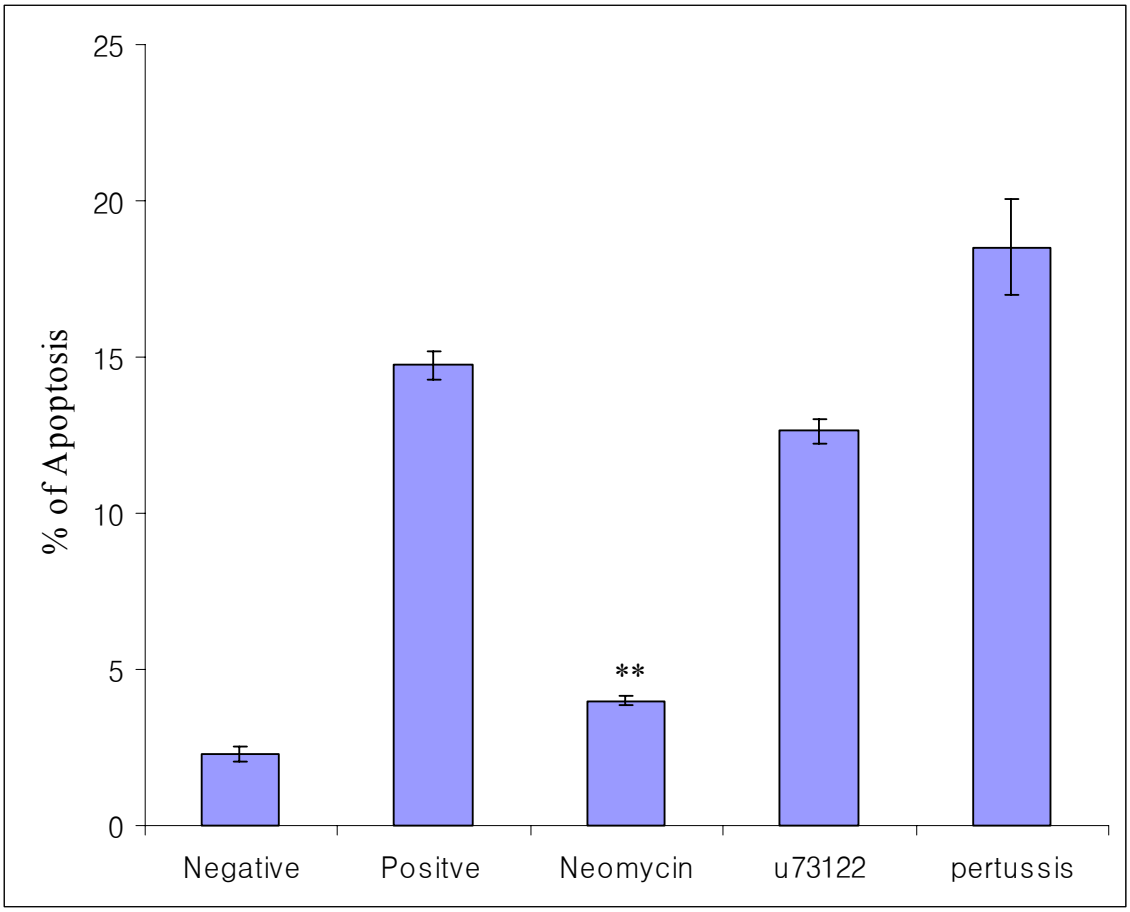

Figure 3-5. Effect of inhibition of G protein activation and PLC activation on cell apoptosis after the high strain rate injury. Negative: No high strain rate applied to SH-SY5Y cells, Positive: High strain rate applied to SH-SY5Y cells, Neomycin: High strain rate applied to SH-SY5Y cells with inhibitor of PLC and adenylyl cyclase added prior to injury, U73122: High strain rate applied to SH-SY5Y cells with inhibitor of PLC added prior to injury, Pertussis: High strain rate applied to SH-SY5Y cells with inhibitor of G protein activation added prior to injury. Error bars represent mean ± standard deviation and n independent measurements where n is 3. **: $p < 0.01$ compared to positive
The treatment of Neomycin, U73122, and Pertussis on unstrained cell had no effect.

The protective effects of Neomycin may originate from the adenylyl cyclase inhibition properties of drug.

Inhibition of apoptosis by blocking calcium transport. Calcium has a pivotal role in neuronal cell signaling and survival. Many reports showed that calcium is associated with apoptosis during TBI (Faden et al., 1989, Graham et al., 1993, McBurney et al., 1997, Leski et al., 1999, Vexler and Ferriero, 2001). We examined the anti-apoptosis activity of calcium transport blockers. We used EGTA, a calcium-chelating agent, ω-Conotoxin, an inhibitor of a voltage gated calcium channel, and AP-5 and MK801 blockers of the NMDA receptor, to prevent calcium entry into cells after injury (Figure 3-6). None of them significantly affected cell apoptosis levels in injured cells.

Inhibition of apoptosis by blocking protein synthesis. As seen in Figure 3-7, we examined the anti-apoptosis activity of a protein synthesis inhibitor. We used cycloheximide to block protein synthesis. As seen in Figure 3-7, cycloheximide could not block cell apoptosis in injured cells.

Application of shear stress to SH-SY5Y cells at a low strain rate leads to apoptosis. During TBI, cells are exposed to tensile stress, shear stress, and compression with both fast and slow dynamics. Our lab has researched low strain rate nerve injury using modification of a cone and plate viscometer (Triyoso and Good, 1999). As seen in Figure 3-8, apoptosis associated with low strain applied to cells increased with the application time of mechanical insult.

Activation of phospholipase C after a low strain rate injury. To identify the activation of PLC after a low strain rate injury, Phospholipase C (PLC) activity was measured. Negative control and 13 minutes of low strain rate to cells showed basal levels of PLC activity. However, shear stress applied for 90 minutes, either steady or

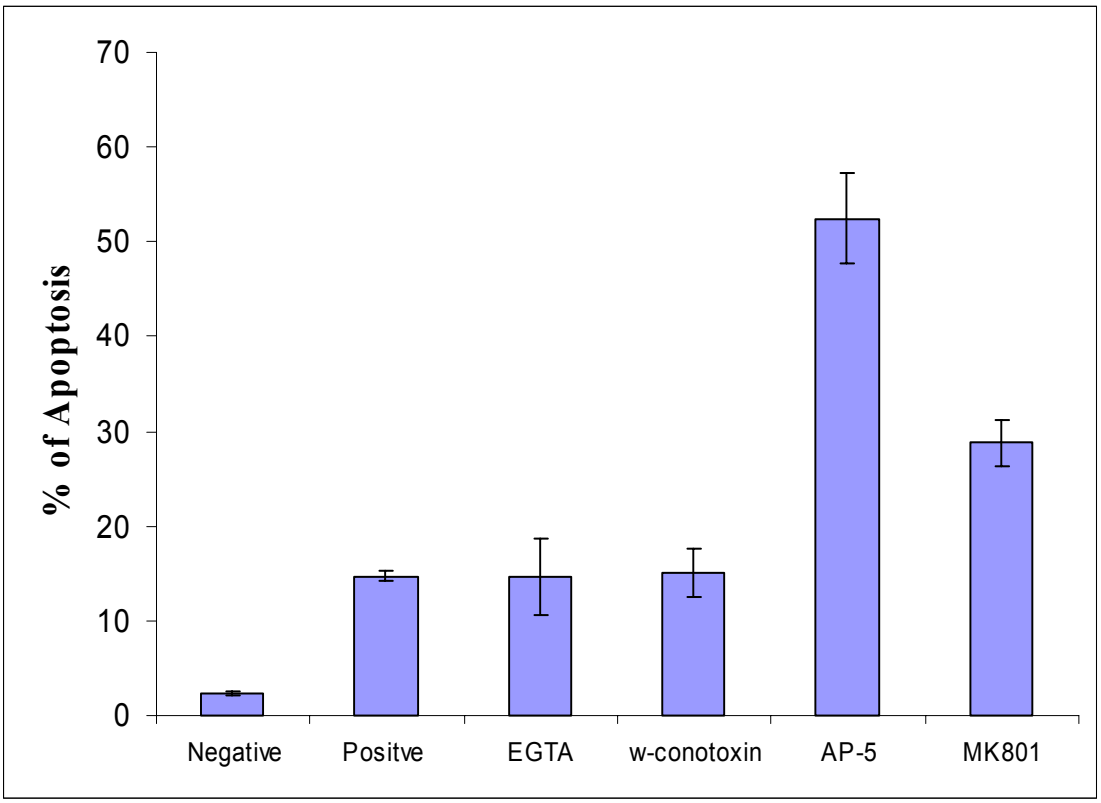

Figure 3-6. Effect of calcium entry inhibitors on apoptosis after injury. Negative: No high strain rate applied to SH-SY5Y cells, Positive: High strain rate applied to SH-SY5Y cells, EGTA : High strain rate applied to SH-SY5Y cells with EGTA added to cells in culture 90minutes prior to injury, ω-Conotoxin: High strain rate applied SH-SY5Y cells with ω-Conotoxin added to cells 0 minute prior to injury, AP-5: High strain rate applied SH-SY5Y cells with AP-5 added to cells 0 minute prior to injury. MK801: High strain rate applied SH-SY5Y cells with MK801 added to cells 0 minute prior to injury. None of the agents applied significantly attenuate apoptosis associated with injury. EGTA caused cell detachment in uninjured cells. Error bars represent mean ± standard deviation and n independent measurements where n is 3. $p < 0.01$ compared to positive

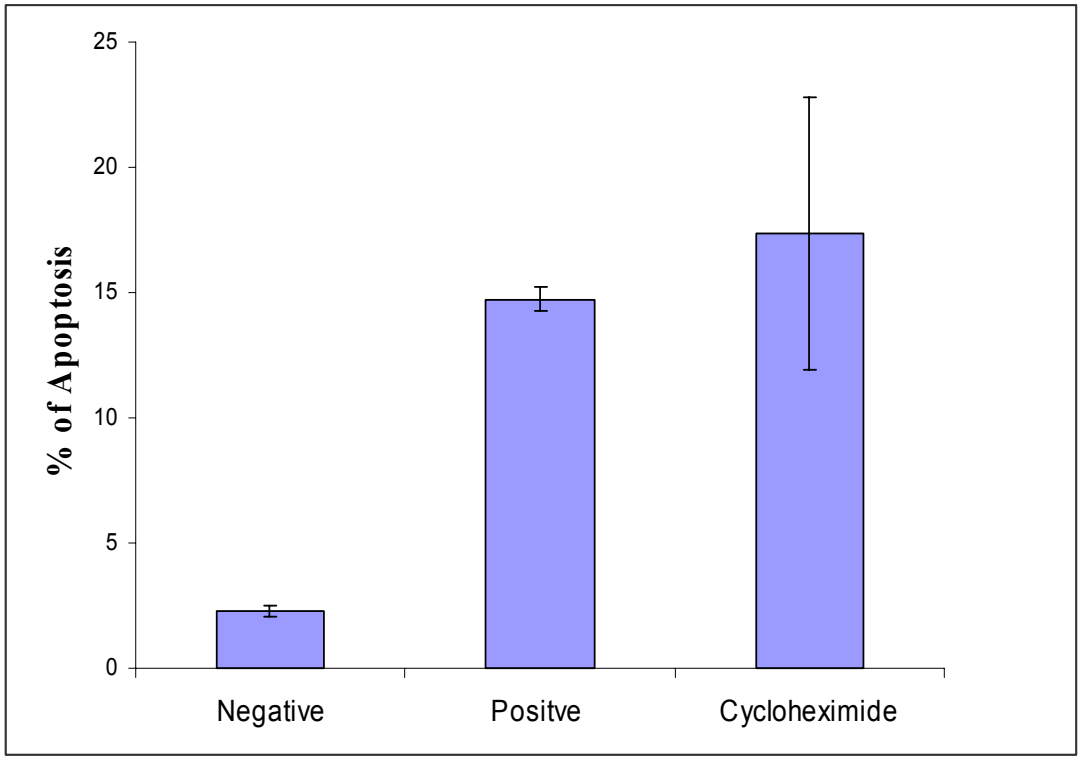

Figure 3-7. Effect of inhibition of new protein synthesis on cell after the high strain rate injury. Positive: High strain rate applied to SH-SY5Y cells, Cycloheximide: : High strain rate applied to SH-SY5Y cells with cycloheximide added to cells in culture 4 hours prior to injury. Error bars represent mean ± standard deviation and n independent measurements where n is 3. $p>0.05$ compared to positive. Cycloheximide treatment of uninjured cells had no effect in apoptosis.

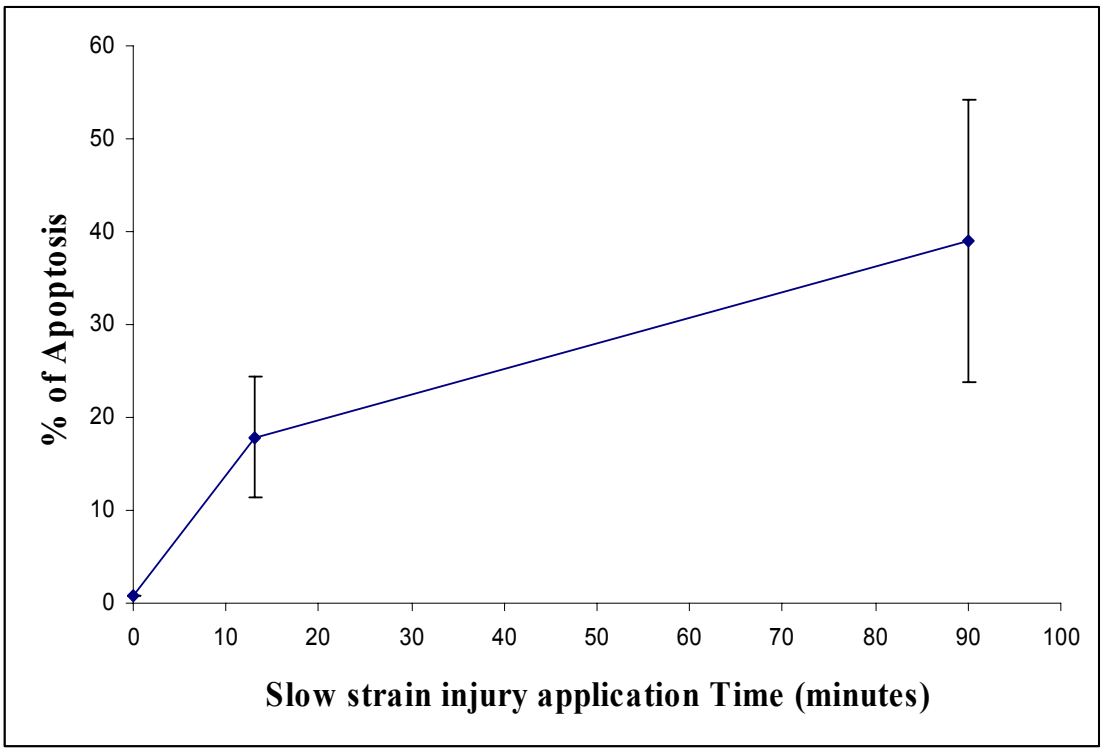

Figure 3-8. Apoptosis in SH-SY5Y after shear induced low strain rate injury. Low strain rate applied for 0 minute, 13minutes, and 90 minutes. Percentage of apoptosis of injured cells was measured right immediately after injury. Error bars represent mean ± standard deviation and n independent measurements where n is 3. $p > 0.05$, 13 minute compared to 90 minute

cyclic, led to an increase in PLC activity (Figure 3-9, Figure 3-10)**.**

Inhibition of apoptosis by blocking activation of phospholipase C. During low strain rate injury, the Phospholipase C (PLC) was activated. We used Neomycin to inhibit apoptosis by blocking PLC activation. As seen in Figure 3-9, neomycin only significantly reduced the level of apoptosis in cells exposed to 90 minutes cyclic shear stress, suggesting that apoptosis under other conditions of low strain rate injury was not associated with the PLC pathway.

Discussion

During TBI, mechanical stresses and strains can trigger neurodegeneration. The duration of the greatest magnitude mechanical insults to brain tissue, as predicted by theoretical simulations of TBI, is from 20ms to 100ms (Margulies et al., 1990, Margulies and Thibault, 1992). Based on these simulations, it is believed that cell strains of 10% or more are achieved during this time period. We used an *in vitro* model of high strain rate mechanical injury, 16% strain in less than 30 ms, to mimic the mechanical insult that occurs during TBI. Differentiated SH-SY5Y cells, human neuroblastoma cell line, were used in the model. Apoptosis was observed in cells within 30 minute after mechanical insult was applied. The flow cytometry results showed that possible involvement of cell cycle related proteins after injury. The flow cytometry result of negative control, differentiated SH-SY5Y cells, showed three peaks which are cell debris peak, G0/G1 peak, and S-G2-M peak. After injury, the S-G2-M peak was moved or/and combined with G1/G0 peak, and peaks were moved to the apoptotic area. These phenomena may be associated with cell cycle positively or negatively. Differentiated SH-SY5Y cells are postmitotic neurons that should not be able to replicate.

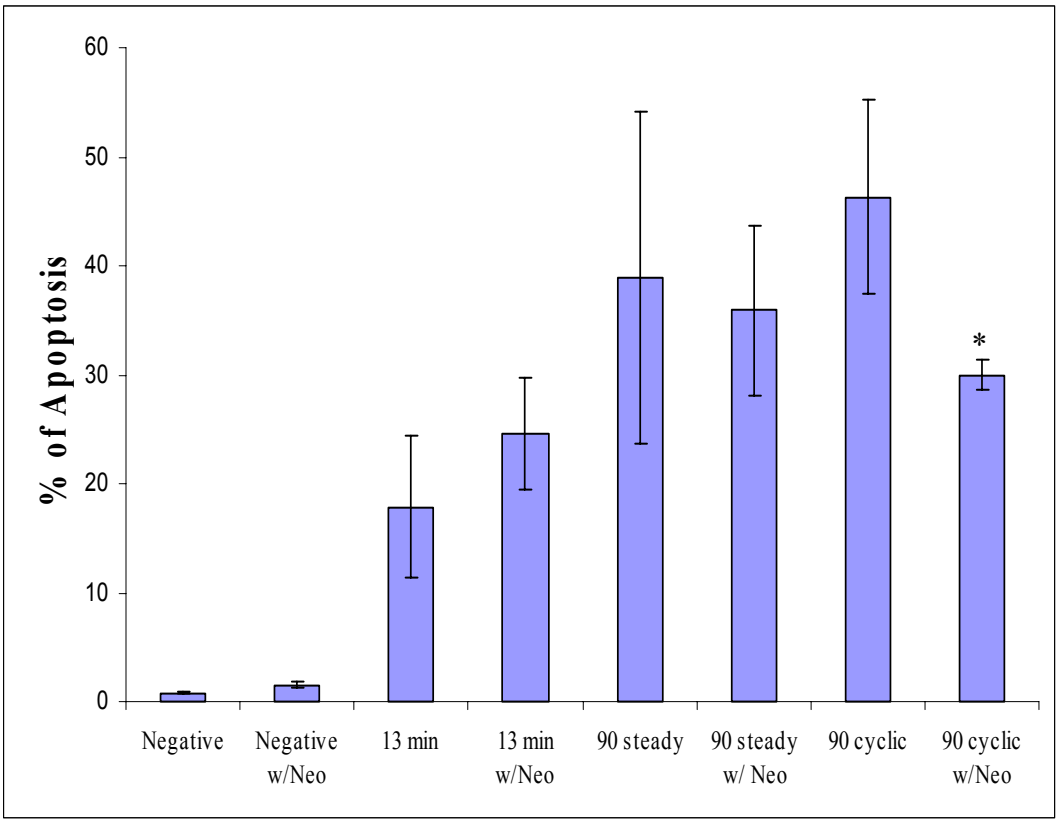

Figure 3-9. Effect of phospholipase C inhibition on cell apoptosis after low strain rate nerve injury. Apoptosis was measured immediately after injury. Negative: No injury applied to cells, Negative w/ Neo: No injury applied to cells incubated with Neomycin, 13 min: 1 dyne/cm^2 of low strain rate applied to cells for 13 minutes, 13 min w/Neo: 1 dyne/cm^2 of low strain rate applied to cells with Neomycin for 13 minutes, 90 min steady: 1 dyne/cm^2 of low strain rate applied to cells for 90 minutes, 90 min steady w/ Neo: 1 dyne/cm^2 of low strain rate applied cells with Neomycin for 90 minutes. 90 cyclic: 1 dyne/cm^2 of low strain rate applied to cells for 6 cycles of 13 minute shearing and 2 minute unshearing, 90 cyclic w/ Neo: 1 dyne/cm^2 of low strain rate applied to cells for 6 cycles of 13 minute shearing and 2 minute unshearing with Neomycin. Error bars represent mean ± standard deviation and n independent measurements where n is 3.*: $p < 0.05$ compared to sample without Neomycin.

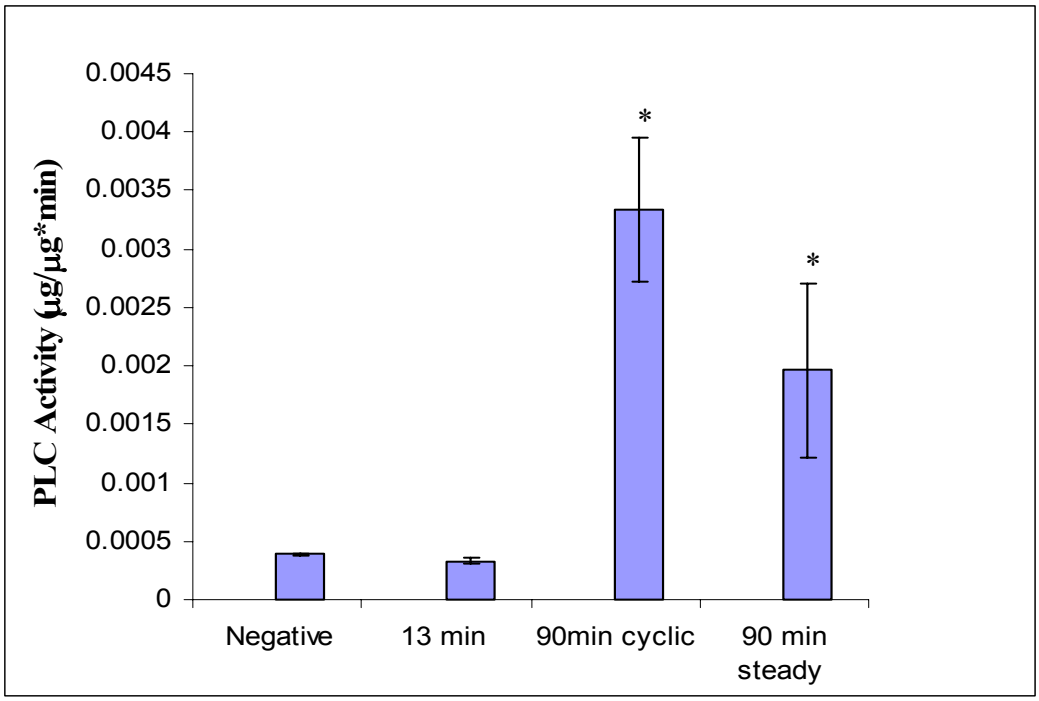

Figure 3-10. Phospholipase C (PLC) activation after slow strain injury. Activity of PLC was measured immediately after injury. Negative: No injury applied to cells, 13 min: 1 dyne/cm^2 of low strain rate applied to cells for 13 minutes, 90 cyclic: 1 dyne/cm^2 of low strain rate applied cells for 6 cycles of 13 minute shearing and 2 minute unshearing, 90 min steady: 1 dyne/cm^2 of low strain rate applied to cells for 90 minutes. Error bars represent mean ± standard deviation and n independent measurements where n is 3. *: $p < 0.05$ compared to negative and 13 minute.

However, during the apoptotic process in damaged neurons, cell cycle related protein can be expressed.

The most probable cell cycle proteins that could be expressed associated with neuron injury would be those associated with the check point system of eukaryotic cells. For protection and proof reading of DNA damage from a variety of insults, eukaryotic cells have developed the checkpoint system which delays cell cycle progression to repair DNA damage since DNA damage can cause abnormal expression and mutation of proteins, and are lethal to cells (Bartek and Lukas, 2001; Walworth, 2001). In mammals, the checkpoint kinase 1 (Chk1) is the most important molecule involved in DNA replication and damage checkpoints. Kaya and coworkers detected the protein expression of Cyclin D1 and cdk4 from the rat brain following cortical contusion (Kaya et al., 1999). Interestingly, Cyclin D1 and cdk4 were not detectable in apoptotic neurons but in non-apoptotic neurons. This may suggest a role for cell cycle proteins in the survival of neurons after TBI. Increasing the expression or activity of cell cycle proteins shortly after TBI may be neuroprotective.

Another important protein is p53 in cell cycle and apoptosis. p53 plays a pivotal role as a detector of DNA damage resulting in growth arrest and apoptosis. DNA damage stimulates stabilization and accumulation of p53 which starts G1cell cycle arrest and apoptosis (Fraser et al., 1996). In the normal cells, the concentration of p53 is low. To activate p53, it should be phosphorylayed by other kinases. Even though p53 can be activated by different kinases, probably, DNA-dependent kinase (DNA-PK) which is substrate of caspase 3 could phosphorylate p53 in TBI. In differentiated neurons, p53 is located in cytoplasm. However, by abnormal insults or stimuli, p53 could be translocated to the nuclei and play a role in apoptosis and cell arrest. Martin et al and Lu et al found increased p53 expression in apoptotic cells in traumatized rat (Lu et al., 2000; Martin et al., 2003).

The retinoblastoma tumor suppressor (Rb) is also important in cell cycle. Rb interacts with many transcription factors includingthe early gene 2 factor (E2F). E2F is a transcription factor that plays major role in cell cycle. Also, Rb and Rb-related

proteins p107 and p130 are the substrates of G_1 CDKs. Phosphorylation and dephosphorylation of Rb regulates E2F–protein binding activity which may activate or deactivate other gene expression. The hypophosphorylated Rb binds to E2F in the G1 phase to repress transcriptional activity. Transcriptional repression is reduced in late G1, when the Rb family of proteins become highly phosphorylated by one or more CDKs. Activated E2F enhances transcription of many genes involved in DNA replication and cell growth control. E2F regulated genes have a role in DNA synthesis, DNA repair, mitosis, and, apoptosis. Di Giovanni and coworkers checked Rb mRNA expression after spinal cord injury (Di Giovanni et al., 2003). They found high levels of Rb and E2F mRNA were expressed after injury. Thus, from the supporting research cited above, we cautiously speculate that the results of flow cytometry might originate from the phenomena of cell phase progression of apoptosis and survival of injured cells regulated by cell cycle related proteins because as seen on Figure 3-1.

The percentage of apoptotic cells did not change significantly at times greater than 1 hour after mechanical insult. Induction of the apoptotic pathway after TBI is consistent with *in vivo* and *in vitro* experiments of TBI (Ellis et al., 1995; Pravdenkova et al., 1996; LaPlaca et al., 1997; LaPlaca and Thibault, 1997; Yakovlev et al., 1997; Fink et al., 1999). Pravdenkova and coworkers induced TBI in rats by dropping a 450-g weight and measured apoptosis at intervals of 3, 10, 24 h, 3 and 10 days after TBI. Apoptosis was detected as early as 3 hours. The maximum level of apoptosis occurred at 10 h after TBI (Pravdenkova et al., 1996). Yakovlev and coworkers used fluid percussion-induced TBI in rats and investigated the potential pathophysiological role of caspase-3-like proteases. DNA fragmentation was observed at 4 hr after TBI (Yakovlev et al., 1997). Fink and coworkers found heavily fragmented DNA at 24 hours after injury (Fink et al., 1999). LaPlaca and coworker induced TBI using hydrodynamic shearing to cultured neurons. They found that apoptosis and necrosis of neurons were stimulated by hydrodynamic shearing (LaPlaca et al., 1997; LaPlaca and Thibault, 1997). However, since lactose dehydrogenase was highly measured after injury, the degrees of shear stress application might be much higher. Ellis and coworkers used

flexible bottomed plate to deform astrocytes and found that necrosis and apoptosis occurred after injury (Ellis et al., 1995).

Apoptosis can be activated by different pathways in neurons (Ankarcrona et al., 1995; Gustafson et al., 1999; Vexler and Ferriero, 2001; Wilson and Gelb, 2002). In TBI, it is believed that apoptosis is activated by the caspases pathway (Choi, 1994; Dugan and Choi, 1994; Marciano et al., 2001). During the experimental TBI, caspase 3 expression was associated with the appearance of TUNEL positive cells as well. As seen on Figure 3-10, activation of caspase 3 could be stimulated by at least two major initiator pathways which are caspase 8 by cell surface death receptors (extrinsic pathway), and a caspase 9 by signals from the mitochondria that lead to formation of an apoptosomal complex (intrinsic pathway). The apoptosis inhibition using the caspases inhibitors (Figure 3-4) suggest that caspases 1 and 3 are activated in cells exposed to high strain rate injury, but no statistical differences were seen between the different caspases enzyme inhibitors used. Our results are consistent with those found in a rat fluid percussion model of TBI (Knoblach et al., 2002) and an *in vitro* low strain rate nerve injury model (Triyoso and Good, 1999). Also, Pike and coworkers measured the calpain and caspase 3 activity in an *in vitro* model of TBI with septo-hippocampal cell cultures (Pike et al., 2000). One hour after injury, lactate dehydrogenase release reached maximum levels and calpain was partially activated. However, caspase 3 was detected 24 hours later after moderate, but not mild or severe injury. This result implies that DNA damage and cellular apoptosis could be activated by activating caspase 3 at early times after TBI.

Physiological role of G protein in the nervous system is as a second messenger playing a role in neurotransmission and in signal transduction from transmembrane receptors to intracellular effectors (Figure 3-11). G proteins can elevate intracellular calcium via activating phospholipase C and can activate diacylglycerols (DAGs). Homayoun and coworkers found that 4 days after TBI in the rat, free fatty acids and

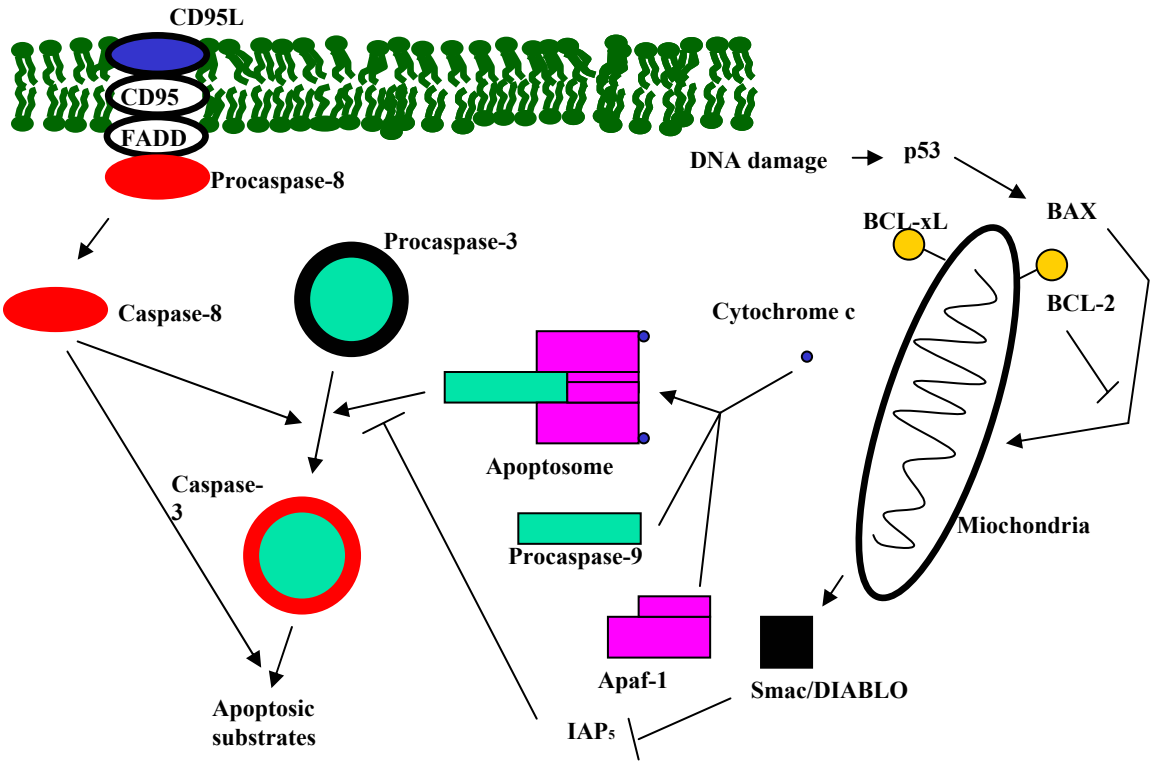

Figure 3-11. Caspase pathway in cell.

The role of diacylglycerol is as a lipid second messenger that triggers cell growth and apoptosis. DAG can be produced during signal transduction by hydrolysis of membrane phospholipids. Excessive production of DAG can lead to degradation of the cell membrane excessive breakdown of membrane phospholipids. Floyd and coworkers found that inositol polyphosphate levels increased up to 10 times relative to those found in normal cells in an *in vitro* model of TBI in which astrocytes were stretched, and inhibition of PLC by Neomycin and U73122 showed a decrease of inositol polyphosphate levels (Floyd et al., 2001). In our experiments, blocking of G protein activation by pertussis did not show anti-apoptotic activity in high strain rate injury (Figure 3-5). However, inhibition of PLC by Neomycin resulted in a dramatic decrease in the percentage of apoptotic cells in high and low strain rate injury but inhibition of PLC by U73122 was only effective in low strain rate injury (Figure 3-5 and 3-9). This is consistent with the results of Floyd and coworkers. It implies that PLC and DAG play a role in TBI, both in low strain rate and high strain rate injuries, activating calcium increase by PLC and membrane phospholipid degradation by DAG. In our experiment, only neomycin showed anti-apoptotic activity after injury. Neomycin can block the activation of PLC and formation of DAG. Thus, the protective effects of neomycin during models of TBI may be associated with the formation of DAGs after injury as opposed to the blockage of activation of calcium increase.

Roles of calcium in nerve cells are numerous (Sattler and Tymianski, 2000). The excessive increase of intracellular calcium levels has been suggested as a common cell death pathway in neurons (Choi et al., 1994; Schanne et al., 1994). In TBI, intracellular calcium increase has been considered as pivotal in the injury pathway (Faden et al., 1989; McBurney et al., 1997; Leski et al., 1999). We used EGTA, ω-Conotoxin, AP-5, and MK801 to block the calcium entry to cells. However, none of these agents were able to prevent apoptosis in our injury model (Figure 3-6). EGTA is a general calcium chelating agent. Xiong and coworkers used EGTA to block the calcium entry into cells in traumatized rat model. They showed that the addition of EGTA to the traumatized rat restored intracellular calcium levels to control levels. ω-Conotoxin is a blocker of the

voltage gated calcium channel. Wolf and coworkers used ω-Conotoxin to block the voltage gate calcium channel in an *in vitro* model of axonal stretch. They showed that blocking of the voltage-gated calcium channel by ω-Conotoxin attenuated the post-traumatic increase in calcium (Wolf et al., 2001). In our hands, we only measured if EGTA and ω-conotoxin could prevent apoptosis, but not how they affected calcium levels. Therefore, our results are not inconsistent with the other investigators, and may suggest that while calcium does enter cells from the extracellular space, entry through the voltage gated calcium channel does not lead directly to cell apoptosis in TBI.

AP-5 and MK801 are potent blockers of the NMDA receptor, which is a glutamate gated calcium channel. In our model of injury, addition of AP-5 and MK801 led to higher numbers of apoptotic cells after injury (Figure 3-6). In TBI, high levels of glutamate are thought to be released, which activate NMDA receptors and lead to increased calcium entry into the cells. Many articles showed the involvement of the NMDA receptor and the anti-apoptotic effect of blocking the NMDA receptor in TBI (Schanne et al., 1979; Lobner and Lipton, 1993; Graham et al., 1993; Vexler and Ferriero, 2001). However, *in vivo* blocking calcium transport through the NMDA receptors in the perinatal rat causes extensive apoptosis (Ikonomidou et al., 1999). This suggests that role of the NMDA receptor in synaptic activity, development, and neuroplasticity in brain is very important. If, in our model, blockade of the NMDA receptor inhibited a neuroplastic response to injury, then our observation of increased apoptotic activity is consistent with observations by Ikonomidou and others.

The inhibition of protein expression by cycloheximde during high strain rate injury showed that blockage of protein expression exacerbates the apoptosis after high strain rate injury (Figure 3-7). However, Allen and coworkers showed antiapoptotic activity of cycloheximde in combined mechanical trauma and metabolic impairment in astrocyte (Allen et al., 1999). Triyoso and coworkers (1999) also observed the anti-apoptotic effects of cycloheximide in a low strain rate nerve injury model. One possible explanation of the difference in results is that in our experiment, because the degree of mechanical insult was low, the majority of neurons initiate repair mechanisms after

mechanical injury, e.g. check point system. Thus, inhibition of protein expression might block the repair mechanisms of injured neurons, resulting in the increase in the apoptosis observed after injury.

The high and low strain rate injury models showed different levels of cell apoptosis after injury (Figure 3-3, 3-8). In low rate strain injury, the percentage of apoptotic cells were higher than the high strain rate injury, and activation of phospholipase C was detected, but only in low strain rate injury of sufficiently long duration (90 minutes as opposed to the 30 ms of high strain rate injury). As seen in Figure 3-5, and 3-6, inhibition of G protein activation, inhibition of PLC and calcium entry into the cells had no effect on apoptosis observed after high strain rate injury. These results suggest that the G protein signaling pathway and calcium entry from the extracellular space are not associated with apoptosis after injury. In low strain rate injury, G proteins (Triyoso and Good, 1999) and PLCs were activated (Figure 3-12) and inhibition of G protein activation and PLC activation showed anti-apoptotic activity. However, in high strain rate injury, inhibition of G protein activation could not reduce the percentage of apoptotic cells. In the low strain rate injury, it has been suggested that shear stress on the cell membrane activates the G protein pathway. The tensile stress applied to cells in our high strain rate device would not have the same physical effects on the cell membrane. Also, as previously mentioned, PLC and DAG could be activated in high strain rate injury. In high strain rate injury, the time of mechanical insult was 30ms. It is possible that the G protein signaling pathway was not activated in this short a time, or only activated at very low levels below our detection capabilities.

In summary, we showed that the high strain rate injury applied to cells caused apoptosis. Inhibition of Caspases 1 and 3 attenuated apoptosis in high strain rate injury, while inhibition of G protein activation, and calcium entry into cells had little effect on apoptosis (Figure 3-13). Inhibition of protein synthesis increased the level of cell apoptosis after injury. Neomycin, a PLC inhibitor, was able to reduce apoptosis levels in

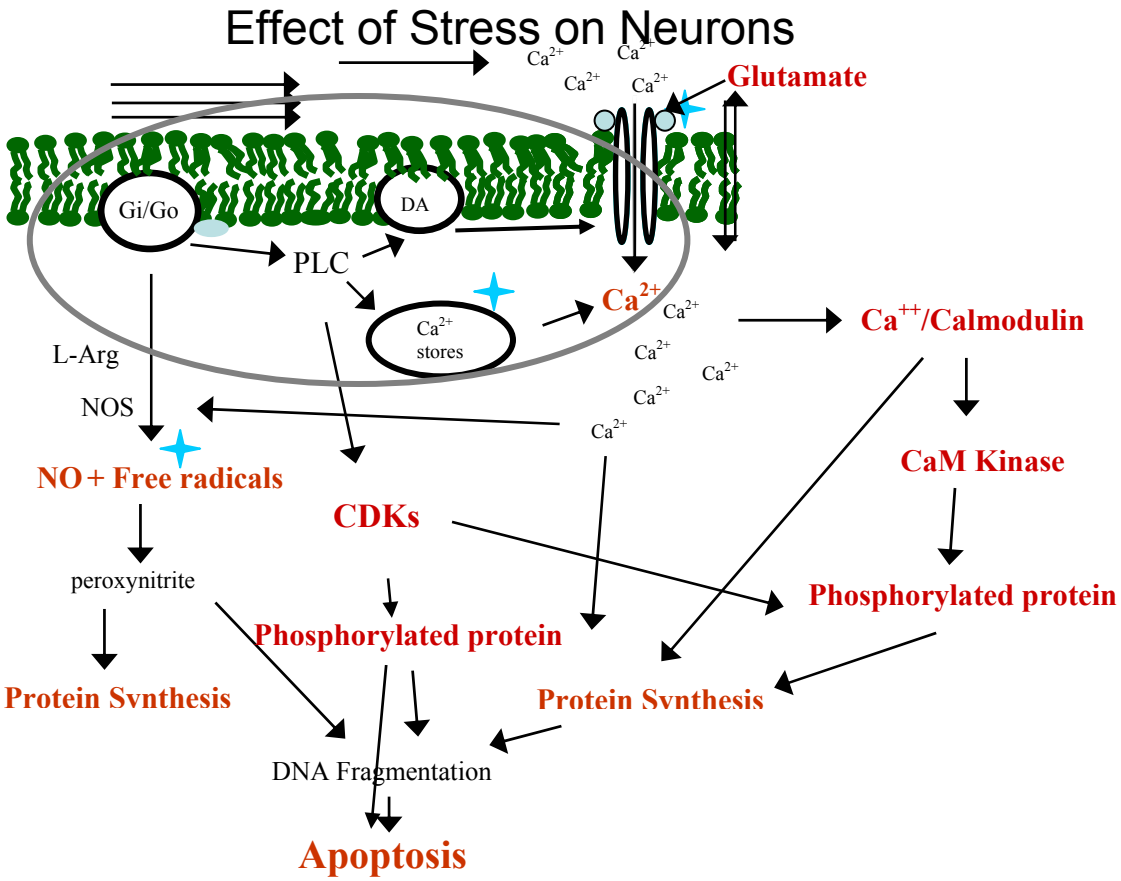

Figure 3-12. The role of phospholipase C activation in the cell (Circled area).

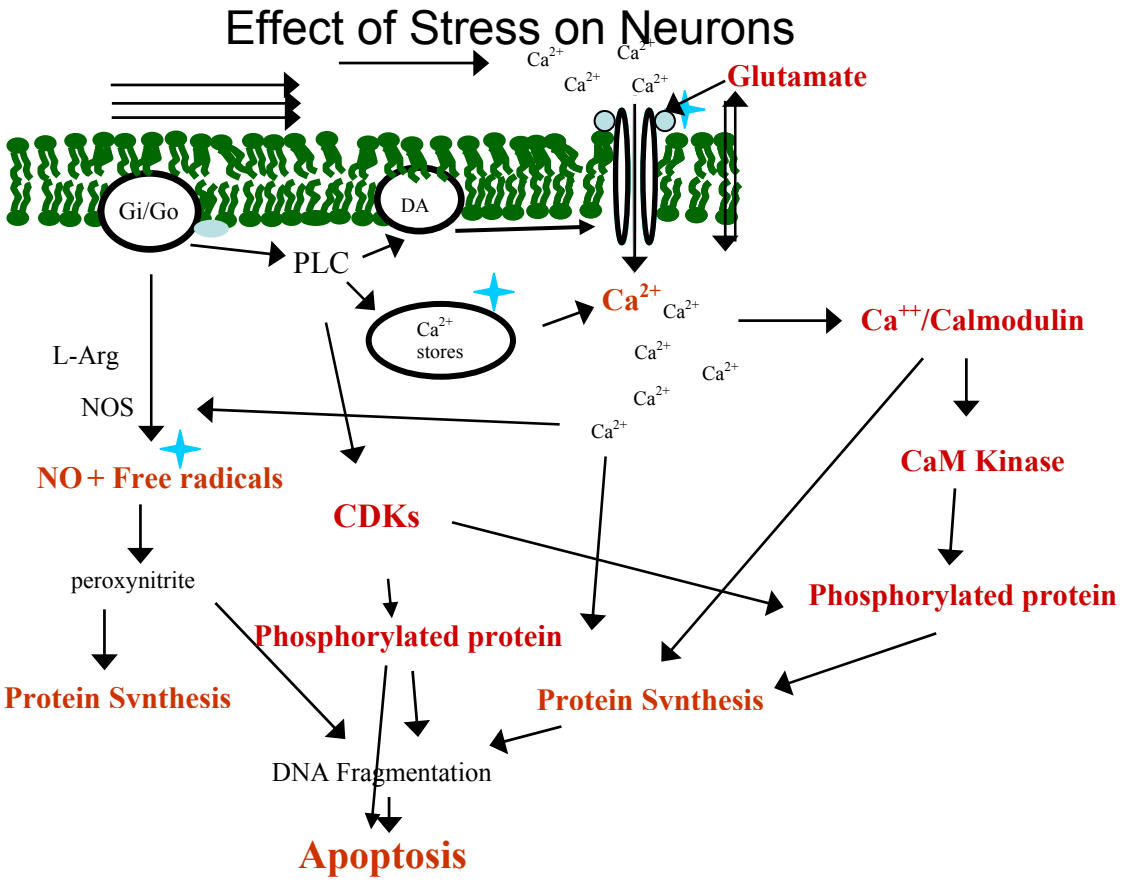

Figure 3-13. Causes of apoptosis in a neuron as a result of mechanical insult.

our high strain rate injury model. The flow cytometry results suggested possible connection with injury and cell cycle progression. One possible interpretation of our results is that cell repair mechanisms were very important for cell survival (and apoptosis prevention) in our high strain rate model of TBI, while in other models of injury, such as the low strain rate and long duration injury model previously developed in the lab, repair is less important as the level of injury is more severe. Precise study of cell cycle and PLC or DAGs in TBI awaits further study.

CHAPTER IV
CHARACTERIZATION OF PROTEIN SYNTHESIS AND PHOSPHORYLATION OF NERVE INJURY INDUCED BY HIGH STRAIN RATE

Introduction

In spite of remarkable achievements in our understanding of the genomic basis of deadly diseases such as cancer, AIDS, Alzheimer disease, and TBI, extensive disparities exist between insight into pathogeneses and development of diagnoses and treatments. One approach to expanding our knowledge of the pathogenesis of TBI and possibly guiding development of treatment is the use of proteomics.

Genomics means a biochemical study of mapping, sequencing, and investigating genomes or genes. Typically, the genomics is studied by examining levels of gene expression or mRNA levels within cells. Proteomics comes from the "Protos" meaning "First" in Greek. Proteomics refer to a study of proteins and their function in the cell. The significance of proteomics originates from the differences between genomics and proteomics. First of all, the human genome consists of about 50,000 genes. In contrast, human body contains more than 100,000 proteins. The quantity of mRNA of a certain gene cannot represent the quantity of a protein, its product, since the lifetime of each protein is different. Cellular pathways can be activated or deactivated by small modifications of proteins such as phosphorylation, methylation, hydroxylation, or the loss of such modifications. These modifications have little connection with the mRNA levels measured with gene arrays. For example, G protein activation in low strain rate injury is connected to apoptosis. G protein activation is associated with GTP binding. The activated G protein can activate phospholipase C and sub-sequential pathways. These cellular changes are very important in response to certain types of injury, but none are associated with changes in gene expression. Only by studying changes in cellular proteins (chemical and structural modifications) using proteomics can these changes be observed. Thus, proteomics is currently most useful tool in biomedical science.

Traumatic brain injury is caused by mechanical insult and acceleration of brain tissue. However, even though many experiments have been done examining nerve cell death by mechanical injury, the characterization of protein expression and modification in early stages of neuronal death has not been done previously. In this chapter, protein expression and protein phosphorylation modification during early stages of neuronal cell death induced by mechanical injury are characterized using pulse chase experiments and proteomics tools. These contribute to our understanding of the molecular level changes that occur during neuronal cell death associated with mechanical insult. This work may guide development of new treatments for TBI.

Materials and Methods

Materials. Eagle's Minimum essential media (MEM) and non-essential amino acids, fetal bovine serum, penicillin, streptomycin, fugizone, and trypsine/EDTA were bought from the GibcoBRL (Grand Island, NY). β–nerve growth factor, acetonitrile, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), urea, iodoacetamide, ammonium persulfate, ammonium bicarbionate, TEMED, silver nitrate, sodium thiosulfate, trypsine, formic acid, sodium dodasyl sulfate (SDS), trizma base, methanol, methionine free minimal essential media, glacial acetic acid, bromophenol, glycerol, acetone, and dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO). Methionine S35 labeled was obtain from ICN. Flexible-bottomed culture plates, BioFlex® Untreated Culture Plates, were obtained from Flexcell International (Hillsborough, NC). Immobiline™ DryStrip pH 3-10 NL, 13cm, and IPG buffer, pH 3-10 NL were obtained from Amersham Biosciences (Piscataway, NJ). Complete™ Mini protease inhibitor cocktail was obtained from Boehringer Mannheim. 30% Acrylamide/Bis solution, 37.5:1 was obtained from Bio-Rad (Hercules, CA). Gelcode blue stain reagent was obtained from Pierce (Rockford, IL). Gel-Dry Drying solution was obtained from Invitrogen (Carlsbad, CA). Pro-Q Diamond phosphoprotein gel stain was purchased from Molecular Probe (Eugene, OG). Blue x-ray film was obtained from Phenix (Hatward, CA).

Cell culture. The human neuronblastoma cell line, SH-SY5Y, was used for all experiments. The SH-SY5Y cell line is subclone of SK-N-SH cell line. Cells were cultured in a humidified 5% (v/v) CO^2/air environment at 37°C in Eagles' minimal essential media with 10% fetal bovine serum, 100U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 3 mM L-glutamine. Prior to stretch experiments, cells were transferred to BioFlex® Untreated Culture Plates at a density of 2,000,000 cells per plate and cells were differentiated in a plate for 7 days by adding 30 μg ml^{-1} human β-nerve growth factor to flexible bottomed plates.

Application of high tensile strain rate to cells. Differentiated SH-SY5Y cells were cultured on the flexible bottomed plate. The flexible bottomed plate was placed on the cell stretching deck and air was pumped out. The cells were stretched for 30 ms. The strain experienced by cells was 16.2%. Device details are described in Chapter II.

Total protein purification. 1 hour later after high strain rate application to cells, cells were scraped and washed with phosphate buffered saline (PBS), pH 7.2. Washed cells were mixed with cell lysis solution (4% CHAPS, 9.5 M urea, 18mM of DTT, and one tablet of Complete™ Mini protease inhibitor cocktail per 1mL of lysis solution) and incubated for 10 minutes at room temperature. After incubation with lysis solution, using the 24 and 21 gauge syringes, mechanical forces were applied to cells to break the chromosomal DNA and cell membranes. The cell lysates were centrifuged at 55,000 rpm for 15 minutes and supernatant of lysate (total protein) was precipitated by cold acetone and stored for further experiments at -20°C. In phosphorylation detection experiments, total proteins were delipidated and desalted by chloroform and methanol after the purification. Supernatants were discarded and pellet was dried in vacuum centrifuge.

2 dimensional electrophoresis. 5µl of IPG buffer and 2.5 µl of bromophenol was added to 250µl of total protein solution. The mixed protein solution with IPG buffer was load to strip holder (Amersham Biosciences, Piscataway, NJ) and Immobiline^{TM} DryStrip pH 3-10 NL, 13cm was placed. Immobiline^{TM} DryStrip was rehydrated for 16 hours and isoelectric focusing was conducted using IPGphore (Pharmacia Biotech, San Francisco, CA). Briefly, Immobiline^{TM} DryStrip pH 3-10 NL, 13cm was focused at 500 volts for 1 hour, 1,000 volts for 1 hour, 8,000 volts for 30 minutes, 8,000 volts for 12 hours and 500 volts for 15 hours. Prior to second dimension of SDS gel electrophoresis, isoelectrically focused Immobiline^{TM} DryStrip pH 3-10 NL, 13cm was processed with the reducing buffer containing 10mg DTT $1mL^{-1}$ and the alkylating buffer containing 25mg iodoacetomide $1mL^{-1}$. Second dimension of SDS gel electrophoresis was performed using 1mm in thickness and 12% acrylamide/Bis Tris-HCl at 25mA for 3 hours till bromophenol blue band exited from the gel. Gels were stained with Blum silver staining, Gel code solution, or Pro-Q diamond phosphoprotein stain case by case.

Pulse chase experiment and metabolic labeling. Prior to the high strain rate experiment, cells were washed with methionine free minimal essential media twice and cells were incubated with 0.02mCi mL^{-1} of methionine S^{35} and 1mCi mL^{-1} of ATP(gamma) labeled. After incubation with labeled S^{35} methionine and 1mCi mL^{-1} of ATP(gamma), a high strain rate was applied to cells for 30ms and cells were incubated before the protein purification. Total proteins were purified as described above. Using a scintillation counter, radioactivity of total protein was measured. To identify the expressed and phosphorylated proteins associated with high strain rate injury, 2 dimensional gel electrophoresis was conducted. After 2 dimensional gel electrophoresis, gels were exposed to an X-ray film for 7 days.

In-gel protein digestion for protein sequencing. A gel slice was removed with a razor blade and placed in an anti-stick tube. The gel was washed with 30% methanol and 100mM of ammonium bicarbonate. 100mM of ammonium bicarbonate containing

2.5mM of DTT was added and incubated for 30 minutes at 60 ^{o}C. 10μl of 100 mM iodoacetamide solution was added and the mixture incubated at room temperature in dark. The gel band was washed with 50μl of ammonium bicarbonate and a mixture of acetonitrile and 50 mM of ammonium bicarbonate. 5-20 μl of trypsin solution that was lyophilized in ammonium bicarbonate was added to the gel and incubated overnight. After trypsin digestion of the gel slice, protein sequence was characterized by mass spectroscopy.

Identification of protein candidate. Using results of mass spectroscopy of proteins, protein candidates were identified with the help of the Profound program (http://129.85.19.192/profound_bin/WebProFound.exe). Profound is a search engine that matches the molecular weights of typic fragments of the unknown protein against those of known proteins in one of the protein databases. The database used was SWISS-PROT. Homo sapience was selected as the taxonomic category because SH-SY5Y cells are of human origin cell line. The allowed missed cleavage by trypsin was set 2 to find undigested fragments. In modification section, iodoacetamide and methionine oxidation were selected since total protein samples were prepared with reducing and alkylating buffers. After running Profound program, possible proterin candidates were selected from protein matches found : Results from the search were ranked by the percent overlap of sequence. From the probable candidates, the most possible candidates were chosen by comparing the isoelectric point and molecular weight of the protein candidates to those of the unknown proteins on the gel, and checking the role of the most possible protein in the neuron.

Schematic experimental procedures of identification of protein synthesis by injured cells were described in Figure 4-1.

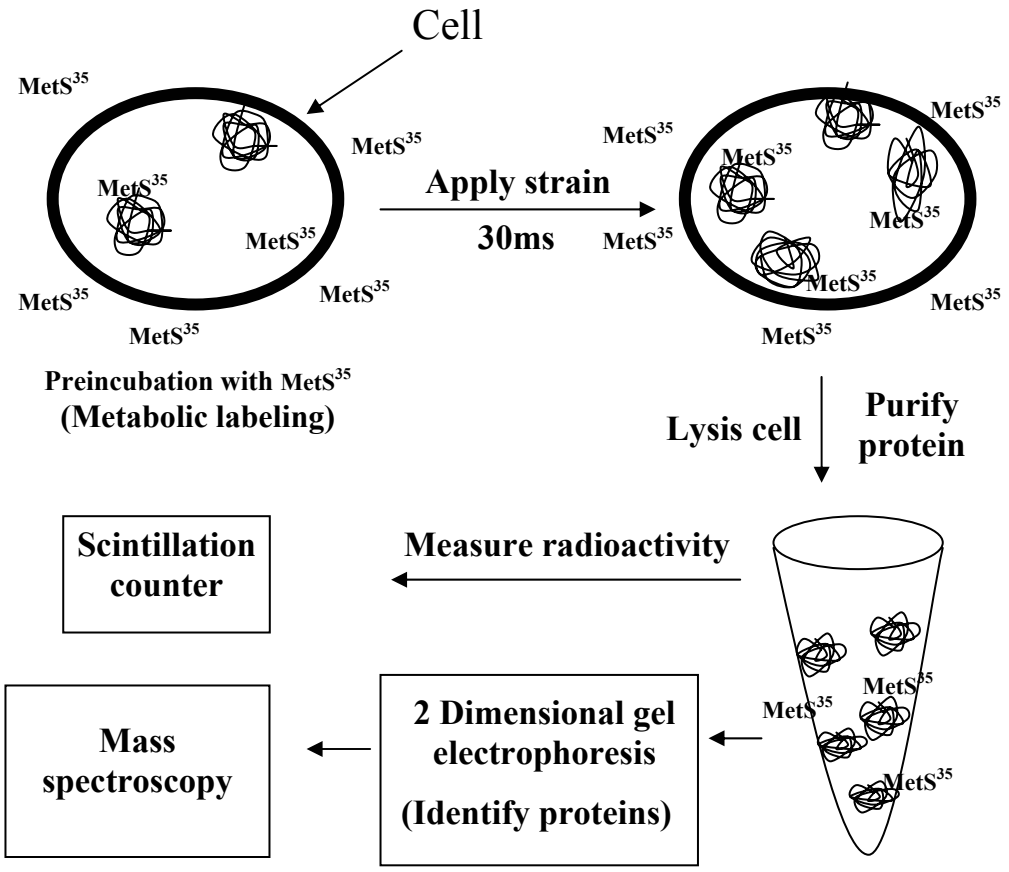

Figure 4-1. Schematic illustration of experimental procedure for metabolic labeling of new protein synthesis.

Results

Pulse-chase experiment. To identify new protein synthesis associated with injury, we performed a pulse-chase experiment with metabolic labeling to assess when and if significant new protein synthesis occurs after injury. As seen in Figure 4-2, the incorporation of S^{35} labeled Methionine in total proteins of injured cells is higher than those of control cells. Methionine is coded as ATG in a gene. ATG is the first codon of all of genes. Thus, higher radioactivity of total proteins of injured cell than those of control cells means higher protein expression in injured cells relative to control cells. In Figure 4-2, protein synthesis increased over the time in both injured and control cells. The rate of protein synthesis in injured cells was greater than that in normal cells.

Two dimensional electrophoresis. To identify differences in protein expression associated with injury, 2 dimensional gel electrophoresis on total cellular proteins was performed and compared with those of normal cells. As seen in Figure 4-3, a different pattern of protein spots and greater number of proteins were visible on silver stained 2 dimensional electrophoresis gels of high strain rate injured cells relative to untreated controls. More than 680($\pm$78, n=3) proteins in normal and 700 ($\pm$72, n=3) proteins in injured cells were clearly identified on silver stained gel. While differences in total protein expression associated with injury were observed via this method, changes in protein levels would be difficult to discern via this method. We then performed coupled experiments of metabolic labeling and 2 dimensional electrophoresis. As seen in Figure 4-4, the patterns of S^{35} labeled proteins from injured cells detected by autoradiograms of spots of 2 dimensional electrophoresis gels were different from that of normal cells. 46

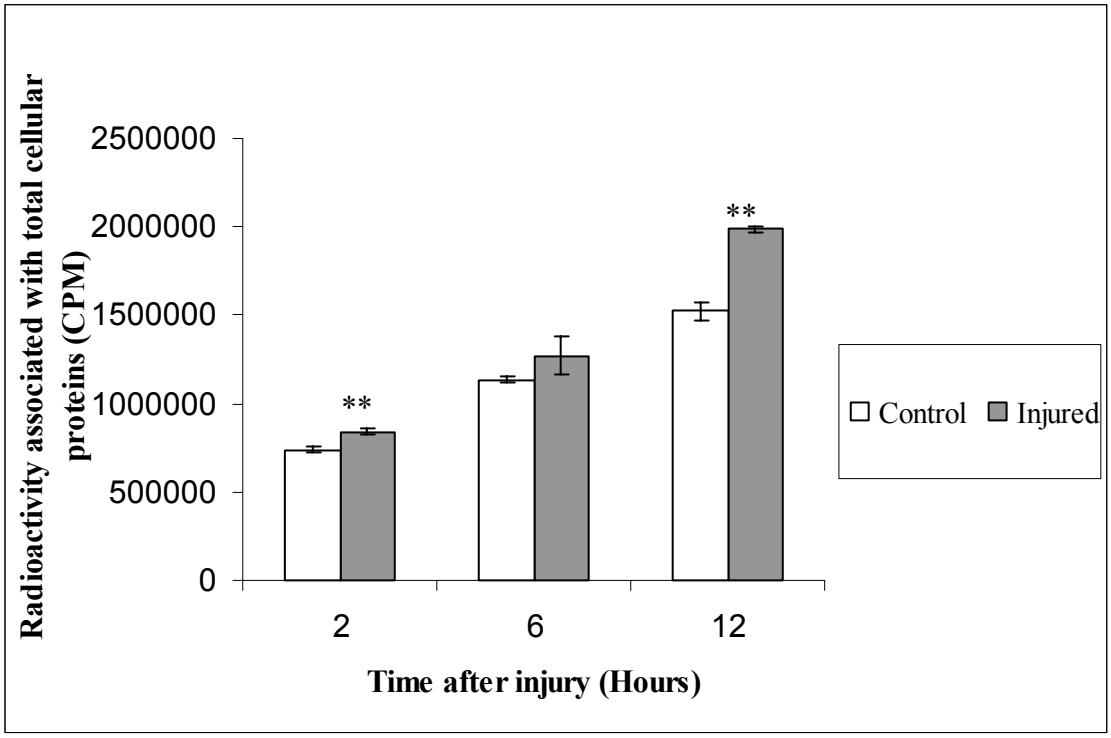

Figure 4-2. Increase in radioactivity as incorporated into newly synthesized proteins after high strain rate injury.

Control cells are treated identically except for application of injury. Methionine S^{35} was added to cells 30 minute before injury. Total protein were collected at various times after injury.

Error bars represent mean ± standard deviation and n independent measurements where n is 3. ** indicates results are statistically different than the control at same time after injury $p<0.01$.

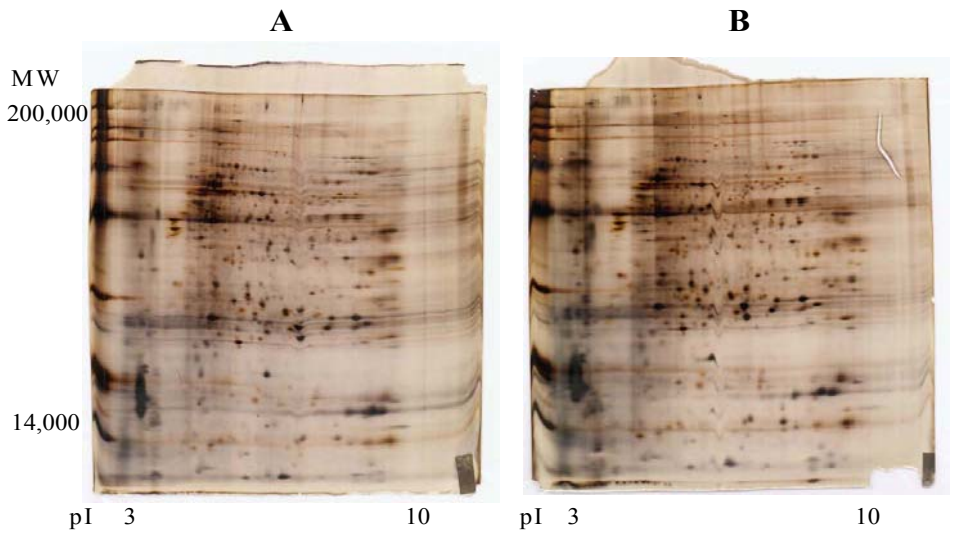

Figure 4-3. Representative silver stained 2 dimensional electrophoresis gels. The range of molecular weight of protein on the gel is from 10,000 to 200,000 daltons. A: Control, B: high strain applied (1 hour later after injury), MW: molecular weight (Dalton), pI: isoelectric point.

(±6, n=3) proteins containing Met S^{35} were detected on X ray film from normal cells and 77 (±11, n=3) proteins containing Met S^{35} were detected from injured cells (Figure 4-4). About 6.7 % of proteins in normal cells and 11% of proteins in injured cells were labeled with Met S^{35}. 7 spots on 2 dimensional gel of injured cells were clearly new proteins. Other spots represent proteins which existed in both normal cells and injured cells. However, the concentration of labeled proteins on 2 dimensional gels in injured cells was much higher than that of normal cells.

Protein spot analysis. There are several patterns of changes in proteins observed on the 2 dimensional electrophoresis gels and X-ray films before and after injury. As seen in Figure 4-5, the circled protein pattern on 2 dimensional electrophoresis gel from injured cells and control cells are comparable. However, the spot intensities on the X-ray films from injured and control cells are different. This could be due to increased expression or upregulation of existing proteins after injury. Fig 4-6 highlights a pattern of proteins that indicate newly synthesized proteins associated with injury. As seen in Figure 4-6, the circled spots on the 2 dimensional electrophoresis gel of injured cells and the X-ray film of the 2 dimensional electrophoresis gels of injured cells are similar. However, the circled spot showed a high intensity on the X-ray film. On the 2 dimensional electrophoresis gel of the injured cells, the spot intensity is very low. It could be associated with new protein synthesis because the incorporation of S^{35} of the red circled protein is much higher than other proteins.

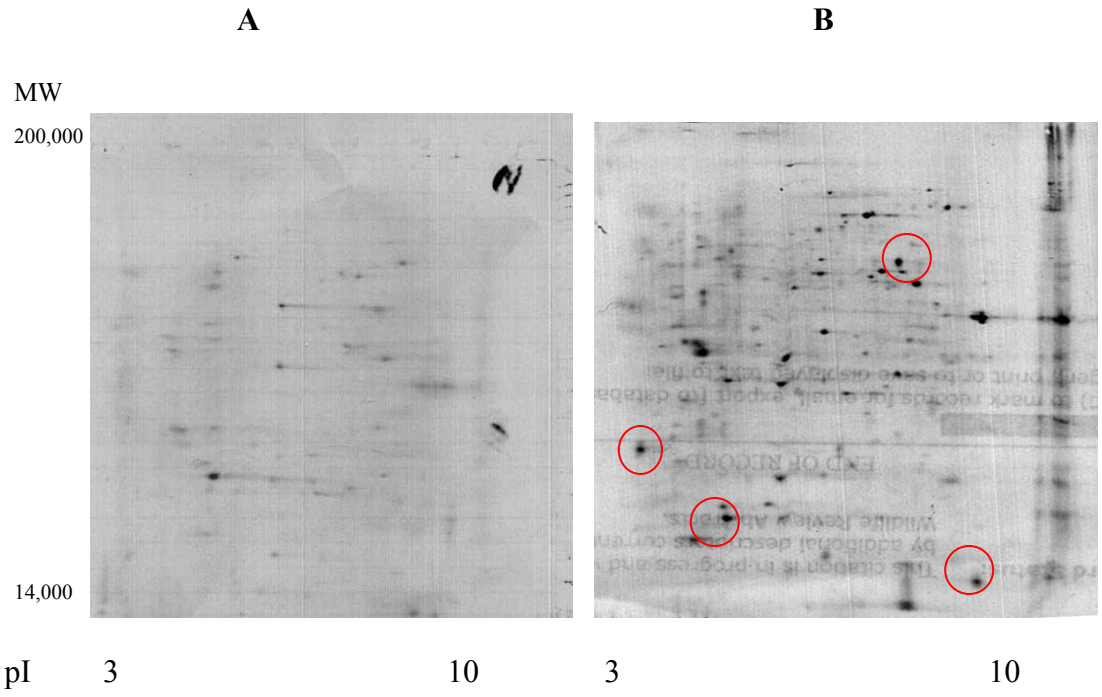

Figure 4-4. Representive autoradiograms of S^{35} labeled proteins after 2 dimensional electrophoresis.

The range of isoelectric point of the gel is pH 3 to 10. The range of molecular weight of protein on the gel is from 10,000 to 200,000 daltons. A: Control, B: Injured (1 hour later). Circled spots are newly synthesized proteins. The quantity of labeled proteins from control cells on the X-ray film: 46 spots, The quantity of labeled proteins from injured cells on the X-ray film: 87 spots, The quantity of newly synthesized protein from injured cells: 7 spots.

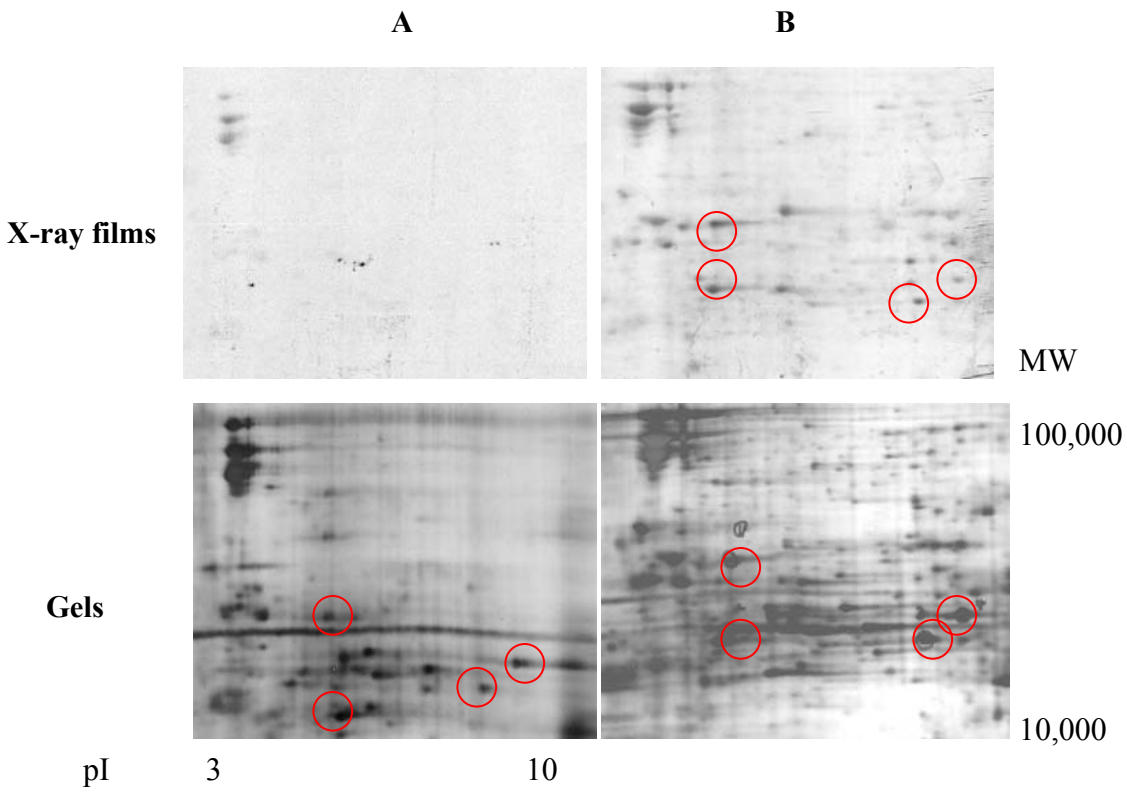

Figure 4-5. Spot patterns of activated existing protein synthesis after injury.
A: control, B: Injured pI of each gel was 3 to 10. The range of protein molecular weight was about 100,000 to 10,000.

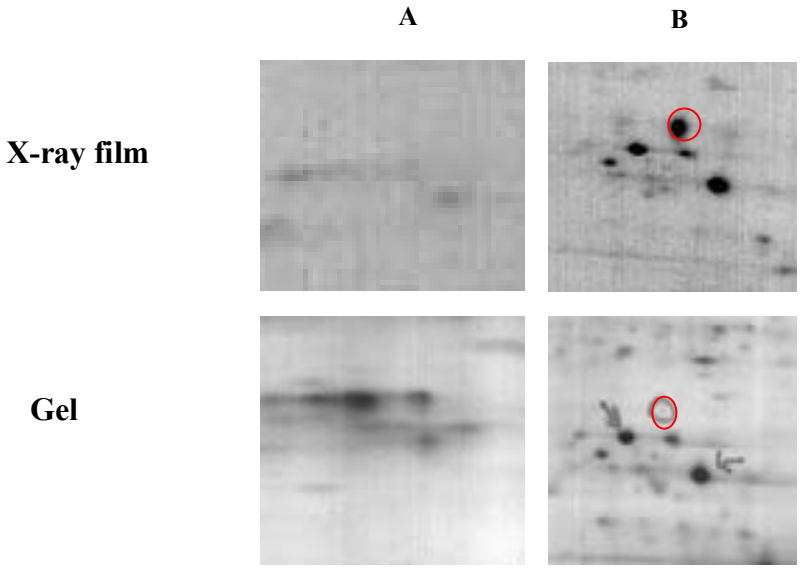

Figure 4-6. Identification of newly synthesized protein from injured cells.
A: control, B: Injured. pI and molecular weight of spotted area is 7.8 and 67,000

Protein sequence identification. Proteins identified as either newly synthesized or upregulated from 2 dimensional gels were then cut out of gels, digested, and the fragments analyzed via mass spectroscopy. Analysis of digestion fragments led to the identification of possible candidate proteins, listed in Table 4-1. The molecular weight and isoelectric point of candidate proteins matched the molecular weight and isoelectric point of protein cut off from the gel of a total of 13 digested proteins. 3 proteins were identified with confidence. Unidentified proteins yielded low numbers of trypic fragment. If there were less than 1 fragment or overlap of total sequence was less than 20%, no protein identification made.

Phosphorylation on protein detection after the high strain rate injury. Phosphorylation is one example of protein modifications. To identify the phorsphorylation of proteins after the high strain rate injury, we separated total cellular proteins via 2 dimensional electrophoresis then stained gel with a specific phosphorylation stain. More than 300 spots were identified on 2 dimensional gels from control and injured. As seen on Figure 4-7, the number of phosphorylated proteins increased after injury. 5 phosphorylated proteins were identified on 2 dimensional gel from normal cells. However, 18 phosphorylated proteins were identified on 2 dimensional gel from injured cells. 5 proteins on 2 dimensional gel from the injured cells were identical to those from normal cells. Thus at least 13 were newly phosphorylated after injury. As shown in Figure 4-8, the protein patterns on the 2 dimensional electrophoresis gels of injured cells and control cells were quite similar. We concluded that the high strain rate increased the protein phosphrylation. Also, from the comparison of gels and transillumated image of phosphorylated spots, a change of protein location on the gel was noted (Figure 4-9). The phosphorylated proteins migrated to higher molecular area and relatively neutral pH area (from 3.5 to 3.7).

Table 4-1. List of protein candidates.

% match: probability calculated by Profound program (the normalized probability that a protein in a database is the protein being analyzed based on data, experimental conditions and other background information).

Protein Candidate	Isoelectrical Point	Molecular Weight	Role in the cellular mechanism.
Cytoplasmic dynein light chain 28%match	5.0	12.66 kDa	A motor for retrograde axonal transport for movement to the neuronal cell body.
Human high mobility group protein (HMG-1) 12% match	5.6	25.05 kDa	Receptor for advanced glycation end products (RAGE) mediated neurite outgrowth.
Profilin II 14% match	5.8	15.41 kDa	Regulate actin, poly-L-proline and phosphatidylinositol 4, 5 bisphosphate ($PIP_{2)}$

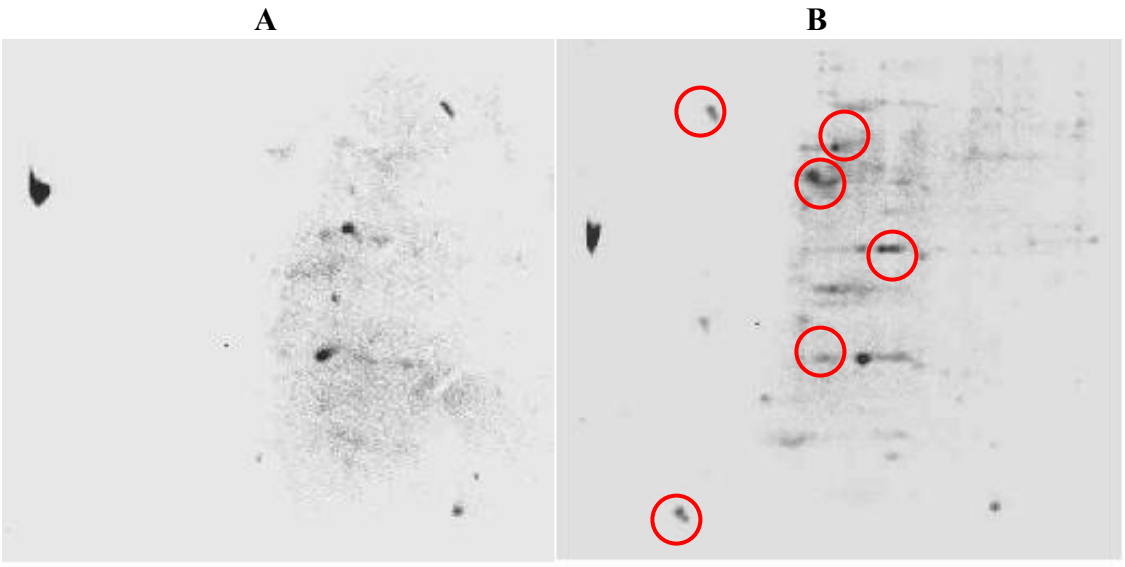

Figure 4-7. Reverse image of Pro-Q diamond stained phosphorylated proteins on 2 dimensional electrophoresis captured by transilluminator.

A: Control, B: Injured. Circled spots were newly phosphorylated after injury. The range of pI and molecular weight are 3 to 10 and 10,000 to 200,000.

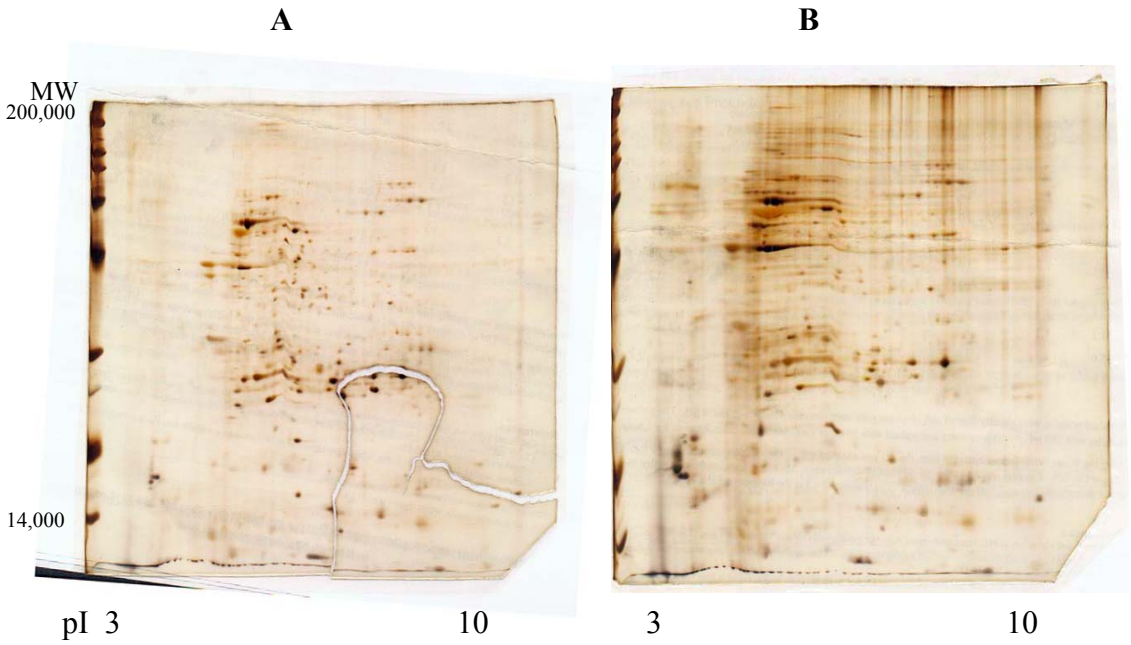

Figure 4-8. Comparison of silver stained 2 dimensional electrophoresis between control and injured after Pro–Q staining. A: control, B: injured.

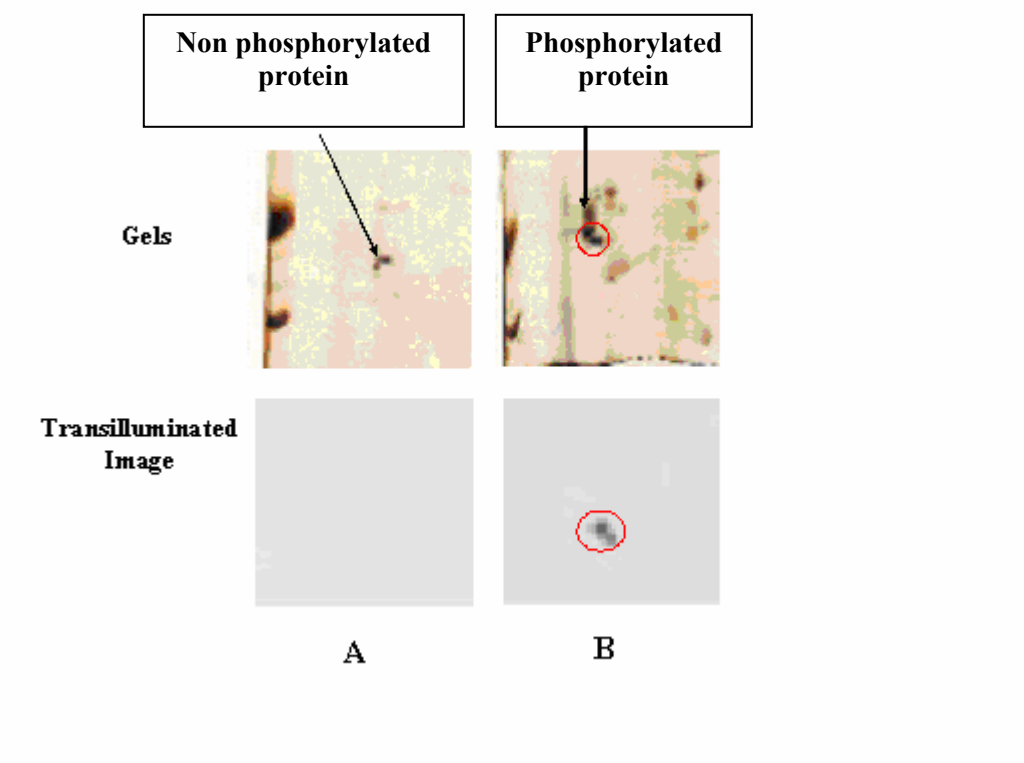

Figure 4-9. The change of protein location by phosphorylation.

A: Control, B: Injured. The molecular weights and isoelectric points of proteins were changed by phosphorylation. pI and molecular weight of phophorylated spot are 4.5 and 20,000.

Discussion

Proteomics tools have great advantages in exploring the protein synthesis and modification associated with disease. Traumatic brain injury is associated with activation of the apoptosis pathways in neurons (Pravdenkova et al., 1996; Yakovlev et al., 1997). In the apoptosis pathway, many proteins are involved (Sugimoto et al., 1994; Hay et al., 1994; Beidler et al., 1995; Datta et al., 1997). We performed 2 dimensional gel electrophoresis to characterize the changes of protein levels associated with our *in vitro* model of TBI. About 700 proteins were identified from the 2 dimensional gels from normal and injured cells. As seen in Figure 4-3, the protein patterns of control and normal cells were nearly identical. However, S^{35} labeled proteins in the gels exposed on X-ray films showed different patterns of protein synthesis. Similar results were obtained from the pulse chase experiments. From Figures 4-5, and 4-6, we identified cases of both upregulation of existing proteins and new protein synthesis after injury. Of all proteins detected, approximately 5% increased in expression after injury and about 1% were newly expressed (there was no detectable level of those proteins prior to injury). Jenkins and coworkers performed a similar proteomic study as ours on hippocampal protein from rat 24hours after moderate controlled cortical impact, an *in vivo* model of head injury (Jenkins et al., 2002). They found about 1,500 proteins on silver stained gel and from the spot matching, about 600 proteins were shown to be same. More than 80% of protein increased in protein expression. They found that changes in expressions of cytoskeleton proteins, cell signaling proteins, and phosphorylation of protein kianse B related proteins. However, in our experiments, we detected about half the number of proteins and saw increased expression in less than 10% of proteins. Differences in total proteins detected most likely reflect the quantity of protein loaded on the gel and other parameters associated with 2 dimensional gel electrophoresis. Differences in percentage of proteins upregulated probably reflect differences in cell population, time after injury, strain rate, and other parameters. Time after injury was 1 hour in our experiments. 24 hours after injury is as opposed to Jenkin's experiments. It is not unexpected that fewer proteins would be expressed early after injury. It is our hope that by identifying these

early proteins, that we will discover new avenues to block those changes that occur within injured cells that commit it to the apoptotic pathway. Differences in model systems of TBI could cause the different results of proteomic results. *In vivo* model, the complex interaction of tissue, blood supply, and oxygen limitation could play a pivotal role in protein expression and modification. However, in *in vitro* model, one may be able to isolate the effect of strain rate and time after injury. Since there are numerous differences between *in vivo* and *in vitro* models of TBI, the levels of protein expression and modification could be different.

In neuronal apoptosis, the role of cell cycle and cell migration related proteins were studied (Kaya et al., 1999; Lu et al., 2000; Barket et al., 2001; Walworth et al., 2001). The enhanced protein expression could be associated with the apoptosis or cell cycle regulation. However, the inhibition of protein synthesis led to a higher percentage of apoptotic cells after injury compared to injured cells where protein synthesis was not inhibited (Chapter III)

Thus, we concluded that excessive or non specific inhibition of protein expressions might be destructive to cells and/or that some protein expression after injury might be associated with repair. Some of the proteins that were synthesized within 1 hour in control cells were for normal maintenance.

There were at least 7 newly synthesized proteins after injury. The amino acid sequences of those proteins were not identified due to the low concentration of proteins on gels even though the levels of radioactivity of spots were higher than the normal spot intensities. These newly synthesized the proteinscould be associated with apoptosis and cell cycle regulation related because those proteins are not normally expressed or/and activated in differentiated neuron. To obtain anti-apoptotic inhibition of protein expression, not only can sequences of proteins be characterized but also the role of expressed protein should be identified.

From the analyses of mass spectroscopy results of highly expressed proteins after injury, we identified several protein candidates. Candidates are high mobility group protein 1 (HMG-1), cytoplasmic dynein, and profilin II. High mobility group protein-1

(HMG-1) is a chromosomal protein which can bind to DNA with histones. The molecular weight of HMG-1 is 25.05 kDa and its isoelectrical point is 5.6. HMG-1 has been reported in gene regulation and cellular differentiation (Huttune et al., 1999; Chou et al., 2001; Kamitani et al., 2001). HMG-1 is an abundant nuclear and cytoplasmic protein. One role of HMG-1 in neurons is as a receptor for advanced glycation end products (RAGE) mediated neurite outgrowth (Huttune et al., 1999; Kamitani et al., 2001). The receptor for advanced glycation end products is a member of the immunoglobulin superfamily of cell surface proteins interacting with a range of ligands. Receptor for advanced glycation end products is reported to play a role in neuronal injury by oxidative stress by activation of NF-kappaB (Huttune et al., 1999). Huttune and coworkers reported that transfection of RAGE to neuroblastoma cells induces extension of filopodia and neurites on amphoterin (a major form of HMG proteins) coated substrates and ligation of RAGE in transfected cells enhances NF-kappaB-dependent transcription. Both the RAGE-mediated neurite outgrowth and activation of NF-kappaB are blocked by deletion of the cytoplasmic domain of RAGE. Thus, HMG-1 appears to enhance the neuronal outgrowth and cellular differentiation. While the role of HMG-1 has not previously been reported to be associated with TBI, it could play a role in repair of axon or other neuritis after injury.

The second mechanism of HMG-1 is p53 mediated transcriptional activation as a transcriptional factor. p53 is a tumor suppressor and transcription factor. p53 regulates gene expressions of proteins such as Bax, Cyclin G, PCNA, and GADD45 which are associated with apoptosis prevention, cell cycle, nucleosome formation, and DNA damage inducible gene, respectively. Also, p53 promotes apoptosis and cell cycle arrest. Banerjee and coworkers found that HMG-1 induces p53 dependent apoptosis in HeLa cell (Banerjee and Kundu, 2003). However, inducement of p53 dependent apoptosis by HMG-1 in neuron has not been reported yet Thus, the role of HMG-1 as a transcriptional regulator in damaged neuron should be studied. *In vivo* and *in vitro* transcription, and DNA finger printing method using the p53 associated gene promoters

are desired to characterize the HMG-1 bound transcription associated factors during the neurodegeneration.

Third mechanism of HMG-1 is DNA binding protein as a chaperone without sequence specificity. The precise mechanism of HMG-1 is unclear. HMG-1 could facilitate the formation of nucleoprotein complex. In our experiment, HMG-1 was highly expressed 1 hour later after injury. Since HMG-1 has various activities in cells. To identify the role of HMG-1 associated with chaperone activity in TBI, the study of high expression of HMG-1 gene in neuron by transfection should be conducted to detect the effect of enhanced HMG-1 expression, or HMG-1 gene disruption experiment to examine the nucleosome(DNA and histon complex) stability after injury because the high or/and low expression of HMG-1 could change the stability of nucleosome after injury.

We could propose that HMG-1 could be related with salvation pathway of injured neuron by repairing the damaged neurite or establishing the lost connection and/ apoptosis activation in neuronal cells via enhancing the p53. If the high expression of HMG-1 in neuron causes the apoptosis, HMG-1 could be associated with p53 activation. However, if the high expression of HMG-1 in neuron enhances neurite outgrowth, HMG-1 could be associated with neurite repair or/and regeneration. In future experiments,we could block the expression of HMG-1 during injury either with anti sense or siRNA, and then examine the effect on cell apoptosis in our model. If HMG-1 playing repair role, the inhibition of its expression should increase the level of injury. While if its expression is associated with apoptosis, then blocking its expression should lower the level of cell apoptosis.

One of the other possible candidates is cytoplasmic dynein light chain. Neuron has a cell body and axons. During the axon formation, axonal proteins are transported (anterograde transport) because most of neuronal proteins are synthesized in cell body. In anterograde movements, membrane organelles, microtubules, and kinesins are involved. In retrograde transport, for example, transport of nerve growth factors from the axon terminal to the cell body, cytoplasmic dynein and microtubule motors are

needed. Cytoplasmic dynein is expressed in the cell body and is transported to axon terminal in inactive form. The activation of cytoplasmic dynein is associated with the phosphorylation of dynein subunits. Cytoplasmic dynein has two cytoplasmic dynein heavy chains, intermediate, light intermediate, and light chains. Cytoplasmic dynein also plays a role in nuclear migration during axon growth and microtubule organization. Cytoplasmic dynein has been reported to be one of key components of motor neuron generation. From the knock-out mouse experiments, mutations of the cytoplasmic dynein heavy-chain protein caused motor neuron degeneration (Andersen, 2003). Li and coworkers performed the scanning analysis of crush-operated rat sciatic nerves. After injury, cytoplasmic dynein synthesis showed that most of the dynein moved in the anterograde direction and proximal accumulation of dynein increased rat sciatic nerves (Li et al., 2000). In our experiments, cytoplasmic dynein light chain was expressed highly 1 hour later after injury. From the roles of cytoplasmic dynein, the high expression of cytoplasmic dynein could be associated with the formation and maintenance of damaged synapses by after injury. To identify the role of cytoplasmic dynein in TBI, dynein driven movement of nerve growth factors, and microtubules should be studied after injury. Also, genetic level study of cytoplasmic dynein gene should be performed to identify the transcriptional activation pathway.

Another protein candidate is profilin II. Profilin is a ubiquitous actin binding protein found in animal cells, plants, and viruses. There are two types of profilins, profilin I and profilin II. They are thought to regulate actin polymerization in response to extracellular signals. Profilin II is a 15.41k Da and was a pI of 5.8. Profilin has the ability to bind actin, poly-L-proline and phosphatidylinositol 4, 5 bisphosphate (PIP_2) (Kneussel and Betz, 2000). In mice, profilin I is expressed ubiquitously while profilin II is expressed at high levels only in brain (Witke et al., 1998). In mice brain, profilin I and profilin II can form complexes with regulators to conduct endocytosis, synaptic vesicle recycling and actin assembly. Profilin I and profilin II can bind to dynamin I, clathrin, synapsin, Rho-associated coiled-coil kinase, the Rac-associated protein NAP1 and a member of the NSF/sec18 family. *In vivo*, profilins binds to dynamin I and

synapsin in axonal and dendritic processes. Because of its affinity for phosphatidylinositol 4,5-bisphosphate, profilin also plays a role in the signal transduction pathway through phospholipase C. One class of molecules in the brain profilin complexes appears to play a role in signaling through the small G proteins Rac and Rho. ROCK 2 which can bind to profilin is a Rho-associated kinase. The targets of ROCK2 are not well understood. Recent biochemical studies have suggested that profilin plays a key role in promoting actin assembly in the plasma membrane that makes cell motility and other actin-linked processes (Tojo et al., 2003). In our experiment, the profilin II expression was enhanced after injury. Due to biological characteristics of profilin II, even though the role of profilin II is not clear in neuron, profilin II could be associated with the actin polymerization of cellular structure and/or axonal regeneration in damaged neuron. To elucidate the role of profilin II in TBI, a profilin knock-out cell line or anti-sense profilin II gene disruption could be useful in neuronal injury. If profilin II plays a role in neurodegeneration after TBI, profilin II deficient cell line would show the less neuron apoptosis.

Phosphorylation is an important protein modification that is associated with changes in protein activity or function. Tau phosphorylation in Alzheimer disease is one of the examples of hazardous phosphorylation to cells (Kobayashi et al., 2003). In our experiment, the phosphorylated protein detection (shown in Figure 4-7) showed that there were higher levels of phosphorylation of proteins in injured cells than in control cells. One possibility is that the increase in phosphorylation is associated with anti-apoptosis. Recently, some papers reported that anti-apoptotic activity of phosphorylation of the serine-threonine kinase, Akt, in mice (Noshita et al., 2002; Martin et al., 2003). The serine-threonine kinase, Akt, has been reported in the survival signaling pathways in many cell systems. Noshita and coworkers examined the Akt and apoptosis after TBI in mice. Immunohistochemistry showed phospho-Akt was decreased in the injured brain 1 h after TBI. Expression of Akt was significantly decreased 1 hour after TBI. However, the phosphorylation of Akt was increased 4 hours after injury. Furthermore, from the overlapping of apoptosis and cell cycle regulation,

checkpoint kinase 1 was investigated in DNA replication and damage checkpoints (Bartek and Lukas, 2001). Checkpoint kinase 1 appears to have anti-apoptotic effects by repairing the damaged cellular structures. Another possible activator of phosphorylation is retinoblastoma tumor suppressor (Rb). Di Giovanni and coworkers checked Rb mRNA expression in spinal cord injury (Di Giovanni et al., 2003). DNA micro array and Western blots showed high level mRNA of Rb and E2F was expressed after injury. Cell cycle related proteins were expressed over 24 hours after injury.

Another possibility of increased phosphorylation is apoptosis associated phosphorylation. It is probable that the protein phosphorylation observed associated with calcium dependent phosphorylation. The elevated intracellular calcium levels seen in TBI could be caused by physical damage to the cell membrane, receptor mediated glutamate toxicity, import from the voltage gated calcium channel, and release from the endoplasmic reticulum through the phosphoinositol pathway (Floyd et al., 2001). Other activators of phosphorylation by calcium are p38 MAP kinase and c-Jun kinase. Calcium uptake by NMDA receptors could cause the activation of p38, MAP kinase and c-Jun kinase (Graham et al., 1993, Vexler and Ferriero, 2001). p38, MAP kinase and c-Jun kinase are considered as transcriptional regulator of apoptosis related gene expression. Mitogen activated protein kinase (MAP), ERK, c-Jun *N*-terminal kinase (JNK), and p38 are known to play a critical role in the regulation of cell growth and differentiation. MAP phosphorylates other intracellular enzymes and transcription factors. Noshita and coworkers measured ERK phosphorylation after transient focal cerebral ischemia using transgenic mice that express copper/zinc superoxide dismutase. In transgenic mice, phosphor ERK was reduced compared with control mice. It showed that phosphorylation of ERK could activate apoptosis after transient focal cerebral ischemia. Also, calcium dependent phosphorylations of proteins are associated with the activation of apoptotic pathway. In high strain rate injury, Floyd and coworkers found that inositol polyphosphate levels in an *in vitro* model of TBI, and inhibition of activation of PLC led a decrease in neurodegeneration (Floyd et al., 2001). Also, some experiments showed that during an apoptotic process, some proteins are phosphorylated

to activate apoptosis (Lei and Davis, 2003; Martin et al., 2003; Xu et al., 2003). One of the phosphorylation related cellular signaling pathway in TBI is cell cycle dependent phosphorylation. Martin et al examined the target deprivation and axotomy-induced apoptosis in rat. They found high phosphorylation of p53 after a neuronal injury. Phosphorylation of p53 in the target deprivation and axotomy-induced apoptosis in the rat model were detected 4 days after injury and the Akt phosphorylation was detected 4 hours after injury (Noshita et al., 2002, Martin et al., 2003).

Thus, the effects of phosphorylation of proteins in TBI could have anti-apoptotic and apoptotic effects. Moreover, the phosphorylated proteins we found on the gel have special significance because those proteins were phosphorylated within 1 hour after the injury. It could determine the fate of damaged neurons. Study of early phosphorylations of proteins by mechanical stress could reveal neuronal responses to injury that are part of restoration pathway and/or death pathway. However, the amino acid sequences of phosphorylated proteins were not identified due to the low protein concentration. Our results indicate that mechanical insult can activate phosphorylases (kinases) and phosphorylation is associated cell injury.

We demonstrated that high strain rate injury can activate protein expression and phosphorylation of proteins after injury. Both new proteins and currently existing proteins were expressed following high strain rate nerve injury. Moreover, phosphorylated proteins were expressed highly after the high strain rate nerve injury. The roles of highly expressed proteins and effects of phosphorylation after the injury should be characterized. We believe that this work will contribute to the discovery of new the therapeutic targets and better treatments for TBI.

CHAPTER V
OVERALL CONCLUSION AND FUTURE WORK

Traumatic brain injury (TBI) is one of the major public health problems in the United States. Traumatic brain injury is associated with the mechanical displacement of brain tissues and acceleration of a head. Mechanical insult associated with TBI causes changes in cellular events and damages cellular structures. Numerous models of TBI have been developed to characterize the effects of mechanical displacement of the brain. Various studies have suggested that mechanical insult to the neuron may cause cell death. Nonetheless, appropriate approaches to treat or prevent the neurondegeneration in TBI remain obscure due to the incomplete understanding of the biochemical mechanism of TBI. Thus, an *in vitro* model of TBI can give us crucial information for upgrading the understanding of the mechanism of TBI at both the cellular and molecular levels. In this chapter, we summarize our experimental results and propose future work. This work could be valuable to complete our understanding of the mechanism of neurodegeneration by TBI.

Conclusion

Development of a device for high strain rate injury to neurons and neuron-like cells. Numerous cell stretching devices have been developed. The evaluated strain rates associated with TBI range from 1 to $5s^{-1}$, or about 10% strain in 20 to 100 ms. Precise control of mechanical insult to neuron will be useful in elucidating the elaborate pathological cascade associated with TBI. The *in vitro* high strain rate application device developed at Cellular Engineering Lab at TAMU was designed to ensure precise control of mechanical deformation parameters in the *in vitro* model of TBI. The high strain rate application device had several advantages over other devices including: the production of a relatively linear and homogeneous strain of the membrane, an optical window that allowed direct microscopic observation of the cells during injury, a simple

way to change strain of membrane, and the ability to change the strain rate in the device for both low strain rate injury and high strain rate injury. The relatively linear and homogeneous strain of the membrane could produce a uniform of high strain injury of neuron. The optical window of a planar cylinder could be used as a proficient way to study the cell behavior during the cell death. The strain of the membrane was controlled by adding a rubber plate between the flexible bottomed plate and the plate loading deck.

We were unable to measure strain rate in real time because of technical difficulty with the CCD camera. Although frames of membrane profile of CCD camera during the high strain application showed that the membrane touched the cylinder, actual deformation profiles of membrane have not been recorded. Indeed, there is a nonlinear strain area on the outside of the membrane. Thus, the actual linear and homogeneous area of membrane should be calculated. Furthermore, in order to get a precise control of strain, the vacuum level of pump should be controlled because the subtle change of vacuum level might cause different strain or strain rate of the membrane.

High tensile strain rate leads to apoptosis in SH-SY5Y cells. *In vitro* models of TBI have great advantages over *in vivo* models in their ability to allow the investigator characterize the cellular and molecular level of neurodegeneration. We used an *in vitro* model of high strain rate mechanical injury to mimic the mechanical insult that occurs during TBI. High strain rate of differentiated SH-SY5Y cells triggered the apoptosis within 1 hour after injury. From the flow cytometry analyses, the cell population in the G1 area was increased after injury, and the cell populations gradually moved to G1 area over hours. This change could be associated with the activation of cell cycle related kinases after injury. To understand the affiliation of cell cycle associated mechanisms and apoptosis in TBI, effects of cell cycle dependent kinases should be examined by blocking the specific cell cycle stage. Also, since cell cycle kinases could be associated with expression of various genes in apoptosis inhibition and activation, cell cycle kinases dependent gene expressions after TBI should be analyzed.

Apoptosis was observed in cells within 30 minute after injury. The fraction of apoptotic cells after injury was relatively constant over the 24 hours and the fraction of apoptotic cells increased immediately after the injury. The percentage of apoptotic cells did not change significantly at times greater than 1 hour after mechanical insult. Also, we found that apoptotic cells during high strain rate injury were triggered by the initial mechanical impact but in low strain injury, the percentage of apoptotic cells showed a steady raise with time. This effect might be associated with the different application time of injury or the different mechanical insult. Apoptotic cell death should be mathematically modeled to predict the effect of mechanical insult on cell death and to find when after injury apoptosis prevention might be most successful.

In TBI, it is believed that apoptosis is activated by the caspases pathaway. The caspases inhibition experiments showed that caspase 1 and 3 were involved in the high strain rate injury of neurons. Experiments blocking caspase 1 and 3 expression at early stages after injury should be performed because caspase 1 and 3 can be activated by different pathways in the early stages of apoptosis.

The inhibition of G protein activation, calcium entry, and protein synthesis could not decrease the fraction of apoptotic cells after the high strain rate injury. However, the inhibition of phospholipase C activation by Neomycin decreased the apoptotic cells in high and low strain rate injuries. The role of PLC pathway is not well characterized in TBI. The anti-apoptotic activity of Neomycin in high strain rate injury might be associated with a different mechanism than that involved in low strain rate injury. To identify the precise G protein signaling and PLC pathway in TBI, the activities of G protein and PLC should be measured with time and apoptosis after TBI. Also, cell cycle dependent diacylglycerol (DAG) and PLC activation should be studied after TBI because the substrate of DAG is phospholids in the cell membrane. Thus, the cell division or membrane disruption could be associated with DAG formation.

To identify the intracellular calcium increase and calcium import from outside associated with apoptosis after TBI, calcium imaging experiments are needed, along with measuring the mRNA levels of apoptotic related genes. The gene array and

calcium imaging data could provide better understanding of calcium associated gene expression in TBI. Moreover, calcium dependent phosphorylation in the cell should be characterized coupled with inhibition experiments of phosphorylation after TBI because the intracellular calcium could be elevated not by importing calcium from outside but by releasing calcium from endoplasmic reticulum. Activation of PLC is the key component of calcium release from endoplasmic reticulum.

Protein synthesis inhibition by a nonspecific translation blocking agent resulted in an increase in the fraction of apoptotic cells after TBI. The increase in apoptosis after protein synthesis inhibition could be associated with blocking the essential protein expression for cell maintenance, and/or anti-apoptotic protein expression. From the proteomic study, the protein expression increased after injury. To decrease the level of apoptosis after TBI, inhibition of translation of specific apoptosis related genes would be required. Also, random protein expression could block the gene expression of salvation or restoration pathway of mechanically damaged neurons.

Our experiment showed slightly different results than other researchers have reported. In order to find alternative perspectives to understand the mechanism of mechanical damage in neurons, it should be investigated how neurons maintain their structure after damage and how the repair mechanism in damaged neurons is associated with apoptosis. Since most TBI researchers have focused on aspects of neurodegeneration, understanding of repair mechanisms associated with TBI could provide us with novel therapeutic stratagems.

Characterization of protein synthesis and phosphorylation after nerve injury induced by high strain. Proteomics study of diseases is one of the most promising methods to find new therapeutics. Many proteins are involved in the neuronal apoptosis pathway. We performed a proteomic study to characterize the protein expression after the injury. The metabolic labeling of injured cells and normal cells and pulse chase experiments indicated that normal and injured cells had different patterns on 2 dimensional gels. The protein synthesis was triggered within 1 hour after an injury.

10% of total proteins were high expressed after injury and some proteins (about 1%) were newly synthesized after injury. 7 newly synthesized proteins were detected. Mechanical injury of neuron enhanced the protein expression. However, the roles of newly expressed proteins were not identified. To identify the role of newly synthesized proteins, genomic studies and higher level of protein loading during the proteomics study might be useful. Protein levels of newly synthesized proteins were very low, and difficult to identify, both because of their abundance and because of the resolution of the mass spectrometer available. Also, even though mechanical insult to neurons caused apoptosis, the roles of newly synthesized and highly expressed proteins might be associated with anti-apoptosis or apoptosis. The coupled experiments of apoptosis inhibition and proteomics study could give us the crude roles of newly synthesized and highly expressed proteins. If some newly synthesized proteins are related to apoptosis, the concentrations of those proteins will be increased.

From the mass spectroscopy analysis, several highly expressed proteins, high mobility group protein (HMG-1), cytoplasmic dynein light chain, and profilin II, were identified. Those proteins were expressed highly after injury. The first candidate is high mobility group protein I. High mobility group protein (HMG-1) plays various roles in cellular mechanism associated with transcription, DNA stability, chaperone, and differentiation. Due to the diverse roles of HMG-1, it is quite difficult to ascertain the specific role of HMG-1 in neurodegeneration by mechanical insult. However, high expression of HMG-1 could imply that neurodegeration by mechanical insult might activate assorted cellular mechanisms. The second candidate is cytoplasmic dynein light chain. Cytoplasmic dynein plays a pivotal role in nuclear migration during the axon growth, axon terminal receptor, and microtubules organization. Cytoplasmic dynein is synthesized in neuronal cell body and it is transported to axon terminal. High expression of cytoplasmic dynein light chain could be involved in the restoration process of the damaged neuronal terminal since the role of cytoplasmic dynein is associated with the transport mechanism of axonal terminal. The third candidate is profilin II. Profilins are ubiquitous actin binding proteins found in animal cells, plants, and viruses. Also, in

brain, profilin complexes appear to play a role in G protein signaling. Due to the complex role of profilin II, the detailed profilin II associated with the mechanism of injury (or repair) could not be characterized. However, by the inhibition of profilin II gene using anti sense method, the role of prpfilin II could be characterized.

Phosphorylation is a crucial protein modification associated with function and activity of proteins in cells. Phosphorylation of a protein can occur without expression of a specific gene. Moreover, some phosphorylation of proteins could activate the cellular apoptotic pathway. In our experiments, we showed that higher levels of phosphorylated proteins were detected in injured cells than in control cells within 1 hour after injury. This result implied that mechanical insult could activate phosphorylases (kinases) associated with neuronal injury. However, the phosphorylated proteins were not identified due to the low amount of protein on gels and the resolution of the available mass spectrometer. To identify the phosphorylation activation pathway in injured cells, inhibition of apoptosis and the cell cycle related pathway should be performed.

Much research has been performed to elucidate the mechanism of injury in *in vitro* models of TBI. The *in vitro* models of TBI have been developed to characterize the process of neurodegeneration in cellular level. We demonstrated that high strain rate could activate apoptosis in differentiated SH-SY5Y cells. We found the possible candidates of the inhibition of apoptosis after injury. Proteomics study showed that protein expression and phosphorylation of proteins after injury were elevated. Both new proteins and currently existing proteins were expressed by high strain rate nerve injury. Though the newly synthesized proteins were not characterized due to the low protein concentration, high mobility group protein (HMG-1), cytoplasmic dynein light chain, and profilin II were identified by analyzing the highly expressed proteins after injury. The phosphorylated proteins were expressed highly after injury. We believe that this work will contribute to the discovery of new therapeutic targets and better treatments for TBI.

REFERENCES

Adams JM, Cory S. 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281:1322-1326.

Aldman B. 1986. An analytical approach to the impact biomechanics of head and neck injury. Ann Conf Am Asso Auto Med 439-454.

Allen JW, Knoblach SM, Faden AI. 1999. Combined mechanical trauma and metabolic impairment *in vitro* induces NMDA receptor-dependent neuronal cell death and caspase-3-dependent apoptosis. FASEB J 13:1875-1882.

Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J. 1996. Human ICE/CED-3 protease nomenclature. Cell 87:171.

Andersen J. 2003. Defects in dynein linked to motor neuron degeneration in mice. Sci Aging Knowl Environ 18:PE10.

Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera P. 1995. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 15:961-973.

Ashkenazi A, Dixit VM. 1998. Death receptors: signaling and modulation. Science 281:1305-1308.

Bandak FA, Vander Vorst MJ, Stuhmiller LM, Mlakar PF, Chilton WE, Stuhmiller JH. 1995. Animaging-based computational and experimental study of skull fracture: finite element model development. J Neurotrauma 12:679-688.

Banerjee S, Kundu TK. 2003. The acidic C-terminal domain and A-box of HMGB-1 regulates p53-mediated transcription. Nucleic Acids Res 31:3236-347.

Banes AJ, Gilbert J, Taylor D, Monbureau O. 1985. A new vacuum-operated stress-providing instrument that applies static or variable duration cyclic tension or compression to cells *in vitro*. J Cell Sci 75:35-42.

Barkats M, Millecamps S, Bilang-Bleuel A. Mallet J. 2002. Neuronal transfer of the human Cu/Zn superoxide dismutase gene increases the resistance of dopaminergic neurons to 6-hydroxydopamine. J Neurochem 82:101-9.

Bartek J, Lukas J. 2001. Mammalian G_1- and S-phase checkpoints in response to DNA damage. Curr Opin Cell Biol 13:738–747.

Beckman JS, Koppenol WH. 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 271:C1424–C1437.

Beidler DR, Tewari M, Friesen PD, Poirier G, Dixit VM. 1995. The baculovirus p35 protein inhibits Fas- and tumor necrosis factor-induced apoptosis. J Biol Chem 270:16526-16528.

Biedler JL, Kelson L, Spengler BA. 1973. Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. Cancer Res 33: 2643-2653.

Berti R, Williams AJ, Moffett JR, Hale SL, Velarde LC, Elliott PJ, Yao C, Dave JR, Tortella FC. 2002. Quantitative real-time RT-PCR analysis of inflammatory gene. J Cereb Blood Flow Metab 22:1068-79.

Blomgren K, Hallin U, Andersson AL. 1999. Calpastatin is up-regulated in response to hypoxia and is a suicide substrate to calpain after neonatal cerebral hypoxia-ischemia. J Biol Chem 274:14046–14052.

Bonfanti L, Strettoi E, Chierzi S, Cenni MC, Liu XH. 1996. Protection of retinal ganglion cells from natural and axotomyinduced cell death in neonatal transgenic mice overexpressing bcl-2. J Neurosci 16:4186–4194.

Boucher JL, Moali C, Tenu JP. 1999. Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for L-arginine utilization. Cellu Mol Life Sci. 55:1015-1028.

Boulanger L, Poo MM. 1999. Presynaptic depolarization facilitates neurotrophin induced synaptic potentiation. Nat. Neurosci. 2:346–351.

Bradshaw DRS, Ivarsson J, Morfey CL, Viano DC. 2001. Simulation of acute subdural hematoma and diffuse axonal injury in coronal head impact. J Biomech 34: 85-94.

Castagne V, Lefevre K, Clarke PG. 2001. Dual role of the NF-kappaB transcription factor in the death of immature neurons. Neurosci 108:517-521.

Castagne V, Clarke PG. 1999. Inhibitors of mitogen-activated protein kinases protect axotomized developing neurons. Brain Res 842:215-219.

Centers for Disease Control and Prevention (CDC), National Center for Injury Prevention and Control. 1999. Traumatic brain injury in the United States. Report to Congress. Atlanta GA.

Chang CH, Chang LT, Chang GL, Huang SC, Wang CH. 2000. Head injury in facial impact--a finite element analysis of helmet chin bar performance. J Biomech Eng 122:640-6.

Chao DT, Linette GP, Boise LH, White LS, Thompson CB, Korsmeyer SJ. 1995. Bcl-XL and Bcl-2 repress a common pathway of cell death. J Exp Med 182:821-828

Choi DW. 1995. Calcium: still center-stage in hypoxic-ischemic neuronal death. Trends Neurosci 18:58–60.

Choi DW. 1994. Glutamate receptors and the induction of excitotoxic neuronal death. Prog Brain Res100:47-51.

Chou DK, Evans JE, Jungalwala FB. 2001. Identity of nuclear high-mobility-group protein, HMG-1, and sulfoglucuronyl carbohydrate-binding protein, SBP-1, in brain. J Neurochem 77:120-131.

Chu C, Lin M, Huang H, Lee M. 1994. Finite element analysis of cerebral contusion. J Biomech. 27:187-194.

Conti AC, Raghupathi R, Trojanowski JQ, McIntosh TK. 1998. Experimental brain injury induces regionally distinct apoptosis during the acute and delayed post-traumatic period. J Neurosci 1:5663-5672.

Craig BF. Clark B. 2001. Uniaxial strain system to investigate strain rate regulation *in vitro*. 2001. Rev. Sci. Instrum. 72: 2415-2419.

Cui Q, Harvey AR. 1995. At least two mechanisms are involved in the death of retinal ganglion cells following target ablation in neonatal rats. J Neurosci 15:8143–8155.

Datta R, Kojima H, Yoshida K, Kufe D. 1997. Caspase-3-mediated cleavage of protein kinase C theta in induction of apoptosis. J Biol Chem 272:20317-20320.

Decker ML, Janes DM, Barclay MM, Harger L, Decker RS. 1997. Regulation of adult cardiocyte growth: effects of active and passive mechanical loading. Am J Physiol 272:2902-2918.

Di Giovanni S, Knoblach SM, Brandoli C, Aden SA, Hoffman EP, Faden AI. 2003. Gene profiling in spinal cord injury shows role of cell cycle in neuronal death. Ann Neurol 53:454-468.

Dirnagl U, Iadecola C, Moskowitz MA. 1999. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22:391–397.

Donna M. Geddes J. 2001. An *in vitro* model of neural trauma: device characterization and calcium response to mechanical stretch. Biomech Eng 123: 247-280.

Dugan LL, Choi DW. 1994.Excitotoxicity, free radicals, and cell membrane changes. Ann Neuro. 35:S17-21.

Ellis EF, McKinney JS, Willoughby KA, Liang S, Povlishock JT. 1995. A new model for rapid stretch-induced injury of cells in culture: characterization of the model using astrocytes. J Neurotrauma 12:325–339.

Faden AI, Demediuk P, Panter SS, Vink R. 1989. The role of excitatory amino acids and NMDA receptors in traumatic brain injury. Science. 244:798-800.

Ferriero DM, Sheldon RA, Black SM. 1995. Selective destruction of nitric oxide synthase neurons with quisqualate reduces damage after hypoxia-ischemia in the neonatal rat. Pediatr Res 38:912–918.

Ferriero DM, Soberano HQ, Simon RP. 1990. Hypoxia-ischemia induces heat shock protein-like (HSP72) immunoreactivity in neonatal rat brain. Brain Res Dev Brain Res 53:145–150 1996.

Fiebich BL, Butcher RD, Gebicke-Haerter PJ. 1998. Protein kinase C-mediated regulation of inducible nitric oxide synthase expression in cultured microglial cells. J Neuroimmun 92:170-8.

Fink KB, Andrews LJ, Butler WE, Ona VO, Li M. Bogdanov M, Endres M, Khan SQ,

Namura S, Stieg PE, Beal MF, Moskowitz MA, Yuan J, Friedlander RM. 1999. Reduction of post-traumatic brain injury and free radical production by inhibition of the caspase-1 cascade. Neurosci 94:1213-8.

Fleming I, Busse R. 1999. Signal transduction of eNOS activation. Cardiovas Res 43:532-541.

Fletcher GC, Xue L, Passingham SK, Tolkovsky AM. 2000. Death commitment point is advanced by axotomy in sympathetic neurons. J Cell Bio 150:741-754.

Floyd CL, Rzigalinski BA, Weber JT, Sitterding HA, Willoughby KA, Ellis EF. 2001. Traumatic injury of cultured astrocytes alters inositol (1,4,5)-trisphosphate-mediated signaling. Glia. 33:12-23.

Fraser A, McCarthy N, Evan GI. 1996. Biochemistry of cell death. Curr Opin Neurobiol 6:71–80.

Fukuda H, Nishida A, Saito H, Shimizu M, Yamawaki S. 1994. Imipramine stimulates phospholipase C activity in rat brain. Neurochem Int 25:567-571.

Galbraith JA, Thibault LE, Matteson DR. 1993. Mechanical and electrical responses of the squid giant axon to simple elongation. J Biomech Eng 115:13-22.

Gennarelli TA. 1996. The spectrum of traumatic axonal injury. Neuropath Appl Neurobiol 22:509-513.

Gennarelli TA. 1983. Head injury in man and experimental animals: clinical aspects. Acta Neurochem 32:1-13.

Gennarelli TA, Thibault LE, Adams JH, Graham DI, Thompson CJ, Marcincin RP. 1982. Diffuse axonal injury and traumatic coma in the primate. Ann Neuro. 12:564-574.

Gibson L, Holmgreen SP, Huang DC, Bernard O, Copeland NG, Jenkins NA, Sutherland GR, Baker E, Adams J,. Cory S. 1996. bcl-w, a novel member of the bcl-2 family, promotes cell survival. Oncogene 13:665-675.

Golding EM, Contant CF Jr, Robertson CB, Bryan RM. 1998. Temporal effect of severe controlled cortical impact injury in the rat on the myogenic response of the middle cerebral artery. J Neurotrauma 15:973-984.

Goldsmith W. 1985. Critical impact and acceleration loading to the head. Physiologist 28:S21-24.

Graham SH, Chen J, Sharp FR. 1998. Limiting ischemic injury by inhibition of excitatory amino acid release. J Cereb Blood Flow Metab 13:88–97.

Greensmith H, Hasan I, Vrbová G. 1994. Nerve injury increases the susceptibility of motoneurons to N-methyl-D-aspartate-induced neurotoxicity in the developing rat. Neurosci 58:727–733.

Gustafson K, Hagberg H, Bengtsson BA. 1999. Possible protective role of growth hormone in hypoxia-ischemia in neonatal rats. Pediatr Res 45:318–323.

Haan JB, Newman JD, Kola I. 1992. Cu/Zn superoxide dismutase mRNA and enzyme activity, and susceptibility to lipid peroxidation, increases with aging in murine brains. Brain Res Mol Brain Res 13:179–187.

Han BH, Holtzman DM. 2000. BDNF protects the neonatal brain from hypoxic-ischemic injury *in vivo* via the ERK pathway. J Neurosci 20:5775–5781.

Hay BA, Wolff T, Rubin GM.1994. Expression of baculovirus P35 prevents cell death in *Drosophila*. Development 120:2121-2129.

Homayoun P, Rodriguez de Turco EB, Parkins NE, Lane DC, Soblosky J, Carey ME, Bazan NG. 1997. Delayed phospholipid degradation in rat brain after traumatic brain injury. J Neurochem 69:199-205.

Huang Q, Tatro JB. 2002. Alpha-melanocyte stimulating hormone suppresses intracerebral tumor necrosis factor-alpha and interleukin-1beta gene expression following transient cerebral ischemia in mice. Neurosci Lett 334:186-190.

Hubbard RP, Melvin JW, Barodawala IT. 1971. Flexure of cranial sutures. J Biomech 4:491-496.

Huelke DF, Nusholtz GS. 1986. Cervical spine biomechanics: a review of the literature. J Ortho Res 4:232-245.

Huttunen HJ, Kuja-Panula J, Sorci G, Agneletti AL, Donato R, Rauvala H. 1999. Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF-kappaB require the cytoplasmic domain of the

receptor but different downstream signaling pathways. J Biol Chem 274:19919-19924.

Igarashi T, Huang TT, Noble LJ. 2001. Regional vulnerability after traumatic brain injury: gender differences in mice that overexpress human copper, zinc superoxide dismutase. Exp Neuro 172:332-41.

Ikonomidou C. 1999. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283:70–74.

Inglis FM, Furia F, Zuckerman KE, Strittmatter SM, Kalb RG. 1998. The role of nitric oxide and NMDA receptors in the development of motor neuron dendrites. Neurosci 18:10493-501.

Ito M, Spatz J, Walker T, Klatzo I. 1975. Experimental cerebral ischemia in Mongolian gerbils. I. Light microscopic observations. Acta Neuropathol. 32:209–223.

Ivarsson J, Viano DC, Lovsund P, Aldman B. 2000. Strain relief from the cerebral ventricles during head impact: experimental studies on natural protection of the brain. J Biomech 33:181-189.

Iwasaki Y, Ikeda K, Shiojima T, Kinoshita M. 1995. CNQX prevents spinal motor neuron death following sciatic nerve transection in newborn rats. 1995. J Neurol Sci 134:21–25.

Jenkins LW, Peters GW, Dixon CE, Zhang X, Clark RS, Skinner JC, Marion DW, Adelson PD, Kochanek PM. 2002. Conventional and functional proteomics using large format two-dimensional gel electrophoresis 24 hours after controlled cortical impact in postnatal day 17 rats. J Neurotrauma 19:715-740

Kamitani W, Shoya Y, Kobayashi T, Watanabe M, Lee BJ, Zhang G, Tomonaga K, Ikuta K. 2001. Borna disease virus phosphoprotein binds a neurite outgrowth factor, amphoterin/HMG-1. J Virol 75:8742-8751.

Kawasaki D. 1997. Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells. J Biol Chem 272:18518–18521.

Kirschner PB, Jenkins BG, Schulz JB. 1996. NGF, BDNF and NT-5, but not NT-3 protect against MPP+ toxicity and oxidative stress in neonatal animals Brain Res 713:178–185.

Kleiven S, von Holst H. 2002. Consequences of head size following trauma to the human head. J Biomech 35:153-160.

Kneussel M, Betz H. 2000. Clustering of inhibitory neurotransmitter receptors at developing postsynaptic sites: the membrane activation model. 2000. Trends Neurosci 23:429-435.

Knoblach SM, Nikolaeva M, Huang X, Fan L, Krajewski S, Reed JC, Faden AI. 2002. Multiple caspases are activated after traumatic brain injury: evidence for involvement in functional outcome. J Neurotrauma 19:1155-1170.

Kobayashi K, Nakano H, Hayashi M, Shimazaki M, Fukutani Y, Sasaki K, Sugimori K, Koshino Y. 2003. Association of phosphorylation site of tau protein with neuronal apoptosis in Alzheimer's disease. J Neurol Sci 208:17-24.

Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW. 1993. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc Nat Acad Sci USA 90:3516-3520.

Krabbel G, Appel H. 1995. Development of a finite element model of the human skull. J Neurotrauma 12:735-742.

LaPlaca MC. Thibault LE. 1998. Dynamic mechanical deformation of neurons triggers an acute calcium response and cell injury involving the N-methyl-D-aspartate glutamate receptor. J Neurosci Res 52:220-229.

Laplaca MC, Lee VM, Thibault LE. 1997. An *in vitro* model of traumatic neuronal injury: loading rate-dependent changes in acute cytosolic calcium and lactate dehydrogenase release. J Neurotrauma 14:355-368.

LaPlaca MC, Thibault LE. 1997. An *in vitro* traumatic injury model to examine the response of neurons to a hydrodynamically-induced deformation. Ann Biomed Eng 25:665-677.

Lee MC, Haut RC. 1989. Insensitivity of tensile failure properties of human bridging

veins to strain rate: implications in biomechanics of subdural hematoma. J Biomech 22:537-542.

Lei K, Davis RJ. 2003. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax- dependent apoptosis. Proc Nat Acad Sci USA 100:2432-2437.

Leski ML, Valentine SL, Coyle JT. 2000. L-type voltage-gated calcium channels modulate kainic FLi JY, Pfister KK, Brady ST, Dahlstrom A. Cytoplasmic dynein conversion at a crush injury in rat peripheral axons. J Neurosci Res 15:151-61.

Lieberman AR. 1971. The axon reaction: A review of the principal features of perikaryal responses to axon injury. Int Rev Neurobiol 14:49–124.

Lin EY, Orlofsky A, Wang HG, Reed JC, Prystowsky MB. 1996. A1, a Bcl-2 family member, prolongs cell survival and permits myeloid differentiation. Blood 87:983-992.

Lin SZ, Yan GM, Koch KE, Paul SM, Irwin RP. 1997. Mastoparan-induced apoptosis of cultured cerebellar granule neurons is initiated by calcium release from intracellular stores. Brain Res 771:184-195.

Lindgren S. 1996. Experimental studies of mechanical effects in head injury. Acta Chirurgica Scandinavica 360:1-100.

Lobner D, Lipton P. 1993. Intracellular calcium levels and calcium fluxes in the CA1 region of the rat hippocampal slice during *in vitro* ischemia: relationship to electrophysiological cell damage. J Neurosci 13:4861-4871.

Lu J, Moochhala S, Kaur C, Ling E. 2000. Changes in apoptosis-related protein (p53, Bax, Bcl-2 and Fos) expression with DNA fragmentation in the central nervous system in rats after closed head injury. Neurosci Lett 25:89-92.

Lund LM, Machado VM, McQuarrie IG. 2002. Increased beta-actin and tubulin polymerization in regrowing axons: relationship to the conditioning lesion effect. Exp Neurol 178:306-312.

Maas AI. 2001. Neuroprotective agents in traumatic brain injury. Expert Opin Invest Drugs 10:753-67.

Marciano P, Eberwine JH, Raghupathi R, McIntosh TK. 2001. The assessment of genomic alterations using DNA arrays following traumatic brain injury. Restor Neuro 18:105-113.

Margulies SS, Thibault LE. 1992. A proposed tolerance criterion for diffuse axonal injury in man. J Biomech 25:917-923.

Margulies SS, Thibault LE, Gennarelli TA. 1990. Physical model simulations of brain injury in the primate. J Biomech 23:823-836.

Martin LJ, Price AC, McClendon KB, Al-Abdulla NA, Subramaniam JR, Wong PC, Liu Z. 2003. Early events of target deprivation/axotomy-induced neuronal apoptosis *in vivo*: oxidative stress, DNA damage, p53 phosphorylation and subcellular redistribution of death proteins. 2003. J Neurochem 85:234-247.

Martin LJ, Nael A, Al-Abdulla, Ansgar M, Brambrink, Kirsch JR, Frederick E, Portera-Cailliau C. 1998. Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: a perspective on the contributions of apoptosis and necrosis. Brain Res Bull. 6:281-309.

Matter A. 1979. Microcinematographic and electron microscopic analysis of target cell lysis Induced by cytotoxic T lymphocytes. Immunol 36:179–190.

Mattson MP, Zhang Y, Bose S. 1993. Growth factors prevent mitochondrial dysfunction, loss of Calcium homeostasis, and cell injury, but not ATP depletion in hippocampal neurons deprived of glucose. Exp Neurol 121:1-13.

Maxwell WL, Povlishock JT, Graham DI. 1997. A mechanistic analysis of non-If disruptive axonal injury. J Neurotrauma 14:419-440.

McBurney RN. 1997. Development of the NMDA ion-channel blocker, aptiganel hydrochloride, as a neuroprotective agent for acute CNS injury. Inter Review Neurobiol. 40:173-95.

McKinney JS, Willoughby KA, Liang S, Ellis EF. 1996. Stretch-induced injury of cultured neuronal, glial, and endothelial cells: Effect of polyethylene glycol-conjugated superoxide dismutase. Stroke 27:934-940.

Meloni BP, Majda BT, Knuckey NW. 2002. Evaluation of preconditioning treatments to

protect near-pure cortical neuronal cultures from *in vitro* ischemia induced acute and delayed neuronal death. Brain Res 928:69-75.

Merrill T, Goldsmith W, Deng YC. 1984. Three-dimensional response of a lumped parameter head-neck model due to impact and impulsive loading. J Biomech 17:81-95

Morrison BI, Meaney D, Mcintosh T. 1998a. Mechanical characterization of an *in vitro* device designed to quantitatively injure living brain tissue. Ann Biomed Eng 26:381-390.

Morrison BI, Saatman KE, Meaney DF, McIntosh TK. 1998b. *In vitro* central nervous system models of mechanically induced trauma. J Neurotrauma 15:911–928.

Murphy EJ, Horrocks LA. 1993. A model for compression trauma: pressure-induced injury in cell cultures. J Neurotrauma 10:431-444.

Nagata S. 1997. Apoptosis by death factor. Cell 88:355-365.

Norton LA, Andersen KL, Arenholt-Bindslev D, Andersen L, Melsen B. 1995. A methodical study of shape changes in human oral cells perturbed by a simulated orthodontic strain *in vitro*. Archi Oral Biol 40:863–872.

Noshita N, Lewen A, Sugawara T, Chan PH. 2002. Akt phosphorylation and neuronal survival After traumatic brain injury in mice. Neurobiol Disease 9:294-304.

Nusholtz GS, Ward C. 1987. Comparison of epidural pressure in live anesthetized and post-mortem primates. Aviation Space Environ Med 58:9-17.

Nusholtz GS, Kaiker PS. 1986. Pelvic stress. J Biomech 19:1003-1011.

Oberhammer W, Bursch W, Parzefall P, Breit E, Erber M, Stadler R, Schulte-Hermann R. 1991. Effect of transforming growth factor β on cell death of cultured rat hepatocytes. Cancer Res 51:2478–2485.

Ommaya AK. 1995. Head injury mechanisms and the concept of preventive management: a review and critical synthesis. J Neurotrauma. 12:527-546.

Panickar KS, Jayakumar AR, Norenberg MD. 2002. Differential response of neural cells to trauma-induced free radical production *in vitro*. Neurochem Res 27:161-6.

Pellegrini-Giampietro DE, Cherici G, Alesiani M, 1988. Excitatory amino acid release from rat hippocampal slices as a consequence of free-radical formation. J Neurochem 51:1960–1963.

Perron JC, Bixby JL. 1999. Distinct neurite outgrowth signaling pathways converge on ERK activation. Mol. Cell. Neurosci. 13:362–378.

Piccioli P, Porcile C, Stanzione S, Bisaglia M, Bajetto A, Bonavia R, Florio T, Schettini G. 2001. Inhibition of nuclear factor-kappaB activation induces apoptosis in cerebellar granule cells. J Neurosci Res 66:1064-1073.

Piechnik SK, Czosnyka M, Richards HK, Whitfield PC, Pickard JD. 2001. Cerebral venous blood outflow: a theoretical model based on laboratory simulation. Neurosurgery. 49:1214-1222.

Pike BR, Zhao X, Newcomb JK, Glenn CC, Anderson DK, Hayes RL. 2000. Stretch injury causes calpain and caspase-3 activation and necrotic and apoptotic cell death in septo-hippocampal cell cultures. J Neurotrauma 17:283-298.

Pintar FA, Kumaresan S, Yoganandan N, Yang A, Stemper B, Gennarelli TA. 2001. Biomechanical modeling of penetrating traumatic head injuries: a finite element approach. Biomed Sci Instru 37:429-434.

Pollock D, Lowery D. 2001. Emergency medicine and public health: new steps in old directions. Ann Emerg Med 38:675-683.

Povlishock JT. 1992. Traumatically induced axonal injury: pathogenesis and pathobiological implications. Brain Pathol 2:1-12.

Povlishock JT, Becker DP, Cheng CL, Vaughan GW. 1983. Axonal change in minor head injury. J Neuropath Exp Neuro 42:225-242.

Pravdenkova SV, Basnakian AG, James SJ, Andersen BJ. 1996. DNA fragmentation and nuclear endonuclease activity in rat brain after severe closed head injury. Brain Res 729:151-155.

Ramon y Cajal,S. 1928. Degradation and Regeneration of the Nervous Systems. New York: Hafner Publisher Co.

Ruan JS. Prasad P. 1995. Coupling of a finite element human head model with a lumped

parameter Hybrid III dummy model: preliminary results. J Neurotrauma. 12:725-734.

Rudel T, Bokoch GM. 1997. Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science 276:1571-1574.

Russell SW, Rosenau W, Lee JC. 1972. Cytolysis induced by human lymphotoxin. Cinemicrographic and electron microscopic observations. Am J Pathol 69:103–118.

Sattler R, Tymianski M. 2000. Molecular mechanisms of calcium-dependent excitotoxicity. J Mol Med. 78:3-13.

Schanne FAX, Kane AB, YoungEE, Farber JL. 1979. Calcium dependence of toxic cell death: a final common pathway. Science 206:700–702.

Schwarzschild MA. 1997. Glutamate, but not dopamine, stimulates stress-activated protein kinase And AP-1-mediated transcription in striatal neurons. J Neurosci 15:3455–3466.

Shen S, Wiemelt AP, McMorris FA, Barres BA. 1999. Retinal ganglion cells lose trophic responsiveness after axotomy. Neuron 23:285–295.

Simonian RL, Getz JC, Leveque C, Konradi, JT. 1996. Kainic acid induces apoptosis in neurons. 75:1047–1055.

Smith DS, Niethammer M, Ayala R, Zhou Y, Gambello MJ, Wynshaw-Boris A, Tsai LH. 2000. Regulation of cytoplasmic dynein behavior and microtubule organization by mammalian Lis1. Nature Cell Biol 2:767-775.

Steller H. 1995. Mechanisms and genes of cellular suicide. Science 267:1445-1449.

Spinnewyn B, Cornet S, Auguet M, Chabrier PE. 1999. Synergistic protective effects of antioxidant and nitric oxide synthase inhibitor in transient focal ischemia. J Cereb Blood Flow Metab 19:139-43.

Stokes BT, Fox P, Hollinden G. 1983. Extracellular calcium activity in the injured spinal cord. Exp. Neurol 80:561-572.

Sugimoto A, Friesen PD. Rothman JH. 1994. Baculovirus p35 prevents developmentally

programmed cell death and rescues a ced-9 mutant in the nematode *Caenorhabditis elegans*. EMBO 13:2023-2028.

Takizawa H, Sugiura K, Baba M, Tachisawa T, Kadoyama S, Kabayama T, Ohno H, Fuseya Y. 1998. Structural mechanics of the blowout fracture: numerical computer simulation of orbital deformation by the finite element method. Neurosurgery 22:1053-1055.

Thordstein M, Bügenholm R, Thiringer K. 1993. Scavengers of free oxygen radicals in combination with magnesium ameliorate perinatal hypoxic-ischemic brain damage in the rat. Pediatr Res 34:23–26.

Thornberry NA, Lazebnik Y. 1998. Caspases: enemies within. Science 281:1312-1316.

Thurman D, Alverson C, Dunn K, Guerrero J, Sniezek J. 1999. Traumatic brain injury in the United States: a public health perspective. J Head Trauma Rehab 14:602-15.

Tojo H, Kaieda I, Hattori H, Katayama N, Yoshimura K, Kakimoto S, Fujisawa Y, Presman E, Brooks CC, Pilch PF. 2003. The Formin family protein, formin homolog overexpressed in spleen, interacts with the insulin-responsive aminopeptidase and profilin IIa. Mol Endocrinol 17:1216-1229.

Tornheim PA, Prioleau GR, McLaurin RL. 1984. Acute responses to experimental blunt head trauma. Topography of cerebral cortical edema. J Neurosur 60:473-480.

Triyoso DH, Good TA. 1999. Pulsatile shear stress leads to DNA fragmentation in human SH-SY5Y neuroblastoma cell line. J Physiol 515:355-365.

Trump BJ, Goldblatt PJ, Stowell RE. 1965. Studies on necrosis of mouse liver *in vitro*. Ultrastructural alterations in the mitochondria of hepatic parenchymal cells. Lab Invest 14:343–371.

Ueno K, Melvin JW, Li L, Lighthall JW. 1995. Development of tissue level brain injury criteria by finite element analysis. J Neurotrauma. 12:695-706

Van Kampen GPJ, Veldhuizen JP, Kuijer R, van de Stadt RJ, Schipper CA. 1998. Cartilage response to mechanical force in high-density chondrocyte cultures. Arthritis Rheumat 28:419–424.

Van Ooyen A, Willshaw DJ. 1999. Competition for neurotrophic factor in the

development of nerve connections Proc. R. Soc. London Ser. B 266:883–892.

Vexler ZS, Ferriero DM. 2001. Molecular and biochemical mechanisms of perinatal brain injury. Semin Neonatol 6:99-108.

Waetzig V, Herdegen TA. 2002. Single c-Jun N-terminal kinase isoform (JNK3-p54) is an effector in both neuronal differentiation and cell death. J Biol Chem 278:567-572.

Wallach D, Kovalenko AV, Varfolomeev EE, Boldin MP. 1998. Death-inducing functions of ligands of the tumor necrosis factor family: a Sanhedrin verdict. Curr Opin Immunol 10:279-288.

Walworth NC. 2001. DNA damage: Chk1 and Cdc25, more than meets the eye.Curr Opin Genet Dev 11:78–82.

Wilson JX, Gelb AW. 2002. Free radicals, antioxidants, and neurologic injury: possible relationship to cerebral protection by anesthetics. J Neurosur Anesthes 14:66-79.

Witke W, Podtelejnikov AV Di Nardo A Sutherland JD, Gurniak CB, Dotti C, Mann M. 1998. In mouse brain profilin I and profilin II associate with regulators of the endocytic pathway and actin assembly. EMBO J 17:967–976.

Wolf JA, Stys PK, Lusardi T, Meaney D, Smith DH. 2001. Traumatic axonal injury induces calcium influx modulated by tetrodotoxin-sensitive sodium channels. 2001. J Neurosci 21:1923-1930.

Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW. 1998. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Develop 12:806-819.

Xiong Y, Gu Q, Peterson PL, Muizelaar JP, Lee CP. 1997. Mitochondrial dysfunction and calcium perturbation induced by traumatic brain injury. J Neurotrauma 14:23-34.

Xu Z, Kukekov NV, Greene LA. 2003. POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis. EMBO J 22:252-261.

Xue D, Shaham S, Horvitz HR. 1996. The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. Genes Develop 10:1073-1083.

Yang DD. 1997. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature 389:865–870.

Yakovlev AG, Knoblach SM, Fan L, Fox GB, Goodnight R, Faden AI. 1997. Activation of CPP32-like caspases contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury. J Neurosci 17:7415-7424.

Yousefian J, Firouzian F, Shanfeld J, Ngan P, Lanese R, Davidovitch Z. 2001. A new Experimental model for studying the response of periodontal ligament cells to hydrostatic pressure. Am J Orthod Dentofacial Orthop 108:402–409.

Zhang F, Sprague SM, Farrokhi F, Henry MN, Son MG, Vollmer DG. 2002. Reversal of attenuation of cerebrovascular reactivity to hypercapnia by a nitric oxide donor after controlled cortical impact in a rat model of traumatic brain injury. J Neurosur 97:963-9.

Zhao EG, Song Q, Cross S, Misko I, Lees-Miller SP, Lavin MF. 1998. Resistance to etoposide-induced apoptosis in a Burkitt's lymphoma cell line. Inter J Cancer 77:755-762.

VITA

IN HONG YANG

Permanent Address

Seochogu Jamwoon Dong A Apt 105-1003

Seoul, Korea

011-82-2-535-8944

PREVIOUS EDUCATION

Master of Science, KyungHee University, Seoul, Korea

Molecular Biology, Feb. 1996

Bachelor of Science, KyungHee University, Suwon, Korea

Genetic Engineering, Feb. 1994