SPECIFIC PROTEINS INDUCTION AND ALTERATION OF ENERGY METABOLISM IN CULTURED NEURAL CELLS IN RESPONSE TO IN VITRO ISCHEMIA

By

W. Y. Fu, B.Sc. (Hons)

A Thesis Presented to
The Hong Kong University of Science and Technology
in Partial Fulfillment
of the Requirements for
the Degree of Master of Philosophy
in Biology Department

Hong Kong, August 1996

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ADP       Adenosine diphosphate
AMP       Adenosine monophosphate
AMPA      Alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid
ATP       Adenosine triphosphate
bFGF      Basic fibroblast growth factor
BSA       Bovine serum albumin
Ca²⁺      Calcium ion
Cl⁻       Chloride ion
CNS       Central nervous system
CO₂       Carbon dioxide
ETC       Electron transfer chain
FCS       Fetal calf serum
GABA      γ-aminobutyric acid
GFAP      Glial fibrillary acidic protein
Hsc 70    Heat shock cognate 70
Hsp 70    Heat shock protein 70
H₂        Hydrogen
HCO₃⁻     Bicarbonate ion
HPLC      High performance liquid chromatography
HRP       Horseradish peroxidase
HS        Horse serum
HSP       Heat shock protein
HSP 70    Heat shock protein 70 family
IEG       Immediate early gene
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<td>IP3</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>KA</td>
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<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Potassium phosphate</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium chloride</td>
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<tr>
<td>MEM</td>
<td>Minimum essential media</td>
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<td>N₂</td>
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<td>Na⁺</td>
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<td>NAD</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NMDA</td>
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<td>NP-40</td>
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<tr>
<td>OD</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethysulfonyl fluoride</td>
</tr>
<tr>
<td>RT</td>
<td>Retention time</td>
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<tr>
<td>SAPKs</td>
<td>Stress activated protein kinases</td>
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<td>SDS</td>
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ABSTRACT

Ischemia can be induced by head trauma, stroke, and cardiac arrest. The causes are multifactorial, including hypoxia, nutrient deprivation and failure to remove toxic metabolic products. The injury of ischemic damage to neural cells is believed to be mediated by the excitotoxicity, calcium accumulation and free radical formation. Neurons are more vulnerable than astrocytes to ischemic damage. Brief exposure to ischemia does not cause severe injury to astrocytes and the responses of astrocytes are suggested to be beneficial to other neural cells in CNS. In this study, two in vitro models were used to study the changes of cellular responses and energy metabolism in cultured astrocytes under ischemia. By using the mineral oil induced ischemia model, c-Fos, c-Jun and the constitutively expressed form of HSP 70 proteins in cultured cortical astrocytes were induced by the injury of ischemia. They may act as specific markers for cortical astrocytes in response to ischemia. The anaerobic chamber induced ischemia model was used to study the alteration of energy metabolism in cortical neurons and astrocytes under ischemia.
There was a direct correlation between the energy metabolism and cell injury in astrocytes under ischemia. The depletion of ATP in astrocytes was more tolerable than neurons to this simulated ischemic injury. The extent of damage to astrocytes by ischemia incubation was determined in this study. Two hour of ischemia did not cause any significant damage to astrocytes. Four hour of ischemia caused a moderate injury. By exposure to 6 and 8 hours of ischemia, the damage to cultured astrocytes became severe. Nine hour of ischemia was lethal to cultured astrocytes. The viable astrocytes after various lengths of ischemia incubations were able to retain their ability of ATP production. This study also suggested that the critical period for astrocytes in response to ischemia was between 4 to 6 hours. By studying the biochemical and molecular events of astrocytes around this period, it may provide essential information about the tolerance of astrocytes and assist in the development of potential therapies to prevent and cure the damage by ischemia.
CHAPTER 1

INTRODUCTION

1.1 Ischemia

Cerebral ischemia is the blockade of blood circulation to the brain that caused by head trauma, stroke and cardiac arrest. Ischemia is a combination of several pathological conditions such as severe hypoxia (lack of oxygen), substrate deprivation and failure to remove toxic metabolic products. There are two types of cerebral ischemia. Focal ischemia only affects locally to a part of the brain and global ischemia affects the entire brain. Both types of ischemia can induce immediate injury to brain cells. Depending on the extent and duration of ischemia, the damage may be transient or permanent. If the injury is severe enough, it will cause an irreversible cell damage that finally leads to cell death in certain populations of neural cells. Normal functions in some parts of the brain would be disturbed.

1.2 Mechanism of Neuronal Cell Injury Under Ischemia

Ischemia induces a selective and delayed injury to certain neuronal populations (Brierley, 1976). Pyramidal neurons in CA1 region of the hippocampal formation (Kirino, 1982; Deshpande et al., 1992), striatal medium size neurons (Francis and Pulsinelli, 1982), neocortical neurons and cerebellar Purkinje neurons are especially vulnerable to ischemic injury. Glial cells and
endothelial cells are more resistant. However, they would also be injured that depend on the severity of the ischemic damage.

Due to the complexity of the Central Nervous System (CNS), the mechanisms involved with cerebral ischemia cell damage are not completely known. The most accepted hypothesis for ischemic neural cell injury involved three major components. They are excitotoxicity (Choi, 1990a, 1992), accumulation of intracellular calcium ions (Choi, 1988; Siesjö and Bengtsson, 1989; Siesjö, 1991) and the free radicals formation (Floyd, 1990; Siesjö, 1981).

1.2.1 Excitotoxicity

Excitotoxicity is the excessive exposure of glutamate and other related amino acids to neural cells (Olney, 1986). After energy depletion, there is an enhancement of K\(^+\) conductance that leads to the accumulation of extracellular K\(^+\) accompanied with a depletion of extracellular of Na\(^+\) and Ca\(^{2+}\). All these cause an immediate membrane depolarization in neuronal cells (Martin et al., 1994). Thus, glutamate released from neural cells to extracellular space is also enhanced (Benveniste et al., 1984; Graham et al., 1990; Wahl et al., 1994) through the reversed operation of glutamate uptake carrier (Szatkowski et al., 1990; Szatkowski and Attwell, 1994; Longuemare and Swanson, 1995). In addition, the removal of glutamate from extracellular spaces that mainly depends on astrocytes (Yu et al., 1989; Huang et al., 1993) are inhibited because of the depletion of high energy phosphate.

Glutamate, the most abundant amino acid in the brain (Fonnum, 1984), is an excitatory amino acid (Watkins and Evans, 1981). Glutamate activates three major families of ionophore-linked receptors (Monaghan et al., 1989; McDonald and Johnston, 1990; Farooqui and Horrocks, 1991). They are N-methyl-D-
aspartate, (NMDA), kainate (KA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). It also activates a family of metabotropic receptors. This is the quisqualate-prefering receptor that is linked via G proteins to phospholipase C (PLC). Activation of the receptor leads to the hydrolysis of phosphotidyl inositol and generation of diacylglycerol and inositol 1,4,5-triphosphate (IP3). Through these activation of receptors, the second messengers of the signal transduction pathway are activated (Nicolletti et al., 1986a, 1986b). All these receptors might involve with the ischemic injury in neural cells to different degrees.

Activation of glutamate ion gated channel receptors (Watkins and Olverman, 1987; Collingridge and Lester, 1989) causes a further influx of extracellular Na\(^+\), Cl\(^-\) and water passively through Na\(^+\) and K\(^+\) channels. It causes a derangement of ion homeostasis in neural cells resulting in an accumulation of intracellular Ca\(^{2+}\), Na\(^+\), Cl\(^-\) and a depletion of intracellular K\(^+\). Loss of ion homeostasis causes an immediate cell swelling (Choi, 1987; Goldberg et al., 1986; Olney et al., 1986; Rothman, 1985) which is potentially lethal and responsible for the immediate cell injury by destruction of membrane stability (Maulucci-Gedde and Choi, 1987) leading to autolysis.

1.2.2 Accumulation of Intracellular Ca\(^{2+}\)

Calcium ions are more important in mediating the delayed neuronal death (Meyer, 1989). Extensive influx of Ca\(^{2+}\) is predominantly via the NMDA receptors (Siesjö and Bengtsson, 1989; Goldberg and Choi, 1993; Hartley et al., 1993). NMDA receptor couples to a cationic channel that has a high permeability to Ca\(^{2+}\) (Nowak et al., 1984; MacDermott et al., 1986). Blockade of NMDA receptors significantly reduces the loss of neuronal cells in focal ischemia (Simon et al., 1984). Activation of non-NMDA receptors may augment injury too (Frandsen et
al., 1989; Kaku et al., 1991; Choi, 1993), especially in certain population of cortical neurons (Weiss et al., 1990). Activation of metabotropic receptors (Eccles and Mcgeer, 1979) causes a massive mobilization of Ca^{2+} from intracellular stores through PLC and accumulation of IP3 (Berridge, 1984).

Excess accumulation of intracellular Ca^{2+} causes a series of derangement processes in the cells. Calcium ions and Ca-calmodulin complexes can modulate the activities of certain proteins such as protein kinase C (PKC) (Domanska-Janik and Zalewska, 1992; Wieloch et al., 1991) and calcium calmodulin kinase II (Siesjö, 1993). These proteins mostly involve in modulating the function of receptors, ion channels, translocators and enzymes. The activities of these proteins are dependent on the phosphorylation and dephosphorylation of their functional domains (Chin et al., 1985). Altering the phosphorylated state of these proteins leads to the alteration of the normal metabolic functions in cells. PKC and calcium calmodulin kinase II are second messenger of signal transduction. Phosphorylation of these proteins causes the alteration of many gene expression such as activation of immediate early gene (IEG) (Abe and Kogure, 1993). The protein products of IEG affect the expression of late response genes (Morgan and Curran, 1989; Sheng and Greenberg, 1990). These may affect the long term response of cell after ischemic injury. Elevation of intracellular Ca^{2+} also activates a variety of degradative enzymes such as calpain I (Ostwald et al., 1994), endonuclease (Nicotera et al., 1989) and phospholipase (Siesjö, 1993). Neutral protease calpain I (Croall and DeMartino, 1991) can degrade major neuronal structural proteins. Endonucleases involve in the DNA degradation and phospholipases involve in the break down of phospholipids of cell membrane and liberating of free fatty acid including arachidonic acid.
1.2.3 Free Radicals Formation

Release of arachidonic acid and its metabolites from damaged membrane leads to the formation of oxygen free radicals (Chan et al., 1985). Oxygen free radicals are formed especially after reperfusion when oxygen is available again. Free radical contains an unpaired electron that is highly reactive to attack other biomolecules. Oxygen free radicals initiate other important destructive processes in neural cells including lipid peroxidation—the oxidative damage to cell membrane. This further enhances the destruction of membrane stability and affects the proper functions of essential membrane proteins such as Na/K+ ATPase (Floyd, 1990). In addition, arachidonic acids will slow down the uptake of glutamate which lead to accumulation of extracellular concentration of glutamate (Yu et al., 1986; Szatlowski et al., 1992). The final consequences of these cellular derangements are the destruction of neuronal cytoskeleton and membrane components. As a result, the neural cells are severely injured and died.

1.3 The Responses of Astrocytic Cells to Ischemic Injury

1.3.1 Normal Functional Roles of Astrocytes

Astrocyte is one of the major classes of glial cells that play an important role in maintaining the normal functions of neuronal cells. Astrocytes provide a structural support to neurons. The end feet of astrocytes contact both the blood capillaries and neurons that may provide a nutritive support for neurons. Astrocytes are important in maintenance of the normal homeostatic environment. Under normal physiological condition, astrocytes around synaptic clefts have high
affinity for neurotransmitter uptake such as glutamate and γ-aminobutyric acid (GABA) that are released from neurons (Kimmelberg et al., 1993). This astrocytic uptake is important for preventing the prolonged excitation in neurons and further firing of other neurons. In addition, astrocytes have a high permeability of K⁺ (Duffy and MacVigur, 1993). They take up K⁺ from extracellular space where excess K⁺ are released from depolarized neurons. Astrocytes can redistribute K⁺ to the extracellular space with relatively low K⁺ concentration or remove K⁺ to vascular compartments if extracellular K⁺ is too high (Gardner-Medwin, 1983).

1.3.2 Early Responses of Astrocyte to Ischemia.

Astrocytes have a unique capacity to tolerate ischemic injury. The immediate response of astrocytes after ischemia is swelling (Kimmelberg and Ransom, 1986). This swelling is reversible if the ischemic injury is brief. However, it would be lethal due to mechanical destruction if ischemia persists. The swelling may be contributed by the extensive influx of K⁺ accompanied with Cl⁻ and HCO₃⁻ into astrocytes. After ischemia, there is an immediate depolarization in neural cells followed with a drastic accumulation of K⁺ in extracellular space. This also causes a depolarization in astrocytes resulting in an activation of astrocytic anion channels. Thereafter, a passive influx of K⁺, Cl⁻ and HCO₃⁻ into astrocytes is enhanced (Walz et al., 1993). Furthermore, uptake of glutamate is inhibited in astrocyte. Under normal physiological condition, glutamate uptake is electrogenic that couples with influx of Na⁺ and efflux of K⁺ (Brew and Attwell, 1987). High extracellular K⁺ causes a reversed operation of glutamate uptake system and results in an enhanced release of glutamate from astrocytes (Swanson, 1992; Huang et al., 1993; Kimmelberg et al., 1995; Longuemare and Swanson, 1995). Prolonged exposure to glutamate may directly cause an injury to astrocyte. In vitro study of
cultured astrocytes has shown that intracellular concentration of Ca\textsuperscript{2+} was observed after exposure to glutamate (Cornell-Bell et al., 1990).

1.3.3 Late Responses of Astrocyte to Ischemia

Astrocytes greatly influence the normal growth and survival of neurons (Banker, 1980). After ischemia, astrocytes are activated with an increasing astroglial-cell proliferation, hypertrophy with stellation and increased expression of glial fibrillary acidic protein (GFAP) (Petito et al., 1990; Tanaka et al., 1992; Jørgensen et al., 1993). These responses occurred before there is a secondary injury in neurons (Kato et al., 1994). This astrogliosis response is beneficial to the injured cells in CNS. They may help in the re-establishment of the extracellular microenvironment after ionic homeostatic derangement. Furthermore, uptake of glutamate in astrocytes is resumed after post-ischemia. The expression of glutamine synthetase, which enzymatically converts glutamate to glutamine, is increased in astrocytes (Tanaka et al., 1992). Reactive astrocytes also upregulate the expression of a large number of molecules such as nerve growth factor (NGF) (Shigeno et al., 1991) and basic fibroblast growth factor (bFGF) (Nakata et al., 1993). Shigeno et al. (1991) have shown that NGF released from astrocyte may ameliorate the survival of neurons. In additions, astroglial-derived growth factors attenuate the toxic effects of microglia (Giulian, 1993; Giulian et al., 1993) and may help to preserve neurons under attack by phagocytic cells.

Astrocytes are resistant to brief exposure of ischemia. The late responses of astrocytes may be beneficial to other injured cells and reduce further injury to other cells in CNS. Although astrocytes are tolerant to ischemia, there is an alteration of their metabolic and biosynthetic mechanisms (Hori et al., 1994). Prolonged exposure to ischemia is also lethal to astrocytes when there is a high extracellular glutamate. It would further attenuate the excitotoxicity to other neural cells.
Therefore, it is worth to search for the critical time period under ischemia for astrocytes to commit irreversible damage or cell death. If the critical period could be determine, one can study the biochemical and molecular events of astrocytes under reversible and irreversible damage. The hope is that understanding these events will assist the development of potential therapies to prevent injury under ischemia.

1.4 Expression of Specific Proteins Under Ischemia and Post-ischemia

One of the essential cellular responses under ischemia is the alteration of gene expression pattern (Abe and Kogure, 1993; Pombo et al., 1994) that leads to the synthesis of a set of proteins. However, total protein synthesis in neural cells (Kleihues and Hossmann, 1971; Hossmann, 1993) are suppressed under ischemia and post-ischemia that may be due to the altered phosphorylation of initiation factor 2. The expression of normally constitutively expressed proteins in neural cells such as tubulin, neuron-specific enolase and brain specific kinase are greatly reduced (Matsumoto et al., 1987). Nevertheless, the expression of some specific proteins was elevated during ischemia and post-ischemia. These proteins may have significant effects on the cell when response to ischemic injury. Two classes of proteins have already been studied extensively under stress including ischemia. A lot of evidences have shown that their genes were induced and their protein expressions were increased after ischemia and post-ischemia. One class is IEG products such as c-Fos and c-Jun where their genes are induced under various stimuli and their proteins are the third messenger for signal transduction (Bohmann et al., 1987). The other class is stress protein such as Heat Shock Protein 70 family (HSP 70) where their proteins were induced under various adverse environments (Hightower, 1991).
Hori et al. (1994) have shown that the total cellular protein synthesis in cultured rat astrocytes was inhibited under hypoxia and reoxygenation but certain proteins were elevated. Yu et al. (1995) have also shown that the genes of c-fos and HSP 70 were induced in cultured rat astrocytes under simulated ischemia and post-ischemia. These gene products may not be translated that may be due to the rapid degradation of their RNAs or suppression of the translational mechanism after ischemic injury. Therefore, the expressions of these two protein products were examined using the similar ischemia model as Yu et al. (1995). The expression of c-Jun protein was also studied too as c-Jun forms a complex with c-Fos to regulate the expression of late response genes. The expression of these proteins in astrocytes may contribute to their tolerance and the astrocytic response to ischemic injury.

1.4.1 Immediate Early Gene Products

C-jun and c-fos proto-oncogenes belong to a family of IEG. C-Jun can form a homodimer or heterodimer with c-Fos protein through the conserved leucine zipper region (Kouzarides and Ziff, 1988; Turner and Tjian, 1989). They are known as the major components of the AP-1 complex of transcriptional regulators (Bohmann et al., 1987) and act as third messenger molecules in the signal transduction system.

It has been found that c-jun and c-fos proto-oncogene products played a critical role in the commitment of cell proliferation (Holt et al., 1986; Kovary and Bravo, 1991a, 1991b) and differentiation (Müller and Wagner, 1984; Hisanaga et al., 1990; Jalava and Mai, 1994). It was hypothesized that c-Jun and c-Fos had an essential role for the response of astrocytes under injury. Under ischemic injury, there is an activation of a signal transduction pathway that involves a novel kinase termed Stress Activated Protein Kinases (SAPKs) (Woodgetti et al., 1996).
SAPKs are able to phosphorylate c-Jun under ischemia and reperfusion (Kyriakis et al., 1994; Pombo et al., 1994). SAPKs have already been present in resting cells, which would facilitate the binding of c-Jun to DNA motif and lead to the activation of RNA polymerase II machinery. This suggests that c-Jun may contribute to the ischemic tolerance by inducing changes in gene expression in astrocytes (Kato et al., 1995). In addition, the expression of c-Fos and c-Jun proteins in reactive astrocytes are involved in the expression of a variety of molecules such as NGF and bFGF (Neveu et al., 1993) that are beneficial to the injured cells in CNS.

1.4.2 Stress proteins

A set of stress proteins with 30, 70 and 90 kDa can be induced in cells by a number of stressful conditions (Subjeck and Shyy, 1986) such as heat shock, viral infection, heavy metal toxicity (Welch and Schan, 1986) and ischemia (Dwyer and Nishimura, 1992). These stress induced proteins are more commonly known as Heat Shock Protein (HSP) family since it was originally discovered under heat shock stress. These proteins may involve in the protection of cells under stress (Hightower, 1991). HSP 70 family is studied intensely since its functions seem to interfere with various neural trauma and degenerative disorders.

The HSP 70 family includes a constitutively synthesized 70 kDa member of heat shock cognate (Hsc 70). Its normal function is involved in the uncoating clathrin-coated vesicles (Ungewickell, 1985). It also binds to newly synthesized peptides for the proper folding of proteins and assists the nascent polypeptide chains for the transportation from ribosome to other organelles (Beckmann et al., 1990). One member of the family is a highly stress inducible heat shock protein 70 (Hsp 70) that is highly synthesized after a variety of stress stimuli. The function of stress-inducible Hsp 70 is not completely known yet. Papadopoulos et al. (1996) have recently shown that over-expression of Hsp 70 in cultured rat astrocytes
greatly reduced the death of astrocytes after 7 hr of oxygen and glucose deprivation. Therefore, Hsp 70 may play an essential role in the cytoprotection after stress in astrocytes.

1.5 Energy Metabolism

A normal level of energy contents is essential for the maintenance of normal cell function and integrity. Adenosine triphosphate (ATP) is the main energy source in all cells. High energy in ATP is stored as phosphoanhydride bonds. The process of hydrolysis of these bonds with the loss of the phosphate groups in ATP released the energy and degraded the ATP to energy poor adenosine diphosphate (ADP) and adenosine monophosphate (AMP). ATP functions are mainly for the proper maintenance of ion homeostasis, translocation of molecules across the cell and organelles membranes and also directly or indirectly involves in all the enzymatic reactions. The activation of the enzymes is depended on the phosphorylation and dephosphorylation by the presence of ATP.

Respiration is the process to synthesis ATP. It can be divided into 3 stages: glycolysis, Krebs cycle and electron transfer chain (ETC). The process of glycolysis is located in the cytoplasm. Glucose molecule is broken down into 2 pyruvic acids and generates 2 molecules of ATP in the process of glycolysis. Then pyruvic acids enter the Krebs cycle where the reaction take place in the matrix of the mitochondria. The Krebs cycle is to remove pairs of hydrogen atoms from pyruvic acid. Then the hydrogen-carrying coenzyme nicotinamide adenine dinucleotide (NAD) takes up the hydrogen atoms and passes them into the inner mitochondrial membrane. Hydrogen atoms are split into protons and electrons. Electrons are passed from one carrier protein to the next through the ETC. This process causes protons to be pumped across the inner mitochondrial membrane. The gradient of
protons across the mitochondrial membrane contains free energy that the back flow of protons across the membrane provides energy for ATP synthesis (James and Mattews, 1991).

Energy depletion is the primary events to cause cellular derangement after ischemic injury. Under normal condition, brain tissue carries aerobic respiration. Brain cells exclusively depend on the metabolism of glucose and they utilize about 20% of the total body oxygen for the synthesizing of ATP to maintain their high metabolic rates. The brain tissues only reserve a very small amount of energy compounds. During ischemia, the reserved energy compounds are only enough to sustain the cells for only a few minutes. It has been found that there were destruction in the structure and disturbance in the normal function of mitochondria (Sims, 1991; Allen et al., 1995; Almeida et al., 1995) following transient ischemia. The dysfunction of mitochondria would lead the cells to mainly depend on anaerobic glycolysis to supply its energy. That is very inefficient in ATP production and also will lead to the accumulation of an undesirable product—lactate. It is believed that the disturbances of energy metabolism is one of the major factors in the initiation of a sequence of undesirable cellular response to ischemic injury.

The capability of restoring high phosphate energy compounds in astrocytes after ischemia is a crucial factor for cell recovery. The energy supply is a necessity to maintain all the essential cellular functions in astrocytes such as ion homeostasis (Ekholm et al., 1993) and membrane stabilization. The responses of astrogliosis also require energy. The dysfunction of the restoration of normal energy metabolites balances after ischemia may be one of the important factors involved in the further astrocytic cell damage. Therefore, it is worth to determine the extent of injury in astrocytes under ischemia and the disturbance of energy metabolism in
viable astrocytes after post-ischemia. It may provide more information about the resistant ability of astrocytes to ischemic injury.

1.6 \textit{In Vitro} Models for Studying the Mechanism of Ischemic Injury in Neural Cells

\textit{In vitro} ischemia model provides a simple system to study ischemia induced damage in an enriched neural cell populations. Though the multiple characteristics of ischemia \textit{in vivo} are usually not completely present in \textit{in vitro} model (Choi, 1990b), this problem is offset by the analytic power and simplicity for studying the injury response in neural cells. \textit{In vitro} set up has the advantage of examining the response of individual cell type without the interaction with other neural cells and also the hormonal and haemodynamic factors occurred \textit{in vivo}. Furthermore, \textit{in vitro} model allows a better manipulation of extracellular environment such as nutrients, ions, pH and addition of chemicals.

In this study, two \textit{in vitro} models were established which simulate \textit{in vivo} ischemia and post-ischemia conditions. One model is created by isolating the culture from air with a layer of mineral oil (Yu \textit{et al.}, 1992, 1995). This model is suitable for studying the process of morphological changes of the cells in cultures under ischemia by Time Lapse Video Recording Microscopy. As mentioned before, this model has been used to study the induction of \textit{c-fos} and HSP 70 gene expressions in astrocytes. Therefore, this model will be used to determine whether these proteins are being translated or not under similar conditions. The anaerobic chamber model has been popularly used (Kaku \textit{et al.}, 1991; Juurlink \textit{et al.}, 1993; Vornov, 1995) for the study of responses of cultured cells to ischemia. The ischemic condition is created by incubating cultured cells in a limited amount of oxygen and glucose deprivation medium inside an anaerobic chamber. This model
Table I. Comparison of the mineral oil induced ischemia model and anaerobic chamber induced ischemia model in cultures to the physiological ischemia.

<table>
<thead>
<tr>
<th>Ischemia Components</th>
<th>Physiological Ischemia</th>
<th>Mineral Oil Model</th>
<th>Anaerobic Chamber Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe Hypoxia</td>
<td>Lack of oxygen supply from fresh blood</td>
<td>Limited amount of dissolved O$_2$ in cultured medium and insulation of the culture from air (source of O$_2$) by mineral oil and argon</td>
<td>Limited amount of dissolved O$_2$ in cultured medium and no O$_2$ inside the atmosphere in the chamber</td>
</tr>
<tr>
<td>Substrate Deprivation</td>
<td>Lack of glucose supply and other nutrients to brain tissue from fresh blood</td>
<td>Limited amount of glucose and other nutrients in cultured medium</td>
<td>No glucose left in cultured medium</td>
</tr>
<tr>
<td>Accumulation of Toxic</td>
<td>Accumulation due to no blood circulation</td>
<td>Accumulation due to limited volume of the incubating medium</td>
<td>Accumulation due to limited volume of the incubating medium</td>
</tr>
<tr>
<td>Metabolites</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P.S. Table was partly adapted from Yu et al. 1995.
allows one to handle a large number of samples at one time in biochemical study of ischemia. However, it is quite difficult to observe the dynamic changes of the cells in cultures under ischemia directly by microscope. The changes of energy contents in cultured neural cells under ischemia were studied by using this anaerobic model. Both models are feasible for extending the ischemia study to post-ischemia simply by adding normal medium to the cultures at the end of the ischemia incubation and incubating the culture under normal condition. In Table I, these models were compared to the physiological ischemia.

1.7 Aims of the Study

Primary cerebral cortical astrocytic and neuronal cultures were used for this study. The aims can be divided into the following 4 parts:

A. To study the expression of specific proteins in astrocytic cultures under ischemia and post-ischemia

The expressions of c-Jun, c-Fos and HSP 70 proteins were studied. Induction of these proteins appears to be useful markers for the changes of pathophysiology in nerve cells under and after ischemia. The mineral oil induced ischemia model is utilized in this set of studies. After ischemia and post-ischemia incubations, the induction of protein expression is studied by using Western blotting analysis or immunocytochemistry.

B. To study the correlation between the depletion of energy metabolites and the extent of cell injury in cultured astrocytes under ischemia
By comparing the depletion of adenine nucleotides and the viability of cultured astrocytes under ischemia, it is hoped that the extent of astrocytes damage by ischemia would be determined under various lengths of ischemia incubations. In this part of study, the anaerobic chamber induced ischemia model was employed. The contents of adenine nucleotides was measured by High Performance Liquid Chromatography (HPLC). The actual cell death under ischemia was monitored by a cell viability assay.

C. *To determine the restoration of energy metabolites in viable astrocytes after post-ischemia*

The level of adenine nucleotides under ischemia and post-ischemia were used to reflect the restoration capacity of energy metabolism in cultured astrocytes after various lengths of ischemia incubations. Anaerobic chamber model was used for this part of study and the adenine nucleotides were determined by HPLC.

D. *To study the depletion of energy metabolites in primary neuronal cultures under ischemia incubations*

The depletion of adenine nucleotides in cortical neurons under ischemia in the anaerobic chamber was determined with the aids of HPLC. The extent of energy metabolites depletion in neurons and astrocytes was compared.
CHAPTER 2

METHODS AND MATERIALS

2.1 Primary Culture of Rat Cerebral Cortical Astrocytes

The primary cortical astrocytic cultures were prepared from the cerebral cortex of new-born Sprague-Dawley rats (Animal Care Centre, HKUST) as described by Yu et al. (1986, 1993) with minor modifications. Cerebral cortices freed of meninges were cut into small cubes (<1 mm$^3$) in Minimum Essential Media (MEM) (GIBCO BRL, Life Technologies, Inc., NY, USA), then mechanically dissociated by vortexing for 1.5 min. Thereafter, the cell suspension was sieved through a 70 and a 10 μm sterile Mesh® nylon filter (Spectra/Mesh®, Spectrum Medical Industries, Inc., TX, USA), respectively. A volume of cell suspension equivalent to approximately one-fiftieth of a cerebrum was plated in a 35 mm Falcon tissue culture dish (Becton Dickinson & Company, Oxnard, CA, USA). Fresh MEM supplemented with 10% fetal calf serum (FCS) (Globe pharm Ltd., UK) was added to yield a final volume of 2 ml. All cultures were incubated in a Napco CO2 incubator (Precision Scientific Inc., Chicago, USA) at 37°C with 95% air/5% CO2 (vol/vol) and a humidity of 95%. The culture medium was changed after 3 days of seeding and subsequently 2 times per week with MEM containing 10% FCS. Cultures were not used for experiments until they were at least 4 weeks old.
2.2 Primary Culture of Mouse Cerebral Cortical Neurons

The primary cerebral cortical neuronal cultures were prepared from the cerebral cortex of 16-day-old ICR mouse embryo (Animal Care Center, HKUST) as described by Yu et al. (1984, 1986) with minor modifications. Cerebral cortices freed of meninges were cut into small cubes (<1 mm³) in a Ca²⁺ and Mg²⁺ free Hank's medium (GIBCO BRL, Life Technologies, Inc., NY, USA), then incubated in 0.2% trypsin (GIBCO BRL, Life Technologies, Inc., NY, USA) on ice for 1 min before incubating at 37°C for 5 min. After inhibition of the trypsin by adding 1 ml of horse serum (HS) (GIBCO BRL, Life Technologies, Inc., NY, USA), tissue was triturated with a pipette and then the cell suspension was centrifuged for 5 min at 900 g. The supernatant was discarded and the pellet was resuspended in serum free MEM (GIBCO BRL, Life Technologies, Inc., NY, USA) with 30 mM glucose instead of 7.5 mM. The cell suspension was sieved through a 70 μm sterile Mesh® nylon filter (Spectra/Mesh®, Spectrum Medical Industries, Inc., TX, USA). A volume of cell suspension equivalent to approximately 1 cerebrum was seeded in a 35 mm Falcon tissue culture dish (Becton Dickinson & Company, Oxnard, CA, USA). The culture dishes were precoated for at least 1 hr with 1 ml of 12.5 μg/ml of poly-D-lysine (Sigma Chemical Co., St. Louis, MO, USA) in water which was drained prior plating. Fresh MEM with high glucose concentration and 10% HS was added to yield a final volume of 2 ml. After 20 min of incubation at 37°C, unattached cells (mostly nonneuronal cells) were removed together with the medium. The cultures were refed with fresh glucose enriched MEM containing 10% HS. These cultures were exposed to 40 μM of cytosine arabinoside (Sigma Chemical Co., St. Louis, MO, USA) for 24 hr to eliminate glial cells and other proliferating cells after 3 days of
culturing. Thereafter, 1 ml of culture medium was carefully removed and 1 ml of fresh glucose enriched MEM with 10% HS (without cytotoxic agent) was added. No further feeding was necessary until experimentation on day 10.

2.3 Mineral Oil Induced Ischemia and Post-ischemia in Astrocytes

The mineral oil induced ischemia model has been established by Yu et al. (1992, 1995). Prior to the experiment, the mineral oil (light white oil, cat# 74H1025, Sigma Chemical Co., St. Louis, MO, USA) was degassed for 30 min before saturated with pure nitrogen for 30 min. The cultures were washed 3 times with serum free MEM which containing 3.5 mM of glucose. A layer of mineral oil was added to the culture medium before draining the medium by a pasteur pipette. The cultures were then incubated at 37°C for the desired period in an argon atmosphere. Since argon is much heavier than air, it is more effective than the commonly used nitrogen in resisting air currents and vortices that may carry air including oxygen to the samples.

For the post-ischemia condition, it was created by the addition of 2 ml normal feeding medium without serum to the ischemic cultures before the removal of mineral oil. Mineral oil is easily removed as it always floats on the surface of medium.
2.4 Anaerobic Chamber Induced Ischemia and Post-ischemia

2.4.1 For Astrocytes Under Ischemia and Post-ischemia

The anaerobic chamber induced ischemia has been used by Kaku *et al.* (1991), Juurlink *et al.* (1993) and many others. The experimental procedure for this study was described below with some modifications. Briefly, the astrocytic cultures were transferred into an anaerobic chamber (Forma Scientific, Inc., Ohio, USA) saturated with an 85/5/10 of N2/CO2/H2 mixture gas. The astrocytic cultures were washed 3 times with glucose and serum free MEM. Eight hundred μl of glucose and serum free MEM was added which was enough to cover all the astrocytes in the culture throughout the experimental period. The medium used was degassed for 30 min and saturated with an 85/5/10 of N2/CO2/H2 mixture gas for 15 min before being stored in the chamber. All cultures were wrapped with paraffin to prevent evaporation during incubation in the humidified 37°C incubator within the anaerobic chamber.

Post-ischemia incubation was created by adding 2 ml of serum free MEM outside the chamber after removing the glucose free medium. Then the cultures were returned to a normal CO2 incubator.

Corresponding controls for ischemia and post-ischemia conditions were set up. The control for ischemia was created by washing the cultures 3 times with normal MEM, then 800 μl of MEM was added and the cultures were incubated in a normal CO2 incubator for designed experimental periods. The controls for post-ischemia were created by refeding the normal cultures with 2 ml of serum free MEM and continue incubating them in a normal CO2 incubator.
2.4.2 For Neurons Under Ischemia Incubation

The procedure for ischemia incubation in cortical neuronal cultures was the same as those for astrocytic cultures except with some minor modifications. The cultures were not wrapped with paraffin as in astrocytes since the longest incubation time for neurons was only up to 90 min. Control incubation for neuronal cultures was as same as for astrocyte culture. Changes of adenine nucleotides under post-ischemia were not studied in neuronal cultures.

2.5 Western Blot Analysis

2.5.1 Preparation of Protein Samples

After different periods of experimental incubation, the cultured cells were washed with ice-cold Phosphate Buffered Saline (PBS) and then the proteins were extracted by 150 µl of lysis buffer [1x PBS, 1% (v/v) NP-40, 0.5% (w/v) decoxylate, 0.1% (w/v) SDS and 1 mM PMSF] at 4°C. The extracted proteins were transferred to an eppendorf tube (Scientific Plastics®, FL, USA) after 30 min of rocking by using a shaker, and then centrifuged with 14,000 g for 5 min at 4°C.

Ten µl of lysate was saved for protein determination (Lowry et al., 1951). One hundred and twenty µl of protein sample was mixed with 30 µl of 5x loading dye [3 M Tris, pH 6.8, 50% (v/v) glycerol, 25% (w/v) β-mercaptoethanol, 10% (w/v) SDS and 1% (w/v) saturated bromophenol blue]. Thereafter, the protein samples
were boiled for 5 min and stored at -80°C until further use. All the chemicals were from Sigma Chemical Co. (St Louis, MO, USA).

2.5.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Electroblotting

Proteins were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using a slab gel electrophoresis system (SE 600, Hoefer Scientific Instruments, CA, USA). The proteins were resolved on a 12.5% SDS-PAGE slab gel [12.5% (w/v) polyacrylamide, 0.3% (w/v) N',N'-methylenebis-acrylamide, 1% (w/v) SDS, 1.5 M Tris base, 0.075% (w/v) ammonium persulfate, 0.01% (v/v) glycerol and 0.025% (v/v) Temed, pH 8.8] with 5% acrylamide stacking gel [5% (w/v) polyacrylamide, 0.5% (w/v) N',N'-methylenebis-acrylamide, 0.1% (w/v) SDS, 0.5 M Tris base, 0.065% (w/v) ammonium persulfate, 0.5% (v/v) glycerol and 0.07% (v/v) Temed, pH 6.8]. The gel was electrophoresed at 60 V for the stacking gel and 120 V for the slab gel. After gel electrophoresis, the proteins were electrophoretically transferred (50 mA/cm² for 1 hr) from the acrylamide gel to a nitrocellulose membrane (Amershan, UK) using SemiPhor Transfer System (Hoefer Scientific Instruments, CA, USA).

2.5.3 Immunoblotting

The protein expression of c-Jun and HSP 70 proteins under ischemia and post-ischemia were visualized by the chemi-luminescent method using indirect labeling with horseradish peroxidase (HRP) conjugated antibody after the proteins were transferred from acrylamide gel to nitrocellulose membrane.
2.5.3.1 c-Jun Protein Expression

After blotting, the membrane was washed once in Tris Buffered Saline (TBS), then blocked in TBS plus 0.1% (v/v) Tween 20 (TBS-T) with 5% (w/v) low fat dried milk and 2% (v/v) goat serum for 2 hr. After that, the membrane was rinsed in TBS-T, then incubated with a 1:1000 dilution of anti-c-Jun rabbit polyclonal antibody (cat# sc-45, Santa Cruz Biotechnology, Inc., USA) in TBS with 2.5% low fat dried milk overnight at 4°C. The membrane was washed 3 times with TBS-T, 15 min once and 5 min twice, then incubated with a 1:1000 dilution of HRP conjugated goat anti-rabbit IgG (Amersham, UK) in TBS with 2.5% (w/v) low fat dried milk for 1 hr at room temperature. The HRP indirectly bound proteins were visualized using chemi-luminescent techniques (ECL Kit, Amersham, UK) and followed by autoradiography with high performance luminescence detection film (Amersham, UK). The film was developed by an X-ray film processor (Fuji RG II, Japan). The expression level of protein was quantified by a densitometer from Molecular Dynamics (CA, USA).

2.5.3.2 HSP 70 Protein Expression

The procedure was similar to c-Jun except with the following modifications. The blot was blocked in TBS-T with 5% (v/v) low fat dried milk for 1 hr at room temperature. The primary antibody incubation was with a 1:2000 dilution of anti-HSP 70 mouse monoclonal antibody (Sigma Chemical Co., St. Louis, MO, USA) for 1 hr at room temperature and then followed by a 1:1000 dilution of HRP labeled goat anti-mouse IgG (Amersham, UK) in TBS with 2.5% (w/v) low fat dried milk for 1 hr at room temperature.
2.6 Immunocytochemistry

2.6.1 Fixation

At the end of ischemia and post-ischemia incubations, the cultures were carefully rinsed with ice-cold PBS twice. Then they were fixed with 1 ml of 4% paraformaldehyde in PBS at room temperature for 30 min. After that, the cultures were washed 3 times with PBS before immunocytochemistry.

2.6.2 Localization of c-Fos Protein

For studying the localization of c-Fos in astrocyte, the fixed cultures were first rinsed with 0.1 M glycine in PBS for 5 min, followed with 10% goat serum, 0.2% (v/v) Triton-X 100 and 3% (w/v) BSA for 20 min. The cultures were then incubated with 1:100 dilution of anti-c-Fos rabbit polyclonal antibody (cat# sc-42, Santa Cruz Biotechnology, Inc., USA) in PBS at room temperature for 2 hr. After the cultures were rinsed with PBS 3 times, affinity purified Rhodamine-conjugated goat anti-rabbit IgG secondary antibody (Calbiochem-Novabiochem Co., CA, USA) at a 1:100 dilution in PBS was added. After 3 washes with PBS, the cultures were mounted in Mowiol (Calbiochem-Novabiochem Co., CA, USA) and observed under a Confocal Laser Scanning Microscope (Bio-Rad Laboratories, MRC-600, CA, USA).

2.6.3 Localization of HSP 70 Protein

For studying the localization of HSP 70 in astrocyte, the fixed cultures were first rinsed with 0.1 M glycine in PBS for 5 min, permeabilized with 0.3% (v/v) NP-40 in PBS for 10 min, and incubated with a 1:100 dilution of anti-HSP 70 mouse monoclonal antibody (Sigma Chemical Co., St. Louis, MO, USA) in PBS
for 1 hr at room temperature. After the cultures were rinsed with PBS 3 times, affinity purified Texas red-conjugated goat anti-mouse IgG secondary antibody (Calbiochem-Novabiochem Co., CA, USA) at a 1:100 dilution in PBS was added. After 3 washes with PBS, the cultures were mounted in Mowiol (Calbiochem-Novabiochem Co., CA, USA) and observed under a Confocal Laser Scanning Microscope (Bio-Rad Laboratories, MRC-600, CA, USA).

2.7 Extraction of Energy Metabolites

After various lengths of experimental incubations, cultured cells were washed twice with iced-cold PBS and then dissolved in 0.5 ml of 0.125 M KOH. The cell extracts were transferred to labeled eppendorf tubes (Scientific Plastics®, FL, USA) and vortexed immediately. After 5 min on ice, 0.4 ml of 1 M KH₂PO₄ was added to adjust the extracts to pH 6. The extracts were lyophilized and stored at -80°C until HPLC analysis. Before HPLC analysis, the dried powder was resuspended in 200 µl of double distilled water. Twenty-five µl of the suspension was saved for protein determination (Lowry et al., 1951). The rest of the suspension was centrifuged for 5 min at 4°C in a table-top Eppendorf Centrifuge (Eppendorf 5414C, Netheler, Hinz GmbH, Hamburg). The supernatant was utilized for the subsequent adenine nucleotides determination. All the chemicals were from Sigma Chemical Co. (St. Louis, MO, USA).

2.8 High Performance Liquid Chromatography

HPLC system (Millipore Corporation, Milford, MA, USA) was used to quantify the changes of nucleotides in neural cell cultures under ischemia and post-
ischemia incubations. The system consisted of a Model 600E multisolvent delivery system, a Model 717 autosampler and a Model 996 photodiode array detector. Retention times and peak areas were recorded by a Millennium 2010 chromatography manager system. Separations were performed on a 5 mm Supelcosil LC 18™ column (25 cm x 4.6 mm I.D.) (Supelco, PA, USA) connected to a Supelguard™ guard column. The mobile phases consisted of two eluents: Buffer A, 0.1 M KH₂PO₄, pH 6 and Buffer B, 0.1M KH₂PO₄, pH 6, with 10% (v/v) methanol. All buffers, after pH adjustment with KOH, were filtered through a 0.45 mm filter (Sigma Chemical Co., St. Louis, MO, USA) and degassed by helium gas (99.995%) for 20 min with a sparge rate of 100 ml/min before use. Thereafter, the sparge rate was kept at 15 ml/min throughout the run. The mobile phase flow rate was 1.3 ml/min. The chromatographic gradient program was initially 9 min of 100% Buffer A, gradual increase of Buffer B to 25% for 6 min, then increased to 90% for 2.5 min and to 100% for the final 2 min. The initial condition was restored for 5.5 min. The column was conditioned with 100% buffer A for an additional 5 min before the next injection. Adenine nucleotides contents were quantified at 254 nm.

Standard stock solution of adenine nucleotides were prepared in 0.1 M KH₂PO₄, pH 6, and stored at -80°C. Figure 1 showed the sample chromatograph for ATP, ADP and AMP standards with amounts of 1 nmol each. Retention times (RT) for ATP, ADP, and AMP were 5.618, 6.488 and 12.415 min, respectively. The calibration curve for each standard was created by integrating 3 amounts. The amounts for ATP were 0.1, 1 and 10 nmol, while the amounts for ADP and AMP were 0.5, 1 and 5 nmol. The height of the peaks for the corresponding standards was integrated and the sample calibration curves for ATP, ADP and AMP were created and showed in Figures 2–4, respectively. The 3 calibration curves showed a straight line. The contents of adenine nucleotides in the samples were all
Figure 1. A sample chromatograph for ATP, ADP and AMP standards measured by HPLC. The amount of each standard was 1 nmol and the absorbances were monitored at 254 nm.
ATP:-

Retention time: 5.618 min

$Y = 2.97e+04X$

$R = 0.999683$  \hspace{1cm} $R^2 = 0.999367$

Standard error = $4.042660e+03$

Figure 2. A sample calibration curve for ATP standard created by integrating 3 amounts of ATP: 0.1, 1 and 10 nmol.
ADP:
Retention time: 6.488 min

\[ y = 1.70 \times 10^4 X \]

\[ R = 0.993224 \quad R^2 = 0.986495 \]

Standard error = 5.262100e+03

Figure 3. A sample calibration curve for ADP standard created by integrating 3 amounts of ADP: 0.5, 1 and 5 nmol.
AMP:

Retention time: 12.415 min

Y = 2.02e+0.4X

R = 0.99992 \quad R^2 = 0.999985

Standard error = 2.001425e+02

Figure 4. A sample calibration curve for AMP standard created by integrating 3 amounts of AMP: 0.5, 1 and 5 nmol.
quantified within the calibration range, thus, the amounts of adenine nucleotides calculated from these 3 calibration curves were reliable.

2.9 Cell Viability Assay

The cell viability assay was performed in ischemic astrocytes. After ischemia incubations, the astrocytic cultures were removed from the anaerobic chamber. They were rinsed 3 times with PBS and then incubated with cell viability assay solution containing 4 µM Calcein-AM and 4 µM ethidium homodimer [Live/Dead® Eukolight™ Viability/Cytotoxicity Kit (L-3224), Molecular Probes, Inc., OR, USA] in MEM without serum for 40 min at 37°C in a normal CO2 incubator. Thereafter, the cultures were rinsed 3 times with PBS and mounted in Mowiol (Calbiochem-Novabiochem Co., La Jolla, CA, USA). The number of Calcein-AM positive and ethidium homodimer positive cells were counted under a fluorescence microscope. Calcein-AM was well retained within live cells (Moore et al., 1990). Live cells have the ubiquitous intracellular esterase enzymatic activity that was able to convert nonfluorescent cell-permeant calcein-AM to intensely fluorescent calcein that produces an intense green fluorescence (about 530 nm). Ethidium homodimer only entered damaged cell membranes and bound to nucleic acid to produce a bright red fluorescence (>600 nm) in dead cells.

2.10 Statistical Analysis

All data were analyzed by StatView, Version 4.0 and presented as the means ± S.E.M. Statistical analysis was performed by Student's t-test. Unpaired
$t$-tests was used and a confident interval of 95\% (i.e. $p \leq 0.05$) was considered being statistical significance.
CHAPTER 3

RESULTS

3.1 Induction of Protein Expressions in Astrocytic Cultures Under Ischemia and Post-ischemia

For studying specific protein expression in astrocytes, the mineral oil induced ischemia model was used. For ischemia, the incubation times were 0, 1, 2, 3 and 4 hr. The post-ischemia incubations were performed only on cultures after 2 hr of ischemia.

3.1.1 Western Blot Analysis

3.1.1.1 Induction of C-Jun Protein

A c-Jun control peptide (cat# sc-45 P, Santa Cruz Biotechnology, Inc., USA) was run along with the protein samples during Western blot analysis. Two bands of around 60 kDa were detected in the control peptide column (Figure 6A). Figure 5 showed the induction of c-Jun protein in astrocytic cultures under ischemia. The level of c-Jun at 1 and 2 hr was increased significantly to about 120% of the control and maintained insignificantly around this level for the rest of ischemia incubation. Figure 6 showed the expression of c-Jun in astrocyte cultures which was further induced under post-ischemia. By 2 hr of post-ischemia, the level of c-Jun protein maintained at the same level as 2 hr of ischemia but it was significantly increased to 140% of control after 4 hr of post-ischemia. After that,
Figure 5. A) Western blot analysis of c-Jun protein in astrocytic cultures after 4 hr of ischemia. B) Densitometric analysis of c-Jun protein expression under ischemia detected by Western blotting. The measured optical densities (OD) were expressed as ratios compared to the culture at 0 hr. Data represented the means ± S.E.M. taken from 3 cultures. (*) meant p≤0.05 as compared to culture at 0 hr.
Figure 6. A) Western blot analysis of c-Jun protein in astrocytic cultures under post-ischemia (after 2 hr of ischemia). P represented the c-Jun control peptide. B) Densitometric analysis of the c-Jun protein expression under post-ischemia detected by Western blotting. The measured OD were expressed as ratios compared to the culture at 0 hr without any treatment. Left side of the dotted line represented the measured OD of cultures at 0 and 2 hr of ischemia and right side represented the post-ischemia incubations. Data represented the means ± S.E.M. taken from 3 cultures. (*) meant $p<0.05$ as compared to culture at 0 hr.
the amount of c-Jun was insignificantly maintained at the same level for the rest of post-ischemia incubation.

3.1.1.2 Induction of HSP 70 Protein

The HSP 70 antibody from Sigma was able to recognize the constitutive form of Hsc 70 and inducible form of Hsp 70. Copin et al. (1995) have shown that the antibody was able to detect a band of 70 kDa in control culture and cultures being heated for 2 hr at 43°C. An additional band with molecular weight 68 kDa was detected in cultures after 4 hr recovery from the heat stress. This band was the inducible form of Hsp 70. Thus, the antibody used in this study was able to detect the two forms of HSP 70 in cultured rat astrocytes.

In this study, only one band was observed in all experimental conditions (Figures 7A and 8A). It suggested only the constitutively form Hsc 70 was expressed in these cultures. Figure 7 showed the induction of Hsc 70 in astrocytic cultures under ischemia. The level of Hsc 70 in 2 hr was increased but insignificantly to 110% of the control and maintained around this level for the rest of ischemia incubations. Figure 8 showed the expression of Hsc 70 in astrocytic cultures that was further induced under post-ischemia. After 2 hr of post-ischemia, the protein was significantly increased to 130% of the control level and maintained at this level after 10 hr of post-ischemia before decreased to the control level after 48 hr of post-ischemia incubation.
Figure 7. A.) Western blot analysis of Hsc 70 protein in astrocytic cultures after 4 hr of ischemia. B) Densitometric analysis of the Hsc 70 protein expression under ischemia detected by Western blotting. The measured OD were expressed as ratios compared to the culture at 0 hr. Data represented the means ± S.E.M. taken from 3 cultures.
Figure 8. A) Western blot analysis of Hsc 70 protein in astrocytic cultures under post-ischemia (after 2 hr of ischemia). B) Densitometric analysis of the Hsc 70 protein expression under post-ischemia detected by Western blotting. The measured OD were expressed as ratios compared to the culture at 0 hr without any treatment. Left side of the dotted line represented the measured OD of cultures at 0 and 2 hr of ischemia and right side represented the post-ischemia incubations. Data represented the means ± S.E.M. taken from 3 cultures. (*) meant p < 0.05 as compared to culture at 0 hr.
3.1.2 Immunocytochemistry

3.1.2.1 Localization of C-Fos Protein

Figure 9 showed the induction of c-Fos protein in astrocytes under ischemia and post-ischemia. C-Fos protein was undetected in control cultured astrocytes (Figure 9A). Only very slight but detectable c-Fos proteins were stained in nuclei by 1 hr of ischemia (Figure 9B). After 2 hr of ischemia (Figure 9C), the intensity for c-Fos staining in nuclei became stronger. The immunoreactivity was further increased by 4 hr of ischemia (Figure 9D). This was especially obvious in some of the nuclei.

In post-ischemia incubation, more nuclei had strong staining for c-Fos antibody (Figure 9E). Figure 9F showed the nuclei after 4 hr of post-ischemia still had high immunoreactivity with c-Fos antibody. By 10 hr of post-ischemia (Figure 9G), the immunoreactivity of some nuclei was further increased. Similar observation was seemed after 24 hr of post-ischemia (Figure 9H) except the processes of some astrocytes showed increased immunoreactivity to c-Fos antibody.

3.1.2.2 Localization of HSP 70 Protein

Figure 10 showed the localization of HSP 70 protein in astrocytes under ischemia and post-ischemia. In Figure 10A, the control culture showed a low but barely detectable staining of Hsc 70 in the nuclei. After 1 hr of ischemia incubation (Figure 10B), a few numbers of astrocytes started to show positive staining for HSP 70 antibody in nuclei. By 2 hr of ischemia (Figure 10C), astrocytes had a very strong staining in both cytoplasms and nuclei. The intensity of the staining became stronger at 4 hr of ischemia (Figure 10D).
Figure 9. Confocal images of astrocytic cultures stained with Rhodamine conjugated with anti-c-Fos rabbit polyclonal antibody. Pictures in left column showed the cultures under ischemic injuries: A) control; B) 1 hr; C) 2 hr; D) 4 hr. Pictures in right column showed the cultures under post-ischemia after exposed to 2 hr of ischemia: E) 0.5 hr; F) 4 hr; G) 10 hr; H) 24 hr. Bar size = 50 μm.
Figure 10. Confocal images of astrocytic cultures stained with Texas red conjugated with anti-HSP 70 mouse monoclonal antibody. Pictures in left column showed the cultures under ischemic injuries: A) control; B) 1 hr; C) 2 hr; D) 4 hr. Pictures in right column showed the cultures under post-ischemia after exposed to 2 hr of ischemia: E) 0.5 hr; F) 3 hr; G) 24 hr; H) 48 hr. Bar size = 50 μm.
The staining of HSP 70 seen in culture at 2 hr of ischemia did not show the same observation as in similar culture after 30 min of post-ischemia incubation (Figure 10E). However, the staining in both nuclei and cytoplasms were intensified again at 3 hr of post-ischemia (Figure 10F). At 24 hr of post-ischemia (Figure 10G), the HSP 70 protein staining in the nuclei was decreased but more cytoplasms being stained. After 48 hr of post-ischemia (Figure 10H), the general staining intensity of HSP 70 in both nuclei and cytoplasms were decreased. There were still a few nuclei stained strongly for HSP 70 protein.

3.2 Extent of Damage in Astrocytes Under Ischemic and Post-ischemic Injuries

The alteration of energy metabolism in astrocytes under ischemia and post-ischemia was investigated by anaerobic chamber model.

3.2.1 Cell Morphology

Figure 11 showed the morphological changes of astrocytes under simulated ischemia induced by anaerobic chamber. Figure 11A showed a control culture of rat astrocyte before it was exposed to ischemia. The culture consisted of a confluent layer of globblestone-like astrocytes with few phase bright microglial cells lying on the surface. Figure 11B showed a similar culture exposed to 1 hr of ischemia, the general integrity and morphology of the cultured cells changed and the astrocytes started to bear long phase dark processes. Some cells contained granular structures in their cell bodies. In 2 hr of ischemia (Figure 11C), the number of phase dark process bearing cells drastically increased. Some cell bodies were shrunked and acquired a star shape morphology. By 4 hr of ischemia (Figure 11D), a group of cells died and left a layer of debris and nuclei on top of a cell layer.
Figure 11. Phase contrast micrographs showed the morphological changes of primary astrocytic cultures under ischemia induced by anaerobic chamber. A) Control = 0 hr; B) 1 hr; C) 2 hr; D) 4 hr; E) 6 hr; F) 8 hr; G) 9 hr and H) 10 hr. Bar size = 50 μm.
These cells at the bottom were still intact and some of them bear long and thick cytoplasmic processes. Same observation was seemed at 6 hr (Figure 11E) of ischemia except the cell processes from the bottom cells layer started to loss its integrity. Figure 11F showed the cultures at 8 hr of ischemia, lots of cells died and many nuclei left on the culture plate. There were some phase dark process bearing cells at the bottom but they did not appear intact. By 9 hr (Figure 11G) and 10 hr (Figure 11H) of ischemia, nearly all the cells died with many nuclei still remained on the culture plate.

3.2.2 Cell Viability Assay in Astrocytic Cultures Under Ischemia

The extent of cell death under ischemic injury was measured by a cell viability assay kit. Figure 12 showed the representative micrographs of astrocytes that stained with Calcein-AM and ethidium homodimer after various periods of ischemia incubations. At 0 hr, astrocytes took up Calcein-AM and converted it to calcein with green fluorescence by the enzymatic activity of intracellular esterase. Figure 12A and D showed the astrocytes with green fluorescence where they were evenly distributed throughout the pictures. At 4 hr of ischemia (Figure 12B and E), some of the cells still had the appearance of green fluorescence. Other cells were intensively stained with ethidium homodimer in nuclei. These cells were already dead so that ethidium homodimer was able to enter the damaged membranes and bound to nucleic acid to produce a bright red fluorescent. After being subjected to 10 hr of ischemia (Figure 12C and F), nearly all the cells were dead as very few green fluorescent astrocytes were observed.

The numbers of live and dead cells were counted under microscope and expressed as percentage of viable cell in Figure 13. No significant loss of viable cells was observed in 2 hr of ischemia. After that, the number of viable cells was
Figure 12. Representative fields of primary astrocytic cultures demonstrating live astrocytes (Calcein AM positive cells with green fluorescence) and dead astrocytes (ethidium homodimer positive cells with orange fluorescence) under control (A, D), 4 hr (B, E) and 10 hr (C, F) of ischemia incubations. Top row was phase contrast micrographs and the bottom row represented the fluorescence pictures of the same field. Photographs were taken with the barrier filter LP 520. Bar size = 50 µm.
Figure 13. The measurement of viable cells in percentage of the culture at 0 hr in primary astrocytic cultures after various lengths of ischemia incubations. Data represented means ± S.E.M. from two separate experiments with n = 4-5. (*) meant $p \leq 0.05$ as compared to the control value at 0 hr.
significantly reduced by more than 60% between 4 and 6 hr. It was further reduced to 10% of control by 8 hr and no further decrease was observed for the rest of ischemia incubations.

3.2.3 Alteration of Energy Metabolites in Astrocytes

The changes of adenine nucleotides in cultured astrocytes were measured with HPLC. Figure 14 showed 3 representative chromatographs for adenine nucleotides measurement of control culture and cultures at 4 hr and 10 hr of ischemia. Figure 14A was a sample chromatograph for control culture. There were 2 peaks with RT at 5.618 and 6.488 min that corresponding to the peaks for ATP and ADP, respectively. The RT for AMP was 12.415 min as determined by standard AMP (Figure 1). No AMP peak was detected at that position in the control cultures. Figure 14B showed the chromatograph for astrocytes at 4 hr of ischemia. Although both peaks of ATP and ADP were decreased, ATP decreased more drastically. No peak for AMP was detected. The chromatograph for 10 hr ischemia was shown in Figure 14C. The peaks for ATP and ADP were decreased to a level nearly undetectable. No peak for AMP was detected in astrocytes throughout this study. This observation might be due to the content of AMP in astrocytic cultures was relatively small and the amount was well below the sensitivity of the detector.

3.2.3.1 ATP And ADP Contents Under Ischemia

The absolute values of ATP and ADP in control astrocytic cultures at 0 hr were 35.10±1.77 and 9.38±0.87 nmol/mg protein, respectively (Table II). All the ATP and ADP values measured in experimental cultures would be presented as percentage to these absolute values. The percent changes of ATP and ADP in astrocytic cultures after being subjected to control and ischemia incubation were
Figure 14. Representative chromatograms for the measurement of adenine nucleotides concentrations in primary astrocytic cultures under A) 0 hr; B) 4 hr and C) 10 hr of ischemia. One-fourth amount of each sample was injected for the HPLC analysis and the absorbances of these samples were monitored at 254 nm. The HPLC program lasted for 35 min.
Table II. The contents of protein and adenine nucleotides in astrocyte cultures under control incubations for 0 and 10 hr and ischemia incubation for 10 hr. Data represented means ± S.E.M. (n = 11).

<table>
<thead>
<tr>
<th>Incubation Condition</th>
<th>Protein Content μg/culture</th>
<th>ATP Content nmol/mg protein</th>
<th>ADP content nmol/mg protein</th>
<th>ATP/ADP Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 hr)</td>
<td>343.83 ± 22.08</td>
<td>35.10 ± 1.77</td>
<td>9.38 ± 0.87</td>
<td>3.78 ± 0.25</td>
</tr>
<tr>
<td>Control (10 hr)</td>
<td>283.23 ± 35.06</td>
<td>39.91 ± 2.98</td>
<td>11.53 ± 0.62</td>
<td>3.47 ± 0.23</td>
</tr>
<tr>
<td>Ischemia (10 hr)</td>
<td>107.50 ± 14.49</td>
<td>3.46 ± 0.78</td>
<td>2.68 ± 0.32</td>
<td>1.05 ± 0.23</td>
</tr>
</tbody>
</table>
Figure 15. The percent changes of ATP and ADP contents in primary astrocytic cultures under control and ischemia incubations. (△) meant ATP contents in control incubation, (▲) meant ATP contents in cultures under ischemia, (□) meant ADP contents in control incubation and (■) meant ADP contents in cultures under ischemia. Data represented the means ± S.E.M. with a minimum of five cultures per time point examined. (⁎) meant p≤0.05, compared to the ATP contents in the control culture at 0 hr. (†) meant p≤0.05, compared to the ADP contents in the control culture at 0 hr.
shown in Figure 15. Within the 10 hr of control incubations, no significant change in ATP or ADP contents was observed. The ATP and ADP values at 10 hr of control incubation were 39.91±2.98 and 11.53±0.62 nmol/mg protein, respectively (Table II). However, after 2 hr of ischemia, astrocytic cultures showed a significant depletion in both ATP and ADP contents. The contents were reduced by 70% for ATP and 40% for ADP by 4 hr of ischemia. The ATP content was further reduced to 15% of the control at 6 hr and further to less than 10% by 8 hr of ischemia. No further reduction in ATP contents was observed in longer ischemia incubation and the content of ATP was 3.46±0.78 nmol/mg protein at 10 hr of ischemia. For ADP, it was reduced by 70% at 6 hr of ischemia and remained at this level for the rest of the ischemia incubation. The value of ADP was 2.68±0.32 nmol/mg protein after 10 hr of ischemia. Both ATP and ADP contents showed a similar trend of depletion but the decrease of ADP contents was slower than ATP.

3.2.3.2 Recoveries of ATP Contents Under Post-ischemia

Figure 16 showed the recoveries of total intracellular ATP contents during post-ischemia incubation in primary cultures of astrocytes exposed to various period of ischemia. Figure 16A showed the changes of ATP contents in astrocytes under control incubations for 36 hr. The ATP content was insignificantly increased by 10% after 4 hr of incubation. Thereafter, the ATP content was insignificantly decreased to 95% of the control at 24 hr and gradually to 90% of control value after 36 hr.

Figure 16B showed that astrocytic cultures exposed to 1 hr ischemia were able to replenish their ATP contents to the control level after 4 hr of post-ischemia. At the end of post-ischemia incubation, the ATP content was 90% of the control with insignificant difference.
Figure 16. The recoveries of total intracellular ATP contents in primary astrocytic cultures after different periods of ischemia incubations were studied. The ischemia incubation times were: A) control; B) 1 hr; C) 2 hr; D) 4 hr; E) 6 hr; F) 8 hr; G) 9 hr and H) 10 hr. The changes in ATP contents were expressed as percentage of the control value at 0 hr. The dotted vertical lines marked the end of ischemia incubations and the starting times for post-ischemia incubations. Left of the dotted lines showed the changes of ATP contents under ischemia which has been shown in more details in Figure 19. Data represented the means ± S.E.M. with a minimum of five measurements. (*) meant $p \leq 0.05$ as compared the ATP contents under post-ischemia incubations to the control value at 0 hr. (†) meant $p \leq 0.05$ as compared the ATP contents under post-ischemia incubations to the value at the end of their corresponding ischemia incubations.
In cultures after 2 hr of ischemia incubation (Figure 16C), their ATP contents returned to 90% of the control value in 4 hr of post-ischemia incubation. The final ATP content was slightly but insignificantly reduced to 80% of the control value at the end of post-ischemia incubation.

Figure 16D showed the recoveries of ATP contents in astrocytes after 4 hr of ischemia. After 4 hr of post-ischemia, the ATP content was significantly increased by 250% as compared to the content just after ischemia, i.e. 80% of the control. In 8 hr of post-ischemia, there was a small depletion in the ATP content but it showed no significant difference as compared to the value at 4 hr post-ischemia. At 24 hr of post-ischemia, these astrocytes were able to replenish their ATP contents to the control level.

The recoveries of ATP contents in astrocytes after 6 hr of ischemia were shown in Figure 16E. Similar observation was seen as in 4 hr of ischemia. Within the first 4 hr of post-ischemia incubation, the ATP content was significantly increased 250% as compared to the contents at the end of ischemia. However, the ATP content was equivalent to only 50% of the control. The content remained at around 40% of the control after 8 hr and then gradually increased to 60% of the control at 24 hr of post-ischemia. The total content of ATP did not return to its control level throughout the post-ischemia incubations.

As shown in Figures 16F, only 10% of ATP content remained after 8 hr of ischemia. After 4 hr of post-ischemia, the content was significantly increased by 400% as compared to the content just after ischemia. The contents were maintained at this level up to 16 hr before gradually returned to 60% of the control at 24 hr of post-ischemia.
The ATP contents remained at both 9 hr (Figure 16G) and 10 hr (Figure 16H) of ischemia were well below 10% of the control ATP contents. For 9 hr ischemia, the ATP contents were significantly increased by 200% in 4 hr of post-ischemia as compared to the content at the end of ischemia. It finally reached to 40% of the control value at 24 hr of post-ischemia. For 10 hr of ischemia, no significant increase in ATP content was observed in the initial 4 hr of post-ischemia and it reached to 30% of the control level at the end of the post-ischemia.

Based on these observations, in 1 hr of ischemia, there was no change of ATP contents in astrocytes during the 24 hr of post-ischemia. By 2 and 4 hr of ischemia, astrocytes were able to recover their ATP contents within 4 hr of post-ischemia and maintained at the control level for the rest of post-ischemia incubations. After 6 hr of ischemia, astrocytes did not return their ATP contents to the control level in 4 hr of post-ischemia and the contents was only 50% of control by 24 hr of post-ischemia. Similar observations were obtained for the rest of ischemia incubations, ATP contents did not return to the control level and they were well below 50% of the control after 24 hr of post-ischemia.

3.2.3.3 Recoveries of ADP Contents Under Post-ischemia

Figure 17 showed the recoveries of ADP contents in ischemic astrocytes under post-ischemia. Trends for the recoveries of ADP contents after various lengths of ischemia were very similar to those of ATP. For cultures under control incubation (Figure 17A) or 1 hr (Figure 17B) of ischemia, no significant change in ADP content was observed.

Astrocytes after 2 hr ischemia (Figure 17C) was further reduced insignificantly by 10% in 4 hr of post-ischemia. The contents returned to 90% of the control at the end of post-ischemia incubation.
Figure 17. The recoveries of total intracellular ADP contents in primary astrocytic cultures after different periods of ischemia incubations were studied. The ischemia incubation times were: A) control; B) 1 hr; C) 2 hr; D) 4 hr; E) 6 hr; F) 8 hr; G) 9 hr and H) 10 hr. The changes in ADP contents were expressed as percentage of the control value at 0 hr. The dotted vertical lines marked the end of ischemia incubations and the starting times for post-ischemia incubations. Left of the dotted lines showed the changes of ADP contents under ischemia which has been shown in more details in Figure 15. Data represented the means ± S.E.M. with a minimum of five measurements. (*) meant $p < 0.05$ as compared the ADP contents under post-ischemia incubations to the control value at 0 hr. (†) meant $p < 0.05$ as compared the ADP contents under post-ischemia incubations to the value at the end of their corresponding ischemia incubations.
After 4 hr of ischemia (Figure 17D), astrocytes were able to replenish their ADP content from 40% to 80% of the control within 4 hr of post-ischemia incubation. The content was returned to the control level by 24 hr of post-ischemia.

For astrocytes after 6 hr of ischemia (Figure 17E), the ADP content was significantly increased by 200% in the 4 hr of post-ischemia as compared to the content at the end of ischemia. However, the content was still significantly below the control level. The ADP contents were further reduced gradually. ADP was slightly decreased from 60% in 4 hr of post-ischemia to 45% of the control by the end of 24 hr of post-ischemia.

After 8 hr of ischemia (Figure 17F), the ADP content was returned to 50% of the control in 4 hr of post-ischemia. This content was reduced to 25% after 16 hr but increased to 45% of the control at the end of the 24 hr post-ischemia incubation.

The ADP contents at both 9 hr (Figure 17G) and 10 hr (Figure 17H) of ischemia were well below 30% of the control level. The cultures seemed to lose their ability to recover the ADP contents. No significant increase in the contents after 4 hr post-ischemia was observed as compared to the values at the end of the ischemia incubations. After 24 hr of post-ischemia, only 40% of the control ADP content was left in cultures exposed to 9 hr and 30% for 10 hr of ischemia.

3.2.3.4 Changes of ATP/ADP Ratios in Astrocytic Cultures Under Ischemia and Post-ischemia

ATP is the major energy source for supporting all the biochemical reactions in cells. It can transfer its energy to certain other molecules by its terminal phosphate groups. It becomes the energy depleted compound ADP but can be
Fig 18. The depletion of ATP/ADP ratios in primary cultures of astrocytes under control (□) and ischemia (◆) incubations were calculated based on the data presented in Figure 15. Data represented the means ± S.E.M. with a minimum of five measurements for each time point. (*) meant $p \leq 0.05$ as compared to the control culture at 0 hr.
reformed by rephosphorylation of the ADP. ATP/ADP is the ratio to reflect the appropriate rephosphorylation of the energy poor compound ADP to ATP, thus, the ratio may act as another index for studying ATP lost by hydrolysis under ischemia and the ability for ATP production by oxidative phosphorylation under post-ischemia. The absolute value of ATP/ADP in control astrocytic culture was 3.78±0.25 (Table II). Figure 18 showed the depletion of ATP contents in primary astrocytic cultures under ischemia being re-expressed as ATP/ADP ratios. Throughout the control incubations, the ratios did not seem to be significant altered. The ratio was 3.47±0.23 at the end of 10 hr control incubation. Within the first 2 hr of ischemia, the ratios were unaffected. After 4 hr of ischemia, the ratio dropped rapidly and significantly to 60% of the control. Only 30% remained at 8 hr of ischemia and this level was maintained for the rest of ischemia incubations. The ratio after 10 hr of ischemia incubation was 1.05±0.23.

Comparing the changes in ATP/ADP ratios to the ATP contents shown in Figure 15 the trends were similar. Significant loss of ATP contents started after 2 hr of ischemia and decreased very fast between 2-6 hr. After 8 hr of ischemia, no significant change of ATP was observed. However, the loss of ATP contents reflected by ATP/ADP ratios was slower compared to the ATP contents in Figure 15. After 4 hr of ischemia, the ATP content was reduced to one-third of the control but the ATP/ADP ratio was reduced to two-third of the control. The ATP content was only 10% of the control after 10 hr of ischemia but there was still 30% of the ATP remained as reflected by the ATP/ADP ratio.

Recoveries of ATP/ADP ratios under post-ischemia in astrocyte cultures were shown in Figure 19. The changes of ATP/ADP ratios in the first 4 hr of ischemia incubation (Figures 19B-D) were the same as those for ATP in Figures 16B-D. The ATP/ADP ratios returned to normal level in the first 4 hr of post-ischemia and remained at the control level for the rest of the incubations. However,
Figure 19. The recoveries of ATP/ADP ratios in primary astrocytic cultures after different periods of ischemia incubations were studied. The ischemia incubation times were: A) control; B) 1 hr; C) 2 hr; D) 4 hr; E) 6 hr; F) 8 hr; G) 9 hr and H) 10 hr. The dotted vertical lines marked the end of ischemia incubations and the starting times for post-ischemia incubations. Left of the dotted lines showed the changes in ATP/ADP ratios under ischemia which were shown in more details in Figure 18. Data represented the means ± S.E.M. with a minimum of five measurements. (*) meant $p \leq 0.05$ as compared the ratios under post-ischemia incubations to the control value at 0 hr. (†) meant $p \leq 0.05$ as compared the ratios under post-ischemia incubations to the value at the end of their corresponding ischemia incubations.
for those cultures under 6 hr of ischemia (Figure 19E), their ATP/ADP ratios returned to the control level within 4 hr of post-ischemia but it continuously increased significantly by 30% of control level at the end of the 24 hr post-ischemia. Similar phenomenon was observed in culture after 8 hr of ischemia (Figure 19F), the ATP/ADP ratio in post-ischemic astrocytes was continuously increased to 90% of the control at the end of post-ischemia. For 9 and 10 hr of ischemia, the abilities for ATP replenishment as reflected by ATP/ADP ratios were seem to be disturbed. Only 60% of the control value was reached after 4 hr of post-ischemia. The ratios gradually returned to the control level at 24 hr of post-ischemia.

The recoveries of ATP/ADP ratios were normal in culture exposed to 4 hr of ischemia incubations. Thereafter, the recovery patterns for ATP contents as reflected by the ATP/ADP ratios were changed after 6 hr of ischemia. The recoveries of ATP/ADP ratios in cultures after 6 hr of ischemia were quite different from those of ATP alone shown in Figure 16. For 6 and 8 hr of ischemia, the ratios were continuously increased and overshoted the control value after 24 hr of post-ischemia. The ratios under post-ischemia were increased to 130% and 190% of the control in cultures exposed to 6 and 8 hr ischemia, respectively. While under 9 and 10 hr of ischemia, the ATP/ADP ratios required longer times to return to control level after post-ischemia.

3.2.4 Changes of Protein Contents in Astrocytic Cultures After Ischemia and Post-ischemia

The protein content in culture can act as an index to reflect the amount of cell protein remained in culture after injury. The absolute protein content in control astrocytic cultures was 343.83±22.08 μg (Table II). Figure 20 showed the protein contents in astrocytic cultures under control, ischemia and 24 hr of post-ischemia
Figure 20. The percent changes of cell proteins in primary astrocytic cultures under control (◻ ), ischemia (◼ ) and 24 hr post-ischemia (◼◼◼ ) incubations. Data represented the means ± S.E.M. with a minimum of 5 measurements. (*) meant $p \leq 0.05$ as compared the protein contents to the control value at 0 hr. (†) meant $p \leq 0.05$ as compared the protein contents of 24 hr of post-ischemia incubation to the corresponding value at the end of ischemia incubation.
after various lengths of ischemia incubations. In the control incubation, the protein content was significantly reduced to 75% of the control value in 2 hr and it was further reduced to about 70% in 8 hr of incubation. The protein contents were returned to the 85% of the control value at the end of the control incubation.

For ischemia, in 1 hr of ischemia, the protein content was significantly reduced to 75% of the control value. It remained at this level for the next 5 hr. By 6 hr, the protein content was 70% of the control. It was drastically reduced to 40% in 8 hr and maintained at the similar level for the rest of incubations.

In 24 hr of control incubation, the protein content was significantly reduced to 70%. Compared 1 hr of ischemia to its 24 hr of post-ischemia, the protein content was increased by 10% as compared to the content after ischemia. This increase was not significant. For 2 hr incubation, the protein content in 24 hr post-ischemia culture was significantly increased by 20% as compared to the amount at the end of ischemia and reached 85% of the control. For 4-8 hr of ischemia, the protein contents in 24 hr post-ischemia remained at the same level as their corresponding ischemic contents. The protein content in 24 hr post-ischemia culture of 9 hr ischemia exposure further decreased significantly to less than 30%. No significant difference was observed in cultures of 10 hr of ischemia as compared to the 24 hr of post-ischemia.
3.3 Depletion of Energy Metabolites in Neuronal Cultures Under Ischemia

3.3.1 Cell Morphology

The disturbance of energy metabolism in neuronal cells under ischemia was studied by incubating the cultures in anaerobic chamber. Figure 21A showed a micrograph of a 10-day-old primary culture of cerebral cortical neurons. Some of the neuronal cell bodies were migrated to form aggregates and linked by a network of neurites. There was no significant morphological change in neuronal culture after being subjected to 60 min of ischemia and micrograph was not shown. After 90 min of exposure to ischemia (Figure 21B), most of the neurons appeared to lose their integrity although some of the neuritic network seemed to be intact. The neuronal cell bodies were swollen.

3.3.2 Depletion of Energy Metabolites

The changes of adenine nucleotides in primary neuronal cultures were studied by separating and quantifying the nucleotides with HPLC. Figure 22 showed the representative chromatographs in control and 90 min of ischemia. Chromatograph in Figure 22A showed 3 peaks with RT 5.618, 6.488 and 12.415 min that represented ATP, ADP and AMP, respectively. By 90 min of ischemia, the chromatograph (Figure 22B) showed that the height of ATP's peak was drastically decreased. No significant change was observed in the ADP's peak but the height for AMP's peak was increased.

The absolute values for ATP, ADP and AMP in a control neuronal culture at 0 hr were 30.06±3.83, 13.12±1.22 and 2.86±0.36 nmol/mg protein, respectively
Figure 21. Phase contrast micrographs showed the morphological changes of primary neuronal cultures under ischemia induced by anaerobic chamber. A) Control and B) 90 min. Bar size = 50 μm.
Figure 22. Representative chromatographs for the measurement of adenine nucleotides concentrations in primary cerebral cortical neuronal cultures under A) 0 and B) 90 min of ischemia. Two-fifth amount of each sample was injected for the HPLC analysis and the absorbances of the samples were monitored at 254 nm. The HPLC program lasted for 35 min.
Table III. The contents of adenine nucleotides in primary cerebral cortical neuronal cultures under control incubations for 0 and 90 min and ischemia incubation for 90 min. Data represented means ± S.E.M. (n=6)

<table>
<thead>
<tr>
<th>Incubation Condition</th>
<th>ATP Content nmol/mg protein</th>
<th>ADP Content nmol/mg protein</th>
<th>AMP Content nmol/mg protein</th>
<th>ATP/ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 hr)</td>
<td>30.06±3.38</td>
<td>8.52±0.66</td>
<td>0.09±0.02</td>
<td>3.57±0.40</td>
</tr>
<tr>
<td>Control (90 min)</td>
<td>13.12±1.22</td>
<td>6.46±0.54</td>
<td>0.92±0.05</td>
<td>2.06±0.28</td>
</tr>
<tr>
<td>Ischemia (90 min)</td>
<td>2.86±0.36</td>
<td>2.84±0.26</td>
<td>1.10±0.04</td>
<td>1.01±0.11</td>
</tr>
</tbody>
</table>
(Table III). The changes of adenine nucleotides under 90 min of control and ischemia incubations were shown in Figure 23.

3.3.2.1 Depletion of ATP Contents

Figure 23A showed the neurons did not have a significant loss in ATP content within 5 min of control incubation. However, it was significantly reduced by 55% within 10 min and only 40% was left at the end of the control incubation. Under ischemia, ATP content was insignificantly reduced to 70% of the control within 5 min and significantly decreased to 35% after 15 min ischemia. At the end of 90 min of ischemia, only 10% of ATP content was left in the neuronal cultures. By comparing the ATP values after different periods of ischemia incubations to the contents of their corresponding period of controls, the depletion of ATP under ischemia was much faster and larger than the control. A significant difference in ATP contents was observed by comparing the contents after 30 min of experimental incubations.

3.3.2.2 Changes of ADP Contents

Under control incubation (Figure 23B), ADP content was significantly increased by 80% within 5 min but returned to the control level by 15 min. For the rest of control incubations, no further significant change in ADP content was observed and 75% of control value was left after 90 min of incubation. By 5 min of ischemia, ADP content was slightly increased by 10%. Thereafter, the ADP contents were gradually decreased and one-third of control was left at the end of ischemia incubation. Unlike ATP contents, there was a significant difference in ADP contents after 5 min of ischemia as compared to the corresponding control incubations. ADP content in 5 min of control incubation was significantly 200% more than at 5 min of ischemia. No significant difference of ADP contents between
Figure 23. The percent changes of adenine nucleotides in primary cerebral cortical neuronal cultures under ischemia incubations. A) ATP: (◯) meant ATP contents under control incubations and (●) meant ATP contents under ischemia; B) ADP: (□) meant ADP contents under control incubations and (■) meant ADP contents under ischemia; C) AMP: (△) meant AMP contents under control incubations and (▲) meant AMP contents under ischemia. Data represented the means ± S.E.M. with n = 5-6. (*) meant p≤0.05 as compared the contents to the control value measured at 0 hr. (†) meant p≤0.05 as compared the contents under ischemia incubations to the contents of their corresponding control incubations.
the 15 min of control and ischemia incubations that implied ADP content in control incubation was drastically returned to control level within 10 min of incubation. For control incubations, after the ADP content returned to the control level in 15 min, no significant change was observed in its contents for the rest of the incubation. While in ischemia, the ADP content was further reduced after 15 min and significantly reduced to 40% of the control by the end of the incubation.

3.3.2.3 Changes of AMP Contents

AMP contents in primary neuronal cultures were relatively low as compared to the other adenine nucleotides (Figure 22). After 5 min of control incubation (Figure 23C), the AMP content was significantly increased to 750% of the control and up to 1100% within 30 min. The content was reduced to 700% of control after 60 min but increased to 1000% again at the end of control incubation. Within 5 min of ischemia, AMP content was drastically increased to 1000% of the control. It further significantly increased to 1800% of the control at 30 min of ischemia but decreased after 30 min to 1200% of the control at the end of 90 min ischemia. Under ischemia, ATP and ADP contents were decreased but AMP content was increased drastically. The AMP content by 15 min of ischemia incubation was significantly higher than incubated under control for 15 min. However, at the end of the incubations, both values were gradually reduced to similar levels.

3.3.2.4 Changes of ATP/ADP Ratios

Figure 24 showed the changes of ATP/ADP ratios in neuronal cultures. The absolute ATP/ADP ratio at 0 hr was 3.57±0.40 (Table III). For both control and ischemia incubations, the ratios were dropped immediately by 5 min. The ratio was significantly reduced to 50% of the control and remained at this level after 30 min of control incubation. After that, it was returned to 60% of the control at the
Figure 24. The changes of ATP/ADP ratios in primary cerebral cortical neuronal cultures under control (△) and ischemia (▲) incubations. Data represented the means ± S.E.M. with n = 5-6. (⁎) meant $p \leq 0.05$ as compared to the control culture at 0 hr. (†) meant $p \leq 0.05$ as compared the ATP/ADP ratios under ischemia to their corresponding control incubations.
end of the 90 min of control incubation. Under ischemia, the ratio dropped slower in the initial 5 min and reduced by 30% of the control. After that, it further reduced to 30% of the control by 15 min of ischemia incubation and maintained at this level for the rest of ischemia incubations. By comparing the ratios after different periods of ischemia incubations to their corresponding control incubations, a significant difference in ATP/ADP ratios was observed after 30 min of experimental incubations.
CHAPTER 4

DISCUSSION

4.1 Proteins Induction Under Ischemia and Post-ischemia

This study demonstrated that the expression of c-Jun, c-Fos and HSP 70 proteins were induced in cultured rat astrocytes under ischemia and post-ischemia. It inferred that the c-fos and HSP 70 genes shown to expressed under similar ischemia and post-ischemia conditions (Yu et al., 1995) could be translated into their proteins. The expressions of these proteins may have significant biological role to astrocytes in response to ischemic injury. The c-Fos expression may act as a signaling of the injury to astrocytes and HSP 70 expression may be the protective responses of astrocytes to ischemic injury.

4.1.1 Immediate Early Gene Products

The signaling pathways for the induction of IEG in astrocytes were via multiple pathways including the activation of protein kinase A, protein kinase C or calcium-calmodulin kinase (Arendander and deVellis, 1992; Hisanaga et al., 1992). The signal transduction mechanism for c-fos and c-jun genes under ischemia are still unknown. There were evidences shown that there was an immediate induction of c-fos and c-jun genes in cultured astrocytes after direct exposure to glutamate (Condorelli et al., 1993), various types of proteases (Neveu et al., 1993), hydrogen peroxide (Pecháň et al., 1992) and xanthine/xanthine oxidase (Naveihan et al., 1994). In this study, we were able to detect the expression of these 2 IEG proteins
in ischemic astrocytes. Since these two genes are transcription regulators, their expression may be essential for the recovery of injured cells by the regulation of the late response genes.

*In vivo* study has shown that c-Jun protein induction in hippocampal astrocytes was contributed to ischemic tolerance (Kato *et al.*, 1995). Ischemic tolerance is a phenomenon that selective vulnerability of neurons can be reduced by preconditioning the brain with sublethal ischemia. Jehan *et al.* (1995) have shown that c-Jun was important for the synthesis of NGF in cultured astrocytes. Many studies have shown that there was a novel synthesis of NGF and bFGF in astrocytes under various kinds of stress environments such as glutamate (Pecháň *et al.*, 1993) and reactive oxygen species (Naveilhan *et al.*, 1994). The induction of NGF under the adverse conditions was associated with the immediate and transient induction of *c-fos* and *c-jun* genes. NGF is believed to have a protective role on injured neurons after ischemia (Shigeno *et al.*, 1991). Many evidences have shown that c-Fos proteins were induced in glial-like cells during traumatic brain injury (Dragunow *et al.*, 1990a) and hypoxia-ischemia (Gunn *et al.*, 1990). It was suggested that c-Fos proteins might involve in the induction of GFAP (Dragunow *et al.*, 1990b) and contribute to the proliferation of astrocytes after ischemia. Evidence has shown that the expression of c-Fos protein in glial cells occurs before there is a maximal glial cell division following trauma (Dragunow and Robertson, 1988).

### 4.1.2 HSP 70 Protein Expression

The expression of stress inducible Hsp 70 protein has been shown to be induced in cultured cortical astrocytes after heat shock injury (Marini *et al.*, 1990; Nishimura *et al.*, 1991). In this study, by Western blot analysis, only the constitutively expressed Hsc 70 protein was elevated and the inducible Hsp 70 was
undetected throughout the ischemic and post-ischemic incubations. This observation was consistent with Copin et al. (1995) that the cultured astrocytes did not express Hsp 70 after exposure to hypoxia. It might imply that the inducible Hsp 70 in astrocytes did not response to this ischemic injury. Copin et al. (1995) suggested that the synthesis of Hsp 70 protein in brain tissue may be contributed by the neurons.

Expression of HSP 70 genes seemed to have protective effect on the survival of the cells that experienced stress. Hsc 70 proteins have been found to be redistributed between nucleus and cytoplasm under and recovering from adverse environment (Welch and Mizzen, 1988). Studying the localization of Hsc 70 in astrocytes may provide more insight on the protective effect provided by astrocytes. The localization of Hsc 70 protein was studied in the mineral oil induced ischemia model.

Western blot analysis in this study showed that only the expression of Hsc 70 was elevated after ischemic injury. Furthermore, Copin et al. (1995) showed that the expression of inducible Hsp 70 protein appeared after 4 hr of heat shock injury (2 hr of 43°C). Thus, most probably, the positive immunoreactivity of HSP 70 protein studied in this part of study was Hsc 70 protein. The Hsc 70 protein in astrocytes was first detected in nuclei after 1 hr of ischemia. Hsc 70 proteins in nuclei may involve in the protection of ribosome biosynthesis and its recovery after ischemia (Pelham, 1984; Welch and Suhan, 1986; Welch and Mizzen, 1988). By 2 hr of ischemia, the immunoreactivity of Hsc 70 in the cytoplasms of astrocytes was detected. By 4 hr of ischemia, the immunoreactivity was further increased. These may be due to the stimulation from the increase of denatured proteins in cytoplasms. Four hr exposure of astrocytic cultures to ischemia could cause a more severe injury than 2 hr of ischemia. More Hsc 70 proteins may be needed in
binding or in appropriate refolding of these denatured proteins (Beckmann et al., 1990).

4.2 The Extent of Ischemic Injury in Cultured Astrocytes

Based on the result from this study, the extent of ischemic damage to astrocytes was determined and could be roughly divided into 4 stages. The first stage was between 0-2 hr of ischemia. The injury in astrocytes created was mild as there was no significant change in viable cell number and disturbance in energy metabolism. The second stage was at 4 hr of ischemia that the injury was moderate. The number of viable cells decreased by 50% and there were significant lost in high phosphate energy compounds (ATP and ADP). The third stage was between 6-8 hr of ischemia. The injury to astrocytes became severe. There was a further depletion of high phosphate energy compounds and a disturbance of energy metabolism. The forth stage was after 8 hr of ischemia. The damage was lethal since more than 90% of cultured astrocytes were dead.

4.2.1 Mild Injury (0-2 hr Ischemia)

Ischemic condition in this study was created by the anaerobic chamber induced ischemia model. Astrocytes experienced hypoxia and hypoglycemia immediately after the medium changing.

One hr of ischemia did not cause any observable or measurable injury in cultured astrocytes. The contents of ATP and ADP remained at the control level and there was no disturbance in the energy metabolism as reflected by the
ATP/ADP ratios. Depletion of ATP/ADP ratio reflects the extent of ATP hydrolysis to ADP. The ratios remained at control level meant that the energy storage in cultured astrocytes was able to support the normal physiological functions. In addition, 1 hr of ischemia did not affect the energy metabolism of astrocytes under post-ischemia. The contents of energy metabolites maintained at the control level for the 24 hr of post-ischemia incubation.

Normally, the metabolism of resting cultured astrocytes is mainly depended on aerobic glycolysis (Kauppinen et al., 1988). Their energy storage can only be sustained for a few min when there is no oxygen and glucose supply. Under ischemic condition, ATP synthesis in cultured astrocytes by aerobic glycolysis and oxidative phosphorylation should be immediately suppressed. However, in this study, the energy metabolism of astrocytes was normal under the first stage of injury. The normal in energy metabolism might be supported by the oxidation of other substrates such as glutamate or ketone bodies (Patel et al., 1981; Kauppinen et al., 1988; Yu et al., 1992).

By 2 hr of ischemia, there was a insignificant lost of viable astrocytes. The energy contents started to decrease slowly. It seemed that the ATP production by anaerobic glycolysis could not support the energy requiring metabolic processes in astrocytes after 1 hr of ischemia. ATP was lost by hydrolysis as reflected by the ratio of ATP/ADP. However, 2 hr exposure to ischemia did not cause any significant injury in energy synthesis system. The shortage in energy storage was non lethal as the astrocytes were able to recover the energy contents immediately within 4 hr of post-ischemia and maintained at the control level for the rest of post-ischemia incubations.

Brief exposure of astrocytes to ischemia did not affect the energy metabolism but it seemed to affect other cellular responses. This was reflected by
the changes in the total protein contents. The protein contents in control cultures were significantly decreased by 30% after 24 hr of incubation. The loss of protein content in the initial control incubation may be due partly to the detachment of other cells such as oligodendrocytes and microglial that are loosely attached on top of the astrocytic cultures. In addition, as cultures were exposed to serum free medium, the astrocytes will release lot of proteins to recondition the new environment. These released proteins will contribute a great part of the proteins lost from the cultures. However, there was no further loss of protein contents after 24 hr recovery from 1 and 2 hr of ischemia. For 2 hr of ischemia, the protein contents after 24 hr of post-ischemia were significantly higher than the content just after ischemic incubation. Furthermore, by comparing the 1 and 2 hr of ischemia incubations, the protein contents after 24 hr of post-ischemia were higher than that of 24 hr of control. The increase of protein contents may be contributed by the proliferation of astrocytes after ischemia. As mentioned before, astrogliosis would occur in response to brief exposure of ischemia that did not cause immediate destruction of astrocytes. In vivo evidence have shown that there was a proliferation of astrocytes after ischemic insult in gerbil brain (du Bois et al., 1985). The contents of GFAP were increased in astrocytes (Petito et al., 1990). Complete hydrolysis of ATP caused the production of ADP and AMP. Adenosines were formed by the deamination of AMP and they were released to the cultured medium. Adenosine and its nucleotides have the effects on inducing proliferation and differentiation of astroglial cells (Rathbone et al., 1992; Ciccarelli et al., 1994). By 2 hr of ischemia, some cells in cultured astrocytes were dead and more nucleotides or nucleosides were released. These additional extracellular adenine nucleotides and nucleosides would further increase the proliferation of astrocytes. This may have considerable biological significance during brain injury. It may be one important factor contribute to the gliotic changes following ischemia in brain.
4.2.2 Moderate Injury (4 hr Ischemia)

Four hr of ischemia caused a moderate injury to cultured astrocytes that less than half of the astrocytes was alive. Sixty percent of cells were dead by immediate destruction in 4 hr of ischemia. It might be a group of astrocytes that was more metabolic active, a fall in ATP content below certain critical value was able to trigger a cascade of complex biochemical reaction and cause immediate ischemic cell death.

By 4 hr of ischemia, it seemed that the substrates to support anaerobic glycolysis were used up or the supply of ATP was not enough to cover the expense of ATP. The ATP content dropped as reflected by ATP/ADP ratio, ATP was lost quickly by hydrolysis. Because energy contents were depleted in the cells, ADP was further hydrolyzed to AMP to support the energy requiring processes. Therefore, both energy contents were decreased in the same trends but the decrease of ADP contents was slower than that of ATP.

The amount of protein content left in cultures can act as an index to reflect the number of cells left. However, the amount of protein contents did not match with the number of viable cells left after 4 hr of ischemia. Under microscope, some dead cells were seemed with darken nuclei but they were still attached to the culture layer. The dead cells also contributed to the protein contents measured in the cultures. Therefore, the protein content may not be a good index to reflect the actual amount of viable cells in the cultures after 4 hr of ischemia. In this study, the contents of ATP and ADP were calculated as per mg protein. This protein included the dead cells that would decrease the actual value of ATP and ADP contents. Thus, the ATP and ADP contents were underestimated.
Though 60% of cells were lost under this injury stage, the remaining viable astrocytes were able to recover their energy metabolism within 4 hr of post-ischemia. The value of ATP/ADP returned to control level within 4 hr. It implied that ATP was synthesized by the phosphorylation of ADP. It also reflected that under this stage, no significant damage was done to the mitochondria function.

4.2.3 Severe Injury (6 and 8 hr of Ischemia)

The injury to astrocytes was more severe after exposure to 6 and 8 hr of ischemia incubations. The number of viable astrocytes and the energy rich phosphate compounds were further decreased. Reflected by the ATP/ADP ratios, about two-third of intracellular ATP was lost showing that an almost complete catabolism of ATP was occurred under this condition.

Exposure of astrocytes to 6 and 8 hr of ischemia did cause a disturbance in the balance of energy metabolism. Nevertheless, the viable astrocytes were able to resume their synthesis of ATP within 4 hr of post-ischemia as the ATP/ADP ratios returned to the control level. However, the ratios were increased continuously and reached nearly doubled of the control at the end of the post-ischemia incubations. It might imply that energy demand in astrocytes was greatly increased in order to re-establish the normal homeostasis conditions. Under normal status, the synthesis of ATP in mitochondria is highly regulated to prevent the wasteful oxidization of substrate. When energy demand in the cells increases, the accumulation of low energy phosphate compounds activates the re-synthesis of ATP to support the high energy demand biochemical processes in astrocytes. The overshoot of ATP/ADP ratio might be due to the rephosphorylation of all the cytosolic ADP after recovery.
4.2.4 Lethal Injury (8-10 hr Ischemia)

After 8 hr of ischemia, only 10% of cultured astrocytes were viable. It can assume that 8 hr of incubation without oxygen and glucose is lethal to astrocytes. The physiological functions of viable astrocytes were inhibited or all the available ATP was completely hydrolyzed as there was no further decrease of ATP/ADP ratios after 8 hr of ischemia.

The synthesis of ATP by oxidative phosphorylation in viable astrocytes was inhibited under post-ischemia. ATP/ADP ratios were only returned to 50% of the control level by 4 hr of post-ischemia and they required 24 hr to reach the control level. It might be because there were a destruction in structure and function of the mitochondria in the remaining but surviving astrocytes. The slow recovery of ATP content after post-ischemia might cause a further damage to the viable cells.

4.2.5 Correlation Between the Depletion of ATP and the Viability of Cultured Astrocytes Under Ischemic Injury

In this part of study, the extent of ischemic injury to cultured astrocytes was determined. Yu et al. (1989) and Gregory et al. (1990) have shown the correlation between the depletion of ATP and cell death which was studied by the activity of extracellular LDH in cultured astrocytes after exposure to hypoxic injury. Released of LDH from cells is an indirect indicator for studying cell injury. In this part of study, we have directly shown that there was a correlation between the depletion of ATP contents and the cell death in astrocytes under ischemic injury. The cell death of astrocytes started after 2 hr of ischemia which corresponds well with the ATP depletion as reflected by the ratios of ATP/ADP. After 4 hr of ischemia, significant lost of about 60% in viable astrocytes was associated with the significant depletion of 30% of ATP. This might imply that the energy reserve in astrocytes was used
up in 2 hr of ischemia. The astrocytes were not able to support the normal homeostatic function. This led to the influx of Na⁺, Ca²⁺ and Cl⁻ accompanied with water into astrocytes and the prolonged exposure to such condition might contribute to the direct mechanical destruction in astrocytes (Olson et al., 1986).

In this study, it also showed that there were many populations of astrocytes that have different tolerance to ischemic insult. The variability in tolerance among astrocytes might be due to the different energy storages in astrocytes and their metabolic stages during the insult (Roseniberg and Dichter, 1987; Juurlink et al., 1993). Nutrient storages were varied from cell to cell. The cultures may also contain a sub-population of astrocytes with younger differentiated stages. There were evidences that undifferentiated astrocytes were more resistant to the insult of hypoxia (Yager et al., 1994) and hypoglycemia (Hertz et al., 1995). Juurlink et al. (1993) also suggested that the release of cytoplasmic components from dead cells was able to support the metabolism of viable astrocytes left under ischemia.

4.2.6 The Restoration of Energy Metabolism in Viable Astrocytes After Post-ischemia

In this study, it seemed that astrocytes survived from the ischemic insult could resume their ATP synthesis ability under post-ischemia. However, the balance of energy metabolism was disturbed during recovery periods in astrocytes exposed to longer ischemia.

The 2 hr of ischemic injury to astrocytes was reversible as reflected by the rapid restoration of energy level. Four hr caused 60% of cell dead, however, the viable astrocytes were able to recover their ATP level within 4 hr of post-ischemia. It seemed that the survived astrocytes were not reached a stage of death and the changes in ATP synthesis was reversible. The rapid recovering of energy
metabolism in astrocytes may explain why astrocytes are more resistant to ischemia than neurons. Ischemia did cause a disturbance of energy metabolism in astrocytes after 4 hr of ischemia incubation as reflected by ATP/ADP ratios. Between 6-8 hr of ischemia, the ratios returned to the control level within 4 hr of post-ischemia but it continuously increased after reaching the control level. The overshooting in ATP productions might be due to the viable astrocytes required a large amount of ATP and all the available ADP was being phosphorylated to ATP.

The ischemic incubation between 4-6 hr may be the critical period for astrocytes in response to ischemic damage. The restoration of energy metabolites between these two time points was quite different after post-ischemia. By using a similar ischemia model, Goldberg et al. (1993) have suggested that cultured astrocytes were irreversibly injured by exposure to 4-6 hr of ischemia. In this study, we could not show the irreversible damage after post-ischemia but we did show the different in responses of energy metabolism between 4 and 6 hr of ischemia.

The restoration of energy metabolism seemed to be suppressed after 8 hr of ischemia. The ATP/ADP ratios could not return to the control level in 4 hr of post-ischemia. The viable astrocytes took more times for the building up of normal ATP/ADP ratios. It is believed that it would cause an irreversible damage to the energy metabolism in astrocytes after 8 hr of ischemia.
4.3 Depletion Of Energy Metabolites In Neuronal Cultures

Neuronal cultures showed a large change in energy contents under both control and ischemia incubations. For the control incubation, the perturbation in the energy metabolism may be due to the mechanical disturbance during the changing of incubating medium. Compared the ATP contents, the mechanical destruction in control was not as severe as ischemic injury to neurons. As reflected by the values of ATP/ADP ratios, the control neuronal cultures had the tendency to re-synthesis ATP by oxidative phosphorylation after 30 min of control incubation.

Changes of AMP contents were increased several folds under both control and ischemia incubations. As shown in Figure 22, the peaks for AMP were very small compared to the peaks for ATP and ADP. Small shift of the AMP peak height might cause a significant change in the AMP contents. Nevertheless, the pattern of adenine nucleotides changes in cortical neurons under ischemia was consistent with other similar studies in spinal cord (Stokes et al., 1991) and hepatocytes (Schwendel et al., 1995).

There was an immediate and dramatic disturbance in the energy metabolism as reflected by the depletion of ATP/ADP ratios. It implied that there was an immediate hydrolysis of ATP to ADP in neurons under ischemia. As ischemic incubation proceeds, the ATP content was reduced to less than 30% of control. It is certainly that ADP was also used to support the energy required for biochemical processes in neuronal cultures under ischemia. Neurons did not sustain ischemia incubation as they did not store energy and the metabolism of other substrates could not compensate for the rapid lost of energy metabolites (Pauwels et al., 1985).
Changes of energy metabolism in cortical neuronal cultures under post-ischemia were not studied. In in vivo study (Pulsinelli and Duffy, 1983), it has shown that neuronal cells in hippocampus regained the normal or near-normal function of mitochondria, in terms of energy metabolism but still had a delayed neural cells death. Therefore, the restoration of energy metabolites in neuronal cells after post-ischemia was not determined in this study.

Many studies had shown that cultured neurons were more susceptible to ischemia than astrocytes (Goldberg and Choi, 1993; Sochocka et al., 1994). In this study, we showed that there was a delayed depletion of ATP in astrocytes. The depletion of ATP was started after 2 hr of ischemia but the depletion of ATP was immediate and drastic in neurons by 5 min of ischemia. This may contribute to the difference in vulnerability between neurons and astrocytes to ischemia.

4.4 Mineral Oil Vs Anaerobic Chamber Induced Ischemia

Two in vitro models were used to simulate in vivo ischemia. The mineral oil model is a more economic system to study the responses of cultured cells in vitro. The only disadvantage is the greasy mineral oil making the experimental set up very clumsy. It is very difficult to handle many cultures at the same time. Anaerobic chamber model has been popularly used in other laboratories and is more convenient when handling a large batch of cultures in one single experiment. Theoretically, the injury effects created by these two models should be the same except the starting conditions were different. In anaerobic chamber model, cultures were fed with glucose and oxygen free medium. Cells immediately experienced hypoxia and hypoglycemia. In cultures under mineral oil model, cells were not washed before adding mineral oil. Therefore, the remaining medium still contained
a limited amount of oxygen and glucose. Therefore, the initial injury may come faster in anaerobic chamber induced ischemia model. The culture medium used in the mineral oil model is about 4 folds less than those in the anaerobic chamber. Thus, the toxic metabolites accumulation effect would be more severe in the mineral oil model. When ischemia incubation proceeds, the oxygen and glucose will be quickly used up in the mineral oil model, the injury to the cultured cells would be enhanced. Nevertheless, the cultures under either models would experience hypoxia, hypoglycemia and damaged by toxic metabolites. We have no intention to compare the outcomes obtained from these two models. Therefore, no effort was put to synchronize the starting points for these two models.
Chapter 5

Conclusion

Mineral oil induced model was used to study the induction of specific protein expressions in cultured astrocytes in response to ischemic injury. Three proteins were studied. C-Fos and c-Jun proteins belong to the products of IEG and Hsc 70 belongs to the family of stress proteins. All these proteins expressions were elevated after ischemic injury. This indicated that c-Fos, c-Jun and HSP 70 proteins may act as good markers for ischemic injury in cultured cortical astrocytes. Their expressions in astrocytes may have significant biological functions in the response to ischemia.

Anaerobic chamber induced ischemia model was used to study the alteration of energy metabolism in neural cells. Cultured cortical astrocytes were more tolerance than cultured cortical neurons to ischemia insult. The direct correlation between the depletion of ATP and cell injury in astrocytes was determined. The study also demonstrated that in culture of astrocytes, there were many populations of astrocytes at different stages of metabolism or development which have different tolerance to ischemic insults. After 6 hr of ischemia, there was a disturbance of energy metabolism in cultured astrocytes under post-ischemia. Most important of all, 4-6 hr of ischemia incubations may be the critical period for astrocytes in response to ischemic damage as indicated by the changes of energy metabolism. By studying the changes of other cellular responses between this period under similar ischemic condition, it will provide essential information about the mechanism of ischemic injury and cell death in neural cells.
References


