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Mechanisms of environmental chemical-induced apoptosis in dopaminergic cells: critical roles of protein kinase C-delta and relevance to Parkinson's disease

Masashi Kitazawa
Iowa State University

Mechanisms of environmental chemical-induced apoptosis in dopaminergic cells: critical roles of protein kinase C-delta and relevance to Parkinson's disease

by

Masashi Kitazawa

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Program of Study Committee:
Anumantha G. Kanthasamy, Major Professor
Walter H. Hsu
Srdija Jeftinija
Jorgen Johansen
Richard J. Martin

Iowa State University

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has met the dissertation requirements of Iowa State University

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Major Professor

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For the Major Program

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LIST OF ABBREVIATIONS

Apaf-1: apoptotic protease activating factor

CARD: caspase recruitment domain

Caspase: cysteinyl aspartate-specific protease

CNS: central nervous system

DAG: diacylglycerol

DAT: dopamine transporter

DED: death effector domain

DOPAC: 3,4-dihydroxyphenylacetic acid

DRD: dopa-responsive dystonia

$\Delta\Psi m$: mitochondrial membrane potential

ETS: electron transport system

$FADH_2$: flavin adenine denucleotide (reduced form)

GSH: glutathione

iCAD: inhibitor of caspase-activated DNAase

GABA: γ-amino-butylic acid

L-DOPA: L-3,4-dihydroxyphenylalanine

MAO: monoamine oxidase

MMT: methylcyclopentadienyl manganese tricarbonyl

MPP^+: 1-methyl-4-pyridinium ion

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NADH: α-nicotineamide adenine dinucleotide (reduced form)

NADPH: nicotineamide adenine dinucleotide phosphate

NGF: Nerve growth factor

NGFI-C: nerve growth factor induced-C

OR: odds ratio

PARP: poly-(ADP-ribose)-polymerase-1

PC12: pheochromocytoma cells

PCB: polychlorinated biphenyl

PD: Parkinson's disease

PDK1: 3-phosphoinositide-dependent protein kinase 1

PKC: protein kinase C

ROS: reactive oxygen species

SNc: substantia nigra pars compacta

SOD: superoxide dismutase

TH: tyrosine hydroxylase

UPS: ubiquitin-proteosome system

VMAT2: vesicular monoamine transporter-2

ABSTRACT

We have investigated the dopaminergic toxicity and cell death signaling mechanisms of the potential environmental risk factors, dieldrin, methylcyclopentadienyl manganese tricarbonyl (MMT), and manganese, for Parkinson's disease (PD) in the dopaminergic rat pheochromocytoma (PC12) and rat mesencephalic ($1RB_3AN_{27}$ or N27) cell lines. Dopaminergic cells were more susceptible to both dieldrin and MMT toxicity as compared to non-dopaminergic cells, such as M213-20 (rat striatal GABAergic) cells, α-TC (rat clonal pancreatic) cells, and HCN-2 (human cortical neuronal) cells. Acute exposure to dieldrin or MMT altered dopamine catabolism, as observed by an increase in DOPAC formation and dopamine release, and subsequent decrease in dopamine content. Also, a rapid generation of reactive oxygen species (ROS) was observed within 5 min of dieldrin (30-300 μM) or MMT (30-200 μM) exposure. This ROS generation was partially blocked by α-methyl-p-tyrosine or selegiline, inhibitors of tyrosine hydroxylase or monoamine oxidase-B, respectively, indicating that the presence of dopamine and disruption of dopamine catabolism and degradation may serve as an additional source of ROS. Dieldrin, MMT or manganese treatment in dopaminergic cells triggered apoptotic cell death process, as measured by mitochondrial depolarization, release of cytochrome c, and caspase-9 and caspase-3 activation. These initial pro-apoptotic processes were almost completely blocked by the over-expression of the anti-apoptotic protein, Bcl-2. Thus, one of the primary cellular targets of dieldrin, MMT, and manganese could be the mitochondria; specifically, mitochondrial function was inhibited to initiate the apoptotic cascade. Interestingly, we observed proteolytic cleavage of the novel protein kinase Cδ (PKCδ) following dieldrin, MMT, and manganese exposure. PKCδ (72-74 kDa) was cleaved into the regulatory (42 kDa) and catalytic (38 kDa) subunits by caspase-3, resulting in increased kinase activity. Other PKC family proteins, including PKCα, PKCβII, and PKCζ, were not cleaved during dieldrin or MMT exposure, indicating that the proteolytic cleavage of

PKCδ was isozyme specific. Both pharmacological and genetic modulation of PKCδ resulted in attenuation of toxicant-induced DNA fragmentation and apoptosis, suggesting that PKCδ plays an important role in the execution of apoptosis. Additional experimental results indicate that PKCδ amplifies the caspase cascade by positive feedback activation during the neurotoxic insult. Another regulatory role of PKCδ was observed during dieldrin or MMT treatment; translocation of PKCδ into mitochondrial membranes was increased, and it was followed by release of pro-apoptotic molecules such as cytochrome c and Smac, and time-dependent activation of caspase-9 and caspase-3. Down-regulation of PKCδ by TPA or pretreatment with rottlerin significantly blocked dieldrin-induced cytochrome c release, yet rottlerin did not inhibit translocation of PKCδ into mitochondria. These results strongly support that PKCδ modulates mitochondrial function and triggers the initiation process of apoptosis. Delivery of recombinant active PKCδ into dopaminergic cells mimicked the effect of dieldrin-induced PKCδ translocation into mitochondria, indicating that PKCδ plays a critical role not only in the execution process, but also in the initiation of apoptosis. Taken together, these experimental results suggest that environmental neurotoxic agents (dieldrin, MMT, and manganese) promote dopaminergic degeneration by sequentially activating the following cellular events: i) generation of oxidative stress to initiate the apoptotic cascade, ii) induction of apoptotic cell death by caspase-3 dependent proteolytic activation of PKCδ, and iii) amplification of the caspase cascade by positive feedback regulation of upstream molecules associated with mitochondrial mediated apoptotic cell death.

CHAPTER I: GENERAL INTRODUCTION

Dissertation Organization

This dissertation is written in an alternative thesis format. It contains a general introduction, six research papers, a general discussion, and an acknowledgement. A list of references cited is included in the end of each chapter. The general introduction (Chapter I) includes a research objective, background information, and literature review of the relationship between environmental factors and Parkinson's disease. Chapter II, "Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptotic cell death in dopaminergic cells", and Chapter III, "Oxidative stress and mitochondrial-mediated apoptosis in dopaminergic cells exposed to methylcyclopentadienyl manganese tricarbonyl (MMT)", have been published in *Free Radical Biology and Medicine* (**31**:1473-1485, 2001) and the *Journal of Pharmacology and Experimental Therapeutics* (**302**:26-35,2002), respectively. Chapter IV, "Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase Cδ in dopaminergic cells: relevance to pathogenesis of Parkinson's disease", has been submitted to *Neuroscience.* Chapter V, "Dieldrin induces apoptosis in a mesencephalic dopaminergic neuronal N27 cell line via caspase-3-dependent proteolytic activation of protein kinase Cδ", Chapter VI, "Role of protein kinase Cδ and Bcl-2 in caspase-3-dependent apoptosis during manganese exposure in dopaminergic cells", and Chapter VII, "Mitochondrial translocation of protein kinase Cδ inactivates Bcl-2 by proteolytic degradation during environmental neurotoxic insult in dopaminergic cells", will be submitted for publication in *Neurotoxicology*, the *Proceedings of National Academy of Sciences*, and the *Journal of Neurochemistry*, respectively.

This dissertation contains the experimental results obtained by the author during his graduate study under the supervision of his major professor, Dr. Anumantha G. Kanthasamy.

Research Objective

Parkinson's disease (PD) is one of the major neurodegenerative disorders affecting approximately 1-2% of the population over the age of 50 in the United States (Aschner, 2000; Shastry, 2001). The prevalence of the disease increases up to 4-5% by the age of 85 (Giasson and Lee, 2001). The etiopathogenesis of dopaminergic neurodegeneration in PD remains unknown. Aging and genetic defects have long been considered the primary risk factors of PD. However, recent epidemiological findings as well as genetic analyses reveal that genetic factors may not be major causal factors of sporadic PD (Golbe and Pae, 1988; Ho et al., 1989; Tanner, 1989; Koller et al., 1990; Tanner and Langston, 1990; Semchuk et al., 1992; Seidler et al., 1996; Liou et al., 1997; Chan et al., 1998a; Gorell et al., 1998; Tanner et al., 1999; Zorzon et al., 2002). Rather, these studies strongly suggest that environmental factors initiate a neurodegenerative process in dopaminergic neurons in the substantia nigra to cause typical pathological features of PD. Exposure of mine workers to manganese produces Parkinsonian-like symptoms known as Manganism, a neurological condition affecting extrapyramidal motor function of the central nervous system (CNS) (Inoue and Makita, 1996). Hence, the recent legalization of a manganese-containing gasoline adduct, methylcyclopentadienyl manganese tricarbonyl (MMT), is of concern due to the possible health hazards resulting from exposure to high levels of manganese. The exact mechanisms of CNS toxicity induced by manganese and MMT have not been well characterized, yet these compounds could be risk factors for dopaminergic degeneration. In addition to transition metals, pesticides have accumulated in brains of PD patients (Fleming et al., 1994; Corrigan et al., 1996; Corrigan et al., 1998). Dieldrin, a chlorinated cyclopentadiene pesticide, has been detected in brains from PD patients and is believed to promote progressive dopaminergic neurodegeneration. Studies in animals have also shown that chronic exposure to dieldrin significantly reduces brain

levels of dopamine and induces transient tremors, pathological and symptomatic features of PD (Sharma et al., 1976; Wagner and Greene, 1978). However, the cellular and molecular mechanisms of dieldrin-induced dopaminergic neurodegeneration have not yet been characterized.

The major objectives of this dissertation are: (i) to determine the involvement of oxidative stress as an initiation factor of environmental chemical-induced dopaminergic toxicity; (ii) to characterize environmental chemical-induced mitochondrial dysfunction and the subsequent activation of cell signaling molecules involved in pro- and anti-apoptotic functions; and (iii) to define the pro-apoptotic function and regulatory role of protein kinase Cδ (PKCδ) during environmental chemical-induced dopaminergic degeneration. Together, these studies will enhance our understanding of cell death mechanisms underlying dopaminergic degeneration and will also give further insight about the role of environmental factors in the pathogenesis of Parkinson's disease.

Background and Literature Review

This section provides background information related to the studies presented in the dissertation: (1) Parkinson's disease; (2) Environmental risk factors; (3) Toxic effects of dieldrin, manganese, and MMT; and (4) Signal transduction mechanisms of apoptosis in neurodegeneration.

Parkinson's disease

Parkinson's disease (PD) was first described by a British physician, James Parkinson in 1817 under the heading of "paralysis agitans" in his *Essay on the Shaking Palsy*. However, evidence suggests a possible case of PD in India in 3000 B.C., indicating that the disease may have been known for thousands of years (Roman et al., 1995). Research progressed slowly until the 1960s, when scientific discoveries linked the disease to the loss of dopaminergic brain neurons in the substantia nigra (SNc), the

brain region that controls motor activity. Currently, PD is characterized by a slow but progressive and selective degeneration of dopaminergic neurons in the nigral-striatal pathway, resulting in irreversible motor dysfunction. In most cases, PD is prevalent among older individuals. In fact, almost 2,000,000 individuals in the United States have been diagnosed with PD, which accounts for approximately 1-2% of the population over the age of 50 (Aschner, 2000; Shastry, 2001). The prevalence increases up to 4-5% by the age of 85 (Giasson and Lee, 2001), indicating that age is an undisputable risk factor of the disease. On the other hand, the age of PD onset has been decreasing in several countries, and PD is currently divided into three major groups: idiopathic PD, young-onset PD (YOPD), and juvenile Parkinsonism (JP). Although the clinical symptoms are similar among the groups, the age of disease onset and some pathological features are distinct. In general, patients who develop PD at 40 years of age and older are considered to have idiopathic PD while patients who develop PD between 21-40 years of age are diagnosed with YOPD. JP develops in patients younger than 21 years of age. The prevalence of YOPD (including JP) is rare, approximately 10-47 cases per 1,000,000 people, in the U.S. and Europe, and accounts for 4-12% of all PD cases. Alternatively, up to 40% of all PD cases are YOPD in certain regions of Japan, indicating that as yet unidentified factors may be involved in the development of YOPD. Despite the extensive research efforts to understand this debilitating disease, the exact pathophysiological mechanism underlying nigral dopaminergic degeneration in PD remains unknown.

The major symptoms of PD are resting tremor, rigidity, bradykinesia, and postural instability. The resting tremor is considered the most common symptom, yet tremors may be absent both in the early and advanced stages of PD, with the first obvious disease manifestation only an intermittent trembling confined to one finger of one hand (Selby, 1990). The trembling may be limited to one finger for two to three years before it progresses to other regions. Usually observed in one side of an arm or leg, the resting tremor is considered a unilateral tremor. Body rigidity is associated

with bradykinesia, but the two symptoms are not parallel to each other (Duvoisin, 1991). Rigidity can be described as muscle stiffness due to resistance to passive movements in opposing muscles. In early and advanced stages of PD, rigidity is detected more frequently than resting tremor (Selby, 1990). The other common manifestation of bradykinesia is associated with the loss of automatic movements, such as the swing of arms while walking, eye blinking, swallowing of saliva, or minor movement for postural adjustment (Duvoisin, 1991). Together with rigidity, bradykinesia contributes to the reduction of emotional facial movements (Selby, 1990). As a result, patients have a mask-like appearance, with little facial expression. All of the above-mentioned symptoms are related to abnormal movements that can be explained by the pathological changes in the PD brain. In addition to the motor disabilities, a significant cognitive decline prevails in the late stages of PD. In the following section, the anatomy and pathology of PD in the substantia nigra and related regions will be described.

In 1664, distinct subcortical structures were clearly identified for the first time, and the basal ganglia was referred to as the corpus striatum (Parent, 1986). The corpus striatum was recognized as the major component of the "extrapyramidal motor system" by S. A. K. Wilson in 1912 (Nauta and Domesick, 1984). This term loosely grouped the corpus striatum with an array of brain stem nuclei and reflected the assumption that this grouping constituted a complete and independent motor unit (Carpenter et al., 1981). The term "basal ganglia" has been generally used to refer to these major anatomical telencephalic subcortical nuclei at the base of the forebrain. More formally, this definition groups the corpus striatum (striatum and globus pallidus) with the substantia nigra and subthalamic nucleus (Figure 1). Information from the cerebral cortex projects to the basal ganglia, and the outputs then funnel back to the frontal areas of the cortex via the thalamus or directly to the motor systems in the midbrain and hindbrain (Houk, 1995). Fine motor activities and modification of movements are controlled by the basal ganglia and cerebellum. The output of the cerebellum is generally excitatory, while the basal ganglia output is inhibitory. The balance between these two systems

allows for smooth, coordinated movement, while a disturbance in either system manifests as movement disorders.

The region most affected pathologically in the development of PD is the substantia nigra in the basal ganglia. PD is described as a selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) (Marsden, 1990; Duvoisin, 1991). The SNc contains approximately 400,000 dopaminergic neurons at birth, which account for 80-90% of the dopaminergic neurons in the body. Every year, about 2,400 dopaminergic neurons in the normal SNc die. Major clinical symptoms of PD are observed when at least 60-85% of the tyrosine hydroxylase (TH)-immunoreactive dopaminergic neurons are lost in the SNc, or when the dopaminergic tone to the striatum has diminished to 50-80% of normal (Fearnley and Lees, 1991; Hornykiewicz, 1998; Betarbet et al., 2002). In particular, the caudorostral and ventrolateral areas of the SNc are the most severely affected, followed by the medioventral, dorsal, and lateral areas (Jellinger, 2001) and the neuromelanins, by-products of dopamine metabolism, are depleted by 45-66% in the SNc (Jellinger, 2001). In the normal brain, 60% of dopaminergic neurons would not be depleted before at least 100 years. In the brain affected by PD, some unknown factors, possibly originating in the environment, genetic background, or both facilitate the abnormal rate of dopaminergic neuronal loss in the substantia nigra. On the other hand, JP patients have preserved, non-degenerated dopaminergic neurons in the SNc but lack neuromelanin, suggesting that JP is caused by a genetic defect in dopamine synthesis. In support of this hypothesis, about 40% of YOPD and almost all JP patients in Japan have a family history of Parkinsonism. In particular, several gene mutations have been identified in patients with a certain type of JP, which will be discussed later in this chapter.

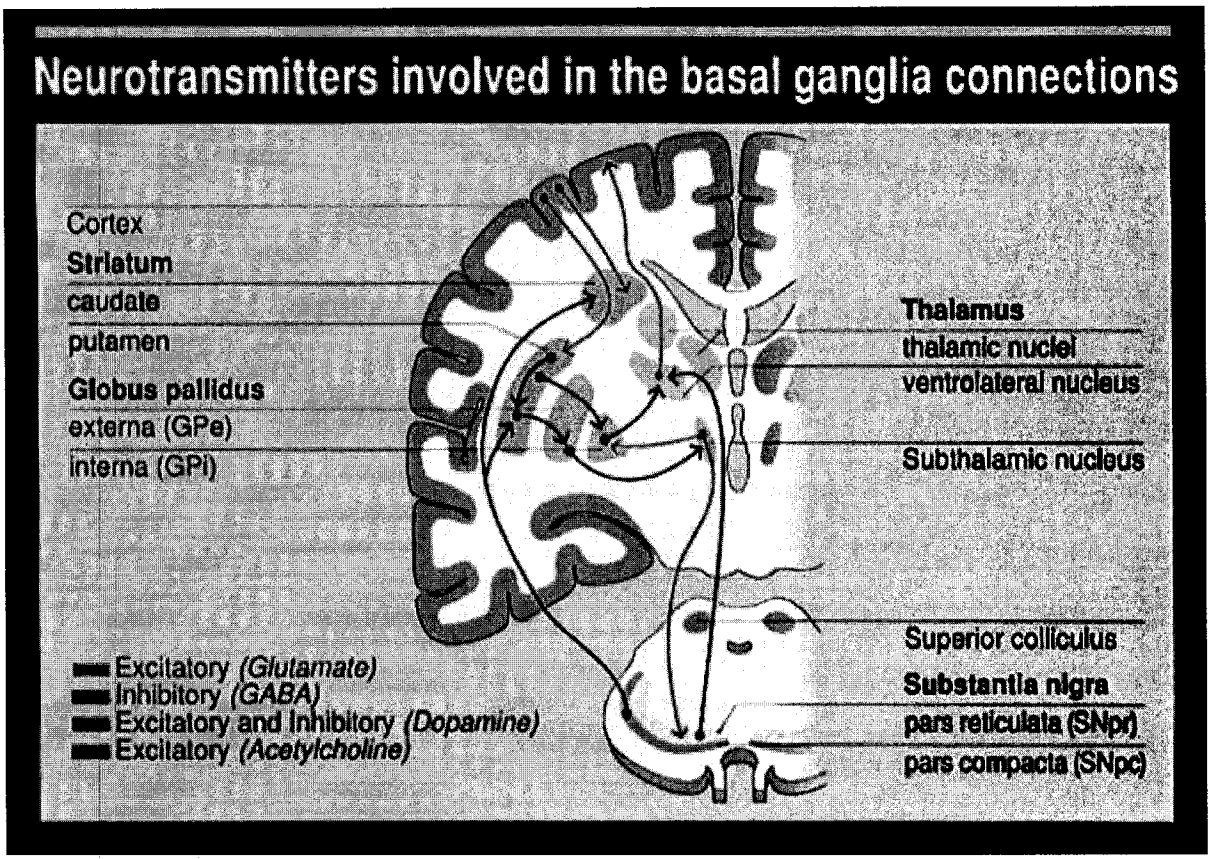


Figure 1: Anatomy of Basal Ganglia (Modified from Kibiuk, 1997)

The dopaminergic neurons in the SNc project to the inhibitory GABAergic neurons with dopamine receptors in the corpus striatum. Dopamine receptors are G protein coupled receptors divided into five D_1 through D_5 subtypes. D_1 and D_5 receptors are Gαs coupled and stimulate adenylate cyclase (AC), whereas D_2, D_3, and D_4 receptors are Gαi/o coupled and inhibit AC activity. D_1 and D_2 receptors are found in the striatum, and the D_1 receptor mediates the striatonigral (direct) pathway, whereas the D_2 receptor mediates the striatopalladial (indirect) pathway. A simple schematic diagram of the basal ganglia based on the description by Webster (1990) and Young and Penney (1988) is shown in Figure 2 (Young and Penney, 1988; Webster, 1990). In the normal brain, dopamine released from the SNc excites GABAergic neurons in the corpus striatum and stimulates (D_1 receptor; direct pathway) or inhibits (D_2 receptor; indirect

pathway) the release of the inhibitory GABA neurotransmitter. In the direct pathway, striatal GABAergic neurons project to the globus pallidus interna (GPi) and release GABA upon stimulation by dopamine to inhibit GABA release from the GPi to the thalamus followed by stimulation of glutamate release to the cortex. In the indirect pathway, dopamine inhibits the release of GABA from the striatum to the globus pallidus externa (GPe) to subsequently stimulate the release of GABA from the GPe to the subthalamic nucleus to inhibit glutamate release from the subthalamic nucleus to the substantia nigra reticulata (SNr). As a result, the release of GABA from the SNr to the thalamus is suppressed, and the signal is transmitted to the cortex.

Once dopaminergic neurons in the SNc are reduced significantly (>70%), the basal ganglia regulatory loops that control motor function will be disturbed, and downstream neural transmission will be altered significantly. Some of the major symptoms of PD can be explained pathophysiologically. Rigidity may result from severe degeneration of dopaminergic neurons in the ventrolateral part of the SNc that project to the D_1 receptor-containing GABAergic neurons in the dorsal putamen (Jellinger, 2001), which eventually increases the inhibitory tone to the ventrolateral/medial thalamus. Furthermore, it is more common to observe the up-regulation of D_2 receptor in striatum in PD patients.

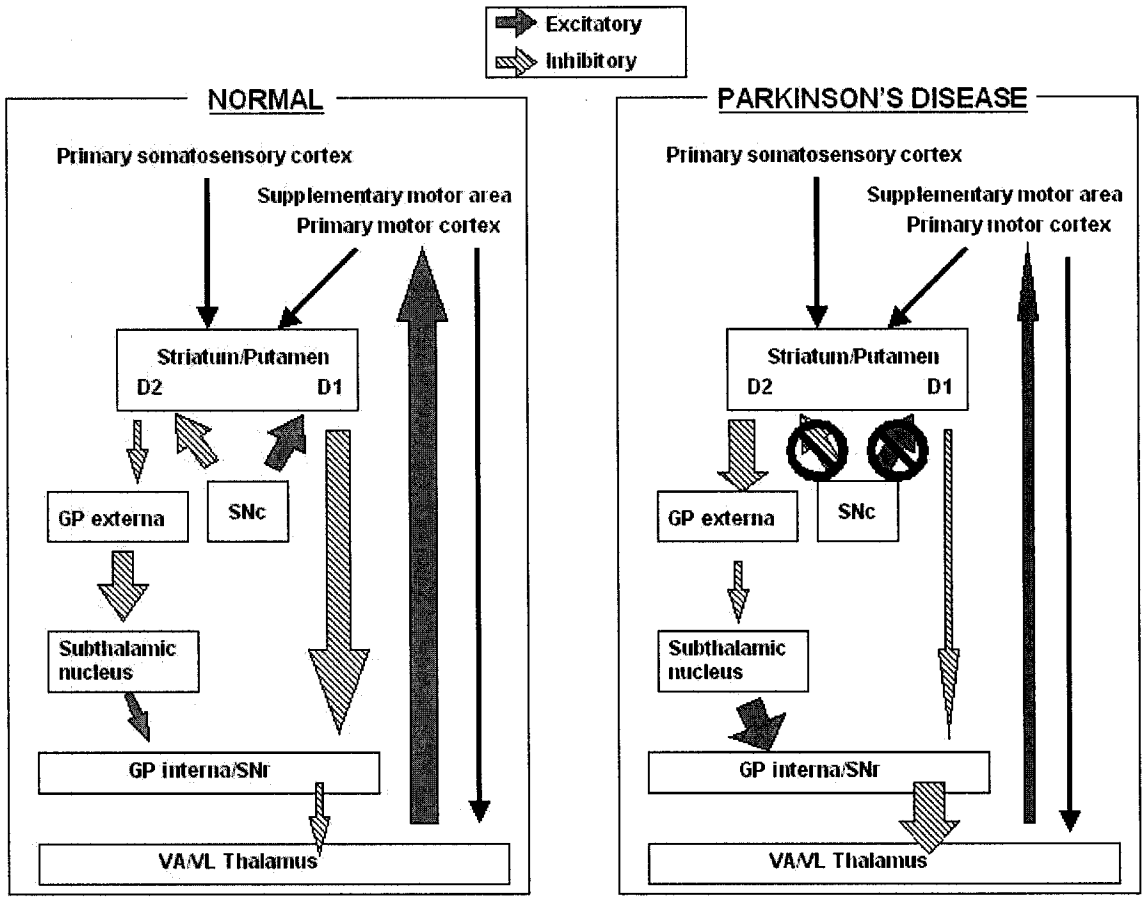


Figure 2: Schematic Diagram of Neuronal Connection in normal and PD

The selective destruction of dopaminergic neurons in the SNc has not been definitively identified, but accumulating data strongly indicate that genetic and/or environmental factors may be responsible for the etiology of PD. Genetic factors and gene mutations have long been investigated for an association with the etiology of PD. Specifically, mutations in tyrosine hydroxylase (TH) and other enzymes responsible for dopamine biosynthesis have been studied because of specific expression patterns in dopaminergic neurons. Thus, tyrosine hydroxylase and its related enzymes were best candidate genes to determine dopaminergic neuron susceptibility. The mutation of GTP cyclohydrolase, a rate-limiting enzyme for the biosynthesis of tetrahydrobiopterin, has been found in dopa-responsive dystonia (DRD), a disease clinically similar to

juvenile Parkinsonian syndrome (Bandmann et al., 1996b; Ichinose and Nagatsu, 1997). Because of malfunction of tetrahydrobiopterin synthesis, a co-factor of TH activity, dopaminergic neurons in DRD patients are incapable of producing dopamine, and patients exhibit abnormal motor activity like PD patients. However, a clinical study of 99 sporadic PD patients and 161 control patients revealed no significant evidence of TH mutations associated with the incidence of PD (Kunugi et al., 1998). In addition, GTP cyclohydrolase was not mutated in PD patients with no family history of DRD (Bandmann et al., 1996a). Furthermore, other possible enzymes, such as glutathione peroxidase, catalase, superoxide dismutase, and amyloid precursor protein were not positively correlated with the etiology of PD (Gasser et al., 1994; Parboosingh et al., 1995). However, the genetic factor hypothesis regained momentum when mutations in the α-synuclein gene were identified (Polymeropoulos et al., 1997; Kruger et al., 1998) and linked to a region on chromosome 2 in familial PD (Gasser et al., 1998). The physiological function of α-synuclein has not been fully identified, yet it appears to be involved in vesicular fusion and/or chaperone-like functions (Davidson et al., 1998; Souza et al., 2000a). Over-expression of wild-type α-synuclein has shown both protective and facilitative effects on dopaminergic cell death (Zhou et al., 2000; Junn and Mouradian, 2002; Kaul et al., 2002). The α-synuclein point mutations alanine to proline at position 30 and alanine to threonine at position 53 cause accumulation and aggregation of α-synuclein and the formation of Lewy bodies, characterized as distinctive protein aggregations in dopaminergic neurons of PD patients. Also, mutations in the parkin protein appear to be associated with the etiology of JP, and has been investigated extensively, especially in Japan (Kitada et al., 1998). Yet, these mutation-related PD cases are rare. In general, genetic-related PD cases account for approximately 5% of the whole population of PD patients (Chan et al., 1998b), and PD caused by mutations of α-synuclein is less than 1%. The hallmarks of genetic factors in PD will be discussed in more detail later in this chapter.

A recent landmark epidemiology study by Tanner and colleagues (1999) of

nearly 20,000 monozygote (MZ) and dizygote (DZ) twins from a WWII veterans health care database determined that the concordance of PD incidence is similar between MZ (0.155) and DZ (0.111) twins, with a relative risk of 1.39, which is not significantly different from the entire population with PD (Table 1) (Tanner et al., 1999). Interestingly, if one of twin is diagnosed with PD at age 50 or under, the concordance and relative risk are significantly higher. Thus, genetic factors may be more important in young-onset PD, and other factors may play a more significant role in the incidence of late-onset or geriatric PD. In addition, more data have accumulated that indicate the significance of exogenous or environmental factors in the etiology of PD.

	Concordant pairs		Discordant pairs		Pairwise concordance		
	MZ	DZ	MZ	DZ	MZ	DZ	Relative risk
Overall	11	10	60	80	0.155	0.111	1.39
First twin diagnosed ≤50 yr	4	2	0	10	1.00	0.167	6.00
First twin diagnosed >50 yr	7	8	58	68	0.108	0.105	1.02

Table 1: Genetic Risk Factors and Parkinson's disease: A Case Study in Twins (Modified from Tanner et al., 1999)

Environmental risk factors and PD

Age has long been considered a potential risk factor of Parkinson's disease because most patients develop Parkinson's disease after age 50 (Burton and Calne, 1990). The environmental factor hypothesis emerged in the PD field following the discovery of the Parkinsonian toxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). In 1977, an unusually high number of young-onset Parkinson's-like syndrome was observed in Virginia as well as in San Francisco, U.S.A.; those afflicted were later found to have used the synthetic designer drug meperidine, which is an analogue of 1-methyl-4-phenyl-propionoxy-piperidine (MPPP). They first suffered from symptoms of visual hallucinations, jerking limbs, and stiffness. In the later stage, they experienced immobility, a flexed posture, and tremor, similar to symptoms often observed in PD patients. These symptoms were remarkably attenuated by

administration of the anti-Parkinsonian drug L-DOPA. Furthermore, positron emission tomography (PET) using 6-fluorodopa showed a significant reduction in activity of dopaminergic neurons in the basal ganglia, as normally seen in PD patients. Scientists isolated MPTP as a contaminant and a possible toxicant for dopaminergic neurons from the synthetic drug (Davis et al., 1979) (Figure 3A-B). The intensive investigation of MPTP toxicity and its association with PD has elucidated the mechanism of dopaminergic neuronal cell death by MPTP. MPTP is not a toxic compound, but its metabolite, 1-methyl-4-phenylpyridine (MPP^+), is a potent neurotoxin selectively toxic to dopaminergic neurons (Schapira, 1993; Hartley et al., 1994). When MPTP is administered to humans and other mammals, it passes through the blood-brain barrier and accumulates in the brain. Astrocytes convert MPTP into the intermediate compound, $MPDP^+$, and then MPP^+ is formed spontaneously in the nerve terminal. Intercellular MPP^+ is selectively taken up by the nerve terminals of dopaminergic neurons via dopamine transporters. As a result, MPP^+ selectively destroys dopaminergic neurons by inhibiting mitochondrial respiratory function (Complex I function) (Figure 3B). The discovery of a potent Complex I inhibitory property of MPP^+ brought additional attention to the potential exogenous causes of PD because patients with idiopathic PD have less functional mitochondrial Complex I activity in dopaminergic cells, which may cause dopaminergic cell death in PD (Parker et al., 1989; Schapira, 1993; Anglade et al., 1997b). Complex I of the mammalian electron transfer chain is composed of at least 43 protein subunits, seven of which are encoded by mtDNA. Complex I catalyzes the transfer of electrons from NADH to ubiquinone and translocates protons from the mitochondrial matrix to the intermembrane space and may also play direct roles in the mitochondrial permeability transition and in cell death pathways (Greenamyre et al., 2001). Currently, several other environmental chemicals that inhibit Complex I are under extensive investigation, which will be briefly described later in this section. Although MPTP and MPP^+ are currently used as a well-established model in PD research, some pathological differences have been

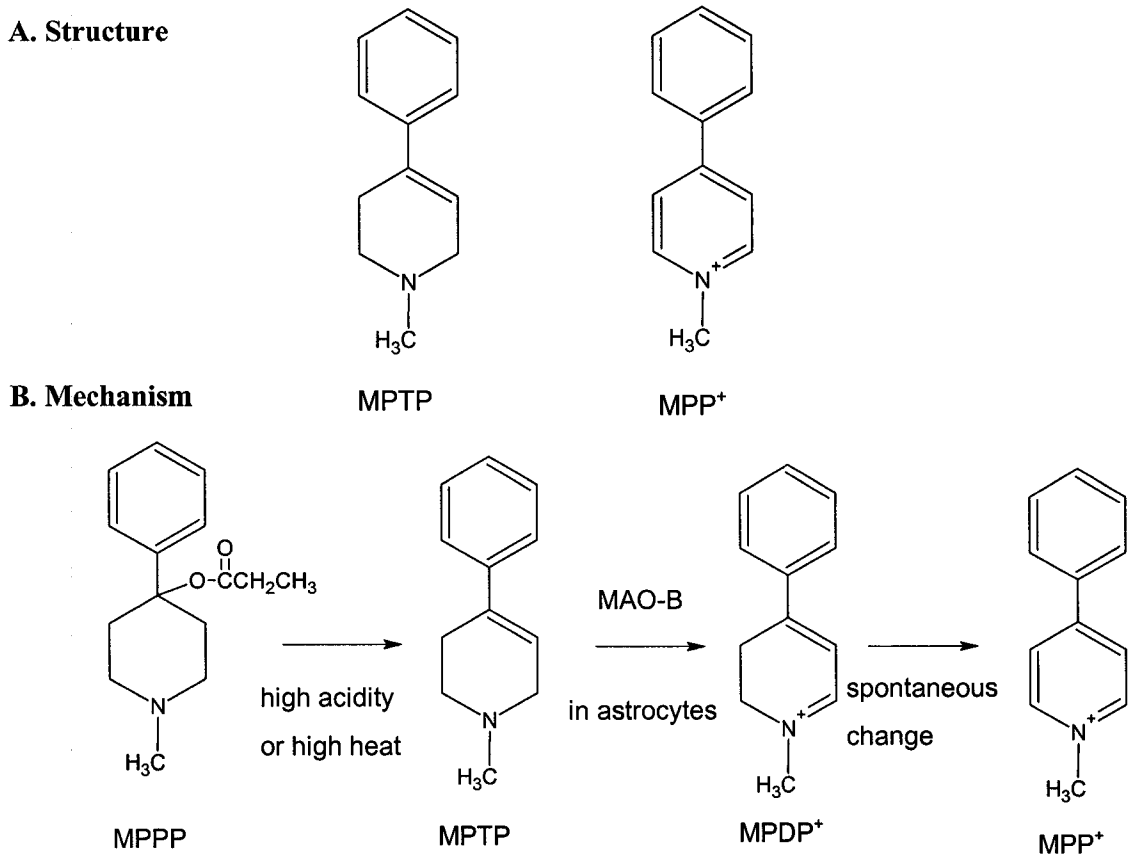
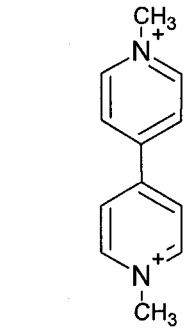


Figure 3: Chemical Structure and Mechanism of MPTP Neurotoxicity

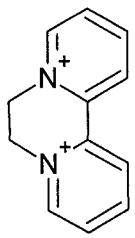
reported between MPTP- or MPP^+-induced dopaminergic neurodegeneration and idiopathic PD. For example, pigmented aggregates known as Lewy bodies are observed in idiopathic PD, but not in MPTP-induced PD; thus, the actual dopaminergic cell death mechanism of idiopathic PD may differ from MPTP-induced dopaminergic cell death.

The discovery that MPTP/MPP^+ causes acute neurotoxicity brought attention to other chemicals and environmental factors possibly involved in the etiology of PD, undermining aging as the primary causal factor of PD. Several pesticides structurally similar to MPP^+, such as the widely used paraquat and diquat, have been examined to establish the role of environmental factors in the pathogenesis of PD. (Figure 4 and

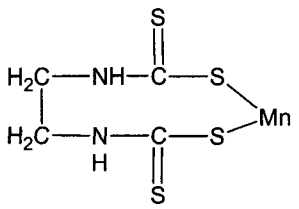
Table 2). Paraquat induced dopaminergic cell death via a mechanism similar to that of MPP^+ in PC12 cells (Li and Sun, 1999; Chun et al., 2001b). Also, repeated, intraperitoneal (i.p.) paraquat injections in mice caused dose- and age-dependent dopaminergic neuronal cell death in the SNc whereas other neurons, including GABAergic neurons, were not significantly affected (McCormack et al., 2002). This dopaminergic neurodegeneration may be due to the up-regulation and increased aggregation of α-synuclein, which may lead to the formation of Lewy bodies (Uversky et al., 2001; Manning-Bog et al., 2002). However, a recent study reported that the dopamine transporter is not involved in paraquat uptake, indicating that paraquat selectivity in dopaminergic cells may not be as high as that of MPP^+ (Miller and Quan, 2002). Diquat, structurally similar to paraquat, has been reported to accumulate significantly in pigmented nerve cells following i.p. injection in frogs (Lindquist et al., 1988), indicating that dopaminergic cells may be one of the target regions due to the high levels of neuromelanin. Additionally, a farmer acutely exposed to diquat (10% solution) for 10 min developed severe PD-like symptoms including bradykinesia 10 days after the exposure, indicating that diquat may mimic the toxic mechanism of MPP^+ (Sechi et al., 1992). Cyperquat, which contains MPP^+, was tested for herbicidal activity during the 1960s because of its structural similarity to paraquat. Unlike paraquat, cyperquat was not widely distributed in agricultural areas. Maneb (manganese ethylene-bis-dithiocarbamate), another chemical used as a fungicide, contains manganese in its chemical structure. Because of the possible association between manganese exposure and the etiology of PD-like syndrome (described in the later section), maneb is suspected to be one of the chemical risks for PD. Several human cases of PD-like syndrome following maneb exposure have been reported in Italy (Meco et al., 1994), and case-control study focusing on maneb exposure and its association with PD-like symptoms was documented in Brazil (Ferraz et al., 1988). These studies strongly suggest that maneb may be a potent dopaminergic neurotoxicant, yet the cellular neurodegenerative mechanism needs to be investigated. Furthermore,



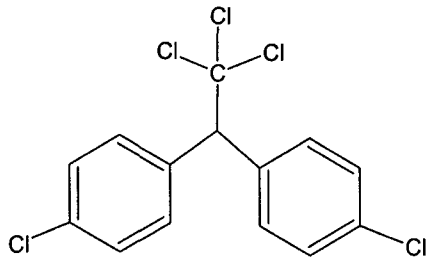
Paraquat (MW 186.26)



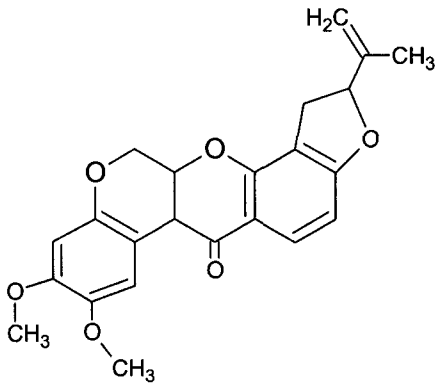
Diquat (MW 184.2)



Maneb (MW 265.29)



p,p-DDT (MW 354.5)



Rotenone (MW 394.43)

Figure 4: Chemical Structures of Selected Pesticide

combined exposure to paraquat and maneb for up to 4 weeks in mice causes an immediate decrease in motor activity and an additive reduction in dopaminergic neurons in the SNc (Thiruchelvam et al., 2000), suggesting that multiple chemical exposures may facilitate dopaminergic degeneration and further enhance the risk of developing PD in humans.

	Use	General Toxicity	Symptoms	Study relative to PD
Paraquat	Herbicide and drying agent	GI, kidney and lung	Decreased locomotor activity	mice - degeneration of dopaminergic neurons
$C_{12}H_{14}N_2$	used on rice and soya bean	No carcinogenic, teratogenic,	reduced ambulatory activity	mice - up-regulation and aggregation of a-synuclein
186.26		but maybe mutagenic		activation of caspase-dependent cell death pathway
				Activation of AP-1 transcription factor (PC12)
Diquat	used to desiccate potato vines	Preliminary kidney and heart	Loss of facial expression,	frog - accumulation of diquat in pigmented neurons
$C_{12}H_{12}N_2$	and seed crops, to control	No carcinogenic, teratogenic,	flexed posture, akinesia	Clinical case: acute diquat causes PD-like symptoms
184.26	flowering of sugarcane, and for	but maybe mutagenic		
	industrial and aquatic weed control.			
	Also use on fruits, grains and			
	vegetables, aquatic areas and cotton.			
Maneb	fungicide used in the control of	No carcinogenic, mutagenic,	Decreased locomotor activity	mice - degeneration of DA neurons with
$C_4H_6N_2S_4Mn$	early and late blights on potatoes,	but teratogenic	when combined with paraquat	combination with paraquat
265.29	tomatoes and many other diseases			maneb exposure causes PD-like symptoms
	of fruits, vegetables, field crops,			
	and ornamentals.			
DDT	insecticide	N/A	N/A	No significant relationship between
$C_{14}H_9Cl_5$	used to kill mosquito and control			DDT toxicity and PD or
354.5	malaria			dopaminergic neurodegeneration
Rotenone	used for control of leaf-eating	N/A	Akinesia, rigidity, tremor,	mice - causes dopaminergic neurodegeneration
$C_{23}H_{22}O_6$	caterpillars, beetles, squash bugs,		flexed posure	and formation of Lewy bodies
394.43	thrips, and scales on woody			causes aggregation of α-synuclein (formation of
	ornamentals and herbaceous perennials			α-synuclein inclusions)

Table 2: Summary of Toxicity in Selected Pesticides

Recently, the house pesticide rotenone has been evaluated in PD research. Rotenone, a natural chemical compound made from tropical legumes, Lonchocarpus, and Derris often referred to as Cube Root, is especially effective against the most troublesome of pests, such as leaf-eating caterpillars, beetles, squash bugs, thrips, and scales on woody ornamentals and herbaceous perennials. Rotenone is a well-known inhibitor of mitochondrial complex I; its chemical structure is shown in the Figure 4. Betarbet et al. (2000) demonstrated that chronic infusion of rotenone to rats reproduces PD pathology including Lewy body formation in the substantia nigra and also causes PD-like symptoms, such as hypokinesia and rigidity (Betarbet et al., 2000). Rotenone treatment induces the aggregation of α-synuclein and depletion of ATP in *in vitro* studies (Uversky et al., 2001; Lee et al., 2002). In SH-SY5Y dopaminergic cells, chronic low dose (5-20 nM) treatment with rotenone causes calcium overload and may result in cell death (Sherer et al., 2001).

Pesticides are currently being evaluated by many research groups as one of the potential risk factors of PD. However, other chemical risk factors also are likely to

contribute to the etiology of PD. In this regard, we must recognize that we are exposed to multiple chemicals from various sources in our daily lives, and these chemicals may interact and potentiate adverse effects. The case-control studies further reveal some other possible risk factors in the environment.

Some epidemiological studies have reported that early onset Parkinson's disease tends to be observed in rural areas where farming is a major occupation (Tanner and Langston, 1990; Jenner, 1998). To identify any possible risk factors for Parkinson's disease, large-scale case control and epidemiological studies have been conducted mainly in rural areas around the world (Golbe and Pae, 1988; Ho et al., 1989; Tanner, 1989; Koller et al., 1990; Tanner and Langston, 1990; Semchuk et al., 1992; Seidler et al., 1996; Liou et al., 1997; Chan et al., 1998a; Gorell et al., 1998; Zorzon et al., 2002). The summary of each study is compared in Table 3. Several differences have been observed among these studies. However, farming and pesticide/herbicide uses were the factors with consistently high and significant odd ratios (OR), ranging up to 5.2 for farming and 3.6 for pesticide/herbicide uses, indicating that these factors have a positive association with the incidence of PD and could increase the risk of PD in humans (Semchuk et al., 1992; Seidler et al., 1996; Liou et al., 1997; Gorell et al., 1998). Seidler et al. (1996) specified that organochlorines and organophosphates were particularly high risk factors of Parkinson's disease. In contrast, Koller et al. (1990) concluded that drinking well water and rural living significantly increased the risk of Parkinson's disease, whereas pesticides and herbicides did not increase the risk. However, since the well water did not undergo chemical analysis, the well water may have been contaminated with pesticides or other agricultural chemicals.

Heavy metals are other important environmental factors. Manganese, copper, iron, lead, and aluminum have been suspected to be risk factors of PD. A more detailed explanation/discussion of manganese appears later in this chapter. Here, other metals associated with PD will be discussed. A case control study conducted by Gorell et al. (1997) revealed that chronic metal exposure significantly increases the risk of PD

(Gorell et al., 1997). Comparing 144 PD patients with 464 controls, they found that PD is positively associated with more than 20 years of exposure to copper (OR = 2.49), manganese (OR = 10.61), lead-copper (OR = 5.24), lead-iron (OR = 2.83), and iron-copper (OR = 3.69). Iron, mercury, and zinc were not statistically associated with PD in this study. Thus, dopaminergic neurodegeneration is not caused by non-specific toxicity of heavy metals, but probably by the specific neurotoxicity of these selected metals. The incidence of PD following manganese exposure is remarkably high. Manganese induces more *in vitro* DNA fragmentation in PC12 cells than nickel, copper, or zinc (Hirata, 2002). Several PD cases have been reported following more than 30 years of lead exposure (Kuhn et al., 1998). However, these cases of PD may not have resulted solely from lead poisoning. Copper, on the other hand, is positively associated with PD as shown above. In addition, copper increases the *in vitro* aggregation of α-synuclein, providing possible insight into the etiopathology of PD (Paik et al., 1999). Aluminum, another abundant metal, has been considered a risk factor for PD because significantly greater accumulation of aluminum was found in the substantia nigra, caudate nucleus, and globus pallidus of PD brains as compared to controls (Good et al., 1992; Yasui et al., 1992). In addition, aluminum in the CNS has been reported in Parkinsonism-dementia in Guam (Perl et al., 1982). Thus, excessive intake or absorption of aluminum may result in deposition of aluminum in the CNS, leading to neuronal cell death. Despite these reports, the cellular/molecular mechanisms of heavy metal-induced dopaminergic neurodegeneration have not yet been identified.

Interestingly, several exogenous factors have been shown to prevent the risk of PD in case control studies (Hellenbrand et al., 1997; Liou et al., 1997; Chan et al., 1998a; Checkoway et al., 2002; Zorzon et al., 2002). These possible factors are cigarette smoke, coffee/tea, and alcohol. The compounds studied most intensively for their protective effects against PD are nicotine and caffeine (Golbe et al., 1986; Benedetti et al., 2000; Ross et al., 2000; Tanner et al., 2002). All the case control studies mentioned above showed significantly lower ORs for smoking, averaging about

one-half of the risk as compared with non-smokers, strongly indicating that smoking has a negative association with PD. Furthermore, the risk of PD decreases up to an OR of 0.2 as the daily amount and/or duration of smoking increases (Hellenbrand et al., 1997; Checkoway et al., 2002). Similar results were reported by Benedetti et al. (2000). The risk of PD was reduced to 0.65 or 0.62 when the number of cigarettes smoked were 1-20 or more than 20 per day, respectively, and 0.63 or 0.59 if the years of smoking were 1-30 years or more than 30 years, respectively. The 'Twin study' revealed that one twin without PD smoked on average 9.7 more packs a year than his twin brother with PD (Tanner et al., 2002). The exact mechanism by which smoking prevents PD is not known. Several lines of evidence have led to possible mechanisms of the protective effects of cigarette smoking. For example, cigarettes reduce brain monoamine oxidase (MAO) A and B, which converts MPTP into the active metabolite MPP^+ in astrocytes in animals as well as humans (Fowler et al., 1996; Mendez-Alvarez et al., 1997). MAO-B activity especially, is believed to play an important role in PD; thus, MAO-B inhibitors, such as selegiline (Deprenyl®), are potential therapeutic drugs for PD (Ebadi et al., 2002). Nicotine has been reported to have an antioxidant effect, which inhibits MPP^+ uptake in dopaminergic neurons, and attenuates MPTP-induced neuronal degeneration (Carr et al., 1992; Maggio et al., 1998). The protective effect of nicotine may be due to activation of one of the nicotine receptor subtypes that might have neuroprotective influences on the nigrostriatal dopaminergic system. Nevertheless, more experimental evidence is needed to delineate the protective effect of nicotine. Emerging experimental results indicate that a certain adenosine receptor subtype in a select brain region might contribute to the protective effect observed from coffee and tea drinking. The association between PD and alcohol consumption is inconclusive because fewer study results are available in this area.

Case-control studies	Case/Control	Rural living	Farming	Well water drinking	pesticide/ herbicide
China (Tanner et al., 1989)	100/200	0.6	0.2	0.74	**2.4**
Hong Kong (Ho et al., 1989)	35/105	**2.1**	**5.2**	N/A	**3.6**
Kansas, U.S.A. (Koller et al., 1990)	150/150	**1.9**	1	**1.7**	1
New Jersey, U.S.A. (Golbe et al., 1988)	106/106	**2**	1	1	**7**
Chicago, U.S.A. (Tanner et al., 1990)	78/78	1	**3**	1	1
Detroit, U.S.A. (Gorell et al., 1998)	144/464	1.2	**3**	1.1	**3.21**
Taiwan (Liou et al., 1997)	120/240	**2.04**	**1.81**	1.1	**2.89**
Germany (Seidler et al., 1996)	380/376	N/A	N/A	N/A	**2.2**
Calgary, Canada (Semchuk et al., 1991)	130/260	0.9	1.2	0.9	N/A
Italy (Zorzon et al., 2002)	136/272	**1.9**	**5.2**	**2.3**	**2**
China (Chan et al., 1998)	215/313	1	0.98	1	**1.8**

Case-control studies	Case/Control	Smoking	Coffee	Alcohol	Tea
Italy (Zorzon et al., 2002)	136/272	0.8	N/A	N/A	N/A
Washington, U.S.A. (Checkoway et al., 2002)	210/347	**0.5**	0.8	0.8	**0.4**
Germany (Hellenbrand et al., 1997)	380/755	**0.5**	N/A	N/A	N/A
China (Chan et al., 1998)	215/313	**0.4**	N/A	N/A	1
Taiwan (Liou et al., 1997)	120/240	**0.4**	N/A	**0.6**	N/A

Table 3: Summary of Case Control Studies associating Environmental Factors and Parkinson's disease

It is extremely difficult to identify exact risk factors from the case control studies because data are strictly based on the memory of patients and family members. However, results suggest that environmental factors, especially pesticides and herbicides, may play an important role in the etiology of Parkinson's disease.

Since pesticides and herbicides were most strongly associated with Parkinson's disease, we selected the pesticide dieldrin, which was used as an insecticide until the mid 1970s, as a possible risk factor of Parkinson's disease. In addition, we have investigated manganese and the manganese-containing organic compound methylcyclopentadienyl manganese tricarbonyl (MMT) as other possible environmental risk factors of PD based on the similarities between PD and Manganism. Intensive investigation of dieldrin toxicity in dopaminergic neurons is necessary to determine its association with Parkinson's disease. In the following section, the chemical and

toxicological properties of dieldrin are reviewed.

Dieldrin

The formal chemical name of dieldrin is 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-endo,exo-1,4:5,8-di-methanonaphthalene (HEOD) (Figure 5). Dieldrin was first synthesized in 1946 in the laboratories of Julius Hyman & Company in Denver, U.S.A. (de Jong, 1991) by the Diels-Alder reaction; thus, dieldrin was named after this reaction (Hassell, 1990). Dieldrin production began two years after the first synthesis and was distributed commercially as an insecticide in 1950. Dieldrin was widely used as an insecticide around the world until the middle 1970s, mainly for the control of soil pests, such as termites, grasshoppers, locusts, beetles, and textile pests, and for the treatment of seeds. Dieldrin was a very effective soil insecticide, especially for perennial crops, such as sugar cane, palm, and banana. Dieldrin has also been used to control tsetse flies and other vectors of tropical diseases, including malaria, yellow fever, Chagas disease, Oraya fever, African sleeping sickness, river blindness, and filariasis (de Jong, 1991). Dieldrin had various industrial uses as well. For example, it was used to protect electricity and telephone cables and preserve timber (de Jong, 1991).

In 1974, The United States Environmental Protection Agency (US EPA) restricted the use of dieldrin due to its possible carcinogenic actions and its bioaccumulation. Henceforth, dieldrin was used only for termite control and other specific applications that did not give rise to residues in food or the environment. At the same time, production of dieldrin in the U.S. was ceased. Other developed countries followed the same action and strictly banned the use of dieldrin in agriculture. Finally, in 1987, the US EPA banned the use of dieldrin for almost all applications. Currently, some developing countries continue to use it as an insecticide (Suwalsky et al., 1997). Dieldrin is classified as one of the 12 most persistent bioaccumulative and toxic (PBT) chemicals by the US EPA (www.epa.gov/opptintr/pbt/cheminfo.htm), and as one

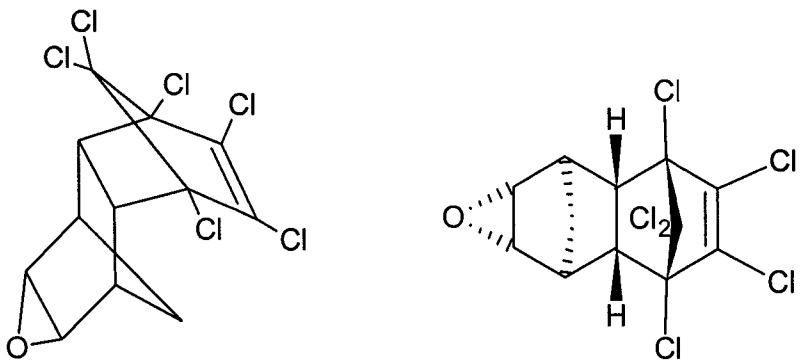


Figure 5: Structure of Dieldrin (Modified from Hassell, 1990)

of the top 20 human hazardous substances by the Agency for Toxic Substances and Drug Registry (ATSDR) (www.atsdr.cdc.gov/cxcx3.html). As described later in this chapter, dieldrin is highly persistent in the environment, and many of people, especially those who live in agricultural regions, risk exposure through food supplies.

Chemical Properties of Dieldrin

Dieldrin is classified as a chlorinated cyclodiene compound with the chemical formula $C_{12}H_{10}Cl_{16}O$ and a molecular weight of 380.92. As mentioned above, dieldrin can be synthesized through the Diels-Alder reaction between a diene and a dienophile to produce a cyclic diene. Aldrin is a chlorinated cyclodiene compound and resembles dieldrin structurally and chemically. Aldrin does not possess an epoxide ring, which differentiates it from dieldrin. Dieldrin's epoxide ring is unusually stable in the environment compared to the epoxide rings of other chemicals (Hassell, 1990). A typical chlorinated compound, dieldrin is highly lipophilic. Along with its lipophilicity, the vapor pressure of dieldrin (3.1×10^{-6} mmHg at 20°C) enhances its stability in the environment. Its low vapor pressure does not allow ready migration. As a result,

dieldrin persists in the same location for years as an environmental pollutant.

Fate of Dieldrin

Dieldrin is largely stored in adipose tissue due to its high lipophilicity (de Jong, 1991). Some dieldrin may cross the blood-brain barrier and remain in brain tissues. Dieldrin is metabolized through enzymatic oxidation or hydrolysis reactions. Three different metabolic processes are common among species (Figure 6) (Hassell, 1990). Mono-oxygenases transfer oxygen to the carbon in the unchlorinated bridge to form 9-hydroxydieldrin. Epoxide hydrolase hydrolyzes the epoxide ring to form aldrin 6,7-trans-dihydrodiol. The last and major metabolite, 2-keto-dieldrin, is formed by oxidation, dechlorination, and molecular rearrangement and is found in the urine of many species (Hassell, 1990). Aldrin is readily metabolized to dieldrin by the NADPH-dependent enzymes in hepatic microsomes by the same metabolic pathways as dieldrin (Hassell, 1990).

In the environment, some microorganisms degrade dieldrin by removing a chlorine atom from the methylene exo-bridge. However, this reaction is extremely slow, and consequently not an effective method of remediation of dieldrin in the environment. Typically, the half-life of dieldrin in the environment is up to 25 years (Meijer et al., 2001).

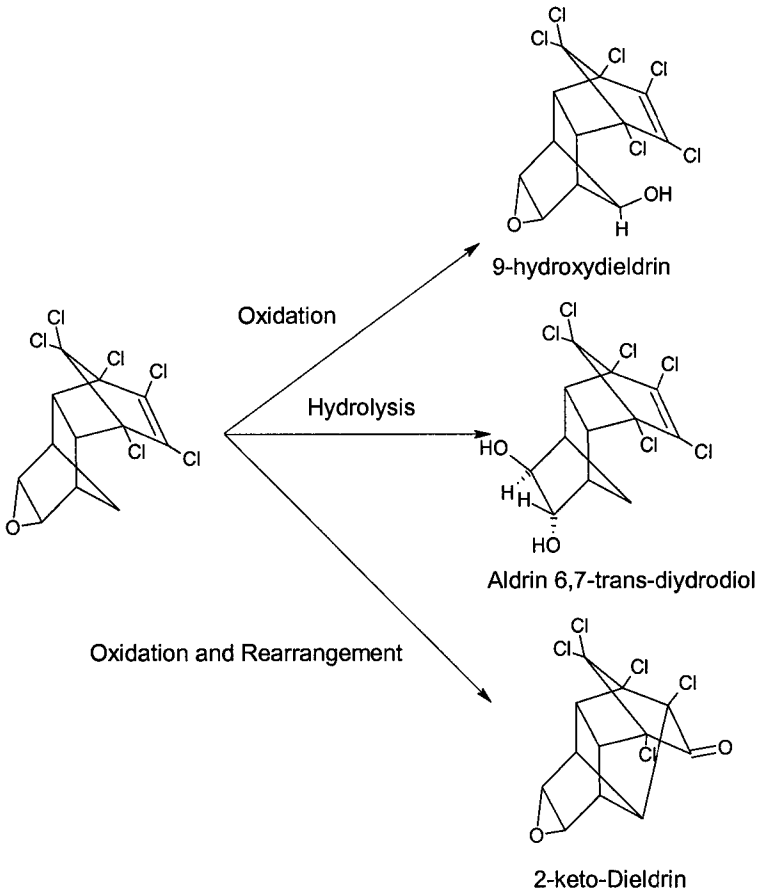


Figure 6: Metabolic Pathways of Dieldrin (Modified from Hassell, 1990)

Toxicity of Dieldrin

The general toxicity of dieldrin has been well documented (Ashwood-Smith, 1981; de Jong, 1991). The most commonly used index of toxicity is the LD_{50}, or lethal dose that kills 50% of the animals in a group. The oral LD_{50} of dieldrin in rats is 37-87 mg/kg body weight and 45-50 mg/kg body weight in rabbits (Treon and Cleveland, 1955; de Jong, 1991). Because of its high lipophilicity, dieldrin is rapidly absorbed through the skin. The dermal LD_{50} in rats is 56-90 mg/kg body weight (Bojanowska and Brzezicka-Bak, 1967; de Jong, 1991) and about 150 mg/kg body weight in rabbits (de Jong, 1991). The main target organ for acute dieldrin intoxication in animals is the liver. Diverse toxicological results have been reported from chronic animal studies, yet,

the main target organ is unquestionably the liver. However, the central nervous system is also affected by dieldrin (de Jong, 1991).

One of the reasons for EPA's ban of dieldrin's use as an insecticide was because of its possible carcinogenicity. The first report of carcinogenicity of dieldrin was in 1962 when Davis and Fitzhugh administered 10 ppm of dieldrin orally to mice for 2 years and observed cancer development in the liver (Davis and Fitzhugh, 1962). The dieldrin-treated mice developed more hepatomas (24%) than the control mice (7%) during the 2-year period. Additionally, other studies have shown that dieldrin causes a dose-dependent increase in the incidence of benign and malignant liver tumors only in mice (Epstein, 1975a, b). The National Cancer Institute (NCI) also tested tumorigenicity of dieldrin on rats by feeding 250 ppm for 104-105 weeks and found no sign of tumors; thus, it concluded that dieldrin-induced liver cancer was species specific, particularly seen only in mice but not in other species (NCI, 1978). Dieldrin has not demonstrated carcinogenicity in humans or other animals besides mice. Different metabolic pathways for dieldrin among species may account for the species-specific carcinogenicity of dieldrin. The liver is the main organ to detoxify foreign compounds following oral administration. Mice may metabolize dieldrin differently and produce carcinogenic intermediates or reactive intermediates that cause DNA adduct formation and mutations, which develop into hepatomas. However, this is a speculation, and scientific evidence demonstrating species-specific metabolic pathways and toxic metabolite production does not exist. This issue remains to be clarified.

Because of its possible carcinogenicity and to reveal the mechanism of tumorigenesis in mice, mutagenicity of dieldrin has also been investigated. Structurally, one epoxide ring in dieldrin enables its mutagenicity (Ashwood-Smith, 1981). In general, epoxide groups are highly reactive due to steric hindrance and electron deficiency. Epoxide groups readily bind to nucleophiles to form covalent bonds. Since DNA is negatively charged and considered nucleophilic, epoxide rings can bind to DNA and form DNA adducts, eventually leading to DNA mutations and development of

cancers. To determine if dieldrin is mutagenic, the commonly used Ames test has been conducted in different laboratories. Dieldrin was not mutagenic in bacterial or yeast test systems with or without S9 microsomal preparations (Hanna and Dyer, 1975). In other Salmonella test systems, such as TA1535, TA1536, TA1537, and TA1538, dieldrin (1 mg/plate) was not mutagenic with or without metabolic activation (Marshall et al., 1976). On the other hand, another report using TA98, TA100, and TA1535 showed positive Ames results following dieldrin (1 μg/ml) exposure (Majumdar et al., 1977). Thus, the mutagenicity of dieldrin is inconclusive from the Ames test.

Dieldrin genotoxicity has been further evaluated both *in vivo* and *in vitro*. Human embryonic lung (WI-38) cells were exposed to 1 μg/ml (2.6 μM) dieldrin for 24 hr, and chromosomal abnormalities, such as DNA breaks and fragmentation, were observed (Majumdar et al., 1976). Also, 24 hr after intraperitoneal dieldrin administration (1 mg/kg) to STS mice, two- to three-fold increases in chromosomal abnormalities were observed in bone marrow cells (Majumdar et al., 1976). In addition, a significant increase in DNA replication in hepatocytes was observed in 8-week-old B6C3F1 mice exposed to 20 mg/kg dieldrin by gavage for 48 hr (Miyagawa et al., 1995). These results strongly indicate that dieldrin may be a potent genotoxic compound. A recent finding suggests that dieldrin-induced DNA damage and adduct formation, such as 8-hydroxy-2'-deoxyguanosine (8-oxodGuo), may be mediated by oxidative stress because dieldrin increases the generation of reactive oxygen species (ROS) in PC12 cells (Stedeford et al., 2001).

Some human exposure data are available. The acute lethal dose in adults is approximately 1.5-5 g based on a clinical case (Steentoft, 1979) although 120 mg/kg dieldrin did not cause death in another clinical report (Black, 1974). Hunter and Robinson (1967) and Hunter et al. (1969) examined changes in blood concentrations of dieldrin over time (Hunter and Robinson, 1967; Hunter et al., 1969). In these experiments, human male volunteers ingested 0, 10, 50, or 211 μg of dieldrin daily for 18 months. The blood dieldrin concentrations were 5 and 15 ng/ml of blood following

the 50 and 211 μg doses, respectively. Hunter and his colleagues also calculated the half-life of dieldrin in human blood to be 396 days. Another group determined dieldrin's half-life as 266 days in human blood in occupationally exposed workers (Jager et al., 1970). Concentrations of dieldrin and other chlorinated hydrocarbons (CHCs) in the fat tissues of 262 German children (183 healthy, 46 with malignant tumors, and 33 with benign tumors) were analyzed, yet tumorigenesis and dieldrin body burdens were not correlated (Teufel et al., 1990). Mortality and liver tumors in workers occupationally exposed to dieldrin were not clearly associated either (Brown, 1992; de Jong et al., 1997), indicating that dieldrin acts via complex toxicological mechanisms in humans exposed to low doses.

The main target organ following both acute and chronic dieldrin exposure in humans is the central nervous system. Dieldrin did not induce any significant liver damage following occupational exposures (de Jong et al., 1997). The major symptoms of dieldrin poisoning are headache, nausea, vomiting, convulsion, and coma.

Mechanisms of Action

The exact mechanisms of action of dieldrin toxicity in the liver (animals) and the central nervous system (animals and human) are not known yet. The insecticide dieldrin as well as several other organochlorine pesticides inhibits the GABA(A) receptor in a manner similar to that of picrotoxin (Ikeda et al., 1998). The GABA(A) receptor channel consists of five subunits, and each subunit has four transmembrane domains (Olsen and Tobin, 1990). Upon inhibition of the GABA(A) receptor, dieldrin causes hyperexcitation and leads to a massive influx of Ca^{2+} via the glutamate receptor-channel. Consequently, cellular enzymatic activities are altered due to elevated intracellular Ca^{2+} and physiological functions are disrupted (Narahashi et al., 1998).

Other physiological function of dieldrin is not clear yet. Since dieldrin does not appear to damage nuclear DNA, it apparently acts on other organelles to destroy

cellular functions. One study showed that dieldrin inhibited mitochondrial oxidative phosphorylation *in vitro* in rat liver mitochondria (Bergen, 1971). Briefly, rat liver mitochondria were isolated from male rats, and mitochondrial activity was determined by measuring the consumption of oxygen in samples. Oxygen consumption was significantly reduced as soon as dieldrin was added to the isolated mitochondria, indicating that mitochondrial oxidative phosphorylation was directly inhibited by dieldrin. Further investigation showed that cytochrome c function was not inhibited by dieldrin, whereas electron flow in other respiratory proteins, such as NADH dehydrogenase, succinate dehydrogenase, and cytochrome b was blocked remarkably. Thus, dieldrin may induce inhibition in or near cytochrome b, which might be close to antimycin's site of inhibition. Preliminary results from the same laboratory indicate decreased mitochondrial respiratory function in liver mitochondria from rats chronically exposed intraperitoneally to dieldrin (Bergen, 1971).

The *in vivo* and *in vitro* results described above indicate that dieldrin impairs mitochondrial function irreversibly when it reaches mitochondria. Once mitochondrial oxidative phosphorylation is impaired, cells cannot produce enough energy (ATP) to maintain cellular integrity. The energy depletion may be especially critical in neurons in the central nervous system (CNS) due to the high dependency of the CNS on aerobic respiration. Along with depletion of energy production, other destructive cascades, such as calcium influx or caspase activation, may cause necrosis or apoptosis.

Dieldrin and Parkinson's disease

As mentioned earlier, some environmental factors may be responsible for the etiology of Parkinson's disease (PD) (Tanner and Langston, 1990; Semchuk et al., 1992; Seidler et al., 1996; Liou et al., 1997; Gorell et al., 1998). Pesticide and herbicide exposure appears to be associated with especially high risk of development of PD. Unfortunately, individual pesticides or herbicides cannot be identified as risk chemicals from these case-control studies. More specific investigation of each potential chemical

hazard is necessary to identify chemicals that increase or facilitate the risk of PD.

Dieldrin has been identified as one of the possible environmental risk factors of PD. Brain concentrations of various organochlorine pesticides were measured from human postmortem brain samples (Fleming et al., 1994). Concentrations of organochlorine compounds detected in brains from PD patients, Alzheimer's disease patients, and an age-matched control group were compared. Only dieldrin and DDT and its metabolites, pp-DDE and pp-DDT, were detected above the minimally detectable limits from brain samples. Dieldrin was detected in six of 20 brains from PD patients and in none of the 14 age-matched control brains. DDT and pp-DDE were found in most brains, and pp-DDT was found more in brains from Alzheimer's disease patients. The only significant correlation was between dieldrin and PD. A similar study conducted by Corrigan and his colleagues (Corrigan et al., 1998) demonstrated detectable levels of dieldrin in both control brains and brains from PD patients, but dieldrin levels in the caudate nucleus from PD patients were significantly higher than those in control brains. They also detected several PCB congeners as well as pp-DDE in most samples. All of the organochlorine compounds tended to be found in higher amounts in brains from PD patients.

The significant levels of dieldrin detected in human brains, even after the ban of dieldrin decades earlier, are attributed to its stability in organisms and the environment. A study conducted in Taiwan revealed that dieldrin was the most abundant of the cyclodiene compounds in selected river sediments (Doong et al., 2002). Also, high levels of its metabolites indicate that dieldrin persists in the environment for a long period of time. In Japan, pesticide residues were measured in selected domestic and imported agricultural products from 1995 to 1999 (Akiyama et al., 2002). Low levels of multiple pesticide residues were found in 32% of domestic products and 51% of imported products. However, dieldrin levels above the legal maximum limit were detected in cucumbers. In India, more than 1 μg dieldrin per 1 g of vegetables was detected (Kannan et al., 1997), indicating higher contamination in developing countries.

World Health Organization sets acceptable daily intake (ADI) of dieldrin as 100 ng/kg body weight and oral reference dose (RfD) as 50 ng/kg-day. Recent studies indicate that dairy products, meat, and seafood are also believed to be primary sources of human exposure to dieldrin, and the daily intake level of dieldrin through these contaminated food sources was estimated at 0.059 μg per average person in Taiwan (Doong et al., 1999) and 1.0-1.3 μg in Poland (Falandysz, 1999), indicating the risk of human exposure to dieldrin is still high. Dietary exposure to dieldrin in the U.S. among over 100,000 adults has been estimated to be 0.5 μg/day (MacIntosh et al., 1996). Because of dieldrin's half-life in humans (approximately 300 days) and its highly lipophilic nature, dieldrin accumulates in the body and persists for a long time. Interestingly, a recent Centers for Disease Control (CDC) investigation revealed that farmers and spouses in Iowa have significantly higher serum dieldrin levels, and dietary dieldrin consumption may reach as high as 0.4-0.5 μg/kg-day, which is 8- to 11-times higher than RfD as well as ADI (Brock et al., 1998). In Table 4, soil, water and food contaminations of dieldrin is summarized (Romero et al., 2000; Amaraneni and Pillala, 2001; Campoy et al., 2001; Jabber et al., 2001; Meijer et al., 2001; Rao and Pillala, 2001; Akiyama et al., 2002; Aktumsek et al., 2002; Doong et al., 2002; Hung and Thiemann, 2002; Schmitt, 2002).

Thus, accumulation of organochlorine compounds, including dieldrin, may cause or facilitate PD. Since dieldrin was the only organochlorine compound significantly correlated with PD, it may act on dopaminergic neurons in the SNc and destroy them selectively. However, a cause-effect relationship between dieldrin and the death of brain dopaminergic neurons has not been clearly substantiated. Experiments with animals or tissue culture are necessary to uncover a cause-effect relationship or possible mechanism.

Doong 2002	river sediment 0.12-5.8 ng/g (highest sigmaHCH 0.57-14.1 ng/g) in Da-han and Erh-jen rivers (Taiwan)
Doong 1999	daily intake 0.059 μg per adult (Taiwan)
Akiyama 2002	0.03 μg/g in cucumber (maximum residue limit MRL 0.02 μg/g) (Japan)
Hung 2002	river water mean 2.19-4.3 ng/L (minor level) (highest DDT 43.7-56.1 ng/L) from Red river and Duong river (Vietnam)
Amaraneni 2001	maximum concentration 1.98 μg/g fish (highest DDT 157.4 μg/g) in India
Campoy 2001	human breast milk dieldrin mean 0.6-5.6 ng/ml (max DDE 30.3-36.8 ng/ml) in Spain
Rao 2001	sediment from Kolleru Lake dieldrin up to 128 μg/kg dry weight (DDT 191 μg/kg) (India)
Meijer 2001	Soil in Broadbalk 9.7 ng/g dw (1986), soil in Luddington 2.9 ng/g dw (1990) highest among other detected pesticides
Romero 2000	human breast milk dieldrin is the 3rd most abundant, mean 0.018 μg/g milk fat (range 0-0.355 mg/g) DDE 2.805 mg/g, DDT 0.129 mg/g
MacIntosh 1996	daily intake 0.5 μg per adult (U.S.A.)
Falandysz 1999	daily intake 1.0-1.3 μg per adult (Poland)
Brock 1998	daily intake 50 ng/kg among Iowa farmers (U.S.A.)
Kannan 1997	more than 1 μg/g dieldrin was detected from several Indian vegetables (India)

Table 4: Level of Dieldrin in Soil, Water, and Food

Several *in vivo* and *in vitro* studies have been conducted to understand the relationship between dieldrin toxicity and PD (Bergen, 1971; Sharma et al., 1976; Wagner and Greene, 1978; Heinz et al., 1980; Sanchez-Ramos et al., 1998). Chronic exposure of cultured mesencephalic cells to dieldrin showed that dieldrin is selectively toxic to dopaminergic neurons compared to other neurons, such as GABAergic neurons (Sanchez-Ramos et al., 1998). Dopaminergic neurons are somehow more sensitive to dieldrin-induced toxicity, but unfortunately, a mechanism of the selective toxicity was not proposed. This observation supports the hypothesis that dieldrin is a potential risk factor that causes or facilitates PD. In addition, a correlation between dieldrin and selective toxicity to dopaminergic neurons has also been reported in *in vivo* models. Ring doves administered low doses of dieldrin in the daily diet for 8 weeks had significantly depleted brain levels of both dopamine and norepinephrine by 58.6% and 38.0%, respectively (Heinz et al., 1980). Dopamine depletion was more severe than norepinephrine depletion. The same effect was observed in rats and ducks exposed to dieldrin chronically (Sharma et al., 1976; Wagner and Greene, 1978). Thus, dieldrin

has a high potential to deplete brain dopamine levels, and may be selectively toxic to brain dopaminergic neurons. This assumption is further supported by the findings of Miller et al. (1999). They measured the expression and activity of the dopamine transporter (DAT) and the vesicular monoamine transporter (VMAT2) in pre-synaptic terminals of dopaminergic neurons in the striatum. Exposure of C57BL mice to an organochlorine pesticide (up to 12 mg/kg) for 2 weeks significantly increased the expression of both the DAT and VMAT2 in the striatum (Miller et al., 1999a). In addition, the organochlorine pesticide inhibited VMAT2 function in an *in vitro* model. These results suggest that levels of free dopamine are not protected by vesicles and can be enhanced in pre-synaptic terminals to possibly enhance the risk of oxidative stress. Another study has shown that DAT binding (27-64%) was increased in the striatum of Sprague-Dawley rats exposed to dieldrin (3 mg/kg-day) (Purkerson-Parker et al., 2001), indicating that dopamine reuptake was facilitated by dieldrin. Together, the above studies indicate that dieldrin alters neurochemical function in the striatum, an area involved in PD. However, data regarding the neurochemical mechanisms underlying the dopaminergic toxicity of dieldrin have not been reported.

As mentioned earlier, dieldrin is a chlorinated cyclodiene compound and belongs to the class of polychlorinated biphenyls (PCBs). To date, 209 congeners have been named in the PCB family. PCBs were manufactured between the 1930s and the 1970s and have been used extensively in industries related to the following: dielectric and heat transfer fluids; hydraulic and lubrication oils; condensers, transformers and capacitors in electrical systems; plasticisers in paints and sealants; putties, waterproof wall coverings and printing inks based on their inflammability and stability. The toxicity of PCBs has been broadly studied and may be involved in human carcinogenicity, endocrine disruption, reproductive toxicity, neurotoxicity, and immune suppression (Safe, 1994; Faroon et al., 2001; Tharappel et al., 2002). Even low levels of PCB exposure can induce neurotoxic responses, including subtle changes in behavior and cognition; the cellular and molecular basis of PCB-induced neurotoxicity remains

unclear at this point. The involvement of Ca^{2+} signaling in PCB-induced neurotoxicity during the development and maturation of neurons has been reported (Tilson and Kodavanti, 1998; Tilson et al., 1998; Inglefield et al., 2001; Jessen-Eller et al., 2002; Kodavanti and Derr-Yellin, 2002). PCB (Arochlor 1254) caused in neocortical cells increased intracellular IP_3 signaling due to phospholipid hydrolysis, resulting in increased intracellular Ca^{2+} through the ER and plasma membrane associated Ca^{2+} channels (store-operated channels or SOC) (Inglefield et al., 2001). However, PCB-induced increased intracellular Ca^{2+} levels do not appear to activate caspase-dependent apoptosis, but these cells undergo necrosis (Inglefield et al., 2001). In non-neuronal cell lines, PCB induces apoptotic cell death via a Ca^{2+} and/or caspase-dependent process (Lee et al., 2001; Jeon et al., 2002; Shin et al., 2002b). Mechanistically, Arochlor 1254 has been reported to produce significant levels of ROS in cerebellar granule cells (Mariussen et al., 2002) and rat synaptosomes (Voie and Fonnum, 2000). Interestingly, Arochlor 1254 inhibits mitochondrial transition pore opening and cytochrome c release (Salvi and Toninello, 2001), which partially explains the PCB-induced caspase-dependent apoptosis (Jeon et al., 2002). Furthermore, activation of protein kinase C (PKC) attenuates PCB-induced apoptotic cell death in HL-60 cells (Shin et al., 2002a). However, the effect of this PCB mixture on the dopaminergic system is not known. To our knowledge, dieldrin is most closely correlated with other PCBs with regard to dopaminergic toxicity.

Manganese

Manganese (Mn) was first discovered in 1771, and its name is derived from the Greek word for magic. It is essential in the function of certain enzymes and membrane transport systems in all mammalian tissues. Mn is present in over 100 common salts and mineral complexes distributed in rocks, soils, lakes, and oceans, which comprise about 0.1% of the Earth's crust. Mn has 11 oxidation states, ranging from 3- to 7+ (Aschner et al., 1999), and the manganous (Mn^{2+}) and manganic (Mn^{4+}) oxidation states

are the most common in the natural environment. However, Mn exists as one of three oxidation states (2+, 3+, and 7+) in mammalian tissues. Mn forms tight complexes with other ligands, thus, the level of free plasma Mn is low. Mn is an abundant metal, essential in blood clotting, energy metabolism, and immune system function in mammals. Mn is a constituent of metalloproteins, including superoxide dismutase, pyruvate carboxylase, and glutamine synthetase. Deficiencies in dietary Mn cause a failure to thrive, congenital abnormalities, impaired reproductive function, ataxia, and defects in lipid and carbohydrate metabolism (Hurley, 1981). In addition, seven human volunteers who ate a Mn-deficient diet (0.11 mg/day) for 39 days experienced the development of dermatitis, hypocholesterolaemia, and elevation of serum calcium and phosphorus (Friedman et al., 1987). On the other hand, excess Mn intake also leads to psychiatric disturbance and movement disorders, described later in this chapter.

In adult humans, average total body Mn levels are approximately 10-20 mg (200-400 μmol) (Andersen et al., 1999), and the required daily dietary intakes for Mn are 0.3-1.0 mg/day in infants, 1.0-3.0 mg/day in children, and 2.0-5.0 mg/day in adults (Keen and Zidenberg-Cherr, 1990). Mn is absorbed in the small intestine by high-affinity, low-capacity active transport (Garcia-Aranda et al., 1983). Infants absorb nearly 99% Mn, but the absorption rate decreases with age, and only 3-5% of total Mn is absorbed in adults (Zlotkin and Buchanan, 1986; Davidsson et al., 1988). Mn is transported to various tissues by carrier proteins, such as transferrin, α2-msvtohlobulins, and albumins (Aschner and Aschner, 1991) and is distributed to various tissues and organs. High concentrations have been found in bone (3.3 ppm), liver (1.68 ppm), kidney (0.93 ppm), pancreas (1.21 ppm), hair (0.80 ppm), brain (0.34 ppm), and gonads (0.09 ppm) (Underwood, 1977). Especially high concentrations up to 25% of the total Mn body content have been found in bone. Mn is excreted through the liver in bile and feces.

Historically, Mn was used for manufacturing glass during the Egyptian and Roman Empires. Today, Mn is one of the most widely used metals in the world, and

industrial uses of Mn include the manufacture of dry cell batteries and various alloys and steels, which contain Mn metal up to 14%. It is also produced as a high-purity salt with many chemical uses, some of which include textile dyeing, oxidation catalysis, paint and varnish drying, paint pigmentation, fertilizer production, food packaging, nutritional additives, pharmaceutical preparation, explosives, and fungicides. The production of organic Mn, including Maneb (manganese ethylenebisdithiocarbamate) as a fungicide and MMT (Methylcyclopentadienyl Manganese Tricarbonyl) as an anti-knock agent in gasoline (Sax and Lewis, 1987; Pal et al., 1999), is also important.

Manganese Exposure and Toxicity

Essential dietary Mn levels are derived from food. As shown in Table 5, the highest Mn concentrations are found in nuts and grains, which contain up to 46 μg of Mn per gram (Pennington et al., 1986). Environmental and accidental exposures to Mn are mainly by ingestion or inhalation, and the most commonly reported Mn exposure is through industrial or mining occupational exposure. Atmospheric Mn concentrations have been measured in various locations. Zayed et al. (1999) collected samples from gas stations, downtown areas, and near an express way in Montreal, Canada, to determine whether the use of an organic Mn anti-knocking agent (MMT, discussed below) increased atmospheric Mn concentrations (Zayed et al., 1999). Their results indicate that the concentrations of atmospheric Mn particles in these locations were 0.103-0.141 $\mu g/m^3$, whereas the US EPA atmospheric reference concentration (R_fC) for Mn is 0.05 $\mu g/m^3$ (Davis et al., 1998), indicating that the Mn concentrations were two- to three-fold higher than the regulatory limit. In the United States, the mean atmospheric Mn concentration in urban areas was reported to be approximately 0.033 $\mu g/m^3$ in 1982 (Lynam et al., 1994), which was lower than that in Canada. Industrial exposures to inorganic Mn occur from Mn mining and during the industrial uses of Mn, as mentioned above. The ambient levels of Mn in a factory in Taiwan were approximately 20,000 $\mu g/m^3$ due to a malfunctioning ventilation control system, and

these levels caused toxicity (Huang et al., 1989). The ambient levels of Mn dust in the vicinity of drilling in a mine might contain as much as 450,000 $\mu g/m^3$ (Rodier, 1955). The smoke from welding might contain more than 25,000 $\mu g\ Mn/m^3$ (Wang et al., 1989). Upon inhalation, Mn is absorbed into blood from lungs and binds to cationic carrier proteins, such as transferrin (Tf), albumin and divalent metal transporter-1 (DMT-1), and then is transported to various tissues including brain (Egyed and Wood, 1996; Davis et al., 1998; Aposhian et al., 1999). Free Mn^{2+}, Tf bound Mn^{3+}, and DMT-1 bound Mn^{2+} readily cross the blood-brain barrier (BBB) and accumulate in certain regions of the brain. High Mn concentrations have been observed in the caudate-putamen, globus pallidus, substantia nigra, and subthalamic nuclei (Scheuhammer and Cherian, 1981; Newland et al., 1989; Olanow et al., 1996). A highly selective accumulation of Mn in the basal ganglia suggests that Mn might be one of the possible causes of selective degeneration of dopaminergic neurons in the substantia nigra, which may result in the development of PD. Furthermore, individuals with chronic liver failure are at risk of Mn accumulation in the brain due to the incomplete excretion of Mn from the body through the biliaric tract. Post-mortem study reveals that high levels of Mn accumulate in the basal ganglia in patients with liver cirrhosis (Krieger et al., 1995).

Exposure to Mn appears to be associated with depletion of striatal dopamine levels due to degeneration of dopamine nerve endings and massive cell loss in the internal segment of the globus pallidus where inhibitory GABAergic neurons are present (Bernheimer et al., 1973; Brouillet et al., 1993). On the other hand, Pal et al. (1999) showed that Mn primarily affects GABAergic neurons in the globus pallidus, and does not cause significant damage to dopaminergic neurons in the substantia nigra (Pal et al., 1999). Furthermore, Shinotoh et al. (1995) described the morphological changes in monkeys exposed to Mn in the globus pallidus (Shinotoh et al., 1995) as Mn-induced Parkinson-like syndrome, pathologically distinct from idiopathic PD. However, a recent clinical case questions the effect of Mn in the globus pallidus. Two patients with a long history of occupational Mn exposure presented with Mn-induced Parkinsonism.

Manganese level in food	ppm (ug/g or mg/L)
Nuts and nut products	18-47
Grains and grain products	0.4-41
Legumes	2.2-6.7
Fruits	0.2-10.4
Fruit juices and drinks	0.05-11.5
Vegetables and vegetable products	0.4-6.6
Desserts	0.04-8
Infant foods	0.2-4.8
Meats, poultry, fish and eggs	0.1-4
Mixed dishes	0.7-3
Condiments, fats, and sweetners	0.04-1.5
Beverages	0-2.1
Soups	0.2-0.7
Milk and milk products	0.02-0.5

Table 5: Manganese Contamination in Selected Food

[$^{(123)}$I]-(1r)-2β-carboxymethoxy-3β-(4-iodophenyl) tropane (β-CIT) single-photon emission computed tomography revealed that significantly low dopamine transporter levels in the striatum, one of the pathological hallmarks of idiopathic PD, were measured in these patients (Kim et al., 2002). This finding suggests a direct effect of Mn in dopaminergic neurons in the SNc, and that Mn toxicity in the globus pallidus may not be the primary causal factor of Mn-induced PD-like syndrome.

Many different researchers have studied the molecular mechanisms of Mn toxicity. The biological half-life of Mn in tissues is 36 to 41 days (Gavin et al., 1990, 1992; Goyer, 1996; Inoue and Makita, 1996). Mn accumulates in mitochondria and inhibits mitochondrial complex I activity (Galvani et al., 1995). In addition, Mn inhibits aconitase and succinate dehydrogenase (Complex II), two important enzymes in oxidative phosphorylation in both *in vivo* and *in vitro* models (Zheng et al., 1998; Malecki, 2001). As a result, the mitochondrial electron transport system (ETS) is impaired, ATP production is decreased, and lactate formation is increased (Brouillet et al., 1993; Hirata et al., 2001). Also, due to the impairment of mitochondrial function,

the unconsumed cellular oxygen level increases, and some is converted to reactive oxygen species (ROS). Oxidative stress appears to play an important role in Mn toxicity (Donaldson et al., 1982; Graham, 1984; Archibald and Tyree, 1987; Liccione and Maines, 1988; Desole et al., 1996; Desole et al., 1997). Increased intracellular ROS levels not only cause imbalance of cellular antioxidant defense mechanisms, such as glutathione (GSH), superoxide dismutase (SOD), and catalase, but also activate the oxidative stress-mediated cell death processes (Medina et al., 1996). Mn exposure in dopaminergic PC12 cells or SN4741 cells induced the early activation of mitogen activated protein (MAP) kinase activity, several caspase activities, and resulting DNA fragmentation (Hirata et al., 1998; Chun et al., 2001b; Hirata, 2002). Also, Mn mobilizes caspase-12 in the endoplasmic reticulum and further facilitates caspase-dependent cell death in the dopaminergic cell line SN4741 or in NIH3T3 cells (Chun et al., 2001a; Oubrahim et al., 2001). On the other hand, Mn-induced apoptotic cell death in dopaminergic PC12 cells was not inhibited by caspase inhibitors, indicating that other cell death pathways may also be important in cell death following Mn exposure (Roth et al., 2000).

Another proposed mechanism of toxicity is secondary excitotoxicity that follows bioenergetic defects in which ROS and Ca^{2+} mediate neuronal damage (Beal et al., 1993). Studies demonstrate that decortication or treatment with MK-801, an NMDA antagonist, prior to Mn administration protects against pathological changes (Brouillet et al., 1993). In many respects, the mechanisms of Mn toxicity are similar to those of other known mitochondrial toxins, including aminooxyacetic acid, malonate, 3-nitro-propionic acid, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and paraquat (Dawson et al., 1995; Cassarino et al., 1999). In addition, mitochondrial defects have recently been found in patients with PD, implicating mitochondrial toxins as possible environmental stressors important in the development of PD.

Manganese exposure and PD

Excessive exposure to Mn generally results from inhalation of high levels of Mn in mine workers and may cause pulmonary or neurological dysfunction. Exposure to high levels of Mn is known to cause Manganism, with neurological symptoms similar to those caused by Parkinson's disease (PD) (Inoue and Makita, 1996). The first reported neurological disturbances associated with exposure to Mn were described by Couper in 1837 in France (Couper, 1837). Five workers in a Mn ore crushing plant developed neurological symptoms, such as muscle weakness, tremor, postural instability, and mumbling. Since this first report, Mn-associated neurological disorders have been observed all over the world including Chile, Cuba, India, Japan, and the U.S. (Schuler et al., 1957; Cotzias, 1958; Balani et al., 1967; Mena et al., 1967; Greenhouse, 1971; Cook et al., 1974; Huang et al., 1989; Montgomery, 1995; Gorell et al., 1999; Mergler et al., 1999). Due to its neurotoxicity, Mn appears to be transported into the brain easily via simple diffusion of Mn^{2+} and transferrin-bound Mn^{3+} (Aschner and Aschner, 1990; Aschner and Gannon, 1994). High levels of Mn are found in the pallidum, thalamic nuclei, and substantia nigra, where high transferring receptors are also found. Animal studies using monkeys have confirmed that low dose exposure to Mn over 1-2 years develops signs of motor dysfunctions and selective damages and significant accumulation (60-80% over control) in globus pallidus and substantia nigra (Bird et al., 1984; Newland and Weiss, 1992). Clinical reports have also shown significantly high levels of Mn in the substantia nigra and cerebellum in PD patients as compared to age-matched control groups (Yase, 1972). Mn levels in cerebellum range from 0.12 to 0.53 μg/g tissue wet weight (Sumino et al., 1975). Mn also selectively targets pathways intrinsic to the basal ganglia, and high Fe^{3+} and/or catecholamine levels potentiate the neurotoxicity of Mn^{2+} (Sloot and Gramsbergen, 1994). Mn is also closely associated with neuromelanin *in vivo*, a phenomenon that could decrease the pigment's scavenging of free radicals (Lyden et al., 1984). However, the exact mechanisms by which Mn is selectively accumulated and neurotoxic in these regions

remain to be uncovered.

The clinical syndrome of Mn related neurotoxicity may be broadly divided into three stages: (i) neurobehavioral changes or the initial phase, (ii) parkinsonian features or the intermediate phase, and (iii) dystonia with severe gait disturbances or the established phase (Pal et al., 1999). The clinical symptoms of the first stage of neurological damage from Mn exposure include fatigue, anorexia, headache, poor memory, reduced concentration, apathy, insomnia, loss of appetite, diminished libido, hallucinations, and psychosis (Huang et al., 1993; Pal et al., 1999). These symptoms are categorized as non-specific symptoms, and the appearance and severity of these symptoms vary among individuals. A generalized slowing of movements during the initial stage is also observed in some cases (Mena et al., 1967). A direct link between psychiatric symptoms and motor dysfunction, called "locura Manganica (manganese madness)", observed in Manganism has not been established, and not every patient shows both types of symptoms during the initial phase. The intermediate phase usually follows the initial phase by 1 to 2 months. Extrapyramidal signs emerge, including monotone speech, expressionless face, and impairment of writing, dexterity, movement, posture, and gait. Speech disturbances include slurring and stuttering, writing disturbances include micrographia, and facial expression becomes blank or mask-like, interrupted by periods of spasmodic laughing or dystonic grimace. Movements are generally slow, clumsy, and uncertain and walking backwards becomes difficult (severe retropulsion), as does turning "en bloc" (Huang et al., 1989). In the established phase of neurological Manganism, patients experience a dystonic posturing of the foot known as "cock-walk", where the patient walks with small steps, elevating the heels and rotating them outward. During this phase, tremors are less frequently observed (Mena et al., 1967; Huang et al., 1989). The disorder becomes progressive when it reaches the established phase, and patients do not recover even if the high exposure levels of Mn are removed.

Chronic Mn exposure has not been verified as a risk factor for idiopathic PD.

In case-control studies, Mn exposure and development of PD were not significantly associated (Semchuk et al., 1992; Seidler et al., 1996). On the other hand, Gorell et al. (1997, 1999) reported that more than 20 years of exposure to Mn were positively associated with an increased incidence of PD (Gorell et al., 1997, 1999). According to these reports, exposure to Mn for more than 20 years significantly increases the risk of PD (odds ratio, OR = 10.61). Furthermore, combinations of other metals, such as lead-copper (OR = 5.24), lead-iron (OR = 2.83) or iron-copper (OR = 3.69), may also be associated with PD. Pathologically, Mn primarily causes toxicity in the globus pallidus rather than the substantia nigra, where dopaminergic neurodegeneration occurs in idiopathic PD (Yamada et al., 1986; Pal et al., 1999). However, recent clinical results reveal a direct toxic effect of Mn on dopaminergic neurons and indicate that Mn-induced PD and idiopathic PD are not different pathologically (Kim et al., 2002). Still, pathological differences have been characterized between PD and Manganism, and the role of Mn in the pathogenesis of these diseases has yet to be elucidated.

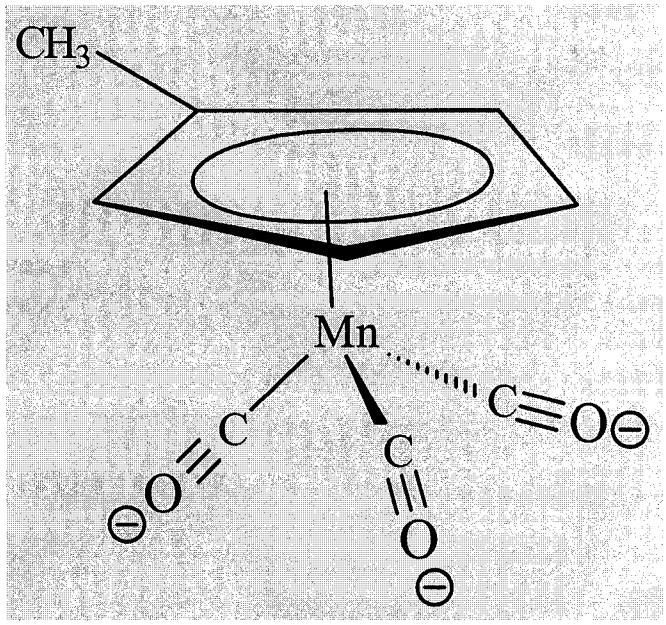
At the cellular level, Mn inhibits tyrosine hydroxylase (TH) activity in dopaminergic cells (Hirata et al., 2001) and accumulates preferentially in neuromelanin-rich cells including pigmented neurons in the substantia nigra (Lyden et al., 1984). Chemically, Mn possesses strong oxidation-reduction properties. Intracellularly, Mn is in dynamic equilibrium between divalent (Mn^{2+}) and trivalent (Mn^{3+}) states, and Mn^{3+} is a strong oxidizing agent. Dopamine, one of the most unstable neurotransmitters in nature, readily gives up an electron under strong oxidizing conditions and forms toxic free radical quinone compounds (Donaldson et al., 1982). These findings imply that Mn exposure can cause dopaminergic degeneration and decrease dopamine production, resulting in decreased dopamine levels in the striatum, and therefore may induce extrapyramidal signs and symptoms resembling idiopathic PD. Mn also depletes dopamine in the striatum when administered to monkeys and rodents (Neff et al., 1969; Brouillet et al., 1993; Sloot and Gramsbergen, 1994). In addition, chronic Mn exposure in rats revealed significant accumulation of Mn in the globus

pallidus but not in the caudate putamen. However, neuronal cells in both the globus pallidus and caudate putamen were significantly destroyed (Salehi et al., 2001). These results further support the correlation between Mn neurotoxicity and the risk of PD.

MMT

Methylcyclopentadienyl manganese tricarbonyl (MMT), first synthesized by Ethyl Corporation in the 1950s, has been used as an anti-knocking gasoline adduct in Canada (Lynam et al., 1999). This tetrahedral compound has an aromatic ring structure and three carbonyls attached to a Mn atom (Figure 7). The methylcyclopentadiene ring has a net charge of -1 due to its delocalized electrons, and it bonds to Mn through the interaction of its π orbital cloud with the d orbitals of Mn. The remaining d orbitals of Mn interact with the three carbonyl groups, all of which are considered neutral ligands. The yellow, volatile, water-soluble liquid has a molecular weight of 218.1. MMT is produced from reaction of $MnCl_2$, cyclopentadiene, and carbon monoxide in the presence of manganese carbonyl (Sax and Lewis, 1987). MMT is thermally stable but photochemically unstable (Hysell et al., 1974), degrading within 15 sec in sunlight to produce a mixture of manganese oxides (Ter Haar et al., 1975). However, significant amounts of MMT have been found in rain water and storm runoff collected along highways (Yang and Chau, 1999), even though it is photochemically unstable. The purity of commercial MMT, called AK-33X or HITEC 3000, ranges from 24.4% to 25.2%. Gasoline contains up to 72 mg of MMT per liter; a recent measurement of 25.8 mg/L MMT was reported in MMT-added gasoline (Zayed et al., 1999). The chemicals emitted from MMT-containing gasoline are manganese tetraoxide, manganese sulfate, and manganese phosphate (Ressler et al., 2000).

A. Chemical structure



B. Combustion of MMT

$$\text{MMT} \xrightarrow[\text{combustion}]{} \text{MnSO}_4 + \text{Mn}_2\text{O}_3 + \text{MnPO}_4$$

Figure 7: Chemical Structure of MMT

MMT is now approved for use in Argentina, Australia, Bulgaria, the U.S., France, Russia, and conditionally in New Zealand. Nevertheless, these countries are using MMT non-intensively while waiting for further evidence indicating no adverse human health effects (Zayed, 2001). MMT was used in gasoline in the U.S. from the early 1970s until passage of the amendments to the Clean Air Act in 1977 (Lynam et al., 1999). Though MMT is a legal gasoline additive in the U.S. now, public health concerns still exist (Frumkin and Solomon, 1997; Davis, 1998). Toxicity data generated by Canadian research groups regarding ambient Mn levels and adverse health effects from MMT exposure have not been useful in U.S. Environmental Protection Agency (EPA) risk assessment of MMT, due to the lack of proper controls in the Canadian studies (Davis, 1998). The USEPA tried to ban the use of MMT as a gasoline additive, but failed in 1996 after a lawsuit by Ethyl Corporation claimed unfair

denial of their request for a waiver of the ban (Davis, 1998). The judgment was awarded based on a legal technicality associated with the North American Free Trade Agreement (NAFTA).

The environmental fate and contamination of MMT and effects of chronic human exposure have not been investigated. In some urban areas, combustion of gasoline containing MMT contributes nearly 8% of the total Mn levels in the air (Loranger and Zayed, 1995). In areas of the U.S., the level of Mn from vehicle emissions varies. For example, automobiles contribute an average of 13 ng Mn/m^3 of atmospheric Mn levels in southern California, whereas automobiles contribute only 3 ng Mn/m^3 in central and northern California (Davis et al., 1988). The absorption, distribution, metabolism, and excretion of MMT in animal models have been evaluated. The biodistribution of MMT differs greatly from that of its inorganic counterparts (Gianutsos et al., 1985; Komura and Sakamoto, 1992, 1994). Cerebellum Mn concentrations were significantly higher in mice treated with MMT (0.5 g/kg for 12 months) than in mice treated with $MnCl_2$ (2.0 g/kg for 12 months) (Komura and Sakamoto, 1994). The differences in bioavailability and biodistribution between MMT and inorganic Mn may be partially explained by the results of Zheng et al. (Zheng et al., 2000). They assessed the toxicokinetics of MMT and inorganic Mn in rats and revealed that the clearance rate of Mn derived from MMT in plasma was much slower (37-fold) than the clearance rate of inorganic Mn, indicating that MMT may have prolonged effects on organisms. MMT is mainly biotransformed in liver and is excreted in the urine as two major metabolites, $(CO)_3MnC_5H_4CO_2H$ and $(CO)_3MnC_5H_4CH_2OH$, which account for 67% and 14%, respectively, of the MMT administered (Hanzlik et al., 1980). The urinary excretion of Mn derived from MMT was only 5% of the daily amount of MMT administered (Komura and Sakamoto, 1992). Organic forms of Mn were apparently absorbed much easier and faster than inorganic forms of Mn. Mn is metabolized mainly by cytochrome P-450 enzymes. Only a small fraction of MMT was found in feces, indicating that MMT was efficiently absorbed systemically. The bioavailability of

MMT in the CNS has not been evaluated. However, due to its high lipophilicity, MMT may accumulate in the brain. MMT induces neurological effects, including agitation and convulsion, as well as pulmonary damage (Penney et al., 1985). Fishman et al. (1987) observed seizure activity following MMT administration to mice, an effect not seen following $MnCl_2$ administration (Fishman et al., 1987). Furthermore, MMT inhibited the binding of t-[^{3}H]t-butylbicycloorthobenzoate (a ligand for the GABA-A-receptor) in mouse brain membranes with an IC_{50} value of 22.8 μM, indicating that MMT-induced seizure activity may result from GABA-A receptor inhibition.

MMT clearly affects the CNS and causes neurological syndromes, but the particular brain regions affected are not known. MMT has been investigated to determine if it is correlated with the development of PD. Chronic MMT treatment (5 months) in rats did not cause significant degeneration of dopaminergic neurons in the substantia nigra although Mn levels were elevated significantly (Yong et al., 1986). Tyrosine hydroxylase activity was not altered either during the treatment period. On the other hand, administration of MMT to mice for 3 weeks decreased dopamine concentrations in the striatum and olfactory tubercles (Gianutsos and Murray, 1982). The GABA content in the striatum was also elevated after either treatment while the cerebellar GABA content did not change. GABA levels in the substantia nigra of MMT-treated mice were also increased, indicating that MMT affects both dopaminergic neurons and GABAergic neurons in the basal ganglia. This is additional evidence that MMT may target nigrostriatal neurons. The acute toxicity of organic Mn is apparently due to its effect on neurotransmitter function, which results in the subsequent manifestation of neurological signs. However, systematic chronic exposure studies in animal models evaluating long-term health effects of MMT have not yet been conducted.

MMT toxicity using *in vitro* systems has not been well established either. MMT inhibits mitochondrial phosphorylation at site I in isolated hepatocytes (Autissier et al.,

1977). MMT affects Na/K-ATPase activity in sciatic nerves and decreases levels of the Na/K-ATPase catalytic alpha1 subunit (Liu et al., 2000), which may partially explain the neurodegenerative process of MMT. However, more extensive research is necessary to understand the biochemical mechanisms of MMT neurotoxicity and the relevance to the etiopathogenesis of PD.

Mechanisms of apoptosis and neurodegeneration

Although the causes of neuronal death in neurodegenerative disorders are still enigmatic, several mechanisms are currently under discussion. These mechanisms include programmed cell death (apoptosis), passive cell death (necrosis), and autophagy (Anglade et al., 1997b). Apoptosis has been evaluated extensively in the quest to understand cellular and molecular mechanisms underlying the pathophysiological mechanisms of many neurodegenerative disorders including PD, Alzheimer's disease, Huntington's disease, amylotrophic lateral sclerosis, and ischemia as well as other diseases such as cancer and autoimmunity (Burke, 1998; Hartmann and Hirsch, 2001; Roth, 2001; Sathasivam et al., 2001). One of the reasons why apoptosis may be more favorable than the other cell death mechanisms is because these neurodegenerative disorders result from specific patterns of cell loss, which is not as easily explained by necrosis or autophagy.

Apoptosis is morphologically and biochemically well characterized as a cell death mechanism. Unlike necrosis, the cellular structural integrity is preserved, the plasma membrane remains intact, and the mitochondrial and nuclear membranes are well preserved until the late stage of apoptosis. Membrane blebbing is often observed, and genomic DNA is most commonly fragmented into approximately 180-200 base-pairs. Also, no inflammatory response is observed throughout the process. Neurons undergoing apoptotic death are stimulated or initiated in several different ways via caspase-dependent and caspase-independent pathways. The caspase-dependent pathway has been investigated intensively, and activation of this pathway is classified

into the death receptor-mediated and the mitochondria-mediated initiation processes. These processes are described in detail in the following paragraphs.

The family of cysteine-directed proteases closely associated with apoptotic cell death is called caspase (**c**ysteinyl **asp**artate-specific prote**ase**). To date, 14 isoforms have been identified (Cohen, 1997; Nicholson and Thornberry, 1997) (Figure 8). The physiological functions of caspases differ somewhat but are broadly categorized into inflammatory responses and apoptotic signaling, as depicted in Figure 8A. All caspases exist in cells as zymogens (pro-caspases), and are activated when pro-caspases are proteolytically cleaved into one large subunit (~20 kDa) and one small subunit (~10 kDa) and form heterotetramers with two identical large and two identical small subunits (Figure 8B). Each active caspase contains two catalytically active sites that are permanently active until degradation (Cohen, 1997). Each caspase recognizes a specific sequence of tetrapeptides (Table 6). Caspases proteolytically cleave aspartate residues at the carboxylic end of tetrapeptide sequences.

Class	Name	Regulatory unit	Adapter molecule	Optimal tetrapeptide
Activators	caspase-2	CARD	RAIDD	DXXD
	caspase-8	DED	FADD	(I/V/L)EXD
	caspase-9	CARD	Apaf-1	(I/V/L)EHD
	caspase-10	DED	FADD	(I/V/L)EXD
Executioners	caspase-3			DEXD
	caspase-6			(I/V/L)EXD
	caspase-7			DEXD
Cytokine processors	caspse-1	CARD	CARDIAK	(W/Y/F)EHD
	caspase-4	CARD		(W/Y/F)EHD
	caspase-5			(W/Y/F)EHD
	caspase-11			
	caspase-12			
	caspase-13			
	caspase-14			
CARD: Caspase recruitment domain				
DED: Death effector domain				

Table 6: Classification of Caspases (Modified from: www.neuro.wustl.edu/neuromuscular/mother/apoptosis.htm)

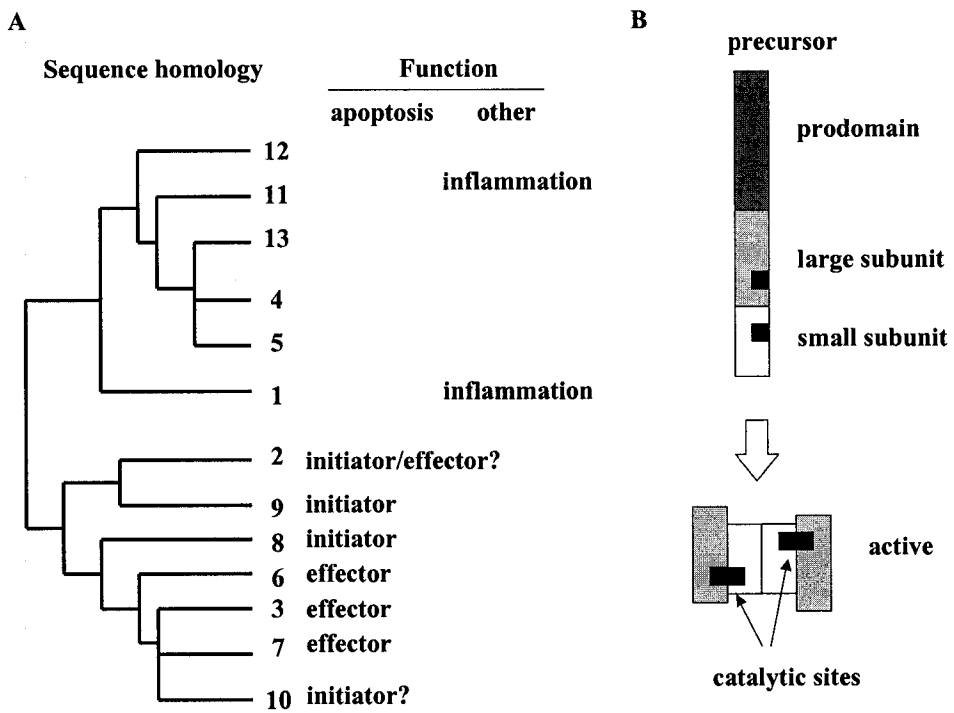


Figure 8: Classification and Activation Caspases (Modified from Thornberry and Lazebnik, 1998)

Apoptosis-related caspases are subtyped based on their activation processes and roles as initiator caspases and effector caspases (Wolf and Green, 1999). Initiator caspases, including caspase-2, caspase-8, caspase-9, and caspase-10, possess specific regulatory sequences or adapter domains, namely the caspase recruitment domain (CARD) or the death effector domain (DED). Effector caspases, including caspase-3, caspase-6, and caspase-7, are activated by initiator caspases (Thornberry and Lazebnik, 1998). Unlike the initiator caspases, effector caspases do not possess known regulatory sequences, and their pro-domains are relatively short. The role of the caspase cascades in cells has been investigated intensively. Two distinct caspase-dependent apoptotic pathways have been identified as the death receptor-mediated apoptotic pathway and the chemical-induced mitochondrial-mediated apoptotic pathway (Figure 9). In death

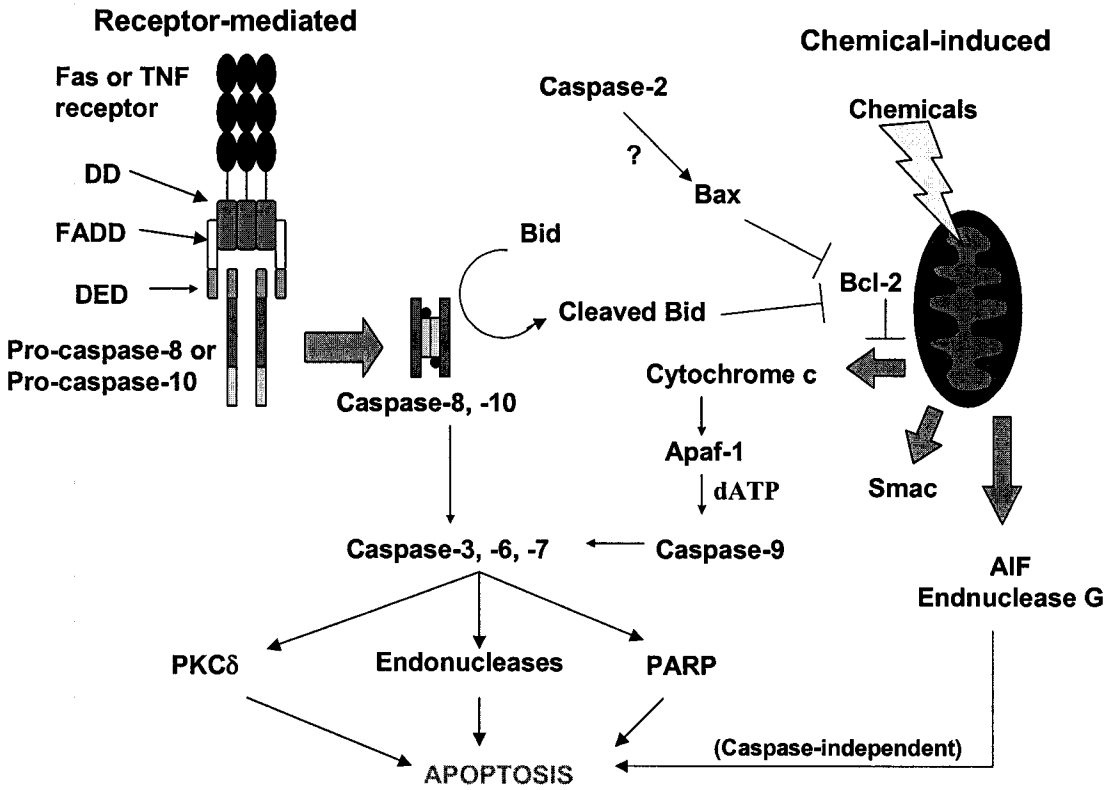


Figure 9: Apoptotic Pathways

receptor-mediated apoptosis, tumor necrosis factor-α (TNFα) or the Fas (APO-1/CD95) receptors are associated with caspase-8 or caspase-10 by DED adaptor domains and undergo conformational changes when ligands bind to the receptors, resulting in activation of initiator caspase-8 or -10. Effector caspases (caspase-3, -6, and -7) are then activated, which further initiates apoptosis by cleaving vital cellular target proteins.

In the chemical-induced apoptotic pathway, mitochondria play central roles in regulating apoptosis (Desagher and Martinou, 2000). When apoptotic stimuli are introduced to cells, mitochondrial functions, particularly respiratory chains, are inhibited. As a result, the mitochondrial permeability transition (MPT) pores located on the inner membrane of mitochondria are opened to reduce the proton gradient, generate reduced forms of electron transport proteins which process NAD^+ and FAD^+ by uncoupling, and

increase oxygen consumption (Crompton, 1999). MPT pore opening, however, results in depolarization of the mitochondrial transmembrane potential ($\Delta\Psi m$), swelling of the matrix, and generation of highly toxic oxygen radicals or reactive oxygen species (ROS). Consequently, several mitochondrial factors are released into cytosol. Mitochondria contain several pro-apoptotic molecules including cytochrome c, Smac/DIABLO, Htr2A (Omi), apoptosis-inducing factor (AIF), and endonuclease G (Desagher and Martinou, 2000; Shi, 2001; Wang, 2001). Some caspases are also localized in mitochondria (Zhivotovsky et al., 1999). AIF and endonuclease G execute apoptosis independently from caspases (Wang, 2001), while cytochrome c and Smac/DIABLO serve as initiation signals of the apoptotic cascade. When Apaf-1 (**a**poptotic **p**rotease **a**ctivating **f**actor-**1**) and cytochrome c form a complex called the apoptosome in the presence of dATP or ATP in the cytosol, caspase-9 is recruited and activated through the CARD adaptor domain (Pan et al., 1998). Caspase-9, like caspase-8, further activates effector caspases and induces apoptosis. The other mitochondrial molecule Smac/DIABLO inhibits the inhibitor of apoptosis protein (IAP) and thereby promotes apoptosis (Srinivasula et al., 2001). Recently, the important role of caspase-2 in mitochondrial-mediated apoptosis was demonstrated (Lassus et al., 2002). The apoptotic function of caspase-2 has been understood for some time, as it was the first mammalian apoptotic caspase to be identified (Kumar et al., 1994; Wang et al., 1994). Caspase-2 acts upstream of the mitochondria in chemical- or UV-induced apoptosis in several different cell lines, such as IMR90 (human fibroblasts), A549 (lung adenocarcinoma), and U2OS (osteosarcoma), and regulates the release of cytochrome c and Smac as well as the translocation of pro-apoptotic Bax into mitochondria by altering mitochondrial membrane permeabilization by unknown mechanisms (Lassus et al., 2002). This finding may change the view of mitochondria as a central regulator in apoptosis. Additionally, caspase-2 may be localized in golgi complexes and cleave golgin-160 (Mancini et al., 2000). Thus, the golgi complex may be another key organelle for the initiation of apoptotic cell death.

Recently, cross-talk between the receptor-mediated and mitochondrial-mediated pathways in apoptosis has been reported. Caspase-8 cleaves one of the pro-apoptotic Bcl-2 family proteins, Bid, upon activation (Li et al., 1998; Gross et al., 1999). Cleaved Bid translocates into the mitochondrial membrane to inhibit Bcl-2 and/or Bcl-X_L anti-apoptotic function, resulting in depolarization of $\Delta\Psi m$ and release of pro-apoptotic molecules including cytochrome c from mitochondria. This cross-talk may work as an amplification mechanism of apoptotic cell death (Gross et al., 1999; Tang et al., 2000).

ROS, on the other hand, reacts with cellular macromolecules, lipids, and nucleotides to disturb normal cellular functions. ROS has also been reported to activate certain stress-activated protein kinases (SAPK) and/or mitogen activated protein kinases (MAPK) to induce the apoptotic process (Kang et al., 1998; Luo et al., 1998; Crenesse et al., 2000). Some of the MAPK are activated in response to oxidative stress (Frasch et al., 1998; Luo et al., 1998; Bhat and Zhang, 1999; Assefa et al., 2000) and activate many redox-sensitive transcription factors including NF-kB and AP-1, which may play critical roles in the execution of apoptosis (Grilli and Memo, 1999; Li and Sun, 1999). Thus, disruption of the oxidation balance in biological systems results in activation of apoptotic cell death.

Several pro- and anti-apoptotic proteins regulate the activation of the caspase cascade. Mitochondria are one of the most important organelles involved in caspase activation and apoptosis. Mitochondrial products rate-limit the activation of caspases and endonucleases, the major executors of apoptosis, in cell-free systems (Zamzami et al., 1995; Ellerby et al., 1997; Kluck et al., 1997; Yang et al., 1997). In addition, downstream caspase activation and other execution processes are significantly blocked when mitochondrial membranes are stabilized (Zamzami et al., 1995; Marchetti et al., 1996). These results suggest that mitochondria or mitochondrial proteins are important in the regulation of apoptosis. Among them, Bcl-2 family proteins play major roles in the initiation phase of apoptosis (Tsujimoto, 1998). Bcl-2 family proteins are abundant

particularly in the outer mitochondrial membrane and are divided into two subgroups based on their pro-apoptotic and anti-apoptotic roles. Pro-apoptotic Bcl-2 proteins include Bad, Bax, Bak, Bid, and Hrk, and anti-apoptotic Bcl-2 proteins include Bcl-2 and Bcl-X_L (Figure 10). Anti-apoptotic Bcl-2 proteins share three or four regions in their structure, depicted as BH1-4 in Figure 10. Also, most of the anti-apoptotic Bcl-2 proteins possess a membrane-spanning domain at the C-terminus, enabling their localization to nuclear membranes, the endoplasmic reticulum (ER), or mitochondrial membranes. The exact mechanisms of these proteins are still under investigation. An important function of Bcl-2 and Bcl-X_L is inhibition of the mitochondrial permeability transition (MPT) pores. In some cases, MPT pore formation plays a significant role in the initiation of apoptosis as the megachannel, multiprotein complex formed at the contact site between the mitochondrial inner and outer membranes that regulate the mitochondrial Ca^{2+} level, pH, transmembrane potential, and mitochondrial volume (Kroemer, 1999). Cellular depolarization of $\Delta\Psi m$ and release of mitochondria associated apoptotic signaling molecules, including Apaf-1, Smac/DIABLO, or cytochrome c result (Shi, 2001). Also MPT pore opening disturbs Ca^{2+} homeostasis by increasing cytosolic Ca^{2+} levels (Macho et al., 1997). Bcl-2 and Bcl-X_L interact with MPT pores to inhibit pore activity (Kroemer, 1999). MPT pores have also been implicated in CNS injuries including excitotoxic neuronal death (Albin and Greenamyre, 1992; Beal et al., 1993; Ellerby et al., 1997; Nicotera et al., 1997). Pharmacologically, MPT pores can be blocked by cellular treatment with cyclosporine A (CsA, 1 μM) or bongkrekic acid (50-100 μM) and rescued from apoptosis (Cao et al., 2001).

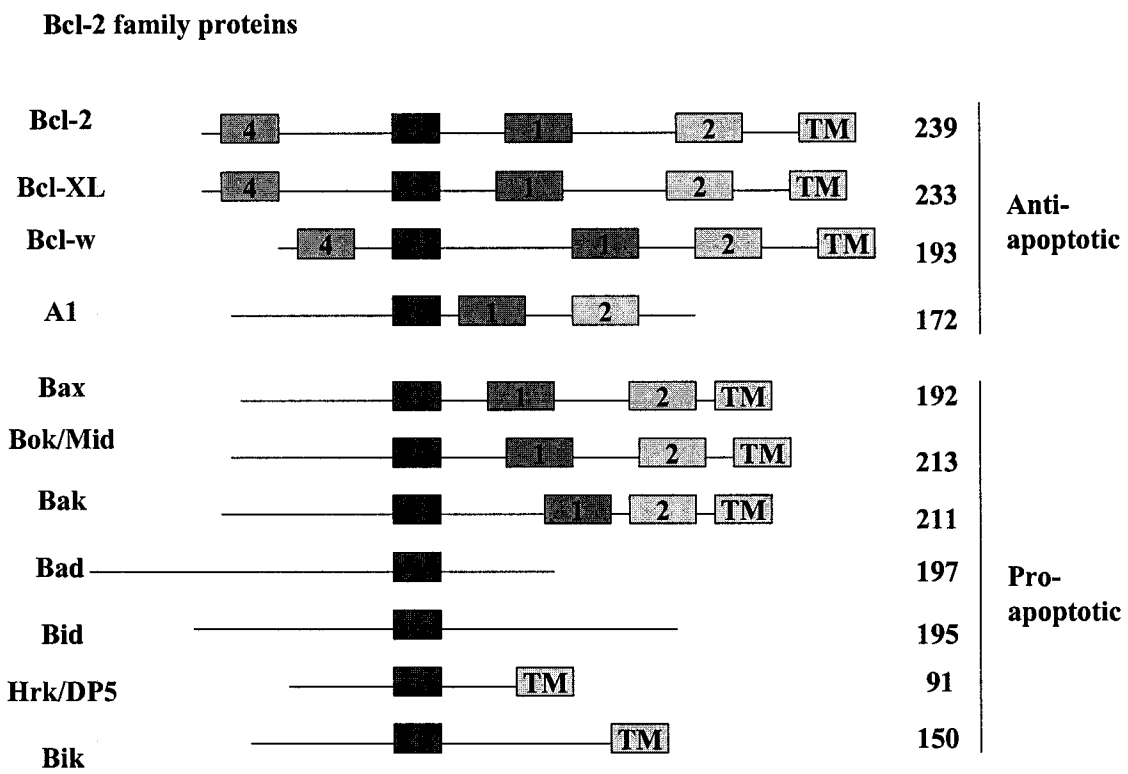


Figure 10: Sequence Homology of Bcl-2 Family Proteins (Modified from Strasser et al., 2000)

However, in some cases, these pharmacological inhibitors do not inhibit depolarization of $\Delta\Psi m$ and apoptosis. CsA-insensitive pores have been found in mitochondria, and pro-apoptotic Bcl-2 proteins may be responsible for the formation of these pores during certain apoptotic processes (Shi, 2001). When pro-apoptotic Bcl-2 proteins, such as Bad and Bax, are abundant in mitochondrial membranes, they form homodiamers or heterodiamers and create small pores (Tsujimoto, 1998; Desagher and Martinou, 2000; Shi, 2001). This pore formation initiates apoptotic signals, similar to the effects caused by MPT pores. Bcl-2 and Bcl-X_L are believed to bind with Bax and Bad to form heterodiamers and prevent mitochondrial depolarization and release of pro-apoptotic signaling molecules (Chiang et al., 2001).

The Bcl-3 homologous (BH3) proteins, such as Bad, Bid, Bim, Bax, Hrk, and

Bik, are the major pro-apoptotic Bcl 2 proteins and initiate cytotoxic stimuli-induced apoptosis. These proteins initiate apoptosis by inhibiting anti-apoptotic Bcl-2 proteins (eg. Bcl2 Bcl-xl)anti-apoptotic functions in mitochondria, (Bouillet and Strasser, 2002). The BH3 domain in these proteins binds to the BH3 domain in anti-apoptotic Bcl-2 proteins to neutralize the anti-apoptotic functions. In neuronal cells, activation of the BH3 protein Bim regulates cytochrome c release and caspase-dependent apoptosis (Putcha et al., 2001), indicating that Bim activation is upstream of mitochondrial effects.

Recently, protein kinases and phosphatases have been suggested to regulate the apoptotic cascade (Berra et al., 1997; Murray and Fields, 1997; Cardone et al., 1998; Whelan and Parker, 1998; Reyland et al., 1999; Reyland et al., 2000; Chiang et al., 2001). Caspase-3 is involved in proteolytic cleavage and activation of certain isoforms of protein kinase C (PKC) and phosphatases (Santoro et al., 1998; Reyland et al., 1999). As mentioned above, Bcl-2 proteins may be primary targets (Kornblau et al., 2000; Chiang et al., 2001). Upon phosphorylation, pro-apoptotic Bcl-2 proteins dissociate from the mitochondrial membrane and bind to chaperone proteins, such as the 14-3-3 protein in cytosol. As a result, upstream apoptotic signaling is suppressed.

Protein kinase Cδ and apoptosis

Protein kinase Cδ (PKCδ), discovered in 1986 by Gschwendt et al., is a novel type of PKC that translocates to the membrane following phorbol ester treatment (Gschwendt et al., 1986). In the following year, PKCδ cDNA was cloned from a rat brain cDNA library, which localized on chromosome 19 (Ono et al., 1987; Kurkinen et al., 2000), enabling the localization of the PKCδ gene on human chromosome 3 and mouse chromosome 14 (Huppi et al., 1994). PKCδ is an isoform in the AGC kinase family. Currently, there are 11 PKC isoforms further classified into three distinct sub-family groups based on their activation patterns (Figure 11). These sub-family categories are conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC). Conventional PKCs include PKCα, βI, βII, and γ, and they are dependent on

intracellular calcium concentrations and activated by diacylglycerol (DAG) or phorbol ester. Novel PKCs include PKCδ, ε, η, θ, and μ, and they are also activated by DAG or phorbol ester but are calcium independent. The last group of atypical PKCs include PKCζ and λ(ι), which are calcium-independent and are not activated by DAG or phorbol ester. The means of activation of enzymes in the different subfamilies are primarily due to differences in the molecular structures of the kinases. All PKC proteins consist of the regulatory domain (N-terminus) and the catalytic domain (C-terminus) (Figure 11). Both cPKC and nPKC contain cysteine-rich sequences that interact with phospholipids and phorbol ester activators (Ono et al., 1989), whereas aPKC enzymes lack these sequences. Furthermore, only cPKC enzymes possess the calcium-binding region (C2) in the regulatory domain and are thus calcium-dependent (Ono et al., 1988). The amino acid sequence homology has been determined to be 82% (βI), 85% (βII), 75% (γ), 58% (δ), 60% (ε), and 51% (ζ) compared to the PKCα isoform (Ono et al., 1988).

PKCδ, one of the nPKC family protein, contains a carboxy-terminal catalytic domain with two conserved regions, an ATP binding region (C3) and a catalytically active/substrate binding region (C4), and an amino-terminal regulatory domain with an inhibitory pseudo-substrate sequence and two cysteine-rich zinc-finger-like sequences (Cys1 and Cys2) in the C1 region (Gschwendt, 1999). Functional studies have revealed that the Cys2 region may play a critical role in the translocation of cytosolic PKCδ into cellular membranes following activation by phorbol esters (Szallasi et al., 1996; Hunn and Quest, 1997). Five of six cysteine residues and two histidine residues interact with Zn^{2+} to form a specific coordination and attract phorbol ester binding (Kazanietz et al., 1995; Zhang et al., 1995).

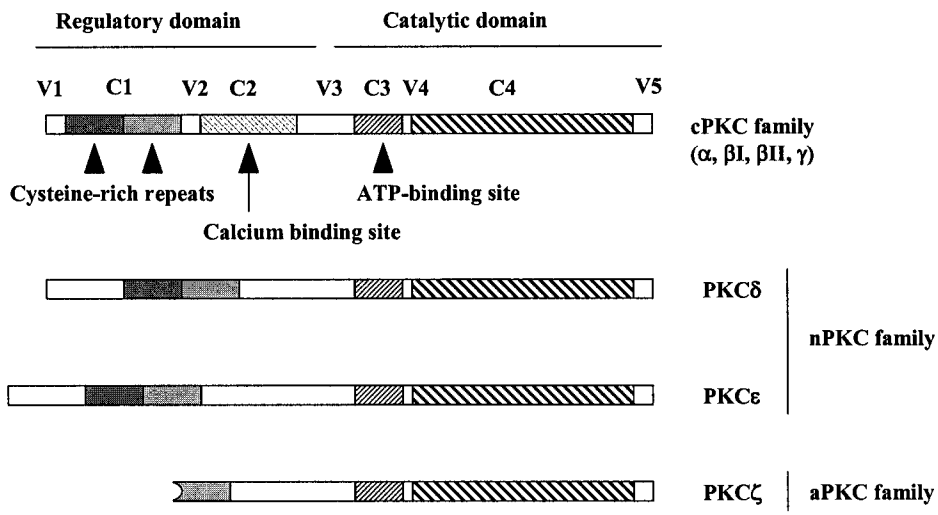


Figure 11: Structural Homology in PKC Family Proteins (Modified from Ono et al., 1988)

Activation of PKCδ requires several signals and changes in its structure. Figure 12 schematically depicts the brief mechanism of PKCδ phosphorylation and activation. Conventionally, PKCδ is activated by phorbol ester (TPA) stimulation and/or generation of phospholipid (PL) or diacylglycerol (DAG) in cellular membranes. Upon binding of the cysteine-rich domain (C1) to TPA, PL or DAG, the catalytic domain is uncovered, allowing substrates to bind to the site. Phosphorylation of S643 appears to be required prior to kinase activation. Mutation of S643A causes more than a 70% decrease in kinase activity (Li et al., 1997). Further investigation revealed that S643 is autophosphorylated, thus enhancing catalytic activity, when PKCδ is in a low activity form (Li et al., 1997; Le Good et al., 1998). However, Stempka et al. (1999) reported that S643A did not reduce kinase activity (Stempka et al., 1999), indicating more studies

are required to draw an accurate conclusion regarding the role of S643A. In addition, several other serine or threonine phosphorylation sites that may be required for activation have been identified in PKCδ. Phosphorylation of T505 in the activation loop and S662 in the hydrophobic C-terminus appears to be important for PKCδ activation because unphosphorylated T505 and S662 sites in PKCδ resulted in less than 1/10 of the normal kinase activity (Le Good et al., 1998). A threonine residue (T505) in the activation loop of PKCδ may be phosphorylated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Hodgkinson and Sale, 2002). These studies suggest that other sites may play a more important role in the activation of PKCδ. Tyrosine phosphorylation and subsequent activation of PKCδ will be discussed later in this section. Another amino acid in the catalytic domain, E500, contributes to the negative charge in the activation loop, which is critical for kinase activity (Stempka et al., 1999). Kinase activity in E500V PKCδ was reduced by about 75% in a mutation study (Stempka et al., 1999). However, the detailed mechanisms are still unclear at this point.

	N-terminal						Position						C-terminal
	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5
PKC isozymes													
PKCα	R	R	R	R	R	K	G	S	F	R	R	K	A
PKCβI	F	K	L	K	R	K	G	S	F	K	K	F	A
PKCβII	Y	K	L	K	R	K	G	S	F	K	K	K	A
PKCγ	R	R	R	R	R	K	G	S	F	K	R	K	A
PKCδ	A	R	R	K	R	K	G	S	F	F	Y	G	G
PKCε	Y	Y	X	K	R	K	M	S	F	F	E	F	F
PKCη	A	R	R	R	R	R	R	S	F	R	R	X	R
PKCζ	R	R	F	K	R	Q	G	S	F	F	Y	F	F

Table 7: Optimal Amino Acid Motifs for Selected PKC Isozymes (Modified from Nishikawa et al., 1997)

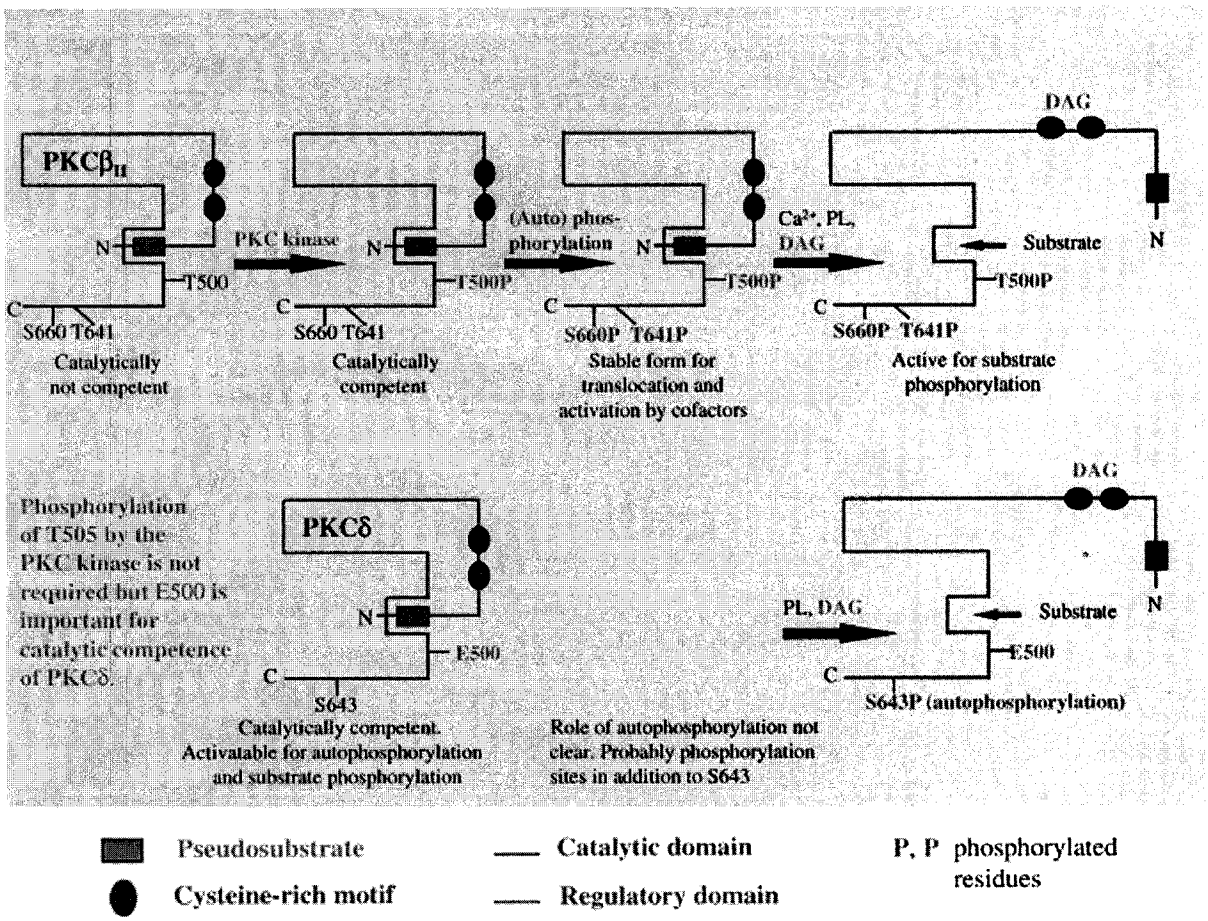


Figure 12: Activation Mechanism of PKCδ (Modified from Gschwendt, 1999)

Upon activation, PKCδ phosphorylates serine/threonine residues in specific substrates. The specific amino acid substrate sequences phosphorylated by PKCδ are not well characterized. Based on a study with synthetic peptides, a general consensus phosphorylation site motif for PKC has been defined as RXX(S/T)XRX, where X could be any amino acid (Pearson and Kemp, 1991). Recently, Nishikawa and his colleagues examined the specific amino acid sequence motifs for major PKC isozymes and identified the common and different substrate amino acid sequences among PKC isozymes (Nishikawa et al., 1997). In particular, PKCδ requires a hydrophobic amino acid at position +1 in the carboxyl-terminal of the phosphorylation site (Ser), basic amino acids at positions -6, -5, -4, and -2, and glycine at position -1. All the PKC

isozymes evaluated require that substrates have arginine at position -3, which reconciles with the consensus sequence motif. Their data also indicate that the optimal amino acid sequence for PKCδ phosphorylation is A(A/R)**R**(K/A)**RKG**<u>**S**</u>**F**F(Y/F)G**G**, where the underlined serine (S) is the phosphorylation site, and the bold letters are particularly important for sequence recognition by PKCδ. Amino acid sequence motifs for other PKC isozymes are shown in Table 7, and some PKC isozymes share common motifs, indicating that PKC phosphorylation sites may be conserved among the isozymes (Nishikawa et al., 1997).

PKCδ is ubiquitously expressed in most tissues and cell types (Leibersperger et al., 1991; Wetsel et al., 1992). The expression of PKCδ in different murine tissues has been examined and extremely high levels of PKCδ were found in epidermis, placenta, uterus, brain, lung, and kidney (Leibersperger et al., 1991). PKCδ as well as PKCα, β, ε, and ζ are present in the CNS at birth, and the expression of PKCδ increases in the brain but decreases in the lung, kidney, and heart as age progresses (Goldberg and Steinberg, 1996). This age-dependent change in specific tissues may affect the responsiveness of these tissues to certain stimuli. Within the CNS, PKCδ is particularly abundant in the cerebellum (Merchenthaler et al., 1993). PKCδ is rapidly induced in certain brain regions following birth or global brain ischemia (Chen and Hillman, 1994; Koponen et al., 2000) (Table 8). The exact mechanism by which PKCδ is rapidly induced following ischemic insult is not known, yet the expression of PKCδ is regulated by several transcription factors. The PKCδ gene encodes the binding sites of AP-1, NF-kB, and nerve growth factor induced-C (NGFI-C), and these transcription factors activate PKCδ (Kurkinen et al., 2000; Kikkawa et al., 2002). AP-1 and NF-kB may be involved in apoptosis (Li and Sun, 1999; Aggarwal, 2000; Garg and Aggarwal, 2002), and NGFI-C is responsible for neurite growth and differentiation (Wernersson et al., 1998). As discussed later in this section, regulation of the PKCδ gene by these transcription factors further indicates that PKCδ can mediate apoptosis, delay cell death as well as control cell differentiation (O'Driscoll et al., 1995; Miettinen et al., 1996;

Sawai et al., 1997; Chen et al., 1999; Li et al., 1999; Koponen et al., 2000; Cerda et al., 2001) (also refer to Table 8).

The physiological roles of PKCδ appear to be diverse, with critical functions in cell differentiation and proliferation as well as in regulation of apoptotic cell death (Table 8). Selective activation of PKCδ facilitates nerve growth factor-induced neurite outgrowth and differentiation in PC12 cells (O'Driscoll et al., 1995) as well as differentiation and growth arrest in human tumor CaCo-2 cells (Cerda et al., 2001). In addition, PKCδ expression is important during pre- and post-natal developmental phases (Chen and Hillman, 1994; Goldberg and Steinberg, 1996), supporting a regulatory role of PKCδ in cell proliferation and differentiation. Endocrine secretion is also regulated by PKCδ. Neurotensin is secreted from pancreatic cells upon translocation of PKCδ into the membrane (Li et al., 2002), indicating that the kinase activity may be an initial signal for the secretion mechanisms of certain endocrines. PKCδ activity is important during apoptosis induced following treatment with hydrogen peroxide, UV-B radiation, etoposide, TNFα, and anti-Fas antibody (Emoto et al., 1995; Ghayur et al., 1996; Konishi et al., 1997; Reyland et al., 1999; Fukunaga et al., 2001; Konishi et al., 2001).

Three distinct PKCδ activation mechanisms have been proposed: translocation of native PKCδ into the cellular membrane; proteolytic cleavage of native PKCδ into the catalytic and regulatory subunits by caspase-3; and tyrosine phosphorylation of PKCδ (Figure 13A). As mentioned above, the most conventional activation mechanism is translocation of PKCδ into membranes upon PL, DAG, or TPA stimulation. Recently, PKCδ was found to translocate into the mitochondrial membrane following TPA treatment and trigger cytochrome c release, an initial apoptotic signaling process (Li et al., 1999; Majumder et al., 2000), suggesting that PKCδ may act as an early pro-apoptotic signal. PKCδ proteolytic cleavage and subsequent activation were first reported by Emoto et al. (1995). Follow-up studies revealed that activation of caspase-3 by exogenous chemicals such as etoposide or TNFα or by radiation insult generates the active PKCδ fragment (Table 8). Genomic results suggest the possible

cleavage site as DIPD at position 324-327 (Figure 13B), yet it has not been confirmed. Tyrosine 52, 155, 187, 311, 332, 512, 523 and 565 are known to be phosphorylated and may play a role in modulating kinase activity (Szallasi et al., 1995; Li et al., 1996; Konishi et al., 1997; Kikkawa et al., 2002). Tyrosine phosphorylation at positions 311, 332 and 512 following hydrogen peroxide or UV-B treatment induces activation of PKCδ in HaCaT or COS-7 cells (Konishi et al., 1997; Fukunaga et al., 2001; Konishi et al., 2001). Tyrosine phosphorylation at these positions may induce conformational changes and open the catalytic domain (Kikkawa et al., 2002), as two major tyrosine phosphorylation sites (Y311 and Y332) are in the hinge region of PKCδ. Upon phosphorylation of these residues, PKCδ may undergo conformational change and expose the catalytic domain, yet further studies are necessary to confirm this hypothesis. Tyrosine phosphorylation is also an important modulator of PKCδ activity in general. Several different tyrosine kinases are involved in phosphorylation of PKCδ (Li et al., 1994; Szallasi et al., 1995; Denning et al., 1996; Joseloff et al., 2002), and some of the phosphorylation sites may be isoform specific. Regulation of kinase activity by tyrosine phosphorylation is particularly important for PKCδ as PKCδ is tyrosine-phosphorylated most efficiently among the PKC family. Tyrosine phosphorylation negatively modulates PKCδ activity in epidermal growth factor (EGF)-treated epidermal cells or following phosphorylation by the Src family enzymes (Denning et al., 1996; Joseloff et al., 2002). On the other hand, tyrosine phosphorylation positively modulates PKCδ activity when cells are treated with phorbol ester, NGF, or substance P (Li et al., 1994; Soltoff and Toker, 1995; Szallasi et al., 1995).

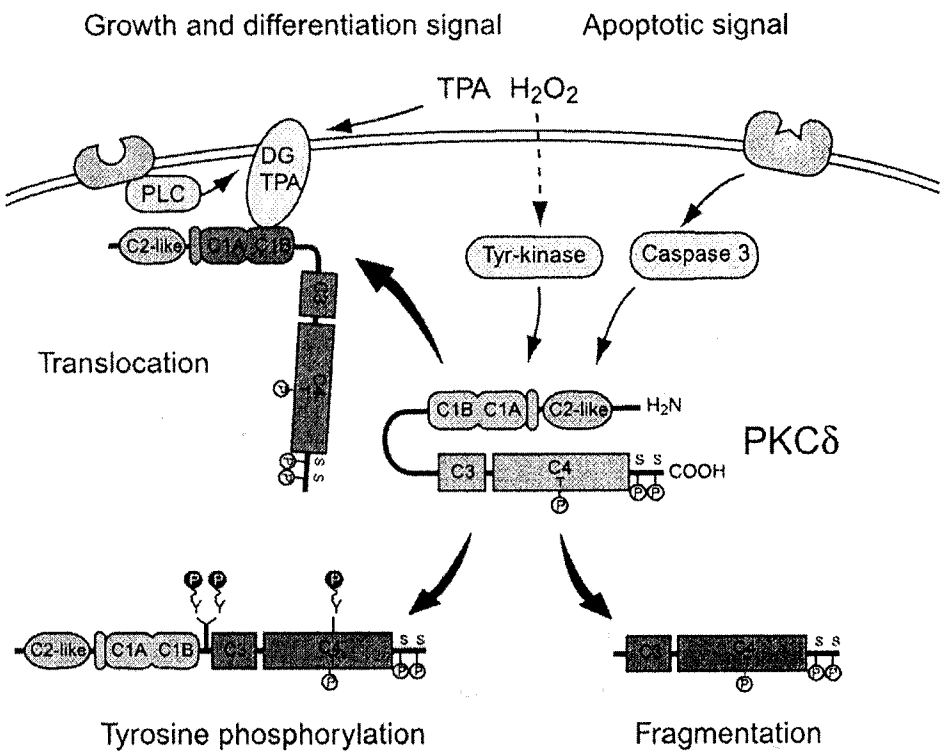
The pro-apoptotic function of PKCδ has been investigated extensively, but the exact mechanism remains unknown. Although the events downstream of PKCδ and those that lead to apoptosis remain unclear, studies from many research groups over the last few years have shown that the catalytically active PKCδ fragment regulates the activity of a host of cell signaling molecules such as scrambalase, an enzyme which induces bi-directional movement of phospholipids across the membrane during

apoptosis (Frasch et al., 2000), DNA protein kinase (DNA-PK), an enzyme essential for the repair of double-stranded DNA breaks (Bharti et al., 1998), small heat-shock proteins-25/27 (Maizels et al., 1998), histone H2B (Ajiro, 2000), and lamin kinase (Cross et al., 2000). In addition, PKCδ phosphorylates other cell signaling molecules such as MAP kinases (Chen and Chen, 1999), the tyrosine kinase Jak2 (Kovanen et al., 2000), and Stat3 signal transducers and activators of transcription (Jain et al., 1999). Most recently, PKCδ has been shown to activate the redox sensitive transcription factor, NF-κB, and thereby promote apoptosis in neutrophils (Vancurova et al., 2001). Furthermore, PKCδ has been shown to translocate to the nucleus, mitochondria, cytoplasm, plasma membrane and other cellular organelles to initiate programmed cell death (Sawai et al., 1997; Chen et al., 1999; Dal Pra et al., 1999; Li et al., 1999; Dempsey et al., 2000; Majumder et al., 2000). Hence, the constitutively active PKCδ fragment results in the loss of regulatory function of many of its substrates, resulting in rapid apoptotic cell death.

Cell line	Treatment	Mode of Activation	Functional Change	Reference
BON cells	TPA	Translocation (membrane)	Neurotensin secretion	Li et al., 2002
CaCo-2	Over-expression	Translocation (membrane)	Differentiation/Apoptosis	Cerda et al., 2001
CI 41	UV-B	Translocation (membrane)	Apoptotic features	Chen et al., 1999
HL-60	Ceramide	Translocation (membrane)	Apoptotic features	Sawai et al., 1997
PC12	NGF	Translocation (membrane)	Differentiation	O'Driscoll et al., 1995
U-937	H_2O_2	Translocation (mitochondria)	Apoptotic features	Majumder et al., 2001
MCF-7	TPA	Translocation (mitochondria)	Apoptotic features	Majumder et al., 2000
Keratinocytes	TPA	Translocation (mitochondria)	Apoptotic features	Li et al., 1999
HeLa cells	Aplidin	Proteolytic cleavage	Apoptotic features	Garcia-Fernandez et al., 2002
HeLa cells	Etoposide	Proteolytic cleavage	Apoptotic features	Cross et al., 2000
Salivary cells	Etoposide	Proteolytic cleavage	Apoptotic features	Rayland et al., 1999
HeLa cells	TNFα	Proteolytic cleavage	Apoptotic features	Ghayur et al., 1996
U-937	Ionized radiation	Proteolytic cleavage	Apoptotic features	Emoto et al., 1995
HaCaT cells	UV-B	Tyrosine phosphorylation	Apoptotic features	Fukunaga et al., 2001
COS-7	H_2O_2	Tyrosine phosphorylation	N/A	Konishi et al., 1997
Animal Studies	Models	Mode of Activation	Functional Change	Reference
Rat	Brain ischemia	Induction	N/A	Koponen et al., 2000
Rat	Brain ischemia	Induction	N/A	Miettinen et al., 1996

Table 8: Mode of Activation and Cellular Function of PKCδ

A. Activation mechanism of PKCδ



B. Amino acid sequence of rat PKCδ

1 MAPFLRISFN SYELGSLQAE DDASQPFCAV KMKEALTTDR GKTLVQKKPT MYPEWKSTFD
61 AHIYEGRVIQ IVLMRAAEDP MSEVTVGVSV LAERCKKNNG KAEFWLDLQP QAKVLMCVQY
121 FLEDGDCKQS MRSEEEAMFP TMNRRGAIKQ AKIHYIKNHE FIATFFGQPT FCSVCKEFVW
181 GLNKQGYKCR QCNAAIHKKC IDKIIGRCTG TATNSRDTIF QKERFNIDMP HRFKVYNYMS
241 PTFCDHCGTL LWGLVKQGLK CEDCGMNVHH KCREKVANLC GINQKLLAEA LNQVTQKASR
301 KPETPETVGI VQGFEKKTAV SGNDIPDNNG TYGKIWEGSN RCRLENFTFQ KVLGKGSFGK
361 VLLAELKGKE RYFAIKYLKK DVVLIDDDVE CTMVEKRVLA LAWENPFLTH LICTFQTKDH
421 LFFVMEFLNG GDLMFHIQDK GRFELYRATF YAAEIICGLQ FLHGKGIIYR DLKLDNVMLD
481 KDGHIKIADF GMCKENIFGE NRASTFCGTP DYIAPEILQG LKYSFSVDWW SFGVLLYEML
541 IGQSPFHGDD EDELFESIRV DTPHYPRWIT KESKDIMEKL FERDPAKRLG VTGNIRLHPF
601 FKTINWNLLE KRKVEPPFKP KVKSPSDYSN FDPEFLNEKP QLSFSDKNLI DSMDQTAFKG
661 FSFVNPKYEQ FLE

Figure 13: Amino Acid Sequence and Activation Mechanism of PKCδ (Modified from Kikkawa et al., 2002)

Progression of Parkinson's disease and biochemical hallmarks

The pathological features of PD are well established, and possible cell death processes have been identified to some extent in recent years. Although the dopaminergic neuronal degeneration has not yet been characterized in idiopathic PD, several lines of evidence strongly suggest that dopaminergic neuronal loss in the SNc has certain positive correlations with mitochondrial dysfunction, activation of caspase-3, and other pro-apoptotic signaling processes (Hartmann et al., 2000). PD is associated with a systemic defect in mitochondrial complex I activity (Schapira, 1993; Haas et al., 1995; Swerdlow et al., 1996). Mammalian complex I in the inner mitochondrial membrane consists of at least 43 proteins, at least seven of which are encoded in mitochondrial DNA (Greenamyre et al., 2001). Mitochondrial complex I mediates an initial electron transfer from NADH to ubiquinone to generate a proton gradient by promoting proton uptake into the inter-membrane mitochondrial space and produce cellular energy. Decreased mitochondrial complex I activity has been observed in PD patients' brains (Mizuno et al., 1989; Parker et al., 1989; Hattori et al., 1991), and treatment with a mitochondrial complex I inhibitor induced PD-like symptoms in animal models (Tipton and Singer, 1993; Betarbet et al., 2000; Betarbet et al., 2002). The defect may result in increased production of ROS and other oxidative stressors, which subsequently activate pro-apoptotic signaling processes. Several environmental toxins, such as rotenone and paraquat as well as MPTP, are known to inhibit mitochondrial complex I activity and are suspected risk factors of PD (Betarbet et al., 2002). Exposure to certain environmental chemicals may cause dysfunction of mitochondrial complex I and result in neurodegeneration. Thus, either genetic or acquired complex I abnormalities may be important in the pathophysiology of PD.

Mitochondria use more than 90% of the total cellular oxygen to produce ATP (Kidd et al., 1988). When mitochondria become dysfunctional, free cellular oxygen may be unused and converted to ROS. In general, the brain is especially susceptible to

oxidative stress. First, antioxidant activities of catalases and peroxidases are normally lower in the brain than in other organs while SOD activity is higher, resulting in the excessive formation of hydrogen peroxide in the brain. Second, the brain consumes much higher levels of oxygen than other organs, contributing to the greater generation of endogenous ROS. The enhanced vulnerability of the substantia nigra to oxidative insults is related to the high levels of dopamine and low levels of GSH. Dopamine, one of the most unstable neurotransmitters in the brain, readily undergoes auto-oxidation to form highly reactive and toxic dopamine quinones (Graham, 1984). One of the most intensively studied dopamine quinones important in PD pathology is 6-hydroxydopamine (6-OHDA). The formation of 6-OHDA is accelerated by metal ions, such as copper, manganese, or zinc (Youdim et al., 1989). In the normal dopamine degradation process, hydrogen peroxide is produced as a by-product during conversion to DOPAC by MAO-B (Stokes et al., 1999). In addition, GSH levels are lower in the SNc than in other brain regions and may also contribute to the enhanced susceptibility of the SNc to oxidative stress (Perry et al., 1982; Jenner, 1998). GSH functions as an antioxidant and as a cofactor of cytochrome P450 enzymes (Youdim et al., 1989; Jenner, 1998). Depletion of cellular GSH could be one of the major causal factors of neurodegeneration. These additional sources of oxidative stress and low antioxidant defense mechanisms make the SNc a primary target of oxidative insults and trigger of apoptotic cell death of dopaminergic neurons in the SNc.

Caspase-3 is in a latent state during the progression of PD. The highest level of activated caspase-3 (68.4% activated caspase-3 positive neurons) was observed in the lateroventral part of the SNc, where the most severe neuronal loss (91%) occurs in PD (Fearnley and Lees, 1991). Activated caspase-3 was also measured in the dorsal (47.2%) and ventromedial (30.4%) parts of the SNc, where 56% and 71% neuronal loss was observed in PD patients, respectively (Hartmann et al., 2000). Although active caspase-3 positive dopaminergic neurons were also detected in control brains, the relative percentage of active caspase-3 positive dopaminergic neurons in PD brains was

five times higher than the control group. Active caspase-3 correlated with apoptosis in postmortem PD brains has also been reported elsewhere (Tatton, 2000). Therefore, the caspase-3 cascade and resulting apoptosis may be one of the possible mechanisms of dopaminergic degeneration in PD. Furthermore, apoptotic dopaminergic neurons in the SNc have been observed in Lewy body-associated disorders (Tompkins et al., 1997), normal aging (Anglade et al., 1997a), and PD (Anglade et al., 1997b; Kingsbury et al., 1998). Interestingly, one of the pro-apoptotic Bcl-2 family proteins, Bax, was also up-regulated in SNc dopaminergic neurons of PD patients (Tatton, 2000). In addition, the SNc from aged PD patients contains lower amounts of the dopamine transporter (DAT) as well as decreased levels of DAT mRNA and vesicular monoamine transporter (VMAT2) in the striatum (Counihan and Penney, 1998; Ma et al., 1999; Miller et al., 1999b).

Several proteins have recently been identified as important in the progression of dopaminergic neurodegeneration and PD. In this section, three proteins, namely α-synuclein, parkin, and ubiquitin carboxy-terminal hydrolase (UCH)-L1, will be discussed. Two mutations in α-synuclein, A30P and A53T, have been identified in familial PD in a German family and a Greek-Italian family, respectively (Polymeropoulos et al., 1997; Kruger et al., 1998). α-synuclein is abundant in the CNS, accounting for approximately 5% of the total synaptic proteins. The human synuclein family consists of α-, β- and γ-synuclein and synoretin (Jakes et al., 1994; Iwai et al., 1995; Buchman et al., 1998). α- and β-synucleins are mainly found in the CNS, whereas γ-synuclein and synoretin are found in the PNS and retina, respectively (Surguchov et al., 1999). α-synuclein consists of 140 amino acids and is characterized by repetitive, imperfect repeats (KTKEGV) distributed throughout most of the amino-terminal half of the peptide and an acidic C-terminus with 3% α-helixes and 23% β-sheets (Souza et al., 2000b). The exact physiological function of α-synuclein is not known, but it may act as a chaperone protein because its N-terminal portion (residue 1-61) shows 40% homology with the 14-3-3 proteins, which are well-known chaperone

proteins (Ostrerova et al., 1999). α-synuclein has been reported to associate with 14-3-3, PKC, BAD, ERK, and tau proteins (Jensen et al., 1999; Ostrerova et al., 1999). Pathologically, α-synuclein plays an important role in the formation of Lewy bodies, and is a major component of these cytoplasmic protein inclusions found in PD. The formation of α-synuclein aggregates is accelerated by the mutations A30P and A53T (Conway et al., 1998; Giasson et al., 1999; Narhi et al., 1999). In addition, nitration of α-synuclein tyrosine residues (Y39, Y125, Y133, and Y136) and resulting formation of dityrosine cross-linkages facilitated fibril and aggregate formation and stabilization in brain (Souza et al., 2000b; Paxinou et al., 2001).

Parkin is responsible for ubiquitin proteosome system (UPS) and may play an important role in the pathogenesis of the common, familial, young-onset PD, known particularly as autosomal recessive juvenile parkinsonism (AR-JP) (Kitada et al., 1998). In most cases, the onset of AR-JP occurs before the age of 40. Parkin is coded in chromosome 6q25.2-q27 and has more than 500 base-pairs and 12 exons, encoding a 465 amino acid protein (Kitada et al., 1998). With a molecular weight of 52 kDa, parkin functions physiologically as an E3 ubiquitin-ligase. Parkin contains a ubiquitin-like sequence at its N-terminus and two RING sequences with an in-between RING finger at its C-terminus, where the E2 ubiquitin-conjugating protein binds. In UPS, three steps are required for protein degradation (Figure 14A). First step is the activation of ubiquitin by ATP and E1 ubiquitin-activating proteins. Activated ubiquitin is passed to E2 ubiquitin-conjugating proteins. Fonally, ubiquitin is conjugated to target proteins by E3 ubiquitin-ligase. These poly-ubiquitinated proteins are unfolded, and ubiquitins are hydrolyzed by (UCH)-L1 for recycling. Unfolded proteins are degraded by proteosomes. Mutations at R42P, K161N, K211N, C212Y, T240R, R256C, R275W, D280N, C289G, G328E, R334C, T415N, G430D, and C431F are considered important in the development of PD, yet not all mutations result in pathological features of idiopathic PD. Among these mutations, R275W results in Lewy body formation in typical regions affected in PD (Farrer et al., 2001). Parkin is

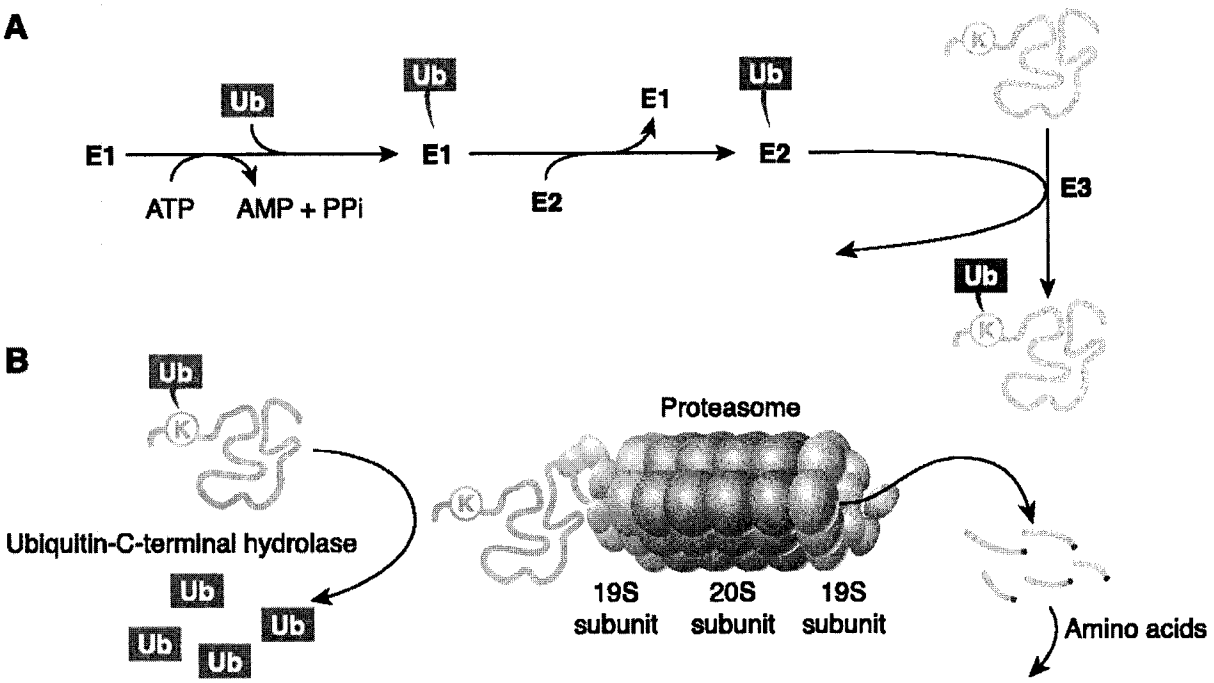


Figure 14: Ubiquitin Proteosome System (Modified from Taylor et al., 2002)

also detected in Lewy bodies along with α-synuclein, cytochrome c, ubiquitins, and heat shock proteins, indicating that parkin may be involved in the pathogenesis of PD (Schlossmacher et al., 2002).

Parkin and the ubiquitin protein degradation system may be involved in α-synuclein degradation. The o-glycosylated form of α-synuclein (αSp22) was recently identified as a different isoform of α-synuclein, and wild-type parkin facilitates the degradation of αSp22 (Shimura et al., 2001). Also, brains from patients with AR-JP (with a mutated parkin gene) were incapable of degrading αSp22, and significant accumulation of αSp22 was detected in Lewy bodies (Shimura et al., 2001). Parkin is proteolytically cleaved by caspase at Asp-126 (Kahns et al., 2002) and is responsible for the degradation of the unfolded parkin-associated endothelin receptor-like receptor (Pael-R) and for accumulation of Pael-R in the endoplasmic reticulum (ER) of dopaminergic neurons. Parkin dysfunction has been observed in AR-JP brains, causes

ER stress, and may be a triggering factor for stress-induced neuronal cell death (Imai et al., 2001; Imai et al., 2002).

Mutation of (UCH)-L1 may be positively associated with the etiology of PD. As mentioned above, (UCH)-L1 is also an important enzyme in the ubiquitin protein degradation process (Figure 14B). Removal of ubiquitin from target proteins is necessary prior to degradation by proteosomes. (UCH)-L1 consists of 230 amino acids and is one of the most abundant proteins in the brain, accounting for approximately 2% of all the brain proteins (Wilkinson et al., 1989; Wilkinson et al., 1992). It is also found in Lewy bodies in PD (Lowe et al., 1990), suggesting its importance in the pathogenesis of PD. Mutation of I93M (isoleucine to methionine at position 93) was identified in a German family with a history of familial PD (Leroy et al., 1998). The mutation site is highly conserved among subtypes as well as species and results in a significant reduction (about 50%) in its catalytic activity, suggesting that the mutation leads to malfunction of the protein degradation and subsequent protein accumulation and aggregation in neurons (Leroy et al., 1998). However, epidemiological evidence indicates that the mutation of I93M in (UCH)-L1 is extremely rare and may not contribute to the etiology of PD (Harhangi et al., 1999; Lincoln et al., 1999). Furthermore, the mutation of S18Y (serine to tyrosine at position 18) in (UCH)-L1 significantly lowers the risk of PD (OR = 0.53), especially young-onset PD (Maraganore et al., 1999). Similar results were reported by other groups (Wintermeyer et al., 2000; Zhang et al., 2000), yet the exact mechanism of the protective effects of this mutation in PD remains unclear at this point.

CHAPTER II: DIELDRIN-INDUCED OXIDATIVE STRESS AND NEUROCHEMICAL CHNAGES CONTRIBUTE TO APOPTOTIC CELL DEATH IN DOPAMINERGIC CELLS

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Masashi Kitazawa, Vellareddy Anantharam, and Anumantha G. Kanthasamy

ABSTRACT

We examined the acute toxicity of dieldrin, a possible environmental risk factor of Parkinson's disease, in a dopaminergic cell model, PC12 cells, to determine early cellular events underlying the pesticide-induced degenerative processes. EC_{50} for 1 hour dieldrin exposure was 143 μM for PC12 cells, whereas EC_{50} for non-dopaminergic cells was 292-351 μM, indicating that dieldrin is more toxic to dopaminergic cells. Dieldrin also induced rapid, dose-dependent releases of dopamine and its metabolite, DOPAC, resulting in depletion of intracellular dopamine. Additionally, dieldrin exposure caused depolarization of mitochondrial membrane potential in a dose-dependent manner. Flow cytometric analysis showed generation of reactive oxygen species (ROS) within 5 min of dieldrin treatment, and significant increases in lipid peroxidation were also detected following 1-hour exposure. ROS generation was remarkably inhibited in the presence of SOD. Dieldrin-induced apoptosis was significantly attenuated by both SOD and MnTBAP (SOD mimetic), suggesting that dieldrin-induced superoxide radicals serve as important signals in initiation of apoptosis. Furthermore, pretreatment with deprenyl (MAO-inhibitor) or α-methyl-L-p-tyrosine (TH-inhibitor) also suppressed dieldrin-induced ROS generation and DNA fragmentation. Taken together, these results suggest that rapid release of dopamine and generation of ROS are early cellular events which may account for dieldrin-induced apoptotic cell death in dopaminergic cells.

Key words: Dieldrin, PC12 Cells, Oxidative Stress, Dopamine, Superoxide, Apoptosis, Parkinson's disease

INTRODUCTION

Parkinson's disease is an age-related neurodegenerative disorder with a lifetime incidence of 1-2%, and every year, 5-24 people per 100,000 of population are diagnosed with this disease.[1, 2] Although the exact etiology of Parkinson's disease remains unknown, the discovery of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which induces a Parkinson-like syndrome, brought attention to the possibility that some exogenous chemicals might contribute to the etiopathogenesis of Parkinson's disease.[3] Recently, a study that examined the extent of genetic contribution to the pathogenesis of Parkinson's disease by comparing thousands of twins concluded that genetic factors play a role in the pathogenesis of young-onset of Parkinson's disease, but not the more common geriatric-onset Parkinson's disease, which further supports the view that environmental factors are dominant risk factors in the etiology of the idiopathic Parkinson's disease.[4] Decreased mean age of onset of Parkinson's disease has been reported in some rural areas, and several epidemiological and case-control studies conducted in rural areas have revealed that certain pesticides and other environmental factors have positive association with the increased incidence of Parkinson's disease.[5-9] On the contrary, a few case-control studies found no association with environmental chemicals, indicating that the role of environmental factors in pathogenesis of Parkinson's disease is still debatable.[10-13] Nevertheless, taking into consideration the variability of study methods, selection of subjects, and geographical regions among these case-control studies, it is remarkable that majority of studies found a significant association between environmental factors and Parkinson's disease.[14]

Dieldrin is a chlorinated cyclodiene compound that was widely used as a pesticide around the world until mid-1970, but is still used in several developing countries. A recent study indicates that dairy products and meats are still believed to be the primary sources of human exposure to dieldrin, and the daily intake level of dieldrin

through these contaminated food was estimated at 0.059 μg per average person.[15] Since the half-life of dieldrin in human blood is estimated to be around 300 days,[16] prolonged exposure to dieldrin through food may greatly increase the accumulation of dieldrin in the brain and other lipophilic tissues.

Evidence from several lines of research, ranging from studies of post-mortem pathology studies to cultured neurons, have implicated a possible relationship between dieldrin and the etiology of Parkinson's disease in humans. Fleming *et al.*[17] measured amounts of various organochlorine pesticides in human postmortem brain samples from Parkinson's disease patients, Alzheimer's disease patients, and age-matched controls. Dieldrin was detected in 6 of 20 brains from Parkinson's disease patients, but in none of the 14 age-matched control brains. Another study reported significant levels of dieldrin in the caudate nucleus from Parkinson's disease patients.[18] Chronic exposure to dieldrin in a mesencephalic cell culture shows that dopaminergic neurons are more susceptible than other neurons to dieldrin toxicity.[19] In addition, *in vivo* studies reported massive dopamine depletions in brains following chronic exposure to dieldrin.[20, 21] These results suggest that chronic exposure to dieldrin, a highly lipophilic compound,[22] could selectively destroy dopaminergic neurons in substania nigra pars compacta (SNc) and could be a risk factor for Parkinson's disease. However, these studies did not examine the mechanisms and processes underlying dieldrin-induced selective dopaminergic degeneration, and no data have been reported regarding the effect of dieldrin on early cellular events. Understanding the early neurochemical and biochemical alterations following acute exposure of a neurotoxic agent might provide mechanistic insights into toxin-induced neurodegenerative processes.

In the present study, we specifically examined the acute effect of dieldrin in dopminergic cells to identify the early cellular events that might contribute to the degenerative process following dieldrin exposure. These early cellular responses play critical roles in determining the fate of cell survival. We have mainly utilized rat pheochromocytoma (PC12) cells in this study because these cells give rise to a homogeneous population with dopaminergic secretory properties. PC12 cells also possess many important neurochemical and signal transduction processes in a manner

similar to dopaminergic neurons.[23, 24] Numerous toxicological studies have utilized PC12 cells as *in vitro* models to examine dopaminergic toxicity of various compounds.[25-28] Herein, we report that dieldrin induces neurochemical changes and oxidative events following acute exposure in PC12 cells, and these initial changes of oxidative stress contribute to apoptotic cell death.

MATERIALS AND METHODS

Chemicals

Dieldrin, β-Nicotinamide adenine dinucleotide, reduced form (NADH), sodium pyruvate, 3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyl tetrazolium bromide (MTT), superoxide dismutase (SOD), α-methyl-L-p-tyrosine (α-MPT), (R)-(-)-deprenyl, and neurotransmitter standards (dopamine, DOPAC, L-DOPA, 5-HIAA, and HVA) were purchased from Sigma (St. Louis, MO). Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) was obtained from OXIS international Inc. (Portland, OR). Fluorescent dyes, hydroethidine, 5-dodecanylamino fluorescein and DiOC6 (3,3'-dihexyloxacarbocyanine iodide), were purchased from Molecular Probes (Eugene, OR). All tissue culture supplies were obtained from Gibco-BRL (Gaithersburg, MD). Other routine laboratory reagents were obtained from Fisher Scientific (Pittsburgh, PA).

Cell culture and treatment

Dopaminergic PC12 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Additionally, two non-dopaminergic cells were used in the cytotoxicity experiments to determine the differential toxic response of dieldrin in dopaminergic cells vs. non-dopaminergic cells. These non-dopaminergic cells were human cortical neuronal (HCN-2) cells which are primarily GABAergic in nature (ATCC, Rockville, MD) and mouse pancreatic α-endocrine (α-TC) cells (generous gift from Dr. W. H. Hsu, Iowa State University). PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat inactivated horse serum, 5% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin and 50 μg/mL

streptomycin. α-TC cells were grown in RPMI1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin and 50 μg/mL streptomycin. HCN-2 cells were grown in DMEM medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin and 50 μg/mL streptomycin. These cells were placed in 75 cm^2 cell culture flasks (Nalge Nunc International, Rochester, NY) at 37°C under an atmospheric condition of 5% CO_2 and 95% air, and 4-6 day old cells were used for the experiments.

Cells were suspended in Krebs-Ringer solution (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 6 mM glucose, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 2.4 mM $CaCl_2$, pH 7.4) or serum-free RPMI1640 or DMEM at a concentration of $2x10^6$ cells/ml. Cells were then treated with dieldrin (30–1000 μM). Dieldrin was first dissolved in dimethylsulfoxide (DMSO) to prepare stock solution, and then it was further diluted in incubation medium to achieve the final concentrations with 1% or less DMSO. After 1-hour incubation with dieldrin at 37°C, dead and live cells were determined by trypan blue exclusion method with an Improved Nebauer Hemacytometer (Fisher Scientific, Pittsburgh, PA). The cell viability was normalized as percent of control.

Lactate dehydrogenase (LDH) assay

LDH activity in the cell-free extracellular supernatant was quantified as an index of cell death. The method originally described by Vassault [29] was modified to a 96 well format. Briefly, 50 μl of the extracellular supernatant was added to 3 ml of 0.08 M Tris buffer (pH 7.2) containing 0.2 M NaCl, 0.2 mM NADH, and 1.6 mM sodium pyruvate. LDH activity was measured continuously by monitoring the decrease in the rate of absorbance at 339 nm by the Beckman DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA). Temperature of cuvettes was maintained at 37°C during reading. Changes in absorbance per minute ($\Delta A/\Delta T$) were used to calculate LDH activity (U/I), using the following equation: $U/I = (\Delta A/\Delta T) \times 9682 \times 0.66$, where 9682 was a coefficient factor, and 0.66 was a correction factor at 37°C.

HPLC analysis of neurotransmitters

Extracellular and intracellular neurotransmitters were analyzed by high-performance liquid chromatography with electrochemical detection (HPLC-EC). A detailed description of this procedure has been described previously.[27, 30] Briefly, the HPLC system consisted of Rainin pressure module and Rainin HPXL solvent delivery system (Rainin Instrument Co. Inc., Woburn, MA) with a Bio-Rad automatic sampler, model AS-48 (Bio-Rad Laboratories, Hercules, CA). Neurotransmitters were separated isocratically by a Microsorb-MV (86-200-E3 C-18 3μm 100A J2 10086-4) reversed phase column (Rainin Instrument Co. Inc., Woburn, MA) with a flow rate of 1 ml/min. Electrochemical detection (EC) system consisted of an ESA coulochem model 5100A with a microanalysis cell model 5014A and a guard cell model 5020 (ESA Inc., Bedford, MA). All systems were controlled by Rainin Dynamax HPLC method manager software program ver. 1.4 (Rainin Instrument Co. Inc., Woburn, MA). The mobile phase contained 0.15 M monochloroacetic acid, 0.13 mM sodium octyl sulfonate, 0.67 mM disodium EDTA, 0.12 M sodium hydroxide, and 1.5% acetonitrile (all chemicals were HPLC grade), and pH of the mobile phase was adjusted to 3.1 with phosphoric acid. The HPLC-EC was calibrated with 3,4-dihydroxyphenyl-L-alanine (L-DOPA), DOPAC, dopamine, 5-hydroxyindoleacetic acid (5-HIAA), and HVA. Loading amount of each sample was 20 μl. The injector was automatically washed after the end of each injection with 50% acetonitrile in deionized water.

3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

MTT assay is widely used to assess cell viability following treatment with various toxic substances.[25, 31, 32] MTT assay measures activity of mitochondrial dehydrogenase enzymes that cleave tetrazolium ring to produce formazan. Thus, the assay can be used as an index of mitochondrial function. After dieldrin treatment, cells were washed once and further incubated in serum-free medium containing 0.25 mg/ml MTT for 3 hours at 37°C. Supernatant was removed, and MTT crystals were solubilized

with acidic isopropanol. Mitochondrial enzyme activity was measured by spectrophotometer at 570 nm with reference wavelength at 630 nm.

Depolarization of mitochondrial membrane potential following dieldrin exposure

Depolarization of mitochondrial membrane potential ($\Delta\Psi m$) were assessed by flow cytometric analysis using DiOC6.[33, 34] 40 nM DiOC6 was added to incubation medium 15 min before the end of treatment period, and the incubation continued at 37°C. Then, the cells were washed once, resuspended with PBS, and analyzed by flow cytometry with excitation at 484 nm and emission at 501 nm.

Detection of reactive oxygen species (ROS) and lipid peroxidation by flow cytometry

Flow cytometry is a powerful tool to quantitate the generation of intracellular ROS and lipid peroxidation.[35-38] The major advantage of flow cytometry over conventional fluorometry is that a flow cytometer measures fluorescence intensity only inside the cells and it does not account for fluorescence in the extracellular medium.[39] All flow cytometric data were collected on a Becton Dickinson FACScan™ flow cytometer (Becton Dickson, San Francisco, CA). Hydroethidine, a sodium borohydride-reduced derivative of ethidium bromide, was used to detect ROS, specifically O_2^{-}.[35-37] When hydroethidine is loaded in the cells, it binds to cellular macromolecules. Once O_2^{-} is generated, it converts hydroethidine to ethidium bromide and increases red fluorescence (620 nm). For lipid peroxidation detection, 5-dodecanoylamino fluorescein was used.[38] The original form of this dye is fluorescent. When it binds to a product of lipid peroxidation, the dye loses its fluorescence; thus, decreased fluorescence intensity is observed in cells with increased lipid peroxidation. A 15-mW air-cooled argon-ion laser was used as an excitation source for both hydroethidine and 5-dodecanoylamino fluorescein at 488 nm. The optical filter was 585/42 nm band pass for hydroethidine and 530/30 nm for 5-dodecanoylamino fluorescein. Cells were detected and distinguished from the background by forward-angle light scattering (FSC) and orthogonal light scattering (SSC) characteristics. All the flow cytometric data were analyzed by

Cellquest™ data analysis software to determine the significant increase or decrease of fluorescence intensity.

PC12 cells were resuspended with Hanks balanced salt solution (HBSS) with 2 mM calcium at a density of 0.5 x 10^6 cells/ml. Cells were then incubated with 10 μM hydroethidine or 1 μM 5-dodecanoylamino fluorescein for 15 min at 37°C in the dark to allow dye loading into the cells. After incubation with dye, excess dye was removed, and the cells were resuspended with HBSS. Following addition of dieldrin (30-100 μM final concentration), and ROS generation was measured at 0, 5, 15, and 30 min after the exposure, and lipid peroxidation was measured after 1 hour of exposure.

Apoptosis assay

When cells undergo apoptosis, several distinct morphological changes can be observed. One of these unique changes is DNA fragmentation, which results in DNA cleavage into ~200 base pairs. Recently, a highly sensitive detection kit called the Cell Death Detection Elisa Plus Assay Kit (Roche Biochemicals) was developed, and it provides quantitative measurement of histone-associated low molecular weight DNA fragments.[40] Apoptotic PC12 cells were measured using this kit exactly as described by the manufacturer. Briefly, following dieldrin exposure, cells were centrifuged and washed once with PBS. Cells were then incubated with lysis buffer (supplied with the kit) for 30 min, centrifuged at 5,000 rpm for 10 min, and then 20 μl of cell lysate was placed in streptavidin-coated 96-well multititer plates. The antibody cocktail was a mixture of anti-histone-biotin directed against histones (H1, H2A, H2B, H3 and H4) and anti-DNA-peroxidase (POD) directed against both single and double stranded DNA in the nucleosomes. After incubation, unbound components were removed by washing with the incubation buffer, and quantitative determination of the amount of nucleosomes retained by anti-DNA-POD in the immunocomplex was determined photometrically with ABTS® as a HRP substrate. Measurements were made at 405 nm against an ABTS® solution as a blank (reference wavelength 490 nm).

Morphological changes of apoptosis can also be assessed by Hoechst 33342 staining as described in previous studies[41, 42]. Since Hoechst 33342 binds to DNA and

condensed chromatin, apoptotic cells are expressed in an optically distinct nucleus. Cells were plated on collagen (6 μg/cm^2) coated cover slides, and treated with dieldrin. After exposure, cells were fixed with 10% buffered formaldehyde for 30 min at room temperature and stained with Hoechst 33342 (10 μg/ml) for 3 min in the dark. Images were taken with a fluorescent microscope (Nikon Inc., Melville, NY) under UV illumination.

Data analysis and statistics

Data were analyzed using one-way ANOVA or student t-test. Dunnett's post-test or Bonferroni's multiple comparison test were performed as post-tests, and $p<0.05$ was considered as significant.

RESULTS

Effect of dieldrin on cell viability

PC12 cells were exposed to 0, 30, 100, 300, 500, 1000 or 3000 μM dieldrin for one hour, and cell viability was measured by trypan blue dye exclusion. Figure 1 shows the relationship between dieldrin concentration (log μM) and percent cell survival relative to the control. Exposure of PC12 cells to various doses of dieldrin for one hour resulted in a dose-dependent decrease in cell viability as evidenced by trypan blue accumulation in damaged cells. The effective concentration of dieldrin (EC_{50}) was calculated to be 143 μM by three-parameter non-linear regression. 95% confidence interval was 104-198 μM. Vehicle treated PC12 cells showed close to 90% viability, and were considered as the baseline cell viability.

To further determine whether dopaminergic cells are more susceptible to dieldrin toxicity, we compared the cytotoxic effect of dieldrin on two non-dopaminergic cell lines, HCN-2 (Human cortical GABAergic cell line) and α-TC (endocrine cells) (Fig. 1). Cytotoxicity was determined by trypan blue following 1 hr exposure to various concentrations of dieldrin, and EC_{50} was calculated as 351 μM and 292 μM for α-TC and HCN-2 cells, respectively. 95% confidence interval of EC_{50} in α-TC cells is 230-

535 μM, and that in HCN-2 cells is 199-427 μM. Both non-dopaminergic cells show significantly higher EC_{50} than dopaminerigic PC12 cells.

Effect of dieldrin on LDH release

To further substantiate the cytotoxic nature of dieldrin, LDH release was measured. Extracellular LDH activity showed a dose-dependent increase with dieldrin treatment ranging from 200 to 700% over control group (Fig. 2). Statistical analysis of cytotoxic exposure revealed a significant ($p<0.05$) toxicity in dieldrin-treated groups at concentrations greater than 100 μM as compared with control or DMSO-treated (vehicle) groups. The final concentration of DMSO (1% or less) used in these experiments did not appear to be cytotoxic to PC12 cells, and no significant increase in LDH release was detected when vehicle-controls were compared with untreated PC12 cells. As observed in cell viability data, LDH release confirmed the dose-dependence of cell death from acute treatment with dieldrin.

Effect of dieldrin on dopamine release and depletion

In order to determine whether or not dieldrin treatment altered cellular dopamine levels, we measured the extracellular release of dopamine as well as intracellular content of dopamine by HPLC-EC analysis. As depicted in Fig. 3A, dieldrin induced a dose-dependent release of dopamine during one-hour exposure. Release of dopamine appeared to be one of the most sensitive events measured with respect to dieldrin neurotoxicity. Levels of release at 30 μM, 100 μM, 300 μM and 500 μM dieldrin were 81%, 167%, 207% and 235% of control level, respectively. 1000 μM dieldrin treatment resulted in a nearly complete release of cellular dopamine (data not shown). Dieldrin-induced dopamine release was accompanied by concomitant depletion of intracellular dopamine content (Fig. 3B), indicating that the synthesis of dopamine was not affected.

Effect of dieldrin on DOPAC content

Major metabolites of dopamine, including DOPAC and HVA, were measured simultaneously by HPLC-EC detection. DOPAC was detected in large quantity and

showed a dose-dependent increase of release (Fig. 4A). This observation is reasonable since DOPAC is the major metabolite of dopamine in PC12 cells, and more cytosolic or free dopamine was available for conversion to DOPAC as doses of dieldrin increased. Another interesting observation was that, unlike dopamine, DOPAC releases were relatively minimal at lower dieldrin exposures. For example, DOPAC release was not statistically significant at 100 μM dieldrin. However, significant increases ($p<0.05$) in DOPAC levels were observed at higher doses of dieldrin. On the other hand, intracellular DOPAC content increased significantly between the doses of 100 μM and 500 μM (Fig. 4B). At 500 μM dieldrin, a maximum intracellular DOPAC level was 1006% over control level.

Effect of dieldrin on mitochondrial function

One of the cellular targets for dieldrin is reported to be mitochondria, as it inhibits the respiratory chain by blocking at or near cytochrome b.[43] Mitochondrial activity was determined by MTT assay. When mitochondria are functioning normally, dehydrogenase enzymes present in mitochondria converts MTT into formazan, and the production of formazan was used as an index of mitochondrial activity. The mitochondrial activity was remarkably inhibited by 1-hour exposure to dieldrin (Fig. 5A). Dieldrin concentrations ranging from 30-500 μM produced a significant ($p<0.05$) decrease in mitochondrial activity.

To further confirm that dieldrin acts on mitochondria and causes mitochondrial dysfunction, we have assessed mitochondrial membrane potential ($\Delta\Psi m$) using DiOC6 (mitochondrial membrane potential sensitive fluorochrome) in flow cytometry. As shown in Figure 5B, PC12 cells underwent depolarization of $\Delta\Psi m$ in a dose-dependent manner. The changes were 85%, 35%, and 11% of control following 1h treatment with 30, 100, and 300 μM dieldrin, respectively.

ROS generation and lipid peroxidation following dieldrin treatment

An immediate increase in ROS generation was detected in dieldrin-treated PC12 cells (Fig. 6). All dieldrin-treated cells showed significant ($p<0.01$) increases in ROS from basal level (0 min) within 5 min of the dieldrin exposure. The lower doses of dieldrin exposure (30 and 100 μM) showed higher levels of ROS generation over a 30 min period, whereas the response of 300 μM dieldrin declined over time.

To test whether or not an antioxidant enzyme suppresses dieldrin-induced ROS generation, we treated the cells with 100 units/ml SOD for 5 min prior to dieldrin exposure, and ROS generation was measured at 0, 5, 15 and 30 min. SOD significantly ($p<0.01$) blocked ROS generation over a 30 min time period in 30 μM (Fig. 7A) and 100 μM (Fig. 7B) dieldrin-treated cells. SOD consistently attenuated 60-70% of dieldrin-induced ROS, suggesting that dieldrin primarily causes cellular O_2^- generation.

Since membrane lipid peroxidation occurs as a result of ROS generation, we measured the level of lipid peroxidation following 1 hour of dieldrin exposure. Decrease in fluorescence intensity of 5-dodecanoylamino fluorescein when compared with vehicle-treated cells, indicated an increase in lipid peroxidation following dieldrin treatment. As depicted in Figure 8, lipid peroxidation was detected in dose-dependent manner, and the significant ($p<0.05$) lipid peroxidation was observed in both 300 μM and 500 μM dieldrin-treated PC12 cells following one hour of exposure. The levels of decrease in fluorescence intensity were 9%, 15%, 35% and 49% in 30 μM, 100 μM, 300 μM, and 500 μM dieldrin treatments, respectively.

Effect of SOD and SOD mimetic on dieldrin-induced necrosis and apoptosis

In order to determine the role of oxidative stress in dieldrin-induced cell death, both necrosis and apoptosis were examined. We have reported that dieldrin exposure showed a dose-dependent increase in LDH release (Fig. 2). Following pretreatment with SOD, LDH release, which is used as a marker of necrotic cell death, did not attenuate dieldrin toxicity over a 2-hour period (Fig. 9). On the other hand, SOD showed a protective effect on dieldrin-induced apoptosis (Fig. 10A). DNA fragmentation assay,

which is the hallmark of apoptotic cell death, revealed that dieldrin-induced DNA fragmentation increased dose-dependently, and it was significant ($p<0.05$) at 100 μM dieldrin exposure (Fig. 10A). Interestingly, SOD significantly ($p<0.01$) reduced dieldrin-induced DNA fragmentation, indicating that SOD is capable of attenuating dieldrin-induced apoptosis, but not necrosis.

To further confirm the anti-apoptotic effect of SOD in dieldrin treatment, a cell permeable SOD mimetic, MnTBAP, was used. Pre-treatment with 2 μM MnTBAP significantly ($p<0.05$) attenuated dieldrin-induced apoptosis (Fig. 10B). Over 30% of dieldrin-induced DNA fragmentation was attenuated by MnTBAP treatment, confirming that superoxide generation contributes to apoptotic cell death following dieldrin exposure in PC12 cells.

To further visualize apoptotic features in dieldrin treated cells, Hoechst 33342 staining for nuclear condensation was performed. As depicted in Figure 11, dieldrin treated cells showed a prominent condensed chromatin, reflecting morphological changes commonly observed in apoptotic cells. In the case of SOD pretreatment (100 unit/ml) dieldrin-induced apoptotic characteristics were attenuated, supporting the antiapoptotic effect of SOD presented above.

Effect of tyrosine hydroxylase (TH) and monoamine oxidase-B (MAO-B) inhibitors on dieldrin-induced ROS generation and apoptosis

To determine whether or not dopamine or dopamine metabolites participate as intrinsic factors in dieldrin-induced dopaminergic toxicity, a series of experiments were conducted using inhibitors of dopamine synthesis and catabolism. Cellular dopamine levels were reduced by inhibiting the rate-limiting enzyme of dopamine synthesis tyrosine hydroxylase using α-methyl-L-p-tyrosine (α-MPT). PC12 cells were treated with 100 or 500 μM α-MPT for 24h, and availability of dopamine was determined by HPLC. As shown in Table 1, intracellular dopamine was dose-dependently depleted, and 500 μM α-MPT caused 84% reduction in dopamine level as compared with the untreated group. Measurement of ROS in dopamine-depleted cells showed a significant ($p<0.05$) reduction as compared with normal PC12 cells (Fig. 12A). Additionally,

dieldrin-induced DNA fragmentation was also significantly ($p<0.05$) attenuated in dopamine depleted cells as compared with cells producing normal dopamine level (Fig. 12B). 500 μM α-MPT did not alter basal DNA fragmentation, but attenuated dieldrin-induced DNA fragmentation from 530% to 360% of control at 1 hour exposure period, indicating that dopamine, at least in part, contributes to the dieldrin-induced apoptotic process.

In the next study, we examined the effect of an MAO-B inhibitor on dieldrin-induced ROS generation and apoptosis to determine whether the increased DOPAC formation during dieldrin exposure contributes to dopaminergic cell death through oxidative stress. Treatment with 100 μM deprenyl (MAO-B inhibitor) for 30 min reduced nearly 80% of DOPAC formation in PC12 cells (Table 1), confirming a significant ($p<0.01$) blockade of MAO-B activity. Subsequent flow cytometric analysis revealed that dieldrin-induced ROS generation was significantly ($p<0.01$) suppressed in deprenyl-treated PC12 cells (Fig. 12A). Consequently, deprenyl also attenuated dieldrin-induced DNA fragmentation from 530% to 300% of control following 1hr treatment (Fig. 12B), indicating that alteration of the dopamine catabolic process involving the formation of DOPAC has a significant role in dieldrin-induced degeneration of dopaminergic cells.

DISCUSSION

In the present study, we have shown that dieldrin depletes dopamine, increases dopamine release, and generates ROS in a dose-dependent manner. We have also demonstrated that dieldrin-induced ROS generation contributes to apoptotic cell death in PC12 cells. Our results reveal that dopaminergic cells are more susceptible to the neurotoxic effect of dieldrin than non-dopaminergic cells during acute exposure. This result is consistent with a previous report in which dieldrin was found to be more toxic in dopaminergic neurons than non-dopaminergic neurons during chronic treatment.[19] By extension, our observation that depletion of dopamine in PC12 cells by the tyrosine hydroxylase inhibitor α-MPT, significantly protected against the generation of ROS and

DNA fragmentation, indicating that dopamine may serve as a susceptibility factor in dieldrin-induced cytotoxicity in dopaminergic cells.

Several organochlorine pesticides have been reported to increase ROS formation in PC12 cells.[44] ROS generation was one of the earliest cellular responses to dieldrin toxicity, and was observed within 5 min of exposure. Consequently, the increase in membrane lipid peroxidation following dieldrin exposure was also observed to be dose-dependent and is thought to result from the excess production of ROS. Products from lipid peroxidation have been shown in many studies to contribute to DNA damage.[45] Recent studies have demonstrated that ROS serves as a signal for stimulation of apoptosis by activating an array of cell signaling molecules including cytochrome c, caspases, kinases and endonucleases.[46-48] Known neurotoxic agents, such as MPTP, paraquat, cyanide, manganese and methylcyclopentadienyl manganese tricarbonyl (MMT) that promote dopaminergic cell death, have been shown to induce apoptosis through the activation of similar cellular signaling mechanisms.[27, 49-52] It has been demonstrated that elevation of intracellular ROS activates transition pore opening on the mitochondrial inner membrane.[53] We demonstrate here that dieldrin treatment elevates intracellular ROS levels and depolarizes $\Delta\Psi m$, which could subsequently activate a series of pro-apoptotic signaling cascades including cytochrome c release and activation of initiator caspase-9, followed by activation of caspase-3, leading to apoptotic cell death. In this regard, our recent observations have demonstrated that dieldrin exposure, indeed, activates an array of apoptotic molecules including cytochrome c, caspase-9 and caspase-3.[54] Furthermore, we have noted from a time-course study that ROS generation precedes the activation of proapoptotic molecules following dieldrin exposure in PC12 cells.

In the present study, we also observed significant increases in DNA fragmentation following 1 hour of dieldrin exposure in PC12 cells. Pretreatment with SOD, an enzyme that effectively scavenges superoxide anions, significantly attenuated dieldrin-induced ROS generation and apoptosis as measured by flow cytometry, which exclusively measures intracellular events. However, SOD did not alter dieldrin-induced necrosis as measured by LDH release. Also, scavenging of superoxide anions by a cell

permeable SOD mimetic, MnTBAP,[55, 56] effectively blocked dieldrin-induced apoptosis. These data strongly suggest that dieldrin exposure increased superoxide production, and that superoxide may act as an initiator of a downstream cellular apoptotic cascade in dopaminergic cells. In agreement with our results, it has recently been demonstrated by other researchers that the addition of exogenous SOD attenuated both generation of ROS and apoptosis in neuronal cells.[55, 57, 58]

Another significant response following dieldrin exposure was massive dopamine release. The inverse relationship between intracellular and extracellular dopamine levels implies that dopamine synthesis is not altered by dieldrin, but rather dieldrin evokes the release of dopamine. Neurotoxicant-induced dopamine release has been reported in both *in vitro* and *in vivo* models with neurotoxic agents that are known to disrupt the dopaminergic system.[27, 30, 50, 59, 60] Excess release and accumulation of released dopamine in the extracellular space often temporally accelerates neurotoxicity,[61] suggesting that dieldrin toxicity may be increased by the massive dopamine release shown in this study. Dieldrin and other organochlorine compounds have been shown to bind and/or affect the function of important neurotransmitter vesicular proteins such as the vesicular monoamine transporter (VMAT2), which may be indicative of an additional target for neurotransmitters.[62] Furthermore, our finding that TH inhibition attenuates dieldrin-induced ROS generation and apoptosis supports the view that excessive dopamine secretion might contribute to the oxidative stress-dependent cell death. In support of our view, recent studies demonstrate that addition of exogenous dopamine not only causes apoptotic cell death but also potentiates other chemical-induced apoptosis.[63-65]

Dieldrin-induced cell death was also significantly suppressed by inhibition of MAO-B. It is known that the catabolic process of dopamine to DOPAC by MAO-B produces ROS as a byproduct, and the acceleration of dopamine catabolism may cause further destruction of cells due to the increase of oxidative stress as described above.[63, 66] Our results showed that DOPAC levels were significantly elevated in all dieldrin-treated groups, and inhibition of DOPAC formation during dieldrin exposure blocked both ROS generation and apoptosis. Although mechanisms underlying toxic effect of dopamine

remain unclear, existing reports suggest that presence of excess dopamine in an ROS-rich environment may augment the oxidative stress and cell death process by formation of highly cytotoxic radicals.[47, 67] Taken together, our data indicate that massive dopamine release and increased ROS through DOPAC formation might further contribute to the onset of dopaminergic neurotoxicity following dieldrin exposure.

In conclusion, dopaminergic cells are relatively more susceptible to dieldrin toxicity. Dieldrin stimulates massive extracellular dopamine release in a manner similar to other dopaminergic neurotoxins such as MPTP, cyanide, MMT and manganese,[27, 30, 50, 68] and generates ROS as an early event in cellular toxicity. Dieldrin promotes an environment of excessive extracellular free dopamine along with ROS which may augment oxidative stress-mediated cell death. Although several reports exist in the literature that do not support the causal role of oxidative stress in Parkinson's disease,[71, 72] recent studies implicate oxidative stress as one of the major factors in the pathogenesis of Parkinson's disease.[69, 70] Additional studies that examine early cellular responses following neurotoxic insults are further needed to resolve the exact role of oxidative stress in the neurodegenerative process. The results of short-term dieldrin exposure studies presented here denote cell death mechanisms that may play a role in earlier stages of the pesticide exposure. Collectively, our results suggest that dieldrin exposure, one of the possible environmental factors in Parkinson's disease, is capable of promoting apoptotic cell death in dopaminergic cells via oxidative stress. Additional mechanistic studies delineating the oxidative stress-dependent cell death pathway are being undertaken, and may shed more light onto the role of environmental factors in the dopaminergic degeneration.

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Treatment	Dopamine (% control)	DOPAC (% control)
Control	100.0 ± 4.2	100.0 ± 18.0
100 μM α-MPT	47.9 ± 3.6**	N.D.
500 μM α-MPT	16.4 ± 2.1**	N.D.
100 μM deprenyl	103.7 ± 7.1	20.3 ± 18.1**

Table 1: Levels of dopamine and DOPAC following TH or MAO-B inhibitors

PC12 cells were treated with 100-500 μM α-methyl-L-p-tyrosine (α-MPT) for 24 hours or 100 μM deprenyl for 30 min, and levels of dopamine and DOPAC were measured by HPLC-EC. Each value is expressed as mean ± SEM. Data represent two separate experiments in triplicate. **$p<0.01$ compared with control group. N.D.= not detected.

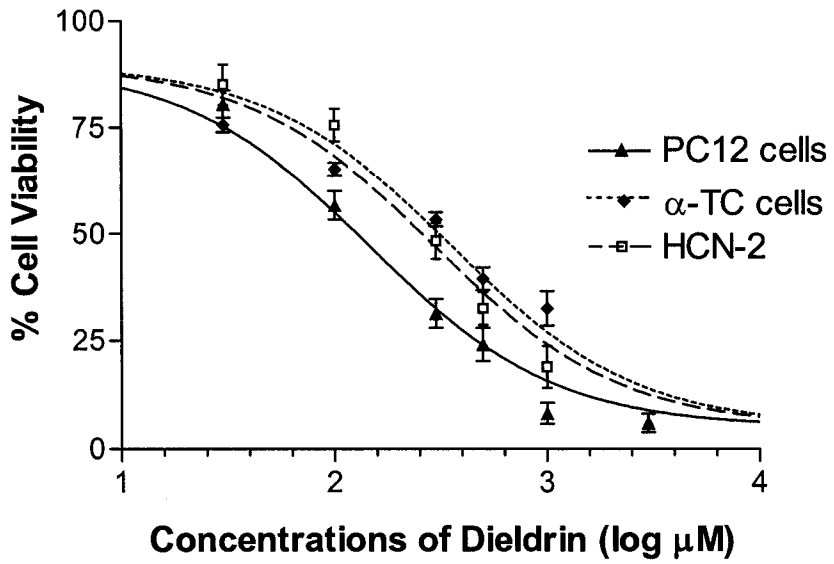

Figure 1: Effect of dieldrin on cell viability in PC12 cells

PC12 cells, α-TC cells, and HCN-2 cells were exposed to 0 - 3000 μM dieldrin for 1 hour at 37°C, and the viability was determined by a trypan blue dye exclusion. Each point represents the mean ± SEM for at least two separate experiments in duplicate. The EC_{50} was calculated by three-parameter nonlinear regression analysis.

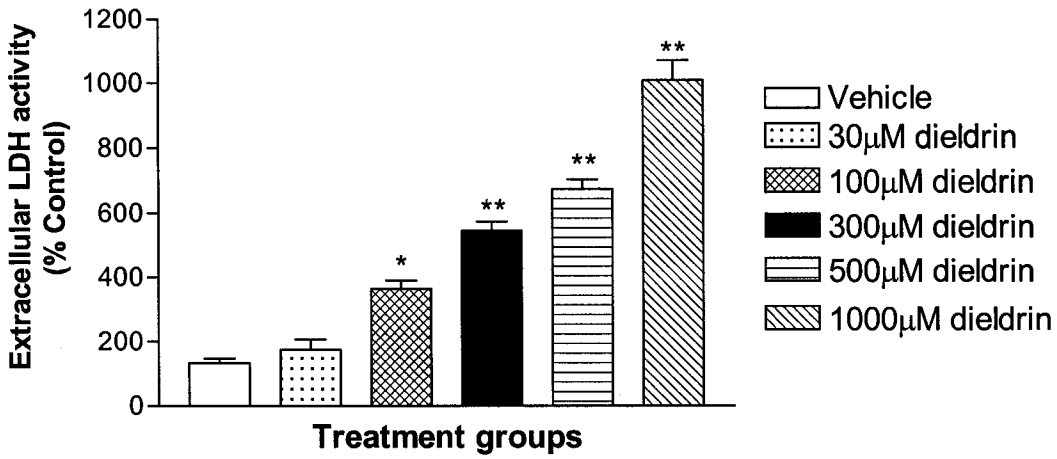

Figure 2: Effect of dieldrin on extracellular LDH release in PC12 cells

PC12 cells were exposed to 0 - 500 μM of dieldrin for 1 hour in Krebs-Ringer at 37°C. After the exposure, cell-free extracellular supernatants were collected, and LDH activity was measured by spectrophotometer. Values represent mean ± SEM for three to five separate experiments in triplicate. Significance was determined by ANOVA followed by Dunnett's post-test between the vehicle-treated group and each treatment group (*$p<0.05$; **$p<0.01$).

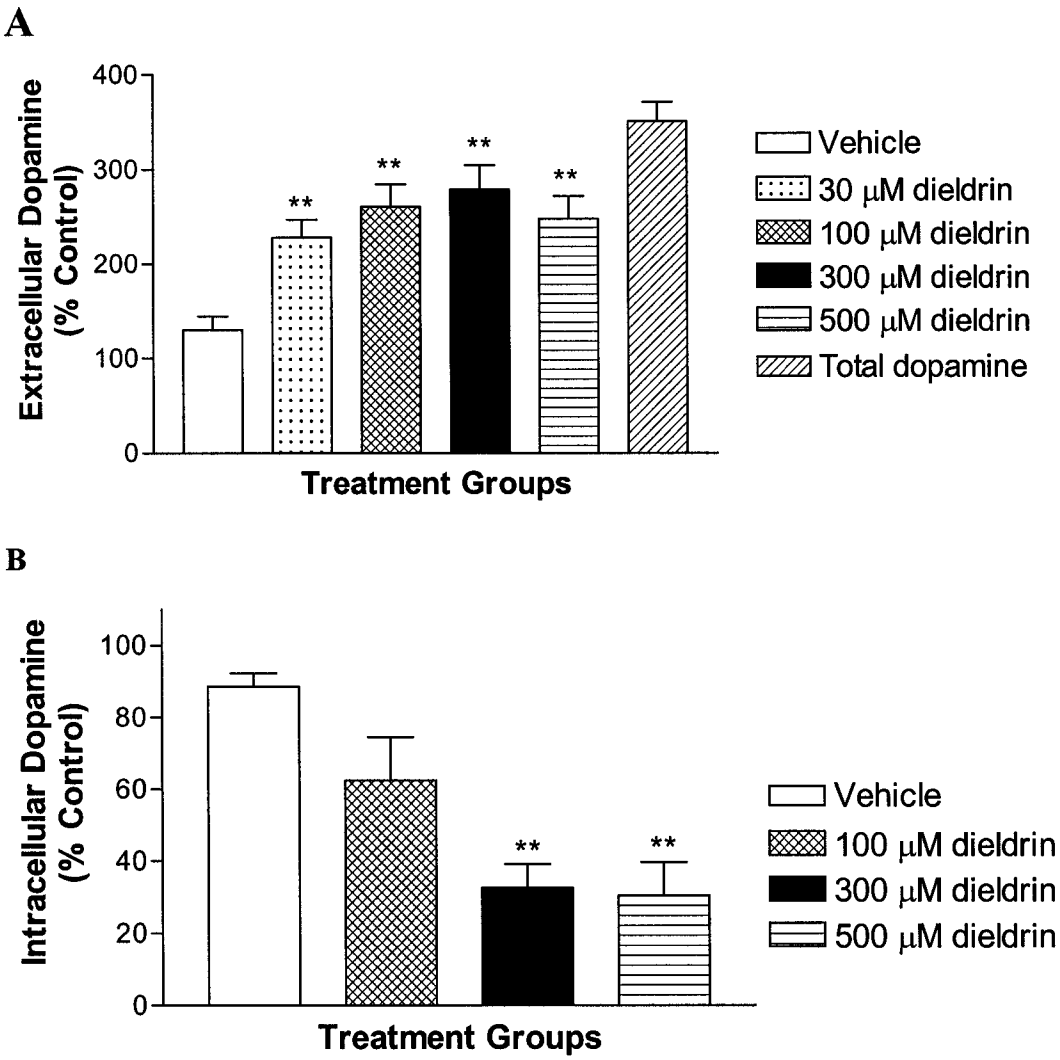

Figure 3: Effect of dieldrin on extracellular and intracellular dopamine levels in PC12 cells

Panels represent extracellular dopamine (A) and intracellular dopamine (B) levels at 1 hour in dieldrin-treated PC12 cells. Dopamine content was analyzed by HPLC-EC. Data represent mean ± SEM for two to three separate experiments performed in triplicate. Significance was determined by ANOVA followed by Dunnett's post-test between the vehicle-treated group and each treatment group (*$p<0.01$).

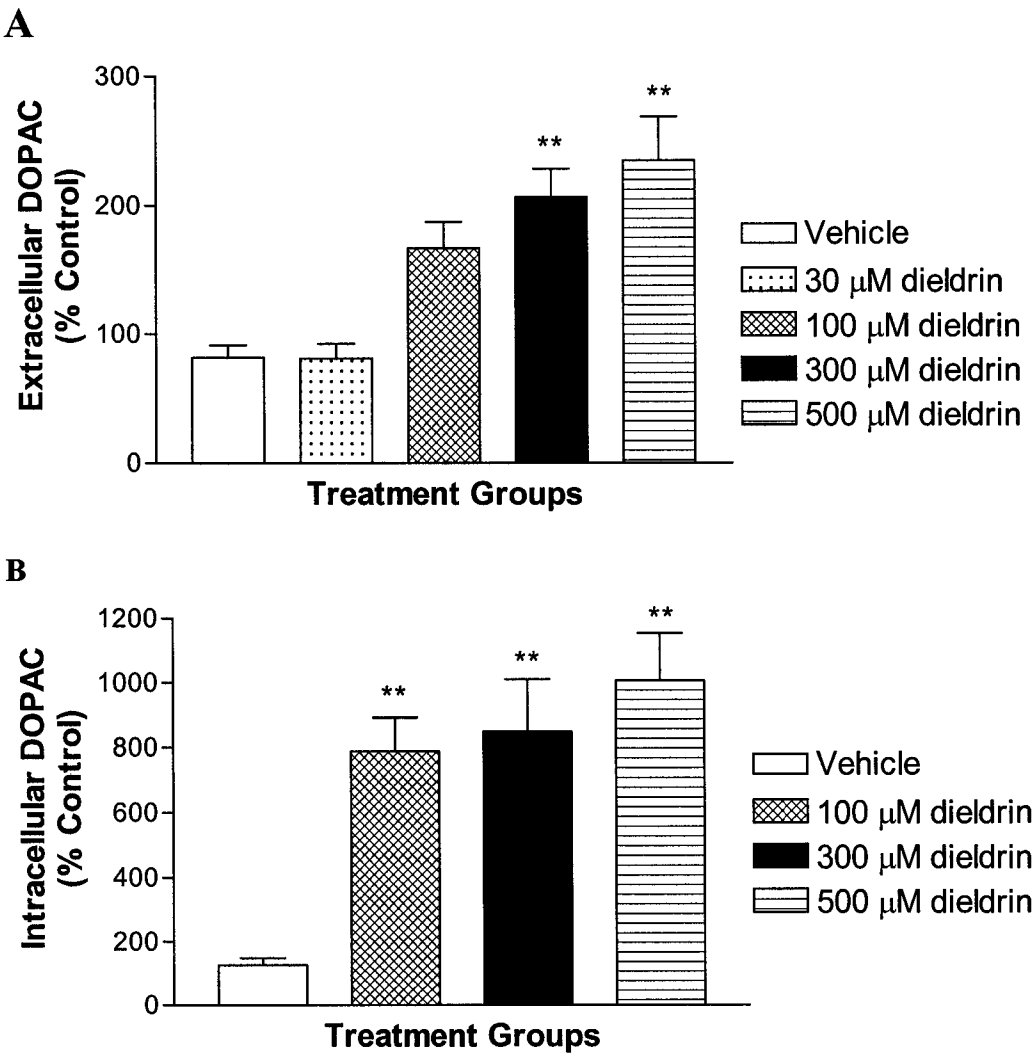

Figure 4: Effect of dieldrin on extracellular and intracellular DOPAC levels in PC12 cells

Panels represent extracellular (A) and intracellular (B) DOPAC levels at 1 hour in dieldrin-treated PC12 cells. DOPAC content was analyzed by HPLC-EC. Data represent mean ± SEM for two to three separate experiments performed in triplicate. Significance was determined by ANOVA followed by Dunnett's post-test between the vehicle-treated group and each treatment group (*$p<0.05$, **$p<0.01$).

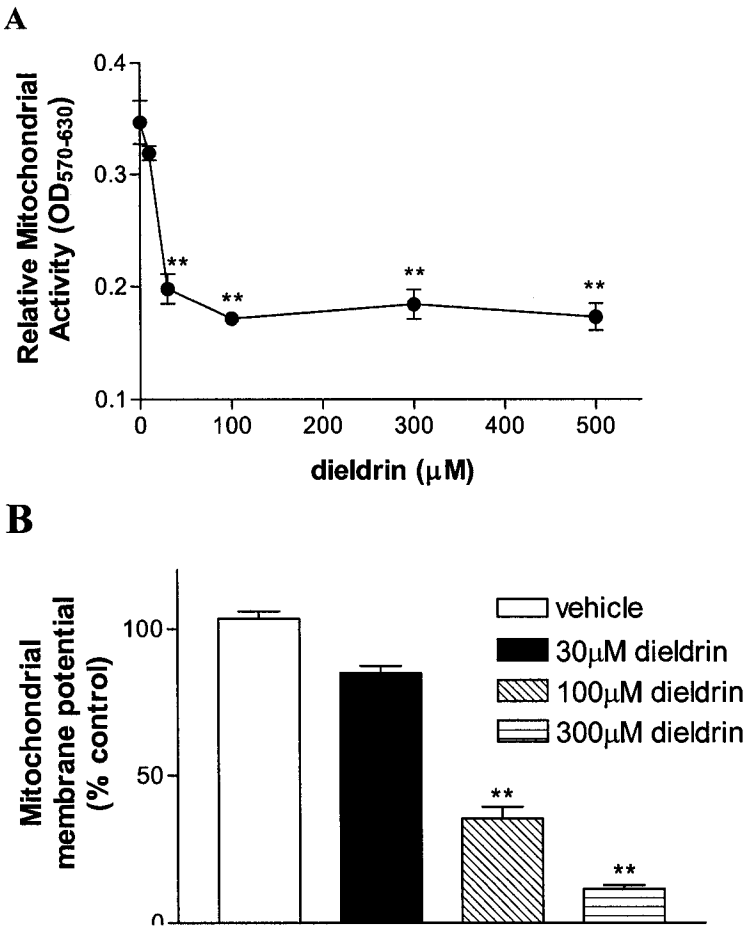

Figure 5: Effect on mitochondrial activity following dieldrin treatment

PC12 cells were treated with dieldrin for 1 hour. (A) MTT assay following dieldrin treatment in PC12 cells. Relative mitochondrial activity was calculated by absorbance at 570 nm and 630 nm. (B) depolarization of mitochondrial membrane potential ($\Delta\Psi m$) was measured by flow cytometer using 40 nM DiOC6. Relative fluorescence intensity was calculated, and depolarization of $\Delta\Psi m$ was expressed as percent of control. Data represent the mean ± SEM for two separate experiments performed in triplicate. Significance was determined by ANOVA followed by Dunnett's post-test between vehicle-treated cells and dieldrin-treated cells (**$p<0.01$).

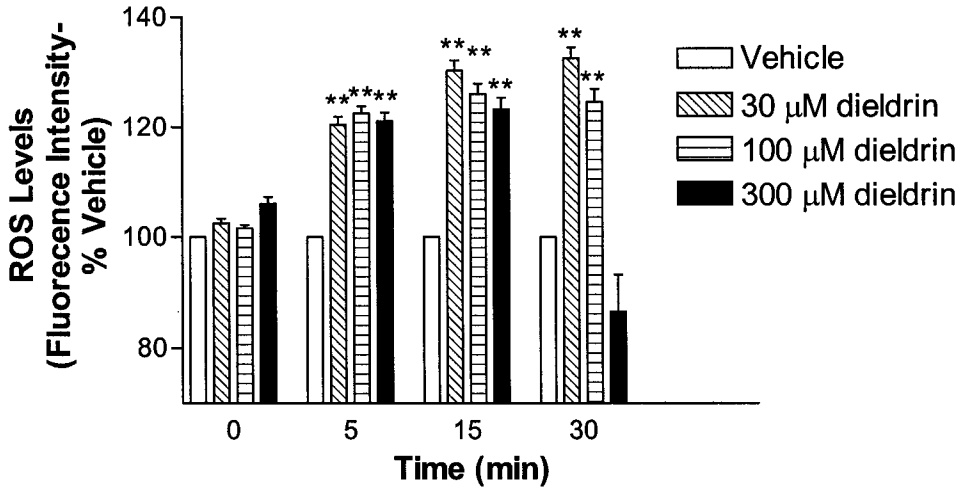

Figure 6: Generation of ROS following exposure to dieldrin in PC12 cells

PC12 cells were treated with varying concentrations of dieldrin (30, 100, 300 μM) for 0-30 min. Hydroethidine fluorescence intensity was measured at various time points (0, 5, 15, 30 min) by a flow cytometry. Data represent the mean ± SEM for three separate experiments performed in triplicate. Significance was determined by ANOVA followed by Dunnett's post-test between the vehicle-treated group and each treatment group (**$p<0.01$).

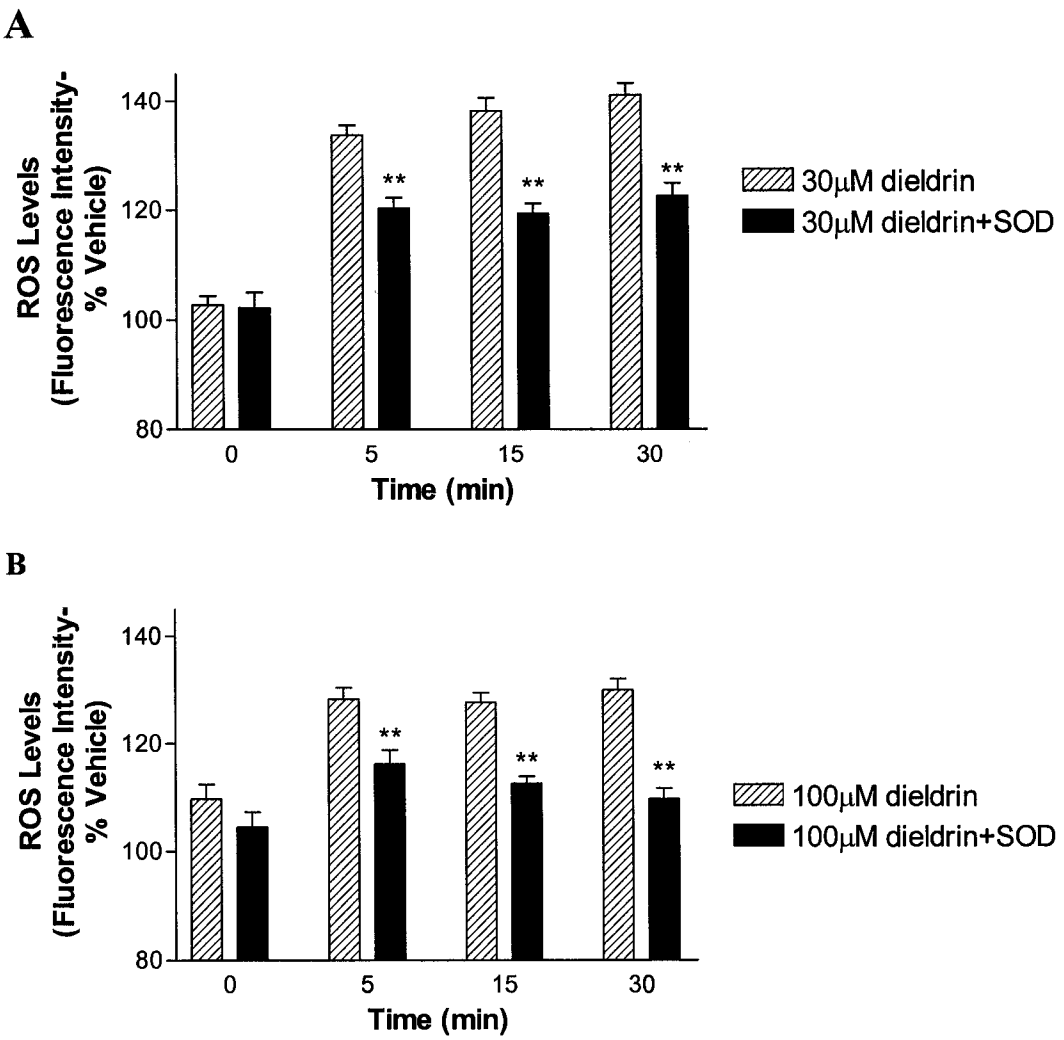

Figure 7: Effect of superoxide dismutase (SOD) on dieldrin-induced ROS generation

Panels represent the effect of SOD pretreatment (100 units, 5 min) on 30 μM (A) and 100 μM (B) dieldrin-treated PC12 cells at time points up to 30 min. Hydroethidine fluorescence intensity was measured by flow cytometry. Data represent the mean ± SEM for three separate experiments performed in triplicate. Significance was determined by ANOVA followed by Dunnett's post-test between the dieldrin treated cells with and without SOD pretreatment (**$p<0.01$).

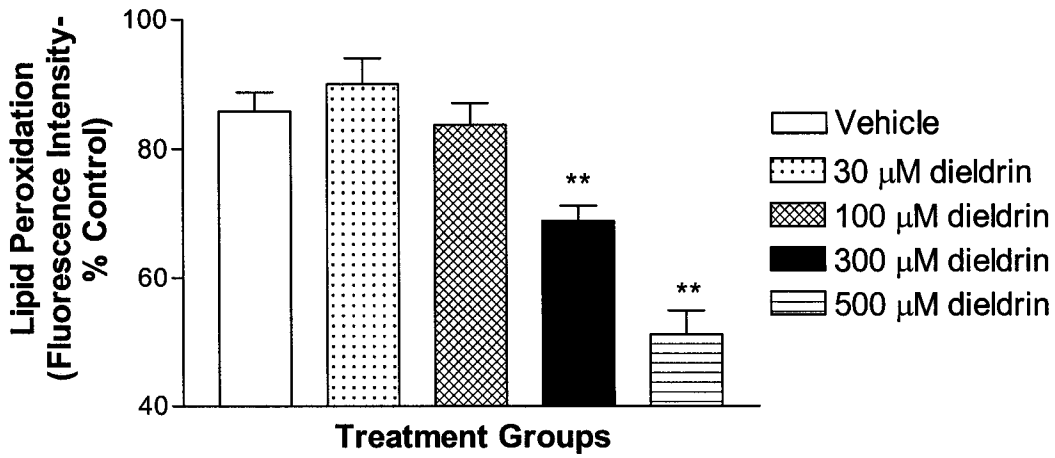

Figure 8: Formation of lipid peroxides following exposure to dieldrin in PC12 cells

PC12 cells were treated with varying concentrations of dieldrin (30, 100, 300 and 500 μM) for 1 hr. Dodecanyl aminofluorescein fluorescence intensity was measured by flow cytometry. Data represent the mean ± SEM for three separate experiments performed in triplicate. Significance was determined by ANOVA followed by Dunnett's post-test between the vehicle-treated group vs each treatment group (**$p<0.01$).

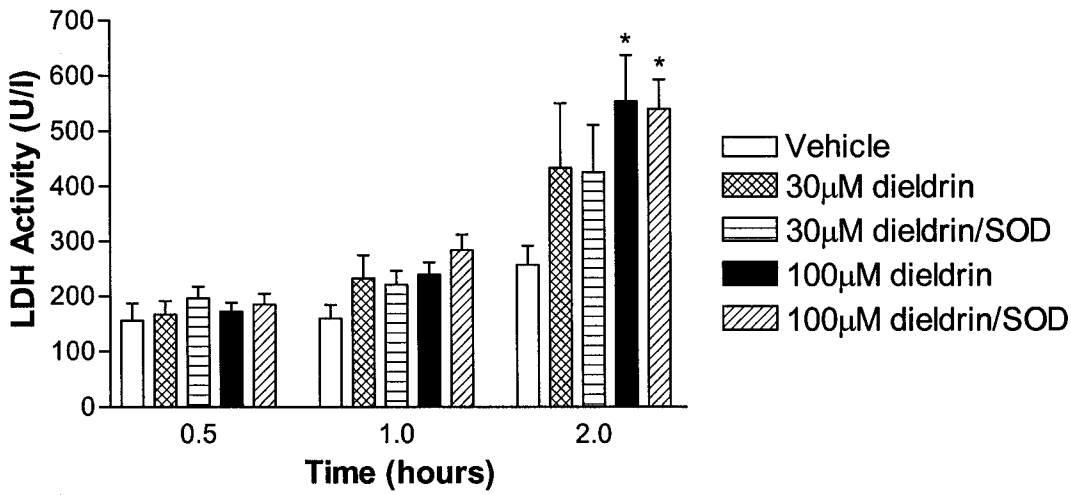

Figure 9: Effect of superoxide dismutase (SOD) on dieldrin-induced cytotoxicity

PC12 cells were exposed to 30 or 100 μM dieldrin for 1 hour, with or without SOD (100 units) pretreatment. LDH activity was measured in the incubation buffer by spectrophotometer in 96-well format. Data represent the mean ± SEM for three separate experiments performed in duplicate. Data were analyzed by ANOVA followed by Dunnett's post-test. SOD pretreatment did not show any significant decrease in LDH activity as compared with groups treated with dieldrin alone.

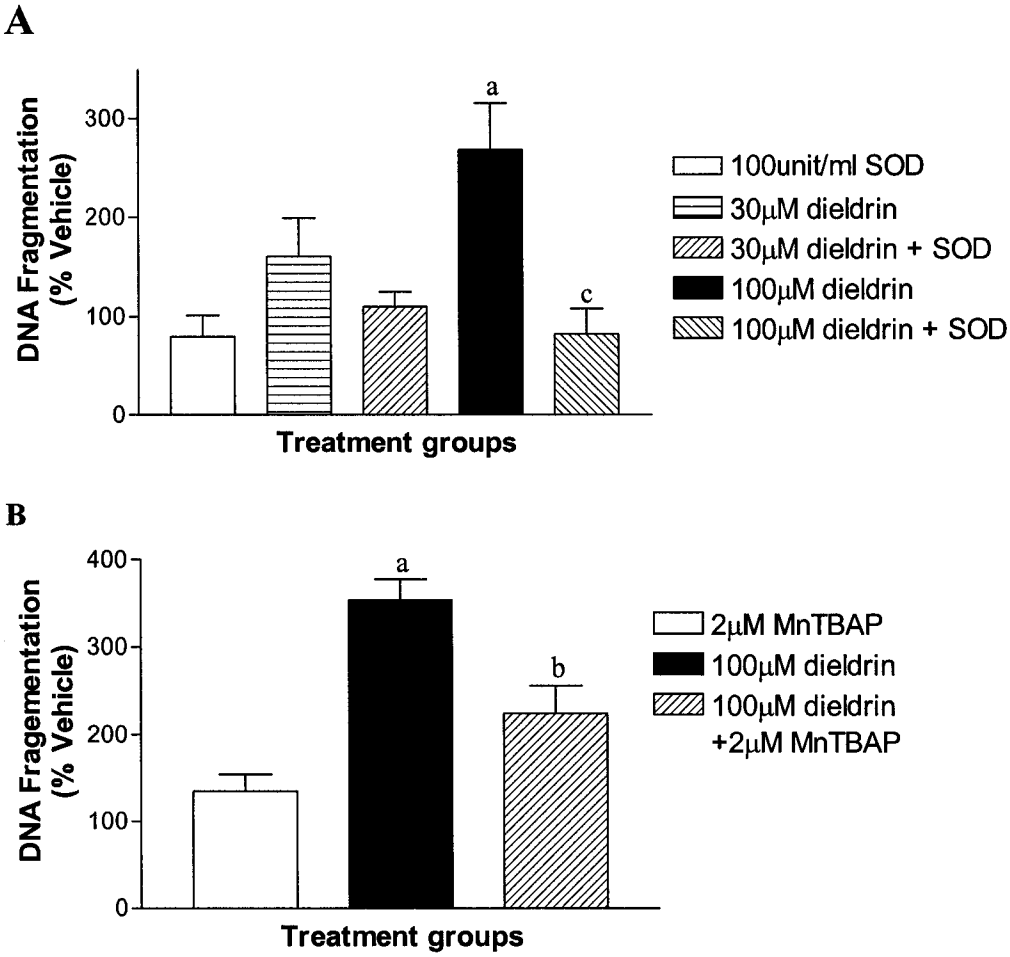

Figure 10: Effect of superoxide dismutase (SOD) on dieldrin-induced DNA fragmentation in PC12 cells

Panels represent results of DNA fragmentation assays in PC12 cells pretreated with SOD (A, 100 units/ml, 5 min) or MnTBAP (B, 2 μM, 30 min). DNA fragmentation was quantified by anti-histone-biotin directed against histones (H1, H2A, H2B, H3 and H4) and anti-DNA-POD directed against both single and double stranded DNA. Data represent the mean ± SEM for two separate experiments performed in triplicate. $^{a}p<0.01$ compared dieldrin-treated groups with vehicle-treated group using ANOVA followed by Dunnett's post-test, and $^{b}p<0.05$ or $^{c}p<0.01$ compared SOD- or MnTBAP-treated groups with dieldrin-treated groups using t-test.

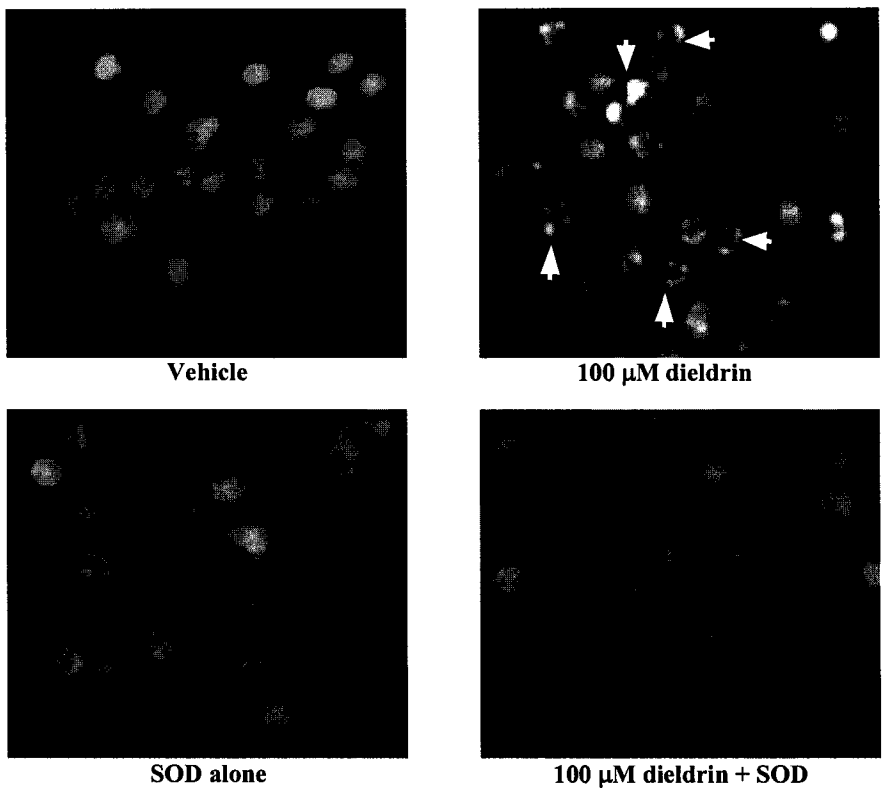

Figure 11: Hoechst 33342 staining of dieldrin-induced apoptosis in PC12 cells

Cultured PC12 cells were treated with dieldrin (100 or 300 μM) alone or in the presence of SOD (100 units) for 1 hour. Hoechst 33342 was used to visualize apoptosis by a fluorescent microscope under UV illumination. Arrows indicate apoptotic features of chromatin condensation. Each image represents in two separate experiments.

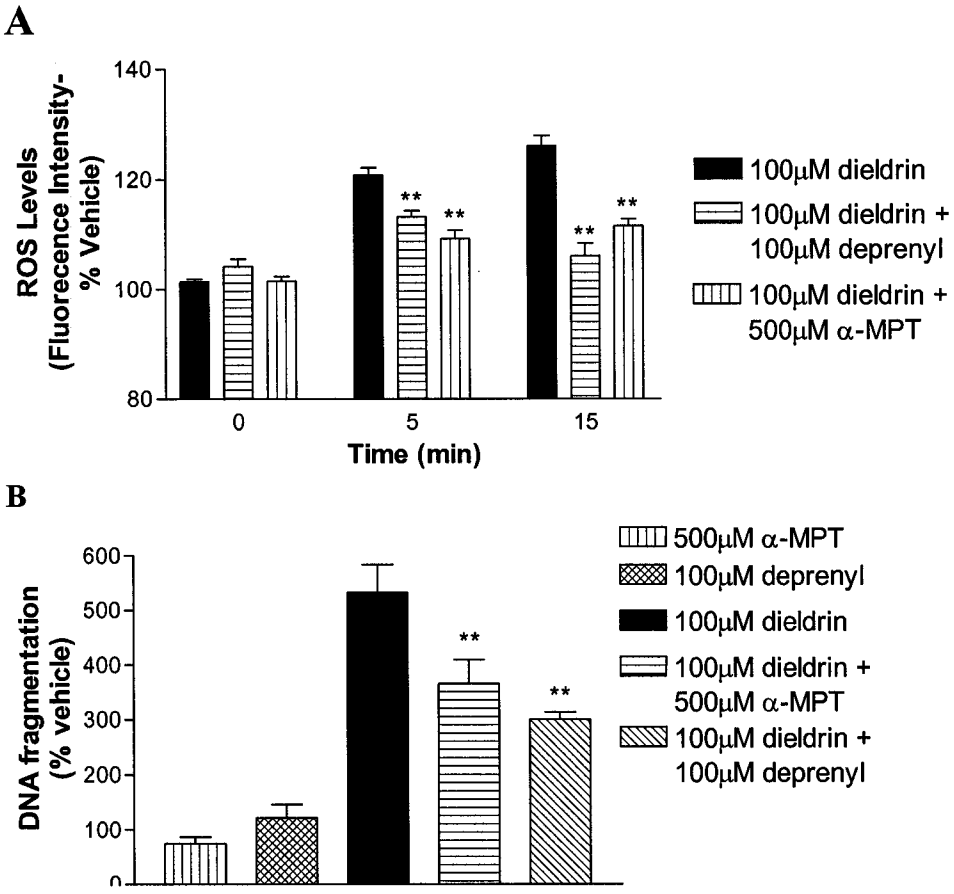

Figure 12: Effect of MAO-B or TH inhibitor on dieldrin-induced ROS generation and apoptosis

(A) PC12 cells were pre-treated with 500 μM α-methyl-L-p-tyrosine (α-MPT) for 24 hours or 100 μM deprenyl for 30 min prior to dieldrin exposure. Intracellular ROS was measured using hydroethidine in a flow cytometer during 0-15 min dieldrin exposure. The data represent mean ± SEM from two separate experiments in triplicate. *$p<0.05$ or **$p<0.01$ compared with dieldrin-treated group. (B) PC12 cells were pre-treated with 500 μM α-MPT for 24 hours or 100 μM deprenyl for 30 min prior to 100 μM dieldrin exposure for 1 hour. DNA fragmentation was quantified as described in "Material and Methods". Data represent the mean ± SEM for two separate experiments performed in triplicate. **$p<0.01$ compared with dieldrin-treated group.

CHAPTER III: OXIDATIVE STRESS AND MITOCHONDRIAL-MEDIATED APOPTOSIS IN DOPAMINERGIC CELLS EXPOSED TO METHYLCYCLOPENTADIENYL MANGANESE TRICARBONYL (MMT)

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Masashi Kitazawa, Jarrad R. Wagner, Michael L. Kirby, Vellareddy Anantharam, Anumantha G. Kanthasamy

ABSTRACT

Methylcyclopentadienyl manganese tricarbonyl (MMT), an organic manganese-containing gasoline additive, was investigated to determine whether MMT potentially causes dopaminergic neurotoxic effects. MMT is acutely cytotoxic and dopamine producing cells (PC12) appeared to be more susceptible to cytotoxic effects than non-dopaminergic cells (striatal GABAergic and cerebellar granule cells). MMT also potently depleted dopamine apparently by cytoplasmic vesicular release to the cytosol, a neurochemical change resembling other dopaminergic neurotoxicants. Generation of reactive oxygen species (ROS), an early effect in toxicant-induced apoptosis, occurred within 15 min of MMT exposure. MMT caused a loss of mitochondrial transmembrane potential ($\Delta\Psi m$), a likely source of ROS generation. The ROS signal further activated caspase-3, an important effector caspase, which could be inhibited by antioxidants (Trolox or N-acetyl cysteine). Pre-depletion of dopamine by using α-methyl-*p*-tyrosine (tyrosine hydroxylase inhibitor) treatment partially prevented caspase-3 activation, denoting a significant dopamine and/or dopamine byproduct contribution to initiation of apoptosis. Genomic DNA fragmentation, a terminal hallmark of apoptosis, was concentration-dependently induced by MMT but completely prevented by pretreatment with Trolox, deprenyl (MAO-B inhibitor) and α-methyl-*p*-tyrosine. A final set of critical experiments were performed to verify the pharmacological studies using a stable Bcl-2 overexpressing PC12 cell line. Bcl-2 overexpressing cells were significantly refractory

to MMT-induced ROS generation, caspase-3 activation, loss of $\Delta\Psi m$ and were completely resistant to MMT-induced DNA fragmentation. Taken together, the results presented here demonstrate that oxidative stress plays an important role in mitochondrial mediated apoptotic cell death in cultured dopamine producing cells following exposure to MMT.

INTRODUCTION

Methylcyclopentadienyl manganese tricarbonyl (MMT), an organic manganese-containing compound, has been recently legalized for use as a lead replacement in fuels in the United States and is marketed as HITEC 3000 or AK-33X and contains around 25% manganese (Zayed *et al.*, 1994; Frumkin and Solomon, 1997). In the past, the major health effects of MMT have centered on possible exhaust products and ambient particulates caused by MMT combustion. Excessive manganese exposure has been reported to cause Parkinsonian-like symptoms, known as Manganism (Calne *et al.*, 1994). Although Manganism differs from Parkinson's disease (PD) in neuropathology, clinical presentations of each disease are similar (Aschner, 2000). Furthermore, numerous epidemiological studies have demonstrated a positive association between environmental risk factors and increased incidence of idiopathic, geriatric-onset PD (Veldman *et al.*, 1998; Tanner *et al.*, 1999). The results of these studies demonstrated that no significant genetic correlate exists in geriatric-onset PD, which by implication suggests an environmental factor, and may contribute to the promotion of the disease. Considering the epidemiological and toxicological evidence collectively, MMT and its manganese combustion products may be considered as potential environmental risk factors for PD and its related disorders.

The manganese atom of MMT is linked to a methylcyclopentadiene ring and three carbonyl groups and these organic substitutions make MMT highly lipophilic, which might increase the bioaccumulation of this organometallic compound. Recently, a comparative toxicokinetic study in rats has demonstrated that the MMT-derived manganese accumulates in plasma 37 times more than inorganic manganese along with a

slower clearance rate (Zheng *et al.*, 2000). Gianutsos and Murray (1982) have also demonstrated MMT-induced dopamine depletion in rat brain, which is suggestive of deleterious cytotoxic effects. Concern has recently been expressed over toxicity of MMT itself due to the possibility of exposure through dermal absorption from accidental spills, deliberate use of gasoline as a solvent cleaner, and solvent abuse such as intentional gasoline fume inhalation (Zayed *et al.*, 1994). Further, Garrison *et al.* (1995) argue that critical exposure sources for MMT do not include engine exhausts, but instead accidental releases during manufacture, handling, transportation, and storage as most likely sources for environmental and human exposure.

Apoptosis, the presumed mechanism of nigrostriatal cell death in PD (Hirsch *et al.*, 1999; Offen *et al.*, 2000), can be initiated by either receptor-stimulated (e.g., Fas-ligand mediated) or toxicant-induced pathways. Both signal pathways share a mitochondrial link to downstream apoptotic events, the specifics of which can vary by initiator stimulus and cell type. A common link between varying detail of apoptotic pathways is the role of Bcl-2 as an inhibitory lock on apoptosis (Voehringer and Meyn, 2000). Overexpression of Bcl-2 in *in vitro* cell line studies has been shown to inhibit cell death and reduce reactive oxygen species (ROS) generation, which directly implicate mitochondria as prime targets in apoptotic cell death.

The present study entails a detailed assessment of MMT-mediated toxic effects in dopamine producing PC12 cells and extends work reported by us previously (Anantharam et al., 2002). PC12 cells have proven to be an *in vitro* experimental model of choice to study effects of various neurotoxic agents including 6-hydroxydopamine, MPP^+, paraquat, and manganese on dopaminergic cells (Shafer and Atchison, 1991; Desole *et al.*, 1997b; Li and Sun, 1999; Viswanath *et al.*, 2001; Anantharam *et al.*, 2002; Park *et al.*, 2002). PC12 cells are electrically excitable and neurosecretory (DA, NE and/or ACh), and contain many membrane-bound and cytosolic macromolecules associated with neurons (Shafer and Atchison, 1991). In the present study, we have examined cytotoxicity, neurotransmitter depletion, ROS generation, caspase-3 activation, depolarization of mitochondrial membrane potential, and DNA fragmentation as toxicological endpoints during acute exposure of MMT to delineate the early cellular

events that might contribute to the degenerative process in dopamine producing cells. Further, we demonstrated protective effects of Bcl-2 protein overexpression, antioxidants, and dopamine synthesis and catabolism inhibitors toward reduction of MMT-induced ROS generation, caspase-3 activation and DNA fragmentation, suggesting causal roles of mitochondrial dysfunction, oxidative stress and dopamine catabolism in MMT-induced cytotoxicity.

MATERIALS AND METHODS

Chemicals. Methylcyclopentadienyl manganese tricarbonyl (MMT; Aldrich, Milwaukee, WI) is a viscous yellow liquid with an herbaceous odor. MMT was prepared fresh and dissolved in DMSO. Trolox was purchased from Aldrich (Milwaukee, WI). Manganese (II) chloride, deprenyl, α-methyl-L-p-tyrosine (α-MPT), cytosine arabinoside and N-acetyl-L-cysteine (NAC) were obtained from Sigma (St. Louis, MO). Caspase-3 substrate, Ac-DEVD-AMC, was purchased from Bachem Biosciences Inc. (King of Prussia, PA). Cell culture media and reagents were purchased from Life Technologies Inc., (Gaithersburg, MD). Dihydroethidine, dichlorofluorescein-diacetate, acridine orange, $DiOC_6$ were purchased from Molecular Probes Inc., (Eugene, OR). Other routine laboratory reagents were obtained from Fisher Scientific (Pittsburgh, PA).

Cell culture. Dopamine producing PC12 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Non-dopaminergic cells were used in the cytotoxicity experiments to determine the differential toxic response of MMT in dopamine producing cells vs. non-dopaminergic cells. Striatal GABAergic cells (M213-20 cells) were a generous gift from Dr. William J. Freed, National Institute on Drug Abuse, Cellular Neurobiology Branch (Baltimore, Maryland). PC12 cells were grown in RPMI-1640 medium containing 10% heat inactivated horse serum, 5% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin and 50 μg/mL streptomycin. M213-20 cells were grown in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin and 50 μg/mL streptomycin. Human Bcl-2 transfected PC12 (PC12HB2-

3) and vector-transfected (PC12V4) cells were generous gifts from Drs. Yutaka Eguchi and Yoshihide Tsujimoto, Osaka University (Osaka, Japan). PC12V4 and PC12HB2-3 cells were grown in DMEM with 7% horse serum and 4% fetal bovine serum. PC12 cells were placed in 75 cm^2 cell culture flasks at 37°C under a humidified atmospheric condition of 5% CO_2 and 95% air, and 3-6 day old cells were used for the experiments. M213-20 cells were grown at 33°C in 5% CO_2 incubator. Cells were suspended in either Krebs-Ringer solution (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 6 mM glucose, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 2.4 mM $CaCl_2$, pH 7.4), serum-free RPMI-1640 or DMEM at a concentration of $2x10^6$ cells/ml depending upon the biochemical assay method.

Cerebellar granule cell cultures were prepared from 7-8 day old rat pup tissues by the method of Gunasekar *et al.* (1995). Cells were cultured in 10% fetal calf serum-amended DMEM, 22 mM glucose, 25 mM KCl and 1 ml penicillin/streptomycin (5000 U/ml)/l at pH 7.4 on poly-L-lysine-coated cover glass in 6-well culture plates. Cytosine arabinoside (10 μM) was added 18 hr later to prevent proliferation of non-neuronal cells. Mature cells (10 days *in vitro*) were used for experiments and ca. 95% of surviving cells were cerebellar granule cells.

Treatment paradigm. PC12 and M213-20 cells were treated with MMT (0–1000 μM) dissolved in DMSO (final concentration in incubates <0.5%). After 1 hr incubation with MMT at 37°C, dead and live cells were determined by trypan blue exclusion method using an Improved Nebauer Hemocytometer. Pretreatment with α-MPT was performed 24 hr prior to MMT treatment, whereas pretreatments with NAC, deprenyl or Trolox were performed 30 min prior to MMT exposure. The cell viability was normalized as percent of vehicle control.

Cerebellar granule cells (10 day) were treated with MMT (0-10 mM) for 1 hr. After 1 hr, the culture medium was removed and cells were lysed in a 0.1 M potassium phosphate buffer containing 0.5% Triton X-100 (pH 7.4). Cell debris was pelleted by centrifugation (10,000xg) and LDH activity was measured as described below.

Lactate dehydrogenase assay. Lactate deydrogenase (LDH) activity in the cell-free extracellular supernatant was quantified as an index of acute cell death in a 96-well format (Kitazawa *et al.,* 2001). Extracellular supernatant (10 µl) was added to 200 µl of 0.08 M Tris buffer (pH 7.2) containing 0.2 M NaCl, 0.2 mM NADH, and 1.6 mM sodium pyruvate. LDH activity was measured continuously by monitoring the decrease in the rate of absorbance at 339 nm using a microplate reader (Molecular Devices, Sunnyvale, CA) and the temperature was maintained at 37°C during reading. Changes in absorbance per minute ($\Delta A/\Delta T$) were used to calculate LDH activity (U/l), using the following equation: $U/l = (\Delta A/\Delta T) \times 9682 \times 0.66$, where 9682 was a coefficient factor, and 0.66 was a correction factor at 37°C. Activity was corrected per mg protein and expressed as percent total LDH signal.

Neurotransmitter determinations. Extracellular and intracellular dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured by high performance liquid chromatography with electrochemical detection (HPLC-EC) as previously described, with slight modification (Kanthasamy *et al.*, 1991). Briefly, PC12 cells were resuspended in Kreb's-Ringer solution at a density of $3\text{-}7 \times 10^6$ cells/ml. MMT was added, and cells were incubated in a shaking water bath for 2 hrs at 37°C. Cells were then centrifuged at 1,500xg for 15 min, and supernatants were collected and stored with antioxidant solution (Na_2EDTA, 15 mM; $Na_2S_2O_5$, 50 mM; $HClO_4$, 4 mM). Cell pellets were resuspended in antioxidant solution and allowed to lyse for 15 min on ice. Samples were stored at –80°C until further analysis. Prior to analysis by HPLC-EC, samples were centrifuged 10,000xg, 15 min, 4°C to pellet cellular debris.

The HPLC method was isocratic with a run time of 10 min at 0.7 ml/min using a Microsorb-MV™ (86-200-E3 C-18 3µm 100Å J2 10086-4) reverse phase column (Varian Analytical Instruments, Walnut Creek, CA) with 20 µl injection volume. Samples were maintained at 4°C prior to column injection. The HPLC system consisted of an electrochemical detector (Coulochem II Model 5200A; ESA, Chelmsford, MA) with a guard cell (ESA Model 5020) and a microdialysis cell (ESA Model 5014B) using

MD-TM™ mobile phase (ESA), with detector settings as follows: electrode 1, -175 mV, 100 μA; electrode 2, 175 mV, 50 nA; guard cell, 375 mV.

Reactive oxygen species (ROS) assay. We used dichlorofluorescein-diacetate (DCF-DA) to measure ROS by fluorometric assay. Briefly, the cells were harvested and washed in Kreb's-Ringer solution, resuspended at 1-3x10^6 cells/ml, and were loaded with DCF-DA, 15 min, 37°C. Cells were pelleted, supernatant containing the excess fluor was removed, and the pellet was then resuspended in 2 ml of Tris buffer (pH 7.4). Production of 2,7-dichlorofluorescein (DCF-H), a fluorescent product of hydrolyzed DCF-DA, was monitored over 1 hr by spectrofluorometer (488/525 nm). MMT was added to the tubes, vortexed and transferred to cuvettes for fluorescent readings (F(0)). After the readings were obtained, the samples were transferred back to the tube and placed in a water bath for 60 min incubation. After 60 min, the tubes were removed and the fluorescence was read again as the F(60) endpoint measurement. Data were expressed as percent of vehicle control.

Additional experiments were performed using a more selective ROS detection agent, dihydroethidine (Kitazawa *et al.,* 2001; Anantharam *et al.,* 2002). Dihydroethidine is a sodium borohydride-reduced hydroethidium dye that fluoresces upon oxidation by superoxide. Briefly, cells were suspended in Hanks balanced salt solution (HBSS) with 2 mM calcium at a density of 1 x 10^6 cells/ml. Cells were then incubated with 10 μM hydroethidine for 15 min in dark. After the incubation with dye, excess dye was washed once with HBSS, and MMT was added. Fluorescence intensity was measured using a flow cytometer (488/585 nm with 42 nm bandpass), and data were analyzed by CellQuest software (Becton Dickinson, San Francisco, CA).

Detection of mitochondrial membrane potential. Depolarization of mitochondrial membrane potential ($\Delta\Psi m$) were assessed by flow cytometric analysis using $DiOC_6$ (Kitazawa *et al.*, 2001). 40 nM $DiOC_6$ was added to incubation medium 15 min before the end of treatment period, and the incubation continued at 37°C. Then, the cells were washed once, resuspended in phosphate-buffer saline (pH 7.4), and analyzed by flow

cytometry with excitation at 484 nm and emission at 501 nm using a flow cytometer. Data were analyzed by CellQuest software (Becton Dickinson, San Francisco, CA).

Caspase-3-like activity assay. Caspase-3 activity was determined by following procedure previously described with slight modification (Yoshimura *et al.*, 1998). Briefly, after the exposure to MMT, cells were washed once with phosphate-buffered saline (pH 7.4) and resuspended in lysis buffer containing 50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 10 μM digitonin. Cells were then incubated at 37°C for 20 min to allow complete lysis. Lysates were quickly centrifuged, and cell-free supernatants were incubated with 50 μM Ac-DEVD-AMC (caspase-3 substrate) at 37°C for 1 hr. Formation of 7-amino-4-methylcoumarine (AMC), as a result of cleavage of substrates by caspase-3, was measured by spectrofluorometer (Molecular Devices) with excitation at 380 nm (slit width 10 nm) and emission at 460 nm (slit width 20 nm). Caspase activity was expressed as fluorescence unit (FU) per mg protein per hr of incubation. Protein content of samples were measured using the Bradford protein assay reagent (BioRad Laboratories, Hercules, CA).

DNA fragmentation assay. DNA fragmentation assay was performed using the Cell Death Detection Elisa Plus Assay Kit (Roche Molecular Biochemicals, Indianapolis, IN). This kit measures amount of histone-associated low molecular weight DNA in the cytoplasm of cells and has recently been used in quantitation of apoptosis because of its reliability and high sensitivity (Anantharam *et al.*, 2002). PC12 cells in antioxidant studies were pretreated with antioxidant for 30 min. Cells were then exposed to 200 μM MMT for 1 hr. Following MMT treatment, cells were pelleted at 200xg for 5 min and washed once with phosphate-buffered saline (pH 7.4). Cells were then incubated with a lysis buffer (supplied with the kit) at room temperature. After 30 min, samples were centrifuged and 20 μl aliquots of the supernatant were then dispensed into streptavidin-coated 96 well microtiter plates followed by addition of 80 μl of antibody cocktail and incubated for 2 hr at room temperature with mild shaking. The antibody cocktail consisted of a mixture of anti-histone biotin and anti-DNA-HRP directed against various

histones and antibodies to both ssDNA and dsDNA, which are major constituents of the nucleosomes. After incubation, unbound components were removed by washing with the incubation buffer supplied with the kit. Quantitative determination of the amount of nucleosomes retained by anti-DNA-HRP in the immunocomplex was determined spectrophotometrically with ABTS as a HRP substrate (supplied with the kit). Measurements were made at 405 nm against an ABTS solution as a blank (reference wavelength 490 nm) using a Molecular Devices Spectramax Microplate Reader.

Western blot analysis of Bcl-2 expression. PC12 cells were centrifuged at 200x g for 5 min. Cell pellets were then washed once with ice-cold Ca^{2+}-free phosphate-buffered saline and resuspended in 2 ml of homogenization buffer (20 mM Tris-HCl, pH 8.0, 10 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 10 μg/ml leupeptin). Suspensions were sonicated for 10 sec, and centrifuged at 100,000 x g for 1 hr at 4°C. Supernatants were discarded and pellets were gently resuspended in ice-cold Ca^{2+}-free phosphate-buffered saline. Protein concentration of each sample was determined using the Bradford protein assay reagent.

Membrane fractions containing equal amounts of protein were loaded in each lane of a 10% SDS-polyacrylamide stacking gel and separated by electrophoresis (23 mA, 3 hr). Proteins were then transferred to nitrocellulose membrane by electro-blotting overnight at 25 V. Non-specific binding sites were blocked by treating the nitrocellulose membranes with 5% non-fat dry milk powder for 2 hr prior to treatment with primary antibodies. The nitrocellulose membranes containing the proteins were incubated with primary antibodies for 1 hr at room temperature with antibody directed against Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibody treatments were followed by treatment with secondary HRP-conjugated antigoat IgG (Santa Cruz Biotechnology) for 1 hr at room temperature. Secondary antibody-bound proteins were detected using Amersham's ECL chemiluminescence kit. To confirm equal protein loading, blots were reprobed with an HSP-60 antibody (Santa Cruz Biotechnology).

***In situ* assessment of apoptosis.** Changes in nuclear morphology and DNA conformation of MMT-treated cells were assessed qualitatively with fluorescent DNA-binding dyes acridine orange. Acridine orange exhibits metachromatic fluorescence that is sensitive to DNA conformation. Apoptotic cells stained with acridine orange show reduced green and enhanced red fluorescence in comparison to normal cells (Kitazawa *et al.*, 2001). PC12 cells were grown on laminin (5 μg/ml) coated slides for 2-3 days in a 37°C, 5% CO_2 incubator. Cells were washed twice with phosphate-buffered saline (pH 7.4) and treated for 1 hr with MMT (200 μM). Cells were incubated with 10 μM acridine orange for 15 min at room temperature in the dark. Cells were again washed with phosphate-buffered saline, mounted with cover slips, and observed under a Nikon DiaPhot microscope with attached SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Data analysis and statistics. Data are expressed as mean ± SEM and statistical significance was determined by analysis of variance (ANOVA) with either Dunnett's test in the case of multiple comparisons with control or Tukey-Kramer means separation test for multiple comparisons between treatment groups. Single comparisons were performed by Student's t-test or Welch-corrected unpaired t-test where appropriate. Differences were accepted as significant at $p<0.05$ or less.

RESULTS

MMT decreases cell viability. PC12 cells were exposed to 0-1000 μM MMT for 1 hr and cell viability was measured by trypan blue dye exclusion. Figure 1A shows the relationship between MMT concentration (log μM) and percent cell survival relative to the control. Exposure of PC12 cells to various concentrations of MMT resulted in a concentration-dependent decrease in cell viability and the EC_{50} of MMT was calculated to be approximately 206 μM by three-parameter non-linear regression. Based upon these results, subsequent measurement of various biochemical indices in key mechanistic studies were performed in PC12 cells treated with 200 μM MMT. Treatment with as

high as 1 mM inorganic Mn^{2+} did not show any significant alteration in cell viability in PC12 cells for 1-hr exposure, and more than 24 hrs were required to induce cytotoxicity in PC12 cells (data not shown).

To determine whether dopamine producing cells are more susceptible to MMT toxicity, we compared the cytotoxic effect of MMT on PC12 cells with a GABAergic cell line (M213-20; Fig. 1A). EC_{50} for 1 hr exposure of M213-20 cells to MMT was approximately 591 μM. The difference in the EC_{50} values of PC12 and M213-20 cells was statistically significant (Fig 1A; $p<0.02$). In a separate study with cultured rat cerebellar granule cells using the trypan blue dye exclusion method showed these cells to be unresponsive to the toxic effects of MMT. Additional experiments were performed with cerebellar granule cells using a more sensitive acute toxicity assessment by measuring extracellular LDH (Fig. 1B). Using this method, cytotoxicity in cerebellar granule cells was only observed at very high concentrations of MMT (10 mM, $p<0.05$ compared with vehicle control). Thus, it appears that dopamine producing cells are more susceptible to MMT toxicity.

MMT causes dopamine depletion. Treatment of PC12 cells with MMT (0-1000 μM) for 1 hr resulted in a significant concentration-dependent depletion of intracellular dopamine (Fig. 2A; $p<0.0001$). Depletion of dopamine by MMT in PC12 cells appeared to be biphasic, with an initial depletion of ca. 44% at concentrations of 30-100 μM MMT, depleting dopamine further with increasing MMT concentration to >95% depletion at concentrations of MMT above 100 μM (Welch-corrected unpaired t-test, $p<0.02$; 300 μM vs. 100 μM MMT). Extracellular dopamine concentrations did not increase across the concentration range of MMT used here, suggesting that MMT does not promote dopamine secretion. Extracellular DOPAC increased 2.3 fold and appeared to increase inversely to cellular dopamine depletion, whereas intracellular DOPAC did not change significantly across the concentration range of MMT used here (Fig. 2B). It is presently unclear whether tyrosine hydroxylase inhibition contributes to observed dopamine depletion because of the short time (1hr) exposure of MMT. Further studies

are needed to determine the exact neurochemical mechanisms underlying MMT-induced dopamine depletion and DOPAC formation.

MMT facilitates ROS generation. Reduction of $\Delta\Psi m$ is an index of mitochondrial dysfunction and mitochondria are considered major sources of oxidative stress. When mitochondria are impaired, more reactive oxygen species (ROS) may be generated (Voehringer and Meyn, 2000). Enhanced generation of ROS was observed in PC12 cells at 1 hr following MMT treatment as measured by the ROS-detecting fluor DCF-DA (Fig. 3A). MMT treatment concentration-dependently increased DCF-H fluorescence, the peroxidized product of DCF-DA. Maximal production of ROS by MMT treatment was 316.3% of vehicle control with an EC_{50} for MMT treatment of 51.55 μM as determined by three-parameter logistic regression (r^2=0.93). Further support of ROS generation was confirmed by hydroethidine fluorescence measurements, which are relatively specific for superoxide radicals. Hydroethidine measurement of superoxide formation revealed a concentration-dependent increase in generation of ROS at 30 min posttreatment (Fig. 3B).

MMT activates caspase-3 activity in PC12 cells. Mitochondrial dysfunction and increased oxidative stress have been implicated in initiation of apoptosis in dopaminergic cells by treatment with various toxicants (Lotharius and O'Malley, 2000; Robertson and Orrenius, 2000; Kitazawa *et al.*, 2001). Since MMT significantly increased intracellular ROS level within 1 hr, other pro-apoptotic molecules may also be activated during the exposure period. We measured the activity of caspase-3, an effector cysteine-aspartate protease and one of the key pro-apoptotic molecules activated by various apoptotic stimuli, using caspase-3 specific fluorescent substrate Ac-DEVD-AMC following 0-200 μM MMT treatment in PC12 cells. Caspase-3 was activated concentration-dependently and caspase-3 activity showed 6- and 15-fold increases from basal level with 150 and 200 μM MMT exposure for 1 hr, respectively (Fig. 4A).

To examine the possible role of oxidative stress in activation of caspase-3, we pretreated PC12 cells with two different antioxidants, 1 mM Trolox or 5 mM N-acetyl-L-

cysteine (NAC), for 30 min, then exposed to MMT for another 1 hr. Cells treated with Trolox significantly ($p<0.05$) reduced both 150 and 200 μM MMT-induced caspase-3 activity (Fig. 4B), with caspase-3 inhibition ranging from 35-50%. Enigmatically, NAC was only effective at 150 μM MMT exposure but not at 200 μM MMT exposure (Fig. 4B).

Results of various studies with dopaminergic cell lines and cultured neurons have indicated that dopamine and/or dopamine oxidation products contribute to the level of oxidative stress and the propensity of cells to undergo apoptosis (Offen *et al.*, 1999; Kitazawa *et al.*, 2001). Since we observed profound changes in dopamine and DOPAC levels following MMT treatment (Fig. 2), we investigated the possibility of dopamine to contribute to oxidative stress by examining the effects of inhibition of dopamine degradation to DOPAC and inhibition of dopamine synthesis. Tyrosine hydroxylase inhibition by 500 μM α-MPT pretreatment for 24 hrs significantly reduced cellular dopamine concentrations (83.6 ± 2.1% reduction; $p<0.01$) and rendered DOPAC concentrations below the detectable limits of HPLC-EC method. MAO-B inhibition by pretreatment with 100 μM deprenyl for 30 min significantly reduced cellular DOPAC formation (79.7 ± 8.1% reduction; $p<0.01$), but did not alter cellular dopamine concentrations as verified by HPLC-EC. Pretreatment with α-MPT partially inhibited MMT-induced caspase-3 activation (Fig. 4B), thus suggesting a contribution of dopamine or dopamine metabolite(s) in apoptotic processes. Deprenyl pretreatment did not inhibit MMT-induced caspase-3 activation (data not shown), suggesting that excessive DOPAC formation may not play a role in initiation of caspase-dependent apoptosis.

MMT induces apoptosis in dopamine producing PC12 cells. Chromatin condensation and DNA fragmentation are unique morphological changes during the terminal phases of apoptotic cell death. Treatment of PC12 cells with MMT (200 μM) caused the formation of uncoiled DNA by qualitative assessment using acridine orange staining (Fig. 5A). Using a more sensitive ELISA-based method to measure DNA fragmentation, we observed approximately a 170% increase in DNA fragmentation with

200 μM MMT exposure within 1 hr (Fig. 5B). Previously, we also demonstrated that caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK effectively attenuated MMT-induced DNA fragmentation, thus caspase-3 plays an important role in apoptosis following MMT exposure (Anantharam *et al.*, 2002).

Mitochondrial dysfunction and the subsequent increase of intracellular ROS levels may be the initial responses for triggering apoptotic cascade. To further determine whether MMT-induced ROS generation plays a role in apoptotic cell death, cells were treated with 1 mM Trolox, which showed profound inhibition of MMT-induced caspase-3 activation, for 30 min prior to exposure to 200 μM MMT. Trolox significantly ($p<0.05$) inhibited the nearly 2-fold MMT-induced increase in DNA fragmentation (Fig. 5B).

We further investigated whether dopamine plays any role in downstream apoptotic cascade, which could help to explain selectivity of MMT for dopamine producing cells. As shown in Fig. 5B, depletion of dopamine by α-MPT treatment prior to MMT exposure significantly inhibited MMT-induced genomic DNA fragmentation. Interestingly, deprenyl (30 min treatment) also attenuated DNA fragmentation observed at 1 hr MMT exposure (Fig. 5B).

Bcl-2 attenuates MMT-altered loss of mitochondrial membrane potential ($\Delta\Psi m$). In order to better substantiate the evidence from pharmacological studies performed here with respect to specific implication of mitochondrial dysfunction as a key factor in initiation of apoptotic cell death, we tested the effect of MMT on a transfected PC12 cell line overexpressing the apoptotic control protein Bcl-2. Verification of the level of Bcl-2 expression in vector-only control (PC12V4) and Bcl-2 overexpressed (PC12HB2-3) cell lines was performed by Western blot (Fig. 6A), with verification of equal protein loading per gel lane by reprobe with anti-HSP60. To measure mitochondrion-specific effects, we utilized $DiOC_6$ to measure reduction of $\Delta\Psi m$ following MMT treatment. Acute (1 hr) MMT exposure concentration-dependently decreased $\Delta\Psi m$, and the reduction was significant ($p<0.01$) at 150 and 200

μM MMT in PC12V4 cells (Fig. 6B). The reduction observed was 58.6% and 48.2% of vehicle-treated group following 150 and 200 μM MMT exposure for 1 hr, respectively, whereas Bcl-2 overexpressed cells showed 106.8% at 150 μM MMT exposure, which completely attenuated the depolarization of $\Delta\Psi m$, and 72.2% at 200 μM MMT. Attenuation of $\Delta\Psi m$ by Bcl-2 was significant ($p<0.05$) at both concentrations of MMT.

Bcl-2 reduces MMT-induced ROS generation. To examine whether attenuation of MMT-induced depolarization of $\Delta\Psi m$ by Bcl-2 was the result of blocked ROS generation, we measured ROS generation in PC12V4 and PC12HB2-3 cells following MMT treatment. It has previously been shown that MMT rapidly increases cellular ROS level within 1 hr, and the peak ROS level is around 15-30 min following MMT treatment (Anantharam *et al.*, 2002). As shown in Figs. 7A and 7B, ROS generation increased following MMT exposure for 15 or 30 min in PC12V4 cells. Conversely, Bcl-2 overexpressed PC12HB2-3 cells showed reduced ROS generation, which is significantly ($p<0.01$) less than the levels in PC12V4 cells. Thus, Bcl-2 protein overexpression appears to attenuate MMT-induced ROS generation.

Bcl-2 overexpression protects against caspase-3 activation and DNA fragmentation. MMT-mediated mitochondrial dysfunction, as measured by the reduction of $\Delta\Psi m$, was effectively attenuated by Bcl-2 overexpression, suggesting that downstream cell death processes could be blocked only if mitochondria regulate MMT-induced apoptotic cell death. As shown in Figure 8A, PC12V4 cells showed 18- and 23-fold increase in caspase-3 activity following 150 and 200 μM MMT exposure, respectively. As we expected, caspase-3 activity in PC12HB2-3 cells was significantly ($p<0.01$) blocked following 1 hr MMT (150 μM) exposure, and increased caspase-3 activity was only observed at 200 μM MMT exposure.

In addition, we tested the involvement of mitochondrial Bcl-2 on MMT-induced DNA fragmentation. MMT exposure for 1 hr (100-200 μM) causes a concentration-dependent increase in DNA fragmentation in PC12V4 cells (Fig. 8B). Bcl-2

overexpressed PC12HB2-3 cells showed almost complete inhibition of MMT-induced DNA fragmentation, which correlates with inhibition of reduction of $\Delta\Psi m$, ROS generation and caspase-3 activity. Taken together with attenuation of caspase-3 activity by antioxidants, mitochondrial dysfunction and oxidative stress contributes to the activation of downstream apoptotic responses during MMT-induced toxicity in dopamine producing cells.

DISCUSSION

Health effects regarding the use of MMT as a tetraethyl lead replacement in automotive fuels have been studied predominantly from the aspect of manganese particulate combustion products. However, data presented here delineates a health risk potential associated with effects of the parent chemical. Accidental exposures through spills or unintended uses of MMT-amended gasoline provides a routine human exposure route which, noting both the demonstrated effects of manganese on dopaminergic neurochemical systems (Olanow *et al.*, 1996; Rodruigez *et al.*, 1998) and long blood plasma half-life of MMT (Zheng *et al.*, 2000), may potentially play a role in environmentally-mediated, geriatric-onset PD. Recent epidemiological and case-control studies support the role of environmental exposure to metals and other organic toxicants such as pesticides in idiopathic PD (Tanner *et al.*, 1999; Gorrel *et al.*, 1999).

One of the earliest cellular responses following MMT exposure is ROS generation. Oxidative stress has also been shown to mediate manganese-induced apoptosis in several *in vitro* models such as PC12 cells and HeLa cells (Desole *et al.*, 1997b; Oubrahim *et al.*, 2001) and *in vivo* in manganese-induced neurotoxicity of the rat (Desole *et al.*, 1997a), which is relevant within the context of combustion products of MMT-amended fuels. Since MMT appears to exert cytotoxic effects also as an organic complex of manganese, the potential danger of MMT-amended fuels is possibly enhanced by both pre-combustion (MMT) and post-combustion (manganese) product exposures (Garrison *et al.*, 1995). In this study, we have shown MMT-induced activation of apoptotic signals in

PC12 cells and have further elucidated the mechanisms of MMT-mediated cell death, which could have implications for effects of MMT on dopaminergic neuronal systems.

The results of the present study clearly demonstrate that the cytotoxic ability of MMT in *in vitro* systems resides in the capacity of MMT to kill cells by a mitochondria-mediated apoptotic mechanism and oxidative stress. One potential additional mechanism for MMT-induced oxidative stress was suggested by the apparent ability of MMT to produce DOPAC formation accompanied with intracellular dopamine depletion. It has been demonstrated previously in a number of studies (Spina and Cohen, 1989; Fabre *et al.*, 1999) that DOPAC metabolism generates H_2O_2 as a byproduct of the reaction, either alone or enhanced in the presence of metals, which could also add to ROS generation observed here.

Previous studies have demonstrated that certain environmental cyclodiene neurtoxicants such as dieldrin (Kitazawa *et al.*, 2001) and heptachlor epoxide (Kirby *et al.*, 2001) evoke dopamine release, which could have deleterious effects towards generation of ROS and activation of apoptotic pathways as a result of cytosolic pooling of recycled dopamine. Apoptosis resulting from cytosolic pooling of recycled dopamine can be attenuated by blocking expression of the presynaptic dopamine transporter (Simantov *et al.*, 1996). However, the results of the present work suggest that MMT-induced dopamine degradation may be an important event in dopaminergic toxicity. Striatal dopamine depletion has been observed in mice treated with MMT (Gianutsos and Murray, 1982) and may occur *in vivo* by this mechanism involving degradation of dopamine. Of additional note, recent work by Lotharius and O'Malley (2000) demonstrates that MPP^+ exerts neurotoxic effects not only by complex I inhibition but also by redistributing dopamine from the vesicular pool to the cytoplasm, which fosters conversion of dopamine to various neurotoxic quinones. However, the authors did not find an MPP^+-mediated reduction in mitochondrial transmembrane potential ($\Delta\Psi m$), in contrast to their results with rotenone, and suggest the existence of a peripheral source of ROS generation leading to catecholamine quinone production. In this respect, the effects of MMT on $\Delta\Psi m$ appear to better resemble the rotenone parkinsonism model (Betarbet

et al., 2000) and may potentially combine the mitochondrial impairment aspect of this model with the catecholamine quinone formation aspect of the MPP^+ model.

MMT cytotoxicity appears to be mediated by oxidative stress produced by both mitochondrial impairment and alteration of dopamine catabolism. Earlier studies by Autissier and colleagues (Autissier *et al.*, 1997a, 1997b) demonstrated direct effects of MMT on mitochondrial complex I and determined that the Mn^{2+} component of MMT is specifically responsible for altering the electronic configuration of the carbonyl groups to promote association with complex I. Additionally, MMT not only interferes with NAD^+-linked substrate energy transfer but also interferes with electron donation to ubiquinone, which results in a decrease in oxidative phosphorylation (Autissier *et al.*, 1977a). In our studies, measurement of the increase in activity of pro-apoptotic messengers (caspase-3) and expression of cytotoxic indices (increased DNA fragmentation) can be collectively inhibited by various protective agents such as Trolox (antioxidant) or α-MPT (tyrosine hydroxylase inhibitor). Deprenyl (MAO-B inhibitor) protected cells from DNA fragmentation, but did not have a measurable effect on reduction of caspase-3 activity which suggests that MMT-induced DNA fragmentation can occur by a caspase-3-independent pathway(s) as observed by other researchers (Volbracht *et al.*, 2001). Regardless, the majority of our results implicate the mitochondria as either the source of or target of ROS and link the process of apoptosis with early events directed at mitochondrial damage. The protective effects of tyrosine hydroxylase inhibition additionally implicate dopamine or dopamine byproducts as contributing components of apoptotic initiation and may partially help to explain the effects of MMT *in vivo* on dopaminergic systems (Gianutsos and Murray, 1982). Additionally, previous studies have demonstrated that inorganic manganese exposure produces selective neurotoxic effects on dopaminergic systems, including the nigrostriatal tract (Olanow *et al.*, 1996; Rodruigez *et al.*, 1998).

We further confirmed the mitochondrial impairment aspect of MMT cytotoxicity by constructing PC12-derived cell lines specifically tailored to overexpress the apoptotic control protein, Bcl-2. In the normal state, phosphorylated Bcl-2 in the mitochondrion forms stable heterodimers with pro-apoptotic control proteins in the same gene family

(Robertson and Orrenius, 2000; Adams and Cory, 2001). Bcl-2 heterodimeric complexes have the ability to inhibit apoptotic processes by direct interference with pro-apoptotic messengers (e.g., Bax, Bak), prevent opening of the mitochondrial permeability transition pore, and by unknown mechanisms fosters increased concentrations of reduced glutathione in the nuclear envelope and augmentation of the NAD(P)H energy pool in mitochondria (Voehringer and Meyn, 2000). The latter two effects also possibly have roles in both down-regulation of apoptotic pathways and increased transcription of anti-apoptotic/homeostatic genes dependent upon the redox status of the cell, a perspective based on several lines of evidence and advocated by Voehringer and Meyn (2000). Indeed, in the present study, stabilization of intracellular redox status by application of antioxidants appears to compliment hypotheses of Bcl-2 function regarding increased expression or activity of ROS-protective mechanisms.

Dephosphorylation of Bcl-2 tends to favor release of Bax and Bak to form homodimers and/or Bax/Bak heterodimers. These Bax and Bak formations promote cytochrome C release and mitochondrial transition pore opening, the latter of which results in loss of $\Delta\Psi m$ (Adams and Cory, 2001). Stabilization of mitochondrial transition pore closed-state in Bcl-2 overexpressed cells was verified by lack of reduction in $\Delta\Psi m$ following MMT exposure, whereas a loss of $\Delta\Psi m$ was observed in vector control cells treated with MMT. We also previously reported that MMT-induced caspase-3 activation is mediated by cytochrome C release from mitochondria (Anantharam *et al.*, 2001, submitted), which follows currently understood toxicant-induced apoptosis models (Tsujimoto, 1998; Robertson and Orrenius, 2000). Following cytochrome C release, Apaf-1 release from dephosphorylated Bcl-2 and subsequent Apaf-1 dimerization to cytosolic cytochrome C promotes cleavage and activation of pro-caspase-9, an important initiator caspase signalling downstream apoptotic events. These capsase-9-dependent events include, but perhaps may not be limited to, cleavage and activation of pro-caspase-3 and pro-caspase-7. Caspase-3 in particular is known to be a critical effector caspase (Abu-Qare and Abou-Donia, 2001; Adams and Cory, 2001), which activates a host of downstream pro-apoptotic effectors (e.g., PKC-δ, DNApk), inactivates a variety of downstream anti-apoptotic effectors (e.g., PARP, ICAD), and cleaves cytoskeletal

structural proteins (e.g., spectrin, actin, lamin). Overexpression of Bcl-2 in its normal phosphorylated state provides an abundant pool for sequestration of mitochondrial pro-apoptotic messengers and further underscores the results of the pharmacological studies reported here which suggest that initiation of apoptosis by MMT occurs through mitochondrial dysfunction.

In conclusion, the results presented here delineate that oxidative stress plays an important role in mitochondrial mediated apoptotic cell death in cultured dopamine producing cells following exposure to MMT. Forthcoming studies conducted in this laboratory will attempt to demonstrate whether nigrostiatal neurons are selectively susceptible to MMT-mediated neurotoxicity.

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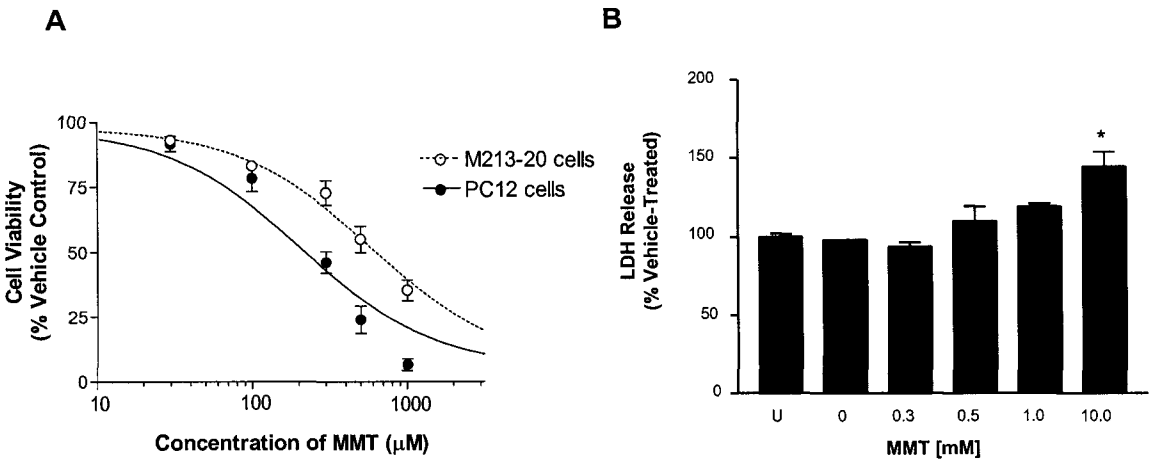

Figure 1: Effect of MMT on cell viability in dopamine-producing and non-dopaminergic cells.

(A) MMT-induced loss of cell viability after 1 hr treatment in dopamine producing PC12 cells and M213-20 cells was determined by trypan blue exclusion method and was analyzed by three-parameter logistic regression (EC_{50} and 95% confidence interval of EC_{50}: PC12 cells, 205.8 μM, 99.9-424.2 ; M213-20 cells, 590.5 μM, 453.4-769.0). EC_{50} values of PC12 and M213-20 cells were significantly different ($p<0.02$, Welch-corrected unpaired t-test). (B) Cytotoxicity of MMT in cerebellar granule cells was determined by measurement of extracellular lactate dehydrogenase (LDH) activity after 1 hr of treatment. 'U' denotes untreated cerebellar granule cells. Data represent results of at least three separate experiments in triplicate and are expressed as percent of vehicle-treated group (mean ± SEM). *$p<0.05$ compared with vehicle-treated group.

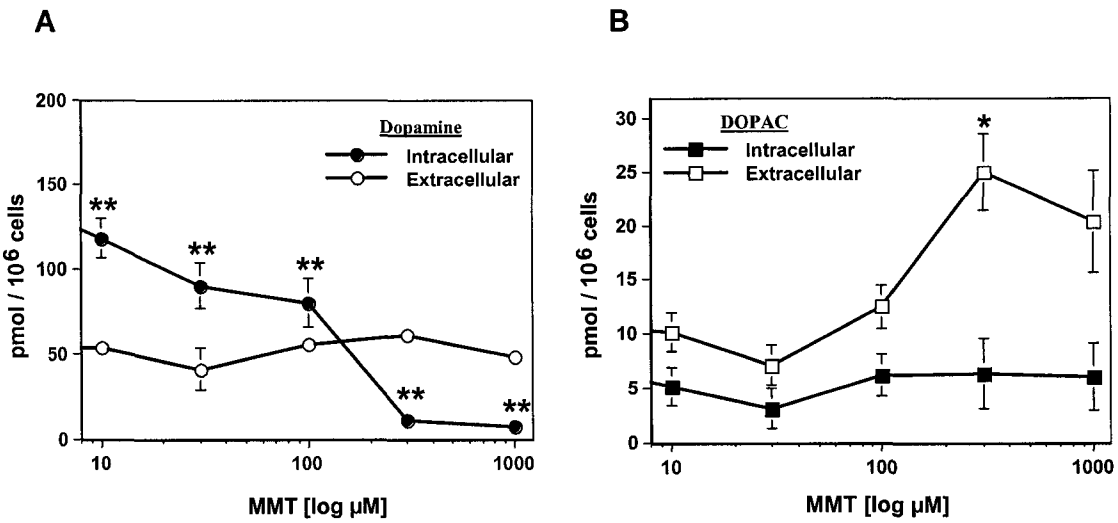

Figure 2: Dopamine depletion in MMT-treated PC12 cells.

Intracellular and extracellular dopamine (A) and 3,4-dihydroxyphenylacetic acid (DOPAC; B) levels were determined by HPLC-EC following 1 hr treatment of PC12 cells with MMT. Significant increases in extracellular DOPAC and decreases in intracellular dopamine were analyzed by ANOVA followed by a Dunnett's test (DA, $p<0.0001$; DOPAC, $p<0.02$). Data represent the mean ± SEM of four separate experiments and are expressed as pmols / 10^6 cells group. *$p<0.05$ and **$p<0.01$ compared with vehicle-treated group, respectively.

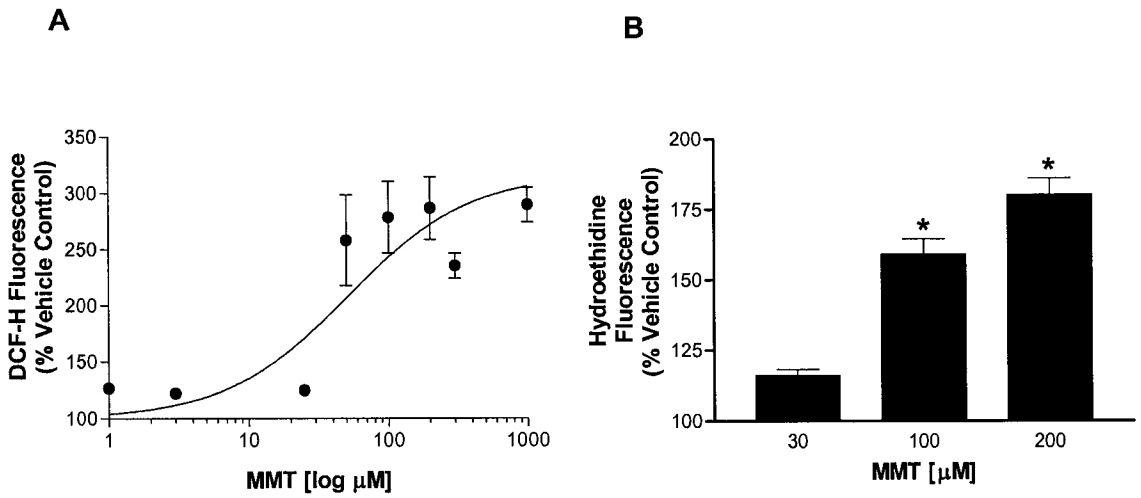

Figure 3: Concentration-dependent ROS generation in MMT-treated PC12 cells.

(A) DCF-H, the fluorescent dye product of peroxidized DCF-DA, was measured fluorometrically in MMT-treated PC12 cells at 1 hr post-treatment and analyzed by three-parameter logistic regression (EC_{50}: 51.52 ± 1.93 μM; r^2=0.94). (B) Hydroethidine, the fluorescent dye product of dihydroethidine, was measured by flow cytometer in MMT-treated PC12 cells at various concentrations up to 30 min. Data represent the mean ± SEM of 3-9 experiments and are expressed as percent vehicle control. *$p<0.01$ compared with vehicle-treated group.

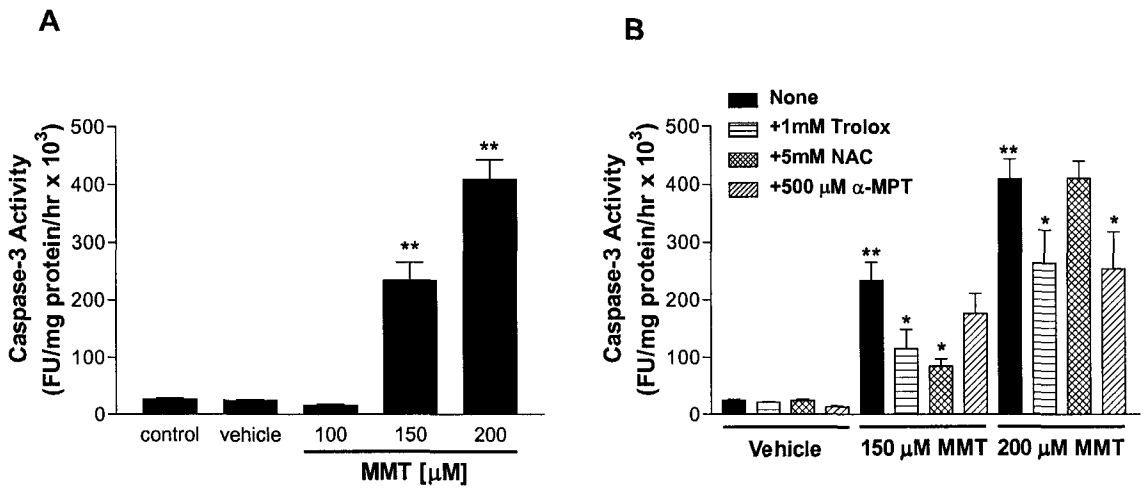

Figure 4: MMT-induced caspase-3 activation.

(A) PC12 cells were exposed to MMT (0-200 μM) for 1 hr, and caspase-3 activity was measured using a caspase-3 specific substrate, Ac-DEVD-AMC. (B) PC12 cells were pretreated with 1 mM Trolox or 5 mM NAC for 30 min or with 500 μM α-MPT for 24 hrs prior to exposure to MMT. Following 1 hr MMT exposure, then cytosolic fraction was collected, and caspase-3 activity was measured. All data represent the mean ± SEM for three separate experiments in triplicate. *$p<0.05$ or **$p<0.01$ compared with vehicle-treated group.

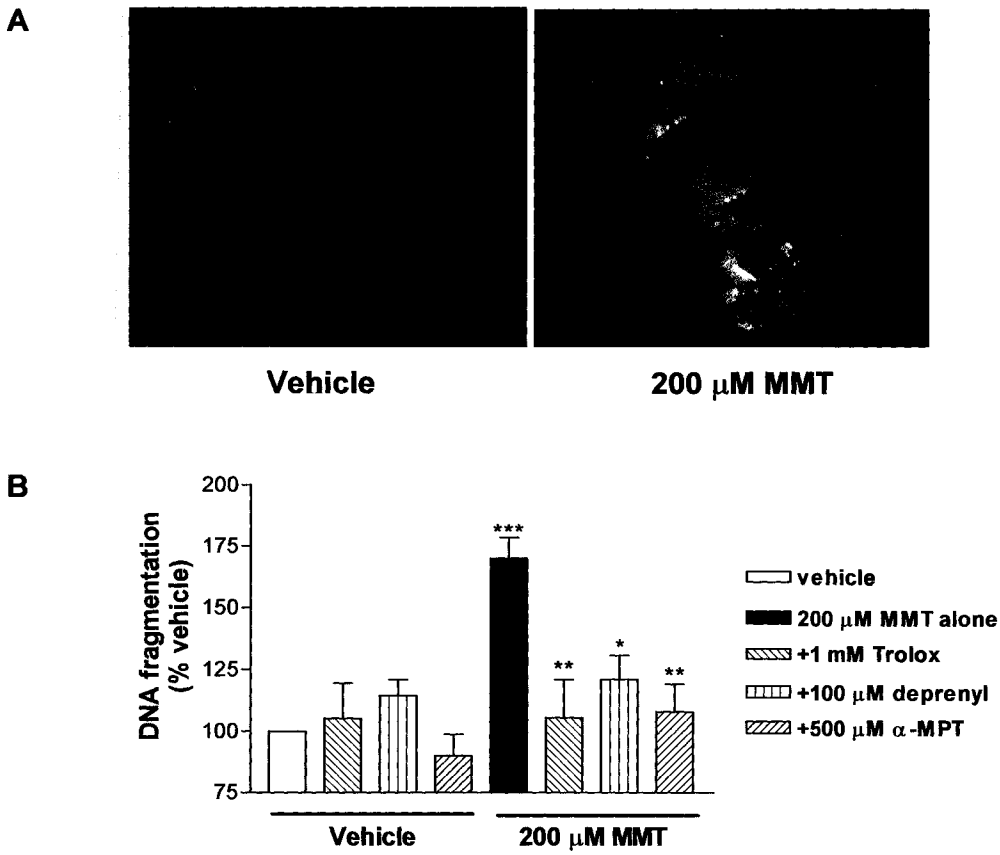

Figure 5: MMT-induced DNA fragmentation in PC12 cells.

(A) Qualitative assessment of nuclear degradation in 200 μM MMT-treated PC12 cells stained with acridine orange at 1 hr. Arrows denote apoptotic morphological changes (B) PC12 cells were treated (30 min) with 1 mM Trolox prior to treatment with 200 μM MMT. DNA fragmentation was measured by DNA ELISA assay following 1 hr of MMT treatment. Asterisks represent results of a Welch-corrected unpaired t-test comparing either Trolox alone or Trolox + MMT with MMT alone. Data are expressed as percent vehicle-treated group (mean ± SEM) of 3-6 experiments. *$p<0.05$ or **$p<0.01$ compared with vehicle-treated group.

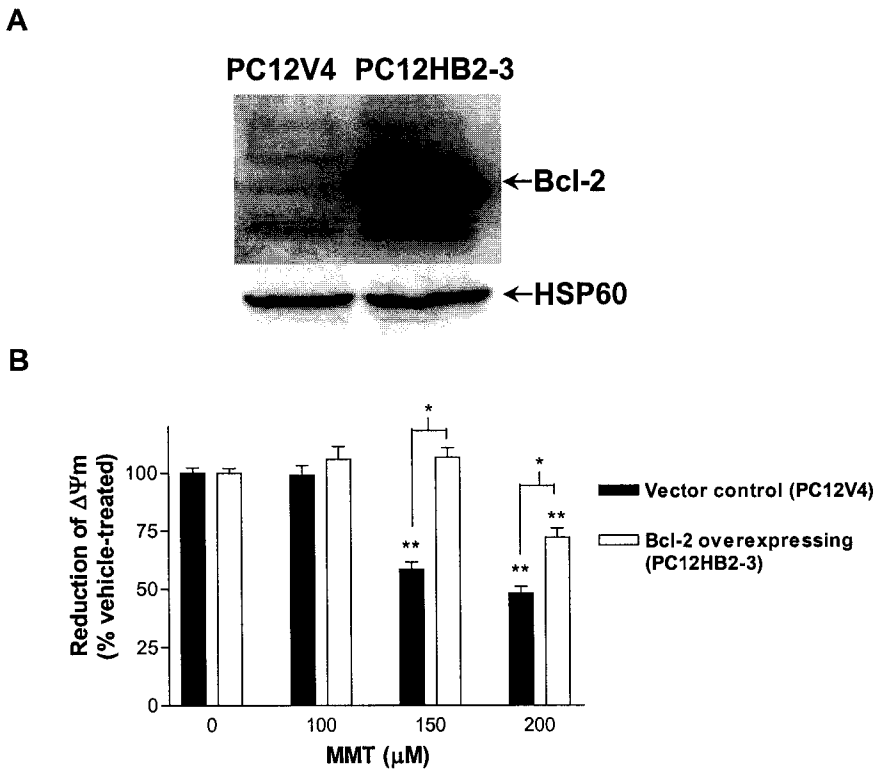

Figure 6: Overexpression of Bcl-2 protects PC12 cells against MMT-induced loss of mitochondrial transmembrane potential (ΔΨm).

(A) Overexpression of Bcl-2 in expression vector control (PC12V4) and Bcl-2 enhanced expression positive (PC12HB2-3) cells as verified by Western blot (top panel). Verification of equal protein loading per gel lane was verified by membrane reprobe for HSP60, a basic mitochondrial protein (bottom panel). (B) PC12V4 and PC12HB2-3 cells were exposed to 0-200 μM MMT for 1 hr, and reduction of ΔΨm was determined by flow cytometer using 40 nM $DiOC_6$. Relative fluorescence intensity was measured and expressed as percent of vehicle-treated group. Data represent the mean ± SEM for two separate experiments in triplicate. *$p<0.05$ or **$p<0.01$ compared with vehicle-treated group or between indicated treatment groups.

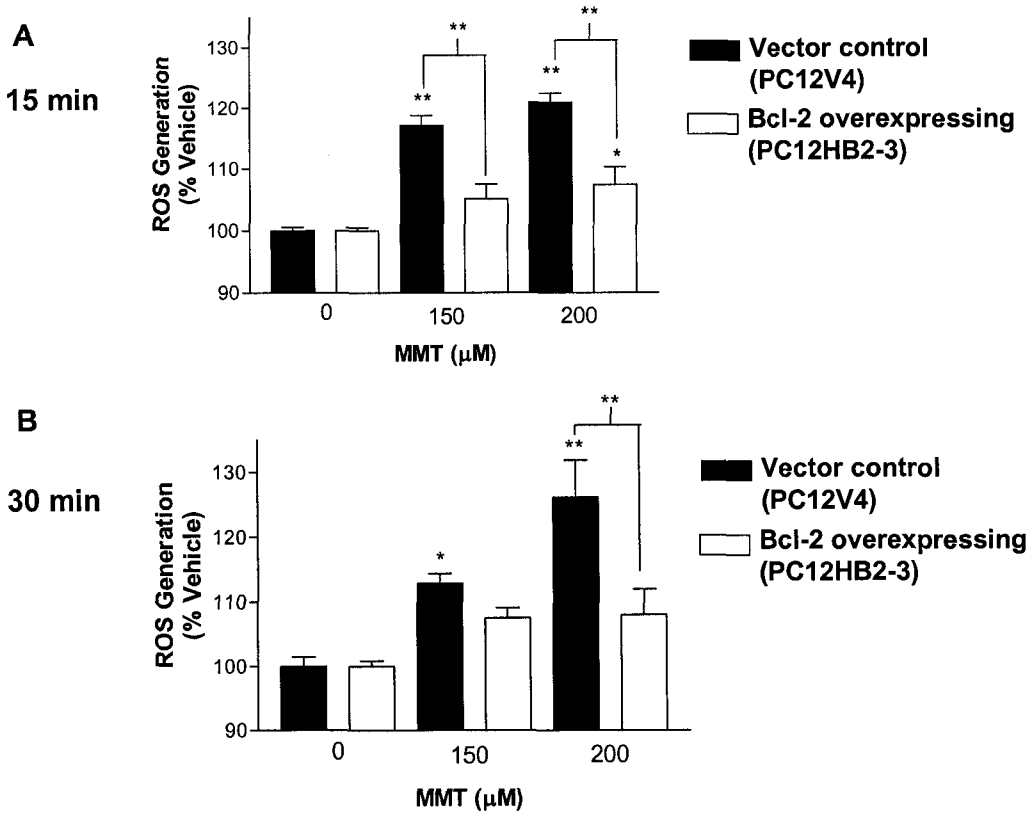

Figure 7: Overexpression of Bcl-2 significantly reduces MMT-mediated reactive oxygen species (ROS) generation.

PC12V4 and PC12HB2-3 cells were exposed to 0-200 μM MMT, and fluorescence intensity of dihydroethidine was measured by flow cytometer at 15 min (A) and 30 min (B) following MMT treatment. Data represent the mean ± SEM of 3 experiments in triplicate and are expressed as percent vehicle control. *$p<0.01$ compared with vehicle-treated group. **$p<0.01$ compared between vector-transfected and Bcl-2 overexpressed PC12 cells.

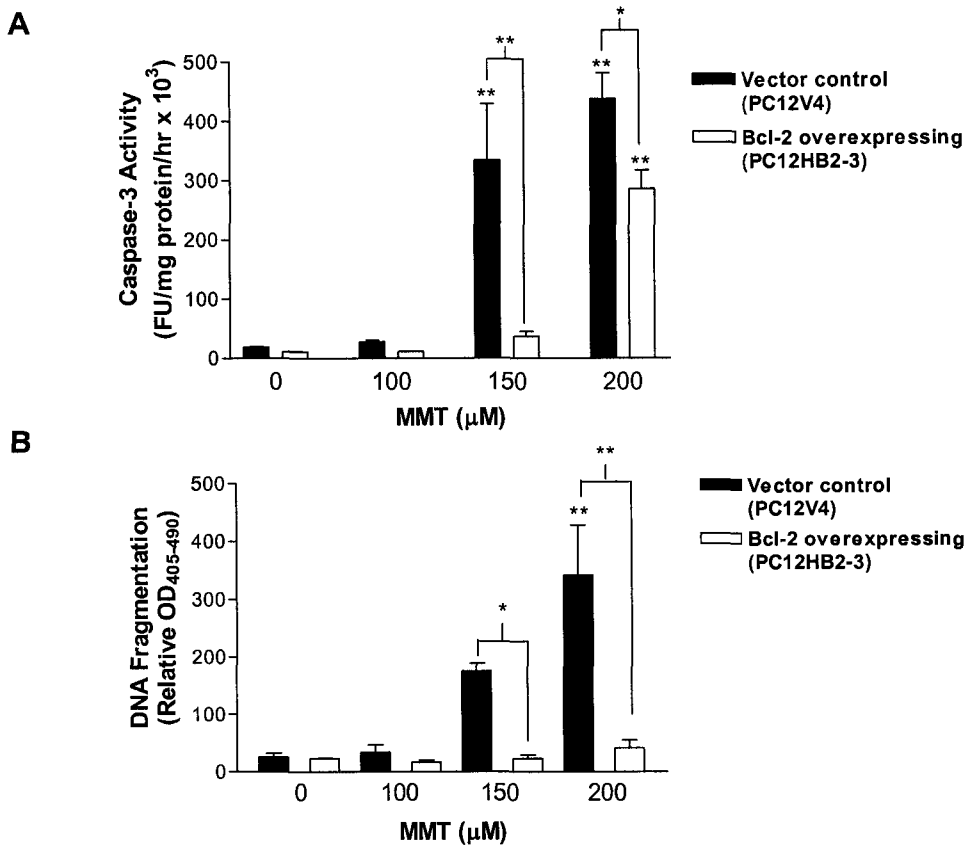

Figure 8: Overexpression of Bcl-2 protects PC12 cells against MMT-induced caspase-3 activation and nuclear DNA fragmentation.

(A) PC12V4 and PC12HB2-3 cells were exposed to MMT (0-200 μM) for 1 hr, and caspase-3 activity was measured. Caspase-3 activity was expressed as relative fluorescence units (FU) per mg protein per hr of incubation with caspase-3 substrate at 37°C. All data represent the mean ± SEM for 3 separate experiments in triplicate. *$p<0.05$ or **$p<0.01$ compared with vehicle-treated group or between indicated treatment groups. (B) PC12V4 and PC12HB2-3 cells were exposed to MMT (0-200 μM) for 1 hr and DNA fragmentation was measured by ELISA method. Data represent the mean ± SEM for 2 separate experiments in duplicate. *$p<0.05$ or **$p<0.01$ compared with vehicle-treated group or between indicated treatment groups.

CHAPTER IV: DIELDRIN INDUCES APOPTOSIS BY PROMOTING CASPASE-3-DEPENDENT PROTEOLYTIC CLEAVAGE OF PROTEIN KINASE Cδ IN DOPAMINERGIC CELLS: RELEVANCE TO OXIDATIVE STRESS AND DOPAMINERGIC DEGENERATION

A paper submitted for publication in Neuroscience

Masashi Kitazawa, Vellareddy Anantharam, and Anumantha G. Kanthasamy

ABSTRACT

We previously reported that dieldrin, one of the potential environmental risk factors for development of Parkinson's disease, induces apoptosis in dopaminergic cells by generating oxidative stress. Here, we demonstrate that the caspase-3-dependent proteolytic activation of protein kinase Cδ (PKCδ) mediates as well as regulates the dieldrin-induced apoptotic cascade in dopaminergic cells. Exposure of PC12 cells to dieldrin (100-300 μM) results in the rapid release of cytochrome C, followed by the activation of caspase-9 and caspase-3 in a time- and dose-dependent manner. The SOD mimetic MnTBAP significantly attenuates dieldrin-induced cytochrome C release, indicating that ROS may contribute to the activation of pro-apoptotic factors. Interestingly, dieldrin proteolytically cleaves native PKCδ into a 41 kDa catalytic subunit and a 38 kDa regulatory subunit to activate the kinase. The dieldrin-induced proteolytic cleavage of PKCδ and induction of kinase activity are completely inhibited by pretreatment with 50-100 μM concentrations of the caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK, indicating that the proteolytic activation of PKCδ is caspase-3-dependent. Additionally, Z-VAD-FMK, Z-DEVD-FMK or the PKCδ specific inhibitor rottlerin almost completely block dieldrin-induced DNA fragmentation. Because dieldrin

dramatically increases (40-80 fold) caspase-3 activity, we examined whether proteolytically activated PKCδ amplifies caspase-3 via positive feedback activation. The PKCδ inhibitor rottlerin (3-20 μM) dose-dependently attenuates dieldrin-induced caspase-3 activity, suggesting positive feedback activation of caspase-3 by PKCδ. Indeed, delivery of catalytically active recombinant PKCδ via a protein delivery system significantly activates caspase-3 in PC12 cells. Finally, overexpression of the kinase-inactive $PKC\delta^{K376R}$ mutant in rat mesencephalic dopaminergic neuronal cells attenuates dieldrin-induced caspase-3 activity and DNA fragmentation, further confirming the pro-apoptotic function of PKCδ in dopaminergic cells. Together, we conclude that caspase-3-dependent proteolytic activation of PKCδ is a critical event in dieldrin-induced apoptotic cell death in dopaminergic cells.

Key Words: neurodegeneration; oxidative stress; mitochondria; caspases; Parkinson's disease; environmental factors

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder among the elderly. Despite the intensive efforts in PD research, the exact cause of selective loss of dopaminergic neurons is poorly understood. The exogenous mitochondrial neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was discovered to cause a Parkinson's-like syndrome (Davis et al., 1979) and brought more attention to the possible role of environmental factors in the pathogenesis of PD. In support of an environmental hypothesis, case-control studies and epidemiological findings have revealed a higher association of PD in individuals exposed to chemicals such as pesticides, herbicides, and heavy metals (Wong et al., 1991, Semchuk et al., 1992, Seidler et al., 1996, Liou et al., 1997, Gorell et al., 1998). Recently, a study conducted on thousands of genetically identical twins concluded that genetic factors may play an important role in the

pathogenesis of young onset of PD, but not in the major form of sporadic PD (Tanner et al., 1999). The results of this study further suggested that environmental factors are dominant risk factors in sporadic PD, which develops in later stage of life.

Dieldrin is an organochlorine pesticide that was widely used agriculturally to control soil pests such as termites, grasshoppers, locusts, beetles, and textile pests. Dieldrin was also used to control tsetse flies and other vectors of tropical diseases including malaria, yellow fever, Chagas disease, Oraya fever, African sleeping sickness, river blindness, and filariasis (de Jong, 1991). After many years of widespread use, the U.S. Environmental Protection Agency (USEPA) restricted the use of dieldrin in 1974 due to its possible carcinogenicity and bioaccumulation. Based on its chemical stability, the USEPA has currently listed dieldrin as one of the top 12 priority persistent bioaccumulative and toxic (PBT) chemicals (www.epa.gov/opptintr/pbt/cheminfo.html). The Centers for Disease Control (CDC) includes dieldrin in the list of the top 20 human hazardous substances (http://www.atsdr.cdc.gov/cxcx3.html). Though dieldrin has been banned, humans continue to be exposed to the pesticide mainly through contaminated foods due to its persistent accumulation in the environment as well as its continued use in some developing countries (Suwalsky et al., 1997, Meijer et al., 2001). High serum dieldrin levels in farmers have recently been reported in some agricultural states in the U.S. (Brock et al., 1998). Potential adverse neurological effects from dieldrin exposure remain a concern. Several lines of evidence indicate that dieldrin exposure is positively associated with an increased incidence of PD (Fleming et al., 1994, Corrigan et al., 1998, Corrigan et al., 2000). Significant levels of dieldrin were detected in brains from PD patients, whereas no dieldrin was detected in age-matched control brains in several postmortem studies (Fleming et al., 1994, Corrigan et al., 1998, Corrigan et al., 2000). Moreover, dieldrin induces Parkinson's-like symptoms, including tremors, transient kinesia, and body rigidity in animals (Sharma et al., 1976, Wagner and Greene, 1978, Heinz et al., 1980). An *in vitro* study also demonstrated that dieldrin targets dopaminergic

neurons selectively (Sanchez-Ramos et al., 1998).

The mechanisms by which various environmental factors including dieldrin promote cell death in dopaminergic neurons are still elusive. In our previous study, we demonstrated that dieldrin induces dopamine release and generates significant levels of reactive oxygen species (ROS) in rat pheochromocytoma (PC12) cells, and our data support that dieldrin-induced ROS plays a causal role in apoptosis because pretreatment with antioxidants effectively attenuated dieldrin-induced apoptotic cell death (Kitazawa et al., 2001). The present study was undertaken to characterize the downstream signaling events of oxidative stress-induced apoptosis following dieldrin exposure. Herein, we report that caspase-3-mediated proteolytic cleavage of PKCδ contributes to apoptosis of dopaminergic PC12 cells following exposure to dieldrin. Furthermore, we demonstrate that activation of PKCδ not only plays a pivotal role in downstream execution process of dieldrin-induced apoptosis but also regulates upstream caspase signaling cascade through positive feedback mechanism.

EXPERIMENTAL PROCEDURES

Materials

Dieldrin, β-nicotinamide adenine dinucleotide, reduced form (NADH), sodium pyruvate, mouse monoclonal β-actin antibody, histone H1, β-glycerophosphate, ATP, Protein-A-sepharose, phosphatidylserine, dioleoylglycerol, human recombinant active PKCδ, Hoechst 33342 propidium iodide, and Fas ligand were purchased from Sigma Chemicals (St. Louis, MO). Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) was obtained from OXIS international Inc. (Portland, OR). Caspase-3 specific inhibitor, Ac-DEVD-CHO, and caspase-3 substrate, Ac-DEVD-AMC and Ac-DEVD-pNA, were purchased from Bachem Biosciences Inc. (King of Prussia, PA). Caspase-3 specific inhibitor, Z-DEVD-FMK, caspase-8 substrate, Ac-IETD-AMC, and caspase-9 substrate, Ac-LEHD-AMC, were purchased from Alexis Biochemicals (San

Diego, CA). Broad specific caspase inhibitor, Z-VAD-FMK, was purchased from Enzyme Systems Products (Livermore, CA). FITC-VAD-FMK was purchased from Promega (Madison, WI). Acridine orange was purchased from Molecular Probes (Eugene, OR). PKCδ specific inhibitor, rottlerin, was purchased from Calbiochem (La Jolla, CA). Antibodies to PKCδ, PKCα, and PKCβII were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse anti-cytochrome C antibody was purchased from BD Pharmingen (San Diego, CA). Cleaved caspase-3 antibody was purchased from Cell Signaling (Beverly, MA). ECL Western blotting analysis kit was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Cell Death Detection Elisa Plus Assay Kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Cytochrome C ELISA kit was obtained from MBL International Corp. (Watertown, MA). BioPORTER protein delivery kit was purchased from Gene Therapy Systems (San Diego, CA). [γ-^{32}P]ATP was purchased from NEN (Boston, MA). RPMI 1640 medium, heat inactivated horse serum, fetal bovine serum, L-glutamine, penicillin/streptomycin, and PCEP4 plasmid were purchased from InVitrogen (San Diego, CA). Other routine laboratory chemicals were obtained from Fisher Scientific (Pittsburgh, PA). Plasmids PKCδ^{K376R}-GFP fusion protein and pEGFP-N1 were kind gifts of Dr. Stuart H. Yuspa, National Cancer Institute (Bethesda, MD). Immortalized rat mesencephalic ($1RB_3AN_{27}$) cell line was a kind gift of Dr. Kedar N. Prasad, Univ. of Colorado Health Sciences Center (Denver, CO).

Cell culture and treatment

PC12 cells (pheochromocytoma, ATCC CRL1721) were cultured in RPMI-1640 medium containing 10% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 μg/ml streptomycin. Immortalized rat mesencephalic cell line ($1RB_3AN_{27}$ cells) was grown in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 μg/ml

streptomycin (Prasad et al., 1998). Both PC12 and $1RB_3AN_{27}$ cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Briefly, cells were resuspended in serum-free RPMI-1640 medium at a density of 2 x 10^6 cells/ml. Dieldrin was dissolved in DMSO and added to the cells. The DMSO concentration was maintained at less than 1% during incubation and used as the vehicle treatment. Caspase inhibitors, the PKCδ inhibitor, and a free radical scavenger (MnTBAP) were added to cell suspensions 30 min prior to the exposure to dieldrin and incubated at 37°C. After the treatment with dieldrin, cell suspensions were quickly centrifuged and cell-free supernatant was collected for extracellular lactate dehydrogenase (LDH) assay. Cell pellets were further processed to determine caspase activity, DNA fragmentation, or cytosolic protein analyses by Western blot. Cells treated with DMSO were used as a vehicle-control treatment for all experiments, and results from vehicle-treated cells were compared with dieldrin-treated cells to determine significance of dieldrin treatments.

Stable transfection

Plasmid pPKCδ^{K376R}-GFP encodes protein kinase Cδ-GFP fusion protein, in which lysine at position 376 is mutated to arginine rendering the kinase catalytically inactive (Li et al., 1995, Li et al., 1996). Plasmid pEGFP-NI encodes the Green Fluorescent Protein alone and used as vector control. pEGFP-N1 and pPKCδ^{K376R} were transfected into $1RB_3AN_{27}$ cells using Lipofectamine Plus reagent according to the procedure recommended by the manufacturer and described previously (Anantharam et al., 2002). To obtain stable cell lines, $1RB_3AN_{27}$ cells were selected in 400 μg/ml hygromycin 48 hr after cotransfection with the PCEP4 plasmid, which conferred hygromycin resistance. Colonies were isolated with trypsin and glass cloning cylinders and then replated and grown to confluence in culture flasks. The stable cell lines were subsequently maintained in 200 μg/ml hygromycin. The stable expression of PKCδ-GFP and GFP fusion protein alone was characterized by Western blot analysis. Antibody

directed against GFP detected ~100 kDa, and 27 kDa bands in cell lines expressing kinase inactive mutant PKCδ^{K376R}-GFP and GFP alone, respectively. Similarly, PKCδ antibody detected ~100 kDa and 72 kDa bands in cell line expressing PKCδ^{K376R}-GFP fusion, where as only a 72 kDa band was detected in cells expressing GFP alone. The 100 kDa, 72 kDa and 27 kDa bands obtained in Western blots correspond to the expression of intact mutant PKCδ^{K376R}-GFP fusion protein, native PKCδ and GFP protein, respectively.

Lactate dehydrogenase (LDH) assay

Cell viability was monitored by LDH activity in the cell-free extracellular supernatant as described previously (Kitazawa et al., 2001). This method of LDH release is a rapid and accurate quantitative procedure that has been widely employed to appraise cytotoxicity (Koh and Choi, 1987, Hartley et al., 1993, Anantharam et al., 2002). Extracellular supernatant was added to 0.08 M Tris buffer (pH 7.2) containing 0.2 M NaCl, 0.2 mM NADH, and 1.6 mM sodium pyruvate. The LDH activity was measured by continuously monitoring the rate of absorbance at 339 nm using a SpectraMAX 250 spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). Temperature was maintained at 37°C during quantification. Changes in absorbance per minute (ΔA/ΔT) were used to calculate U/I, which was a common unit to express the LDH activity. U/I was calculated by (ΔA/ΔT) x 9682 x 0.66, where 9682 was a coefficient factor, and 0.66 was a correction factor of U/I at 37°C.

Detection of cytochrome C release

Dieldrin-induced cytochrome C release was measured using a cytochrome C ELISA commercial kit (MBL International Corp., Watertown, MA). This is a fast, highly sensitive, and reliable assay for the detection of early changes in cytochrome C levels. Briefly, PC12 cells were resuspended in serum-free RPMI-1640 at a cell density of 5 x 10^6/ml. Cell suspensions were exposed to 100 μM or 300 μM dieldrin for 15-30 min at

37°C. After exposure, cells were collected and homogenized, and cytochrome C release in the cytoplasmic fraction was measured by an ELISA assay kit strictly following the protocol provided by the manufacturer. The absorbance in each well was then measured at 450 nm using a microplate reader (Molecular Devices Corp., Sunnyvale, CA). The concentration of cytochrome C was calibrated from a standard curve based on reference standards. Also, the control cytosolic preparation was analyzed for cytochrome C and mitochondrial protein marker cytochrome C oxidase by Western blot analysis to verify that the cytosolic isolation procedure does not cause mitochondrial damage in a non-specific manner.

Caspase activity assay

Caspase activities were determined as previously described (Yoshimura et al., 1998, Anantharam et al., 2002). After dieldrin treatment, cells were lysed and the resulting supernatants were incubated with 50 μM Ac-DEVD-AMC (caspase-3 substrate), 50 μM Ac-IETD-AMC (caspase-8 substrate), or 50 μM Ac-LEHD-AMC (caspase-9 substrate) at 37°C for 1 hr, and caspase activity was measured by spectrofluorometry (Molecular Devices Corp., Sunnyvale, CA) with excitation at 380 nm and emission at 460 nm. The Fas ligand was used as a positive control for caspase-8 activity (Facchinetti et al., 2002). Caspase activity was expressed as fluorescence unit (FU) per mg protein per hr. For measurement of caspase-3 activity in GFP-transfected cells, colorimetric substrate Ac-DEVD-pNA was used. 50 μM Ac-DEVD-pNA was added to the cytosolic extract and incubated for 4 hr at 37°C. The absorbance was measured by using Spectromax spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) at 405 nm.

Activated caspase-3 was also determined by Western blot analysis. Following dieldrin exposure, cells (~10 x 10^6 cells) were resuspended in cell lysis buffer (25 mM HEPES pH 7.5, 20 mM β-glycerophosphate, 0.1 mM Na_2VO_4, 0.3 M NaCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 2 mM EGTA, 0.5 mM DTT, 10 mM NaF, 1 mM PMSF, 25 μg/ml

aprotinin, 10 μg/ml leupeptin, and 0.1% Triton X-100), and kept in ice for 30 min. Samples were then centrifuged at 10,000 xg for 10 min, and supernatants were collected. 90 μg proteins were resolved by 12% SDS-PAGE, and cleaved caspase-3 was detected using cleavage-specific caspase-3 antibody (Cell Signaling, Beverly, MA).

***In-situ* labeling of caspase activity**

For this study, we used Promega's CaspACE kit to label PC12 cells. The kit utilizes FITC-VAD-FMK which is a fluoroisothiocyanate (FITC) conjugate of the cell permeable peptide sequences, VAD-FMK, which is cleaved by activated caspase and serves as an *in situ* marker for apoptosis. PC12 cells were grown on the cover slip coated by type I rat tail collagen (6 $\mu g/cm^2$) for 1 day in a 37°C, 5% CO_2 incubator. Cells were then exposed to 50, 100 or 300 μM dieldrin for 3 hr. After the exposure, the cells were washed once with PBS and treated with 10 μM FITC-VAD-FMK for 40 min at 37°C in dark. Cells were rinsed with PBS and fixed in 10% buffered formalin for 30 min at room temperature in dark. After fixing, the cells were washed three times with PBS for 5 min each to remove formalin and then mounted on slides and observed under Leica TCS-NT confocal microscope (Leica Microsystems Inc., Exton, PA). The caspase-activated cells displayed green fluorescence under the excitation at 488 nm.

Isolation of cytosolic fraction

After the exposure to dieldrin, cell pellet was washed once with ice-cold PBS and resuspended in 2 ml homogenization buffer containing 20 mM Tris/HCl (pH 8.0), 2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 10 μg/ml leupeptin. The suspension was then sonicated for 10 sec and centrifuged at 100,000 x g for 1 hr at 4°C. The resulting supernatant was used as a cytosolic fraction. Protein concentration of each cytosolic fraction was determined using a Bradford protein assay dye reagent (Bio-Rad Laboratories; Hercules, CA). Samples

were diluted with homogenization buffer according to the protein concentration estimated by the assay to equalize protein concentration for gel loading. Each sample was then mixed with 2x gel loading buffer containing 10% SDS and 200 mM DTT and placed in boiling water for 5 min.

Immunoblotting

Proteins were separated on 8-12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membrane. Non-specific binding sites were blocked by 5% non-fat milk blocking solution, then the membrane was treated with primary antibody for PKCδ (1:2000 dilution), cytochrome C (1:1000 dilution), PARP (1:750 dilution), or β-actin (1:5000 dilution). Secondary horseradish peroxidase-conjugated anti-mouse (1:2000 dilution) or anti-rabbit (1:2000 dilution) antibody treatment was followed, and antibody-bound proteins were detected by enhanced chemiluminescence (ECL) detection kit. Band intensity was analyzed using Quantity One 4.2.0 software (Bio-Rad Laboratories).

Protein kinase Cδ activity assay

PKCδ enzymatic activity was measured using an immunoprecipitation kinase assay as described in Reyland *et al.* (Reyland et al., 1999). Following dieldrin exposure, PC12 cells were washed once with PBS and resuspended in PKC lysis buffer (25 mM HEPES, pH7.5, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X 100, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 10 mM NaF, 4 μg/ml aprotinin, and 4 μg/ml leupeptin). The cell lysate was allowed to sit on ice for 30 min, centrifuged at 10,000 x g for 5 min and the supernatants were collected as cytosolic fraction. Protein concentration was determined using a Bradford protein assay. Cytosolic protein (0.25-0.5 mg) was immunoprecipitated overnight at 4°C using 2 μg of anti-PKCδ antibody. Immunoprecipitates were then incubated with Protein-A–sepharose for 1 hr at

4°C. The Protein-A-bound antigen-antibody complexes were then washed 3 times with PKC lysis buffer, 3 times with 2x kinase buffer (40 mM Tris, pH7.4, 20 mM $MgCl_2$, 20 μM ATP, and 2.5 mM $CaCl_2$), and resuspended in 20 μl of 2x kinase buffer. Reaction was started by adding 20 μl of reaction buffer containing 0.4 mg histone H1 and 5 μCi of [γ-^{32}P]ATP (4,500 Ci/mM) to the immunoprecipitated samples and incubated for 10 min at 30°C. SDS gel loading buffer (2x) was added to terminate the reaction, the samples were boiled for 5 min and the products were separated on a 12.5% SDS-PAGE gel. The H1 phosphorylated bands were detected using a Personal Molecular Imager (FX model, Bio-Rad Laboratories) and quantification was done using Quantity One 4.2.0 software (Bio-Rad Laboratories).

***In-situ* labeling of apoptosis**

Hoechst 33342 has been widely used to obtain nuclear condensation, which is one of distinct morphological changes observed in apoptotic cells (Siman et al., 1999, Saito et al., 2001). In combination with propidium iodide staining, we are able to distinguish necrotic cell death and apoptotic cell death under UV illumination using a fluorescent microscope. PC12 cells were grown on cover slips coated by type I rat tail collagen (6 μg/cm^2) for 1 day in a 37°C, 5% CO_2 incubator. Attached PC12 cells were treated with 100 or 300 μM dieldrin for indicated periods at 37°C. After the exposure, cells were washed once with PBS and treated with 10 μg/ml Hoechst 33342 for 5 min at room temperature in dark. Cells were then washed once with PBS and mounted on perfusion chamber with HBSS buffer and observed under Nikon DiaPhot microscope with the excitation wavelength at 380 nm. Fluorescent images were captured with a SPOT digital camera and analyzed by MetaMorph software (Universal Imaging Corp., Downingtown, PA). Quantitative analyses of stained cells were determined by counting cells in three to five randomly selected microscopic fields.

DNA fragmentation assay

DNA fragmentation was measured using a Cell Death Detection Elisa Plus Assay Kit as described previously (Reyland et al., 1999, Anantharam et al., 2002). Cells were pre-treated with various doses of caspase inhibitors or rottlerin for 20 min and then exposed to 100 or 300 μM dieldrin for 1 hr at 37°C. After the exposure, cells were centrifuged and washed once with PBS. DNA fragmentation was measured in cell lysates according to the manufacturer's protocol. The absorbance of the ELISA reaction was measured at 490 nm and 405 nm using a SpectraMAX Pro 250 spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

Intracellular delivery of active PKCδ

PC12 cells (~1-2 x 10^5 cells/well) were subcultured in 24-well tissue culture plate for 24 hr. Human recombinant active PKCδ was delivered into cells using BioPORTER reagent as strictly following manufacture's protocol. This reagent has recently been used for delivery of various bioactive molecules, including antibodies, enzymes (caspase-3, caspase-8, β-galactosidase, kinases and granzyme B), cytochrome C, dextran sulfates, phycobiliproteins and albumins into the cytoplasm of numerous adherent and suspension cells (Reyland et al., 1999). Briefly, 5 ng of catalytically active human recombinant PKCδ (Sigma Chemicals, St. Louis) or heat-inactivated PKCδ (15 min in boiling water) was mixed with BioPORTER reagent and delivered into cells for 4 hr in serum-free DMEM. Following the completion of delivery, cells were collected, and caspase-3 activity was measured as described above. The efficiency of the protein delivery system was determined by FITC-labeled control protein (supplied by the kit) uptake. PC12 cells were treated 5 ng of FITC-labeled goat IgG in serum-free medium for 4 hrs at 37°C. Cells were washed 3 times with PBS and observed under fluorescent microscope at emission 488 nm. To determine the delivery efficiency, fluorescent cells and non-fluorescent cells were counted in 5 randomly selected regions. The delivery efficiency of BioPORTER reagent

was approximately 70%.

Data analysis and statistics

Data were first analyzed using one-way ANOVA. Dunnett's post-test or Bonferroni's multiple comparison test was then performed to compare treated samples, and $p<0.05$ was considered to be significant.

RESULTS

Dieldrin-induced cytotoxicity and apoptosis

First, we characterized dieldrin-induced cytotoxicity over a 5 hr period to determine the sequence of cell death signaling mechanisms activated during dieldrin exposure in PC12 cells. To assess dieldrin-induced necrosis and apoptosis, we measured LDH and DNA fragmentation, respectively. Cytotoxicity proportionally increased over time during dieldrin exposure, indicating that cells were undergoing necrosis or late apoptosis (Fig. 1A). To further distinguish necrosis and apoptosis, we examined DNA fragmentation, which is one of the unique characteristics of apoptosis. As shown in Figure 1B, treatment with 100 μM and 300 μM dieldrin for 1 hr increased DNA fragmentation 129% and 180% over control levels, respectively, indicating that dieldrin acted as a potent apoptotic cell death inducer.

To further determine the phase of cell death during the 5-hr period of dieldrin exposure, qualitative analysis of apoptotic cell death was performed using Hoechst 33342. Hoechst 33342 staining revealed that dieldrin-treated cells were undergoing apoptosis in a time-dependent manner, evident from the condensation of chromatin (Fig. 2). The number of apoptotic cells increased over time, and the percent of apoptotic cells reached 55% and 68% following exposure to 100 μM and 300 μM dieldrin, respectively. The vehicle-treated PC12 cells had normal membrane integrity and no indication of apoptosis

during the incubation period.

Dieldrin-induced cytochrome C release from mitochondria

Increased ROS production has been shown to trigger mitochondrial cytochrome C release into cytosol (Simon et al., 2000, Junn and Mouradian, 2001), which subsequently initiates apoptotic signaling processes (Kuida, 2000). Dieldrin-treated PC12 cells showed both dose- and time-dependent increases in cytochrome C release as determined by Western blot analysis (Fig. 3A). A profound level of cytosolic cytochrome C release was observed at 3 hr of dieldrin exposure. In contrast, non-treated and vehicle-treated PC12 cells did not show detectable levels of cytosolic cytochrome C during the entire treatment period. To determine if dieldrin-induced cytochrome C release occurs at an earlier time point, we used a more sensitive method to measure cytochrome C release. As shown in Figure 3B, ELISA-based cytochrome C detection revealed that dieldrin significantly ($p<0.05$) increased cytosolic cytochrome C as early as 15 min post-exposure. Cytochrome C levels were increased 80-100% and 210-230% after 15 min of treatment with 100 μM and 300 μM dieldrin, respectively, compared to the vehicle-treated levels.

To further determine if dieldrin-induced cytochrome C release is dependent on ROS generation, we examined the effect of a ROS scavenger on cytochrome C release. A new cell permeable superoxide dismutase (SOD) mimetic, namely MnTBAP, has been shown to attenuate ROS generation and apoptosis in dopaminergic cells (Lotharius and O'Malley, 2000, Anantharam et al., 2002). In our recent study, we showed that dieldrin generates predominantly superoxides, and MnTBAP protects against dieldrin-induced apoptosis (Kitazawa et al., 2001). As shown in Figure 3C, pretreatment with 2-5 μM MnTBAP dose-dependently attenuated dieldrin-induced cytochrome C release. Although MnTBAP did not completely suppress the dieldrin-induced cytochrome C release, the effect was statistically significant ($p<0.05$, 2 μM MnTBAP; $p<0.01$, 5 μM MnTBAP).

Together, these results suggest that dieldrin-induced ROS is responsible at least in part for the release of the initial proapoptotic signal, cytochrome C, from mitochondria.

Activation of initiator caspases following dieldrin exposure

When cytochrome C is released into cytosol, it forms a complex with apoptotic activating factor-1 (Apaf-1) and activates caspase-9 (Kuida, 2000). Caspase-9 is one of the initiator caspases and cleaves downstream effector caspases to further drive apoptotic cascade. To determine whether caspase-9 was activated following the release of cytochrome C, we have measured the enzyme activity using fluorescent-tagged caspase-9 specific substrate, Ac-LEHD-AMC. Caspase-9 activity increased 2- to 5-fold in 1-2 hr and returned to the basal level at 5 hr in 100 μM dieldrin-treated cells, whereas its activity was still significantly ($p<0.05$) elevated at 5 hr in 300 μM dieldrin-treated cells (Fig. 4A). To further confirm whether dieldrin-induced apoptosis is distinctly mediated through a mitochondrial cytochrome C-caspase-9 activation pathway, we measured caspase-8 activity, another major initiator caspase activated primarily by death receptors. As shown in Figure 4B, caspase-8 was not significantly increased over the entire exposure period following dieldrin treatment. In order to further verify that dieldrin does not activate the caspase-8 cascade, we used the known caspase-8 activator Fas ligand (FasL) as a positive control in PC12 cells (Felderhoff-Mueser et al., 2000). Treatment with 50-500 ng/ml of FasL dose-dependently increased caspase-8 activity; cells treated with 500 ng/ml showed a 2.2-fold increase over control cells (data not shown). Together, these results indicate that dieldrin primarily activates the initiator caspase-9 but not caspase-8 in dopaminergic cells.

Activation of caspase-3 by dieldrin in PC12 cells

Initiator caspase-9 cleaves downstream effector caspases including caspase-3, -6, and -7. Among these, caspase-3 is one of the most studied effector caspases and plays a

critical role in execution of apoptosis (Dodel et al., 1998, Kitamura et al., 1998, Ochu et al., 1998, Hartmann et al., 2000, Turmel et al., 2001). Caspase-3 proteolytically cleaves the carboxyl site of an aspartate residue in a specific amino acid sequence, Asp-Glu-Val-Asp (DEVD). Activation of caspase-3-like activity can be used as an index of caspase-mediated apoptosis. Figure 5A demonstrates that dieldrin induces the activation of caspase-3 in a dose- and time-dependent manner. Caspase-3 was significantly ($p<0.05$) activated as early as 30 min following dieldrin exposure, and its activity increased substantially over 10- to 46-fold and 15- to 82-fold in 100 and 300 μM dieldrin exposure, respectively. The magnitude of caspase-3 activity was far more substantial than that of caspase-9, which showed 2- to 5-fold increase over basal level (Fig. 5A). The peak activation of caspase-3 was observed between 2 and 3 hr post exposure. At 5 hr, the activity of caspase-3 was diminished but still significantly ($p<0.05$) greater than vehicle-treated cells. The time sequence of caspase-3 and caspase-9 activation indicates that initiator caspase-9 activation precedes downstream effector caspase-3 activation. An *in situ* caspase fluorescence imaging using FITC-VAD-FMK also demonstrated that dieldrin-treated PC12 cells expressed active caspases within 3 hr of exposure period, and caspases were activated in 58-82% cells following dieldrin treatment (Fig. 5B).

Dieldrin-induced proteolytic cleavage of PKCδ

Active caspase-3 proteolytically cleaves various endogenous molecules that are vital for cell survival or apoptosis. Recently, we and others have identified the caspase-3 activation proteolytically cleaves PKCδ (Reyland et al., 1999, Anantharam et al., 2002). Exposure to dieldrin over a 5 hr time period resulted in the proteolytic cleavage of native PKCδ (72 and 74 kDa) into two fragments, 38 kDa regulatory and 41 kDa catalytic subunits (Fig. 6). The band intensity of native PKCδ decreased, whereas that of the proteolytic cleavage PKCδ fragmentation increased in dose- and time-dependent manner. Cleaved PKCδ was detected as early as 1 hr after treatment with 300 μM dieldrin and

reached maximum at 3 hr of the exposure in both 100 and 300 μM dieldrin-treated PC12 cells. Control or vehicle-treated PC12 cells did not show any proteolytic cleavage of PKCδ during the entire exposure period. In addition, dieldrin exposure did not induce translocation of PKCδ from the cytosol to plasma membrane (data not shown), which is another process of kinase activation (Powell et al., 1996, Majumder et al., 2000).

Dieldrin-induced proteolytic cleavage of PKCδ is mediated by caspase-3

To determine whether dieldrin-induced proteolytic cleavage of PKCδ was mediated by caspase-3, the specific inhibitors for caspase-3, Ac-DEVD-CHO and Z-DEVD-FMK, and a broad spectrum caspase inhibitor, Z-VAD-FMK, were used. Pre-treatment with Ac-DEVD-CHO (100 to 300 μM) or Z-VAD-FMK (30 to 300 μM) for 30 min blocked proteolytic cleavage of PKCδ in a dose-dependent manner (Fig. 7A-B). In addition, pre-treatment with various concentrations of Z-DEVD-FMK (10 to 100 μM), an irreversible caspase-3 inhibitor, also dose-dependently inhibited dieldrin-induced proteolytic cleavage of PKCδ (Fig. 7C).

In order to determine the specificity of proteolytic cleavage of PKCδ during dieldrin-induced cell death, the proteolytic cleavage of other PKC isoforms were examined. No proteolytic cleavage of PKCα or PKCβII was detected in dieldrin-treated PC12 cells as well as vehicle-treated cells over 5 hr of exposure (data not shown). Only the native forms of these enzymes were present in both dieldrin-treated and non-treated cells. Thus, it appears that dieldrin-induced caspase-3-mediated proteolytic cleavage of PKCδ is isoform-specific. In addition, both PKCα and PKCβII did not show increase in translocation to plasma membrane (data not shown). Collectively, these data indicate that PKCδ may be the major isoform of protein kinase that is activated during dieldrin exposure of PC12 cells.

Proteolytic cleavage of PKCδ results in increased PKCδ kinase activity

To further examine whether dieldrin-induced proteolytic cleavage of PKCδ reflects an increase in kinase activity, we determined PKCδ activity by ^{32}P phosphorylation assay following immunoprecipitation of PKCδ. ^{32}P phosphorylation was increased in cells treated with dieldrin for 3 hr (Figure 8A), reconciling with increased proteolytic cleavage of PKCδ observed above. Densitometric analysis of phosphorylated histone H1 bands revealed that 100 μM and 300 μM dieldrin exposure resulted in 145% and 149% increase in kinase activity over vehicle-treated cells, respectively. To further confirm that the increased PKCδ activity was due to proteolytic cleavage of PKCδ, we examined PKCδ activity with or without caspase-3 inhibitor (Z-DEVD-FMK) or PKCδ specific inhibitor (rottlerin). Rottlerin is widely used as a PKCδ inhibitor (Datta et al., 1999, Junn and Mouradian, 2001). We have previously confirmed that rottlerin at concentrations 3-20 μM specifically inhibits PKCδ activity as determined by kinase assay (Anantharam et al., 2002). As depicted in Figure 8B, PKCδ activity in 100 μM dieldrin-treated PC12 cells was completely blocked by 50 μM Z-DEVD-FMK as well as 20 μM rottlerin. Since the caspase inhibitor almost completely blocked dieldrin-induced PKCδ activity to basal level, it indicates that PKCδ activation during dieldrin exposure was mediated by caspase-3-dependent proteolytic cleavage.

Role of PKCδ in dieldrin-induced DNA fragmentation

PKCδ is one of the key signaling molecules activated by caspase-3 dependent apoptotic processes under conditions of dieldrin exposure. To determine the physiological and functional role of proteolytic activation of PKCδ in dieldrin-induced apoptosis, we pretreated PC12 cells with rottlerin to test whether dieldrin-induced DNA fragmentation was attenuated. DNA fragmentation assay showed that pretreatment with 10 μM rottlerin in PC12 cells significantly suppressed dieldrin-induced apoptosis (Fig. 9A). DNA fragmentation was reduced from 216% to 124% of control, which was nearly the basal

DNA fragmentation level. In addition, we also examined whether caspase inhibitors attenuated the dieldrin-induced DNA fragmentation since these inhibitors completely blocked the proteolytic cleavage of PKCδ (Fig. 7A-C) and its kinase activity (Fig. 8B). Caspase inhibitors, Z-DEVD-FMK (50 μM) and Z-VAD-FMK (100 μM) significantly ($p<0.01$) attenuated dieldrin-induced DNA fragmentation to the basal level (Fig. 9A) consistent with other caspase-3 dependent processes denoted above. Inhibitors alone did not alter DNA fragmentation in PC12 cells (data not shown), indicating the concentrations of inhibitors used in the experiment did not cause any toxicity to the cells. We further confirmed DNA fragmentation by qualitative analysis of apoptosis. Hoechst 33342 staining showed nuclear condensation, one of the distinct morphological changes during apoptosis, following 3 hr dieldrin exposure (Fig. 9B). Pretreatment with rottlerin remarkably reduced dieldrin-induced chromatin condensation from 47% to 18%. Together, these results suggest that proteolytic activation of PKCδ plays an important role in execution of apoptosis.

PKCδ is involved in feedback regulation of caspase-3

Since we observed a dramatic increase in caspase-3 (40-80 fold, Fig. 5A) as compared with a moderate increase in caspase-9 (2-5 fold; 4A) activity in PC12 cells following dieldrin exposure, we further examined whether additional regulatory mechanisms are involved in activation of caspase-3. It has recently been reported that certain protein kinases, such as protein kinase B (PKB), interact with caspases and other pro-apoptotic molecules to modulate apoptotic signaling pathways (Cardone et al., 1998, Datta et al., 1999). Therefore, we investigated whether PKCδ is capable of activating caspase-3 by a positive feedback modification of certain molecules involved in dieldrin-induced apoptosis. To address this hypothesis, we used a PKCδ specific inhibitor, rottlerin, to examine changes in caspase-3 activity and proteolytic cleavages of PKCδ. Treatment with 3 to 20 μM rottlerin significantly ($p<0.01$) and dose-dependently inhibited

dieldrin-induced caspase-3 activity by 30% to 89% and 44% to 90% of control in 3-hr exposure to 100 and 300 μM dieldrin, respectively (Fig. 10A-B). Treatment of control PC12 cells with rottlerin alone did not alter basal caspase-3 activity, indicating that the influence of rottlerin on dieldrin-induced caspase-3 activity was due to feedback regulatory mechanism. To further rule out any direct effect of rottlerin on caspase-3 activity, we demonstrated the changes of active caspase-3 by Western blot. PC12 cells were treated with rottlerin (3-20 μM) for 30 min and then exposed to dieldrin (100 and 300 μM) for additional 3 hr. As shown in Fig. 10C, 17 kDa active caspase-3 fragment decreased as rottlerin concentration increased, and 20 μM rottlerin almost completely abolished cleaved caspase-3. This result further confirms that dieldrin-induced caspase-3 activation may be regulated by activity of PKCδ.

To further confirm whether the feedback activation is due to catalytic activity of PKCδ as the result of proteolytic cleavage, we delivered a catalytically active PKCδ into cells and measured caspase-3 activity. Protein delivery was confirmed using FITC-labeled antibody supplied by the kit, and approximately 60-70% cells were labeled under the fluorescent microscope. Following 4-hr incubation, cells with catalytically active PKCδ showed 2-fold increase in caspase-3 activity compared with control (reagent alone), whereas cells with heat-inactivated PKCδ protein were not significantly different from control (Fig. 10D). This result clearly provides supporting evidence that PKCδ is capable of mediating caspase-3 activation.

Dieldrin-induced proteolytic cleavage of PKCδ was also found out to be blocked dose-dependently by pre-treatment with rottlerin (Fig. 11A). Appearance of the catalytic subunit (41 kDa) was significantly diminished by 20 μM rottlerin pre-treatment, the concentration of which causes approximately 90% reduction in dieldrin-induced caspase-3 activity. Dieldrin-induced PKCδ kinase activity was also significantly ($p<0.01$) reduced by pretreatment with rottlerin (Fig. 8B), which corresponds to the reduced catalytic subunit. Through inhibition of PKCδ kinase activity, rottlerin indirectly

suppressed caspase-3 activity and inhibited proteolytic cleavage of PKCδ. These findings indicate that PKCδ has a positive feedback regulatory mechanism to enhance the apoptotic signals.

Attenuation of dieldrin-induced caspase-3 and DNA fragmentation in mesencephalic cells over-expressing PKCδ^{K376R} (kinase-inactive) mutant

To further substantiate the proapoptotic function of PKCδ in dopaminergic cell death, we examined whether the over-expression of a kinase inactive PKCδ mutant (dominant negative) suppresses dieldrin-induced apoptosis. We stably transfected dopaminergic rat mesencephalic ($1RB_3AN_{27}$) cells with plasmids pPKCδ^{K376R}-GFP and pEGFP-N1 to express a dominant negative PKCδ mutant protein (Fig. 12A). Single amino acid mutation from lysine to arginine of PKCδ at position 376, which is located in the active site, completely eliminates kinase activity (Li et al., 1995, Li et al., 1996) (Li et al, 1996). The plasmid pPKCδ^{K376R}-GFP codes for a kinase inactive PKCδ mutant fused to the green fluorescent protein (GFP), and the plasmid pEGFP-N1 encodes GFP alone and was used as a vector control. Figure 12B shows stable GFP expression in $1RB_3AN_{27}$ cells transfected with GFP alone or kinase inactive mutant PKCδ^{K376R}-GFP. Determination of PKCδ kinase activity in dominant negative cells showed almost a complete suppression of the kinase activity in dieldrin-treated cells as compared to vector-transfected cells (Fig. 12C), indicating that the mutant PKCδ provides a dominant pool of inactive PKCδ.

We then compared the extent of caspase-3 activation and DNA fragmentation in PKCδ mutant-expressed cells and vector cells following dieldrin exposure. As shown in Fig 13A, 100 μM and 300 μM dieldrin exposure for 3 hr in vector-transfected $1RB_3AN_{27}$ cells increased caspase-3 activity to 235% and 347% of vehicle control, respectively. Dieldrin-induced caspase-3 activity was significantly ($p<0.01$) reduced in PKCδ mutant cells. Thus, the attenuation of dieldrin-induced caspase-3 activation in PKCδ mutant cells

further supports our hypothesis that PKCδ influences caspase-3 activity via positive feedback activation during dieldrin-induced apoptosis in dopaminergic cells. Measurement of DNA fragmentation in vector-transfected cells showed 165% and 162% of vehicle control following 100 and 300 μM dieldrin exposure, respectively (Fig. 13B). Conversely, PKCδ mutant-expressed cells almost completely blocked dieldrin-induced DNA fragmentation at both 100 and 300 μM concentrations. These data strongly support the pro-apoptotic role of PKCδ in dieldrin-induced dopaminergic degeneration.

DISCUSSION

Previously, we demonstrated that dieldrin increases ROS as early as 5 min post-exposure, and that ROS generation participates in dieldrin-induced apoptotic cell death in PC12 cells (Kitazawa et al., 2001). In the present study, we demonstrate that dieldrin activates series of signaling molecules, and these molecules are highly organized to execute apoptotic cell death. Major findings from our present study are: (i) dieldrin activates a cytochrome C, caspase-dependent cell death signaling cascade; (ii) caspase-3 produces a persistent activation of PKCδ by proteolytic cleavage; (iii) proteolytic activation of PKCδ participates in execution of apoptosis; and (iv) PKCδ plays an important role in the positive feedback regulation of caspase cascade. Collectively, these findings demonstrate that caspase-3 mediated proteolytic activation of PKCδ is a key downstream signaling event in the execution as well as amplification of apoptosis in the dopaminergic cells following exposure to environmental neurotoxic agent.

In the present study, we observed release of cytochrome C into cytosol as early as 15 min following dieldrin exposure in a dose- and time-dependent manner over 5-hr period. Cytochrome C acts as a pro-apoptotic molecule and is released from mitochondria into cytoplasm under conditions of increased oxidative stress or mitochondrial damage in both neuronal and non-neuronal cells (Petit et al., 1998, Blackstone and Green, 1999,

Cassarino et al., 1999, Hollensworth et al., 2000, Lee and Wei, 2000). We previously demonstrated that dieldrin increases ROS production within 30 min and significantly reduces mitochondrial membrane potential in PC12 cells (Kitazawa et al., 2001). The present study further demonstrates that dieldrin-induced ROS triggers cytochrome C release from mitochondria to initiate the apoptotic cascade. The influence of dieldrin on mitochondrial function may be related to its inhibitory effect on the mitochondrial respiratory chain, possibly at or near cytochrome b on complex III (Bergen, 1971). Taken together, our study indicates that dieldrin potentially affects mitochondria and is capable of inducing cytochrome C release by a mechanism similar to that of other dopaminergic toxicants such as MPP^+, 6-hydroxydopamine (6-OHDA), paraquat, or MMT (Dodel et al., 1999, Gomez et al., 2001, Anantharam et al., 2002).

An initial cell death signaling molecule, cytosolic cytochrome C forms a complex with Apaf-1 (apoptotic protease activating factor-1) in the presence of dATP (Kuida, 2000) and subsequently activates an initiator caspase, caspase-9 (Liu et al., 1996, Li et al., 1997, Cassarino et al., 1999). Caspase-9 then proteolytically cleaves procaspase-3 into catalytically active caspase-3 (Budihardjo et al., 1999). We observed a dramatic increase in caspase-9 and caspase-3 activity in dieldrin-treated PC12 cells. The sequence of caspase signaling cascade identified in dieldrin-induced apoptosis reconciles with the known caspase cascade induced by several apoptosis inducers in various cells (Dodel et al., 1999, Gamen et al., 2000, Liu et al., 2000, Yamazaki et al., 2000). Recently, it has been demonstrated that caspase-3 is a vulnerability factor and a critical effector in apoptotic cell death induced in both MPTP mouse model and in human patients with Parkinson's disease (Hartmann et al., 2000). Apoptotic neuronal cell death has recently been recognized as a prominent mode of dopaminergic neurodegeneration in substantia nigra in idiopathic PD (Mattson et al., 1999, Albers and Beal, 2000, Chun et al., 2001). Despite evidence from other studies which suggest that all key pro-apoptotic molecules are located within close proximity to each other, this study indicates that dieldrin is

capable of activating a specific cell death pathway involving mitochondrial cytochrome C, caspase-9 and caspase-3.

One significant outcome of the current study is selective activation of PKCδ by caspase-3 through proteolytic cleavage. PKC family members are involved in activation of downstream regulatory mechanisms of apoptosis in non-neuronal systems (Shao et al., 1997, Chen et al., 1999). Recent reports indicate that certain PKC isoforms play specific and often divergent roles in apoptotic cell death (Ruvolo et al., 1998, Gschwendt, 1999, Li et al., 1999, Reyland et al., 1999, Cross et al., 2000, Musashi et al., 2000, Reyland et al., 2000). PKCα has been shown to phosphorylate anti-apoptotic proteins, such as PKB and Bcl-2, and serves as a survival signal (Ruvolo et al., 1998, Li et al., 1999, Deng et al., 2000). In contrast, PKCδ is known to translocate to plasma membrane during phorbol ester treatment or UV-B irradiation and induces apoptosis (Emoto et al., 1995, Chen et al., 1999, Cross et al., 2000). Our present results demonstrate that PKCδ, but not conventional PKC family members including PKCα or PKCβII, was proteolytically cleaved and activated during dieldrin exposure. Blockade of proteolytic cleavage of PKCδ by pre-treatment with caspase-3 specific inhibitors demonstrated that caspase-3 cleaves PKCδ into its active form following exposure to dieldrin in dopaminergic cells. Recently, caspase-3-mediated PKCδ activation was also observed during apoptosis in other cell types including U-937 cells, neutrophils, and etoposide-treated salivary gland acinar cells (Emoto et al., 1995, Ghayur et al., 1996, Pongracz et al., 1999, Reyland et al., 1999). Our results further demonstrate that proteolytic cleavage of PKCδ results in persistent activation of the kinase activity in dopaminergic cells. Although the downstream events involving PKCδ and those that specifically lead to apoptotic cell death remain to be defined, DNA protein kinase (DNA-PK), an enzyme essential for the repair of DNA strand breaks, has been shown to be regulated by PKCδ through phosphorylation (Bharti et al., 1998, Pongracz et al., 1999).

To further clarify the proteolytic activation of PKCδ and its role in apoptotic cell

death following dieldrin exposure, a series of experiments was conducted to elucidate whether PKCδ performs a critical function in dieldrin-induced apoptosis. Pretreatment with caspase-3 inhibitors and a PKCδ-specific inhibitor almost completely suppressed dieldrin-induced apoptosis, indicating that PKCδ participates in apoptotic cell death. Additionally, we performed dominant negative mutant experiments by stably transfecting an inactive PKCδ^{K376R} gene in a dopaminergic cell model and examining dieldrin-induced cell death pathways. Dieldrin exposure produced a significant increase in DNA fragmentation in vector-transfected cells, whereas no significant DNA fragmentation was observed in PKCδ^{K376R} mutant cells. Taken together with inhibitor studies, our data provide convincing evidence that PKCδ plays a key role in execution of apoptosis in dopaminergic cells.

In addition to a role of PKCδ in apoptosis, the results from experiments demonstrating suppression of caspase-3 activity by PKCδ inhibitor rottlerin or activation of caspase-3 activity by catalytically active PKCδ indicate the existence of a positive feedback interaction between caspase-3 and PKCδ. Our finding corroborates a recent report showing a similar regulatory mechanism in salivary gland acinar cells following etoposide exposure (Reyland et al., 1999). Recently, PKCδ has been shown to be translocated to the mitochondrial membrane and promotes cytochrome C release (Majumder et al., 2000). In this regard, PKCδ may act on upstream apoptotic molecules and facilitate apoptotic cascade.

In conclusion, dieldrin, a putative environmental risk factor for development of PD, induces caspase-3-dependent apoptosis through a mitochondrial-mediated activation pathway. The results obtained in this series of studies help to build a proposed mechanism for dieldrin-induced apoptosis in dopaminergic cells (Fig. 14). Dieldrin-induced ROS promotes cytochrome C release into the cytosol to initiate the caspase cascade. Activation of caspase-9 is followed by activation of caspase-3, which in turn cleaves PKCδ to an active catalytic product. PKCδ participates in the feedback activation of the

dieldrin-induced caspase cascade, thereby amplifying downstream apoptotic signaling molecules. Thus, PKCδ serves as an obligatory regulator for caspase-3 in directing cells to apoptosis. For the first time, this study establishes an important role for PKCδ in apoptotic cell death in *in vitro* models of Parkinson's disease. However, further studies are needed to understand the exact mechanisms by which PKCδ regulates the apoptotic pathway in dopaminergic cells following exposure to environmental toxins.

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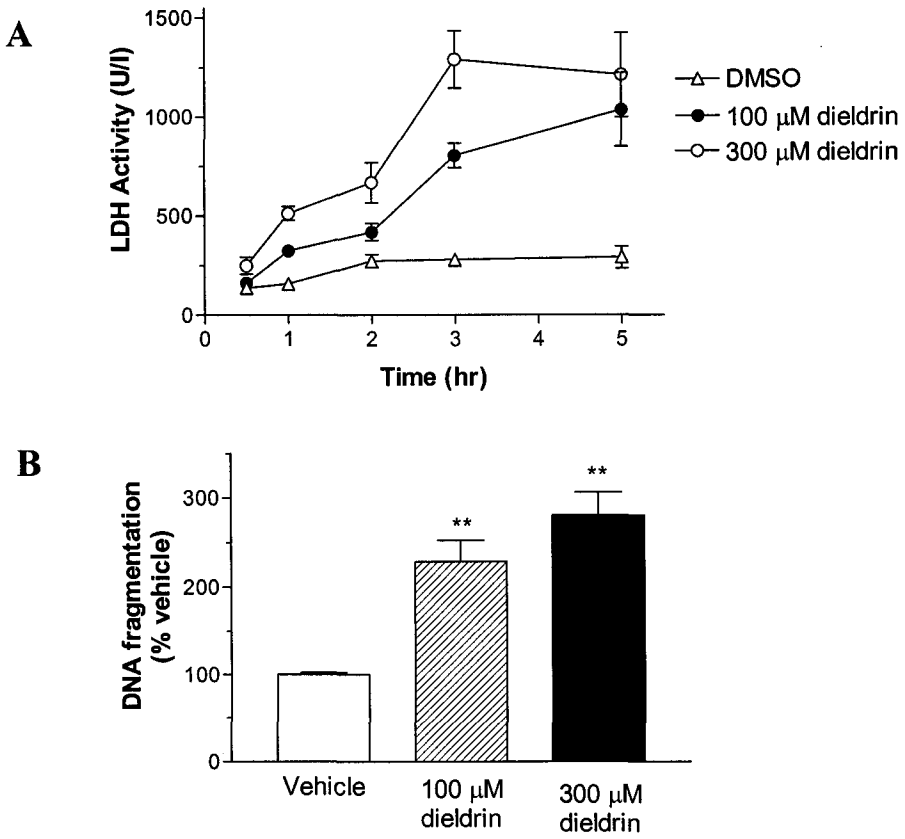


FIG. 1. Dieldrin-induced cytotoxicity and apoptosis in PC12 cells.

(**A**) PC12 cells (2 x 10^6 cells/ml) were exposed to 100 or 300 μM dieldrin for 1, 2, 3, or 5 hr at 37°C. After the exposure, the cell-free supernatants were collected, and LDH activity was measured by UV-spectrophotometer. Each data point represents mean ± SEM from at least three separate experiments in triplicate. (**B**) PC12 cells were exposed to 100 or 300 μM dieldrin for 1 hr, and DNA fragmentation was assayed using the Cell Death Detection ELISA-Plus assay. The data are expressed as the percentage of DNA fragmentation observed in vehicle-treated PC12 cells. Each point represents mean ± SEM from three separate experiments in duplicate. The average baseline control value for DNA fragmentation is 23 optical density (O.D.)/mg protein. **$p<0.01$ compared with vehicle-treated group.

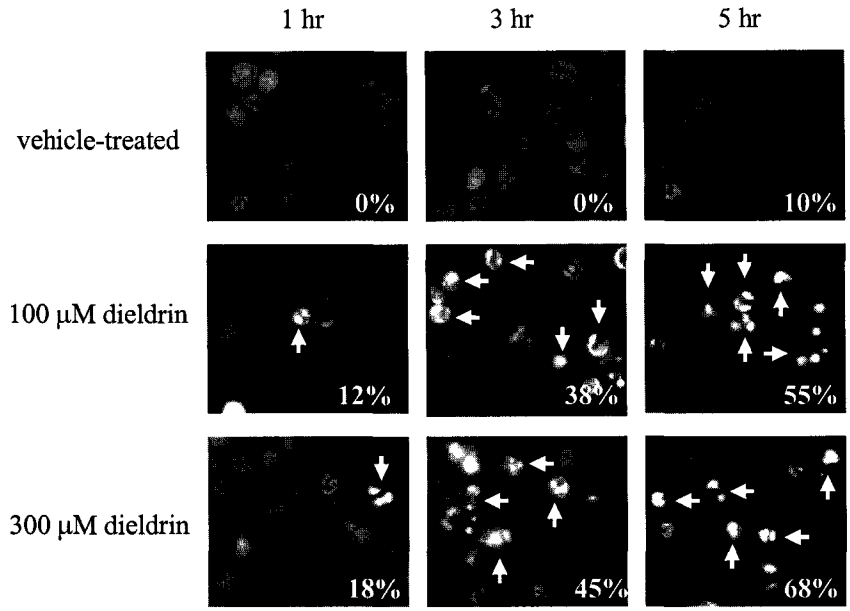
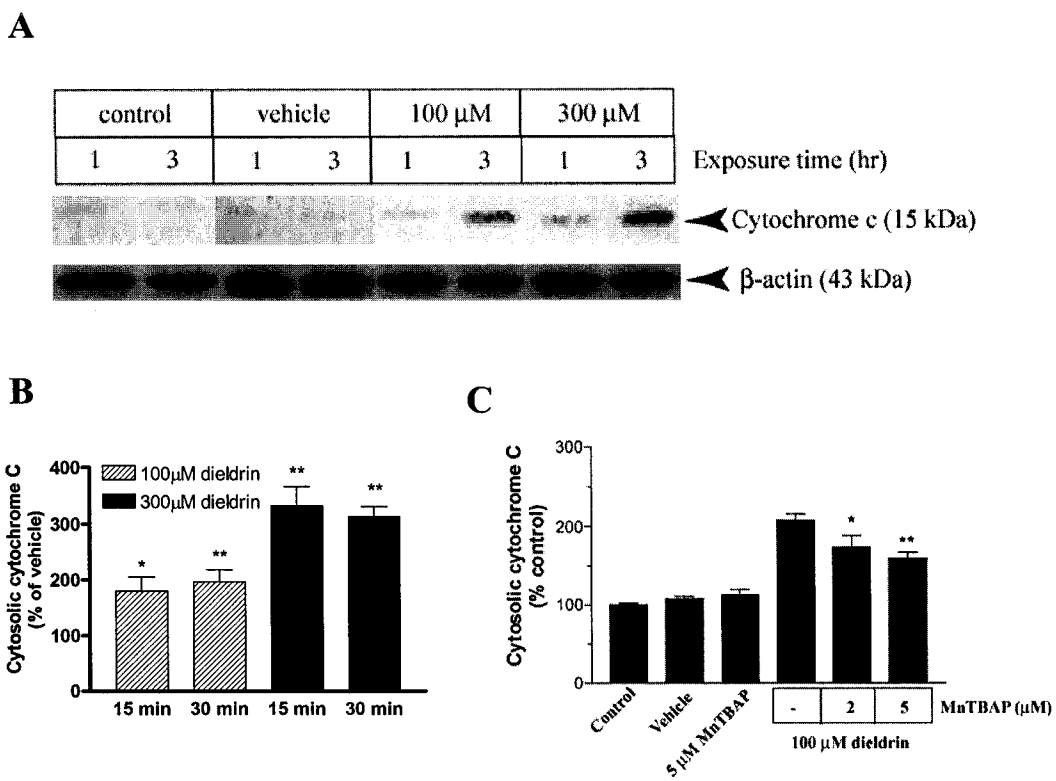


FIG. 2. Apoptotic morphology of PC12 cells following dieldrin exposure.

PC12 cells were grown on type I rat-tail collagen-coated (6 μg/cm^2) cover slips for 24 hr at 37°C. The cells were exposed to 100 or 300 μM dieldrin for 1, 3, or 5 hr, treated with 10 μg/ml Hoechst 33342, and observed under a fluorescent microscope with excitation at 380 nm as described under "Experimental procedures." Arrows indicate chromatin condensation typically observed in apoptotic cells. Percentage of apoptotic cells is shown in the bottom right of each panel. This experiment was repeated twice with similar results.

FIG. 3. Cytochrome C release following exposure of PC12 cells to dieldrin.

(A) PC12 cells (~1 x 10^7 cells) were exposed to 100 μM or 300 μM dieldrin for 1 to 3 hr at 37°C. Cytochrome C release into cytosol was determined by Western blot analysis as described under "Experimental Procedures." 2 μg of cytosolic samples were resolved by 12.5% SDS-polyacrylamide gel. Arrowhead indicates cytosolic cytochrome C (~15 kDa). Levels of β-actin (43 kDa) confirm equal protein loading in each lane. **(B)** PC12 cells (~1 x 10^7 cells) were exposed to 100 μM or 300 μM dieldrin for 15 to 30 min at 37°C, and cytosolic fraction was analyzed using cytochrome C ELISA kit. Each data point is expressed as percent of vehicle-treated group and represents mean ± SEM from two separate experiments in duplicate. *$p<0.05$ or **$p<0.01$ compared with vehicle-treated group. **(C)** PC12 cells were pretreated with 2-5 μM MnTBAP for 30 min and then exposed to 100 μM dieldrin for another 30 min. Cytosolic cytochrome C was measured using an ELISA kit. Each data point is expressed as percent of control group of two separate experiments in triplicate. *$p<0.05$ or **$p<0.01$ compared with 100 μM dieldrin-treated group. Baseline value (control) is 0.414 ± 0.03 O.D./10^6 cells, and vehicle-treated group shows similar value (0.441 ± 0.005).


A
control
vehicle
100 µM
300 µM
1 3 1 3 1 3 1 3
Exposure time (hr)
Cytochrome c (15 kDa)
β-actin (43 kDa)
B
Cytosolic cytochrome C (% of vehicle)
100µM dieldrin
300µM dieldrin
15 min 30 min 15 min 30 min
C
Cytosolic cytochrome C (% control)
Control
Vehicle
5 µM MnTBAP
- 2 5 MnTBAP (µM)
100 µM dieldrin

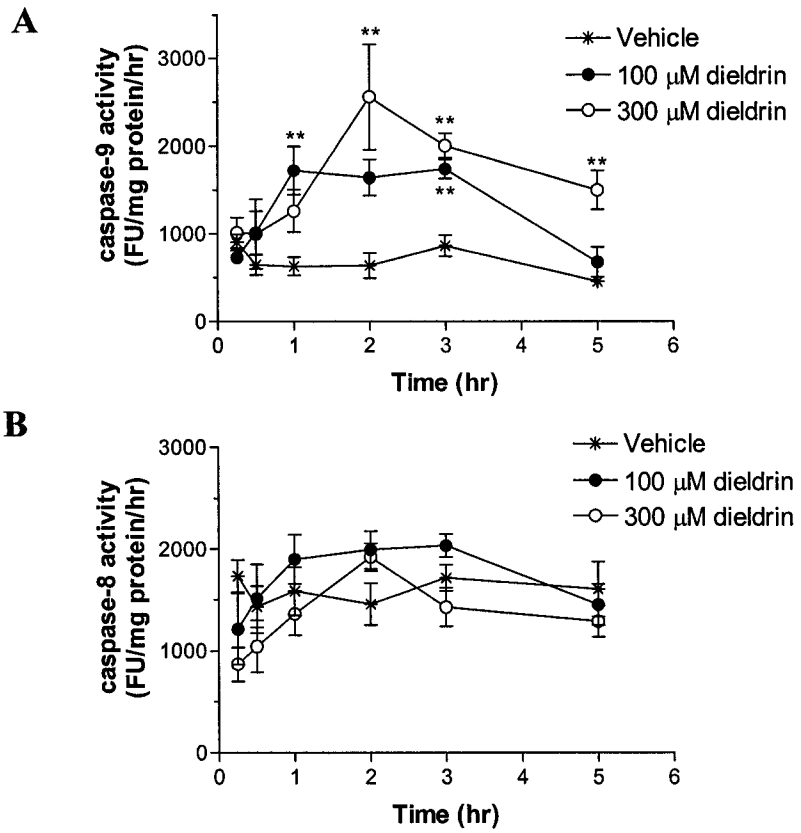


FIG. 4. Activation of initiator caspases following exposure of PC12 cells to dieldrin. PC12 cells (2 x 10^6 cells/ml) were exposed to 100 or 300 μM dieldrin for 30 min, 1, 2, 3, or 5 hr at 37°C, and (**A**) caspase-9 or (**B**) caspase-8 activity was measured using caspase-9 or caspase-8 specific substrates, Ac-LEHD-AMC or Ac-IETD-AMC, respectively as described under "Experimental Procedures." The data are expressed as fluorescence unit (FU) per mg protein per hr of incubation. Each point represents mean ± SEM from at least three separate experiments in triplicate. The baseline activities of caspase-9 and caspase-8 were not significantly changed between untreated (control) and vehicle treated cells (caspase-9: control = 857.9 ± 74.7 vs vehicle = 685.7 ± 67.8 FU/mg protein/hr, and caspase-8: control = 2285 ± 92 vs vehicle = 1589 ± 51 FU/mg protein/hr). **$p<0.01$ compared with vehicle-treated groups.

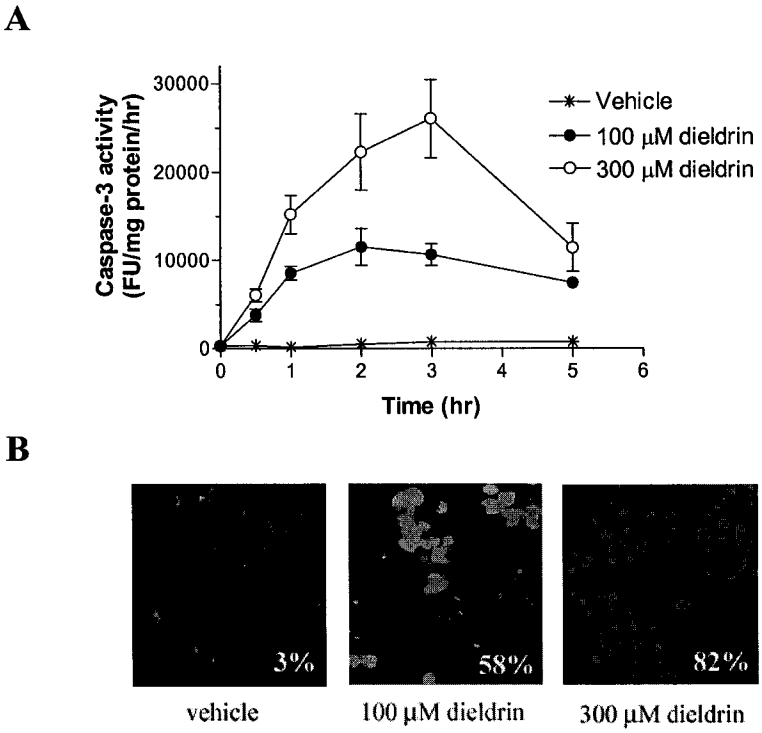


FIG. 5. Activation of caspase-3 following exposure of PC12 cells to dieldrin.

PC12 cells (2 x 10^6 cells/ml) were exposed to 100 or 300 μM dieldrin for up to 5 hr at 37°C, and caspase-3 activity was measured using caspase-3 specific substrate, Ac-DEVD-AMC as described under "Experimental Procedures." The data are expressed as FU per mg protein per hr of incubation. Each point represents mean ± SEM from at least three separate experiments in triplicate. The caspase-3 activity in control cells was similar to that of vehicle-treated cells (control = 481.1 ± 121.6 vs vehicle = 480.8 ± 97.1 FU/mg protein/hr). Dieldrin-treated cells showed statistically significant ($p<0.01$) compared with vehicle-treated cells in all time point. **(B)** PC12 cells were grown on type I rat-tail collagen coated (6 μg/cm^2) cover slip for 24 hr at 37°C. The cells were exposed to 100 or 300 μM dieldrin for 3 hr, treated with FITC-VAD-FMK, and observed under a fluorescent microscope with excitation at 488 nm as described under "Experimental procedures." The percent of cells that show activated caspases is indicated in the bottom right of each image.

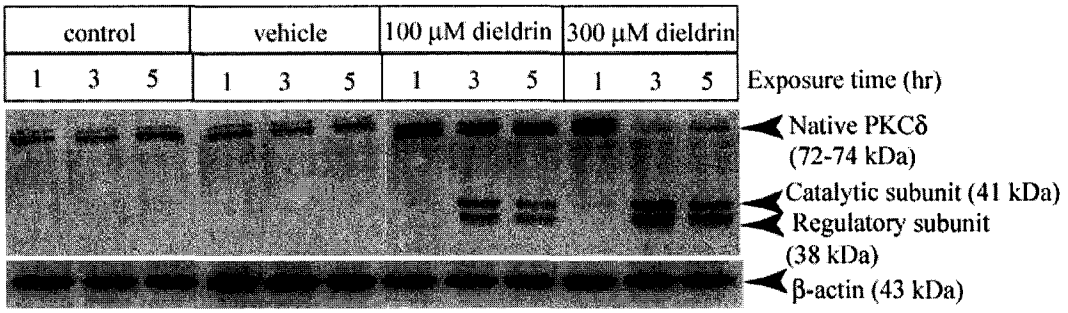
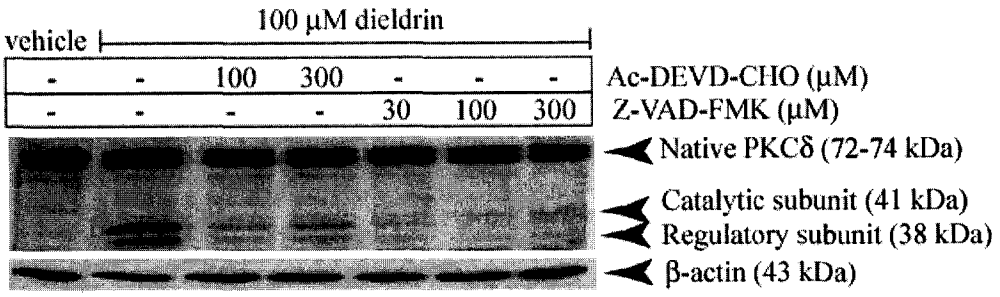


FIG. 6. Proteolytic cleavages of PKCδ following exposure of PC12 cells to dieldrin. PC12 cells (~1 x 10^7 cells) were exposed to 100 μM or 300 μM dieldrin for 1, 3 or 5 hr at 37°C, and cytosolic proteins were collected as described under "Experimental Procedures." Approximately 5 μg of cytosolic proteins were resolved by 10% SDS-polyacrylamide gel and determined proteolytic cleavage of PKCδ. Proteolytic cleavage of PKCδ was observed as early as 3 hr following dieldrin treatment. Arrowheads indicate native PKCδ (72-74 kDa), catalytic subunit (41 kDa) and regulatory subunit (38 kDa) resulting in proteolytic cleavage of PKCδ. β-actin (43 kDa) confirms equal protein loading in each lane.

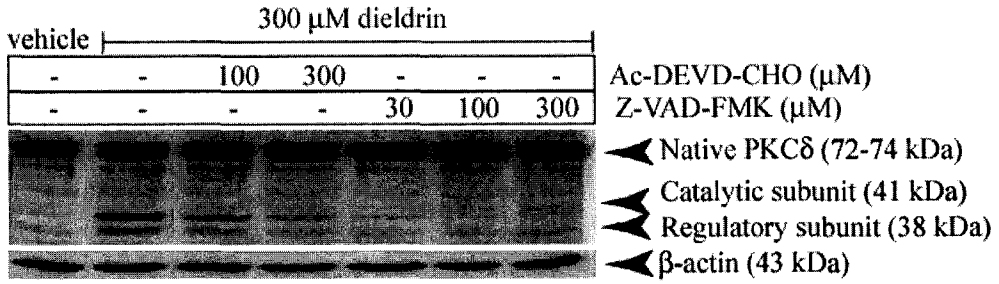
FIG. 7. Dieldrin-induced proteolytic cleavage of PKCδ is inhibited by caspase inhibitors in PC12 cells.

PC12 cells were pre-treated with Ac-DEVD-CHO, Z-VAD-FMK (**A** and **B**), or Z-DEVD-FMK (**C**) for 30 min, then exposed to 100 μM or 300 μM dieldrin for 3 hr, and cytosolic proteins were collected as described under "Experimental Procedures." Approximately 2 μg of cytosolic proteins were resolved by 10% SDS-PAGE and detected dieldrin-induced proteolytic cleavage of PKCδ. Arrowheads indicate native PKCδ and cleaved subunits, as indicated in the figure. β-actin confirms equal protein loading in each lane.

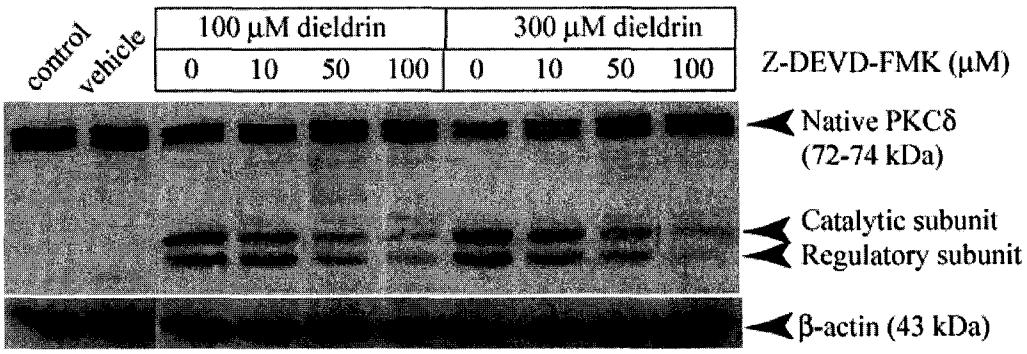
A


vehicle
100 μM dieldrin
- - 100 300 - - - Ac-DEVD-CHO (μM)
- - - - 30 100 300 Z-VAD-FMK (μM)
Native PKCδ (72-74 kDa)
Catalytic subunit (41 kDa)
Regulatory subunit (38 kDa)
β-actin (43 kDa)

B


vehicle
300 μM dieldrin
- - 100 300 - - - Ac-DEVD-CHO (μM)
- - - - 30 100 300 Z-VAD-FMK (μM)
Native PKCδ (72-74 kDa)
Catalytic subunit (41 kDa)
Regulatory subunit (38 kDa)
β-actin (43 kDa)

C


control
vehicle
100 μM dieldrin
300 μM dieldrin
0 10 50 100 0 10 50 100 Z-DEVD-FMK (μM)
Native PKCδ (72-74 kDa)
Catalytic subunit
Regulatory subunit
β-actin (43 kDa)

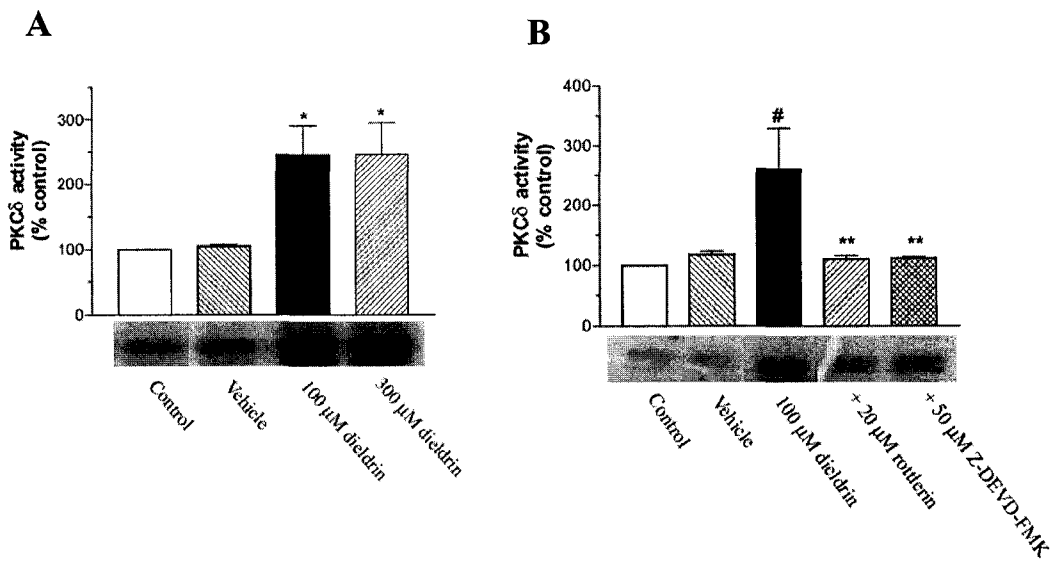


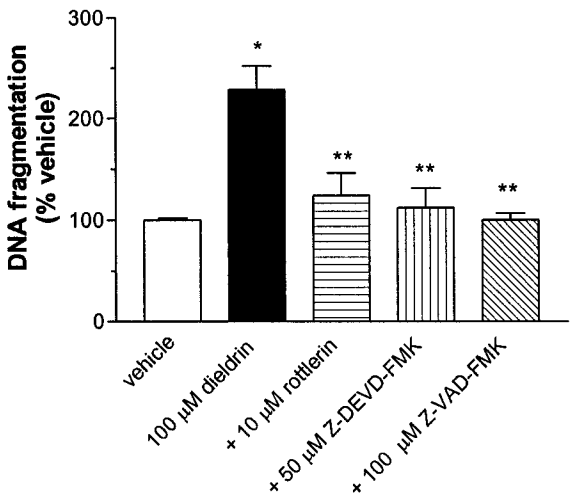
FIG. 8. PKCδ kinase activity during caspase-3-dependent proteolytic cleavage in dieldrin-treated PC12 cells.

(**A**) PC12 cells (~1 x 10^7 cells) were exposed to dieldrin for 3 hr, and PKCδ activity was assayed using an immunoprecipitation kinase assay as described under "Experimental Procedures." Densitometric analysis of phosphorylated histone in each treatment group is expressed as percent of control. The data represent three separate experiments. *$p<0.05$ compared with vehicle-treated groups. (**B**) PKCδ activity was assayed as in panel A. PC12 cells were pretreated for 30 min with 20 μM rottlerin or 50 μM Z-DEVD-FMK and exposed to 100 μM dieldrin for 3 hr. This experiment was repeated twice with similar results. Densitometric analysis of phosphorylated histone in each treatment group is expressed as percent of control. #$p<0.05$ compared with vehicle-treated groups, and **$p<0.01$ compared with dieldrin-treated groups.

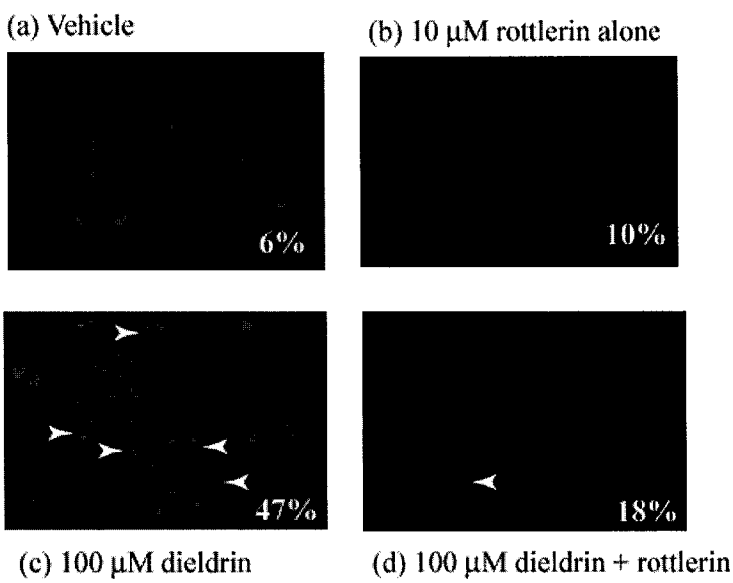
FIG. 9. DNA fragmentation and apoptosis in PC12 cells exposed to dieldrin.

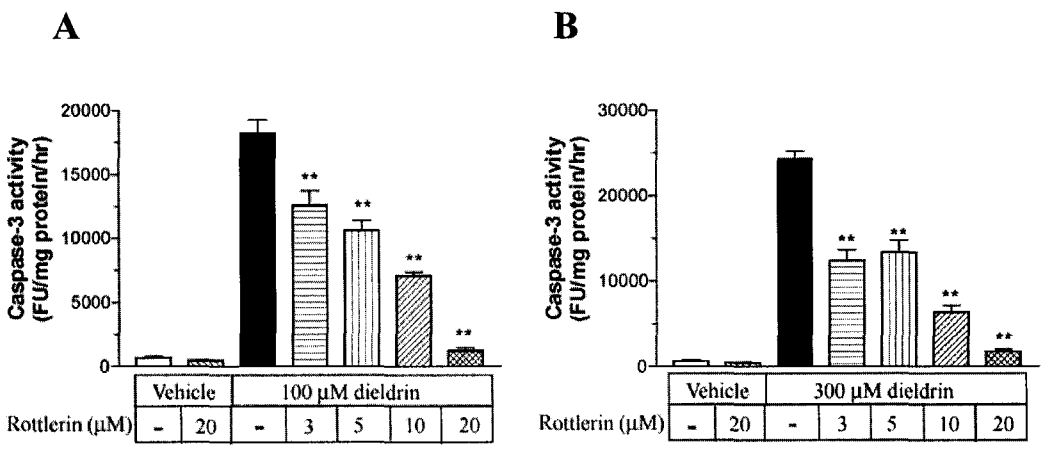
(**A**) PC12 cells (2 x 10^6 cells/ml) were exposed to 100 μM dieldrin for 1 hr with or without the 30 min pretreatment with rottlerin (10 μM), Z-DEVD-FMK (50 μM), or Z-VAD-FMK (100 μM). DNA fragmentation was assayed using the Cell Death Detection ELISA-Plus assay which measures DNA fragmentation as described under "Experimental Procedures." The data are expressed as the percentage of DNA fragmentation observed in vehicle-treated PC12 cells. Each point represents mean ± SEM from three separate experiments in duplicate. Baseline DNA fragmentation is 35 ± 9 (control), and vehicle-treated group shows similar value (29 ± 8). *$p<0.01$ compared with vehicle-treated group, or **$p<0.01$ compared with 100 μM dieldrin-treated group. (**B**) PC12 cells were grown on type I rat-tail collagen coated (6 $\mu g/cm^2$) cover slip for 24 hr at 37°C. Apoptotic morphology of PC12 cells following 3 hr of (a) vehicle-treated, (b) 10 μM rottlerin alone, (c) 100 μM dieldrin-treated, and (d) 100 μM dieldrin with 10 μM rottlerin pretreatment, was examined using Hoechst 33342 (10 μg/ml), and observed under a fluorescent microscope with excitation at 380 nm as described under "Experimental procedures." Arrowheads indicate nuclear condensation typically observed in apoptotic cells.

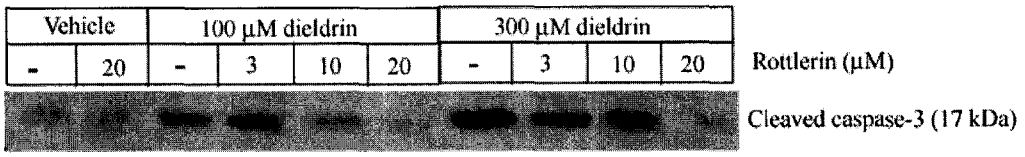
A

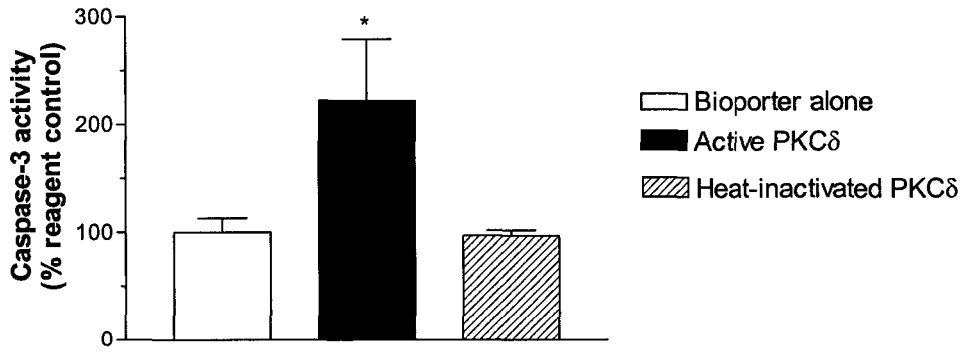

DNA fragmentation (% vehicle)
300
200
100
0
*
**
**
**
vehicle
100 μM dieldrin
+ 10 μM rottlerin
+ 50 μM Z-DEVD-FMK
+ 100 μM Z-VAD-FMK

B


(a) Vehicle
(b) 10 μM rottlerin alone
6%
10%
47%
18%
(c) 100 μM dieldrin
(d) 100 μM dieldrin + rottlerin


A
B
Caspase-3 activity (FU/mg protein/hr)
20000
15000
10000
5000
0
30000
20000
10000
**
Vehicle
100 μM dieldrin
300 μM dieldrin
Rottlerin (μM)
-
20
3
5
10


C
Vehicle
100 μM dieldrin
300 μM dieldrin
Rottlerin (μM)
Cleaved caspase-3 (17 kDa)


D
Caspase-3 activity (% reagent control)
300
200
100
0
*
Bioporter alone
Active PKCδ
Heat-inactivated PKCδ

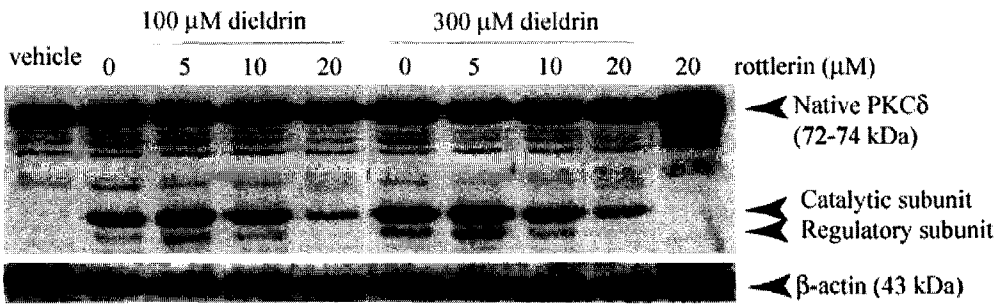


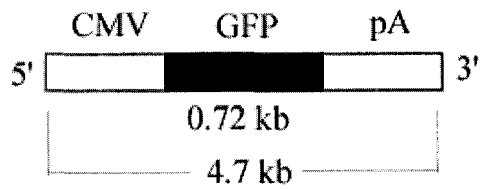
FIG. 11. Inhibition of dieldrin-induced proteolytic cleavages of PKCδ by rottlerin in PC12 cells.

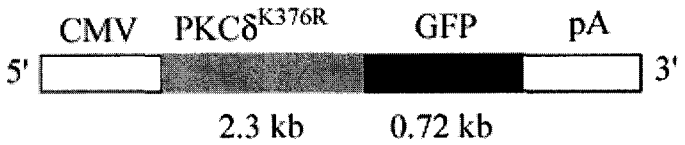
PC12 cells (~1 x 10^7 cells) were pre-treated with 0-20 μM rottlerin for 90 min, then exposed to 100 or 300 μM dieldrin for 2 hr. Cytosolic proteins were collected as described under "Experimental Procedures." Approximately 2-5 μg of cytosolic proteins were resolved by 8-10% SDS-polyacrylamide gel and detected PKCδ. Solid and open arrowheads indicate native and cleaved proteins, respectively. β-actin confirms equal protein loading in each lane.

FIG. 12. Overexpression of catalytically inactive PKCδ protein in immortalized dopaminergic neuronal cell line ($1RB_3AN_{27}$).

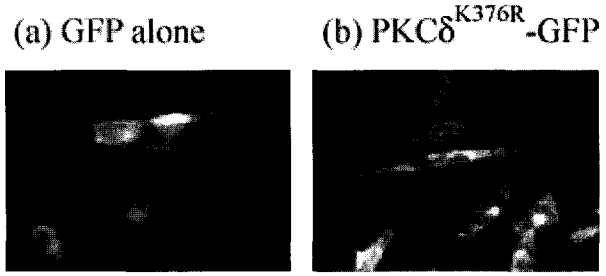
(**A**) Description of plasmid: pEGFP-NI construct codes for the Green Fluorescent Protein (GFP) mRNA transcribed under the 5' Human cytomegalovirus (CMV) immediate early promoter and the mRNA is stabilized with the 3' SV40 mRNA polyadenylation signal (pA) and was used as vector control. $PKC\delta^{K376R}$-GFP construct codes for the kinase inactive PKCδ-GFP fusion transcript. (**B**) Stable expression of GFP (a) and $PKC\delta^{K376R}$-GFP fusion protein (b) in $1RB_3AN_{27}$ cells was confirmed under a fluorescence microscope, and images were obtained with a SPOT digital camera. (**C**) (a) Vector- and (b) $PKC\delta^{K376R}$-transfected $1RB_3AN_{27}$ cells were treated with 100 μM dieldrin for 3 hr, and cytosolic fraction was collected. PKCδ kinase activity assay was performed following immunoprecipitation of PKCδ. The data represent a mean ± SEM of two separate experiments. $^{**}p<0.01$ compared with control.

A

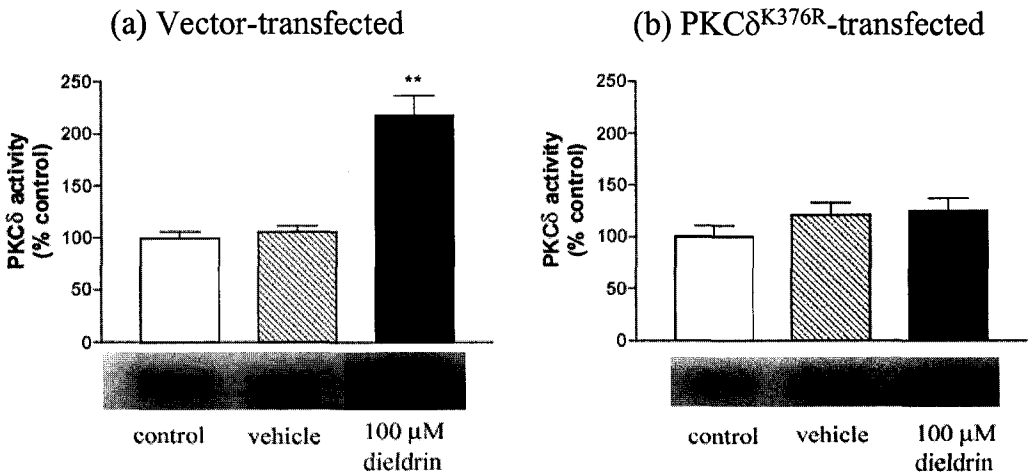

CMV
GFP
pA
5'
3'
0.72 kb
4.7 kb


CMV
PKCδ^K376R
GFP
pA
5'
3'
2.3 kb
0.72 kb

B


(a) GFP alone
(b) PKCδ^K376R-GFP

C


(a) Vector-transfected
(b) PKCδ^K376R-transfected
PKCδ activity (% control)
250
200
150
100
50
0
**
control
vehicle
100 μM dieldrin

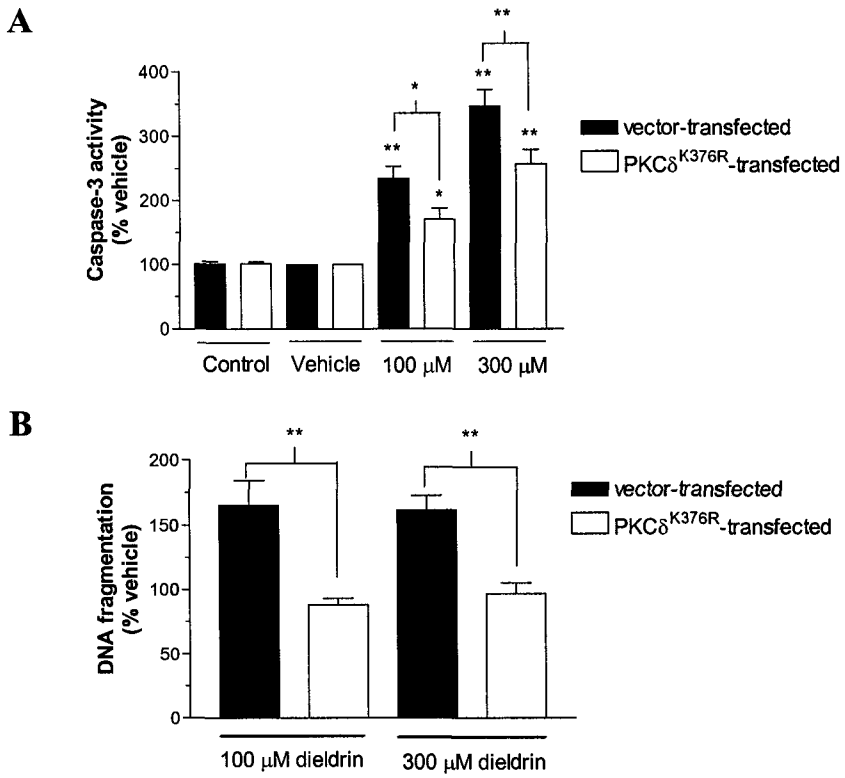


FIG. 13. Overexpression of catalytically inactive PKCδ protein blocks dieldrin induced caspase-3 and DNA fragmentation

Subconfluent cultures of undifferentiated $1RB_3AN_{27}$ cells stably expressing vector or $PKC\delta^{K376R}$-GFP fusion protein were treated with 100 and 300 μM dieldrin for 3 hr. **(A)** Caspase-3 activity was determined using colorimetric substrate, Ac-DEVD-pNA, and **(B)** DNA fragmentation was assayed using DNA ELISA assay as described under "Materials and Methods". The data are expressed as percentage of caspase-3 activity (average baseline value is 1.4 O.D./mg protein/hr) or DNA fragmentation (average baseline value is 28 O.D./mg protein) observed in vehicle-treated cells. The data represent a mean ± SEM of six individual measurements from two separate experiments. Single asterisk denotes $p<0.05$, and double asterisks indicates $p<0.01$.

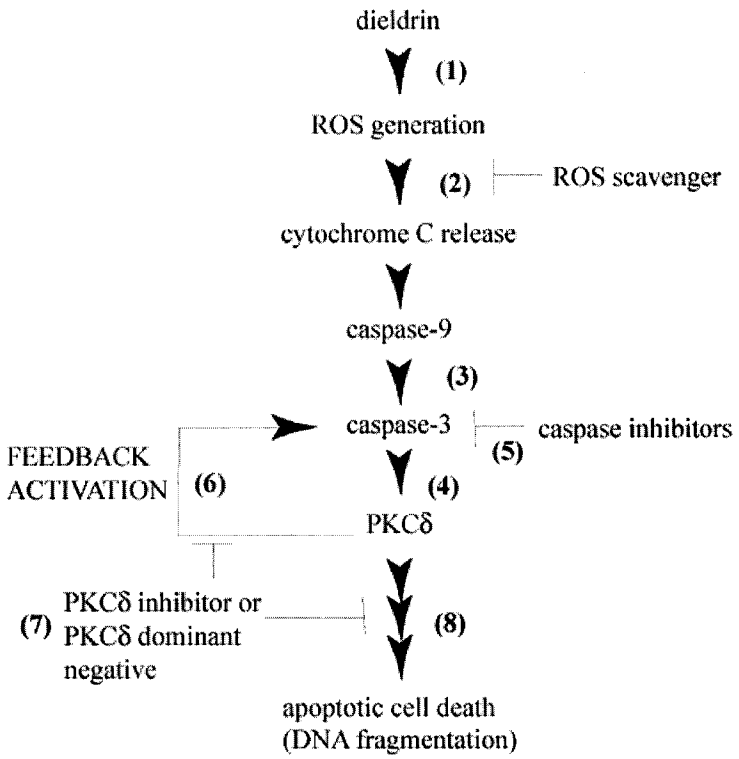


FIG. 14. A proposed mechanism for dieldrin-induced apoptotic signaling molecules in dopaminergic cells.

Based on our studies, the following signaling pathways occur in dopaminergic cells exposed to dieldrin: 1) dieldrin increases ROS levels in the cell; 2) cytochrome C is released from the mitochondria into the cytosol, and cytochrome C release can be blocked by a ROS scavenger, such as MnTBAP; 3) cytosolic cytochrome C activates caspase-3 via caspase-9; 4) caspase-3 activates PKCδ by proteolytic cleavage; 5) dieldrin-induced proteolytic cleavage of PKCδ is blocked by pretreatment with the caspase-3 inhibitors, Ac-DEVD-CHO, Z-VAD-FMK & Z-DEVD-FMK; 6) the catalytically active PKCδ fragment positively regulates caspase-3 activity; 7) pretreatment with PKCδ inhibitor or overexpression of $PKC\delta^{K376R}$ (dominant negative mutant) reduces caspase-3 activity in dieldrin-treated PC12 cells; and 8) PKCδ further executes apoptotic cell death by activating downstream signaling molecules.

CHAPTER V: DIELDRIN INDUCES APOPTOSIS IN A MESENCEPHALIC DOPAMINERGIC NEURONAL N27 CELL LINE VIA CASPASE-3-DEPENDENT PROTEOLYTIC ACTIVATION OF PROTEIN KINASE Cδ

A paper to be submitted for publication in Neurotoxicology

Masashi Kitazawa, Yongjie Yang, Siddharth Kaul, Michael L. Kirby, Vellareddy Anantharam, and Anumantha G. Kanthasamy

ABSTRACT

Previously, we have investigated dieldrin cytotoxicity and its signaling cell death mechanisms in dopaminergic PC12 cells. Dieldrin has been reported to be one of the environmental factors of Parkinson's disease and may selectively destroy dopaminergic neurons. Here, we further investigated dieldrin toxicity in dopaminergic neuronal cells, namely N27 cells. We have observed that dieldrin-treated N27 cells underwent a rapid and significant increase in reactive oxygen species followed by cytochrome c release into cytosol. The cytosolic cytochrome c activated caspase-dependent apoptotic pathway, and the increased caspase-3 activity was observed following 3-hr dieldrin exposure in dose-dependent manner. Furthermore, as previously reported, dieldrin caused the caspase-dependent proteolytic cleavage of protein kinase Cδ (PKCδ) into 41 kDa catalytic and 38 kDa regulatory subunits. PKCδ plays a critical role in the execution of apoptotic process in dieldrin-treated dopaminergic neuronal cells. Pretreatment with rottlerin, or transfection and over-expression of catalytically inactive $PKC\delta^{K376R}$ significantly attenuate dieldrin-induced DNA fragmentation and chromatin condensation, unique morphological features for apoptosis. Together, we conclude that caspase-3-dependent proteolytic activation of PKCδ is a critical event in dieldrin-induced apoptotic cell death in dopaminergic neuronal cells.

INTRODUCTION

Epidemiological studies of Parkinson's disease (PD) over the past decade have promoted the conclusion that idiopathic, geriatric-onset PD is an environmentally-mediated neurodegenerative disorder (Veldman et al., 1998; Stoessl, 1999; Engel et al., 2001; Herishanu et al., 2001; Anca et al., 2002). PD-associated factors most often cited include residence in a rural area, use of well water as a drinking water source and occupational use of pesticides, all of which can be linked to pesticide exposures, and have been reported in numerous epidemiological studies (Langston, 1996; Schulte et al., 1996; Liou et al., 1997; Chan et al., 1998; Marder et al., 1998; Smargiassi et al., 1998; Le Couteur et al., 1999; Taylor et al., 1999; Priyadarshi et al., 2000; Ritz and Yu, 2000; Tuchsen and Jensen, 2000; Priyadarshi et al., 2001). A recent landmark epidemiology study by Tanner and colleagues (Tanner et al., 1999) of nearly 20,000 twin pairs from a WWII veterans health care database determined that no clear genetic correlate exists to explain the incidence of PD and concluded that PD is an environmentally-mediated disorder. Postmortem studies of PD have reported significantly higher brain concentrations of chlorinated hydrocarbons and among these cyclodiene insecticides (Fleming et al., 1994; Corrigan et al., 1998; Corrigan et al., 2000), thus further suggesting a direct link between environmental exposure to neurotoxicants and PD.

Cyclodiene insecticides are heavily chlorinated toxicants that are known to primarily act as antagonists of the $GABA_A$ receptor ionophore (Gant et al., 1987; Bloomquist, 1993). Since the majority of GABA projections in brain are inhibitory in function, cyclodienes are pharmacologically defined as pro-convulsant chemicals (Bloomquist, 1992; Hawkinson and Casida, 1992; Bloomquist, 1993). Pharmacokinetically, cyclodienes and similar lipophilic chlorinated cage toxicants, collectively termed polychlorocycloalkanes, have been shown to accumulate in fatty tissues and brain (Matsumura, 1985; Murphy and Harvey, 1985). Dieldrin, specifically, is one of the most environmentally persistent insecticides known (Joy, 1994). Polychlorocycloalkanes were used extensively due to their excellent latent kill activity against crop and structural pests and the low cost of their manufacture, however bioaccumulation and

biomagnification issues in non-target species led to the banning of these chemistries in the 1970's, with a few exceptions (e.g., g-HCH, endosulfan, methoxychlor). Approximately 3 billion tons of these chemistries were manufactured and used commercially for insect control to date (Casida et al., 1988). Regardless of the current restricted use polychlorocycloalkanes in Western countries, ongoing human exposure through either direct contact with environmental residues, ground water exposure, or consumption of imported products from countries where these chemistries are still legal for agricultural and industrial use continues to pose a significant human health risk. Daily dietary exposure to dieldrin, according to a study of 120,000 U.S. adults, is estimated to be in excess of EPA minimum safety standards (MacIntosh et al., 1996).

Attempts to link dieldrin to emerging models of disease progression in idiopathic PD have been reported from our laboratory (Kitazawa et al., 2001; Kitazawa et al., 2002) and laboratories of other researchers (Chun et al., 2001). Previously, we demonstrated the existence and regulation of a selective toxicant-evoked apoptotic pathway in PC12 cells which incorporates a signal amplification loop between caspase-3 and protein kinase Cδ (Kitazawa et al., 2002). Herein we report additional work towards characterization of dieldrin-specific pro-apoptotic effects in a rat mesencephalic cell line (N27) with dopaminergic characteristics. The present work strongly reflects results reported in PC12 cell studies which indicated that dieldrin initiates apoptosis in cells by a mitochondrial mechanism that facilitates early onset reactive oxygen species generation, cytochrome c release to the cytoplasm, caspase cascade activation and PKCδ cleavage and activation. The central role of PKCδ as a link between initiation and end-point effects in dieldrin-induced apoptosis, as shown previously in our PC12 studies, is supported by evidence presented here and is discussed in the present work.

MATERIALS AND METHODS

Materials. Dieldrin, Hoechst 33342, and mouse monoclonal anti-β-actin antibody were purchased from Sigma (St. Louis, MO, USA). Caspase-3 substrate, Ac-DEVD-AMC was purchased from Bachem Biosciences Inc. (King of Prussia, PA). Caspase-3

specific inhibitor, Z-DEVD-FMK was purchased from Alexis Biochemicals (San Diego, CA). Hydroethidine was purchased from Molecular Probes (Eugene, OR, USA). Rabbit polyclonal anti-nPKCδ antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). ECL Western blotting analysis kit was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Cell Death Detection Elisa Plus Assay Kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Cytochrome c ELISA kit was obtained from MBL International Corp. (Watertown, MA). All tissue culture supplies were purchased from Gibco-BRL (Gaithersburg, MD, USA). Other routine laboratory reagents were purchased from Fisher Scientific (Pittsburg, PA, USA). Plasmids PKCδ^{K376R}-GFP fusion protein and pEGFP-N1 were kind gifts of Dr. Stuart H. Yuspa, National Cancer Institute (Bethesda, MD). Immortalized rat mesencephalic (N27) cell line was a kind gift of Dr. Kedar N. Prasad, Univ. of Colorado Health Sciences Center (Denver, CO).

Animals. Adult male Sprague Dawley rats (125-150 g; Zivic Miller Laboratory, Alison Park, PA) were used in all experiments with animal tissues. Rats were housed one per cage in a temperature-controlled room (23°C) with a 12:12 L:D cycle. Animals were fed standard laboratory diet and water *ad libitum*. Experimental procedures used here were approved by the Institutional Animal Care and Use Committee at Iowa State University. The Iowa State University vivarium is an AAALAC approved facility.

Cell lines. Immortalized rat mesencephalic cells ($1RB_3AN_{27}$, abbreviated here as N27 cells) were grown in RPMI medium supplemented with 10% fetal bovine serum, 1% L-glutamine, penicillin (100 U/ml), and streptomycin (100 U/ml), maintained at 37°C in a humidified atmosphere of 5% CO_2 (Clarkson et al., 1999; Anantharam et al., 2002). Vector-transfected (N27-pEGFP-NI) and PKCδ dominant negative mutant (N27-pPKCδ^{K376R}) were maintained in the supplemented medium above amended with 200 μg/ml hygromycin.

Stable transfection. Plasmid pPKCδ^{K376R}-GFP encodes protein kinase Cδ-GFP fusion protein, the number K376R refers to the mutation of lysine residue at position 376 to arginine in the catalytic site of PKCδ rendering it inactive (Li et al., 1999). Plasmid pEGFP-NI encodes the Green Fluorescent Protein alone and used as vector control. pEGFP-NI and pPKCδ^{K376R} were transfected into N27 cells using Lipofectamine Plus reagent according to the procedure recommended by the manufacturer. In brief, 8 μg of DNA, 24 μl of lipid and 24 μl of Plus reagent were used to transfect N27 cells in 100-mm tissue culture dishes at 50% confluence in 4 ml of culture medium without serum. Fresh medium containing serum was added 3 hr later. For stable cell lines, the N27 cells were selected in 400 μg/ml hygromycin, 48 hr after cotransfection with PCEP4 plasmid, which confers hygromycin resistance. Colonies were isolated with trypsin and glass cloning cylinders, and they were then re-plated and grown to confluence in T75 flasks and subsequently, the stable cell lines were maintained in 200 μg/ml hygromycin.

Isolation of cytosolic fraction in N27 cell homogenates. Cells were pelleted by centrifugation at 200 x g, 10 min, 4°C. The cell pellet was washed once with ice-cold PBS and resuspended in 2 ml homogenization buffer (20 mM Tris HCl, 2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotonin, 10 μg/ml leupeptin). The suspension was then sonicated for 10 sec and centrifuged at 100,000 x g for 1 hr at 4°C. Resulting supernatant was used as a cytosolic fraction. Protein concentration of each cytosolic fraction was determined using a Bradford protein assay dye reagent (BioRad Laboratories; Hercules, CA, USA). Samples were diluted with homogenization buffer according to the protein concentration estimated by the assay to equalize protein concentration for gel loading. Each sample was then mixed with 2x gel loading buffer containing 10% SDS and 200 mM DTT and placed in boiling water for 5 min.

Cell treatment methods. After 2-4 days in culture, N27 cells were harvested and resuspended in serum-free growth medium at a cell density of 1-3 x 10^6/ml. Cell suspensions were treated with DMSO (0.1% final concentration) or DMSO containing

varying concentrations of dieldrin (30-300 μM) over a period of 5 min to 3 hr at 37°C. In inhibitor studies, Z-DEVD-FMK (caspase-3-specific inhibitor, 50 μM) was added 30 min prior to the addition of dieldrin. The reaction samples were removed at various time points, centrifuged at 200 x g (5 min, 4°C), and cell pellets were used for assessing cytochrome c release, caspase-3 enzymatic activities, PKCδ cleavage and DNA fragmentation. Cell samples used for flow cytometry were further treated with visualization fluors and are described in methods below.

Brain slice preparation and treatment. Sprague Dawley male rats (125-150g) were euthanized by ether and decapitated. Brains were removed by brain case dissection to a cold table, dura and pia mater were removed by forceps, and brains were rinsed with 0.9% sterile saline. Brain sections (300 μm) were cut in 4°C carboxygenated (5% CO_2/95% O_2) artificial cerebrospinal fluid slicing medium (1.4 mM KCl, 685 μM NaH_2PO_4, 14 mM $NaHCO_3$, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 50 mM sucrose, 2.5 mM dextrose) using a Lancer Vibratome (model 1000; The Vibratome Co., St. Louis, MO, USA). Sections were transferred to carboxygenated artificial cerebrospinal fluid (ACSF; 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2.5 mM dextrose) and allowed to recover from trauma for 2 hr at 37°C prior to treatment with toxicants. At 2 hr, the incubation medium was refreshed with 37°C carboxygenated ACSF and DMSO (0.033% final concentration) or DMSO containing dieldrin (30-100 μM final concentration) was added to the medium and allowed to incubate with slices for 3 hr at 37°C. Following incubations, slices were removed to 1.5 ml tubes, centrifuged briefly at 1000 x g, the supernatant was discarded, and tissues were prepared for western blot by Dounce homogenization (15 strokes) in a modified lysis buffer (25 mM HEPES, 100 μM Na_2VO_4, 300 μM NaCl, 1.5 mM $MgCl_2$, 200 μM EDTA, 50 mM dithiothreitol, 10 μl Triton X-100, 20 mM NaF, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 50 μg/ml leupeptin) at 400 μl/slice. Homogenates were centrifuged (12,000 x g, 20 min, 4°C) and protein concentrations of supernatants were determined using a Bradford protein assay dye reagent (BioRad Laboratories, Hercules, CA, USA). Samples were diluted to

protein concentrations appropriate for gel loading and boiled 5 min in 2x gel loading buffer containing 10% SDS and 20 mM dithiothreitol. Samples were stored at –80°C until used for western blot analysis.

Reactive oxygen species (ROS) flow cytometry. Flow cytometry analysis was performed on a Becton Dickenson FACScan™ flow cytometer (Becton Dickinson, San Francisco, CA). Hydroethidine, a sodium borohydride-reduced derivative of ethidium bromide, is used to detect ROS produced specifically O_2^- inside the cell (Narayanan et al., 1997). Hydroethidine loaded to cells binds to cellular macromolecules and reacts with O_2^- as it is generated, converting hydroethidine to ethidium bromide, increasing red fluorescence (620 nm). A 15-mW air-cooled argon-ion laser was used as an excitation source for hydroethidine at 488 nm and the optical filter was 585/42 nm bandpass. Cells were detected and distinguished from the background by forward-angle light scattering (FSC) and orthogonal light scattering (SSC) characteristics. All the flow cytometric data were analyzed by Cellquest™ data analysis software to determine the significant increase or decrease of fluorescence intensity.

Cytochrome c release assay. Dieldrin-induced cytochrome c release was measured using a cytochrome c ELISA kit as described previously (Anantharam et al., 2002). Briefly, N27 cells (5 x 10^6 cells) were resuspended in serum-free RPMI-1640. Cell suspensions were exposed to 100 μM or 300 μM dieldrin for 15-30 min at 37°C. After exposure, cells were collected, washed once with ice-cold phosphate-buffered saline (PBS; pH 7.4) and resuspended in 1 ml of ice-cold homogenization buffer (10 mM Tris HCl pH 7.5, 0.3 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 10 μg/ml leupeptin). Following homogenization, cells were centrifuged at 10,000xg for 60 min at 4°C. Resulting supernatants were collected as cytoplasmic fraction and used to measure cytochrome c release by cytochrome c ELISA assay kit strictly following the protocol provided by the manufacturer (MBL, Watertown, MA, USA). Optical density of each well was then measured at 450 nm using a microplate reader (Molecular Devices

Corp., Sunnyvale, CA, USA). Concentration of cytochrome c was calibrated from a standard curve based on reference standards.

Caspase-3 activity. Caspase activities were determined as previously described (Anantharam et al., 2002). Briefly, after the exposure to dieldrin, cells were washed once with PBS and resuspended in lysis buffer containing 50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 10 μM digitonin. Cells were then incubated at 37°C for 20-30 min to allow complete lysis. Lysates were quickly centrifuged and cell-free supernatants were incubated with 50 μM Ac-DEVD-AMC (caspase-3 substrate) at 37°C for 1 hr. Caspase activity was then measured using a microplate reader (Molecular Devices Corp., Sunnyvale, CA) with excitation at 380 nm and emission at 460 nm. Caspase activity was expressed as fluorescence unit (FU) per mg protein per hr.

Western blot. Cytoplasmic fractions or brain tissue samples containing equal amounts of protein (5-10 μg) were loaded in each lane and separated on a 10% SDS-polyacrylamide gel. Proteins were then transferred to nitrocellulose membrane by electro-blotting overnight (4°C, 25 V). Non-specific binding sites were blocked by treating the nitrocellulose membranes with 5% non-fat dry milk powder for 2 hr prior to treatment with primary antibodies. Nitrocellulose membranes containing the proteins were incubated with rabbit anti-PKCδ for 1 hr at RT (1:2000 dilution). Primary antibody treatments were followed by treatment with secondary HRP-conjugated antir-abbit IgG (1:2000 dilution) for 1 hr at RT. Secondary antibody-bound proteins were detected using Amersham's ECL chemiluminescence kit. To confirm equal protein loading, blots were re-probed with a β-actin antibody (1:5000 dilution). Gel photographs were taken with a gel imaging system and quantification of bands was performed using Scion Image.

Annexin V and propidium iodide flow cytometry. Flow cytometry analysis of apoptotic and necrotic N27 cells following 3 hr exposure to dieldrin (100 μM) was performed by Annexin V-FTIC and propidium iodide (PI) staining kit (BD PharMingen)

as per the manufacturer's specifications. Annexin V binds to phosphatidylserine (PS) and other negatively charged phospholipids producing fluorescence primarily indicative of PS translocation from the inner to the outer cell membrane leaflet reflective of aminophospholipid translocase activity in apoptotic cells (Bratton et al., 1997). PI is a nucleic acid dye that penetrates the nuclear envelope of necrotic cells and was used here as a counter stain to differentiate between live, apoptotic, late stage apoptotic/early stage necrotic, and necrotic cells. Flow cytometry analysis was performed on a Becton Dickenson FACScan™ flow cytometer (Becton Dickinson, San Francisco, CA). N27 cells were washed 2x with cold phosphate-buffered saline (pH 7.4) and resuspended in a binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$; pH 7.4) at a concentration of 0.5 x 10^6 cells/ml. Cell aliquots of 100 µl were incubated with Annexin V-FITC (5 µl) and PI (2 µl) for 15 min at RT in the dark. After 15 min, incubates were diluted with 400 µl of binding buffer and analyzed by flow cytometry. A 15-mW air-cooled argon-ion laser was used as an excitation source for Annexin V-FITC at 488 nm with optical filter at 530/15 nm bandpass. PI fluorescence was measured with optical filter at 650/42 nm bandpass. Cells were detected and distinguished from the background by forward-angle light scattering (FSC) and orthogonal light scattering (SSC) characteristics. All the flow cytometric data were analyzed by Cellquest™ data analysis software to determine the significant increase or decrease of fluorescence intensity.

DNA fragmentation analysis. DNA fragmentation assay was performed using a recently developed Cell Death Detection Elisa Plus Assay Kit (Roche Applied Science, Basel, Switzerland). N27 cells were exposed to DMSO (0.1% final concentration) or DMSO containing dieldrin (30-100 µM) for 3 hr at 37°C. Following treatment, cells were centrifuged 200 x g, 5 min, 4°C and washed once with 1X phosphate-buffered saline (pH 7.4). Cells were then incubated with a lysis buffer (supplied with the kit) at RT for 30 min. Incubates were centrifuged 10,000 x g, 20 min, 4°C and 20 µl aliquots of supernatant were dispensed to streptavidin-coated 96 well microtiter plates followed by addition of 80 µl of antibody cocktail. Plates were incubated for 2 hr at RT with mild

shaking. The antibody cocktail consisted of a mixture of anti-histone biotin and anti-DNA-HRP directed against various histones and antibodies to both single strand DNA and double strand DNA, which are major constituents of the nucleosomes. After incubation, unbound components were removed by washing with the incubation buffer supplied with the kit. Quantitative determination of the amount of nucleosomes retained by anti-DNA-HRP in the immunocomplex was determined spectrophotometrically with ABTS as a HRP substrate (supplied with the kit). Measurements were made at 405 nm against an ABTS solution as a blank (reference wavelength ~490 nm) using a microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA).

Immunocytochemistry. N27 cells were grown on collagen (6 $\mu g/cm^2$) coated slides for 2-3 days in a 37°C, 5% CO_2 incubator. Cells were washed twice with phosphate-buffered saline (pH 7.4) and treated for 3 hr with DMSO (0.1% final concentration) or DMSO containing dieldrin (100 μM). Cells were again washed with phosphate-buffered saline and were fixed with 10% buffered formaldehyde for 30 min at room temperature, followed by staining with Hoechst 33342 (10 $\mu g/ml$) for 3 min in the dark. Cells stained with Hoechst 33342 dye fluoresce bright blue upon binding to DNA in the nucleus. The nucleus of apoptotic cells exhibits strong blue staining and staining pattern is heterogeneous and occurs in patches, indicative of chromatin condensation, whereas the nucleus of non-apoptotic cells exhibit more diffused, weak and homogenous staining (Shimizu et al., 1996; Du et al., 1997). Slide-mounted cells were observed under a Nikon DiaPhot microscope under UV illumination, and pictures were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Data analysis. Data were first analyzed using one-way ANOVA. Dunnett's post-test or Bonferroni's multiple comparison test was then performed to compare treated samples, and $p<0.05$ was considered to be significant.

RESULTS

Dieldrin-induced reactive oxygen species (ROS). Exposure of N27 cells to dieldrin resulted in a rapid, transient increase in generation of ROS as measured by flow cytometric analysis of hydroethidium dye oxidation (Fig. 1A). A significant shift of fluorescent intensity indicates the massive generation of intracellular ROS in time-dependent manner. Moderate concentrations of dieldrin (30 μM) produced a significant time-dependent increase of ca. 65% in ROS levels 5 min after treatment ($p<0.01$), which appeared to reach signal saturation by 15 min at ca. 200% of vehicle control (Fig. 1B). Comparison of exposure of N27 cells to various concentrations of dieldrin (30-200 μM) indicated that approximately similar levels of ROS generation were reached at 5 min post-treatment (ca. 145-165% vehicle control) and subsequently reversed at concentrations of dieldrin greater than 100 μM beyond 5 min exposure (data not shown).

Dieldrin promotes mitochondrial cytochrome c release. Dieldrin-mediated cytochrome c release, an early event in apoptosis, measured colorimetrically by ELISA indicated time- and dose-dependent increases in the appearance of cytochrome c in cytosol over a 30 min dieldrin exposure period (Fig. 2). Dieldrin significantly increased cytochrome c release 50% and 140% at 15 and 30 min post-treatment, respectively ($p<0.01$). Exposure of N27 cells to 300 μM dieldrin evoked a significant increase in accumulation of cytosolic cytochrome c 110% and 260% following 15 min and 30 min of dieldrin exposure, respectively ($p<0.0001$).

Dieldrin-mediated activation of caspase-3. Dieldrin increased N27 cell activity of caspase-3, an important effector caspase in apoptosis, in a dose-dependent manner after 3 hr of exposure as measured by Ac-DEVD-AMC fluorometry ($p<0.001$; Fig. 3). Increases in caspase-3 activities measured were 210%, 260% and 340% of vehicle control for 30, 100 and 300 μM dieldrin, respectively. No significant vehicle effect (DMSO, 0.2% final concentration) was observed, suggesting that measured effects were directly attributable to dieldrin exposure. Concentrations of dieldrin above 100 μM

appeared to produce similar levels of caspase-3 activities, whereas 30 μM dieldrin-induced increases in caspase-3 activities were intermediate with respect to concentrations above 100 μM and the vehicle control.

Caspase-3-dependent dieldrin-induced cleavage and activation of PKCδ. Concentrations of dieldrin (100 and 300 μM) effecting maximum increases in caspase-3 activities increased the cleavage and activation of PKCδ in a concentration-dependent manner 3 hr following treatment as measured by western blot analysis (Fig. 4A). Previously, it has been documented that PKCδ is selectively activated by caspase-3 under conditions of toxicant exposure (Emoto et al., 1995; Reyland et al., 1999; Anantharam et al., 2002). Incubation with a selective caspase-3 inhibitor, Z-DEVD-FMK (50 μM), for 30 min prior to 3 hr treatment of N27 cells with 100 μM dieldrin markedly reduced PKCδ cleavage and activation (ca. 70% reduction; Fig. 4B) approxmating basal levels of cleaved products observed in controls, confirming proteolytic cleavage of PKCδ is caspase-3 dependent.

Preliminary results from incubations of 300 μm coronal sections of rat midbrain tissue with concentrations of dieldrin (30 μM) indicated results similar to that seen above in N27 cells (Fig. 4C). Dieldrin at 30 μM produced a 75.3% increase in cleaved products of PKCδ, reminiscent of changes observed with 100 μM dieldrin in N27 cells (see Fig. 4B) and suggested that similar proteomic processes may be activated *in situ* following dieldrin exposure. Also, brain tissues appeared to be more sensitive to dieldrin neurotoxicity, indicating that dieldrin could cause greater adverse effect in dopaminergic neurons in actual situation.

Annexin V and propidium iodide indicate dieldrin-mediated apoptosis. Flow cytometric analysis of N27 cells incubated 3 hr with DMSO (0.1% final concentration) or DMSO containing dieldrin (100 μM) produced marked increases in both apoptotic (annexin V, 51%) and apoptotic/necrotic or late apoptotic (annexin V and propidium iodide, 31%) indices, whereas vehicle-treated cells did not increase apoptosis as indicated to be 9%

and 13% on apoptotic and late apoptotic, respectively (Fig. 5). Chi square analysis of the distribution of annexin V FITC and propidium iodide positive cells indicated a positive trend toward apoptosis in dieldrin-treated N27 cells (χ^2=69.12, p<0.0001).

Dieldrin-mediated DNA fragmentation and apoptosis. We measured dieldrin-induced DNA fragmentation by ELISA method in N27 cells treated with 100 μM dieldrin for another 3 hr with or without pretreatment of 1-3 μM rottlerin, a selective PKCδ specific inhibitor, for 30 min, to further confirm the role of PKCδ activity during dieldrin-induced apoptosis. As shown in Fig. 6A, rottlerin dose-dependently protected dieldrin-induced DNA fragmentation. Especially, 3 mM rottlerin significantly (p<0.01) attenuated dieldrin toxicity, and nearly 30% reduction of DNA fragmentation was observed. The levels of chromatin condensation observed by Hoechst 33342 were 64%, 40%, and 28% in 100 μM dieldrin only, dieldrin + 1 μM rottlerin, and dieldrin + 3 μM rottlerin, respectively.

We have also utilized genetic approach to characterize the pro-apoptotic function of PKCδ. Catalytically inactive PKCδ mutant ($PKC\delta^{K376R}$) was stably transfected to N27 cells, dieldrin-induced DNA fragmentation was measured. Vector-transfected N27 and PKCδ mutant-transfected N27 cells were treated with 100 μM dieldrin for 3 hr. DNA fragmentation was indicated a significant dieldrin concentration-dependent increase (Fig. 7A; N27-vector, p<0.0001; N27-$PKC\delta^{K376R}$, p<0.001). Maximal increases in DNA fragmentation were observed with 100 μM dieldrin treatment (N27-vector, 346.7%; N27- $PKC\delta^{K376R}$, 212.4%). Comparison of the N27-vector and N27-$PKC\delta^{K376R}$ cell lines treated with 100 μM dieldrin revealed a significant decrease in DNA fragmentation (134.3% decrease, p<0.01) in the dominant negative mutant PKCδ cell line. Our previous report that cells expressing a dominant negative form of PKCδ were nearly completely resistant to DNA fragmentation following methylcyclopentadienyl manganese tricarbonyl exposure was performed in cells transiently expressing a catalytically inactive mutant of PKCδ (Anantharam et al., 2002). In the present study, the N27-DN cell line stably expresses catalytically inactive PKCδ

at lower levels than that achieved with transient expression and is likely the reason for the partial effect observed here. Chromatin condensation indicative of apoptosis were also examined by Hoechst 33342 staining of 3 hr dieldrin-treated N27-DN cells (Fig. 7B).

DISCUSSION

Results presented here further support our hypothesis that dieldrin contributes to apoptotic cell death in dopaminergic neuronal cells. Previously, we and other researchers have shown the selective toxicity of dieldrin on dopaminergic cells (Sanchez-Ramos et al., 1998; Kitazawa et al., 2001) as well as characterized the subsequent signaling cell death mechanism in dopaminergic PC12 cells (Kitazawa et al., 2002). Here, we have used clonal dopaminergic neuronal cells, which were more relevant to link between the effect of dieldrin and dopaminergic neurodegeneration. The cell death pathway observed in dopaminergic neuronal cells following acute exposure to dieldrin was identical to that observed in PC12 cells; that is, i) initial and rapid increase of reactive oxygen species (ROS), ii) possible mitochondrial damage and subsequent release of cytochrome c, iii) caspase-3 activation and proteolytic cleavage of PKCδ, and iv) apoptotic cell death as a result of activation of these pro-apoptotic molecules.

Generation of ROS was a rapid response to dieldrin toxicity. Within 5 min after the exposure, cells increased intracellular ROS by 50% from the basal level, suggesting dieldrin somehow interacts with certain cellular molecules to potentiate the production of ROS as soon as it gets into the cells. Possible candidates could be mitochondrial membrane proteins responsible for cellular respiration. It has been reported that dieldrin inhibits mitochondrial electron transport system (ETS) near the complex III (Bergen, 1971). Termination of ETS causes accumulation of reduced form of electron carrier proteins and unused oxygen, resulting in uncoupling in mitochondria and conversion of oxygen into ROS. Since the generation of ROS was observed so rapidly, the primary target of dieldrin could be mitochondria as also shown previously (Kitazawa et al., 2001).

Subsequently, significant release of cytochrome c was observed. The release of cytochrome c was dose- and time-dependent, and it was as early as 15 min following dieldrin exposure. The release of cytochrome c and other pro-apoptotic factors, such as apaf-1 from mitochondria have been known to activate caspase-9 (Li et al., 1997; Kuida, 2000). Caspase-9 serves as an initiator caspase, and it further proteolytically cleaves and activates effector caspases including caspase-3, -6, and -7 (Budihardjo et al., 1999). We have observed significant increase in caspase-3 activity following 3-hr dieldrin exposure in dose-dependent manner. Both 100 and 300 μM dieldrin showed significance over control and vehicle treated cells, indicating the level of dieldrin-induced cytochrome c release is sufficient to promote mitochondrial-mediated apoptotic cell process in dopaminergic cells.

Accumulating evidence strongly suggested the pro-apoptotic role of PKCδ during apoptosis (Emoto et al., 1995; Reyland et al., 1999; Matassa et al., 2001). We have verified that the proteolytic activation of PKCδ was due to caspase-3 activation. Caspase-3 specific inhibitor, Z-DEVD-FMK, blocked the proteolytic cleavage of PKCδ by 70%, indicating majority of PKCδ cleavage was due to caspase-3. Previously, we have also shown that PKCδ plays an essential role in environmental chemical-induced apoptotic cell death in PC12 cells (Anantharam et al., 2002; Kitazawa et al., 2002). In these reports, PKCδ not only facilitates downstream apoptotic process including DNA fragmentation, but also modulate upstream process including caspase-3 activity, with unknown mechanism. The regulatory role of PKCδ has also been documented in elsewhere (Reyland et al., 1999), but the exact mechanism and function of PKCδ in regulatory role remain to be elucidated.

In the present paper, we focus on the execution role of PKCδ. We have shown dieldrin-induced apoptosis using annexin-V-FITC. Further experiments were conducted to characterize whether PKCδ played an important role in dieldrin-induced DNA fragmentation. To answer this question, we have utilized catalytically inactive PKCδ mutant ($PKC\delta^{K376R}$)-expressed dopaminergic cells and measured DNA fragmentation using ELISA technique. The kinase activity of mutant cells has been documented

previously by our laboratory (Kitazawa et al., 2002). Mutant cells showed partially protective following dieldrin exposure, indicating that PKCδ is modulating DNA fragmentation somehow. Furthermore, pretreatment with PKCδ specific inhibitor, rottlerin, also dose-dependently reduced nuclear condensation. These results match with previously published data.

In conclusion, we have verified that dieldrin is a potent apoptosis induced in dopaminergic neuronal cells. Comparing with our previous data from dopaminergic PC12 cells, neuron-derived cells seem to be more sensitive to dieldrin toxicity. Further research will be necessary to understand dieldrin neurotoxicity and the impact to pathogenesis of neurodegenerative disorders including Parkinson's disease.

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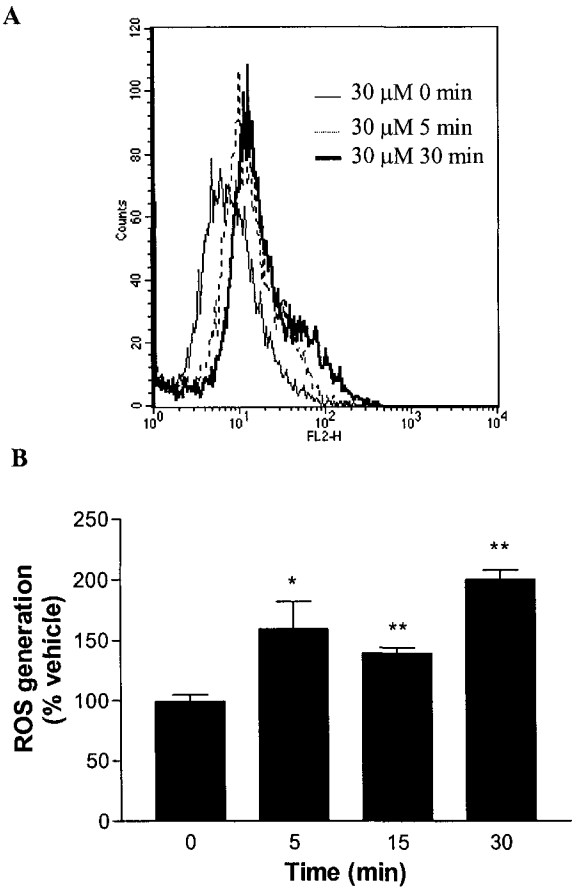


Figure 1: Dieldrin-induced ROS generation in N27 cells.

N27 cells (~1 x 10^6 cell/ml) were treated with 30 μM dieldrin for 0-30 min. Hydroethidine fluorescence intensity was measured at various time points (0, 5, 15, 30 min) by a flow cytometry. (A) representative shift of fluorescent intensity during dieldrin treatment. (B) Quantitative analysis of ROS generation. Data represent the mean ± SEM for three separate experiments performed in triplicate. Significance was determined by ANOVA followed by Dunnett's post-test between the vehicle-treated group and dieldrin-treated group (*$p<0.05$ and **$p<0.01$).

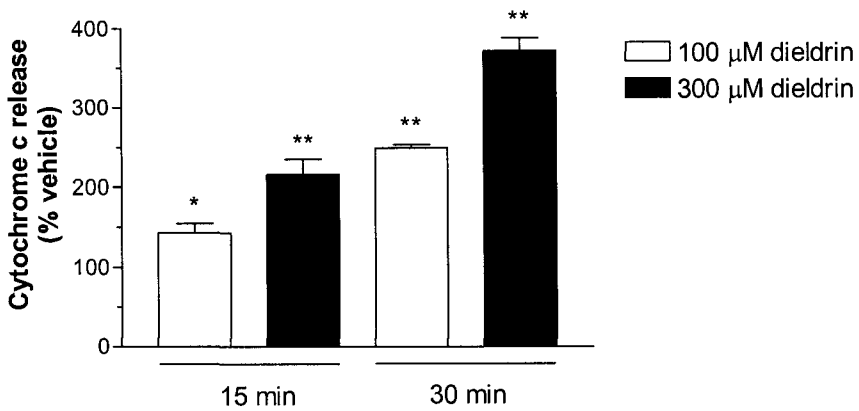


Figure 2: Dieldrin-induced cytochrome c release in N27 cells.

N27 cells (5 x 10^6 cells) were exposed to 100 or 300 μM dieldrin for 15-30 min. Mitochondria-free cytosolic fraction was collected as described in "Materials and Methods", and cytosolic cytochrome c was measured using ELISA cytochrome c assay. Data represent the mean ± SEM for three separate experiments performed in triplicate. *$p<0.05$ or **$p<0.01$ compared with vehicle-treated group in each time point.

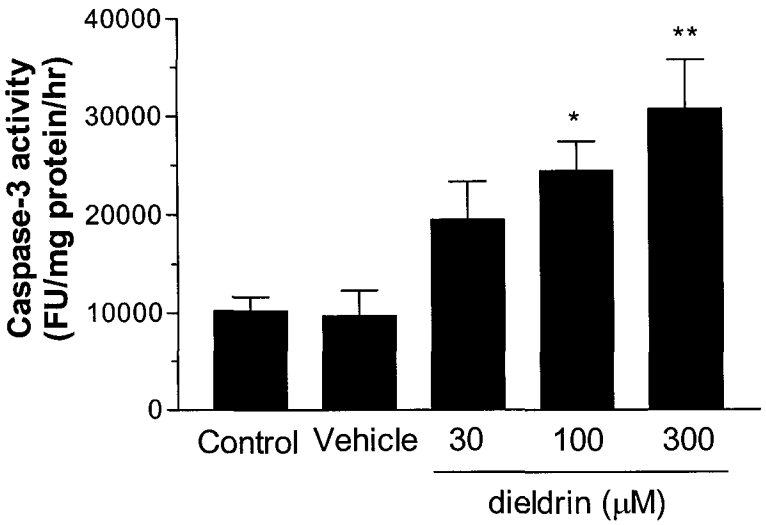
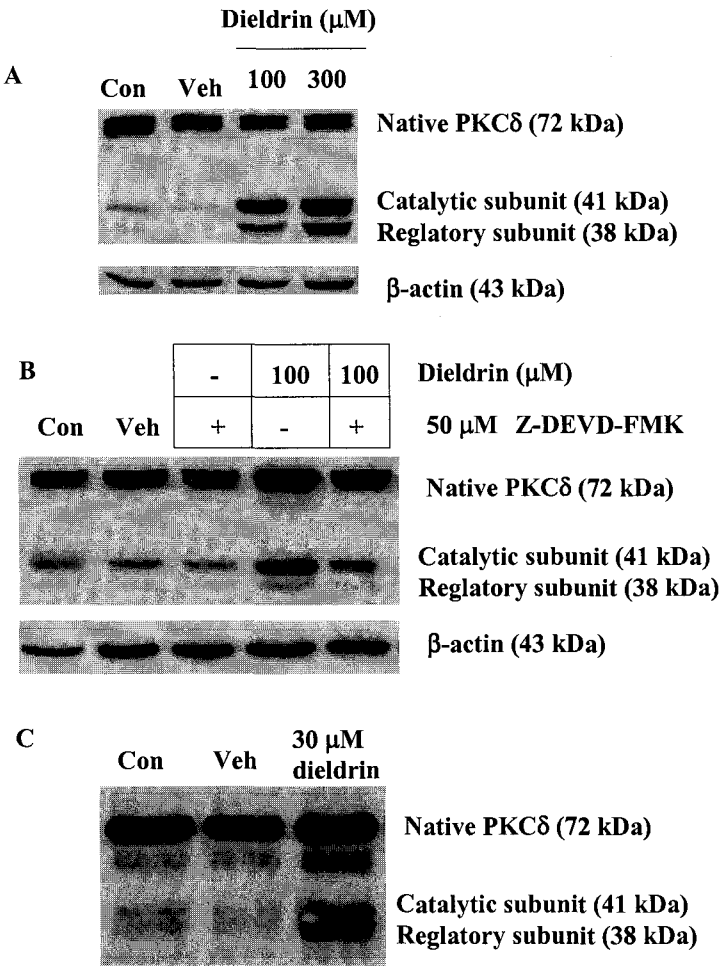


Figure 3: Dieldrin-induced caspase-3 activity in N27 cells.

N27 cells (2 x 10^6 cells/ml) were exposed to 30-300 μM dieldrin for 3 hr at 37°C, and caspase-3 activity was measured using caspase-3 specific substrate, Ac-DEVD-AMC as described under "Materials and Methods." The data are expressed as fluorescent unit (FU) per mg protein per hr of incubation. Each point represents mean ± SEM from two separate experiments in triplicate. *$p<0.05$ or **$p<0.01$ compared with vehicle-treated cells.

Figure 4: Proteolytic cleavage of PKCδ following dieldrin treatment in N27 cells.

(A) N27 cells (~1 x 10^7 cells) were exposed to 100 μM or 300 μM dieldrin for 3 hr at 37°C, and cytosolic proteins were collected as described under "Materials and Methods." Approximately 5 μg of cytosolic proteins were resolved by 10% SDS-PAGE and determined native PKCδ (72 kDa), catalytic subunit (41 kDa) and regulatory subunit (38 kDa) of proteolytically cleaved PKCδ. (B) N27 cells were pretreated with caspase-3 specific inhibitor, Z-DEVD-FMK (50 μM), for 30 min, then exposed to 100 μM dieldrin for another 3 hr. The equal protein loading was confirmed by reprobing with β-actin (43 kDa). (C) Brain slices (300 μm) from Sprague Dawley male rats were treated with 30-100 μM dieldrin for 3 hr. Cytosolic fraction was collected, and proteins were resolved by 10% SDS-PAGE. Native PKCδ (72 kDa) and proteolytically cleaved PKCδ (38-41 kDa) were detected using PKCδ antibody (Santa Cruz Biotechnology).


Dieldrin (μM)
A
Con
Veh
100
300
Native PKCδ (72 kDa)
Catalytic subunit (41 kDa)
Reglatory subunit (38 kDa)
β-actin (43 kDa)
B
-
100
100
Dieldrin (μM)
Con
Veh
+
-
+
50 μM Z-DEVD-FMK
Native PKCδ (72 kDa)
Catalytic subunit (41 kDa)
Reglatory subunit (38 kDa)
β-actin (43 kDa)
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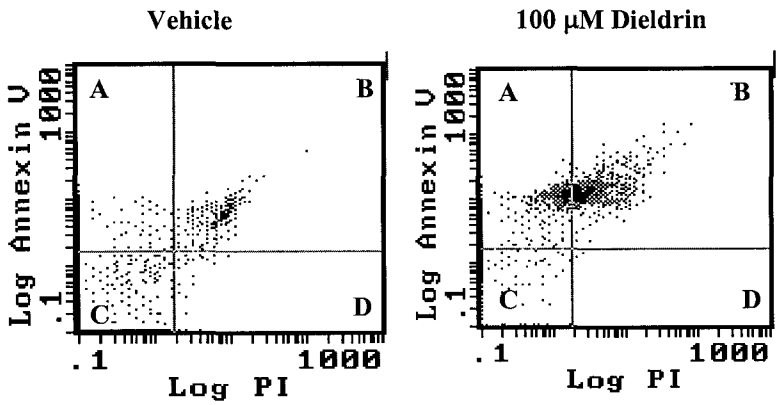
Catalytic subunit (41 kDa)
Reglatory subunit (38 kDa)



	A	B	C	D
	Apoptotic	Late apoptotic	Live	Necrotic
Vehicle	9%	13%	75%	3%
Dieldrin	51%	31%	18%	0%

Figure 5: Dieldrin-induced apoptosis in N27 cells.

N27 cells (~1 x 10^6 cells/ml) were treated with 100 μM dieldrin for 3 hr, and apoptotic cells were detected by flow cytometry as described in "Materials and Methods." By the dual staining of cells with annexin-V-FITC and propidium iodide, detected cells were divided into four regions (A, B, C, and D). Region A is apoptotic cells, B is apoptotic and necrotic cells, C is live or healthy cells, and D is necrotic cells. Experiment was repeated three times and data represent the average of each region.

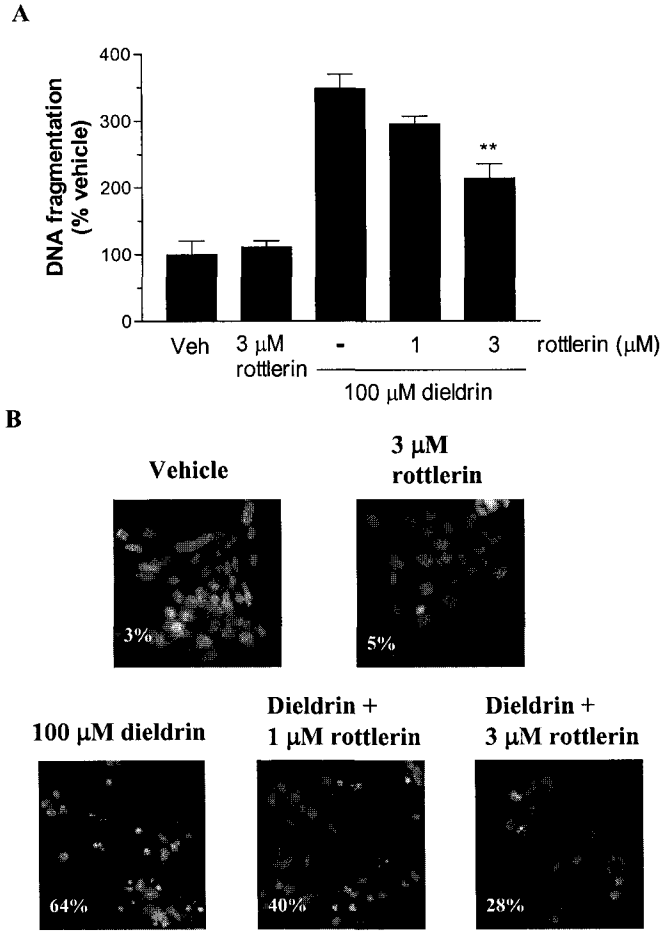


Figure 6: The role of PKCδ in dieldrin-induced DNA fragmentation and nuclear condensation in N27 cells.

N27 cells were pretreated with 1-3 μM rottlerin for 30 min then treated with 100 μM dieldrin for another 3 hr. (A) DNA fragmentation was quantitatively measured by ELISA DNA fragmentation assay kit. Each bar represents mean ± SEM for two separate experiments in triplicate. **$p<0.01$ compared with dieldrin-treated group. (B) Chromatin condensation was observed using Hoechst 33342 staining. The percentage of nuclear condensation was calculated by counting positive cells in three to five randomly selected regions. The experiment was repeated three times, and similar results were obtained.

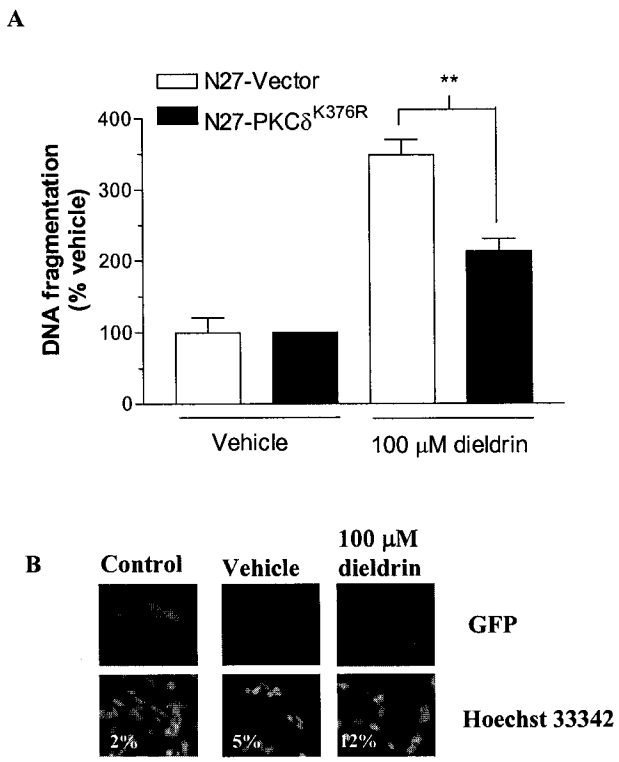


Figure 7: The role of PKCδ in dieldrin-induced DNA fragmentation and nuclear condensation in N27 cells.

Vector-transfected N27 cells and PKCδ^{K376R}-transfected N27 cells were exposed to dieldrin for 3 hr. DNA was extracted and DNA fragmentation was measured using ELISA DNA fragmentation assay kit as described in "Materials and Methods." Each bar represents mean ± SEM. *$p<0.05$ or **$p<0.01$ compared with vehicle-treated cells or between indicated groups. (B) PKCδ^{K376R}-transfected N27 cells were treated with 100 μM dieldrin for 3 hr, and nuclei were stained using 10 μg/ml Hoechst 33342. The percentage of chromatin condensation was calculated by counting positive cells in three to five randomly selected regions. The experiment was repeated three times, and similar results were obtained.

CHAPTER VI: ROLE OF PROTEIN KINASE Cδ AND BCL-2 IN CASPASE-3-DEPENDENT APOPTOSIS DURING MANGANESE EXPOSURE IN DOPAMINERGIC CELLS

A paper to be submitted for publication in Proceedings of National Academy of Sciences

Masashi Kitazawa, Vellareddy Anantharam, Yoko Hirata, and Anumantha G. Kanthasamy

ABSTRACT

Chronic inorganic manganese exposure causes selective toxicity to the nigrostriatal dopaminergic system resulting in a Parkinsonian-like neurological condition known as Manganism. However, the mechanisms underlying manganese-induced dopaminergic cell death are not well characterized. Herein, we examined the manganese-induced apoptotic cell death process in two cell culture models of Parkinson's disease, PC12 cells and mesencephalic dopaminergic neuronal cells. Chronic exposure of PC12 cells to manganese induced a sequential activation of mitochondrial dependent pro-apoptotic events including mitochondrial membrane depolarization, cytochrome c release, caspase-3 activation, and DNA fragmentation. Overexpression of Bcl-2 in PC12 cells remarkably attenuated manganese-induced mitochondrial membrane depolarization, cytochrome c release, caspase-3 activity, and DNA fragmentation, indicating that the mitochondrial dependent apoptotic cascade contributes to manganese-induced apoptosis. Furthermore, we identified that protein kinase Cδ (PKCδ) is an important downstream cellular target of caspase-3, which proteolytically cleaves the kinase to cause persistent activation. The manganese-induced proteolytic cleavage of PKCδ was also significantly blocked by

Bcl-2 overexpression. Administration of active recombinant PKCδ induced DNA fragmentation in PC12 cells, suggesting an important role of PKCδ in apoptotic cell death. We also evaluated the role of PKCδ in dopaminergic neuronal cells (N27 cells) expressing a catalytically inactive $PKC\delta^{K376R}$ protein (PKCδ dominant negative mutant) after manganese exposure. DNA fragmentation was significantly reduced in $N27PKC\delta^{K376R}$ cells as compared to fragmentation in vector-transfected cells following manganese treatment. Together, these results suggest that the mitochondrial dependent apoptotic cascade mediates apoptosis via proteolytic activation of PKCδ in manganese-induced dopaminergic toxicity.

Key Words: manganese, Bcl-2, caspase-3, mitochondria, environmental factors, Parkinson's disease.

INTRODUCTION

Chronic exposure to high levels of manganese is known to cause neurological symptoms similar to idiopathic Parkinson's disease (PD) in both humans and laboratory animals (1). The hallmark of idiopathic PD is the selective degeneration of dopaminergic neurons in the substantia nigra pars compacta, resulting in debilitating motor impairment. Manganese exposure is associated with many occupations such as mining, automobile garage maintenance, dry cell battery manufacturing, and agricultural application of manganese containing pesticides (Maneb) (2). However, the manganese containing organic compound methylcyclopentadienyl manganese tricarbonyl (MMT), used as an anti-knocking agent in gasoline and emitted as inorganic manganese upon combustion (3, 4), was recently legalized in the U.S.. Consequently, exposure to manganese is likely to increase. Manganese induces a variety of cellular changes including dopamine depletion, impairment of the antioxidant system, and increased

oxidative stress (1, 5).

Apoptosis is recognized as a major cell death process in neurodegenerative disorders including Parkinson's disease (6, 7). Manganese induced apoptosis in human B cells (8) and in rat pheochromocytoma (PC12) cells (9, 10). In addition, manganese enhances oxidative stress-mediated L-DOPA toxicity, indicating that manganese is a potent dopaminergic neurotoxicant (11). The mitochondrion is one of the important cellular targets in manganese-induced apoptotic cell death in dopaminergic cells (12-14). The Bcl-2 family proteins have central regulatory roles in mitochondrial dependent apoptotic cell death (15). Overexpression of Bcl-2 proteins attenuates apoptosis induced by various chemicals (8, 16, 17), demonstrating the important anti-apoptotic function of Bcl-2 proteins.

The downstream targets of activated caspase-3 in manganese-induced apoptosis that result in DNA fragmentation are not well characterized. In the present study, we examined the sequential activation of apoptotic signaling molecules in two dopaminergic cell models, pheochromocytoma (PC12) cells (18, 19) and immortalized rat mesencephalic dopaminergic neuronal (N27) cells (20, 21). Herein, we demonstrate that manganese activates primarily the mitochondrial-dependent apoptotic cascade in dopaminergic cells, and that proteolytic cleavage of protein kinase Cδ (PKCδ) by caspase-3 is key in the mediation of apoptotic cell death.

MATERIALS AND METHODS

Chemicals

Manganese chloride, mouse monoclonal β-actin antibody, propidium iodide, and human recombinant active PKCδ protein were purchased from Sigma Chemical Co. (St. Louis, MO). Phorbol-12-myristate-13-acetate (TPA) was purchased from Calbiochem (La Jolla, CA). The caspase-3 substrate Ac-DEVD-AMC was purchased from Bachem Biosciences Inc. (King of Prussia, PA). The caspase-9 substrate

Ac-LEHD-AMC and the caspase-3 inhibitor Z-DEVD-FMK were purchased from Alexis Biochemicals (San Diego, CA). The caspase inhibitor Z-VAD-FMK was purchased from Enzyme Systems Products (Livermore, CA). Acridine orange was purchased from Molecular Probes (Eugene, OR). Rabbit polyclonal anti-nPKCδ antibody and mouse monoclonal Bcl-2 antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The ECL Western blotting analysis kit was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The cytochrome c ELISA kit was obtained from MBL International Corp. (Watertown, MA). The cell Death Detection ELISA Plus Assay Kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). The cytotoxicity detection kit was purchased from Roche (Mannheim, Germany). BioPORTER was purchased from Gene Therapy Systems (San Diego, CA). Dulbecco's modified Eagle medium (DMEM) was purchased from Mediatech, Inc. (Herndon, VA). Heat inactivated horse serum and fetal bovine serum were purchased from Gibco BRL Products (Gaithersburg, MD). Other routine laboratory chemicals were obtained from Fisher Scientific (Pittsburgh, PA). The Bcl-2-transfected PC12 cells (PC12HB2-3) and vector-transfected PC12 cells (PC12V4) were generous gifts from Drs. Yutaka Eguchi and Yoshihide Tsujimoto, Osaka University (Osaka, Japan). The immortalized rat mesencephalic ($1RB_3AN_{27}$ or N27) cell line was a kind gift from Dr. Kedar N. Prasad, University of Colorado Health Sciences Center (Denver, CO). Plasmids $PKC\delta^{K376R}$-GFP fusion protein and pEGFP-N1 were kind gifts from Dr. Stuart H. Yuspa, National Cancer Institute (Bethesda, MD).

Cell culture

PC12V4 and PC12HB2-3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 7% heat-inactivated horse serum and 4% fetal bovine serum. The immortalized rat mesencephalic cell line (N27 cells) was grown in RPMI-1640

medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 μg/ml streptomycin. Both cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The MTT assay is another method used to assess cytotoxicity (9, 22). N27 cells (~10,000 cells/well) were plated onto a 96-well plate for 24 hr and treated with various concentrations of $MnCl_2$ for 24 hr. After the treatments, cells were washed once and then incubated in serum-free medium containing 0.25 mg/ml MTT for 3 hr at 37°C. Supernatants were removed and MTT crystals were solubilized with acidic isopropanol. The cytotoxicity index was measured by spectrophotometry at 570 nm with the reference wavelength at 630 nm, as described previously (18).

Cytochrome c detection assay

Manganese-induced cytochrome c release was measured using a cytochrome c ELISA kit following the step-by-step procedure as described in the manufacturer's protocol. Briefly, PC12 cells (~5 x 10^6 cells) were exposed to 1 mM $MnCl_2$, cytoplasmic fractions were collected as described previously, and levels of cytosolic cytochrome c were analyzed by an ELISA method (23). The optical density of each well was then measured at 450 nm using a microplate reader (Molecular Devices Corp., Sunnyvale, CA). The concentration of cytochrome c was calibrated from a standard curve based on reference standards.

Mitochondrial membrane potential detection assay

Mitochondrial membrane potential($\Delta\Psi m$) depolarization was assessed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Francisco, CA) (24). PC12 cells (~1 x 10^6 cells) were exposed to 1 mM $MnCl_2$ for up to 40 hr, and 40 nM

3,3'-dehexyloxacarbocyanine ($DiOC_6$) was added for 15 min. The cells were then washed once and resuspended with phosphate buffered saline (PBS), and $\Delta\Psi m$ was measured by flow cytometry with excitation at 484 nm and emission at 501 nm. Measurement was completed when 10,000 cells were analyzed.

Caspase-3 activity assay

Cells (~1-2 x 10^5 cells/well) were subcultured in a 24-well tissue culture plate for 24 hr and treated with $MnCl_2$ for 0 to 48 hr. Cells were washed once with PBS (pH 7.4) and resuspended in lysis buffer containing 50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 10 μM digitonin. Cells were then incubated at 37°C for 20 min to allow complete lysis. Lysates were quickly centrifuged at 10,000 x g, and cell-free supernatants were collected. Caspase-3 activity was measured using the caspase-3 specific fluorescent substrate Ac-DEVD-AMC, as described previously (23, 25). Formation of 7-amino-4-methylcoumarine (AMC), resulting from caspase substrate cleavage, was measured by spectrofluorometry (Molecular Devices, Sunnyvale, CA) with excitation at 380 nm (slit width 10 nm) and emission at 460 nm (slit width 20 nm). Caspase activity was expressed as fluorescence unit (FU) per mg protein per hr.

Isolation of cytosolic fractions

Cells (~1 x 10^7 cells) were exposed to $MnCl_2$ at 37°C for the indicated periods. Cells were washed once with ice-cold PBS and resuspended in homogenization buffer containing 20 mM Tris/HCl (pH 8.0), 2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM sodium fluoride, and 50 μM sodium orthovanadate. Cells were then sonicated for 10 sec and centrifuged at 100,000 x g for 60 min at 4°C to produce the supernatant cytosolic fractions. To collect membrane fractions, pellets were dissolved in homogenization buffer containing 1% Triton X-100 to form suspensions and then

sonicated and centrifuged at 10,000 x g for 30 min. The protein concentration of each cytosolic fraction was determined using a Bradford protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA). Cytosolic fraction samples were mixed with 2x gel loading buffer containing 10% SDS and 200 mM DTT and placed in boiling water for 5 min.

Western blotting

Proteins in cytosolic fractions were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membrane (Bio-Rad Laboratories). Non-specific binding sites were blocked by 5% non-fat milk blocking solution (Amersham Pharmacia Biotech). The membrane was then treated with anti-PKCδ (1:2000 dilution) antibody, followed by secondary horseradish peroxidase-conjugated anti-rabbit (1:2000 dilution) antibody. Antibody-bound proteins were detected by an enhanced chemiluminescence (ECL) system. To confirm equal protein in each lane, antibodies were stripped from membranes with stripping buffer (Geno Technology, St. Louis, MO) and reprobed with β-actin (1:5000 dilution).

***In situ* apoptotic labeling**

Acridine orange and propidium iodide double staining was performed to assess DNA damage in apoptotic cells (26). PC12 cells were grown on cover slips coated with type I rat tail collagen (6 $\mu g/cm^2$) for 24 hr at 37°C, and $MnCl_2$ was added for the indicated periods. After the exposures, cells were washed once with PBS and stained with 5 μM acridine orange and 5 μM propidium iodide for 10 min or 10 μM Hoechst 33342 for 5 min at room temperature in the dark. The cells were washed once with PBS and mounted on a perfusion chamber with HBSS buffer and observed under a Nikon DiaPhot microscope (Nikon Inc., Melville, NY) with the excitation wavelength at 360 nm, 488 nm, or 540 nm for Hoechst 33342, acridine orange, or propidium iodide,

respectively. Fluorescent images were captured with a SPOT digital camera.

DNA fragmentation assay

DNA fragmentation was measured using a Cell Death Detection ELISA Plus Assay Kit, as described previously (23, 27). Cells (~1 x 10^6 cells) were subcultured in 6-well culture plates for 24 hr and exposed to $MnCl_2$ with or without various inhibitors for the indicated periods at 37°C. The number of apoptotic PC12 cells was determined exactly as described by the manufacturer, and DNA fragmentation was expressed as a percentage of control.

***In vitro* delivery of catalytically active PKCδ**

PC12V4 cells (~1-2 x 10^5 cells/well) were subcultured in 24-well tissue culture plates for 24 hr. Human recombinant catalytically active PKCδ protein was delivered into cells using BioPORTER reagent, strictly following the manufacturer's protocol. Briefly, cells were incubated for 4 hr with 5 ng of recombinant PKCδ or heat-inactivated recombinant PKCδ (15 min in boiling water) with 3 μl BioPORTER reagent in serum-free DMEM. Cells were counted and equal numbers of cells were analyzed by DNA fragmentation assay as described above.

Data analysis and statistics

Data from MTT, caspase activity, cytochrome c release and DNA fragmentation assays were first analyzed using one-way ANOVA. Dunnett's post-test or Bonfferoni's multiple comparison test was then performed to compare treated samples, and $p<0.05$ was considered significant.

RESULTS

Dose- and time-dependent manganese-induced cytotoxicity in vector PC12 cells (PC12V4) and Bcl-2 overexpressing PC12 cells (PC12HB2-3)

First, we performed a dose-response cytotoxicity study to determine the optimal manganese concentrations for additional mechanistic studies in PC12V4 (vector control) and PC12HB2-3 (Bcl-2-overexpressing) cells. As shown in Figure 1, exposure to various doses of manganese (0-1 mM) over 72 hr produced dose-dependent cytotoxicity in both PC12V4 and PC12HB2-3 cells. However, manganese (over 400 μM) induced significantly less toxicity in Bcl-2 overexpressing cells ($p<0.01$) as compared to the vector control cells. We found that 1 mM manganese produced approximately 50% cell death in PC12HB2-3 cells and 75% cell death in PC12V4 cells at 72 hr. We also found that 1 mM manganese showed time-dependent increase in cell death, and did not significantly cause necrotic cell death at earlier time point (measured by LDH assay; data not shown). Therefore, we used 1 mM manganese in subsequent experiments evaluating the cellular mechanism of dopaminergic cell death during manganese exposure at up to 48 hr.

Manganese affects mitochondrial function and releases pro-apoptotic cytochrome c

Manganese reportedly accumulates in mitochondria and inhibits mitochondrial Complex I and/or II activity (14, 28, 29). We examined the effect of manganese on mitochondrial functions and found that the mitochondrial membrane potential ($\Delta\Psi m$) was significantly ($p<0.05$) depolarized in a time-dependent manner following 1 mM manganese treatment in PC12V4 cells (Fig. 2A). The significant depolarization of $\Delta\Psi m$ was detected after 24 hr and continued up to 40 hr. Bcl-2 overexpression effectively suppressed and delayed the manganese-induced depolarization of $\Delta\Psi m$.

Mitochondrial insults often result in release of cytochrome c into cytoplasm to initiate the apoptotic caspase cascade. As shown in Figure 2B, manganese caused

significant ($p<0.05$) release of cytochrome c in the cytosol at 28 hr post-exposure, the time point just proceeding the significant $\Delta\Psi m$ depolarization. However, cytosolic cytochrome c levels were not significantly increased in PC12HB2-3 cells, indicating that Bcl-2 overexpression protects against manganese toxicity at the mitochondrial level.

Manganese induces the caspase-3 cascade in PC12 cells

Since cytochrome c release is known to initiate the caspase cascade following binding to apoptosis activating factor-1 (Apaf-1), the next series of experiments was designed to examine whether or not manganese exposure induces the effector caspase-3 in dopaminergic cells. Exposure of PC12V4 cells to manganese (1mM) time-dependently increased caspase-3 activity 1.3- to 4.3-fold above basal levels at 24 to 48 hr post-exposure, but not earlier than 24 hr (Fig. 3A). Thus, the caspase-dependent cell death process was initiated between 24 and 28 hr after manganese exposure, when depolarization of $\Delta\Psi m$ and cytochrome c release occurred. We also found that the manganese-induced caspase-3 activation between 28-48 hr was significantly suppressed ($p<0.05$) in cells overexpressing Bcl-2; To determine if caspase-3 activation was mediated by initiator caspase-9, we evaluated the effect of the caspase-9 specific inhibitor Z-LEHD-FMK. As shown in Figure 3B, pretreatment with Z-LEHD-FMK (50 μM or 100 μM) dose-dependently inhibited manganese-induced caspase activity, indicating that manganese-induced caspase-3 activation is mainly dependent on caspase-9. Capase-8, another initiator caspase linked to receptor-mediated apoptosis, could not be measured during the entire manganese exposure (data not shown).

Manganese-induced proteolytic cleavage of PKCδ

We recently showed that PKCδ is an important substrate for caspase-3, which proteolytically cleaves and activates the kinase to promote apoptosis in dopaminergic cells (23, 30). We monitored PKCδ proteolytic cleavage every 4 hr for 48 hr following

manganese (1mM) treatment in both PC12V4 and PC12HB2-3 cells. As shown in Figure 4A, the native PKCδ (74 kDa) was proteolytically cleaved into a catalytically active fragment (41 kDa) starting 24 hr post-exposure to manganese in PC12V4 cells. The PKCδ cleavage increased in a time-dependent manner over a 48 hr period, and slight cleavage was noted at the 48 hr time point. The temporal pattern (time course) of PKCδ cleavage paralleled caspase-3 activation. In PC12HB2-3 cells, the proteolytic cleavage of PKCδ was not detected at all during the entire 48 hr manganese exposure period (Fig. 4B), indicating that Bcl-2 can suppress the caspase-mediated apoptotic cell death process.

To confirm that manganese-induced proteolytic cleavage of PKCδ was caspase-mediated, we treated PC12V4 cells with the pan caspase inhibitor Z-VAD-FMK and the caspase-3 specific inhibitor Z-DEVD-FMK along with manganese. Pretreatment with Z-VAD-FMK (50-100 μM) or Z-DEVD-FMK (50-100 μM) significantly inhibited manganese-induced proteolytic cleavage of PKCδ in a dose-dependent manner, suggesting that the manganese-induced proteolytic activation of PKCδ was caspase-3-dependent (Fig. 4C and 4D). Manganese treatment did not induce translocation of PKCδ to the membrane, which is a common mechanism of PKCδ activation, in either PC12V4 or PC12HB2-3 cells (data not shown). Therefore, the increased PKCδ activity during manganese exposure was mainly through a caspase-mediated proteolytic activation mechanism.

To further characterize capase-3 activation and proteolytic cleavage of PKCδ following manganese treatment, we performed dose-response studies in PC12V4 cells following exposure to various doses (0-600 μM) of manganese over a 72 hr period. As shown in Figure 5A, caspase-3 activity dose-dependently increased up to 4-fold following a 72-hr manganese treatment in PC12V4 cells, but not in PC12HB2-3 cells; these findings agree with the cytotoxicity data shown in Figure 1. In addition, manganese (400-600 μM) induced the proteolytic cleavage of PKCδ in a dose- and

time-dependent manner, indicating that the proteolytic cleavage of PKCδ may be an obligatory event in the apoptotic cell death process during chronic manganese exposure.

Manganese-induced DNA fragmentation in PC12 cells

We examined the effect of Bcl-2 on manganese-induced DNA fragmentation, the final stage of the apoptotic cell death process. DNA fragmentation was detected in PC12V4 cells as early as 24 hr after exposure to 1 mM manganese, and it increased time-dependently by 8-, 15-, and 21-fold at 24, 36, and 48 hr, respectively (Fig. 6A). DNA fragmentation was dramatically attenuated at all time points in manganese-treated PC12HB2-3 cells, indicating that Bcl-2 can rescue dopaminergic cells from apoptotic cell death during manganese exposure.

We further confirmed manganese-induced apoptotic cell death using acridine orange/propidium iodide double staining or Hoechst 33342 nuclear staining. Both acridine orange and Hoechst 33342 are used to detect chromatin integrity, which is an index of apoptosis (26), and propidium iodide detects cells undergoing necrosis or the late stage of apoptosis or necrosis. As shown in Figure 6B, apoptotic cell death increased from 24 hr of manganese (1 mM) exposure in PC12V4 cells, and necrosis followed at the later time point. However, membrane integrity was normal in PC12HB2-3 cells and very few of the cells were apoptotic during the entire 48 hr of manganese exposure. Taken together, Bcl-2 overexpression protected against both necrosis and apoptosis following manganese exposure.

We next examined the effect of caspase inhibitors on DNA fragmentation during manganese treatment. In Figure 6C, manganese-induced DNA fragmentation was significantly ($p<0.01$) suppressed by pretreatment with 100 μM Z-VAD-FMK, a potent caspase inhibitor, at 24- and 36-hr post-exposure in PC12V4 cells. This concentration of Z-VAD-FMK completely blocked manganese-induced caspase-3 activity at 36 hr (data not shown). In addition, 100 μM Z-DEVD-FMK also

significantly ($p<0.01$) attenuated manganese-induced DNA fragmentation at 36 hr, although not as effectively as Z-VAD-FMK. The caspase inhibitors alone did not significantly alter basal DNA fragmentation levels during the entire experimental period (data not shown).

Pro-apoptotic role of PKCδ in dopaminergic cells

The next series of experiments was designed to examine whether PKCδ activation plays any role in manganese-induced apoptosis. Chronic treatment with low dose TPA down-regulates PKCs (31, 32). PC12V4 cells were treated with 0.2 μM TPA for 24 hr to down-regulate PKC proteins, and cells were then exposed to manganese (1 mM) for 24 to 36 hr. As shown in Figure 7A, 24 hr of TPA treatment resulted in a prolonged down-regulation of PKCδ for more than 72 hr. Other PKC isoforms except PKCζ, an atypical PKC class, were also down-regulated in the same manner (data not shown). We found that these PKC-deficient dopaminergic cells are significantly ($p<0.01$) more resistant to manganese-induced DNA fragmentation as compared to control cells (Fig. 7B). Although we recognized that TPA down-regulates most of the PKC isoforms, this initial experiment in PKC-deficient cells provided support that PKCs are important in manganese-induced apoptosis.

To further determine the specific role of PKCδ in apoptosis in dopaminergic cells, we delivered the active recombinant PKCδ protein into cells and then monitored DNA fragmentation. We recently demonstrated, along with others, that various biologically active proteins including enzymes can be delivered into cells using a new lipid mediated protein delivery system known as BioPORTER (23, 33). Human recombinant PKCδ was administered (5 ng) to cells by the BioPORTER system. The delivery effectiveness of BioPORTER was determined using FITC-labeled antibody (supplied by kit), and treated cells were observed under a fluorescence microscope (30). In our previous study, we confirmed the biological activity of the recombinant PKCδ

administered to cells (34). As shown in Figure 7C, DNA fragmentation was increased 2.5-fold in active recombinant PKCδ-delivered cells over the BioPORTER reagent treated (control) cells, whereas DNA fragmentation was increased only slightly in heat-inactivated PKCδ protein-administered cells. Thus, catalytically active PKCδ plays an important role in the execution of apoptotic cell death.

Protein kinase Cδ is a critical effector of manganese-induced apoptosis

Since PKCδ displayed pro-apoptotic functions in PC12 cells, we further characterized the role of PKCδ in neuronal apoptosis during manganese exposure in mesencephalic dopaminergic neuronal (N27) cells. We found that this clonal dopaminergic neuronal cell line was more susceptible to manganese toxicity than PC12 cells. Exposure of N27 cells to various concentrations (0-1000 μM) of manganese produced dose-dependent cytotoxicity (Fig. 8A). The LC_{50}, or death in 50% of the cells, following 24 hr of manganese treatment was calculated to be 345 μM based on the cell viability data. We thus selected two concentrations (100 and 300 μM) for the following experiments to clarify the role of PKCδ in manganese-induced apoptosis. Treatment of N27 cells with 300 μM manganese for 24 hr induced profound proteolytic cleavage of PKCδ (Fig. 8B), indicating that manganese activated similar or identical apoptotic mechanisms in N27 cells as in PC12 cells.

To determine if PKCδ activation is important in manganese toxicity in dopaminergic neuronal cells, we examined the effect of the specific PKCδ inhibitor rottlerin on manganese-induced DNA fragmentation. Rottlerin concentrations between 1-5 μM effectively inhibit PKCδ activity, as determined by *in vitro* measurement of phosphorylation (23, 35). N27 cells were pretreated with rottlerin (1-5 μM) for 30 min prior to manganese (300 μM) treatment for an additional 24 hr. Manganese-induced DNA fragmentation was significantly ($p<0.01$) reduced to 65% and 46% in N27 cells pretreated with 3 and 5 μM rottlerin, respectively (Fig. 8C).

To further substantiate the pro-apoptotic function of PKCδ in manganese-induced apoptotic cell death in dopaminergic neuronal cells, we adopted a genetic approach to determine if overexpression of a kinase inactive PKCδ mutant (dominant negative) suppresses manganese-induced DNA fragmentation. Catalytically inactive PKCδ ($PKC\delta^{K376R}$) fused with GFP was stably transfected in N27 cells. Previously, we showed loss of the kinase activity in $PKC\delta^{K376R}$ overexpressing N27 cells (N27-$PKC\delta^{K376R}$) as compared to the vector (pEGFP-N1) transfected control (N27-vector) (30), thereby confirming the dominant negative property of these dopaminergic neuronal cells. As shown in Figure 9A, N27-$PKC\delta^{K376R}$ cells underwent significantly ($p<0.01$) less DNA fragmentation for 24 hr as compared to N27-vector cells following manganese exposure (100-300 μM). To determine if upstream apoptotic events were altered by the overexpression of mutant PKCδ, we also measured caspase-3 activity in N27-$PKC\delta^{K376R}$ following manganese treatment. As shown in Figure 9B, caspase-3, however, did not significantly differ between N27-vector and N27$PKC\delta^{K376R}$ cells, indicating that reduction of DNA fragmentation was due to inactivation of PKCδ kinase activity. In conclusion, PKCδ is a critical effector molecule in manganese-induced apoptosis in dopaminergic neuronal cells.

DISCUSSION

Manganese primarily targets the nigrostriatal dopaminergic system in the CNS; however, the cellular mechanisms underlying manganese-induced cell death are not well characterized. The present study demonstrates that (i) manganese induces apoptotic cell death in dopaminergic cells, and mesencephalic dopaminergic neuronal cells are highly sensitive to the neurotoxic effect; (ii) manganese depolarizes the mitochondrial membrane potential to promote the release of the initial pro-apoptotic factor cytochrome c; iii) manganese activates the caspase-3 dependent apoptotic cascade; iv) mitochondrial

anti-apoptotic protein Bcl-2 negatively modulates the manganese-induced apoptotic cascade; and v) caspase-3 dependent proteolytic activation of PKCδ is a critical downstream contributor to apoptotic cell death in dopaminergic neuronal cells. Furthermore, these findings indicate that mitochondria promote the manganese-induced apoptotic cell death process via sequential activation of the apoptotic signaling pathway depicted in Figure 10.

Manganese has been reported to accumulate in mitochondria and inhibit mitochondrial complex I activity (28, 36, 37). Reactive oxygen species (ROS) are generated upon mitochondrial inhibition. In a recent study, manganese increased ROS generation in isolated mitochondrial fractions from mouse brain (38). Recently, we demonstrated that ROS plays a causal role in apoptotic cell death following exposure to the organic manganese compound methylcyclopentadienyl manganese tricarbonyl (MMT) (39). ROS in a dopamine rich environment contribute to oxidative stress by forming dopamine derived quinone radicals (40, 41). Therefore, the increased susceptibility of dopaminergic neurons to manganese-induced neurotoxic insult can be attributed to the enhanced sensitivity of dopaminergic neurons to oxidative stress. Although we did not measure ROS generation in the present study, the observed mitochondrial membrane depolarization indicates the possible involvement of oxidative insult in manganese-induced neurotoxicity.

Mitochondrial membrane depolarization results in the rapid release of the pro-apoptotic factor cytochrome c into cytoplasm to initiate the caspase cascade. Our result is consistent with other recent reports of caspase-3 activation observed during manganese toxicity in various *in vitro* models (10, 42, 43). However, Oubrahim et al. (2001) reported that manganese-induced caspase-3 activation and apoptosis in HeLa cells (derived from human epithelial adenocarcinoma) were not mediated by mitochondria because manganese did not alter the mitochondrial membrane potential ($\Delta\Psi m$). Mitochondria from cancer cells appear to be resistant to manganese toxicity.

Another study by Roth et al. (2000) concluded that manganese-induced apoptosis is a caspase-independent process because the caspase inhibitor Z-VAD-FMK failed to attenuate manganese-induced cell death in PC12 cells at 24 hr (10). Since the investigators did not measure DNA fragmentation directly, the functional role of caspase-3 in the apoptotic process is difficult to ascertain. Also, they examined the effect of Z-VAD-FMK on manganese toxicity only up to 24 hr, and apoptosis could have occurred at a later time point. Our data show that caspase-3 activation starts to rise at 24 hr and peaks around 36 hr, whereas maximal DNA fragmentation occurs around 36-48 hr manganese post-treatment. Thus, the mode of the manganese-induced cell death process appears to be specific to cell type and time-dependent. Nevertheless, our data clearly demonstrate that chronic manganese exposure targets mitochondria and activates caspase-dependent apoptosis in dopaminergic neuronal cells, which are known to be selectively affected in manganese-induced neurotoxicity. Furthermore, attenuation of manganese-induced depolarization of $\Delta\Psi m$, cytochrome c release, caspase-3 activation, and apoptosis by the mitochondrial anti-apoptotic protein Bcl-2 indicates that mitochondria may serve as an initial regulator of manganese-induced apoptosis in dopaminergic cells. Furthermore, recent evidences strongly suggest that manganese may affect endoplasmic reticulum (ER) and induce caspase-12-dependent apoptotic cascade (42, 44). Manganese may target multiple organelles and promotes cell death.

Caspase-dependent apoptosis has been reported in dopaminergic degeneration resulting from exposure to various dopaminergic neurotoxins (including MPP^+) treatment (23, 30, 34, 45, 46) as well as in brains of PD patients (47). However, the key downstream events that contribute to DNA fragmentation are not well characterized. One significant finding of the current study is identification of PKCδ proteolytic activation upon manganese exposure. PKCδ belongs to the novel isoform family of PKCs and is activated in a Ca^{2+} independent manner. Traditionally, PKCs have been

considered to have anti-apoptotic functions, but PKCδ is emerging as a key pro-apoptotic factor. Lipid-dependent translocation and proteolytic activation are two major activation mechanisms of PKCδ (27, 48-50). Recently, we demonstrated that PKCδ proteolytic activation mediates apoptotic cell death in dopaminergic cells following exposure to environmental neurotoxic chemicals (23, 30). In these studies as well as in the present study, PKCδ was not activated by translocation, indicating that proteolytic activation is the primary mode of activation in dopaminergic cells. Induction of DNA fragmentation by delivery of the catalytically active PKCδ protein and attenuation of manganese-induced DNA fragmentation by pharmacological and genetic modulation of PKCδ clearly show that PKCδ is a key downstream substrate of caspase-3. The cellular substrates of PKCδ that specifically mediate apoptotic cell death have not yet been defined. However, several signaling molecules such as DNA protein kinase (DNA-PK), MAP-kinase, scrambalase, and NF-kappa transcription factor have been proposed to function in apoptosis (51-54). Previously, Hirata et al. (1998) reported activation of the JNK pathway by phosphorylation of a serine-threonine kinase in PC12 cells (9).

In conclusion, chronic manganese exposure in dopaminergic cells induces caspase-3-dependent PKCδ activity, and the active PKCδ may contribute to downstream apoptotic events, including DNA fragmentation. Further identification of critical cellular targets of PKCδ important in apoptotic cell death in dopaminergic cells following manganese exposure may provide insight into manganese-induced dopaminergic degeneration.

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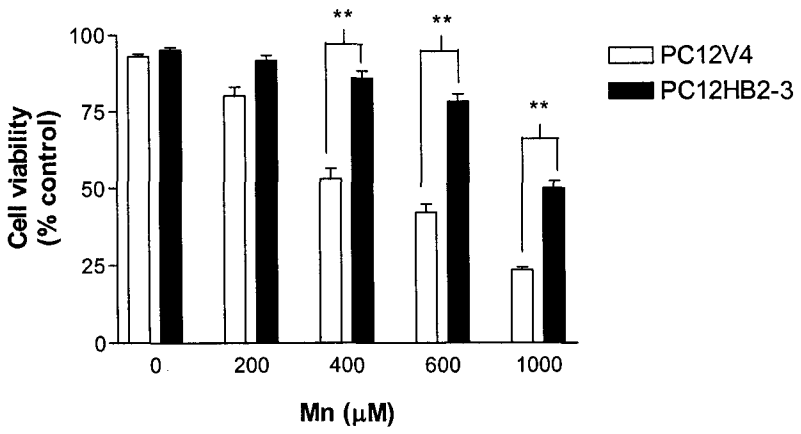


Fig. 1. Manganese-induced cytotoxicity in PC12 cells

PC12V4 and PC12HB2-3 cells were treated with 200-1000 μM manganese for 72 hr, and cytotoxicity was determined using trypan blue exclusion by counting live vs. dead (stained) cells in three to five randomly selected fields. Each point represents mean ± SEM for two separate experiments in triplicate. **$p<0.01$ compared between cells in the same manganese treatment groups.

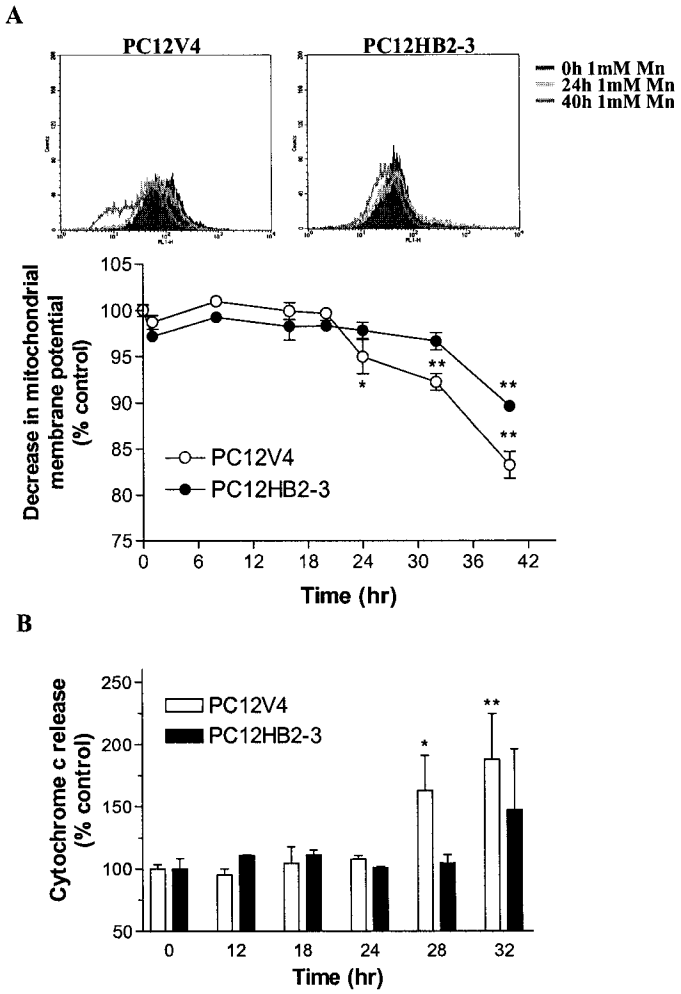


Fig. 2. Effect of manganese on mitochondrial function and cytochrome c release (A) Both PC12V4 and PC12HB2-3 cells (~1 x 10^6 cells/ml) were exposed to 1 mM manganese for 0-40 hr, then mitochondrial membrane potential ($\Delta\Psi m$) was measured by using $DiOC_6$ (40 nM). Histogram is the representative data for depolarization of $\Delta\Psi m$ at 0, 24, and 40 hr. The graph represents the reduction of $\Delta\Psi m$ as percent of control. Each point represents mean ± SEM for two separate experiments in triplicate. *$p<0.05$ or **$p<0.01$ compared with 0 hr treatment (control) group in each cell line. (B) Release of cytochrome c into cytosol was detected using a cytochrome c ELISA kit. Each bar represents mean ± SEM for two separate experiments in triplicate. *$p<0.05$ or **$p<0.01$ compared with 0 hr treatment group in each cell line.

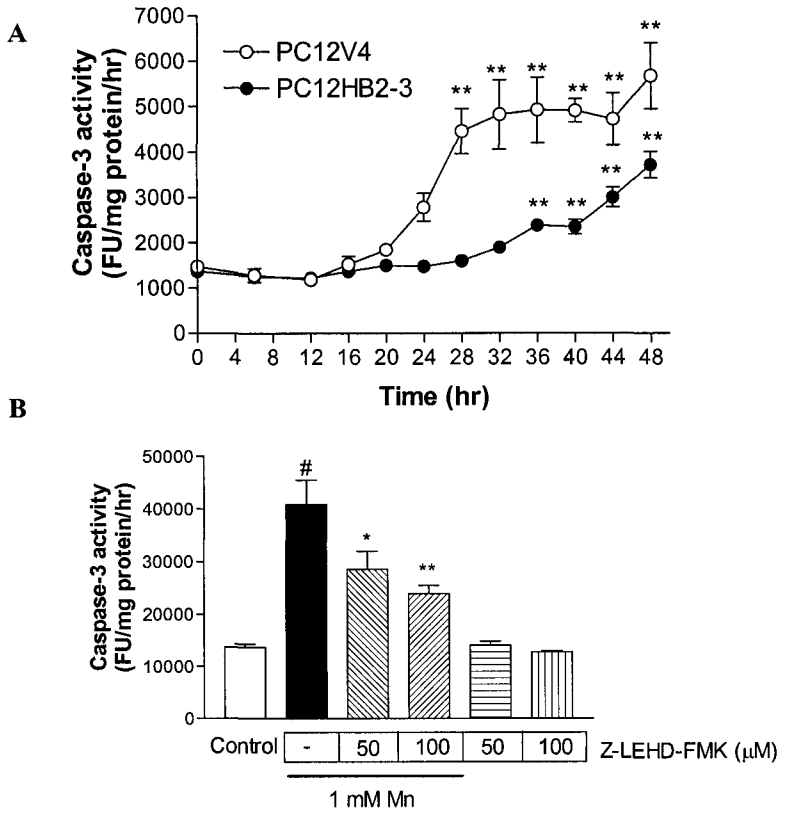
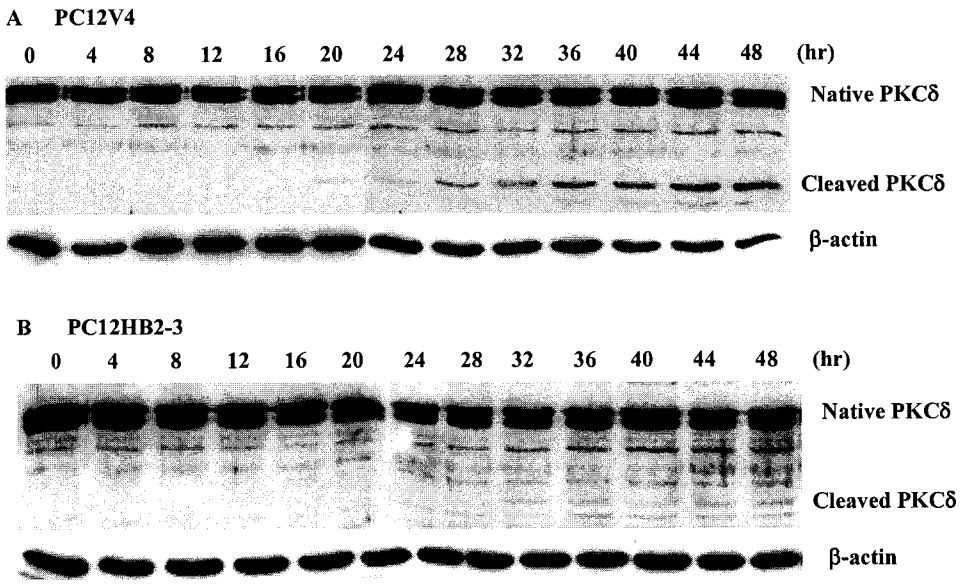
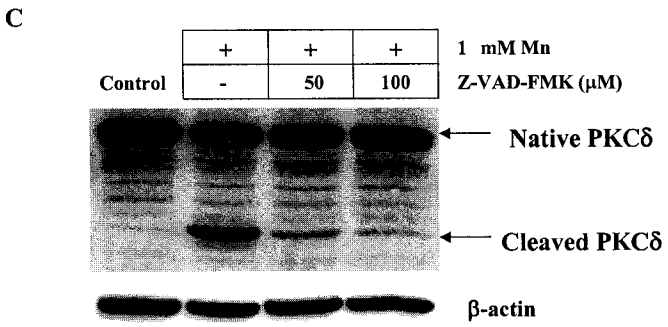


Fig. 3. Manganese-induced caspase-3 activity in PC12V4 and PC12HB2-3 cells (A) Both PC12V4 and PC12HB2-3 cells were treated with 1 mM manganese for 0-48 hr. Cytosolic supernatants were collected and caspase-3 activity was measured by incubating the supernatant with 50 μM Ac-DEVD-AMC, a fluorogenic caspase-3 substrate, for 1 hr at 37°C, and expressed as fluorescent unit (FU) per mg protein per hr incubation. Each point represents mean ± SEM for at least three separate experiments in triplicate. **p<0.01 compared with control. (B) PC12V4 cells were pretreated with 50-100 μM of the caspase-9 specific inhibitor Z-LEHD-FMK for 30 min and exposed to 1 mM manganese for 28 hr. Caspase-3 activity was measured as described above. Each bar represents mean ± SEM for at least two separate experiments in triplicate. #p<0.01 compared with control and *p<0.05 or **p<0.01 compared with 1 mM manganese treated group.

Fig. 4. Manganese induced proteolytic cleavage of PKCδ in PC12 cells

PC12V4 or PC12HB2-3 cells (1 x 10^7 cells) were treated with 1 mM manganese for 0-48 hr. Cytosolic fractions were collected, and equal amounts of proteins were resolved in 10% SDS-PAGE followed by polyclonal PKCδ antibody. Time-course study of PKCδ in PC12V4 cells (A) or PC12HB2-3 cells (B). Native PKCδ appears at 72-74 kDa and the cleaved, catalytically active PKCδ appears at around 41 kDa. (E and F) PC12V4 cells were treated with 1 mM manganese with or without 50-100 μM Z-VAD-FMK, a pan-caspase inhibitor (C) or 50-100 μM Z-DEVD-FMK, a caspase-3 specific inhibitor (D). The cells were pretreated with the caspase inhibitors for 30 min prior to the treatment with 1 mM manganese for 36 hr. Cytosolic fractions were isolated and proteins were resolved in 10% SDS-PAGE. Native PKCδ (72-74 kDa) and cleaved PKCδ (38-41 kDa) were detected. Equal protein loading (5 μg) was confirmed by re-probing with β-actin.


A PC12V4
0 4 8 12 16 20 24 28 32 36 40 44 48 (hr)
Native PKCδ
Cleaved PKCδ
β-actin
B PC12HB2-3
0 4 8 12 16 20 24 28 32 36 40 44 48 (hr)
Native PKCδ
Cleaved PKCδ
β-actin


C
Control
+ + + 1 mM Mn
- 50 100 Z-VAD-FMK (μM)
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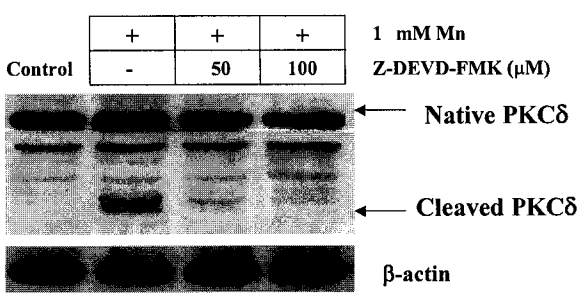
Cleaved PKCδ
β-actin


D
Control
+ + + 1 mM Mn
- 50 100 Z-DEVD-FMK (μM)
Native PKCδ
Cleaved PKCδ
β-actin

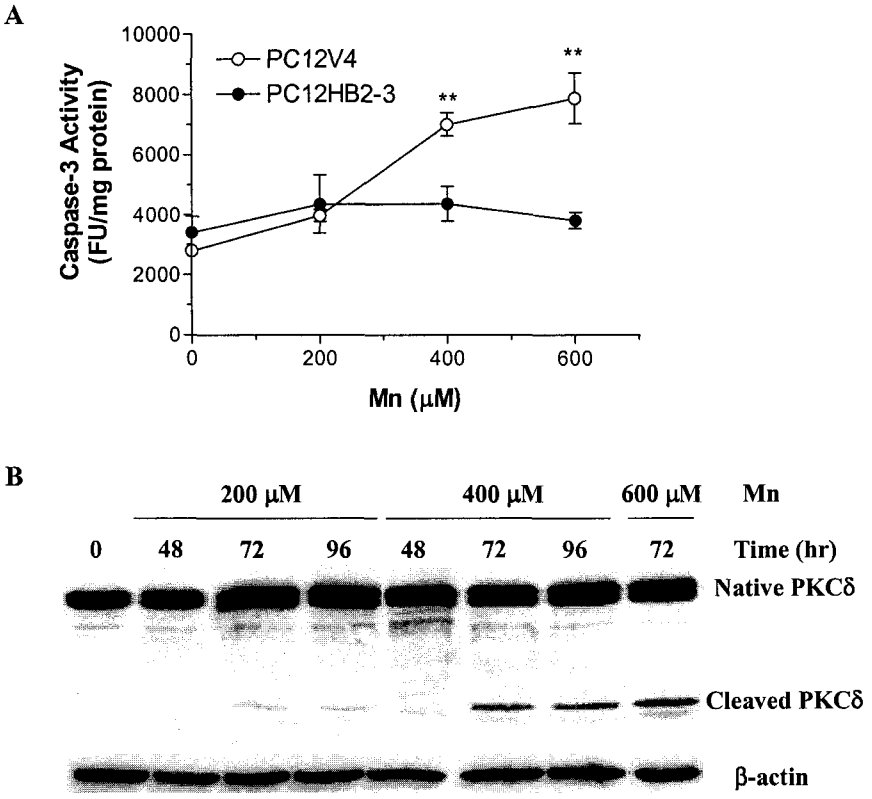


Fig. 5. Caspase-3 activation and proteolytic cleavage of PKCδ induced by chronic low-dose manganese exposure in PC12 cells

(A) Chronic exposure (72 hr) to lower doses of manganese dose-dependently increased caspase-3 activity in PC12V4 cells. Cells were treated with 200, 400, or 600 μM manganese for 72 hr and caspase-3 activity was measured. Each bar represents mean ± SEM for two separate experiments performed in triplicate; *$p<0.05$ or **$p<0.01$ compared with each control group. (D) Chronic exposure (48-96 hr) to lower doses of manganese also induced the proteolytic cleavage of PKCδ in PC12V4 cells. After the treatment, cytosolic fractions were isolated and proteins were resolved in 10% SDS-PAGE. Native PKCδ (72-74 kDa) and cleaved PKCδ (38-41 kDa) were detected. Equal protein loading (5 μg) was confirmed by re-probing with β-actin.

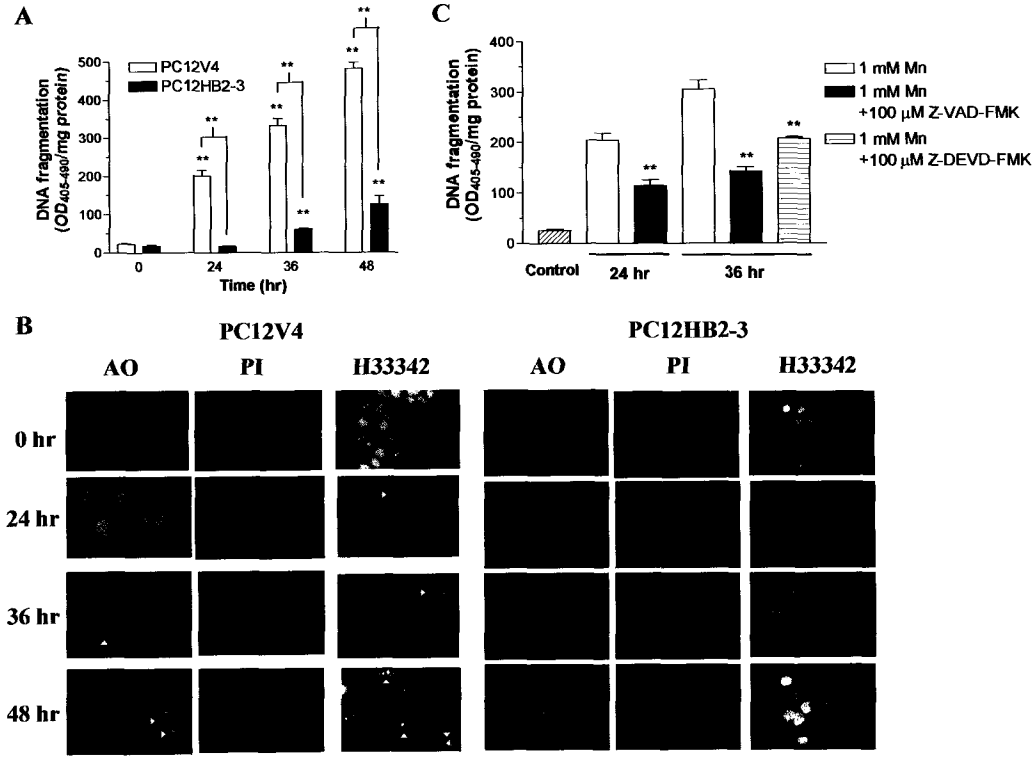
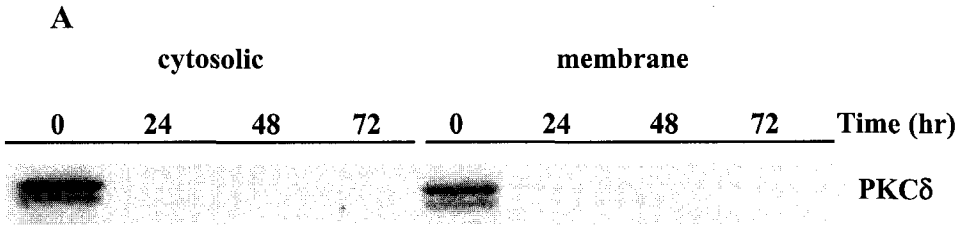


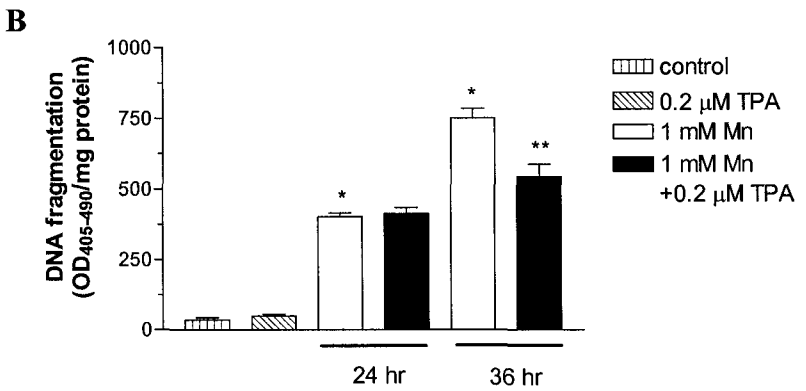
Fig. 6. Manganese-induced apoptosis in PC12 cells

(A) Apoptotic cell death was determined by measuring DNA fragmentation. Cells were treated with 1 mM manganese for 0-48 hr, and the level of DNA fragmentation was analyzed using a DNA ELISA assay kit. Each bar represents mean ± SEM for three separate experiments in duplicate. **$p<0.01$ compared with control (0 hr) or between PC12V4 and PC12HB2-3 cells. (B) Qualitative detection of manganese-induced apoptosis. Cells were plated on collagen-coated cover slips and exposed to 1 mM manganese for 0-48 hr. Cells were then stained with 5 μg/ml acridine orange and 5 μg/ml propidium iodide or 10 μg/ml Hoechst 33342, and observed under a fluorescence microscope. Arrows indicate cells undergoing apoptosis. (C) PC12V4 cells were exposed to 1 mM manganese with or without 100 μM Z-VAD-FMK, a pan-caspase inhibitor, or 100 μM Z-DEVD-FMK, a caspase-3 specific inhibitor, for 24 or 36 hr. DNA fragmentation was determined using a DNA ELISA assay kit. Each bar represents mean ± SEM for two separate experiments in duplicate. **$p<0.01$ compared with 1 mM manganese-treated cells.

Fig. 7. The catalytically active PKCδ protein plays a critical role in the execution of DNA fragmentation

(A) PC12V4 cells were treated with 0.2 μM TPA for 24 to 72 hr, and down-regulation of PKCδ was determined by Western blot. (B) PC12V4 cells were treated with 0.2 μM TPA for 24 hr, exposed to 1 mM manganese for 24 or 36 hr, and DNA fragmentation was measured by ELISA assay. Each bar represents mean ± SEM for two separate experiments in triplicate. *$p<0.01$ compared with control group and **$p<0.01$ compared with 1 mM manganese-treated PC12V4 cells without TPA pretreatment. (C) PC12V4 cells were treated with 10 μl BioPORTER reagent and 5 ng of human PKCδ catalytically active protein in serum-free DMEM for 4 hr to successfully deliver active PKCδ protein into the cells. For comparison, 5 ng of heat-inactivated PKCδ protein (15 min in boiling water) or solvent (PBS) was delivered with the BioPORTER reagent. After the 4 hr treatment, equal numbers of cells were collected and processed to determine DNA fragmentation levels using a DNA ELISA assay kit. DNA fragmentation was expressed as percent control (10 μl BioPORTER reagent + PBS). Each bar represents mean ± SEM for two separate experiments in triplicate. *$p<0.05$ or **$p<0.01$ compared between indicated treatment groups.


A
cytosolic
membrane
0
24
48
72
0
24
48
72
Time (hr)
PKCδ


B
DNA fragmentation
(OD405-490/mg protein)
1000
750
500
250
0
*
*
**
control
0.2 μM TPA
1 mM Mn
1 mM Mn
+0.2 μM TPA
24 hr
36 hr

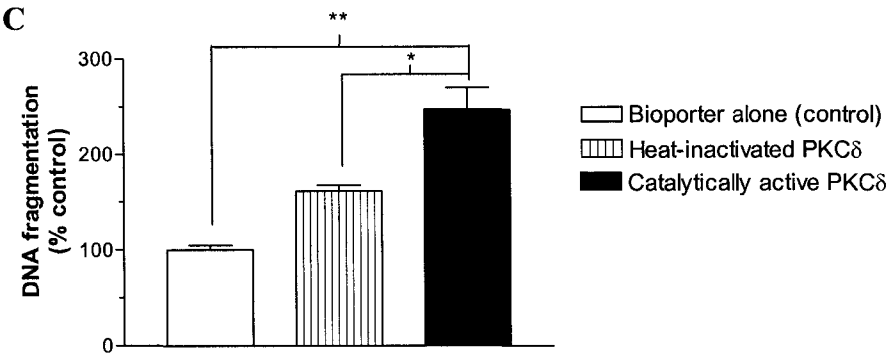
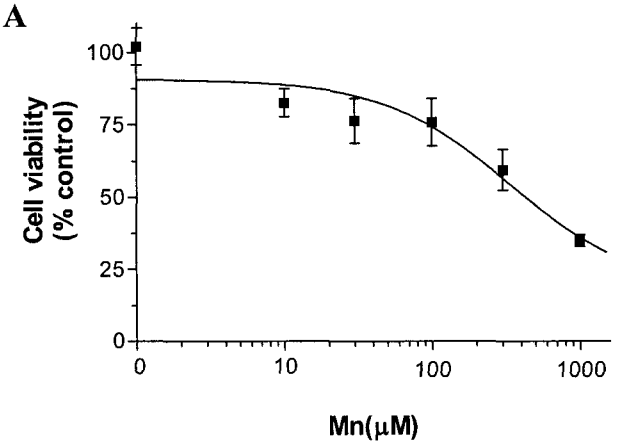
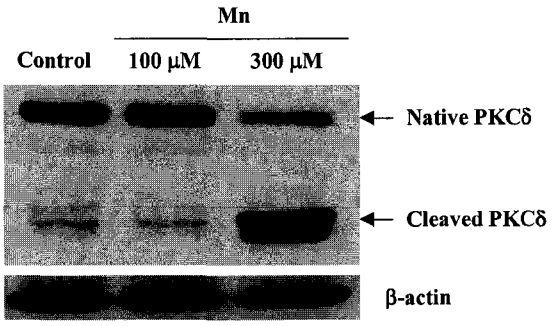

C
DNA fragmentation
(% control)
300
200
100
0
**
*
Bioporter alone (control)
Heat-inactivated PKCδ
Catalytically active PKCδ

Fig. 8. Effect of manganese on mesencephalic dopaminergic (N27) neuronal cells (A) Cell viability following manganese treatment for 24 hr in N27 cells was assessed using an MTT assay. The data were plotted against logarithmic doses of manganese, and the EC_{50} was calculated by three-parameter nonlinear regression analysis. Each point represents mean ± SEM for two separate experiments in triplicate. (B) N27 cells were treated with 100 or 300 μM manganese for 24 hr, and proteolytic cleavage of PKCδ was determined by Western blot. Equal protein loading (5 μg) was confirmed by reprobing with β-actin. (C) N27 cells were pretreated with the PKCδ specific inhibitor rottlerin (1-5 μM) for 30 min prior to 300 μM manganese treatment for 24 hr. DNA fragmentation was assayed using a DNA ELISA assay kit. Each bar represents mean ± SEM for two separate experiments in triplicate. **$p<0.01$ compared with manganese-treated cells.

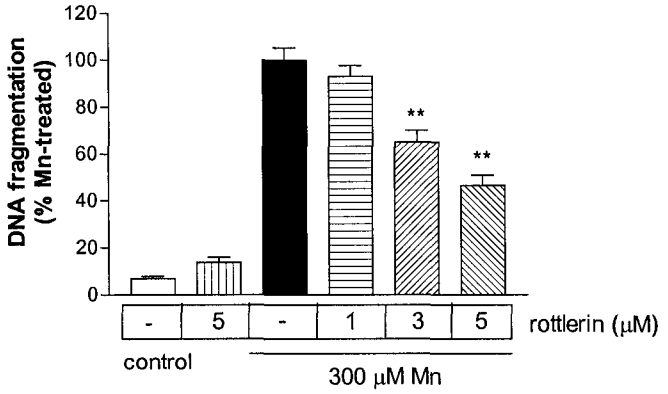
A


Cell viability
(% control)
100
75
50
25
0
0
10
100
1000
Mn(μM)

B


Mn
Control
100 μM
300 μM
Native PKCδ
Cleaved PKCδ
β-actin

C


DNA fragmentation
(% Mn-treated)
120
100
80
60
40
20
0
**
**
-
5
-
1
3
5
rottlerin (μM)
control
300 μM Mn

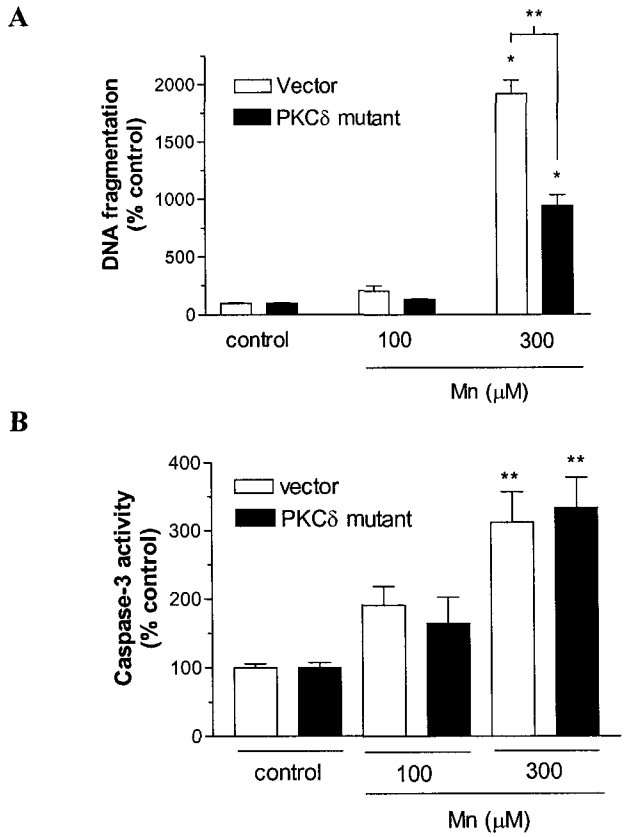


Fig. 9. Proapoptotic role of PKCδ in manganese-induced apoptosis in N27 cells Catalytically inactive PKCδ^{K376R} and its vector were stably transfected in N27 cells. Cells were exposed to 100-300 μM manganese for 24 hr and DNA fragmentation was assayed using a DNA ELISA kit (A), or caspase-3 activity was measured using the colorimetric substrate Ac-DEVD-pNA (B). Each bar represents mean ± SEM for two separate experiments performed in triplicate. *$p<0.05$ or **$p<0.01$ compared with control or between vector- and PKCδ^{K376R}-transfected cells.

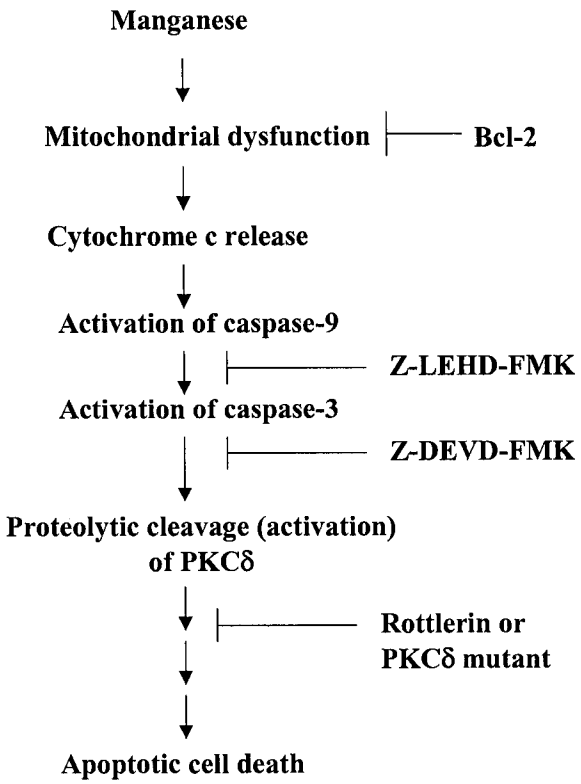


Fig. 10. Schematic diagram of manganese-induced apoptotic pathway in dopaminergic cells

Based on our present findings, we propose the following apoptotic cell signaling pathway during manganese exposure in dopaminergic neuronal cells. Manganese treatment depolarizes the mitochondrial membrane potential to induce cytochrome c release, which subsequently promotes activation of caspase-3. Activated caspase-3 proteolytically cleaves PKCδ to promote the downstream execution process of apoptosis. Mitochondrial Bcl-2 overexpression, pharmacological caspase inhibition (Z-LEHD-FMK, Z-DEVD-FMK, and Z-VAD-FMK), or genetic modulation of PKCδ activity significantly attenuates the manganese-induced apoptotic process.

CHAPTER VII: MITOCHONDRIAL TRANSLOCATION OF PROTEIN KINASE Cδ INACTIVATES BCL-2 BY PROTEOLYTIC DEGRADATION DURING ENVIRONMENTAL NEUROTOXIC INSULT IN DOPAMINERGIC CELLS

A paper to be submitted for publication in Journal of Neurochemistry

Masashi Kitazawa and Anumantha G. Kanthasamy

ABSTRACT

Previously, we demonstrated that exposures to environmental neurotoxic agents such as MPP^+, dieldrin, and methylcyclopentadienyl manganese tricarbonyl (MMT) induce proteolytic activation of protein kinase Cδ (PKCδ) via a caspase-3-dependent pathway in dopaminergic cells. We further characterized that PKCδ activation not only mediates apoptosis but also regulates the upstream caspase cascade through positive feedback activation. Herein, we have investigated the pro-apoptotic role of PKCδ in the upstream caspase cascade following dieldrin or MMT treatment in dopaminergic PC12 cells. Following acute exposure to dieldrin (100 μM) or MMT (200 μM), native PKCδ rapidly translocated into mitochondria within 1 hr, resulting in release of key mitochondrial pro-apoptotic molecules including cytochrome c and second mitochondrial activation of caspases (Smac). The PKCδ specific inhibitor rottlerin blocked cytochrome c release without affecting PKCδ translocation, indicating that PKCδ kinase effects on mitochondria are important in initiating the apoptotic cascade. Additionally, we examined the regulatory effect of catalytically active recombinant PKCδ on mitochondria. Delivery of active recombinant PKCδ into dopaminergic cells induced depolarization of mitochondrial membrane potential, cytochrome c release, and

caspase-9 activation in dopaminergic cells, further confirming that PKCδ is an important regulator of mitochondrial pro-apoptotic function. Immunoprecipitation analyses revealed that PKCδ associated with Bcl-2 and caspase-3 during dieldrin or MMT exposure, and an *in vitro* phosphorylation assay indicated that PKCδ phosphorylated Bcl-2, possibly resulting in the loss of Bcl-2 anti-apoptotic function. Interestingly, Bcl-2 was proteolytically cleaved in PC12 cells 3 hr after dieldrin or MMT treatment, and the cleavage was blocked by PKCδ and a caspase-3 inhibitor. Together, our results suggest that mitochondrial translocation of PKCδ is an important pro-apoptotic event that promotes proteolytic inactivation of Bcl-2 by caspase-3 during the environmental chemical-induced dopaminergic degenerative process.

Keywords: mitochondria, Smac, caspase-3, phosphorylation, dieldrin, MMT

INTRODUCTION

Recent reports indicate that both environmental and genetic components are important in the pathogenesis of Parkinson's disease (Polymeropoulos et al. 1997; Kruger et al. 1998; Tanner et al. 1999; Lim et al. 2002). A recent landmark epidemiology study by Tanner et al (1999) suggests that genetic factors may be important in the young-onset PD (YOPD) but not in the major form of late-onset sporadic PD. In support of environmental hypothesis in the sporadic PD, several epidemiological and case control studies show that pesticides and heavy metals are dominant chemical risk factors of PD (Ho et al. 1989; Gorell et al. 1997; Liou et al. 1997; Gorell et al. 1998). Exposure to certain pesticides and metal containing compounds has been shown to replicate some extent the behavioral, neurochemical and pathological futures of PD (Couper 1837; Sharma et al. 1976; Wagner and Greene 1978; Sechi et al. 1992). However, the cellular mechanism involved in dopaminergic degeneration process following environmental chemical exposure remains elusive. We

and others have shown that the mitochondria are important cell target of neurotoxic agents in inducing cell death process in dopaminergic cells (Choi et al. 1999; Stoessl 1999; Kitazawa et al. 2002a; Kitazawa et al. 2002b). Despite apoptosis is recognized as a major mode of the cell death process in neurotoxin exposure, the event downstream of caspase-3 is not well characterized.

Protein kinase C (PKC) family proteins are serine/threonine protein kinases and classified into three subgroups based on the activation mechanisms (Mellor and Parker 1998; Musashi et al. 2000; Way et al. 2000:Nishizuka, 1988 #39). Conventional PKCs (PKCα, βI, βII, and γ) are $Ca2^{+}$ and phospholipids dependent isoforms whereas the activation of novel PKCs (PKCδ, ε, η, and θ) is independent of intracellular $Ca2^{+}$. The atypical isoforms (PKCζ and λ/ι) require neither Ca^{2+} nor lipid for their activation. Most of PKC isoforms are expressed in the central nervous system, and they have been known to play an important role in various physiological and pathological conditions including cell differentiation, proliferation, development, synaptic plasticity, epilepsy, ischemia and neurodegeneration (Dekker et al. 1989; Mailhos et al. 1994; Miettinen et al. 1996; Newton 1997; Mellor and Parker 1998; Gschwendt 1999; Franklin and McCubrey 2000; Naik et al. 2000). PKCδ has recently been recognized as a pro-apoptotic kinase in non-neuronal cells during chemical induced apoptotic cell death (Reyland et al. 1999; Fujii et al. 2000; Majumder et al. 2000; Reyland et al. 2000). In this context, we have recently demonstrated that PKCδ, in dopaminergic cells, is proteolytically cleaved and activated by caspase-3, which subsequently mediates apoptotic cell death in a known Parkinsonian toxin MPP^{+} induced apoptosis as well as other environmental chemicals that have been thought to be risk factors of PD namely dieldrin (a organochlorine pesticide) and MMT (methylcyclopentadienyl manganese tricarbonyl; an organic manganese compound) (Sanchez-Ramos et al. 1998; Anantharam et al. 2002; Kitazawa et al. 2002a; Kitazawa et al. 2002b). We also found that the toxin-induced PKCδ activation amplifies upstream caspase cascade via positive feedback activation.

The purpose of present study was to examine the cellular and molecular basis of

regulatory mechanism of PKCδ at the level of mitochondria during environmental chemical-induced apoptosis in dopaminergic cells. Herein, we report that PKCδ translocates to mitochondria and activates pro-apoptotic events including mitochondrial depolarization, cytochrome c release, caspase-9 and caspase-3 activation and inactivation of bcl2 by caspase-3 dependent proteolytic cleavage in dopaminergic cells following exposure dieldrin or MMT.

MATERIALS AND METHODS

Chemicals

Mouse monoclonal β-actin antibody, human recombinant Bcl-2, active human recombinant caspase-3, and active human recombinant PKCδ were purchased from Sigma Chemicals (St. Louis, MO). Phorbol-12-myristate-13-acetate (PMA) is purchased from Calbiochem (La Jolla, CA). The caspase-3 substrate Ac-DEVD-AMC was purchased from Bachem Biosciences, Inc. (King of Prussia, PA). The caspase-9 substrate Ac-LEHD-AMC was purchased from Alexis Biochemicals (San Diego, CA). The caspase-3 inhibitor Z-DEVD-FMK was purchased from Enzyme Systems Products (Livermore, CA). Rabbit polyclonal nPKCδ antibody, caspase-3 antibody, caspase-9 antibody, and mouse monoclonal Bcl-2 antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The ECL Western blotting analysis kit was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The cytochrome c ELISA kit was obtained from MBL International Corp. (Watertown, MA). The Cell Death Detection Elisa Plus Assay Kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). BioPORTER was purchased from Gene Therapy Systems (San Diego, CA). ^{32}P-ATP was purchased from NEN. Dulbecco's modified Eagle medium (DMEM) was purchased from Mediatech, Inc. (Herndon, VA). Heat-inactivated horse serum and fetal bovine serum were purchased from Gibco BRL Products (Gaithersburg, MD). Other routine laboratory chemicals were obtained from Fisher Scientific (Pittsburgh, PA). Bcl-2-transfected PC12 cells (PC12HB2-3) and

vector-transfected PC12 cells (PC12V4) were generous gifts from Drs. Yutaka Eguchi and Yoshihide Tsujimoto, Osaka University (Osaka, Japan).

Cell culture and treatment regiment

PC12V4 (vector-transfected PC12 cells) and PC12HB2-3 (Bcl-2-transfected PC12 cells) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 7% heat-inactivated horse serum and 4% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Subconfluent cells were used in experiments. Cells were resuspended in serum-free DMEM and dieldrin or MMT was added. The final concentration of DMSO (used as vehicle) was less than 0.5%.

For PKCδ delivery studies, cells were subcultured on 24-well culture plate at a density of 0.2 x 10^6 cells/well. After the cells were subconfluent, a mixture of BioPORTER reagent and human recombinant PKCδ (5 ng) in serum-free DMEM was added, and the cells were incubated for 4 hr, as described previously (Anantharam et al. 2002; Kitazawa et al. 2002a). After incubation, cells were used for following experiments.

Determination of mitochondrial membrane potential

Depolarization of mitochondrial membrane potential ($\Delta\Psi m$) was measured using flow cytometry and $DiOC_6$ (Kitazawa et al. 2002b). Briefly, 40 nM $DiOC_6$ was added to the cells 15 min before the end of treatment period, and the incubation continued at 37°C. Cells were then washed once, resuspended in PBS (pH 7.4), and analyzed by flow cytometry with excitation at 484 nm and emission at 501 nm. Data were analyzed by CellQuest software (Becton Dickinson, San Francisco, CA).

Cytochrome c assay

Cytosolic cytochrome c release was measured using a cytochrome c ELISA kit (MBL International Corp). We followed the procedure as described previously

(Anantharam et al. 2002; Kitazawa et al. 2002a). Briefly, cells were washed once with PBS (pH 7.4) and resuspended in homogenization buffer containing 10 mM Tris HCl (pH 7.5), 0.3 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, and 10 μg/ml leupeptin. Cells were dounce homogenized and centrifuged. The resulting supernatants were collected as mitochondria-free cytoplasmic fractions. The cytoplasmic extracts were processed for cytochrome c detection as per the manufacturer's ELISA protocol. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices Corp., Sunnyvale, CA).

Caspase-9 activity assay

Caspase-9 activity was determined by following the procedure previously described by our laboratory (Anantharam et al. 2002; Kitazawa et al. 2002a). After the delivery of PKCδ, cells were washed once with PBS (pH 7.4) and resuspended in lysis buffer containing 50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 10 μM digitonin. Cells were then incubated at 37°C for 20 min to allow complete lysis. Lysates were quickly centrifuged, and cell-free supernatants were incubated with 50 μM of the caspase-9 substrate Ac-LEHD-AMC at 37°C for 1 hr. Caspase-9 activity was measured by spectrofluorometry (Molecular Devices) with excitation at 380 nm (slit width 10 nm) and emission at 460 nm (slit width 20 nm). The activity was expressed as fluorescence unit (FU) per mg protein per hr of incubation with substrate. Protein estimation was measured using the Bradford protein assay reagent (BioRad Laboratories, Hercules, CA).

Subcellular fractionation

Cells (~1 x 10^7 cells) were exposed to dieldrin at 37°C for the indicated periods. Cells were washed once with ice-cold PBS and resuspended in homogenization buffer containing 20 mM Tris/HCl (pH 8.0), 2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 10 μg/ml leupeptin, 5

mM sodium fluoride, and 50 μM sodium orthovanadate. Cells were kept on ice for 30 min then homogenized using a Dounce homogenizer. Homogenates were centrifuged at 1,000 x g for 10 min at 4°C to isolate nuclei and unlysed cells. Supernatants were centrifuged at 10,000 xg for 15 min at 4°C to isolate the mitochondrial membrane rich fractions. The supernatants were further centrifuged at 100,000 x g for 60 min at 4°C to collect cytosolic fractions. The protein concentration of each cytosolic fraction was determined using a Bradford protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA). Cytosolic fraction samples were mixed with 2x gel loading buffer containing 10% SDS and 200 mM DTT and placed into boiling water for 5 min.

Immunoprecipitation

Each cell extract (500 μg/ml) was incubated with PKCδ antibody or Bcl-2 antibody (10 μl per 200 μl of sample) at 4°C overnight. After the incubation, 100 μl protein A-sepharose (for PKCδ IP) or protein G-sepharose (for Bcl-2 IP) (Sigma Chemicals) was added to each sample and further incubated for 1-2 hr at 4 °C. Protein A- or protein G-bound antigen-antibody complex was collected and resuspended with homogenization buffer or kinase reaction buffer for immunoblotting or kinase activity assay, respectively.

Immunoblotting

Proteins in cytosolic fractions were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membrane (Bio-Rad Laboratories). Non-specific binding sites were blocked by 5% non-fat milk blocking solution (Amersham Pharmacia Biotech); the membrane was then treated with anti-PKCδ (1:2000 dilution) antibody, followed by secondary horseradish peroxidase-conjugated anti-rabbit (1:2000 dilution) antibody. Antibody-bound proteins were detected by the enhanced chemiluminescence (ECL) system. To confirm equal amount of protein in each lane, antibodies were stripped from membranes with stripping

buffer (Geno Technology, St. Louis, MO) and reprobed with β-actin (1:5000 dilution) to confirm equal protein loading in each lane.

In vitro PKCδ kinase activity assay

PKCδ enzymatic activity was measured using an immunoprecipitation kinase assay as described by Reyland *et al.* (Reyland et al. 1999). The protein-A-bound antigen-antibody complexes collected by immunoprecipitation were washed three times with homogenization buffer, three times with 2x kinase buffer (40 mM Tris, pH 7.4, 20 mM $MgCl_2$, 20 μM ATP, and 2.5 mM $CaCl_2$), and resuspended in 20 μl of 2x kinase buffer. Reaction was started by adding 20 μl of reaction buffer containing 10 μg histone H1 and 5 μCi of [γ-^{32}P]ATP (4,500 Ci/mM) to the immunoprecipitated samples and incubated for 10 min at 30°C. For detamination of Bcl-2 phosphorylation by PKCδ, human recombinant Bcl-2 protein (3 μg) was used as a substrate instead of histone and reacted with purified PKCδ from cell extracts. SDS gel loading buffer (2x) was added to terminate the reaction, the samples were boiled for 5 min, and the products were separated on a 12.5% SDS-PAGE gel. The H1 phosphorylated bands were detected using a Personal Molecular Imager (FX model, Bio-Rad Laboratories) and quantified using Quantity One 4.2.0 software (Bio-Rad Laboratories).

Data analysis and statistics

Caspase activity and DNA fragmentation data were first analyzed using one-way ANOVA and then Dunnett's post-test or Bonfferoni's multiple comparison test was performed to compare differences between the treatment groups; $p<0.05$ or less was considered significant.

RESULTS

PKCδ translocation into mitochondria is an initial apoptotic signal in dieldrin- or MMT-induced apoptosis

Previously, we demonstrated that dieldrin or MMT activates mitochondrial dependent apoptotic cascade involving cytochrome c release and caspase-9 and caspase-3 activation in dopaminergic cells (Anantharam et al. 2002; Kitazawa et al. 2002a). We also found that PKCδ is proteolytically activated by caspase-3 and then acts downstream in the apoptotic process and upstream to amplify caspase signaling through a possible feedback mechanism. In the present study, we found a significant level of native PKCδ (72-74 kDa) translocated into mitochondria following dieldrin or MMT treatment in PC12 cells (Figure 1A-B). The translocation of PKCδ was rapid, starting as early as 0.5 hr after dieldrin (100 μM) or MMT (200 μM) exposure, and maximal migration occurred 1-2 hr post exposure. Especially, the PKCδ band intensity in mitochondrial membrane increased up to 217%, 213%, 707% and 749% at 0.5, 1, 2, and 3 hr following MMT treatment, respectively. The caspase-3-dependent proteolytically cleaved PKCδ products (38-41 kDa) were observed in cytosol starting 1 hr after the treatments, whereas noticeable translocation to mitochondria occurred 3 hr after dieldrin or MMT treatment, indicating that the translocation of native PKCδ into mitochondria is the initial signaling event during dieldrin- or MMT-induced apoptosis, and may alter mitochondrial function.

Mitochondrial translocation of PKCδ promotes release of the mitochondrial proapoptotic factor cytochrome c

In the next set of experiments, we examined the impact of PKCδ translocation on mitochondrial function. Cytochrome c release from mitochondria is one of the early proapoptotic events known to trigger the caspase cascade, and we previously demonstrated increased cytosolic cytochrome c levels in PC12 cells following both

dieldrin and MMT treatment (Anantharam et al. 2002; Kitazawa et al. 2002a). Since acute stimulation of cells with the synthetic lipid analog TPA is known to cause translocation of PKCδ to mitochondria (Majumder et al. 2000), we compared the effect of TPA, dieldrin, or MMT on cytochrome c release. Stimulation of PC12 cells with 1 μM TPA for 30 min caused pronounced translocation of PKCδ to mitochondria and increased the accumulation of cytochrome c in the cytosolic fraction (Figure 2A). Dieldrin treatment (100 μM) also increased the cytosolic cytochrome c levels as compared to untreated control cells.

To further verify that translocation of PKCδ into mitochondria mediates cytochrome c release, we generated PKCδ–deficient PC12 cells by down-regulating the kinase. In contrast to acute TPA stimulation, chronic treatment with low-dose TPA (0.2 μM for 24 hr) down-regulates endogenous PKC proteins (Morino et al. 2001). Previously, we showed that treatment with TPA (0.2 μM) for 24 hr almost completely eliminated PKCδ levels for over 72 hr (Chapter VI). As shown in Figure 2A, dieldrin treatment did not induce mitochondrial translocation of PKCδ or cytochrome c accumulation in TPA-down-regulated PC12 cells.

To determine if PKCδ kinase activity is required for the mitochondrial release of cytochrome c, we pretreated PC12 cells with the PKCδ specific inhibitor rottlerin (5 μM) for 30 min, and then treated the cells with dieldrin or MMT for another 3 hr. We have shown that 5 μM rottlerin inhibits PKCδ kinase activity by more than 60%, as determined in an *in vitro* ^{32}P phosphorylation assay (Anantharam et al. 2002; Kitazawa et al. 2002a). As depicted in Figure 2B, pretreatment with rottlerin significantly inhibited dieldrin- or MMT-induced cytochrome c release without affecting PKCδ translocation into mitochondria. These data strongly suggest the translocation and activation of PKCδ are important in mediating cytochrome c release during dieldrin or MMT treatment.

In addition to cytochrome c release, second mitochondrial activation of caspases (Smac), often referred to as DIABLO, is another proapoptotic molecule that

was released in a time-dependent manner from mitochondria following dieldrin or MMT treatment (Figure 2C). As shown in Figure 1C, release of Smac occurred immediately after PKCδ translocation into mitochondria.

Delivery of active recombinant PKCδ alters mitochondrial function in dopaminergic cells

PKCδ regulation of mitochondrial function was also confirmed by delivering human recombinant PKCδ using a lipid mediated protein delivery system. In our recent studies, we observed 60-70% delivery efficiency (Anantharam et al. 2002) and a dose-dependent increase in PKCδ intracellular kinase activity (unpublished observation). In the present study, mitochondrial membrane potential ($\Delta\Psi m$), cytochrome c release (50%), and caspase-9 activity were measured in active PKCδ-delivered PC12 cells. As shown in Figures 3A, 3B, and 3C, delivery of active human PKCδ produced significant increases ($p<0.01$) in mitochondrial membrane depolarization, cytochrome c release, and caspase-9 activity. In comparison, these parameters were not significantly changed in cells delivered heat-inactivated recombinant PKCδ, further supporting our finding that PKCδ has an important proapoptotic role in mitochondrial function.

Bcl-2 associates with PKCδ during dieldrin- or MMT-induced apoptosis

We sought to determine whether PKCδ phosphorylates certain mitochondrial proteins and thereby alters mitochondrial function. Since phosphorylation of the major anti-apoptotic protein Bcl-2 inhibits activation of the proapoptotic process involving cytochrome c release (Haldar et al. 1995; Attalla et al. 1998; Tashiro et al. 1998; Fan et al. 2000; Torcia et al. 2001), we examined whether PKCδ phosphorylates Bcl-2 to inactivate its anti-apoptotic effect. We treated Bcl-2 over-expressing PC12 (PC12HB2-3) cells with dieldrin (100 and 300 μM) or MMT (100 and 200 μM) for 3 hr and then performed immunoprecipitation studies in the cell lysates. PKCδ was immunoprecipitated, and resulting samples were immunoblotted with Bcl-2. As shown

in Figure 4A, Bcl-2 levels were increased in PKCδ-immunoprecipitated samples following both dieldrin and MMT treatments. To confirm the positive association between Bcl-2 and PKCδ, we performed reverse immunoprecipitation studies in which samples were immunoprecipitated with Bcl-2 and then immunoblotted with PKCδ. As shown in Figure 4B, Bcl-2 and PKCδ were not associated in control or vehicle treated PC12HB2-3 cells, whereas PKCδ was detected in dieldrin or MMT treated cells in a dose-dependent manner, further confirming the association between PKCδ and Bcl-2. Bcl-2 (IP PKCδ) or PKCδ (IP Bcl-2) bands were not detected in vector-transfected PC12 (PC12V4) cells, confirming that the protein bands were specific for Bcl-2 and PKCδ (Figure 4C).

Since we observed PKCδ translocation to mitochondria and its association with Bcl-2, the next logical step was to determine if PKCδ phosphorylates Bcl-2. We evaluated Bcl-2 phosphorylation by PKCδ using an *in vitro* kinase assay. Recombinant Bcl-2 was used as the substrate for PKCδ in the kinase assay and the ^{32}P-ATP reaction was carried out in the presence or absence of the PKCδ inhibitor rottlerin (10 μM). The phosphoimage showed phosphorylated Bcl-2 bands, indicating that PKCδ phosphorylated the Bcl-2 protein (Figure 4D). Rottlerin significantly inhibited Bcl-2 phosphorylation, suggesting that the phosphorylation of Bcl-2 was mediated by PKCδ.

Bcl-2 is proteolytically cleaved by caspase-3 during dieldrin- or MMT-induced apoptosis

In our previous study, we found that PKCδ contributes to the dramatic increase (40-60 fold) in caspase-3 levels via positive feedback activation during dieldrin or MMT treatment (Anantharam et al. 2002; Kitazawa et al. 2002a). Since the present study demonstrated an interaction between PKCδ and Bcl-2, we further evaluated whether caspase-3 plays a role in the interaction. Immunoprecipitation of dieldrin or MMT treated cell lysates with PKCδ antibody followed by caspase-3 immunoblotting showed that caspase-3 is associated with PKCδ (Figure 5A). However, when

PKCδ-immunoprecipitated samples were immunoblotted for caspase-9, PKCδ and caspase-9 were not associated (data not shown).

We further examined the functional consequence of PKCδ, Bcl-2, and caspase-3 interactions during dieldrin- or MMT-induced neurotoxicity. Amino acid sequence analysis using NIH database revealed a possible caspase-3 cleavage site (DAGD) in the Bcl-2 protein (Figure 5A). Also, recent studies have demonstrated that caspase-3 cleaves Bcl-2 in non-neuronal cells (Liang et al. 2002). Therefore, we examined the proteolytic cleavage of Bcl-2 following dieldrin or MMT treatment in Bcl-2 over-expressed PC12 (PC12HB2-3) cells. After a 3 hr exposure period, Bcl-2 cleavage product (23 kDa) was observed in a dose-dependent manner (Figure 5B), whereas vehicle treated cells showed only native Bcl-2 protein (26 kDa). Pretreatment with the caspase-3 inhibitor Z-DEVD-FMK (50-100 μM) almost completely inhibited Bcl-2 cleavage, indicating that caspase-3 cleaves Bcl-2 (Figure 5C-D). Furthermore, pretreatment with the PKCδ inhibitor rottlerin (5-10 μM) dose-dependently inhibited the Bcl-2 cleavage (Figure 5C-D), suggesting that PKCδ may play an important role in the proteolytic cleavage of Bcl-2. Taken together with the Bcl-2 phosphorylation studies, the results suggest that phosphorylation of Bcl-2 by PKCδ may be required for its proteolytic cleavage by caspase-3.

DISCUSSION

In our previous studies, we have demonstrated the caspase-3-dependent proteolytic activation of PKCδ and the important pro-apoptotic function of PKCδ in dieldrin- or MMT-induced dopaminergic cell death. We have discovered that inhibition of PKCδ attenuated caspase-3 activity, indicating that PKCδ might have positive association with caspase-3 activity. Our present study further investigated the regulatory role of PKCδ in upstream apoptotic processes at the level of mitochondria, and the results demonstrate that dieldrin or MMT treatment in dopaminergic cells

activates PKCδ that (i) rapidly translocates to mitochondria, (ii) subsequently promotes release of mitochondrial proapoptotic factors cytochrome c and smac, (iii) phosphorylates Bcl-2 and promotes cleavage by caspase-3. To our knowledge this is the first report that demonstrates an important regulatory proapoptotic function for PKCδ in the mitochondria following exposure to environmental chemical exposure in dopaminergic cells.

PKC activation normally has been shown to be anti-apoptotic role in various cell model, however, we and other recently demonstrated that activation of PKCδ, a key member of novel PKC isoform family, promotes apoptosis in neuronal cells as well as non-neuronal cells (Chen et al., 1999; Li et al., 1999; Reyland et al., 1999; Cross et al., 2000; Fukunaga et al., 2001; Majumder et al., 2001; Anantharam et al., 2002; Garcia-Fernandez et al., 2002; Kitazawa et al., 2002a). One of the proapoptotic action of PKCδ is augmentation of mitochondrial mediated caspase cascade (Li et al., 1999; Majumder et al., 2001; Garcia-Fernandez et al., 2002), but cellular mechanisms underlying the positive regulation of the apoptotic signaling is not well characterized. Our results indicate that translocation of PKCδ and subsequent release of proapoptotic factors by inactivating the major mitochondrial anti-apoptotic protein Bcl-2 via proteolytic cleavage by caspase-3.

Mitochondrial membrane-associating Bcl-2 family proteins play an important role in apoptotic process. Pro-apoptotic Bcl-2 proteins include Bax, Bad, Hrk, Bim and Bid, and all possess Bcl-3 homology (BH3) domain, which regulates the translocation to mitochondrial membranes (Strasser et al., 2000). Among them, Bad and Bax have been reported to be regulated by phosphorylation and dephosphorylation. For example, Akt and PKA are known to phosphorylate Bad at Ser 136 and Ser 112, respectively, and inactivate pro-apoptotic function of Bad by dissociating from mitochondrial membranes and interacting with 14-3-3 protein (Zha et al., 1996; Datta et al., 1997). On the other hand, anti-apoptotic Bcl-2 proteins including Bcl-2, Bcl-XL, and Bcl-w, are only found in mitochondrial membranes and prevent depolarization of $\Delta\Psi m$ during apoptosis.

Potentiation of pro-apoptotic signaling mechanism through inactivation of anti-apoptotic function of Bcl-2 has been documented previously (Haldar et al., 1995; Attalla et al., 1998; Tashiro et al., 1998; Fan et al., 2000; Torcia et al., 2001). In cell extract, Bcl-2 are phosphorylated at serine residues, and Bcl-2 overexpressed cells did not prevent okadaic acid- or taxol-induced apoptosis, strongly suggesting phosphorylation of serine residues in Bcl-2 abolishes its anti-apoptotic property (Haldar et al., 1995). Further studies indicate that MAPK, especially c-Jun N-terminal kinase (JNK) phosphorylates and inactivates Bcl-2 during vinblastine-induced cell cycle arrest (Tashiro et al., 1998; Fan et al., 2000). On the other hand, the translocation of p38 MAPK, not JNK, into mitochondria is responsible for phosphorylation of Bcl-2 during nerve growth factor deprivation-induced apoptosis in memory B cells (Torcia et al., 2001). These reports convince the fact that apoptosis-related protein kinases play an important regulatory role in Bcl-2 function. Specifically, three amino acid residues (Ser70, Ser 87, and Thr69) in Bcl-2 were determined to be phosphorylated during paclitaxel-induced G2/M cell cycle arrest in Jurkat T cells, and resulted in inactivation of Bcl-2 (Yamamoto et al., 1999). They have identified that JNK was responsible for the phosphorylation of these three amino acid residues in Bcl-2. Srivastava et al. (1998) reported PKA primarily phosphorylates Ser70 of Bcl-2 during apoptotic process, further indicating the direct modulation of Bcl-2 anti-apoptotic function by phosphorylation during apoptosis (Srivastava et al., 1998). In addition, recently caspase-3-mediated Bcl-2 proteolytic cleavage and inactivation was observed in neocarzinostatin (NCS)-induced apoptosis in Bcl-2 overexpressed PC12 cells (Liang et al., 2002), further indicating the enhancing effect of apoptosis by directly interfering Bcl-2 function. However, these reports did not describe detail mechanisms underlying the inactivation of Bcl-2 during apoptosis.

Thus, we have hypothesized a novel mechanism of Bcl-2 inactivation and intiation/potentiation of apoptosis in dieldrin or MMT treatment as shown in Figure 6. Mitochondrial translocation of PKCδ is one of the earliest signaling mechanisms, and

PKCδ appears to phosphorylate Bcl-2 protein and inactivate its anti-apoptotic function following translocation. In addition, caspase-3 also cleaves Bcl-2 to further facilitate pro-apoptotic signaling processes. Our present data strongly suggest that Bcl-2 and PKCδ associate together following dieldrin or MMT treatment, and PKCδ plays an important role in modulating the anti-apoptotic function of Bcl-2 at earlier phase of dieldrin- or MMT-induced apoptosis in dopaminergic cells.

ACKNOWLEDGEMENT

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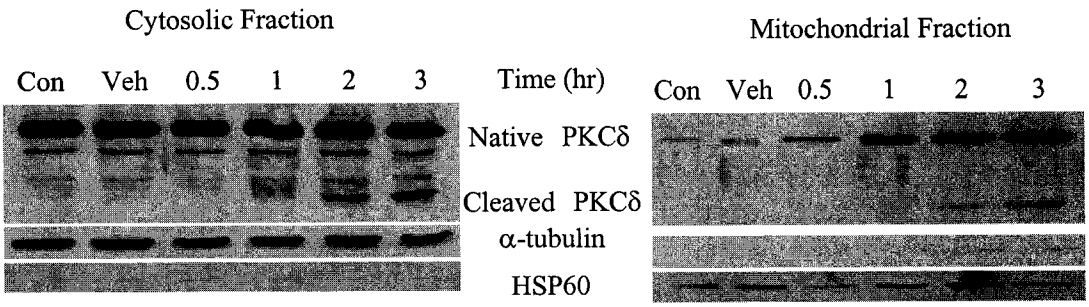
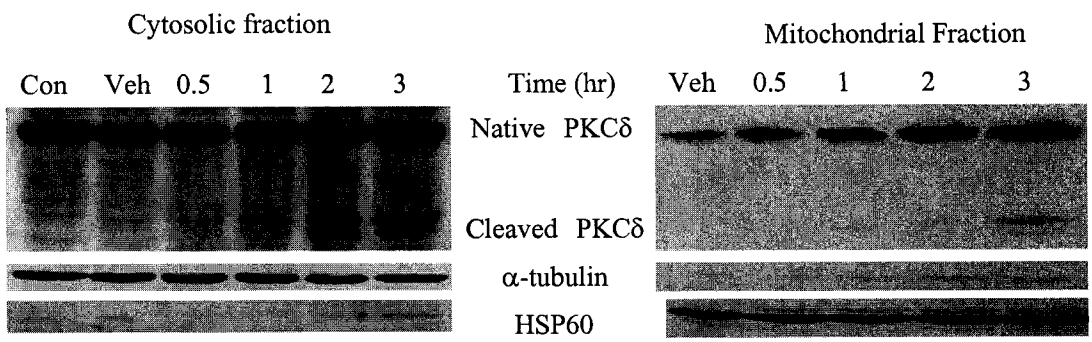
Figure 1: Dieldrin- or MMT-induced translocation of PKCδ into mitochondria and release of cytochrome c or Smac in PC12 cells.

PC12V4 cells (~1 x 10^7 cells) were treated with 100 μM dieldrin (A) or 200 μM MMT (B) for 0.5, 1, 2, and 3 hr. Mitochondrial fraction and cytosolic fraction were isolated and approximately 10 μg proteins per sample were resolved by 10% SDS-PAGE. Native PKCδ and proteolytically activated PKCδ are found in 72 kDa and 41 kDa, respectively. Reprobing the membranes with α-tubulin (50 kDa) and HSP60 (60 kDa) confirms purity of each subcellular fraction. (C) Cytosolic fractions were further used to determine the release of cytochrome c and/or Smac, major pro-apoptotic signaling molecules found in mitochondria. 17 kDa cytochrome c and/or 26 kDa Smac were detected time-dependently in dieldrin- or MMT-treated PC12 cells. α-tubulin and HSP60 confirmed no contamination of mitochondrial fraction.

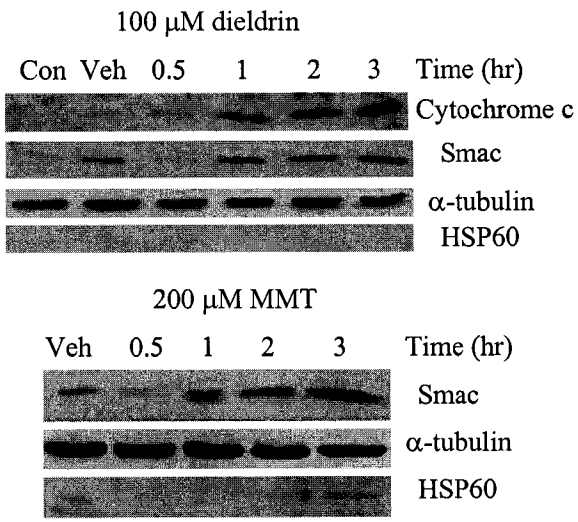
A 100 μM dieldrin



B 200 μM MMT



C



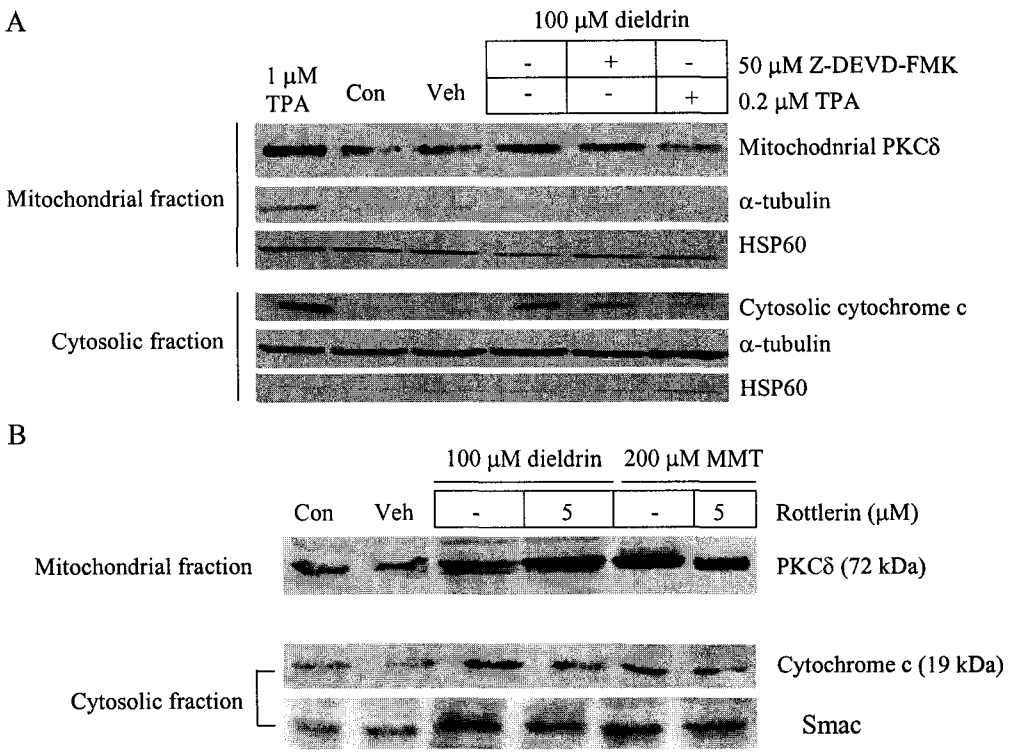


Figure 2: Down-regulation of PKCδ attenuated dieldrin-induced cytochrome c release.

(A) PC12V4 cells (~1 x 10^7 cells) were pretreated with 0.2 μM TPA for 24 hr or with 50 μM Z-DEVD-FMK for 30 min. The cells were then treated with 100 μM dieldrin for 3 hr, and mitochondrial fractions and cytosolic fractions were collected. Approximately 10 μg proteins were resolved in 10% SDS-PAGE, and cytosolic cytochrome c (17 kDa) and mitochondrial PKCδ (72 kDa) were detected. 1 μM TPA treatment for 30 min was used as a positive control for mitochondrial translocation of PKCδ and cytochrome c release. α-tubulin and HSP60 confirmed the purity of cytosolic fraction. (B) PC12V4 cells (~1 x 10^7 cells) were pretreated with 5 μM rottlerin for 30 min, then exposed to 100 μM dieldrin or 200 μM MMT for 3 hr. After the completion of exposure, mitochondrial and cytosolic fractions were collected, and mitochondrial translocation of PKCδ and cytosolic release of cytochrome c were measured by Western blot.

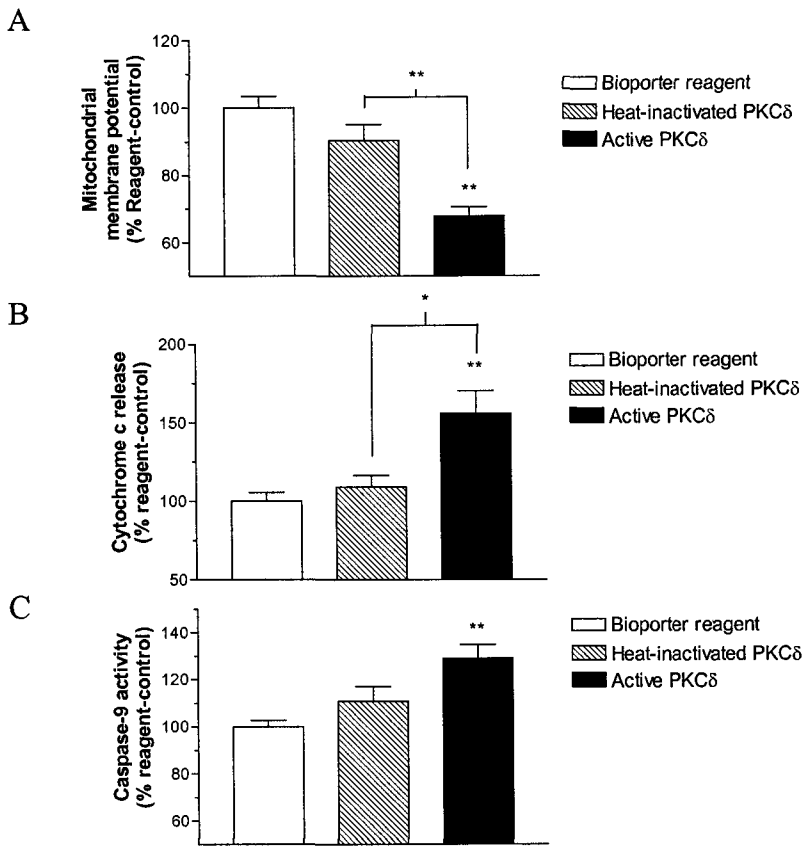
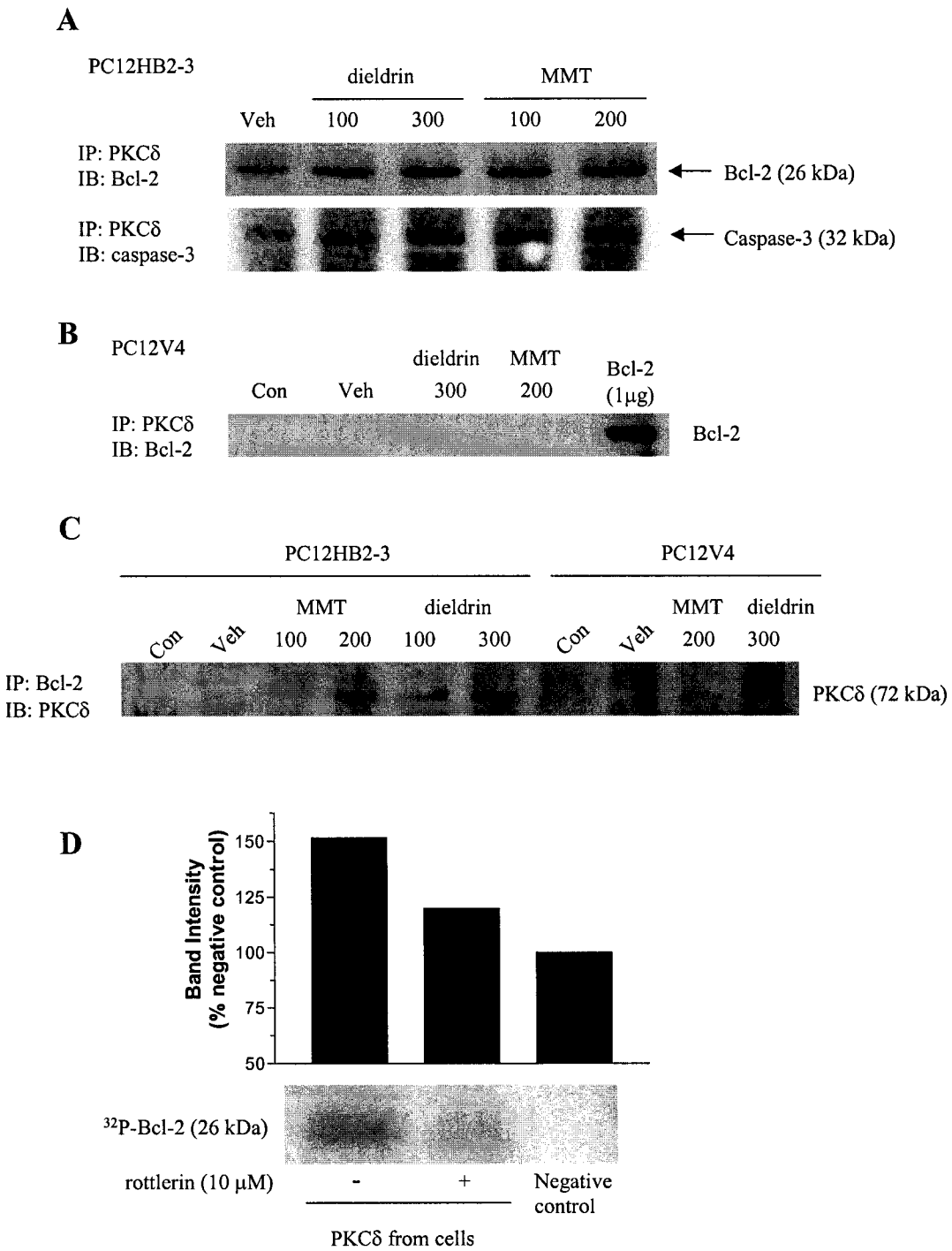


Figure 3: Alteration of mitochondrial membrane potential ($\Delta\Psi m$), cytochrome c release and caspase-9 activation following delivery of active PKCδ protein in PC12 cells.

PC12V4 cells were subcultured on 24-well culture plate at a density of 1-2 x 10^6 cells/well. BioPORTER reagent was used to deliver active human recombinant PKCδ protein (5 ng/well). After 4 hr incubation with BioPORTER reagent with PKCδ protein, cells were collected, and (A) depolarization of $\Delta\Psi m$ was measured by flow cytometry, (B) cytochrome c release was measured by ELISA cytochrome c assay, or (C) caspase-9 activity was measured using fluorogenic substrate, Ac-LEHD-AMC. Each bar represents mean ± SEM for two separate experiments in triplicate. *$p<0.05$ or **$p<0.01$ compared with reagent-control group or between indicated groups.

Figure 4: Association of PKCδ with Bcl-2 during dieldrin or MMT treatment in Bcl-2 overexpressed PC12 cells.

(A) Bcl-2 overexpressed PC12 (PC12HB2-3) cells (1 x 10^7 cells) were treated with dieldrin (100 or 300 μM) or MMT (100 or 200 μM) for 3 hr, and cytoplasmic (cytosolic + mitochondrial) fractions were collected. Samples (500 μg/ml) were immunoprecipitated with PKCδ, and immunoblotted with Bcl-2 (26 kDa), caspase-3 (32 kDa) and caspase-9 (30 kDa). (B) Vector-transfected PC12 (PC12V4) cells (1 x 10^7 cells) were treated with 300 μM dieldrin or 200 μM MMT for 3 hr, and cytoplasmic fractions were collected. Samples (500 μg/ml) were immunoprecipited with PKCδ and immunoblotted with Bcl-2 (26 kDa). 1 μg human recombinant Bcl-2 protein was also loaded as a positive control. (C) Cytoplasmic samples (500 μg/ml) were now immunoprecipitated with Bcl-2 and immunoblotted with PKCδ to confirm the specificity of association between Bcl-2 and PKCδ. (D) PKCδ was immunoprecipitated from cell extract and incubated in the presence of human recombinant Bcl-2 protein (3 μg) and ^{32}P-ATP for 10 min at 30 °C without (lane 1) or with (lane 2) 10 μM rottlerin. Negative control (lane 3) contains only human recombinant Bcl-2 protein and ^{32}P-ATP. Bar graph represents the relative intensity of phosphorylated Bcl-2 expressed as percent of negative control.


A
PC12HB2-3
dieldrin
MMT
Veh
100
300
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IP: PKCδ
IB: Bcl-2
Bcl-2 (26 kDa)
IP: PKCδ
IB: caspase-3
Caspase-3 (32 kDa)
B
PC12V4
Con
Veh
dieldrin
300
MMT
200
Bcl-2
(1μg)
IP: PKCδ
IB: Bcl-2
Bcl-2
C
PC12HB2-3
PC12V4
MMT
dieldrin
MMT
dieldrin
Con
Veh
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100
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Con
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IP: Bcl-2
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PKCδ (72 kDa)
D
Band Intensity
(% negative control)
150
125
100
75
50
32P-Bcl-2 (26 kDa)
rottlerin (10 μM)
-
+
Negative
control
PKCδ from cells

Figure 5: Dieldrin- or MMT-induced proteolytic cleavage of Bcl-2 in Bcl-2 overexpressed PC12 cells.

(A) Human Bcl-2 amino acid sequence is shown. Amino acid sequence at 31-34 (DAGD) could be a possible tetrapeptide for caspase-3 cleavage. Also, gray letters (S and T) indicate possible amino acid for phosphorylation by PKCδ. (B) Bcl-2 overexpressed PC12 (PC12HB2-3) cells (1 x 10^7 cells) were treated with dieldrin (100 or 300 μM) or MMT (100 or 200 μM) for 3 hr, and whole cell lysates were collected. Approximately 3 mg proteins were loaded to each lane and resolved in 15% SDS-PAGE. 26 kDa native Bcl-2 and its cleaved fragment (approximately 23 kDa) were detected in dose-dependent manner. (C and D) Bcl-2 overexpressed PC12 (PC12HB2-3) cells (1 x 10^7 cells) were pretreated with 5-10 μM rottlerin or 50-100 μM Z-DEVD-FMK for 30 min, then treated with 200 μM MMT (C) or 300 μM dieldrin (D) for 3 hr. After the treatment period, whole cell lysates were collected. Approximately 3 mg proteins were loaded to each lane and resolved in 15% SDS-PAGE. Cleaved Bcl-2 decreased in rottlerin- or Z-DEVD-FMK-pretreated PC12 cells.

A

```
  1 MAHAGRTGYD NREIVMKYIH YKLSQRGYEW DAGDVGAAPP GAAPAPGIFS
 51 SQPGHTPHTA ASRDPVARTS PLQTPAAPGA AAGPALSPVP PVVHLTLRQA
101 QDDFSRRYRR DFAEMSRQLH LTPFTARGRF ATVVEELFRD GVNWGRIVAF
151 FEFGGVMCVE SVNREMSPLV DNIALWMTEY LNRHLHTWIQ DNGGWDAFVE
201 LYGPSMRPLF DFSWLSLKTL LSLALVGACI TLGAYLGHK
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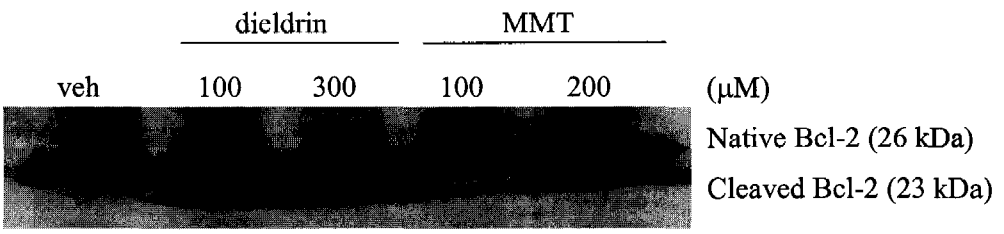
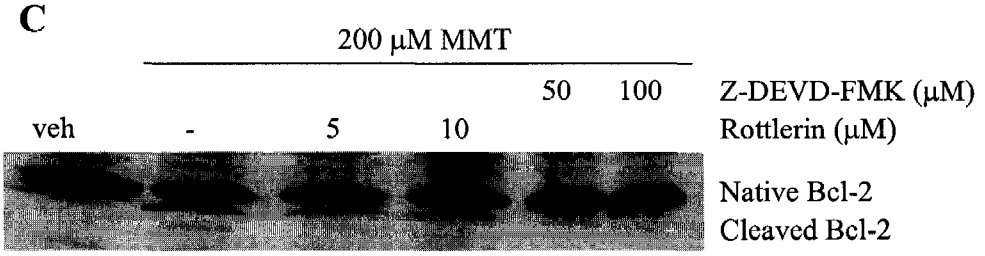
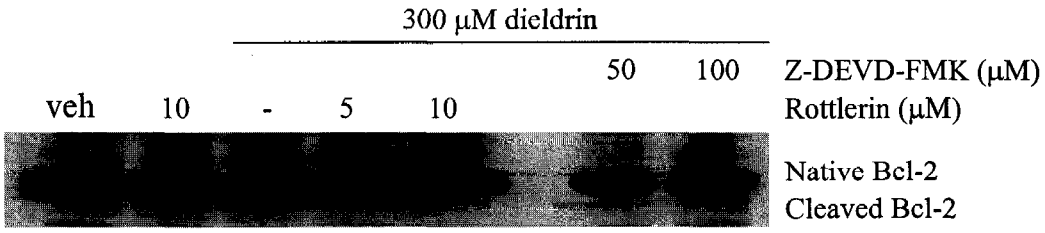
B



C



D



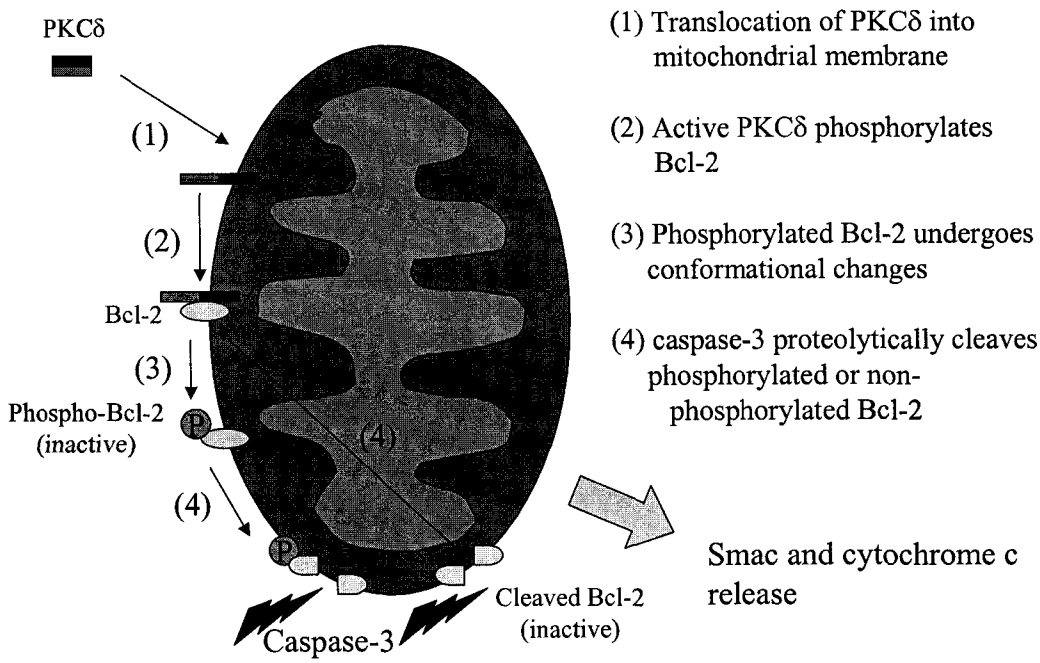


Figure 6: Proposed mechanism of inactivation of Bcl-2 by PKCδ and caspase-3 following dieldrin or MMT treatment in PC12 cells.

Based on the present results, we have proposed a possible inactivation processes of Bcl-2 anti-apoptotic function by PKCδ and caspase-3. (1) PKCδ translocates into mitochondrial membrane following dieldrin or MMT insults, (2) PKCδ is activated and phosphorylates Bcl-2, (3) phosphorylated Bcl-2 loses anti-apoptotic function, resulting in depolarization of mitochondriam membrane potential, cytochrome c release and activation of caspase cascade, and (4) upon activation of caspase-3, phosphorylated and/or non-phosphorylated Bcl-2 proteins are further proteolytically cleaved and lose anti-apoptotic function.

CHAPTER VIII: GENERAL CONCLUSIONS

The major findings of my research described in this dissertation have already been discussed in the Discussion sections of each chapter. The overall conclusions derived from these studies and a proposed environmental chemical-induced apoptotic cell death mechanism in dopaminergic neurons and its relevance to the pathogenesis of Parkinson's disease (PD) will be discussed here.

Susceptibility of dopaminergic cells to environmental chemical toxicity

Dopaminergic neurons are among the most vulnerable cells in the brain due to their unique biochemical and physiological properties. Although cellular mechanisms underlying the 'selective vulnerability' of the substantia nigra have not yet been characterized, nigrostriatal dopaminergic neurons appear to be intrinsically vulnerable due to their relatively low levels of catalase and cellular GSH (Chance et al., 1979; Perry et al., 1982; Percy, 1984; Jenner, 1998). Furthermore, this brain region possesses high concentrations of pro-oxidant metal iron and serves as a 'sink' for accumulation of many transition metals due to high levels of iron binding protein (Riederer et al., 1989). Furthermore, the presence of dopamine and its metabolic system (MAO-B) contribute to the generation of oxidative stress in dopaminergic neurons. Thus, oxidative stress may play an initial role in triggering dopaminergic neurodegeneration and pathogenesis of PD. We first determined whether dopamine and oxidative stress play major roles in environmental chemical-induced apoptosis. We demonstrated that dopaminergic PC12 cells were more susceptible to acute dieldrin or MMT as compared to non-dopaminergic cells, such as cerebellar granule cells, striatal GABAergic (M213-20) cells, human cortical neuronal (HCN-2) cells, and mouse pancreatic α-endocrine (α-TC) cells. In addition, rat mesencephalic dopaminergic neuronal ($1RB_3AN_{27}$ or N27) cells were more susceptible to dieldrin and MMT than dopaminergic PC12 cells (unpublished observation). These results support our hypothesis that dopaminergic cells are more

vulnerable to environmental insults than non-dopaminergic cells. Next, we examined the cellular mechanisms underlying the environmental chemical-induced dopaminergic degenerative process in detail.

To understand the mechanisms behind the enhanced susceptibility, we focused on the neurochemicals present in dopaminergic cells, dopamine and its metabolites. Dopamine is one of the most unstable chemicals in nature, and it is readily oxidized in oxygen-rich environments to form highly reactive dopamine quinones (Graham, 1984; Stokes et al., 1999). Also, during normal catabolic processes, dopamine generates hydrogen peroxide (H_2O_2) as a byproduct through MAO-B reaction when it is catabolized to DOPAC, the major breakdown metabolite of dopamine. Both dieldrin and MMT significantly increased dopamine release and catabolism resulting in DOPAC formation, strongly suggesting that more ROS was generated. The resulting ROS oxidizes vesicle-free dopamine to exacerbate oxidative stress. To confirm whether the presence of dopamine and/or the alteration of dopamine catabolism contributed to generation of ROS following dieldrin- or MMT-treatment in PC12 cells, we depleted the dopamine content with the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine (α-MPT) or inhibited MAO-B activity with selegiline (Deprenyl®) and measured ROS generation during dieldrin or MMT toxicity. ROS generation was significantly blocked by these pretreatments, and surprisingly, downstream caspase-3 activity and DNA fragmentation were also significantly attenuated. The same effect was observed following pretreatment with antioxidants (MnTBAP, SOD, or trolox). Our results strongly suggest that environmental chemicals (dieldrin and MMT) induce oxidative stress by altering the dopamine system, and the subsequent elevation of cellular oxidative stress triggers the caspase cascade in dopaminergic cells. These findings explain why dopaminergic cells are more susceptible to toxicants and generate more ROS than non-dopaminergic cells.

Dieldrin, MMT, and manganese as mitochondrial toxicants

Dieldrin, MMT, and manganese have been reported to block mitochondrial oxidative phosphorylation by inhibiting electron transport complexes (Bergen, 1971; Autissier et al., 1977; Gavin et al., 1992; Galvani et al., 1995). MPTP is a well-known chemical that induces PD-like syndromes in humans and other mammals and inhibits mitochondrial Complex I activity. MPP^+, an active metabolite of MPTP, selectively enters dopaminergic cells/neurons via the dopamine transporter (DAT) because of its similar structure to dopamine. Thus, MPTP is selectively and potently toxic to dopaminergic cells but not other cells or neurons. It is not known if dieldrin and MMT are selectively toxic to dopaminergic cells. Dieldrin blocks at or near the Complex III or cytochrome b, and MMT and manganese interact with and inhibit the Complex I activity as well as aconitase and succinate dehydrogenase activities (Zheng et al., 1998; Malecki, 2001). The exact mechanisms by which these chemicals act on specific sites of mitochondrial complexes remain unclear. However, in the case of MMT, the manganese ion component of MMT may be specifically responsible for altering the electronic configuration of the carbonyl groups to promote association with complex I. Additionally, MMT not only interferes with NAD^+-linked substrate energy transfer but also interferes with electron donation to ubiquinone, resulting in decreased oxidative phosphorylation (Autissier et al., 1977). Although dieldrin, MMT, and manganese are potent mitochondrial toxicants and may act via similar mechanisms as MPTP in side the dopaminergic cells, the role of these chemicals in the activation of apoptosis and the development of PD is not yet understood .

In the present study, we observed the depolarization of mitochondrial membrane potential ($\Delta\Psi m$) following treatments with these toxicants. When mitochondrial electron transport is blocked, electron carriers such as NADH and $FADH_2$ cannot pass electrons and the ratio of oxidized and reduced forms of these electron carriers are altered. As a result, cells shift their energy production from an aerobic to an anaerobic pathway to consume oxidized NADH or $FADH_2$ and to produce energy for

survival. Simultaneously, oxygen consumption is reduced in cells due to impaired mitochondrial function, and mitochondria initiate mitochondrial transition pore (MTP) opening to uncouple the proton gradient and compensate for the use of oxygen molecules (Skulachev, 1996; Cassarino et al., 1999). These changes eventually cause mitochondria to swell and depolarize the transmembrane potential. The depolarization of $\Delta\Psi m$ observed in our studies further suggests that dieldrin, MMT, and manganese are mitochondrial toxicants and inhibit mitochondrial complexes.

MTP opening not only causes a loss of $\Delta\Psi m$ but also induces the release of mitochondrial proteins into cytosol. Mitochondria contain several critical pro-apoptotic as well as anti-apoptotic proteins (Kroemer, 1999; Desagher and Martinou, 2000) and therefore, are considered one of the most important organelles regulating cell survival and death. The major pro-apoptotic proteins found in mitochondria are cytochrome c, apoptosis activating factor-1 (Apaf-1), Smac/DIABLO, AIF, and caspase-9 (Shi, 2001). We showed a significantly increased release of cytochrome c into cytosol following depolarization of $\Delta\Psi m$. Furthermore, cytochrome c release was blocked by overexpression of the anti-apoptotic mitochondrial protein Bcl-2, indicating that dieldrin, MMT, and manganese do not release cytochrome c by simply disrupting the mitochondrial membrane. Rather, mitochondria are functionally changed and MTP opens to release pro-apoptotic proteins. Released cytochrome c along with Apaf-1 and ATP activate caspase-9 (Cain et al., 2002). As a result, downstream effector caspases including caspase-3, -6, and -7 are proteolytically cleaved and activated. Also, Smac interacts with inhibitor of apoptosis protein (IAP) to further enhance caspase activity. A dramatic activation of caspase-3 was observed in cells treated with these three chemicals, indicating these dopaminergic neurotoxins are potent inducers of apoptosis.

In addition, the acute studies with dieldrin and MMT revealed the rapid increase in reactive oxygen species (ROS) within a few minutes of exposures. Mitochondria may be an additional source of ROS generation, especially when their normal physiological function is disrupted (Beal et al., 1993; Kowaltowski et al., 2001). The

mitochondrion is the major organelle utilizing oxygen molecules, accounting for more than 90% of the total cellular oxygen consumption, and impairment of mitochondrial function results in increased levels of unconsumed oxygen molecules. The significantly increased levels of superoxide anion (O_2^-) observed, possibly derived directly from oxygen molecules following dieldrin or MMT exposure, may have resulted from impaired mitochondrial function. Bcl-2 overexpressed PC12 cells generated significantly reduced levels of ROS and had preserved $\Delta\Psi m$, indicating mitochondrial protection.

Taken together, the primary target of dieldrin, MMT, and manganese is possibly the mitochondrion and the dysfunction of mitochondrial activity results in the generation of ROS and initiation of the mitochondrial-mediated caspase-dependent apoptotic pathway. Further studies are needed to verify the effect of these neurotoxic agents in isolated mitochondria.

Caspase-dependent apoptosis and significance of PKCδ proteolytic cleavage on apoptotic execution

In neuronal apoptosis, downstream effectors of caspase-3 that contribute to apoptotic cell death are not well characterized. The present study identified PKCδ as a key effector molecule during neurotoxic insult in dopaminergic cells. The exact physiological roles of PKCδ in neurons are not well understood yet. PKCδ has been reported to play an important role in cell proliferation and differentiation in early developmental stages in non-neuronal cells (Gschwendt, 1999). PKCδ was recently found to be involved in apoptotic cell death in various non-neuronal models.

PKC family proteins are classified into three subfamilies: conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC). The activation process and physiological function of each subfamily is different. Intracellular calcium elevation is important for the activation of cPKCs, and their physiological functions are stimulation, synthesis, and secretion of certain hormones for digestion (Kawakita et al., 1995). Also,

some cPKCs (PKCα) play critical roles in apoptosis, mainly to prevent apoptosis and rescue cells (Deng et al., 2000; Ruvolo, 2001). On the other hand, nPKC does not require intracellular calcium elevation for activation but is activated through activation of PLC and formation of DAG in the plasma membrane. Recently, nPKCs were identified as substrates for caspase-3 during apoptosis (Shao et al., 1997; Reyland et al., 1999; Cross et al., 2000). As shown in the present study, proteolytic cleavage and activation of PKCδ occurs following caspase-3 activation after dieldrin, MMT, and manganese treatments in dopaminergic cells. Caspase-3-like-protease-mediated PKCδ cleavage has also been reported in non-neuronal cells (Emoto et al., 1995; Reyland et al., 1999). Once PKCδ is proteolytically cleaved into the regulatory and catalytic subunits, the kinase of the catalytic subunit remains persistently active, as demonstrated in the present study.

PKCδ may phosphorylate other apoptosis-related molecules and facilitate the execution process of apoptosis. Phosphorylation and dephosphorylation act as a biological switch controlling physiological functions of enzymes and other molecules. Thus, PKCδ is speculated to modulate pro- and/or anti-apoptotic molecules upon activation by caspase-3. The target substrate of PKCδ responsible for promoting DNA fragmentation has not yet been identified. The results in this dissertation demonstrate that DNA fragmentation is significantly attenuated by the PKCδ specific inhibitor rottlerin and in PKCδ mutant overexpressed cells following dieldrin, MMT, or manganese exposures. Thus, PKCδ may directly or indirectly regulate the activity of other key molecules involved in DNA fragmentation. Considering the abundance of PKCδ in neuronal cells and the heightened susceptibility of dopaminergic cells to environmental toxins, xenobiotics may play an important role in the pathogenesis of PD. During apoptosis, caspase-dependent DNase (CAD) causes DNA fragmentation by translocating into the nucleus to cleave DNA into small base pairs (Korn et al., 2002). PKCδ was recently reported to translocate into the nucleus during apoptosis and subsequently phosphorylate transcription factors to promote apoptosis (Cross et al.,

2000). Though not proven, PKCδ may phosphorylate activated CAD and potentiate its activity as a possible mechanism of regulating DNA fragmentation. However, the present study also demonstrates an alternative regulatory role of PKCδ during apoptosis and DNA fragmentation: PKCδ's feedback mechanism in the caspase cascade.

Role of PKCδ in feedback activation of the caspase cascade

Several lines of evidence suggest that PKCδ regulates upstream apoptotic events upon activation. Pharmacological or genetic inhibition of PKCδ results in decreased caspase-3 activity and the subsequent attenuation of DNA fragmentation (Reyland et al., 1999; Reyland et al., 2000; Anantharam et al., 2002). Two possible mechanisms of PKCδ pro-apoptotic effects on upstream events are proposed. First, PKCδ may phosphorylate upstream pro-apoptotic molecules and modulate caspase-3 activity directly or indirectly. Our data showed that PKCδ associated with caspase-3 following dieldrin or MMT treatment, and caspase-3 may be one of the substrates for PKCδ, and upon phosphorylation by PKCδ, caspase-3 protease may be enhanced.. Caspase-9 is regulated through phosphorylation by Akt (Cardone et al., 1998); likewise, caspase-3 activity may be modulated by phosphorylation. PKCδ translocates into the mitochondrial membrane following TPA and H_2O_2 treatments, and this translocation process is essential for cytochrome c release and activation of the apoptotic mechanism (Li et al., 1999; Majumder et al., 2000). Inactive PKCδ overexpressed cells failed to release cytochrome c into cytosol following chemical stimulation, strongly suggesting that PKCδ strictly regulates the initiation process of apoptosis. Furthermore, PKCδ functions not downstream, but upstream to initiate apoptosis. Yet, the exact mechanism by which PKCδ regulates the release of cytochrome c from mitochondria is not well known.

In the present study, we also observed early translocation of PKCδ into mitochondrial membranes to promote cytochrome c and Smac release into cytosol. An immunoprecipitation study revealed that PKCδ associates with Bcl-2, an anti-apoptotic

Bcl-2 family protein. Bcl-2 is a highly important molecule associated with PKCδ because Bcl-2 as well as Bad are regulated by phosphorylation and dephosphorylation. Mitogen activated protein (MAP) kinase phosphorylates Bad. Phosphorylated Bad then associates with the chaperone protein 14-3-3, resulting in the inactivation of its pro-apoptotic function by its movement into cytosol (Masters et al., 2001; Masters et al., 2002). On the other hand, Bcl-2 is inactivated upon phosphorylation, yet the kinases responsible for Bcl-2 inactivation have not been identified (Haldar et al., 1995; Tashiro et al., 1998; Fan et al., 2000; Torcia et al., 2001).

In the present study, *in vitro* delivery of recombinant active PKCδ into PC12 cells results in the release of cytochrome c, activation of caspase-9 and caspase-3, and subsequent increase in DNA fragmentation. Furthermore, dieldrin promotes translocation of native PKCδ into the mitochondrial membrane, independent of caspase-3 activity. Cytochrome c release was attenuated in cells treated with low-dose (200 nM) TPA treatment to down-regulate PKC (Bjaaland et al., 1988; Morino et al., 2001) and in inactive PKCδ overexpressed dopaminergic cells following dieldrin treatment. Thus, translocation of native PKCδ into the mitochondrial membrane is important to induce the subsequent cytochrome c release as an initiation step of apoptotic cell death. However, current evidence suggests phosphorylation of Bcl-2 by PKCδ and proteolytic cleavage of Bcl-2 by caspase-3, leading to inactivation of Bcl-2 and promotion of apoptosis. Further studies are required to better understand the regulatory mechanism of PKCδ in mitochondria during apoptosis.

In summary, we identified the major apoptotic cell death mechanism following exposure to environmental chemicals (Figure 14). During the initial phase, dopaminergic cell responses to dieldrin or MMT may differ in terms of its sensitivity, but not in terms of neurotoxic mechanisms. Dopamine release is promoted, and DOPAC formation is increased, resulting in increased generation of oxidative radicals intracellularly, which triggers mitochondrial dysfunction. Dieldrin and MMT may act directly on mitochondria and inhibit oxidative phosphorylation, leading to depolarization

of mitochondrial membrane potential and dysfunction. Additionally, dieldrin and MMT promote translocation of PKCδ into mitochondria by an unknown mechanism, and translocated PKCδ phosphorylates Bcl-2 to inactivate its anti-apoptotic function. These initial processes facilitate the release of pro-apoptotic molecules including cytochrome c and Smac into cytosol. Once cytochrome c and/or Smac are released, the caspase cascade is activated via Apaf-1. Caspase-9 activation by Apaf-1/cytochrome c complex (apoptosome) is followed by caspase-3 activation. Activated caspase-3 proteolytically cleaves its substrates including PKCδ, which plays a critical role in both upstream and downstream steps in the apoptotic pathway. PKCδ induces DNA fragmentation and cells undergo apoptotic cell death. Activated PKCδ also associates with caspase-3 and possibly enhances enzymatic caspase-3 activity by phosphorylation. In addition, caspase-3 proteolytically cleaved Bcl-2 further potentiates the apoptotic cascade. Although some evidence indicates that PKCδ translocates into mitochondria after proteolytic cleavage, the involvement of the cleaved fragment in mitochondria in modulating mitochondrial function is not clear. Inhibitor studies further confirmed the proposed apoptotic pathway. Tyrosine hydroxylase (TH) and MAO-B inhibitors significantly reduce dieldrin- or MMT-induced ROS generation, caspase-3 activity, and DNA fragmentation, indicating dopamine plays an important role in this pathway. Antioxidants and Bcl-2 over-expression inhibit cytochrome c release, and caspase inhibitors almost completely suppress downstream apoptotic events. PKCδ inhibition blocks not only DNA fragmentation, but also cytochrome c release, supporting the dual role of PKCδ in both downstream and upstream processes. Finally, both PKCδ and caspase-3 inhibitors inhibit inactivation of Bcl-2.

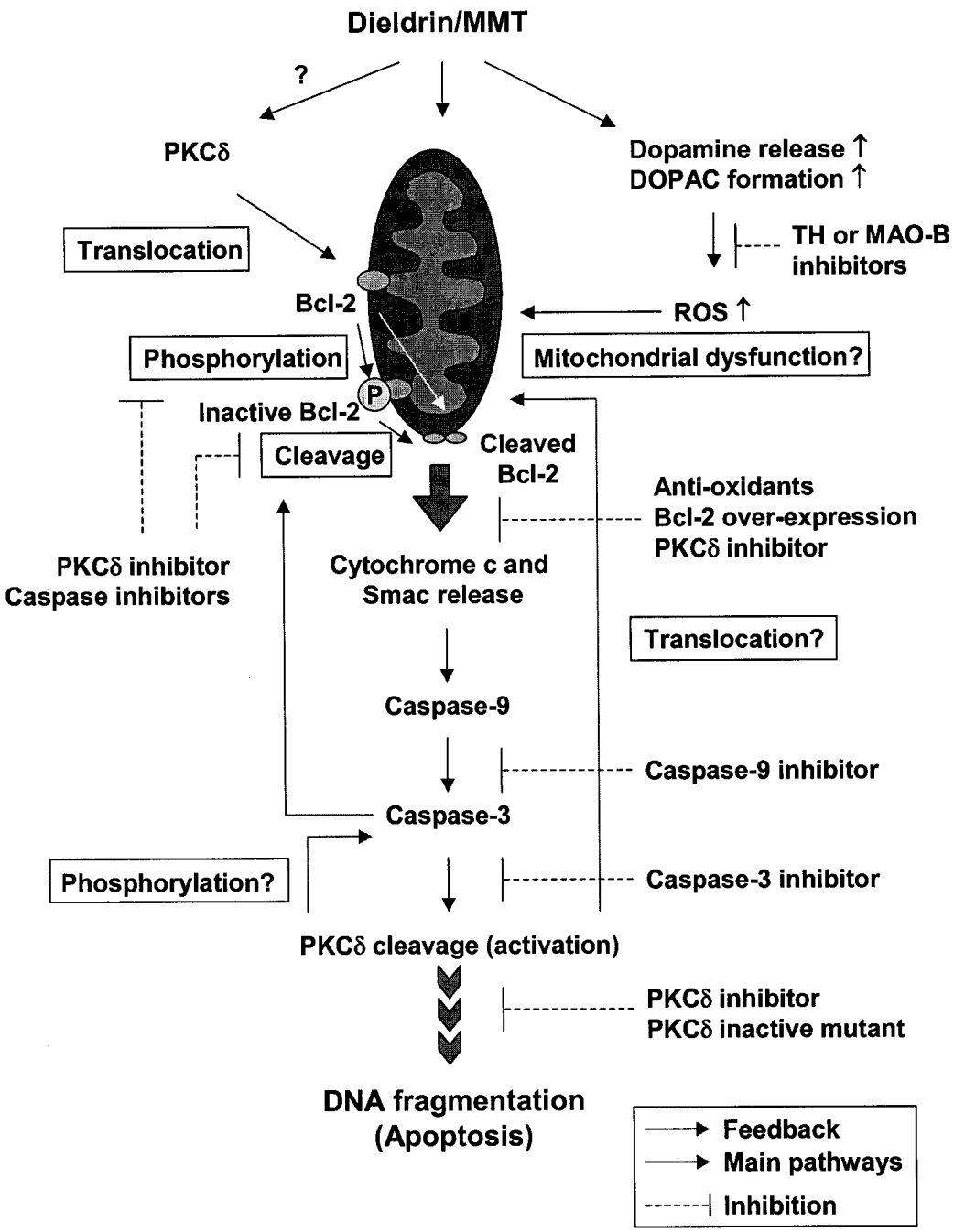


Figure 14: Integrated Cell Death Pathway Induced by Environmental Chemicals

Relevance of environmental chemical exposure to Parkinson's disease

From the present study, these environmental chemicals induce a common cell death mechanism in dopaminergic cells. The enhanced susceptibility of dopaminergic cells to these environmental chemicals due to the presence of dopamine may support but not prove the causality of environmental factors in PD. Furthermore, dopaminergic neurons in the SNc seem to be more susceptible to oxidative insults due to their insufficient antioxidant defense mechanisms. Cellular GSH levels in the SNc are lower than in other brain regions. Also, dopamine degradation and oxidation result in extra sources of oxidative stress, such as H_2O_2 and dopamine quinones. In general, catalase activity is significantly low in the brain, whereas SOD activity is high. This imbalanced ROS scavenging activity further results in the accumulation of H_2O_2 in neurons. Thus, exposure to certain environmental chemicals that alter dopamine catabolism and/or cause oxidative insults may facilitate dopaminergic neurodegeneration. Recent histological studies in human PD cases demonstrating oxidative markers, caspase-3, PKCs, and apoptosis in Lewy bodies, a hallmark of PD pathology, support our finding that oxidative stress and apoptosis are critical in dopaminergic degeneration. Also, results from post-mortem and case-control studies indicate that environmental chemicals are positively associated with the pathogenesis of PD. Dieldrin, especially, was significantly high in PD brains. Manganese induces the Parkinson-like syndrome known as Manganism following chronic exposure. Though the current evidence is incomplete, MMT may be a potential risk factor because it is structurally similar to dieldrin's cyclodiene moiety, as it contains manganese in its structure. Current evidence partially supports the positive association with PD by means of the cell death signaling mechanisms induced by these chemicals in dopaminergic cells. However, chronic toxicological studies and cell/molecular biological evidence supporting the association are needed to establish a more solid conclusion about the potential risk of chemicals in PD pathogenesis. Future intensive investigations will provide insight into the molecular mechanism of dopaminergic degeneration and etiopathogenesis of PD.

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