Anti-Apoptotic Actions of R-2HMP in Cerebellar Granule Cells: Changes of Mitochondrial Membrane Potential and Sub-cellular GAPDH Protein

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ABSTRACT

(R)-*N*-(2-heptyl)-*N*-methylpropargylamine (R-2HMP) has been shown to reduce neuronal death through an action at the same site as R-deprenyl: this site has been proposed to be glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Using cultured cerebellar granule cells, two modes of apoptosis were induced by adding cytosine arabinoside (Ara-c) (p53-dependent) or by lowering the extracellular concentration of potassium (p53-independent). A fluorescent probe, chloromethyl-tetramethylrhodamine methyl ester (CMTMR), was introduced to the medium 0, 4, 6, 12, 18, or 24 hours after the induction of apoptosis. Chromatin condensation was visualized with bis-benzamide. Treated and untreated cells at 6, 12 or 24 hours were collected, subcellular fractions obtained and Western blotting of GAPDH performed. Northern Blot analysis was done to examine the effect of R-2HMP on the expression of GAPDH. The results indicate that: 1) A decrease in mitochondrial membrane potential is an early event, occurring between 4 and 6 hours after Ara-c induced apoptosis; 2) Increased nuclear and mitochondrial GAPDH protein is a later event, occurring between 6 and 12 hours after Ara-c induced apoptosis; 3) GAPDH mRNA is increased 1 hour following Ara-c; 4) There was no decrease of mitochondrial membrane potential or subcellular changes of GAPDH associated with low K^+ induced

apoptosis; 5) R-2HMP prevents Ara-c induced apoptosis, prevents mitochondrial membrane potential changes and GAPDH protein and mRNA changes, but has no effect on low K^+ induced apoptosis. Taken together, these results provide good evidence that some forms of apoptosis involve a change of mitochondrial membrane potential followed by altered subcellular GAPDH. R-2HMP can prevent the changes associated with Ara-c induced apoptosis, and appears to act very early in the apoptotic cascade.

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$\Delta \psi_m$	mitochondrial membrane potential
δp	proton electromotive force
AD	Alzheimer's Disease
ADP	Adenosine Diphosphate
AdNT	adenine nucleotide translocator
AIDS	acquired immune deficiency syndrome
AIF	apoptosis initiating factor
Ap4A	dinucleoside polyphosphate, P1,P4-di(adenosine-5') tetraphosphate
Apaf-1	apoptosis protease-activating factor-1
Ara-c	cytosine arabinoside
ATP	adenosine triphosphate
bcl-2	B-cell lymphoma/leukemia-2
BSA	bovine serum albumin
C. elegans	Caenorhabditis elegans
CDK	cyclin dependent kinase
Ced-3	nematode pro-apoptotic gene (homologous to ICE family of cysteine protease)
Ced-9	nematode anti-apoptotic gene (homologous to bcl-2 family of proteins)
CGC	cerebellar granule cell
CHO	Chinese hamaster ovary
CMTMR	chloromethyl-tetramethylrhodamine methyl ester
DSP-4	*N*-(2-chloroethyl)-*N*-ethyl-bromobenzylamine
FPLC	fast phase liquid chromatography
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H&E	haematoxylin & eosin
HD	Huntington's disease
hr	hour
ICE	interleukin-1β-converting enzyme
JNK	c-Jun N-terminal kinase
ISEL	in situ end labeling
Kbp	one thousand base pairs
LamP	lamin protease

L-Dopa	(-)-3-(3,4-dihydroxyphenyl)-L-alanine (levodopa)
MAO	monoamine oxidase
MAP-2	microtubule associated protein-2
MPT	mitochondrial permeability transition
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
NAD+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
PARP	[poly (ADP-ribose)] polymerase
PBR	peripheral benzodiazepine receptor
PBS	phosphate buffered saline
PCD	programmed cell death
PD	Parkinson's disease
PRE	post-transcriptional regulatory cis-element
PrICE	protease resembling ICE
R-2HMP	(R)-*N*-(2-heptyl)-*N*-methylpropargylamine
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNc	Substantia nigra pars compacta
SOD	superoxide dismutase
TNF	tumor necrosis factor
UDG	uracil DNA glycosylase
UTR	untranslated region
WAF1	p53 wild type activating factor-1

1. INTRODUCTION

1.1 Overview of Apoptosis

Although the term 'apoptosis' was coined by Kerr et al. from a Greek word meaning 'falling off' (1972), descriptions of development cell death date back to 1925. Ernest (1925) and Gluckman (1930) provided the first descriptions of widespread neuronal death in embryonic tissues (cited in Raff et al., 1994). Nevertheless, it is only within the last decade that scientists have truly recognized the importance of apoptosis in a variety of biological processes and disease states. Increased apoptosis has been shown to be involved in many human diseases (Raff et al., 1994). In contrast, decreased or suppression of apoptosis contributes to the development of cancer or malformation of tissues (Raff et al., 1994). Since an in-depth study of apoptosis has only emerged recently, the intracellular processes involved in apoptosis are only presently being resolved.

Apoptosis is also referred to as "programmed cell death", since it is a highly ordered form of cellular death in which a cell executes a series of internal events to destroy itself. The decision to activate these death mechanisms or cascades may arise from signals internal or external to the cell itself. Apoptosis is a form of "altruistic suicide", since in essence, an

individual cell makes a decision to live or die which is for the ultimate good of the whole organism.

Morphologically, apoptosis is characterized by cellular and nuclear shrinkage, convolution of both nuclear and plasma membranes, plasma membrane "blebbing" and chromatin condensation (Bredesen, 1995). The cells' organelles stay intact, and the plasma membrane appears to be well conserved. One of the most archetypal features of apoptosis is the activation of an endonuclease that cuts the cell's nuclear DNA into fragments of about 180 bp but does not affect mitochondrial DNA (mtDNA), due to degradation occurring within the boundary of an intact nuclear envelope. When this DNA is separated on an agarose gel, it appears as a ladder-like series of bands – a trademark of many types of cellular apoptosis (Arends et al., 1990). Eventually both the nuclear and cytoplasmic fragments form distinct organelles known as apoptotic bodies, which are phagocytozed by neighbouring macrophages (Bredesen, 1995). Since the contents of the cell do not leak into the extracellular space, there is no inflammatory response, and this lack of inflammation is also considered to be a hallmark feature of apoptosis. Hence, apoptosis can be generally conceptualized as an orderly process by which individual cells die with insignificant damage to neighbouring cells or tissues. These

features should be distinguished from the other type of cell death "necrosis". Morphologically, necrosis is characterized by clumping of the chromatin, swelling of the endoplasmic reticulum and dispersal of ribosomes (Bredesen, 1995). Basically, the cell appears to swell and lyse, the cell membrane integrity is compromised early and the cell's contents are released into the extracellular space, causing an inflammatory response. A comparison between apoptosis and necrosis is provided in Table 1.1.

It is now apparent that there is some variation in apoptotic features from organism to organism, and even within the same organism. For instance, while nuclear DNA is often broken into oligonucleosome-sized (180 bp) fragments, this is not always seen (Raff, 1992). Further, some types of apoptosis are dependent on the synthesis of new proteins, while others are not (Martin et al., 1988). Thus while certain features of apoptosis are apparent in some situations, they may not be seen in others. As such, apoptosis should be defined based upon a number of criteria, rather than simply on the appearance or not of an individual feature such as DNA ladder formation, which may not occur in any given situation. These variations are probably related to the recent descriptions of multiple, independently regulated, apoptotic pathways (Park et al., 1998).

Table 1.1 Summary of Necrosis and Apoptosis

Necrosis	Apoptosis
Loss of cellular homeostasis	No initial major changes
Altered membrane permeability Potassium loss; sodium entry; fall in membrane potential	Not seen, at least initially No sodium influx; no change in potassium in cell
Swelling of all cytoplasmic compartments	Cytosol condensation
Destruction of mitochondria and other organelles	Generally intact organelles Protruberances from cell surface separate to form apoptotic bodies
Depletion of cellular energy (ATP)	No depletion of cellular energy
Lowered macromolecular synthesis	Macromolecular synthesis activation is required
Affects tracts of contiguous cells	Affects scattered individual cells
Loose aggregates of chromatin	Highly condensed granular aggregates of chromatin
Passive atrophy	**Active degeneration**

1.2 Signal Transduction in Apoptosis

1.2.1 Initiation and effector phases of apoptosis

As mentioned earlier, apoptosis can be activated by internal or external signals which may initiate a cascade of events which eventually ends with the death of a cell. A variety of cells, including many relatively long-lived cell types, will rapidly undergo apoptosis when removed from their in vivo environment (Raff, 1992). This suggests that all cells of the body are continuously receiving signals from their local environment that determines the apoptotic threshold of the cell, and that the nature of these signals is different in different tissues. For instance, members of the nerve growth factor (NGF) family maintain the survival of neurons during development by remaining bound to the trkA or high-affinity NGF receptor (Raff, 1992). Interestingly, the situation is complicated by the presence of the low affinity NGF receptor, p75. It was found that NGF binding to mature oligodendrocytes expressing p75, but not trkA, resulted in an enhanced expression of intracellular ceramide and c-Jun amino-terminal kinase (JNK) activity, which are thought to be involved in a signal transduction pathway leading to apoptosis (Casaccia-Bonnefil et al., 1996).

If trkA and p75 were co-expressed, however, inhibition of the ceramide pathway occurred, resulting in cell survival.

In contrast to trkA, the binding of Fas ligand to the Fas receptor, and the binding of tumor necrosis factor-α (TNF α) to the TNF receptor, induces apoptosis (Nagata and Golstein, 1995). The TNF and Fas receptors share a related intracellular sequence known as the death domain which is 60-70 amino acids long and critical for the induction of apoptosis via these receptors (Golstein et al., 1995). Whether from the removal or presence of a stimulus, the cell-surface receptor-mediated response usually acts via a signal transduction system. The transduction system generally entails the stimulation of the receptor, the activation of protein kinase/phosphatase cascades, and the liberation of second messengers such as ceramide, which can either enhance or repress the transcription of particular genes (Hale et al., 1996). Also of key importance are the ICE (interleukin converting enzyme) or ICE-like proteases which play a role in most forms of apoptosis. For instance, an antibody to the Fas receptor leads to apoptosis in thymocytes, which can be blocked by inhibitors specific for ICE-like proteases (Kuida et al., 1995). These enzymes will be discussed in more detail below (Section 1.2.2.1).

1.2.2 Pro-apoptotic genes/proteins

In recent years, a number of genes and their protein products have been uncovered, which can influence or determine the progression of apoptosis and serve as markers of the process. Oncoproteins, anti-oncogenes, and several protease families have received particular attention as mediators or modifiers of apoptosis.

1.2.2.1 ICE related proteins

ICE, a member of the cysteine protease family, was investigated for its potential involvement in mammalian apoptosis because it contains 29% amino acid homology to Ced-3, a pro-apoptotic protein in *Caenorhabditis elegans* (Yuan et al., 1993). ICE-like proteases are termed caspases, the *c* denoting cysteine protease and the *aspase* referring to the tendancy of the enzymes to cleave after an aspartic acid residue (Cohen, 1997; Villa et al., 1997). To date, 13 different caspases have been identified and named caspase 1-10 (Thornbery and Lazebnik, 1998). The caspases are synthesized as inactive precursors and cleaved either by self or other caspases. Several of the caspases, particularly caspase 3, have been shown to be activated in models of neuronal apoptosis (Armstrong et al., 1997; Du et al., 1997; McCarthy et al., 1997). Specific caspases have been shown to

contribute to the cleavage of the cytoskeletal protein actin that occurs during apoptosis (Nath et al., 1996; Jordan et al., 1997a) and caspase inhibitors block actin cleavage during apoptosis (Gressner et al., 1997). Generally, caspases can be classified into two groups: activator caspases (e.g. caspase 9) and execution caspases (e.g. caspase 3).

1.2.2.2 p53

Originally identified as a tumor suppressor, p53 is a DNA transcription factor that is involved in the control of cell proliferation and DNA repair (Wang et al., 1995). Normally, p53 is thought to inhibit growth by arresting the cell cycle, allowing time for DNA repair before division (Somasundaram, 2000). Wild-type p53 has been shown to prevent tumor development by increasing the sensitivity of abnormally proliferating cells to apoptosis (Donehower et al., 1992). Mutations in p53 have been found in more than 50% of all human cancers (Hollstein et al., 1991) which is thought to allow the continued proliferation of cells which would normally undergo apoptosis. Because p53 acts as a transcriptional activator of the *bax* gene, p53 activation may lead to an increase in the bax/bcl-2 ratio and may therefore enhance sensitivity to apoptosis (Miyashita and Reed, 1995). Though p53 protein expression is associated with apoptosis, it is not

essential for apoptosis (Donehower et al., 1992); for example, DNA damage (UV irradiation) causing thymocyte apoptosis is p53-dependent (Clark et al., 1993), while DNA damage causing T-cell hybridoma apoptosis is p53-independent (Woronicz et al., 1994). P53-dependent and p53-independent pathways also exist in the same cell type. Ara-c induced apoptosis in cultured cerebellar granule cells is p53-dependent whereas low K^+ induced apoptosis is p53-independent (Enokido et al., 1996a, b). Further, thymocytes undergo p53-independent apoptosis following dexamethasone treatment but a p53-dependent apoptosis after γ-irradiation (Clarke et al., 1993). Recently, a large number of p53-induced genes have been discovered, some of which are speculated to mediate apoptosis (Polyak et al., 1997).

1.2.2.3 Immediate early genes

There is now evidence for the activation of immediate early genes during apoptosis in neural cells. Enhanced expression of the transcription factors c-Jun and c-Fos (Estus et al., 1994; Ferrer et al., 1996), increased levels of *c-jun* mRNA (Miller and Johnson, 1996), and phosphorylation of c-Jun on its N-terminal transactivation domain, indicating the involvement of AP-1 (Ham et al., 1995), were observed during neural apoptosis.

Activation of the p38 and c-Jun N-terminal kinase (JNK) group of mitogen-activated protein kinases was reported in apoptosis induced by glutamate (Kawasaki et al., 1997), as well as ischemia (Ozawa et al., 1999). Mice lacking the *JNK-3* gene are resistant to kainic acid-induced apoptosis and show reduced levels of phosphorylated c-Jun and AP-1 transcription factor complex (Yang et al., 1997). Thus, expression of c-Jun and c-Fos proteins, their phosphorylation, and formation of the AP-1 factor appear to be essential for neuronal apoptosis. It is likely that AP-1, which coordinates gene expression, is involved in the commitment of neurons to apoptosis.

1.2.3 Anti-apoptotic genes/proteins

1.2.3.1 The bcl-2 family

The mammalian homologue of *ced-9* is the protooncogene *bcl-2* (Vaux et al., 1988). Overexpression of bcl-2 produces the same protective effect as does overexpression of ced-9 in *C. elegans* cells and prevents death among mutant cells that have lost the function of ced-9 (Hengartner and Horvitz, 1994). The bcl-2 protein blocks apoptosis induced by ICE (Miura et al., 1993), Ich (Wang et al., 1994), and c-myc (Bissonnette et al., 1992). As discussed below, studies suggest that increased bcl-2 expression

imparts survival to cells that would otherwise die by apoptosis; therefore, it can promote cancer cell expansion and contribute to chemoresistance. Its mechanism of action may be linked to an antioxidant activity (Hockenbery et al., 1993) and its ability to prevent the release of cytochrome c, a proapoptotic mediator, from mitochondria (Yang, J. et al., 1997) and keep the mitochondrial permeability transition (MPT) pore closed (Zamzami et al., 1996).

Like ICE, bcl-2 belongs to a family of proteins that can regulate the apoptotic threshold. Some of these proteins, such as bcl-x_L, function as inhibitors of cell death, whereas others, such as bax, bad, bak and bcl-x_s, increase sensitivity to apoptotic stimuli (Oltvai et al., 1993; Yang et al., 1995). It has been shown that bcl-2 can physically interact with several of these homologous proteins to form hetero-dimers. Dimerization of bcl-2 with bax seems to be a critical interaction. A higher concentration of bax, compared with bcl-2, enhances cell susceptibility to apoptosis. Cells continue to survive if bcl-2 predominates over bax (Oltvai et al., 1993). Mutations of bcl-2 that inhibit its ability to bind to bax result in a decreased ability to prevent apoptosis (Yin et al., 1994). Thus, bax may promote cell death unless it is neutralized by bcl-2 or bcl-x_L (Yang et al., 1995).

1.2.4. Mitochondria as a commitment point

It has recently been suggested that apoptosis is associated with a sequence of events in which mitochondria are intimately involved (Tatton and Chalmers-Redman, 1996). Mitochondria have been shown to play a critical role in the decisional phases of many forms of apoptosis. These events include: a) a fall in mitochondrial membrane potential ($\Delta\Psi m$); b) opening of the MPT pore; c) release of small mitochondrial proteins, which signal the initiation of apoptosis. These events will be discussed in detail below.

1.2.4.1. Mitochondrial membrane potential

The maintenance of mitochondrial membrane potential ($\Delta\Psi m$) across the mitochondrial inner membrane is critical to energy production and the control of apoptosis. An electrochemical proton gradient normally exists across the inner mitochondrial membrane resulting in a $\Delta\Psi m$ of approximately –150 mV inside and a proton concentration difference across the mitochondrial membrane. The $\Delta\Psi m$ is dependent on the capacity of three mitochondrial respiration complexes (I, III and IV) to use

the energy in the carrier molecules NADH, ubiquinone, and cytochrome c, to pump protons across the inner mitochondrial membrane. The outward pumping of protons produces an electron gradient that is biochemically reflected by a pH difference (ΔpH) and electrically by a voltage across the inner mitochondrial membrane termed the $\Delta\Psi m$ (Sherratt, 1991). The difference between $\Delta\Psi m$ and the proton concentration (ΔpH), contributes to a proton electromotive force (δp) which drives the conversion of ADP to ATP by complex V (ATP synthase). Since $\Delta\Psi m$ is by far the greater contributor to δp, $\Delta\Psi m$ can be assumed to vary almost linearly with the ATP/ADP ratio (Jacotot et al., 1999). Therefore $\Delta\Psi m$ is critical for ATP formation.

Since the uptake of Ca^{2+} into mitochondria is driven by $\Delta\Psi m$, marked increases in cytosolic Ca^{2+} cause relatively large amounts of Ca^{2+} to be sequestered into mitochondria and thereby cause $\Delta\Psi m$ to decrease. Situations in which Ca^{2+} can freely pass across mitochondrial membranes or in which high levels of Ca^{2+} accumulate in mitochondria due to high cytosolic levels will result in a complete loss of $\Delta\Psi m$ and of mitochondrial energy production.

1.2.4.2 MPT Pore and Apoptosis-Initiating Factors (AIFs)

A decrease in $\Delta\Psi m$, in the presense of increased intramitochondrial Ca^{2+}, induces opening of a MPT pore which spans the inner and outer mitochondrial membranes. Details on the in vitro reconstruction of the MPT pore have recently been provided (Marzo et al., 1998; Zamzami et al., 1998), although the precise structure is uncertain (Zoratti and Szabo, 1995). The adenine nucleotide translocator (AdNT) is a critical element of the MPT pore (Zoratti and Szabo, 1995), but whether it forms the pore itself or is just closely associated with a pore-forming protein is unknown. The MPT pore also includes a voltage-dependent anion channel and a peripheral benzodiazepine receptor (Jacotot et al., 1999).

The MPT pore opens in response to a decrease in $\Delta\Psi m$ in the presence of an increase in intramitochondrial Ca^{2+} (Scorrano et al., 1997b). Increases in Ca^{2+}, increased oxidative radical levels, or failure of respiratory complexes, acting either individually or together, can induce a fall in $\Delta\Psi m$ (Richter, 1993). Complete opening of the MPT pore allows free exchange between the mitochondrial matrix and extramitochondrial cytosol of solutes and proteins (Zorrati and Szabo, 1995). This can result in mitochondrial swelling and rupture of the outer mitochondrial membrane

with the release of heat labile Apoptosis Initiating Factors (AIFs), such as cytochrome c, from the intermembrane space into the cytoplasm (Marchetti et al., 1996; Susin et al., 1996; Zamzami et al., 1996). While cytochrome c does not transit through the MPT pore, it can be released into the cytosol through fractures in the mitochondrial membrane (Marchetti et al., 1996; Susin et al., 1996; Zamzami et al., 1996). Cytochrome c released from mitochondria acts as a cofactor for activation of caspase-9-like proteases (Liu et al., 1996). The cytosolic partner of cytochrome c in the apoptotic cascade has recently been identified as apoptosis protease-activating factor (Apaf-1) (Li, P. et al., 1997; Zou et al., 1997). Apaf-1 and cytochrome c, along with dATP, allow activation of caspase-9. Activated caspase-9 mediates cleavage of pro-caspase-3 to active caspase-3 (Li, P. et al., 1997; Zou et al., 1997), one of the principal proteases in mammalian apoptosis.

Several factors are known to influence the opening or closure of the MPT pore. Cyclosporin A binds to the MPT pore and maintains it in a closed state. It also promotes pore closure by binding cyclophilins that in the presence of Ca^{2+}, induce MPT pore opening through binding to the AdNT (Bernardi et al., 1994; Scorrano et al., 1997a). Factors like glutathione, ADP levels, and reactive oxygen species (ROS) when present in the mitochondrial matrix modulate the opening of MPT pore but are not

sufficient themselves to open the pore (Chernyak and Bernardi, 1996). The anti-apoptotic protein bcl-2 maintains closure of MPT pore possibly by binding to the peripheral benzodiazepine receptor (PBR) (Carayon et al., 1996). bcl-2 has been shown to localize to the outer mitochondrial membrane (Hockenbery et al., 1990; Lithgow et al., 1992; Monaghan et al., 1992) near the PBR (Carayon et al., 1996). Truncated bcl-2, which cannot dock in mitochondrial membranes and remains in the cytosol, is less effective in reducing apoptosis (Hockenbery et al., 1993). bcl-2 has also been shown to reduce apoptosis by maintaining $\Delta\Psi m$ (Richter, 1993). Thus, opening of the MPT pore is a critical step in many forms of apoptosis and is proposed to constitute an irreversible step in the process.

1.2.5 Degradation phase of apoptosis

The vast number of effector mechanisms involved in apoptosis suggests a number of parallel processes working together which ultimately lead to cellular death. These processes include the destruction of energy metabolism, increases in intracellular Ca^{2+}, changes in cell membrane structure, loss of anabolic functions, degradation of cellular molecules and

phagocytosis of cellular debris (Bredesen, 1995). Obviously, depending on the type of apoptotic model in question, not all of the processes mentioned will necessarily be present, but they do represent conditions typically observed. A brief description of these events follows.

The breakdown of energy metabolism resulting from mitochondrial failure plays a major role in certain types of apoptosis. The loss of ATP compromises the maintenance of electrochemical gradients and membrane integrity. Once membrane functions are compromised, cytosolic concentrations of ions change, which further assists in cellular destruction (McConkey et al., 1994; Trump and Berezesky, 1995). Most importantly, Ca^{2+} levels increase which activates endonucleases, such as DNase I, leading to DNA fragmentation (Polzar et al., 1993). Also cooperating in the destruction of the nucleus are lamin proteases, such as LamP, which destroy lamins or intermediate filaments holding the nucleoskeleton together (Earnshaw, 1995). LamP may be regulated by ICE-like proteases, such as prICE (Earnshaw, 1995). ICE-like enzymes may further compromise nuclear functioning by cleaving PARP (poly (ADP-ribose) polymerase), which typically functions in cellular-DNA repair (Kaufmann et al., 1993; Lazebnik et al., 1994). Moreover, Ca^{2+} may also influence the cytoplasmic event of apoptotic body formation. While quite hypothetical,

evidence suggests that Ca^{2+}-dependent transglutaminases increase during apoptosis (Hale et al., 1996), and can lead to the appearance of cytoplasmic changes (apoptotic bodies) which are characteristic of apoptosis. The transglutaminases are a family of Ca^{2+}-dependent glutamine and glutamyl transferases which stabilize the microfilament network holding a cell's cytoskeleton in place (see Hale et al., 1996 for review). It would seem that the re-arrangement of the cytoskeleton during the formation of apoptotic bodies via transglutaminases, may also reduce or prevent the leakage of cell contents into the extracellular space which would cause an inflammatory response.

Ultimately, changes in the cytoskeleton and alterations in the proteins expressed on the dying cells' surface allow for its identification and removal by macrophages and nonphagocytic adjacent cells. In particular, the surface expression of phosphatidyl serine appears critical to phagocytic action (Koopman et al., 1994).

1.3 Glyceraldehyde-3-phosphate dehydrogenase

1.3.1 Role in glycolysis

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) has been considered intensively as a glycolytic protein (Rossman et al., 1975). It is comprised of a polypeptide chain of 330 amino acids. As an active glycolytic enzyme it is isolated as a tetramer of approximately 150 kDa composed of four identical 37 kDa subunits (Sirover, 1999). GAPDH binds one NAD^+ molecule to individual active sites on each subunit (Sirover, 1999). Critical amino acids include cys^{149} and his^{176} (Sirover, 1999).

GAPDH catalyzes the glycolytic reaction:

D-glyceraldehyde-3-phosphate + NAD^+ + $P_i \leftrightarrow$ 1,3-diphosphoglycerate + NADH + H^+.

GAPDH has been isolated from numerous organisms and is ubiquitous in nature. Sequences are highly conserved across species. As a

very abundant protein, it may constitute between 10-20% of total cellular protein (Sirover, 1999). Structural studies identified two important regions, the NAD^+ binding site (Rossman fold) and the glyceraldehyde-3-phosphate binding site (Sirover, 1999).

1.3.2 Non-glycolytic functions

As can be seen from above, GAPDH has been considered a house keeping protein involved in basic cell catabolic processes. However, independent studies from a number of different laboratories have reported a variety of diverse biological properties of the GAPDH protein (Sirover, 1999).

1.3.2.1 Membrane binding functions of GAPDH

Membrane fusion fulfills an important role in a number of basic cell functions. For example, it is involved in cell division, the immune response, synaptic transmission as well as the response to environmental change. As such, it has been suggested that proteins which display membrane fusogenic activity (the ability to merge phospholipid bilayers),

play a critical role in normal cell function. Further, alteration of proteins affecting these functions would present a severe challenge to the cell. Early studies identified GAPDH as a membrane bound protein (Tanner and Gray, 1971; Wooster and Wrigglesworth, 1976). It was determined that 60-70% of total erythrocyte GAPDH was membrane associated (Kant and Steck, 1973). Ensuing studies identified that GAPDH colocalized with the band 3 anion exchanger (AE) protein which is a membrane protein (Ercolani et al., 1992). Its glycolytic activity was diminished by this protein/protein interaction. A further indication of the role of GAPDH in membrane fusion was identified recently by Hessler et al. (1998). In neutrophil mediated degeneration, a significant aspect of immunoregulation, membrane fusion is required for invagination of the plasma membrane to engulf foreign particles. In these studies, human neutrophil cytosol was examined for fusion activity. Significantly, fusogenic activity was quantitated after depletion of annexin by Ca^{2+}-dependent affinity chromatography. Subsequently, remaining fusogenic activity was purified through ion exchange and fast phase liquid chromatography (FPLC). Size exclusion studies revealed a single protein which contained the majority of the non-annexin fusion activity. Sequence analysis identified this protein as

GAPDH. Glycolytic activity but not fusogenic activity was lost after FPLC (Hessler et al., 1998).

Similarly, GAPDH was identified as a tubulin binding protein, catalyzing tubulin polymerization (Muronetz et al., 1994). In skeletal muscle, GAPDH/tubulin binding resulted in triad junction formation (Caswell and Corbett, 1985). Tubulin binding by GAPDH was inhibited by ATP (Huitorel and Pantaloni, 1985). The functional role of tubulin bound GAPDH was demonstrated by analysis of mutant Chinese hamster ovary (CHO) cells deficient in endocytosis (Robbins et al., 1995). These studies revealed that a mutation in microtubule-associated GAPDH was the causative agent. Sequence analysis of the mutant GAPDH cDNA indicated that a single amino acid change was responsible for the endocytotic deficiency.

1.3.2.2 Cytoplasmic (soluble) functions of GAPDH

A variety of experimental protocols have been used to identify specific proteins that interact with cellular mRNA at 5'- and 3'-UTR sequences. N-terminal sequencing has identified one binding protein as GAPDH (Nagy and Rigby, 1995). It was shown that GAPDH uniquely bound to AU rich 3'-UTR of lymphokine mRNA (Nagy and Rigby, 1995),

to the 5'-UTR stem-loop IIIA region of the hepatitis A virus (Schultz et al., 1993), and to the 3'-genome sequence element of human parainfluenza I virus type 3 (De et al., 1996). A common element in these studies is the identification of pyrimidine rich regions in the RNA molecule as the putative GAPDH binding site.

Competition experiments indicated that GAPDH/RNA binding was inhibited by poly (U) or NAD^+. These results suggest the involvement of the NAD^+ binding site within GAPDH in binding to RNA. Further studies suggested (Sioud and Jespersen, 1996) that GAPDH increased the activity of the tumor necrosis factor alpha (TNF-α) ribozyme. This enhancement was observed both in vitro and in vivo. In accord with the mRNA studies described above, the GAPDH binding site was contained within pyrimidine rich regions of the TNF-α ribozyme.

1.3.2.3 Nuclear functions of GAPDH

Initial structural investigations identified GAPDH as a DNA binding protein (Tsai and Green, 1973; Perucho et al., 1977) as well as a non-histone nuclear protein (Morgenegg et al., 1986). Recent studies have indicated three new nuclear functions of GAPDH. First, Singh and Green (1993) identified GAPDH as a nuclear tRNA export protein. This study

used normal and export-defective tRNA in mobility shift assays. The purification of the unknown export protein and N-terminal sequencing identified the unknown protein as GAPDH. Competition for binding was observed using NAD^+. Sequence analysis indicated G57 uracil as an important residue for GAPDH binding. Another study also indicates the role of GAPDH in nuclear RNA transport. Zang et al. (1998) examined the mechanisms of action of a post-transcriptional regulatory cis-element (PRE) in the hepatitis B virus infection. PRE appears to be necessary for the increase in viral transcripts in the cell cytoplasm. PRE fragments bound to two proteins, one of them identified as human GAPDH. The GAPDH/PRE complex was localized in the nucleus and was immunoprecipitated by anti-GAPDH antibody.

Second, Baxi and Vishwanatha (1995) investigated the role of Ap_4A in DNA replication and DNA repair. In their studies, photoaffinity probes were used to identify Ap_4A binding proteins. These studies detected unique binding of a 37 kDa nuclear protein. The nuclear protein was identified as GAPDH. The physiological significance of this structural interaction is indicated by previous studies demonstrating the physical association of mammalian GAPDH with replicating DNA (Lee and Sirover, 1989). The role of GAPDH/Ap_4A interactions in vivo however, is unclear at the

present time. However, diadenine nucleotides are thought to be involved in a number of cell processes. Early studies on Ap_4A identified it as binding to a subunit within the multiprotein DNA poly α complex (Baril et al., 1983). Later studies demonstrated its binding to cell membranes (Edgecombe et al, 1996). Of note, several reports link diadenine nucleotides to apoptosis (Gasmi et al., 1996). The potential significance of this new observation will be considered in a later section (Section 1.3.3) on the role of GAPDH in apoptosis.

Third, two different laboratories have reported a role of human GAPDH in DNA repair. DNA repair is an important function in vertebrate cells. A nuclear activity, it is responsible for the removal of DNA lesions which alter the cells genetic information. The DNA repair enzyme uracil DNA glycosylase (UDG) removes uracil as the free base resulting in the formation of an apyrimidinic site in DNA. Initially, nuclear human GAPDH was identified as UDG based on its ability to release uracil from a poly (dA)-poly (dU) substrate (Meyer-Siegler et al., 1991). Subsequently, this report was confirmed by Baxi and Vishwanatha (1995). They demonstrated that their Ap_4A binding protein, exhibited UDG activity. The functions of GAPDH are summarized in Table 1.2

Table 1.2 Functions of Mammalian Glyceraldehyde-3-Phosphate Dehydrogenase

Localization	Cell function	Activity	Molecular mechanism
Membrane	Macromolecular transport	Endocytosis	Microtubule binding protein
Cytoplasm	Energy generation Control of gene translation	Glycolysis Translational regulation	ATP production 5'UTR, 3'UTR mRNA binding protein
Nuclear	Control of gene expression Transfer of genetic information Prevention of genomic instability	Transcriptional regulation DNA replication DNA repair	Nuclear tRNA export protein $A_{p4}A$ binding protein Uracil DNA glycosylase

1.3.3 GAPDH and apoptosis

1.3.3.1 Indirect evidence

Oxidative stress is one of the best documented inducers of apoptosis (Tong et al., 1998). In isolated rabbit aorta, oxidative stress has been reported to induce a marked increase in GAPDH mRNA with no concomitant change in glycolytic enzyme activity (Ito et al., 1996). Unfortunately, this study did not investigate whether the increase in GAPDH mRNA following oxidative stress results in an increase in GAPDH protein levels.

In vivo and in vitro hypoxia/ischemia is a popular model for the study of apoptosis. Increases in GAPDH mRNA have been reported in response to hypoxia/ischemia both in vivo (Feldhaus & Liedtke, 1998) and in vitro (Graven & Farber, 1998). The presence or absence of apoptosis however, was not determined by these two studies, although previous studies have shown apoptosis to occur following hypoxia/ischemia (Tamatani et al., 1998).

Hindlimb unweighting in rats is a model system for muscular changes occurring during prolonged periods of inactivity, such as that seen during limb immobilization, prolonged bed rest, or in conditions of reduced

gravity. An up-regulation in GAPDH mRNA which followed a similar temporal profile to the associated muscular atrophy has been demonstrated (Cros et al., 1999). Apoptosis is known to be involved in this atrophy process (Allen et al., 1997) with the number of apoptotic nuclei reaching a maximum at 3 to 7 days of hindlimb unweighting (Allen et al., 1997), in good agreement with the maximum increase in GAPDH mRNA between 4 to 8 days (Cros et al., 1999).

Another interesting study was done on changes in GAPDH in hibernating animals (Soukri et al., 1995, 1996). Hibernating animals have prolonged muscular inactivity; their body temperature drops, possibly resulting in prolonged hypoxia. All these would normally result in apoptosis (see above), but this does not appear to occur. It was found that GAPDH protein levels in muscular tissue decreased under these conditions (Soukri et al., 1995). Decreases of GAPDH protein levels however, were not seen in all tissues. In liver, the specific activity of GAPDH was 2-3-fold lower than in the euthermic animal but there was no change of GAPDH protein levels (Soukri et al., 1996). The resulting decrease of enzyme activity could contribute to the wide depression of the glycolytic metabolic flow associated with mammalian hibernation.

The above studies can be summarized as follows. In general, increased levels of GAPDH are seen under conditions in which apoptosis occurs, GAPDH levels were decreased however when apoptosis was prevented from occurring.

Apoptosis has been shown to be involved in many neurodegenerative diseases. GAPDH interacts with a number of proteins implicated in the etiology of these diseases, including β-amyloid precursor protein (Schulze et al., 1993), and proteins containing polyglutamine repeats, in particular huntingtin (Burke et al., 1996; Koshy et al., 1996; Gentile et al.,1998). The binding of GAPDH to β-amyloid precursor protein is of particular interest. Antibodies raised against Alzheimer's disease β-amyloid plaques identify GAPDH (Sunaga et al., 1995), suggesting that GAPDH is present in these plaques. β-amyloid is known to be pro-apoptotic (Estus et al., 1997).

An abnormal expansion of polyglutamine regions is associated with several degenerative diseases, including Huntington's disease. The huntingtin protein may be directly involved in the initiation of apoptosis (Zeitlin et al., 1995), an effect which appears to be due to the polyglutamine insert (Ikeda et al., 1996). These inserts bind a number of proteins including caspases (Goldberg et al., 1996) and GAPDH (Burke et al., 1996; Gentile et al., 1998).

The final piece of indirect evidence to link GAPDH to apoptotic cascades comes from investigations of the mechanism(s) of action of anti-apoptotic compounds. R-deprenyl has become the standard to which other anti-apoptotic compounds are compared although the actual active compound may be its metabolite R-desmethyl deprenyl (Mytilineou et al., 1997b). R-desmethyl deprenyl and CGP3466 (Kragten et al., 1998), and 17-β estradiol (Joe et al., 1999) all bind to GAPDH with high affinity and are anti-apoptotic (Goodman et al., 1996; Dubal et al., 1998; Kragten et al., 1998). The affinity for GAPDH binding (nM) appears to be equal to their anti-apoptotic activities.

1.3.3.2 Direct evidence

It is only within the last 4-5 years, that direct evidence of a linkage of GAPDH to apoptosis has been brought into focus, thanks in large part to the work of Chuang and co-workers. The first report suggesting a direct role for GAPDH in apoptotic mechanisms showed that low extracellular K^+ induced apoptosis in cerebellar granule cell cultures resulted in an increase in both GAPDH mRNA and protein (Sunaga et al., 1995). The increase in GAPDH protein was associated with the particulate fraction. That this increased expression of GAPDH was integral to the induction of apoptosis

was suggested by the prevention of not only GAPDH changes, but also apoptosis, by pre-incubation of cultures with a GAPDH anti-sense oligodeoxynucleotide (Sunaga et al., 1995). An increase in particulate GAPDH expression was also associated with age-induced apoptosis of cerebellar granule cells (Ishitani et al., 1996b) and cerebro-cortical neurons (Ishitani et al., 1996a), and apoptosis induced by cytosine arabinoside (Ara-c) in cerebellar granule cells (Ishitani & Chuang, 1996). Again, apoptosis (Zhang et al., 1999) and changes in GAPDH mRNA (Ishitani et al., 1996a; Ishitani et al., 1996b) and protein (Ishitani & Chuang, 1996) were prevented by pre-incubation with GAPDH anti-sense, but not sense oligonucleotides. Similar effects have also been reported in non-neuronal cells (Messmer & Brune, 1996; Sawa et al., 1997).

This increase in particulate GAPDH is independent of glycolytic activity (Saunders et al., 1997) and associated with the nucleus (Ishitani et al., 1998; Saunders et al., 1999; Sawa et al., 1997; Shashidharan et al., 1999). A concomitant decrease in supernatant GAPDH protein appears to depend on the age in culture of the cells (Saunders et al., 1997). Interestingly polyglutamine containing disease proteins (see section 1.3.3.1), which selectively bind GAPDH are associated with a nuclear translocation, which is thought to be important for cell death to occur.

The mechanism by which GAPDH initiates apoptosis is unknown at present, although it may involve specific GAPDH isozymes (Saunders et al., 1999). The mechanism does not appear simply to represent a loss of glycolytic activity due to loss of cytoplasmic GAPDH (Saunders et al., 1997), and it also appears to be independent of the uracil DNA glycosylase activity (Saunders et al., 1999) of GAPDH in the nucleus.

1.4 Physiologic relevance of apoptosis

1.4.1 Development

As early as 1951, it was known that nerve cell death played a major role in vertebrate embryogenesis (Glucksmann, 1951). It is now known that a massive death of neurons occurs during vertebrate brain development and that this is related to failed competition for trophic factors (Oppenheim, 1989) and represents a form of apoptosis (Kerr et al, 1972). The death process in these developing neurons was termed programmed cell death (PCD) since it was shown to be dependent on the activation of an intrinsic program of gene expression that led to self destruction through the synthesis of 'killer proteins' (Oppenheim et al., 1990). The dependence on

new protein synthesis was similar to that found in the apoptosis that occurs in cultured sympathetoblasts deprived of NGF (Martin et al., 1988). It has subsequently become clear that new protein synthesis is not necessary for all forms of neuronal apoptosis (Dragunow et al., 1995a; Johnson et al., 1995).

A leading theory on apoptosis states that during development cells inherently die unless they are continuously signaled not to commit suicide (Raff, 1992). The nervous system during embryogenesis serves as a good example of this hypothesis. Naturally occurring neuronal death is a well-established phenomenon in the developing vertebrate nervous system. Many more neurons are generated than are eventually required and, according to the neurotrophic theory, the surplus cells die during synaptogenesis because of deprivation of growth factors produced by the target cells. The neurotrophic theory supports the idea that the target tissue regulates the number of neurons that innervate it. Such a mechanism ensures that all targets are innervated and that the right amount of each kind of neuron is correctly matched to the appropriate target (Raff et al., 1994).

Several in vitro and in vivo studies have provided evidence that neurotrophic factors promote the survival of developing neurons by

suppressing cell death (Martin et al., 1988; Oppenheim et al., 1990). It is generally thought that developmental neuronal death is apoptotic (Johnson et al., 1995). Apoptosis was not however the only form of cell death found in these studies. Martin et al. (1988) found neurons that did not have condensed chromatin. Other morphological patterns have been identified in dying neurons deprived of trophic support. Moribund postnatal rat superior colliculus neurons (Giordano et al., 1980) and embryonic chick ciliary ganglion cells (Pilar and Landmesser, 1976) displayed apoptotic morphological features but exhibited swollen endoplasmic reticulum, Golgi apparatus, and mitochondria. In addition, partition of the cytoplasm into apoptotic bodies was not found. Apoptosis, as defined by its classical morphological features, is only one of many types of cell death that may occur during neuronal development.

1.5 Pathologic relevance of apoptosis

1.5.1 Apoptosis and neurodegeneration

In developing neurons, apoptosis was thought to provide a counter balance for overexuberant cell replication. It therefore seemed unlikely to

involve adult nerve cells, since they are unable to replicate. It is now understood that neuronal apoptosis can occur in response to a variety of insults, many of which are relevant to the pathogenesis of human neurodegenerative diseases. For example, neuronal apoptosis can be caused by exposure to excitatory amino acids (Mitchell et al., 1994; Bonfoco et al., 1995), MPTP (Tatton and Kish, 1997) and MPP^+(Dipasquale et al., 1991; Mochizuki et al., 1994; Mutoh et al., 1994), ß-amyloid protein (Forloni et al., 1996; Estus et al., 1997), mutant presenilin 2 (Wolozin et al., 1996), H_2O_2 (Slater et al., 1995), 6-hydroxydopamine (Walkinshaw and Waters, 1995), mitochondrial complex I inhibitors (Hartley et al., 1994), high levels of dopamine (Ziv et al., 1994) or levodopa (Walkinshaw and Waters, 1995), and the AIDS protein gp 120 (Muller et al., 1992).

Despite the demonstration that apoptosis is an important form of neuronal death, it did not receive recognition as a key factor in neuronal degeneration until the last few years. One important reason for this is the difficulty in demonstration of apoptotic cells because of their relatively short existence. In vivo, apoptotic nuclei and bodies are phagocytozed and digested within hours. The presence of a small number of apoptotic nuclei in a tissue section at a single point in time may therefore reflect large numbers of apoptotic deaths if the process is ongoing over years (Allen et

al., 1997). Thus, this creates the impression that apoptosis plays an inconsequential role. In recent years, apoptosis has been described in a variety of human neurodegenerative disorders primarily based on the use of ISEL (in situ end labelling) techniques to detect neuronal nuclei with apparent DNA cleavage in post-mortem brain tissue. Findings consistent with increased apoptosis have been reported in post-mortem brain samples of patients with Parkinson's disease (PD) (Cotman et al., 1994; Agid, 1995; Mochizuki et al., 1996), Alzheimer's disease (AD) (Dragunow et al., 1995b; Lassmann et al., 1995; Smale et al., 1995; Li et al., 1997), Hungtington's disease (HD) (Dragunow et al., 1995b; Thomas et al., 1995) and ALS (Thomas et al., 1995). For example, approximately 1-2% of individual melanin-containing neurons in the Substantia nigra pars compacta (SNc) of PD patients stained positively with ISEL compared to only 0.2% in age-matched controls (Tatton and Chalmers-Redman, 1998).

1.5.2 Apoptosis and cancer

Apoptosis also participates in the prevention of neoplastic growth. This can be considered a natural way of ensuring that cells that are damaged do not become cancerous and instead die. Apoptosis occurs in

healthy tissue after exposure to DNA-damaging agents such as ionizing radiation or chemotherapeutic drugs (Potten, 1977). Failure to die could result in the propagation of DNA damage to progeny cells and therefore contribute to high mutation rates. It can be argued that the process of carcinogenesis includes interference with the regulation of apoptosis, rather than merely uncontrolled proliferation.

Mutation or deletion of genes that regulate progression through the cell cycle is a central theme in cancer biology. Oncogenic mutations that increase the proliferative rate of the cell are increasingly being found to alter the apoptotic threshold (Rudin and Thompson, 1997). The crosstalk between cell-cycle regulation and the apoptotic pathway serves as a screen for the inappropriate proliferation that typifies malignancy (Rudin and Thompson, 1997). A central factor in the functioning of this screen is the tumor suppressor gene *p53*. The p53 gene product has been reported to participate in both G1-S and G2-M checkpoints regulating cell-cycle progression and induces cell-cycle arrest in response to DNA damage, allowing repair enzymes to function (Sheikh and Fornace, 1999). However, in a cell being driven through the cell cycle by expression of cellular or viral oncogenes, a p53-dependent apoptotic response may result (Lowe et al., 1993; Symond et al., 1994). Thus, p53 appears to play a key role in

preventing propagation of potentially oncogenic variants, deleting cells that demonstrate disregulated replication by triggering apoptosis. The central nature of this mechanism in preventing outgrowth of putative transformants may explain the remarkably high frequency of inactivating p53 mutations in hematopoietic and solid tumors (Sheikh and Fornace, 1999), and the predisposition toward malignancy in patients heterozygous for p53. The loss of p53 would permit replication of these malignant cells and, by failing to enforce cell-cycle checkpoints, may contribute to a higher mutation frequency in the resulting tumor.

In addition to the role of inhibition of apoptosis in oncogenesis, it is becoming clear that the same process is responsible for the chemotherapeutic resistance of many cancers. Inactivation of p53 correlates with poor prognosis in most human malignancies (Harris and Hollstein, 1993). Using genetically defined tumors in immunocompromised mice, p53 mutation has been associated with treatment resistance to both chemotherapy and γ irradiation, and with tumor relapse (Lowe et al., 1994). Bcl-2 and bcl-x_L can both confer high-level resistance to chemotherapeutic agents of several drug classes, and to radiation (Minn et al., 1995). Bcl-2 expression correlates with poor response to chemotherapy in acute myeloid leukemia, and bcl-x_L

expression correlates with negative prognostic features in breast cancer (Campos et al., 1993). Inhibition of apoptosis confers resistance to a wide array of toxins, including DNA-damaging agents, metabolic inhibitors, microtubule assembly inhibitors, topoisomerase inhibitors, and others, leading to a reinterpretation of the way chemotherapeutic agents and ionizing radiation work in killing tumor cells. Antineoplastic therapy appears to kill cells not by direct and irreversible damage to cell structures but rather by the indirect triggering of an inherent apoptotic pathway. The mechanical damage to the cell, such as DNA strand breaks introduced by ionizing radiation, are not inherently fatal and can be repaired if apoptosis is prevented.

1.6 Antiapoptotic compounds

1.6.1 (R)-Deprenyl

1.6.1.1 Inhibition of monoamine oxidase-B

(R)-deprenyl is an irreversible inhibitor of monoamine oxidase (MAO)-B frequently used as an adjuvant therapy in the treatment of Parkinson's disease (PD) (for review see Berry et al., 1994). It is

commonly believed that (R)-deprenyl provides symptomatic relief by improving dopaminergic neurotransmission. (R)-deprenyl treatment was found to result in a small to moderate decrease in levodopa requirement and to decrease levodopa induced "on-off" disabilities (Lees et al., 1977; Yahr et al., 1983). The basis for these clinical benefits is uncertain. Human dopaminergic neurons contain MAO-A rather than MAO-B (Westlund et al., 1985) so that intra-neuronal dopamine metabolism should be reduced by MAO-A inhibition, and not by MAO-B inhibition. Furthermore, although chronic (R)-deprenyl treatment has been shown to decrease striatal dopamine metabolism in primates (Kaseda et al., 1999), acute administration of (R)-deprenyl does not alter dopamine metabolism in the monkey Substantia nigra pars compacta (SNc) (Paterson et al., 1995) and microdialysis studies do not reveal any acute effect of (R)-deprenyl on extracellular dopamine concentrations in the striatum of rat brain (Butcher et al., 1990). Chronic treatment with (R)-deprenyl results in a loss of MAO-B specificity and inhibition of MAO-A (Felner and Waldmeier, 1979). Taken together, the above observations have raised the possibility that the clinical benefits found for combined levodopa and (R)-deprenyl treatment result from MAO-A inhibition rather than MAO-B inhibition.

Deprenyl may reduce oxidative radical damage by inhibiting MAO-B. The finding that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced PD-like deficits in animals and humans (Langston et al., 1983, 1984; Burns et al., 1984) opened the door to a second possible therapeutic benefit of (R)-deprenyl in PD, namely protection of neurons from damage caused by reactive oxygen species (ROS). MPTP has been shown to be converted to 1-methyl-4-phenylpyridinium (MPP^+) by MAO-B in astroglia (Chiba et al., 1984), and MPP^+ is accumulated in catecholaminergic neurons by the dopamine transporter (Chiba et al., 1984). In neurons, it inhibits NADH dehydrogenase (Nicklas et al., 1985; Mizuno et al., 1987; Ramsay et al., 1987) of complex I of the mitochondrial respiratory chain, which results in a decrease in ATP levels (Di Monte et al., 1986). Reduction in ATP levels can promote an increase in cytosolic Ca^{2+} and dopamine release from synaptic vesicles (Chang and Ramirez, 1986), both of which lead to ROS production resulting from oxidation of free dopamine (Chiueh et al., 1994). Furthermore, mitochondrial respiratory chain failure in the presence of increased Ca^{2+} levels results in ROS production (Dykens, 1994; Fernandez-Checa et al., 1998). High ROS levels promote nucleic acid, protein and lipid peroxidation with resultant apoptosis (Gibb et al., 1988). Since (R)-deprenyl is a MAO-B inhibitor, this suggested that the

protective action of (R)-deprenyl was the result of a prevention of MPTP conversion to MPP^+.

1.6.1.2 Antiapoptotic actions of (R)-deprenyl

Though circumstantial evidence and speculation regarding (R)-deprenyl's ability to spare neurons has existed in the literature for about 20 years, it has only been in the last 6-7 years that researchers have specifically examined the phenomenon in in vitro and in vivo models. Much of this interest is attributable to the work of Tatton and his co-workers who observed that (R)-deprenyl prevents neurodegeneration in the MPTP model of Parkinsonism (Tatton and Greenwood, 1991; Salo and Tatton, 1993). They discovered that (R)-deprenyl increases Substantia nigra pars compacta (SNc) neuronal survival by a mechanism independent of MAO-B inhibition, and proposed that (R)-deprenyl may be slowing the progression of PD by rescuing neurons.

Stemming from Tatton and Greenwood's findings, a number of other studies employing in vitro and in vivo models have been published regarding the nature of (R)-deprenyl's ability to increase neuronal survival. This effect of (R)-deprenyl has been shown in vitro with PC12 cells (Tatton et al., 1994) , mescencephalic dopaminergic neurons (Roy and Bedard,

1993; Mytilineou et al., 1997a), cultured cerebellar granule cells after adding high concentrations of the mitotic inhibitor cytosine arabinoside (Ara-c) (Paterson et al., 1998) and in vivo in the rat hippocampus following hypoxia-ischemia (Paterson et al., 1997a), dopaminergic neurons in the Substantia nigra following MPTP treatment (Tatton and Greenwood, 1991; Yu et al., 1994), and in facial motoneurons following facial nerve axotomy (Salo and Tatton, 1992; Ansari et al., 1993). In all of these studies, it has been shown that MAO-B inhibition is not required for the neuronal sparing effect. Most of the insults described above for neuronal death models that are R-deprenyl responsive have been found to induce apoptosis. Prominent examples include MPTP (Le et al., 1997; Tatton and Kish, 1997; Klevenyi et al., 1999; Saporito et al., 1999), hypoxia-ischemia (Tamatani et al., 1998; MacManus et al., 1999), motoneuron axotomy (Rossiter et al., 1996; Baba et al., 1999) and Ara-c treated cerebellar granule cells in culture (Dessi et al., 1995). The antiapoptotic action of deprenyl is highly stereospecific: (R)-deprenyl can prevent apoptosis of facial motoneurons at low doses, whereas (S)-deprenyl is without anti-apoptotic effect, even at high doses (Ansari et al., 1993; Paterson et al., 1998).

Administration of kainic acid, an excitotoxin, to rats results in seizure activity, which originates in the hippocampus (Sperk, 1994). If

these seizures are sufficiently severe then apoptotic cell death can be demonstrated in the hippocampus during the 5 days following kainic acid administration (Gelowitz and Paterson, 1999). When administered 5 h after the onset of seizure activity and then daily for a further 5 days, (R)-deprenyl significantly improved the survival of CA3 neurons. Again, this effect was stereospecific and was not related to a decrease in epileptogenic activity; 5 h after kainic acid administration, seizure activity had almost completely abated (Gelowitz and Paterson, 1999). Further, it was suggested that treatment for 14 days after kainic acid was sufficient to give a permament functional recovery (Gelowitz and Paterson, 1999). This suggests that (R)-deprenyl has a true neural rescue effect.

The antiapoptotic mechanisms of (R)-deprenyl are under investigation. In relation to the MPTP model, apoptosis induced by MPTP or MPP^+ is associated with a loss of $\Delta\Psi m$ (Chalmers-Redman et al., 1999) and MPT pore opening (Cassarino et al., 1999), likely due to reduced proton pumping at complex I. A reduction in $\Delta\Psi m$, similar to that caused by a partial loss of complex I activity, has been shown to induce MPT pore opening (Scorrano et al., 1997b). Accordingly, an initial partial loss of $\Delta\Psi m$ caused by MPP^+-induced complex I inhibition induces MPT pore opening and a resultant collapse of $\Delta\Psi m$ due to dissipation of the

transmembrane proton gradient. Proteins or agents which facilitate MPT pore closure and maintain $\Delta\Psi m$, like bcl-2 (Marzo et al., 1998), reduce apoptosis caused by MPTP or MPP^+ (Yang et al., 1998; Cassarino et al., 1999).

(R)-deprenyl has been found to reduce apoptosis through maintenance of $\Delta\Psi m$ (Wadia et al., 1998). The basis for the action of (R)-deprenyl on $\Delta\Psi m$ is uncertain but may depend on the induction of synthesis of proteins like bcl-2 (Tatton and Chalmers-Redman, 1996; Paterson and Tatton, 1998). Agents that block either transcription or translation prevent (R)-deprenyl from reducing apoptosis (Tatton et al., 1994). It therefore seems that changes in new protein synthesis induced by (R)-deprenyl include increases in the synthesis of proteins that facilitate MPT pore closure.

Kragten et al. (1998) first showed that deprenyl-related compounds bind to GAPDH and postulated the GAPDH binding was responsible for the anti-apoptotic action of (R)-deprenyl. It therefore seems that binding of (R)-deprenyl to GAPDH reduces the capacity of the protein to contribute to the series of signaling events in some forms of apoptosis.

Based on these studies, it can be concluded that (R)-deprenyl spares neurons in a manner independent of MAO-B inhibition, at least in part, from a capacity to slow neuronal apoptotic death.

1.6.2 Aliphatic propargylamines

1.6.2.1 Inhibition of MAO-B

In 1989, Yu showed that aliphatic amines were excellent substrates for MAO-B (Yu, 1989). By analogy with (R)-deprenyl, a large number of propargyl analogs of these amines were synthesized and tested for MAO-B inhibitory activity (Yu et al., 1992). These compounds were shown to be good MAO-B inhibitors. R-2HMP has become the standard aliphatic propargylamine to which other compounds are compared.

R-2HMP was initially developed as a novel, highly potent inhibitor of MAO activity with selectivity toward MAO-B. This compound showed a selectivity for MAO-B inhibition, with MAO-B being inhibited at 1000-fold lower concentrations than MAO-A (Yu et al., 1992). In vivo R-2HMP maintains MAO-B-inhibitory selectivity following either oral or intraperitoneal administration. In mice, oral administration of a high dose (10 mg/kg) of racemic 2HMP results in a selective inhibition of brain

MAO-B activity (Yu et al., 1994). Intraperitoneal administration of racemic 2HMP gives an ED_{50} of 0.5 mg/kg for inhibition of brain MAO-B (Yu et al., 1992).

1.6.2.2 Antiapoptotic activity

The primary in vitro screen for antiapoptotic activity of aliphatic propargylamines has been using primary cerebellar granule cell (CGC) cultures. This is a very well defined system (Yan and Paul, 1997), which can be induced to undergo apoptosis by a number of factors (Enokido et al., 1996a,b; Scorziello et al., 1997; Yan and Paul, 1997; Berry, 1999a). Cytosine arabinoside (Ara-c) (Dessi et al., 1995) results in the induction of a p53-dependent pathway (Enokido et al., 1996a,b), while lowering media K^+ levels to physiological concentrations (Taylor et al., 1997) induces a p53-independent pathway (Enokido et al., 1996a,b).

Under such conditions, it was shown that (R)-deprenyl, but not (S)-deprenyl, protected CGCs from apoptosis induced by Ara-c but not low K^+ (Paterson et al., 1998). Similarly R-2HMP resulted in a dose-dependent protection of CGCs from Ara-c induced apoptosis but was completely without effect on low K^+-induced apoptosis. S-2HMP, like (S)-deprenyl,

was completely devoid of any antiapoptotic activity over a dose range of 10 μM to 10 nM (Paterson et al., 1998). S-2HMP was, however, shown to dose-dependently antagonize the effects of R-2HMP, and also antagonized the antiapoptotic effects of (R)-deprenyl with an identical dose-response curve (Paterson et al., 1998). Similar effects were observed with (S)-deprenyl (Paterson et al., 1998). The above results suggested the existence of a specific pharmacological binding site through which R-2HMP exerts its antiapoptotic actions. Furthermore, this would appear to be the same site through which R-deprenyl is active.

Hippocampal slices briefly incubated under hypoxic/hypoglycemic conditions show an increase in apoptotic nuclei (Small et al., 1995). A 10-min hypoxic/hypoglycemic insult, followed by incubation for 4 h in artificial CSF in the presence or absence of R-2HMP, has shown that R-2HMP results in a significant increase in cell survival as assessed by confocal microscopy following staining with a LIVE/DEAD kit (Zuo et al., 1997; Yu et al., 1998). This effect showed stereospecificity being restricted to the R isomer (Yu et al., 1998). Additional studies in the hippocampal slice preparation where [^{3}H]thymidine incorporation was measured are in agreement with the above neuroprotective effect. In these experiments, an increase in [^{3}H]thymidine incorporation was observed in R-2HMP treated

slices following hypoxic/hypoglycemic insult (Zuo et al., 1997; Yu et al., 1998). Such an effect is consistant with an increase in DNA-repair mechanisms in R-2HMP treated slices, although the possibility that the effect is due to a proliferation of nonneuronal cells in the slice has yet to be examined.

Thymocytes represent a nonneuronal population which can be induced to undergo p53-dependent and independent forms of apoptosis (Clarke et al., 1993).The effects of R-2HMP were examined on dexamethasone-induced apoptosis in cultured thymocytes. These experiments revealed R-2HMP to be without effect (Yu et al., 1998). This was not surprising since dexamethasone induces a p53-independent apoptosis (Clarke et al., 1993). Subsequent experiments in cultured thymocytes have examined the effects of γ-irradiation-induced apoptosis, which is p53-dependent (Lowe et al., 1993). Surprisingly, R-2HMP was unable to protect thymocytes from undergoing apoptosis in response to γ-irradiation (Yu et al., 1998). This suggests that at least two forms of p53-dependent apoptosis exist, which can be distinguished between by R-2HMP.

Neuroprotective/neural rescue effects of R-2HMP have also been demonstrated in vivo. DSP-4 [*N*-(-chloroethyl)-*N*-ethyl-2-

bromobenzylamine] is a highly potent and selective toxin of the locus coeruleus noradrenergic system (Fritschy and Grzanna, 1989). R-2HMP has been shown to at least partially protect against DSP-4 toxicity in vivo (Yu et al., 1994; Zhang et al., 1996). This effect can be demonstrated both neurochemically (Yu et al., 1994) and morphologically (Zhang et al., 1995, 1996). These protective effects are not due to an interaction with noradrenergic uptake systems (Yu et al., 1994), nor due to MAO-B-inhibitory activity (Yu et al., 1994). Furthermore, there is evidence for a neural rescue effect. R-2HMP administered after DSP-4 administration continues to increase cell survival (Yu et al., 1995).

R-2HMP has been tested in a modified Levine preparation (Paterson et al., 1997b). In this model, R-2HMP exhibited a dose-related protection of CA1 neurons following subcutaneous administration (Paterson et al., 1997b). These effects were stereospecific with S-2HMP being devoid of activity (Paterson et al., 1997b). The neuronal death in this model has been shown to be apoptotic (Paterson et al., 1997b). Similarly, R-2HMP has recently been shown to rescue neurons in a unilateral focal ischemia model when administered either orally or i.p. 3 hrs following lesion (Berry et al., 2000).

The effects of R-2HMP in the galactose-fed *Drosophila melanogaster* aging model have also been examined (Jordens et al., 1999). In this system, replacing sucrose in the food media with galactose results in a significant decrease in *D. melanogaster* life span (Jordens et al., 1999). Inclusion of R-2HMP in the food media, while having no effect in sucrose-fed flies, significantly increased the life span of the galatose-fed flies (Jordens et al., 1999). This is similar to the effect previously reported in other aging models for R-deprenyl (Kitani et al., 1993; Knoll et al., 1989; Freisleben et al., 1997).

1.7 Aims of the thesis

In light of a paucity of information surrounding R-2HMP's ability to spare neurons in vitro, the present investigation was conducted in order to study this phenomenon using two modes of apoptosis in cultured cerebellar granule cells (CGCs): Ara-c induced and low K^+-induced apoptosis. The experiments addressed the following questions:

1.does R-2HMP spare neurons from Ara-c-induced or low K^+-induced apoptosis in cultured CGCs?

2. since Ara-c-induced apoptosis is p53-dependent, and low K^+-induced apoptosis is p53-independent, what are the differences between these two apoptotic pathways in the same cell type?

3. at which level of the apoptotic cascade is R-2HMP active at?

2. METHODS

2.1 Animals

All procedures involving animals were performed with the approval of the University of Saskatchewan Animal Care Committee, in accordance with the guidelines of the Canadian Council on Animal Care and the National Institute of Health. Female timed pregnant Wistar rats were obtained from Charles River (Montreal, Canada), housed individually at a constant temperature on a 12-hr light-dark cycle, with free access to food and water. Animals were inspected daily, and the date of birth of pups was recorded.

2.2 Cell culture

2.2.1 CGC culture as a model of neuronal apoptosis

Cerebellar granule neurons, the principal interneurons of the cerebellum, are among the most abundant neuronal phenotype in the mammalian CNS (Ito, 1984). These neurons contain glutamate as their primary neurotransmitter and express both NMDA and non-NMDA glutamate receptors on their dentrites and perikarya (Garthwaite et al., 1986; Burgoyne and Cambray-Deakin, 1988). During cerebellar

development, granule neurons are generated postnatally in the external germinal layer where they differentiate, migrate in approximately 3 days to the granule neuron layer and are finally innervated by mossy fiber axons (Altman, 1972). There is evidence that mossy fiber axons from the dorsal pontine nuclei of the brainstem, which innervate cerebellar granule neurons, utilize acetylcholine as a primary neurotransmitter (Ito, 1984). Granule neurons in turn innervate Purkinje cell dendrites via their long, parallel fiber axons (Ito, 1984). Postmitotic granule neurons can be readily maintained in vitro in their fully differentiated state for several weeks if depolarized with high concentrations of K^+ (25 mM) (Gallo et al., 1987) or by exposure to the excitatory amino acids, glutamate, or NMDA (Balazs et al., 1988). Given the relative homogeneity of these cultures, and the ease with which apoptosis can be reliably induced, cultured cerebellar granule neurons have proven to be a fascile in vitro model system for studying neuronal apoptosis.

Cultured cerebellar granule cells can be induced into apoptosis by several mechanisms, including aging related excitotoxicity induced by NMDA (Ishitani et al., 1996b), high-K^+ deprivation (D'Mello et al., 1993), high doses of Ara-c (Ishitani and Chuang, 1996), staurosporine (Taylor et al., 1997), β-amyloid (Estus et al., 1997) or ethanol (Zhang et al., 1998).

2.2.2 CGC preparation

Pups were removed from the mother on post-natal day 7 and used immediately for the preparation of primary cerebellar granule cell cultures. Cultures were prepared by a modification of the method of Paterson et al. (1998). Pups were decapitated, and the skull cap and meninges carefully removed. Cerebelli were aseptically removed and placed in a culture dish containing Solution A (phosphate-buffered saline, pH 7.4, containing 1mM Mg^{2+}, 13mM glucose, and 0.3% [w/v] bovine serum albumin). Dissected cerebelli were transferred to a dry culture dish, finely diced and suspended in 5 ml of Solution A. This suspension was centrifuged at 300 g × 1 min, the supernatant carefully removed and the pellet resuspended in 8 ml Solution B (Solution A + 0.025% [w/v] trypsin). The suspension was incubated at 37°C in a 5% CO_2 atmosphere for 20 min with occasional inversion, 4 ml Solution C (Solution A + 0.006% [w/v] DNase I + 0.06% [w/v] soybean trypsin inhibitor) added and the suspension centrifuged at 300 g × 5 min. The resultant pellet was resuspended in 1 ml Solution C, followed 5 min later by 3 ml neuron culture media (modified Eagle's medium (MEM) pH 7.4 supplemented with: 10% [v/v] fetal bovine serum, 0.8 mM glutamine, 30 mM glucose, 24 mM KCl, 80 μM 5-fluoro-5'-

deoxyuridine, 50 IU penicillin, 50 μg streptomycin). The suspension was centrifuged at 300 g × 5 min and the pellet resuspended in 1 ml neuron culture media. A total cell count was performed and the suspension diluted to a final concentration of 3.89×10^6 cells/ml. This suspension was aliquoted (300 μl) onto 22-mm^2 poly-L-lysine-coated German borosilicate glass coverslips (Carolina Biologicals, Burlington NC) in six-well culture dishes (Falcon, Franklin Lakes, NJ). Cultures were placed in a biological incubator (37°C, 5% CO_2) for 24 hr.

2.2.3 Drug treatment

Twenty-four hours after seeding, either neuron culture medium or low K^+ (KCl concentration 5mM) medium was added to each dish to bring the total volume to 2 ml. This was followed immediately by 20 μl of required drugs or vehicle. All drugs were dissolved in distilled water. Ara-c was added to a final concentration of 300 μM, along with R-2HMP or S-2HMP, as required, at final concentrations of 10^{-7} M and 10^{-5} M respectively. The concentrations of the enantiomers of 2HMP were chosen based on previous studies showing these to give maximum effects (Paterson et al., 1998).

Following addition of media and drugs, cultures were returned to the incubator for 24 hours.

2.3 Assessment of apoptosis

The cells were fixed with FAM fixative (40% formaldehyde, glacial acetic acid, and methanol in a ratio of 1:1:8) and mounted onto microscope slides using an aqueous mountant (Dako R; Dako, Carpinteria, CA) containing bis-benzamide (5μg/ml). The slides were stored in the dark at 4°C for at least 12 h before assessment of the level of apoptosis. Cultures were examined using a microscope with UV fluoresence (Olympus BS2) and visualized using a video camera and image analysis system (Image 1; Empix Imaging, Mississauga, Ontario, Canada). Nuclei were considered to be apoptotic if they exhibited chromatin condensation (increased brightness in a smaller area) in round, crescent, annular, or lobed structures (see Appendix I). Normal and apoptotic nuclei from at least four random fields of view, giving a total nuclear count greater than 100, were counted for each culture. Total nuclear counts for each culture were expressed as percent apoptotic nuclei, with each treatment group consisting of at least four cultures in each of three or more independent experiments.

2.4 Assessment of mitochondrial membrane potential

Recently, fluorescent probes for assessing $\Delta\Psi m$ have been developed that are retained in cells at the time of fixation and can be used to examine $\Delta\Psi m$ together with levels and localization of specific proteins or nuclear DNA fragmentation (see Macho et al., 1996). One of these dyes, chloromethyl-tetramethylrhodamine ethyl ester (CMTMR, Mito Tracker CMTMRos, Molecular Probes, Eugene, OR) enters mitochondria proportionally to the difference between the negativity of the cytoplasmic compartment and mitochondrial matrix, and the chloromethyl group reacts with thiols on proteins and peptides to form aldehyde-fixable conjugates. Once $\Delta\Psi m$ is lost, CMTMR remains bound even after permeabilization and fixation (Chen and Cushion, 1994; Calarco, 1995). Therefore, CMTMR fluoresence represents the highest level of potential difference in the mitochondria during the period of dye exposure before fixation.

CMTMR accumulation was linear with time between 10 and 40 min (See figure 2.1), so an incubation period of 30 min was chosen for these experiments.

At 0, 4, 6, 12, 18 and 24 hours after adding Ara-c or low K^+ medium, CMTMR (1μM) was added to the media and incubated at 37°C for 30 minutes. Cultures were then sequentially washed with fresh neuron culture medium for 20 minutes and warm PBS for 10 minutes (Zhang et al., 1999).

The mitochondrial membrane potential of cultures was assessed by measuring the average fluorescent intensity of CMTMR in the cytoplasm from at least 100 neurons, from 4 random fields of view, per culture. CMTMR fluorescence was induced using a blue exciter filter (wavelength 490 nm) attachment for the fluorescence microscope. The neuronal diameter (visualized with bis-benzamide) of these neurons was also measured.

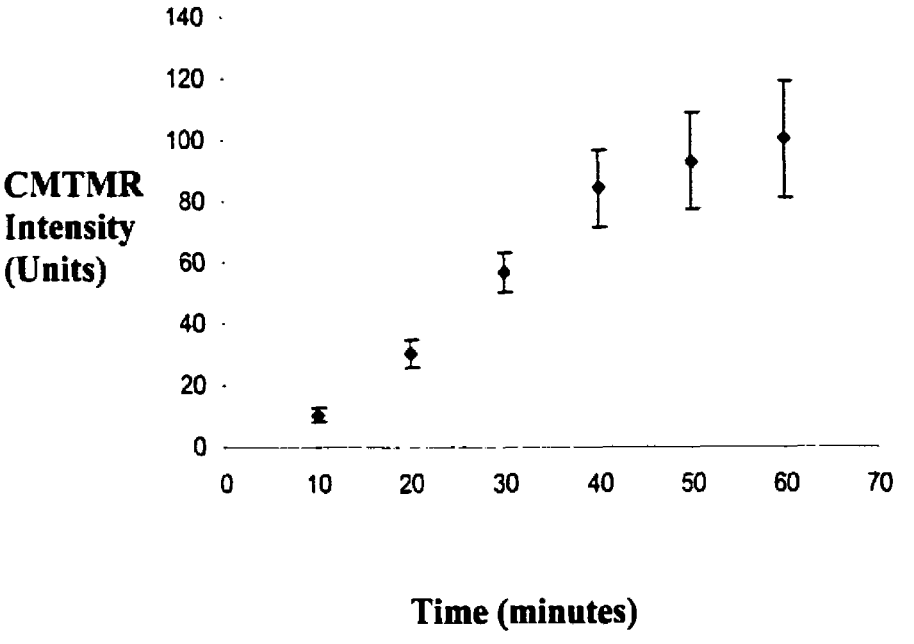


Figure 2.1 Changes in CMTMR (1 μM) accumulation with increasing duration of incubation. Data are mean ± SD (bars) value for CMTMR intensity (gray scale value) of 400-500 cells from four cultures

2.5 Assessment of GAPDH

2.5.1 Subcellular fractionation

Six, 12 and 24 hours after the induction of apoptosis, CGCs from 24 cultures were scraped, pooled and ruptured by sonication in 0.32 M sucrose. Subcellular fractions were isolated by a modification of the method of Gray and Whittaker (1962). The homogenate was centrifuged for 10 min at 1,000g to produce a pellet (P_1; nuclear), which was washed once by re-suspension in an equal volume of 0.32 M sucrose and re-centrifuged at the same speed. The original supernatant was then centrifuged at 15,000g for 25 min to produce a pellet (P_2; mitochondrial) and a supernatant (S; cytosolic). The final pellets (P_1 and P_2) were re-suspended by sonication in freezing buffer (10% glycerol, 4mM EGTA, 1 mM $MgCl_2$, 6mM Tris-HCl, pH 7.4) and, along with the supernatant, were stored at -70 °C until use.

2.5.2 Protein determinations

Protein concentration was determined by the Bradford method using the Bio-Rad protein assay kit (Hecules, CA), using bovine serum albumin

(BSA) as a standard. Basically, a series of standards were prepared containing 0, 100, 300, 500, 700, 900 μg/ml BSA, along with a series of tests containing 20 μl of samples. The absorbance of the solutions were read at 595 nm using a UV spectrophotometer. The BSA standards were used to construct a calibration curve, from which the protein content of the test samples could be determined.

2.5.3 Western blotting

Equal amounts of total protein (3 μg nuclear; 3 μg mitochondrial, 5 μg cytosolic) were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) gels as described by Laemmli (1970). After electrophoresis, the protein was electroblotted onto a nitrocellulose membrane (Bio-Rad, Hecules, CA) at 30 V for 3 hours. The membranes were then blocked in PBST (PBS + Tween 20) containing 5% nonfat milk overnight at 4°C. The membrane was probed with mouse monoclonal anti-GAPDH (1:500, Chemicon, Temecula, CA) for 2 hours at room temperature, and then washed twice for 5min each in PBS containing 1% Triton X-100 and once for 5 min in PBS. The membranes were then probed with goat biotin conjugated anti-mouse IgG (1:600, Sigma, St. Louis, MO) for 1 hour and visualized using an ABC kit

(Vector, Burlingame, CA) for 1h, followed by incubation in PBS containing 3,3′-diaminobenzidine (0.56% w/v) and 3% NiCl for 5 min. The membranes were visualized by adding H_2O_2 (30 µl).

2.5.3.1 Quantification of Western blots

Densitometry was performed by capturing images of the membranes using a VDS camera (ImageMaster VDS, Amersham Pharmacia Biotech, San Fransico, CA) and ImageQuant (Molecular Dynamics, Sunnyvale, CA) software. The linearity of the quantification with total protein is shown in figure 2.2.

2.5.4 RNA extraction and determination

Cerebellar granule cells were harvested at 1, 2 and 4 hours after drug treatments. Briefly, total cellular RNA was prepared by extraction in GITC buffer (4 M guanidinium isothiocyanate, 5 mM sodium citrate, 0.5% *N*-lauroylsarcosine, and 0.1 M ß-mercaptoethanol, pH 5.5) and collected by ultracentrifugation through 5.7 M CsCl. The RNA was chloroform-extracted, ethanol-precipitated, resuspended in diethyl pyrocarbonate

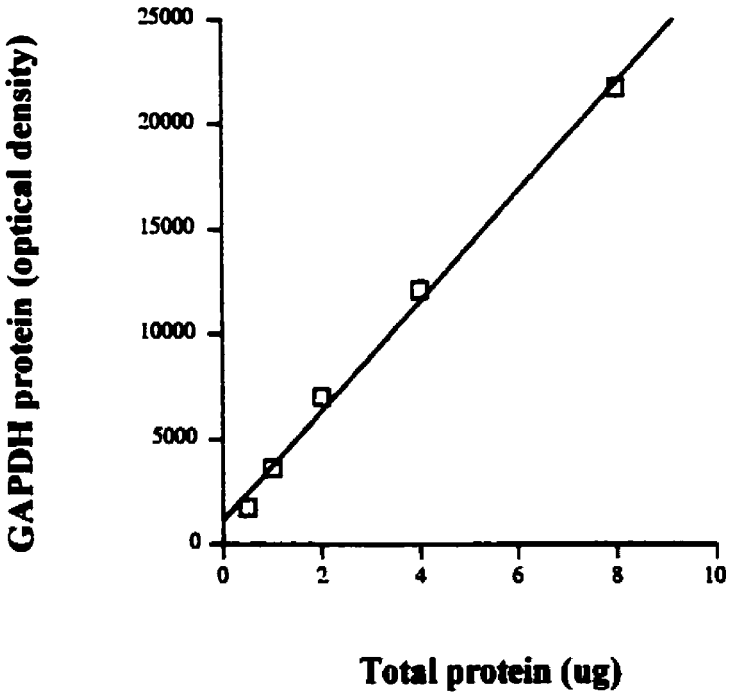


Figure 2.2 Linearity of quantification of Western blot with total protein (nuclear fraction)

(DEPC) treated water, and stored at -70°C until use. RNA concentration was measured spectrophotometrically by absorbance at 260 nm (Li. XM, et al., 1997).

2.5.5 Northern blotting

The total RNA was denatured at 65°C for 15 min in MOPS buffer (20 mM 3-(4-morpholino) propanesulfonic acid, 5mM sodium acetate, and 1 mM EDTA), pH 7.0, containing 50% formamide and 2.2 M formaldehyde.

Equal amounts (12 μg) of total RNA was loaded and separated by electrophoresis in a 1% agarose gel containing MOPS buffer. Following electrophoresis, the RNA was transferred to nylon membranes using a slot-blot manifold apparatus (Bio-Rad, Richmond, CA). RNA was cross-linked in a UV stratalinker 2400 (Stratagene, La Jolla, CA). The GAPDH cDNA probe (Clontech, Palo Alto, CA) (1.1 kb) was labeled by random primer synthesis with [α-^{32}P] dCTP. Hybridization with labeled GAPDH cDNA probe was performed at 65°C for 18 hr. After hybridization, membranes were washed at room temperature twice in 2 X SSPE (1 X=0.18 M NaCl,

0.001 M sodium phosphate, and 0.001 M EDTA)-0.1% (sodium dodecyl sulphate) SDS and once in 0.1 X SSPE -0.1% SDS at 60°C.

2.5.5.1 Quantification of Northern blots

The membranes were exposed to Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and the blots were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The linearity of the quantification with total RNA is shown in figure 2.3.

2.6 Data analysis

Apoptotic nuclei were expressed as a percentage of total nuclei for each culture and each treatment group consisted of 4 cultures. The percentage data was analysed by one-way analysis of variance and comparisons between individual groups was made using the Newman-Keuls test or student's t test. For Western and Northern blot analysis, the Newman-Keuls test or student's t test was used to compare individual groups.

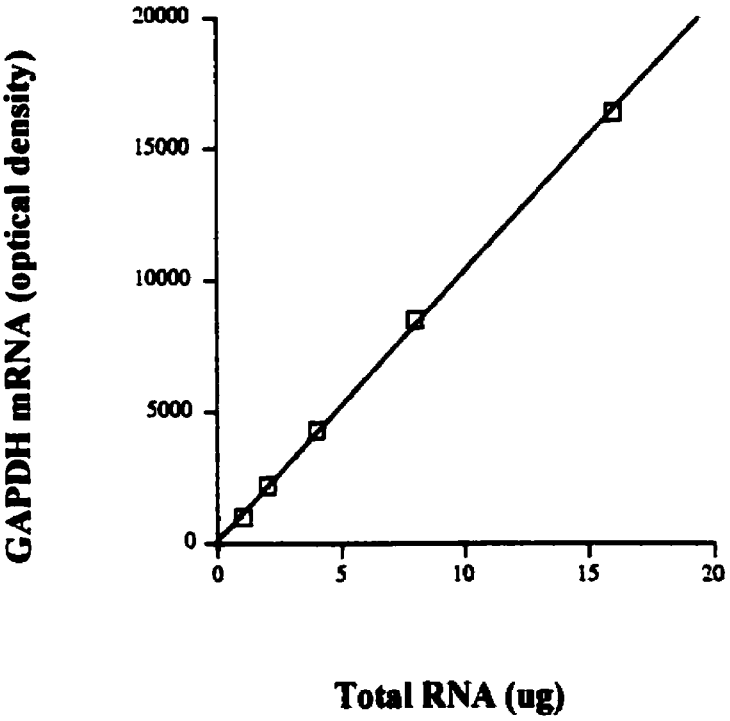


Figure 2.3 Linearity of quantification of Northern blot with total RNA

3. RESULTS

3.1 Assessment of apoptosis

3.1.1 Temporal effects of Ara-c and low K^+ induced apoptosis

The nuclei of the cells were assessed for changes associated with apoptosis as revealed by bis-benzamide staining. There was a steady increase in the number of apoptotic nuclei between 6 and 24 hours following insult. At 6 hours however, there was no difference in the number of apoptotic nuclei detected by bis-benzamide staining compared with control cultures (Figure 3.1). The validity of assessing apoptosis with bis-benzamide staining was verified by comparison to identical cultures stained with hematoxylin and eosin (H & E) or the Apoptag Kit (Oncor, Gaithersbrug, MD). No differences in the level of apoptosis detected by the three methods was observed (see also Berry, 1999a).

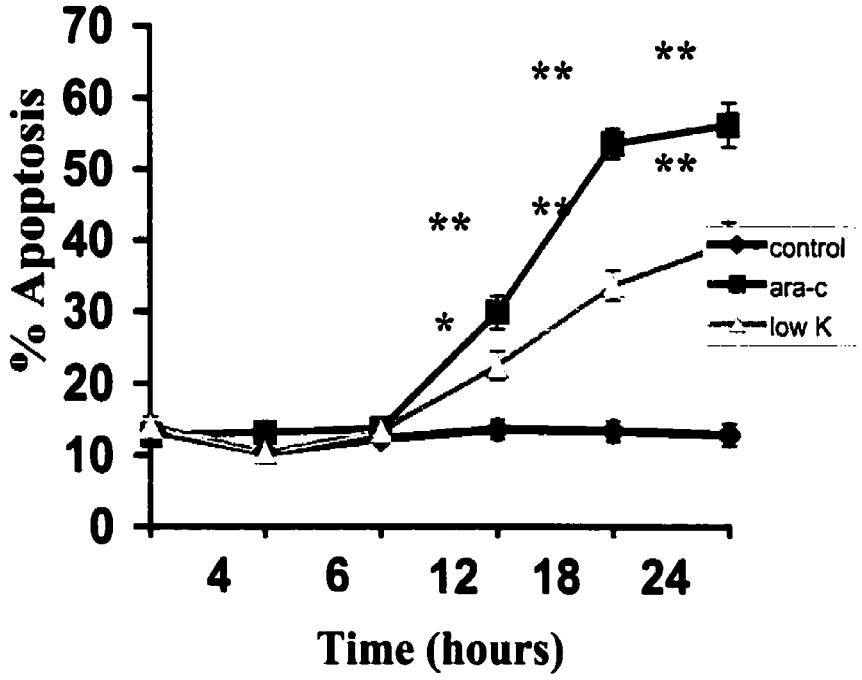


Figure 3.1. Time course of the death of CGCs exposed to Ara-c and low K^+ medium. Results are expressed as the mean ± s.e.m. from 4 cultures. * $p < 0.05$, ** $p < 0.01$ compared with corresponding time point untreated control, student's t test.

3.1.2 Effect of R-2HMP on apoptosis in CGCs

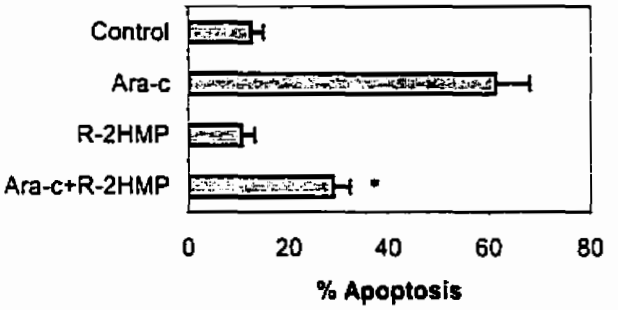
At 24 hours, control cultures of CGCs exhibited a basal level of apoptosis, of approximately 10-15%. Addition of 300 μM Ara-c increased this to 50-60% while low K^+ media induced about 30-40% apoptosis, at 24 hours (Figure 3.2). Addition of R-2HMP (10^{-7} M) substantially reduced the Ara-c-induced apoptosis (Figure 3.2, A) but had no effect on the number of apoptotic nuclei in control cultures or low K^+-induced apoptosis (Figure 3.2. B).

3.2 Assessment of mitochondrial membrane potential

3.2.1 Temporal effects on mitochondrial membrane potential

A time-dependent loss of CMTMR accumulation was seen in cultures treated with Ara-c but not those exposed to low K+ media (Figure 3.3). This reduction in CMTMR staining, which was seen in cells not showing nuclear shrinkage, can be detected as early as 6 h after adding Ara-c, but not at 4 h (Figure 3.3).

A



B

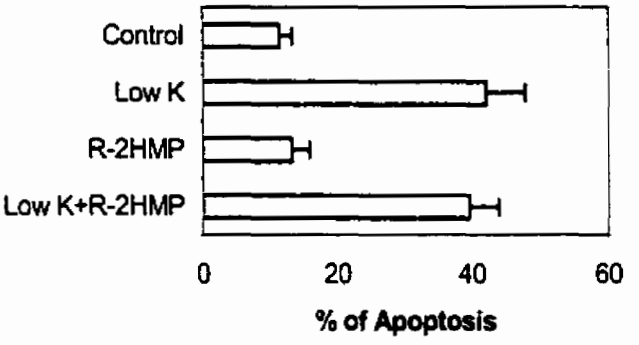


Figure 3.2. Effect of R-2HMP on apoptosis induced by 300 μM Ara-c (A) and low K^+ medium (B) at 24 hours. Percentages of apoptotic nuclei were assessed with bis-benzamide staining. Bars represent the mean ± s.e.m. of eight independent experiments (four cultures per experiments; n=32). * $p < 0.01$ with respect to the Ara-c alone condition (Newman-Keuls test).

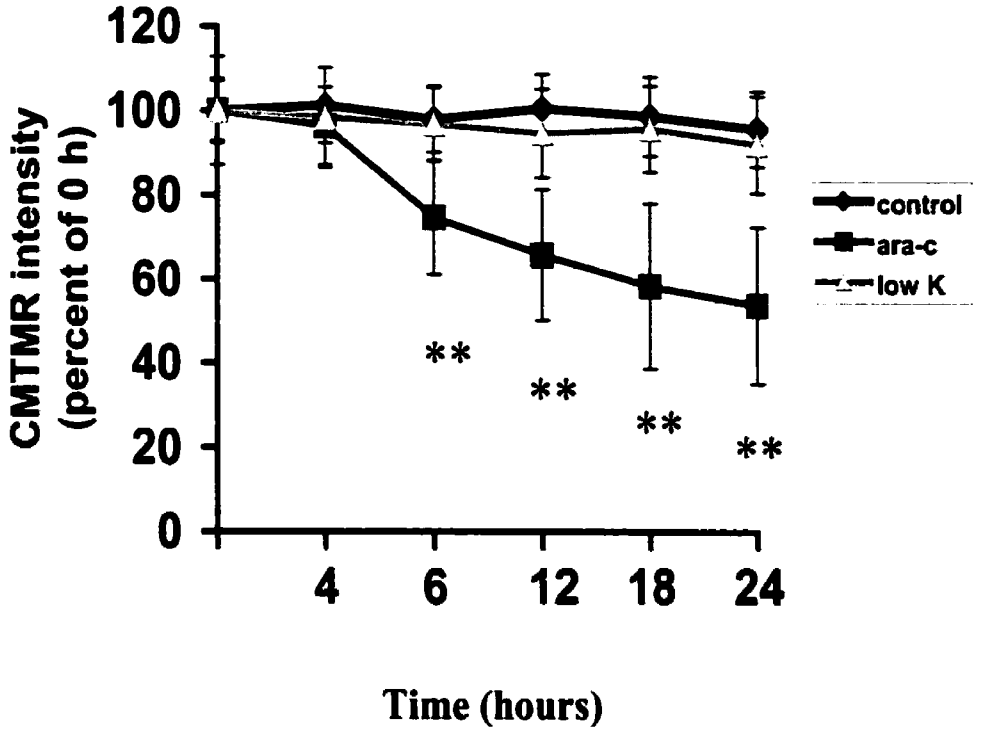


Figure 3.3. Temporal changes in CMTMR accumulation. Each point represents the mean ± S.D. CMTMR intensity (expressed as a percentage of values at 0 h) of 400-500 cells (cell populations II and III in figure 3.4) from 4 cultures. ** $p < 0.01$ with respect to 0 h, student's t test.

3.2.2 Changes of mitochondrial membrane potential at 6 hr

Six hours is the earliest time point at which we can detect a significant decrease in CMTMR accumulation in Ara-c treated cells (Figure 3.3). This time point, which precedes nuclear condensation (Figure 3.1), was therefore chosen to examine in more detail the changes in mitochondrial membrane potential occuring following Ara-c or low K^+-induced apoptosis. CMTMR fluorescence was measured in the cytoplasm of all cells in 4 random fields of view per culture (n=4 cultures). The nuclear diameter of the same cells was also measured. As can be seen from figure 3.4, three distinctive populations of cells can be observed. I: Cells with reduced nuclear diameter and mitochondrial membrane potential (apoptotic). II: Cells with normal nuclear diameter and mitochondrial membrane potential (normal). III: Cells with normal nuclear diameter but reduced mitochondrial membrane potential (pre-apoptotic). In Ara-c treated cultures (Figure 3.4B), we can detect a downward shifting of CMTMR staining in cells with normal nuclear morphology (cell population III) as compared with control cultures (Figure 3.4A). There was no such effect in low K^+ (Figure 3.4C) treated cultures.

3.2.3 Effects of R-2HMP on mitochondrial membrane potential and apoptosis

R-2HMP reduces Ara-c induced apoptosis (Figure 3.2) and causes a shifting of cell population III to II at 6 hours (Figure 3.5) and cell populations I and III to II at 24 hours (Figure 3.6). R-2HMP had no effect on changes of mitochondrial membrane potential in low K+ treated cultures (Table 3.1).

3.2.4 Enantiomeric antagonism of the effects of R-2HMP

The anti-apoptotic effect of R-2HMP (10^{-7} M) is known to be antagonised by S-2HMP at 10^{-5} M (Paterson et al., 1998). Addition of S-2HMP antagonised the effect of R-2HMP on mitochondrial membrane potential in Ara-c treated cultures (Table 3.1). S-2HMP alone had no effect on mitochondrial membrane potential (Table 3.1).

Figure 3.4

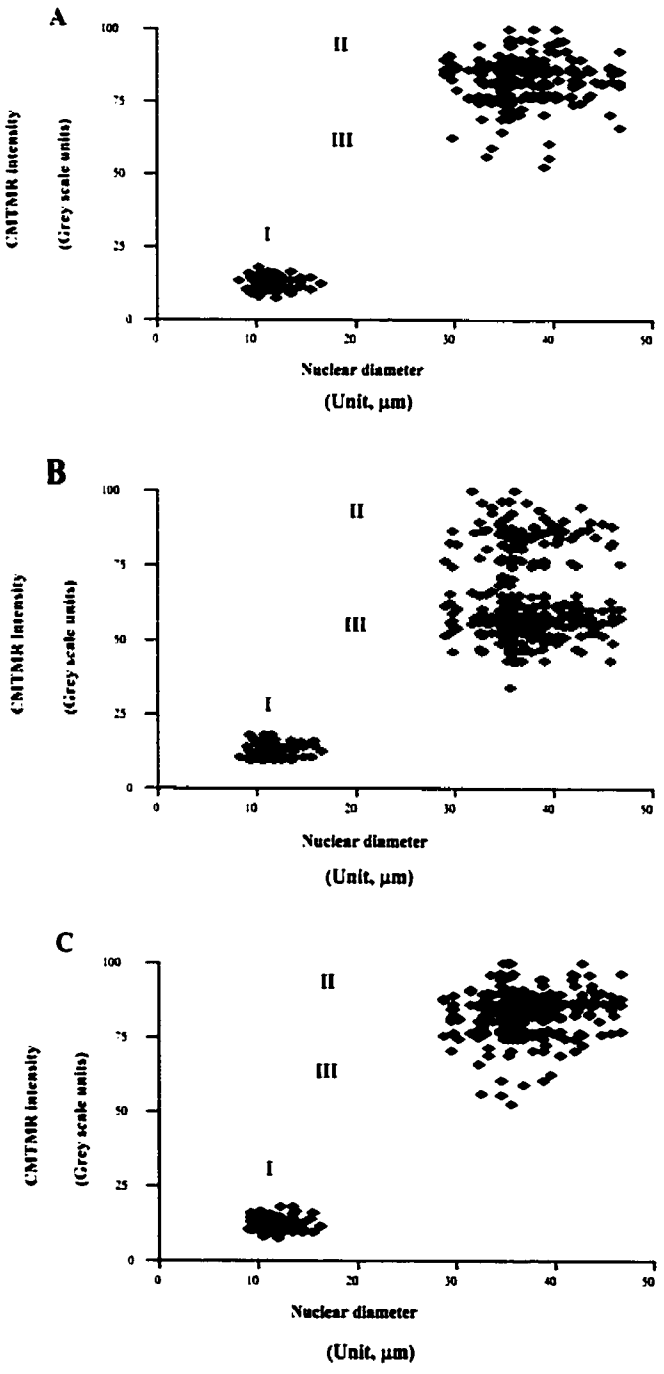
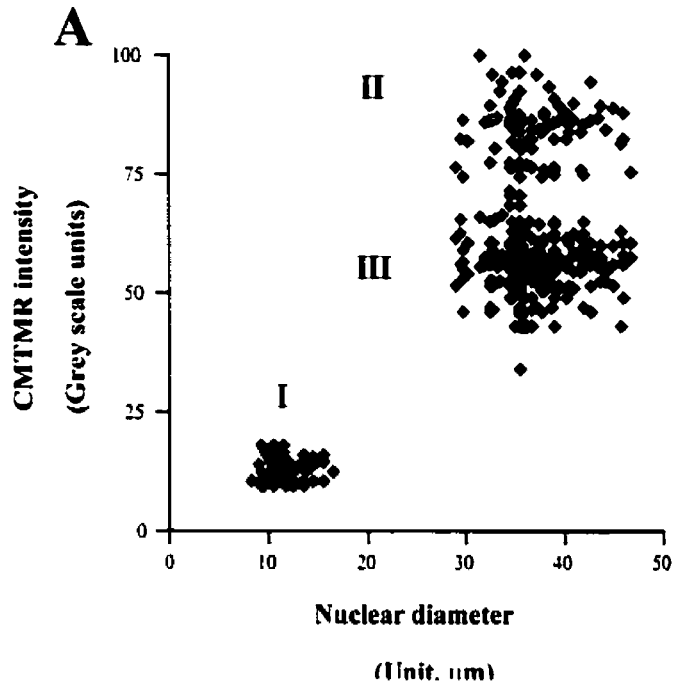

A
B
C
I
II
III
CMTMR intensity
(Grey scale units)
Nuclear diameter
(Unit, μm)

Figure 3.4. CMTMR intensity and nuclear diameter in control (A), Ara-c (B) and low K^+ (C) treated cells at 6 hours. CMTMR fluorescence was measured in the cytoplasm of all cells in 4 random fields of view per culture (n=4). The nuclear diameter of the same cells was also measured. Three distinctive populations of cells can be observed. I: Cells with reduced nuclear diameter and mitochondrial membrane potential (apoptotic). II: Cells with normal nuclear diameter and mitochondrial membrane potential (normal). III: Cells with normal nuclear diameter but reduced mitochondrial membrane potential (pre-apoptotic). Each point represents one cell. The percentage of cells in population I of total cells as follows: Control, 11.3%; Ara-c, 10.6%; Low K+, 9.8%. Population II: Control, 87.9%; Ara-c, 41.8%; Low K+, 88.1%. Population III: Control, 0.8%; Ara-c, 47.6%; Low K+ 2.1%.

Figure 3.5


A
100
75
50
25
0
II
III
I
CMTMR intensity
(Grey scale units)
0
10
20
30
40
50
Nuclear diameter
(Unit. um)

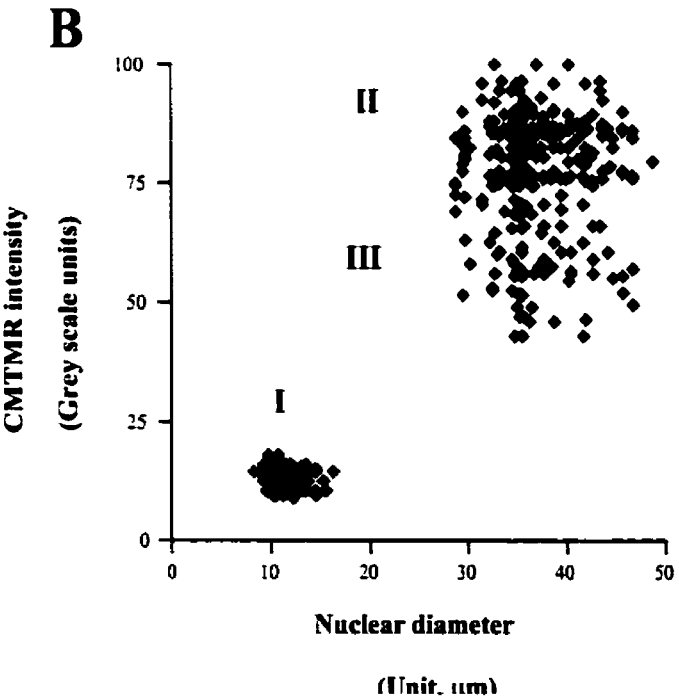

B
100
75
50
25
0
II
III
I
CMTMR intensity
(Grey scale units)
0
10
20
30
40
50
Nuclear diameter
(Unit. um)

Figure 3.5. Effects of R-2HMP on mitochondrial membrane potential in Ara-c treated cells at 6 hours. Each point represents one cell. A=Ara-c B=Ara-c + R-2HMP. CMTMR intensity and nuclear diameter were measured as previously described. The percentage of cells in population I of total cells as follows: Ara-c, 10.6%; Ara-c + R-2HMP, 12.1%. Population II: Ara-c, 41.8%; Ara-c + R-2HMP, 63.5%. Population III: Ara-c, 47.6%; Ara-c + R-2HMP 24.4%.

Figure 3.6

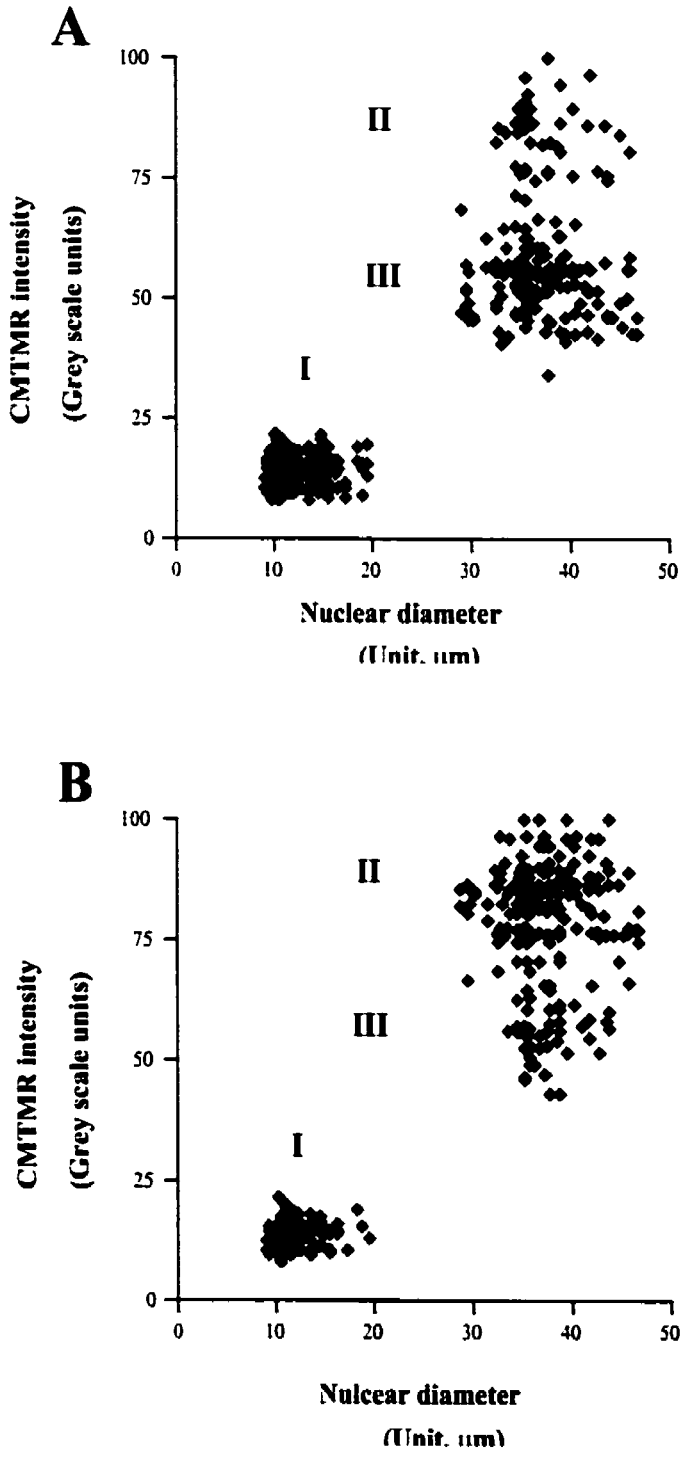

A
100
75
50
25
0
II
III
I
CMTMR intensity
(Grey scale units)
0
10
20
30
40
50
Nuclear diameter
(Unit. um)
B
100
75
50
25
0
II
III
I
CMTMR intensity
(Grey scale units)
0
10
20
30
40
50
Nulcear diameter
(Unit. um)

Figure 3.6. Effects of R-2HMP on mitochondrial membrane potential in Ara-c treated cells at 24 hours. Each point represents one cell. A=Ara-c B=Ara-c + R-2HMP. CMTMR intensity and nuclear diameter were measured as previously described. The percentage of cells in population I of total cells as follows: Ara-c, 55.3%; Ara-c + R-2HMP, 32.1%. Population II: Ara-c, 21.4%; Ara-c + R-2HMP, 54.1%. Population III: Ara-c, 47.6%; Ara-c + R-2HMP 13.8%.

Table 3.1. Effect of R-2HMP on CMTMR Accumulation 6 hr After the Induction of Apoptosis

Treatment	Control	Ara-c (300 μM)	Low K^+
Saline	83 ● 7.1	62 ● 13.5**	80 ± 10.5
R-2HMP	82 ± 8.2	78 ● 11.5^{++}	77 ± 9.4
S-2HMP	78 ± 9.4	64 ± 15.2**	79 ± 8.7
R+S-2HMP	79 ± 8.8	65 ± 14.3**	81 ± 11.8

Only cells with normal nuclear morphology (corresponding to cell populations II & III) are included. Values represent average CMTMR fluorescence intensity (grey scale units) mean ± S.D., n=4 cultures, 100-150 cells per culture. ** $P < 0.01$ with respect to control cultures, $^{++}$ $P < 0.01$ with respect to Ara-c/saline & Ara-c/ R-2HMP (10^{-7} M) + S-2HMP (10^{-5} M), student's t test.

3.3 Assessment of GAPDH

3.3.1 Subcellular changes of GAPDH protein 6, 12 or 24 hrs after the induction of apoptosis.

Differential centrifugation was performed to generate pellets at 1000 and 15,000g , referred to as P_1 (nuclear enriched) and P_2 (mitochondrial enriched) fractions respectively. The final supernatant was designated as the S (cytosolic; soluble) fraction. Western blotting of these subcellular fractions revealed a single immunoreactive band of GAPDH with a molecular mass of approximately 38 kDa, consistent with the known molecular mass of GAPDH (Saunders et al., 1997). Ara-c treated cells were associated with an increase in GAPDH protein in nuclear and mitochondrial fractions, but not in the cytosolic fraction (Figure 3.7). There was no such effect in low K^+ treated CGCs (Figure 3.7). Twenty-four hours is the time point at which we can detect highly advanced apoptosis. At 6 hours, however, there was no difference in the number of apoptotic nuclei detected by bis-benzamide staining compared to control cultures. At this time point, there were no changes of GAPDH levels in subcellular fractions in any condition (Figure 3.8). GAPDH was, however, increased at 12 hours

(Figure 3.9) in nuclear and mitochondrial fractions following Ara-c administration. A summary of changes of sub-cellular GAPDH protein at different time points following Ara-c is seen in figure 3.10.

3.3.2 The effects of R-2HMP on subcellular changes of GAPDH protein

Cells were treated with R-2HMP (10^{-7} M) to determine if the protective effect of the drug was correlated with changes in the distribution of GAPDH protein. Figure 3.7 and 3.9 show that R-2HMP (10^{-7} M) significantly reduced the Ara-c-induced increase in GAPDH protein levels in the nuclear and mitochondrial fractions back toward the control levels. At both 12 (Figure 3.9) and 24 (Figure 3.7) hours, R-2HMP (10^{-7} M) alone had no effect on GAPDH levels. No effect of R-2HMP was observed in low K^+ treated or control cultures.

3.3.3 Enantiomeric antagonism of the effects of R-2HMP on Ara-c-induced subcellular changes of GAPDH protein at 12 hr

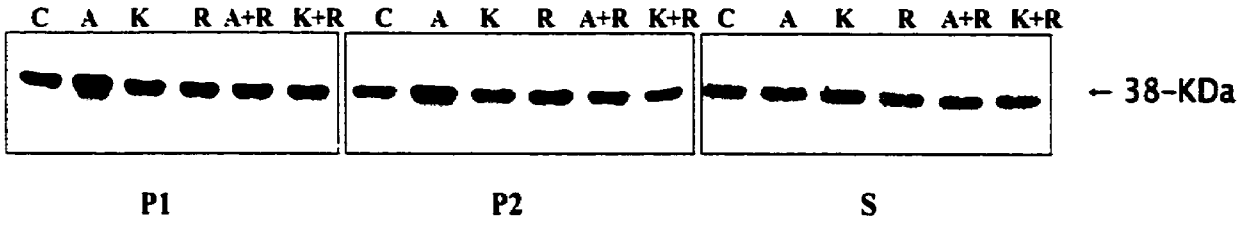
Addition of S-2HMP (10^{-5} M) antagonized the effects of R-2HMP (10^{-7} M) on Ara-c-induced subcellular changes of GAPDH 12 hours after

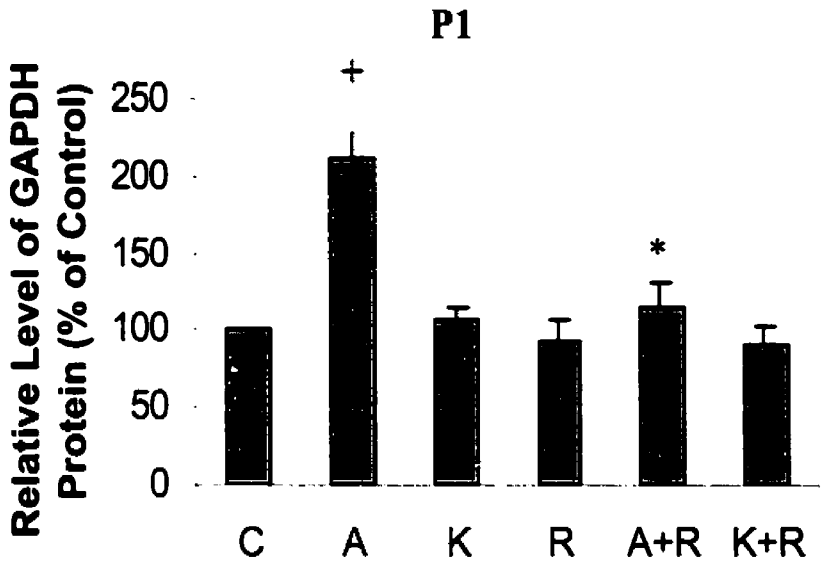
the induction of apoptosis (Figure 3.9). S-2HMP alone had no effect (Figure 3.9).

3.3.4 Effects of R-2HMP on GAPDH mRNA levels

The neuroprotective effects of GAPDH anti-sense (Ishitani and Chuang, 1996; Zhang et al., 1999) suggested that Ara-c-induced apoptosis involved an increased transcription of GAPDH. An increased level of GAPDH mRNA was detected as early as 1 hour after Ara-c treatment. At 2 hours, the levels of GAPDH mRNA had decreased to ~120% of control. The GAPDH mRNA level was close to control levels at 4 hours (Figure 3.11A). There was no change in mRNA levels in low K^+ treated cells. (Figure 3.11A). Addition of R-2HMP (10^{-7} M) effectively suppressed the mRNA increase seen at 1 hour after Ara-c treatment, but had no effect on GAPDH mRNA levels in low K^+ treated and control cultures (Figure 3.11B).

Figure 3.7


C A K R A+R K+R C A K R A+R K+R C A K R A+R K+R
← 38-KDa
P1
P2
S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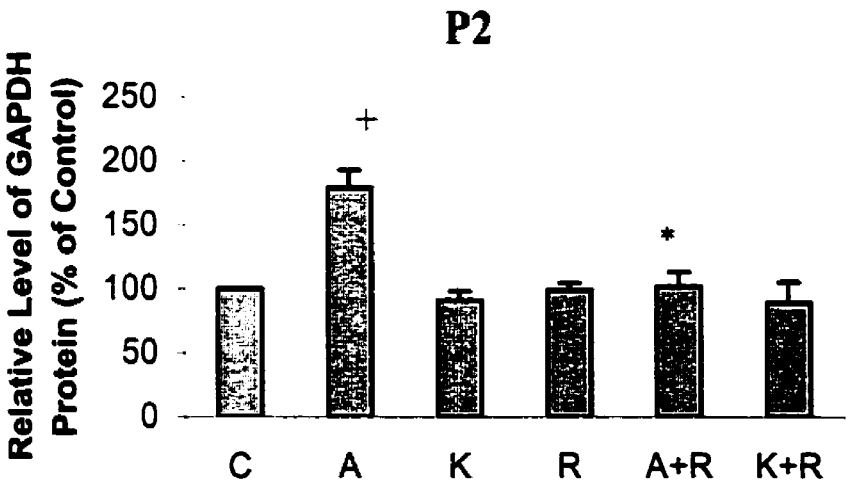
200
150
100
50
0
+
*
C A K R A+R K+R


P2
Relative Level of GAPDH Protein (% of Control)
250
200
150
100
50
0
+
*
C A K R A+R K+R

S

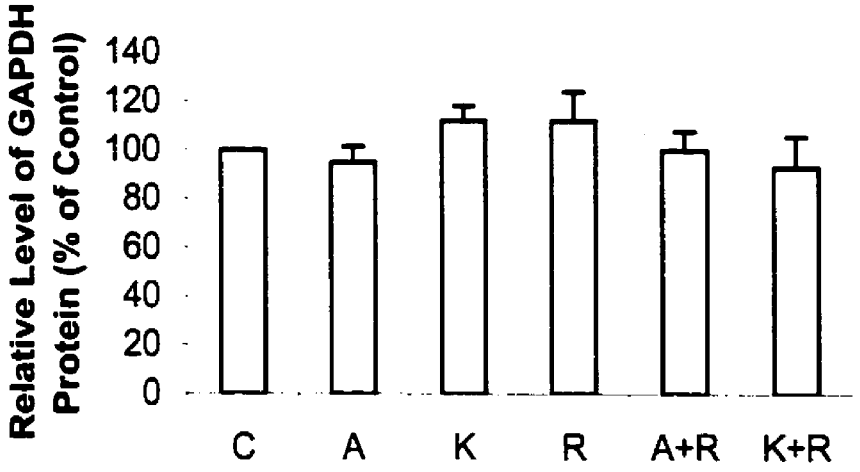
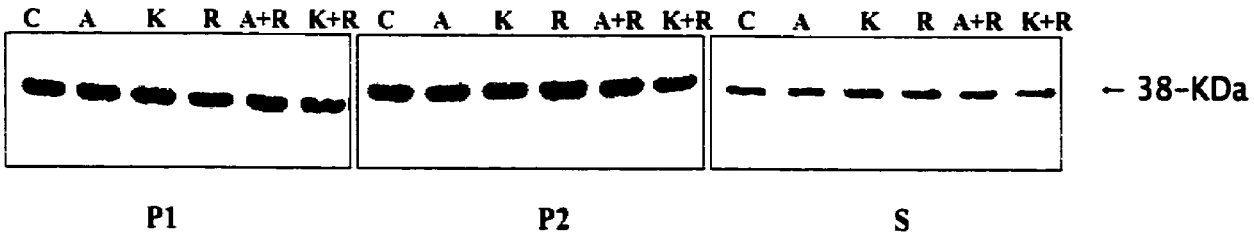


Figure 3.7 Effects of R-2HMP on Ara-c (300 μM) and low K^+ (5 mM)-induced sub-cellular changes of GAPDH protein at 24 hours. Levels of quantified GAPDH proteins are expressed as relative values compared to control. Values are mean ± s.e.m. of three independent experiments. $^+$ $p<0.01$ compared with control; * $p<0.01$ compared with Ara-c alone condition using Student's *t* test. C, control; A, Ara-c; K, low K^+; R, R-2HMP (10-7 M); A+R, Ara-c+R-HMP; K+R, low K^++R-2HMP.

Figure 3.8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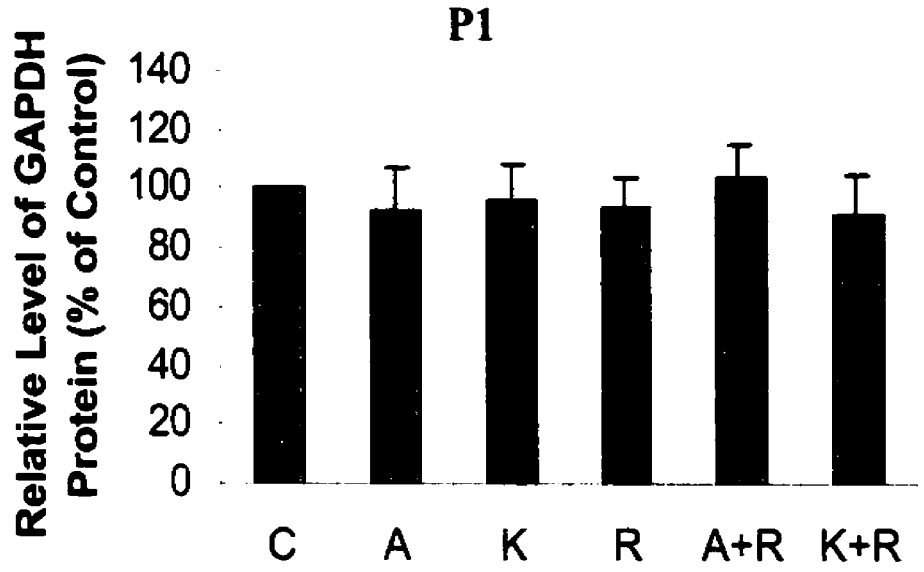
← 38-KDa
P1
P2
S


P1
Relative Level of GAPDH Protein (% of Control)
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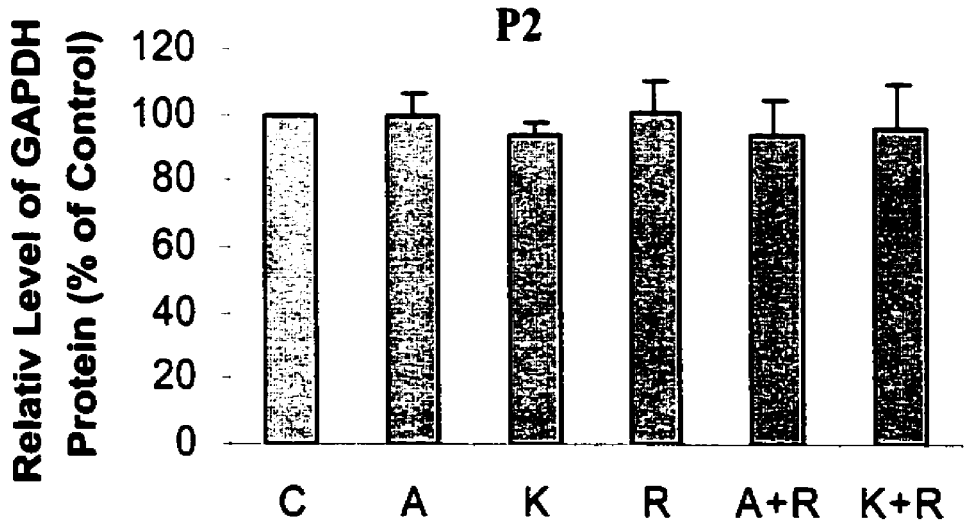
120
100
80
60
40
20
0
C A K R A+R K+R


P2
Relativ Level of GAPDH Protein (% of Control)
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100
80
60
40
20
0
C A K R A+R K+R

S

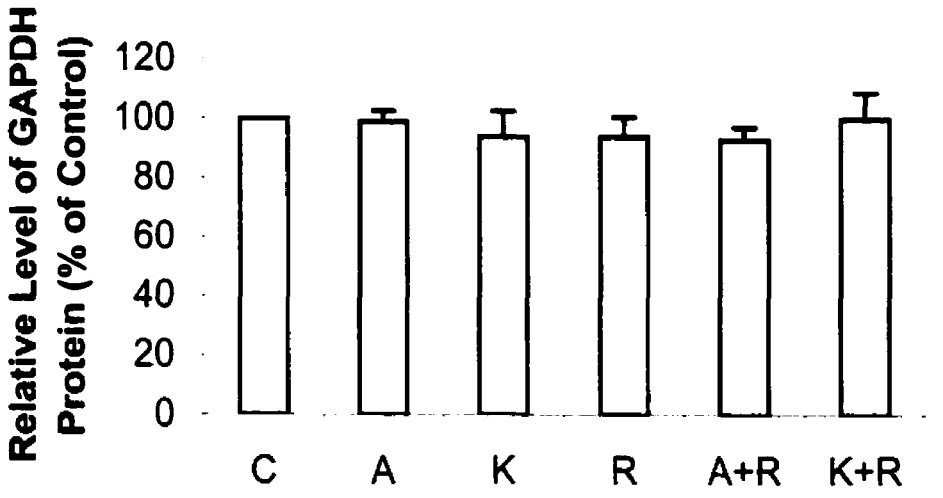
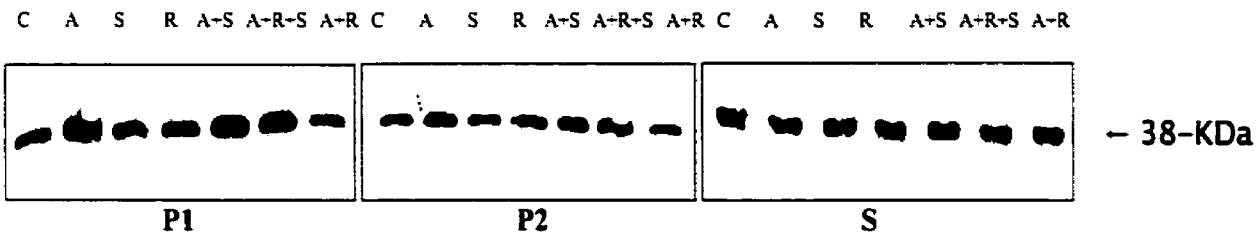
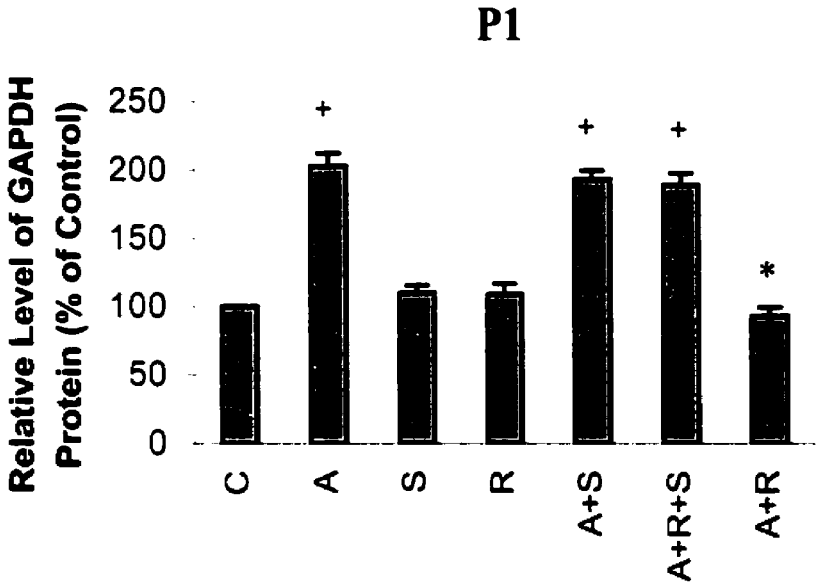


Figure.3.8 Effects of R-2HMP on Ara-c and low K^+ induced sub-cellular changes of GAPDH protein at 6 hours. Levels of quantified GAPDH proteins expressed as relative values compared with the control. Values are mean ± s.e.m. of three independent experiments. C, control; A, Ara-c; K, low K^+; R, R-2HMP (10-7 M); A+R, Ara-c+R-HMP; K+R, low K^++R-2HMP.

Figure 3.9


C A S R A+S A+R+S A+R C A S R A+S A+R+S A+R C A S R A+S A+R+S A+R
← 38-KDa
P1
P2
S


P1
Relative Level of GAPDH Protein (% of Control)
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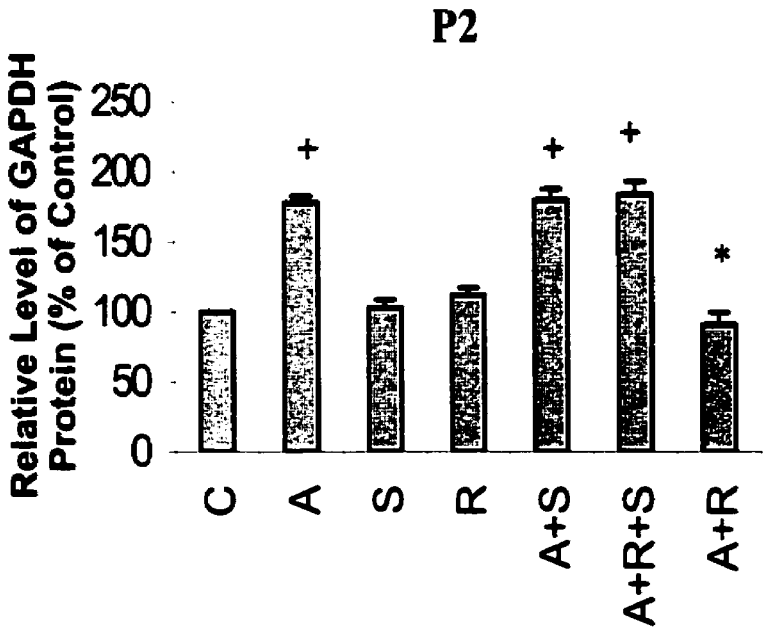
200
150
100
50
0
+
+
+
*
C
A
S
R
A+S
A+R+S
A+R


P2
Relative Level of GAPDH Protein (% of Control)
250
200
150
100
50
0
+
+
+
*
C
A
S
R
A+S
A+R+S
A+R

S

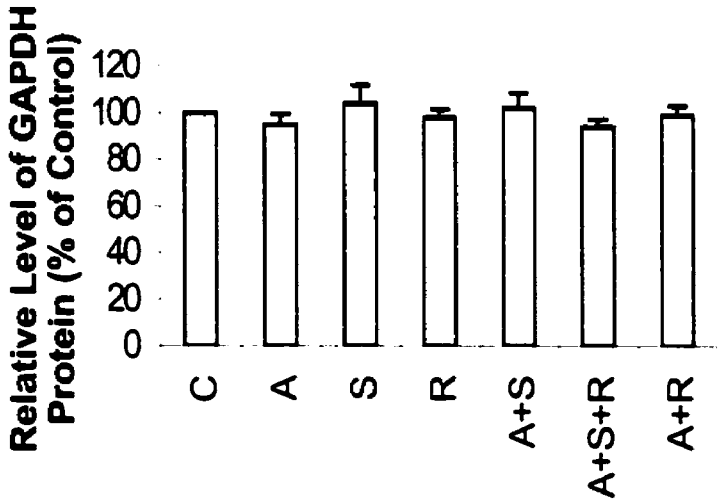


Figure 3.9 Effects of R-2HMP (10^{-7} M) and S-2HMP (10^{-5} M) on Ara-c-induced changes of GAPDH protein levels in sub-cellular fractions at 12 hours. Levels of quantified GAPDH proteins expressed as relative values compared with the control. Values are mean ± s.e.m. of three independent experiments. $^{+}$ $p<0.01$ compared with control condition, * $p<0.01$ compared with Ara-c alone, Ara-c plus S-2HMP and Ara-c plus R-2HMP, S-2HMP conditions using Student's *t* test. C, control; A, Ara-c; S, S-2HMP; R, R-2HMP; A+S, Ara-c+S-2HMP; A+R+S, Ara-c+R-2HMP+S-2HMP; A+R, Ara-c+R-2HMP

Figure 3.10

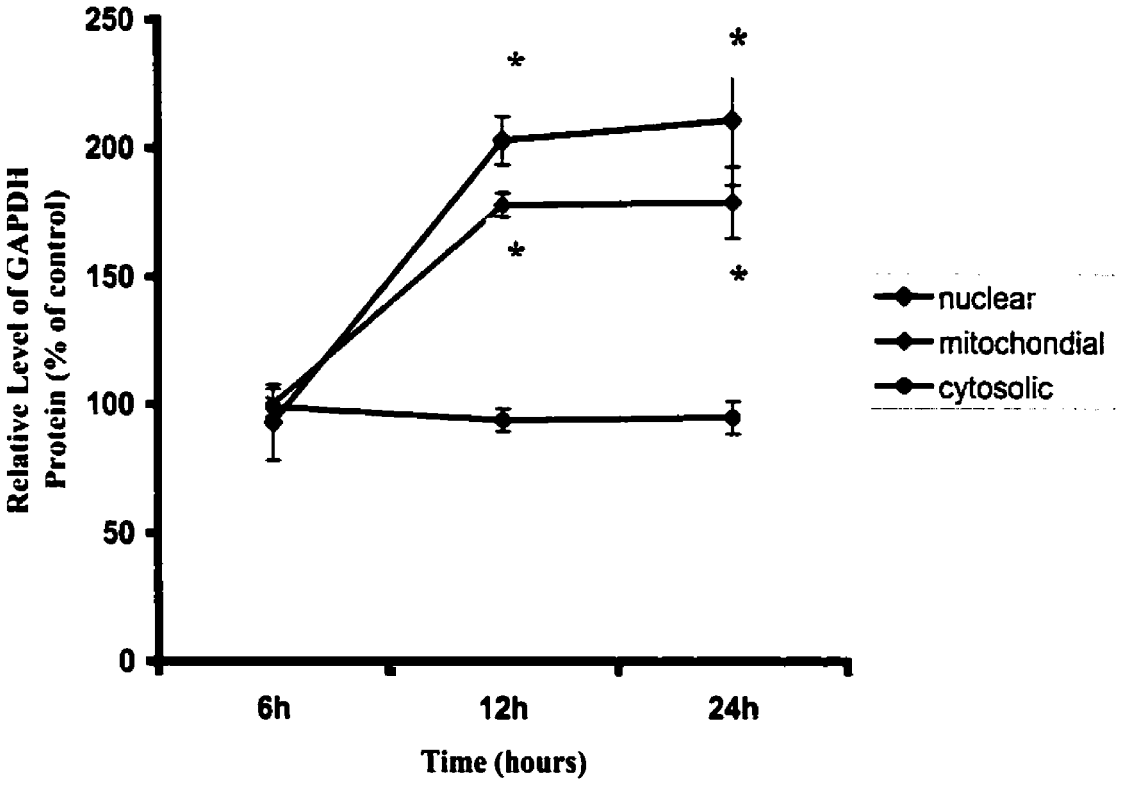
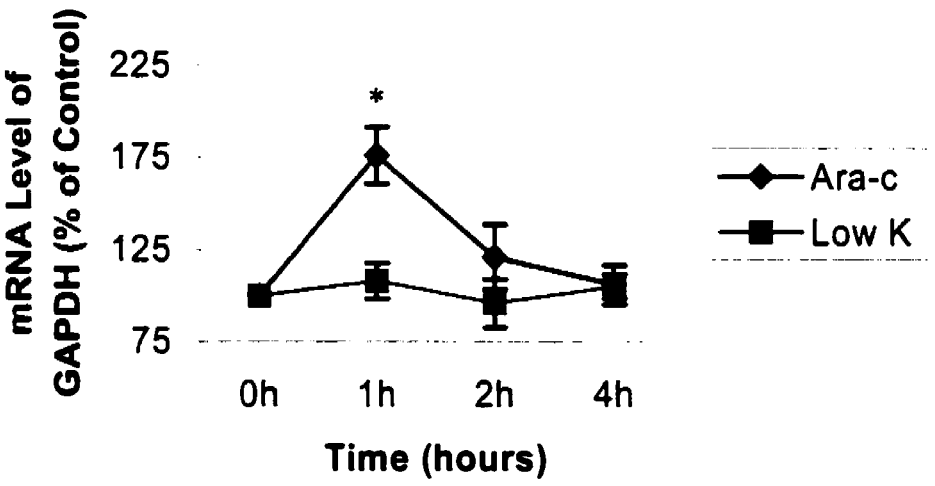


Figure 3.10 Time course of changes of sub-cellular GAPDH protein levels in Ara-c induced apoptosis. Levels of quantified GAPDH proteins are expressed as relative values compared to control. Values are mean ± s.e.m. of three independent experiments. * $p < 0.01$ compared with control using Student's *t* test.

Figure 3.11

A



B

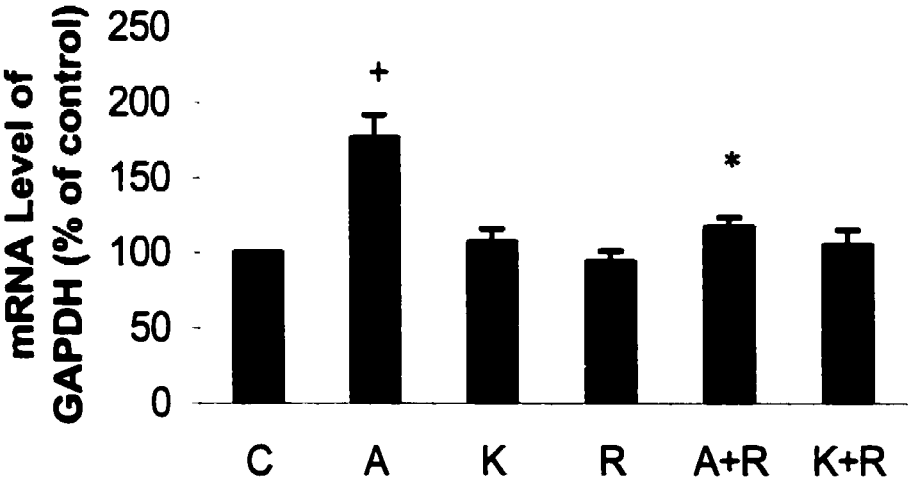


Figure 3.11 Northern blot analysis of GAPDH mRNA levels following induction of apoptosis: Time course (A) and effects of R-2HMP (B). Levels of quantified GAPDH mRNA are expressed as values relative to the control. A, Time course of changes in GAPDH mRNA levels in CGCs after exposure to 300 μM Ara-c. * $p<0.01$ compared with the control. B, Effects of R-2HMP on the overexpressed GAPDH mRNA levels induced by Ara-c (300 μM) for 1 hour. Values are mean ± s.e.m. of three independent experiments. $^{-}$ $p<0.01$ compared with control; *$p<0.01$ compared with Ara-c alone condition using student's *t* test. C, control; A, Ara-c; K, low K^{+}; R, R-2HMP; A+R, Ara-c+R-2HMP; K+R, low K^{+}+R-2HMP.

4. DISCUSSION

4.1 R-2HMP as a rescue agent

Cerebellar granule cells in primary culture, like many cell types, can be induced into apoptosis by a variety of mechanisms (D'Mello et al., 1993, Dessi et al., 1995, Taylor et al., 1997). Ara-c-induced and low K^+-induced cerebellar granule cell death has been previously characterized as apoptotic (D'Mello et al., 1993, Dessi et al., 1995) though it has been reported recently that low K^+ media in combination with serum withdrawal induces both apoptosis and necrosis (Villalba et al., 1997). In the present study, we used nuclear condensation as the main index of neuronal death. That this is valid has been shown by comparison to Apoptag- and H & E-stained cultures (Berry, 1999a). It was found that there is a steady increase of apoptosis between 6 and 24 hours after adding Ara-c and low K^+ media (Fig. 3.1).

The results from this study clearly show that R-2HMP (10^{-7} M) prevents Ara-c-induced but not low K^+-induced apoptosis in 1-day old cerebellar granule cells (Fig. 3.2) and this finding is consistent with the

earlier report from our lab (Paterson et al., 1998) in that the effect of this compound is a potent and selective neurorescue agent. It is interesting that R-2HMP prevents Ara-c-induced apoptosis but did not affect low K^+-induced apoptosis or the basal level of apoptosis seen in primary cultures of cerebellar granule cells. While both basal apoptosis and low K^+-induced apoptosis are seen in cultures of cerebellar granule cells from $p53^{-/-}$ mutant mice, Ara-c-induced apoptosis only occurs in mice with normal p53 (Enokido et al., 1996a, b), suggesting that R-2HMP acts selectively on p53-dependent apoptosis. In support of this, R-2HMP can prevent ischemia-induced apoptosis in vivo (Paterson et al., 1997a,b), and p53 mutant mice have been shown to be relatively resistant to ischemia (Crumrine et al., 1994). R-2HMP also prevents MPTP induced toxicity (Berry, 1999b) and this has been shown to be p53-dependent (Trimmer et al., 1996). Conversly, R-2HMP failed to prevent dexamethasone-induced apoptosis in thymocytes (Fang et al., 1995), which is a p53-independent apoptosis (Clarke et al., 1993).

The involvement of p53 suggests that Ara-c and low K^+ induced apoptosis in cultured CGCs may occur by two different apoptotic pathways, each exhibiting different apoptotic commitment points. Multiple,

independently regulated apoptotic programmes are known to exist. One study which substantially supports this hypothesis was done by Park et al. (1998). He compared the pathways by which DNA-damaging agents, NGF deprivation, and superoxide dismutase 1 (SOD1) depletion evoke apoptosis of sympathetic neurons. DNA-damaging agents such as camptothecin, Ara-c and UV treatment lead to apoptosis by a mechanism that, as in the case of NGF deprivation, includes activation of cell cycle components (eg. Cyclin-dependent kinase (CDK)). CDK inhibitors, flavopiridol and olomoucine, however, had no effect on death induced by SOD1 depletion, suggesting that CDKs do not play a role in this paradigm of neuronal death. To compare further the mechanisms of death evoked by NGF withdrawal, SOD1 depletion, and DNA-damaging agents, he investigated their responses to inhibitors of caspases, elements of apoptotic pathways. V-ICE_{inh} and BAF, two peptide inhibitors of caspases, protected neurons in all three death paradigms. In contrast, the caspase inhibitory peptide zVAD-fmk conferred protection from NGF withdrawal and SOD1 depletion, but not DNA damaging agents, whereas acYVAD-cmk protected only from SOD1 depletion. So based on this study, the capacity of different initiating events to cause apoptotic death in the same type of neuron raises several issues, including 1) the extent to which the pathways leading to apoptosis

in each case are distinct and 2) the degree to which they share common elements. Apoptotic death of neurons, even of the same type of cell, can be evoked by different initiating events. These multiple apoptotic pathways are thought to converge into a few stereotypic pathways, at which point the cell finally passes point(s) of no return and is irreversibly committed to death. As such, an anti-apoptotic compound may prevent one or more apoptotic pathways depending on the level at which it is active.

Previously, (R)-deprenyl was shown to be an effective neurorescue agent (Tatton and Kish, 1997; Paterson et al., 1998). This however, is not due to MAO-B inhibition (Tatton and Chalmers-Redman, 1996; Boulton et al., 1998). As discussed before (Section 1.6.1.1), (R)-deprenyl was shown to reduce the reductions of striatal dopamine caused by MPTP exposure in both monkeys and mice (Heikkila et al., 1984). The inhibiton of MAO-B was thought to block the conversion of MPTP to its active radical MPP^+ within astrocytes and thereby protect the dopaminergic neurons from MPP^+ damage. At that time, little attention was paid to the finding until (R)-deprenyl was shown to reduce the loss of murine dopaminergic nigrostriatal neurons when first administered 72 hours after MPTP treatment (Tatton and Greenwood, 1991). Since MPTP is fully converted to

MPP^+ within 4 hours in mice and maximal striatal damage has occurred by 72 hours (Tatton and Greenwood, 1991), the finding seemed to support the earlier research showing that (R)-deprenyl could maintain dopamine levels in culture independently of MAO-B inhibition. Using the same 72-hour delay in (R)-deprenyl administration, doses as small as 0.01 mg/kg every 2 days were found to cause a marked increase in the numbers of surviving dopaminergic neurons after MPTP exposure (Tatton and Greenwood, 1991). This dose was shown to be insufficient to inhibit MAO-A or MAO-B, even after 20 days of administration. This conclusively established that dopaminergic neurons could be rescued from MPTP exposure without MAO inhibition. Furthermore, the desmethyl metabolite of (R)-deprenyl is active as an anti-apoptotic compound but does not inhibit MAO-B (Tatton and Chalmers-Redman, 1996; Mytilineou et al., 1997b). Like (R)-deprenyl, R-2HMP has been shown to be neuroprotective in several systems (Section 1.6.2.2) by a mechanism independent of MAO-B inhibition (Paterson et al., 1997b; Boulton et al., 1998; Paterson and Tatton, 1998).

The observation that the S isomers (S)-deprenyl and S-2HMP can block the action of both (R)-deprenyl and R-2HMP (Paterson et al., 1998) suggests that (R)-deprenyl and R-2HMP are acting at the same

phamacological site. Antagonism of the antiapoptotic action of (R)-deprenyl and R-2HMP by their S-enantiomers is also interesting because it is in complete contrast to the effect of the S-enantiomers in MAO inhibition where they are somewhat weaker, less selective inhibitors of MAO-B than the R-enantiomers (Robinson, 1985; Yu et al., 1992, 1993), clearly suggesting that this antiapoptotic action occurs through a different mechanism.

Kragten et al. (1998) first showed that deprenyl-related compounds bind to GAPDH and postulated that the GAPDH binding was responsible for the antiapoptotic action of (R)-deprenyl. It seems that binding of these compounds to GAPDH reduces the capacity of the protein to contribute to the series of signaling events in some forms of apoptosis. These effects will be discussed in more detail below (see section 4.3).

4.2 Role of mitochondria in apoptosis

Considerable evidence has accumulated showing that failure of mitochondria is an early event in apoptosis (Zamzami et al., 1995; Tatton and Chalmers-Redman, 1996) and that it may represent a commitment point in the apoptotic death program. Mitochondria have been shown to

play a critical role in the effector phase of apoptosis. The current study shows that loss of mitochondrial membrane potential occurs before the nuclear changes in Ara-c induced apoptosis (Figure 3.1 and 3.3) but not in low K^+ or basal apoptosis. This suggests that these two forms of apoptotic cell death have different commitment points. A failure of mitochondrial function can lead to a loss of mitochondrial membrane potential, opening of the permeability transition pore and release of a 50-kDa factor that induces chromatin condensation and DNA fragmentation (Susin et al., 1996). Opening of the permeability transition pore also allows the escape of mitochondrial factors that induce apoptotic degradation (Susin et al., 1996). One of these factors is cytochrome c, which is normally located between the inner and outer mitochondrial membranes, but when released into the cytosol activates the caspase cascade (Slee et al., 1999), via activation of caspase 9 and then CPP32 (Liu et al., 1996; Li, P. et al., 1997), resulting in cleavage of nuclear DNA. Tatton and his colleagues have shown that the anti-apoptotic compound, (R)-deprenyl, prevents loss of mitochondrial membrane potential in trophically deprived PC12 cells (Wadia et al., 1998) by altering selectively the expression of several genes that act in concert to preserve mitochondrial function and prevent the opening of the permeability transition pore (see Tatton and Chalmers-

Redman, 1996). Among these factors which (R)-deprenyl induces, is bcl-2, which blocks the release of cytochrome c from mitochondria (Kluck et al.,1997; Yang et al., 1997).

In the current study, R-2HMP was shown to exert its anti-apoptotic action at least in part, following a stabilization of mitochondrial membrane potential. R-2HMP prevented loss of mitochondrial membrane potential and apoptosis in Ara-c treated cells (Figure 3.2A, 3.5B, 3.6B). R-2HMP did not prevent low K^+ induced apoptosis (Figure 3.2B) and it alone had no effect on mitochondrial membrane potential (Table 3.1). So, stabilization of mitochondrial membrane potential by R-2HMP is closely related to its anti-apoptotic effect. Further, S-2HMP antagonized the effects of R-2HMP on mitochondrial membrane potential changes induced by Ara-c (Table 3.1). S-2HMP alone had no effect (Table 3.1). This suggests that the site of action of R-2HMP and S-2HMP is either at the level of mitochondria or further upstream.

Mitochondrial membrane potential has been assessed in the current study with CMTMR. This compound has the same basic structure as rhodamine123 with the addition of a chloromethyl moiety, which reacts

with thiol groups on peptides. CMTMR enters mitochondria proportionally to the potential difference between the cytoplasmic compartment and the mitochondrial matrix, and the chloromethyl group reacts with thiols on proteins to form aldehyde-fixable conjugates. Once mitochondrial membrane potential is lost, rhodamine123 is washed out of the mitochondria, whereas CMTMR remains bound even after fixation (Calarco, 1995; Chen and Cushion, 1994). Therefore, CMTMR fluorescence can be used to assess mitochondrial membrane potential in systems requiring subsequent fixation, thereby allowing cells to be dual labelled with CMTMR and an apoptosis detection stain. CMTMR staining represents the highest level of potential difference in the mitochondria during the period of dye exposure prior to fixation.

The results in Figure 3.3 appear to contradict the findings of Nardi et al. (1997), who reported that there is a rapid loss of ATP and mitochondrial function following combined K^+ and serum withdrawal from mouse cerebellar granule neurons. There are however several differences between the two studies. Nardi used 6-7 day-old mouse CGC cultures and insulted them with a combination of K^+ and serum withdrawal, producing a very rapid loss of neuronal survival. This has been shown elsewhere to result in

both apoptotic and necrotic cell death (Villalba et al., 1997). We have used 1-day-old rat CGCs and insulted the cells with only K^+ withdrawal to produce a slower, less extensive, but selective induction of apoptosis. Further, Nardi reported that the decreases in ATP levels occur later than the loss of neuronal viability and, in low K^+ induced apoptosis, we see no change in CMTMR accumulation unless there is also nuclear shrinkage (Figure 3.4C). As such the loss of $\Delta\Psi m$ reported by Nardi et al. (1997) may have been a function of cells which had already died. Three distinctive populations of cells can be observed in the present study. I: Cells with reduced nuclear diameter and mitochondrial membrane potential (apoptotic). II: Cells with normal nuclear diameter and mitochondrial membrane potential (normal). III: Cells with normal nuclear diameter but reduced mitochondrial membrane potential (pre- apoptotic). For low K^+ treated cultures, there is no increase of cell population III (pre-apoptotic) at 6 hours (Figure 3.4C), but there is an increase of cell population I (apoptotic) at 24 hours. It is likely, therefore, that the commitment to apoptosis following K^+ withdrawal does not involve a loss of mitochondrial function, but that this occurs secondary to neuronal death. Ara-c induced apoptosis, however, does involve a loss of mitochondrial function early in the apoptotic program

(Figure 3.3). These results are consistent with the activation of different apoptotic pathways, each involving a different commitment point.

More recently, it has also been shown elsewhere that loss of mitochondrial membrane potential is not required for some forms of neuronal apoptosis (Krohn et al., 1999). Using the protein kinase inhibitor staurosporine-induced cell death in cultured rat hippocampal neurons, Krohn et al. demonstrated that cytochrome c release, caspase activation, and nuclear apoptosis proceeded independently of loss of mitochondrial membrane potential. This suggests that mitochondrial depolarization is not a decisive event in all forms of neuronal apoptosis. This effect is consistent with the previous demonstration that Ara-c induced and low K^+ induced apoptosis use different commitment points.

In this respect, it is worth noting that while multiple apoptotic pathways are known to exist (Park et al., 1998), they may converge to a common execution step. Kluck and Yang (1997) have reported that cytochrome c can be released into the cytosol without collapse of the mitochondrial membrane potential. Cytochrome c is known to allow activation of CPP32 (Liu et al., 1996), and CPP32 has been reported to be

involved in both Ara-c induced (Ibrado et al., 1996) and low K^+ induced apoptosis in CGCs (Ni et al., 1997). It is therefore conceivable that cytochrome c is released from mitochondria resulting in activation of CPP32 in both Ara-c and low K^+ induced apoptosis, but that the upstream signals for this are different.

In summary, the conclusions from the experiments are:

1. A decrease of mitochondrial membrane potential is involved in Ara-c induced apoptosis, whereas a decrease of mitochondrial membrane potential is not involved in low K^+ induced apoptosis in cultured CGCs.
2. The decrease of mitochondrial membrane potential occurs before the nuclear changes in Ara-c induced apoptosis.
3. Maintenance of mitochondrial membrane potential in the low K^+ induced apoptosis demonstrates that upstream signals (before mitochondrial membrane potential failure) are different and suggests that multiple apoptotic pathways exist although they may converge to a common executation step.
4. The antiapoptotic drug R-2HMP blocks neuronal death and prevents loss of mitochondrial membrane potential in Ara-c induced p53-dependent apoptosis in CGCs.

5. S-2HMP antagonizes the effect of R-2HMP on mitochondrial membrane potential in Ara-c treated cultures suggesting that the specific site at which these compounds act is either at the level of mitochondria or further upstream.

4.3 Role of GAPDH in apoptosis

The previous described studies show that the site of action of R-2HMP is upstream of the reduction in mitochondrial membrane potential associated with Ara-c induced apoptosis. The nature of this site has yet to be determined. We have good reason, however, to suspect that this site may be GAPDH. R-2HMP is known to act at the same site as (R)-deprenyl, on the basis of cross-antagonism of the respective S-isomers (Paterson et al., 1998). The site of (R)-deprenyl's anti-apoptotic effect has recently been suggested to be GAPDH (Kragten et al., 1998; Tatton, 1999).

GAPDH is a glycolytic enzyme that exists as multiple forms in various subcellular compartments and has been shown to have many nonglycolytic functions (for review see Sirover, 1999). During the last 4-5 years, accumulating evidence has suggested that GAPDH protein is

involved in apoptosis (Berry and Boulton, 2000). The results from this study show that there was an increase of GAPDH protein levels in nuclear and mitochondrial fractions in Ara-c induced apoptosis (Fig. 3.7 and 3.9) consistent with previous results (Saunders et al., 1997). Low K^+ induced apoptosis in CGCs however, does not involve altered subcellular distribution of GAPDH (Fig 3.7 and 3.9).

In the system used here we do not see GAPDH changes in low K^+-induced apoptosis, which contradicts the result from Ishitani's group. This may be because of differences in the protocols used. Ishitani et al. (1996a,b, 1997) used Sprague-Dawley rat pups and induced apoptosis at 7-8 days in culture. We here used Wistar rat pups, and induce apoptosis after 1 day in culture. The age of cerebellar granule cells in culture is known to influence apoptotic responses (Ishitani et al., 1996a,b; Saunders et al., 1997). Thus, while GAPDH clearly appears to be involved in at least some forms of apoptosis, this may not be a universal change associated with all apoptotic programmes.

That the increased expression of GAPDH is integral to the induction of apoptosis is suggested by the prevention of apoptosis by pre-incubation

of cultures with a GAPDH anti-sense oligodeoxynucleotide (Ishitani et al., 1996a; Zhang et al., 1999). This suggests that new GAPDH protein is synthesized and required during Ara-c induced apoptosis, and the changes observed do not represent simply a translocation of existing GAPDH among the different cellular compartments. This is further supported by the result showing that GAPDH mRNA levels are increased at 1 hour after Ara-c-induced apoptosis (Fig 3.11A). The mechanism by which GAPDH initiates apoptosis is unknown at present, but it may involve specific GAPDH isozymes (Saunders et al., 1999). There are more than 300 copies of the GAPDH gene in the genome (Tso et al., 1985) and GAPDH protein is located in multiple cellular compartments, including the plasma membrane, mitochondria, cytoskeleton, and nucleus (Rogalski et al., 1989). The specific isozyme observed by Saunders et al. (1999) may be nuclear targeted, with the appearance of nuclear GAPDH being the trigger for apoptosis initiation. Overexpressed GAPDH is however, translocated from the cytosol to the nucleus in neurons (Saunders et al., 1997, 1999). One may surmise that GAPDH-dependent apoptosis is caused by a perturbation of nuclear functions. In this context, Ara-c induced GAPDH nuclear translocation occurs concurrently with a nuclear loss of dehydrogenase and uracil glycosylase activities (Saunders et al., 1999), suggesting that

GAPDH undergoes structural and functional changes after transport to the nucleus.

The results here demonstrate that GAPDH is induced in the Ara-c induced neuronal apoptotic model. This phenomenon is thought to be a general one (not specific to neurons) because GAPDH accumulates in the particulate fraction of dying nonneuronal cells such as macrophages (Messmer and Brune, 1996), thymocytes, lymphomas, and pheochromocytomas (Sawa et al., 1997). The signaling pathways that lead to increased GAPDH levels in the nucleus in early apoptosis are not known. P53 overexpression induces apoptosis that is associated with downstream increases in the expression of a large number of genes, including GAPDH (Polyak et al., 1997; Chen et al., 1999). Accordingly, a p53-dependent signaling pathway may contribute to GAPDH-associated apoptosis. Consistent with this, Ara-c induced apoptosis is p53-dependent and is associated with an increased GAPDH expression. In contrast, low K^+ induced apoptosis is p53-independent and is not associated with an increased GAPDH expression.

It has been proposed that the induction of p53 in neurons in vivo is a marker of neuronal apoptosis (see Sakhi and Schreiber, 1997). The role of p53 is of interest because of the accumulating evidence that apoptotic neurons reproduce at least some of the elements for cell cycle transition and mitosis but fail to pass the G1/S boundary. p53 plays a crucial role in arresting cell cycle transition at the G1 checkpoint by inducing $p21^{WAF1}$ which inhibits cyclin-dependent kinases and may contribute to the induction of apoptosis by increasing the expression of bax (Miyashita et al, 1994) which is seen to be increased in hippocampal neurons following cerebral ischaemia (Chen et al, 1996). Recently however, it has been shown that overexpression of p53 can induce neuronal apoptosis but this effect is not mimicked by the overexpression of $p21^{WAF1}$, suggesting that the role of p53 in apoptosis is independent of cell cycle arrest (Jordán et al, 1997).

It has independently been shown before that R-2HMP selectively reduces Ara-c-induced but not low K^+-induced apoptosis (Paterson et al., 1998). The results from this study show that R-2HMP substantially reduces Ara-c-induced apoptosis in CGCs (Fig. 3.2) and prevents all GAPDH changes including GAPDH mRNA (Fig. 3.11B) and protein (Fig 3.7, 3.9). Such

findings suggest that R-2HMP acts early in the induction phase of apoptosis although the mechanism underlying its neuroprotective effects remain unclear. It is however not due to MAO inhibition (Boulton et al., 1997, 1998). The cross-antagonism of the S-isomers of deprenyl and 2HMP to the R enantiomeric forms (Paterson et al., 1998) suggest that R-2HMP and (R)-deprenyl act at the same pharmacological site(s). Recently, (R)-deprenyl has been shown to bind strongly to GAPDH when protecting neuroblastoma cells from apoptotic insults (Kragten et al., 1998). GAPDH normally exists as a tetramer with minor populations of dimers and monomers (Wright et al., 1972; Kim et al., 1995). (R)-desmethyl deprenyl has been proposed to bind to the tetrameric form of GAPDH resulting in a splitting of the tetramer into dimers (Carlile et al., 2000). Since (R)-desmethyl deprenyl also prevented the nuclear accumulation of GAPDH associated with the initiation of apoptosis, it was suggested that in the free dimeric form GAPDH is unable to translocate to the nucleus, whereas in its free tetrameric form, translocation is possible (Tatton, 1999). Under normal conditions tetrameric GAPDH is thought not to be free, due in part to an interaction with AUUUA rich RNA (Nagy & Rigby, 1995).

This however, does not appear to be consistant with our results of GAPDH mRNA changes and their prevention by R-2HMP. Saunders et al. (1999) have shown that one or more specific GAPDH isozymes appear in the nucleus during Ara-c induced apotosis. Further, at least some of the GAPDH that accumulates in the nucleus was previously resident in the cytosol and was not newly synthesized (Shashidharan et al., 1999). A unifying hypothesis would be that an initial release of cytoplasmic tetrameric GAPDH occurs, which translocates to the nucleus where it then initiates novel nuclear GAPDH isozyme transcription. Nuclear GAPDH has been shown to be an activator of transcription (Morgenegg et al., 1986). The specific, nuclear targeted, GAPDH isozyme then triggers p53-dependent apoptosis. While an exact mechanism remains be determined, of possible relevance is the recent demonstration that GAPDH transcription can be regulated by p53 (Polyak et al., 1997; Chen et al., 1999).

In summary, the conclusions from this study are:

1. Ara-c induced, p53-dependent apoptosis is associated with an increase in GAPDH protein in nuclear and mitochondrial fractions. This suggests that the levels of GAPDH protein in particulate fractions are selectively altered in p53-dependent apoptosis.

2. Pretreatment with GAPDH anti-sense prevents Ara-c induced apoptosis (Ishitani et al., 1996a; Zhang et al., 1999) suggesting the increased level of GAPDH in particulate fractions might represent an overexpression of GAPDH, rather than simply a translocation of pre-existing protein. Northern blot analysis of GAPDH mRNA confirms this.
3. R-2HMP prevents the altered pattern of GAPDH protein expression (GAPDH mRNA and protein) associated with Ara-c induced apoptosis. This is may be consistent with the proposal that R-2HMP exerts its anti-apoptotic action through an interaction with GAPDH protein. In this situation, R-2HMP prevents initial translocation of GAPDH to the nucleus and thus prevents activation of nuclear targeted GAPDH isoenzyme transcription which triggers p53-dependent apoptosis
4. S-2HMP antagonizes the effect of R-2HMP on subcellular GAPDH changes in Ara-c treated cultures suggesting that the specific site at which these compounds act is either at the level of GAPDH or upstream.

4.4 Conclusions

In conclusion, this thesis shows that loss of mitochondrial membrane potential and changes in GAPDH mRNA & protein are involved in p53-

dependent apoptosis, but not in a p53-independent form. Further, R-2HMP prevents p53-dependent apoptosis by preventing loss of mitochondrial membrane potential and subcellular GAPDH changes. These changes can be explained by R-2HMP being active through binding to the same pharmacological site as (R)-deprenyl, namely, GAPDH.

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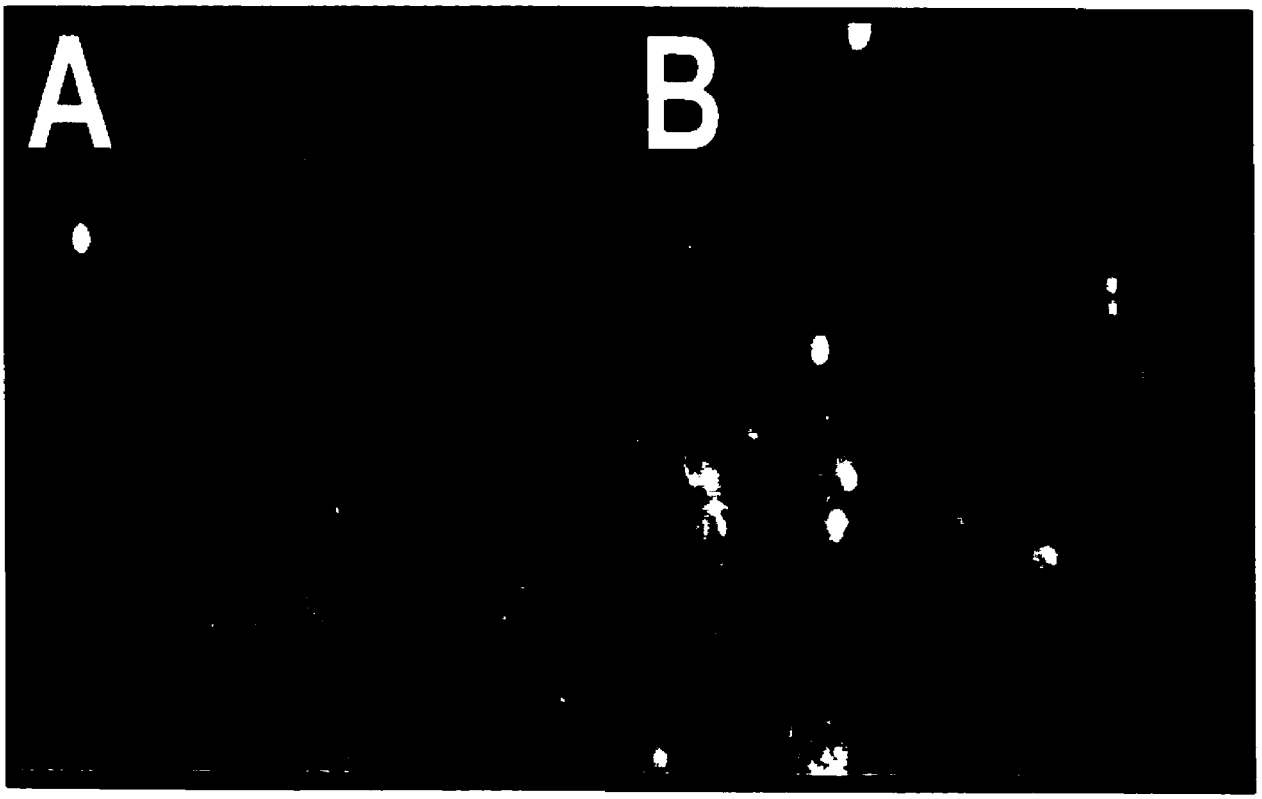
Appendix I Photomicrograph of bis-benzamide stained cerebellar granule cells



Control **Ara-c**

Nuclei were considered to be apoptotic if they exhibited chromatin condensation (increased brightness in a smaller area) in round, crescent, annular, or lobed structures. See increased apoptotic nuclei in Ara-c treated cultures.

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Publications

Papers:-

2000 D. Zhang, M.D. Berry, I.A. Paterson, X.-M. Li and A.A. Boulton
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Abstracts:-

1999

D. Zhang. M.D. Berry, I.A. Paterson and A.A. Boulton
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Changes in mitochondrial membrane potential are dependent on the apoptotic programe activated. *Joint ISN/ESN meeting* (Berlin, Germany)

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R-2HMP prevents changes in mitochondrial membrane potential and apoptosis. *XIII International Congress on Parkinson's Disease* (Vancour, Canada)

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1998

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