ผลของ Insulin-like growth factor I ต่อการบาดเจ็บของเซลล์อันเกิดจากภาวะขาดออกซิเจนในเซลล์ประสาทแกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัมของหนูขาว

นางสาว อัญชุกร เจริญศิริ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF INSULIN-LIKE GROWTH FACTOR I ON HYPOXIA-INDUCED CELLULAR INJURIES IN CULTURED RAT CEREBELLAR GRANULE NEURONS

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การวิจัยนี้มีวัตถุประสงค์ในการทดสอบผลของ Insulin-like growth factor I ในการป้องกันการบาดเจ็บของเซลล์ประสาทจากภาวะขาดออกซิเจน โดยใช้เซลล์ประสาทแกรนูลเพาะเลี้ยงจากสมองส่วนซีรีเบลลัมของหนูขาวเป็นโมเดลทดสอบให้อยู่ในภาวะ physical hypoxia (100% ไนโตรเจน) หรือ chemical hypoxia (rotenone) และใช้การวิเคราะห์ทางชีวเคมี ได้แก่ MTT reduction และ LDH release เป็นเครื่องมือชี้วัดความบาดเจ็บของเซลล์

ผลการทดลองแสดงว่าภาวะ physical และ chemical hypoxia ชักนำให้เกิดการบาดเจ็บของเซลล์ประสาทแกรนูลเพาะเลี้ยงจากซีรีเบลลัม ในลักษณะที่ขึ้นกับเวลาสัมผัสและความเข้มข้นตามลำดับ อย่างไรก็ดีภาวะขาดออกซิเจนทั้งสองแบบที่ใช้ในการศึกษานี้ไม่ทำให้เซลล์ประสาทตาย การให้ IGF-I สามารถลดความบาดเจ็บของเซลล์อันเกิดจากภาวะขาดออกซิเจนได้อย่างชัดเจน เมื่อให้ก่อนและระหว่างการสัมผัสกับภาวะขาดออกซิเจน แต่จะลดประสิทธิผลลงเมื่อให้ภายหลังการขาดออกซิเจน นอกจากนั้น IGF-I ยังลดการเพิ่ม lipid peroxidation อันเกิดจากภาวะขาดออกซิเจน ดังนั้นจึงน่าเชื่อว่า IGF-I มีผลปกป้องเซลล์ประสาทจากการบาดเจ็บอันเกิดจากภาวะขาดออกซิเจน โดยอาจทำให้เกิดผลดังกล่าวผ่านการลด lipid peroxidation ได้อีกทางหนึ่ง

ภาควิชา สหสาขาวิชาสรีรวิทยา	ลายมือชื่อนิสิต.....….....
สาขาวิชา สรีรวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา.....….....
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ANCHUKORN JAROINSIRI : EFFECTS OF INSULIN-LIKE GROWTH FACTOR I ON HYPOXIA-INDUCED CELLULAR INJURIES IN CULTURED RAT CEREBELLAR GRANULE NEURONS. THESIS ADVISOR: ASSOC. PROF. BOONYONG TANTISIRA, Ph.D., CO-ADVISOR; ASSIST. PROF. SURACHAI UNCHERN, Ph.D., 116 pp. ISBN 974-17-1304-5.

The objective of this study was to test whether IGF-I is protective against neuronal cell injuries induced by hypoxia. Cultured rat cerebellar granule neurons were used as the testing model and exposed to physical hypoxia (100% N_2) or chemical hypoxia (rotenone). End points for cell injury were quantitated by biochemical assays, MTT reduction and LDH release

The experimental results suggest that physical and chemical hypoxia induce cell injuries in cultured cerebellar granule neurons in the exposure time- and concentration-related manners, respectively. However, both types of hypoxic conditions used in this study did not lead to neuronal cell death. Treatment with IGF-I markedly attenuated hypoxia-induced cellular injuries when administered before (pre-treatment) and during (co-treatment) neuronal exposure to hypoxia. Cytoprotection was less effective when IGF-I was administered after (post-treatment) the hypoxic condition. In addition, IGF-I also attenuated hypoxia-induced increases in lipid peroxidation. Therefore, it is likely that IGF-I has protective effect against hypoxia-induced neuronal cell injuries which may be mediated, at least partly, by reduction of the lipid peroxidation.

Department Inter-department of Physiology	Student's signature.....….
Field of study Physiology	Advisor's signature……..….
Academic year 2002	Co-advisor's signature……..……..

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List of Abbreviations

α	=	Alpha
β	=	Beta
γ	=	Gamma
δ	=	Delta
ε	=	Epsilon
ξ	=	xi
ρ	=	Rho
μ	=	Micro
μg	=	Microgram
μl	=	Microlitre
μm	=	Micrometre
μM	=	Micromolar
%	=	Percent
AA	=	Arachidonic acid
Ach	=	Acetylcholine
ACTH	=	Adrenocorticotrophic hormone
ALS	=	Amyotrophic lateral sclerosis
ANT	=	Adenine nucleotide translocator
APAF-1	=	Apoptosis-activating factor 1
ADP	=	Adenosine diphosphate
ATP	=	Adenosine triphosphate
ATPase	=	Adenosine triphosphatase

BBB	=	Blood-brain barrier
BDNF	=	Brain-derived neurotrophic factor
bFGF	=	Basic fibroblast growth factor
BH	=	Bcl-2 homology
BSS	=	Balanced salt solution
^{o}C	=	Degree Celsius
Ca^{2+}	=	Calcium ion
Cl^{-}	=	Chloride ion
cm	=	Centimetre
CNS	=	Central nervous system
CO_2	=	Carbon dioxide
DMEM	=	Dulbecco's modified Eagle's medium
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
DNase I	=	Deoxyribonuclease I
DPBS	=	Dulbecco's phosphate-buffered saline
EC_{50}	=	Median effective concentration
ECM	=	Extracellular matrix
EEG	=	Electroencephalogram
e.g.	=	Exampli gratia (for example)
EGL-1	=	EGg Laying defective 1
eIF	=	Eukaryotic initiation factor
ER	=	Endoplasmic reticulum
EST	=	Expressed sequence tag

et al.	=	et alii (and other)
FBS	=	Fetal bovine serum
Fig	=	Figure
FSH	=	Follicular stimulating hormone
g	=	Gram
GH	=	Growth hormone
GraB	=	Granzyme B
GRB2	=	Growth factor receptor bound protein 2
hr	=	Hour
HBSS	=	Hank's balanced salt solution
HCl	=	Hydrochloric acid
HSP70	=	Heat Shock Protein 70
ICE	=	Interleukin-1-β-converting enzyme
IGF-I	=	Insulin-like growth factor I
IGF-II	=	Insulin-like growth factor II
IGFBP	=	Insulin-like growth factor binding protein
IRS-I	=	Insulin receptor substrate-I
K^+	=	Potassium ion
KCN	=	Potassium cyanide
kDa	=	Kilodalton
kg	=	Kilogram
L	=	Litre
LDH	=	Lactate dehydrogenase
M	=	Molar

MAP	=	Mitogen-activated protein
MDA	=	Malondialdehyde
mg	=	Milligram
Mg^{2+}	=	Magnesium ion
min	=	Minute
ml	=	Millilitre
mm	=	Millimetre
M6P	=	mannose-6-phosphate
mRNA	=	messenger ribonucleic acid
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium
MW	=	Molecular weight
N_2	=	Nitrogen
Na^+	=	Sodium ion
NaCN	=	Sodium cyanide
NADH	=	Reduced nicotinamide adenine dinucleotide
NADPH	=	Reduced nicotinamide adenine dinucleotide phosphate
NaOH	=	Sodium hydroxide
NGF	=	Nerve growth factor
ng	=	Nanogram
NMDA	=	N-methyl-D-aspartate
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
NPXY	=	Asn-Pro-X-Tyr

NSS	=	Normal saline solution
NT	=	Neurotrophin
OD	=	Optical density
P	=	Postnatal
PBS	=	Phosphate-buffered saline
PI3-kinase	=	Phosphoinositide 3-kinase
PKB	=	Protein kinase B
PNS	=	Peripheral nervous system
p.m.	=	post meridian (afternoon)
PDGF	=	Platelet-derived growth factor
PLA_2	=	Phospholipase A_2
PT	=	Permeability transition pore
ROS	=	Reactive oxygen species
sec.	=	Second
SEM	=	Standard error of the mean
SH2	=	Src homology 2
SHC	=	Src homology 2 domain-containing
SOD	=	Superoxide dismutase
TBARS	=	Thiobarbituric acid reactive substance
TNF	=	Tumor necrosis factor
TNFR-1	=	tumor necrosis factor receptor-1
Tyr	=	Tyrosine
VDAC	=	Voltage-dependent anion channel

Chapter I

Introduction

Neurotrophic factors and neuronal death

Neurotrophic factors include a large family of target-derived proteins that play an important role in the regulation of neuronal life and death. This is true for both the central and peripheral nervous systems, especially during brain development and under pathologic conditions such as hypoxia-ischemia.

1. Role of neurotrophic factors in naturally-occurring neuronal death

A previously held view is that naturally occurring neuronal death depends simply on the absence of appropriate survival signals such as neurotrophic factors. In the past decade, numerous neurotrophic factors and their mechanisms of action have been elucidated and this has greatly improved our understanding of the role of neurotrophic factors in neuronal survival and death. Current literature has shown that, on the one hand, neuronal survival depends on neurotrophic factors; insufficient amounts of such neurotrophic factors can lead to cell death. For example, nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) are essential for survival of sympathetic neurons during development (Bamji *et al*., 1998; Sondell *et al*., 1999). On the other hand, some of these neurotrophic factors such as brain-derived neurotrophic factor (BDNF) (Bamji *et al*., 1998) can mediate apoptotic signals and thus facilitate neuronal apoptosis. Therefore, naturally occurring neuronal death is determined by the balance of survival and apoptotic signals elicited by a variety of trophic factors (Bamji *et al*., 1998; Behrens *et al*., 1999).

2. Role of neurotrophic factors in hypoxic/ischemic apoptosis

Several lines of evidence have suggested that neurotrophic factors are up-regulated in the nervous system and are involved in hypoxic/ischemic cascades (Bossenmeyer-Pourie *et al*., 1999; DeCoster *et al*., 1999; Rong *et al*., 1999). In response to hypoxic-ischemic stress, neurotrophic factors may regulate neuronal survival and death in two ways: (a) direct participation in pathophysiological cascades and neuronal

apoptosis and (b) activation of inhibitory mechanisms of neuronal apoptosis. For example, there is a line of evidence showing that BDNF expression was increased in adult rat forebrain following limbic seizures (Cheng and Mattson, 1994; Mitchell *et al*., 1999; Nitta *et al*., 1999). Since BDNF may activate apoptotic signals, it is possible that the increased BDNF expression is one of the mechanisms underlying epileptic neuronal loss. Indeed, a number of neurotrophic factors has shown a protective role in neuronal apoptosis under pathological conditions. For instance, Insulin-like growth factor I (IGF-I) was capable of promoting cell survival and inhibiting apoptosis (Baker *et al*., 1999; Ye and D'Ercole, 1999)

IGF-I

Activity of IGF-I like proteins has been discovered in 1957. It was firstly named sulfatation factor (Salmon and Daughaday, 1957) later the term somatomedin C had been used (Daughaday *et al*., 1972; Daughaday and Rotwein, 1989). The precise action of IGF-I was poorly understood until the production of recombinant human IGF-I in the 1980s. Firstly, it had been well characterized as a factor mediating the action of growth hormone. It had been extensively studied in the development of bone and cartilage (Johansson *et al*., 1993) and proposed that it could play a significant role in the repair process in patients suffering from arthritis (Dor'e *et al*., 1994). IGF-I is found in high levels in the blood and believed to originate mainly from the liver , where its production is induced by growth hormone (Daughaday and Rotwein, 1989). Other organs, including the brain and spinal cord (Rotwein *et al*., 1988), also synthesize this peptide. Furthermore, systemic IGF-I enters the brain (Armstrong *et al*., 2000; Carro *et al*., 2000; Reinhardt and Bondy, 1994). Therefore, both locally produced and systemic IGF-I may affect brain function.

The structure of IGF-I, a peptide of 70 amino acids, is very much similar to that of insulin and IGF-II (Dor'e, 1997c) (Fig. 1). Interestingly, given the evidence that insulin level in the brain is quite low, researchers have suggested that IGF-I could exert insulin-like activity in the central nervous system. In fact, IGF-I has the capacity to bind to the family of IGF-like receptors including the insulin receptor (Fig. 2).

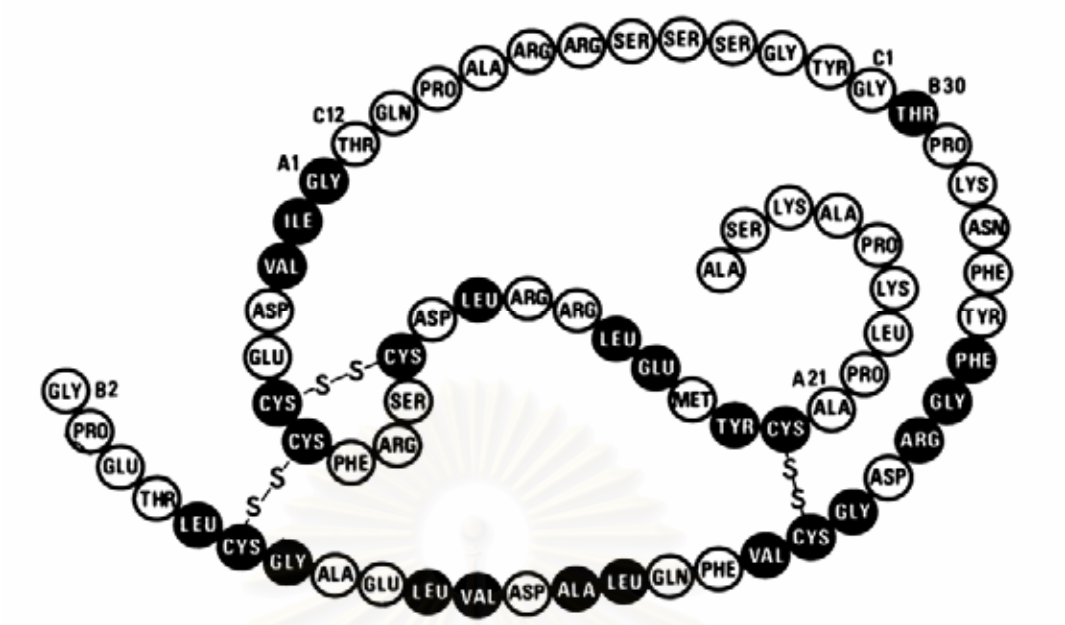


Fig. 1. Amino acid sequence of IGF-I. Key features are S-S bridges between A and B chains as observed for insulin (Dor'e *et al*., 2000).

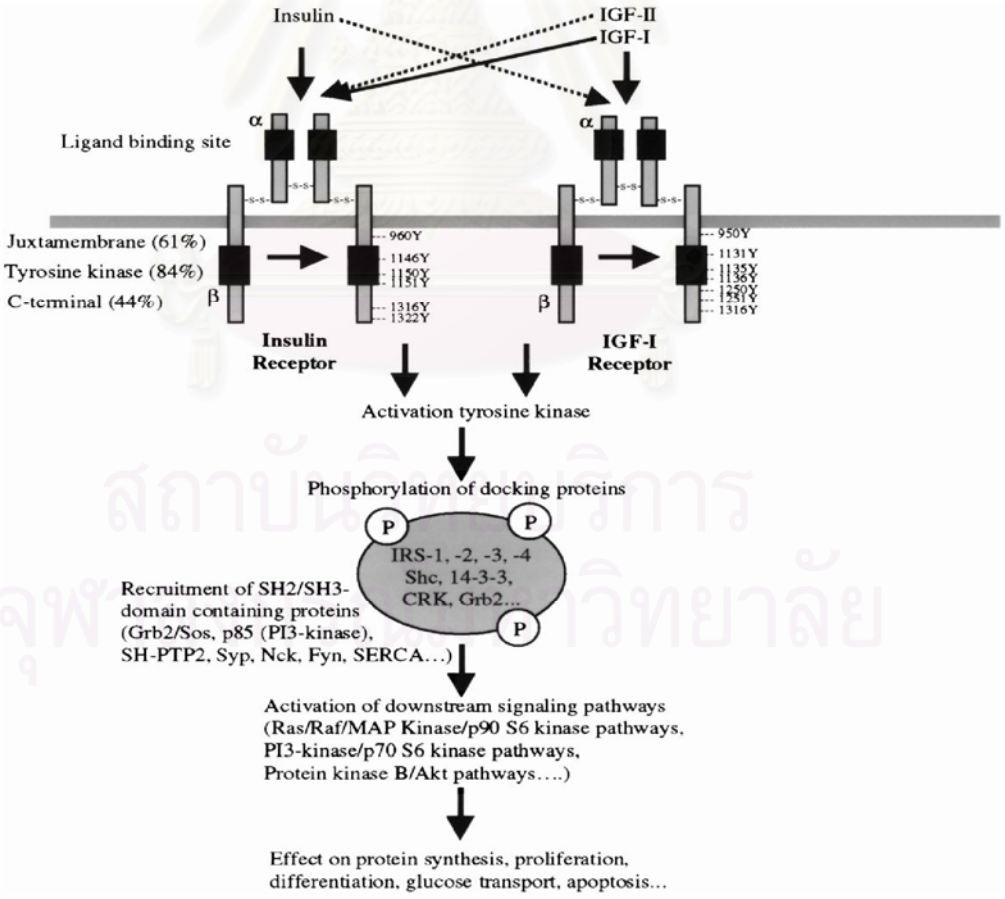


Fig. 2. Insulin and IGF-I receptors. Receptor domains are described on the left and the percentage of homology is indicated. Tyrosine residues undergoing phosphorylation upon receptor activation is also

show. Binding of the ligand to the extracellular α-subunits leads to autophosphorylation of specific tyrosine residues on the intracellular part of the β-subunits through a transphosphorylation mechanism that results in the activation of their tyrosine kinase domain. These enable the receptor kinase to phosphorylate intermediate docking proteins which subsequently recruit various intracellular proteins. This results in the activation of downstream signaling pathways (Dor'e *et al*., 2000).

Mechanisms of action of IGF-I

The IGFs are expressed in a highly regulated manner (Jones and Clemmons, 1995; de Pablo and de la Rosa, 1995). The biological functions of the Insulin-like growth factors (IGFs) and insulin are mediated by specific plasma membrane receptors, designated as the IGF-I (type-I IGF receptor), IGF-II (type-II IGF receptor) and insulin receptors.

IGF-I like immunoreactivity has been shown to be selectively localized to various regions of the brain and its physiological responses are presumed to be mediated by specific interactions with cell surface receptors (LeRoith *et al*., 1995; Dor'e *et al*., 1997b). The actions of IGF-I are mediated by the IGF receptors and modulated by a family of at least six IGF-binding proteins (IGFBP1-6) (Jones and Clemmons, 1995; Dor'e *et al*, 1997b). These IGFBPs can bind IGF-I and IGF-II, but not insulin, with high affinities. IGF-I and insulin mediate their actions by binding to insulin and/or IGF-I receptors that are structurally related disulfide-bonded heterotetrameric membrane glycoproteins of about 330–350 kDa (Fig. 2). The IGF-I receptor is a 1367 residue protein having higher affinity for IGF-I than for IGF-II or insulin, and consists of a heterotetramer consisting of two α- (115 kDa) and two β- (94 kDa) subunits joined by disulfide bridges (LeRoith *et al*., 1995). The binding of the ligand to the two α extracellular subunits induces conformational changes that lead to the phosphorylation of certain residues (Tyr-1131, 1135 and 1136) and the activation of intracellular effector molecules. The phosphorylation of Tyr-950 localized in the NPXY motif is required for IGF-I receptor internalization. The adaptor proteins SHC and Insulin receptor substrate-I (IRS-I) are also known to bind to this site. These events stimulate the activation of a cascade of intracellular signaling pathways.

The IGF-II/mannose-6-phosphate (M6P) receptor has a higher affinity for IGF-II than IGF-I and does not bind insulin. It comprises a single polypeptide chain with a

large extracellular domain and a short cytoplasmic tail (LeRoith *et al*., 1995). Furthermore, while the role of the IGF-II receptor in transporting lysosomal enzymes and internalizing various compounds appears to be well established, its function in signal transduction, unlike that of the IGF-I receptor, remains controversial (LeRoith *et al*., 1995; Dor'e *et al*., 1997b; Kar *et al*., 1997).

The process of receptor internalization is also strikingly different between the IGF-I and IGF-II receptors. Since the IGF-I receptor is coupled to tyrosine kinase activity, tyrphostin 47, a specific tyrosine kinase inhibitor, was used in cultured hippocampal cells and shown to inhibit [^{125}I] IGF-I, but not [^{125}I] IGF -II, receptor internalization (Dor'e *et al*., 1997b). Accordingly, IGF-I is apparently internalized mostly via the IGF-I tyrosine kinase receptor while IGF-II is not.

The activation of the IGF-I tyrosine kinase receptor by its endogenous agonist leads to an association with the src homology 2 (SH2) domain of proteins, and transducing the signal to a downstream cascade. Such a protein for both the IGF-I and insulin receptors is the IRS-1. Recently, several proteins with similar structures and different tissue distributions have been cloned (IRS-2,-3,-4) (Butler *et al*., 1998). IGF-I receptor/IRS-1 association subsequently activates phosphoinositide 3-kinase (PI3-kinase), phosphotyrosine phosphatases, S6 kinase, Ras-mitogen-activated protein (MAP) kinase and transcription factors, leading to alterations in Ca^{2+} mobilization and mitochondrial respiration (Lienhard, 1994; Jones and Clemmons, 1995; LeRoith *et al*., 1995).

The stimulation of IGF-I receptor results in the activation of pathway implicating PI3/Akt kinase by binding the p85 subunit directly (Tartare-Deckert *et al*., 1996) (Fig. 3). This pathway is believed to regulate many cellular processes, including apoptosis, glucose transport and metabolism, protein synthesis, mitosis and differentiation (Marte *et al*., 1997).

The use of pharmacological inhibitors of the PI-3 kinase pathway such as LY294002 and wortmannin and dominant negative PI-3 kinase mutants has shown that this pathway is an important mediator of the anti-apoptotic effects of IGF-I (D'Mello *et al*., 1997; Dudek *et al*., 1997; Kulik *et al*., 1997; Parrizas *et al*., 1997a). The activation of Akt is an important step in the inhibition of apoptosis, since the prevention of apoptosis by IGF-I

is diminished in cells expressing dominant negative Akt mutants (Dudek *et al*., 1997; Kulik *et al*., 1997). Two recent studies have led to better understanding of the role that Akt has in inhibiting apoptosis (Datta *et al*., 1997; del Peso *et al*., 1997). Current models of the regulation of apoptosis suggest that the balance between members of the Bcl-2 family plays a critical role in determining whether a cell survives or activates the cell death machinery. Several members of the Bcl-2 family (Bcl-2, Bcl-xL, MCL-1, A1, and BAG-1) appear to promote survival, whereas others (Bcl-x_S, BAD, BAX, and BAK) promote apoptosis (Zha *et al*., 1996). The balance between hetero- and homodimerization of the various Bcl-2 family proteins is believed to be critical in determining cell fate (Minshall *et al*., 1997; Parrizas and LeRoith, 1997b). The stimulation of Akt activity following IGF-I receptor activation culminates in the phosphorylation of BAD on ser-136. Serine-phosphorylated BAD can then form a complex with 14-3-3 ξ, thereby sequestering BAD and preventing its proapoptotic actions (Datta *et al*., 1997; del Peso *et al*., 1997). IGF-I can also promote cell survival by increasing the expression of Bcl-2 family members that inhibit apoptosis (Minshall *et al*., 1997; Parrizas and LeRoith, 1997b).

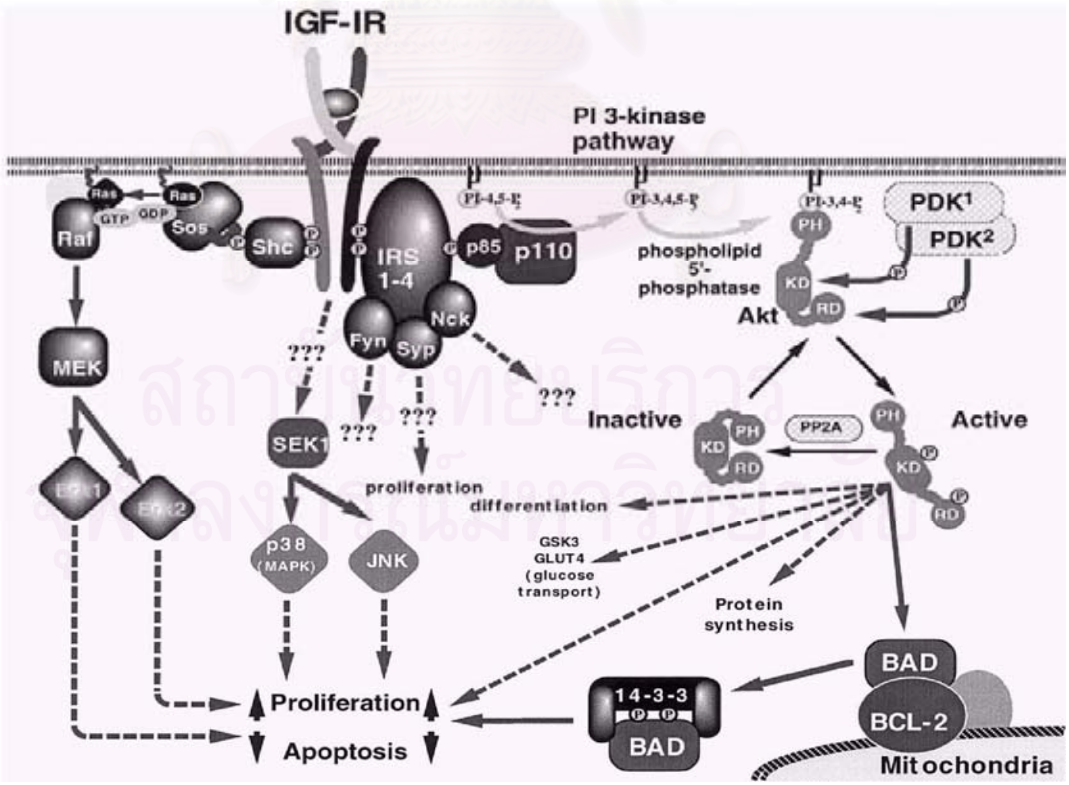


Fig. 3. Signal transduction cascades activated by the IGF-I receptor. Activation of the IGF-I receptor (IGF-IR) induces the binding and subsequent tyrosine phosphorylation of the SHC and IRS adapter

proteins to the intracellular region of the IGF-I receptor b subunit. Tyrosine phosphorylation of SHC and the IRS proteins creates binding sites for other proteins in the signal transduction cascade such as GRB2 (SHC, IRS), p85, Nck, Syp, and Fyn. The binding of the p85 and p110 subunit of phosphoinositol 3-kinase (PI 3-kinase) to members of the IRS family activates this pathway, ultimately resulting in the activation of the serine:threonine kinase Akt. A recently proposed model for the inhibition of apoptosis by Akt phosphorylation of BAD is shown (Butler *et al*, 1998).

A number of *in vitro* studies link IGF-I actions to the Bcl family of proteins, but these findings are somewhat discrepant. For example, IGF-I inhibition of apoptosis is associated with:

(1) increased Bcl-x_L expression and action (Singleton *et al*., 1996; Parrizas and LeRoith, 1997b);

(2) increased Bcl-2 expression (Minshall *et al*., 1997);

(3) suppressed interleukin-1β-converting enzyme (caspase)-mediated cell death, apparently through a mechanism independent of the expression of Bcl-2, Bcl-x_L, and Bax (Jung *et al*., 1996), and

(4) an inactivation of Bad by phosphorylation (del Peso *et al*., 1997; Dudek *et al*., 1997).

Therefore, IGF-I inhibition of apoptosis and these IGF-I actions may be regulated by alterations in the expression of genes for the Bcl family of proteins (Chrysis *et al*., 2001).

Potential Uses of IGF-I

Neurotrophic factors regulate the growth and survival of selected populations of neurons in the central nervous system (CNS) and peripheral nervous system (PNS). Many of them have recently been investigated extensively for their potential usefulness in the treatment of various neurodegenerative diseases, peripheral neuropathies and brain tumors (Lindsay *et al.,* 1994; Yuen and Mobley, 1996). IGF-I is a trophic factor with a wide spectrum of actions on different CNS and PNS tissues (de Pablo and dela Rosa, 1995; LeRoith *et al*., 1995) (Table 1).

TABLE 1. Demonstrated actions of IGF-I in the nervous system (Dor'e *et al*, 1997c)

Organ	Action of IGF-I
Brain	
	Promotes division, differentiation, maturation and assures survival or reduces apoptosis of neuronal (olfactory bulb, septum, cortex, hypothalamus, hippocampus, mesencephalon, brainstem, cerebellum) and glial (Schwann, oligodendrocytes, astrocytes) cells
	Protects neurons against toxicity induced by: iron, colchicine, Ca^{2+} destabilizers, H_2O_2, amyloid β peptides, human amylin and cytokines, pharmacological lesions
	Modulates release of neurotransmitters: acetylcholine (Ach), dopamine, serotonin, glutamate, neuropeptide Y
	Induces the expression of neurofilament, tubulin, myelin basic protein
	Directs the sprouting of spared afferents into a deafferented hippocampus
	Actions on glucose metabolism
Spinal cord	
	Modulates choline acetyltransferase activity and attenuates loss of cholinergic phenotype
	Enhances motoneuron sprouting
	Increases myelination and inhibits demyelination
	Stimulates interstitial cell proliferation
	Reduces apoptosis in motoneurons during normal development, spinal transection, deafferentiation
	Potential chemotactic properties
	Stimulates motoneuron proliferation and differentiation from precursor cells
	Promotes survival of Schwann cell precursors
	Promotes Schwann cell division, maturation and growth
	Increases the rate of regeneration of sciatic sensory and motor nerves
	Faster recovery after crush or freeze injury of the nerve
	Prevents peripheral neuropathies induced by cancer chemotherapy
Muscle	
	Induces Ach receptor cluster formation
	Increases neuromuscular function and muscle strength
	Positive effects on *wobbler* mouse

For many years, IGF-I had been considered for use in, or used in, young and elderly patients who suffer from growth deficiency, osteoporosis, diabetes and various catabolic conditions (Dor'e *et al*., 1997c). IGF-I was also used topically to accelerate wound healing, and some athletes even use it to enhance their performance (Dor'e *et al*., 1997c). Most recently, a clinical trial has suggested the beneficial effects of IGF-I in the treatment of amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disorder of motoneurons (Yuen and Mobley, 1996). The limited side effects of IGF-I, together with recent advances in our understanding of its physiological importance in the maintenance of normal CNS and PNS functions, now support the potential therapeutic use of this growth factor in the treatment of other neurological disorders or trauma (Table 2).

TABLE 2. Disorders of the nervous system with potential for therapeutic use of IGF-I (Dor'e *et al*, 1997c)

Amyotrophic lateral sclerosis or motor neuron disease
Multiple sclerosis, ataxia, autoimmune neuritis, autoimmune encephalomyelitis
Myotonic dystrophy and Duchenne's disease
Chemotherapy-induced peripheral neuropathy
Post-polio syndrome
Diabetic neuropathy
Alzheimer's disease
Stroke, head trauma
Olivopontocerebellar atrophy
Parkinson's disease
Neurofibromatosis
Ceroid lipofuscinosis
Anorexia nervosa

Many studies of cultured cells (Matthews and Feldman, 1996), including those of cultured neurons and oligodendrocytes (Baker *et al*., 1999; Ye and D'Ercole,

1999), demonstrated that IGF-I was capable of promoting cell survival. Investigations into whether IGF-I acts *in vivo* to inhibit apoptosis, however, are few. A number of studies indicated that intraventricular IGF-I administration could protect against neuron injury after hypoxia–ischemia (Guan *et al*., 1996; Guan *et al*., 2000; Johnston *et al*., 1996), and demonstrated that topical IGF-I application on the cerebral cortex can ameliorate apoptosis after ischemia (Wang *et al*., 2000). Recent evidence indicated that systemically administered IGF-I could cross the blood–brain barrier (BBB) (Armstrong *et al*., 2000). Furthermore, systemically administered IGF-I appeared capable of protecting neurons from injury-induced apoptosis (Tagami *et al*., 1997). Therefore, IGF-I might be capable of protecting against injury-induced neuron death and thus could be useful in the therapy of a variety of neural injuries and disorders.

Actions of the IGFs *in vitro*

IGF-I receptors are present in a wide variety of cell types and mediate most of the effects of IGF-I and IGF-II *in vitro,* as well as effects of insulin when IGF-I is present in sufficiently high concentrations. In general, the effects of the IGFs *in vitro* are either acute anabolic effects on protein and carbohydrate metabolism, or long-term effects on cell replication and differentiation. The effects of the IGFs in multiple cell types have been reviewed recently (Giudice, 1992; Cohick and Clemmons, 1993).

1. Effects on cell cycle progression. The most widely studied effect of the IGFs *in vitro* is the stimulation of DNA synthesis and cell replication, by causing cells to traverse the successive phases of the cell cycle. IGF-I had been shown to function as a progression factor in the cell cycle. Treatment of the cells with IGF-I allows progression through G_1 and continuation through the cell cycle, resulting in DNA synthesis and cell proliferation.

2. Effects on cell proliferation. IGF-I receptors may be critical in regulating the progression of cells through the cell cycle. It is therefore not surprising that an extremely wide variety of cells demonstrated a mitogenic response to stimulation with IGF-I. In addition to fibroblasts, IGF-I stimulated a mitogenic response in chondrocytes, osteoblasts, keratinocytes, thyroid follicular cells, smooth muscle cells, skeletal muscle cells, neuronal cells, mammary epithelial cells, mesangial cells, erythroid progenitor cells,

thymic epithelium, oocytes, granulosa cells, spermatogonia, Sertoli cells, and several cancer cell lines (Giudice, 1992; Cohick and Clemmons, 1993). Proliferation of primary cultures of chick chondrocytes under serum-free conditions was stimulated by IGF-I, which was much more potent in this assay than basic fibroblast growth factor (bFGF) or platelet-derived growth factor (PDGF) (Bohme *et al.*, 1992). Proliferation of primary cultures of human keratinocytes was stimulated by IGF-I, which had been identified as the active paracrine growth promoting factor that was secreted by feeder layers of fibroblasts, which were commonly used to promote and maintain the growth of keratinocytes *in vitro* (Barreca *et al.*, 1992). IGF-I stimulated DNA synthesis and hormone secretion in thymic epithelial cells (Timsit *et al.*, 1992). Proliferation of human erythroid progenitor cells and T lymphocytes was stimulated by IGF-I and IGF-II, by means of the IGF-I receptor (Merchav *et al.*, 1992; Merchav *et al.*, 1993; Kooijman *et al.*, 1992a). DNA synthesis in rat spermatogonia was also found to be stimulated by IGF-I and IGF-II in serum-free cultures of seminiferous tubules (Soder *et al.*, 1992).

3. Effects on cell death. A cellular action of the IGFs that is complementary to their stimulation of cell proliferation is their capacity in certain cells to inhibit cell death. This action has been best characterized in hematopoietic cells. In these cells, programmed cell death by apoptosis is believed to have an important role in the regulation of blood cell production by growth factors (Williams *et al.*, 1990). In human erythroid progenitor cells, DNA breakdown from apoptosis due to serum deprivation was suppressed by IGF-I, to an extent that was comparable to that seen with stem cell factor but less than that seen with erythropoietin (Muta and Krantz, 1993).

4. Effects on cell differentiation. Florini and Magri (1989) had demonstrated that the IGFs were potent inducers of myoblast terminal differentiation. Recently, this group had shown that IGF-I induced expression of the myogenin gene and that the induction of myoblast differentiation by IGF-I could be specifically blocked with antisense oligonucleotides complementary to the sequence of this gene, strongly suggesting that the induction of this muscle determination gene mediated this action of IGF (Florini *et al.*, 1991).

5. Effects on cell function. Hormone secretion from many cell types is regulated by the IGFs. IGF-I and IGF-II stimulate hormone synthesis and secretion in

ovarian granulosa and theca cells, and synergistic effects are seen when they are combined with follicular stimulating hormone (FSH) and estrogen (Giudice, 1992). Thymulin secretion by thymic epithelium was also stimulated by IGF-I (Timsit *et al*., 1992). Treatment of adrenal fasciculata cells with IGF-I increased adrenocorticotrophic hormone (ACTH) receptor number and potentiated steroid hormone secretion in response to ACTH (Penhoat *et al*., 1989a). Conversely, treatment of these cells with ACTH stimulated IGF-I secretion, which was then available as an autocrine factor to further potentiated ACTH action (Penhoat *et al*., 1989b). IGF-I also stimulated hormone secretion from Ledig cells and thyroid follicular cells (Lowe, 1991).

The IGFs can affect a wide variety of cell-specific functions. In cultured pituitary somatotrophes, IGF-I directly inhibited growth hormone (GH) secretion (Yamasaki *et al*., 1991a). Moreover, this inhibitory effect was potentiated by overexpression of recombinant IGF-I receptor (Yamasaki *et al*., 1991b). Cytotoxic T cell function *in vitro* was potentiated by IGF-I (Kooijman *et al*., 1992b). IGF-I and IGF-II, acting through the IGF-I receptor, potentiated the release of histamine from basophils in response to immunoglobulin E (Hirai *et al*., 1993). IGF-I inhibited the glutamate-stimulated release of amino butyric acid from Purkinje cells and may function *in vivo* as a neuromodulator for these cells (Castro-Alamancos and Torres-Aleman, 1993). An IGF-I gradient had been reported to increase chemotactic migration in T lymphocytes (Tapson *et al*., 1988), bronchial epithelial cells (Shoji *et al*., 1990), endothelial cells (Grant *et al*., 1987), melanoma cells (Stracke *et al*., 1989), and in retinal pigment epithelial cells (Grant *et al*., 1990). IGF-I-induced chemotaxis in the latter two cell lines was shown to be specifically mediated by the IGF-I receptor. However, in human rhabdomyosarcoma cells, the IGF-II receptor had been shown to mediate a chemotactic response to IGF-II (Minniti *et al*., 1992). IGF-I also stimulated the nondirectional migration of keratinocytes (Ando and Jensen, 1993).

In most cells with functional IGF-I receptors, the IGFs stimulate to some degree amino acid and glucose uptake and general protein synthesis. In skeletal muscle *in vitro,* IGF-I had insulin-like stimulatory effects on glucose uptake, glycolysis, and glycogen synthesis, but unlike insulin did not stimulate glucose oxidation (Dimitriadis *et al*., 1992). IGF-I stimulated chondrocytes (Hill *et al*., 1992), osteoblasts, fibroblasts, and

endothelial cells (Lowe, 1991) to increase synthesis of extracellular matrix (ECM) proteins, most notably collagens and proteoglycans.

Hypoxia

Human survival is critically dependent on the availability of oxygen. Since the key organ, the brain, has no oxygen storage capacity, it needs a continuous supply. An absence of brain tissue oxygenation, therefore, will lead to a loss of nerve cell function in a matter of seconds. Following cardiac arrest, for example, the electroencephalogram (EEG) becomes flat within half a minute. For many years, it was believed that a failure of energy and fuel supply for about 5 minute caused irreversible brain damage (Wauquier, 1984).

Oxygen can become unavailable to the brain through a loss of blood flow (ischemia) following cardiac arrest or occlusion of intracranial vessels, or through an insufficient oxygen concentration in the blood (hypoxia) (Nieber, 1999).

Hypoxic-ischemic brain injury continues to be the third leading cause of death in the United State, affecting over half a million new victims each year. Of these, nearly one-third will die and another one-third will be left with severe and permanent disability. Unlike ischemic injury to many other tissues, the severity of disability is not predicted well by the amount of brain tissue lost. For example, damage to a small area in the medial temporal lobe may lead to severe disability, such as loss of speech, while damage to a greater volume elsewhere has little effect on function. The degree of disability does not simply reflect the severity or distribution of impaired blood supply. Populations of cells lying side by side in the brain can display dramatically different vulnerabilities to equivalent degrees of ischemia (Laura and Dennis, 1999).

Hypoxic injury in the fetal and newborn brain results in neonatal morbidity and mortality, as well as long-term sequelae such as mental retardation, seizure disorders and cerebral palsy (Vannucci, 1990; Volpe, 1995).

It is well known that within the intact brain, a transient critical reduction of oxygen triggers various pathophysiological changes. It has been accepted recently that ischemic cell injury arises from complex interactions between multiple electrophysiological, hemodynamic, and biochemical cascaded. This includes

disturbances in energy metabolism (Paschen and Djuricic, 1995) and modifications in the synaptic interactions (Zhu and Krnjevic, 1994; Luhmann, 1996). The disturbed ion and sodium (Na^+) and calcium (Ca^{2+}) influx is followed by a substantial extracellular acidosis (Tombaugh and Sapolsky, 1993), free radical formation (Perez-Velazquez *et al*., 1997), cell swelling (Payne *et al*., 1996), and inhibition of protein synthesis (Raley-Susman and Lipton, 1990; Raley-Susman and Murata, 1995).

Prominent functional modifications induced by hypoxia consist of substantial membrane depolarization and rapid failure of synaptic transmission. The depolarization occurs shortly after the beginning of severe hypoxia is a trigger signal for the induction of neuronal hyperexcitability, irreversible cellular dysfunction, and cell death (Balestrino, 1995). The depolarization and the rapid failure of synaptic transmission during hypoxia are caused by different mechanisms. Until now, the interest has been focused on the question, which factors are initially triggering these processes. Two major hypotheses attempt to explain the mechanisms responsible for the synaptic depression. One hypothesis is known as the excitotoxic hypothesis. It postulates a central role of glutamate and its receptors in the neuronal damage (Choi, 1988; Choi, 1990). It has been discussed that the excessive opening of ionotropic glutamate receptor channels contributes to the hypoxic brain damage. However, the hypothesis that energy failure during hypoxia causes glutamate receptor-mediated toxicity (Szatkowski and Attwell, 1994) is oversimplified and conflicted with a number of findings. For example, although it is well established that glutamate is toxic to cultured neurons, however, extremely high concentrations of glutamate are required for depolarization and neurotoxicity to occur *in vivo* (Obrenovitch *et al.,* 1996).

The second hypothesis postulates that the accumulation of free cytosolic Ca^{2+} (loss of Ca^{2+} homeostasis) plays a key role in neuronal vulnerability (Siesjo, 1988) (Fig 4). A high cytosolic concentration of Ca^{2+} is potentially harmful because it may overstimulate Ca^{2+} dependent proteases, phospholipases, and endonucleases (Lipton and Rosenberg, 1994). It has been suggested that the increase in intracellular Ca^{2+} concentration after hypoxia may be caused by various mechanisms:

1. decreased extrusion of Ca^{2+} by the Ca^{2+}-ATPase due to a diminished adenosine tri phosphate (ATP) level (Kass and Lipton, 1986),

2. increased Ca^{2+} influx through voltage-gated Ca^{2+} channels (Choi, 1990),
3. increased activity of the Na^{+}/Ca^{2+} exchanger caused by a prolonged depolarization and Na^{+} influx (Frandsen and Schousboe, 1993), and
4. increased release of Ca^{2+} from intracellular stores (Frandsen and Schousboe, 1991).

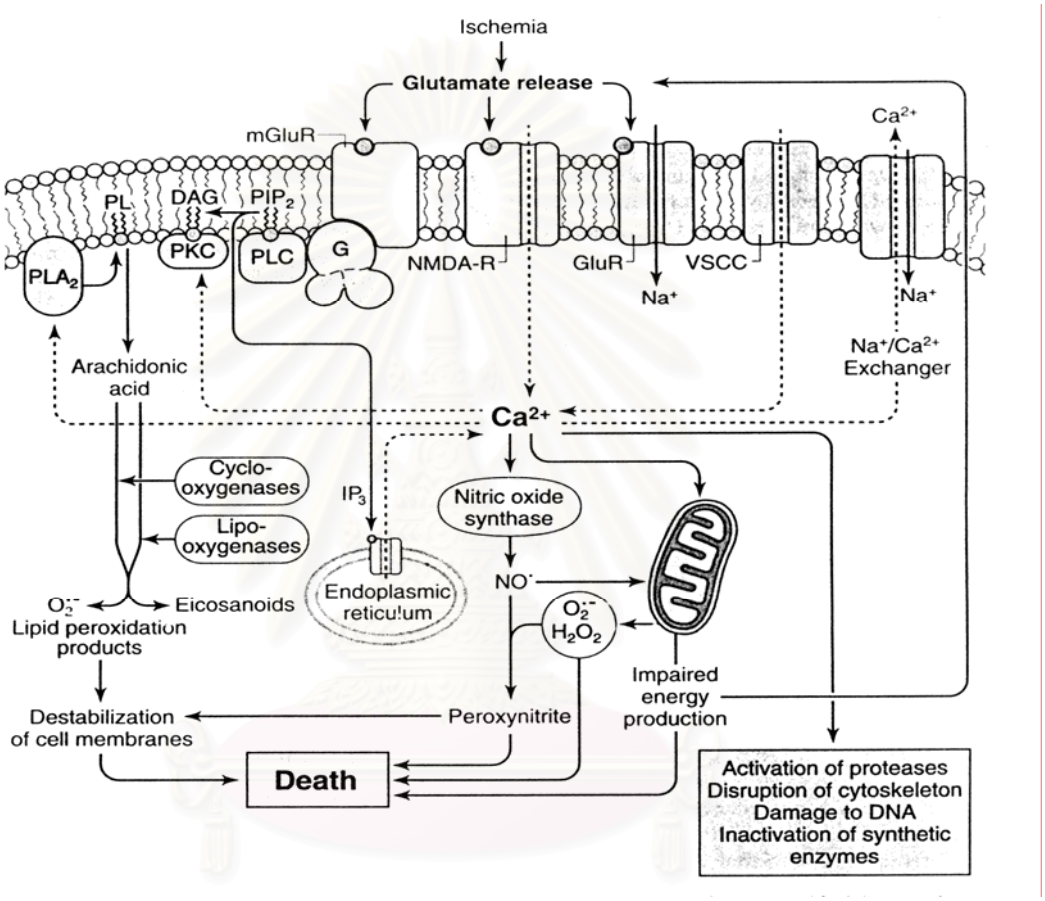


Fig. 4 Mechanisms contributing to neuronal injury during ischemia-reperfusion. Simplified diagram showing several pathway believed to contribute to excitotoxic neuronal injury in ischemia. *mGluR*, metabotropic glutamate receptor; *NMDA-R*, N-methyl-D-aspartate receptor; *GluR*, AMPA/Kainate type of glutamate receptor; *PL*, phospholipids; PLA_2, phospholipase A_2; *DAG*, diacylglycerol; *PLC*, phospholipase C; *PKC*, protein kinase C; G, G protein; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; $NO^{\bullet}$, nitric oxide; $O^{\bullet}{}_2^-$, superoxide radical; H_2O_2, hydrogen peroxide; *VSCC*, voltage-sensitive Ca^{2+} channel (Laura and Dennis, 1999).

These compartmental Ca^{2+} shifts are associated with activation of the phosphatase calcineurin, phospholipase A_2 and phospholipase C. Dephosphorylation by calcineurin activates nitric oxide syntase (NOS) and releases Bad from sequestering proteins. Phospholipases liberate free fatty acids, particularly arachidonate, from

membranes. Arachidonate causes depletion of endoplasmic reticulum (ER) Ca^{2+}, inducing the ER unfolded protein response that includes activation of an eukaryotic initiation factor-2 (eIF2α) kinase resulting in a rapid increase in eIF2α(P), which inhibits initiator methionine introduction for peptide synthesis.

Oxidative metabolism of arachidonate is associated with a burst of excess oxygen radical production, iron is released from storage proteins by superoxide-mediated reduction, and nitric oxide (NO) is generated by NOS. These events result in peroxynitrite generation, inappropriate protein nitrosylation, and lipid peroxidation, which together cause membrane damage.

Lipid peroxidation inhibits anti-apoptotic growth factor survival signaling. Dephosphorylated Bad sequesters Bcl-2, with consequent liberation of Bax, which induces mitochondrial release of cytochrome c and caspase 9 to interact with apoptosis-activating factor 1 (APAF-1) for activation of caspase 3 and apoptosis. Thus, the neurons die from radical and proteolytic injury to which they are unable to respond because of failed growth factor signaling and loss of translational competence (Fig 5).

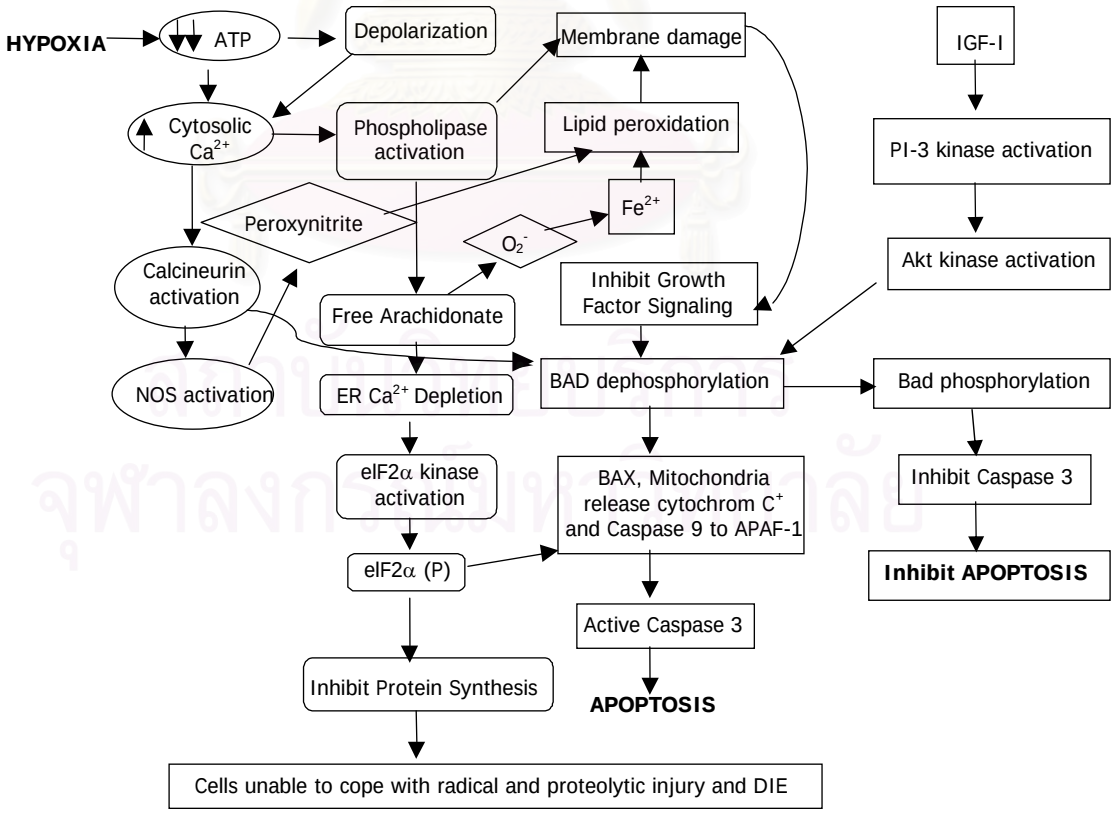


Fig 5. Mechanisms underlying hypoxia-induced neuronal apoptosis (adapted from White *et al.*, 2000).

Models for studying hypoxic changes *in vitro*

The vulnerability of mammalian neurons to oxygen deprivation varies widely among different regions within the CNS. Therefore, it is difficult to decide which of these changes are related directly to irreversible cell damage. An *in vitro* hypoxia/anoxia allows the study of cellular and environmental changes in relatively intact mammalian brain tissue without the need to manipulate systemic parameters, such as variations in regional blood flow during the onset of ischemia or reperfusion. A large number of studies had shown that *in vitro* hypoxia caused neuronal alterations that might reflect the actual processes under ischemia *in vivo* (Rothman, 1984; Goldberg *et al.,* 1986; Goldberg and Choi, 1993).

Hypoxic changes were also investigated in neuronal cell cultures from different brain regions with varying susceptibility to hypoxic effects (Tombaugh and Sapolsky, 1990; Rosenbaum *et al.,* 1994; Friedmann and Haddad, 1994; Kusumoto *et al*., 1996; Rogers and Hunter, 1997).

Hypoxic condition can be induced by:

1. Replacement of oxygen with 100% nitrogen (N_2) and subsequent incubation (Fujiwara *et al*., 1987; Leblond and Krnjevic, 1989; Aitken *et al*., 1991; Rosen and Morris, 1991; Tombaugh and Sapolsky, 1990).
2. Replacement of oxygen with highly inert argon gas (Kusumoto *et al*., 1996).
3. Superfusion of the cells with 95% N_2 and 5% carbondioxide (CO_2) equilibrated buffer solution (Friedmann and Haddad, 1994).
4. Oxygen deprivation is accompanied by a glucose deprivation (Grigg and Anderson, 1989; Dux *et al.,* 1992).
5. Oxygen and glucose deprivation (Kalda *et al*., 1998; Kalda and Zharkovsky, 1999; Kalda *et al*., 2000; Kaasik *et al*., 2001).
6. Metabolic inhibitors, such as rotenone (Hartley *et al*., 1994; Monica *et al*., 1999; Betarbet *et al*., 2000; Christine *et al*., 2000; Jenner, 2001; Todd *et al*., 2001), potasssium cyanide (KCN) (So *et al*., 1999), sodium cyanide (NaCN) (Rajdev and Reynolds, 1994), Iodoacetate (Uto *et al*., 1995).

Additionally, numerous laboratories have studied hypoxic mechanisms in slice preparations of brain regions that are more resistant to oxygen and glucose

deprivation, such as pons (Nieber *et al.,* 1995; Yang *et al*., 1997), striatum (Ghribi *et al.,* 1994; Calabresi *et al.,* 1995a,b; Milusheva *et al.,* 1996), and brain stem (hypoglossal, dorsal vagal) neurons (O'Reilly *et al.,* 1995; Ballanyi *et al*., 1996).

Furthermore, a line of evidence indicates that the hypoxia-induced changes in membrane parameters and recovery processes after reoxygenation differ in their magnitude, depending on the length of the oxygen deprivation and the age of the animal (Haddad and Donnelly, 1990; Luhmann and Kral, 1997). The majority of data reviewed in the following sections was obtained in brain slices from adult animals, but some results were found in organotypic cell cultures.

Rotenone

Chemical hypoxia was induced by the addition of rotenone, a naturally occurring compound derived from the roots of certain plant species, that is commonly used as an insecticide in vegetable gardens, and is also used to kill or sample fish populations in lakes and reservoirs. It is widely believed to be a safe, natural alternative to synthetic pesticides (Monica *et al*, 1999; Betarbet *et al*., 2000; Jenner, 2001).

Rotenone is a specific inhibitor of mitochondrial complex I (NADH: ubiquinone oxidoreductase) of the electron transport chain in many tissues including brain, muscle, and platelets (Cooper *et al*., 1995; Janetzky *et al*, 1994). The inhibition of complex I activity of the mitochondrial respiratory chain, with consequent ATP depletion and a loss of mitochondrial transmembrane potential (Seaton *et al*., 1998).

Mitochondria are the source of oxidative phosphorylation providing energy to power cellular activities. Recently, however, mitochondria have been found to play an intimate role in a number of other cellular processes in addition to ATP production. For example, mitochondria participate in a number of cellular signaling pathways including the regulation of calcium dynamics (Lawrie *et al*, 1996; Werth and Thayer, 1994). Mitochondria may also regulate the activity of transcription factors and impact nuclear gene expression (Biswas *et al*, 1999). Finally, mitochondria represent an important component of the apoptotic pathway since factors released from mitochondria initiate apoptotic cell death (Yang *et al*, 1997). Changes in complex I activity may alter the ability of mitochondria to participate in many critical cellular processes such as calcium

signaling, the production of free radicals, nuclear gene expression and provide insights into the mechanisms of cell death in late-onset progressive neurodegenerative diseases (Hartley *et al*, 1994; Christine *et al*, 2000)

Mitochondrial dysfunction and oxidative stress have been implicated in neurodegenerative disorders such as Alzheimer's, Amyotrophic lateral sclerosis, Huntington's and Parkinson's diseases (Monica *et al*, 1999).

Regulation of hypoxia-induced neuronal apoptosis

Bcl-2 and related proteins

The bcl-2 family is a growing group of proteins regulating cell death (Table 2). To date, more than 15 bcl-2 family members have been identified (Chao and Korsmeyer, 1998). Each member of this gene family contains one or more of four conserved regions, known as the bcl-2 homology domains (BH1-BH4), that control the ability of these proteins to dimerize as well as to regulate apoptotic cell death (Sato *et al*., 1994; Sedlak *et al*., 1995). Bcl-2 family members that inhibit apoptosis contain at least both the BH1 and BH2 domains (Chittenden *et al*., 1995a, 1995b; Muchmore *et al*., 1996). These bcl-2 proteins have also been divided into two groups based on their structural domains: (a) those are structurally similar to bcl-2 and possess the BH1, BH2, and BH3 domains such as Bax, Bak, and Bok and (b) those only possess the BH3 domain such as EGL-1 (Chittenden *et al*., 1995a, 1995b; Cosulich *et al*., 1997).

Table 3 Mechanism(s) of action of bcl-2 family members (Chao and Korsmeyer, 1998)

Gene	Function	Mechanism(s) of Action
bcl-2	Anti-apoptotic	Inhibits mitochondrial permeability transition; heterodimer formation with pro-apoptotic proteins; inhibition of reactive oxygen species (ROS) generation; regulation of Ca^{2+} flux; regulation of intracellular pH
bcl-xl	Anti-apoptotic	Inhibits mitochondrial permeability transition; heterodimer formation with pro-apoptotic proteins

Table 3 (Continue)

Gene	Function	Mechanism(s) of Action
bag-1	Anti-apoptotic	Enhances raf-1 kinase activity; stimulate Hsp70 and Hsp70 ATPase activity
BI-1	Anti-apoptotic	Interacts with bcl-2 and bcl-xl to inhibit bax activity
bfl-1	Anti-apoptotic	Inhibits p53- and tumor necrosis factor (TNF)-mediated apoptosis; cell cycle regulation
boo	Anti-apoptotic	Complexes apaf-1 and caspase-9
bcl-w	Anti-apoptotic	Unknown
bax	Pro-apoptotic	Promotes mitochondrial permeability transition
bak	Pro-apoptotic	Promotes mitochondrial permeability transition; heterodimer formation;
bad	Pro-apoptotic	Heterodimer formation; ceramide generation
bid	Pro-apoptotic	Induces structural change in bax
bim	Pro-apoptotic	Microtubule binding
diva	Pro-apoptotic	Binds to Apaf-1
bcl-xs,blk,bod, bok,btf,hara-kiri	Pro-apoptotic	Unknown

Bcl-2 and Bcl-xl

Bcl-2 is the first of a growing family of regulators of apoptosis that was initially discovered in the t(14:18) chromosomal breakpoint found in B-cell lymphomas. (Tsujimoto and Croce, 1986). In comparison to other oncoproteins, bcl-2 is unique in that it promotes cell survival rather than cell proliferation and this is achieved via inhibition of apoptosis in multiple cell types, including neurons. Bcl-xl represents one of three known splice variants of the bcl-x gene product (Gonzalez-Garcia *et al*., 1995; Ban *et al*., 1998). Bcl-xl is similar in size and structure to bcl-2 and, like bcl-2, functions as a suppressor of apoptosis (Chao *et al*., 1995).

Bcl-2 and bcl-xl are complex proteins localized to the membranes of the endoplasmic reticulum, nuclear envelope and the outer mitochondrial membrane (Hockenbery *et al*., 1990; Hsu *et al*., 1997a, 1997b) indicating that these proteins may play a role in ion and/or protein transport across membrane surfaces. Indeed, it had been

shown that recombinant bcl-2 and bcl-xl formed pores in liposomes and conductance channels in lipid bilayers (Muchmore *et al*., 1996; Schendel *et al*., 1997; Schlesinger *et al*., 1997). The formation of these channels occurs at acid pH and the resulting channels are cation selective at physiological pH (Schendel *et al*., 1997). The bcl-2 channel is selective for potassium while the bcl-xl channel conducts sodium (Schendel *et al*., 1997; Lam *et al*., 1998). The ability to form channels *in vitro* suggests that bcl-2 and bcl-xl regulate the mitochondrial permeability transition pore (PT) and release of proteins from the mitochondrion, e.g., cytochrome c, that are critical to the activation of cellular proteases (Yang *et al*., 1997; Clem *et al*., 1998; Zamzami *et al*., 1998; Finucane *et al*., 1999; Gross *et al*., 1999).

The mitochondrial PT is a large (~2.9 nm) cyclosporin-sensitive channel present in the inner mitochondrial membrane and opens during the process of apoptotic cell death. The opening of this channel has been associated with release of free radicals, calcium and mitochondrial proteins critical to caspase activation into the cytosol (Bernardi *et al*., 1994; Zorati and Szabo, 1994) (Fig. 6). Bcl-2 and bcl-xl have been localized to the outer mitochondrial membrane predominantly at sites of contact between the inner and outer mitochondrial membrane (Hockenbery *et al*., 1990). Bcl-2 and bcl-xl prevent mitochondrial membrane depolarization via the mitochondrial permeability transition in apoptosis (Yang *et al*., 1997; Clem *et al*., 1998; Zamzami *et al*., 1998; Finucane *et al*., 1999; Gross *et al*., 1999) (Fig. 6). In addition, these proteins inhibit the release of cytochrome c from the mitochondrion and prevent the activation of caspases. However, the exact mechanisms by which bcl-2 regulates the PT is yet to be elucidated. Regulation of the PT by bcl-xl occurs as a consequence of binding to the voltage-dependent anion channel (VDAC) subunit of the PT and subsequent closure of the VDAC (Shimizu *et al*., 1998). Bcl-xl may also interact with the adenine nucleotide translocator (ANT) subunit of the PT demonstrating that mitochondrial adenylate transport is under active regulation. Furthermore, bcl-xl expression allows cells to maintain sufficient mitochondrial ATP/ADP exchange to sustain coupled respiration (Heiden *et al*., 1999), a mechanism that may enable mitochondria to adapt to the changes in metabolic demand during apoptotic cell death.

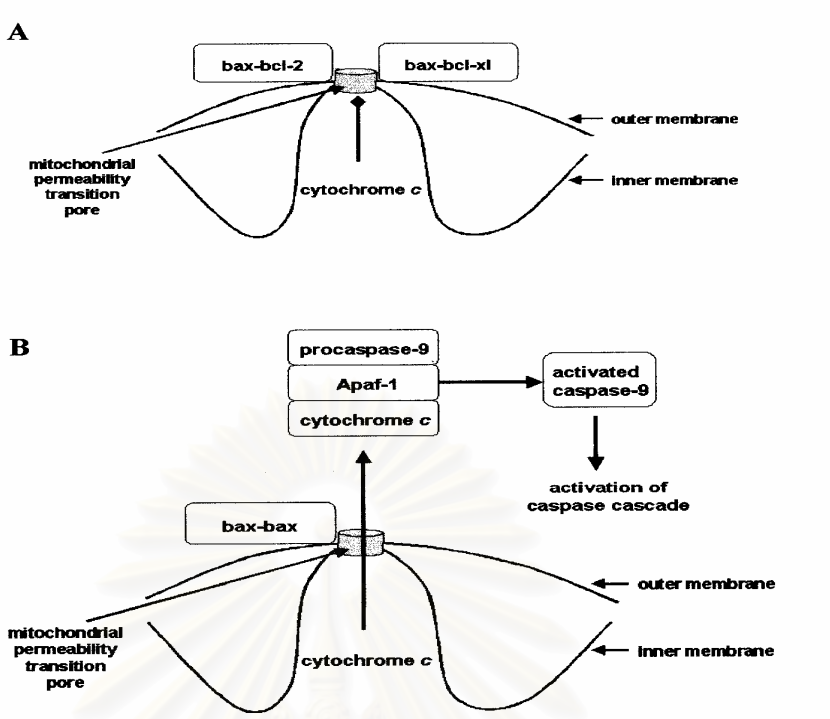


Fig. 6. Regulation of the mitochondrial permeability transition pore (PT). (A) Bcl-2-bax or bcl-xl-bax heterodimers maintain the PT in a closed state, preventing mitochondrial release of cytochrome c. Under conditions that lead to an increase in bax relative to bcl-2 or bcl-xl, bax homodimers are formed (B). The bax homodimer alters the conformation of the PT allowing mitochondrial release of cytochrome c. Cytochrome c then forms a trimeric complex with apaf-1 and procaspase-9. This complex cleaves procaspase-9 producing caspase-9 in the initial step of the mitochondrial-mediated caspase cascade (Bernardi *et al.*, 1994; Zorati and Szabo, 1994).

Although the effects of bcl-2 and bcl-xl on mitochondrial permeability transition may be critical to its regulation of cell survival during apoptosis, the presence of these proteins within other cell membranes suggests that bcl-2 and bcl-xl have other apoptosis regulatory functions (Hsu *et al.*, 1997a, 1997b). Overexpression of bcl-2 *in vitro* has been shown not only to protect cells from oxidative agents but also to prevent the generation of reactive oxygen species (Satoh *et al.*, 1996; Fabisiak *et al.*, 1997) in response to apoptotic stimuli. Bcl-2 has also been shown to prevent apoptotic cell death associated with increases in intracellular calcium (Distelhorst and McCormick, 1996; Marin *et al.*, 1996; Kruman *et al.*, 1998) by regulating intracellular calcium in several ways. Firstly, bcl-2 prevents depletion of intracellular calcium from the endoplasmic reticulum (Distelhorst and McCormick, 1996; Wei *et al.*, 1998) by enhancing calcium uptake (Kuo *et al.*, 1998). Secondly, bcl-2 overexpression potentiates the uptake of

calcium by mitochondria (Murphy *et al*., 1996). Thirdly, bcl-2 inhibits calcium entry into the nucleus (Marin *et al*., 1996). There is also growing evidence that bcl-2 may participate in intracellular pH regulation. Intracellular acidification is an important event in the activation of endonucleases and cysteine proteases during apoptosis (Morana *et al*., 1996; Furlong *et al*., 1998). Overexpression of bcl-2 had been shown to prevent intracellular acidification-mediated caspase activation (Furlong *et al*., 1997; Ishaque and Al-Rubeai, 1998). The specific mechanism by which bcl-2 prevents intracellular acidification is yet to be elucidated.

1.2 Bax

Bax is one of the first members of the bcl-2 family found to promote apoptosis. To date, there are six known isoforms of bax, all of which have been shown to induce apoptosis (Zhou *et al*., 1998; Shi *et al*., 1999). Bax has a structure similar to that of bcl-2 and bcl-xl. Unique to bax, however, is its ability to form oligomers but the functional significance of this biochemical property is unknown (Tan *et al*., 1999). Like bcl-2 and bcl-xl, bax forms membrane channels and binds to other bcl-2 family members, properties that are important to its pro-apoptotic functions (Simonen *et al*., 1997). Bax exists predominantly in the cytosol under unstressed conditions but translocates to mitochondrial and other membranes in response to apoptotic stimuli (Hsu *et al*., 1997a, 1997b; Goping *et al*., 1998). Processing of bax by one or more caspases appears to be necessary for this translocation (Goping *et al*., 1998).

The membrane-associating property of bax led to the observation that bax, like bcl-2 and bcl-xl, forms ion channels in lipid bilayers (Antonsson *et al*., 1997). These channels formed by bax are mildly chloride selective, maximally active at acidic pH (Antonsson *et al*., 1997; Schlesinger *et al*., 1997), and are antagonized by bcl-2 (Antonsson *et al*., 1997). Bax also appears to alter the intrinsic properties of lipid bilayers. Introduction of bax into phospholipid bilayers leads to destabilization and rupture of membranes and their subsequent breakdown (Basanez *et al*., 1999).

The translocation of bax to the mitochondrial membrane and its destabilizing effect are important factors that influence mitochondrial function during apoptosis. Once bax translocates to the mitochondrial membrane, it cross-links as a

homodimer leading to loss of mitochondrial membrane potential, cytochrome c release, and caspase activation (Narita *et al*., 1998; Saikumar *et al*., 1998; Finucane *et al*., 1999; Priault *et al*., 1999) (Fig. 6). These mitochondrial changes appear to occur via two mechanisms. Firstly, cytochrome c loss may occur via opening of the mitochondrial permeability transition pore in a process that is calcium-dependent (Narita *et al*., 1998) and enhanced by bax binding to the ANT subunit (Priault *et al*., 1999). The release of cytochrome c through the permeability transition pore by bax appears to require ATP and mitochondrial F1/F0 ATPase activity (Narita *et al*., 1998; Priault *et al*., 1999). Secondly, bax-dependent cytochrome c release may also occur via a magnesium-dependent mechanism (Eskes *et al*., 1998). The effects of bax on cytochrome c and caspase activation are inhibited by both bcl-2 and bcl-xl as described above (Otter *et al*., 1998; Saikumar *et al*., 1998; Priault *et al*., 1999).

The binding of bax to other members of the bcl-2 family is critical to its regulation of cell survival. The formation of heterodimers versus homodimers is dependent on the relative amounts of bax and bcl-2 and is important in regulation of caspase activity and cell fate (Zha *et al*., 1997; Otter *et al*., 1998).

1.3 BAD

Bad promotes apoptosis via several mechanisms. First, bad displaces bax from bcl-2 or bcl-xl heterodimers, allowing free bax to carry out its death promoting functions (Yang *et al*., 1997; Zha *et al*., 1997). Formation of heterodimers by bad may be prevented by phosphorylation (Zha *et al*., 1996; Scheid and Duronio, 1998; Zundel and Giaccia, 1998). In contrast, calcium-activated protein phosphatase calcineurin dephosphorylates bad, allowing it to translocate from the cytosol to the mitochondrial membrane where it interacts with bcl-xl (Wang *et al*., 1999a, Wang *et al*., 1999b) (Fig. 6;). Bad may also induce apoptosis through mechanisms mediated by ceramide (Basu *et al*., 1998).

Bad appears to exert its pro-apoptotic effects by binding and sequestering Bcl-2 (Zha *et al*., 1996). The activity of Bad is regulated by phosphorylation; the dephosphorylated form binds Bcl-2. Bad is phosphorylated on serines 112 and 136 by Akt or protein kinase B (PKB), a central element in survival signaling, that is activated

through the growth-factor-mediated PI-3 kinase pathway (Datta *et al*., 1997). Phosphorylated Bad is sequestered in the cytoplasm by 14-3-3 proteins (Datta *et al*., 1999). A major effect of maintaining Bad in its phosphorylated state appears to be preventing the release of cytochrome *c* from mitochondria, and Akt is unable to inhibit the mechanism responsible for cytochrome *c* release when Bad ser-112 and ser-136 are mutated to alanine (Kennedy *et al*., 1999). Bad is dephosphorylated by the Ca^{2+}/calmodulin-activated phosphatase calcineurin (Wang *et al*., 1999a), which is also implicated in NOS activation.

Caspases

During the process of apoptosis, there are a number of biochemical events that are critical to the ultimate degradation of the cell. One of these events is the activation of proteolytic enzymes, the cysteine proteases or caspases. The role of caspases in apoptotic cell death was realized with the discovery of Ced-3, a requisite cell death gene in Caenorhabditic elegans homologous to mammalian interleukin-1-β-converting enzyme (ICE; caspase 1) (Thornberry *et al*., 1992; Yuan *et al*., 1993). It was the first caspase initially discovered as a cytokine-processing enzyme. Due to the rapid expansion of the expressed sequence tag (EST) database and the presence of the conserved pentapeptide sequences QACR (N/Q) G at the caspase active site, over 14 new caspases have been cloned in a short period of time (Thornberry and Lazebnik 1998). Caspase-1 and caspase-11 have been shown to function mainly in cytokine processing (Li *et al*., 1995, Kuida *et al*., 1995). In the other hand, caspase-2, -3, -6, -7, -8, -9, -10 are involved in the regulation and execution of apoptosis (Kuida *et al*., 1996, 1998; Hakem *et al*., 1998; Varfolomeev *et al*., 1998; Bergeron *et al*., 1998). The functions of the remaining caspases are largely unknown at this moment.

Caspases activation

All apoptotic caspases exist in normal cells as inactive enzymes analogous to the zymogens involved in the regulation of blood clotting. When cells undergo apoptosis, these caspases become activated through one or two sequential proteolytic events that cleave the single peptide precursor into the large and small fragments that constitute the active enzyme (Thornberry and Lazebnik 1998). There are

several different routes to the activation of the apoptotic program, however, they can for simplicity be divided into two pathways - an intrinsic and an extrinsic one (Fig.7). The intrinsic pathway is driven by activation of caspase 9 upon release of cytochrome c from the mitochondria and is responsible for the initiation of apoptosis in response to stimuli such as ionizing radiation, chemotherapeutic drugs, and certain developmental cues. The extrinsic pathway, driven by activation of caspases 8 and 10, is mainly initiated in response to ligation of transmembrane, cell surface receptors belonging to the tumor necrosis factor receptor-1 (TNFR-1) family. A separate extrinsic pathway is initiated by delivery of granzyme B (GraB) from cytotoxic cells to sensitized targets. Signifcantly, all pathways are thought to converge on the activation of the executioner caspases 3 and 7 (Stennicke and Salvesen, 2000).

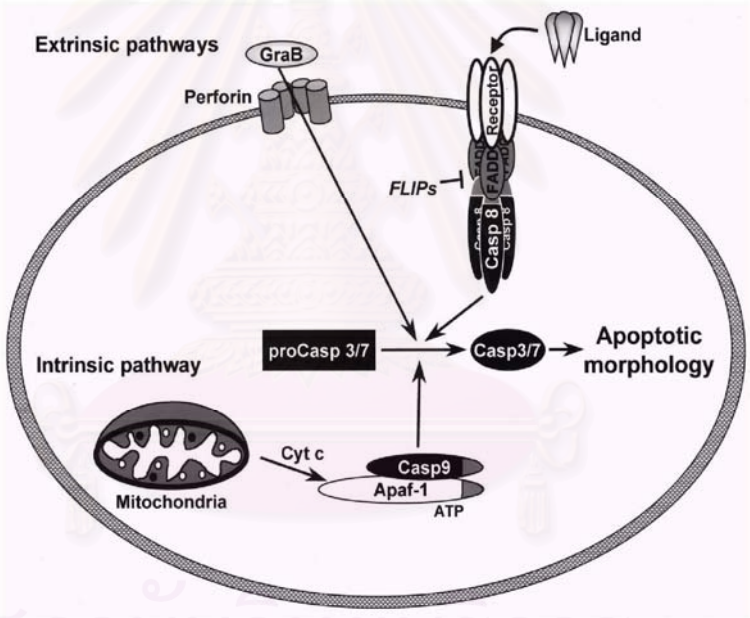


Fig. 7; (Stennicke and Salvesen, 2000) Currently there exist three recognized points at which apical proteases are activated to initiate apoptosis. These may be divided into intrinsic and extrinsic pathways depending on whether the apoptotic signal originates from the inside or the outside of the cell. The extrinsic pathways include delivery of granzyme B to the cells as well as receptor ligation. Following TNFR-1 or Fas receptor ligation, the initiator caspase 8 is activated by adapter-mediated recruitment to the receptor's cytosolic face. Alternatively in the intrinsic pathway, the initiator caspase 9 is activated following release of mitochondrial components to form the Apaf complex (Li *et al*., 1997; Zou *et al*., 1997). Both activated initiators converge on the proteolytic activation of caspase 3. In death receptor triggered apoptosis the main pathway is direct activation of pro-caspase 3 by caspase 8. The importance of the mitochondrial pathway in death receptor triggered apoptosis is unknown, but apparently subordinate to the dominant, direct pathway in most cell types (Kuida *et al*., 1998).

Role of caspases in hypoxia-induced apoptosis

It is becoming clearer that caspases are likely the critical effector molecules in hypoxia-induced apoptosis. Activation of caspase-3 has been shown in apoptotic cells following hypoxic insults (Chen *et al*., 1998; Namura *et al*., 1998). Inhibitors of caspase-3 have shown some protective effect against hypoxic neuronal injury (Cheng *et al*., 1998).

Caspases contribute to the genesis of apoptosis by: (a) activation of caspases and other death promoting agents, (b) destruction of cytoskeletal elements, and (c) inactivation of anti-apoptotic proteins. For example, caspase-3 is converted from its inactive, or procaspase, form by caspase-8 following Fas receptor activation (Muzio *et al*., 1997; Takahashi *et al*., 1997). Similarly, following mitochondrial cytochrome c release and caspase-9 activation, the activated form of caspase-9 cleaves procaspase-3, producing activated caspase-3 (Li *et al*., 1998). The activated form of caspase-3 targets multiple proteins for proteolysis. Actin is a cytoskeletal protein shown to be cleaved by caspase-3, a mechanism that may be important to the structural changes observed in apoptotic cells (Thornberry and Lazebnik, 1998). Caspase-3 has been also shown to cleave the anti-apoptotic protein, bcl-2, releasing a fragment that promotes apoptosis (Adams and Cory, 1998). Determination of the exact pathway(s) leading to caspase activation in neurons exposed to hypoxia is still under investigation. However, it is likely that both pathways will be involved.

Objectives of the study

Hypoxia induced neuronal injury, especially the progressive neuronal injury. In this connection, hypoxia is an important component of a number of neurodegenerative diseases such Alzheimer's disease, Parkinson's disease and Amyotrophic lateral sclerosis.

IGF-I is broadly distributed within the CNS (Baskin *et al*., 1988) and has been shown to be a potent neurotrophic factor. IGF-I is induced endogenously in damaged regions in experimental animal models (Gluckman *et al*., 1992; Lee *et al*., 1993) and can prevent neuronal injury. IGF-I may also have a role in the treatment of

various neurodegenerative diseases such stroke, perinatal asphyxia and other form of acute brain injury is obvious.

Therefore, objectives of the present study are as follows:

1. to verify cytotoxic effects of hypoxia on cultured neurons;
2. to investigate whether IGF-I is neuroprotective against hypoxia-induced cell injuries;
3. to compare the effect of IGF-I when added before, during, and after physical and chemical hypoxia conditions;
4. to clarify some of possible mechanisms underlying neuroprotective effects of IGF-I against hypoxia-induced cellular injury

In order to achieve these objectives, various models and tools were used in this study including;

1. cultured rat cerebellar granule neurons, a homogeneous population of neurons *in vitro*
2. physical and chemical hypoxia, experimental models of hypoxic insults
3. MTT reduction — an indication of cell metabolic activity, especially mitochondrial dehydrogenase activity.
4. LDH release — an indication of cell membrane damage and death.
5. TBARS — a marker of lipid peroxidation products, especially malondialdehyde (MDA), a cytotoxic consequence of hypoxic condition.

Chapter II

Materials and Methods

Experimental animals

Timed-pregnant Wistar albino strain rats of gestation day 18 were supplied by the National Laboratory Animal Centre, Mahidol University, Salaya, Nakornpathom, Thailand. The pregnant rats were maintained under natural light/dark cycle at controlled temperature (25 °C) and were allowed free access to both food (F.E. Zeulig, Thailand, Co., Ltd.) and water in the Animal House, Faculty of Pharmaceutical Sciences, Chulalongkorn University, until the day of delivery. After delivery, the rat pups were kept with their mother until postnatal day 8 (P8). These pups were then used in the experiments. All animal care and handling were approved with the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Chemicals

n-Butanol [Sigma, U.S.A.]

Butylated hydroxytoluene [Sigma, U.S.A.]

Cytosine-β-D-arabinofuranoside [Sigma, U.S.A.]

Dimethyl Sulfoxide (DMSO) [BDH, England]

5-5' dithiobis-(2-nitrobenzoic acid) [Sigma, U.S.A.]

Dnase I [Sigma, U.S.A.]

Dulbecco's Modified Eagle's Medium (DMEM) [Sigma, U.S.A.]

Dulbecco's Phosphate-buffered Saline (DPBS) without Calcium chloride [Sigma, U.S.A.]

Equine Serum [Hyclone, U.S.A.]

Ethanol [Merck, Germany]

Fetal Bovine Serum (FBS) [Hyclone, U.S.A.]

Insulin-like growth factor I [Sigma, U.S.A.]

In vitro Toxicology Assay Kit (Lactate Dehydrogenase Based) [Sigma, U.S.A.]

Methanol [BDH, England]

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) [Sigma, U.S.A.]

Penicillin G [Sigma, U.S.A.]

Phosphotungstic acid [Sigma, U.S.A.]

Progesterone [Sigma, U.S.A.]

Putrescine [Sigma, U.S.A.]

Poly-D-lysine Hydrobromide (MW 150,000-300,000) [Sigma, U.S.A.]

Pyruvic Acid Sodium Salt [Fluka, Japan]

Rotenone [Sigma, U.S.A.]

Sodium Selenite [Sigma, U.S.A.]

Sodium dodecyl sulfate [Sigma, U.S.A.]

Streptomycin sulfate [Sigma, U.S.A.]

Thiobarbituric acid [Sigma, U.S.A.]

Transferrin, Human [Sigma, U.S.A.]

Trypsin [Sigma, U.S.A.]

Trypan Blue 0.04% [Sigma, U.S.A.]

Preparation of Culture Media and Drug Solutions

Culture Media

Dulbecco's modified Eagle's medium (DMEM, high glucose) was supplemented with 99 μg/ml sodium pyruvate, 0.29 mg/ml L-glutamine, 3.7 mg/ml sodium bicarbonate, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. Where indicated, 10% (v/v) heat-inactivated fetal bovine serum (FBS) was added. Serum-free DMEM was normal DMEM without FBS.

Drug solution

IGF-I solution was prepared by dissolving IGF-I in 0.1 M acetic acid according to the manufacturer's instructions. Rotenone was dissolved in 100% dimethyl sulfoxide (DMSO). Both solutions were prepared as stock solutions and directly added to the culture medium. Unless otherwise stated, the final concentration of DMSO was less than 0.5%.

Equipments

Adjustable pipette : 10-100 μl [Nichiryo]

Adjustable pipette : 200-1000 μl, 1-5 ml [Labsystems]

Aluminum Foil [Tops]

Bunsen burner

Carbon dioxide incubator [Forma Scientific]

Cell culture dish : diameter 35 mm, diameter 100 mm [Nunc]

24-well cell culture plate [Nunc]

Cell strainer : 40 μm Nylon [Becton Dickinson]

Conical tube : 15 ml, 50 ml [Nunc]

Disposable glass Pasteur pipettes : 230 mm [Volac]

Hemocytometer (Depth 0.100 mm) [Improved Neubauer]

Inverted microscope, Axiovert 135 [Zeiss]

Latex Free Syringe : 10 ml [Becton Dickinson]

Laminar air flow hood [Hepaco]

96-well microtiter plate [Nunc]

Microplate reader [Bio Rad model 3550]

Pipette tip : 1-200 μl, 200-1000 μl, 1-5 ml [Labsystems]

Sterile Millex – GV (0.22 μm filter unit) [Millipore]

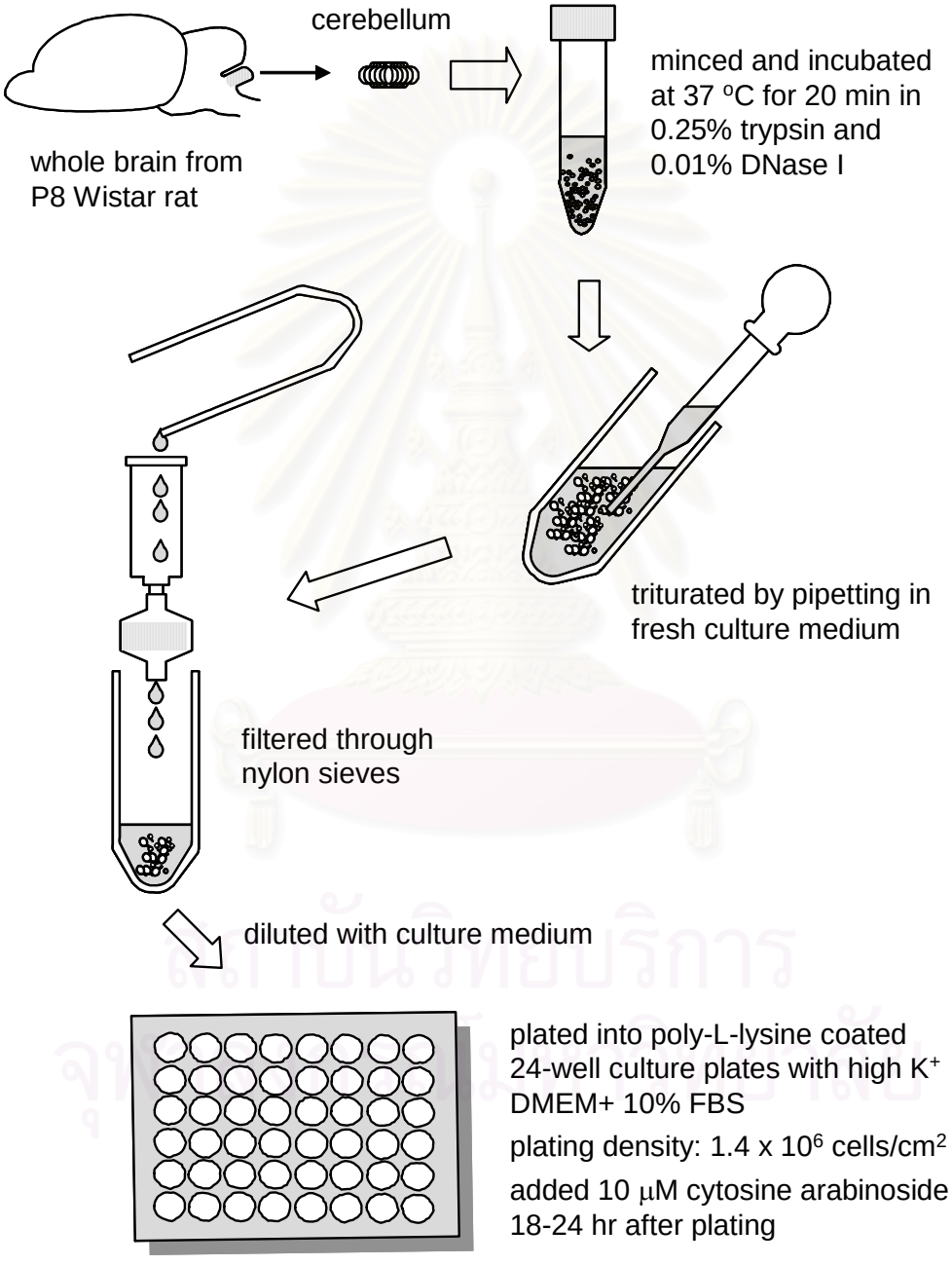
Sterivex – GS (0.22 μm filter unit with filling bell) [Millipore]

Experimental methods

1. Cerebellar granule neuron cultures

Cultures of cerebellar granule neurons were prepared from pooled cerebella of postnatal rats. The method described by Unchern (1997) was used in this study. Rat pups were anesthetized by ether. The cerebella were removed aseptically from the brains of 8-day-old Wistar rats, a time when many of the granule neurons were still at an early post-mitotic stage of differentiation. After removal of the meninges the tissue was cut into cubes of about 0.4 mm. side dimensions, and incubated for 20 min at 37 °C with 0.25% trypsin and 0.01% Dnase I in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution (HBSS). The incubation was terminated by the addition of heat-inactivated horse serum and tissue fragments were centrifuged at 1,000 rpm for 10 min. The tissue pellet was gently rinsed and resuspended in high K^+ (25 mM KCl) DMEM containing 10% FBS. The single cells were dissociated by gentle passing the suspension through a 10-ml plastic pipette tip and then through a flame-polished Pasteur pipette tip. The cell suspension was filtered through two sheets of nylon net (40 μm-mesh) to remove cell lumps, and was further diluted as appropriate with high K^+ DMEM containing 10% FBS and plated on poly-D-lysine (100 μg/ml). The cultured cells were counted in a hemocytometer, and cell viability was determined with 0.4% trypan blue. A density of 1.4 x 10^6 cells/cm^2 was plated in 24-well culture plates. The cultures were grown in a CO_2 incubator with humidified 5% CO_2 – 95% air atmosphere at 37 °C. At 18-24 hr after plating, cytosine arabinoside was added to the medium to a final concentration of 10 μM; this curtails the number of astrocytes that develop in the cultures. No subsequent medium change was made since this compound degrades rapidly at 37 °C and since cerebellar granule neurons do not appear to tolerate medium changes well, particularly when glutamine is present in the medium. In routine cultures, medium changes were made every 3 days. The cultures were incubated in the CO_2 incubator until day 8 *in vitro* (DIV 8) at which the medium was changed to serum-free high K^+ DMEM plus desired concentrations of IGF-I.

Fig. 8 Preparation of cerebellar granule neuronal cultures



2. Physical and Chemical Hypoxia

Physical Hypoxia

After 8 days *in vitro*, culture medium was changed to serum-free high K^+ DMEM. High levels of extracellular K^+ ensure proper development and prolong survival of cerebellar granule neurons in culture. Neuronal cell cultures were divided to 5 experiment groups. Four experiment groups were exposed to physical hypoxia by placing the 24-well culture plate into an air-tight plastic chamber and then perfused the chamber with 100% N_2 for 10 min. After sealing, the plastic chamber was returned to the aerobic CO_2 incubator for 4, 12, 18 and 24 hr. To terminate the hypoxic condition, the 24-well culture plate was taken out of the plastic chamber and analyzed for cell injury. A control group was maintained under routine conditions in the aerobic CO_2 incubator.

Chemical Hypoxia

After 8 days *in vitro*, culture medium was changed to serum-free high K^+ DMEM. Neuronal cell cultures were divided to 5 experiment groups. Four experiment groups were exposed to chemical hypoxia by adding rotenone into the medium at final concentrations of 0.1 μM, 0.5 μM, 5 μM and 25 μM. These cultures were kept in the CO_2 incubator for 6 hr, whereas a control group was maintained under routine conditions in the aerobic CO_2 incubator.

3. Neuronal Exposure to IGF-I

After 8 days *in vitro*, culture medium was changed to serum-free high K^+ DMEM. Neuronal cell cultures were divided to 5 experiment groups. Four experiment groups were exposed to IGF-I by adding IGF-I into the medium at final concentrations of 0.3, 1, 3 and 6 ng/ml. These groups were kept in the CO_2 incubator for 24 and 48 hr, whereas a control group was maintained under routine conditions in the CO_2 incubator.

4. Neuronal Exposure to IGF-I Before, During, and After Physical Hypoxia

Pre-treatment with IGF-I

After 8 days *in vitro*, culture medium was changed to serum-free high K^+ DMEM. Neuronal cell cultures were divided to 6 experiment groups. Five experiment groups were exposed to IGF-I in concentrations of 0, 0.3, 1, 3 and 6 ng/ml for 48 hr and then exposed to physical hypoxia for 18 hr (50% decrease from control value in MTT reduction assay). A control (normal) culture was maintained under routine conditions in the CO_2 incubator.

Co-treatment with IGF-I

After 8 days *in vitro*, culture medium was changed to serum-free high K^+ DMEM. Neuronal cell cultures were divided to 6 experiment groups. Five experiment groups were exposed to IGF-I in concentrations of 0, 0.3, 1, 3 and 6 ng/ml in combination with physical hypoxia for 18 hr. A control (normal) culture was maintained under routine conditions in the CO_2 incubator.

Post-treatment with IGF-I

After 8 days *in vitro*, culture medium was changed to serum-free high K^+ DMEM. Neuronal cell cultures were divided to 6 experiment groups. Five experiment groups were exposed to physical hypoxia for 18 hr then IGF-I was added into the medium in final concentrations of 0, 0.3, 1, 3 and 6 ng/ml. These groups were kept in the CO_2 incubator for 48 hr. A control (normal) culture was maintained under routine conditions in the CO_2 incubator.

5. Neuronal Exposure to IGF-I Before, During, and After Chemical Hypoxia

Pre-treatment with IGF-I

After 8 days *in vitro*, culture medium was changed to serum-free high K^+ DMEM. Neuronal cell cultures were divided to 6 experiment groups. Five experiment groups were exposed to IGF-I in final concentrations of 0, 0.3, 1, 3 and 6 ng/ml. These groups were kept in the CO_2 incubator for 48 hr then the medium was replaced with serum-free high K^+ DMEM containing 5 μM rotenone. These groups were returned to the CO_2 incubator for 6 hr. A control group was maintained under routine conditions in the aerobic CO_2 incubator.

Co-treatment with IGF-I on physical hypoxia-induced injury on cultured granule neurons

After 8 days *in vitro*, culture medium was changed to serum-free high K^+ DMEM. Neuronal cell cultures were divided to 6 experiment groups. Five experiment groups were exposed to IGF-I in concentrations of 0, 0.3, 1, 3 and 6 ng/ml in combination with 5 μM rotenone for 6 hr. A control (normal) culture was maintained under routine conditions in the CO_2 incubator.

Post-treatment with IGF-I on physical hypoxia-induced injury on cultured granule neurons

After 8 days *in vitro*, culture medium was changed to serum-free high K^+ DMEM. Neuronal cell cultures were divided to 6 experiment groups. Five experiment groups were exposed to 5 μM rotenone. These groups were kept in the CO_2 incubator for 6 hr then the medium was replaced with serum-free high K^+ DMEM containing IGF-I in final concentrations of 0, 0.3, 1, 3 and 6 ng/ml. These groups were returned to the CO_2 incubator for 48 hr. A control (normal) culture was maintained under routine conditions in the CO_2 incubator.

6. Colorimetric MTT-based cytotoxicity assay

The original procedure (Mosmann, 1983) using the tetrazolium salt MTT to measure mitochondrial function was used. MTT is a purported indicator of the mitochondrial activity in living cells (Shearman *et al.*, 1995). The colorimetric MTT assay based on the selective ability of living cells to reduce the yellow-colored tetrazolium salt, MTT to formazan. Mitochondrial dehydrogenases in viable cells convert the soluble yellow form of the salt into an insoluble, intracellular purple formazan which is quantitated spectrophotometrically by solubilization in an organic solvent.

MTT reduction was analysed by adding 16 μl of the MTT stock solution, 5 mg/ml in phosphate-buffered saline (PBS), to the medium in each well (final concentrations were 100 μg/ml). Cultures were incubate in a CO_2 incubator at 37 °C for a period of 1 hr and the medium in each well was aspirated off without disturbing the formazan precipitate. Then 400 μl of DMSO was added to each well to solubilize the formazan crystals. Following thorough formazan solubilization, 200 μl aliquots of soluble formazan were transferred to a 96-well microtiter plate. The absorbance in each well was read at 595/655 nm with a BIO-RAD Model 550 dual wavelength microplate reader. The cellular reduction of MTT which reflects metabolic activity and viability was expressed in term of the absorbance (optical density: OD) unit/well or the percentage of absorbance in control cultures.

7. Lactate dehydrogenase release assay

Neuronal cell injury was also quantitatively assessed by the measurement of lactate dehydrogenase (LDH) activity released from damage or destroyed cells into the medium. LDH is a stable cytoplasmic enzyme present in all cells and it represents an excellent marker of metabolic compromise that ultimately leads to neuronal degeneration and cell death (Hansen *et al.*, 1989). It is rapidly released into the medium upon damage of the plasma membrane. With the use of the Cytotoxicity detection kits, LDH activity can easily be measured in medium according to the manufacture's instructions. Briefly, medium LDH was assayed by pipetting 100 μl of culture medium from each well into a 96-well microtiter plate. Cellular LDH in cultures was measured by carefully aspirated off the

remaining culture medium, solubilized cells with 1 ml of 0.5% Triton X-100 in PBS and incubated the cells in CO_2 incubator for 10 min after which 100 μl aliquots were pipetted into a 96-well microtiter plate. The reaction was started by adding 50 μl of substrate solution into each well. The reaction mixtures were left at room temperature for 30 min (should be protected from light) after which 50 μl of stopping solution (0.5N HCl) was added into each well. The light absorbance in each well was measured at 490/690 nm with a BIO-RAD Model 550 dual wavelength microplate reader. The total activity of LDH in control cultures was considered to represent 100% cell viability. The LDH release which reflects cell death was presented as percentage of total LDH activity by the following formula:

$$\% \text{ LDH release} = \frac{\text{LDH activity in medium}}{\text{LDH activity in medium + LDH activity in cells}} \times 100$$

In most cases, comparative LDH release in test conditions was expressed as the percentage of that in control conditions.

8. Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation is a well-established mechanism of cellular injury and is used as an indicator of oxidative stress in cells and tissue. Lipid peroxides are unstable, and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals upon decomposition. Measurement of MDA and 4-hydroxyalkenals has been used as an indicator of lipid peroxidation. Lipid peroxides were quantified using the thiobarbituric acid-reactive substances (TBARS) assay according to the method of Ohkawa *et al*. (1979). In brief, cerebellar granule cultures grown in 24-well plates were lysed with 250 μl of 2% sodium dodecyl sulfate for 30 min. The lysates (250 μl) were added serially with 12.5 μl of butylated hydroxytoluene (4% in ethanol), 250 μl of phosphotungstic acid (10% in 0.5 M sulfuric acid), and 125 μl of thiobarbituric acid (0.7% in 0.05N NaOH). The mixtures were incubated at 95°C for 60 min, cooled over iced water and then 300 μl of *n*-

butanol was added to extract the TBARS. After centrifugation at 3,000 rpm for 15 min, the supernatant was collected (samples from two culture wells were pooled), and the amounts of TBARS were measured at 540 nm using a microplate reader.

Calculation and statistical analysis

All results are expressed as the means $\pm$ standard error of mean (S.E.M.) of 6 to 8 samples within the same sister culture from a representative experiment. All studies were repeated in at least two independent experiments. Differences between means were analysed using a two-tailed Student's *t*-test. Differences among means were analysed using one-way ANOVA. When ANOVA showed significant differences, pairwise comparisons between means were analyzed by Tukey HSD *post hoc* testing ($p < 0.05$).

Chapter III

Results

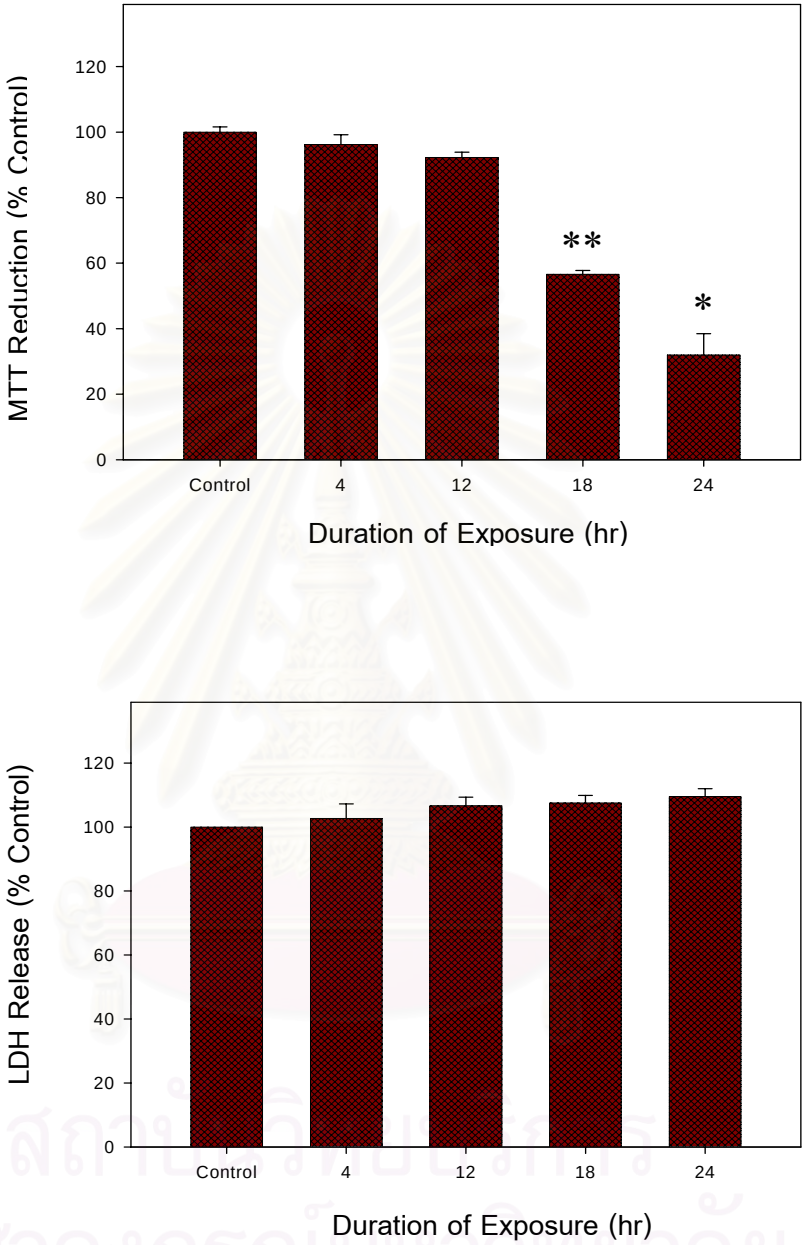
1. Injurious effect of physical hypoxia on cultured granule neurons

In order to study the injurious effect of physical hypoxia on cultured granule neurons, MTT reduction assay was used to measure cellular metabolic activity. After exposure to physical hypoxia, there was a gradual decline of mitochondrial activity (MTT assay) (When exposed to physical hypoxia 0, 4, 12, 18 and 24 hr, MTT reduction assay were 100%, 96.23%, 92.24%, 56.59% and 32.99%, respectively) and the magnitude of decrease was dependent on the duration of exposure (Fig. 9) in which the time to observe approximately 50% decrease in MTT reduction assay (from control value) was 18 hr after exposure to physical hypoxia.

In addition to cell injury, physical hypoxia-induced cell death was also quantitated by measuring release of LDH into the medium. In contrary to the effect of physical hypoxia on MTT reduction, LDH release was not significantly different from control at all times after exposure to physical hypoxia (up to 24 hr) (Fig. 9).

Visual inspection of neurons by phase-contrast microscopy at 18 hr after exposure to physical hypoxia revealed that cultured granule neurons displayed small degree of morphological changes as compared to control cultures (Fig 10).

Fig. 9 Time course of physical hypoxia-induced cellular injuries on cultured granule neurons

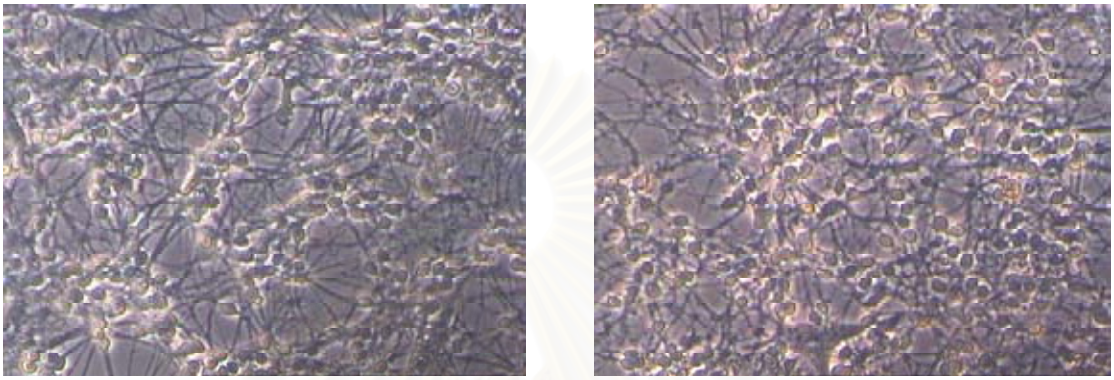


Cultured granule neurons were exposed to physical hypoxia (up to 24 hr) after which cellular injuries were determined. A: Cellular MTT reduction. B: Cytoplasmic LDH release. Values are means $\pm$ SEM [MTT: (n = 8) and LDH: (n = 6)]. *p<0.05 and **p<0.01 vs control (ANOVA and Tukey HSD test). The time to observe approximately 50% decrease in MTT reduction (from control value) was 18 hr after exposure to physical hypoxia.

Fig. 10 Physical hypoxia-induced morphological changes in cultured granule neurons

A. Control

B. Physical Hypoxia



100 μm

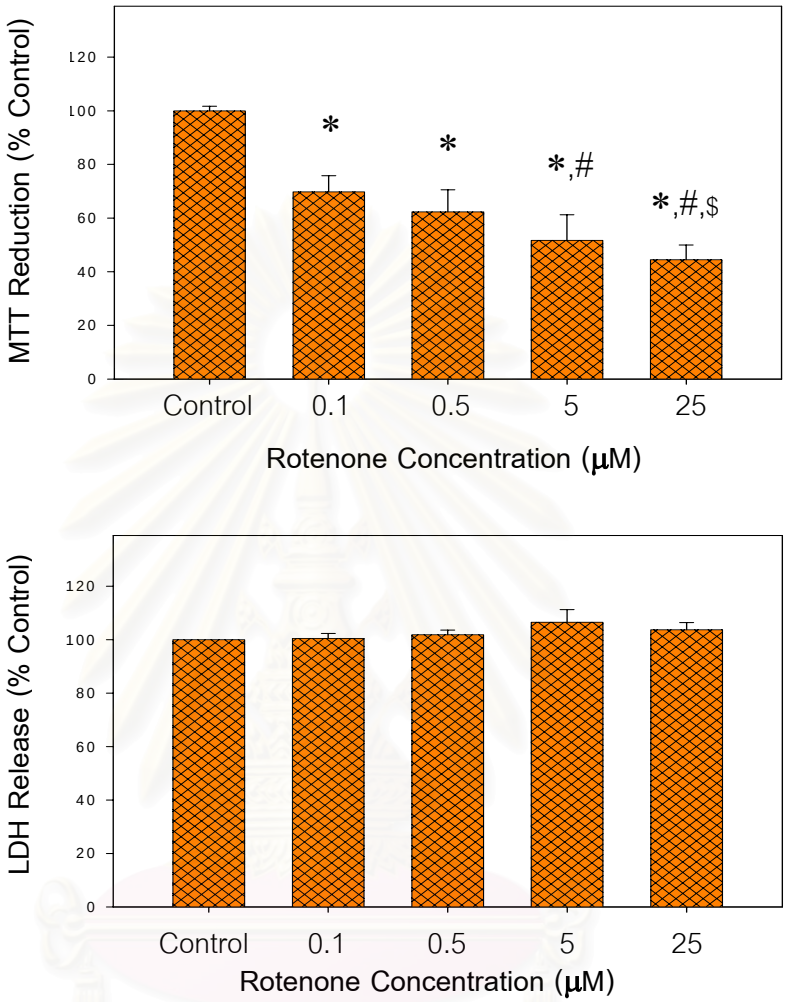
After exposure to physical hypoxia for 18 hr, cultured granule neurons were observed under phase-contrast microscopy. A: Control culture. B: 18 hr exposure to physical hypoxia.

2. Injurious effect of chemical hypoxia on cultured granule neurons

After exposure to chemical hypoxia, there was a significant decline of mitochondrial activity (MTT assay) (When exposed to rotenone 0, 0.1, 0.5, 5 and 25 μM, MTT reduction assay were 100%, 69.72%, 62.33%, 51.68% and 44.49%, respectively), the magnitude of decrease was dependent on the final concentration of rotenone (Fig. 11) in which the EC_{50} was approximately 5 μM. In similarity to physical hypoxia, LDH release after exposure to chemical hypoxia was not significantly different from that of control (Fig. 11).

Visual inspection of neurons by phase-contrast microscopy after the addition of 5 μM rotenone showed that neurons displayed morphological features of cell injury as compared to control cultures (Fig 12).

Fig. 11 Injurious effect of chemical hypoxia on cultured granule neurons



Cultured granule neurons were exposed to rotenone for 6 hr after which cell injuries were determined. A: Cellular MTT reduction. B: Cytoplasmic LDH release. Values are means ± SEM (n = 6). Significant difference was determined by ANOVA and Tukey HSD test. The EC_{50} values was approximately 5 μM rotenone.

* $p < 0.05$ denotes statistically significant difference from control

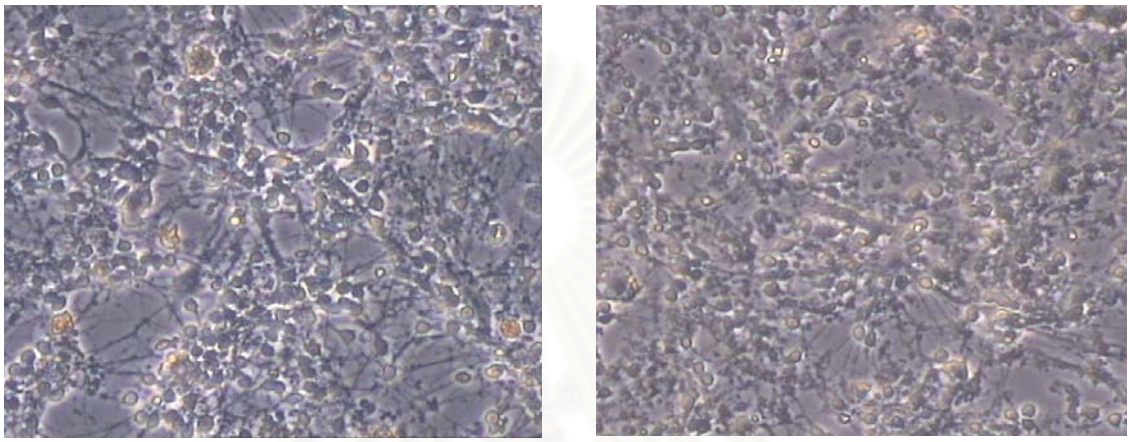
\# $p < 0.05$ denotes statistically significant difference from 0.1 μM rotenone

$ $p < 0.05$ denotes statistically significant difference from 0.5 μM rotenone

Fig. 12 Chemical hypoxia-induced morphological changes in cultured granule neurons

A. Control

B. 5 μM Rotenone



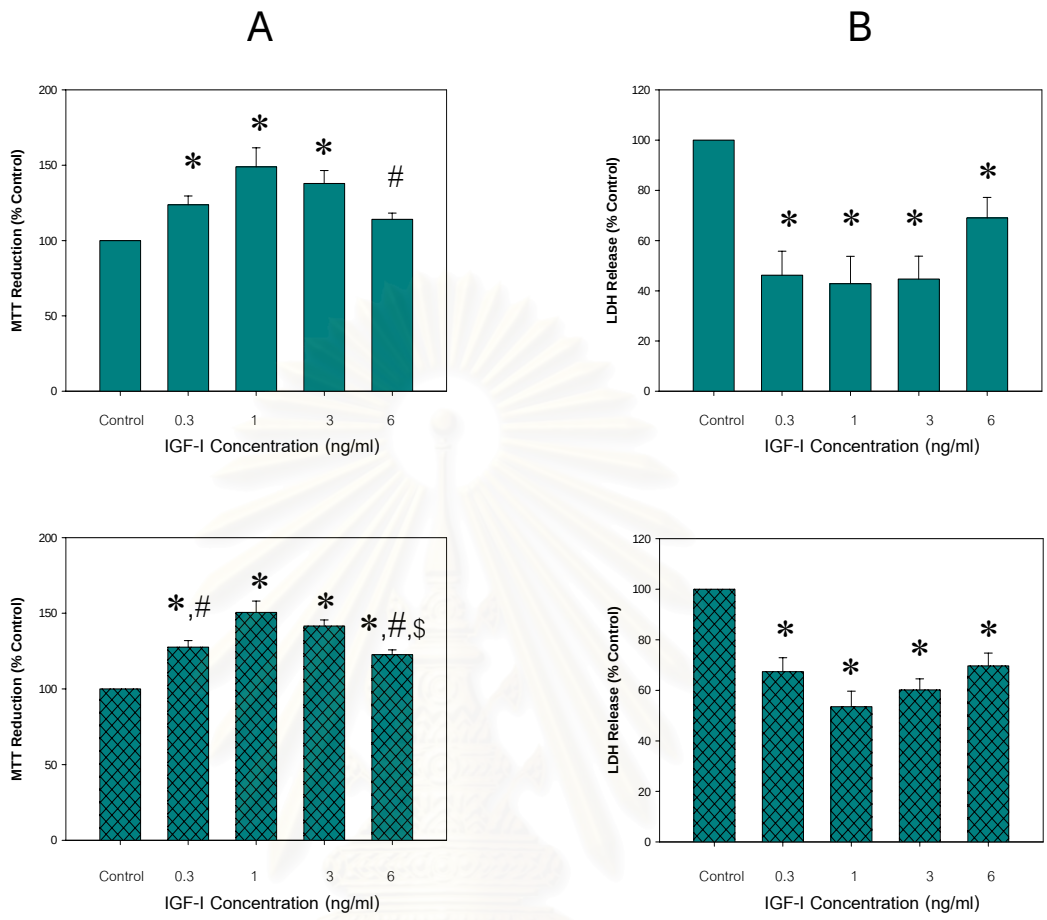
After exposure to 5 μM rotenone for 6 hr, cultured granule neurons were observed under phase-contrast microscopy. A: Control culture. B: Rotenone-exposed culture.

3. Effect of IGF-I on the survival of cultured granule neurons

A marked increase in neuronal survival/metabolic activity was observed when IGF-I was added into the medium for 24 hr (Fig. 13), as shown by the MTT reduction assay (When exposed to IGF-I 0, 0.3, 1, 3 and 6 ng/ml, MTT reduction assay were 100%, 123.86%, 148.88%, 138.03% and 114.16%, respectively) and for 48 hr, (When exposed to IGF-I 0, 0.3, 1, 3 and 6 ng/ml, MTT reduction assay were 100%, 127.61%, 150.30%, 141.79% and 122.54%, respectively). IGF-I exerted maximal effect at the final concentration around 1 ng/ml under both exposure conditions (Fig. 13). Moreover, LDH release was decreased from control value when IGF-I was added into the medium for 24 and 48 hr and the maximal effect was seen at IGF-I concentration around 1 ng/ml under both exposure conditions (Fig. 13).

Visual inspection of neurons by phase-contrast microscopy after the addition of 1 ng/ml IGF-I into the medium for 48 hr showed that cultured granule neurons displayed more healthy morphology as compared to control cultures (Fig 14).

Fig. 13 Effect of IGF-I on the survival of cultured granule neurons



Cultured granule neurons were exposed to IGF-I for 24 hr and 48 hr after which cell viability was determined. A: Cellular MTT reduction. B: Cytoplasmic LDH release. Values are means ± SEM [MTT: (n = 8) and LDH: (n = 6)]. Significant difference was determined by ANOVA and Tukey HSD test. The maximal effect was seen at concentrations around 1 ng/ml IGF-I.

* $p < 0.05$ denotes statistically significant difference from Control

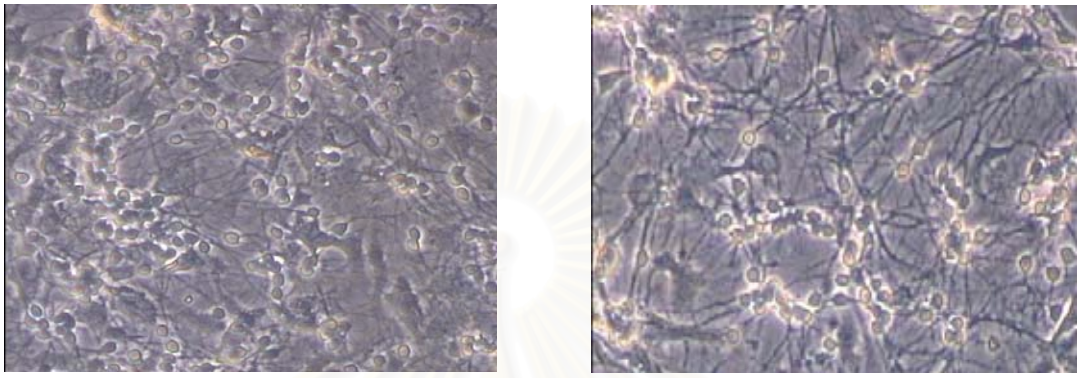
# $p < 0.05$ denotes statistically significant difference from 1 ng/ml IGF-I

$ $p < 0.05$ denotes statistically significant difference from 3 ng/ml IGF-I

Fig. 14 Effects of IGF-I on the morphology of cultured granule neurons

A. Control

B. 1 ng/ml IGF-I

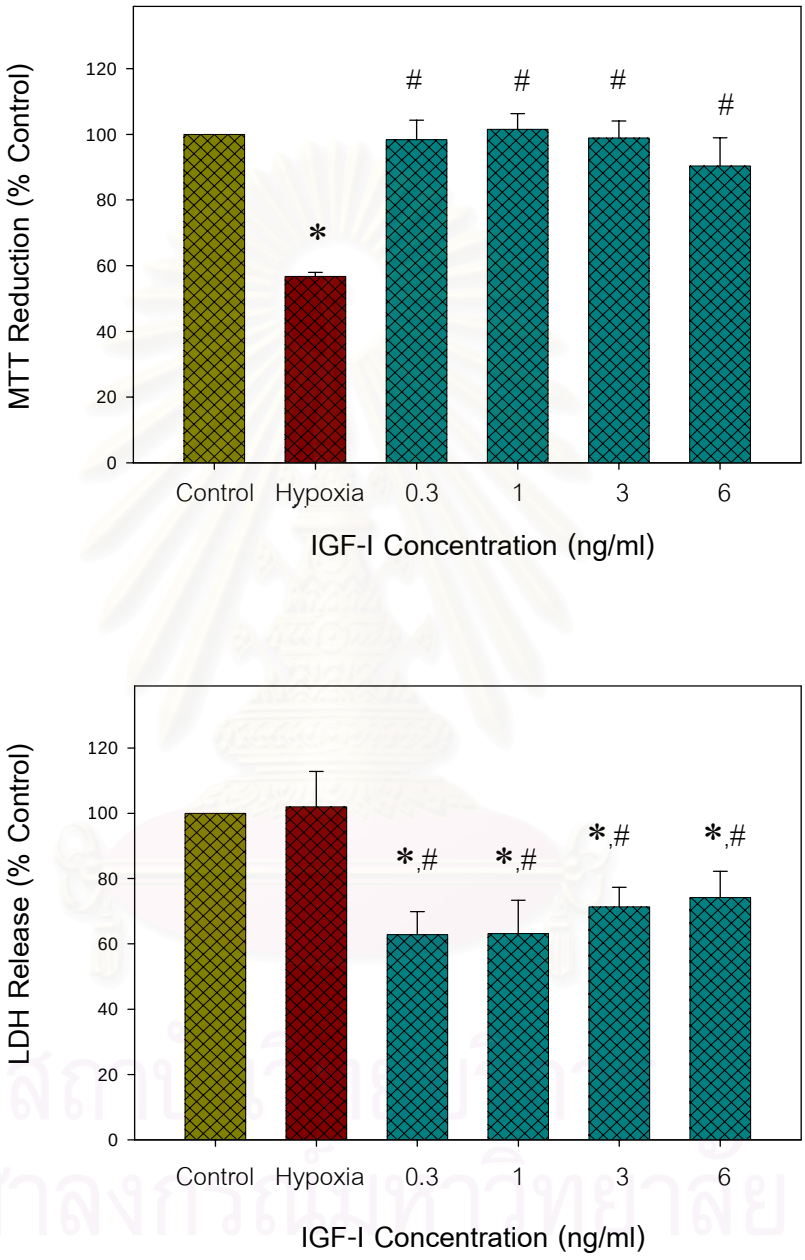


Cultured neurons were exposed to IGF-I for 48 hr before the observation under phase-contrast microscopy. A: Control culture. B: 1 ng/ml IGF-I-treated culture (48 hr).

4. Effect of IGF-I pre-treatment on physical hypoxia-induced injuries on cultured granule neurons

After pre-treatment of cultured granule neurons with IGF-I for 48 hr before the exposure to physical hypoxia for 18 hr, there was a marked increase in MTT reduction in comparison with control hypoxia (up to the values in normal cultures) (When exposed to control, physical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before physical hypoxia, MTT reduction assay were 100%, 56.76%, 98.54%, 101.53%, 98.98% and 90.52%, respectively), as shown in Fig. 15. In addition, treatment with IGF-I before the physical hypoxia reduced the LDH release in a similar manner to that observed with IGF-I alone(When exposed to control, physical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before physical hypoxia, LDH release assay were 100%, 102.01%, 62.82%, 63.26%, 71.19% and 74.04%, respectively),. In both assays, IGF-I provided the maximal neuroprotective effect at concentrations around 1 ng/ml (Fig. 15).

Fig. 15 Effects of IGF-I pre-treatment on physical hypoxia-induced injuries on cultured granule neurons



Cultured granule neurons were incubated with IGF-I for 48 hr and then exposed to physical hypoxia for 18 hr after which cell viability was determined. A: Cellular MTT reduction activity. B: Cytoplasmic LDH release. Values are means $\pm$ SEM [MTT: (n = 8) and LDH: (n = 6)]. Significant difference was determined by ANOVA and Tukey HSD test. The maximal neuroprotective effect of IGF-I was seen at concentrations around 1 ng/ml.

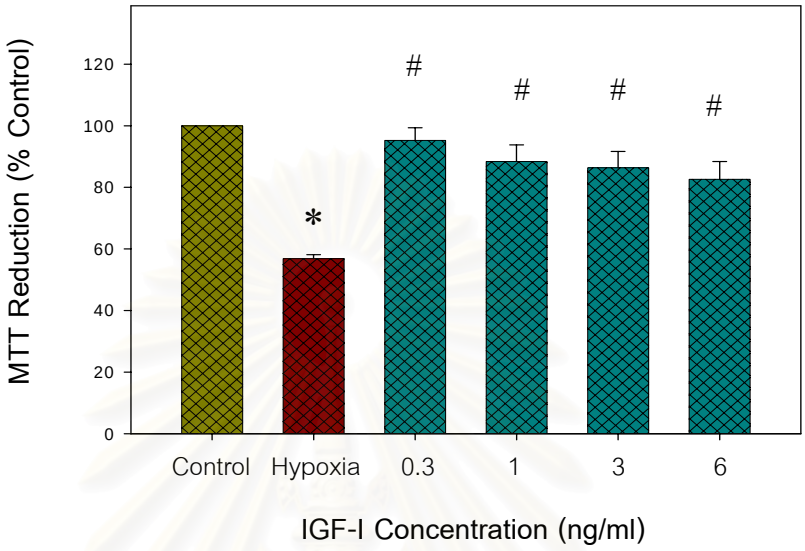
$^{*}p < 0.05$ denotes statistically significant difference from Control

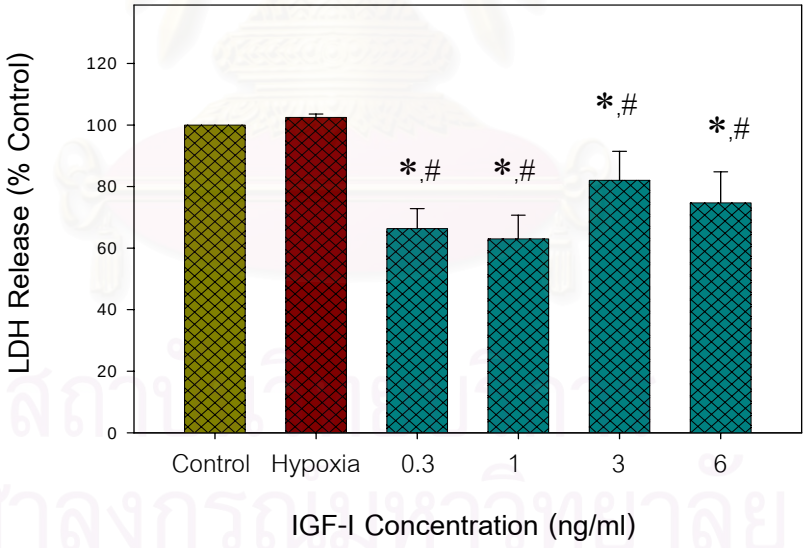
$^{\#}p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

5. **Effect of IGF-I co-treatment on physical hypoxia-induced injuries on cultured granule neurons**

After simultaneous treatment of cultured granule neurons with IGF-I and physical hypoxia for 18 hr, there was a marked increase in MTT reduction in comparison with control hypoxia (close to the values in normal cultures) (When exposed to control, physical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before physical hypoxia, MTT reduction assay were 100%, 56.76%, 95.38%, 88.49%, 86.55% and 82.66%, respectively), as shown in Fig. 16. In addition, treatment with IGF-I during the physical hypoxia reduced the LDH release in a similar manner to that observed with IGF-I alone (When exposed to control, physical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before physical hypoxia, LDH release assay were 100%, 102.32%, 66.48%, 63.01%, 81.89% and 74.78%, respectively). In both assays, IGF-I provided the maximal neuroprotective effect at concentrations around 1 ng/ml (Fig. 16).

Fig. 16 Effects of IGF-I co-treatment on physical hypoxia-induced injuries on cultured granule neurons





Cultured neurons were incubated with IGF-I and physical hypoxia for 18 hr after which cell viability was determined. A: Cellular MTT reduction activity. B: Cytoplasmic LDH release. Values are means $\pm$ SEM [MTT: (n = 8) and LDH: (n = 6)]. Significant difference was determined by ANOVA and Tukey HSD test. The maximal neuroprotective effect was seen around 1 ng/ml IGF-I.

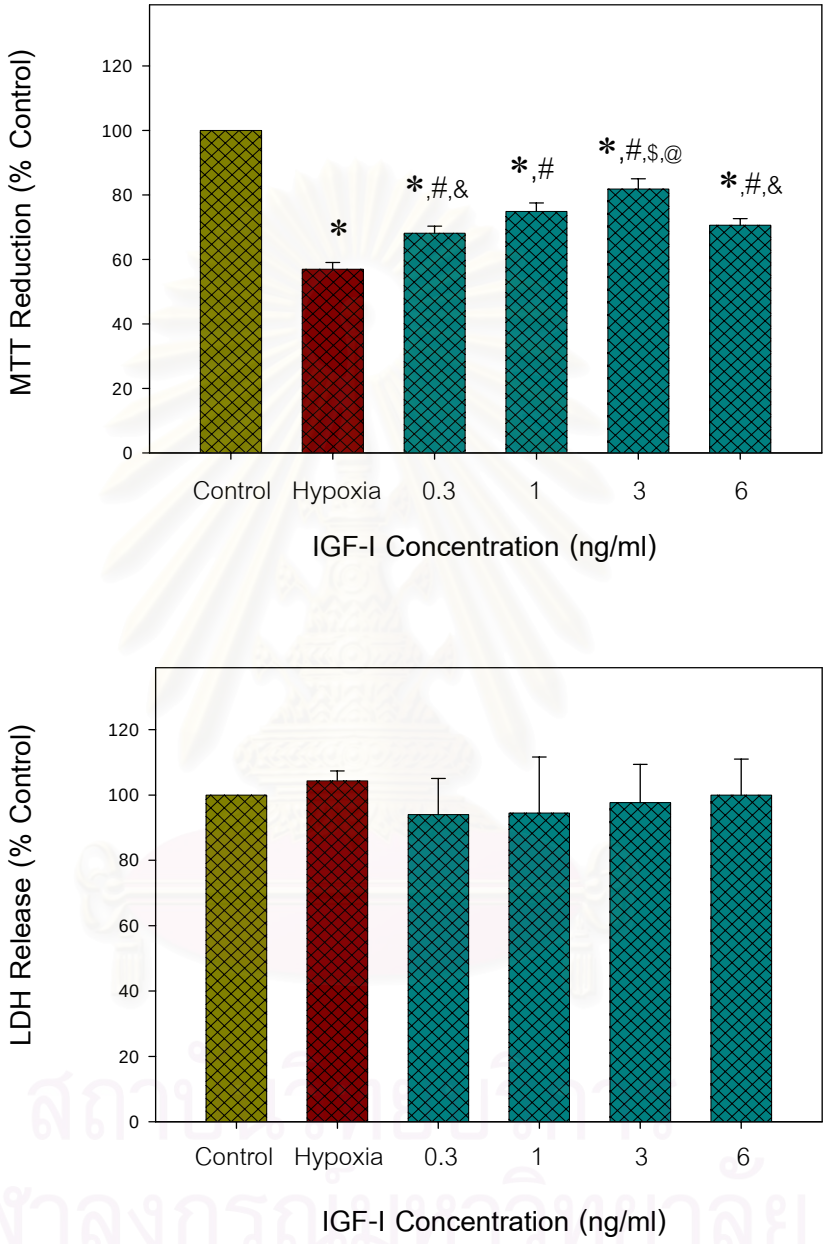
$^{*}p < 0.05$ denotes statistically significant difference from Control

$^{\#}p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

6. **Effect of IGF-I post-treatment on physical hypoxia-induced injuries on cultured granule neurons**

After post-treatment of cultured granule neurons with IGF-I for 48 hr following the exposure to physical hypoxia for 18 hr, there was a small increase in MTT reduction in comparison with control hypoxia (When exposed to control, physical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before physical hypoxia, MTT reduction assay were 100%, 57.18%, 68.06%, 74.70%, 81.87% and 70.47%, respectively), as shown in Fig. 17. In addition, treatment with IGF-I after the physical hypoxia modestly reduced the LDH release from that observed in control hypoxia (When exposed to control, physical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before physical hypoxia, LDH release assay were 100%, 104.37%, 94.09%, 94.62%, 97.54% and 100.06%, respectively).

Fig. 17 Effects of IGF-I post-treatment on physical hypoxia-induced injuries on cultured granule neurons



Cultured neurons were exposed to physical hypoxia for 18 hr and then incubated with IGF-I 48 hr after which cell viability was determined. A: Cellular MTT reduction activity. B: Cytoplasmic LDH release. Values are means $\pm$ SEM [MTT: (n = 8) and LDH: (n = 6)]. Significant difference was determined by ANOVA and Tukey HSD test.

* $p < 0.05$ denotes statistically significant difference from Control

$^{\#}$ $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

$^{\$}$ $p < 0.05$ denotes statistically significant difference from 0.3 ng/ml IGF-I

$^{\&}$ $p < 0.05$ denotes statistically significant difference from 1 ng/ml IGF-I

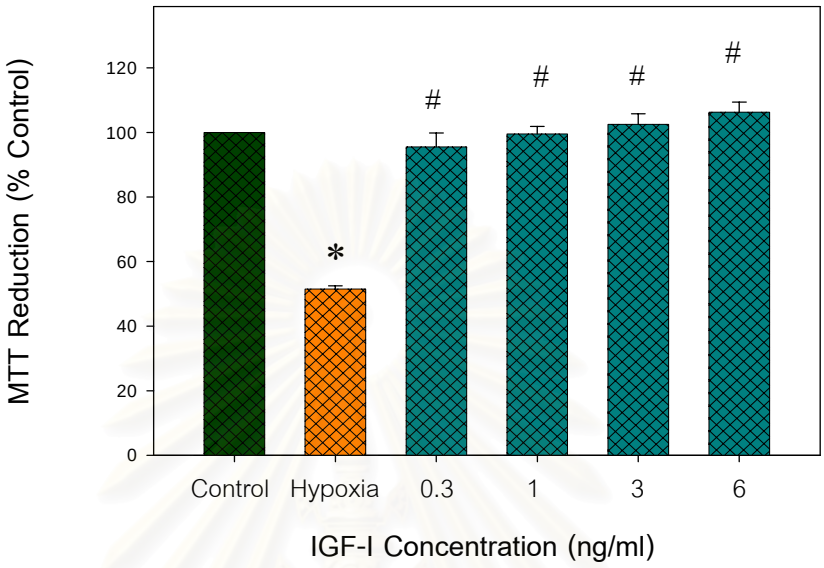
$^{@}$ $p < 0.05$ denotes statistically significant difference from 3ng/ml IGF-I

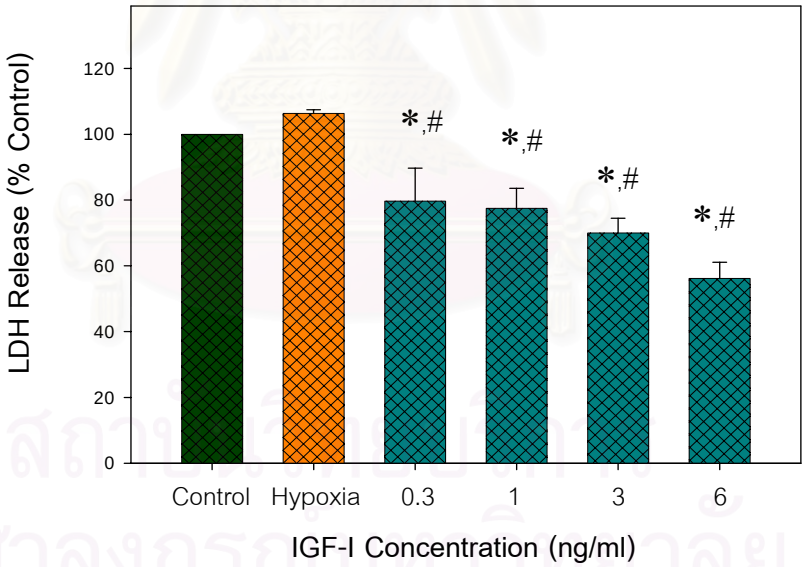
$^{\Phi}$ $p < 0.05$ denotes statistically significant difference from 6 ng/ml IGF-I

7. **Effect of IGF-I pre-treatment on chemical hypoxia-induced injuries on cultured granule neurons**

After pre-treatment of cultured granule neurons with IGF-I for 48 hr before the exposure to chemical hypoxia for 6 hr, there was a marked increase in MTT reduction in comparison with control hypoxia (up to the values in normal cultures) (When exposed to control, chemical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before chemical hypoxia, MTT reduction assay were 100%, 51.70%, 95.46%, 99.41%, 102.50% and 106.38%, respectively), as shown in Fig. 18. In addition, treatment with IGF-I before the chemical hypoxia reduced the LDH release in a similar manner to that observed with IGF-I alone. In both assays (When exposed to control, chemical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before chemical hypoxia, MTT reduction assay were 100%, 106.23%, 79.67%, 77.50%, 69.99% and 56.18%, respectively), IGF-I provided the maximal neuroprotective effect at concentrations around 6 ng/ml (Fig. 18).

Fig. 18 Effects of IGF-I pre-treatment on chemical hypoxia-induced injuries on cultured granule neurons





Cultured neurons were incubated with IGF-I 48 hr and then exposed to 5 μM rotenone for 6 hr after which cell viability was determined. A: Cellular MTT reduction activity. B: Cytoplasmic LDH release. Values are means ± SEM [MTT: (n = 8) and LDH: (n = 6)]. Significant difference was determined by ANOVA and Tukey HSD test. The maximal neuroprotective effect was seen at concentrations around 6 ng/ml IGF-I.

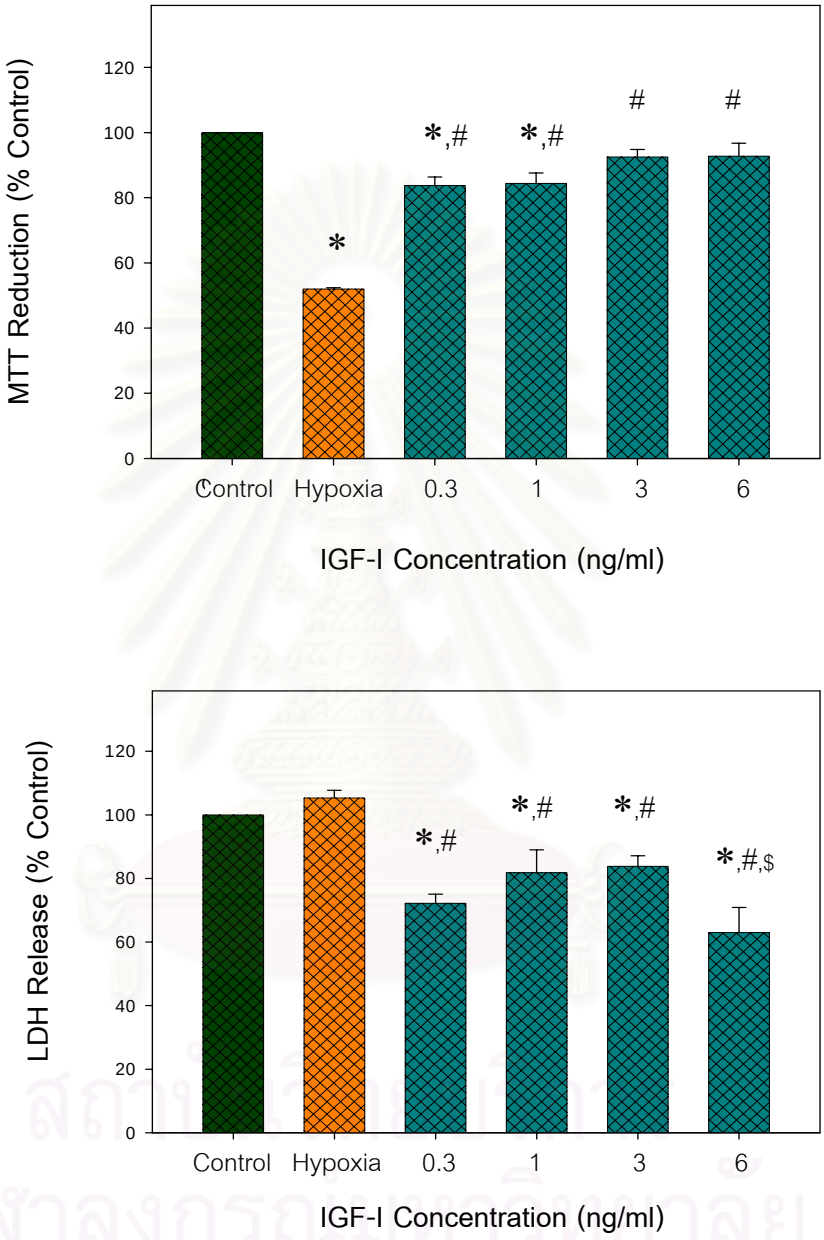
* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

8. **Effect of IGF-I co-treatment on chemical hypoxia-induced injuries on cultured granule neurons**

After simultaneous treatment of cultured granule neurons with IGF-I and chemical hypoxia for 6 hr, there was a marked increase in MTT reduction in comparison with control hypoxia (close to the values in normal cultures) (When exposed to control, chemical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before chemical hypoxia, MTT reduction assay were 100%, 51.74%, 83.82%, 84.32%, 92.33% and 92.83%, respectively), as shown in Fig. 19. In addition, treatment with IGF-I during the chemical hypoxia reduced the LDH release in a similar manner to that observed with IGF-I alone (When exposed to control, chemical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before chemical hypoxia, LDH release assay were 100%, 105.25%, 72.18%, 81.80%, 83.88% and 62.94%, respectively). In both assays, IGF-I provided the maximal neuroprotective effect at concentrations around 6 ng/ml (Fig. 19).

Fig. 19 Effects of IGF-I co-treatment on chemical hypoxia-induced injuries on cultured granule neurons



Cultured granule neurons were coincubated with IGF-I and 5 μM rotenone for 6 hr after which cell viability was determined. A: Cellular MTT reduction activity. B: Cytoplasmic LDH release. Values are means ± SEM [MTT: (n = 8) and LDH: (n = 6)]. Significant difference was determined by ANOVA and Tukey HSD test. The maximal neuroprotective effect was seen at concentrations around 6 ng/ml IGF-I.

* $p < 0.05$ denotes statistically significant difference from Control

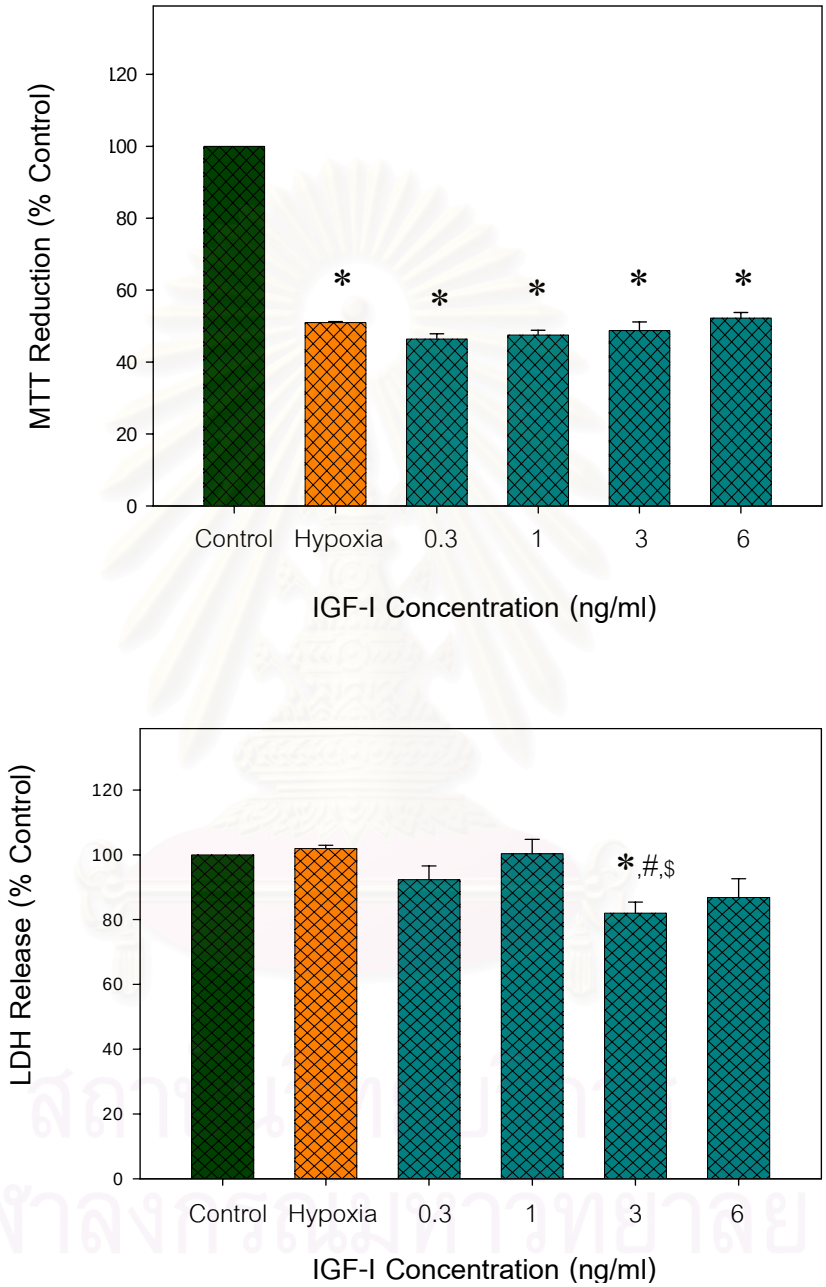
# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

$ $p < 0.05$ denotes statistically significant difference from 3 ng/ml IGF-I

9. **Effect of IGF-I post-treatment on chemical hypoxia-induced injuries on cultured granule neurons**

After post-treatment of cultured granule neurons with IGF-I for 48 hr following the exposure to chemical hypoxia for 6 hr, there was no increase in MTT reduction in comparison with control hypoxia (When exposed to control, chemical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before chemical hypoxia, MTT reduction assay were 100%, 51.03%, 46.56%, 47.56%, 48.89% and 52.42%, respectively), as shown in Fig. 20. In addition, treatment with IGF-I after the chemical hypoxia did not reduce the LDH release from that observed in control hypoxia (When exposed to control, chemical hypoxia, IGF-I 0.3, 1, 3, and 6 ng/ml before chemical hypoxia, LDH release assay were 100%, 101.78%, 92.50%, 100.27%, 82.15% and 86.78%, respectively).

Fig. 20 Effects of IGF-I post-treatment on chemical hypoxia-induced injuries on cultured granule neurons



Cultured granule neurons were exposed to 5 μM rotenone for 6 hr and then incubated IGF-I 48 hr after which cell viability was determined. A: Cellular MTT reduction activity. B: Cytoplasmic LDH release. Values are means ± SEM [MTT: (n = 8) and LDH: (n = 6)]. Significant difference was determined by ANOVA and Tukey HSD test.

* $p < 0.05$ denotes statistically significant difference from Control

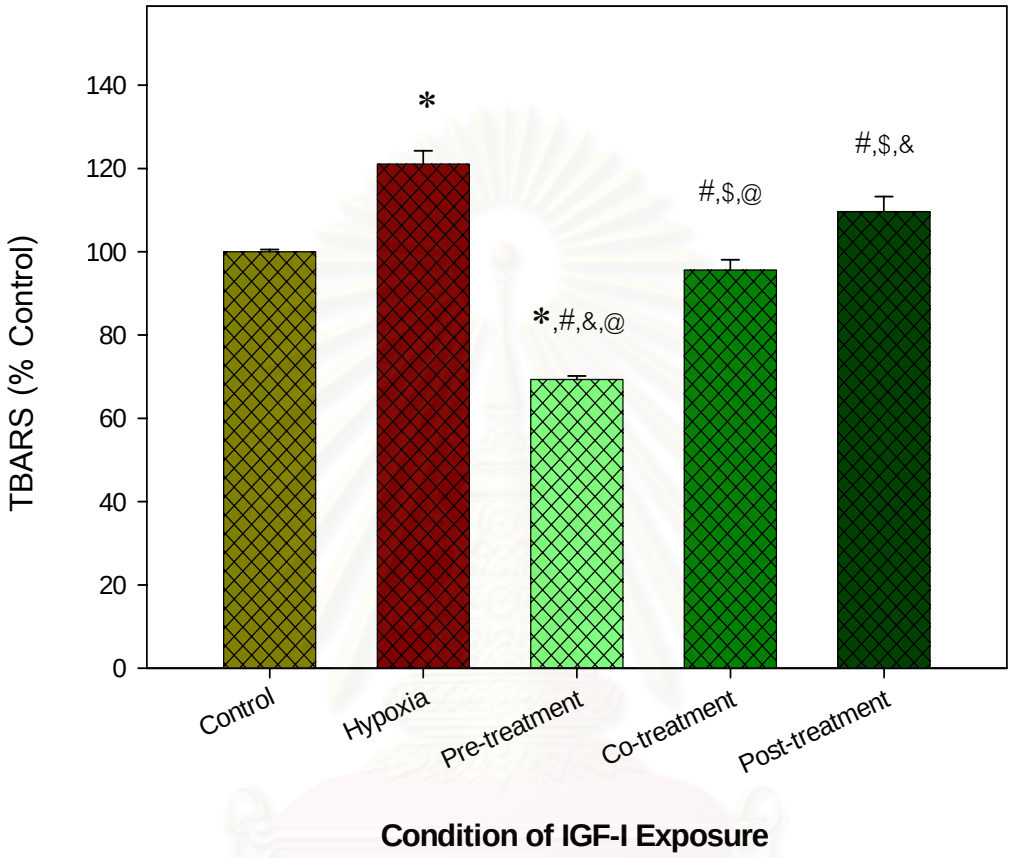
# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

$ $p < 0.05$ denotes statistically significant difference from 1 ng/ml IGF-I

10. **Effect of IGF-I on physical hypoxia-induced lipid peroxidation in cultured granule neurons**

Physical hypoxia for 18 hr caused a significant increase in thiobarbituric acid reactive substance (TBARS), an indicator for lipid peroxidation (Fig. 21). However, pre-, co-, and post-treatment with 1 ng/ml IGF-I diminished the physical hypoxia-induced increase in TBARS. In particular, pre-treatment with IGF-I led to a marked decrease in TBARS even below the control value (When exposed to control, physical hypoxia, pre-, co-, and post-treatment with 1 ng/ml IGF-I and IGF 1 ng/ml, TBARS were 100%, 121.05%, 69.30%, 95.61%, 109.65% and 75.44%, respectively) (Fig. 21).

Fig. 21 Comparative effects of IGF-I on physical hypoxia-induced lipid peroxidation in cultured granule neurons



Cultured granule neurons were treated with pre-, co- and post-treatment of 1 ng/ml IGF-I in addition to physical hypoxia for 18 hr. Then lipid peroxidation was assessed by TBARS assay. Values are means ± SEM (n = 8). Significant difference was determined by ANOVA and Tukey HSD test.

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from Physical hypoxia

$ $p < 0.05$ denotes statistically significant difference from pre-treatment of 1 ng/ml IGF-I

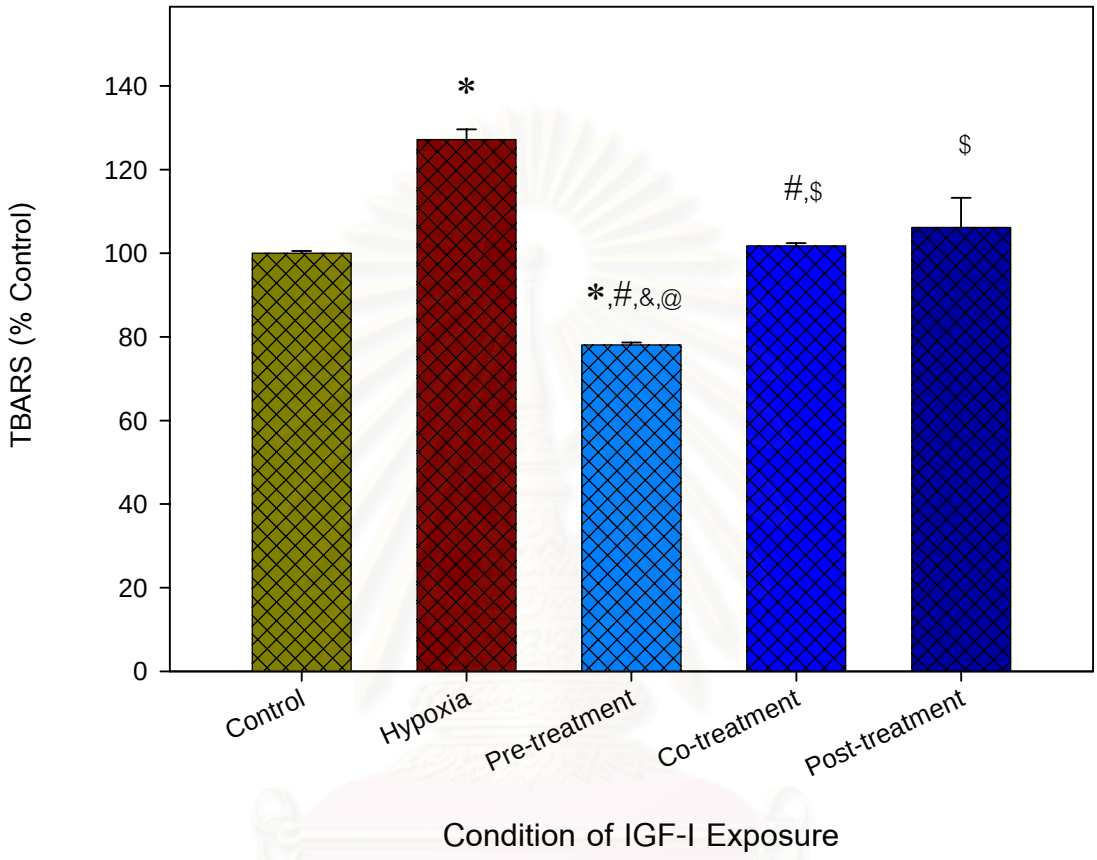
& $p < 0.05$ denotes statistically significant difference from co-treatment of 1 ng/ml IGF-I

@ $p < 0.05$ denotes statistically significant difference from post-treatment of 1 ng/ml IGF-I

11. Effect of IGF-I on chemical hypoxia-induced lipid peroxidation in cultured granule neurons

Chemical hypoxia for 6 hr caused a significant increase in TBARS (Fig. 22). However, pre-, co-, and post-treatment with 1 ng/ml IGF-I diminished the chemical hypoxia-induced increase in TBARS. In particular, pre-treatment with IGF-I led to a marked decrease in TBARS even below the control value (When exposed to control, chemical hypoxia, pre-, co-, and post-treatment with 1 ng/ml IGF-I and IGF 1 ng/ml, TBARS were 100%, 127.19%, 78.07%, 101.75%, 106.14% and 75.44%, respectively) (Fig. 22).

Fig. 22 Comparative effects of IGF-I on chemical hypoxia-induced lipid peroxidation in cultured granule neurons



Cultured granule neurons were treated with pre-, co- and post-treatment of 1 ng/ml IGF-I in addition to 5 μM Rotenone for 6 hr. Then lipid peroxidation was assessed by TBARS assay. Values are means ± SEM (n = 8). Significant difference was determined by ANOVA and Tukey HSD test.

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from Physical hypoxia

$ $p < 0.05$ denotes statistically significant difference from pre-treatment of 1 ng/ml IGF-I

& $p < 0.05$ denotes statistically significant difference from co-treatment of 1 ng/ml IGF-I

@ $p < 0.05$ denotes statistically significant difference from post-treatment of 1 ng/ml IGF-I

Chapter IV

Discussion

During postnatal development, granule cells constitute the most abundant neuronal population in the mammalian brain. In the present study, granule neurons were cultured from postnatal day 8 (P8) rat cerebella. When cultured from early postnatal rats (days 4-10), granule cells differentiate *in vitro*, acquiring several morphological, biochemical, and electrophysiological characteristics of mature neurons (Gallo *et al*., 1987; Cull-Candy *et al*., 1988). In view of their relative abundance, cerebellar granule cells provide an excellent primary cell culture system to investigate the mechanisms underlying neuronal cell death in the CNS. The same cells are capable of dying in distinct patterns, necrosis or apoptosis, which almost certainly involve distinct mechanisms (D'Mello *et al*., 1993). Cerebellar granule neurons therefore are a convenient system to investigate the similarities and differences in the molecular mechanisms underlying these degenerative processes (D'Mello *et al*., 1993).

Survival of a variety of neuronal types, including cerebellar granule neurons can be maintained by IGF-I. Although IGF-I is not produced in cerebellar granule neurons at any stage of development, Purkinje cells in the cerebellum synthesize and secrete this polypeptide, especially during early postnatal life (Andersson *et al*., 1988; Bondy, 1991). During this period, immature granule cells migrate through the Purkinje cell layer to reach the internal granule layer, whereupon they differentiate. It is likely that the IGF-I secreted by the Purkinje cells is taken up by granule cells and influences the terminal differentiation of these neurons. Indeed, granule cells migrating through the molecular layer transiently display immunoreactivity to IGF-I (Andersson *et al*., 1988). Most importantly, granule neurons express the IGF-I receptor (Bondy, 1991). The previously mentioned observations with IGF-I support the conclusion that the effect of IGF-I is mediated by the IGF-I receptor.

Many studies in rats and mice have revealed that the developing brain expresses the components of IGF-I in the developing cerebellum (Lee *et al*., 1992;

Torres-Aleman *et al*., 1994) and its receptors in cerebellar granular layers (Lee, 1992; Torres-Aleman, 1994). These studies had shown that overexpression of IGF-I increases proliferation and survival of granule cells (Ye *et al*., 1996). Beside its beneficial effect on granule cells, IGF-I promotes the *in vitro* survival and neurite outgrowth of various sensory, sympathetic, cortical, and motor neurons (Neff *et al*., 1993).

Furthermore, IGF-I is involved in the response of neural tissues to injury (Beilharz *et al.,* 1998) and protects neurons against various neurodegenerative stimuli. For instance, IGF-I is a trophic factor for motoneurons (Di Giulio *et al*, 2000) and protects motoneurons as well as retinal ganglion neurons (Kermer *et al*, 2000) from axotomy-induced death. IGF-I is also protective against hypoxia–ischemia (Guan *et al*., 2000; Wang *et al*., 2000) and protects a variety of neurons such as cerebellar granule cells from neurotoxic insults (Leski *et al*., 2000) and hilar hippocampal neurons (Azcoitia *et al*., 1999) from kainic acid toxicity, hippocampal neurons from β-amyloid (Dor'e *et al*., 1997) and iron toxicity, inferior olivary nucleus neurons against 3-acetyl pyridine (Fermandez *et al*., 1999), striatal neurons against quinolinic acid (Alexi *et al*., 1999)

Death of granule cells by lowering of K^+ can be prevented by IGF-I but not by several other growth factors including fibroblast growth factor (FGF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (D'Mello *et al*., 1993). Several pieces of evidence suggest that IGF-I may be physiologically important for the development of granule neurons (Bondy, 1991; Ye *et al*., 1996). Due to the abundance and homogeneity of cultured cerebellar granule cells and documented regulatory roles of IGF-I on granule neuron development, the potential benefit of this neurotrophic factor on hypoxia-induced cell injuries is tested by using granule cell culture model.

In this study, hypoxia-induced cellular injuries in cultured cerebellar granule neurons were initiated by two different conditions, namely, physical and chemical hypoxia. Physical hypoxia was created by subjecting cultured granule neurons to 100% N_2 for various exposure times and chemical hypoxia was created by incubating cultured granule neurons with various concentrations of a metabolic inhibitor,

rotenone. Cultured granule neurons exposed to physical hypoxia displayed exposure time-related neuronal injury (Fig. 9A), and those exposed to chemical hypoxia displayed concentration-related neuronal injury (Fig. 11A). Decreases in cellular MTT reduction which coincided with the onset of neuronal injury suggested that compromised mitochondrial metabolism may be, at least partly, responsible for the observed hypoxia-induced neuronal injury. However, there was no significant increases in LDH release from cultured neurons under both hypoxic conditions. Therefore, it was apparent that the severity of hypoxic insults used in this study was only moderate and did not lead to remarkable neuronal cell death (Figs. 9B and 11B). It is conceivable that this *in vitro* degenerative process in cultured neurons may be of practical value due to their similarity to the *in vivo* pathology occurred in many progressive neurodegenerative disorders (Rothman, 1984; Goldberg et al, 1986; Goldberg and Choi, 1993).

The objective of this study was to test whether IGF-I is protective against neuronal cell injuries induced by physical hypoxia and chemical hypoxia. The experimental results demonstrated that progressive neuronal injury was significantly reduced by pre- and co-treatment with IGF-I (Figs. 15, 16, 18 and 19). However, IGF-I was less effective when added after neuronal exposure to hypoxia (Figs. 17 and 20). These findings indicate that IGF-I may possess the ability to limit the ongoing neuronal injury but it has limited capacity to rescue neurons which were already injured from hypoxic insults. A possible role for IGF-I in controlling neuronal degeneration had been raised (Hsu *et al*., 1994) and the results of this study are in accordance with that hypothesis.

There has been a line of evidence suggesting that IGF-I is neuroprotective and may be used to inhibit neuronal apoptosis. For example IGF-I had been shown to block developmental apoptosis in chick spinal motoneurons (Gluckman *et al*, 1998). Furthermore, in pathological paradigms, IGF-I had been shown to block apoptosis in neurons subject to hypoglycemia, hypoxia, osmotic stress or β-amyloid *in vitro* (Tamm and Kikuchi, 1990). IGF-I has been well demonstrated as an anti-apoptotic agent in the CNS both *in vitro* and *in vivo* (D'Mello *et al*., 1993; D'Mello *et al*., 1997).

It is tempting to speculate that the mechanisms involved in hypoxia-mediated injury of granule neurons in culture are similar to those operated during neuronal injury *in vivo*, e.g., following a cerebrovascular stroke and ischemia-reperfusion. In addition, these mechanisms may also be activated in other neurodegenerative diseases. Evidently, hypoxia leads to disrupted cellular energy metabolism, especially on mitochondrial respiration, and generation of cytotoxic free radicals. Free radicals can then initiate many damaging consequences including membrane lipid peroxidation.

The experimental results demonstrated that hypoxic conditions induced lipid peroxidation reaction. After exposure to both physical and chemical hypoxia, lipid peroxidation in granule neurons was significantly increased as compared with controls (Figs. 21 and 22). A remarked increase in MDA production suggested that neuronal injuries under hypoxic conditions might be due to the consequence of lipid peroxidation such as membrane damages and the generation of more free radicals.

It is likely that IGF-I prevents injurious effects of hypoxia by reduction of lipid peroxidation (Figs. 21 and 22). Apparently, this peptide has neuroprotective effect when administered before (pre-treatment) and during (co-treatment) physical and chemical hypoxia but it is less effective than when administered after (post-treatment) hypoxic conditions. Therefore, the neuroprotective effect of IGF-I on hypoxia-induced neuronal injuries may mediate, at least partly, through reduction of membrane lipid peroxidation in cultured granule neurons.

The protective effect of IGF-I on granule neuron apoptosis had also been noted in dissociated cell cultures of rat cerebellar granule neurons (D'Mello *et al*., 1993). It is reasonable that IGF-I may have a role in the treatment of neurodegenerative diseases such as stroke, perinatal asphyxia and other forms of acute brain injury. IGF-I is broadly distributed within the CNS (Baskin *et al*., 1988) and has been shown to be a potent neurotrophic factor. IGF-I is induced endogenously in damaged brain regions in experimental animal models (Gluckman *et al*., 1992; Lee *et al*., 1993) and can prevent neuronal injury when administered before and during hypoxic exposure.

In conclusion, the experimental results suggest that IGF-I is markedly neuroprotective against hypoxia-induced injuries in cultured granule neurons when added before and during the insult. However, it has limited capacity to rescue the injured neurons after hypoxic exposure. The mechanisms underlying neuroprotective effects of IGF-I on cultured granule neurons are still unclear but they may be related, at least partly, to reduction of lipid peroxidation.

Chapter V

Conclusion

In conclusion, the present study illustrated toxic effects of hypoxic conditions on cultured cerebellar granule neurons. The experimental results indicated that physical and chemical hypoxia used in this study induced remarked cellular injuries without significant cell death. The extent of cellular injuries was dependent on the severity of hypoxic conditions, namely, duration of exposure and concentration of insulting agent. IGF-I possesses neurotrophic activity that effectively prevented or antagonized injurious effects of physical and chemical hypoxia on cultured granule neurons. However, it has limited capacity to rescue already injured neurons after exposure to hypoxia. The underlying mechanism of IGF-I-mediated neuroprotection against hypoxic injuries is still unclear but it may involve IGF-I-induced decrease in lipid peroxidation.

References

Adams, J. M. and Cory, S. The bcl-2 protein family: arbiters of cell survival. Science. 281 (1998): 1322-1326.

Aitken, P. G., Jing, J., Young, J. and Somjen, G. G. Ion channel involvement in hypoxia-induced spreading depression in hippocampal slices. Brain Res. 541 (1991): 7-11.

Alexi, T., Hughes, P. E., van Roon-Mom, W. M., Faull, R. L., Williams, C. E., Clark, R. G. and Gluckman, P. D. The IGF-I amino-terminal tripeptide glycine-proline-glutamate (GPE) is neuroprotective to striatum in the quinolinic acid lesion animal model of Huntington's disease. Exp. Neurol. 159 (1999): 84-97.

Andersson, I. K., Edwall, D., Norstedt, G., Rozell, B., Skottner, A. and Hensson, H. A. Differing expression of insulin-like growth factor I in the developing and in the adult rat cerebellum. Acta Physiol. Scand. 132 (1988): 167-173.

Ando, Y. and Jensen, P. J. Epidermal growth factor and Insulin-like growth factor I enhance keratinocyte migration. J. Invest. Dermatol. 100 (1993): 633-639.

Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J. J., Mazzei, G., Maundrell, K., Gambale, F., Sadoul, R. and Martinou, J. C. Inhibition of Bax channel-forming activity by Bcl-2. Science. 277 (1997): 370-372.

Armstrong, C. S., Wuarin, L. and Ishii, D. N. Uptake of circulating insulin like growth factor-I into the cerebrospinal fluid of normal and diabetic rats and normalization of IGF-II mRNA content in diabetic rat brain. J. Neurosci. Res. 59 (2000): 649-660.

Azcoitia, I., Sierra, A. and Garcia-Segura, L. M. Neuroprotective effects of estradiol in the adult rat hippocampus: interaction with insulin like growth factor-I signalling. J. Neurosci. Res. 58 (1999): 815-822.

Baker, N. L., Russo, V. C., Bernard, O., D'Ercole, A. J. and Werther, G. A. Interactions between bcl-2 and the IGF system control apoptosis in the developing mouse brain. Brain Res. Dev. Brain Res. 118 (1999): 109-118.

Balestrino, M. Pathophysiology of anoxic depolarization: new findings and a working hypothesis. J. Neurosci. Methods. 59 (1995): 99-103.

Ballanyi, K., Doutheil, J. and Brockhaus, J. Membrane potentials and microenvironment of rat dorsal vagal cells in vitro during energy depletion. J. Physiol. 495 (1996): 769-784.

Bamji, S. X., Majdan, M., Pozniak, C. D., Belliveau, D. J., Aloyz, R. Kohn, J., Causing, C. G. and Miller, F. D. The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. J. Cell Biol. 140 (1998): 911-923.

Ban, J., Eckhart, L., Weninger, W., Mildner, M. and Tschachler, E. Identification of a human cDNA encoding a novel Bcl-x isoform. Biochem. Biophys. Res. Commun. 248 (1998): 147-152.

Barreca, A., De Luca, M., Del Monte, P., Bondanza, S., Damonte, G., Cariola, G., Di Marco, E., Giordano, G., Cancedda, R. and Minuto, F. In vitro paracrine regulation of human keratinocyte growth by fibroblast-derived insulin-like growth factors. J. Cell Physiol. 151 (1992): 262-268.

Basanez, G., Nechushtan, A., Drozhinin, O., Chanturiya, A., Choe, E., Tutt, S., Wood, K. A., Hsu, Y., Zimmerberg, J. and Youle, R. J. Bax, but not Bcl-xL, decreases the lifetime of planar phospholipid bilayer membranes at subnanomolar concentrations. Proc. Natl. Acad. Sci. USA. 96 (1999): 5492-5497.

Baskin, D. G., Wilcox, B. J., Figlewicz, D. P., Dorsa, D. M. Insulin-like growth factor in the CNS. Trends Neurosci. 11 (1988): 107-111.

Basu, S., Bayoumy, S., Zhang, Y., Lozano, J. and Kolesnick, R. BAD enables ceramide to signal apoptosis via Ras and Raf-1. J. Biol. Chem. 273 (1998): 30419-30426.

Behrens, M. M., Strasser, U., Lobner, D. and Dugan, L. L. Neurotrophin-mediated potentiation of neuronal injury. Micr. Res. Tech. 45 (1999): 276-284.

Beilharz, E. J., Russo, V. C., Butler, G., Baker, N. L., Connor, B., Sirimanne, E. S., Dragunow, M., Werther, G. A., Gluckman, P. D., Williams, C. E. and Scheepens, A. Co-ordinated and cellular specific induction of the components

of the IGF/IGFBP axis in the rat brain following hypoxic–ischemic injury. Mol. Brain Res. 59 (1998): 119-134.

Bergeron, L., Perez, G. I., Macdonald, G., Shi, L. and Sun, Y. Defects in regulation of apoptosis in caspase-2-deficient mice. Genes Dev. 12 (1998): 1304-1314.

Bernardi, P., Broekemeier, K. M. and Pfeiffer, D. R. Recent progress on regulation of the mitochondrial permeability transition pore: a cyclosporin-sensitive pore in the inner mitochondrial membrane. J. Bioenerget. Biomembr. 26 (1994): 509-517.

Betarbet, R., Todd, B. S., Gullian, M., Monica, G., Alexander, V. and Timothy, G. J. Chronic systemic pesticide exposure reproduce features of Parkinson's disease. Nature Neurosci. 3 (12) (2000): 1301-1305.

Biswas, O. A., Adebanjo, B. D., Freeman, H. A., Anandatheerthavarada, C., Vijayasarathy, M., Zaidi, M., Kotlikoff, N. G. and Avadhani. Retrograde calcium signaling in C2C12 skeletal myocytes in response to mitochondrial genetic and metabolic stress: a novel mode of inter-organelle cross-talk. EMBO J. 18 (1999): 522-533.

Bohme, K., Conscience Egli, M., Tschan, T., Winterhalter, K. H. and Bruck-ner, P. Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: the role of insulin-like growth factor-I, insulin, or thyroxine. J. Cell Biol. 116 (1992): 1035-1042.

Bondy, C. A. Transient IGF-I gene expression during the maturation of functionally related central projection neurons. J. Neurosci. 11 (1991): 3442-3455.

Bossenmeyer-Pourie, C., Chihab, R., Schroeder, H. and Daval, J. L. Transient hypoxia may lead to neuronal proliferation in the developing mammalian brain: from apoptosis to cell cycle completion. Neuroscience 91 (1999): 221-231.

Butler, A. A., Yakar, S., Gewolb, I. H., Karas, M., Okubo, Y. and LeRoith, D. Insulin-like growth factor-I receptor signal transduction: at the interface between physiology and cell biology. Comp. Biochem. Physiol. Biochem. Mol. Biol. 121 (1998): 19-26.

Calabresi, P., Pisani, A., Mercuri, N. B. and Bernardi, G. Hypoxia-induced electrical changes in striatal neurons. J. Cereb. Blood Flow Metab. 15 (1995a): 1141-1145.

Calabresi, P., Pisani, A., Mercuri, N. B. and Bernardi, G. On the mechanisms underlying hypoxia-induced membrane depolarization in striatal neurons. Brain. 118 (1995b): 1027-1038.

Carro, E., Nunez, A., Busiguina, S. and Torres-Aleman, I. Circulating insulin-like growth factor I mediates effects of exercise on the brain. J. Neurosci. 20 (2000): 2926-2933.

Castro-Alamancos, M. A. and Torres-Aleman, I. Long-term depression of glutamate-induced gamma-aminobutyric acid release in cerebellum by insulin-like growth factor I. Proc. Natl. Acad. Sci. USA. 90 (1993): 7386-7390.

Chao, D. T. and Korsmeyer, S. J. Bcl-2 family: regulators of cell death. Annu. Rev. Immunol. 16 (1998): 395-419.

Chao, D. T., Linette, G. P., Boise, L. H., White, L. S., Thompson, C. B. and Korsmeyer, S. J. Bcl-XL and Bcl-2 repress a common pathway of cell death. J. Exp. Med. 182 (1995): 821-828.

Chen, J., Nagayama, T., Jin, K., Stetler, R. A., Zhu, R. L., Graham, S. H. and Simon, R. P. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J. Neurosci. 18 (1998): 4914-4928.

Cheng, B. and Mattson, M.P. NT-3 and BDNF protect CNS neurons against metabolic/excitotoxic insults. Brain Res. 640 (1994): 56-67.

Cheng, Y., Deshmukh, M., D'Costa, A., Demaro, J. A., Gidday, J. M., Shah, A., Sun, Y., Jacquin, M. F., Johnson, E. M. and Holtzman, D. M. Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. J. Clin. Invest. 101 (1998): 1992-1999.

Chittenden, T., Harrington, E. A., O'Connor, R., Flemington, C., Lutz, R. J., Evan, G. I. and Guild, B. C. Induction of apoptosis by the Bcl-2 homologue Bak. Nature. 374 (1995a): 733-736.

Chittenden, T., Flemington, C., Houghton, A. B., Ebb, R. G., Gallo, G. J., Elangovan, B., Chinnadurai, G. and Lutz, R. J. A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. EMBO J. 14 (1995b): 5589-5596.

Choi, D. W. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Neurosci. 11 (1988): 465-469.

Choi, D. W. Calcium: Still center-stage in hypoxic-ischemic neuronal death. Trends Neurosci. 18 (1995): 58-60.

Choi, D. W. Cerebral hypoxia: some new approaches and unanswered questions. J. Neurosci. 10 (1990): 2493-2501.

Christine, T., William, L. J. and Donato, A. D. Increased striatal dopamine turnover following acute administration of rotenone to mice. Brain Res. 885 (2000): 283-288.

Chrysis, D., Calikoglu, A. S., Ye, P. and D'Ercole, A. J. Insulin-like growth factor-I overexpression attenuates Cerebellar apoptosis by altering the expression of Bcl family proteins in a developmentally specific manner. J. Neurosci. 21(5) (2001): 1481-1489.

Clem, R. J., Cheng, E. H., Karp, C. L., Kirsch, D. G., Ueno, K., Takahashi, A., Kastan, M. B., Griffin, D. E., Earnshaw, W. C., Veliuona, M. A. and Hardwick, J. M. Modulation of cell death by Bcl-XL through caspase interaction. Proc. Natl. Acad. Sci. USA. 95 (1998): 554-559.

Cohick, W. S. and Clemmons, D. R. The insulin-like growth factors. Annu. Rev. Physiol. 55 (1993): 131-153.

Cooper, J. M., Daniel, S. E., Marsden, C. D. and Schapira, A. H. L-dihydroxyphenylalanine and complex I deficiency in Parkinson's disease brain. Movement Disord. 10(3) (1995): 295-297.

Cosulich, S. C., Worrall, V., Hedge, P. J., Green, S. and Clarke, P. R. Regulation of apoptosis by BH3 domains in a cell-free system. Curr. Biol. 7 (1997): 913-920.

Cull-Candy, S. G., Howe, J. R. and Ogden, D. C. Noise and single channels activated by excitatory amino acids in rat cerebellar granule neurons. J. Physiol. (Lond). 400 (1988): 189-222.

Datta, S. R., Brunet, A. and Greenberg, M. E. Cellular survival: a play in three Akts. Genes Dev. 13 (1999): 2905-2927.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H. S. and Gotoh, Y. Akt phosphorylation of Bad couples survival signals to the cell intrinsic death machinery. Cell 91 (1997): 231-241.

Daughaday, W. H. and Rotwein, P. Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structure, serum and tissue concentrations. Endocr. Rev. 10 (1989): 68-91.

Daughaday, W. H., Hall, K., Raben, M. S., Salmon, Jr. W. D., Brande, J. L. and Wyk, J. J. Somatomedin: proposed designation for sulphation factor. Nature. 235 (1972): 107.

DeCoster, M. A., Schabelman, E., Tombran-Tink, J. and Bazan, N. G. Neuroprotection by pigment epithelial-derived factor against glutamate toxicity in developing primary hippocampal neurons. J. Neurosci. Res. 56 (1999): 604-610.

del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R. and Nunez, G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science. 278 (1997): 687-689.

de Pablo, F. and de la Rosa, E. The development CNS: a scenario for the action of proinsulin, insulin and insulin-like growth factors. Trends Neurosci. 18 (1995): 143-150.

Di Giulio, A. M., Germani, E., Lesma, E., Muller, E. and Gorio, A. Glycosaminoglycans co-administration enhance insulin-like growth factor-I neuroprotective and neuroregenerative activity in traumatic and genetic models of motor neuron disease: a review. Int. J. Dev. Neurosci. 18 (2000): 339–346.

Dimitriadis, G., Parry Billings, M., Bevan, S., Dunger, D., Piva, T., Krause, U., Wegener, G. and Newsholme, E. A. Effects of Insulin-like growth factor I on the rates of glucose transport and utilization in rat skeletal muscle in vitro. Biochem. J. 285 (1992): 269-274.

Distelhorst, C. W. and McCormick, T. S., Bcl-2 acts subsequent to and independent of Ca^{2+} fluxes to inhibit apoptosis in thapsigargin- and glucocorticoid-treated mouse lymphoma cells. Cell Calcium 19 (1996): 473-483.

D'Mello, S. R., Borodezt, K., Soltoff, S. P. Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI 3-kinase in IGF-1 signaling. J. Neurosci. 17(5) (1997): 1548-1560.

D'Mello S. R., Galli C., Ciotti T. and Calissano P. Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. Proc. Natl. Acad. Sci. USA. 90 (1993): 10989-10993.

Dor'e, S., Kar, S. and Quirion, R. Insulin-like growth factor I protects and rescues hippocampal neurons against β-amyloid and human amylin-induced toxicity. Proc. Natl. Acad. Sci. USA. 94 (1997a): 4772–4777.

Dor'e, S., Kar, S. and Quirion, R. Presence and differential internalization of two distinct insulin-like growth factor receptors in rat hippocampal neurons. Neuroscience. 78 (1997b): 373-383.

Dor'e, S., Kar, S. and Quirion, R. Rediscovering an old friend, IGF-I: potential use in the treatment of neurodegenerative diseases. Trends Neurosci. 20(8) (1997c): 326-331.

Dor'e, S., Kar, S., Zheng, W. H. and Quirion, R. Rediscovering good old friend IGF-I in the new millinium: possible usefulness in Alzheimer's disease and stroke. Pharmaceutica Acta Helvetiae 74 (2000): 273-280.

Dor'e, S., Pelletier, J. P., DiBattista, J. A., Tardif, G., Brazeau, P. and Martel-Pelletier. Human osteoarthritic chondrocytes possess an increased number of insulin-like growth factor I binding sites but are unresponsive to its stimulation: possible role of IGF-I-Binding proteins. J. Arthritis Rheum. 37 (1994): 253-263.

Dudek, H., Datta, S. R. and Franke, T. F. Regulation of neuronal survival by the serine–threonine protein kinase Akt. Science. 275(5300) (1997): 661-665.

Dux, E., Schubert, P. and Kreutxberg, G. W. Ultrastructural localization of calcium in ischemic hippocampal slices: the influence of adenosine and theophylline. J. Cereb. Blood Flow Metab. 12 (1992): 520-524.

Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A., Martinou, J. C. Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg^{2+} ions. J. Cell Biol. 143 (1998): 217-224.

Fabisiak, J. P., Kagan, V. E., Ritov, V. B., Johnson, D. E. and Lazo, J. S. Bcl-2 inhibits selective oxidation and externalization of phosphatidylserine during paraquat-induced apoptosis. Am. J. Physiol. 272 (1997): 675-684.

Fernandez, A. M., Gonzalez de la Vega, A., Planas, B. and Torres-Aleman, I. Neuroprotective actions of peripherally administered insulin-like growth factor I in the injured olivo-cerebellar pathway. Eur. J. Neurosci. 11 (1999): 2019-2030.

Finucane, D. M., Bossy-Wetzel, E., Waterhouse, N. J., Cotter, T. G. and Green, D. R. Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. J. Biol. Chem. 274 (1999): 2225-2233.

Florini, J. R., Ewton, D. Z. and Roof, S. L. Insulin-like growth factor-1 stimulates terminal myogenic differentiation by induction of myogenin gene expression. Mol. Endocrinol. 5 (1991): 718-724.

Florini, J. R. and Magri, K. A. Effects of growth factors on myogenic differentiation. Am. J. Physiol. 256 (1989): C701-C711.

Frandsen, A. and Schousboe, A. Dantrolene prevents glutamate cytotoxicity and Ca^{2+} release from intracellular stores in cultured cerebral cortical neurons. J. Neurochem. 56 (1991): 1075-1078.

Frandsen, A. and Schousboe, A. Excitatory amino acid-mediated cytotoxicity and calcium homeostasis in cultured neurons. J. Neurochem. 60 (1993): 1202-1211.

Friedmann, J. E. and Haddad, G. G. Removal of extracellular sodium prevents anoxia-induced injury in freshly in dissociated rat CA1 hippocampal neurons. Brain Res. 641 (1994): 57-64.

Fujiwara, N., Higashi, H., Shimoji, K. and Yoshimura, M. Effects of hypoxia on rat hippocampal neurones *in vitro*. J. Physiol. 384 (1987): 131-151.

Furlong, I. J., Ascaso, R., Rivas, A. L. and Collins, M. K. L. Intracellular acidification induces apoptosis by stimulating ICE-like protease activity. J. Cell Science. 110 (1997): 653-661.

Furlong, I. J., Lopez-Mediavilla, C., Ascaso, R., Lopez-Rivas, A. and Collins, M. K. Induction of apoptosis by valinomycin: mitochondrial permeability transition causes intracellular acidification. Cell Death Differ. 5 (1998): 214-221.

Gallo, V., Kingsbury, A., Balazs, R. and Jorgensen, O. S. The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. J. Neurosci. 7 (1987): 2203–2213.

Ghribi, O., Callebert, J., Plotkine, M. and Boulu, R. G. Competitive NMDA receptor blockers reduce striatal glutamate accumulation in ischaemia. Neuroreport. 5 (1994): 1253-1255.

Giudice, L. C. Insulin-like growth factors and ovarian follicular development. Endocr. Rev. 13 (1992): 641-669.

Gluckman, P. D., Guan, J., Williams, C., Scheepens, A., Zhang, R., Bennet, L. and Gunn, A. Asphyxial brain injury-the role of the IGF system. Molecular and Cellular Endocrinology. 140 (1998): 95-99.

Gluckman, P. D., Klempt, N. D., Guan, J., Mallard, E. C., Sirimanne, E., Dragunow, M., Klempt, M., Singh, K, Williams, C. E. and Nikolics, K. A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. Biochem. Biophys. Res. Commun. 182 (1992): 593-599.

Goldberg, M. P. and Choi, D. W. Combined oxygen and glucose deprivation in cortical cell culture:calcium-dependent and calcium-independent mechanisms of neuronal injury. J. Neurosci. 13 (1993): 3510-3524.

Goldberg, W. J., Kadino, R. M. and Barrett, J. N. Effects of ischemia-like conditions on cultured neurons: protection by low Na^{+}, low Ca^{2+} solutions. J. Neurosci. 6 (1986): 3144-3151.

Gonzalez-Garcia, M., Garcia, I., Ding, L., O'Shea, S., Boise, L. H., Thompson, C. B. and Nunez, G. Bcl-x is expressed in embryonic and postnatal neural tissues and

functions to prevent neuronal cell death. Proc. Natl. Acad. Sci. USA. 92 (1995): 4304-4308.

Goping, I. S., Gross, A., Lavoie, J. N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S.J. and Shore, G.C. Regulated targeting of BAX to mitochondria. J. Cell Biol. 143 (1998): 207-215.

Grant, M., Guay, C. and Marsh, R. Insulin-like growth factor I stimulates, migration and plasminogen activator release by human retinal pigment epithelial cells. Curr. Eye Res. 9 (1990): 323-335.

Grant, M., Jerdan, J. and Merimee, T. J. Insulin-like growth factor I modulates endothelial cell chemotaxis. J. Clin. Endocrinol. Metab. 65 (1987): 370-371.

Grigg, J. J. and Anderson, E. G. Glucose and sulfonylureas modify different phases of the membrane potential change during hypoxia in rat hippocampal slices. Brain Res. 489 (1989): 302-310.

Gross, A., Yin, X. M., Wang, K., Wei, M. C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P. and Korsmeyer, S.J. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J. Biol. Chem. 274 (1999): 1156-1163.

Guan, J., Bennet, L., George, S., Waldvogel, H. J., Faull, R. L. M., Gluckman, P. D. Keunen, H., Gunn, A. J. Selective neuroprotective effects with insulin-like growth factor-1 (IGF-I) in phenotypic striatal neurons following ischemic brain injury in fetal sheep. Neuroscience 95 (2000): 831-839.

Guan, J., Williams, C. E., Skinner, S. J., Mallard, E. C. and Gluckman, P. D. The effects of insulin-like growth factor (IGF)-1, IGF-2, and des-IGF-1 on neuronal loss after hypoxic-ischemic brain injury in adult rats: evidence for a role for IGF binding proteins. Endocrinology 137 (1996): 893-898.

Haddad, G. G. and Donnelly, D. F. O_2 deprivation induces a major depolarization in brain stem neurons in the adult but not in the neonatal rat. J. Physiol. 429 (1990): 411-428.

Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M. Differential requirement for caspase-9 in apoptotic pathways *in vivo*. Cell. 94 (1998): 339-352.

Hansen, M. B., Nielsen, S. E. and Berg, K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods. 119 (1989): 203-210.

Hartley, A., Stone, J. M., Heron, C., Cooper, J. M. and Schapira, A. H. V. Complex I inhibitors induce dose-dependent apoptosis in PC 12 cells: Relevance to Parkinson's disease. J. Neurochem. 63 (1994): 1987-1990.

Heiden, M. G., Chandel, N. S., Schumacker, P. T. and Thompson, C. B. Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Mol. Cell. 3 (1999): 159-167.

Hill, D. J., Logan, A., McGarry, M. and de Sousa, D. Control of protein and matrix-molecule synthesis in isolated ovine fetal growth-plate chondrocytes by the interactions of basic fibroblast growth factor, Insulin-like growth factor I and –II, insulin and transforming growth factor-beta 1. J. Endocrinol. 133 (1992): 363-373.

Hirai, K., Miyamasu, M., Yamaguchi, M., Nakajima, K., Ohtoshi, T., Koshino, T., Takaishi, T., Morita, Y. and Ito, K. Modulation of human basophil histamine release by insulin-like growth factors. J. Immunol. 150 (1993): 1503-1508.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., Korsmeyer, S. J. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature. 348 (1990): 334-336.

Hsu, M., Sik, A., Gallyas, F., Horvath, Z., Buzsaki, G. Short-term and long-term changes in the postischemic hippocampus. Ann. N.Y. Acad. Sci. 743 (1994): 121-134.

Hsu, S. Y., Kaipia, A., McGee, E., Lomeli, M. and Hsueh, A. J. Bok is a pro- apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. Proc. Natl. Acad. Sci. USA. 94 (1997a): 12401-12406.

Hsu, Y. T., Wolter, K. G. and Youle, R. J. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc. Natl. Acad. Sci. USA. 94 (1997b): 3668-3672.

Ishaque, A. and Al-Rubeai, M. Use of intracellular pH and annexin-V flow cytometric assays to monitor apoptosis and its suppression by bcl-2 over-expression in hybridoma cell culture. J. Immunol. Methods. 221 (1998): 43-57.

Janetzky, B., Hauck, S., Youdim, M. B., Riederer, P., Jellinger, K., Pantucek, F., Zochling, R., Boissl, K .W. and Reichmann, H. Unaltered aconitase activity, but decreased complex I activity in substantianigra pars compacta with Parkinson's disease. Neurosci. Lett. 169 (1994): 126-128.

Jenner, P. Parkinson's disease, pesticides and mitochondrial dysfunctions. Trends Neurosci. 24(5) (2001): 245-247.

Johansson, A. G., Lindh, E. and Ljunghall, S. IGFs: Function and clinical importance 4 growth hormone, insulin-like growth factor I, and bone: A clinical review. J. Intern. Med. 234 (1993): 553–560.

Johnston, B. M., Mallard, E. C., Williams, C. E. and Gluckman, P. D. Insulin like growth factor-1 is a potent neuronal rescue agent after hypoxic ischemic injury in fetal lambs. J. Clin. Invest. 97 (1996): 300-308.

Jones, J. I. and Clemmons, D. R. Insulin-like growth factors and their binding proteins: biological actions. Endocr. Rev. 16 (1995): 3-34.

Jung, Y. K., Miura, M. and Yuan, J. Suppression of interleukin-1 betaconverting enzyme-mediated cell death by insulin-like growth factor. J. Biol. Chem. 271 (1996): 5112-5117.

Kaasik, A., Kalda, A., Jaako, K. and Zharkovsky, A. Dehydroepiandrosterone sulphate prevents oxygen-glucose deprivation-induced injury in cerebellar granule cell culture. Neuroscience 102(2) (2001): 427-432.

Kalda, A., Elo Eriste, E., Vassiljev, V. and Zharkovsky, A. Medium transitory oxygen-glucose deprivation induced both apoptosis and necrosis in cerebellar granule cells. Neurosci. Lett. 240 (1998): 21-24.

Kalda, A., Kaasik, A. Vassiljev, V., Pokk, P. and Zharkovsky, A. Neuroprotective action of group I metabotropic glutamate receptor agonists against oxygen-glucose deprivation-induced neuronal death. Brain Res. 853 (2000): 370-373.

Kalda, A. and Zharkovsky, A. Metabotropic glutamate receptor agonists protect from oxygen-glucose deprivation and colchicine-induced apoptosis in primary cultures of cerebellar granule cells. Neuroscience 92(1) (1999): 7-14.

Kar, S., Seto, D., Dore, S., Hanisch, U. and Quirion, R. Insulin-like growth factor-I and –II differentially regulate endogenous acetylcholine release from the rat hippocampal formation. Proc. Natl. Acad. Sci. USA. 94 (1997): 14054-14059.

Kass, I. S. and Lipton, P. Calcium and long-term transmission damage following anoxia in dentate gyrus and CA1 regions of the rat hippocampal slices. J. Physiol. 378 (1986): 313-334.

Kennedy, S. G., Kandel, E. S., Cross, T. K. and Hay, N. Akt/ protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. Mol. Cell Biol. 19 (1999): 5800-5810.

Kermer, P., Klocker, N., Labes, M. and Bahr, M. Insulin-like growth factor-I protects axotomized rat retinal ganglion cells from secondary death via PI3-K-dependent Akt phosphorylation and inhibition of caspase-3 *in vivo*. J. Neurosci. 20 (2000): 722-728.

Kooijman, R., Willems, M., De Haas, C. J., Rijkers, G. T., Schuurmans, A. L., Van Buul Offers, S. C., Heijnen, C. J. and Zegers, B. J. Expression of type I insulin-like growth factor receptors on human peripheral blood mononuclear cells. Endocrinology 131 (1992a): 2244-2250.

Kooijman, R., Willems, M., Rijkers, G. T., Brinkman, A., Van Buul offers, S. C. Heijnen, C. J. and Zegers, B. J. Effects of insulin-like growth factors and growth hormone on the in vitro proliferation of T lymphocytes. J. Neuroimmunol. 38 (1992b): 95-104.

Kruman, I., Guo, Q. and Mattson, M. P. Calcium and reactive oxygen species mediate staurosporine-induced mitochondrial dysfunction and apoptosis in PC12 cells. J. Neurosci. Res. 51 (1998): 293-308.

Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P. and Flavell, R.A. Cell. 94 (1998): 325-337.

Kuida, K., Lippke, J. A., Ku, G., Harding, M. W. and Livingston, D. J. Altered cytokine export and apoptosis in mice deficient in interleukin-1β converting enzyme. Science. 267 (1995): 2000-2002.

Kuida, K., Zheng, T. S., Na, S., Kuan, C. Y. and Yang, D. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature. 384 (1996): 368-372.

Kulik, G., Klippel, A. and Weber, M. J. Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol. Cell. Biol. 17(3) (1997): 1595-1606.

Kuo, T.H., Kim, H.R., Zhu, L., Yu, Y., Lin, H.M. and Tsang, W. Modulation of endoplasmic reticulum calcium pump by Bcl-2. Oncogene. 17 (1998): 1903-1910.

Kusumoto, M., Duc, E., Pachen, W. and Hossmann, K. A. Susceptibility of hippocampal and cortical neurons to argon mediated in vitro ischemia. J. Neurochem. 67 (1996): 1613-1621.

Lam, M., Bhat, M. B., Nunez, G., Ma, J. and Distelhorst, C. W. Regulation of Bcl-xl channel activity by calcium. J. Biol. Chem. 273 (1998): 17307-17310.

Lamothe, B., Baudry, A., Desbois, P., Lamotte, L., Bucchini, D., De Meyts, P. and Joshi, R.L. Genetic engineering in mice: impack aon insulin signalling and action. Biochem. J. 335 (1998): 193-204.

Laura, L. D. and Dennis, W. C. Hypoxic-Ischemic brain injury and oxidative stress. Basic Neurochem. (1999): 711-729.

Lawrie, A. M., Rizzuto, R., Pozzan, T. and Simpson, A. W. M. A role for calcium influx in the regulation of mitochondrial calcium in endothelial cells. J. Biol. Chem. 271 (1996): 10753-10759.

Leblond, J. and Krnjevic, K. Hypoxic changes in hippocampal neurons. J. Neurophysiol. 62 (1989): 1-14.

Lee, W. H., Javedam, S. and Bondy, C. A. Coordinate expression of insulin-like growth factor system components by neurons and neuroglia during retinal and cerebellar development. J. Neurosci. 12 (1992): 4737-4744.

LeRoith, D., Werner, H., Beitner-Johnson, D. and Roberts, C.T. Jr. Molecular and cellular aspects of the Insulin-like growth factor I receptor. Endocr. Rev. 16 (1995): 143–163.

Leski, M. L., Valentine, S. L., Baer, J. D. and Coyle, J. T. Insulin-like growth factor I prevents the development of sensitivity to kainate neurotoxicity in cerebellar granule cells. J. Neurochem. 75 (2000): 1548–1556.

Li, P., Allen, H., Banerjee, S., Franklin, S. and Herzog, L. Mice deficient in IL-1β-converting enzyme are defective in production of mature IL-1β and resistant to endotoxic shock. Cell. 80 (1995): 401-411.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula S. M., Ahmad, M., Alnemri, E. S. and Wang, X. Cell. 91 (1997): 479-489.

Li, H., Zhu, H., Xu, C. J. and Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. 94 (1998): 491-501.

Lienhard, G. E. Life without the IRS. Nature. 372 (1994): 128-129.

Lindsay, R. M. Neurotrophic factors: from molecule to man. Trends Neurosci. 17 (1994): 182-194.

Lipton, S. A. and Rosenberg, P. A. Excitatory amino acids as a final common pathway for neurologic disorders. N. Engl. J. Med. 330 (1994): 613-622.

Lowe, W. L. Biologic actions of the insulin-like growth factor. In: LeRoith D. (eds) Insulin-like growth factors: Molecular and Cellular Aspects. CRC Press Boca Raton, (1991): 49-85.

Luhmann, H. J. Ischemia and lesion induced imbalances in cortical function. Prog. Neurobiol. 48 (1996): 131-166.

Luhmann, H. J. and Kral, T. Hypoxia-induced dysfunction in developing rat neocortex. J. Neurophysiol. 78 (1997): 1212-1221.

Marin, M. C., Fernandez, A., Bick, R. J., Brisbay, S., Buja, L. M., Snuggs, M., McConkey, D. J., von Eschenbach, A. C., Keating, M. J. and McDonnell, T. J. Apoptosis

suppression by bcl-2 is correlated with the regulation of nuclear and cytosolic Ca^{2+}. Oncogene. 12 (1996): 2259-2266.

Marte, B. M., Downward, J. and Deprez, J. PKB:Akt : connecting phosphoinositide 3-kinase to cell survival and beyond. Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other kinases of the insulin signalling cascade. Trends Biochem. Sci. 22(9) (1997): 355-358.

Matthews, C. C. and Feldman, E. L. Insulin-like growth factor I rescues SH-SY5Y human neuroblastoma cells from hyperosmotic induced programmed cell death. J. Cell Physiol. 166 (1996): 323–331.

Merchav, S., Graif, Z. and Skottner, A. In-vitro response of erythroid progenitors from children with thalassaemia major to human growth hormone and insulin-like growth factor I. Clin. Endocrinol. (Oxf). 39 (1993): 207-211.

Merchav, S., Silvian Drachsler, I., Tatarsky, I., Lake, M. and Skottner, A. Comparative studies of the erythroid-potentiating effects of biosynthetic human insulin-like growth factors-I and –II. J. Clin. Endocrinol. Metab. 74 (1992): 447-452.

Milusheva, E. A., Doda, M., Baranyi, A. and Vizi, E. S. Effect of hypoxia and glucose deprivation on ATP level, adenylate energy charge and $[Ca^{2+}]_O$-dependent and independent release of [^{3}H]-dopamine in rat striatal slices. Neurochem. Int. 28 (1996): 501-507.

Minniti, C. P., Kohn, E. C., Grubb, J. H., Sly, W. S., Oh, Y., Muller, H. L., Rosenfeld, R. G. and Helman, L. J. The insulin-like growth factor II (IGF-II)/mannose 6-phosphate receptor mediates IGF-II-induced motility in human rhabdomyosarcoma cells. J. Biol. Chem. 267 (1992): 9000-9004.

Minshall, C., Arkins, S. and Straza, J. IL-4 and insulin-like growth factor-I inhibit the decline in Bcl-2 and promote the survival of IL-3-deprived myeloid progenitors. J. Immunol. 159(3) (1997): 1225-1232.

Mitchell, J. J., Paiva, M., Walker, D. W. and Heaton, M. B. BDNF and NGF afford in vitro neuroprotection against ethanol combined with acute ischemia and chronic hypoglycemia. Dev. Neurosci. 21 (1999): 68-75.

Monica, L. L., Charlene, M. H., Jen, H. L. and Marianne, S. J. Inosine and guanosine preserve neuronal and glial cell viability in mouse spinal cord cultures during chemical hypoxia. Brain Res. 821 (1999): 426-432.

Morana, S. J., Wolf, C. M., Li, J., Reynolds, J. E., Brown, M. K. and Eastman, A. The involvement of protein phosphatases in the activation of ICE/CED-3 protease, intracellular acidification, DNA digestion, and apoptosis. J. Biol. Chem. 271 (1996): 18263-18271.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65 (1983): 55-63.

Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S. L., Ng, S. L. and Fesik, S. W. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature. 381 (1996): 335-341.

Murphy, A. N., Bredesen, D. E., Cortopassi, G., Wang, E. and Fiskum, G. Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. Proc. Natl. Acad. Sci. USA. 93 (1996): 9893-9898.

Muta, K. and Krantz, S. B. Apoptosis of human erythroid colony forming cells is decreased by stem cell factor I as well as erythropoietin. J. Cell Physiol. 156 (1993): 264-271.

Muzio, M., Salvesen, G. S. and Dixit, V. M. FLICE-induced apoptosis in a cell-free system, Cleavage of caspase zymogens. J. Biol. Chem. 272 (1997): 2952-2956.

Namura, S., Zhu, J., Fink, K., Endres, M., Srinivasan, A., Tomaselli, K. J., Yuan, J. and Moskowitz, M. A. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J. Neurosci. 18 (1998): 3659-3668.

Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H. and Tsujimoto, Y. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc. Natl. Acad. Sci. USA. 95 (1998): 14681-14686.

Neff, N. T., Prevette, D., Houenou, L. J., Lewis, M. E., Glicksman, M. A., Yin, Q. W. and Oppenheim, R. W. Insulin-like growth factors: putative musclederived trophic agents that promote motoneuron survival. J. Neurobiol. 24 (1993): 1578-1588.

Nieber, K., Sevcik, J. and Illes, P. Hypoxia changes in rat locus coeruleus neurons *in vitro*. J. Physiol. 486 (1995): 33-46.

Nieber, K. Hypoxia and neuronal function under *in vitro* conditions. Pharmacol. Ther. 82(1) (1999): 71-86.

Nitta, A., Ohmiya, M., Sometani, A., Itoh, M., Nomoto, H., Furukawa, Y. and Furukawa, S. Brain-derived neurotrophic factor prevents neuronal cell death induced by corticosterone. J. Neurosci. Res. 57 (1999): 227-235.

Obrenovitch, T. P., Urenjak, T. and Zilkha, E. Excitotoxicity in cerebral ischemia: alternative hypothesis to high extracellular glutamate. In: Pharmacology of Cerebral Ischemia. (1996): 9-23.

Ohkawa H., Ohishi N. and Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction . Anal. Biochem. 95 (1979): 351-358.

O'Reilly, J. P., Jiang, C. and Haddad, G. G. Major differences in response to graded hypoxia between hypoglossal and neocortical neurons. Brain Res. 683 (1995): 179-186.

Otter, I., Conus, S., Ravn, U., Rager, M., Olivier, R., Monney, L., Fabbro, D. and Borner, C. The binding properties and biological activities of Bcl-2 and Bax in cells exposed to apoptotic stimuli. J. Biol. Chem. 273 (1998): 6110-6120.

Parrizas, M. and LeRoith, D. Insulin-like growth factor 1 inhibition apoptosis is associated with increased expression of the Bcl-xL gene product. Endocrinology 138(3) (1997b): 1355-1358.

Parrizas, M., Saltiel, A. R. and LeRoith, D. Insulin-like growth factor 1 inhibits apoptosis using the phosphatidylinositol 3-kinase and mitogen-activated kinase pathways. J. Biol. Chem. 272(1) (1997a): 154-161.

Paschen, W. and Djuricic, B. Comparison of *in vitro* ischemia – induced disturbances in energy metabolism and protein synthesis in the hippocampus of rats and gerbils. J. Neurochem. 65 (1995): 1692-1697.

Payne, R. S., Schurr, A. and Rigor, B. M. Cell swelling exacerbates hypoxic neuronal damage in rat hippocampal slices. Brain Res. 723 (1996): 210-213.

Penhoat, A., Naville, D., Jaillard, C., Chatelain, P. G. and Saez, J. M. Hormonal regulation of insulin-like growth factor I secretion by bovine adrenal cells. J. Biol. Chem. 264 (1989a): 6858-6862.

Penhoat, A., Jaillard, C. and Saez, J. M. Synergistic effects of corticotropin and insulin-like growth factor I on poeticotropin receptors and corticotropin responsiveness in cultured bovine adrenocortical cells. Biochem. Biophs. Res. Commun. 165 (1989b): 355-359.

Perez-Velazquez, J. L., Frantseva, M. V. and Carlen P. L. In vitro ischemia promotes glutamate-mediated free radical generation and intracellular calcium accumulation in hippocampal pyramidal neurons. J. Neurosci. 17 (1997): 9085-9094.

Priault, M., Camougrand, N., Chaudhuri, B. and Manon, S. Role of the C- terminal domain of Bax and Bcl-XL in their localization and function in yeast cells. FEBS Lett. 443 (1999): 225-228.

Rajdev, S. and Reynolds, I. J. Glutamate-induced intracellular calcium changes and neurotoxicity in cortical neurons *in vitro*: effect of chemical ischemia. Neuroscience. 62 (1994): 667-679.

Raley-Susman, K. M. and Lipton, P. In vitro ischemia and protein synthesis in the rat hippocampal slices: the role of calcium and NMDA receptor activation. Brain Res. 515 (1990): 27-38.

Raley-Susman, K. M. and Murata, J. Time course of protein changes following in vitro ischemia in the rat hippocampal slice. Brain Res. 695 (1995): 94-102.

Reinhardt, R. R. and Bondy, C. A. Insulin-like growth factors cross the blood–brain barrier. Endocrinology 135 (1994): 1753-1761.

Rogers, D. C. and Hunter, A. J. Dissociation of effects of glutamate receptor antagonists on excitotoxic and hypoxic neuronal cell death in a novel rat cortical culture system. Brain Res. Bull. 44 (1997): 131-139.

Rong, P., Bennie, A.M., Epa, W.R. and Barrett, G.L., Nerve growth factor determines survival and death of PC12 cells by regulation of the bcl-x, bax, and caspase-3 genes. J. Neurochem. 72 (1999): 2294-2300.

Rosen, A. S. and Morris, M. E. Depolarizing effects of anoxia on pyramidal cells of the rat neocortex. Neurosci. Lett. 124 (1991): 169-173.

Rosenbaum, D. M., Kalberg, J. and Kessler, J. A. Superoxide dismutase ameliorates neuronal death from hypoxia in culture. Stroke. 25 (1994): 857-863.

Rothman, S. Synaptic release of excitatory amino acid neurotransmitter mediate anoxic neuronal death. J. Neurosci. 4 (1984): 1884-1891.

Rotwein, P., Burgess, S. K., Milbrandt, J. D. and Krause, J. E. Differential expression of insulin-like growth factor genes in rat central nervous system. Proc. Natl. Acad. Sci. USA. 85 (1988): 265-269.

Saikumar, P., Dong, Z., Patel, Y., Hall, K., Hopfer, U., Weinberg, J. M. and Venkatachalam, M. A. Role of hypoxia-induced Bax translocation and cytochrome c release in reoxygenation injury. Oncogene. 17 (1998): 3401-3415.

Salmon, W. D. and Daughaday, W. H. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. J. Lab. Clin. Med. 49 (1957): 825-836.

Sato, T., Hanada, M., Bodrug, S., Irie, S., Iwama, N., Boise, L. H., Thompson, C. B., Golemis, E., Fong, L. and Wang, H. G. Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. Proc. Natl. Acad. Sci USA. 91 (1994): 9238-9242.

Satoh, T., Sakai, N., Enokido, Y., Uchiyama, Y. and Hatanaka, H. Free radical-independent protection by nerve growth factor and Bcl-2 of PC12 cells from hydrogen peroxide-triggered apoptosis. J. Biochem. (Tokyo) 120 (1996): 540-546.

Scheid, M. P. and Duronio, V. Dissociation of cytokine-induced phosphorylation of Bad and activation of PKB/akt: involvement of MEK upstream of Bad phosphorylation. Proc. Natl. Acad. Sci. USA. 95 (1998): 7439-7444.

Schendel, S. L., Xie, Z., Montal, M. O., Matsuyama, S., Montal, M., Reed, J. C. Channel formation by antiapoptotic protein Bcl-2. Proc. Natl. Acad. Sci. USA. 94 (1997): 5113-5118.

Schlesinger, P. H., Gross, A., Yin, X. M., Yamamoto, K., Saito, M., Waksman, G. and Korsmeyer, S. J. Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. Proc. Natl. Acad. Sci. USA. 94 (1997): 11357-11362.

Sedlak, T. W., Oltvai, Z. N., Yang, E., Wang, K., Boise, L. H., Thompson, C. B. and Korsmeyer, S. J. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. Proc. Natl. Acad. Sci. USA. 92 (1995): 7834-7838.

Seaton, T. A. Cooper, J. M. and Schapira, A. H. V. Cyclosporin ingibition of apoptosis induced by mitochondrial complex I toxins. Brain Res. 809 (1998): 12-17.

Shearman, M. S., Hawtin, S. R. and Tailor, V. J. The intracellular component of cellular 3-(4,5- Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium bromide (MTT) reduction is specifically inhibited by β-amyloid peptides. J. Neurochem. 65 (1995): 218-227.

Shi, B., Triebe, D., Kajiji, S., Iwata, K. K., Bruskin, A. and Mahajna, J. Identification and characterization of baxepsilon, a novel bax variant missing the BH2 and the transmembrane domains. Biochem. Biophys. Res. Commun. 254 (1999): 779-785.

Shimizu, S., Eguchi, Y., Kamiike, W., Funahashi, Y., Mignon, A., Lacronique, V., Matsuda, H. and Tsujimoto, Y. Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. Proc. Natl. Acad. Sci. USA. 95 (1998): 1455-1459.

Shoji, S., Ertl, R. F., Linder, J., Koizumi, S., Duckworth, W. C. and Rennard, S. I. Bronchial epithelial cells respond to insulin and insulin-like growth factor I as a chemoattractant. Am. J. Respir. Cell Mol. Biol. 2 (1990): 553-557.

Siesjo, B. K. Historical overview. Calcium, ischemia, and death of brain cells. Ann. N.Y. Acad. Sci. 522 (1988): 638-661.

Simonen, M., Keller, H. and Heim, J. The BH3 domain of Bax is sufficient for interaction of Bax with itself and with other family members and it is required for induction of apoptosis. Eur. J. Biochem. 249 (1997): 85-91.

Singleton, J. R., Dixit, V. M. and Feldman, E. L. Type I insulin-like growth factor receptor activation regulates apoptotic proteins. J. Biol. Chem. 271 (1996): 31791-31794.

Stennicke, H. R. and Salvesen, G. S. Caspases-controlling intracellular signals by protease zymogen activation. Biochimica et Biophysica Acta. 1477 (2000): 299-306.

So, Y. S., Eun, Y. K., Harriet, K. and Byoung, J. G. Neuroprotective effect of high glucose against NMDA, free radical and oxygen-glucose deprivation through enhanced mitochondrial potentials. J. Neurosci. 19(20) (1999): 8849-8855.

Soder, O., Bang, P., Wahab, A. and Parvinen, M. Insulin-like growth factors selectively stimulate spermatogenesis. Endocrinology 131 (1992): 2344-2350.

Sondell, M., Lundborg, G. and Kanje, M. Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J. Neurosci. 19 (1999): 5731-5740.

Stennicke, H. R. and Salvesen, G. S. Caspases-controlling intracellular signals by protease zymogen activation. Biochimica et Biophsica Acta. 1477 (2000): 299-306.

Stracke, M. I., Engel, J. D., Wilson, L. W., Rechler, M. M., Liotta, L. A. and Schiffmann, E. The type I insulin-like growth factor receptor is a motility receptor in human melanoma cells. J. Biol. Chem. 264 (1989): 21544-21549.

Szatkowski, M. and Attwell, D. Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. Trends Neurosci. 17 (1994): 359-365.

Tagami, M., Ikeda, K., Nara, Y., Fujino, H., Kubota, A., Numano, F. and Yamori, Y. Insulin-like growth factor-1 attenuates apoptosis in hippocampal neurons caused by cerebral ischemia and reperfusion in strokeprone spontaneously hypertensive rats. Lab. Invest. 76 (1997): 613-617.

Takahashi, A., Hirata, H., Yonehara, S., Imai, Y., Lee, K. K., Moyer, R. W., Turner, P. C., Mesner, P. W., Okazaki, T., Savai, H., Kishi, S., Yamamoto, K., Okuma, M. and Sasada, M. Affnity labeling displays the stepwise activation of ICE-related proteases by fas, staurosporine, and CrmA-sensitive caspase-8. Oncogene. 14 (1997): 2741-2752.

Tamm, I. and Kikuchi, T. Insulin-like growth factor-1 (IGF-1), insulin, and epidermal growth factor (EGF) are survival factors for density-inhibited, quiescent Balb:c-3T3 murine fibroblasts. J. Cell Physiol. 143 (1990): 494-500.

Tan, Y. J., Beerheide, W. and Ting, A. E. Biophysical characterization of the oligomeric state of Bax and its complex formation with Bcl-XL. Biochem. Biophys. Res. Commun. 255 (1999): 334-339.

Tapson, V. F., Boni Schnetzler, M., Pilch, P. F., Center, D. M. and Berman, J. S. Structural and functional characterization of the human T lymphocyte receptor for insulin-like growth factor I in vitro. J. Clin. Invest. 82 (1988): 950-957.

Tartare-Deckert, S., Murdaca, J., Sawka-Verhelle, D., Holt, K. H., Pessin, J. E. and Van Obberghen, E. Interaction of the molecular weight 85K regulatory subunit of the phosphatidylinositol 3-kinase with the insulin receptor and the insulin-like growth factor-1 (IGF-1) receptor: comparative study using the yeast two-hybrid system. Endocrinology 137(3) (1996): 1019-1024.

Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R. and Aunines, J. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature. 356 (1992): 768-774.

Thornberry, N. A. and Lazebnik, Y. Caspases: enemies within. Science. 281 (1998): 1312-1316.

Timsit, J., Savino, W., Safieh, B., Chanson, P., Gagnerault, M. C., Bach, J. F., Dardenne, M. Growth hormone and insulin-like growth factor-I stimulate hormonal function and proliferation of thymic epithelial cells. J. Clin. Endocrinol. Metab. 75 (1992): 183-188.

Todd, B. S., Patrica, A. T., Kate, B., Janice, K. P., James, P. B. and Jeremy, B. T. Chronic reduction in complex I function alters calcium signaling in SH-SY5Y neuroblastoma cells. Brain Res. 891 (2001): 94-105.

Tombaugh, G. C. and Sapolsky, R. M. Evolving concepts about the role of acidosis in ischemic neuropathology. J. Neurochem. 61 (1993): 793-803.

Tombaugh, G. C. and Sapolsky, R. M. Mechanistic distinctions between excitotoxic and acidotic hippocampal damage in an *in vitro* model of ischemia. J. Cereb. Blood Flow Metab. 10 (1990): 527-535.

Torres-Aleman I., Pons S. and Ar'evalo M. A. The insulin-like growth factor I system in the rat cerebellum: developmental regulation and role in neuronal survival and differentiation. J. Neurosci. Res. 39 (1994): 117-126.

Tsujimoto, Y. and Croce, C. M. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc. Natl. Acad. Sci. USA. 83 (1986): 5214-5218.

Unchern S. Neurotoxicity of piperine on cultured brain neurons. Doctoral dissertation, Department of Chemical Pharmacology, Pharmaceutical Sciences, The University of Tokyo, (1997).

Uto, A., Dux, E., Kusumoto, M. and Hossmann, K. A. Delayed neuronal death after brief histotoxic hypoxia *in vitro*. J. Neurochem. 64 (1995): 2185-2192.

Vannucci, R. C. Experimental biology of cerebral hypoxia-ischemia: Relation to perinatal brain damage. Pediatr. Res. 27 (1990): 317-326.

Varfolomeev, E. E., Schuchmann, M., Luria V., Chiannilkulchai N. and Beckmann J. S. Targeted disruption of the mouse caspase-8 gene ablates cell death induction by the TNF receptors, Fas/Apo-1, and DR3 and is lethal prenatally. Immunity. 2 (1998): 267-276.

Volpe, J. J. Neurology of the newborn, 3rd ed. Philadelpia: W. B. Saunders, (1995).

Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F. and Reed, J. C. Ca^{2+}-induced apoptosis through calcineurin dephosphorylation of BAD. Science. 284 (1999a): 339-343.

Wang, L., Zu, D., Dai, W. and Lu, L. An ultraviolet-activated K^+ channel mediates apoptosis of myeloblastic leukemia cells. J. Biol. Chem. 274 (1999b): 3678-3685.

Wang, J. M., Hayashi, T., Zhang, W. R., Sakai, K., Shiro, Y. and Abe, K. Reduction of ischemic brain injury by topical application of insulin-like growth factor-I after transient middle cerebral artery occlusion in rats. Brain Res. 859 (2000): 381-385.

Wauquier, A. Hypoxia of the brain: an introduction. Clinical neuropharmacology. 7 (1984): 486-487.

Wei, H., Wei, W., Bredesen, D. E. and Perry, D. C. Bcl-2 protects against apoptosis in neuronal cell line caused by thapsigargin-induced depletion of intracellular calcium stores. J. Neurochem. 70 (1998): 2305-2314.

Werth, J. L. and Thayer, S. A. Mitochondria buffer physiological calcium loads in cultured rat dorsal root ganglion neurons. J. Neurosci. 14 (1994): 348-358.

White, B. C., Sullivan, J. M., DeGracia D. J., O'Neil, B. J., Neumar, R. W., Grossman, L. I., Rafols, J. A. and Krause, G. S. Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. J. Neurosci. 179 (2000): 1-33.

Williams, G. T., Smith, C. A., Spooncer, E., Dexter, T. M. and Taylor, D. R. Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature. 343 (1990): 76-79.

Yamasaki, H., Prager, D., Gebremedhin, S., Moise, L. and Melmed, S. Binding and action of insulin-like growth factor I in pituitary tumor cell. Endocrinology 12(8) (1991a): 857-862.

Yamasaki, H., Prager, D., Gebremedhin, S., Moise, L. and Melmed, S. Insulin-like growth factor I (IGF-I) attenuation of growth hormone is enhanced by overexpression of pituitary IGF-I receptors. Mol. Endocrinol. 5 (1991b): 890-896.

Yang, J. J., Chou, Y. C., Lin, M. T. and Chiu, T. H. Hypoxia-induced differential electrophysiological changes in rat locus coeruleus neurons. Life Sci. 61 (1997): 1763-1773.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibraco, A. M., Peng, T. I., Jones, D. P. and Wang, X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science. 275 (1997): 1081-1082.

Ye, P. and D'Ercole, A. J. Insulin-like growth factor I protects oligodendrocytes from tumor necrosis factor-alpha-induced injury. Endocrinology 140 (1999): 3063-3072.

Ye, P., Xing, Y., Dai, Z. and D'Ercole, A. J. *In vivo* actions of insulin-like growth factor-I (IGF-I) on cerebellum development in transgenic mice: evidence that IGF-I increases proliferation of granule cell progenitors. Dev. Brain. Res. 95 (1996): 44-54.

Yuen, E. C. and Mobley, W. C. Therapeutic potential of neurotrophic factors for neurological disorders. Ann. Neurol. 40 (1996): 346–354.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., Horvitz, H. R., The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta converting enzyme. Cell. 75 (1993): 641-652.

Zamzami, N., Brenner, C., Marzo, I., Susin, S. A. and Kroemer, G. Subcellular and submitochondrial mode of action of Bcl-2 like oncoproteins. Oncogene. 16 (1998): 2265-2282.

Zha, J., Harada, H., Osipov, K., Jockel, J., Waksman, G. and Korsmeyer, S. J. BH3 domain of BAD is required for heterodimerization with BCL-XL and pro-apoptotic activity. J. Biol. Chem. 272 (1997): 24101-24104.

Zha, J. P., Harada, H., Yang, E., Jockel, J. and Korsmeyer, S. J. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell. 87 (1996): 619-628.

Zhou, M., Demo, S. D., McClure, T. N., Crea, R. and Bitler, C. M. A novel splice variant of the cell death-promoting protein BAX. J. Biol. Chem. 273 (1998): 11930-11936.

Zhu, P. J. and Krnjevic, K. Anoxia selectively depresses excitatory synaptic transmission in hippocampal slices. Neurosci. Lett. 166 (1994): 27-30.

Zorati, M., Szabo, I. Electrophysiology of the inner mitochondrial membrane. J. Bioenerget. Biomembr. 26 (1994): 543-553.

Zou, H., Henzel, W. J., Liu, X., Lutschg, A. and Wang, X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell. 90 (1997): 405-413.

Zundel, W. and Giaccia, A. Inhibition of the anti-apoptotic PI(3)K/Akt/Bad pathway by stress. Genes Dev. 12 (1998): 1941-1946.

APPENDIX

Effects of Hypoxia induced cellular injuries in culture rat cerebellar granule neurons

Table 1. Effects of Physical Hypoxia induced cellular injuries in cerebellar granule neurons : MTT Reduction

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	0.4516	0.6123	0.2164
Hypoxia 4 hr	8	0.4345	0.1941	0.0687
Control	8	0.3478	0.1293	0.0457
Hypoxia 12 hr	8	0.3209	0.1428	0.0505
Control	8	0.3340	0.3644	0.1288
Hypoxia 18 hr **	8	0.1889	0.1754	0.0620
Control	8	0.3475	0.1871	0.0662
Hypoxia 24 hr *	8	0.1118	0.1639	0.0579

* $p < 0.05$ denote statistically significant difference from Control

** $p < 0.01$ denote statistically significant difference from Control

Table 2. Effects of Physical Hypoxia induced cellular injuries in cerebellar granule neurons : LDH Release

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	6	100	0.000	0.000
4 hr	6	102.6667	11.1475	4.5509
12 hr	6	106.6667	6.532	2.6667
18 hr	6	107.5	5.8224	2.377
24 hr	6	109.5	6.091	2.4866

Table 3. Effects of Chemical Hypoxia induced cellular injuries in cerebellar granule neurons : MTT Reduction

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	6	100	0.000	0.000
Rot 0.1 μM *	6	69.8333	7.9352	3.2395
Rot 0.5 μM *	6	54.1667	3.1885	1.3017
Rot 5 μM *,#	6	51.5000	3.0166	1.2315
Rot 25 μM *,#,$	6	44.3333	4.4121	1.8012

* $p < 0.05$ denotes statistically significant difference from control

# $p < 0.05$ denotes statistically significant difference from 0.1 μM rotenone

$ $p < 0.05$ denotes statistically significant difference from 0.5 μM rotenone

Table 4. Effects of Chemical Hypoxia induced cellular injuries in cerebellar granule neurons : LDH Release

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	6	100	0.000	0.000
Rot 0.1 μM	6	100.5	4.3704	1.7842
Rot 0.5 μM	6	101.8333	4.1673	1.7013
Rot 5 μM	6	106.5	11.606	4.7381
Rot 25 μM	6	103.667	6.7429	2.7528

Effects of IGF-I in culture rat cerebellar granule neurons

Table 5. Effects of IGF-I induced cellular injuries in cerebellar granule neurons 24 hr : MTT Reduction

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0.3 ng/ml*	8	123.75	16.5422	5.8485
IGF-I 1 ng/ml *	8	148.875	35.8028	12.6582
IGF-I 3 ng/ml *	8	137.875	24.2925	8.5887
IGF-I 6 ng/ml#	8	114.125	11.4572	4.0507

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 1 ng/ml IGF-I

Table 6. Effects of IGF-I induced cellular injuries in cerebellar granule neurons 24 hr : LDH release

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	6	100	0.000	0.000
IGF-I 0.3 ng/ml*	6	46.1667	23.4642	9.5792
IGF-I 1 ng/ml*	6	42.8333	26.7239	10.91
IGF-I 3 ng/ml*	6	44.6667	22.4113	9.1494
IGF-I 6 ng/ml*	6	69	20.04	8.1813

* $p < 0.05$ denotes statistically significant difference from Control

Table 7. Effects of IGF-I induced cellular injuries in cerebellar granule neurons 48 hr : MTT Reduction

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0.3 ng/ml *,#	8	127.625	11.9993	4.2424
IGF-I 1 ng/ml *	8	150.5	21.4009	7.5664
IGF-I 3 ng/ml *	8	141.625	11.0704	3.914
IGF-I 6 ng/ml *,#,$	8	122.625	9.0544	3.2012

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 1 ng/ml IGF-I

$ $p < 0.05$ denotes statistically significant difference from 3 ng/ml IGF-I

Table 8. Effects of IGF-I induced cellular injuries in cerebellar granule neurons 48 hr : LDH release

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	6	100	0.000	0.000
IGF-I 0.3 ng/ml *	6	67.3333	13.6333	5.5658
IGF-I 1 ng/ml *	6	53.5	15.0698	6.1522
IGF-I 3 ng/ml *	6	60.1667	10.6286	4.3391
IGF-I 6 ng/ml *	6	69.6667	12.3882	5.0574

* $p < 0.05$ denotes statistically significant difference from Control

Effects of IGF-I on Hypoxia-induced cellular injuries in cultured granule neurons

Table 9. Effects of IGF-I Pre Physical Hypoxia-induced cellular injuries in cultured granule neurons : MTT reduction

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0 ng/ml *	8	56.75	3.4538	1.2211
IGF-I 0.3 ng/ml #	8	98.375	16.7241	5.9129
IGF-I 1 ng/ml #	8	101.5	13.6277	4.8181
IGF-I 3 ng/ml #	8	98.875	14.6915	5.1942
IGF-I 6 ng/ml #	8	90.375	24.3307	8.6022

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

Table 10. Effects of IGF-I Pre Physical Hypoxia-induced cellular injuries in cultured granule neurons : LDH release

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0 ng/ml	8	102	26.4726	10.8074
IGF-I 0.3 ng/ml *,#	8	62.8333	17.3138	7.0683
IGF-I 1 ng/ml *,#	8	63.1667	24.8951	10.1634
IGF-I 3 ng/ml *,#	8	71.3333	14.7468	6.0203
IGF-I 6 ng/ml *,#	8	74.1667	19.6918	8.0391

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

Table 11. Effects of IGF-I Co Physical Hypoxia-induced cellular injuries in cultured granule neurons : MTT reduction

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0 ng/ml *	8	56.875	3.4821	1.2311
IGF-I 0.3 ng/ml #	8	95.25	11.5728	4.0916
IGF-I 1 ng/ml #	8	88.375	15.2965	5.4081
IGF-I 3 ng/ml #	8	86.375	14.9851	5.298
IGF-I 6 ng/ml #	8	82.625	16.3265	5.7723

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

Table 12. Effects of IGF-I Co Physical Hypoxia-induced cellular injuries in cultured granule neurons : LDH release

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0 ng/ml	8	102.5	2.6646	1.0878
IGF-I 0.3 ng/ml *,#	8	66.3333	15.9457	6.5098
IGF-I 1 ng/ml *,#	8	63	18.942	7.733
IGF-I 3 ng/ml *,#	8	82	23.1517	9.4516
IGF-I 6 ng/ml *,#	8	74.6667	24.8408	10.1412

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

Table 13. Effects of IGF-I Post Physical Hypoxia-induced cellular injuries in cultured granule neurons : MTT reduction

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0 ng/ml *	8	57	5.8064	2.0529
IGF-I 0.3 ng/ml *,#,&	8	68.125	6.1745	2.183
IGF-I 1 ng/ml *,#	8	74.875	7.4726	2.642
IGF-I 3 ng/ml *,#,$,@	8	81.875	8.9032	3.1478
IGF-I 6 ng/ml *,#,&	8	70.625	5.7802	2.0436

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

$ $p < 0.05$ denotes statistically significant difference from 0.3 ng/ml IGF-I

& $p < 0.05$ denotes statistically significant difference from 1 ng/ml IGF-I

@ $p < 0.05$ denotes statistically significant difference from 3ng/ml IGF-I

Table 14. Effects of IGF-I Post Physical Hypoxia-induced cellular injuries in cultured granule neurons : LDH release

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0 ng/ml	8	104.3333	7.4476	3.0405
IGF-I 0.3 ng/ml	8	94	27.0259	11.0333
IGF-I 1 ng/ml	8	94.5	42.0131	17.1518
IGF-I 3 ng/ml	8	97.6667	28.6193	11.6838
IGF-I 6 ng/ml	8	100	26.8775	10.9727

Table 15. Effects of IGF-I Pre Chemical Hypoxia-induced cellular injuries in cultured granule neurons : MTT reduction

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0 ng/ml *	8	51.5	2.7775	0.982
IGF-I 0.3 ng/ml #	8	95.5	12.2007	4.3136
IGF-I 1 ng/ml #	8	99.5	6.59	2.3299
IGF-I 3 ng/ml #	8	102.5	9.2118	3.2569
IGF-I 6 ng/ml #	8	106.25	8.8761	3.1382

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

Table 16. Effects of IGF-I Pre Chemical Hypoxia-induced cellular injuries in cultured granule neurons : LDH release

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0 ng/ml	8	106.3333	2.7325	1.1155
IGF-I 0.3 ng/ml *, #	8	79.6667	24.5574	10.0255
IGF-I 1 ng/ml *, #	8	77.5	14.856	6.0649
IGF-I 3 ng/ml *, #	8	70	10.8628	4.4347
IGF-I 6 ng/ml *, #	8	56.1667	12.1395	4.9559

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

Table 17. Effects of IGF-I Co Chemical Hypoxia-induced cellular injuries in cultured granule neurons : MTT reduction

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0 ng/ml *	8	52	1.069	0.378
IGF-I 0.3 ng/ml *,#	8	83.75	7.4594	2.6373
IGF-I 1 ng/ml *,#	8	84.375	9.1173	3.2234
IGF-I 3 ng/ml #	8	92.5	6.5683	2.3223
IGF-I 6 ng/ml #	8	92.75	11.2345	3.972

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

Table 18. Effects of IGF-I Co Chemical Hypoxia-induced cellular injuries in cultured granule neurons : LDH release

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0 ng/ml	8	105.3333	5.8195	2.3758
IGF-I 0.3 ng/ml *,#	8	72.1667	7.0261	2.8684
IGF-I 1 ng/ml *,#	8	81.8333	17.5547	7.1667
IGF-I 3 ng/ml *,#	8	83.8333	8.1833	3.3408
IGF-I 6 ng/ml *,#,$	8	63	19.2873	7.874

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

$ $p < 0.05$ denotes statistically significant difference from 3 ng/ml IGF-I

Table 19. Effects of IGF-I Post Chemical Hypoxia-induced cellular injuries in cultured granule neurons : MTT reduction

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	0.000	0.000
IGF-I 0 ng/ml *	8	51	0.7559	0.2673
IGF-I 0.3 ng/ml *	8	46.375	4.1726	1.4752
IGF-I 1 ng/ml *	8	47.5	3.8914	1.3758
IGF-I 3 ng/ml *	8	48.75	6.7981	2.4035
IGF-I 6 ng/ml *	8	52.25	4.3012	1.5207

* $p < 0.05$ denotes statistically significant difference from Control

Table 20. Effects of IGF-I Post Chemical Hypoxia-induced cellular injuries in cultured granule neurons : LDH release

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	6	100	0.000	0.000
IGF-I 0 ng/ml	6	102	2.3664	0.9661
IGF-I 0.3 ng/ml	6	92.3333	10.4817	4.2791
IGF-I 1 ng/ml	6	100.3333	11.003	4.492
IGF-I 3 ng/ml *,#,$	6	82	8.438	3.4448
IGF-I 6 ng/ml	6	86.8333	14.2185	5.8047

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from 0 ng/ml IGF-I (Hypoxia)

$ $p < 0.05$ denotes statistically significant difference from 1 ng/ml IGF-I

Table 21. Effects of Physical Hypoxia-induced cellular injuries in cultured granule neurons : TBARS

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	1.4899	0.5263
Hypoxia *	8	121.053	9.0975	3.1884
Pre -treatment *,#,@	8	69.298	2.2670	0.8861
Co -treatment #,$,@	8	95.614	6.5995	2.451
Post -treatment #,$,&	8	109.649	10.0962	3.6

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from Physical hypoxia

$ $p < 0.05$ denotes statistically significant difference from pre-treatment of 1 ng/ml IGF-I

& $p < 0.05$ denotes statistically significant difference from co-treatment of 1 ng/ml IGF-I

@ $p < 0.05$ denotes statistically significant difference from post-treatment of 1 ng/ml IGF-I

Table 22. Effects of Chemical Hypoxia-induced cellular injuries in cultured granule neurons : TBARS

Group	count	The Mean	Standard deviation	Standard Error of the Mean
Control	8	100	1.4899	0.5346
Hypoxia *	8	127.193	6.7680	2.3895
Pre-treatment *,#,&,@	8	78.0701	1.6949	0.6040
Co -treatment #,$	8	101.754	2.0636	0.7402
Post-treatment $	8	106.140	1.5347	1.1470

* $p < 0.05$ denotes statistically significant difference from Control

# $p < 0.05$ denotes statistically significant difference from Physical hypoxia

$ $p < 0.05$ denotes statistically significant difference from pre-treatment of 1 ng/ml IGF-I

& $p < 0.05$ denotes statistically significant difference from co-treatment of 1 ng/ml IGF-I

@ $p < 0.05$ denotes statistically significant difference from post-treatment of 1 ng/ml IGF-I

Curriculum Vitae

Miss Anchukorn Jaroinsiri was born on 29th December 1974, in Burirum, Thailand. She received the Bachelor of Nursing from Mahidol University in 1999. Since her graduation, she had an experience in nursing care about a year at the department of emergency room, Kasemras Bangkhae Hospital, Bangkok, Thailand.