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Characterization of the Mechanisms by Which the Organophosphorus Compound Malathion Induces Apoptosis in L929 Murine Fibroblasts

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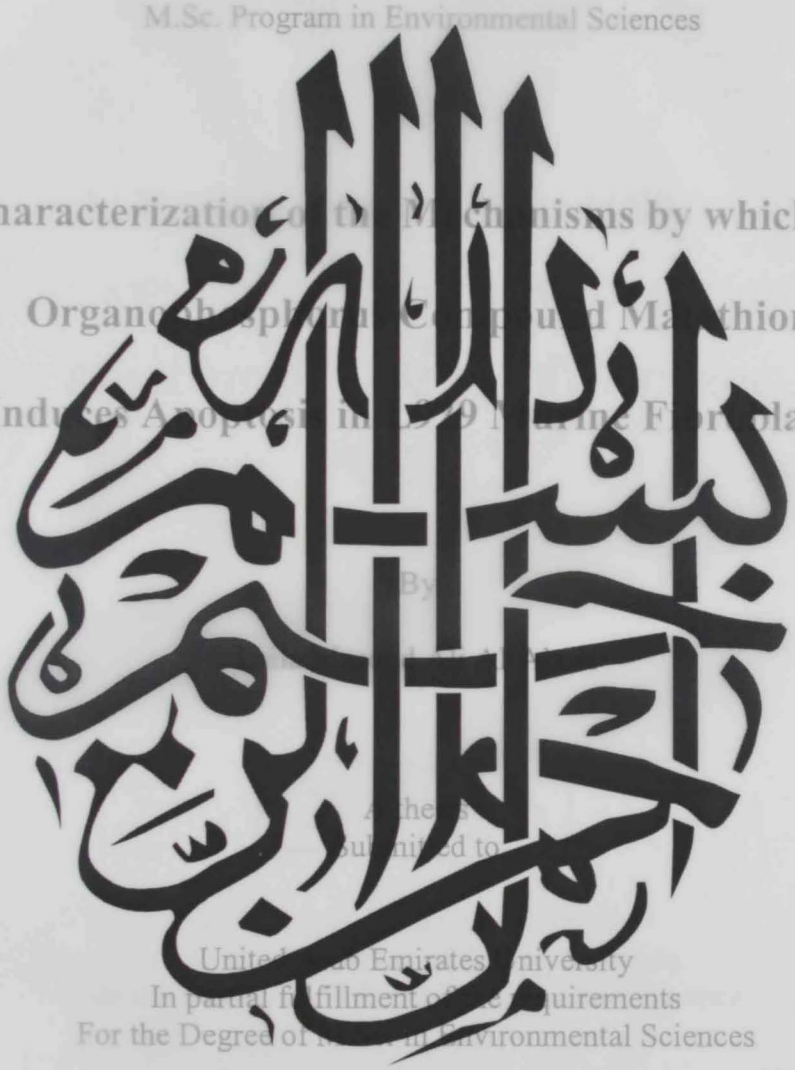

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By
[redacted]

A thesis
submitted to

United Arab Emirates University
In partial fulfillment of the requirements
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Supervisors

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2004-2005

The Thesis of Laila Masoud Ali Al-Alawi for the Degree of Master of Science in Environmental is approved.

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Dean of the Graduate Studies, Professor James E. Fletcher

United Arab Emirates University
2004/2005

DEDICATION

This thesis is dedicated to my mother and father;

your names and love will always remain there in

my heart.

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Completing a master is truly a marathon event, and I would not have been able to complete this journey without the aid and support of countless people. I must first express my gratitude towards my supervisor, Dr. Ayman Saleh, Assistant Professor, in the Department of Biochemistry, for his enthusiastic supervision and for providing excellent facilities. I will never forget his guiding me to the things that I needed to achieve my dream.

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Abstract

While acute organophosphorous compound poisoning due to inhibition of acetylcholinesterase is a well-established clinical entity, the existence of chronic poisoning due to exposure to low levels of organophosphorous compounds (below the threshold required for cholinergic clinical symptoms) is a hotly debated issue. In this study, we have evaluated the effects of noncholinergic doses of malathion (0.01-20 μM) on apoptosis of murine L929 fibroblasts. Employing flow cytometric and caspase activation analyses, we demonstrate that malathion induces apoptosis in L929 cells in a dose- and time-dependent manner. The initiator caspases (caspase-8 and caspase-9) as well as the effector caspase (caspase-3) were activated by the treatment of L929 cells with malathion. Exposure of L929 cells to malathion in the presence of a general inhibitor of caspase, z-VAD-FMK, abolished the apoptotic effect of the compound. In addition, malathion induced an increase in the expression of the pro-apoptotic protein p53. However, the induction of p53 expression was subsequent to activation of the caspase cascades. The present findings suggest that the cytotoxicity of malathion at noncholinergic doses is mediated through caspase-dependent apoptosis.

By employing caspase specific inhibitors, we extended our observations to elucidate the sequence of events involved in malathion-stimulated apoptosis. Pretreatment of L929 cells with the caspase-9 specific inhibitor zLEHD-fmk attenuated malathion induced apoptosis in a dose-dependent manner, whereas the caspase-8 inhibitor, zIETD-fmk, had no effect. Furthermore, the activation of caspase-9, -8, and –3 in response to malathion treatment was completely inhibited in the presence of zLEHD-fmk, implicating the involvement of caspase 9-dependent mitochondrial pathways in malathion-stimulated apoptosis. Indeed, under both *in vitro* and *in vivo* conditions, malathion triggered a dose- and time-dependent

translocation of cytochrome *c* from mitochondria into the cytosol, as assessed by Western blot analysis. Investigation of the mechanism of cytochrome *c* release revealed that malathion disrupted mitochondrial transmembrane potential, induced formation of reactive oxygen species and caused loss of mitochondrial cardiolipin independent of the activation of caspase cascades. Finally, malathion treatment also resulted in a time-dependent up-regulation and translocation of the pro-apoptotic molecule Bax to mitochondria. Inhibition of this event by zVAD-fmk suggests that the activation and translocation of Bax to mitochondria is subsequent to activation of the caspase cascades. The results indicate that malathion induces apoptosis in L929 cells through direct effects on mitochondrial functions, causing the release of cytochrome *c* into the cytosol and subsequent activation of caspase-9. Inhibition of this specific pathway might provide a useful strategy to minimize organophosphate-induced poisoning.

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ABBREVIATIONS:

OPCs:	organophosphorus compounds
AChE :	acetylcholinesterase
IC_{50}:	inhibitory concentration 50 (concentration of a chemical required to reduce the enzyme activity by 50%)
ROS:	reactive oxygen species
Bax:	Bcl-2 associated x protein
Bad:	Bcl-2 associated death agonist
Bid:	BH3 interacting domain death agonist
Bak:	Bcl-2-antagonist/killer
Bcl-2:	B-cell lymphoma 2
Bcl-x_L:	long form of Bcl-x
Caspase:	cysteine specific aspartate protease
VDAC:	voltage-dependent anion-selective channel proteins
ANT:	adenine nucleotide translocator
PARP:	poly (ADP-ribose) polymerase
PI:	propidium iodide
PMSF:	phenylmethylsulfonylfluoride
zDEVD-fmk:	benzyloxy -Asp-Glu-Val-Asp-fluoromethylketone
zIETD-fmk:	benzyloxy -Ile-Glu-Thr-Asp-fluoromethylketone
zLEHD-fmk:	benzyloxy -Leu-Glu-His-Asp-fluoromethylketone
zVAD-fmk:	benzyloxy-valine-alanine-aspartate-O-methyl-fluoromethylketone
NAC:	*N*-acetyl-L-cysteine
DCFH-DA:	2′,7′-Dichlorofluorescein diacetate

CHAPTER 1:

INTRODUCTION

1.1 Introduction

Struggle for survival: under this slogan man has waged violent battles against many thousands of environmental enemies termed injurious pests. During this time man developed many different weapons to control these pests. These weapons are known as pesticides. Pesticide is defined as any substance or mixture of substances intended for preventing, destroying or controlling any pest, such as insects, weeds, rodents, fungi, bacteria or other organisms. The term includes insecticides, herbicides, rodenticides, disinfectants, fumigants, and wood preservatives, as well as growth regulators, defoliants, and desiccants (FAO, 2003; Ware, 1983; Roberson and Nolan, 1988; Bohmont, 1997).

During the past four decades some 15,000 individual compounds and more than 35,000 different formulations have come into use as pesticides. Amongst these, insecticides constitute an important group, and organophosphorus compounds (OPCs) are the major class of insecticides applied in the world today (Karalliedde and Senanayake, 1989; Aspelin, 1994; Bardin et al., 1994). Malathion (*O,O*-dimethyl S-1,2-bis-ethoxy carbonyl ethyl phosphorodithioate), is one of the most widely used organophosphorus pesticides for agriculture and public health programmes (Banerjee et al., 1998 a,b, 1999; Baselt and Cravey, 1989). The acute toxicity of malathion is mediated by the inhibition of acetylcholinesterase (AChE) (Nigg and Knaak, 2000; Overstreet and Djuric, 2001; Kwong, 2002).

In spite of extensive research related to cholinergic effects of OPCs, little is known about health effects from long term exposure to very low levels of OPCs on other biochemical pathways that do not involve AChE inhibition. It is well recognized that many compounds are not directly cytotoxic; rather they cause sublethal damages, which may trigger an innate suicidal sequence of activities in the cell (Hampton and

Orrenis, 1998). Recent studies have suggested the involvement of OPCs in apoptotic processes (Carlson and Ehrich, 2001; Carlson et al., 2000, Carlson and Ehrich, 1999). However, the mechanisms by which they modulate this process are poorly investigated. Apoptosis or programmed cell death is a physiological process activated to eliminate unwanted, damaged, aged, or misplaced cells during embryonic development and tissue homeostasis (Ravagnan et al., 2002; Borner, 2003). The two pathways of apoptosis include the cell receptor pathway, which mediates transduction of the death signal, and the mitochondria-initiated pathway, which involves membrane permeabilization and release of several mitochondrial proteins, such as cytochrome c and procaspases (Pessayre et al., 1999; Zamzami et al., 1998; Maeda, 2000). The most important apoptotic effectors are the caspases (Cain, 2000) that are synthesized and stored as inactive precursors and activated by proteolysis upon induction by various apoptotic stimuli (Pallardy et al., 1999). Progression of the caspase cascade ends with the activation of the effector caspase-3, which finally cleaves various vital substrates in the cell (Alnemri, 1997). Before presenting the results of this thesis on the effects of non-cholinergic doses of malathion (0.01-20μM) on apoptosis of murine L929 fibroblasts and the role of a mitochondrial pathway in the induction of apoptosis by malathion, I will summarize the knowledge on OPCs, using malathion and its toxicological effects as an example. In addition, the two pathways of apoptosis and the previous studies on the effect of different pesticides on apoptosis will be discussed, as results from this work indicate that malathion induces apoptosis in L929 cells through direct effects on mitochondrial functions causing the release of cytochrome *c* into the cytosol and subsequent activation of caspase-9. These findings suggest that inhibition of this specific pathway might provide a useful strategy to minimize organophosphate-induced poisoning.

1.2 Organophosphorus Compound Usage

Organophosphorus compounds (OPCs) are extensively used in agriculture as pesticides, in industry and technology as plasticizers in products like paints, lacquers, and varnishes, as additives in lubricants and hydraulic fluids, plastic softeners, stabilizers, antifoaming, antioxidants, wetting agents and flame retardants in plastic, textiles and building materials (Mochida et al., 1988; Katoh et al., 1990; Mortensen and Ladefoged, 1992; Ware, 1994; WHO, 1998; Weiner and Jortner, 1999; Marklund et al., 2003). They are also used in veterinary practice as ectoparasiticides (Kwong, 2002), in human medicine for eradication of human body lice (Elston, 2002; Roberts, 2002), and in food preparation and processing areas (Savage et al., 1981). In addition, some OPCs are applied in military technology as chemical warfare agents (Karalliedde, 1999, Karalliedde et al., 2000).

However, these economic and health benefits are not achieved without potential risk and possible adverse health effects to humans, domesticated animals and the environment. It has been estimated that 85-90% of the pesticides applied in agriculture never reach their target organisms, but instead are dispersed in the air, water, and soil (Repetto and Baliga, 1996). Based upon such estimates, pesticide exposure is likely for non-target organisms.

Most studies on toxicity of OPCs have focused on the enzyme alterations, immunotoxic, reproductive and developmental effects, mutagenic and potential genotoxic properties of these agents. Only recently have the potential of pesticides to induce apoptosis received adequate attention. More information is, however, required regarding the mechanisms through which these agents induced apoptosis.

1.3 Toxicity of Organophosphorus Compounds

Organophosphorus (OP) poisonings continue to be a major cause of morbidity and mortality in the third world countries (Peter and Cherian, 2000). The World Health Organization (WHO) reported that more than one million casualties occur annually due to OP exposure (Namba, 1971). Numerous OP poisonings are actually accidental and have occurred among pesticide workers (Baker et al., 1978) and children (Hayes, 1982).

Over a ten-year period from 1987 to 1996, hospitals in Western Australia encountered 69 cases of OP poisoning (Emerson, 1999). There were 47 cases of OP insecticide poisoning in Turkey between 1990 and 2000 (Sungur and Guven, 2001). In addition, from 1991 to 1992, there were 133 cases of OP insecticide poisoning in Tanzania (Ngowi et al., 2001).

Toxicity of organophosphorus compounds stems mainly from the accumulation of acetylcholine in the nervous system due to inhibition of acetylcholinesterase (AChE, Pope, 1999). Many studies have demonstrated the toxicity of organophosphorus compounds. These studies were done in different organ systems, either *in vitro* or *in vivo*.

Neurotoxic effects of OP pesticides and related compounds fall into three categories either in humans or animals (Ray, 1998). The acute or cholinergic syndrome, which results from AChE inhibition, occurs within a few minutes to hours after exposure (Karalliedde et al., 2000; Brown and Brix, 1998). It produces a complex mixture of muscarinic and nicotinic signs which vary according to the severity with target organ tissue, dose and agent (Marrs, 1993). The second neurotoxic effect of OPs is an intermediate syndrome (Karalliedde et al., 2000), described as a late complication of some cases of severe acute poisoning (Ray, 1998).

It is characterized by weakness of respiratory neck and proximal limb muscles (Lotti, 2002). OP-induced delayed neurotoxicity (OPIDN) or polyneuropathy is a third type neurotoxicity, which is characterized by paralysis of the lower limbs, partial sensory loss, and degeneration of long axons in the spinal cord and peripheral nerves, evident 10-14 days after exposure. It is attributed to phosphorylation and aging of neuropathy target esterase (NTE, Johnson, 1992).

Several studies have addressed the potential of low-dose OPs to produce a variety of effects in man (Jamal, 1997) and in animals (Ray and Richards, 2001). Furthermore, repeated exposure to low-level of OPs can lead to chronic damage to the nervous system (Stephens and Spurgeon, 1995), such as neurological syndrome characterised by headache, fatigue, skeletal muscle, weakness and nausea (Karalliedde, 1999).

The toxic effects of OPs have also been investigated in fish and birds (Walker and Johnston, 1989). Animals that are exposed to organophosphates show that low single doses on a critical day of development can cause hyperactivity and permanent changes in neurotransmitter receptor levels in the brain (Ahlbom et al., 1995). Furthermore, organophosphates such as flupyrazofos, chlorpyrifos and dichlorvos induce maternal toxicity in experimental animals (Baksi, 1978; Breslin et al., 1996; Chung et al., 2002).

1.4 Non-Esterase Cellular Effects of OPs

However, the toxicity of OPs is not restricted only to AChE inhibition. It has been shown that parathion was capable of inducing toxic effects in FG-9307 cells, and causing alteration of mitochondria, rough endoplasmic reticulum, nuclear membranes and production of numerous lysosomes (Li and Zhang, 2001). Also, it has been

demonstrated that oxidative stress may occur in OP toxicity (Pena-Llopis et al., 2002). Several studies have proved oxidative stress is induced by OPs in rats (Gultekin, 2000; Gupta et al., 2001; Akhgari et al., 2003) and humans (Banerjee et al., 2001; Ranjbar et al., 2002).

There are a number of *in vitro* studies where OP sensitivity of a number of proteins was proposed. For example, calmodulin activity (Pala et al., 1991) and phosphoinositol phosphodiesterase (Davies and Holub, 1983) are both influenced by OP esters at extremely high concentrations. Diazinon is an organophosphorus compound has been shown to increase protein metabolism (Ceron et al., 1996), inhibit protein synthesis (Marinovich et al., 1994) and alter the metabolic pathways for specific amino acids (e.g. L-tryptophan, Seifert and Pewnim, 1992). A potential genotoxic effect of OPs was observed in fish treated with organophosphorus insecticides, methyl parathion and phosphamidon, which resulted in sister chromatid exchanges and chromosomal aberration (Das and Joh, 1999). In another study, Shukla et al., (2000) showed that topical exposure of Quinalphos on mouse skin may initiate tumorigenic potential. Furthermore, organophosphates such as flupyrazofos, chlorpyrifos and dichlorvos induce maternal toxicity in experimental animals (Baksi, 1978; Breslin et al., 1996; Chung et al., 2002).

Defined mechanisms implicated in OPC elicited *in vitro* such as cell death are not widely described, but the implication of apoptotic processes is observed (Akbarsha and Sivasamy, 1998; Hamm et al., 1998).

1.5 Malathion

Malathion [S-1,2-bis(ethoxycarbonyl)ethyl O, O-dimethyl phosphorodithioate; CAS Registry No. 121-75-5] is a widely used organophosphorus insecticide applied in

agriculture as a pesticide, in veterinary practice as ectoparasiticide (Flessel et al., 1993), in eradication of human body lice (Elston, 2002; Roberts, 2002) and in food preparation and processing areas (Savage et al., 1981). Malathion is classified as class III (slightly toxic) in terms of risk according to the WHO (WHO, 2000). In the USA alone, it an estimated 16.7 million pounds of malathion are applied annually. Its chemical structure is shown in Figure 1.1. Approximately 12.5 million pounds are used in agriculture, mostly for cotton, through the USDA Boll Weevil Eradication program, and 3.4 million pounds are applied to non-agricultural uses, such as for the med fly quarantine, mosquito abatement, golf courses and home owner outdoor control (USEPA, 2000a). In 1999, malathion was also utilized in the West Nile Virus eradication effort (USEPA, 2000b, 2001).

In the UAE in 2000, malathion was used during the malaria control programme (Ministry of Health, 2003).

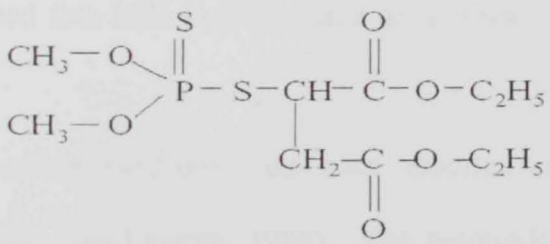


Figure.1.1 The chemical structure of malathion (adapted from Dikshith, 1991).

1.5.1. Toxicological Effects

1.5.1.1. Poisoning Statistics

Acute toxic effects induced by malathion pesticides are mainly caused by inhibition of acetylcholinesterase (AChE) in the nervous tissue with a consequent

increase in the levels of the neurotransmitter acetylcholine in synapses (Nigg and Knaak, 2000; Overstreet and Djuric, 2001; Kwong, 2002).

Malathion has been implicated in several cases of accidental or suicidal poisoning, some of which were fatal. In Japan, from 1957 to 1961 and 1965 to 1966, there were 63 accidental poisonings resulting in ten fatalities, and 480 suicide or homicide attempts, of which 404 were deaths (Matsumura, 1975). In 1973, Nalin, detected an epidemic of 264 suicides that resulted from malathion poisoning in Guyana (Nalin, 1973).

In Pakistan among malaria workers, malathion accounted for 2800 cases of poisoning (Baker et al., 1978). Petros (1990) reported on nine cases of malathion poisoning admitted to a hospital in Ethiopia in 1986. Hayes and Vaughn (1977) reported that for five years surveyed (1956, 1961, 1969, 1973, and 1974), malathion was responsible for 11 deaths due to accidental causes. Furthermore, malathion was one of two insecticides (endosulfan being the other) identified as causing 64% of the pesticide related fish kills in US coastal areas between 1980 and 1989 (Pait et al., 1992).

Intermediate syndrome has been reported in humans after exposure to malathion (Gallo and Lawryk, 1991). The cytotoxic effects of malathion (50μM) have also been demonstrated in human, rat hepatocytes and HaCaT cells (Delescluse et al., 1998).

1.5.1.2. Mutagenicity and Genotoxicity Effects

Malathion can be regarded as a potential mutagen/ carcinogen agent (Blasiak et al., 1999). Use of malathion by farmers has been linked to an increased risk of one type of cancer, non-Hodgkin's lymphoma (Cantor et al., 1992). In experimental

animal study using three acute doses (2.5, 5, 10mg/kg), malathion was reported to produce chromosomal aberration, sperm abnormalities, and delay in cell cycle at higher doses (Giri et al., 2002). A low concentration of malathion, was found to be genotoxic in mouse spleen (Amer et al., 1996). Several *in vitro* studies in human cells have also shown an increase in chromosome aberrations and/or chromatid exchanges after malathion exposure (Herath et al., 1989; Garry et al., 1990; Walter et al., 1980; Sobti et al., 1982; Nicholas et al., 1979). Kamrin (1997) summarized results of mutagenicity tests of malathion and other selected organophosphours pesticides: malathion produced detectable mutagenesis in three different types of cultured human cells.

1.5.1.3. Reproductive and Developmental Effects

Malathion has developmental effects on the male reproductive system and on embryogenesis. Several studies have addressed the potential of malathion to cause Sertoli cell changes in animals (Contreras et al., 1999; Banerjee et al., 1998). Male mice injected i.p. with 240mg/kg/day of commercial malathion for 40 days show significant decreases in sperm count, and plasma testosterone level, as well as altered sperm tail morphology (Bustos-Obregon and Gonzalez-Hormazabal, 2003).

Embryotoxicity, as indicated by decreases in body weight and skeletal size and a lag in development has been reported in mice after administration of malathion at about 15-50% of the oral LD_{50} values, but no indication of maternal toxicity was provided (Asmatullah et al., 1993). Embryonic abnormalities and mortality have been induced by malathion when injected into yolk sac of fertile chicken eggs (Pourmirza, 2000). There is also evidence that malathion induce morphological abnormalities such as vertebral deformities in fish (Lien et al., 1997). Similarly, administration of

malathion to toad embryos reduces glutathione (GSH) level, glutathione S-transferase (GSH S-transferase) activity, and produces morphologic abnormalities and mortality (Anguiano et al., 2001).

Developmental defects were also observed in Xenopus embryos exposed to malathion (Snawder and Chambers, 1990, 1993). A potential reproductive effect was demonstrated in Culex quinquefasciatus (Aguilera et al., 1995). Additionally sublethal doses of malathion (5 and 20µl/l) were found to cause a sex hormonal imbalance in fish (Singh and Singh, 1992).

1.5.1.4. Immunotoxic effects

Malathion is known to modulate or suppress immune responses at specific dose levels (Johnson et al., 2002; Beaman et al., 1999; Rodgers and Xiong, 1997). Non-cholinergic doses of malathion elevate the macrophage, proliferative and humoral immune responses in MRL-Ipr mice (Rodgers, 1997). Non-cholinergic doses of malathion as low as 0.1mg/kg per day (LD50=2800 mg/kg) stimulate the immune system by inducing mast cell degranulation (Rodgers and Xiong, 1997); whereas cholinergic doses of malathion (>720 mg/kg) suppress immune function by reducing anti-sheep red blood cell (SRBC) antibody production (Casale et al., 1983).

Moreover, it has been reported that malathion suppresses generation of nitric oxide and TNF-α by rat peritoneal macrophages under *in vitro* conditions over 24 h (Ayub et al., 2003). In addition, a decrease in cellular immune responses has been reported for malathion (Banerjee et al., 1998).

1.5.1.5. Other Health Effects of Sublethal Doses

Chowdhury et al. (1980), treated rats with a single oral dose (1g/kg body wt) of malathion and reported reduced glucose and glycin absorption, as well as depressed on brush border enzymes including sucrase, lactase, alkaline phosphatase, Mg2+-ATPase and lactate dehydrogenase in rat intestine. Malathion induced alterations in the lipid profile and the rate of lipid peroxidation in rat brain and spinal cord (Haque et al., 1987). In an other study, application of malathion in rat hepatoma-derived Fa32 cells resulted in lysosomal attack and mitochondrial dysfunction as the primary intoxication mechanism (Dierickx, 2000). Administration of malathion for 4 weeks increased the activities of catalase and superoxide dismutase in erythrocytes and liver of rats exposed to malathion (Akhgari et al., 2003). Furthermore, malathion, has been found to cause oxidative stress in rats and mice (Ahmed et al., 2000; Yarsan et al., 1999).

Sublethal doses of malathion affect ovarian lipid metabolism in catfish (Singh, 1992), and the carbohydrate metabolism of crustaceans (Reddy et al., 1986). Use of sublethal doses of malathion, was found to produce variation in the serum protein fractions of fish (Richmonds and Dutta, 1992), and inhibit lipid and protein synthesis by the liver (Saxena et al., 1989). Thyroid physiology impairment by malathion has also been investigated in fish (Sinha et al., 1992).

Most studies on toxicity of malathion either *in vitro* or *in vivo* have focused on the neurotoxic, immunotoxic, reproductive and developmental effects, as well as mutagenic and potential genotoxic properties of this agent. However, there is an area regarding malathion's toxicity which must be explored, which is the effect of malathion on induction of apoptosis.

1.6 Apoptosis

Apoptosis or programmed cell death is a physiological process that is activated to eliminate unwanted, damaged, aged, or misplaced cells during embryonic development and tissue homeostasis (Ravagnan et al., 2002; Borner, 2003). Apoptosis has unique morphological and biochemical features characterised morphologically by shrinkage of the cell, membrane blebbing into membrane-bound apoptotic bodies and rapid phagocytosis by neighbouring cells (Saraste and Pulkki, 2000; Poulaki et al., 2001). Internucleosomal fragmentation of genomic DNA has been the biochemical hallmark of apoptosis for many years (Saraste and Pulkki, 2000).

Apoptosis is orchestrated by the activation of a family of aspartate-specific cysteine proteases known as caspases, which normally exist as inactive proenzymes (Cohen, 1997). Two major apoptosis pathways have been identified: the death receptor pathway and the mitochondrial pathway.

The death receptor pathway is initiated through the binding of death ligands, such as Fas ligand and tumor necrosis factor (TNF), to their specific receptors on the cell surface. This stimulation results in the recruitment and activation of caspase-8 through the related adaptor molecules (FADD and FLASH). Active caspase-8 farther activates downstream effector caspases such as caspase-3, 6 and 7 (Cohen, 1997). The other major route leading to apoptosis is the mitochondrial pathway. Mitochondria play a major role in the apoptotic death by liberating apoptogenic proteins, including cytochrome c, into the cytoplasm (Martinou and Green, 2001; Zamzami and Kroemer, 2001). Once in the cytoplasm, cytochrome c, in the presence of ATP or dATP, associates with Apaf-1 (a poptotic protease-activating factor) and procaspase-9 in a complex known as the apoptosome. Apoptosome formation leads to activation of

caspase-9, which then directly cleaves and activates procaspase-3 (Saleh et al., 1999; Green, 2000).

Although the precise molecular mechanisms by which cytochrome c is librated from mitochondria to the cytosol are still unclear, it is believed that members of the Bcl-2 family proteins modulate the release process. The members of this family can be subdivided into two groups: one with anti-apoptotic proteins that inhibit apoptosis, such as Bcl-2 and Bcl-XL, and pro-apoptotic proteins that promote apoptosis, such as Bax and Bak (Antonsson, 2001; Huang and Strasser, 2000).

1.7 Pesticides and Apoptosis

Pesticides among several other factors can induce apoptosis (Carlson et al., 2000; Kannan et al., 2000; Warren et al., 2000). However, studies in this field are scarce. For example, organophosphates of chlorinated hydrocarbon insecticides have been demonstrated to induce apoptosis (Carlson et al., 2000; Kannan et al., 2000; Rought et al., 2000). N-nitroso metabolite of carbofuran (carbamate pesticides) induces cell cycle arrest and apoptosis in CHL cells (Yoon et al., 2001).

In vitro exposure to OP compounds such as tri-ortho tolyl phosphate, triphenyl phosphite, and parathion have been shown to produce changes in mitochondrial transmembrane potential in human neuroblastoma cells (Carlson and Erich, 1999). Subsequently, Carlson et al., (2000) proposed that cytotoxicity of organophosphorus compounds could be due to induction of apoptosis by various routes, including mitochondrial permeability pores mediated caspase pathways, or serine protease activation.

Pena-Llopis et al. (2002) studied the association between the glutathione levels in marine bivalves and survival through exposure to the OP pesticide

fenitrothion. They reported that an impairment in the glutathione redox status could result in an induction of the cell death, either by apoptosis or necrosis, leading eventually to the death of the organism. In another study, a single topical application of permethrin insecticide in mice resulted in thymocyte apoptosis (Prater et al., 2002). In addition, mitochondrial depolarization, caspase-3 activation and apoptosis have been observed in cultured cells (dopaminergic PC12) following exposure to organochlorine pesticide, dieldrin (Kitazawa et al., 2001; 2003).

The OP insecticide dichlorvos induced *in vivo* neurotoxicity and apoptosis, respectively, has been demonstrated by a variety of techniques including depletion of glutathione metabolism, inhibition of AChE and caspase-3- like activity (Pena-Llopis et al. 2003).

Biochemical changes other than acetylcholinesterase inhibition have been reported *in vitro* after exposure to OP compounds, including inhibition of protein synthesis (Harvey and Sharma, 1980; Reddy et al., 1990), fragmentation of DNA (Carlson and Ehrich, 1999), leakage of lactate dehydrogenase (Bagchi et al., 1995), and inhibition of glucose metabolism (Harvey and Sharma, 1980). Recent studies have indicated that, through non-cholinergic mechanisms, chlorpyrifos affects brain cell development involving alterations in the expression and function of transcription factors that control cell replication, differentiation, and apoptosis (Crumpton et al. 2000; Garcia et al. 2001; Schuh et al. 2002). Furthermore, ultrastructural changes of rat hepatoma, mitochondria and lysosomes after malathion treatment were also observed by Dierickx (2000).

Results from these studies conducted *in vivo* and *in vitro* models indicate that the OP compounds can induce apoptotic cell death.

1.8 Objectives

AIM 1: To test the effect of malathion pesticides on the induction of apoptosis in L929 cell line.

AIM 2: To evaluate the toxic effect of malathion on mitochondrial function.

CHAPTER 2:

EFFECT OF MALATHION ON APOPTOSIS OF MURINE L929 FIBROBLASTS: A POSSIBLE MECHANISM FOR TOXICITY IN LOW DOSE EXPOSURE*

* A version of this chapter has been published.

Masoud, L., Vijayasarathy, C., Fernandez-Cabezudo, M., Petroianu, G., and Saleh, A.M. (2003).Effect of malathion on apoptosis of murine L929 fibroblasts: a possible mechanism for toxicity in low dose exposure. *Toxicol.* 185, 89-102.

2.1. Introduction

Malathion [*S*-1,2-bis(ethoxycarbonyl)ethyl *O,O*-dimethyl phosphorodithioate; CAS Registry No. 121-75-5] is one of the most widely used organophosphate compounds (OPCs) applied in agriculture as a pesticide, in veterinary practice as an ectoparasiticide (Flessel et al., 1993), in eradication of human body lice (Elston, 2002; Roberts, 2002) and in food preparation and processing areas (Savage et al., 1981). Acute toxic effects induced by malathion pesticides are mainly caused by inhibition of acetylcholinesterase (AChE) in the nervous tissue with a consequent increase in the levels of the neurotransmitter acetylcholine (Nigg and Knaak, 2000; Overstreet and Djuric, 2001; Kwong, 2002). Malathion can be metabolized to non-toxic intermediates by carboxyesterases enzymes (Gupta et al., 1983; Nigg and Knaak, 2000). However, its widespread use in agriculture and household practices has raised concern over its potential to cause adverse health effects in humans, animals, wildlife and fish (Flessel et al., 1993; Wolfe and Seiber, 1993). Occupational exposure to malathion is a cause for concern. Dermal exposure to malathion following aerosol application has been estimated to be as high as >5 mg/kg per day (Wolfe et al., 1967 & 1978).

Recent studies have indicated that at noncholinergic (sub-lethal acute) doses, malathion affects several biochemical pathways that do not involve modulation of AChE activity. Both under *in vitro* and *in vivo* conditions, malathion (< 10 mg/Kg body weight ≈ 30 μM) has been shown to induce DNA damage, chromosomal aberrations (Blasiak et al., 1999; Giri et al., 2002) and malignant transformation (Cabello et al., 2001). Malathion has also been shown to modulate oxidative stress and immune response in experimental animals (John et al., 2001; Johnson et al., 2002).

Cells undergo apoptosis (programmed cell death) in response to various stimuli including chemical stress. Apoptosis, which is distinct from necrosis, another form of cell death (Raffray and Cohen, 1997; Pallardy et al., 1999; Bratton and Cohen, 2001), is mediated by the intracellular aspartate specific cysteine proteases designated as caspases (Alnemri, 1997). Caspases are synthesized and stored as inactive precursors (procaspases) and activated by proteolysis upon induction by various apoptotic stimuli (Pallardy et al., 1999).

Of the two pathways that activate caspases, the receptor-mediated pathway involves the ligation of death receptors resulting in recruitment and activation of caspase-8 (Scaffidi et al., 1998), while the stress induced mitochondria dependent pathway leads to activation of caspase-9 (Saleh et al., 1999; Zou et al., 1999). Following activation of the initiator caspase-8 or -9, the two pathways converge on the activation of the effector caspase-3, which finally cleaves various vital substrates in the cell (Alnemri, 1997). Apart from these two pathways, p53, a molecule involved in cell surveillance, also activates the mitochondrial pathway of apoptosis in response to DNA damage (Luu et al., 2002; Trinei et al., 2002). Although these pathways appear to be distinct from each other, a cross talk between them ensures effective elimination of injured or damaged cells.

Although the cytotoxicity of malathion has been shown to involve several biochemical pathways, studies on apoptosis following exposure to this compound have not received much attention as yet. In the present study, we assessed the cytotoxic effect of very low non-cholinergic doses of malathion (concentrations that do not inhibit AChE activity: 0.01-20 μM) on apoptosis of cultured mouse L929 fibroblasts using flow cytometry, caspase activation and DNA fragmentation

techniques. Our results demonstrate that malathion induces a caspase-dependent apoptosis in a time and dose-dependent manner in cultured mouse L929 fibroblasts.

2.2. Materials and Methods

2.2.1. Reagents

Malathion with 95% purity was obtained from PolyScience (Division of Preston Industries, Inc. Niles, IL). Stock solution of malathion (100 mM) was prepared in dimethyl sulfoxide (DMSO) and stored at –80°C. The working dilutions in phosphate buffered saline (PBS) were prepared just before use. Protease inhibitors, [PMSF, pepstatin A, leupeptin and aprotinin] were acquired form Sigma Chemical Company (Sigma Aldrich Chemie GmbH Steinheim, Germany). The colorimetric tetrapeptide substrates for caspase-8 (Ac-IETD-ρNA), caspase-9 (Ac-LEHD-ρNA) and caspase-3 (Ac-DEVD-ρNA) were purchased from Calbiochem (San Diego, CA). Caspase-8 inhibitor (Ac-IETD-CHO), caspase-9 inhibitor (z-LEHD-FMK), caspase-3 inhibitor (Ac-DEVD-CHO) and the general caspase inhibitor (z-VAD-FMK) were also obtained from Calbiochem. The following polyclonal antibodies were obtained from various sources as indicated: anti-caspase-8, anti-p53, anti α-tubulin and anti-PARP (Santa Cruz Biotechnology, CA), anti-caspase-9 and anti-caspase-3 (Stressgen Biotechnolgies, Victoria, BC, Canada).

2.2.2. Cell Culture

L929 mouse fibroblast cells were obtained from American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in RPMI 1640 medium containing L-glutamine (Sigma, St. Lois, MO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL, Grand Island, NY), 100 U/ml penicillin and 100 µg/ml streptomycin, and were kept at 37°C in humidified 5% CO_2/95% air. Prior to confluence, the cells were harvested and seeded onto plastic 6–well culture plates or 100-mm culture dishes at 5 $X10^5$ cells/ml. The cells were then allowed to

grow for 2-3 days and exposed to varying concentrations of malathion (0.01 to 20 μM) for 16 h. Following treatment, the cells were harvested using 0.25% trypsin/EDTA (GIBCO BRL), centrifuged at 1000 rpm for 5 min, washed with PBS and subsequently used for various biochemical investigations. The specificity of caspase-mediated apoptosis was determined by including z-VAD-FMK, a general inhibitor of caspases, in the medium. In those experiments where the inhibitors were used, the cells were pretreated with 25 or 50 μM z-VAD-FMK for 6 h, followed by treatment with malathion for 16 h prior to harvesting.

2.2.3. Analysis of Apoptosis by Flow Cytometry

Loss of plasma membrane asymmetry is one of the earliest features of apoptosis. In apoptotic cells, the membrane phospholipid, phosphatidylserine (PS) is exposed to the external cellular environment as a result of translocation from the inner to the outer leaflet of the plasma membrane (Okamoto et al., 2002). To identify early apoptotic cells, we used the Annexin V-FITC (fluorescein isothiocyanate) staining kit from BD Biosciences (Franklin Lakes, NJ). Annexin V has a high affinity for PS and binds to cells with exposed PS (Vermes et al. 1995). Propidium Iodide (PI) was used to differentiate apoptotic cells with preserved membrane integrity ($Annexin^+$, PI^-) from necrotic cells that lost membrane integrity ($Annexin^-$, PI^+). The assay was performed following the manufacturer's procedure. Briefly, malathion-treated cells ($1X10^6$/ml) were harvested and washed twice with ice-cold PBS. About $1X10^5$ cells were stained with Annexin V-FITC and PI as per the manufacturer's protocol. 10,000 cells were analyzed by flow cytometry (Facs Vantage, Becton, Dickinson, USA). Cells with positive Annexin V stain ($Annexin^+$, PI^-) were counted as apoptotic and their number expressed as a percentage of the total cells.

2.2.4. Western Blot Analysis

Malathion-treated cells were harvested, washed twice with PBS and suspended in a lysis buffer containing 100 mM HEPES, pH 7.5, 10% sucrose, 10 mM DTT, 0.1% CHAPS, 150 mM NaCl and protease inhibitors (1 mM PMSF, and 1 μg/ml leupeptin, aprotinin and pepstatin A). Cells were lysed by four repeated cycles of freeze thawing and centrifuged at 4 °C for 30 min at 14,000 xg. The supernatant was collected and stored at -80°C or used immediately. The samples were analyzed for total protein by a protein assay kit based on Bradford (1976) colorimetric reaction (BioRad, USA). Cell lysates (50 μg protein per lane) were separated on 8 and 12% SDS-polyacrylamide gels and electroblotted onto a PVDF membrane (Millipore, USA) using standard techniques (Laemmli, 1970; Towbin et al., 1979). After electrophoretic transfer, the membranes were blocked by incubation for 1 h with 5% non-fat dry milk in PBS containing 0.1% Tween-20. The blots were then incubated for 2 h at room temperature or overnight at 4°C with one of the following antibodies diluted in PBS containing 0.1% Tween-20 and 2% non-fat dry milk: anti-caspase-8 (1:1000), anti-caspase-9 (1:3000), anti-caspase-3 (1:1000), anti-PARP (1: 1000) and anti-p53 (1: 500). The blots were then incubated with a horseradish peroxidase-conjugated secondary antibody against rabbit IgG (1:2000 dilution; Sigma). The antigen-antibody complexes on the blots were detected by SuperSignal chemiluminescence kit as described in the manufacturer's protocol (Pierce Biotechnology, Rockford, IL) and visualized by autoradiography. To confirm equal loading of proteins, the blots were also immunoprobed with a rabbit polyclonal antibody against the cytoskeletal protein α-tubulin (1: 2500 dilution). The consistent equal signals of α-tubulin from the different extracts also indicate that malathion does not interfere with protein synthesis in L929 cells.

2.2.5. Assay of Caspase-8, -9 and -3 Activities

Caspase-3, one of the principal caspases in apoptotic cells, is activated during the apoptotic signaling by upstream caspases including caspase-8 and –9 (Bratton and Cohen, 2001). The activities of caspase-8, -9 and -3 were determined using the colorimetric tetrapeptide substrates Ac-IETD-*p*NA, Ac-LEHD-*p*NA and Ac-DEVD-*p*NA, respectively, as previously described by Srinivasula et al. (2001). Lysates (30 μg protein) from malathion treated cells were incubated with 0.2 mM of caspase-3, -8 or –9 colorimetric peptide substrates in a ½ volume microtiter plate at 30°C. The assays were performed both in the presence and absence of the caspase-8 (Ac-IETD-CHO), caspase-9 (z-LEHD-FMK) or caspase-3 (Ac-DEVD-CHO) specific inhibitors (20 μM) to eliminate non-specific activities in the lysates. Caspase activity was monitored spectrophotometricaly, with a microtiter plate reader by measuring the increase in absorbance at 405 nm, which corresponds to the amount of *p*-Nitroaniline (*p*NA) liberated from the peptide substrates. The absorbance change was converted into units of enzyme activity using a standard curve generated with free *p*NA. One unit of caspase-3, -8, or –9 activity corresponds to the amount of enzyme that will release 1 pmol of *p*NA from 0.2 mM DEVD-*p*NA, IETD-*p*NA or Ac-LEHD-*p*NA per min, respectively.

2.2.6. DNA Fragmentation Assay

DNA fragmentation was analysed using agarose gel electrophoresis. Genomic DNA was prepared using the method of Gong et al. (1994) with slight modification. After treatment of L929 fibroblasts with malathion, about $2x10^6$ cells were washed in ice-cold PBS and prefixed in ice-cold 70% ethanol. The partially degraded oligonucleosomal DNA was selectively extracted with 100 μl of 0.2 M phosphate-

citrate buffer at pH 7.8. The extracts were then treated with NP-40 (0.015%), RNase A (0.06 mg/ml) and Proteinase K (0.06 mg/ml). The DNA was extracted and purified sequentially with phenol: chloroform (1:1) and chloroform followed by precipitation in 100% ethanol. The samples were then air dried and resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.5). DNA samples (5 μg each) were resolved by electrophoresis for 4 h at 40 Volts on a 1.5% agarose gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 7.5). The gels were stained with ethidium bromide and the banding patterns were visualized with the Foto/ Eclipse UV transilluminator and photographed.

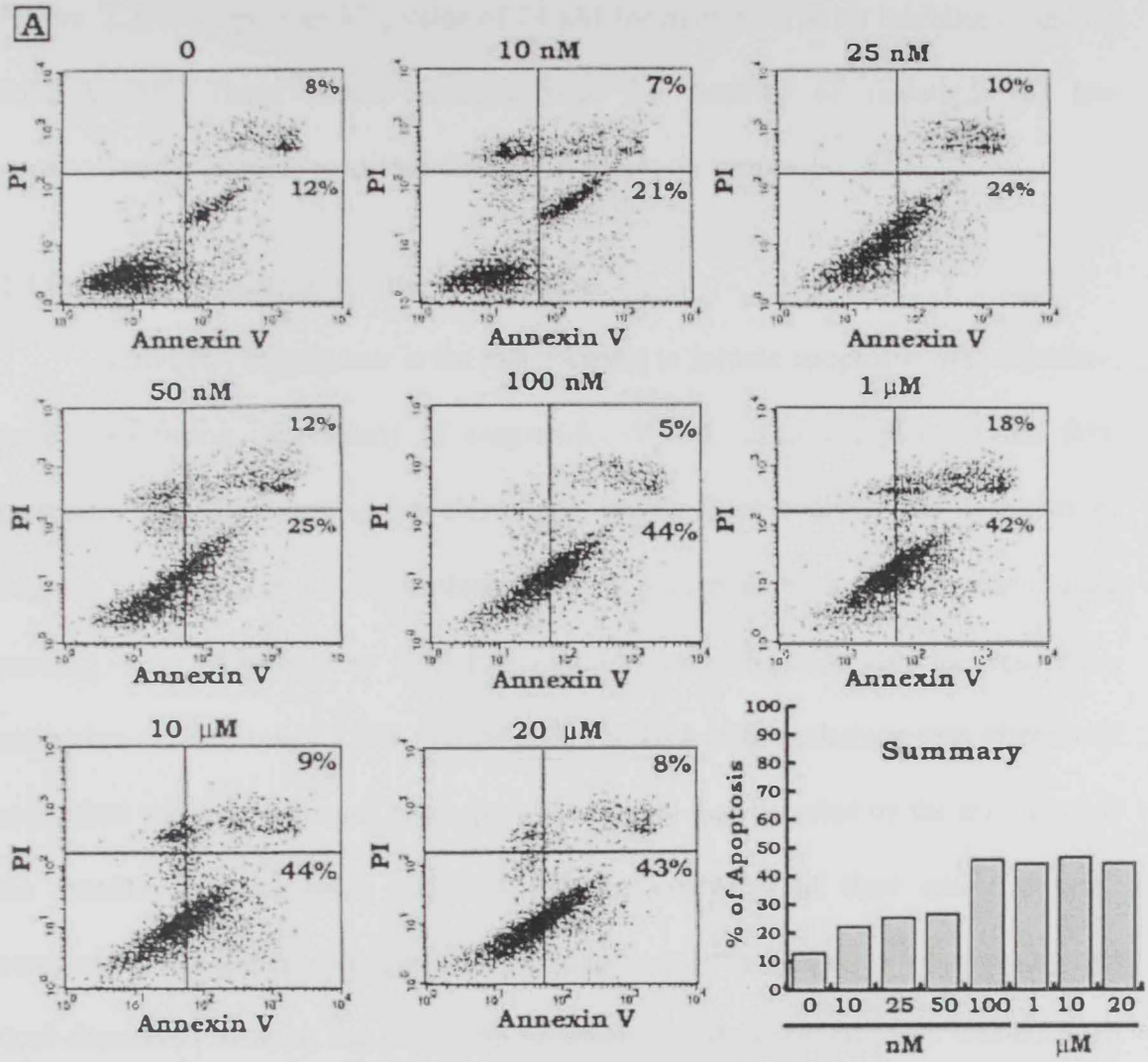
2.2.7. RBC-AChE Activity

The effect of malathion (0-50 μM) on AChE activity was measured in diluted whole blood samples in the presence of the selective butyrylcholinesterase inhibitor, ethoproprazine, as previously described (Worek et al., 1999). The assay, which is based on Ellman's method, measures the reduction of dithiobis-nitrobenzoic acid (DTNB) to nitrobenzoate (TNB^-) by thiocholine, the product of acetylthiocholine (ASch) hydrolysis. Freshly drawn venous blood samples from three male mice were diluted in 0.1 M phosphate buffer (pH 7.4) and incubated with DTNB (10mM) and ethopropazine (6 mM) for 10 min at 37 °C prior to addition of ASch. The change in the absorbance of DTNB was measured at 436 nm. The AChE activity was calculated using an absorption coefficient of TNB^- at 436 nm (ε = 10.6 mM^{-1} cm^{1}). The values were normalized to the hemoglobin (Hb) content (determined as cyanmethemoglobin) and expressed as mU/μmol/Hb. IC_{50} values were derived from a plot that indicates percent AChE activity as a function of malathion concentration.

2.3. Results

2.3.1. Evaluation of Apoptosis by Flow Cytometry

First, we investigated whether malathion induces apoptosis. To assess this effect, we incubated L929 cells with various concentrations of malathion overnight and measured apoptosis by counting the Annexin V stain positive and PI negative cells (Annexin $^{+}$, PI^{-}). As shown in figure. 2.1A, malathion induced apoptosis in L929 cells in a dose-dependent manner. As compared to untreated cells, apoptosis became apparent (Annexin^{+}, PI^{-}; 10% above the background apoptosis seen in the control) at doses of malathion as low as 10 nM and reached maximum (30%) in cells treated with 1 μM of malathion. Higher concentrations of malathion up to 20 μM did not cause any further increase in the percentage of apoptotic cells. At all concentrations of malathion tested, the number of necrotic cells that lost their plasma membrane as determined by PI stain (Annexin^{-}, PI^{+}) were not significant. However there was a dose-dependent increase in the number of cells with positive Annexin V and PI signal (Annexin^{+}, PI^{+}). Since there is a possibility that the necrotic stage cells also acquire Annexin V signal and could not be distinguished from apoptotic cells, the Annexin^{+}, PI^{+} cells were not included in the analysis (Figure. 2.1A, B). To further assess the effect of malathion-induced apoptosis, we determined the percentage of apoptotic cells after incubation with 1 μM of malathion at different intervals of time (Figure. 2.1B). Malathion induced apoptosis in a time-dependent manner. Apoptosis (Annexin^{+}, PI^{-}) was apparent as early as 2 h of incubation with malathion (~10%) and reached maximum (~45%) between 16 and 20 h of treatment. These results indicate that malathion is a potent apoptosis-inducing agent at concentrations as low as ≤ 1 μM. To relate the potential of malathion to induce apoptosis at such low concentrations to it's cholinergic effect, we determined the IC_{50} value for malathion. As shown in


A
0
8%
12%
10 nM
7%
21%
25 nM
10%
24%
50 nM
12%
25%
100 nM
5%
44%
1 μM
18%
42%
10 μM
9%
44%
20 μM
8%
43%
PI
Annexin V
Summary
% of Apoptosis
0 10 25 50 100 1 10 20
nM
μM

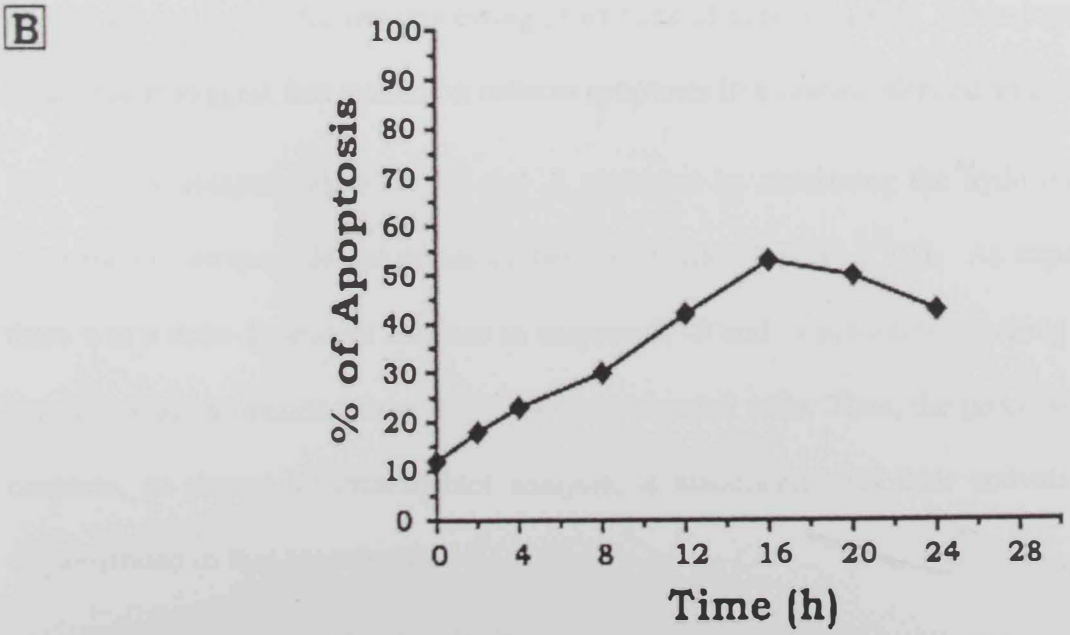

B
% of Apoptosis
Time (h)

Figure 2.1: Malathion induces apoptosis in a dose- and time-dependent manner.

A. L929 cells were treated with various concentrations of malathion (0-20 μM) overnight. After treatment, cells were harvested, stained with Annexin V-FITC and PI and analyzed by flow cytometry. The percentages of cells in early ($Annexin^+$, PI^-; lower right quadrant) and late apoptotic-necrotic stages ($Annexin^+$, PI^+; upper right quadrant) are indicated. The results are representative of two independent experiments. Cells with positive Annexin stain ($Annexin^+$, PI^-) were counted as apoptotic and their number expressed as a percentage of the total cells.

B. L929 cells were incubated with 1 μM malathion for different periods of time (0-24 h) and apoptosis was analyzed by flow cytometry as described in (A). The control (0) represents cells incubated under similar conditions in the presence of the carrier DMSO. The results represent the average of two independent experiments.

Figure. 2.2, we report an IC_{50} value of 24 μM for malathion as an inhibitor of mouse RBC-AcChE. These results indicate that the toxicity of malathion at low noncholinergic doses is mediated through its effect on apoptosis.

2.3.2. Processing and Activation of Caspases

Activation of caspases is the major signal to initiate apoptosis. We, therefore, tested processing (activation) of caspase-8, -9 and -3 to complement the flow cytometry results. Figure 2.3A shows the immunoblot analysis for detection of caspase processing in L929 fibroblasts treated with malathion. Cell lysates (50 μg protein) were separated by SDS-PAGE and immunoblotted with the respective antibodies. Treatment of L929 murine cells for 16 h with malathion was effectively associated with processing of procaspase-8, –9, and -3 as detected by the formation of the smaller subunits (p20, p35 and p19, respectively) of their active enzyme complexes. While there was a gradual increase in caspase-8, -9 and –3 processing in a dose-dependent manner, the maximum processing of these proenzymes was detected after treatment with 1 μM of malathion. Consistent with the apoptosis percentages observed by AnnexinV staining assay, increasing the malathion concentrations up to 20 μM did not induce further processing of any one of these caspases. Taken together, these results suggest that malathion induces apoptosis in a caspase-dependent fashion.

We directly assayed caspase-8, -9 and -3 activities by measuring the hydrolysis of colorimetric tetrapeptide substrates of these enzymes (Figure. 2.3B). As expected, there was a dose-dependent increase in caspase-8, -9 and -3 activities, reaching peak levels in lysates obtained from 1μM malathion-treated cells. Thus, the processing of caspases, as shown by immunoblot analysis, is associated with their activation as demonstrated in this experiment.

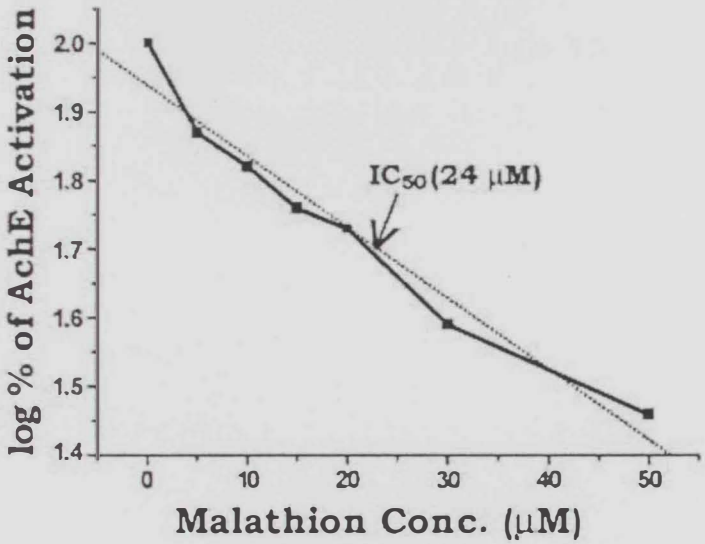

log % of AchE Activation
2.0
1.9
1.8
1.7
1.6
1.5
1.4
IC50 (24 μM)
0
10
20
30
40
50
Malathion Conc. (μM)

Figure 2.2: IC_{50} value for malathion as inhibitor of mouse RBC-AChE.

The effect of malathion (0-50μM) on AChE activity was measured in mouse blood samples as described in the Materials and Methods, and the IC_{50} values were calculated from a plot that indicates percent AChE activity as a function of malathion concentration. The results are representative of two independent experiments.

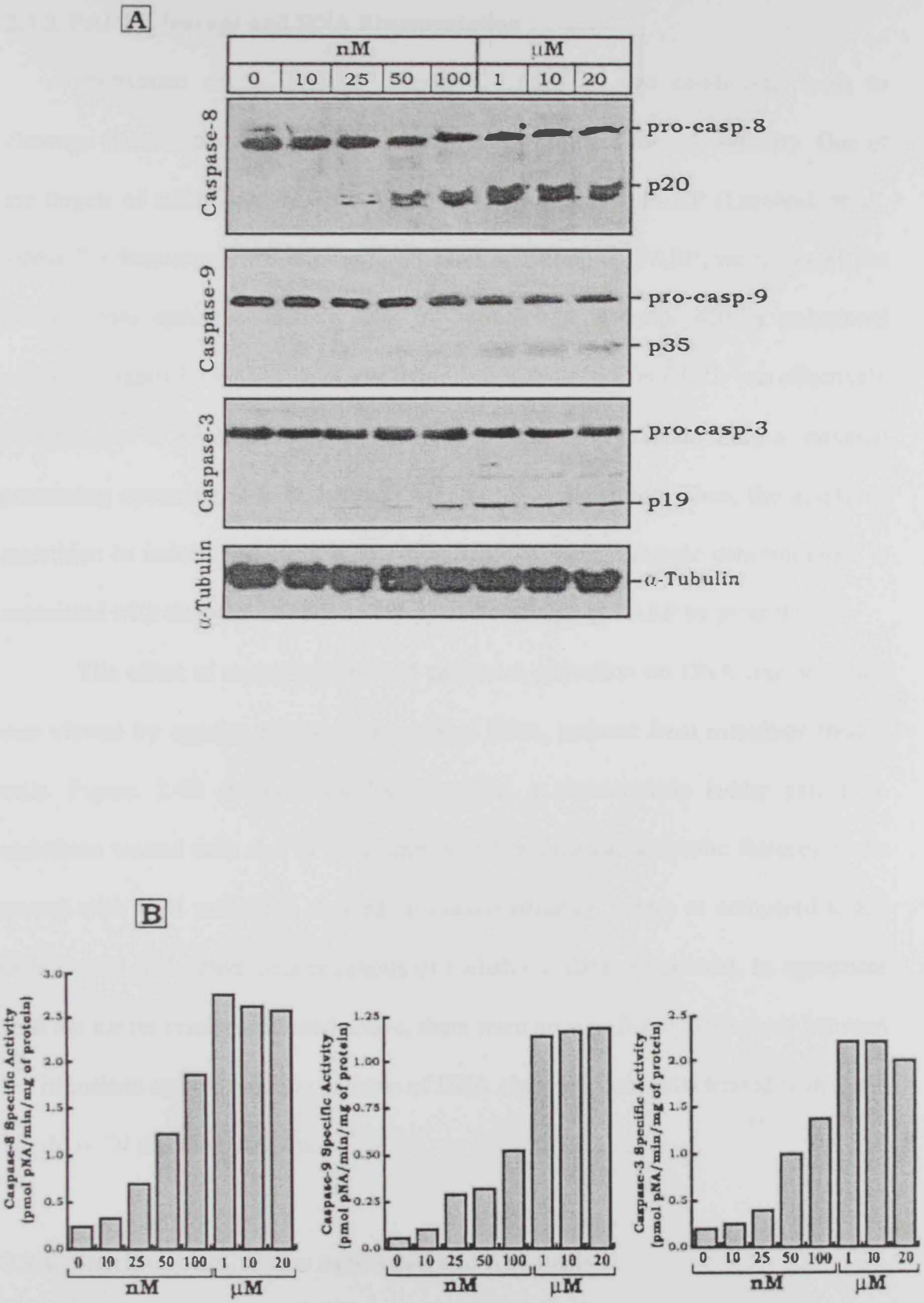

A
nM
μM
0
10
25
50
100
1
10
20
Caspase-8
pro-casp-8
p20
Caspase-9
pro-casp-9
p35
Caspase-3
pro-casp-3
p19
α-Tubulin
α-Tubulin
B
Caspase-8 Specific Activity (pmol pNA/min/mg of protein)
Caspase-9 Specific Activity (pmol pNA/min/mg of protein)
Caspase-3 Specific Activity (pmol pNA/min/mg of protein)
nM
μM

Figure 2.3: Malathion promotes caspase-8, -9 and –3 activation in L929 cells.

A. Whole cell extracts (50 μg), obtained from L929 cells after treatment with various concentrations of malathion for 16 h as indicated on top of each lane, were analyzed by Western blotting with antibodies against caspase-8, -9 and –3, respectively. The unprocessed forms of caspase-8 (pro-casp-8; 54 kDa), caspase-9 (pro-casp-9; 46 kDa), caspase-3 (pro-casp-3; 32 kDa) and their respective cleavage products p20, p35 and p19 of the active enzymes are indicated. The same membranes were also probed with an antibody against α-tubulin as a loading control. The results are representative of two independent experiments.

B. Extracts (30 μg) from cells treated with various concentrations of malathion were analyzed for caspase-8, -9 and -3 catalytic activities using their specific colorimetric tetrapeptide substrates Ac-IETD-*p*NA, Ac-LEHD-*p*NA and Ac-DEVD-*p*NA, respectively. The assays were performed both in the presence and absence of the caspase-8 (Ac-IETD-CHO), caspase-9 (z-LEHD-FMK) or caspase-3 (Ac-DEVD-CHO) specific inhibitors (20 μM) to eliminate nonspecific activities in the lysates. The specific enzyme activities, which represent the average of two independent experiments, were measured as described in the methods.

2.3.3. PARP Cleavage and DNA Fragmentation

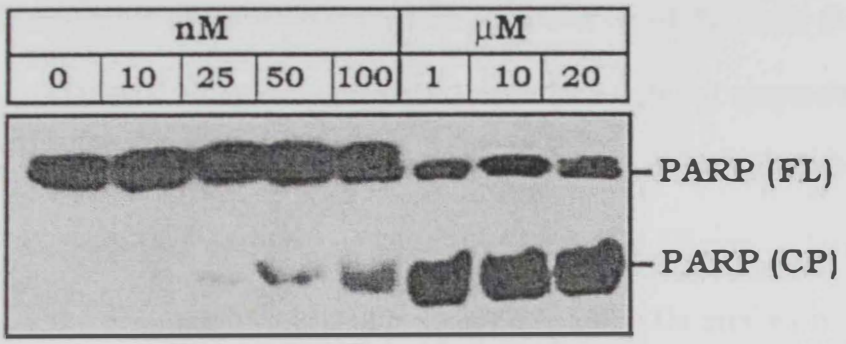
Activation of the effector caspase-3, under *in vivo* conditions, leads to cleavage (inactivation) of essential target proteins required for cell viability. One of the targets of active caspase-3, is the DNA repair enzyme, PARP (Lazebnik et al., 1994). To demonstrate the effect of caspase-3 activation on PARP, we subjected the lysates from malathion-treated cells to immunoblot analysis with a polyclonal antibody against PARP. As shown in figure. 2.4A, the 116 kDa PARP was effectively cleaved into a 89 kDa fragment in a dose-dependent manner with a maximal processing occurring in cells treated with 1 μM of malathion. Thus, the ability of malathion to induce apoptosis at low but effective noncholinergic concentrations is associated with elevated caspase-3 activity and cleavage of PARP by proteolysis.

The effect of malathion-induced caspase-3 activation on DNA fragmentation was viewed by agarose gel electophoresis of DNA, isolated from malathion treated cells. Figure. 2.4B shows DNA fragmentation, a characteristic ladder pattern in malathion treated cells that is consistent with the classical apoptotic features. Cells treated with 1μM malathion showed an intense banding pattern as compared to the cells treated with lower concentrations of malathion (data not shown). In agreement with the earlier results discussed above, there were no significant differences between the intensities and the banding patterns of DNA obtained from cells treated with 1 μM, 10 μM or 20 μM of malathion.

2.3.4. Specificity of Caspase Activation and Apoptosis

The above results suggest that the ability of malathion to induce apoptosis is dependent on triggering the activation of caspase cascades. To investigate this possibility, we compared the ability of malathion to induce apoptosis in the presence

A



B

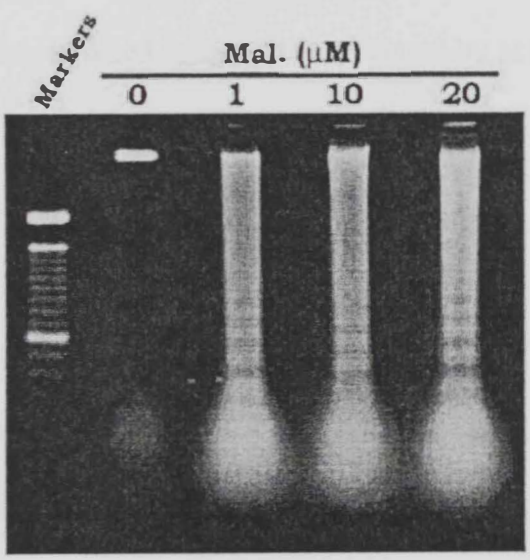


Figure 2.4: Malathion induces PARP cleavage and DNA fragmentation in L929 cells.

A. 50 μg protein of whole cell extracts, obtained from cells treated with various concentrations of malathion, as indicated on top of each lane, were loaded onto a 8% SDS-PAGE, followed by Western blotting with a polyclonal antibody against PARP. The 116 kDa full-length (FL) and the 89 kDa cleavage product (CP) immunoreactive bands of PARP are labeled.

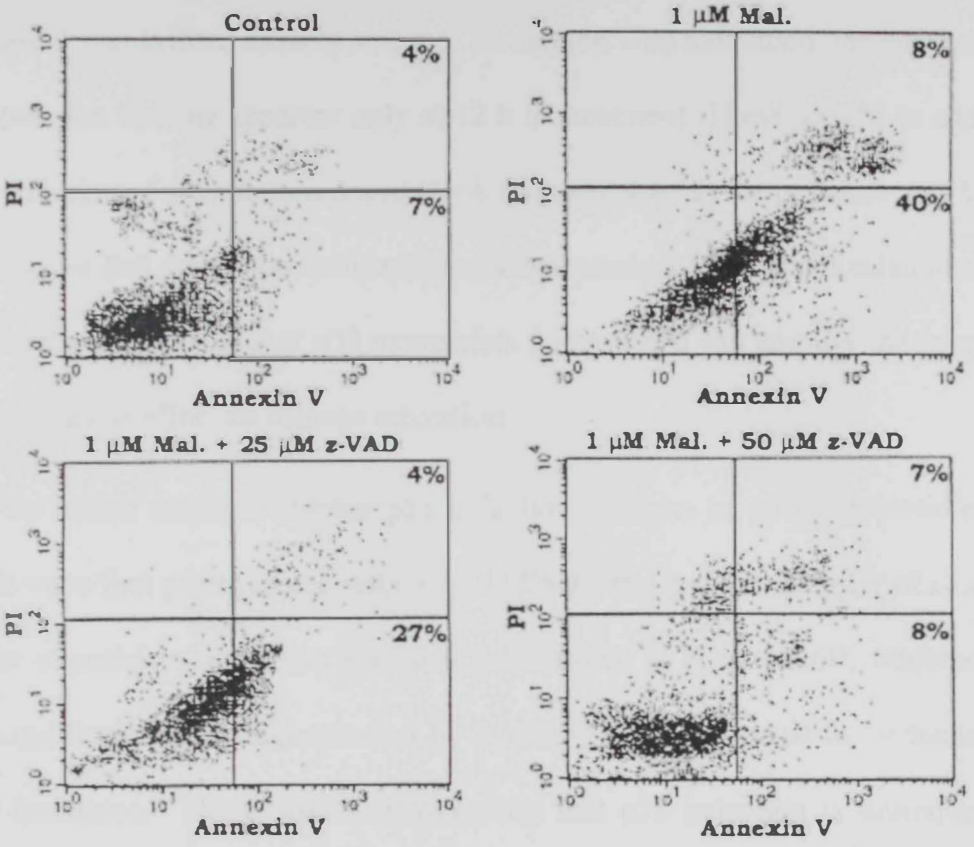
B. Oligonucleosomal DNA was selectively extracted from cells treated with different concentrations of malathion as described in the methods. 5 μg of the extracted DNA was resolved onto 1.5% agarose gels and the DNA bands were visualized on a UV transilluminator after staining with ethidium bromide. A DNA size marker (marker) was run along with the samples as indicated. The results are representative of two independent experiments.

and absence of the broad-range caspase inhibitor, z-VAD-FMK (Figure. 2.5A). L929 cells were treated with 1 μM malathion alone or in combination with 25 μM or 50 μM z-VAD-FMK and apoptosis was assessed by the flow cytometry method describe earlier. As compared to untreated control and malathion alone treated cells, 25 μM z-VAD-FMK decreased the ability of malathion to induce apoptosis by ~40%, while the presence of 50 μM z-VAD-FMK completely prevented malathion induced apoptosis. Inhibition of the malathion-mediated apoptosis in the presence of z-VAD-FMK correlated well with a substantial decrease in DNA fragmentation (Figure. 2.5B). Under these conditions, the cleavage of full-length PARP into a 89 kDa product was also inhibited in the presence of z-VAD-FMK (not shown). These observations indicate that the malathion-induced apoptosis is mediated by activation of the caspase cascades in L929 fibroblasts.

2.3.5. p53 Expression and Caspase Activation

It was reported that malathion is capable of inducing DNA damage in human peripheral blood lymphocytes as well as in purified bacterial plasmids (Griffin and Hill, 1978; Richardson and Imamura, 1985; Pluth et al., 1996; Blasiak et. al., 1999). This effect was attributed to its ability to act as a strong positive alkylating agent. Cells respond to DNA damage by increasing the production of several DNA repair enzymes/proteins, including p53. The protein molecule p53 is known to induce apoptosis through its effect on mitochondrial pathway (Robles et al., 1999; Chao et al., 2000). To examine the possible relationship between malathion-induced apoptosis and p53, we examined the kinetics of processing of caspase-3 and expression of p53

A


Control
4%
7%
PI
Annexin V
1 μM Mal.
8%
40%
PI
Annexin V
1 μM Mal. + 25 μM z-VAD
4%
27%
PI
Annexin V
1 μM Mal. + 50 μM z-VAD
7%
8%
PI
Annexin V

B

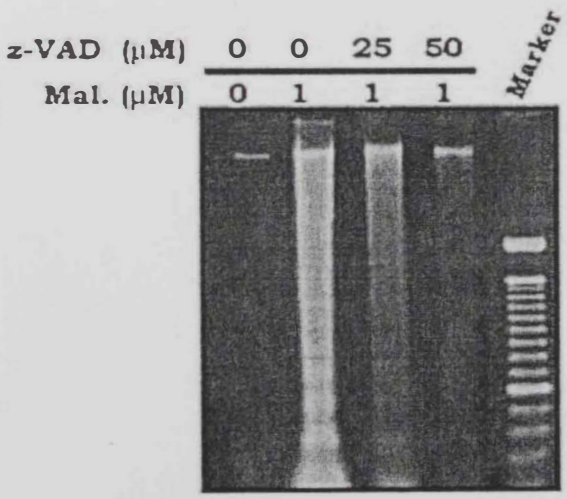

z-VAD (μM) 0 0 25 50
Mal. (μM) 0 1 1 1
Marker

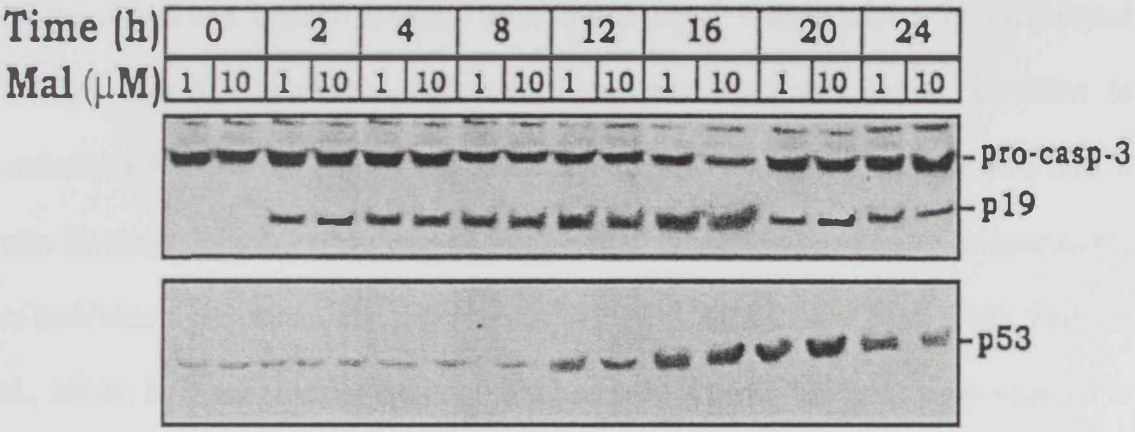
Figure 2.5: Malathion induces a caspase-dependent apoptosis in L929 cells.

A. L929 cells were cultured for 16 h without (control) or with 1 μM malathion alone, or in combination with 25 μM or 50 μM z-VAD-FMK for 6h. After staining with Annexin V-FITC and PI, apoptotic cells were counted by flow cytometry as described above (Figure.2.1 A). The results are representative of three independent experiments. B. Oligonucleosomal DNA was extracted from L929 cells treated with malathion alone or in combination with z-VAD-FMK as indicated on top of each lane. DNA fragmentation was analyzed by agarose gel electrophoresis as described in Figure. 2.4 B. The results are representative of two independent experiments.

by immunoblotting (Figure. 2.6A). While the appearance of the cleaved p19 product of caspase-3 was evident as early as 2 h of incubation with malathion, the increase in p53 expression became apparent only at 12 h of treatment. These results, in addition to the inhibition of malathion-induced DNA fragmentation in the presence of z-VAD-FMK, suggest that malathion induces a caspase-dependent DNA fragmentation which subsequently promotes higher p53 expression. In turn, p53 can amplify the apoptotic signal through its effect on caspase activation.

To further confirm whether p53 induction is a pre- or post- apoptotic event, the cells were first preincubated with z-VAD-FMK, the general inhibitor of caspase, and later exposed to malathion for 16 h. As shown in figure. 2.6B, inhibition of caspase mediated DNA fragmentation by z-VAD-FMK suppressed the induction of p53 by malathion. These results thus confirm that p53 induction is subsequent to caspase-mediated DNA fragmentation.

A


Time (h)
0
2
4
8
12
16
20
24
Mal (μM)
1
10
pro-casp-3
p19
p53

B

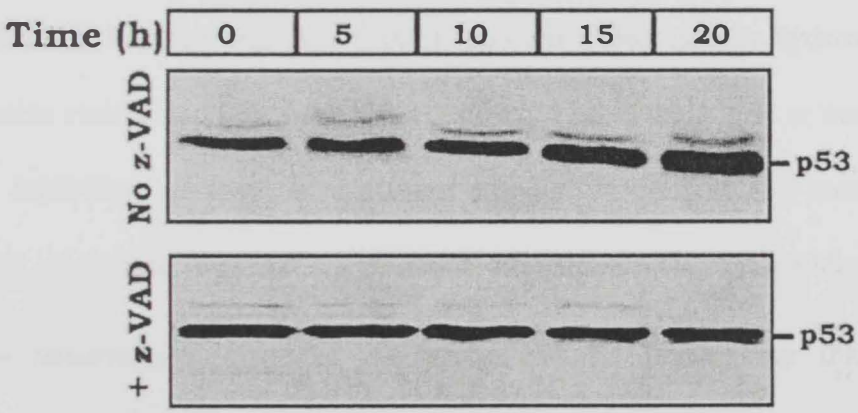

Time (h)
0
5
10
15
20
No z-VAD
p53
+ z-VAD
p53

Figure 2.6: Malathion-induced expression of p53 is subsequent to caspase activation.

A. 50 μg protein of whole cell extracts, obtained from cells treated with 1 μM or 10 μM malathion at different time points, as indicated on top of each lane, were loaded onto a 12% SDS-PAGE, followed by Western blotting analysis with polyclonal antibodies against caspase-3 and p53. The immunoreactive bands of the unprocessed form of caspase-3 (pro-casp-3), p19 cleavage product of the active caspase-3 enzyme and p53 are labeled.

B. 50 μg protein of whole cell extracts, obtained from cells treated with 50 μM of z-VAD-FMK for 6h prior to incubation with 1 μM malathion at different time points, were loaded onto a 12% SDS-PAGE and the expression level of p53 was visualized by Western blotting with a polyclonal antibody against the protein. The results are representative of two independent experiments.

2.4. Discussion

Recent studies on OPCs toxicity have focused on chronic intoxication, environmental contamination and diseases not immediately related to their toxic potential on AChE such as Parkinson's disease, skin, lung and immune diseases. Most of these diseases appear as long term and delayed health effects in agricultural workers and in populations exposed to environmental sources. In an effort to understand the pathophysiology of these non-cholinergic effects, both *in vitro* and *in vivo* investigations have been carried out, using concentrations of OPCs comparable to their chronic exposure levels (Blasiak et al., 1999; Samimi and Last., 2001; Saleh et al., 2002). In these studies, the dosage schedules (<0.001-125 μM) were selected to model the environmental and occupational exposure levels and were kept far below the levels found in the blood of individuals (530-1560 μM) who were dead following an overdose of malathion (Jadhav et al., 1992; Blasiak et al., 1999). These studies have provided insights into the mechanisms of action of OPCs that are independent of AChE inhibition. Samimi and Last (2001) reported a decrease in hydroxylation of collagen lysine residues in fetal lung fibroblasts exposed to malathion or malaoxon (0-125 μM). Inhibition of lysyl hydroxylase activity will affect the maturation of collagen that might lead to skin diseases and teratogenic effects of malathion.

The observations made in the present study demonstrate that at non-cholinergic doses (0.01-20 μM) malathion induces apoptosis in cultured L929 mouse fibroblasts. The cells exposed to malathion exhibited both time- and dose-dependent morphological and biochemical changes that are characteristic of classical apoptosis. This finding is of great physiological relevance as OPCs, including malathion, are readily absorbed through the skin, respiratory or gastrointestinal tract and via eyes (Marty et al., 1994). Dermal exposure to pesticide applicators has been estimated to

be as high as > 5 mg malathion per kg per day (Wolfe et al., 1967). Immune cells such as T-lymphocytes are also widely used as a sensitive tool to examine the subclinical effects of chemical exposures (Luster et al., 1988). At low, non-cholinergic doses, malathion also induced apoptosis in EL4 murine T-lymphocytes (data not shown). In a previous study we have also reported that at doses below IC_{50} (12.5 nM for mouse RBC-AChE), paraoxon is a potent inducer of apoptosis both in cultured EL4 T-lymphocytes and mouse fibroblasts (Saleh et al., 2002). These results, as well as a growing body of recent evidence (Carlson and Ehrich, 2001) indicates that apoptosis might be one of the mechanisms by which the cells respond to chronic exposure to low doses of OPCs.

The acute toxicity of OPCs is mediated by the inhibition of AChE. However, the inhibition appears to decrease as the OPC concentration increases (Kardos and Sultatos, 2000). The molecular basis of the biphasic dose response is poorly understood. In our study, the response to non-cholinergic doses of malathion (10 nM-20 μM) exhibited a hyperbolic curve reflecting saturation kinetics with a maximal percentage of apoptosis occurring at 1 μM level, a value much less than the observed IC_{50} value of 24 μM for malathion as an inhibitor of mouse RBC-AChE.

The chemical properties of the OPCs including malathion, support their potential to cause cell injury. The cells respond to such an injury by undergoing apoptosis. Recent studies have demonstrated the ability of malathion to act as a strong positive alkylating and cause genotoxic effects (Griffin and Hill, 1978; Richardson and Imamura, 1985; Pluth et al., 1996; Blasiak et al., 1999; Amer et al., 2002; Giri et al., 2002). Further, the lipophilic nature of OPCs facilitates their interaction with the membrane and lead to perturbations of the phospholipid bilayer structure (Videira et al., 2001). Such a phenomenon occurring in mitochondrial inner

membrane might affect electron delivery and signal apoptosis as a result of mitochondrial injury (Moreno and Madeira, 1990).

Caspase activation is a hallmark of classical apoptosis and our results show the activation of both the initiator caspases, namely caspase-8 and caspase-9. Furthermore, we demonstrate that there is a coordinated induction of apoptotic signals in malathion treated cells. The time courses of caspase-3 activation and p53 induction in malathion treated cells indicate that during the initial phase of malathion-mediated cell injury, there is an activation of caspase cascade that will execute apoptosis by DNA fragmentation. Caspase-mediated DNA fragmentation leads to the induction of p53 expression, which can amplify the apoptotic processes through its effect on mitochondria.

In summary, the results of this study indicate that at non-cholinergic doses, malathion induces a caspase-mediated apoptosis. By using caspase specific inhibitors we are currently investigating the molecular mechanisms by which malathion and other OPCs trigger apoptosis. Such studies will provide further insights into the molecular mechanisms behind OPCs poisoning, and might suggest new diagnostic and therapeutic approaches to their toxicity.

CHAPTER 3:

THE ROLE OF A MITOCHONDRIAL PATHWAY IN THE INDUCTION OF APOPTOSIS BY THE ORGANOPHOSPHORUS COMPOUND MALATHION

3.1. Introduction

Malathion is one of the most widely used organophosphate compounds (OPCs) employed in agriculture as a pesticide, in veterinary practice as an ectoparasiticide (Flessel et al., 1993), in eradication of human body lice (Elston, 2002) and in the food industry (Savage et al., 1981). Acute toxic effects induced by malathion pesticides are mainly caused by inhibition of acetylcholinesterase (AChE) in nervous tissues with a consequent increase in the levels of the neurotransmitter acetylcholine (Kwong, 2002; Overstreet and Djuric, 2001). The widespread use in agriculture and household practices has raised concern over its potential to cause adverse health effects in humans, domestic animals, wildlife and fish (Flessel et al., 1993; Wolfe and Seiber, 1993).

Recent studies have indicated that malathion affects several biochemical pathways that do not involve modulation of AChE activity. Both under *in vitro* and *in vivo* conditions, malathion has been shown to induce lipid peroxidation (Ahmed et al., 2000; Datta et al., 1994; Hazarika et al., 2003), DNA damage, chromosomal aberrations (Blasiak et al., 1999; Giri et al., 2002) and malignant transformation (Cabello et al., 2001). Malathion has also been shown to modulate oxidative stress and immune response in experimental animals (John et al., 2001; Johnson et al., 2002).

Our recent studies have revealed that at non-cholinergic concentrations (up to 1 μM), malathion induces apoptosis in cultured murine L929 fibroblasts (Masoud et al., 2003). Malathion-induced cell death showed features similar to classical apoptosis as revealed by caspase-3 activation and DNA fragmentation. However, the mechanism of malathion-stimulated processing and activation of caspase-3 was not characterized.

Effector caspases, including caspase-3, may be activated via mitochondria-independent or –dependent pathways (Joza et al., 2002; Shi, 2002). The mitochondria-independent pathway is activated upon binding of ligands of the tumor necrosis-factor receptor (TNF-R) family, such as Fas and TNF, to their agonist receptors and requires the direct cleavage of caspase-3 by activated caspase-8 (Stennicke et al., 1998).

Alternatively, caspase-3 can be activated through a mitochondria-dependent pathway. Mitochondria have been recognized to play an important role in apoptosis by releasing cytochrome *c* from its intermembrane space into the cytoplasm (Reed, 1997). In the cytoplasm, cytochrome *c*, in the presence of ATP or dATP, associates with a complex of apoptotic protease activating factor 1 (Apaf-1) and caspase-9, which leads to autocatalytic cleavage and activation of caspase-9 in this complex (Saleh et al., 1999; Qin et al., 1999). In turn, caspase-9 can then directly cleave and activate the proform of caspase-3.

The mechanisms by which cytochrome *c* is translocated from mitochondria into the cytosol are not fully understood. Current theories involve transient opening of the mitochondrial permeability transition pore causing slight swelling as well as formation of pores in the outer membrane by proapoptotic members of the bcl-2 family, *e.g.* Bid, BAX, Bad and BAK (Budihardjo et al., 1999; Wei et al., 2001). These mechanisms mediate the passage of unbound cytochrome *c* through the mitochondrial outer membrane. However, the mechanism by which cytochrome *c* dissociates from the inner membrane is less clear. Cytochrome *c* is bound to the outer surface of the inner membrane phospholipids, particularly cardiolipin, by electrostatic forces (predominating at neutral pH). Dissociation from the inner membrane is a necessary first step before cytochrome *c* can pass through release channels and ultimately reach the cytosol (Garcia-Fernandez et al., 2002; Petrosillo et al., 2003).

Thus, a decrease in cardiolipin synthesis and/or its oxidation by free oxygen radicals (ROS), generated by mitochondria in stress-induced apoptosis, may directly affects cytochrome *c* release from the mitochondrial inner membrane into the cytosol (Petrosillo et al., 2003).

In this study we have extended our observations on the apoptotic effect of malathion in cultured murine L929 cells. Employing caspase specific inhibitors as well as indicators to study mitochondrial membrane permeability changes, we have delineated the sequence of events involved in malathion-stimulated apoptosis by immunoblot, flow cytometry and enzyme activity analyses. The results indicate that malathion stimulated apoptosis by altering mitochondrial transmembrane potential, inducing ROS formation and reducing cardiolipin content in mitochondria of L929 treated cells. These events subsequently caused the release of cytochrome *c* into the cytosol which leads to activation of the Apaf-1/caspase-9 apoptosome.

3. 2. Materials and Methods

3.2.1. Reagents

Malathion [*S*-1,2-bis(ethoxycarbonyl)ethyl *O,O*-dimethyl phosphorodithioate; CAS Registry No. 121-75-5] was obtained from PolyScience (Division of Preston Industries, Inc. Niles, Illinois). As stock solution of malathion (100 mM) was prepared in dimethyl sulfoxide (DMSO) and stored at -80°C. The working dilutions in phosphate buffered saline (PBS) were prepared just before use. Protease inhibitors (PMSF, pepstatin A, leupeptin and aprotinin), *N*-acetyl-L-cysteine (NAC) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were acquired from Sigma Chemical Company (Sigma Aldrich Chemie GmbH Steinheim, Germany). The colorimetric tetrapeptide substrates for caspase-8 (Ac-IETD-*p*NA), caspase-9 (Ac-LEHD-*p*NA) and caspase-3 (Ac-DEVD-*p*NA) were purchased from Calbiochem, San Diego, CA, (USA). Caspase-8 inhibitor (zIETD-fmk), Caspase-9 inhibitor (zLEHD-fmk), Caspase-3 inhibitor (zDEVD-fmk), and the general caspase inhibitor (zVAD-fmk) were obtained from Alexis Corporation (Switzerland). The following polyclonal or monoclonal antibodies were obtained from various sources as indicated: anti-caspase-8, anti-p53, anti-cytochrome *c*, anti-α-tubulin, anti-VDAC, anti-ANT, anti-Bax, Anti-Bad, Anti-Bak, anti-Bcl-2 and anti-Bcl-x_L antibodies (Santa Cruz Biotechnology, CA, USA), anti-caspase-9 and anti-caspase-3 antibodies (Stressgen Biotechnologies, Victoria, BC, Canada) and anti-Bid antibody from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA).

3.2.2. Cell Culture

Mouse L929 cells (ATCC, Manassas, VA, USA) were grown in monolayers in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco BRL,

Grand Island, NY, USA), 100 Units/ ml penicillin and 100 μg/ml streptomycin and were kept at 37°C in humidified 5% CO_2/95% air. Prior to confluence, the cells were harvested and seeded onto plastic 6–well culture plates or 100-mm culture dishes at 5 x 10^5 cells/ml and allowed to grow for 2-3 days. Following this, they were incubated with various concentrations (10-60 μM) of inhibitors specific to caspase-8 (zIETD-fmk) or caspase-9 (zLEHD-fmk), or with 50 μM of the general inhibitor of caspases zVAD-fmk for 4 h. The cells were then exposed to malathion at a concentration of 1 μM for 16 h. The control cells, i.e. cells without malathion or caspase specific inhibitors, were incubated with the carrier solvent of these compounds (DMSO). Following the treatment, cells were harvested by centrifugation at 500g for 5 min, washed with phosphate buffered saline (PBS) and subsequently used for various biochemical investigations.

3.2.3. Analysis of Apoptosis by Flow Cytometry

Apoptosis in the L929 cells subjected to various treatments was determined with the Annexin V-FITC (fluorescein isothiocyanate) staining kit from BD Biosciences (Franklin Lakes, NJ, USA). Propidium Iodide (PI) was used to differentiate apoptotic cells with preserved membrane integrity ($Annexin^+$, PI^-) from necrotic cells that had lost membrane integrity ($Annexin^-$, PI^+). The assay was performed following the manufacturer's procedure. After staining, the percentage of apoptotic cells under various treatments were analyzed by flow cytometry (FACSCalliber, BectonDickinson, USA) as described recently (Masoud et al., 2003; Saleh et al., 2003).

3.2.4. Western Blot Analysis

Malathion-treated cells were harvested, washed twice with PBS and suspended in a lysis buffer containing 100 mM HEPES, pH 7.5, 10% sucrose, 10 mM DTT, 0.1% CHAPS, 150 mM NaCl and protease inhibitors (1 mM PMSF, and 1 μg/ml leupeptin, aprotinin and pepstatin A). The cells were lysed by 4-5 repeated cycles of freeze thawing and were centrifuged at 4°C for 30 min at 14,000g. The supernatant was collected and stored at -80°C or used immediately. The samples were analyzed for total protein by a protein assay kit based on the Bradford colorimetric reaction (Bradford, 1976, BioRad, Hercules, CA, USA). Cell lysates (50 μg protein per lane) were separated on 12% SDS-polyacrylamide gels and electroblotted onto a PVDF membrane (BioRad, USA) by standard techniques as described in our recent publications (Masoud et al., 2003; Saleh et al., 2003). After electrophoretic transfer, the membranes were blocked by incubation for 2 h in PBS buffer containing 5% non-fat dry milk and 0.1% Tween-20. The blots were then incubated for 2 h at room temperature or overnight at 4°C with one of the following polyclonal antibodies (except for anti-Bax, which is a monoclonal antibody) diluted in PBS containing 0.1% Tween-20 and 2% non-fat dry milk. The following were the dilutions of the antibodies: anti-caspase-8 (1:1000), anti-caspase-9 (1:3000), anti-caspase-3 (1:2000), anti-cytochrome *c* (1:2000), anti-VDAC (1:1000), anti-ANT (1:1000), anti-Bcl-2 (1:1000), anti-Bcl-x_L (1:1000), anti-Bax (1:500), anti-Bad (1:1000), anti-Bak (1:500), anti-Bid (1:700) and anti-p53 (1:500). The blots were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution). The antigen-antibody complex on the blots was detected by a SuperSignal chemiluminescence kit as described in the manufacturer's protocol (Pierce Biotechnology, Rockford, IL, USA), and visualized by autoradiography. To confirm

equal loading of proteins, the blots were also immunoprobed with a rabbit polyclonal antibody against the cytoskeletal protein α-tubulin (1:2500 dilution). The consistently equal signals of α-tubulin from the different extracts also indicate that malathion and the caspases inhibitors do not interfere with protein synthesis in L929 cells (data not shown).

3.2.5. Assays of Caspases-8, -9 and –3 Activities

The activities of caspase-8, -9 and -3 in cell lysates (30 μg) were determined spectrophotometricaly at 405 nm with a microtiter plate reader. The assays were performed by incubating 0.2 mM of the caspase specific colorimetric tetrapeptide substrates, Ac-IETD-*p*NA (caspase-8) or Ac-LEHD-*p*NA (caspase-9) or Ac-DEVD-*p*NA (caspase-3) for 1 h at 37°C as described by Masoud et al. (2003). The results were expressed as percentages of total activity obtained from the lysates of cells treated with malathion alone.

3.2.6. Isolation of Mitochondrial and Cytosolic Fractions

Mitochondrial and cytosolic fractions from malathion-treated L929 cells were prepared by differential centrifugation at 4°C (Yang et al., 1997). The cell pellets were washed twice with PBS (pH 7.4) and resuspended in five volumes of H medium (70 mM sucrose, 220 mM mannitol, 2.5 mM HEPES, pH 7.4, 2 mM EDTA, 1mM DTT and 0.1 mM PMSF). The cells were homogenized in a glass Dounce homogenizer (20 strokes). The homogenates were centrifuged twice at 600g for 10 min to remove nuclei and the debris. The resulting supernatant was centrifuged at 12,000g for 15 min and the mitochondria recovered in the pellets were washed and resuspended in H medium. The supernatant was used as the cytosolic fraction.

3.2.7. Assessment of Changes in Mitochondrial Transmembrane Potential

The intactness of mitochondrial transmembrane potential in cells treated with malathion was measured by a MitoCapture™ apoptosis detection kit (Alexix Biochemicals, USA) as per manufacturer's protocol. The kit utilizes MitoCapture™, a cationic dye that fluoresces differently in healthy and apoptotic cells. Healthy cells, in which MitoCapture™ accumulates and aggregates in the mitochondria, give off a bright red fluorescence. In apoptotic cells, the green fluorescing MitoCapture™ remains in the cytosol due to the altered mitochondrial membrane potential. Briefly, the cells were either treated with various concentrations of malathion or exposed to malathion for varying periods of time. Following this, the cells ($1x10^6$) were incubated in 1 ml of incubation buffer containing MitoCapture™ dye (1μg/ml) for 20 min at 37°C in humidified 5% CO_2/95%/air. Later, the cells were collected by centrifugation and the percentage of green fluorescing apoptotic cells under different treatments was determined by flow cytometry (FACSCalliber, BectonDickinson, USA). Mitochondria containing MitoCapture aggregates in healthy cells are detectable in the PI channel (usually FL2), and MitoCapture monomers in apoptotic cells are detectable in the FITC channel (usually FL1). Therefore, the cells fluorescing green represent apoptotic cells.

3.2.8. Release of Cytochrome c

The *in vivo* release of cytochrome *c* from mitochondria into the cytoplasm of malathion treated L929 cells was monitored by probing the blots containing mitochondrial (30 μg) or cytosolic (50 μg) fractions with the anti-cytochrome *c* antibody as described above, by Western blot analysis. To assess the direct effect of malathion on mitochondria, freshly isolated mitochondria (100 μg/100 μl) suspended

in H medium (70 mM sucrose, 220 mM mannitol, 2.5 mM HEPES, pH 7.4, 2 mM EDTA, 1 mM DTT and 0.1 mM PMSF, Yang et al., 1997) were incubated at 30°C for 30 min in 10 mM Tris-HCl buffer pH 7.4 containing 0.15 M KCl, 5 mM succinate and various concentrations of malathion (0.025-1.00 μM). The incubated samples were centrifuged at 8000g for 10 min at 4°C. Aliquots of supernatants (20 μl) were dissolved in Laemmli buffer, boiled for 5 min at 100°C and subjected to SDS-PAGE followed by immunoblotting with anti-cytochrome *c* antibody.

3.2.9. Determination of Intracellular ROS Formation

Formation of intracellular peroxides was detected with an oxidant-sensing fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA), which is de-esterified within cells by endogenous esterases to the ionized free acid, 2',7'dichlorofluorescin. 2',7'-dichlorofluorescin is then oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF) by hydroperoxides (Hempel et al. 1999). A 10 mM DCFH-DA stock solution was prepared in dimethylsulfoxide (DMSO). L929 cells (~1 X 10^6) were plated in 100-mm dishes and cultured overnight. After treatment with 1 μM malathion for the indicated periods of time, cells were incubated with 10 μM DCFH-DA for an additional 15 min at 37°C. Cells were then washed with ice-cold PBS, detached by trypsinization and suspended in PBS containing 10 mM EDTA. The fluorescence of DCF formed by the reaction of DCFH-DA with ROS of more than 10,000 viable cells from each sample was analyzed by recording the increase in FL-1 fluorescence with the FACS Calliber flow cytometer. In certain cases, cells were pre-incubated with 5 mM NAC for 2 h before treatment with malathion.

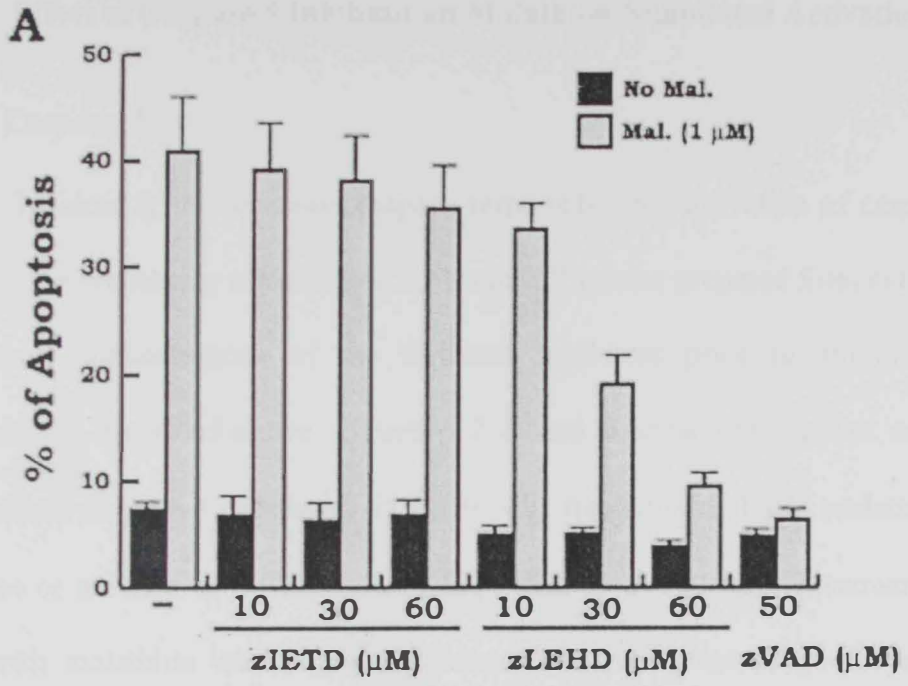
3.2.10. Measurment of Mitochondrial Cardiolipin Content

Mitochondria prepared from cells previously exposed or not to different concentrations of malathion in the presence or absence of 50 μM zVAD-fmk were prepared as described above. Aliquots of the mitochondria were disrupted by ultrasonication in 0.2 ml of a solution containing 0.1 M KH_2PO_4, 0.15 M KCl and 0.1 mM EDTA (pH 7.4). The lysed material was transferred to glass tubes containing 2 ml of methanol and mixed. After 5 minutes, 4 ml of chloroform was added and the tubes remixed, followed after 10 minutes by 1 ml of 0.05 M KCl solution. The tubes were capped and centrifuged to separate the phases. The upper aqueous-methanol phase and the interphase protein pellet were discarded and the chloroform phase was transferred to a clean glass tube and concentrated to about 20 μl under nitrogen. The concentrated lipid extract was applied at one corner of a 20 x 20 cm thin layer of silica gel H containing 1 % (w/w) magnesium acetate and two dimensional chromatography was carried out firstly with chloroform: methanol: 25 % ammonia (65: 35: 5, by vol) and then chloroform: methanol: acetic acid: methanol: water (50: 20: 10: 10: 5, by vol). Separated phospholipids were detected by exposing the dried chromatogram to iodine vapour and spots corresponding to cardiolipin and phosphatidylcholine were identified by comparison with authentic standards (Sigma Chem Co., St. Louis, USA). After evaporation of the iodine, the areas of silica gel containing these phospholipids were scraped from the plates into thick-walled glass tubes and their lipid phosphorus content measured as described by Bartlett (1959). The results are expressed as the ratio of lipid phosphorus in cardiolipin to that in phosphatidylcholine.

3.3. Results

3.3.1. Effect of Various Caspase Inhibitors on Malathion-Induced Apoptosis

To characterize the upstream events involved in both caspase-3 activation and apoptosis induced by malathion, we first investigated the effect of caspase specific inhibitors on malathion-induced apoptosis. Prior to exposure to malathion (1 μM) for 16 h, the L929 cells were first pre-incubated for 4 h in the presence or absence of the caspase-8 specific inhibitor zIETD-fmk, or the caspase-9 specific inhibitor zLEHD-fmk, or the broad range inhibitor of caspases, zVAD-fmk. The percentages of apoptosis in these cells were analyzed by the Annexin V/PI flow cytometric assay (Figure. 3.1A and 3.1B). The caspase-9 specific inhibitor (zLEHD-fmk) caused a dose-dependent reduction in malathion-induced apoptosis. Inhibition was marginal at an inhibitor concentration of 10 μM and was more marked at a concentration of 60 μM. Malathion-induced apoptosis was also inhibited to a similar extent by the general caspase inhibitor zVAD-fmk (50 μM). The increase in apoptosis mediated by malathion was ~6-fold in the absence of these inhibitors, but only 1.4- and 1.1-fold in the presence of zLEHD-fmk (60 μM) and zVAD-fmk (50 μM), respectively. In contrast, even at a concentration of 60 μM, the caspase-8 inhibitor (zIETD-fmk) had a minimal inhibitory effect on malathion-induced apoptosis (5-fold vs. 6-fold). Increasing the concentration of zIETD-fmk up to 100 μM did not cause any further decrease in malathion-induced apoptosis (data not shown). It is important to note that the effects of these inhibitors are specific because the vehicle dimethyl sulfoxide (DMSO), used at a similar concentration, had no effect on malathion-stimulated apoptosis. These observations suggest that malathion-induced apoptosis is dependent on caspase-9 activation.


A
% of Apoptosis
No Mal.
Mal. (1 μM)
0
10
20
30
40
50
–
10
30
60
10
30
60
50
zIETD (μM)
zLEHD (μM)
zVAD (μM)

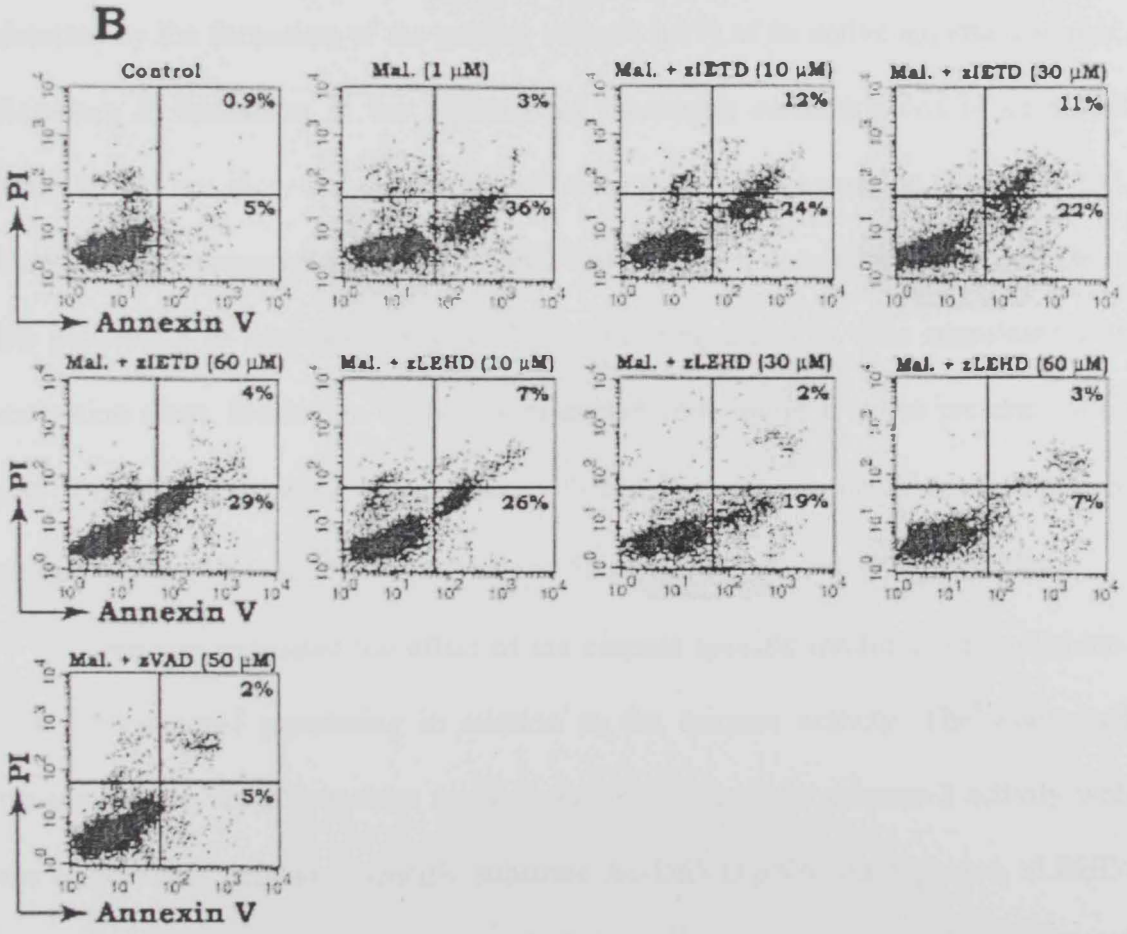

B
Control
0.9%
5%
Mal. (1 μM)
3%
36%
Mal. + zIETD (10 μM)
12%
24%
Mal. + zIETD (30 μM)
11%
22%
Mal. + zIETD (60 μM)
4%
29%
Mal. + zLEHD (10 μM)
7%
26%
Mal. + zLEHD (30 μM)
2%
19%
Mal. + zLEHD (60 μM)
3%
7%
Mal. + zVAD (50 μM)
2%
5%
PI
Annexin V

Figure 3.1: Malathion induced apoptosis in L929 cells in a caspase-9 dependent manner.

A. L929 cells were incubated for 4 h with various concentrations (0-60 μM) of: caspase-8 inhibitor (zIETD-fmk) or caspase-9 inhibitor (zLEHD-fmk) or 50 μM of the broad range caspase inhibitor zVAD-fmk. Malathion (1 μM) was added and the cells were incubated for an additional 16 h. After the various treatments, the cells were harvested, stained with Annexin V-FITC and PI and analyzed by flow cytometry. Annexin V stained cells that were PI negative were counted as apoptotic and their numbers expressed as a percent of total cells. The values are means ± SD of three independent experiments.

B. Representation of the flow cytometry data obtained for the various treatments.

3.3.2. Effect of Caspase-9 Inhibitor on Malathion-Stimulated Activation of Caspase-3

To identify the upstream caspase responsible for activation of caspase-3, we assessed the processing of caspase-3 in whole cell lysates prepared from cells exposed to various concentrations of the different inhibitors prior to stimulation with malathion as described above. Figures 3.2 A and B show immunoblot analysis for detection of caspase-3 processing in L929 cells treated with 1 μM malathion in the presence or absence of zIETD-fmk, zLEHD-fmk or zVAD-fmk. Treatment of these cells with malathion was effectively associated with processing of caspase-3 as detected by the formation of the smaller subunit (p19) of its active enzyme complex. Exposure to malathion in the presence of increasing concentrations of caspase-8 inhibitor did not show any significant effect on caspase-3 processing (Figure. 3.2A). However, the caspase-9 inhibitor zLEHD-fmk caused a dose-dependent decrease in the processing of caspase-3 (Figure. 3.2B). In comparison to cells stimulated with malathion alone, incubation of malathion-treated cells for 16 h in the presence of 60 μM of zLEHD-fmk caused approximately 9-fold decrease in processing of caspase-3, similar to the effect seen in the presence of 50 μM of zVAD-fmk (Figure. 3.2B).

We also evaluated the effect of the caspase specific inhibitors on malathion-induced caspase-3 processing in relation to the enzyme activity. The whole cell lysates used in the immunoblot analysis were also tested for caspase-3 activity with the colorimetric caspase-3 specific substrate Ac-DEVD-*p*NA. As expected, zLEHD-fmk caused a dose-dependent decrease of caspase-3 activation in malathion-treated cells (Figure. 3.2B). As compared to cells treated with malathion alone, preincubation of L929 cells with 60 μM of zLEHD-fmk caused a 5.5-fold decrease in malathion-

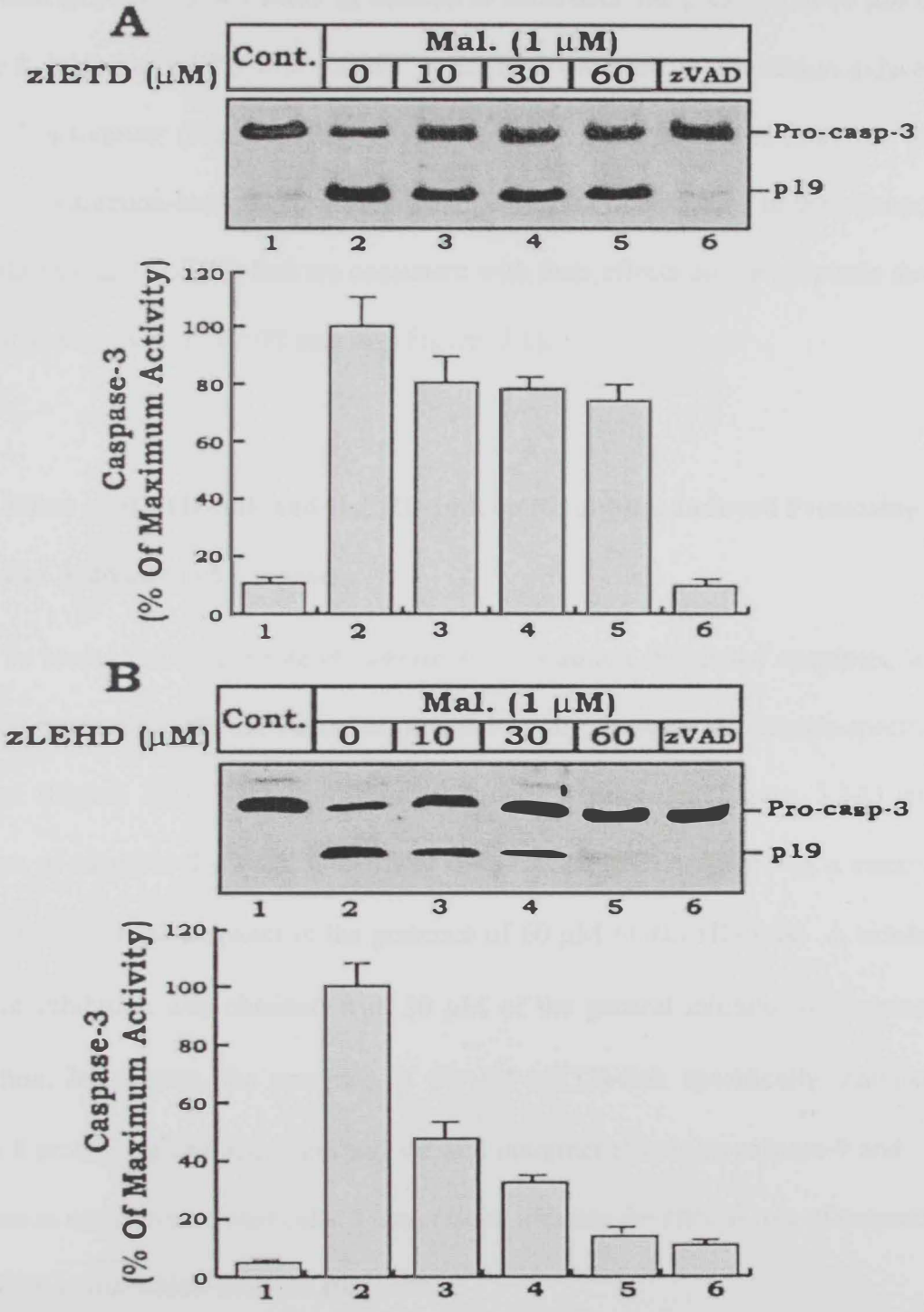

A
zIETD (μM)
Cont.
Mal. (1 μM)
0
10
30
60
zVAD
Pro-casp-3
p19
1
2
3
4
5
6
Caspase-3
(% Of Maximum Activity)
120
100
80
60
40
20
0
B
zLEHD (μM)
Cont.
Mal. (1 μM)
0
10
30
60
zVAD
Pro-casp-3
p19
1
2
3
4
5
6
Caspase-3
(% Of Maximum Activity)
120
100
80
60
40
20
0

Figure 3.2: Malathion induced a caspase-9-dependent activation of caspase-3. L929 cells were treated with 1 μM malathion in the presence of various concentrations of caspase-8 inhibitor zIETD-fmk **(A)** or caspase-9 inhibitor zLEHD-fmk **(B)** as described in figure 3.1. After treatment, whole cell extracts were prepared and analyzed for caspase-3 processing by immunoblotting with an anti-caspase-3 antibody. The unprocessed forms of caspase-3 (pro-casp-3; 32 kDa), and the cleavage product (p19) of the active enzyme are indicated. The zVAD-fmk lane indicates processing of caspase-3 from cells treated with 50 μM zVAD-fmk for 4 h prior to exposure to malathion. The activity of caspase-3 was measured in the same cell extracts (30 μg) by use the caspase-3 specific tetrapeptide substrate Ac-DEVD-*p*NA. The values are expressed as a percentage of the maximum activity (100%) obtained from cells treated with malathion alone. The values are means ± SD of three independent experiments.

induced activation of caspase-3. This inhibitory effect on malathion-stimulated caspase-3 activation was comparable to the one seen in cells treated with 50 μM of zVAD-fmk (5.5-fold vs. 6.7-fold). In contrast to these data, the presence of 60 μM of caspase-8 inhibitor zIETD-fmk caused a 1.3-fold decrease in malathion-induced caspase-3 activation (Figure. 3.2A). Taken together, the results indicate that the kinetics of malathion-mediated processing and activation of caspase-3 in the presence of zIEHD-fmk and zLEHD-fmk are consistent with their effects on the apoptosis data as demonstrated by Annexin/PI staining (Figure. 3.1).

3.3.3. Effect of zIETD-fmk and zLEHD-fmk on Malathion-Induced Processing and Activation of Caspases

To assess further the role of caspase-9 in malathion-stimulated apoptosis, we examined processing and activation of caspases in the presence of caspase-specific inhibitors (Figure. 3.3). While malathion stimulated processing (Figure. 3.3A) and activation of caspases-8, -9 and -3 in L929 cells (Figure. 3.3B), there was a marked inhibition of all these caspases in the presence of 60 μM of zLEHD-fmk. A similar degree of inhibition was obtained with 50 μM of the general inhibitor of caspases zVAD-fmk. In contrast, the presence of 60 μM zIETD-fmk specifically inhibited caspase-8 processing and activation and showed marginal effects on caspase-9 and –3 activation in malathion-treated cells. These results indicate the critical role of caspase-9 activation in malathion-induced apoptosis.

3.3.4. Effect of Malathion on Release of Cytochrome c From Mitochondria

Activation of caspase-9 in the Apaf-1 apoptosome is predominantly triggered

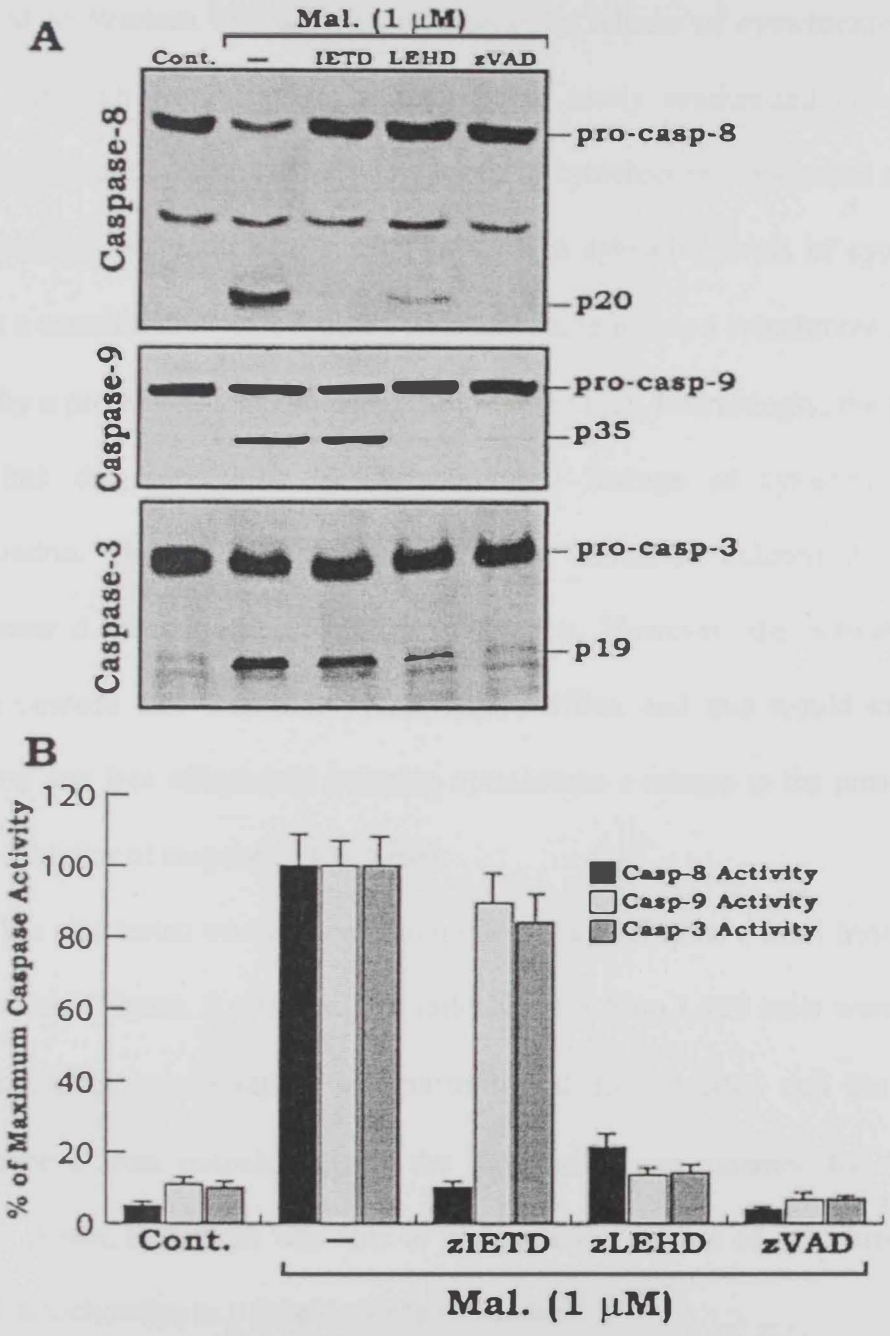

A
Mal. (1 μM)
Cont.
—
IETD
LEHD
zVAD
Caspase-8
pro-casp-8
p20
Caspase-9
pro-casp-9
p35
Caspase-3
pro-casp-3
p19
B
% of Maximum Caspase Activity
120
100
80
60
40
20
0
Casp-8 Activity
Casp-9 Activity
Casp-3 Activity
Cont.
—
zIETD
zLEHD
zVAD
Mal. (1 μM)

Figure 3.3: Inhibition of caspase-8 did not abolish malathion-mediated activation of caspase-9 and caspase-3.

A. L929 cells were treated with 1 μM malathion in the presence or absence of 60 μM of caspase-8 inhibitor (zIETD-fmk) or caspase-9 inhibitor (zLEHD-fmk) or 50 μM of the general inhibitor of caspases zVAD-fmk as indicated on top of each lane. Whole cell extracts (50 μg), obtained from the same cells, were analyzed by Western blotting with antibodies against caspases-8, -9 and -3, respectively. The unprocessed forms of caspase-8 (pro-casp-8; 54 kDa), caspase-9 (pro-casp-9; 46 kDa), caspase-3 (pro-casp-3; 32 kDa) and their respective cleavage products p20, p35 and p19 of the active enzymes complexes are indicated. The blots are representative of two independent experiments.

B. The same extracts (30 μg) were analyzed for caspases-8, -9 and -3 catalytic activities by use of their specific colorimetric tetrapeptide substrates Ac-IETD *p*NA, Ac-LEHD-*p*NA and Ac-DEVD-*p*NA, respectively. The values are expressed as percentages of the maximum activity (100%) obtained from cells treated with malathion alone. The values are means ± SD of three independent experiments.

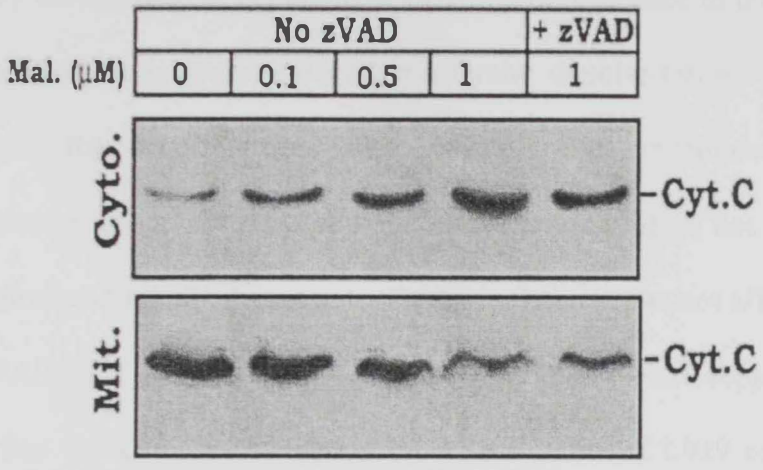
by the release of cytochrome *c* from mitochondria into the cytoplasm (Saleh et al., 1999; Qin et al., 1999). Accordingly, the cytosolic and mitochondrial fractions, prepared from cells treated with increasing concentrations of malathion, were subjected to Western blot analysis to assess the release of cytochrome *c* (Figure. 3.4A). Although not related to apoptosis, the newly synthesized non-heme pool contributes to the presence of very low levels of cytochrome *c* in control cell cytosol. Malathion induced a dose-dependent increase in cytosolic levels of cytochrome *c*. Even at a concentration as low as 0.1 μM, malathion induced cytochrome *c* release as shown by a prominent immunoreative band on the blot. Interestingly, the presence of zVAD-fmk did not inhibit malathion-induced leakage of cytochrome *c* from mitochondria. The latter finding suggests that malathion induced the release of cytochrome *c* independent of caspase activation. However, the activation of the caspase cascade also amplifies cytochrome *c* efflux and this would explain why malathion was less effective in inducing cytochrome *c* release in the presence of the general inhibitor of caspases, zVAD-fmk.

We also tested whether malathion releases cytochrome *c* from freshly isolated mitochondria (Figure. 3.4B). Purified mitochondria from L929 cells were incubated with increasing concentrations of malathion (0 to 1.0 μM) and the efflux of cytochrome *c* from mitochondria to the supernatant was assessed by immunoblot analysis. Again, malathion was able to stimulate the release of cytochrome *c* from isolated mitochondria in a dose-dependent manner.

3.3.5. Effect of Malathion on Mitochondrial Membrane Potential of L929 Cells

One of the possible mechanisms responsible for the release of cytochrome *c* from mitochondria involves changes in the mitochondrial membrane potential ($\Delta\psi$)

A


No zVAD
+ zVAD
Mal. (μM)
0
0.1
0.5
1
1
Cyto.
Cyt.C
Mit.
Cyt.C

B

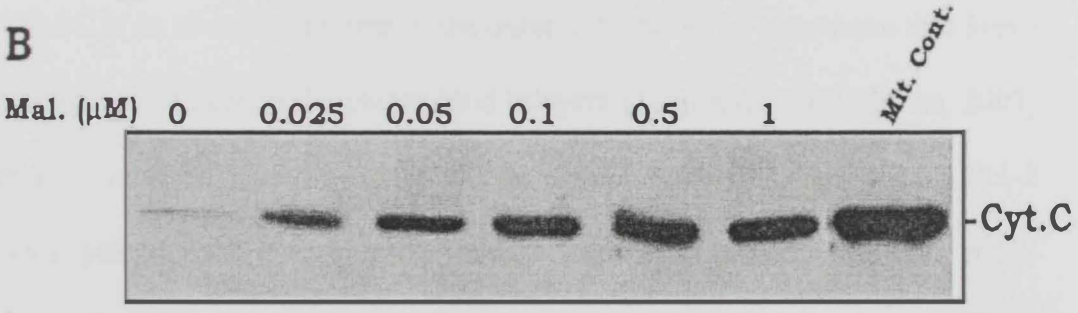

Mal. (μM)
0
0.025
0.05
0.1
0.5
1
Mit. Cont.
Cyt.C

Figure 3.4: Malathion induced release of cytochrome *c* from mitochondria into the cytoplasm of L929 cells independent of caspases activation.

A. Mitochondrial (30 μg) and cytosolic (50 μg) fractions were isolated from L929 cells treated with various concentrations of malathion in the presence or absence of zVAD-fmk (50 μM) as indicated on top of each lane. The presence of cytochrome *c* in both fractions was detected by immunoblotting with a specific antibody to the protein. The results are representative of two independent experiments.

B. Freshly isolated mitochondria (100 μg/100 μl) from L929 cell were incubated with various concentrations of malathion (0.025-1 μM) for 30 min at 30°C in a reaction buffer as described in Materials and Methods. After incubation, the mitochondria were centrifuged and the leakage of cytochrome *c* from mitochondria into the reaction buffer was analyzed by Western blotting with an antibody against cytochrome *c*. The control lane represents 20 μl of the untreated reaction mixture. The results are representative of two independent experiments.

(Brenner et al., 2000). Consequently, we monitored changes in the mitochondrial membrane potential by staining malathion treated L929 cells with the cationic dye MitoCapture™. Malathion induced depolarization of the mitochondrial membrane potential in a time- (Figure. 3.5A) and concentration- (Figure. 3.5B) dependent manner, as shown by the increase in the intensity of green fluorescence in the cytosol of malathion-treated cells. Malathion-induced membrane depolarization was also confirmed by following the concurrent time- and concentration-dependent decrease in the reddish fluorescence given by non-apoptotic mitochondria (data not shown). Disruption of the mitochondrial membrane potential by malathion was not affected by the presence of zVAD-fmk (Figure. 3.5A). This finding once again supports our earlier observation that the action of malathion on mitochondria of L929 cells does not require the activation of caspases.

3.3.6. Effect of Malathion on Expression of VDAC, ANT, Bcl-2, Bcl-x_L, Bad, Bak, Bid, Bax and p53

VDAC is an abundant protein in the outer mitochondrial membrane that forms a large voltage-gated pore in the planar lipid bilayers (Tsujimoto and Shimizu, 2002) and seems to serve, in combination with the proapoptotic members of the Bcl-2 family, as a pathway for cytochrome *c* release from mitochondria (Kuwana et al., 2002; Shi et al., 2003). To test this hypothesis, we examined the induction of VDAC and its regulatory proteins ANT (adenine nucleotide translocator), Bcl-2, Bcl-x_L, Bad, Bak, Bid and Bax. Malathion was found to increase the expression of Bax and induce truncation of Bid in a dose-dependent manner in L929 cells (Figure. 3.6A). However, the levels of VDAC, Bcl-2, Bcl-x_L, Bad and Bak were unchanged in malathion-treated cells. Thus, the increased expression of Bax and truncation of Bid may be attributed to

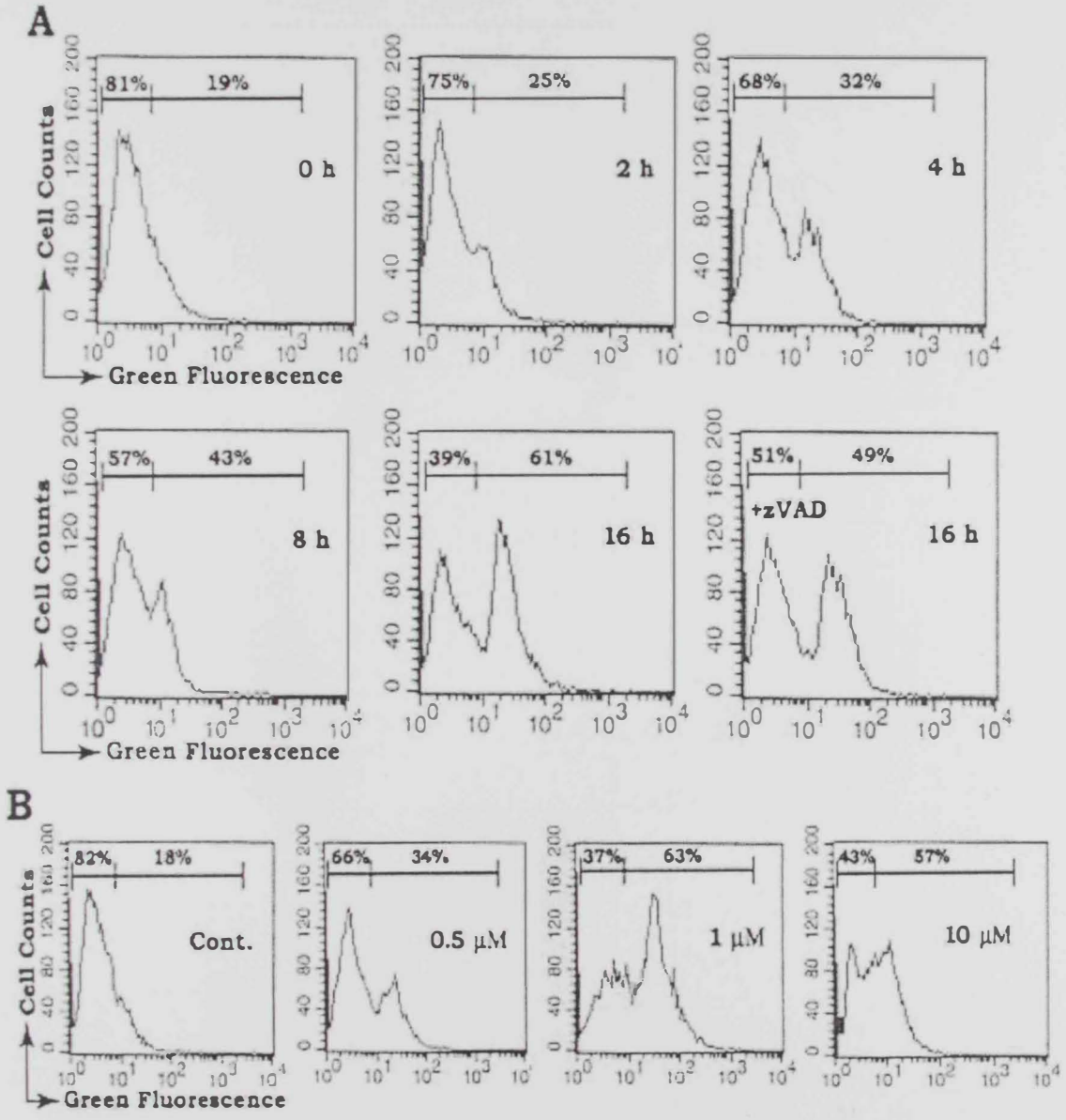

A
Cell Counts
Green Fluorescence
81%
19%
0 h
75%
25%
2 h
68%
32%
4 h
57%
43%
8 h
39%
61%
16 h
51%
49%
+zVAD
16 h
B
82%
18%
Cont.
66%
34%
0.5 μM
37%
63%
1 μM
43%
57%
10 μM

Figure 3.5: Malathion induced a time- and concentration-dependent disruption of mitochondrial transmembrane potential.

A. L929 cells treated with 1 μM malathion for various periods of time (0-16 h) in the presence or absence of 50 μM zVAD-fmk as indicated in each profile. After incubation, cells were stained with the MitoCapture™ cationic dye and the increase in the percentage of green fluorescence was monitored by flow cytometry.

B. L929 cells were exposed to various concentrations of malathion (0-10 μM) for 16 h, stained with MitoCapture™ cationic dye and analyzed by flow cytometry as described in (A). The flow cytometry data shown are representative of two independent experiments.

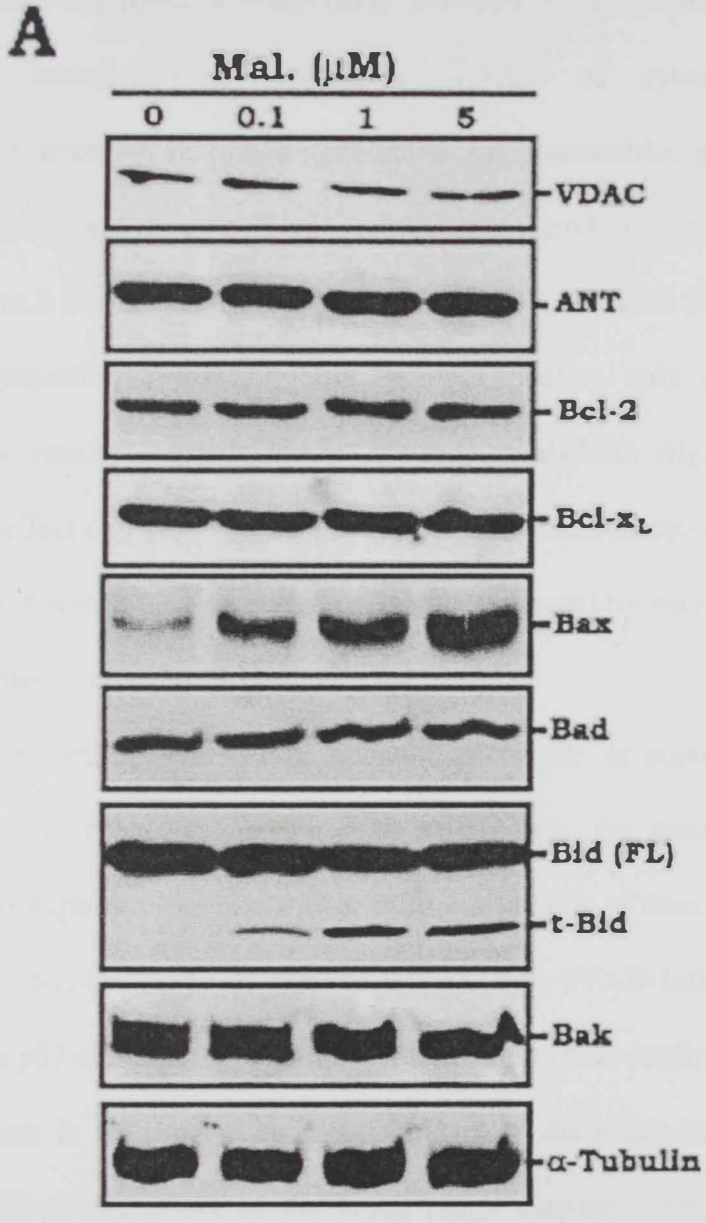

A
Mal. (μM)
0
0.1
1
5
VDAC
ANT
Bcl-2
Bcl-xL
Bax
Bad
Bid (FL)
t-Bid
Bak
α-Tubulin

Figure 3.6: Malathion promoted truncation of Bid as well as expression and translocation of Bax from the cytoplasm to mitochondria of L929 cells.

A. Whole cell extracts (50 μg) from L929 cells, treated with various concentrations of malathion (0-5 μM), (as indicated on top of each lane), were analyzed for the expression of VDAC, ANT, Bcl-2, Bcl-x_L, Bax, Bad, Bid and Bak by Western blotting with their respective antibodies.

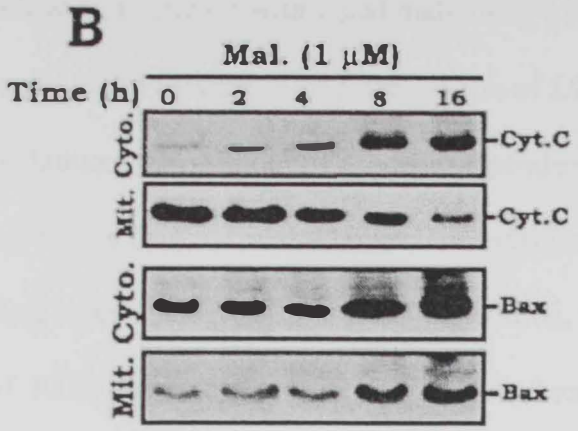
the ability of malathion to release cytochrome *c* from mitochondria into the cytoplasm of L929 cells.

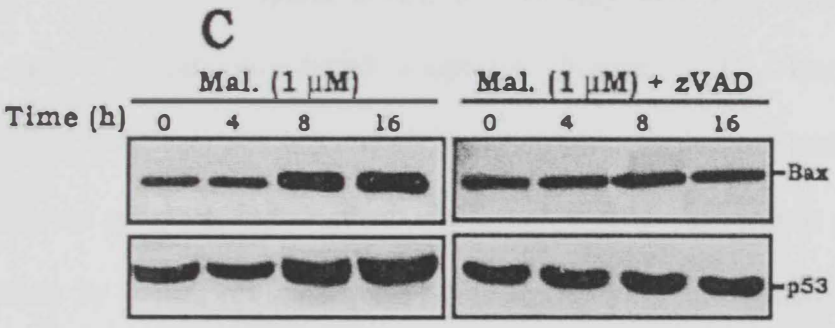
To examine the possible relationship between malathion-induced apoptosis and Bax, we examined the kinetics of release of cytochrome *c* and expression/translocation of Bax to mitochondria by immunoblot analysis (Figure. 3.6B). While the leakage of cytochrome *c* from mitochondria into the cytosol was evident as early as 2 h of incubation with malathion, the increase in Bax expression as well as its translocation to mitochondria became apparent only at 8 h of post-treatment. These results suggest that (i) initially, malathion triggered apoptosis through a direct effect on mitochondria and caused the release of cytochrome *c* and (ii) subsequent downstream events amplified the apoptotic signal by way of translocation of Bax from cytosol to mitochondria.

To confirm further whether Bax induction was a pre- or post-apoptotic event, the L929 cells were first preincubated with zVAD-fmk, the general inhibitor of caspase, and later exposed to malathion for different periods of time (0 to 16 h). As shown in figure 3.6C, inhibition of caspase activation by zVAD-fmk suppressed the induction of both p53 and Bax by malathion. These results thus confirm that induction of Bax expression is an event that is subsequent to malathion-mediated caspase activation. Similar to the effect of the broad range caspase inhibitor zVAD-fmk, inhibition of caspase-9 activation in malathion treated cells suppressed both the induction of expression of Bax and cleavage of Bid (Figure. 3.6D).

3.3.7. Induction of ROS Generation by Malathion

To investigate whether ROS is involved in malathion-mediated apoptosis, we measured the level ROS within the L929 cells by using the fluorometric probe DCF.


B
Mal. (1 μM)
Time (h) 0 2 4 8 16
Cyto.
Cyt.C
Mit.
Cyt.C
Cyto.
Bax
Mit.
Bax


C
Mal. (1 μM)
Mal. (1 μM) + zVAD
Time (h) 0 4 8 16 0 4 8 16
Bax
p53

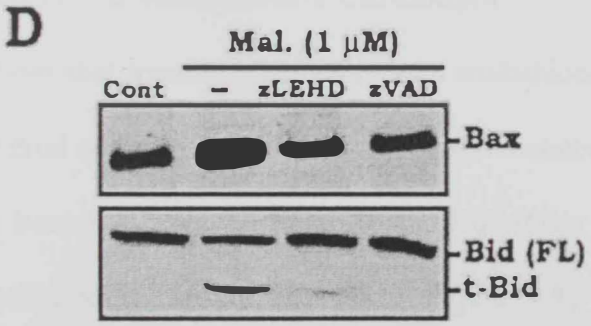

D
Mal. (1 μM)
Cont – zLEHD zVAD
Bax
Bid (FL)
t-Bid

Figure 3.6: Malathion promoted truncation of Bid as well as expression and translocation of Bax from the cytoplasm to mitochondria of L929 cells.

B. Immunoblot analysis of cytochrome *c* and Bax in cytosolic (50 μg) and mitochondrial (30 μg) fractions obtained from L929 cells treated with 1 μM malathion for different periods of time (0-16 h).

C. Western blot analysis of Bax and p53 expression in whole cell extracts (50 μg) of L929 cells exposed to 1 μM malathion for different time points (0-16 h) in the presence or absence of 50 μM zVAD-fmk as indicated on top of the lanes.

D. Western blot analysis of Bax and Bid expression in whole cell extracts (50 μg) of L929 treated or not treated (control) with 1 μM malathion in the presence or absence of zLEHD-fmk (60 μM) or zVAD-fmk (50 μM). The bands corresponding to the full-length Bid (FL) and its cleavage product (truncated Bid, t-Bid) are indicated.

As shown in Figure 3.7, the basal level of DCF-sensitive ROS in L929 cells was not readily detectable. Following treatment with 1 μM malathion, a significant generation of ROS appeared as early as 2 h (Figure. 3.7A). Incubation of L929 cells with various concentrations of malathion also showed a dose-dependent increase in ROS generation, as reflected by the increase in the green fluorescence of DCF dye (Figure. 3.7B). Given the critical roles of malathion in ROS generation, we next investigated whether inhibition of ROS production could rescue malathion treated cells from apoptosis. While pretreatment of L929 cells with 5 mM NAC almost completely prevented ROS formation (Figure. 3.7C), it was only able to provide a partial protection against malathion-induced apoptosis (Figure. 3.7D). Increasing the concentration of NAC up to 20 mM did not provide further protection against malathion-induced apoptosis (data not shown). Taken together, the results suggest that ROS generation by malathion contributes to its ability to induce apoptosis in L929 cells.

3.3.8. Effect of Malathion on Mitochondria Cardiolipin

Figure 3.8 shows that treating L929 cells with malathion caused a substantial decrease of mitochondrial cardiolipin in a dose-dependent manner. This effect was not a caspase-dependent because the broad-range caspase inhibitor zVAD-fmk was not capable of inhibiting changes in cardiolipin content (Figure. 3.8).

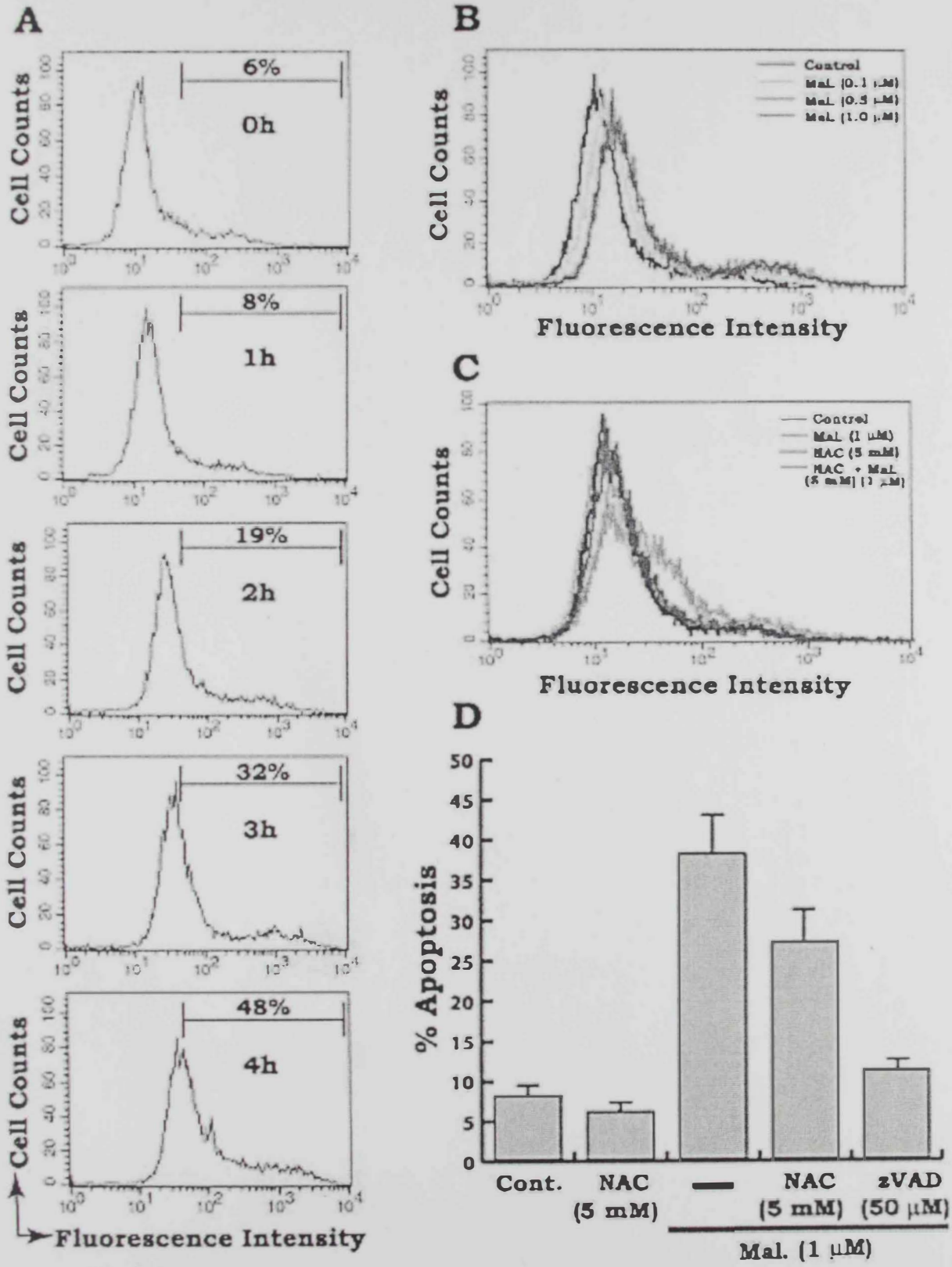

A
6%
0h
8%
1h
19%
2h
32%
3h
48%
4h
Cell Counts
Fluorescence Intensity
B
Control
Mal. (0.1 μM)
Mal. (0.5 μM)
Mal. (1.0 μM)
Cell Counts
Fluorescence Intensity
C
Control
Mal. (1 μM)
NAC (5 mM)
NAC + Mal.
Cell Counts
Fluorescence Intensity
D
% Apoptosis
Cont.
NAC (5 mM)
—
NAC (5 mM)
zVAD (50 μM)
Mal. (1 μM)

Figure 3.7: Malathion induced ROS production in a time- and concentration-dependent manner, which was blocked by the antioxidant NAC.

A. L929 cells treated with 1 μM malathion for various periods of time (0-4 h) as indicated in each profile. After incubation, cells were stained with the DCFH-DA reagent and the increase in the percentage of FL1 fluorescence was monitored by the flow cytometry.

B. Similarly, L929 cells were exposed to various concentrations of malathion (0-1 μM) for 4 h, stained with DCFH-DA and analyzed by flow cytometry as described in (A).

C. Cells were exposed to malathion for 4 h in the presence or absence of 5 mM NAC and analyzed for ROS formation as described above. The flow cytometry data shown in A, B and C are representative of two independent experiments.

D. L929 cells were exposed to 1μM malathion in the presence or absence of 5 mM NAC or 50 μM zVAD-fmk and analyzed for apoptosis using the Annexin/PI method as described in figure 3.1. The values are means ± SD of three independent experiments.

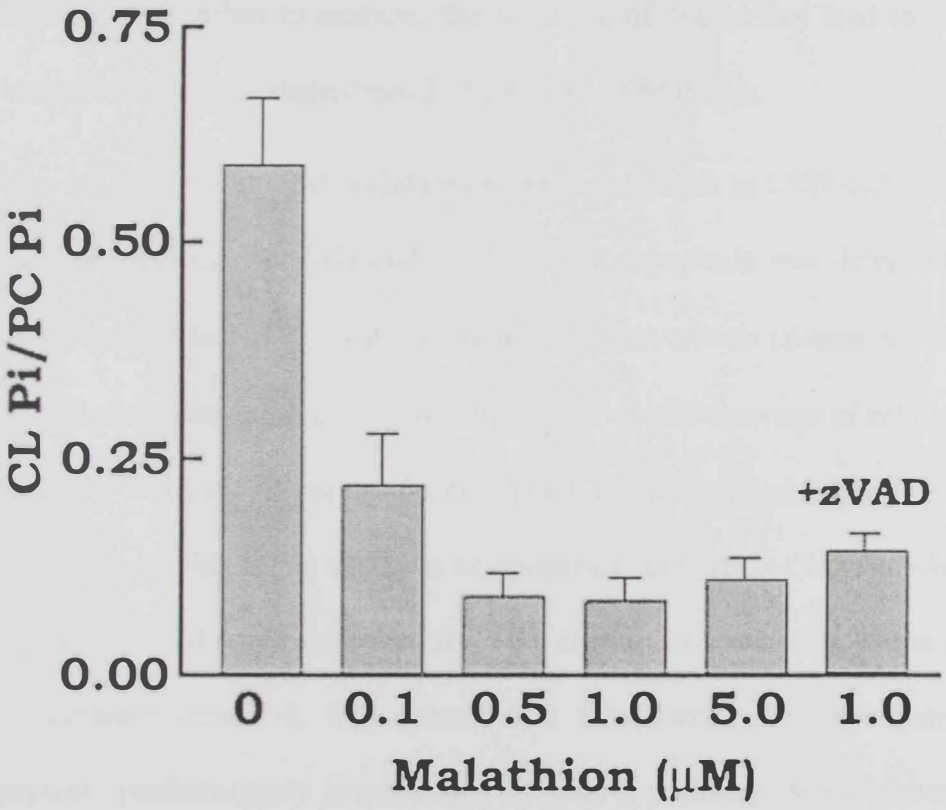

0.75
0.50
0.25
0.00
CL Pi/PC Pi
+zVAD
0
0.1
0.5
1.0
5.0
1.0
Malathion (μM)

Figure 3.8: Effect of malathion on mitochondria cardiolipin.

Mitochondria prepared from L929 cells previously exposed or not to different concentrations of malathion in the presence or absence of 50 μM zVAD-fmk were prepared, and lysed by ultrasonication. Phospholipids were extracted from the lysed mitochondria and cardiolipin content was determined as described in the Methods section. The results were expressed as the ratio of lipid phosphorus (pi) in cardiolipin (CL) to that in phosphatidylcholine (PC). The values are means ± SD of three independent experiments.

3.4. Discussion

Caspases, found in mammalian cells as inactive protease precursors, are grouped into upstream initiator caspases and downstream effector caspases. Inactive initiator caspases (caspases-8 and -9) are first activated in response to apoptotic stimuli and are responsible for processing and activation of effector caspases such as caspases-3, -6 or –7. Activated effector caspases, subsequently, execute apoptosis by cleaving various cellular substrates that are vital for cell survival (Shi, 2002). In this study we have used integrated approaches to examine the sequence of events that lead to the activation of caspases in the malathion-treated L929 mouse fibroblasts.

Recently, we have shown that malathion induces apoptosis in L929 cells in a dose- and time-dependent manner (Masoud et al., 2003). Apoptosis was detectable following a 16 h exposure to 1 μM malathion by four distinct criteria (Annexin V/PI staining, DNA laddering, activation of caspases-8, -9, and -3 and cleavage of cellular substrates such as the DNA repair protein PARP). Since specific proteases belonging to the caspase family are the major effectors of apoptosis, the type of intracellular apoptotic pathways involved may be deduced from the class of initiator caspase that is activated, i.e. caspase-9 versus -8. Our current data demonstrate that malathion-stimulated apoptosis predominantly involves the caspase-9 pathway. We observed that inhibition of caspase-9 activation by zLEHD-fmk decreased malathion-induced apoptotic events. In malathion-treated cells, zLEHD-fmk caused a dose-dependent reduction in morphological changes of plasma membrane asymmetry as demonstrated by Annexin V staining. zLEHD-fmk also inhibited caspase-8, -9 and –3 processing and activation. In contrast, the caspase-8 specific inhibitor zIEHD-fmk had only a marginal effect on malathion-stimulated apoptosis and activation of caspase-9 and –3. It is well known that activation of the upstream initiator caspase, caspase-9, can

trigger a cascade that culminates in the activation of downstream effector caspase, caspase-3 (Saleh et al., 1999; Qin et al., 1999). Once activated, caspase-3 has also been shown to process and activate the upstream initiator caspase, casapase-8, leading to the amplification of the apoptotic cascade (Slee et al., 1999). Thus, the observed activation of caspase-8 in malathion-stimulated cells is mainly due to the activation of caspase-3 by active caspase-9.

Activation of caspase-9 usually occurs downstream of cytochrome *c* release from mitochondria (Saleh et al., 1999). Our results demonstrate, for the first time, the activation of a specific mitochondrial apoptotic pathway in a fibroblastic cell line following exposure to malathion. We document a concentration- and time-dependent release of cytochrome *c* into the cytosol, cleavage of Bid and up-regulation and translocation of Bax to mitochondria upon stimulation with malathion. The efflux of cytochrome *c* from the mitochondrial compartment into the cytosol occurred at 2 h following incubation with 1 μM malathion and gradually increased at 8 h. On the other hand, transcriptional up-regulation of Bax and its translocation to mitochondria were only evident at 8 h post-treatment. These results indicate that the leakage of cytochrome *c* precedes activation and translocation of Bax to mitochondria. This interpretation suggests that induction and translocation of Bax to mitochondria is dependent on further amplification of the caspase cascade and entry of the cell into the execution phase of apoptosis. During this phase, induced-expression of p53, in response to DNA fragmentation, triggers the transcriptional up-regulation of Bax (Miyashita and Reed, 1995), and thus disturbs the ratio of pro-survival heterodimer (Bcl-2-Bax) to pro-apoptotic homodimer (Bax-Bax) proteins (Borner, 2003). Excess Bax homodimers will translocate to the mitochondrial outer membrane leading to leakage of cytochrome *c* through its pore-forming activity (Wei et al., 2001). This

scheme of events is consistent with our results and explains the time lag between caspase activation and the activation as well as translocation of Bax to mitochondria. It is important to note that during this phase, no significant changes were observed in the expression levels of Bcl-2 and Bcl-x_L. Our observation that malathion-induced DNA fragmentation was dependent on activation of caspases (Masoud et al., 2003), suggests that activation and translocation of Bax to mitochondria may represent an amplification mechanism rather than being a major trigger of apoptosis in response to malathion treatment. Furthermore, the up-regulation of both p53 and Bax as well as generation of tBid from its precursor protein in malathion treated cells were inhibited in the presence of zVAD-fmk and zLEHD-fmk. These results provide additional evidence that the actions of Bax and tBid on release of cytochrome *c* are subsequent to activation of caspases triggered by the direct effect of malathion on mitochondria of L929 cells.

Our results demonstrate that malathion disrupts mitochondrial transmembrane potential and causes the release of cytochrome *c* under both *in vitro* and *in vivo* conditions. In L929 cells, malathion stimulated the efflux of cytochrome *c* from mitochondria into the cytosol even in the presence of the general inhibitor of caspases zVAD-fmk. These results indicate a direct action of malathion on mitochondria. Although the biochemical mechanisms by which malathion induces the release of cytochrome *c* from mitochondria remain incompletely understood, reactive oxygen species (ROS) have been suggested candidates that trigger mitochondrial membrane permeabilty transitions (Morkunaite-Haimi et al., 2003). Recent studies have implicated ROS as mediators of OPC-induced cytotoxicity (Banerjee et al., 2001). The OPC-induced membrane depolarization by ROS probably involves oxidation of critical amino acid residues (such as cysteine) in the pore voltage sensor (Carlson and

Ehrich, 1999) causing alterations in membrane permeability that leads to release of apoptogenic factors from mitochondria. The results presented here indicate that malathion induced ROS formation. Interestingly, although the antioxidant NAC inhibited the generation of ROS in malathion treated cells, it conferred only partial protection against malathion-induced apoptosis. The limited protective role of NAC suggests that malathion-induced apoptosis may involve additional effects other than induction of oxidative stress. Such effects may include damage to cellular proteins (Harvey and Sharma, 1980; Marinovich et al., 1996) and DNA (Blasiak et al., 1999; Richardson and Imamura, 1985) and alterations in metabolic pathways (Harvey and Sharma, 1980).

In addition to the role of ROS, translocation of cytochrome *c* to the cytosol is stimulated by changes in the lipid composition of mitochondrial membranes, particularly cardiolipin. The lipophilic nature of malathion and other organophosphorus pesticides facilitates their interactions with mitochondrial membranes leading to alterations in the composition of these structures (Videira et al., 2001). The release of cytochrome *c* into the cytoplasm during apoptosis depends on disruption of its electrostatic interactions with the mitochondrial inner membrane lipid cardiolipin (Garcia-Fernandez et al., 2002). Our results show that malathion causes a dramatic loss of this lipid from the inner membrane of mitochondria of L929 treated cells. This effect, in turn, may induce dissociation of cytochrome *c* from the mitochondrial inner membrane. Changes in the oxidation status of cardiolipin and/or alterations in its synthesis are responsible for disturbing its electrostatic interaction with cytochrome *c* (Garcia-Fernandez et al., 2002). Thus, the ability of malathion to induce changes in mitochondrial cardiolipin content may be due to oxidative damage by ROS or to alteration in its biosynthetic pathway. Our finding that inhibition of

malathion-induced ROS formation by NAC conferred only a partial restoration of the mitochondrial cardiolipin content, indicates that malathion may also interfere with the biosynthesis of this phospholipids (data not shown).

In summary, we have shown that malathion induces apoptosis via the mitochondrial pathway. Malathion stimulates a dose- and time-dependent disruption of mitochondrial membrane potential, ROS formation, loss in cardiolipin content and translocation of Bax and Bid to mitochondria of treated cells. These events, collectively, cause release of cytochrome *c* from mitochondria into the cytoplasm of cultured L929 cells to induce a caspase-9-dependent apoptosis. Thus, inhibition of this specific pathway might provide a useful strategy to minimize organophosphate-induced poisoning.

CHAPTER 4:

CONCLUSIONS

AND

FUTURE DIRECTIONS

4.1. Conclusions and Future Directions

The general public are exposed to low level of OP insecticide through ingestion of residuces on food and contaminated water supplies as well as through inhalation and dermal contact.

In the present study we demonstrate that at concentrations below the IC_{50} for AChE inhibition in red blood cells, malathion induces cell death in murine L929 fibroblasts by apoptotic pathways. This is the first demonstration that very low doses of OPCs affected non-neuronal cells and induced apoptosis independent of AChE inhibition. At doses below the IC_{50}, malathion also induced apoptosis in EL4 murine T-lymphocytes (data not shown). In a previous study we have also reported that at low, non-cholinergic doses, paraoxon is a potent inducer of apoptosis both in cultured EL4 T-lymphocytes and mouse fibroblasts (Saleh et al., 2002). These results, as well as a growing body of recent evidence (Carlson and Ehrich, 2001) indicate that apoptosis might be one of the mechanisms by which the cells respond to chronic exposure to low doses of OPCs.

In this study we have used integrated approaches to examine the sequence of events that lead to the activation of caspases in the malathion-treated L929 mouse fibroblasts.

Organophosphorus compounds are usually esters, amides or thiol derivatives of phosphonic acid. They form a large family of ~50,000 chemical agents with biological properties that have important and sometimes unique implications for man. Thus, the ubiquitous organophosphates present a continuing health hazard in agriculture, public health eradication programs and as chemical warfare agents (Kamanyire and Karalliedde, 2004). The major concern is that there is insufficient

information regarding the molecular mechanisms of their toxicity for implementing appropriate preventive measures in occupational exposures.

Most of the ill-health following exposure to organophosphorus compounds has been attributed to the inhibition of cholinesterases. However, the current literature (Monnet-Tschudi et al., 2000) and the work presented in this thesis have justifiably challenged this view, as the inhibition of cholinesterases by itself cannot account for the wide range of disorders that have been reported following organophosphorus poisoning. It is becoming apparent that, although inhibition of cholinesterases plays a key role in the toxicology of organophosphates, individual susceptibility, the inhibition of other enzyme systems and the direct effects of organophosphates on cells tissues are also important.

The findings of this study that malathion induces apoptosis through its ability to release cytochrome c from mitochondria to the cytoplasm of mammalian cells, suggest the possible use of this phenomenon as a diagnostic strategy for organophosphate poisoning. In dying cells, cytochrome c may leak out to the plasma of organophosphate poisoned animal. To test this possibility *in vivo*, we have recently exposed five groups of rats to different concentrations of the organophosphate malathion, parathione or paraoxon for various period of times and the level of cytochrome c in the plasma of these animals was semi-quantitatively determined by Western blotting (unpublished data). Indeed, the results indicate that the leakage of cytochrome c from tissue cells to the plasma of the animals is proportional to the toxicity levels. Therefore, our results introduce a novel assay to evaluate the levels of OPCs toxicity of exposed individuals.

In addition to the diagnostic assay, molecular characterization of the malathion-induced apoptosis in L929 cells allowed us to screen for potential treatment

for organophosphate-induced toxicity. Recently, we have found that the benzamide derivative metoclopromide, which is clinically used as anti-emetic drug, is able to inhibit all the apoptotic events induced by malathion and paraoxon. These findings suggest the potential use of this drug to treat poisoning with organophosphates. Recently, in collaboration with professor G. Petroianu's laboratory (Department of Pharmacology, Faculty of Medicine, UAE University), we have found that metoclopromite was able to alleviate the inhibitory effect of organophosphates on ACHE activity (Petroianu, et al., 2003), decrease the leakage of cytochrome c from dead tissue cells to the plasma and substantially reduce the mortality rates of animals poisoned with OPCs (unpublished results).

In addition to the above advantages gained from this study, our results indicate that malathion induces apoptosis at doses lower than the one required to inhibit cholinesterase activities. These findings suggest that malathion is highly toxic to organisms. Therefore, the use of this compound in agriculture to control pests should be restricted. Our *in vitro* apoptosis assays provide sensitive techniques to screen for alternative safer pesticides.

This study concentrated on the effect of malathion on the induction of apoptosis in mammalian cells. It provides potential diagnostic and therapeutic strategies for malathion and other OPCs poisoning. However, work is still required to address the effect of these compounds on other cellular systems to implement appropriate preventive measures and optimum treatment interventions for poisoning with OPCs.

In summary, we have shown that malathion induces apoptosis via the mitochondrial pathway (Figure 4.1). Malathion stimulates a dose- and time-dependent disruption of mitochondrial membrane potential, ROS formation, loss in cardiolipin

content and translocation of Bax and Bid to mitochondria of treated cells. These events, collectively, cause release of cytochrome *c* from mitochondria into the cytoplasm of cultured L929 cells to induce a caspase-9-dependent apoptosis.

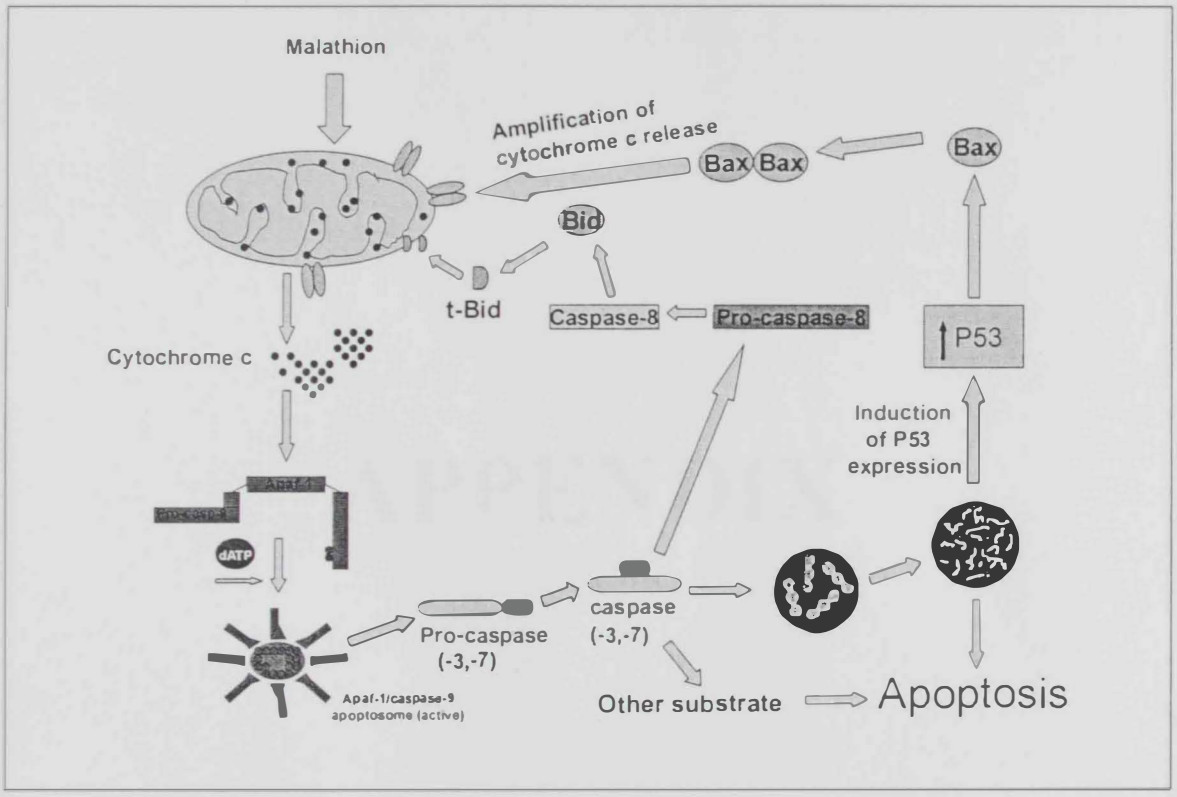


Figure 4.1: A proposed mechanism for malathion-induced apoptotic pathway in murine L929 cells.

APPENDIX

ELSEVIER

13 August 2004

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Dr L Masoud Laila Masoud Al-Alawi
Moozan2002@yahoo.com

Dear Dr Masoud Laila Masoud Al-Alawi

TOXICOLOGY, Vol 185, No 1-2, 2003, Pages 89-102, L Masoud et al "Effect of malathion on apoptosis ..."

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الملخـــص

من المعروف والمثبت طبيا بأن التسمم الحاد للمركبات الفسفورية ينتج عن تثبيط لإنزيم الأستيل كولين استيريز، في حين أن التسمم المزمن الناتج من التعرض لهذه المركبات بدرجات منخفضة (تحت حد العتبة لإحداث أعراض طبية بالجهاز العصبي) مازال موضع نقاش حاد. من خلال الدراسة الحالية قدرنا تأثير الجرعات الغير مؤثرة بالجهاز العصبي من مركب الملاثيون عند تركيزات تتراوح من (٠ر١٠-٢٠ ميكرو مولر) على موت الخلايا المبرمج لخلايا الفأر الL929 الفيبروبلاستية. وذلك بإستخدام تقنية الفلوسيتومترى وتحليل لنشاط إنزيم ال caspase . هذا وقد أثبتت الدراسة بأن مركب الملاثيون يحفز عملية موت الخلايا المبرمج لخلايا الL929 . وبمعالجة خلايا الL929 بالملاثيون تم تنشيط انزيمات ال caspases الأولية وهي (إنزيم caspase-8 وإنزيم caspase-9)، هذا بالإضافة إلى تنشيط الإنزيم المستجيب caspase-3 .

بتعريض خلايا ال L929 إلى مركب الملاثيون في وجود المثبط العام لإنزيم ال caspase وهو Z-VAD-FMK ، يؤدى ذلك إلى إبطال عملية موت الخلايا المبرمج الناتجة عن تأثير مركب الملاثيون. إضافة إلى أن مركب الملاثيون يحفز زيادة معدل بروتين ال p53 . وهي مرحلة لاحقة لنشاط إنزيمات ال caspases. وتقترح هذه الدراسة، بأن السمية الخلوية لمركب الملاثيون عند جرعات (noncholinergic) تؤدى إلى موت الخلايا المبرمج عبر إنزيمات ال caspase .

ولتوضيح تسلسل أحداث عملية الموت المبرمج للخلايا الناتج عن تأثير مركب الملاثيون، استخدمت مثبطات معينة لإنزيمات الcaspase. أدت المعالجة الأولية لخلايا الL929 مع مثبط إنزيم الcaspase-9 (z-LEHD-fmk)، إلى إضعاف تحفيز مركب الملاثيون لعملية موت الخلايا المبرمج. بينما لم يظهر أي تأثير بإستخدام مثبط إنزيم ال caspase-8 (z-IETD-fmk). هذا بالإضافة إلى أن نشاط إنزيمات الcaspase-9,-8 والcaspase-3، بفعل معالجة الخلايا بمركب الملاثيون قد ثبطت تماما في وجود مثبط إنزيم الcaspase-9 (z-LEHD-fmk)، نستنتج من ذلك دور إنزيم الcaspase-9 لتحفيز موت الخلايا المبرمج بواسطة مركب الملاثيون والتى تتم عبر عضية الميتوكندريا.

في الواقع، بأن مركب الملاثيون تحت كلتا الظروف ال(in vivo و الin vitro) يعمل على تحفيز إنتقال بروتين الcytochrome-c من عضية الميتوكندريا إلى السيتوبلازم، كما هو موضح بتحليل ال(Westren blot). بتحري ميكانيكية إطلاق بروتين الcytochrome-c من الميتوكندريا، تبين أن مركب الملاثيون يعمل على إخلال الجهد الكهرباني لل(trasmembrane) في عضية الميتوكندريا، محفزة بذلك تكوين للشوارد الحرة (Reactive Oxygen Spescies) وفقدان لل(cardiolipin) الموجود في عضية الميتوكندريا.

هذه النتائج أظهرت بأن مركب الملاثيون يعمل على تحفيز موت الخلايا المبرمج لخلايا ال L929، من خلال التأثيرالمباشر لوظائف عضية الميتوكندريا مسببة إطلاق بروتين ال cytochrome c إلى السيتوبلازم، ومن ثم يتم تنشيط إنزيم الcaspase-9. وعليه فإن تثبيط هذه الميكانيكية من الممكن أن تساهم في تزويدنا بإستراتيجية مفيدة للإقلال من التسمم الناتج عن المركبات الفسفورية العضوية.

جامعـــة الإمارات العربية المتحـــدة
عمـــادة الدراسات العليا
برنامـــج ماجستير علوم البيئـــــة

تحديد ميكانيكية عمل المركب العضوي الفسفوري الملاثيون لتحفيز موت الخلايا المبرمج في خلايا الفأر الفيبروبلاستية (أل ٩٢٩)

رسالة مقدمة من/

ليلـــى مسعـــود علـــى مسعـود العلـــوى

مقدمة إلى/
جامعة الإمارات العربية المتحدة
إستكمالا لمتطلبات الحصول على درجة الماجستير في علوم البيئة

٢٠٠٤-٢٠٠٥