

Ubiquitin-proteasome system dysfunction in experimental models of Parkinson's disease

by

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ABSTRACT

The present study investigates cellular mechanisms underlying the pathogenic role of ubiquity proteasome system (UPS) dysfunction in dopaminergic degeneration following exposure to Parkinsonian neurotoxins. Mutations or overproduction of α -synuclein have been shown to be associated with familial Parkinson's disease (PD), and wild type α -synuclein is the major component of Lewy bodies, the protein inclusion bodies characteristic of PD. The organochlorine pesticide dieldrin has been implicated as an environmental risk factor for PD. The gene-environment interaction between α -synuclein and dieldrin impaired the proteolytic function of UPS leading to profound formation of α -synuclein aggregates, and enhanced apoptotic cell death in dopaminergic neuronal N27 cells. Proteasome inhibition by MG-132 elicited severe dopaminergic neurotoxicity in both an immortalized dopaminergic cell model of Parkinson's disease (N27 cells) and primary mesencephalic culture. Stereotaxic injection of MG-132 into the substantia nigra resulted in marked nigrostriatal degeneration *in vivo*, as demonstrated by prominent loss of nigral dopamine neurons and depletion of striatal dopamine. The proteasome inhibitor MG-132 was utilized as a pharmacologic tool to mimic UPS dysfunction in the remaining studies to investigate the molecular and cellular mechanisms underlying UPS impairment-induced dopaminergic degeneration. Proteasome inhibition by MG-132 depolarized mitochondrial membrane potential, caused mitochondrial release of proapoptotic molecules, and triggered apoptotic cell death exclusively proceeding through the mitochondria-dependent pathway in N27 cells. Caspase-3-dependent proteolytic cleavage of PKC δ into catalytic fragment (PKC δ -CF) and regulatory fragment (PKC δ -RF), plays a crucial role in MG-132-induced dopaminergic

apoptosis; ROS generation was not found to be important in MG-132-related cell death. PKC δ proteolytic cleavage resulted in a substantial increase in its kinase activity. The PKC δ -specific inhibitor rottlerin, but not SOD mimetic MnTBAP, significantly alleviated caspase-9 and -3 activation, indicating that proteolytically activated PKC δ amplified mitochondrial apoptosis cascades. In agreement, mitochondrial translocation of PKC δ -CF led to caspase-3 activation and DNA fragmentation. Suppression of PKC δ proteolytic cleavage by caspase-3 cleavage resistant mutant PKC δ^{D327A} effectively inhibited MG-132-induced activation of mitochondrial apoptosis. Further study into the mechanisms of proteasome inhibition activating mitochondrial apoptosis yielded some exciting new findings. The mitochondria may be a key sensor of polyubiquitin stress because ubiquitinated proteins preferentially accumulate in mitochondria as compared to cytosol. Additionally, overexpression of ubiquitin^{K48R} mutant effectively rescues cells from MG-132-induced mitochondrial apoptosis without altering antioxidant status of cells, whereas ubiquitin^{K63R} mutant augmented the proapoptotic effect of MG-132. Additionally, ubiquitin^{K48R} conferred neuronal resistance to a variety of dopaminergic neurotoxins that impair UPS including dopamine, MPP⁺ and dieldrin.

These results suggest that UPS impairment consequent to neurotoxin exposure plays a crucial contributory role in dopaminergic degeneration and that exposure to neurotoxic agents and gene-environment interactions could elicit dopaminergic neurotoxicity by converging to impair UPS function. Additionally, the findings of this work not only provide novel insights into cellular mechanisms of ubiquitin stress in dopaminergic neuronal cells but also serve as a foundation for future study ascertaining biochemical and functional links

between UPS dysfunction and mitochondrial apoptosis in the degenerative process of Parkinson's disease.

CHAPTER 1: GENERAL INTRODUCTION

Dissertation organization

Alternative thesis format is used for this thesis, which includes modified version of several manuscripts either published, or to be submitted for publication. It contains a general introduction, 5 research papers, a general conclusion and an acknowledgement. References for each individual section are listed at the end of corresponding chapters, with the background and literature review part as an exception, as the references for this part are listed together with those for the general conclusion part. In the general introduction part (Chapter I), an introduction provides concise information for the current knowledge of the etiopathogenesis of Parkinson's disease and describes the overview of the research objective. Background and literature review I provides background information of the Parkinson's disease, particularly focusing on a dysfunctional ubiquitin proteasome system in the etiopathogenesis of Parkinson's disease. This part has been accepted for publication by the journal of *Pharmacology & Therapeutics* (114: 327-344, 2007). Background and literature review II of the chapter summarizes the current knowledge regarding pathophysiological roles of α -synuclein in the dopaminergic degeneration, and this part is to be published as a book chapter in *Parkinson's Disease Pathogenic and Therapeutic Insights from Toxin and Genetic Models* (editors Richard Nass and Serge Przedborski, Elsevier Inc.). Chapter II "Dieldrin induces ubiquitin-proteasome dysfunction in α -synuclein overexpressing dopaminergic neuronal cells and enhances susceptibility to apoptotic cell death" is a research paper published in *Journal of Pharmacology and Experimental Therapeutics* (315: 69-79, 2005). Chapter III "Proteasome Inhibitor MG-132 Induces Dopaminergic Degeneration in Cell Culture and Animal Models" has been published in *Neurotoxicology* (27: 807-815, 2006). Chapter IV "Mitochondria are key sensor of polyubiquitin overloading stress and polyubiquitination sites Lys-48 and 63 differentially regulate the stress-induced apoptotic cell

death”, chapter V “Proteasome inhibitor MG-132-induces mitochondria apoptosis via proteolytic activation of PKC δ in dopaminergic neuronal cells” and chapter VI “Novel neuroprotective role of Lysine48 polyubiquitination site of ubiquitin during neurotoxicants-induced ubiquitin-proteasome dysfunction in Parkinson’s disease models” are research papers to be submitted to *Journal of cell biology*, *Journal of biological chemistry*, and *Journal of neurochemistry* respectively.

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

Introduction

Parkinson's disease (PD) is a common neurodegenerative movement disorder characterized by selective degeneration of dopaminergic neurons in the substantia nigra. Neurochemical and neuropathological analyses clearly suggest that oxidative stress, mitochondrial dysfunction and impairment of ubiquitin-proteasome system (UPS) are major mechanisms of dopaminergic degeneration because mitochondria dysfunction, increased oxidative markers, defects in mitochondrial electron transport and presence of protein aggregations are consistently observed in PD patients (McNaught and Olanow 2003; Betarbet et al. 2005; Moore et al. 2005; Przedborski and Ischiropoulos 2005). Additionally, numerous studies conducted in cell culture, animal models and *post mortem* human brain tissues have demonstrated that apoptotic cell death is the major form of cell death responsible for selective and irreversible loss of nigral dopaminergic neurons (Heidenreich 2003; Tatton et al. 2003; Vila and Przedborski 2003).

Epidemiological studies imply both environmental neurotoxins and genetic predisposition (genetic mutations) as risk factors for PD (Di Monte 2003; Warner and Schapira 2003), though the mechanisms underlying selective dopaminergic degeneration remain unclear. Several genes involved in UPS, mitochondrial function and oxidative stress are mutated in familial PD (McNaught and Olanow 2003; Betarbet et al. 2005). *Post mortem* and experimental studies in cell culture and animal models suggest pathogenic roles of impaired proteolysis by the ubiquitin-proteasome system (UPS) in PD. Evidence for a causal role of dysfunctional UPS in PD includes reduced proteasomal activities (McNaught et al. 2003), selective loss of proteasome subunits in substantia nigra of *post mortem* human brain

samples (McNaught et al. 2002), and mutation of several genes involved in the UPS degradation pathway including *Parkin* and *Uch-L1* (McNaught and Olanow 2003; Moore et al. 2005). As a defining pathology of PD, Lewy bodies contain a variety of proteins including α -synuclein, ubiquitin, proteasome subunits, some chaperone proteins, signaling proteins like kinases and some neurofilament proteins. Accumulation of ubiquitinated proteins in the protein aggregates indicates the failure of the clearance of target proteins by UPS. In animal models, it has been shown that exposure to proteasome inhibitors results in dopaminergic degeneration and formation of protein aggregates, which recapitulate the characteristic abnormalities of PD (McNaught et al. 2004).

α -Synuclein is the major component of Lewy bodies, thus α -synuclein overproduction or mutations may cause familial PD (Cookson 2005). However, studies with transgenic animal models suggest α -synuclein overproduction alone is not sufficient to induce dopaminergic neuron loss, thus implicating environmental influences as possible factors contributing to PD pathogenesis. The organochlorine pesticide dieldrin has been implicated as risk factor for PD, due to its presence at detectable levels in *post mortem* brain of PD patients, especially in substantia nigra and caudate nuclei tissues (Fleming et al. 1994; Corrigan et al. 1998; Corrigan et al. 2000). Experimental studies have demonstrated the relatively selective toxicity of dieldrin to dopaminergic neurons (Sanchez-Ramos et al. 1998; Kitazawa et al. 2001), and depletion of brain dopamine in animal models treated with dieldrin (Sharma et al. 1976; Heinz et al. 1980). Our initial study demonstrated that α -synuclein overproduction and dieldrin exposure impaired UPS function and sensitized dopamine neuronal cells to apoptotic cell death, suggesting essential pathogenic roles of UPS dysfunction underlying the gene-environment interaction in PD (Sun et al. 2005). In

addition, studies showed that exposure to some commonly used Parkinsonian neurotoxins including MPTP/MPP⁺, rotenone, 6-OHDA, and dopamine compromises UPS function (Sun et al. 2007). Nevertheless, it is unclear whether impaired UPS contributes to dopaminergic degeneration or if it is secondary to other cellular events such as mitochondrial dysfunction and oxidative stress. To date, cellular mechanisms underlying proteolytic stress-induced dopaminergic neurotoxicity consequent to UPS impairment remain to be characterized.

Recent findings demonstrated that proteasome inhibition resulted in mitochondria dysfunction followed by increased mitochondria ROS generation and failure to remove defective mitochondria (Sullivan et al. 2004). In agreement, decreased abundance of several proteins involved in mitochondria function, reduced respiratory capacity, and elevated oxidative stress were revealed in mitochondria from midbrains of Parkin (an E3 ligase) knock-out mice (Palacino et al. 2004). These observations clearly suggest that UPS-mediated proteolysis is essential for maintaining normal mitochondria function. In support of this view, overexpression of Parkin prevents mitochondria swelling and apoptosis cascades, suggesting the presence of putative substrates of UPS in mitochondria (Darios et al. 2003). In addition, the neuroprotective role of Parkin against the challenge of numerous neurotoxicants has been repeatedly and extensively documented. The present studies investigate cellular mechanisms underlying the pathogenic role of ubiquitin-proteasome system (UPS) dysfunction in dopaminergic degeneration by examining ubiquitination-related biochemical abnormalities of mitochondria and its potential involvement in mitochondria-mediated apoptotic cell death in cell culture models of PD.

Background and Literature Review I

Environmental Neurotoxic Chemicals-Induced Ubiquitin Proteasome Dysfunction (UPS) in the Pathogenesis and Progression of Parkinson's Disease

Parkinson's disease (PD), originally documented by James Parkinson in 1817, is the second most common neurodegenerative disorder. The disease affects over one million people in North America (Lang & Lozano, 1998), and four million worldwide (von Bohlen und Halbach et al., 2004). The neuropathological and neurochemical changes of PD are characterized by prominent loss of pigmented dopamine (DA) neurons in the substantia nigra pars compacta region, the presence of intracellular proteinaceous inclusions in the remaining DA neurons, and profound striatal DA depletion. The typical clinical symptoms of PD include bradykinesia, resting tremors, rigidity and postural instability (Dauer & Przedborski, 2003). These major signs manifest when less than 60% of DA neurons remain in SNpc, and greater than 80% loss of striatal DA (Dauer & Przedborski, 2003). Additionally, several autonomic symptoms such as salivation, constipation, loss of smell, bladder disturbances, cardiovascular dysfunction are noted during the very early stages of PD (Magerkurth et al., 2005; Chaudhuri et al., 2006).

Etiology studies involving monozygotic and heterozygotic twins implicated a major role of environmental factors and a minor role of genetic factors in PD pathogenesis (Tanner, 2003; Warner & Schapira, 2003). The etiopathogenesis of PD, which has been researched intensively during the past several decades, is becoming increasingly understood, particularly after the finding that accidental MPTP exposure led to Parkinsonism in humans (Langston & Ballard, 1984). Subsequent studies showed that MPTP consistently produces key signs of PD in mice and nonhuman primates, which yielded critical insight into PD pathogenesis,

including mitochondrial dysfunction, oxidative stress and cell death mechanisms (Dauer & Przedborski, 2003; von Bohlen und Halbach et al., 2004; Bove et al., 2005). Several epidemiological studies showed environmental factors such as rural living, well-water drinking, prolonged pesticide/insecticide exposure and metal exposure increase the risk of developing PD (Di Monte et al., 2002; Lai et al., 2002).

Toxicological studies showed that subacute exposure to the common pesticides rotenone and paraquat induces behavioral and pathological changes characteristic of PD in animal models (Dauer & Przedborski, 2003; von Bohlen und Halbach et al., 2004; Bove et al., 2005; Dinis-Oliveira et al., 2006). The positive association between environmental neurotoxicant exposure and PD indicates the important role of environmental factors in the process of nigrostriatal degeneration in PD. Clinical and toxicological studies will generate needed experimental data to determine the putative causal role of environmental dopaminergic neurotoxins in PD etiopathogenesis.

In addition to environmental factors, increased levels of extrasynaptic dopamine and its auto-oxidation products in striatum could also be detrimental to neurons, as intrastriatal injection of dopamine or repeated methamphetamine (inducer of dopamine release) administration causes degeneration of nigrostriatal projection in rodents (Bozzi & Borrelli, 2006). Due to the structural similarity to dopamine, 6-hydroxyl dopamine (6-OHDA) could be actively absorbed into catecholaminergic neurons through dopamine transporters (DAT) or norepinephrine transporters (NET). 6-OHDA was the first compound identified capable of eliciting selective dopaminergic neurotoxicity, which has been well-characterized in rodents and nonhuman primates (Bove et al., 2005). Currently, MPTP and 6-OHDA models are commonly used for studying PD pathogenesis, or for evaluating neuroprotective agents for PD (Kanthasamy & Kaul, 2006).

During the last 10 years, several gene mutations have been found to be associated with familial PD. The finding of α -synuclein mutation in familial PD has lent more support

for the contribution of genetic factors to PD, especially after the finding that wild type α -synuclein is the major component of Lewy bodies in both familial and sporadic PD. Although less than 10% of PD cases are heritable, genetic information acquired from familial PD has yielded clues to the possible molecular mechanisms of PD pathogenesis. Characterization of the normal function and subcellular localization of the gene products implicates mitochondrial dysfunction/oxidative stress (PINK 1, DJ-1, Parkin, and LRRK2) and ubiquitin proteasome system (UPS) impairment (Uch-L1, Parkin and α -synuclein) as key events associated with PD (Moore et al., 2005). Post-mortem analysis of PD brains and toxin-induced studies in PD models have yielded consistent and explicit evidence supporting the pathogenic role of mitochondria dysfunction, and the resulting oxidative injury, in PD (Bove et al., 2005). Dopamine metabolism (both enzymatic and auto-oxidation) and mitochondrial complex I inhibition represent the two major processes leading to ROS generation in dopaminergic neurons; the promotion of ROS generation by the mitochondrial complex I inhibitors MPP⁺, rotenone, and 6-OHDA has been demonstrated to produce key features of PD *in vitro* and *in vivo* (Shen & Cookson, 2004; Bove et al., 2005). Much attention has been directed toward the role of oxidative stress in PD; however, the importance of UPS dysfunction in the pathogenesis of PD is also gaining recognition (Betarbet et al., 2005). This review summarizes the recent progress with regard to the involvement of UPS impairment in neurotoxin-induced dopaminergic degeneration *in vitro* and *in vivo*.

Ubiquitin proteasome system (UPS): UPS is the principal cellular proteolysis machinery involving ubiquitin, cascades of enzymes for ubiquitination, and the proteolysis complex 26S proteasome (Glickman & Ciechanover, 2002). Numerous proteins participating in a variety of cellular processes, such as the cell cycle, signal transduction, and apoptosis, normally undergo proteolytic degradation in UPS.

Ubiquitin, a highly conserved 76-amino acid protein, contains seven internal lysine residues (Lys 6, 11, 27, 29, 33, 48, and 63). Five out of the seven lysine residues (Lys 6, 11, 29, 48, and 63) are believed to generally serve as a site for polyubiquitin chain extension (Kirkpatrick et al., 2005). However, a study by Peng and coworkers suggests polyubiquitination could occur at all seven internal lysine residues (Peng et al., 2003). Polyubiquitin chains via lys 6 and lys 63 linkage, and monoubiquitin are involved in signaling processes other than proteolysis, such as DNA repair, inflammatory response, protein trafficking and protein translation (Pickart & Fushman, 2004). Polyubiquitin chains

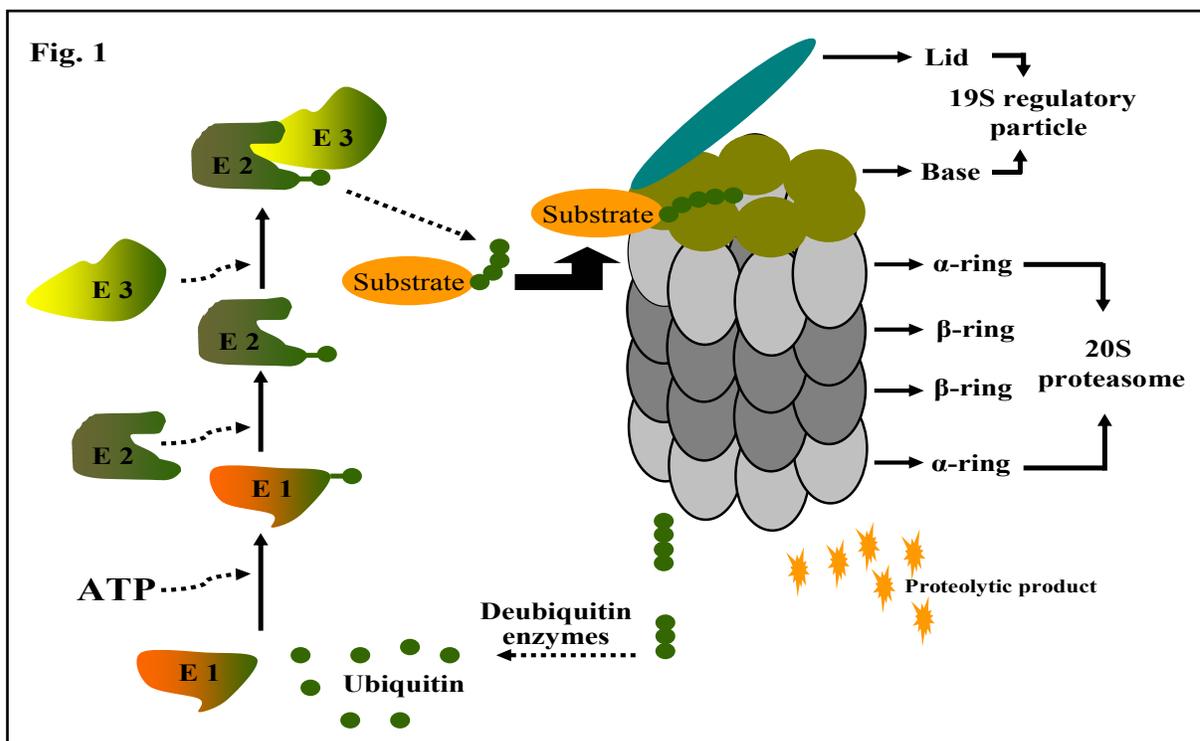


Figure 1: Proteolytic degradation by ubiquitin proteasome system (UPS).

specifying degradation by the proteasome are predominately formed via lysine residue 48 (K48) linkage; these are the most abundant forms of polyubiquitin chains. Some evidence suggests that the polyubiquitin chains of lys 11 and lys 29 linkage could also target proteins

for proteasome degradation (Pickart & Fushman, 2004). Ubiquitination and polyubiquitination involve activation and transfer of ubiquitin to targets, in which a cascade of enzymes, E1 (ubiquitin activating enzyme, Uba), E2 (ubiquitin conjugating enzyme, Ubc), and E3 (ubiquitin ligase), are essential for the formation of the isopeptidyl bond between the carboxyl group of glycine 76 of ubiquitin and the ϵ -amino group of an internal lysine residue (Glickman & Ciechanover, 2002; Passmore & Barford, 2004; Pickart & Eddins, 2004). Bioinformatic analysis of the human genome indicated that human cells have more than 40 E2 and 500 E3s (Sun & Chen, 2004). E3s are characterized by the presence of either a HECT (Homologous to E6-AP Carboxy Terminus) domain or RING (Real Interesting New Gene) finger. Parkin, a recessive gene mutation identified in early onset PD, belongs to E3 ligase of the RING domain family (Shimura et al., 2000; Zhang et al., 2000).

Tagging target proteins with K48 polyubiquitin is essential for their recognition and degradation by the 26S proteasome complex (Fig.1). The 26S complex is made up of the functionally and structurally distinct 20S core particle and 19S regulatory particle (also called proteasome activator 700, PA 700). The proteasome 20S core particle contains two inner β -rings and two outer α -rings. In mammalian cells, the inner β -ring possessing proteolytic activities consists of seven homologous β subunits (β 1- β 7). Five major types of proteolytic activities of the proteasome are predicted based on its cleavage of chromogenic substrates: chymotrypsin-like (C-L), trypsin-like (T-L), peptidyl-glutamyl peptide-hydrolyse (PGPH), branched chain amino acid-preferring (BrAAP), and small neutral amino acid-preferring (SNAAP) activity (Groll & Huber, 2004). Seven homologous subunits α 1- α 7 comprise the outer α -ring, which associates with the base of the 19S regulatory particle. Six homologous ATPase (Rpt1-6) and three non-ATPase subunits (Rpn1, 2 and 10) constitute the base of the 19S regulatory core. The lid of 19S regulatory core is made of eight non-ATPase subunits, Rpn 3, 5-9, 11, and 12 (Glickman & Ciechanover, 2002). Rpn 10 (S5a, its human counterpart) has high affinity for poly-ubiquitinated proteins, and is essential for recognition

and degradation by the 26S proteasome, which mediates ATP- and ubiquitin-dependent degradation (Hartmann-Petersen & Gordon, 2004). In addition, the 20S proteasome could associate with ATP-independent proteasome activator 28 (PA 28 or 11 S) at one or both ends, and the binding of PA 28 could exponentially increase the speed of proteolysis of the 20S proteasome. The biological role of the 20S-PA 28 complex, which likely mediates ubiquitin-independent degradation, including the removal of oxidatively modified proteins (Davies, 2001), is not as well understood as 26S proteasome (Rechsteiner & Hill, 2005).

UPS dysfunction in PD: As is the common defining feature of several neurodegenerative diseases, aggregation of misfolded proteins during neurodegeneration is evocative of deficient protein processing and degradation, although the relevance of protein aggregation to neuronal death or survival is still uncertain. As a defining pathology of PD, cytoplasmic protein aggregates, known as Lewy bodies, contain a variety of proteins including α -synuclein, ubiquitin, proteasome subunits, chaperone proteins, and neurofilament proteins (von Bohlen und Halbach et al., 2004). Accumulation of the ubiquitinated proteins in Lewy bodies is indicative of incomplete clearance of the target proteins by UPS. In line with the idea of defective UPS in PD pathogenesis, *post mortem* analysis of sporadic PD brain samples demonstrated relatively low proteasomal activities in the substantia nigra region (McNaught et al., 2003). The reduction in proteasome activity in this region may be attributed to the profound loss of 20S proteasome core components, and proteasome activators PA700 and PA28 (McNaught et al., 2002a).

Association of several genes involved in the UPS degradation pathway with familial PD has provided compelling evidence for possible involvement of defective UPS in PD. Mutation of Parkin, a RING domain E3 ligase, has been suggested to account for 50% of autosomal-recessive early onset PD (Jain et al., 2005). Physical interaction between Parkin and the 26S proteasome subunit Rpn 10 (S5a), which is required for the proteasomal

recognition of polyubiquitin chain tagged substrates, supports the idea that Parkin plays a role in UPS degradation (Sakata et al., 2003). The accumulation of Parkin-specific substrates, as a consequence of the loss of E3 ligase activity in Parkin mutants, might underlie the neurotoxicity of the Parkin mutant to dopaminergic neurons. Putative Parkin substrates include α -synuclein (Shimura et al., 2001), synphilin-1 (Chung et al., 2001), Pael-R (Yang et al., 2003), p38/JVT-1 (Corti et al., 2003; Ko et al., 2005), α/β tubulin (Ren et al., 2003), CDCrel-1 (Zhang et al., 2000), synaptotagmin IX (Huynh et al., 2003), and far upstream binding protein 1 (Ko et al., 2006). Accumulation of these protein substrates may very well promote protein aggregation, which is a hallmark of UPS dysfunction in the CNS.

Transgenic and knockout animal models have revealed the functional consequences of specific mutations in PD. Recent studies showed that nigral dopamine neurons are well-preserved in Parkin knockout (KO) mouse and *Drosophila* models, but these animals suffer from some neurochemical, mitochondrial and behavioral deficits relevant to PD pathogenesis (Goldberg et al., 2003; Palacino et al., 2004). However others have failed to reproduce the similar neurochemical and behavioral phenotype in mice, suggesting that Parkin knockout may not be an ideal model for future PD etiopathological studies in mice (Perez & Palmiter, 2005). The exact reason for this discrepancy between studies is not clear, but perhaps the noticeable neuronal cell death may occur during the late stages of life in Parkin KO animals (Shen & Cookson, 2004). This suggestion may explain the observed reduction in dopamine uptake in striatum and midbrain regions without nigral degeneration in asymptomatic human Parkin heterozygotes (Khan et al., 2005).

Typically, Parkin increases neuronal resistance to a variety of neuronal stimuli including dopamine (Jiang et al., 2004), proteasome inhibitor (Chung et al., 2004), mitochondrial inhibitors (Darios et al., 2003), 6-OHDA (Darios et al., 2003) α -synucleinopathies (Petrucci et al., 2002; Oluwatosin-Chigbu et al., 2003; Haywood & Staveley, 2004; Lo Bianco et al., 2004; Yamada et al., 2005; Haywood & Staveley, 2006),

tauopathies (Klein et al., 2006), overexpression of Pael-R (Yang et al., 2003), p38/JVT-1 (Ko et al., 2005), and manganese (Higashi et al., 2004). Altered degradation (Wang et al., 2005) or nitrosylation of Parkin (Chung et al., 2004; Yao et al., 2004) likely contributes to nigrostriatal dopaminergic degeneration in sporadic PD by neutralizing Parkin's neuroprotective function.

Among the four ubiquitin carboxyl-terminal hydrolases (Uch-L1-4) found, Uch-L1 actually possesses both deubiquitin and ligase activities (Liu et al., 2002) and is neuron-specific (Nishikawa et al., 2003). Mutation of Uch-L1 was recently discovered in a rare case of familial PD (Leroy et al., 1998). The relevance of Uch-L1 mediated ubiquitination/deubiquitination or its mutation to dopaminergic degeneration is still not clear. There is evidence that hydrolase activity of Uch-L1 likely confers neuronal resistance, and the mutation diminishing the hydrolase activity may be a risk factor for PD development (Leroy et al., 1998; Nishikawa et al., 2003). The disease-related Uch-L1 mutant has been shown to render cells more prone to formation of aggresomes in response to proteasome inhibitor treatment (Ardley et al., 2004). Oxidation of Uch-L1, which is seen in PD brains, has been shown to cause the reduction of its hydrolase activity (Nishikawa et al., 2003; Choi et al., 2004).

α -Synuclein is thought to have a significant role in PD pathophysiology. In sporadic PD, fibrillar α -synuclein is the major structural component of Lewy bodies. Additionally, α -synuclein mutations, including gene locus triplication, A30P, A53T, and E46K, have been found in some familial PD cases (Bennett, 2005). Studies attempting to investigate synucleinopathies using transgenic animals or virus-mediated overexpression revealed significant variability in reproduction of the pathological and behavioral deficits characteristic of PD, and none of the models actually shows the nigral neuronal loss (Maries et al., 2003; Fernagut & Chesselet, 2004). Variation in the α -synuclein levels likely underlies the observed variability (Maries et al., 2003; Fernagut & Chesselet, 2004), or possibly

additional neurotoxic challenge is necessary for α -synuclein to induce nigrostriatal degeneration (Fernagut & Chesselet, 2004). The critical role of α -synuclein in PD physiopathology was also underscored by α -synuclein upregulation in MPTP animal models (Purisai et al., 2005) and the increased resistance of nigral dopamine neurons to MPTP in α -synuclein knockout mice (Dauer et al., 2002; Klivenyi et al., 2006). The neurotoxicity of α -synuclein is now generally believed to be partially attributed to its inhibition of proteasome activities. Proteasome inhibition by wild-type, and more effectively by oxidated-, mutant- or oligomeric- α -synuclein has been demonstrated in cell-free systems, cell cultures and animal models (Ghee et al., 2000; Tanaka et al., 2001; Snyder et al., 2003; Lindersson et al., 2004; Chen et al., 2005; Cole et al., 2005; Chen et al., 2006). Other possible interpretations of the neurotoxicity of α -synuclein include loss of function due to mutation or oligomerization, fibrillation (Sidhu et al., 2004), or increased penetration of the cell membrane by the oligomerized α -synuclein (Volles et al., 2001; Volles & Lansbury, 2002; Furukawa et al., 2006). Recently, we showed that overexpression of α -synuclein in a nigral dopaminergic cell line reduced proteasome activity (chymotrypsin-like) and protected against chemical-induced neurotoxicity up to 12hr, and then exacerbated the neurotoxic response (Sun et al., 2005).

Etiological agents involved in UPS impairment in PD: Although less than 10% of PD cases are heritable, genetic information acquired from familial PD cases has yielded clues to understanding the possible molecular mechanisms of PD pathogenesis. The identified PD genes implicate defective UPS, mitochondrial dysfunction and oxidative stress in PD pathogenesis. Mitochondrial deficit and oxidative injury have consistently been demonstrated as key features in PD pathogenesis by *post mortem* analysis of PD brains and experimental studies utilizing dopaminergic toxins. Since PD is primarily a sporadic disorder, the predominant etiological roles of dopaminergic neurotoxins and UPS dysfunction must be elucidated. The following section summarizes the current knowledge

regarding the effects of putative dopaminergic neurotoxins on UPS components and/or function, and how proteasome inhibition alters neuronal vulnerability to the toxins.

MPP⁺ and MPTP: The critical importance of α -synuclein in the pathogenesis of PD is underscored by its involvement in both familial and sporadic PD, and its upregulation of expression in response to the classical dopaminergic neurotoxin MPP⁺/MPTP challenge *in vitro* and *in vivo* (Gomez-Santos et al., 2002; Kalivendi et al., 2004; Purisai et al., 2005). α -Synuclein upregulation has been shown to be associated with exacerbated mitochondrial pathology in MPTP-challenged α -synuclein transgenic mice (Song et al., 2004). Knockdown or knockout of α -synuclein in rodents confers resistance to neurotoxicity of MPTP (Dauer et al., 2002; Drolet et al., 2004; Hayashita-Kinoh et al., 2006; Klivenyi et al., 2006). It remains to be determined whether modulation of neuronal vulnerability by α -synuclein is related to its capacity for proteasome inhibition (Moore et al., 2005). Although conventional dosing of animals with systemic administration of MPTP fails to produce Lewy body-like pathology, continuous MPTP infusion via minipump has recently been shown to be more effective at producing UPS dysfunction and protein aggregation. However, proteasome inhibition and nigrostriatal degeneration are markedly attenuated in α -synuclein knockout mice (Fornai et al., 2005), implying that proteasome inhibition mediated by α -synuclein may be important in MPTP neurotoxicity. It is important to recognize that high abundance of α -synuclein at neuronal synapses suggests an important physiological function of this protein in CNS (Lotharius & Brundin, 2002).

Experimental evidence indicates α -synuclein may play a role in synaptic plasticity, neurotransmission, and neuroprotection (Di Rosa et al., 2003; Sidhu et al., 2004). We showed that overexpression of α -synuclein protects against Parkinsonian toxin MPP⁺-induced apoptotic cell death by suppressing the proteolytic activation of the proapoptotic kinase PKC δ in dopaminergic cells (Kanthasamy et al., 2003; Kaul et al., 2005). Additionally, our recent study demonstrated that overexpression of α -synuclein in a nigral dopaminergic cell

line reduced proteasome activity (chymotrypsin-like) and enhanced chemical-induced neurotoxicity, which exacerbated the neurotoxic response (Sun et al., 2005). In general, α -synuclein appears to be protective against neurotoxic responses of environmental toxicants at the early stages of exposures, but the toxicity is exacerbated when α -synuclein loses its protective function.

To examine the intrinsic link between mitochondria dysfunction and proteasome inhibition, Hoglinger and colleagues demonstrated that exposure to MPP⁺ leads to the reduction of hydrolase activities of the proteasome in rat primary mesencephalic culture via ATP depletion (Hoglinger et al., 2003). However, a similar MPP⁺ treatment paradigm by Sawada and coworkers yielded opposite results with regard to proteasomal activity changes (Sawada et al., 2004). Further detailed analysis is necessary to resolve these discrepancies. Possibly, upregulation of UPS function could occur adaptively as a stress response to restore cellular homeostasis by eliminating the oxidized or misfolded proteins. Suppression of proteasomal degradation could conceivably exacerbate neurotoxicity of MPP⁺ due to the accumulation of damaged proteins, as previously reported (Hoglinger et al., 2003). A recent study showed that MPTP exposure leads to both functional and structural alterations of proteasome in nonhuman primates, similar to effects observed in sporadic PD (Zeng et al., 2006).

A decrease in the availability of soluble Parkin has been linked to increased risk of PD (Wang et al., 2005). Although soluble Parkin could shift to the insoluble form as a consequence of aging (Pawlyk et al., 2003), the process could be precipitated by neurotoxin insults, such as MPP⁺, which cause the reduction in the soluble fraction of Parkin, elevation in the insoluble fraction, and promotion of Parkin aggregation (Wang et al., 2005). It is still unclear whether altering the solubility of Parkin is related to its S-nitrosylation, which has been seen in PD brains and MPTP mouse models. Both solubility alteration and S-

nitrosylation could compromise the possible neuroprotective function of Parkin (Chung et al., 2004; Yao et al., 2004).

Rotenone: Mitochondrial inhibition by rotenone leads to suppression of proteasomal activity through ATP depletion, as in the case of MPP⁺ (Hoglinger et al., 2003). However, if the cellular energy status is partially maintained, cells retain the capacity to upregulate proteasomal activity as a defense mechanism to deal with cellular stress caused by prolonged rotenone exposure. Increases in proteasomal activity were found to be concurrent with elevation of ubiquitinated proteins and ROS generation (Zeevalk & Bernard, 2005). Proteasome function could vary adaptively as a secondary response to the altered cellular redox status. However, increases in 4-hydroxy-2-nonenal (HNE)-modification of cellular proteins, including the proteasome itself, impairs proteasome function during oxidative stress (Okada et al., 1999). As a result of mitochondrial inhibition by rotenone, ROS production could lead to oxidative modification of the β -subunits of the proteasome, thus affecting its proteolytic capacity (Shamoto-Nagai et al., 2003), and potentially leading to protein aggregations.

Along with the ATPase subunit of 19S regulatory particles, Rpt6 (S6') is one of the major intracellular targets for ROS attack, and its oxidation diminishes its ATPase activity and lessens the capacity of the 26S proteasome to remove ubiquitinated substrates (Ishii et al., 2005). Exposure to rotenone (10 nM to 10 μ M) for 24 hr results in significant and dose-dependent reductions in proteasomal activity in SK-N-MC neuroblastoma cells; persistent suppression of proteasome activity was also observed following prolonged rotenone exposure (Wang et al., 2006). A recent study showed that chronic rotenone exposure causes selective nigrostriatal degeneration concurrent with dysfunctional UPS, DJ-1 oxidation, and α -synuclein accumulation *in vivo* (Betarbet et al. 2006).

Despite the association of UPS alterations with neurotoxicity of rotenone as described, addressing whether the UPS deficit represents the secondary consequence of rotenone

toxicity, or if it actively participates in rotenone-induced dopaminergic degeneration is experimentally difficult. Overexpression of α -synuclein has an inhibitory effect on proteasome degradation; overexpression also increases the vulnerability of dopaminergic neurons in *C. elegans* to rotenone (Ved et al., 2005). The E3 ligase activity of Parkin is central to its neuroprotection against a diversity of neuronal insults including rotenone (Wang et al., 2005), suggesting a pathogenic role of defective UPS in rotenone-induced neuronal cell death (Darios et al., 2003). Also, mitochondrial-localized Parkin likely harbors the neuroprotective function against neurotoxicity elicited through induction of mitochondria dysfunction, since overexpression of Parkin confers resistance to a broad range of neurotoxins (Darios et al., 2003; Wang et al., 2005). Similar to MPP⁺/MPTP, rotenone also induces solubility changes (Wang et al., 2005) and S-nitrosylation of Parkin (Yao et al., 2004), both of which diminish the ligase activity of Parkin. Loss of Parkin function increases the susceptibility of mice to rotenone-induced nigral dopaminergic neuronal death, which might involve microglia activation (Casarejos et al., 2006). Despite the known neuroprotective action of Parkin protein, Parkin knockout mice show only minor behavioral and neurochemical defects, and no apparent nigral dopaminergic neuronal loss (Goldberg et al., 2003). The mechanism underlying this paradoxical finding is not entirely clear. Some compensatory responses in Parkin knockout animals may compensate for the protective function of Parkin.

Paraquat: Case control and epidemiological studies indicate that paraquat could be an environmental neurotoxin associated with increased risk of developing PD (Bove et al., 2005; Dinis-Oliveira et al., 2006). CNS accumulation of paraquat had been debated due to the impermeability of the blood-brain barrier to paraquat. However, active CNS uptake of paraquat has been demonstrated via a dopamine transporter (Shimizu et al., 2001) or L-neutral amino acid transporters (McCormack & Di Monte, 2003). Systemic administration of paraquat results in nigrostriatal degeneration (Brooks et al., 1999; McCormack et al., 2002),

accompanied by the upregulation and aggregation of α -synuclein *in vivo* (Manning-Bog et al., 2002). *In vitro* incubation of α -synuclein with paraquat expedites the fibrillation process (Uversky et al., 2001b), though it is unknown how this is related to altered vulnerability of nigral neurons to paraquat, as α -synuclein transgenic mice studies have yielded inconsistent outcomes (Manning-Bog et al., 2003; Thiruchelvam et al., 2004). Inhibition of proteasome activity by paraquat has been reported in SH-SY5Y neuroblastoma cells (Ding & Keller, 2001b). The paraquat-induced proteasome inhibition apparently contributes to oxidative stress-related neuronal cell death, since upregulation of proteasome function induced by transient exposure to low dose proteasome inhibitors renders cells more resistant to the neurotoxicity of paraquat (Lee et al., 2004). Conversion of soluble and functionally-competent Parkin into inactive and insoluble Parkin seems to occur in response to a broad-spectrum of neuronal insults, including paraquat, implying that the loss of Parkin function may also contribute to paraquat-induced nigral neuronal death (Wang et al., 2005).

Dopamine: Although depletion of striatal dopamine represents the key neurochemical feature of PD, neurotoxicity of dopamine, which is elicited via oxidative stress, and formation of metabolic intermediates such as neuromelanin and dopamine-o-quinone during the process of dopamine metabolism, have been linked to the pathogenesis of PD (Jimenez del Rio & Velez-Pardo, 2000; Barzilai et al., 2001; Asanuma et al., 2004). The interaction between α -synuclein and dopamine appears to be very important and complicated in PD pathogenesis; α -synuclein appears to participate in maintaining dopamine homeostasis, as observed by its roles in regulation of TH activity (Perez et al., 2002), vesicle storage and release of dopamine (Abeliovich et al., 2000; Cabin et al., 2002; Lotharius & Brundin, 2002; Yavich et al., 2004), dopamine uptake by DAT (Wersinger & Sidhu, 2003) and the vesicular dopamine transporter VMAT2 (Lotharius et al., 2002). Cell-free *in vitro* studies have yielded crucial insight into the involvement of dopamine in synucleinopathies. Covalent modification of α -synuclein by metabolites of dopamine and other catecholamines inhibits α -

synuclein fibrillation (Conway et al., 2001), and facilitates its oligomerization process (Cappai et al., 2005), thus favoring its presence in the soluble, toxic oligomer form. Destabilization and breakdown of α -synuclein fibrils by dopamine may be partially responsible for its preference for the oligomer form rather than the fibrillar form (Li et al., 2004). A recent study indicated that interaction between C-terminal amino acid residues 125-129 of α -synuclein and the dopamine metabolite dopaminochrome possibly explains the altered α -synuclein kinetics in the presence of dopamine (Norris et al., 2005).

A study by Keller and colleagues demonstrated that elevated extracellular dopamine levels cause proteasome inhibition, which could be alleviated by suppressing dopamine uptake or synthesis (Keller et al., 2000), suggesting that excess intracellular free dopamine levels adversely affect proteasome function. To further support this idea, a pharmacological inhibitor of tyrosine hydroxylase (the rate-limiting enzyme for dopamine synthesis) upregulates proteasome activity in PC12 cells (Yoshimoto et al., 2005), though the pathophysiological relevance of proteasome modulation by dopamine remains to be determined. Proteasome inhibition by dopamine might contribute to its effects in several areas: 1) Elevation of the α -synuclein protofibril level in the presence of dopamine likely potentiates proteasome inhibition by α -synuclein, as the oligomeric α -synuclein is a more effective inhibitor than the monomeric form (Snyder et al., 2003; Lindersson et al., 2004). 2) Dopamine-derived ROS compromises proteasome function, which could be alleviated by antioxidants (Keller et al., 2000). 3) The dopamine oxidation product aminochrome is also among the candidates exerting strong inhibitory effects on the proteasome in dopaminergic neurons (Zafar et al., 2006). 4) Neuromelanin, which is synthesized from dopamine metabolites, interferes with the proteolytic capacity of the 26S proteasome by depleting structural component of the 19S regulatory particles, likely via promotion of ion-mediated oxidative stress (Shamoto-Nagai et al., 2006).

Given the neuroprotective role of Parkin against neurotoxicity produced by a range of dopaminergic neurotoxins, Parkin may also play an essential role in dopamine detoxification within dopamine-producing neurons. This concept was substantiated by the observation that Parkin suppresses dopamine-induced apoptosis in human neuroblastoma cells (Jiang et al., 2004), and that nigral expression of the Parkin substrate CDCrel-1 causes nigral degeneration, depending on dopamine synthesis (Dong et al., 2003). However, the neuroprotection conferred by Parkin against dopamine neurotoxicity might be at the expense of Parkin, since dopamine facilitates the conversion of soluble Parkin into the insoluble form, as detected in PD brains (Wang et al., 2005). A recent study by LaVoie and coworkers revealed that insolubility, oligomerization, and functional inactivation of Parkin are concurrent with dopamine-related covalent modification of Parkin, implying that Parkin inactivation either by endogenous dopamine, or due to gene mutation, represents the unifying mechanism mediating the selective nigral dopamine neuronal death in sporadic and familial PD (LaVoie et al., 2005).

6-OHDA: Severe oxidative stress following 6-OHDA exposure could mediate proteasome failure, as in the case of dopamine, and cells could actively promote degradation in response to mild oxidative stress in order to remove oxidatively damaged proteins, as manifested by elevated proteasome activity (Hoglinger et al., 2003; Elkon et al., 2004) and increased ubiquitinated proteins (Elkon et al., 2001). Inhibition of the increase in proteasome function potentiates the neurotoxicity of 6-OHDA, presumably by abolishing UPS-related detoxification (Hoglinger et al., 2003). A study with 6-OHDA-induced rat models of PD revealed the association of nigrostriatal degeneration with ubiquitin upregulation in 6-OHDA-injected striatum, indicating the involvement of UPS in the nigrostriatal degeneration process (Pierson et al., 2005). As previously described, alteration in Parkin solubility occurs in response to a variety of stimuli, including 6-OHDA, suggesting that common mechanisms might be responsible for the change in Parkin (Wang et al., 2005). Although neuroprotection

against the dopaminergic neurotoxicity of 6-OHDA by exogenously introduced Parkin has been well documented in *in vitro* and *in vivo* studies (Darios et al., 2003; Jiang et al., 2004; Vercammen et al., 2006), the neuroprotective role of endogenous Parkin at the physiological level following 6-OHDA challenge remains unknown. In fact, Parkin inactivation does not increase susceptibility of nigral neurons to 6-OHDA treatment (Perez et al., 2005).

Metals: Ferrous/ferric iron and manganese: Epidemiological, case control and *post mortem* studies have suggested the possible involvement of heavy metals in the etiology of PD (Uversky et al., 2001a). Of the metals suspected to be involved in development of PD, iron attracts more attention than others; numerous studies have indicated that overload of nigral iron is involved in PD pathophysiology (Berg et al., 2001; Wolozin & Golts, 2002; Kaur & Andersen, 2004). The pathogenic roles of iron in PD have been researched intensively, with particular focus on its involvement in synucleinopathies and oxidative stress. Fe (III) and Fe (III)-related ROS have been shown to induce α -synuclein aggregation *in vitro* (Hashimoto et al., 1999; Golts et al., 2002) and in cell cultures (Ostrerova-Golts et al., 2000).

In a systematic analysis of several metals, Uversky and colleagues showed that incubation with metals such as aluminum (III), copper (II), iron (III), cobalt (III), and manganese (II) markedly promoted the conformational change and fibrillation of α -synuclein in cell free systems (Uversky et al., 2001a). Oxidation of α -synuclein catalyzed by transition metals Fe (III) or Cu (II) favors the formation of the α -synuclein oligomer, which has an inhibitory effect on proteasome (Cole et al., 2005). In addition, iron-mediated ROS (Shamoto-Nagai et al., 2006) also contribute to proteasomal inhibition following Fe (II) exposure in cell culture (Ding & Keller, 2001b; Lev et al., 2006; Shamoto-Nagai et al., 2006), whereas Fe (II, III) exposure appears to increase 20S proteasomal activity in cell-free *in vitro* systems (Amici et al., 2002). The negative effect of Fe (II) on UPS function is also illustrated by the decrease in Parkin solubility in the presence of Fe (II) (Wang et al., 2005).

Occupational and clinical studies have suggested that manganese exposure also causes PD-like syndromes, although brain lesions caused by Mn accumulation are mostly confined to the globus pallidus, instead of the nigrostriatal system as in PD (Olanow, 2004; Jain et al., 2005). *In vitro* incubation of α -synuclein with Mn enhances the formation and stabilization of α -synuclein intermediates with partially folded conformation (Uversky et al., 2001a; Andre et al., 2005); the interaction between α -synuclein and manganese may be important for the neurotoxicity of manganese, since α -synuclein overexpression renders cells more susceptible to manganese-triggered cell death (Piffl et al., 2004). As the major component of the pesticide Maneb, which is linked experimentally to nigral degeneration, manganese ethylene-*bis*-dithiocarbamate triggers cytotoxicity in mesencephalic neuronal cells, concurrently with α -synuclein aggregation and reduction of proteasomal activity (Zhou et al., 2004). Rescue of dopaminergic neuronal cells from manganese cytotoxicity by Parkin suggests the causal association between impaired UPS degradation and manganese-induced cell death, even without significant change in the proteasomal peptidase activity (Higashi et al., 2004).

Dieldrin and other pesticides: With regard to pesticides and PD etiology, the organochlorine pesticide dieldrin, in addition to rotenone, paraquat and Maneb, is of particular concern based on *post mortem* analysis of PD brains and epidemiological and experimental studies (Kanthasamy et al., 2005). Higher levels of dieldrin were detected in many PD brains as compared to control brains (Fleming et al., 1994; Corrigan et al., 2000; Kanthasamy et al., 2005). Dieldrin is selectively neurotoxic to dopamine neurons in primary mesencephalic culture (Sanchez-Ramos et al., 1998) and to dopaminergic cells (Kitazawa et al., 2001). Previous studies by our lab showed that dopamine oxidation may play a role in the increased sensitivity of dopaminergic cells to dieldrin toxicity (Kitazawa et al., 2001), and that proteolytic activation of protein kinase C delta (PKC δ), an oxidative stress-sensitive kinase, contributes to the neurotoxicity of dieldrin (Kanthasamy et al., 2003; Kitazawa et al.,

2003). Dieldrin also negatively affects the proteasomal activity, with significant loss of chymotrypsin-like proteasomal activity and profound α -synuclein aggregation; as observed in dopaminergic cells after exposure to sublethal concentrations of dieldrin. The dieldrin-induced UPS dysfunction appears to contribute to apoptotic cell death in dopaminergic cells, since cumulative proteasomal inhibition by the interaction between α -synuclein and dieldrin exacerbates UPS dysfunction and the neurotoxicity of dieldrin (Sun et al., 2005). α -Synuclein oligomerization facilitated by dieldrin likely contributes to sensitization of the neuronal cells to dieldrin-induced UPS deficit and cell death (Uversky et al., 2001b). A study by Wang and coworkers showed that exposure to six of 25 pesticides examined, including rotenone, ziram, diethyldithiocarbamate, endosulfan, benomyl, and dieldrin, caused decreases in proteasomal activity at nM to μ M concentrations in cell culture. However, none of the six pesticides had an inhibitory effect on the peptidase activity of isolated 20S proteasome (Wang et al., 2006), which implies that some common cellular events mediate proteasomal inhibition resulting from pesticide exposure.

Table 1. Summary of key findings regarding the effects of environmental toxins on UPS

Neurotoxins	Key findings and model systems
MPTP	α -Syn \uparrow (nonhuman primates) ^{a1} ; PA \downarrow (mice, non-human primates) ^{a2,3} ; Susceptibility \downarrow in α -Syn KO (Mice) ^{a4, 5, 6} ; Susceptibility \uparrow in Tg α -Syn (Mice) ^{a7} ; Parkin nitrosylation \uparrow (mice) ^{a8, 9}
MPP ⁺	α -Syn \uparrow (NB) ^{b1, 2} ; Parkin solubility \downarrow (NB, mice) ^{b3,1} ; PA \downarrow (MC) ^{b4 m} or PA \uparrow (MC) ^{b5}
Rotenone	PA \downarrow (MC, mice) ^{b4 .c1} or PA \uparrow with ubiquitin conjugates \uparrow (MC) ^{c2} ; Proteasome oxidation (NB) ^{c3} ; Parkin nitrosylation \uparrow (mice) ^{a8,9} ; Parkin solubility \downarrow (NB) ^{b3} ; Susceptibility in Parkin KO \uparrow (mice) ^{c4} ; α -Syn \uparrow (mice) ^{c1}
Paraquat	PA \downarrow (NB) ^{d1} ; Protection by increased PA (neocortical neurons) ^{d2} ; Parkin solubility \downarrow (NB, mice) ^{b3}
Dopamine	α -Syn fibrillation \downarrow (CFiv) ^{e1} & oligomerization \uparrow (CFiv) ^{e2} ; α -Syn fibril breakdown \uparrow (CFiv) ^{e3} ; PA \downarrow (PC) ^{e4, 5} ; Parkin solubility \downarrow (NB) ^{b3} ; Parkin modification & inactivation (neuronal cells) ^{e6} ; Protection by Parkin overexpression (NB) ^{e7}
6-OHDA	PA \downarrow (PC) ^{f1} or PA \uparrow (MC or PC) ^{b4, f1} with ubiquitin conjugates \uparrow (PC) ^{f2} ; Ubiquitin \uparrow (rat) ^{f3} ; Parkin solubility \downarrow (NB, mice) ^{b3} ; Protection by Parkin (NB, PC, rat) ^{e7, f4, 5} ; No change in susceptibility in Parkin KO (mice) ^{f6}
Irons	Fibrillation (CFiv) ^{g1} ; Oligomerization, Oxidation of α -Syn and resulting PA \downarrow (CFiv) ^{g2} ; PA \downarrow (NB) ^{d1, g3, 4} ; Parkin solubility \downarrow (NB) ^{b3}
Manganese	Increased susceptibility by α -Syn overexpression (NB) ^{h1} ; PA no change (NB) ^{h2} or decrease (NB) ^{h3} ; Protection by Parkin (NB) ^{h2}
Dieldrin	PA \downarrow (neuronal cells) ^{i1,2} ; ubiquitin conjugates \uparrow & \uparrow susceptibility by α -Syn overexpression (Neuronal cells) ⁱ¹

Note: α -Syn: α -synuclein; PA: proteasomal activity; KO: Knockout; Tg: Transgenic; NB: Neuroblastoma; MC: Mesencephalic culture; CFiv: Cell-free *in vitro*; PC: PC12 cells; \uparrow : Increase; \downarrow : Decrease; ^{a1}Purisai, et al., 2005; ^{a2}Fornai, et al., 2005; ^{a3}Zeng et al., 2006; ^{a4}Dauer et al., 2002; ^{a5}Drolet et al., 2004; ^{a6}Klivenyi, et al., 2006; ^{a7}Song et al., 2004; ^{a8}Chung et al., 2004; ^{a9}Yao et al., 2004; ^{b1}Gomez-Santos et al.; ^{b2}Kalivendi et al., 2004; ^{b3}Wang et al., 2005; ^{b4}Hoglinger et al., 2003; ^{b5}Sawada et al., 2004; ^{c1}Betarbet et al., 2006; ^{c2}Zeewalk and Bernard 2005; ^{c3}Shamoto-Nagai et al., 2003; ^{c4}Casarejos et al., 2006; ^{d1}Ding and Keller, 2001; ^{d2}Lee et al., 2004; ^{e1}Conway et al., 2001; ^{e2}Cappai et al., 2005; ^{e3}Li et al., 2004; ^{e4}Keller et al., 2000; ^{e5}Yoshimoto et al., 2005; ^{e6}Lavoie et al., 2005; ^{e7}Jiang et al., 2004; ^{f1}Elkon et al., 2004; ^{f2}Elkon et al., 2001; ^{f3}Pierson et al., 2005; ^{f4}Darios et al., 2003; ^{f5}Vercammen et al., 2006; ^{f6}Perez et al., 2005; ^{g1}Uversky et al., 2001a; ^{g2}Cole et al., 2005; ^{g3}Lev et al., 2006; ^{g4}Shamoto-Nagai et al., 2006; ^{h1}Piffl et al., 2004; ^{h2}Higashi et al., 2004; ^{h3}Zhou et al., 2004; ⁱ¹Sun et al., 2005; ⁱ²Wang et al., 2006

Fig. 2

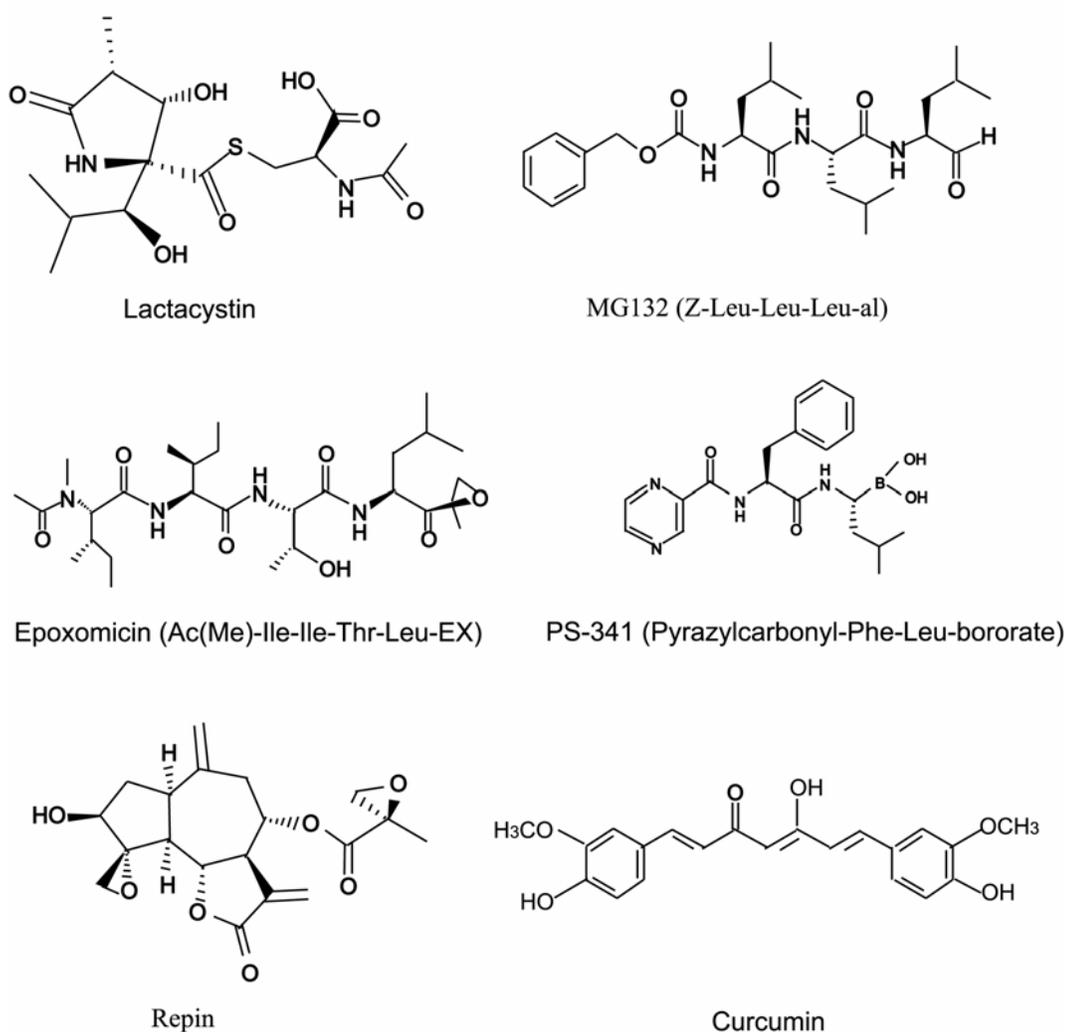


Figure 2: The chemical structures of repin, curcumin and several proteasome inhibitors: lactacystin, MG132, epoxomicin, and S-341.

Exposure to environmental proteasome inhibitors as risk factor for PD. Proteasome inhibitors have been used as an important class of anticancer drugs. The promising potential of UPS as the target for drug development in cancer has evoked unprecedented enthusiasm in the search for proteasome inhibitors of natural origin or synthetic analogs (Kisselev & Goldberg, 2001; Tsukamoto & Yokosawa, 2006). The proteasome inhibitor bortezomib (PS-341) has recently been approved by the FDA for multiple myeloma treatment, while some other inhibitors, such as *clasto*-lactacystin β -lactone, have entered clinical trials (Voorhees & Orłowski, 2006). Diverse synthetic proteasome inhibitors, which structurally fall into different categories: peptide aldehyde, peptide boronates, and peptide vinyl sulfones, have been generated for biomedical and biochemical research (Myung et al., 2001). Meanwhile, an increasing number of proteasome inhibitors have been derived from natural sources such as bacteria, fungi and plants. Lactacystin, epoxomicin and belactosin (Fig. 2) are examples of proteasome inhibitors produced by bacteria of the *Actinomycetes* family (Myung et al., 2001; Tsukamoto & Yokosawa, 2006). These bacteria are universally present in soil, and some species can infect the soil-associated parts of plants. The fungus *Apiospora montagnei*, the source of proteasome inhibitors TMC-95A, B, C and D (Kisselev & Goldberg, 2001; Groll & Huber, 2004), has been shown to infect wheat (Koguchi et al., 2000) and green tea plants (Nam et al., 2001). A long list of natural compounds capable of inhibiting either proteasomal activity or ubiquitin-activating enzyme (E1), ubiquitin ligases (E3) or deubiquitinating enzymes have been isolated from diverse sources (Tsukamoto & Yokosawa, 2006). The structural profile of the natural compounds provides important clues for synthesis of new chemical entities sharing a similar active pharmacophore.

The increased susceptibility of dopaminergic neurons to neurotoxic effects of proteasome inhibitors raises great concern if our potential exposure to environmental (i.e., sources from bacteria/fungi contaminated foodstuffs, environmental contamination from chemical/pharmaceutical industries, etc.) proteasome inhibitors poses additional risk for

development of PD. In this regard, infection of the brain with *Nocardia asteroid*, a bacterium of the *Actinomycetes* family, causes nigral dopaminergic degeneration and Parkinsonism in animal models (Tam et al., 2002). Also, consumption of Russian knapweed (*Acroptilon repens*) results in nigral degeneration in horses. One of the suspected active ingredients responsible for the degeneration process, repin (Fig. 2), shares a similar lactone structure as a well known proteasome inhibitor lactacystin (Stevens et al., 1990; Fenteany et al., 1994). Very recently, we tested whether repin inhibits proteasome activity in dopaminergic cells and found a significant inhibition of proteasome activity (unpublished observation). Paradoxically, the proteasome inhibitor curcumin (Fig. 2 has been shown to be protective in various neurodegenerative models (Yang et al. 2005), suggesting certain proteasome inhibitors may target other signal transduction pathways (for example: NF- κ B) to exert their neuroprotective action.

Recently, systemic administration of the naturally occurring proteasome inhibitor epoxomicin or peptide proteasome inhibitor PSI in rodents produced some key behavioral and pathological features of PD (McNaught et al., 2004). The proteasome inhibitor-treated animals showed striatal dopamine depletion, degeneration and cytoplasmic inclusions in key brain regions including nigra, locus ceruleus, dorsal motor nucleus, and nucleus basalis of Meynert, and the affected animals were also responsive to the antiparkinsonian drug l-dopa (McNaught et al., 2004). Unfortunately, recent studies show that the proteasome inhibitor-induced model is not easily reproduced in other laboratories (Kordower et al., 2006; Manning-Bog et al., 2006).

Summary. The effect of various neurotoxicant exposures on UPS and activation of key biochemical mechanisms associated with degenerative processes in the dopaminergic system are summarized in Fig. 3. Neurotoxic insults could converge to impair the proteolytic efficiency of the ubiquitin proteasome system either by targeting the 26S proteasome or

interfering with the ubiquitination step. Neurotoxins such as 6-OHDA, dopamine, MPP⁺, rotenone, paraquat, or iron could augment intracellular ROS production and oxidative stress.

Fig. 3

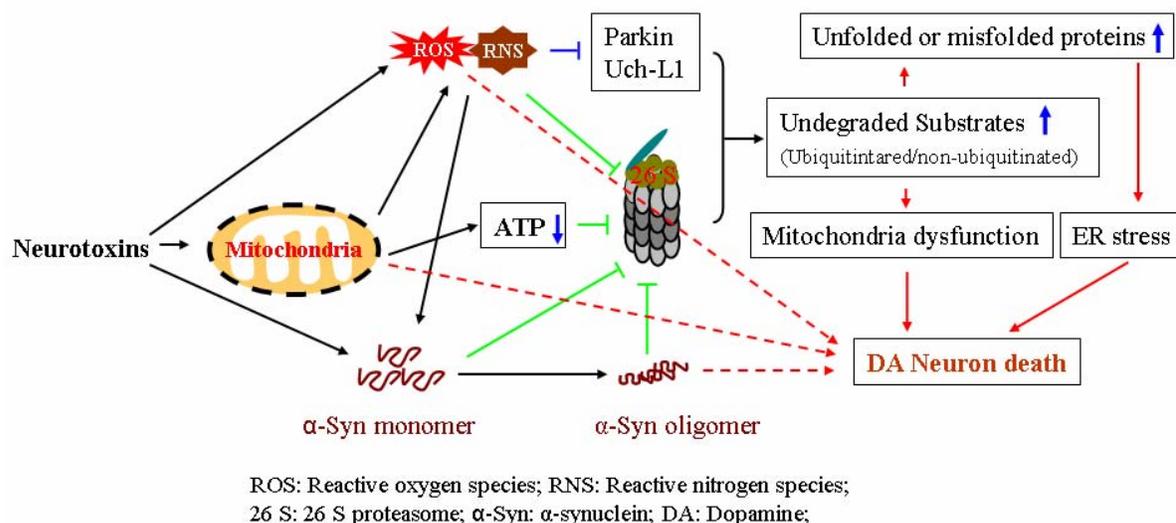


Figure 3: Mechanisms of neurotoxicant-induced proteasome dysfunction and dopaminergic degeneration.

Neurotoxic agents impair the proteolysis function of UPS either by targeting the 26S proteasome (solid green line) or interfering with ubiquitination. 1) Neurotoxicant exposure can augment intracellular ROS production and oxidative stress. Sustained elevation of protein oxidation overwhelms the proteolytic efficiency of the proteasome, and oxidative modification of the subunits of the proteasome also contributes to compromise proteasome function. 2) Mitochondria inhibition by neurotoxins causes loss of intracellular ATP, thus adversely affecting ATP-dependent proteasome degradation. 3) Oligomerization of α-synuclein and upregulation of α-synuclein expression have inhibitory effects on the proteasomal activities. 4) Nitrosylation, reduced solubility or covalent modification of Parkin diminishes its ubiquitin ligase activity, and Uch-L1 oxidation reduces Parkin's hydrolase activity. Proteolytic stress or accumulation of particular substrate proteins resulting from UPS dysfunction interferes with normal mitochondrial function, or induces endoplasmic reticulum stress and eventually leads to the demise of dopaminergic neurons (solid red lines). Additionally, mitochondrial dysfunction, neurotoxicity of α-synuclein or oxidative stress can expedite the dopaminergic degeneration process independent of UPS (red dashed line).

Sustained elevation of protein oxidation overwhelms the proteolytic capacity of the proteasome, and oxidative modification of the subunits of the proteasome may also contribute to proteasome dysfunction. Mitochondrial inhibition by neurotoxins such as MPP⁺, rotenone, paraquat, and dieldrin can deplete intracellular ATP levels, and thus

adversely affect ATP-dependent proteasomal degradation. Oligomerization of α -synuclein following exposure to metals, pesticides, dopamine or dopamine metabolites, and upregulation of α -synuclein expression (e.g.; MPTP, MPP⁺, and rotenone exposure) have inhibitory effects on the proteasomal activities. Nitrosylation (e.g., via MPTP or rotenone exposure), altered solubility (e.g. MPP⁺, rotenone, paraquat, 6-OHDA, or iron treatment) or covalent modification of Parkin (dopamine) diminishes its ubiquitin ligase activity, and Uch-L1 oxidation reduces its hydrolase activity. Thus, the interplay between UPS and mitochondrial dysfunctions promotes the degenerative processes in dopaminergic neurons.

In addition to the various neurotoxic agents described, aging could be an additional factor leading to gradual decline in ubiquitin-proteasome function (Chondrogianni & Gonos, 2005). Impaired UPS degradation has been consistently observed in aged human or animal tissues including the central nervous system (Ding & Keller, 2001a; Gray et al., 2003). The decreased proteolytic capacity of UPS from oxidative stress has been hypothesized to be one of the fundamental changes promoting neurodegeneration (Ding et al., 2006; Halliwell, 2006). A study by Zeng and colleagues showed dramatic reduction in proteasome activities in aged animals in a brain region-specific manner, with substantia nigra being most severely affected, suggesting the contributory role of aging-related UPS dysfunction in nigrostriatal degeneration (Zeng et al., 2005).

As important degradation machinery, UPS also plays a crucial role in the removal of unfolded/misfolded proteins. Coupling of proper protein folding in the endoplasmic reticulum with UPS-mediated protein degradation in the cytosol provides a reliable quality control system for intracellular proteins (Sitia & Braakman, 2003). However, interference with protein folding in the ER, or failure of proteasome degradation could result in the accumulation of unfolded protein, which triggers an unfolding protein response, such as upregulation of HSP 70 and other cytosolic chaperone proteins. In the ER accumulation of unfolded proteins could trigger an ER-stress response, such as upregulation of ER-localized

chaperone proteins Bip. Profiling of gene expression in primary neuronal cells showed that exposure to proteasome inhibitors upregulated the expression of chaperone proteins (Yew et al., 2005), whereas the failure of upregulation of chaperone proteins in dopamine neurons was suggested to sensitize the neurons to apoptotic cell death upon proteasome inhibition (Rideout et al., 2005). Misfolding and aggregation of Parkin substrate Pael-R appears to trigger ER stress and thus promotes nigral dopamine neuron death (Imai et al., 2002). A53T α -synuclein also promotes cell death partially via ER stress in PC12 cells (Smith et al., 2005). The unfolded protein response is extensively observed in dopaminergic cells challenged with PD mimetics 6-OHDA, MPP⁺ and rotenone, which also supports the involvement of UPS dysfunction in PD development (Chen et al., 2004; Holtz & O'Malley, 2003; Ryu et al., 2002).

Puzzles and future directions: Data derived from genetic and biochemical analyses have implied that a defective UPS may play a contributory role in nigrostriatal degeneration. Consistent with this idea, UPS impairment is observed in dopaminergic neurotoxicity provoked by neurotoxins including MPTP/MPP⁺, dopamine, 6-OHDA, metals, rotenone, paraquat and dieldrin. Altered UPS function might occur as a secondary response to nigrostriatal degeneration, or it may be a key cellular event responsible for degeneration (Zeng et al., 2006). In order to establish the effect of dysfunctional UPS on nigrostriatal degeneration, several important questions must be addressed. Firstly, could suppression of UPS degradation effectively model PD? Proteasome inhibitors can produce some features of PD, particularly the formation of proteinaceous inclusions in cell culture, and cell death (McNaught et al., 2002b; McNaught et al., 2002c; Rideout & Stefanis, 2002; Ardley et al., 2003; Rideout et al., 2004; Tanaka et al., 2004; Bandopadhyay et al., 2005). However, it is undetermined whether systemic administration of proteasome inhibitors would yield a promising and reproducible PD model *in vivo*. McNaught and coworkers, and other

researchers have reported that systemic administration of proteasome inhibitors generates models characterizing the key features of PD (McNaught et al., 2004; McNaught & Olanow, 2006; Schapira et al., 2006). However, other labs failed to reproduce the behavioral, pathological or neurochemical alterations characteristic of PD in mice, rat and monkey models using protocols similar to those of McNaught and coworkers (Bove et al., 2006; Kordower et al., 2006; Manning-Bog et al., 2006). Unfortunately, only one out of the six studies listed provided information on the nigral proteasomal activity in animals receiving proteasome inhibitor treatment (Schapira et al., 2006). To resolve the apparent discrepancy, blood brain barrier permeability of the compounds and nigral/striatal UPS function must be monitored in future research to ensure that systemically-administered proteasome inhibitors indeed impair UPS degradation. In contrast, stereotaxic delivery of proteasome inhibitors to substantia nigra or striatum produces nigral dopaminergic degeneration, as demonstrated by several groups including ours (McNaught et al., 2002b; Miwa et al., 2005; Zhang et al., 2005; Sun et al., 2006), suggesting the improved reproducibility in modeling PD with this regimen compared to systemic administration.

The second important question is whether dopaminergic neurons are more vulnerable to proteasome inhibition, since UPS deficit is the common feature of several neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, prion disease, and PD. The presence of protein inclusions in the affected brain regions has been commonly observed in these neurodegenerative disorders (Ding & Keller, 2001a; Halliwell, 2006). The protein aggregations universally observed in different neurodegenerative diseases are clear signs of proteolysis in neurons. The major constituents of the protein aggregations, such as β -amyloid protein, α -synuclein, Huntingtin, mutant SOD and prion, have been shown to hamper proteasome function (Ding et al., 2006; Widmer et al., 2006). Additionally, the involvement of defective UPS function as a common feature of neurodegeneration was also

highlighted by the fact that several E3 ligases have been implicated in the pathogenesis and progression of different neurodegenerative disorders (Ardley & Robinson, 2004).

In addition to nigrostriatal damage, several other brain regions including locus coeruleus, Basalis Megalalis, and nucleus accumbens are affected in PD. The extent of proteasomal dysfunction in these brain regions needs to be determined. If nigral dopaminergic neurons are truly more susceptible to UPS dysfunction, this will provide important clues for ascertaining the precise mechanism responsible for selective nigral dopaminergic degeneration. Thus far, the studies addressing this issue have yielded inconsistent results. A study by McNaught and colleagues showed that the proteasome inhibitor lactacystin and Uch-L inhibitor ubiquitin aldehyde elicit more significant neurotoxicity in mesencephalic dopamine neurons than in GABAergic neurons (McNaught et al., 2002c). In contrast, Kikuchi and coworkers reported that dopamine neurons appear to be slightly resistant to proteasome inhibition by epoxomicin compared to GABAergic neurons in primary mesencephalic culture (Kikuchi et al., 2003). By using a variety of proteasome inhibitors, Reaney and colleagues showed that dopamine neurons are moderately more susceptible to proteasome inhibition (Reaney et al., 2006). This study agrees with the finding that tyrosine hydroxylase-positive neurons in embryonic mesencephalic culture preferentially undergo apoptotic cell death upon proteasome inhibition, possibly due to failure of upregulation of HSP70 (Rideout et al., 2005).

The third question of interest is how the ubiquitin ligase activity of Parkin fits into the hypothesis of mitochondrial dysfunction in PD. The ligase activity of Parkin is vital to its neuroprotection against a broad range of neuronal insults, as previously discussed. A small portion of Parkin associated with the mitochondrial membrane likely mediates the degradation of mitochondrial substrates, and thus suppresses mitochondrial apoptotic events (Darios et al., 2003). Loss of mitochondrial proteins participating in electron transfer/oxidative phosphorylation, compromised mitochondrial respiration, and cellular

detoxification in Parkin knockout mice provide some evidence for the roles of Parkin in preserving mitochondrial physiological function (Palacino et al., 2004; Periquet et al., 2005), although the precise underlying mechanisms remain to be elucidated. Intriguingly, studies in *Drosophila* by two independent groups indicate Parkin likely functions downstream of PINK1, implying cross-talk between Parkin and PINK1 in the same signaling pathway which maintains mitochondrial structure and function (Clark et al., 2006; Park et al., 2006). Future efforts should focus on understanding how Parkin substrates fit in the mitochondria hypothesis, since none of these putative substrates are actually mitochondria-resident proteins. In this regard, *Drosophila* appears to be an alternative model organism to dissect the various pathways involved in dopamine degeneration, including those related to Parkin and other pathways (Bilen & Bonini, 2005; Cauchi & Van Den Heuvel, 2006; Whitworth et al., 2006). Intensive studies with the fly model have yielded novel insight into the mechanisms underlying dopaminergic degeneration, which includes modeling dopamine neuron loss in adult flies expressing α -synuclein (Feany & Bender, 2000), modeling of involvement of chaperone proteins (Auluck et al., 2002; Auluck et al., 2005), oxidative stress (Meulener et al., 2006; Yang et al., 2005b) in dopamine neuron survival and death, interaction of Parkin and PINK1 in maintenance of normal mitochondria function (Yang et al., 2003; Park et al., 2006), and neuroprotection of Parkin. However, one of the problems with the fly models overexpressing α -synuclein or Parkin is toxicity in the flies; this is likely the result of the excess proteins overwhelming UPS. Validation of results from the fly models with mammalian models will advance our understanding of PD pathogenesis, and contribute to the development of potential manipulation strategies.

Finally, a detailed analysis of key overlapping biochemical signaling associated with mitochondria and UPS will help us to elucidate the possible interactive role of mitochondrial and UPS dysfunctions in many neurodegenerative diseases, including PD.

Background and Literature Review II

Role of α -Synuclein in Neuroprotection and Neurotoxicity of Dopaminergic Neurons

Abstract: α -Synuclein is a presynaptic protein that may participate in neuronal plasticity, neurotransmission and maintenance of dopamine homeostasis; although the exact physiological function remains unclear. As the major component of Lewy bodies, the hallmark of the Parkinson's disease (PD), α -synuclein also plays a crucial role in the pathogenesis of PD. A number of α -synuclein mutations are associated with some familial PD. Examination of the effects of α -synuclein on neuronal viability has yielded discrepant results, indicating that the role of α -synuclein varies depending on multiple factors such as expression level, cell types and duration of neurotoxic insults. Among varieties of cell lines used for PD studies, we adopted immortalized rat mesencephalic dopaminergic neuronal cells, N27 cells, as an experimental model for studying cell death mechanisms in PD. Using this cell model, we demonstrated that PKC δ (protein kinase C delta), a member of novel PKC isoform, proteolytically activated to induce apoptosis in dopaminergic cell death following exposure to Parkinsonian neurotoxicants such as MPP⁺ (1-methyl-4-phenylpyridinium ion), 6-OHDA (6-hydroxyl dopamine), manganese and dieldrin. Overexpression of human α -synuclein in N27 cells protects cells from MPP⁺ toxicity and acute dieldrin treatment, presumably via interaction with PKC δ and BAD. However, α -synuclein significantly potentiates the neurotoxicity of prolonged dieldrin treatment in N27 cells, possibly involving α -synuclein misfolding and aggregation. The opposing roles of α -

synuclein observed in the N27 cells are in agreement with discrepancies previously reported in several cell types. Herein, we first summarize previous studies that examined neuroprotective as well as neurotoxic effects of α -synuclein and then describe our observation on the effect of neurotoxicants on wild type and α -synuclein-overexpressing cells. We also demonstrates how N27 cells can be used as an experimental model system to ascertain the physiological and pathological roles of α -synuclein in dopaminergic degeneration.

α -Synuclein was originally identified in cholinergic vesicles of *Torpedo californica*, the Pacific torpedo ray (Maroteaux et al., 1988). The protein was named as α -synuclein, because of its predominant cellular localization at synapse and the nuclear envelope of neurons. Subsequently, the mammalian homologue of the *Torpedo* synuclein was isolated and named as γ -synuclein. α -Synuclein is highly expressed in the central nervous system, especially in the substantia nigra, caudate nucleus, amygdala, and hippocampus. It is currently known that both α - and γ -synuclein belong to the same gene family which also includes β -synuclein and synoretin (Suh and Checler, 2002). All the synuclein family proteins contain the KTKEGV consensus domains. Human α -synuclein, a natively unfolded protein with 140 amino acids, consists of 3 structurally distinct motifs: an N-terminal amphipathic region, a central NAC domain, and a C-terminal acidic tail (Recchia et al., 2004). As shown in Fig. 1, the N-terminal amphipathic region, containing a majority of the repeats of the KTKEGV consensus sequence, has the capacity to associate with negatively charged phospholipids (Cookson, 2005). Upon binding to lipid, native unfolded α -synuclein changes to an α -helix configuration. All three point mutations of α -synuclein: A30P, E46K, and A53T, are exclusively located in this region. The E46K and A53T mutations of α -

synuclein potentiate its lipid binding and accelerate filament formation, while A30P reduces the binding capacity and slows down the formation of fibrillar species. This suggests that the amphipathic helix conformation favors the formation of α -synuclein aggregation (Choi et al., 2004).

Fig.1 Structure of human α -synuclein

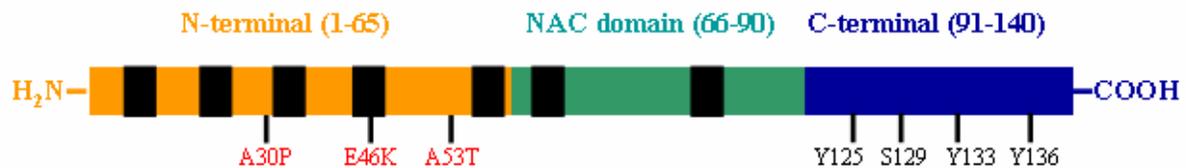


Fig. 1 Structural features of human α -synuclein proteins. The three structural domains of α -synuclein include a N-terminal amphipathic region (1-65), a central NAC domain (66-90), and a C-terminal acidic tail (91-140). The majority of the signature consensus domains (imperfect KTKEGV sequence, black solid box) of synuclein family proteins are located at the N-terminus. A30P, E46K and A53T are the 3 human mutations associated with familial PD. The acidic C-terminus contains several amino acids (Y125, S-129, Y133 and Y136), that could bear post-translational modifications such as phosphorylation and nitration.

The highly negatively charged C-terminal tail of α -synuclein has several phosphorylation sites: Tyr-125, 133 and 136, and Ser-129. Approximately 90% of α -synuclein in the urea-insoluble fraction prepared from brain samples of synucleinopathy is phosphorylated on Ser-129 (Fujiwara et al., 2002). The post-translational modifications in the C-terminus include the nitration on Tyr-125, 133 and 136, and possible glycosylation at an unidentified position. The inhibitory effect of the acidic C-terminus on aggregation is based on the observation that the C-terminal truncated form of α -synuclein more readily forms fibrillar filaments (Murray et al., 2003). Central NAC (Non-A β component) region (66-95) initially was identified as a major component secondary to A β in Alzheimer's plaques. The NAC domain of α -synuclein, which is absent from β - and γ -synuclein, is hydrophobic, and amyloidogenicity of NAC is crucial for formation of the β -sheet structure of α -synuclein.

Likely, β -sheet structure promotes oligomerization of protein to form the so-called protofibril and the subsequent filament, which eventually lead to the protein aggregation in Lewy bodies (Bodles et al., 2001; Giasson et al., 2001).

Physiological function of α -synuclein

Currently, the physiological function of α -synuclein is under intense investigation. Consistent with its high expression at the presynaptic terminals, α -synuclein has been thought to play a role in synaptic transmission. It is postulated that α -synuclein may play a role in dopamine synthesis, vesicle transport and release of dopamine including dopamine uptake by DAT and VMAT in the dopaminergic system. Neurochemical studies revealed impairment in paired stimuli-triggered dopamine release at the nigrostriatal terminals, and reduced striatal dopamine levels in α -synuclein knock-out mice (Abeliovich et al., 2000). Similarly, suppression of α -synuclein expression reduces the number of synaptic vesicles, especially the vesicles of reserve pool in hippocampal neurons, suggesting the important regulatory roles of α -synuclein in presynaptic vesicle formation and maintenance (Cabin et al., 2002). A recent study by Larsen and colleagues indicated that α -synuclein interferes with secretory exocytosis of transmitter release (Larsen et al., 2006). In line with α -synuclein's role in neurotransmission, knockout or mutation of α -synuclein has been shown to lower the capacity of the dopamine storage pool (Yavich et al., 2004). Modulation of phospholipase D2 by α -synuclein in clathrin-mediated endocytosis for the presynaptic vesicle recycling has been suggested to be the underlying regulatory role of α -synuclein in the process (Lotharius and Brundin, 2002a).

Chaperone activity of α -synuclein

Because of its homology and interaction with 14-3-3, α -synuclein has also been postulated to function as a chaperone protein (Ostrerova et al., 1999). Protein-protein

interactions often dictate much of the cellular signaling influenced by α -synuclein within various cellular systems. Indeed α -synuclein has been shown to regulate the activity and function of several proteins associated with dopamine homeostasis and cellular signaling (Table 1).

Table 1: Summary of Proteins that Interact with α -Synuclein.

PROTEIN	REFERENCE
<i>PLD2 (Phospholipase D 2)</i>	(Jenco et al., 1998; Payton et al., 2004)
<i>UCH-L1 (Ubiquitin Ligase 1)</i>	(Liu et al., 2002)
<i>Parkin</i>	(Choi et al., 2001; Oluwatosin-Chigbu et al., 2003; Shimura et al., 2001)
<i>Synphilin</i>	(Engelender et al., 1999; Ribeiro et al., 2002)
<i>14-3-3</i>	(Ostrerova et al., 1999)
<i>PKC, BAD, ERK</i>	(Ostrerova et al., 1999)
<i>Elk-1/Erk-2 Complex</i>	(Iwata et al., 2001b)
<i>MAPK</i>	(Iwata et al., 2001a)
<i>Tubulin</i>	(Alim et al., 2002)
<i>Cytochrome Oxidase IV (COX IV)</i>	(Elkon et al., 2002)
<i>Dopamine Transporter (DAT)</i>	(Kobayashi et al., 2004)
<i>Aβ, Tau</i>	(Jensen et al., 1997; Yoshimoto et al., 1995)
<i>Calmodulin</i>	(Lee et al., 2002a; Martinez et al., 2003)
<i>Protein kinase Cδ</i>	(Kaul et al., 2005a)
<i>PP2A</i>	(Peng et al., 2005)

Tyrosine hydroxylase and dopamine synthesis

Tyrosine hydroxylase (TH) is the rate limiting enzyme in the synthesis of dopamine, and is activated when phosphorylated on any of its serine residues: Ser 19, Ser 31 and Ser 40. Various protein kinases like MAPK and ERK1/2 regulate this reversible phosphorylation of TH on residue Ser 31 (Royo et al., 2004). Protein kinase A phosphorylates TH on Ser 31 and

Ser 40 and Ca⁺⁺-dependent protein kinase C (PKC) modulates activity of TH in other models (Albert et al., 1984; Cahill et al., 1989; Kobori et al., 2004; Sura et al., 2004). Recently, we showed PKC δ negatively regulates TH-ser40 phosphorylation and dopamine synthesis via phosphatase 2B (Zhang et al., 2007). We noted that α -synuclein overexpression suppresses PKC δ levels (unpublished observation), but it is yet to be determined whether PKC δ have any role in the modulating dopaminergic neurotransmission. Chaperone proteins 14-3-3 and α -synuclein have been suggested to interact with tyrosine hydroxylase, and exert opposite regulatory effects on TH activity and dopamine synthesis (Sidhu et al., 2004a). Preferential interaction of 14-3-3 with phosphorylated TH stabilize the protein to its active conformation, and thus maximizes enzymatic activity for dopamine synthesis; whereas association of α -synuclein with dephosphorylated TH likely suppresses TH activation by stabilizing TH in its inactive form (Sidhu et al., 2004a). Additionally, Peng and coworkers showed that activation of PP2A by α -synuclein results in the dephosphorylation of TH, and thus decreased dopamine synthesis (Peng et al., 2005). Due to its chaperone activity, α -synuclein has also been postulated to directly interact with kinases associated with dopamine homeostasis such as MAPK and PKCs (Baptista et al., 2003; Iwata et al., 2001a; Ostrerova et al., 1999). Furthermore, overexpression of familial α -synuclein mutants have been shown to affect dopamine homeostasis in both cell culture and animal models of dopaminergic degeneration (Lotharius et al., 2002; Orth et al., 2004). This effect of α -synuclein on DA metabolism can lead to excessive release or production of DA. Excessive DA production can result in the formation of free radicals due to auto-oxidation of DA, which can be deleterious to the neurons (Jenner, 2003; Lotharius and O'Malley, 2001; Luo et al., 1998).

Vesicular transport and trafficking

In addition to its regulatory role of TH activity, α -synuclein also participates in the other cellular events maintaining dopamine homeostasis, such as modulation of the plasma

membrane dopamine transporter (DAT). Dopamine, once synthesized, is stored in lipid-bound synaptic vesicles that protect the dopaminergic neurons from its auto-oxidative effects. In dopamine neurons, sequestration of cytosolic dopamine into synaptic vesicles by vesicular monoamine transporter 2 (VMAT2) is essential for the neurons to avoid the neurotoxicity of dopamine (DA). The uptake and storage of dopamine in the synaptic vesicles is regulated by the vesicular monoamine transporter 2 (VMAT2) (Weihe and Eiden, 2000). VMAT2 also attenuates the neurotoxicity of MPP⁺, a known dopaminergic toxin, by sequestering it safely in synaptic vesicles (Gainetdinov et al., 1998a) Human positron emission tomography (PET) studies have revealed an enhanced loss of VMAT2 in dopaminergic neurons, indicating that vesicular dysfunction might be an important contributing factor in PD (Lee et al., 2000). α -Synuclein negatively controls dopamine release by acting on VMAT2 activity through its inhibitory action on phospholipase D 2 (PLD2) (Lotharius and Brundin, 2002b; Sidhu et al., 2004b). Overexpression of human A53T α -synuclein mutant has been shown to down-regulate the expression of VMAT, thus impairing the vesicular storage and cytosolic accumulation of dopamine (Lotharius et al., 2002). Also, disruption of the integrity of the vesicular membrane, presumably as the result of the formation of α -synuclein protofibrils, has been suggested to account for the cell type-specific neurotoxicity in dopamine neurons, since α -synuclein overexpression appears to dissipate the proton gradient across the vesicle membrane and remarkably elevates the cytosolic dopamine level (Mosharov et al., 2006).

Dopamine transporter function

The dopamine receptor (DAT) belongs to the Na⁺/Cl⁻-dependent transporter family of monoamine transporters involved in dopamine homeostasis through clearance of excess neurotransmitter from the synaptic clefts (Gallant et al., 2003; Mortensen and Amara, 2003). Similar to α -synuclein, DAT is expressed in the pre-synaptic terminals and is crucial for effective maintenance of dopamine neurotransmission in dopaminergic nerve terminals

(Chen and Reith, 2004; Gainetdinov et al., 1998b). DAT function involves the phosphorylation of certain N-terminal residues by various protein kinases including PKC, resulting in redistribution of the transporter between the plasma membrane and the cytoplasm (Daniels and Amara, 1999; Foster et al., 2002; Melikian and Buckley, 1999; Pristupa et al., 1998). Recent studies have shown that over-expression of the human wild-type α -synuclein led to a reduction in DAT activity due to reduced DA uptake, but not due to DAT trafficking or transcriptional regulation (Wersinger et al., 2003a). The opposite effect of α -synuclein on DAT-mediated dopamine uptake has been reported; α -synuclein attenuates the activity of coexpressed DAT, and suppresses the dopamine-related oxidative stress in the neurons (Wersinger et al., 2003b; Wersinger and Sidhu, 2003). However, other studies have shown that α -synuclein positively regulates DAT activity and enhances the neurotoxicity of dopamine and MPP⁺ (Lee et al., 2001).

α -Synuclein mutations in Parkinson's disease

Several lines of evidence suggest that, in both sporadic and familial forms of PD, protein aggregates within dopaminergic neurons of the substantia nigra are a common feature. Although several proteins have been found in the Lewy bodies, fibrillar α -synuclein is the major component of the intracellular protein inclusions (Choi et al., 2001). Familial PD has been linked to missense and genomic multiplication mutations of the α -synuclein gene. Autosomal dominant mutations in the α -synuclein gene have been shown to be associated with familial PD. Three different missense mutations, A53T, A30P, and E46R have been found in patients of familial PD (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). Triplication and duplication of the α -synuclein locus has also been found in several families with PD (Chartier-Harlin et al., 2004; Singleton et al., 2003). Though the gene triplication/duplication occurs in rare cases of PD, gene multiplication apparently may result in the elevation of the α -synuclein protein level and insoluble protein

aggregates, which may mediate PD pathogenesis (Hofer et al., 2005; Johnson et al., 2004; Miller et al., 2004). A recent study also linked α -synuclein promoter's susceptibility to sporadic PD (Pals et al., 2004). These studies clearly suggest that overproduction of α -synuclein can be a risk factor for PD.

α -Synuclein phosphorylation

The Ser-129 phosphorylation appears to be a very important post-translational modification associated with Lewy bodies (Anderson et al., 2006), and the pathological relevance of the modification is manifested by its role in promoting α -synuclein fibrillation or ubiquitination (Anderson et al., 2006; Hasegawa et al., 2002). Ser 129 is constitutively phosphorylated in transfected HEK293 and PC12 cells; this may be mediated by kinases such as casein kinase (CK I and CK II) and kinase down stream of the G-protein coupled receptors (GPCRs) (Okochi et al., 2000; Pronin et al., 2000). α -Synuclein interaction with PLD2 is important for regulation of vesicle release of dopamine into the synaptic environment, and Ser-129 phosphorylation can attenuate this interaction, thus altering dopamine homeostasis (Lotharius et al., 2002). Recently, however, investigators have also suggested that vesicle trafficking could be PLD2 independent (Abeliovich et al., 2000). Y39, Y125, Y133 and Y136 tyrosine residues are well conserved in all the α -synuclein homologues, as well as in the β -synuclein paralogs, indicating that these residues are important in synuclein functioning. Activation of Pyk2/RAFTK in COS7 cells can phosphorylate α -synuclein via the src kinase family of enzymes, and this tyrosine phosphorylation can serve as a neuroprotective mechanism in the case of deleterious nitrosylation of synuclein at Y125 (Nakamura et al., 2002; Takahashi et al., 2003). Tyrosine phosphorylation has also been suggested to have an effect on the regulation of synaptic vesicles in lieu of the fact that Tau, synuclein and src PTK members interact with each other at various levels of cellular signaling (Lee et al., 1998; Trojanowski and Lee, 2000). Hypothetical suggestions regarding

the importance of these interactions are based on the fact that tau might help bring Src-PTKs in close proximity to α -synuclein and then lead to tyrosine phosphorylation, which plays an important role in the development of synaptic plasticity (Clayton and George, 1999). Recently, Wakamatsu and colleagues reported accumulation of phosphorylated α -synuclein in dopaminergic neurons of transgenic mice that express human α -synuclein (Wakamatsu et al., 2007). It has been reported that substitution of Ser-129 of α -synuclein with alanine (S129A) reduces the formation of intracellular protein aggregation (Smith et al., 2005b). Phosphorylation of α -synuclein at Ser-129 leads to an increase in formation of its insoluble aggregated oligomers. Further, serine hyperphosphorylated forms have been isolated from human brain tissues, transgenic mice and fly neurons (Fujiwara et al., 2002; Kahle et al., 2002; Neumann et al., 2002; Takahashi et al., 2002).

α -Synuclein aggregation

Several studies have linked ubiquitin proteasomal dysfunction to α -synuclein aggregation in primary mesencephalic neurons, dopaminergic neuronal cells and in animal models (McNaught et al., 2002a; McNaught et al., 2002b; Rideout et al., 2001, Sun et al., 2006). Extensive studies also suggest various factors that could promote α -synuclein aggregation. First, as the major component, α -synuclein tends to self-aggregate; cross-linking of nitrated tyrosine by dinitrated bond can form urea/detergent-insoluble dimers or trimers of α -synuclein (El-Agnaf et al., 1998b; Giasson et al., 2000; Souza et al., 2000; Takahashi et al., 2002). Transglutaminase, found in Lewy bodies, has been shown to induce intramolecular cross-linking of α -synuclein (Andringa et al., 2004; Junn et al., 2003). Mitochondrial inhibition has also been shown to result in the formation of α -synuclein aggregation in cell culture and animal models (Fornai et al., 2005; Lee et al., 2002b; Sherer et al., 2003).

Neuroprotective effect of α -synuclein in dopaminergic neurons

To understand the role of α -synuclein in dopaminergic degeneration in PD, numerous groups have examined the effect of overexpression of either wild-type or mutant α -synuclein on dopaminergic neurons in cell culture, as well as in transgenic and knockout animals. In cell culture studies, overexpression of wild-type, but not A53T or A30P α -synuclein mutants, protected against caspase-3 activation and apoptotic cell death induced by several neurotoxicants in the TSM1 neocortical cell line (Alves da Costa et al., 2006). We showed that overexpression of wild type α -synuclein but not mutant α -synuclein attenuated PKC δ dependent apoptotic cascade in dopaminergic cells (Kaul et al., 2005). Similarly, α -synuclein overexpression was also shown to protect a human dopaminergic cell line (SH-SY5Y cells) from cytotoxicity from Parkin knockdown and dopamine treatment (Colapinto et al., 2006; Machida et al., 2005). Studies have shown α -synuclein exerts its neuroprotective effect via inactivation of Jun kinase or inhibition of caspase-3 activation (Hashimoto et al., 2002; Li and Lee, 2005). Others have shown that nanomolar concentrations of α -synuclein can activate the PI3/Akt cell survival signal pathway, which renders neurons more resistant to serum deprivation, oxidative stress, and excitotoxicity.

The effect of α -synuclein on neuronal viability has been suggested to be dependent on several other factors, such as its intracellular abundance, cell types or the types of stimuli (Seo et al., 2002; Xu et al., 2002; Zourlidou et al., 2003). In addition, α -synuclein has been shown to play a protective role in animal PD models (Hashimoto et al., 2002; Lee et al., 2006; Manning-Bog et al., 2003). Animals treated with the herbicide paraquat showed increased α -synuclein expression and aggregation in the brains. This increased expression and aggregation of α -synuclein results in neuroprotection. In neuronal cells overexpressing α -synuclein, the intracellular retrograde transport system has been shown to play a crucial role in aggregate formation, and that these aggregates are thought to represent a

neuroprotective response (Hasegawa et al., 2004). Increased α -synuclein expression in response to dopaminergic toxins, e.g. MPTP, rotenone, paraquat, suggests that an increase of α -synuclein represents an adaptive response to toxic stimuli and α -synuclein overexpression in transgenic mice does not consistently result in neuronal damage, nor does it exacerbate neurodegeneration caused by MPTP or other dopaminergic toxins (Lee et al., 2006; Masliah et al., 2000; Matsuoka et al., 2001). Therefore, the neuroprotective property of α -synuclein may be used for cell survival strategies (Lee et al., 2006). α -Synuclein is abundantly present at synapse and therefore it is not surprising that the normal level of α -synuclein has some neuroprotective functions in CNS. Table 2 summarizes key studies on the neuroprotective effects of α -synuclein.

Table 2. Neuroprotection by α -synuclein.

Key findings	Model	Reference
Wt α -synuclein, not A53T mutant, protects cells from apoptosis	TSM1 neuronal cells	(Alves da Costa et al., 2006)
Wt α -synuclein protects SH-SY5Y cells from apoptosis and dopamine metabolite accumulation as the result of Parkin loss	SH-SY5Y	(Machida et al., 2005)
Wt α -synuclein alleviates cytotoxicity of dopamine in SH-SY5Y cells, and upregulates DJ-1 expression	SH-SY5Y	(Colapinto et al., 2006)
Wt α -synuclein inactivates JNK by upregulating JNK-interacting protein JIP-1b/IB1 during oxidative stress	GT1-7 murine hypothalamic tumor cell line	(Hashimoto et al., 2002)
Wt, A30P human α -synuclein, but not A53T α -synuclein, β -synuclein or mouse α -synuclein, protect cells from apoptosis via inhibiting caspase-3	SH-SY5Y	(Li and Lee, 2005)
Wt α -synuclein, not A53T mutant, suppressed MPP ⁺ -induced activation of apoptosis via interaction with PKC δ and BAD	N27 cells	(Kaul et al., 2005a)

Neurotoxic effect of α -synuclein

Overproduction and/or accumulation of α -synuclein in cultured neuronal cells causes selective degeneration in dopaminergic neurons but not in non-dopaminergic neurons, suggesting selective toxicity (Xu et al., 2002). Also, in mice expressing the A53T human α -synuclein mutation there is an early onset of neurodegeneration and α -synuclein aggregation

in the brain (Lee et al., 2002c). In cell culture models, direct neurotoxicity of α -synuclein was manifested by the increased cell death of SH-SY5Y cells following exposure to mutant, aggregated α -synuclein or NAC fragment (El-Agnaf et al., 1998a; Sung et al., 2001). Endocytotic uptake of α -synuclein involving Rab5A was hypothesized to be crucial for its observed neurotoxicity (Sung et al., 2001). Expression of wild-type, A30P or A53T mutant human α -synuclein induces apoptosis in the mouse nodose ganglion neurons (Saha et al., 2000). Studies with neuronal cell lines indicated that different types of cell death including mitochondria related, endoplasmic reticulum stress cell death or autophagic cell death are involved in the neurotoxicity of either wild-type or mutant α -synuclein (Hsu et al., 2000; Smith et al., 2005a; Stefanis et al., 2001). α -Synuclein also appears to enhance the vulnerability of cells to a variety of neurotoxins. Overexpression of human α -synuclein in human SH-SY5Y neuroblastoma cells, especially the C-terminal truncated form, A30P and A53T mutants, significantly potentiates the oxidative damage and cell death triggered by MPP⁺ or H₂O₂ (Kanda et al., 2000). Consistently, expression of mutant α -synuclein in human BE-M17 neuroblastoma cells results in more profound neuronal death following exposure to iron, which promotes free radical generation (Ostrerova-Golts et al., 2000). Inducible expression of mutant α -synuclein inhibits proteasome activity and renders PC12 cells more susceptible to proteasome inhibitor-induced apoptosis (Tanaka et al., 2001). Coexpression of wild-type or A30P α -synuclein with a dopamine transporter in SH-SY5Y cells revealed dopamine-dependent cell death accompanied by collapse of cellular membrane potential, oxidative stress, and mitochondria abnormalities (Moussa et al., 2004). Table 3 summarizes some key studies that describe the neurotoxic properties of α -synuclein.

Table 3. Neurotoxicity of α -synuclein.

Key findings	Model	Reference
α -Synuclein, especially aggregated form, directly provokes neurotoxicity	SH-SY5Y cells	(El-Agnaf et al., 1998a)
Rab5A-specific endocytosis of α -synuclein mediates the	Rat hippocampal	(Sung et al.,

neurotoxicity of exogenous α -synuclein	neuronal cells H19-7	2001)
Wt, A30P or A53T mutant human α -synuclein, not γ -synuclein, induces apoptosis	mouse nodose ganglion neurons	(Saha et al., 2000)
Formation of α -synuclein aggregates, compromised mitochondria activity, increased ROS generation in α -synuclein overexpressing cells	hypothalamic neuronal cell line (GT1-7)	(Hsu et al., 2000)
A53T mutant, but not wild-type α -synuclein, impairs ubiquitin proteasome and lysosomal degradation and enhances autophagy cell death	PC12 cells	(Stefanis et al., 2001)
A53T mutant α -synuclein induces mitochondria-mediated and ER stress-mediated apoptosis	PC12 cells	(Smith et al., 2005b)
C-terminal truncated, A30P and A53T mutant α -synuclein potentiates oxidative damage and cell death triggered by MPP ⁺ or H ₂ O ₂	SH-SY5Y	(Kanda et al., 2000)
Synuclein increases the vulnerability of cells to neurotoxicity of iron (A53T > A30P > wild-type), and iron induces α -synuclein aggregation in the same order	human BE-M17 neuroblastoma cells	(Ostrerova-Golts et al., 2000)
A30P α -synuclein inhibits proteasomal activity and sensitizes cells to mitochondria apoptosis	PC12 cells	(Tanaka et al., 2001)
Wt α -synuclein inhibits proteasomal activity and enhances dieldrin-induced apoptosis	N27 cells	(Sun et al., 2005)
Selective neurotoxicity of A53T α -synuclein, but not wt, to dopamine neurons in mesencephalic primary culture	Human mesencephalic primary culture	(Zhou et al., 2002)
Dopamine-dependence of the neurotoxicity of wt α -synuclein	Human mesencephalic primary culture	(Xu et al., 2002)
Coexpression of wt or A30P α -synuclein with DAT causes mitochondria pathologies, oxidative stress and dopamine-dependent neuron death	SH-SY5Y	(Moussa et al., 2004)

In essence, the neuroprotective form of α -synuclein can be readily converted to toxic gain-of-function forms, under certain conditions such as overproduction, oxidative modification, and oligomerization, thus demonstrating the observed opposing roles of α -synuclein.

Immortalized mesencephalic cell line (N27) as a model system for elucidating α -synuclein function

We have recently established an immortalized rat mesencephalic dopaminergic neuronal cell line as an excellent model system for studying dopaminergic degeneration. The N27 (1RB₃AN₂₇) cell line was initially developed by Dr. Prasad and his coworkers; these cells exhibited and retained most of the key features of dopaminergic neurons such as the

expression of neuron-specific enolase, nestin, tyrosine hydroxylase, and dopamine transporter, and production of homovanillic acid, a dopamine metabolite (Prasad et al., 1994). Upon differentiation, N27 cells acquire morphologic and functional features of the post-mitotic dopamine neurons, such as enlargement of the cell body, growth of neuronal processes up-regulation of TH and DAT, and increased DA production (Fig. 2). A study by Clarkson and colleagues demonstrated that differentiated N27 cells are even more vulnerable to MPP⁺ and 6-OHDA-induced neurotoxicity than undifferentiated cells (Clarkson et al., 1999). We subsequently established that undifferentiated N27 cells are highly susceptible apoptotic cell death induced by dopaminergic toxins similar to the primary neuronal culture.

In N27 cells, we recently showed that oxidative stress, multiple caspases and PKC δ mediate apoptotic cell death induced by several dopaminergic toxins including MMT, dieldrin, MPP⁺ and manganese (Anantharam et al., 2002; Kaul et al., 2003; Kitazawa et al., 2003; Latchoumycandane et al., 2005). We clearly established that PKC δ is an oxidative stress-sensitive kinase in this cell culture PD model (Kanthasamy et al., 2003). Oxidative stress activates PKC δ by proteolysis in which caspase-3 cleaves the native kinase (72-74-kDa) resulting in 41-kDa catalytically active and 38-kDa regulatory fragments, to persistently activate the kinase. Phosphorylation of PKC δ at tyrosine residue 311 is essential for the proteolytic cleavage of the kinase during oxidative stress (Kaul et al., 2005b). The proteolytic activation of PKC δ plays a key role in promoting apoptotic cell death in various cell types including neuronal cells (Brodie and Blumberg, 2003; Kanthasamy et al., 2003; Kikkawa et al., 2002). Studies from this cell line are consistent with reduced cellular antioxidant capacity, increased oxidative stress and impaired mitochondrial function as observed during dopaminergic degeneration. Overexpression of loss-of-function dominant-negative mutant PKC δ^{D327A} (caspase-cleavage resistant), PKC δ^{K376R} (kinase inactive) and PKC δ^{Y311F} (phosphorylation defective) proteins also attenuates dopaminergic neurons from MPP⁺- and oxidative stress-induced apoptotic cell death. Suppression of caspase-3-

dependent proteolytic activation of PKC δ by small interfering RNA (siRNA) also prevented MPP⁺-induced dopaminergic degeneration (Yang et al., 2004). In addition to the proapoptotic role, PKC δ may also amplify apoptotic signaling via positive feedback activation of the caspase cascade (Kanthasamy et al., 2003). Thus, the dual role of PKC δ as a mediator and amplifier of apoptosis was established in this cell culture model and may be important in the pathogenesis of PD. PKC δ is also highly expressed in these cells, compared to several other non-dopaminergic neuronal cells (unpublished observations) and also colocalizes with tyrosine hydroxylase in these cells. These results were subsequently confirmed in the mouse nigral tissue, where PKC δ is also highly expressed and colocalizes with TH (Zhang et al., 2007). Further investigation revealed PKC δ negatively regulates TH activity and dopamine synthesis by enhancing protein phosphatase-2A activity in N27 cells (Zhang et al., 2007). Many results obtained in N27 cells were able to readily reproduce in animal models, indicating that N27 cells are very reliable cell culture model of PD.

After we established the N27 cell line as a model system for studying dopaminergic degeneration, we generated stable N27 cell lines overexpressing wild-type and A53T mutant α -synuclein (Kaul et al., 2005a). N27 cells over-expressing wild-type α -synuclein were highly resistant to MPP⁺-induced cytotoxicity, mitochondrial cytochrome c release, and subsequent caspase-3 activation, without affecting reactive oxygen species (ROS) generation (Kaul et al., 2005a). Co-immunoprecipitation studies revealed MPP⁺ treatment induced the physical association of α -synuclein with pro-apoptotic proteins PKC δ and BAD, but not with the anti-apoptotic protein Bcl-2. The physical association between PKC δ and α -synuclein did not involve direct phosphorylation. On the contrary, in A53T α -synuclein mutant expressing cells, MPP⁺-induced apoptotic cell death signaling including activation of caspase-3, PKC δ and DNA fragmentation, was exacerbated. These results suggested that normal level of wild-type α -synuclein is neuroprotective whereas A53T α -synuclein is

neurotoxic and may mediate the effects via interaction with pro-apoptotic molecules BAD and PKC δ . Unlike MPP⁺, human α -synuclein exacerbated dieldrin-induced increases in caspase-3 activity and DNA fragmentation compared to vector expressing cells (Sun et al., 2005). In the N27 model system we demonstrated that human α -synuclein can be neuroprotective or neurotoxic, depending on the duration and type of neurotoxin exposed (Kaul et al., 2005a; Sun et al., 2005).

Fig. 2 N27 cells

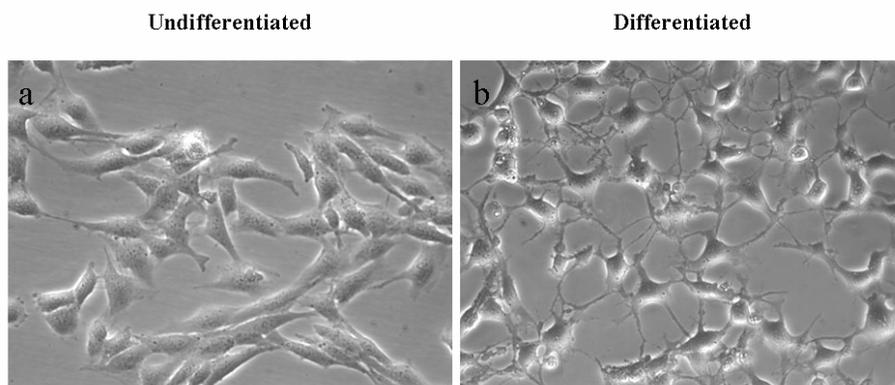


Fig. 2 Rat mesencephalic dopaminergic neuronal cells (N27 cells). The phase contrast image (20 X) shows undifferentiated N27 cells (panel a) and differentiated N27 cells (panel b). Dibutyryl 3,5-cyclic adenosine monophosphate (dbcAMP, 2.0 mM) was supplemented in the growth medium to induce morphological and biochemical alterations characteristic of differentiation of N27 cells.

Since recent evidence indicate that abnormal accumulation and aggregation of α -synuclein and ubiquitin-proteasome system (UPS) dysfunction can contribute to the degenerative processes of PD, we examined the effect of human α -synuclein on dieldrin-induced impairment in UPS dysfunction in N27 cell line (Sun et al., 2005). Baseline proteasomal activity in human α -synuclein overexpressing cells was 50% less than vector expressing N27 cells, suggesting that α -synuclein overexpression significantly attenuated

baseline proteasomal activity. Further, overexpression of human α -synuclein also exacerbated dieldrin-induced decreases in proteasomal activity by more than 60% compared to vector expressing N27 cells. Confocal microscopic analysis revealed that α -synuclein-positive protein aggregates colocalized with ubiquitin protein in dieldrin-treated cells, and these aggregates were distinct from autophagosomes and lysosomes. The dieldrin-induced proteasomal dysfunction in α -synuclein cells also resulted in significant accumulation of ubiquitin protein conjugates; proteasomal inhibition preceded cell death. In these studies human α -synuclein overexpression predisposed N27 cells to proteasomal dysfunction, which can be further exacerbated by the pesticide dieldrin (Sun et al., 2005).

Fig. 3

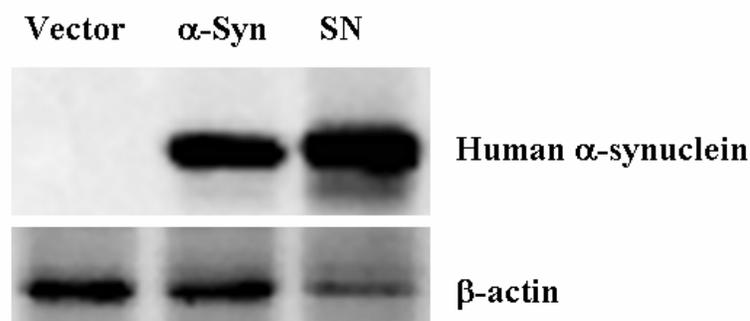


Fig. 3 Expression of human wild-type α -synuclein in N27 dopaminergic cells. pCEP4 expression vector containing coding sequence for human α -synuclein were transfected into N27 cells using Lipofectamine Plus reagent. Stable expression was achieved with prolonged hygromycin screening. Cell lysates were obtained from vector transfected cells (Vec, lane 1), or α -synuclein expressing cells (α -Syn, lane 2) for Western blot analysis with α -synuclein antibody. Lysate of the rat substantia nigra (SN) was included to show that exogenously introduced α -synuclein is expressed at physiological levels comparable to the substantia nigra region (Lysate, lane 3). Protein amount: 20 μ g.

Recently we observed that human α -synuclein expression levels in N27 cells are comparable to that of rat substantia nigra (Fig. 3). Overexpression of human α -synuclein

also did not have any effect on protein expression levels of LRRK2, a kinase implicated in familial PD (Fig. 4A) and MEK1/2, an ERK kinase activator (Fig. 4B), both of which have been implicated in the pathogenesis of PD.

Fig.4

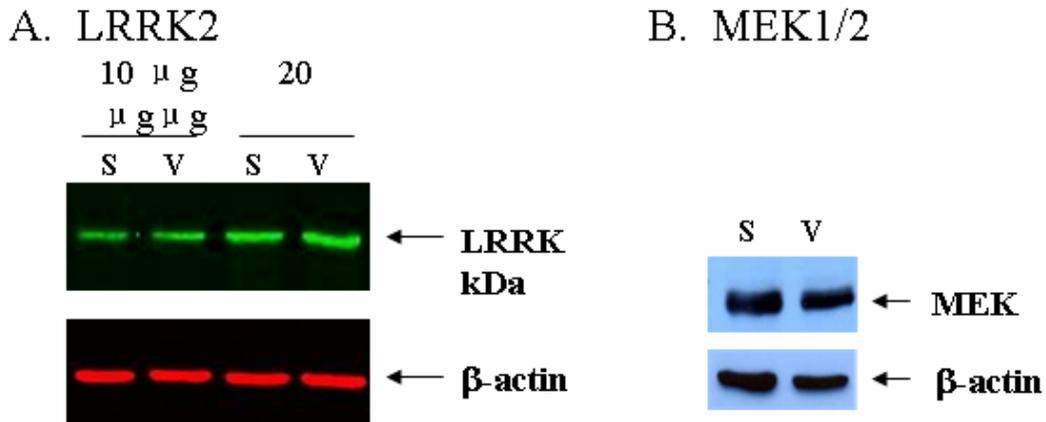


Fig. 4 Lack of effect of α -synuclein on LRRK2 (A) and MEK1/2 (B) protein expression. Western blot was performed on whole cell lysates obtained from α -synuclein-expressing (S) and vector control (V) N27 cells. β -Actin was used as a loading control. Overexpression of human α -synuclein did not have an effect on protein expression levels of LRRK2 (Abcam, ab37178, rabbit polyclonal) and MEK1/2. Both proteins have been implicated in the pathogenesis of PD.

In conclusion, α -synuclein expressing cells appear to be slightly resistant to cytotoxicity induced by acute exposure to dopaminergic toxins, probably by sequestering proapoptotic molecules PKC δ and BAD (Kaul et al., 2005a). However, the α -synuclein cells can also be more susceptible to apoptotic cell death induced by chronic exposure to dopaminergic toxins presumably because of impairment in UPS dysfunction, protein misfolding and aggregation (Sun et al., 2005). However, α -synuclein appears to be detrimental to dopamine neurons, considering the self-aggregation propensity of the protein during toxic insults and the possibility of chronic exposure to environmental factors, which is a dominant risk factor for PD.

**CHAPTER II: DIELDRIN INDUCES UBIQUITIN-PROTEASOMAL DYSFUNCTION
IN α -SYNUCLEIN OVEREXPRESSING DOPAMINERGIC NEURONAL CELLS
AND ENHANCES SUSCEPTIBILITY TO APOPTOTIC CELL DEATH**

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ABSTRACT

Exposure to pesticides is implicated in the etiopathogenesis of Parkinson's disease (PD). The organochlorine pesticide dieldrin is one of the environmental chemicals potentially linked to PD. Since recent evidence indicates that abnormal accumulation and aggregation of α -synuclein and ubiquitin-proteasome system (UPS) dysfunction can contribute to the degenerative processes of PD, in the present study we examined whether the environmental pesticide dieldrin impairs proteasomal function and subsequently promotes apoptotic cell death in rat mesencephalic dopaminergic neuronal cells overexpressing human α -synuclein. Overexpression of wild-type α -synuclein significantly reduced the proteasomal activity. Dieldrin exposure dose-dependently (0-70 μ M) decreased proteasomal activity, and 30 μ M dieldrin inhibited activity by more than 60% in α -synuclein cells. Confocal microscopic analysis of dieldrin treated α -synuclein cells revealed that α -synuclein positive protein aggregates colocalized with ubiquitin protein. Further characterization of the aggregates with the autophagosomal marker monodansyl cadaverine (MDC) and the lysosomal marker (LAMP-1) and dot-blot analysis revealed that these protein oligomeric aggregates were distinct from autophagosomes and lysosomes. The dieldrin-induced proteasomal dysfunction in α -synuclein cells was also confirmed by significant accumulation

of ubiquitin protein conjugates in the detergent-insoluble fraction. We found that proteasomal inhibition preceded cell death following dieldrin treatment and that α -synuclein cells were more sensitive than vector cells to the toxicity. Furthermore, measurement of caspase-3 and DNA fragmentation confirmed the enhanced sensitivity of α -synuclein cells to dieldrin-induced apoptosis. Collectively, our results suggest that increased expression of α -synuclein predisposes dopaminergic cells to proteasomal dysfunction, which can be further exacerbated by environmental exposure to certain neurotoxic compounds like dieldrin.

INTRODUCTION

Dieldrin, a long-lasting organochlorine pesticide, was widely used agriculturally before it was banned by the United States Environmental Protection Agency in 1974. The persistent accumulation of dieldrin in the environment as well as pesticide contaminated food remains a major source of dieldrin exposure to humans even 30 years after its use was banned (Kanthasamy et al., 2005). Dieldrin is highly lipophilic and therefore accumulates in lipid containing tissues including the CNS over a prolonged period of time, with a half life of approximately 300 days. A recent investigation by the Centers for Disease Control (CDC) showed very high serum levels and a high dietary consumption level of dieldrin in farmers and their spouses in Iowa (Brock et al., 1998). Dieldrin exposures to the general population occur through various food sources such as meat, milk products, fruits and fishes (Kanthasamy et al., 2005). A recent study reported a significant amount of dieldrin exposure through consumption of farm-raised salmon as compared to North Atlantic salmon (Hites et al., 2004).

One of the most common neurodegenerative diseases, Parkinson's disease (PD) affects over a million people in the USA, and the prevalence of the disease increases by approximately 70,000 individuals each year. Progressive and selective degeneration of dopaminergic neurons in the substantia nigra is characteristic of PD, which is accompanied by the formation of the cytoplasmic inclusions known as Lewy bodies (Dawson and Dawson, 2003). Although the causes and mechanisms underlying PD are not completely understood, accumulating evidences suggest that both environmental and genetic factors contribute to selective dopaminergic degeneration (Le Couteur et al., 2002). Among the genetic factors, at least 10 distinct loci are responsible for the familial forms of PD including mutations in α -synuclein gene (A53T, A30P and E46K), α -synuclein loci triplication, parkin, ubiquitin C-terminal hydrolase-L1 (UCHL1), DJ-1, PTEN induced kinase 1 (PINK1) and leucine-rich repeat kinase 2 (LRRK2) (Review: Dawson and Dawson, 2003; Moore DJ et al., 2005). Among the environmental factors, pesticides are one of the potential risk factors of Parkinson's disease, as revealed by recent epidemiological studies (Priyadarshi et al., 2000; Kanthasamy, 2005).

Dieldrin is implicated as one of the possible etiological factors for PD because of its detectable levels in the brains of some PD patients, but not in the brains of non-PD patients (Fleming et al., 1994); significantly higher levels of dieldrin were detected in the caudate and substantia nigra of PD patients compared to controls (Corrigan et al., 2000). Animal studies have demonstrated that feeding dieldrin resulted in significantly decreased dopamine levels in brains of doves (Heinz et al., 1980). The relatively selective toxicity of dieldrin to dopaminergic neurons over GABAergic neurons has been reported in primary cultured neurons (Sanchez-Ramos et al., 1998). Recently, we showed that dieldrin impairs

mitochondrial function and induces oxidative stress and apoptotic cell death in dopaminergic cells (Kitazawa et al., 2001). We also demonstrated that caspase-3-dependent proteolytic activation of the proapoptotic protein kinase PKC δ contributes to apoptotic cell death (Kitazawa et al., 2003), and that the DNA repair enzyme PARP is inactivated by proteolytic cleavage in PC12 cells challenged by dieldrin (Kitazawa et al., 2004).

Impairment of ubiquitin-proteasome function and protein aggregation is an emerging area of investigation because genetic analysis of familial PD cases has elucidated mutation of key genes including α -synuclein, Parkin and UCH-L1 (Le Couteur et al., 2002), some of which are important in protein processing and degradation. α -Synuclein has been identified as the major component of Lewy bodies in PD (Dawson and Dawson, 2003). Wild type α -synuclein in monomeric and aggregated forms has been reported to interact with the S6' subunit of 19S cap and inhibit proteasomal function (Snyder et al., 2003).

The recent discovery that an increased level of the α -synuclein gene due to the triplication of the α -synuclein locus causes PD in some individuals strongly suggests that overexpression of this gene could be a risk factor for PD (Singleton et al., 2003). The combination of α -synuclein overexpression and exposure to environmental pesticides may likely contribute to increased vulnerability of nigral dopaminergic neurons. Therefore, in the present study, we investigated the effect of dieldrin, a pesticide with suspected involvement in PD pathogenesis, on proteasomal function and apoptotic cell death in the α -synuclein overexpressing mesencephalic dopaminergic neuronal cell model (N27 cells).

MATERIALS AND METHODS

Chemicals. Dieldrin, lactacystin, thioflavin S, monodansyl cadaverine (MDC), Hoechst 33342 were purchased from Sigma Chemicals (St. Louis, MO). Methamphetamine was a generous gift from National Institute of Drug Abuse, Baltimore, Maryland. The substrate used to measure proteasomal activity, Suc-Leu-Leu-Val-Try-AMC, was purchased from Calbiochem (San Diego, CA). The caspase-3 substrate, Ac-DEVD-AMC, was obtained from Bachem Bioscience (King of Prussia, PA). An enhanced chemiluminescence (ECL) Western blotting analysis kit was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The Cell Death Detection ELISA Plus Assay Kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). RPMI 1640 medium, fetal bovine serum, L-glutamine, penicillin/streptomycin and hygromycin B were purchased from Invitrogen (San Diego, CA). Sytox green and Prolong antifade reagents were obtained from Molecular Probe (Eugene, OR). The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA).

Cell Culture and Stable Expression of α -Synuclein. The immortalized rat mesencephalic dopaminergic cell line (referred to as N27 cells) was a kind gift of Dr. Kedar N. Prasad, University of Colorado Health Sciences Center (Denver, CO). N27 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C as previously described (Kaul et al., 2003). The pCEP4 expression vector containing the full-length human α -synuclein sequence, α -synuclein-pCEP4, was kindly provided by Dr. Eliezer Masliah (UCSD, San Diego). α -Synuclein-pCEP4 and empty pCEP4 vector conferring

hygromycin resistance were transfected into N27 cells using Lipofectamine Plus reagent (Invitrogen, Gaithersburg, MD) by following the procedure recommended by the manufacturer and described recently (Kaul et al., 2005). For the stable transfection, N27 cells were selected in 400 $\mu\text{g/ml}$ hygromycin 48 h after transfection; supplementation of 200 $\mu\text{g/ml}$ hygromycin in the growth medium maintained the stable transfection.

Treatment Paradigm. Vector expressing N27 cells and α -synuclein expressing cells were treated with different concentrations of dieldrin or lactacystin dissolved in dimethyl sulfoxide (final concentration in the medium was no higher than 0.5%) for the duration of the experiments. After treatment, cells were collected by trypsinization, spun down at 200 x g for 5 min, and washed with ice cold phosphate buffered saline (PBS). The lysates from the cell pellets were used for various assays including proteasome peptidase activity, caspase-3 activity, and measurement of DNA fragmentation.

Proteasomal Peptidase Activity Assay. The proteasome enzymatic assay was performed as described previously (Snyder et al., 2003), with slight modification. Briefly, after treatment, cells were collected, washed and lysed with hypotonic buffer (10 mM HEPES, 5 mM MgCl_2 , 10 mM KCl, 1% sucrose, 0.1% CHAPS). The lysates were then incubated with fluorogenic Suc-LLVY-AMC (75 μM) in the assay buffer (50 mM Tris-HCl, 20 mM KCl, 5 mM MgOAc and 10 mM DTT, pH 7.6) at 37°C for 30 min. The cleaved fluorescent product was measured at the excitation wavelength of 380 nm and emission wavelength of 460 nm using a fluorescence plate reader (Gemini Plate Reader, Molecular Devices Corporation). The protein concentration was determined by the Bradford method. The enzymatic activity was

normalized by protein concentration. Lysates from cells treated with 10 μ M lactacystin for 12 h were used as the positive control for the assay.

Immunofluorescence Staining of Protein Aggregation. Immunofluorescence Staining of Protein Aggregation. Immunofluorescence staining was performed essentially as described previously (Lee et al., 2002a). Briefly, after dieldrin treatment, α -synuclein overexpressing N27 cells grown on coverslips pre-coated with poly-L-lysine were washed with PBS and fixed in 4% paraformaldehyde. Coverslips were then washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBS and then incubated with blocking buffer (5% BSA, 5% goat serum in PBS) to block the nonspecific binding sites. Thioflavin S staining was performed by incubating the cells with 0.4% Thioflavin S followed by washing with 80% alcohol before processing the cells for α -synuclein immunochemical analysis. For ubiquitin and α -synuclein double staining, cells were incubated overnight with antibodies against α -synuclein (Santa Cruz, CA, Mouse monoclonal Ab, 1:500) and ubiquitin (DAKO, Carpinteria CA, Rabbit polyclonal Ab, 1:100). α -Synuclein and ubiquitin were visualized with Cy3 conjugated goat anti-mouse and Alexa 488-conjugated goat anti-rabbit secondary antibodies, respectively. For visualization of α -synuclein aggregates and lysosomes (Wilson et al., 2004), polyclonal α -synuclein antibody (Biomol, PA, 1:500) and monoclonal LAMP-1 antibody (Calbiochem, CA, 1:500) were used. Cy3-conjugated anti-rabbit and Alexa 488-conjugated anti-mouse antibodies were used for the visualization of α -synuclein and LAMP-1 respectively. Nuclei were counterstained with Hoechst 33342 for 3 min at final concentration of 10 μ g/ml. Finally, cells were washed once in PBS and mounted onto a slide

with mounting medium containing Prolong antifade reagent. In cases of autophagosome staining, live cells were incubated with 50 μ M monodansyl cadaverine (MDC, Sigma) for 10 min at 37°C prior to fixation and α -synuclein immunostaining (Larsen et al., 2002). The images were analyzed by either Nikon C1 confocal microscopy (Model TE-2000U) or by Leica confocal microscopy (Model TCS NT). Areas of α -synuclein immunopositive aggregates were measured in 14 randomly chosen cells from each group using Metamorph 5.07 image analysis software.

Western Blot Analysis of Ubiquitin-conjugated Proteins. Low-detergent soluble and insoluble fractions were separated according to the procedure described previously, with slight modification (Rideout and Stefanis, 2002). After exposure to dieldrin or lactacystin, cells were collected and washed once with ice-cold PBS. The cell pellets were resuspended in a low-detergent lysis buffer (protease inhibitors and 0.5% Triton X-100 in PBS). The lysates were ultracentrifuged at 100,000 x g for 40 min. The detergent soluble fraction was obtained by collecting the resulting supernatant. The detergent insoluble pellets were washed once with the lysis buffer and resuspended in PBS containing protease inhibitors and 2% SDS and then sonicated for 20 seconds. Equal amounts of protein from the detergent soluble and equal volumes of the suspension of the detergent insoluble fractions were resolved on 8% SDS-PAGE and transferred onto nitrocellulose membrane. Nonspecific binding to the membranes was blocked by 5% non-fat milk blocking solution and then the membranes were probed with ubiquitin antibody (DAKO, 1:500) overnight at 4 °C. Incubation with HRP-conjugated secondary anti-rabbit or anti-mouse IgG (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, 1:2000) for an additional 1 h was followed by the detection of the antibody-

bound proteins using the ECL detection kit. The membranes were reprobed with β -actin antibody (Sigma Chemicals, St. Louis, MO, mouse monoclonal, 1:5000) to confirm equal protein loading. The densitometric analysis of ubiquitin conjugates was performed with Kodak 1D image analysis software.

Dot-blot Analysis. Formation of protein oligomers was determined by dot-blot measurements using A11 antibody (rabbit polyclonal, BioSource, Camarillo CA) which recognizes oligomers of proteins independent of amino acid sequence (Kayed et al., 2003). Dot-blot analysis using this antibody has recently been used for identification of protein oligomers in various neurodegenerative models (Glabe, 2004; Kayed et al., 2003). The procedure for dot-blot analysis was followed as described by the manufacturer. Briefly, vector and α -synuclein cells were harvested after dieldrin treatment and cell lysates were prepared as described above for Western blots. Cell lysates containing equal protein (5-15 μ g) were spotted on the nitrocellulose membrane and air dried for 30 min. Membranes were incubated with A11 anti-oligomer antibody (dilution 1:2000) for 1 h at room temperature and then treated with HRP-conjugated secondary anti-rabbit IgG for 1 h. Antibody-bound proteins were detected with an ECL detection kit, and densitometric analysis of dots representing the oligomeric protein aggregates was performed with Kodak 1D image analysis software.

Assessment of Cell Death by Sytox Green. Cell death was assessed with Sytox green, a membrane-impermeable DNA dye which enters dead cells due to altered membrane permeability and intercalates into the nucleic acid (Kaul et al., 2005). DNA bound Sytox

green can be detected at excitation wavelength of 485 nm and emission wavelength of 538 nm using a fluorescence microplate reader (Molecular Devices Corporation, Gemini Plate Reader). Cells grown in 24-well plates were incubated with 1 μ M Sytox green for 20 min, and then treated with 30 μ M dieldrin or vehicle as a control. To quantify cell death, fluorescence intensity was monitored after the experiments were conducted and normalized by the time-matched control.

Caspase-3 Enzymatic Activity Assay. Caspase-3 activity was measured as previously described (Kitazawa et al., 2003). Briefly, cell lysates were obtained by suspending the cells in 50 mM Tris-HCl lysis buffer containing 1 mM EDTA, 10 mM EGTA and 10 μ M digitonin after cells were collected and washed with PBS. The supernatants from lysates collected after centrifugation at 14,000 x g were incubated with 50 μ M Ac-DEVD-AMC at 37°C for 1 h, and caspase-3 activity was measured using a fluorescence plate reader (Molecular Devices Corporation) with excitation at 380 nm and emission at 460 nm. Protein concentration was determined by the Bradford protein assay.

DNA Fragmentation Assay. DNA fragmentation was measured using a Cell Death Detection ELISA Plus Assay Kit as described previously (Kaul et al., 2003). This method measures the amount of histone-associated low molecular weight DNA in the cytoplasm of cells and is more sensitive than DNA ladder analysis. Dieldrin treated cells were washed with PBS, and the cell pellets were then resuspended with the lysis buffer provided in the assay kit. The lysate was spun down at 200 x g, and 20 μ l of supernatant was incubated for 2 h with the mixture of HRP-conjugated antibody cocktail that recognizes histones, single and

double-stranded DNA. After washing away the unbound components, the final reaction product was measured colorimetrically with ABTS as an HRP substrate using a spectrophotometer at 405 nm and 490 nm. The difference in absorbance between 405 and 490 nm was used to determine the amount of DNA fragmentation in each sample.

Data Analysis. Data are presented as mean \pm S.E.M., and the data analysis was performed with Prism 3.0 software (GraphPad software, San Diego). p Values were determined by one-way ANOVA followed by either Dunnett's post test to compare dieldrin treatment groups with the control group or by Bonferroni's Multiple Comparison Test to compare all pairs of groups. Single comparisons were made using the Student's t-test. A significant difference was accepted if $p < 0.05$.

RESULTS

α -Synuclein Overexpression Impairs Proteasomal Activity in Dopaminergic Neuronal Cells. First we examined the effect of overexpression of human α -synuclein on proteasomal activity in mesencephalic rat dopaminergic neuronal cells (N27 cells). Stable expression of human α -synuclein in N27 cells was determined by Western blot using an antibody that recognizes only the exogenously expressed human α -synuclein or an antibody that detects both exogenously expressed human α -synuclein and endogenous rat α -synuclein as described previously (Kaul et al., 2005). There was an 11-fold increase in stable α -synuclein expression compared to vector-expressing N27 cells (data not shown). Enzymatic activity of 20S/26S proteasome was evaluated in α -synuclein overexpressing and vector

transfected N27 cells with the specific fluorogenic substrate Suc-LLVY-AMC. As shown in Fig. 1, α -synuclein overexpression significantly ($p < 0.001$) inhibited proteasomal activity as compared to vector expressing cells. Almost 50% reduction in proteasomal activity was observed in α -synuclein overexpressing cells. The well known proteasome inhibitor lactacystin (10 μ M) was used as a positive control which inhibited over 90% of proteasomal activity in N27 cells.

Dieldrin Impairs Proteasomal Activity in a Dose- and Time-dependent Manner.

Next we examined the effect of dieldrin exposure on proteasomal activity in vector and α -synuclein overexpressing N27 dopaminergic neuronal cells. Fig. 2A shows a dose-dependent decrease in the proteasomal activity in both vector cells and α -synuclein overexpressing cells following 0-70 μ M dieldrin exposure for 24 h. The EC_{50} values for vector and α -synuclein overexpressing cells were 50 μ M and 32 μ M, respectively. Exposure to 30 μ M of dieldrin for 12 and 24 h caused significant proteasomal dysfunction in both vector and α -synuclein transfected cells, so this dose was used for further studies. We conducted a detailed time course analysis of proteasomal activity following 30 μ M dieldrin exposure to determine the earliest time point at which dieldrin impairs proteasomal activity (Fig. 2B). Proteasomal activity significantly decreased within 12 h in vector transfected and α -synuclein overexpressing cells ($p < 0.01$), and remained reduced during the entire treatment period (Fig. 2B).

To determine if the effects of dieldrin and α -synuclein on proteasome activity are additive or synergistic, we exposed vector cells to 300 nM lactacystin for 3 h to reduce the

baseline UPS activity to the level of α -synuclein expressing cells, then these cells were incubated with 30 μ M dieldrin for an additional 24 h and measured UPS activity. As shown in Fig 2C, exposure of vector cells to 300 nM lactacystin decreased the UPS activity by 53%, which approximated the baseline UPS activity in α -synuclein expressing cells. A 24 h exposure to 30 μ M dieldrin further decreased UPS activity by 18% and 13% in vector cells and in lactacystin-treated vector cells, respectively. Since the extent of UPS inhibition by dieldrin is similar in vector and α -synuclein cells, these results suggest that the effects of α -synuclein and dieldrin are additive and not synergistic.

Dieldrin Exposure Induces the Formation of Intracellular Inclusions Containing α -Synuclein. The biological consequences of impaired proteasomal function are accumulation of proteins, formation of intracellular protein inclusions and upregulation of ubiquitin-conjugated proteins. Accumulation of α -synuclein in the form of intracellular inclusions and increased levels of ubiquitinated protein are typical pathological changes associated with PD. In this study, we examined whether the inhibition of proteasomal function by dieldrin exposure promotes the formation of intracellular protein inclusions and ubiquitinated protein accumulation. Confocal analysis revealed the formation of α -synuclein positive aggregation in a time-dependent manner, with small aggregates appearing as early as 12 h and progressively increasing over 24 h in dieldrin-treated cells (Fig. 3A). Vehicle only treated cells for a period of 24 h did not show any significant formation of α -synuclein aggregates. However, as anticipated, the proteasome inhibitor lactacystin induced a profound aggregation in α -synuclein overexpressing cells (data not shown). Quantitative analysis of

α -synuclein positive aggregates using Metamorph image analysis software is shown in Fig 3B. The results show that dieldrin exposure for 12 and 24 h significantly increased the number of intracellular inclusion bodies (Fig. 3B). In this experiment, we observed that the protein aggregates were negative for thioflavin-S staining. Furthermore, we did not observe any protein inclusions in either dieldrin-treated vector cells or untreated α -synuclein cells. We attribute this finding to recent reports which suggest thioflavin-S stains large perinuclear inclusions but not the small oligomeric aggregates (Lee et al., 2002a).

Dieldrin induces the formation of soluble oligomer proteins in α -synuclein cells.

Neurodegenerative diseases are associated with the accumulation of misfolded proteins in the form of fibrillar protein aggregates (Kayed et al., 2003; Glabe, 2004). The formation of oligomers from misfolded monomeric proteins has been suggested to precede fibrillar formation. Recent development of an antibody A11 which recognizes amino acid sequence-independent oligomers of proteins including β -amyloid, α -synuclein, polyglutamine proteins and prion peptide 106-126 has enabled the determination of protein oligomers in many experimental neurodegenerative models (Kayed et al., 2003; Glabe, 2004). Thus, in order to further verify dieldrin induced protein aggregation, we utilized the A11 antibody in dot-blot experiments. Vector and α -synuclein cells were exposed to 30 μ M dieldrin for 12 h and 24 h. As shown in Fig. 4A, dieldrin treatment for 12 and 24 h significantly increased the protein oligomer levels in α -synuclein cells. The vector treated cells showed only a background staining. Densitometric analysis of the dot-blot revealed 170% and 217% increases in oligomeric protein staining at 12 and 24 hr, respectively, in dieldrin treated α -synuclein

expressing cells (Fig. 4B) as compared to 91% and 115% increases in dieldrin treated vector cells. Taken together, these results with confocal experiments indicate that dieldrin induces protein aggregates in α -synuclein expressing cells.

Dieldrin-induced α -Synuclein Inclusions Colocalize with Ubiquitin but not with Autophagosomes or Lysosomes. Since dieldrin treatment impairs UPS activity, we also determined whether ubiquitin colocalizes with α -synuclein inclusions. Confocal microscopic analysis of anti-ubiquitin immunohistochemical images revealed that the α -synuclein positive aggregates tended to be ubiquitin immunoreactive, as seen by the colocalization of ubiquitin with α -synuclein positive aggregates (Fig. 5A). To further distinguish the α -synuclein inclusions from autophagosomes, we performed autophagosome marker monodansyl cadaverine (MDC) staining. As shown in Fig. 5B, dieldrin-treated cells showed a moderate increase in autophagic vacuoles but these vacuoles did not colocalize with α -synuclein aggregates, indicating that the protein aggregates observed following dieldrin treatments are not autophagosomes. Methamphetamine was used as a positive control because it induced autophagy in mouse primary dopaminergic neurons (Larsen et al., 2002) and in the N27 dopaminergic cell model used in this study (unpublished observations). MDC labeling revealed large autophagic vacuoles in methamphetamine-treated cells (Fig. 5B), and these vacuoles clearly excluded the α -synuclein staining. To further distinguish the α -synuclein aggregates from enlarged lysosomes (Wilson et al., 2004), we performed lysosomal marker LAMP-1 immunohistochemical analysis. As shown in Fig. 5C, confocal microscopic analysis of anti-LAMP-1 immunocytochemical images revealed that dieldrin-

induced α -synuclein positive aggregates did not colocalize with LAMP-1 immunoreactivity, as demonstrated by the distinct staining patterns of LAMP-1 and α -synuclein aggregates.

Accumulation of High Molecular Weight (HMW) Ubiquitin-conjugated Proteins during Dieldrin Exposure. Impairment of the proteasomal machinery leads to accumulation of ubiquitinated protein in the cytosol due to the reduced clearance of proteins by the proteasome (Rideout and Stefanis, 2002). High molecular weight ubiquitin-conjugated proteins accumulated dramatically in the low detergent-insoluble fraction from both vector and α -synuclein overexpressing cells following 24 h of dieldrin exposure (Fig. 6A). However, the accumulation of insoluble HMG ubiquitin-conjugates in α -synuclein overexpressing cells was much higher than in vector transfected cells. Densitometric analysis of the HMW bands from Western blots revealed 136% and 121% of the proteins in vector cells as compared to 172% and 301% in α -synuclein cells following 30 μ M dieldrin treatment for 12 and 24 h, respectively (Fig. 6B). The slight decrease in HMG ubiquitin conjugates in vector cells at 24 h measured by Western blot was not statistically significant. In addition to the HMW ubiquitin conjugates in insoluble fractions, dieldrin treatment also produced a significant increase in the level of HMW ubiquitin conjugates in the soluble fraction of both α -synuclein cells and vector transfected cells (Fig. 6C). However, the level of ubiquitin conjugates did not differ significantly between α -synuclein and vector cells (Fig. 6D). These results suggest that dieldrin induces a time-dependent increase in insoluble HMW ubiquitin conjugates in vector and α -synuclein cells. Treatment with 10 μ M lactacystin for

12 h dramatically increased the level of the HMW ubiquitin conjugates in both soluble and insoluble fractions, and was used as a positive control (Fig. 6A, B).

Overexpression of α -Synuclein Increases the Sensitivity of N27 Cells to Dieldrin-induced Neurotoxicity. In order to determine whether the formation of insoluble protein aggregates and accumulation of ubiquitin proteins during dieldrin treatments enhances the neurodegenerative processes in α -synuclein overexpressing cells, we investigated the temporal dieldrin cytotoxicity in vector and α -synuclein overexpressing cells. Cell death was assessed using the membrane impermeable Sytox green fluorescence dye at various time intervals following 24 h of dieldrin treatment. As shown in Fig. 7, α -synuclein overexpressing cells showed enhanced cytotoxicity at 24 h, whereas cytotoxicity was not significantly increased in vector transfected cells up to 24 h. We also noted a consistent decrease in dieldrin-induced cytotoxicity in α -synuclein overexpressing cells as compared to vector cells up to 12 hr time point.

Dieldrin Induced Caspase-3 Activation and DNA Fragmentation in Vector and α -Synuclein Overexpressing Cells. To determine the functional consequence of dieldrin-induced proteasomal inhibition and protein aggregation on cell survival, we determined caspase-3 activation and DNA fragmentation following dieldrin exposure. As shown in Fig. 8A, measurement of caspase-3 activity using Ac-DEVD-AMC as a substrate revealed that dieldrin activated caspase-3 to 205.1% at 12 h and 311.5% at 24 h in α -synuclein overexpressing N27 cells, whereas only a minimal increase of 138.1% and 168.5% was

observed in vector cells at 12 and 24 h post-treatment, respectively. Also, the classical proteasome inhibitor lactacystin dramatically increased caspase-3 activation (Fig. 8A).

Furthermore, to determine the extent of apoptotic cell death in dopaminergic neuronal cells following dieldrin exposure, we measured DNA fragmentation using the ELISA method. As shown in Fig. 8B, dieldrin significantly (* $p < 0.05$; *** $p < 0.001$) increased DNA fragmentation in both vector and α -synuclein overexpressing cells, but dieldrin-induced DNA fragmentation was more pronounced in α -synuclein overexpressing cells than in vector cells (## $p < 0.01$), indicating that α -synuclein overexpressing cells are more sensitive to apoptotic cell death.

DISCUSSION

Our study clearly demonstrates that dieldrin impairs ubiquitin-proteasome function additively with α -synuclein and triggers apoptosis in dopaminergic neuronal cells. The direct consequence of the proteasome inhibition is the abnormal accumulation of ubiquitinated protein and the formation of intracellular protein aggregates (Rideout and Stefanis, 2002). In this study, we showed that dieldrin exposure promotes the formation of the α -synuclein positive intracellular inclusions and accumulation of high molecular weight ubiquitin conjugated proteins. Overall, our data suggest that the combination of environmental exposure to neurotoxic chemicals like pesticides and α -synuclein overexpression may enhance the susceptibility to apoptotic cell death by impairing the ubiquitin-proteasome system.

Epidemiological studies increasingly implicate pesticides as an important risk factor of PD (Priyadarshi et al., 2000) and suggest that exposure to the organochlorine class of

pesticide may be of particular concern in the development of PD (Corrigan et al., 2000; Kanthasamy et al., 2005). The specific organochlorine pesticide dieldrin has been implicated in PD by epidemiological studies and case control findings in PD brains (Fleming et al., 1994; Corrigan et al., 2000) and experimental studies in cell culture (Sanchez-Ramos et al., 1998; Kitazawa et al., 2001; Kitazawa et al., 2003; Kitazawa et al., 2004) and animal models (Heinz et al., 1980; Kanthasamy et al., 2005). Dieldrin is a highly lipophilic compound and accumulates significantly in the CNS (Fleming et al., 1994; Corrigan et al., 2000). The concentration of dieldrin detected in postmortem PD brain tissue was approximately 50 ppm (Fleming et al., 1994), and the blood dieldrin level was up to 250 ng/ml in workers manufacturing or using aldrin/dieldrin (Nair et al., 1992). A high level of dieldrin accumulates in the body over a lifetime because of the extremely low clearance of this lipophilic neurotoxic compound from the body. Based on reports (MacIntosh et al., 1996; Doong et al., 1999; Campoy et al., 2001), we calculated the cumulative lifetime exposure to dieldrin of approximately 30 μM . We deduced this based on a daily dietary intake of 1.3 μg dieldrin over a 50 year-exposure period, resulting in intake of 41 μM . The 30 μM concentrations used in the present study are lower than concentrations used in previous neurotoxicological studies in the PC12 cell culture model (Kitazawa et al., 2001; Kitazawa et al., 2003; Kitazawa et al., 2004). The EC_{50} of 12 μM dieldrin for primary dopaminergic neurons and 85 μM for non-dopaminergic neurons (Sanchez-Ramos et al., 1998) indicates an increased susceptibility of the dopaminergic system to the neurotoxic effect of dieldrin.

We demonstrate that exposure to a subtoxic concentration of dieldrin (30 μM dieldrin, 11.2 $\mu\text{g}/\text{ml}$) resulted in decreased proteasomal activity and the subsequent accumulation of ubiquitin-conjugated proteins and formation of α -synuclein/ubiquitin

immunopositive inclusions in α -synuclein overexpressing cells. We also show that dieldrin induces formation of oligomeric aggregates in a time-dependent manner in α -synuclein expressing cells. Although our results demonstrate that dieldrin-induced α -synuclein positive aggregates do not colocalize with either autophagosomes or lysosomes for up to 24 h of dieldrin treatment, prolonged accumulation of protein aggregates following chronic exposure of dieldrin may be degraded via the lysosomal pathway. Additionally, our results are in agreement with a recent study demonstrating that a prolonged exposure to dieldrin can change the conformation of α -synuclein to produce protein fibrils in a cell free system (Uversky et al., 2001). Additionally, an interaction between filamentous α -synuclein and subunits of the 20S proteasome core has been shown to decrease its proteolytic activity (Lindersson et al., 2004). Our study clearly suggests that α -synuclein contributes to the dysfunction of the ubiquitin-proteasome system (UPS) since accumulated insoluble HMW ubiquitin-conjugated proteins were predominantly observed only in α -synuclein overexpressing N27 cells but not in vector N27 cells during dieldrin treatment. Previous studies have shown that the overexpression of α -synuclein A53T mutant impairs UPS in cultured cells (Lee et al., 2002b). Recently, we demonstrated that overexpression of A53T α -synuclein mutant potentiates MPP⁺-induced apoptotic cell death in N27 cells (Kaul et al., 2005). Therefore, it is likely that overexpression of A53T mutant may result in greater dieldrin-induced UPS dysfunction than impairment in wild type α -synuclein expressing cells.

Recently, oxidative stress and nitrative stress have been shown to contribute to various neurodegenerative diseases including PD (Ischiropoulos, 2003). In support of this view, studies have demonstrated that reactive oxygen species and reactive nitrogen species

formation can promote α -synuclein aggregation in *in vitro* models (Paxinou et al., 2001; Ischiropoulos, 2003). Since ROS generation and oxidative stress have also been linked to proteasome inhibition (Okada et al., 1999), dieldrin-induced oxidative stress may play a role in the impairment of proteasomal function (Kitazawa et al., 2001). However, the antioxidant trolox and the SOD mimetic MnTBAP did not block dieldrin-induced UPS impairment in preliminary studies (unpublished observations). Recently, administration of paraquat, a pesticide known to generate superoxide radicals induced α -synuclein aggregation in transgenic mice models (Manning-Bog et al., 2002). ATP depletion caused by the mitochondrial complex I inhibitor rotenone reduced proteasomal activity and formation of intracellular inclusions, which could be prevented by promoting ATP production (Hoglinger et al., 2003). Dieldrin is a mitochondrial electron transport inhibitor, which inevitably impairs oxidative ATP production (Kanthasamy et al., 2005). Therefore, a reduction in energy production may also contribute to proteasomal dysfunction during dieldrin exposure. However, further mechanistic studies are needed to establish the exact cellular mechanism underlying dieldrin-induced proteasomal dysfunction in dopaminergic neurons.

The impairment of proteasome function and protein aggregation during dieldrin treatment contributes to cell death. A time course analysis revealed that the proteasomal dysfunction and protein aggregation precedes the cellular toxicity. The α -synuclein aggregation starts to occur as early as 6 h following dieldrin treatment, whereas significant toxicity is noted only after 12 h of dieldrin treatment. Additionally, dieldrin-induced cell death occurs only in α -synuclein overexpressing N27 cells but not in vector N27 cells, suggesting that α -synuclein aggregation plays a causal role in the cytotoxic response. The time course analysis of the apoptotic marker caspase-3 further indicates that dieldrin-induced

protein aggregation precedes caspase-3 activation. Additionally, we showed that the selective proteasome inhibitor lactacystin activates caspase-3. Measurement of DNA fragmentation by ELISA following dieldrin treatment in α -synuclein overexpressing dopaminergic cells indicates that dieldrin-induced protein aggregation promotes apoptotic cell death. We also noted an increase in autophagosomes following dieldrin treatment, but detailed studies are needed regarding the individual contributions of apoptosis and autophagy during dieldrin-induced cell death.

The exact proapoptotic mechanisms downstream of the proteasome inhibition remain unclear in neuronal cells, though various signaling molecules involved in the regulation of apoptotic cell death have been identified as substrates of UPS, including p53, I κ B, Smac, the Bcl 2 family of proteins (Jesenberger and Jentsch, 2002). The UPS is the major cellular proteolytic machinery for the degradation of intracellular proteins. Identification of the mutant Parkin and ubiquitin C-terminal hydrolase (UCH-L1) genes in familial PD, as well as the impaired function and altered component levels of proteasome in the substantia nigra region of sporadic PD patients together suggest a critical role of UPS dysfunction in PD. Dopaminergic neurons are particularly susceptible to proteasome inhibition, and α -synuclein fibrillar inclusion is a characteristic pathological feature of PD (Dawson and Dawson, 2003). A recent study in transgenic flies revealed that overexpression of one of the Parkin substrates, Pael-R, caused selective degeneration of dopaminergic neurons, which could be suppressed by the coexpression of Parkin, which has E3 ligase activity (Yang et al., 2003). Recently, administration of the proteasome inhibitor epoxomicin in rats has been shown to induce delayed symptoms and pathology similar to PD (McNaught et al., 2004). Notably, impaired proteasome function and formation of Lewy bodies were observed in this model,

indicating enhanced vulnerability of the dopaminergic system to impairment of UPS. Recently, the interesting discovery of multiple copies of the α -synuclein gene in some PD patients (Singleton et al., 2003) suggests that overexpression of α -synuclein can increase the risk of dopaminergic generation, and it was shown that in wild-type human α -synuclein transgenic mice, the loss of dopaminergic terminals was accompanied by the formation of intracellular inclusions (Masliah et al., 2000). Our data demonstrate that overexpression of human α -synuclein can dramatically inhibit proteasomal activity in dopaminergic neuronal cells. Importantly, our results also suggest that exposure to environmental chemicals in individuals with increased copies of α -synuclein may enhance their vulnerability to PD.

In summary, we demonstrate for the first time that dieldrin and α -synuclein-cumulatively induce impairment of ubiquitin-proteasome function to promote apoptotic cell death in dopaminergic neuronal cells. This study also reveals a close interaction between environmental factors and genetic defects in the promotion of dopaminergic degeneration involved in PD.

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FIGURE 1.

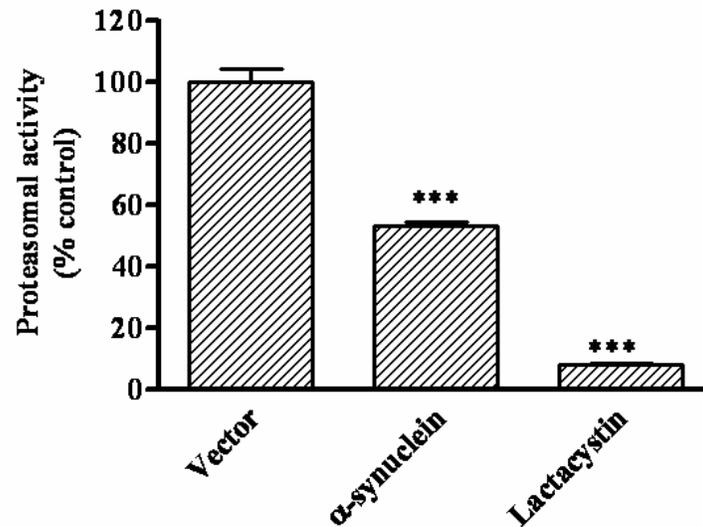


Fig. 1. Overexpression of α -synuclein inhibits proteasomal activity in dopaminergic neuronal cells (N27 cells).

Proteasomal activity was measured in vector and α -synuclein overexpressing N27 cells using a specific fluorogenic peptide substrate, Suc-LLVY-AMC (75 μ M), as described in the methods section. Activity was normalized by the protein concentration and expressed as the percentage of vehicle treated vector control cells. All data represent the mean \pm S.E.M. for six samples in each group. Asterisks (***) p <0.001, Student's t-test) indicate statistically significant differences compared with vector transfected N27 cells.

FIGURE 2.

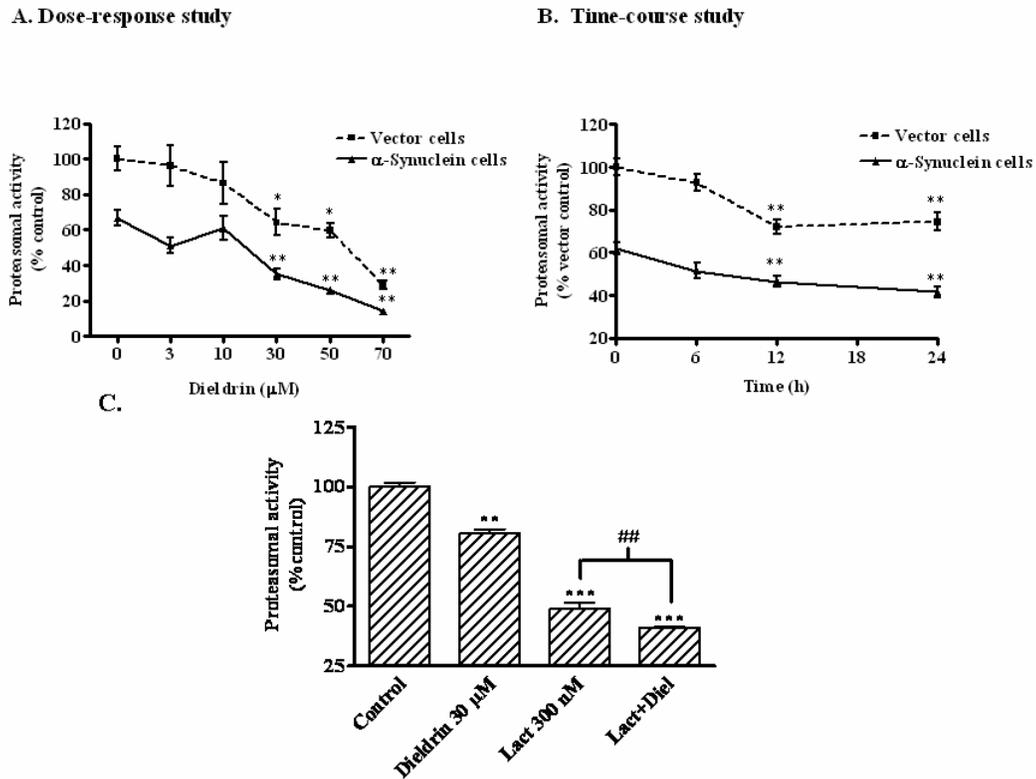


Fig. 2. Dieldrin exposure decreases proteasomal activity in dopaminergic neuronal cells.

A) Dose response studies. Vector transfected and α -synuclein overexpressing N27 cells were exposed to various concentrations of dieldrin ranging from 3 μM to 70 μM for 24 h. Proteasomal activity was measured using the fluorogenic substrate Suc-LLVY-AMC (75 μM). Enzymatic activity was normalized by protein concentration and expressed as the percentage of vehicle treated vector control cells. The data represent mean \pm S.E.M. from six samples in each group. B) Time-course studies. Vector transfected and α -synuclein

overexpressing N27 cells were exposed to 30 μ M dieldrin and the proteasomal activity was measured at 6, 12 or 24 h post-exposure. The values represent mean \pm S.E.M. from two to three separate experiments. C). Cumulative inhibition of proteasomal activity. Vector transfected N27 cells were pretreated with 300 nM lactacystin for 3 h and then exposed to 30 μ M dieldrin for an additional 24 h. Cells were also treated with lactacystin or dieldrin alone for 24 h. After treatment samples were processed for proteasomal activity as described in the methods section. The data represent mean \pm S.E.M. from six samples in each group. Statistical significance between the control group and each treatment group was determined by ANOVA followed by Dunnett's post-test (* p <0.05, ** p <0.01, *** p <0.001). Student's t -test was used for comparison between indicated groups (## p <0.01).

FIGURE 3.

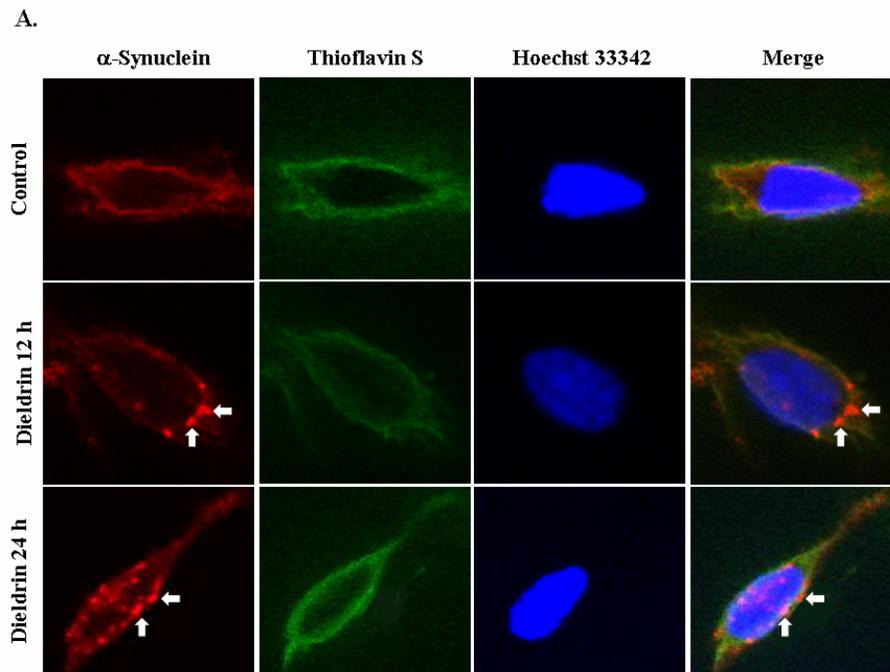
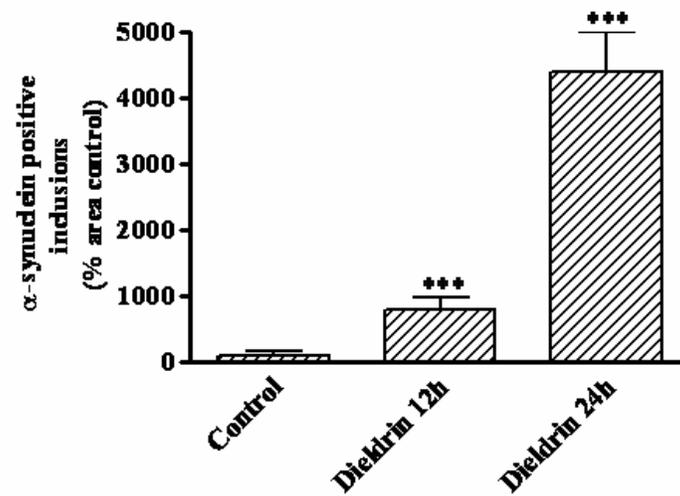


Fig. 3. Dieldrin treatment induces α -synuclein positive protein aggregates in dopaminergic neuronal cells.

B.



A) Confocal image analysis of α -synuclein and Thioflavin-S staining. Arrows indicate the α -synuclein positive inclusions. Briefly, α -synuclein overexpressing N27 cells grown on poly-lysine coated coverslips were treated with 30 μ M dieldrin for 12 or 24 h. Immunocytochemistry was performed using mouse monoclonal α -synuclein primary antibody and Cy3 conjugated secondary antibody (red fluorescence). Protein aggregation was also stained by Thioflavin-S. Arrows indicate the α -synuclein positive inclusions. B) Quantitative analysis of α -synuclein positive protein aggregates. Areas of α -synuclein immunopositive aggregates were measured in 14 randomly chosen cells from each group with Metomorph image analysis software. Statistical significance between the control group and each treatment group was determined by ANOVA followed by Dunnett's post-test (** $p < 0.001$).

FIGURE 4.

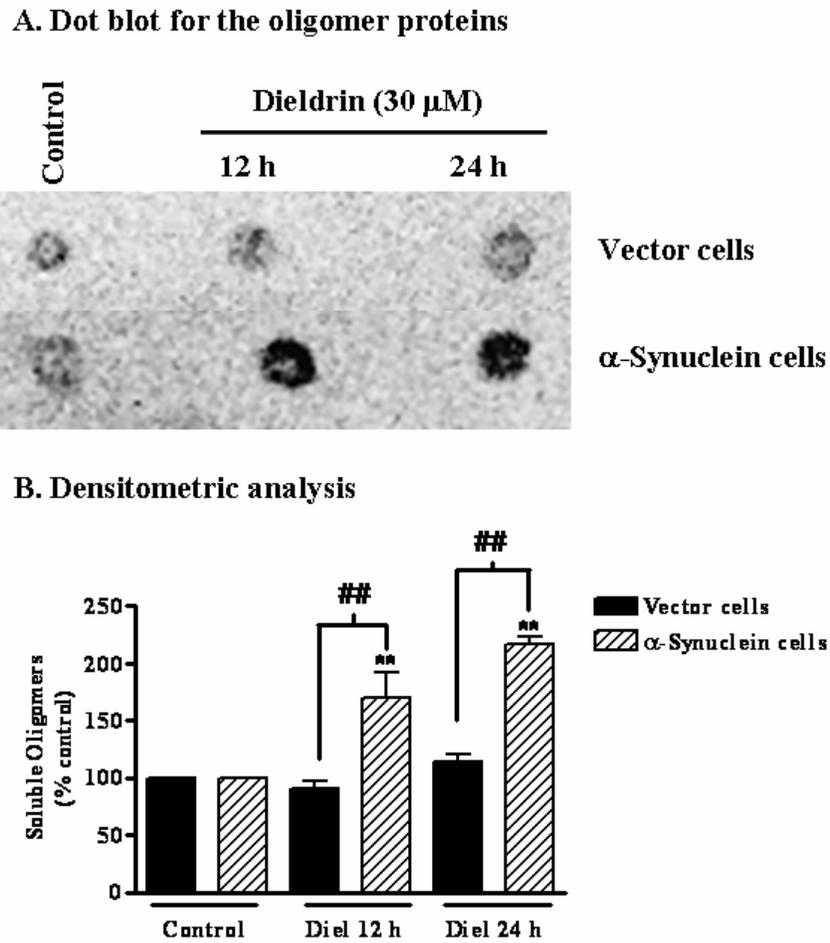
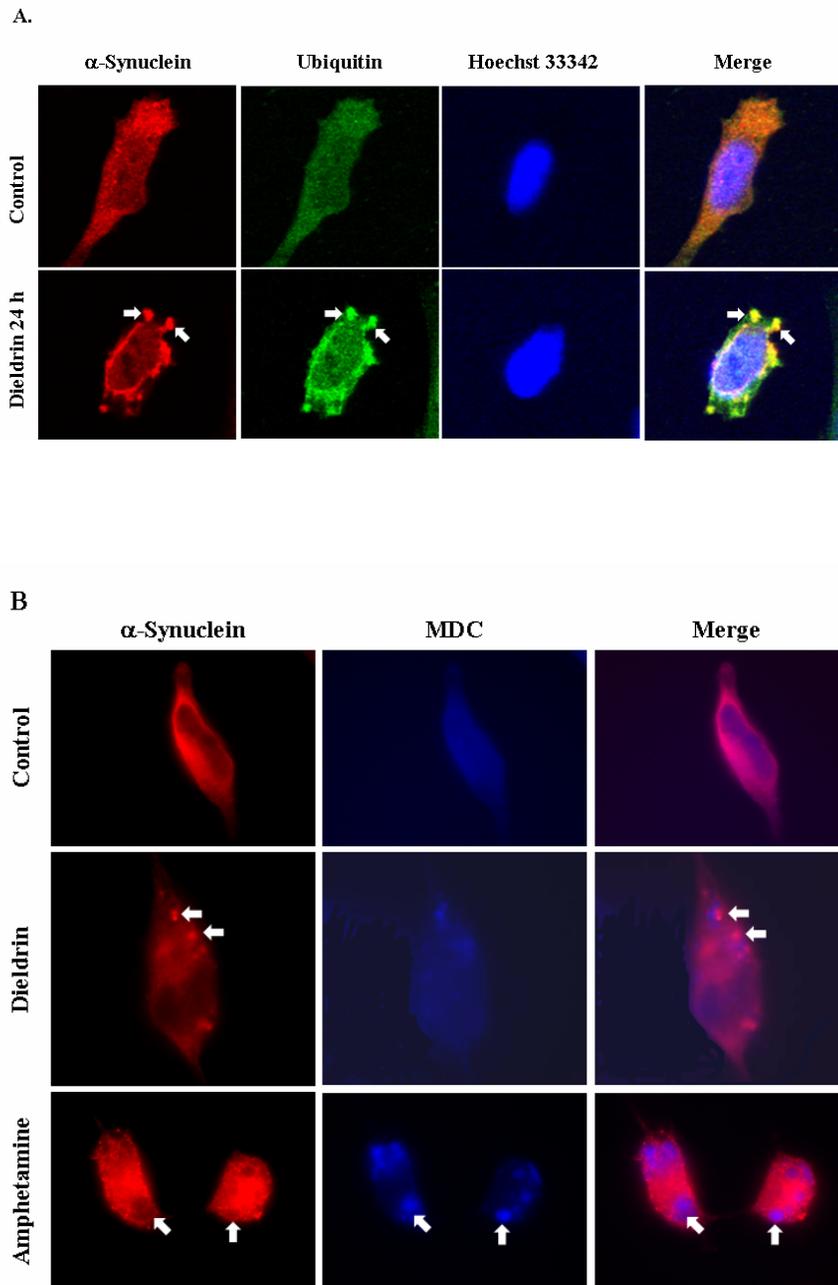


Fig. 4. Effect of dieldrin on formation of oligomeric protein in α -synuclein cells. A) Dot-blot analysis.

A) Vector and α -synuclein overexpressing cells were treated with 30 μ M dieldrin for 12 or 24 h and the formation of protein oligomers in these samples was determined by dot-blot analysis as described in the methods section. Cell lysates containing 5 μ g protein were

spotted on to nitrocellulose membrane, and protein oligomers were detected using A11 anti-oligomer antibody and followed by ECL detection. B) Densitometric analysis of the dot-blot. The levels of soluble oligomers were quantified by densitometry followed by statistical analysis using one-way ANOVA and Dunnett's multiple comparison tests (N=5). Asterisk indicates ** $p < 0.01$ significant differences between untreated cells and the dieldrin treatment groups at each time point, and pound sign ## $p < 0.01$ indicates significant differences between dieldrin treated vector cells and dieldrin treated α -synuclein cells.

FIGURE 5.



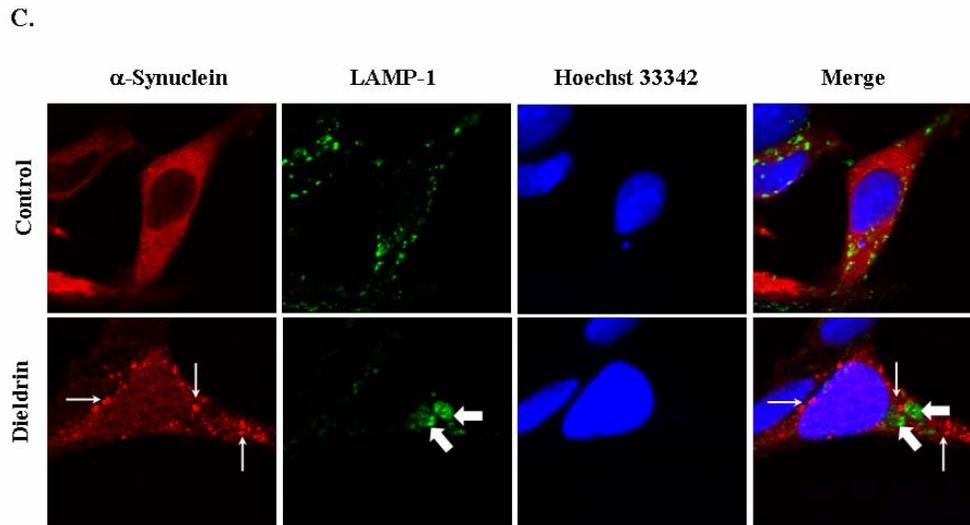


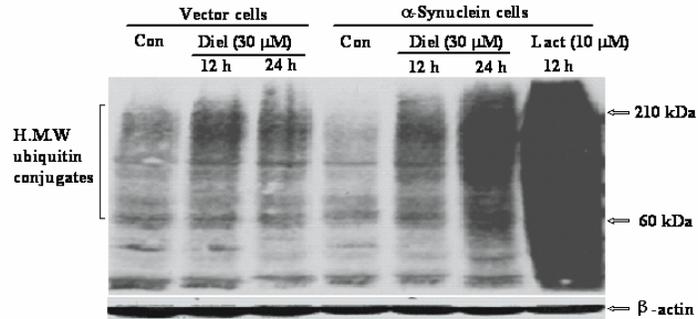
Fig. 5. α -Synuclein protein aggregates colocalize with ubiquitin but not with autophagosomes or lysosomes.

A) α -Synuclein and ubiquitin double staining. Double immunohistochemical analysis was performed using mouse monoclonal α -synuclein primary antibody and rabbit polyclonal ubiquitin antibody. The secondary antibodies used were Cy3 conjugated anti-mouse (red = α -synuclein) and Alexa-488 conjugated anti-rabbit (green = ubiquitin). Confocal image analysis of α -synuclein and ubiquitin staining was performed in the colocalization study. Arrows indicate the α -synuclein and ubiquitin positive inclusions. B). MDC autophagy staining. α -Synuclein overexpressing N27 cells were grown on poly-lysine coated coverslips and treated with 30 μ M dieldrin for 24 h or 2 μ M methamphetamine (positive control) for 12 h. Cells were stained with the autophagosome marker monodansyl

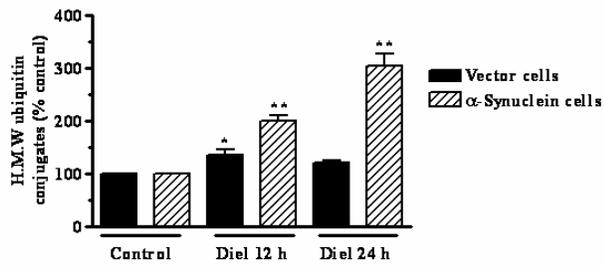
cadaverine (MDC, blue) before fixation and immunostaining with α -synuclein antibody (Mouse) and Cy3 conjugated secondary antibody (red). Arrows indicate protein aggregation following dieldrin treatment and autophagosomes following methamphetamine treatment. C. LAMP-1 immunostaining. Double immunohistochemical analysis was performed using rabbit polyclonal α -synuclein antibody and mouse monoclonal LAMP-1 antibody. The secondary antibodies used were Cy3 conjugated anti-rabbit (red = α -synuclein) and Alexa-488 conjugated anti-mouse (green = lysosomes). Confocal image analysis of α -synuclein and LAMP-1 staining was performed in the colocalization study. The small arrows indicate the α -synuclein aggregates and the large arrows indicate enlarged lysosomes.

FIGURE 6.

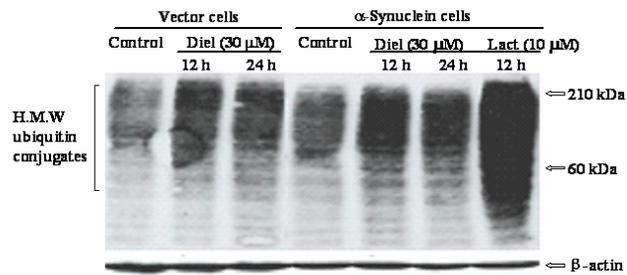
A. Detergent insoluble fraction



B. Densitometric analysis



C. Soluble fraction



D. Densitometric analysis

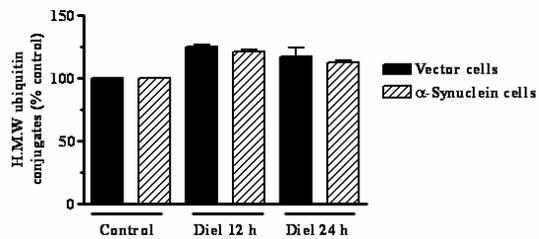


Fig. 6. Accumulation of high molecular weight ubiquitin-conjugated protein in dopaminergic neuronal cells following dieldrin treatment.

Vector overexpressing and α -synuclein overexpressing N27 cells were treated with 30 μ M dieldrin for 12 or 24 h. Detergent soluble and insoluble fractions were determined as described in the methods sections. Equal amounts of proteins from the detergent insoluble fractions (A) and soluble fractions (C) were resolved on 8% SDS-PAGE and blotted with ubiquitin antibody. Square brackets indicate the regions with high levels of ubiquitin-conjugated proteins. 10 μ M lactocystin was used as a positive control. The membrane was reprobed with β -actin antibody. The levels of ubiquitin conjugates in detergent insoluble (B) and soluble fractions (D) were quantified by densitometry followed by statistical analysis (N=4, ANOVA followed by Dunnett's post-test, * p <0.05, ** p <0.01).

FIGURE 7.

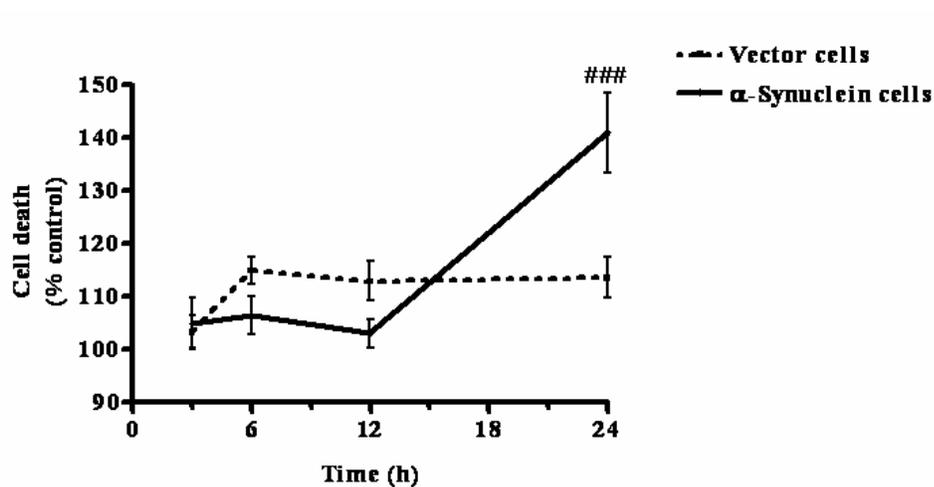


Fig. 7. Overexpression of α -synuclein increases sensitivity to dieldrin-induced cytotoxicity.

Vector transfected and α -synuclein overexpressing N27 cells were treated with 30 μ M dieldrin. The cytotoxicity was measured at 3, 6, 12 and 24 h by Sytox green assay. Cell death was expressed as the percentage of the time-matched control groups. The results represent mean \pm S.E.M. from eight to twelve samples. Significance was determined by ANOVA followed by Bonferroni's Multiple Comparison Test. ### $p < 0.001$, vector transfected cells compared with α -synuclein overexpressing cells.

FIGURE 8.

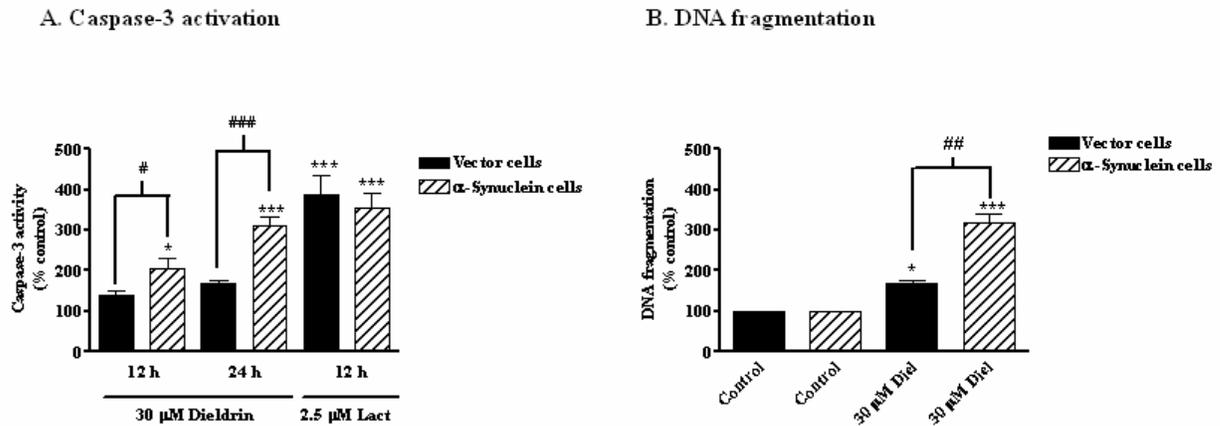


Fig. 8. Dieldrin-induced proteasomal dysfunction enhances apoptotic cell death in α -synuclein overexpressing dopaminergic neuronal cells.

A) Caspase-3 activity, Cells were exposed to dieldrin (30 μ M) for 12 or 24 h and then caspase-3 activity was measured with the fluorogenic substrate Ac-DEVD-AMC. Exposure to classical proteasome inhibitor lactacystin (2.5 μ M) for 24 h also significantly activates caspase-3 activity. The results represent mean \pm S.E.M. from 7 samples for dieldrin treatment and 3 samples for lactacystin treatment. B) Dieldrin-induced DNA fragmentation. Dieldrin (30 μ M) was exposed for 24 h and then DNA fragmentation was assayed using the ELISA assay. Values were expressed as the percentage of the control group. Values represent mean \pm S.E.M. from four individual samples. Data are expressed as the percentage of the control group. * p <0.05 and *** p <0.001 (Student's t-test) in comparison with the untreated control group. # p <0.05 ## p <0.01 and ### p <0.001 (Student's t-test) in comparison with the vector transfected cells.

CHAPTER III: PROTEASOME INHIBITOR MG-132 INDUCES DOPAMINERGIC DEGENERATION IN CELL CULTURE AND ANIMAL MODELS

A paper published in Neurotoxicology

Faneng Sun, Vellareddy Anantharam, Danhui Zhang, Calivarathan Latchoumycandane, Arthi Kanthasamy and Anumantha G. Kanthasamy

ABSTRACT

Impairment in ubiquitin-proteasomal system (UPS) has recently been implicated in Parkinson's disease, as demonstrated by reduced ubiquitin proteasomal activities, protein aggregation and mutation of several genes associated with UPS. However, experimental studies with proteasome inhibitors failed to yield consensus regarding the effect of proteasome inhibition on dopaminergic degeneration. In this study, we systematically examined the effect of the proteasome inhibitor MG-132 on dopaminergic degeneration in cell culture and animal models of Parkinson's disease. Exposure of immortalized dopaminergic neuronal cells (N27) to low doses of MG-132 (2-10 μ M) resulted in dose- and time-dependent cytotoxicity. Further, exposure to MG-132 (5 μ M) for 10 min led to dramatic reduction of proteasomal activity (>70%) accompanied by a rapid accumulation of ubiquitinated proteins in these cells. MG-132 treatment also induced increases in caspase-3 activity in a time-dependent manner, with significant activation occurring between 90 min and 150 min. We also noted a 12-fold increase in DNA fragmentation in MG-132 treated N27 cells. Similarly, primary mesencephalic neurons exposed to 5 μ M MG-132 also induced > 60% loss of TH positive neurons but only a minimal loss of non-dopaminergic cells. Stereotaxic injection of MG-132 (0.4 μ g in 4 μ l) into the substantia nigra compacta (SNc) in

C57 black mice resulted in significant depletion of ipsilateral striatal dopamine and DOPAC content as compared to the vehicle-injected contralateral control sides. Also, we observed a significant decrease in the number of TH positive neurons in the substantia nigra of MG-132-injected compared to the vehicle-injected sites. Collectively, these results demonstrate that the proteasomal inhibitor MG-132 induces dopamine depletion and nigral dopaminergic degeneration in both cell culture and animal models, and suggest that proteasomal dysfunction may promote nigral dopaminergic degeneration in Parkinson's disease.

INTRODUCTION

The ubiquitin proteasome system (UPS) is the primary proteolytic complex responsible for the elimination of unwanted and misfolded intracellular proteins. The UPS is critical for various cellular functions including cell development, survival, apoptosis and intracellular signaling (Glickman and Ciechanover, 2002). The degradation of cellular proteins by UPS is tightly regulated by a system in which ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligase (E3) act sequentially to attach the polyubiquitin chain to the target proteins, and specify the degradation by 26S proteasome. Impairment in UPS function interferes with its proteolysis capacity, and leads to inadequate protein degradation.

Parkinson's disease (PD) is a major neurodegenerative disorder affecting over 1.5 million people in the US. The mechanisms underlying the selective and progressive loss of nigral dopaminergic neurons in PD are still unclear. Several studies have implicated UPS dysfunction in the pathogenesis of PD (Dawson and Dawson, 2003; Betarbet et al., 2005). Impairment of UPS is demonstrated by the decrease in proteasomal enzyme activities as well

as decreased protein levels of the α -subunit of proteasome in the substantia nigra of patients with idiopathic PD (McNaught and Jenner, 2001; McNaught and Olanow, 2003; Betarbet et al., 2005). Additionally, mutations in genes associated with protein processing and degradation, namely parkin, α -synuclein and ubiquitin C-terminal hydrolase-L1 (UCH-L1), have been found in patients with familial PD (Dauer and Przedborski, 2003; Dawson and Dawson, 2003). In support of this view, dopamine (Keller et al., 2000), 6-OHDA (Elkon et al., 2004), and the mitochondria complex I inhibitors MPP⁺ (Sawada et al., 2004) and rotenone (Hoglinger et al., 2003) have been shown to result in decreased proteasomal activity in various cell culture models including PC12 cells and primary mesencephalic cultures. Recently, we demonstrated that exposure to the environmental neurotoxin dieldrin inhibits proteasomal activity to induce alpha-synuclein aggregation and cell death in dopaminergic neuronal cells (Sun et al., 2005). However, some recent studies yielded inconsistent results regarding effects of proteasome inhibitors on dopaminergic degeneration. Systematic administration of the proteasomal inhibitor epoxomicin produces delayed and progressive neurological and neuropathological changes similar to those associated with PD in rodents (McNaught et al., 2004), whereas the proteasome inhibitors protected dopaminergic neurons in a rat 6-OHDA PD model (Inden et al., 2005). In this study, we examined the effect of the well known proteasome inhibitor MG-132 in dopaminergic neuronal cultures and animal models to determine the role of proteasomal inhibition in nigrostriatal dopaminergic degeneration.

MATERIALS AND METHODS

Cell Cultures. The immortalized rat mesencephalic dopaminergic neuronal cells (N27) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 µg/ml streptomycin. Cell cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C as previously described (Yang et al., 2004). We and others have extensively used N27 cells as a useful model to study the neurotoxic mechanisms pertaining to Parkinson's disease (Clarkson et al., 1999; Kaul et al., 2003; Miranda et al., 2004; Kaul et al., 2005a; Kaul et al., 2005b; Peng et al., 2005).

Cytotoxicity Assay with Sytox Green. Assessment of cytotoxicity was conducted using Sytox green as described previously (Latchoumycandane et al., 2005). Membrane-impermeable DNA dye Sytox green can readily enter the cells with altered membrane permeability, resulting in increased fluorescence. The intensity of fluorescence is directly proportional to the amount of dead cells, and this method is more efficient and sensitive than other cytotoxic measurements (Kitazawa et al., 2004). Twenty-four hr after cells were grown in 24-well plates, cells were incubated with 1 µM Sytox simultaneously with 5.0 µM MG-132 or vehicle (1% DMSO) as a control. DNA bound Sytox green (Ex 485 nm and EM 538 nm) was detected using a fluorescence microplate reader (Molecular Devices Corporation, Gemini Plate Reader). Fluorescence intensity was monitored and normalized by the time-matched control to quantify cell death.

Proteasomal Peptidase Activity Assay. Chymotrypsin-like proteasomal activity was assessed with the method described previously (Sun et al., 2005). After collection and lysis

of cells with lysis buffer (10 mM HEPES, 5 mM MgCl₂, 10 mM KCl, 1% sucrose, 0.1% CHAPS) on ice for 20 min., 50 µl lysates were used for assay by incubation with 75 µM fluorogenic Suc-LLVY-AMC (Calbiochem, San Diego, CA) in the assay buffer (50 mM Tris-HCl, 20 mM KCl, 5 mM MgOAc and 10 mM DTT, pH 7.6) at 37°C for 30 min. Fluorescence intensity of the enzymatically cleaved product was measured using a fluorescence plate reader (Gemini Plate Reader, Molecular Devices Corporation). Protein concentration was determined by the Bradford method. Enzymatic activity was normalized by protein concentration.

Caspase Enzymatic Activity Assay. Assessment of caspase activation was conducted as described previously (Sun et al., 2005) using Ac-DEVD-AFC (Bachem Bioscience, King of Prussia, PA) as substrate for the enzymatic activity assay. The cleaved product by caspase-3 was measured (Ex 400 nm and Em 505 nm) using a fluorescence plate reader (Molecular Devices Corporation). Bradford protein assay was used for determination of protein concentration.

SDS-PAGE and Western Blot. Western blot analysis was performed as described previously (Sun et al., 2005). Cells were collected and washed once with ice-cold PBS before lysis with buffer (protease inhibitors and 0.5% Triton X-100 in PBS). The lysates were ultracentrifuged at 100,000 x g for 40 min. The resulting supernatants were collected for protein assay. Equal amounts of protein were resolved on 8% SDS-PAGE and transferred onto nitrocellulose membrane. A standard Western blot procedure was followed for immunoblot with polyclonal ubiquitin antibody generation (DAKO, Carpinteria CA,

1:500). An ECL kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) was used for detection of ubiquitinated proteins. The blot was reprobbed with monoclonal β -actin antibody (Sigma Chemicals, St. Louis, MO, 1:5000) to confirm equal protein loading.

DNA Fragmentation Assay. Cell Death Detection ELISA Plus Assay Kit (Roche Molecular Biochemicals, Indianapolis, IN) was used for analysis of DNA fragmentation by quantification of histone-associated low molecular weight DNA in the cytoplasm of cells (Anantharam et al., 2002). Briefly, cell pellets were lysed with the lysis buffer provided in the assay kit. After being spun down at 200 x g, 20 μ l of supernatant was incubated with the mixture of HRP-conjugated antibody-recognizing histones and fragmented single- and double-stranded DNA. After unbound components were removed by washing, bound HRP-conjugates were assessed colorimetrically with ABTS as substrate using a spectrophotometer at 405 nm, and the optical density at 490 nm was used as reference. Protein concentration was determined by the Bradford protein assay.

Primary Mesencephalic Culture. Preparation of primary mesencephalic neuronal cultures was conducted as described previously (Yang et al., 2004). Ventral mesencephalon was first dissected out from 15-17-day-old mouse embryos, and cell dissociation was achieved by incubating dissected tissues in trypsin-EDTA (0.25%) for 20 min. The dissociated mesencephalic cells obtained from four litters were suspended in serum-free neurobasal medium supplemented with B-27, L-glutamine, penicillin, and streptomycin (Life Technologies) before cells were grown on poly-L-lysine coated coverslips in 24-well plates. The plating density was around 40,000 cells per ml of culture medium per well. Each well

represents an experimental unit (n). Twenty-four hr after cells were in culture, 10 μ M cytosine arabinoside was added to suppress glial cell proliferation. The cells were maintained in a humidified CO₂ incubator (5% CO₂, 37°C) for approximately 6–7-days before treatments. Tyrosine hydroxylase (TH) immunostaining yields approximately 30-40 TH positive cells, accounting for less than 0.1% of the total population of cells in each culture well.

Immunocytochemistry. Immunostaining of the tyrosine hydroxylase (TH) marker of dopaminergic neurons was performed in primary mesencephalic neurons derived from C57 black mice [2]. Briefly, after treatment, primary neurons grown on poly-L-Lysine-coated glass cover slips were double stained with TH antibody and Hoechst staining to determine the number of TH⁺ and TH⁻ neurons and the experiments were blinded. Nuclei were counterstained with Hoechst 33342 at a final concentration of 10 μ g/ml. Primary neurons were fixed with 4% paraformaldehyde, permeabilized, and non-specific sites were blocked with 5% normal goat serum containing 0.4% BSA and 0.2% Triton-X 100 in PBS for 20 min. Cells were then incubated with antibodies directed against TH (1:500 dilution) overnight at 4°C followed by incubation with Cy3-conjugated (1:1000) secondary antibody for 1 hr at RT. Then the cover slips containing cells were washed with PBS, mounted on a slide, viewed under a Nikon inverted fluorescence microscope (Model TE-2000U) and images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Stereotaxic Injection of MG-132. C57 black mice were maintained in a temperature/humidity-controlled environment with free access to food and water. After mice

were anesthetized, MG-132 (0.4 μg in 4 μL) and vehicle (1% DMSO in PBS) were stereotaxically injected into the substantia nigra at the target site (Bregma AP, -3.2 mm, ML, \pm 2.0 mm, DV, -4.7 mm) in the right and left sides, respectively (Hommel et al., 2003). Twelve days after injection, mice were either sacrificed for dissection of the striatum for dopamine and DOPAC measurement or perfused intracardiacally with 4% paraformaldehyde for immunohistological study.

Neurotransmitter Analysis. Striata were dissected from mouse brain on an ice-cold glass platform, and the weight of each striatal tissue was measured. The samples were homogenized in buffer containing 0.2 M perchloric acid, 0.5 mg/ml Na_2EDTA and 1 $\mu\text{g}/\text{ml}$ $\text{Na}_2\text{S}_2\text{O}_5$ and subjected to $13,200 \times g$ centrifugation. The supernatant was analyzed for dopamine and DOPAC by HPLC-EC detection as described in our previous publication (Kitazawa et al., 2001). The HPLC system included a pressure module, a solvent delivery system (Rainin Instrument Co. Inc., Woburn, MA, USA), and an automatic AS-48 sampler (Bio-Rad Laboratories, Hercules, CA, USA) controlled by Rainin Dynamax HPLC method manager software (ver. 1.4, Rainin Instrument Co. Inc.). A C-18 reversed-phase column (Rainin Instrument Co. Inc.) was used to separate neurotransmitters isocratically with the mobile phase (pH 3.1, 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) at the flow rate of 1 ml / min. Measurement of the neurotransmitters was achieved with an electrochemical detection (EC) system consisting of an ESA coulochem model 5100A and a guard cell model 5020 (ESA Inc., Bedford, MA). Calibration of the HPLC-EC with DOPAC and dopamine was performed before each use. The sample injector was programmed to wash automatically

after each injection with 50% acetonitrile in deionized water. The dopamine and DOPAC levels were normalized by the wet tissue weight, and normal levels of dopamine and DOPAC in control animals were approximately 15.0 ng and 5.0 ng per mg wet tissue, respectively.

Quantification of TH and Non-TH Cell count. We used Metamorph software (Universal Imaging, Version 5.0) for measurement of TH⁺ neurons in primary cell culture and *in vivo* sections. The total number of TH⁺ cell count and neurite processes were counted in five to seven cover slips obtained from two separate experiments for primary neurons. The total number of TH⁺ cells averaged 75/coverslip in untreated controls. For *in vivo* sections, fixed brain tissues were cut into sections of 30 μ m thickness using cryostat sectioning, and the free floating nigral sections were stained with tyrosine hydroxylase (Rabbit, 1: 2000) and counterstained with nucleus dye Hoechst 33342. Quantification of TH positive neurons at nigral sections was performed with sections at the caudorostral level of the third cranial nerve as described previously (Kanthasamy et al., 1997). For measurement of TH and non-TH cell count, the images were first thresholded, and then neuronal count and volume were measured using the Integrated Morphometry Analysis (IMA) function in the Metamorph Image analysis software (Molecular Devices, Downingtown, PA). The data were logged to an Excel spreadsheet with defined row and column positions and then analyzed. The data were exported to Graph Pad Prism 4.0 software and analyzed.

Data Analysis. All data analysis was performed with Prism 4.0 software (GraphPad software, San Diego). One-way ANOVA was used for multiple comparisons. Single

comparisons were made using the Student's t-test. A significant difference was accepted if $p < 0.05$.

RESULTS

Exposure to Proteasome Inhibitor MG-132 Induces Cytotoxicity in Dopaminergic Neuronal Cells

Immortalized mesencephalic neuronal cells (N27 cells) were exposed to MG-132 (2.5 μM) for 7 hr and cell viability was monitored by staining with Sytox green dye, a membrane-impermeable DNA binding fluorescent dye which preferentially stains dead cells. Exposure to MG-132 induced a dose- and time-dependent increase in cytotoxic cell death (Fig. 1). Significant cell death was observed starting at 5 hr and progressively increased up to 7 hr.

Inhibition of Proteasomal Activity and Accumulation of Ubiquitin-Conjugated Proteins in MG-132 Treated Cells

Since MG-132 is a proteasomal inhibitor, we examined the inhibitory effect of MG-132 on proteasomal activity in dopaminergic neuronal cells. We assayed the enzymatic activity of the 20S/26S proteasome using the specific fluorogenic substrate Suc-LLVY-AMC in MG-132-treated N27 cells. Fig. 2A shows a rapid decrease in the proteasomal activity within 10 min of 5 μM MG-132 exposure. Less than 25% proteasomal activity remained after the 10 min exposure ($p < 0.001$), indicating that MG-132-induced proteasomal inhibition precedes cell death. Following inhibition of proteasomal enzymatic activity, the levels of ubiquitinated proteins increase in the cytosol due to the reduced clearance of proteins by the UPS (Rideout and Stefanis, 2002) and therefore, we determined levels of high molecular

weight poly-ubiquitinated proteins (200 kDa and 20 kDa) following MG-132 treatment. As shown in Fig. 2B, MG-132 treatment resulted in rapid accumulation of poly-ubiquitinated proteins as determined by Western blot. Densitometric analysis of the level of ubiquitinated proteins (20-200 kDa) revealed the accumulation of the poly-ubiquitinated proteins within the first 10 min of MG-132 treatment and continued to increase over time. However, the percent increase was not statistically different from the 10 min time point. β -Actin was used as the internal control for equal protein loading (Fig. 2B).

MG-132 Treatment Induces Caspase-3 Activation and Apoptotic Cell Death

To determine whether caspase mediated apoptotic cell death plays any role in MG-132-induced dopaminergic cell death, we measured caspase-3 enzyme activity and DNA fragmentation in MG-132-treated N27 cells. As shown in Fig. 3A, exposure to 5 μ M MG-132 resulted in a time-dependent increase in caspase-3 enzyme activity, with significant activation occurring at between 90 to 180 min (10- to 25-fold, $p < 0.001$). Exposure to 5 μ M MG-132 for 2 hr also resulted in a 12-fold increase in DNA fragmentation as measured by an ELISA-sandwich assay (Fig. 3B). These results clearly demonstrate that treatment with the proteasomal inhibitor MG-132 dramatically activates apoptotic cell death in dopaminergic neuronal cells.

MG-132 Induces TH-positive Neuronal Loss in Primary Mesencephalic Cultures

Next we extended our neurotoxic studies with N27 dopaminergic clonal cells to mouse primary neuronal cultures. We determined the effect of MG-132 on the survival of TH neurons in primary nigral dopaminergic neuronal cultures. Primary mesencephalic

dopaminergic neuronal culture cells were exposed to 5 μ M MG-132. After a 5 hr exposure, primary neurons were fixed and stained for tyrosine hydroxylase (TH), a marker for dopaminergic neurons. MG-132 treatment profoundly altered the morphology of dopaminergic neurons. As shown in Fig. 4A, cell bodies of TH-positive neurons shrunk following MG-132 exposure, indicating ongoing degeneration. Also, a significant loss of dopaminergic neurons was observed following MG-132 treatment in primary cultures. Quantitative analysis revealed about 60% loss of TH positive cell count in MG-132-treated cells as compared to untreated primary neurons (Fig. 4B). However, quantification of the total population of cells present in the mesencephalic culture showed only 37% reduction, which is less profound than the loss of TH neurons in the culture (Fig 4 C). These observations suggest that proteasomal inhibition can induce neurotoxic insult to dopaminergic neurons in primary mesencephalic cultures.

Stereotaxic Injection of MG-132 Causes Striatal Dopamine Depletion and Promotes Dopaminergic Neuronal Loss in Mouse Substantia Nigra

Finally, we examined whether inhibition of proteasomal function in the nigra promotes dopaminergic neuronal degeneration in animal models. Vehicle and MG-132 (0.4 μ g) were stereotaxically injected into the left and right mouse substantia nigra as depicted in Fig. 5A, and after 12 days the brains were dissected from the animals and mid brain sections were immunostained for TH. As depicted in Fig. 5B, a marked decrease was observed in the number of TH+ neurons in the MG-132-injected sides of the substantia nigra as compared to vehicle-injected control sides. Quantitative analysis of TH positive neurons indicated that microinjection of MG-132 to substantia nigra led to significant reduction in the number of

nigral TH neurons (Fig 5C, $p < 0.05$), whereas the reduction in the number of Hoechst stained nuclei present in the same nigral regions was not statistically significant (Fig. 5D). Further, consistent with the nigral dopaminergic degeneration, HPLC analysis of striatal tissue revealed a significant depletion in striatal dopamine (Fig. 6A) and its metabolites DOPAC (Fig. 6B) in the MG-132 injected ipsilateral side as compared to the vehicle injected contralateral side.

DISCUSSION

Our studies in cell culture models demonstrate that the proteasome inhibitor MG-132 impairs ubiquitin-proteasome function (UPS) in dopaminergic neuronal cells and promotes degeneration of dopaminergic neurons in mouse mesencephalic primary culture. The time course study revealed that MG-132 induced inhibition of proteasomal activity and accumulation of UPS activity before cell death, suggesting that the impairment in ubiquitin proteasome-mediated protein degradation possibly triggers the neurotoxic response in dopaminergic neuronal cells. Activation of caspase-3 and DNA fragmentation during MG-132 treatment indicate that proteasomal dysfunction triggers the apoptotic cell death cascade. Our results from microinjection of MG-132 show a significant depletion of dopamine and DOPAC with concurrent loss of nigral dopaminergic neurons, and suggest that inhibition of nigral proteasome function can induce nigral dopaminergic degeneration similar to that in Parkinson's disease.

Several lines of recently generated evidence suggest that dysfunction of UPS is one of the causal factors of PD. Studies with postmortem brain samples revealed reduced proteasomal activities (McNaught et al., 2003) and selective loss of α -subunits of proteasome

in the substantia nigra of PD patients (McNaught et al., 2002a; McNaught et al., 2002b). Mutation of some genes involved in the UPS degradation pathway, including parkin, Uch-L1, and α -synuclein, has been found in familial PD (McNaught et al., 2003; McNaught and Olanow, 2003; Moore et al., 2005). A pathogenic role of a dysfunctional ubiquitin-proteasome system in PD is supported by the inhibition of proteasomal activity in cell culture models of PD by dopamine (Keller et al., 2000), 6-OHDA (Elkon et al., 2004), MPP⁺ (Sawada et al., 2004) and rotenone (Hoglinger et al., 2003), and wild-type and mutant human α -synucleins (Dawson and Dawson, 2003; Betarbet et al., 2005). Also, we recently showed that α -synuclein overexpression decreases proteasomal activity and sensitizes dopaminergic N27 neuronal cells to environmental neurotoxin-induced apoptotic cell death (Sun et al., 2005).

The relationship between UPS dysfunction and apoptotic cell death in dopaminergic neurons has not been clearly studied in detail. In the present study, we show that MG-132 inhibits proteasomal activity within 10 min, resulting in the accumulation of intracellular ubiquitinated proteins. This rapid inhibition of UPS triggers a dramatic activation of the key effector proapoptotic protease caspase-3 and DNA fragmentation. In a recent study, we observed about three-fold activation of caspase-3 and DNA fragmentation after 24 hr of treatment with 300 μ M MPP⁺, a Parkinsonian toxin, in N27 cells (Kaul et al., 2003; Kaul et al., 2005a). In comparison, in the present study, we observed an approximate 25-fold increase in caspase-3 activity and a 12-fold increase in DNA fragmentation following 2 hr of 5 μ M MG-132 treatment in N27 cells, suggesting that dopaminergic neuronal cells appear to be sensitive to proteasome inhibition. Also, the magnitude of MG-132-induced TH positive neuronal loss in a primary mesencephalic culture within 5 hr is comparable to the neuronal

loss observed following 10 μ M MPP⁺ treatment for 24 hr, further supporting the increased vulnerability of nigral dopaminergic neurons to UPS dysfunction. It's still a controversy as to whether dopaminergic neurons are more susceptible to UPS dysfunction. Findings of the mutation of Parkin, UCH-L1 in familial PD, protein aggregation and accumulation of neurotoxic PaeR as Parkin substrate in dopamine neurons (Yang et al., 2003), as well as the increased sensitivity of dopaminergic neurons to oxidative stress upon proteasome inhibition (Mytilineou et al., 2004), suggests particular vulnerability of dopamine neurons to proteasome inhibition. The cellular mechanisms underlying the exacerbated toxicity from proteasomal inhibition in dopaminergic neurons are not currently known. A recent study demonstrated that mesencephalic dopaminergic neurons are particularly susceptible to proteasome inhibition-induced apoptosis due to failure to upregulate the expression of chaperone proteins HSP70 in response to proteolytic stress (Rideout and Stefanis, 2002). The HSP70 upregulation failure might underlie the susceptibility of dopamine neurons to proteasome inhibition, which has also been observed by others (McNaught et al., 2002a; McNaught et al., 2002b; Petrucelli et al., 2002). It is also possible that a number of other signaling proteins may play a role in the proteasome inhibitor induced cell death because the levels of proapoptotic and anti-apoptotic proteins are tightly regulated by UPS (Dawson and Dawson, 2003; Hattori and Mizuno, 2004; Ross and Pickart, 2004; Layfield et al., 2005).

Further examination of the effect of MG-132 on nigral dopaminergic degeneration *in vivo* indicated that stereotaxic injection of MG-132 into substantia nigra led to significant depletion of ipsilateral striatal dopamine and its metabolite DOPAC level, which is accompanied by profound loss of dopamine neurons at MG-132 injected substantia nigra regions (Fig. 5 B, panel a). Alternatively, the quantitative analysis of the nuclei present in

the nigral sections indicated that the percentage loss of nuclei (Figure 5B and D) was less profound compared to that in TH neurons (Fig. 5B and C) indicating enhanced vulnerability of dopaminergic neurons to proteasomal dysfunction. Our data clearly shows that proteasomal inhibition by single injection of the proteasome inhibitor MG-132 can cause dopaminergic neuronal death in substantia nigra. Recently, McNaught et al. (2004) demonstrated that repeated systemic administration of the naturally occurring proteasome inhibitor epoxomicin and a synthetic proteasome inhibitor known as PSI in rats can replicate several features of PD including delayed motor deficits, a progressive nigrastriatal degeneration and protein aggregation (McNaught et al., 2004). This will be a highly useful animal model to study the pathogenic mechanisms of PD; however, the model is yet to be easily replicated in other laboratories. There are some inconsistencies observed between *in vitro* and animal studies with regard to the neurotoxic effect of proteasome inhibitors. A recent study showed that injection of proteasome inhibitors protected dopamine neurons from the neurotoxic effect of 6-OHDA in a rat model (Inden et al., 2005), while treatment with proteasome inhibitors in PC12 cells potentiated 6-OHDA toxicity (Elkon et al., 2004). In the present study, the *in vitro* results obtained in N27 cells and animal studies consistently showed that proteasome inhibition can promote dopaminergic degeneration.

In conclusion, our results demonstrate that proteasomal inhibition by MG-132 induces neurotoxicity in nigral dopaminergic neurons both in cell culture and animal models and that proteasome inhibition in dopaminergic neuronal cells activates the apoptotic cascade to induce cell death. Also, our results suggest that proteasomal dysfunction may play a key role in the dopaminergic degenerative processes associated with Parkinson's disease.

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Yang Y, Nishimura I, Imai Y, Takahashi R and Lu B Parkin Suppresses Dopaminergic Neuron-Selective Neurotoxicity Induced by Pael-R in *Drosophila*. *Neuron* 2003; 27:911-24.

FIGURE 1.

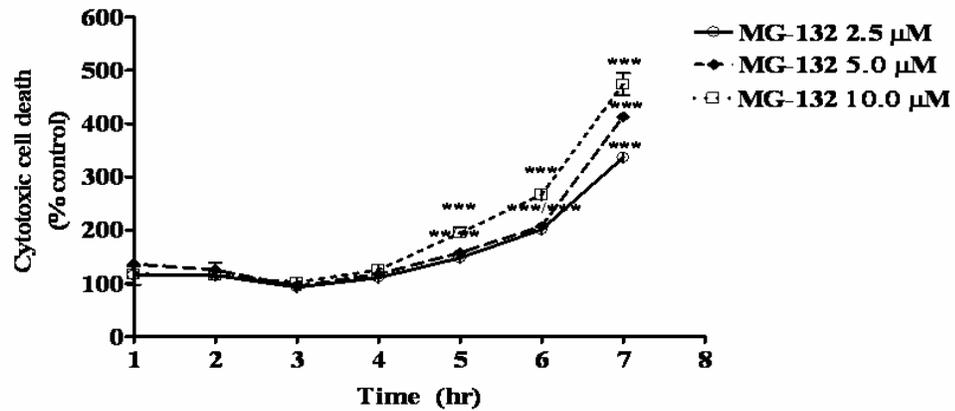


Fig. 1. Cytotoxicity of MG-132 in dopaminergic neuronal (N27) cells.

N27 cells were treated with 5.0 μ M MG-132. The neurotoxicity was assessed at various time points over a 7 hr period using the Sytox green assay. Cell death was expressed as the percentage of the time-matched control groups. The results represent mean \pm S.E.M. from six samples in each group (** $p < 0.01$, *** $P < 0.001$).

FIGURE 2.

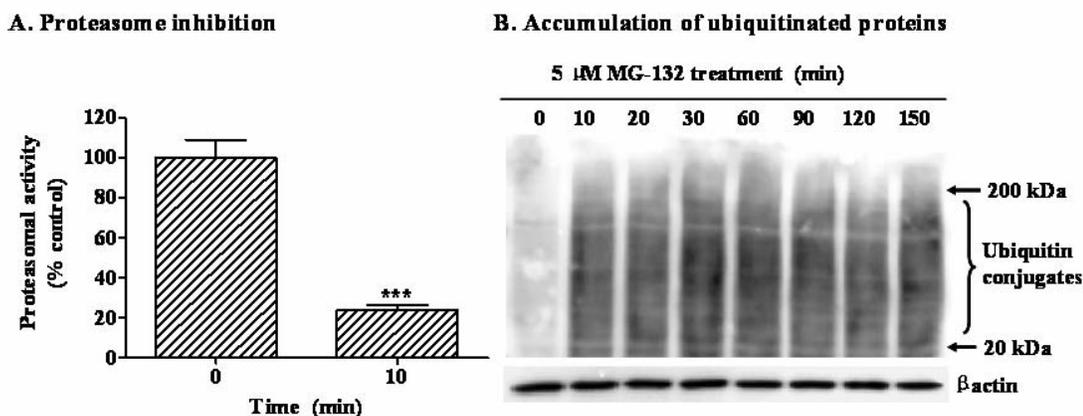


Fig. 2. MG-132 causes rapid proteasome inhibition and accumulation of ubiquitinated proteins.

A: Proteasome inhibition. Proteasomal activity was measured 10 min after N27 cells were exposed to 5.0 μ M MG-132 using the fluorogenic substrate Suc-LLVY-AMC. Enzymatic activity was normalized by protein concentration and expressed as the percentage of vehicle-treated cells. The data represent mean \pm S.E.M. from six samples in each group (***) $p < 0.001$, Student's t-test). **B:** Accumulation of ubiquitinated proteins. N27 cells were exposed to 5.0 μ M MG-132 for various durations ranging from 10 to 150 min as indicated. Cytosolic fractions were prepared as described in the method section, resolved on 8% SDS-PAGE and blotted with ubiquitin antibody. Membranes were also reprobed with β -actin antibody to ensure equal protein loading.

FIGURE 3.

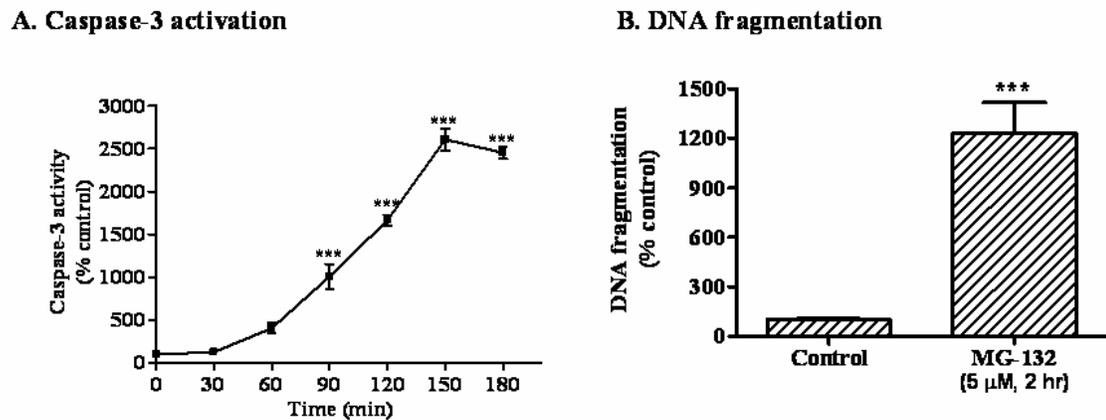


Fig. 3 MG-132 induces apoptosis in dopaminergic neuronal cells.

A: Caspase-3 activity. Cells were treated with 5.0 μ M MG-132 and then caspase-3 activity was measured with the fluorogenic substrate Ac-DEVD-AFC. The results represent mean \pm S.E.M. from eight samples. Statistical significance between the control group and each treatment group was determined by ANOVA followed by Dunnett's post-test (** $p < 0.001$). **B:** DNA fragmentation. DNA fragmentation was assayed using the ELISA assay in N27 cells treated with 5.0 μ M MG-132 for 120 min. Data were expressed as the percentage of the control group. Values represent mean \pm S.E.M. from eight individual samples. ** $p < 0.001$.

FIGURE 4.

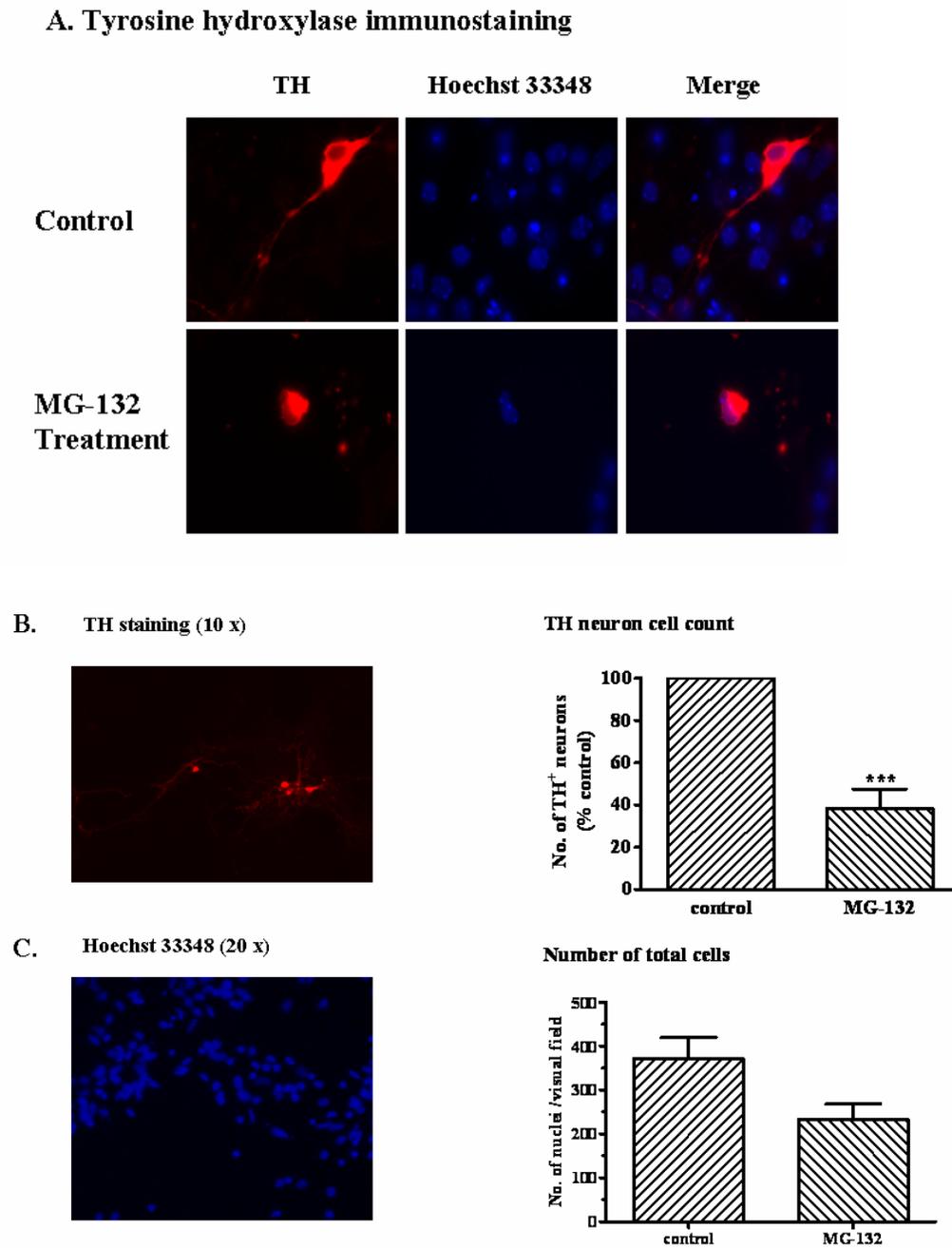
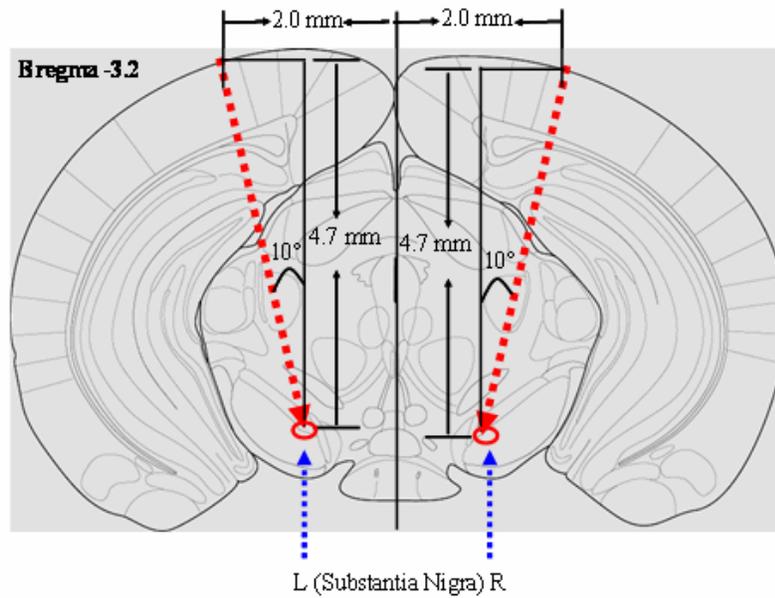


Fig. 4. MG-132 induced morphological changes and dopaminergic neuronal loss in primary mesencephalic culture.

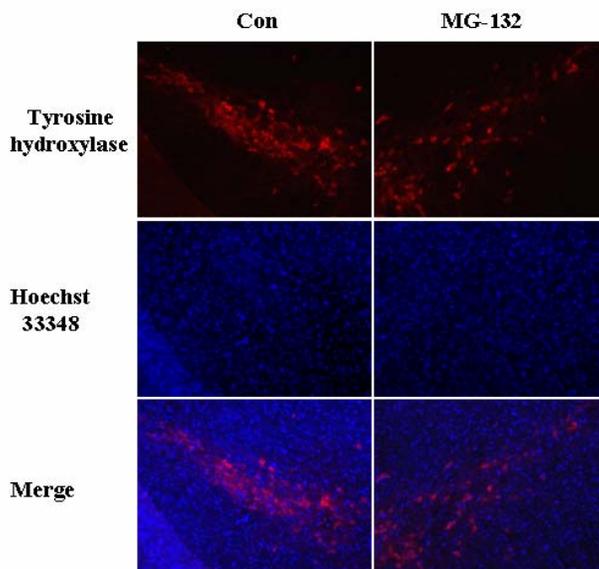
A: Immunostaining for tyrosine hydroxylase (TH). After 6 to 7 hr in culture, the primary mesencephalic culture was treated with 5.0 μ M MG-132 for up to 5 hr. Immunocytochemistry was performed using mouse monoclonal TH primary antibody and Cy3 conjugated secondary antibody. **B:** Quantification of TH positive neurons (approximately 35-40 TH neurons observed per each well in control group). TH positive neurons present in control and MG-132 treated samples were visualized with CY3 under 10 X objective and quantified using Metamorph image analysis software. Data were expressed as the percentage of the control group. Values represent mean \pm S.E.M. from 5-7 individual litter brains. *** $p < 0.001$. **C:** Quantification of mesencephalic culture. Nuclei in the mesencephalic culture were stained with Hoechst 33342, and the nuclei present in 12 randomly selected visual fields were quantified under 20 X objective ($p < 0.05$).

FIGURE 5.

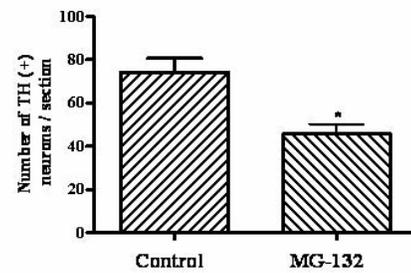
A. Site of stereotaxic injection into Substantia nigra



B. Staining of TH neuron and Nuclei



C. Quantification of nigral TH neurons



D. Quantification of nigral nuclei

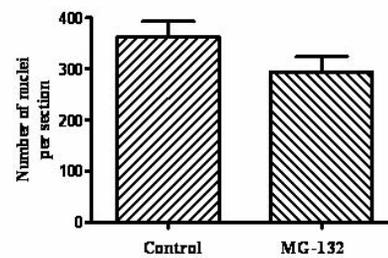


Fig. 5 Intranigral stereotaxic injection of MG-132 causes dopaminergic neuronal loss in a mouse model.

A. Schematic diagram depicting the stereotaxic microinjection site into the left (L) and right (R) substantia nigra (Bregma AP, -3.2 mm, ML, \pm 2.0 mm, DV, -4.7 mm). Red lines represent injection routes, red arrows indicate the needle distance, and the blue arrow indicates the site of injection (substantia nigra). Immunohistochemical analysis of nigral sections. MG-132 (0.4 μ g in 4 μ l) and vehicle were injected stereotaxically into mouse substantia nigra at right and left sides, respectively. Twelve days following the injection, brains were fixed and processed for TH immunohistochemical analysis as described in the methods section. **B:** Visualization of nigral TH neurons and nuclei under 10X objective; **C:** Quantification of TH neurons in nigral sections, * p <0.05, n =5; **D.** Quantification of nuclei present in the nigral regions (n =4).

FIGURE 6.

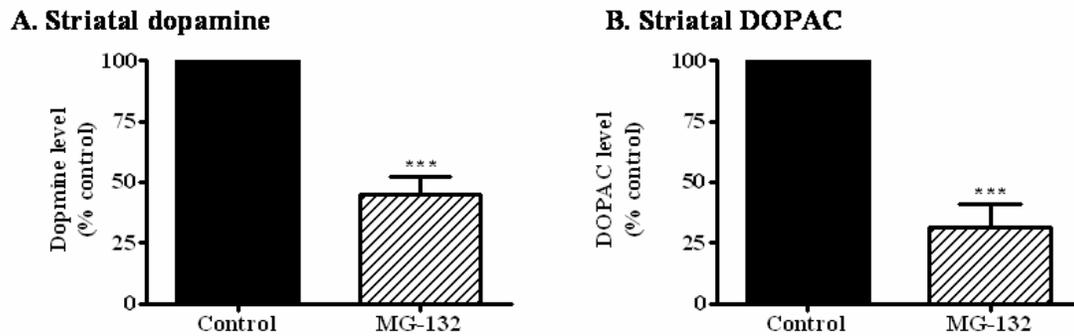


Fig. 6. Striatal dopamine DOPAC depletion following microinjection of MG-132 to substantia nigra.

Mouse striatums were isolated from intranigral vehicle injected or MG-132 injected sides and then analysis of **A.** dopamine and **B.** DOPAC were performed using HPLC. Dopamine and DOPAC levels were approximately 15 ng and 5.0 ng per mg weight nigral tissue respectively. The data were expressed as the percentage of the vehicle control group. Data represent mean \pm S.E.M. N=6, ***p<0.001.

**CHAPTER IV. MITOCHONDRIA ARE KEY SENSORS OF POLYUBIQUITIN
OVERLOADING STRESS AND POLYUBIQUITINATION SITES LYS-48 AND 63
DIFFERENTIALLY REGULATE THE STRESS INDUCED APOPTOTIC CELL
DEATH**

Faneng Sun, Qinglin Li, Vellareddy Anantharam, Arthi Kanthasamy and Anumantha G.

Kanthasamy

ABSTRACT

Impairment of ubiquitin proteasome function results in accumulation of polyubiquitinated proteins eventually culminating overloading of polyubiquitin, which has recently been recognized as a key feature of many neurodegenerative conditions including Parkinson's disease. Nevertheless, cellular mechanisms underlying polyubiquitin overloading stress-mediated cell death remain enigmatic. Herein, we report a novel finding that polyubiquitinated proteins preferentially accumulate in mitochondria during ubiquitin stress, and that polyubiquitin sites Lys-48 and 63 are key determinants of cell survival and death during ubiquitin stress. Exposure to the proteasome inhibitor MG-132 caused a rapid accumulation of polyubiquitinated proteins in mitochondria of a dopaminergic cell model of Parkinson's disease, indicating mitochondria as early sensors of ubiquitin stress. Overexpression of ubiquitin^{K48R} mutant effectively rescued cells from MG-132-induced mitochondrial apoptosis without altering antioxidant status of cells, whereas ubiquitin^{K63R} mutant augmented the proapoptotic effect of MG-132. Together, these findings yield novel insights into cellular mechanisms of ubiquitin stress in dopaminergic neuronal cells.

Introduction

Ubiquitin proteasome system (UPS) is a vital cellular machinery responsible for degradation of intracellular proteins participating in diverse biological processes (Glickman and Ciechanover, 2002). Proteolysis by UPS involves polyubiquitination of target proteins and proteolytic degradation of polyubiquitin tagged target proteins by 26 S proteasome. For polyubiquitination, ubiquitin first forms isopeptidyl bond between its carboxyl group of the glycine 76 and the ϵ -amino group of an internal lysine residue of the target proteins. Then, a second ubiquitin is covalently linked to the internal lysine of the preceding ubiquitin via isopeptidyl bond. Progressive addition of the ubiquitin results in the extension of polyubiquitin chains. All 7 internal lysine residues of ubiquitin could potentially serve as polyubiquitination sites, with K48 and K63 polyubiquitin being two most abundant forms (Pickart and Eddins, 2004). K48 polyubiquitin functions to target the substrates to 26 S proteasome for degradation; whereas K63 polyubiquitin is involved in signal pathways other than proteolysis (Pickart and Fushman, 2004).

Parkinson's disease is a primary neurodegenerative movement disorder, pathologically characterized by selective loss of nigral dopamine neurons. The supporting evidence for pathogenic role of mitochondria dysfunction includes modeling PD with mitochondria complex I inhibitors and mitochondria complex I deficit in the substantia nigra and platelet of PD patients (Abou-Sleiman et al., 2006). Mitochondria dysfunction has been suggested to result in excessive free radicals production and thus oxidative injury (Bove et al., 2005). In addition, PD genes PINK1, DJ-1 and Parkin participate in maintaining mitochondria normal function or dealing with oxidative stress (Abou-Sleiman et al., 2006).

Another direction towards dopamine degeneration is the defective ubiquitin proteasome degradation, as PD genes Parkin and Uch-L1 function in the UPS pathway (Sun et al., 2007). Compromised proteasome function was also reported in the substantia nigra of *post mortem* brain samples from subjects with sporadic PD (Olanow and McNaught, 2006). Currently, little is known how UPS impairment and mitochondria dysfunction are mutually related in dopamine neuron death, despite proteasomal inhibition by mitochondria complex I inhibitors (Hoglinger et al., 2003) and profound mitochondria pathology in cells exposed to low dose of proteasome inhibitor (Sullivan et al., 2004). Other evidence for the essential roles of UPS in maintaining normal mitochondria activities includes functional and structural preservation of mitochondrial function by Parkin (Clark et al., 2006; Park et al., 2006), down-regulation of functionally important mitochondrial proteins in Parkin knockout mice (Palacino et al., 2004). Neuroprotection against a broad range of neurotoxins by Parkin has been extensively reported (Review, Sun et al., 2007). To ascertain how the compromised UPS degradation affects the mitochondria related cell death, we characterized ubiquitination-related biochemical alterations of mitochondria and how this is potentially involved in modulation of dopaminergic neuronal cell viability in this study.

Results and discussion

Proteasome inhibitor MG-132 activates mitochondria apoptosis

Varieties of proteins modulating apoptosis (IAPs, Mcl-1, flip, Bax, Smac, P53 etc), have been shown to the proteolytic substrates of UPS (Zhang et al., 2004), and proteasome inhibitor could be either anti-apoptotic or pro-apoptotic depending on the cellular context. Study by Rideout showed that dopamine neurons sensitively undergo apoptosis in the

mesencephalic culture upon proteasomal inhibition (Rideout et al., 2005). In this study, we found that exposure to proteasome inhibitor MG-132 activated the mitochondria apoptotic cascades in mesencephalic rat dopaminergic neuronal cells (N27 cells), as manifested by the mitochondrial release of cytochrome c and the activation of initiator caspase-9 (Fig. 1B, C), which was preceded by the rapid and dramatic proteasomal inhibition 5 min after exposure to MG-132 (Fig. 1A).

Proteasome inhibition by MG-132 causes mitochondrial accumulation of ubiquitinated proteins

Expression of Parkin, a small portion of which is associated with mitochondria, prevents neuronal cells from mitochondria-mediated apoptosis (Darios et al., 2003), indicating the essential roles of UPS in preserving normal mitochondria function, consistent with the gross mitochondria pathology revealed in the Parkin knockout mice (Palacino et al., 2004). However, it remains unclear how proteasome inhibition affects mitochondria function and mitochondria apoptosis in dopaminergic neuronal cells. Western blot analysis for the mitochondria in this study yielded novel findings, which, for the first time, demonstrated dramatic elevation of the polyubiquitinated protein in the mitochondria from the cells exposed to proteasome inhibitor (Fig. 2A). It's conceivable that elevated ubiquitin conjugates in the mitochondria resulted from the accumulation of K48 polyubiquitin on the undegraded proteins, as it happened following proteasome inhibition. Since the crude mitochondria used for the Western blot analysis likely contained contamination of other vesicles such as lysosome, the crude mitochondria were further separated with sucrose gradient to improve the purity. Western blot analysis of the fractions collected showed that

mitochondria marker COX IV are predominantly detected in the fraction 3 to 5, especially in the fraction 4 (Fig. 2B), the interface approximately half down the gradient solutions, which is consistent with previous studies utilizing similar mitochondria purification procedure (Kim et al., 2004). Immunoblotting with ubiquitin antibody showed that ubiquitin conjugate of high molecular weight was distributed in a pattern similar to COX VI, with strongest immunoreactivity revealed in fraction 4 (Fig. 2B). This result clearly suggested that polyubiquitinated proteins were mainly present at mitochondria. Likely, mitochondrial accumulation of ubiquitin conjugates represents a key early cellular response during neuronal stress, since ubiquitin conjugates have also been reported to accumulate in the mitochondria of cortical and especially hippocampus neurons following cerebral ischemia (Hayashi et al., 1992). Mitochondria ubiquitination also appears to be a key cellular event maintaining mitochondria inheritance by targeting the parental sperm mitochondria for destruction after fertilization.

The novel finding of mitochondrial accumulation of polyubiquitinated proteins, taken together with presence of several mitochondria associated E3 ligase (Darios et al., 2003; Yonashiro et al., 2006) and deubiquitinating enzyme (Kinner and Kolling, 2003) implies that mitochondrial proteins could be the substrates for ubiquitination. An *in vitro* cell free ubiquitination assay for the isolated mitochondria indicated that ubiquitin immunoreactivity dramatically increased in the mitochondria (Fig. 2 C, lane 4), while the ubiquitination could not proceed without fraction A and B, which contain E1, E2 and E3 (lane 3). Interestingly, we found that the substitution for wt ubiquitin with ubiquitin^{K48R} partially reduced mitochondrial ubiquitin immunoreactivity, suggesting that appreciable portion of ubiquitination occurred as the result of the extension of K48 polyubiquitin chains (lane 5 and

6). In consistence, several mitochondrial proteins have been shown to undergo ubiquitination modification, such as prohibitin (Thompson et al., 2003), aconitate hydratase, ATP synthase alpha chain, isocitrate dehydrogenase precursor, aspartate aminotransferase precursor, malate dehydrogenase precursor etc (Weekes et al., 2003), mitochondrial protein hFis1 and Drp1 (Yonashiro et al., 2006). Likely, the mitochondrial translocation of the ubiquitinated cytosolic proteins also contributes to the observed elevation of mitochondrial ubiquitin conjugates. Recent study by Marchenko et al showed that monoubiquitination of p53 promotes its mitochondrial translocation and enhances mitochondria apoptosis (Marchenko et al., 2007).

Establish cells stably expressing His₆-tagged wild type, K48R or K63R ubiquitin/GFP

In light of crucial roles of UPS in preserving physiological function of mitochondria and suppression of mitochondria apoptosis by E3 ligase Parkin, it's intriguing to ascertain how mitochondrial accumulation of K48 polyubiquitinated proteins affects mitochondria apoptosis following proteasome inhibition. First of all, we established the cells stably expressing His₆-tagged wt, or mutant ubiquitin. The plasmids encoding His₆-ubiquitin/GFP fusion proteins were kind gifts from Dr. Gray, who developed the innovative strategy to construct a linear fusion of His₆-ubiquitin and GFP, as ubiquitin is natively expressed either as fusion or polyubiquitin proteins, which are post-translationally processed to release functional monomer ubiquitin. The expressed His₆-ubiquitin/GFP fusion proteins have been previously shown to be precisely processed to yield functional His₆-ubiquitin and GFP (Hyun et al., 2004; Tsigotis et al., 2001). To achieve stable expression and constitutive expression, the coding sequence for His₆-ubiquitin/GFP (wt, K48R or K63R ubiquitin) was subcloned

into pCEP4 vector with CMV promoter for mammalian expression. Fig. 3A showed that after prolonged hygromycin B screening, the majority of cells derived from single clones are positive as manifested by GFP expression at a notable level. Analysis of the His₆-tagged proteins enriched from the transfected cells by SDS-PAGE and Commassie blue stain revealed the presence of His₆-tagged ubiquitin only in transfected cells, with comparable expression levels observed among cells transfected with wt ubiquitin or its mutants. This also corroborates the precise processing of the fusion proteins into His₆-tagged ubiquitin, as reported previously (Hyun et al., 2004; Tsigotis et al., 2001).

Determination of mitochondrial superoxide and cellular glutathione

Mitochondria deficit/oxidative stress represents another direction towards the pathogenesis of PD besides dysfunctional UPS (Abou-Sleiman et al., 2006; Bove et al., 2005). Impaired mitochondrial electron transfer capacity and increased ROS production have been previously reported in the neuronal cells after chronic exposure to proteasome inhibitor (Sullivan et al., 2004). His₆-ubiquitin^{K48R} expressing cells were reported to suffer from elevated oxidative damage (Hyun et al., 2004). In this study, we analyzed mitochondria superoxide production and cellular GSH level in 3 lines of the stable cells. Confocal analysis of mitochondria superoxide using MitoSox red showed that neither the expression of ubiquitin mutants nor MG-132 exposure significantly altered mitochondrial ROS generation, comparing to wt ubiquitin (Fig. 4A). This agreed with the similar glutathione levels detected in 3 different types of cells (Fig. 4B). The data indicate that expression of mutant ubiquitin using pCEP4 vector does not impair the intracellular redox status or alter mitochondria ROS generation in the dopaminergic neuronal cells.

Effect of K48R mutant human ubiquitin on mitochondria apoptosis

To determine how mitochondrial accumulation of K48 polyubiquitinated proteins affects MG-132-induced apoptosis, mitochondria-mediated caspase activation was examined in the cells expressing His₆-tagged wild type, K48R or K63R ubiquitin/GFP. As shown in Fig. 5A, MG-132 treatment triggered profound mitochondrial release of cytochrome c in the wt and K63R His₆-ubiquitin cells; whereas only minimal elevation of cytosolic cytochrome c was noted in K48R His₆-ubiquitin cells. Assembly of cytochrome c with other cofactors to form complex of apoptosome is the key event activating initiator caspase-9. Consistent with less cytochrome c release, activation of caspase-9 and -3 and DNA fragmentation as endpoint event of apoptotic cell death were significantly attenuated in ubiquitin^{K48R} cells compared to cells expressing wt ubiquitin (Fig 5B, C, D), suggesting the assembly of K48 polyubiquitin chains could have causal effect on activation of mitochondria-mediated apoptosis. It's of note that anti-apoptotic effect of the ubiquitin^{K48R} is Lys48 ubiquitination site specific, since ubiquitin^{K63R} expression renders the dopaminergic cells more susceptible to the MG-132 induced apoptosis. The Lys63 polyubiquitin chains play roles in the cellular events such as DNA damage repair, NFκB activation (Pickart and Fushman, 2004). Presumably, interference with the cellular processes by ubiquitin^{K63R} poses additional neuronal stress predisposing cells to apoptosis. In consistence with the observed proapoptotic effect of ubiquitin^{K63R}, study by Tsirigotis has previously showed that stable expression of ubiquitin^{K63R} sensitizes mouse HT4 neuroblastoma cells to the neurotoxicity of cadmium and canavanine (Tsirigotis et al., 2001). However, increase in neuronal resistance of ubiquitin^{K48R} expressing cells is opposite to previous reports, which showed that expression of

ubiquitin^{K48R} renders cells more vulnerable to neurotoxins (Hyun et al., 2004; Tsirigotis et al., 2001). Although the reasons for the apparent difference remain elusive, the distinct effect could reflect the fact that the roles of ubiquitin^{K48R} on the cell viability depend on the cellular context. Alternatively, different promoters (human ubiquitin promoter vs CVM promoter) utilized for ubiquitin^{K48R} expression likely accounts for discrepancy, as exogenously introduced human ubiquitin promoter could possibly compete for the translational regulatory machinery for endogenous ubiquitin expression, thus reduces availability of endogenous native ubiquitin and predispose cells to the subsequent neuronal insults. It's of note that the neuroprotection of ubiquitin^{K48R} is reproducible, since expression of His₆-tagged yeast ubiquitin^{K48R} using lentivirus-mediated transfection also significantly suppresses caspase-9 and -3 activation in N27 cells following exposure to MG-132 (Data not shown).

Elevation of mitochondria ubiquitin conjugates and suppression of mitochondria apoptosis by ubiquitin^{K48R} together suggest that preferential mitochondrial accumulation of ubiquitinated proteins could be proapoptotic. This agrees with previous studies demonstrating that suppression of ubiquitination by dominant negative yeast ubiquitin conjugating enzyme *cdc34* (*Ubc3*) protects cortical neurons from proteasome inhibitor-induced apoptosis (Rideout and Stefanis, 2002). However, it remains to determine whether ubiquitination of mitochondria proteins could be a direct causal event sufficient to activate mitochondria apoptosis, since it's also likely that activation of mitochondria apoptosis requires mitochondrial translocation of some ubiquitinated cytosolic factors, such as the p53 (Marchenko et al., 2007). Elevated CBP level, due to its insufficient UPS degradation, was hypothesized to underlie the neuroprotection of ubiquitin^{K48R} in transgenic mice (Tsirigotis et al., 2006). However, no appreciable change in CBP level was observed in the N27 cells

transfected with His₆-ubiquitin^{K48R}, although proteasome inhibition by MG-132 effectively increased the cellular CBP level (Data not shown), consistent with ubiquitin dependent proteasome degradation of CBP (Sanchez-Molina et al., 2006).

This study revealed the preferential accumulation of polyubiquitinated proteins in the mitochondria and suppression of mitochondria apoptosis by His₆-ubiquitin^{K48R} in dopaminergic neuronal cells exposed to proteasome inhibitor MG-132. The elevation of mitochondrial ubiquitin conjugates is attributed to failure in proteasomal removal of K48 polyubiquitin tagged proteins in mitochondria, as this occurs as consequence of proteasome inhibition. Mitochondrial accumulation of K48 polyubiquitin appears to actively contribute to the activation of mitochondria apoptosis cascades following proteasome inhibition, since expression of His₆-tagged human or yeast ubiquitin^{K48R}, albeit in two different vectors, confers similar neuronal resistance to MG-132 induced apoptosis. Interestingly, the neuroprotection of ubiquitin^{K48R} is polyubiquitination site specific, since expression of ubiquitin^{K63R}, which prevents the K63 polyubiquitin chain extension, renders dopaminergic neuronal cells more susceptible to MG-132-induced mitochondria apoptosis. Future effort will focus on identifying mitochondria substrates of UPS, and thus deciphering the molecular mechanism of mitochondria apoptosis upon proteasome inhibition in dopaminergic neuronal cells. Unraveling the relationship between UPS impairment and mitochondria apoptosis in dopamine neurons will facilitate the development of manipulating strategies for PD.

Materials and methods

Cell culture

The immortalized rat mesencephalic dopaminergic neuronal cells (N27 cells) were grown in RPMI 1640 medium with 10% FBS, 2 mM L-glutamine, 50 units penicillin, and 50 µg/ml streptomycin (Sun et al., 2006).

Plasmid construction and stable expression

The coding sequence for His₆-ubiquitin / GFP in the vectors from Dr. Gray (Ottawa Health Research Institute, Ontario, Canada) was subcloned into pCEP4 vector at Xho I and Hind III sites. AMAXA kit was used to transfect the constructs in N27 cells. Single clones were picked up and screened with hygromycin for stable expression.

DNA fragmentation and enzymatic assays for proteasome and caspases Quantification of DNA fragmentation using ELISA kit and assay for chymotrypsin-like proteasomal activity, caspase-3 and caspase-9 activities using fluoregenic substrates were conducted as described previously (Sun et al., 2006).

Subcellular fraction, mitochondria purification and Western blot

N27 cells were homogenized in mitochondria isolation buffer (MIB, 250 mM sucrose, 1 mM EDTA, 50 mM Tris, 1 mM DTT, 1 mM PMSF and protease inhibitors, pH 7.4) with a glass Dounce homogenizer. The resulting supernatant (1,000 g x 10 min) of homogenates was centrifuged at 10,000 x g at 4 °C for 25 min to obtain pellet and supernatant as crude mitochondria and cytosolic fraction respectively (Qin et al., 2001). To improve purity, crude

mitochondria suspension was laid on the top of sucrose gradient (2.0 ml of 1.2 M and 1.6 M sucrose) and centrifuged at 40,000 x *g* for 1 h at 4°C (Kim et al., 2004). Fractions were collected for Western blot analysis of cytochrome c (Pharmingen), β -actin, (Sigma), COX 4 (Invitrogen), and ubiquitin (DAKO).

Analysis of His₆-ubiquitin expression

The cells were homogenized in buffer (HEPES 20 mM, NaCl 300mM, imidazole 5.0 mM and protease inhibitors, pH 8.0). The supernatant was incubated with Proaffinity Ni-IMAC resin (Bio-Rad), and the bounded proteins were eluted and separated in SDS-PAGE for Commassie blue staining.

***In vitro* ubiquitination**

Ubiquitination kits (Boston Biochem) contains energy source, ubiquitin and ubiquitination enzymes. Mitochondria (80 μ g) were incubated with fraction A (9.6 μ g) and B (9.6 μ g), ubiquitin aldehyde and ubiquitin (8.0 μ g) for 2 hr at 30 °C, then washed with MIB and lysed for Western blot analysis with ubiquitin and cytochrome c antibodies.

Confocal analysis of mitochondria superoxide and assay for glutathione

MitoSOX Red (Invitrogen), a mitochondrial superoxide indicator, could be selectively targeted to mitochondria. Oxidization product of MitoSOX Red by superoxide stains mitochondria DNA and exhibits red fluorescence. N27 cells are incubated with MitoSOX Red (5.0 μ M), then washed with HBSS before confocal analysis (Nikon, Model TE-2000U).

Thiol-reactive probe monochlorobimane fluorescence after conjugated to thiols. To measure glutathione, cells were lysed with buffer (50 mM Tris, 1.0 mM EDTA, 10.0 mM EGTA, and 1.0% NP-40, pH 7.4). The supernatant (16,000 g for 10 min) of cell lysates was incubated with 2.0 mM monochlorobimane for 15 min at 37 °C. The fluorescence intensity as monitored with Ex/Em at 380 nm/460 nm.

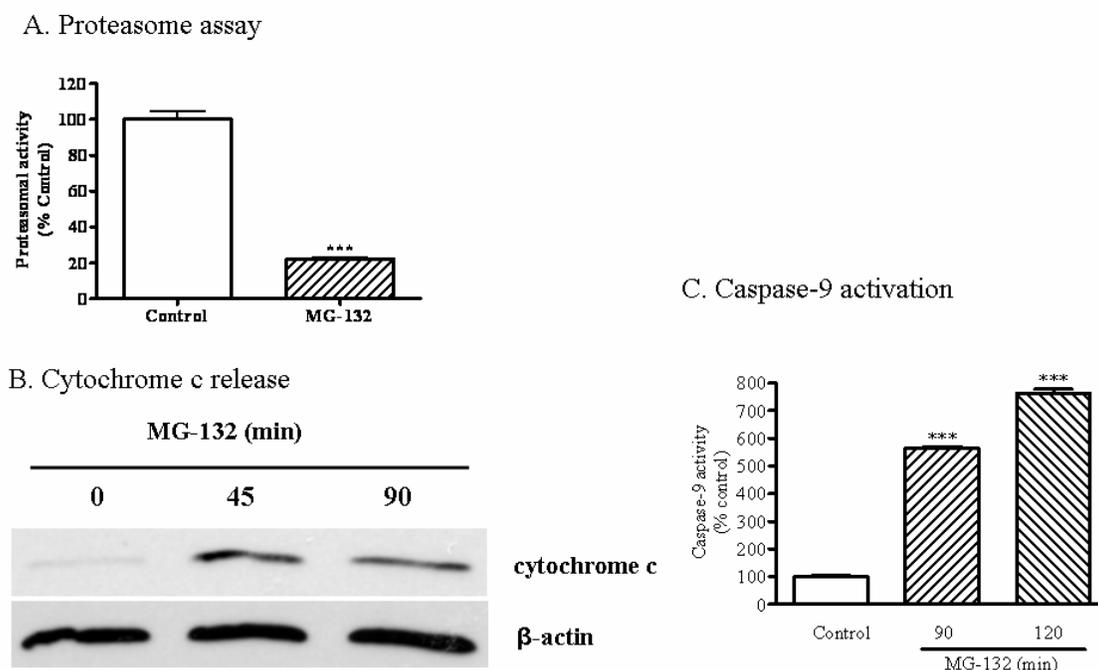
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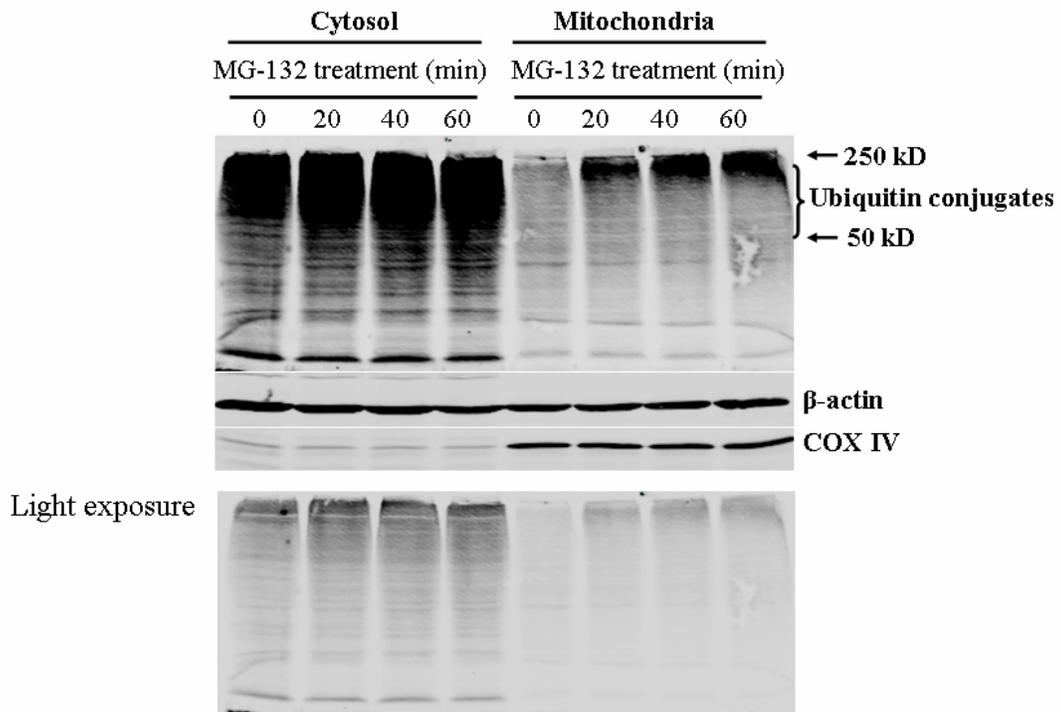
FIGURE 1**Fig.1 Proteasome inhibitor MG-132 activates mitochondria apoptosis.**

A: Proteasome inhibition. After N27 cells were exposed to 2.5 μ M MG-132 for 5 min chymotrypsin-like proteasomal activity was determined using the fluorogenic substrate Suc-LLVY-AMC. Enzymatic activity was expressed as the percentage of vehicle-treated control group. The data represent mean \pm S.E.M., N=6, ***p<0.001, (Student's t-test). **B: Cytochrome c release.** N27 cells were treated with MG-132 for 45 or 90 min, and the level of cytosolic cytochrome c was examined by Western blot using cytochrome c antibody. The membranes were reprobbed and blotted with β -actin antibody as estimation of protein amount. **C: Caspase-9 activation.** Caspase-9 activity was assayed for the cells exposed to MG-132 for 90 or 120 min with LEHD-AFC as substrate. The activity was expressed as the

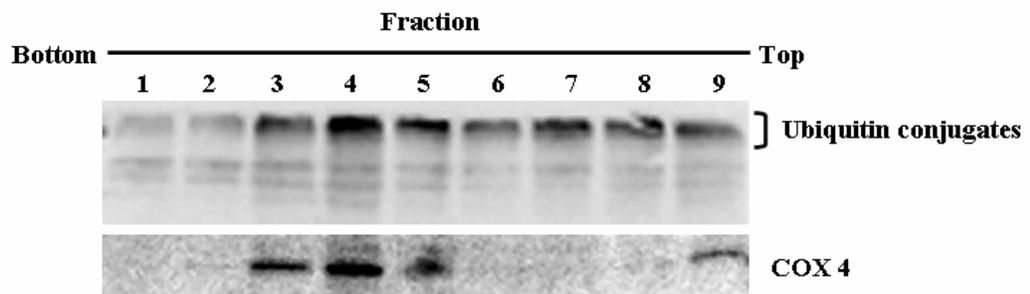
percentage of vehicle-treated cells. N=6, ***p<0.001, (one-way ANOVA followed by Dunnett's test to compare treatment groups with control group).

FIGURE 2

A. Western blot analysis with ubiquitin antibody



B. Verification of mitochondria ubiquitination by sucrose gradient



C. Ubiquitination of mitochondrial proteins in vitro

<i>Components</i>	<i>Volume (μL)</i>						
Mitochondria	20	-	20	20	20	20	20
MIB	-	20	-	-	-	-	-
HEPES buffer	38	-	26	-	-	-	
Fraction A	-	12	-	12	12	12	
Fraction B	-	12	-	12	12	12	
Ubiquitin	-	4	4	4	2.5	1	
Ubiquitin ^{K48R}	-	-	-	-	1.5	3	
Ubi-Aldehyde	-	4	4	4	4	4	
Energy 10 x	-	6.4	6.4	6.4	6.4	6.4	
Sucrose 2.0 M	5.6	5.6	5.6	5.6	5.6	5.6	

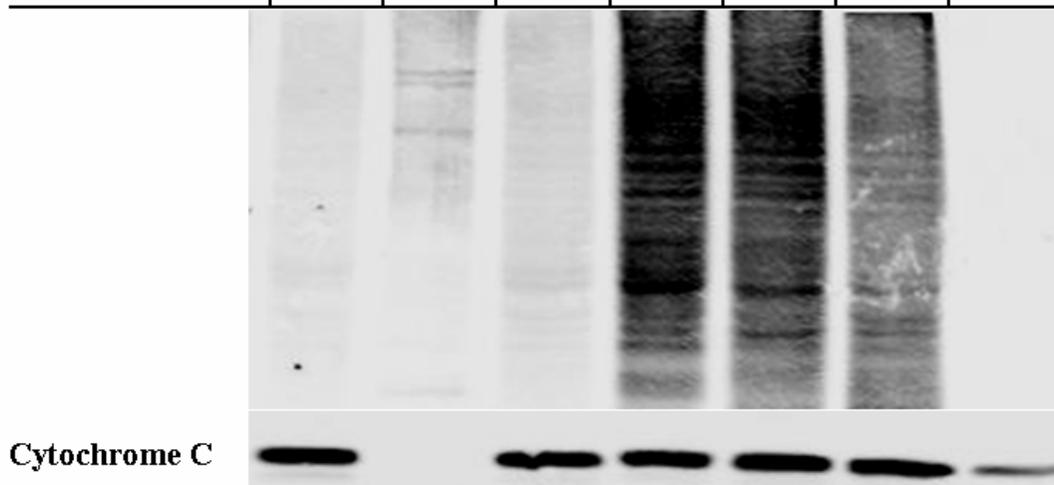


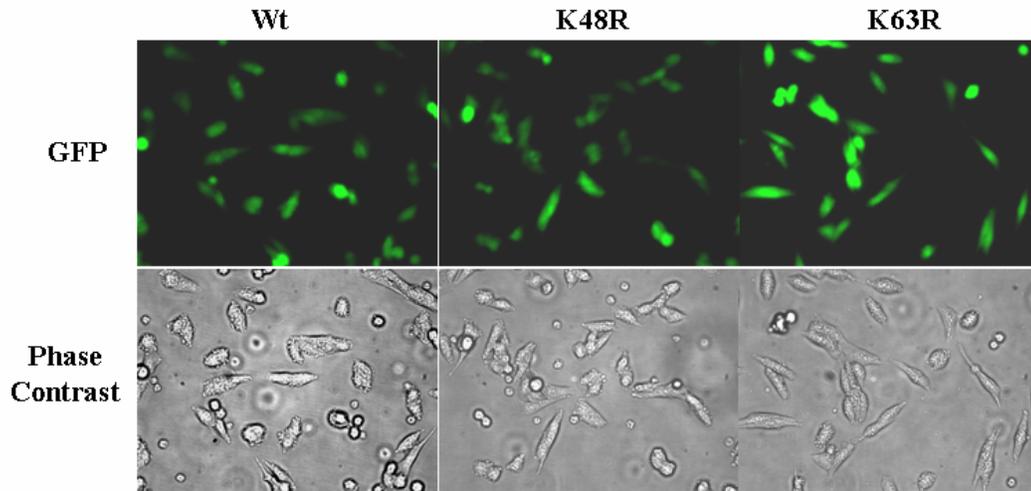
Fig.2 Proteasome inhibition by MG-132 causes mitochondrial accumulation of ubiquitinated proteins.

A). Mitochondrial accumulation of ubiquitin conjugates. N27 cells were treated with 2.5 μ M MG-132 for 20, 40 or 60 min. The cells were collected and processed to obtain cytosolic fraction and crude mitochondria, both of which were resolved on SDS-PAGE and blotted with antibodies for ubiquitin, COX IV or β -actin. **B). Sucrose gradient for mitochondria separation.** Crude mitochondria isolated from N27 cells exposed to MG-132 (2.5 μ M for 40 min) were subjected to sucrose gradient separation as described in method section. All the

fractions collected were resolved on SDS-PAGE and blotted with antibodies for ubiquitin and COX IV. **C). Ubiquitination of mitochondrial proteins.** The reaction was carried out by incubating mitochondria suspension (4.0 mg/ml) with ubiquitination enzymes (9.6 μg for fraction A and B), ubiquitin (8.0 μg), energy source and ubiquitin aldehyde. Mitochondria were then recovered from the reaction mixture for Western blot using antibodies recognizing ubiquitin or cytochrome c.

FIGURE 3

A. Fluorescence and phase contrast images.



B. Comassie staining for enriched His₆ fusion proteins

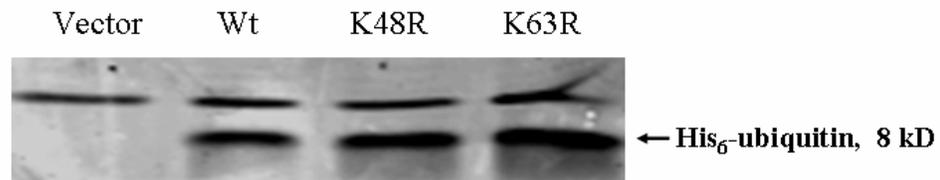
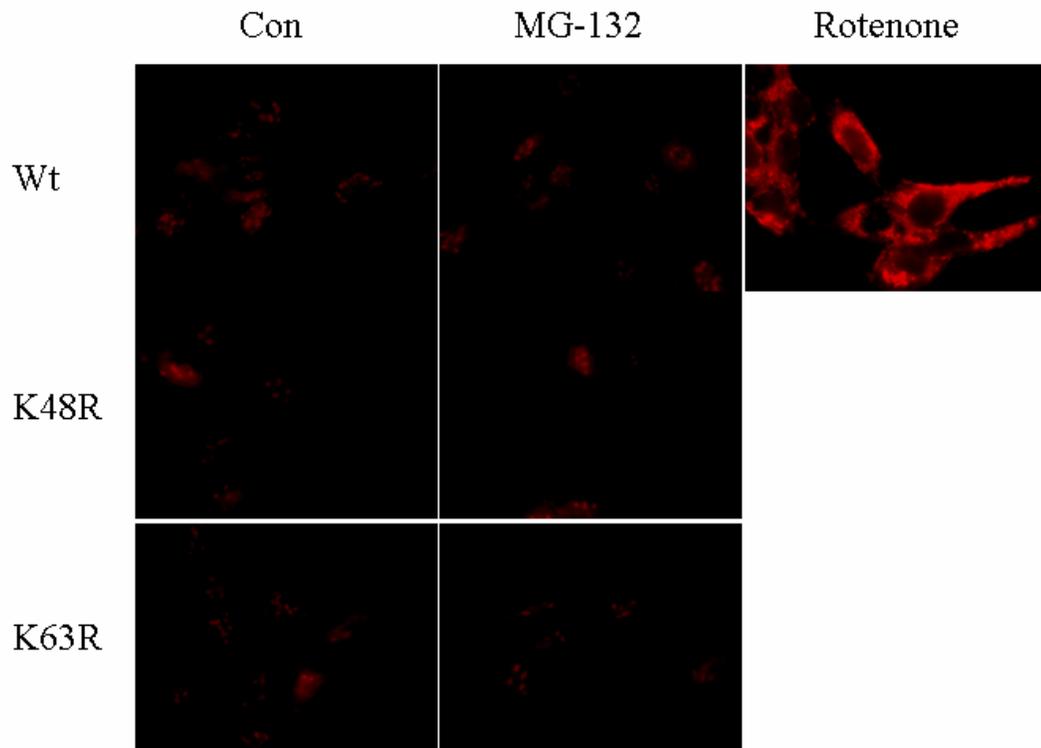
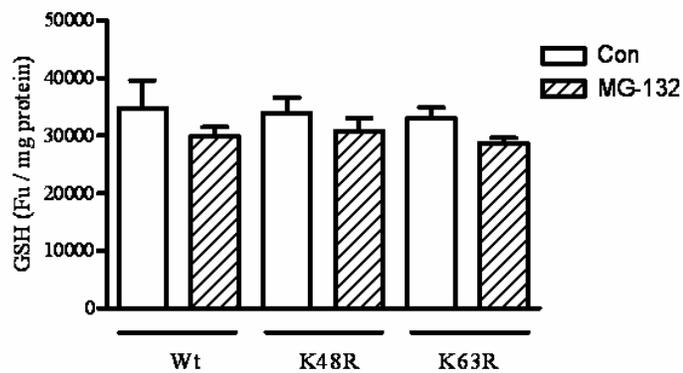
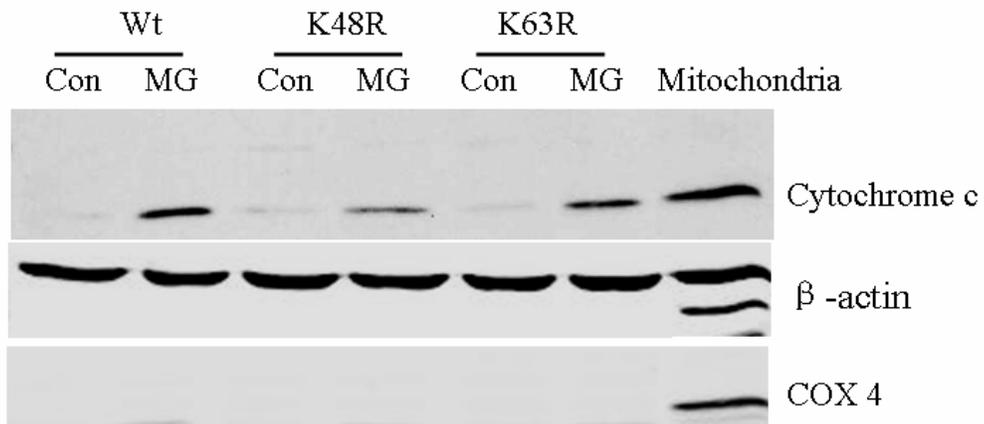
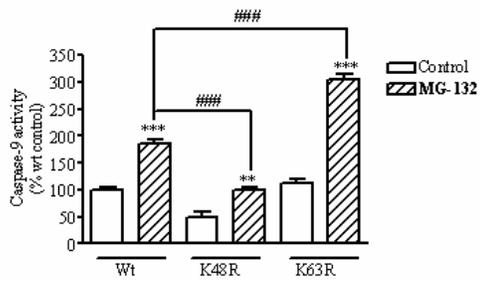
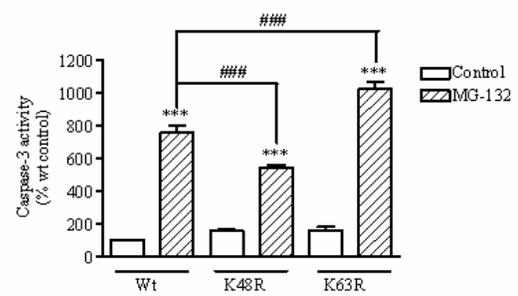
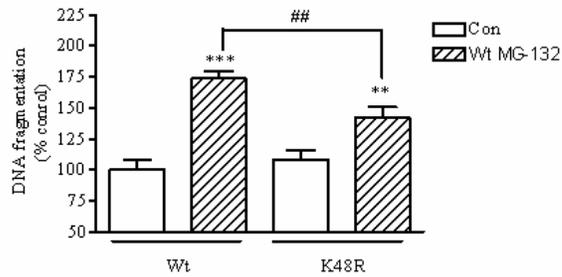


Fig.3 N27 cells stably expressing His₆-tagged wt, K48R or K63R ubiquitin/GFP.

A). Fluorescence and phase contrast images of stable cells. Fluorescence images, as the indication of GFP expression, were compared with phase contrast images at the same visual field. It appears that majority of cells stably transfected the linear fusion of wt or mutant ubiquitin/GFP express GFP at notable levels. **B): Comassie staining for enriched His₆ tagged proteins.** His₆-tagged proteins were enriched from 3 lines of stable cells using Ni-IMAC resin, and resolved on SDS-PAGE before Comassie blue staining for visualization. The arrow indicated the protein of about 8.0 kD expressed in the cells, and the size roughly matches the molecular weight of His₆-ubiquitin.

FIGURE 4.**A. Mitochondria superoxide****B. Cellular glutathione level****Fig. 4 Determination of mitochondrial superoxide and cellular glutathione.**

A): Live images of MitoSOX staining. Cells stably expressing His₆-tagged wt, K48R or K63R ubiquitin/GFP are treated either with 2.5 μ M MG-132 or 1.0 μ M rotenone for 1 hr before the MitoSOX incubation. The live images were then analyzed with confocal microscopy. **B): Cellular glutathione measurement.** Cells expressing His₆-tagged wt, K48R or K63R ubiquitin/GFP are treated with 2.5 μ M MG-132 for 1 hr. The cellular glutathione level was determined with monochlorobimane as described in material and methods. Data represents results of 2 experiments in triplicate.

FIGURE 5**A. Cytochrome c release****B. Caspase-9 activity****C. Caspase-3 activity****D. DNA fragmentation****Fig 5. Effect of ubiquitin mutant on mitochondria apoptosis.**

A): Effect of ubiquitin mutants on cytochrome c release. His₆-tagged wt, K48R or K63R ubiquitin/GFP expressing cells are treated with 2.5 μ M MG-132 for 45 min, and the cytosolic cytochrome c level was determined using Western blot analysis. The membranes were reprobbed and blotted with β -actin antibody to ensure equal amount of protein loaded. **B and C): Effect of ubiquitin mutants on caspase-9 and -3 activation.** Three lines of stable cells were treated with MG-132 for 120 min. Caspase-9 and -3 activities were determined using LEHD-AFC and DEVE-AFC as substrate for caspase-9 and caspase-3 respectively. Enzymatic activities were expressed as the percentage of vehicle-treated wt ubiquitin control group. Data for caspase-9 represent results of 2 experiments with N=5 and 6 respectively; whereas the data for caspase-3 were derived from 3 experiments with N=5, 5 and 6 respectively. **D): Suppression of MG-132-induced DNA fragmentataion by ubiquitin^{K48R}** Following exposure to 2.5 μ M MG-132 for 120 min, cells were collected for analysis of DNA fragmentation using ELISA kit described in materials and methods. Values were expressed as the percentage of vehicle-treated wt ubiquitin control group (N=5). **p<0.01, ***p<0.001 compared with control groups, ##p<0.01, ###p<0.001 (one-way ANOVA followed by Bonferroni test to compare selected groups).

CHAPTER V. PROTEASOME INHIBITOR MG-132 INDUCES MITOCHONDRIA APOPTOSIS VIA PROTEOLYTIC ACTIVATION OF PKC δ IN DOPAMINERGIC NEURONAL CELLS

Faneng Sun, Vellareddy Anantharam, Arthi Kanthasamy, Yongjie Yang and Anumantha G. Kanthasamy

ABSTRACT

Impaired protein degradation by the ubiquitin proteasome system has been implicated in Parkinson's disease; however, cellular mechanisms underlying dopaminergic degeneration following proteasomal dysfunction are yet to be characterized. The present study revealed that mitochondrial translocation of the proteolytically activated PKC δ played an essential role in the full activation of mitochondrial apoptosis in dopaminergic neurons (N27 cells) following exposure to the proteasome inhibitor MG-132. Activation of mitochondrial apoptosis was demonstrated by mitochondrial depolarization, mitochondrial release of proapoptotic proteins, and activation of caspase-9 and -3. Caspase-3-dependent proteolytic activation of PKC δ , but not ROS, appeared to be the key mediator of dopaminergic apoptosis upon proteasome inhibition, since expression of kinase active catalytic fragment of PKC δ (PKC δ -CF) results in caspase-3 activation, and PKC δ -specific inhibitor rottlerin robustly alleviated caspase-9 and -3 activation following MG-132 exposure. Time-dependent accumulation of PKC δ -CF in the mitochondrial fraction possibly underlies its amplifying effect on mitochondrial apoptosis, since mitochondria-targeted expression of PKC δ -CF triggers caspase-3 activation and DNA fragmentation as revealed by TUNEL staining. Consistently, inhibition of PKC δ proteolytic cleavage by a caspase-3 cleavage-resistant mutant effectively attenuated MG-132-triggered caspase-9, -3 activation and DNA

fragmentation. Collectively, the present study demonstrates that PKC δ plays a key role in dopaminergic apoptosis following proteasome dysfunction.

INTRODUCTION

Ubiquitin proteasome system (UPS) is one of the major intracellular proteolysis systems responsible for degradation of damaged or misfolded proteins and proteins involved in various cellular processes including apoptosis. Polyubiquitination of target proteins, which is essential for their recognition and degradation by the 26S proteasome complex, involves a cascade of enzymes including ubiquitin activating enzyme, ubiquitin conjugating enzyme, and ubiquitin ligase (Glickman and Ciechanover, 2002).

Parkinson's Disease (PD) is the most common neurodegenerative movement disorder, affecting over 4 million people worldwide, and with prevalence increasing each year. The disease is characterized by the selective and progressive loss of nigral dopaminergic neurons, with the underlying neuronal death remaining elusive (Sun et al., 2007). Lines of evidence for pathogenic roles of dysfunctional UPS in PD include reduced proteasomal activities, selective loss of proteasome subunits in substantia nigra of patients with sporadic PD, and mutation of several genes involved in UPS degradation pathway in familial PD (Moore et al., 2005; Olanow and McNaught, 2006; Sun et al., 2007). Accumulation of ubiquitinated proteins in Lewy bodies, presumably due to failure of the clearance of target proteins by UPS, is indicative of impaired UPS function in PD.

Exposure to pharmacological inhibitors of the proteasome replicates some biochemical and pathological characteristics of PD *in vitro* or *in vivo*. Proteasome inhibition has been previously shown to result in α -synuclein protein aggregation and cell death in

PC12 cells (Rideout et al., 2001), and in mesencephalic primary culture (McNaught et al., 2002). Recently we and others demonstrated that microinjection of proteasome inhibitors into substantia nigra or striatum effectively reproduces nigrostriatal dopamine degeneration (McNaught et al., 2002; Miwa et al., 2005; Sun et al., 2006). However, the effect of systemically administered proteasome inhibitors on dopaminergic degeneration is still unclear (Sun et al., 2007).

Parkinsonian neurotoxicants including 6-OHDA, dopamine and mitochondria complex I inhibitors MPP⁺ and rotenone have been shown to negatively affect proteasomal degradation in *in vitro* models of PD (Sun et al., 2007). A study by Betarbet and coworkers showed that chronic rotenone exposure led to reduction in proteasomal activity, and accumulation of α -synuclein and ubiquitinated proteins in the ventral midbrain during nigrostriatal degeneration in rats (Betarbet et al., 2006). MPTP exposure has been shown to cause severe UPS dysfunction and protein aggregation in the substantia nigra (Fornai et al., 2005; Zeng et al., 2006). By using an *in vitro* model, we recently found that interaction between α -synuclein and dieldrin, an organochlorine pesticide suspected as a risk factor for PD, promotes dopaminergic degeneration by impairing UPS function. This indicates the role of dysfunctional UPS in dopaminergic degeneration as the result of a gene-environment interaction (Sun et al., 2005). Despite extensive observations of defective UPS degradation in PD pathogenesis, the cellular and molecular mechanisms leading to dopamine neuronal death following proteasomal dysfunction remain to be characterized.

Protein kinase C δ (PKC δ), a member of the novel PKC family, has a structurally and functionally distinct N-terminal regulatory fragment, C-terminal catalytic fragment and a medial hinge region (Steinberg, 2004). Proteolytic cleavage of PKC δ at the hinge region by

caspase-3 represents one of the primary means of its activation, in addition to membrane translocation or phosphorylation (Kanthasamy et al., 2006). Proteolytic activation of PKC δ has previously been revealed as a key mediator of apoptotic cell death in oxidative stress-induced dopaminergic apoptosis (Anantharam et al., 2002; Kaul et al., 2003; Kitazawa et al., 2003; Yang et al., 2004; Latchoumycandane et al., 2005), since active PKC δ appears to amplify caspase cascades via mechanisms not yet characterized. The present study revealed that mitochondrial translocation of proteolytically activated PKC δ plays an essential role in feedback amplification of mitochondrial apoptosis during proteasome dysfunction in mesencephalic dopaminergic neuronal cells.

MATERIALS and METHODS

Cell Culture and Treatment Paradigm. The immortalized rat mesencephalic dopaminergic cell line (N27 cells) was grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C (Yang et al., 2004; Kanthasamy et al., 2006). Cells were treated with different concentrations of MG-132 dissolved in dimethyl sulfoxide (final concentration = 0.1%) for the indicated duration in the experiments.

Mitochondria Depolarization Assay. Cationic lipophilic fluorescent dye JC-1 enters the matrix of intact mitochondria through cross-membrane potential established in the mitochondria of healthy cells. Upon mitochondrial accumulation, JC-1 appears as aggregates, which fluoresce red. However, JC-1 can not accumulate in mitochondria with collapsed membrane potential, and thus exists in cytoplasm at low concentration as a monomer, which

fluoresces green. The intensity of red and green fluorescence provides a reliable measurement of mitochondria membrane potential. N27 cells grown in 6-well plates were treated with MG-132 for indicated durations prior to incubation with JC-1 dye (Invitrogen Carlsbad, CA) for 20 min at a final concentration of 2 $\mu\text{g/ml}$. Red and green fluorescence were determined for the treated cells using flow cytometry, and the ratio between red/green was used as indicator of mitochondria potential.

Caspase Enzymatic Activity Assay. Caspase activities were assessed as described previously (Kanthasamy et al., 2006). Cells were lysed with 10 μM digitonin in Tris buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA). The supernatants (14,000 x g, 5 min) of the lysates were incubated with fluorogenic substrates DEVD-AFC, IEHD-AFC and LEHD-AFC (Biomol International, Plymouth Meeting, PA) for determination of caspase-3, -8 and -9 activities, respectively, using a fluorescence plate reader (Molecular Devices Corporation, Ex/Em: 400/505 nm). Protein concentration was determined by the Bradford method.

Subcellular Fractionation, Preparation of Cell Lysate and Western Blot. Mitochondria isolation was conducted as described previously (Luo et al., 1998) with minor modification. Cells were resuspended in homogenization buffer (pH 7.5, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitors), and incubated in ice for 30 min before homogenized with a glass Dounce homogenizer. Unlysed cells, cell debris and nuclei were removed by centrifugation at 1,000 x g for 10 min. The supernatant was further centrifuged at 10,000 x g for 25 min to obtain supernatant fraction and pellet as cytosolic and

mitochondrial fractions. For whole cell lysates, cells were homogenized by sonication in homogenization buffer (pH 8.0, 20 mM Tris, 2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, protease inhibitors), and then centrifuged at 16,000 x g for 40 min. For Western blot, samples were resolved on SDS-PAGE and then transferred to nitrocellulose membranes for immunoblotting with antibodies recognizing PKC δ (Santa Cruz, Santa Cruz, CA, 1:2000), V5 (Invitrogen, Carlsbad, CA, 1:5000), cytochrome c (BD Pharmingen, San Jose, CA, 1:500), Smac (ProSci, Poway, CA 1:500) or COX 4 (Invitrogen Carlsbad, CA, 1:1500).

***In vitro* Mitochondria Release Assay.** Mitochondria were isolated using the procedure described previously and resuspended in the same isolation buffer at concentration of 2.0 mg/ml. For the release assay (Luo et al., 1998), 40 μ L mitochondria suspension was incubated with 5.0 or 15.0 μ M MG-132 at 30 °C for 60 min. Triton X-100 (0.2%, v/v) was included as positive control to release cytochrome c. After incubation, mitochondria were spun down and the supernatant was collected for the SDS-PAGE and immunoblotted for cytochrome c (BD Pharmingen, San Jose, CA, 1:500).

PKC δ Kinase Assay. The enzymatic activity of PKC δ was measured with an immunoprecipitation kinase assay as described previously (Kitazawa et al., 2003). After MG-132 treatment, N27 cells were lysed with lysis buffer (25 mM HEPES pH 7.5, 20 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 10 mM NaF, 4 μ g/ml aprotinin, and 4 μ g/ml leupeptin). The cell lysate was centrifuged at 10,000 x g for 20 min to obtain the supernatant

as cytosolic fraction. Cytosolic protein (500 μ g) was immunoprecipitated with 2 μ g PKC δ antibody. The immunoprecipitates were then washed 3 times with 2 \times kinase buffer (40 mM Tris pH 7.4, 20 mM MgCl₂, 20 μ M ATP, and 2.5 mM CaCl₂), and resuspended in 20 μ L of the same buffer. The PKC δ -mediated phosphorylation is initiated by adding 20 μ L of reaction buffer (0.4 mg Histone H1, 50 μ g/mL phosphatidylserine, 4.1 μ M dioleoylglycerol, and 5 μ Ci of [γ -³²P] ATP) to the resuspended immunoprecipitates. After incubation for 10 min at room temperature, samples were separated on 12% SDS-PAGE. The radioactively labeled histone H1 was detected using Phosphoimage system (Personal Molecular Imager, FX model, Bio-Rad Laboratories) and analyzed with Quantity One 4.2.0 software.

Plasmid Construction. Full-length wild-type (wt) PKC δ -GFP and PKC δ ^{D327A}-GFP in pEGFP-N1 vector were obtained from Dr. Mary Reyland (University of Colorado, Boulder, CO). Full-length (PKC δ -FL), regulatory fragment (PKC δ -RF) and catalytic fragment (PKC δ -CF) of PKC δ were amplified from wt-PKC δ -GFP in pEGFP-N1 vector, and PKC δ ^{D327A} (caspase-3 cleavage-resistant mutant, PKC δ -CRM) was amplified from PKC δ ^{D327A}-GFP in pEGFP-N1 vector by PCR. The PCR product was then cloned into plenti6/V5-D-TOPO expression vector by following the procedure provided by the manufacturer (Invitrogen, Carlsbad, CA). The primers used were: 5'-CACCATGGCACCCTTCCTGCTC3' (forward primer for PKC δ -FL, PKC δ -CRM and PKC δ -RF) and 5'-AATGTCCAGGAATTGCTCAAAC-3' (reverse primer for PKC δ -FL, PKC δ -CRM and PKC δ -CF), 5'-ACTCCCAGAGACTTCTGGCTT-3' (reverse primer for PKC δ -RF), and 5'-CACCATGAACAACGGGACCTGTGGCAA-3' (forward primer for PKC δ -CF). To achieve mitochondria-targeted expression, PKC δ -RF, PKC δ -CF were cloned into pCMV/Myc/Mito

vector (Invitrogen) at Sal I and Not I sites by following standard cloning procedure. LacZ was cloned into the same vector to serve as a control. The primers used include: 5'-ATATGGGTCGACATGGCACCCCTTCCTGCGCA-3' (forward primer for PKC δ -RF), 5'-ATATATGTCGACATGAACAACGGGACCTATGGCAAGA-3' (forward primer for PKC δ -CF), 5'-ATATAGCGGCCGCAATGTCCAGGAATTGCTCAAAC-3' (reverse primer for PKC δ -FL and PKC δ -CF), and 5'-ATATATGCGGCCGCACTCCCAGAGACTTCTGGCT-3' (reverse primer for PKC δ -RF).

Cell Transfection. The expression vectors (pLenti-PKC δ -CRM and pLenti-LacZ) were cotransfected with packaging plasmids provided by manufacturer into 293 FT cells provided in the kit using Lipofectamine™ 2000 reagent for virus production (Invitrogen, Carlsbad, CA). The lentivirus-derived transfected 293 FT cells were used for transfection of pLenti-PKC δ -CRM and pLenti-LacZ in N27 cells. For stable transfection, single clones were isolated and selected with blasticidin (10.0 μ g/ml) in the growth medium. The stable expression was confirmed by immunostaining of V5 epitope fused at the C-terminal of PKC δ .

Transient transfection was conducted using either AMAXA Nucleofector reagent (Amaya Inc., Gaithersburg, MD) or jetPEI™ DNA *in vitro* transfection reagent (Polyplus-transfection Inc. New York, NY) by following the procedure provided by the manufacturers. For PKC δ -CF and PKC δ -RF, approximately 2 million cells were suspended in 100 μ l prepared Nucleofector™ solution V, and then mixed well with 8.0 μ g DNA. The mixture solution was transferred into a nucleofection cuvette for electroporation. Transfection efficiency was

determined by pmaxGFP transfection, which was used as control group for caspase-3 assay. For transfection of mitochondria-targeted vectors, plasmids (2.0 μ g) were first mixed with 100 μ L sterile sodium chloride (150 mM) to make the plasmid solution, then spun down briefly. The jetPEI™ solution was made by mixing 4.0 μ L jetPEI™ reagent with 100 μ L sterile sodium chloride. After a brief spin-down, the jetPEI™ solution was added to the plasmid solution and mixed well. After spin-down, 200 μ L jetPEI™/DNA mixture was incubated at room temperature for 25 min before being added into culture wells. The cells were cultured for another 24 h.

DNA Fragmentation Assay. DNA fragmentation was measured using a Cell Death Detection ELISA Plus Assay Kit (Roche Applied Science, Indianapolis, IN) as previously described (Kaul et al., 2003). The method, which measures the amount of histone-associated low molecular weight DNA in the cytoplasm, is more sensitive than conventional DNA ladder analysis. After treatment, cells were resuspended with the lysis buffer provided in the assay kit. The lysate was centrifuged at 200 x g, and 20 μ L of supernatant was incubated for 2 h with the mixture of HRP-conjugated antibody cocktail that recognizes histones, and single and double-stranded DNA. After washing away the unbound components, the final reaction product was measured colorimetrically, with ABTS as an HRP substrate using a spectrophotometer at 405 nm (490 nm as reference). Protein concentration is determined by the Bradford protein assay.

ROS Generation Assay. Flow cytometric analysis of reactive oxygen species (ROS) in N27 cells was performed using dihydroethidine (Kaul et al., 2003), a reduction production of ethidium bromide. In cytosol, blue fluorescent dihydroethidium can be dehydrogenated by superoxide (O_2^-) to form ethidium bromide, which intercalates within DNA of cells and

results in staining of nuclei that emits a bright red fluorescence. N27 cells were collected by trypsinization and resuspended in Earle's balanced salt solution (EBSS) with 2 mM calcium at a density of 1.0×10^6 cells/mL. The cell suspension then was incubated with 10 μ M hydroethidine at 37 °C in the dark. Following addition of MG-132, ROS generation in N27 cells were measured at 0, 20, 40, and 60 min. Treatment with H₂O₂ was used as positive control. ROS levels were normalized as percentage of time-matched control.

Immunocytochemistry and TUNEL Staining. Immunofluorescence staining was conducted as described previously (Sun et al., 2005). Briefly, 24 h after plasmid transfection, N27 cells cultured on coverslips pre-coated with poly-L-lysine were washed with PBS, and fixed with 4% paraformaldehyde. After permeabilization with 0.2% Triton X-100, cells were incubated with blocking buffer (5% BSA, 5% goat serum in PBS) to minimize nonspecific binding. For double staining, cells were incubated overnight with antibodies recognizing Myc tag (Abcam, Mouse monoclonal Ab 1:200) and cleaved caspase-3 (Cell signaling, Rabbit monoclonal Ab, 1:100). Then Myc tagged fusion proteins and cleaved caspase-3 were visualized with Cy3 conjugated anti-mouse and Alexa 488-conjugated anti-rabbit secondary antibodies, respectively. The images were analyzed using Nikon C1 confocal microscopy.

TUNEL staining for the transfected cells was conducted by following the protocol described by the manufacturer (Roche Applied Science, Indianapolis, IN). The immunostaining with Myc tag antibody was performed as described above following the TUNEL staining. The images were analyzed with Nikon inverted fluorescence microscopy (Model TE-2000U).

Data Analysis. Results are presented as mean \pm S.E.M., and Prism 4.0 software (GraphPad software, San Diego) was used for data analysis. P-values were determined using Student's t-test for single comparisons of two samples. One-way ANOVA was completed, and followed by Dunnett's test to compare all groups with the control group or Bonferroni's test for comparison of selected groups. A significant difference between groups was defined as $p < 0.05$.

RESULTS

1. Proteasome Inhibition by MG-132 Precedes Mitochondria Depolarization.

We assayed chymotrypsin-like proteasomal activity and conducted a detailed time course analysis for mitochondria membrane potential following MG-132 treatment. As shown in Fig. 1A, MG-132 exposure led to a rapid and significant inhibition of proteasomal activity, with less than 40% activity remaining within 5 min ($p < 0.001$). Mitochondria membrane potential was quantified with JC-1. The dye accumulates in the matrix of mitochondria with high membrane potential, and forms aggregates that fluoresce red, whereas monomers of JC-1 fluoresce green. The ratio between red and green fluorescence intensity provides a reliable measurement of the mitochondria membrane potential. Following MG-132 treatment, gradual depolarization of mitochondria membrane potential was detected. Significant reductions in red/green ratio were noted at 30, 60, 90, and 120 min (17, 34, 47, and 60% reduction, respectively) compared to control ratios (Fig.1B). These data indicate proteasomal inhibition preceded the dissipation of mitochondria membrane potential, since 5 min of treatment led to a significant decrease in proteasomal activity.

2. Proteasome Inhibition by MG-132 Triggers Mitochondria-mediated Apoptosis. Mitochondria depolarization is commonly observed in cells undergoing mitochondrial apoptosis, and it has been hypothesized to play a causal role in mitochondrial release of proapoptotic molecules in some apoptosis models (Ly et al., 2003). In N27 cells, MG-132-induced dissipation of mitochondria membrane potential was accompanied by mitochondrial release of cytochrome c and Smac into the cytosol in similar temporal pattern (Fig. 2A). No detection of mitochondria inner membrane protein COX 4 in the cytosolic fraction indicated that the cytosolic fraction was free of mitochondria contamination. Additionally, incubation of isolated mitochondria with MG-132 followed by an *in vitro* release assay indicated that mitochondrial release of cytochrome c happened as a consequence of proteasome inhibition by MG-132, but not due to the direct stimulatory effect of MG-132 on mitochondria (Fig. 2B).

Formation of the apoptosome complex by mitochondria-released cytochrome c, Apaf-1, and dATP/ATP is essential for the activation of initiator caspase-9, which then activates downstream effector caspase-3. As shown in Fig. 2C, caspase-9 activity significantly increased following MG-132 treatment for 90 min (74%, $p < 0.05$), 120, 150 and 180 min (200%, 361%, and 388%, $p < 0.001$). The same MG-132 treatment also resulted in dramatic increase in caspase-3 activation from 90 to 150 min (10 to 25 fold, $p < 0.001$) (Fig. 2D); whereas minimal increase in the caspase-8 activity was observed only after 120 min (Fig. 2C). It appears that caspase-8 activation followed caspase-3 activation. Additionally, caspase-3 activation could be completely blocked by caspase-9 inhibitor LEHD-fmk (Fig. 2E), indicating that caspase-9 is the exclusive upstream caspase responsible for MG-132-induced caspase-3 activation.

3. Proteasomal Inhibition by MG-132 Leads to Proteolytic Activation of PKC δ .

Emerging evidence suggests that PKC δ serves as a crucial mediator of apoptosis in different types of cells, though the underlying mechanisms vary markedly (Brodie and Blumberg, 2003). Recently it was demonstrated that this proapoptotic kinase is highly expressed in mouse nigral dopamine neurons (Zhang et al., 2007), and previous studies using *in vitro* model revealed the essential role of proteolytically activated PKC δ in oxidative stress-induced dopaminergic degeneration (Anantharam et al., 2002; Kaul et al., 2003; Kitazawa et al., 2003; Yang et al., 2004; Latchoumycandane et al., 2005). Western blot analysis revealed proteolytic cleavage of PKC δ in N27 cells following exposure to the proteasome inhibitor MG-132 (Fig. 3A), indicative of potential regulatory role of PKC δ in dopaminergic degeneration following UPS dysfunction. It appears that proteolytic cleavage is caspase-3 dependent, since it was diminished markedly by the pan-caspase inhibitor ZVAD-fmk or caspase-3 inhibitor DEVD-fmk, (Fig. 3A). To assess the effect of proteolytic cleavage of PKC δ on its kinase activity, the PKC δ immunoprecipitated from cell lysates was incubated with [32 P]-ATP and histone H2B as *in vitro* substrate. Analysis of the intensity of radioactively-labeled histone H1 bands indicated that MG-132 exposure results in a 282% increase in kinase activity of PKC δ (Fig. 3B). Inhibition of PKC δ proteolytic cleavage either by caspase-3 inhibitors DEVD-fmk (50 μ M) or rottlerin or pan-caspase inhibitor ZVAD-fmk (100 μ M) diminished its kinase activity, indicating that caspase-3 mediates PKC δ proteolytic cleavage and significantly activates its kinase activity (Fig. 3B).

4. Activation of Apoptosis Cascade by MG-132 Independent of ROS Generation.

Oxidative stress has been implicated in dopaminergic neuronal cell death in PD. ROS generation has been previously shown to induce PKC δ proteolytic activation in N27 cells exposed to MPP⁺ (Kaul et al., 2003; Yang et al., 2004), and in PC12 cells treated with 6-OHDA (Hanrott et al., 2006). Induction of ROS during proteasome inhibition was previously reported to contribute to apoptosis in several cell lines (Lauricella et al., 2003; Fribley et al., 2004; Lee et al., 2005). However, in the present study using dopaminergic neuronal cells, no significant elevation of ROS level was noted within 60 min following MG-132 treatment (Fig. 4A). MnTBAP, a superoxide dismutase (SOD) mimic that markedly alleviates oxidative stress and apoptosis induced by MPP⁺ in N27 cells (Kaul et al., 2003), failed to attenuate caspase-3 activation following MG-132 exposure (Fig. 4B). This indicates a negligible role of ROS in MG-132-induced mitochondrial apoptosis in the dopaminergic neuronal cells.

5. Activated PKC δ as Mediator for MG-132-induced Mitochondrial Apoptosis.

To directly examine the role of proteolytically activated PKC δ in apoptosis, N27 cells were transiently transfected with PKC δ -CF or PKC δ -RF. The transfection efficiency was estimated by the cotransfected GFP plasmids (Fig. 5A). Enzymatic assessment of the transfected N27 cells revealed a significant increase in the caspase-3 activity in PKC δ -CF-transfected cells, compared to RF-transfected or GFP-transfected cells, suggesting that kinase active PKC δ -CF is responsible for its proapoptotic effect in dopaminergic cells (Fig. 5B). In an attempt to determine whether PKC δ activation indeed contributes to caspase activation following exposure to proteasome inhibitor MG-132, we found that pretreatment with PKC δ -

specific inhibitor rottlerin significantly attenuated MG-132-induced caspase-9 and -3 activation (Fig. 5C-D). This finding suggests that the proapoptotic effect of PKC δ proceeds through the mitochondrial apoptosis pathway, which is consistent with our finding of caspase-9 as the exclusive upstream caspase responsible for caspase-3 activation (Fig. 2E).

6. Mitochondrial Translocation of Active PKC δ Activates Caspase-3. In light of the mitochondrial dependence of proapoptotic PKC δ , we examined the mitochondrial localization of PKC δ , since subcellular translocation could be important for PKC δ function by controlling accessibility of the substrates to the kinase. As shown in Fig. 6A, MG-132 treatment resulted in substantial accumulation of cleaved PKC δ in the mitochondria fraction, with only slight elevation of full-length PKC δ observed. To understand whether mitochondrial translocation of PKC δ -CF is functionally related to its proapoptotic effect, mitochondria-targeted expression of PKC δ -CF and PKC δ -RF was achieved using pCMV/myc/mito vector. Double immunostaining for myc tag (red) and active caspase-3 (green) revealed the presence of active caspase-3 in the PKC δ -CF transfected cells, but not in the PKC-RF or LacZ transfected cells (Fig. 6B). Also, high proportion of cells transfected with PKC δ -CF, but not PKC δ -RF or LacZ, appeared to be TUNEL positive (Fig.6C).

7. Suppression of PKC δ Proteolytic Activation Protects Cells from Mitochondria-mediated Apoptosis Following Proteasome Inhibitor Exposure. PKC δ kinase activity could be modulated by mechanisms other than proteolytic cleavage, such as plasma membrane translocation or phosphorylation (Steinberg, 2004). To further substantiate that proteolytic activation is primarily responsible for the feedback amplification of the

caspase signal during the dopaminergic apoptosis, a caspase-3 cleavage-resistant mutant of PKC δ (PKC δ^{D327A} , PKC δ -CRM), was introduced into N27 cells using a lentivirus expression system. Immunoblotting with antibody against PKC δ^{D327A} -V5 fusion proteins stably expressed in N27 cells showed that PKC δ^{D327A} was non-cleavable following MG-132 treatment (Fig. 7A). Meanwhile, stable expression of PKC δ -CRM efficiently inhibited the proteolytic cleavage of endogenous PKC δ following exposure to proteasome inhibitor via mechanisms yet to be characterized (Fig. 7B). The CRM cells also appear to be more resistant to MG-132-induced mitochondrial apoptosis, as indicated by the significant reduction of caspase-9, caspase-3 activation and DNA fragmentation compared to LacZ transfected cells (Fig. 7C-E).

DISCUSSION

The present study revealed an important regulatory role of PKC δ in mitochondrial apoptosis in mesencephalic dopaminergic neuronal cells following proteasome inhibition. We demonstrated activation of the mitochondrial apoptosis cascade and proteolytic activation of PKC δ during proteasome inhibition. Importantly, we found that proteolytic activation and mitochondrial translocation of PKC δ underlie its positive feedback amplification of mitochondrial apoptosis during proteasome dysfunction in mesencephalic dopaminergic neuronal cells. This mitochondria-dependent proapoptotic capacity of PKC δ also sheds light on the mechanisms of PKC δ as a key mediator in oxidative stress-induced dopaminergic apoptosis observed previously (Anantharam et al., 2002; Kaul et al., 2003; Yang et al., 2004).

Dysfunctional UPS has been implicated in the pathogenesis of Parkinson's disease in addition to mitochondria dysfunction and oxidative stress. Previous studies have revealed

that the substantia nigra particularly suffers from UPS dysfunction in the brains of patients with sporadic PD (Moore et al., 2005; Olanow and McNaught, 2006; Sun et al., 2007). Mutation in Parkin and UchL-1 in familial PD has provided further evidence for the contributory roles of impaired UPS function in PD (Moore et al., 2005; Olanow and McNaught, 2006; Sun et al., 2007). Proteasome inhibitors have been shown to reproduce some key features of PD, including neuronal death (Rideout et al., 2001; McNaught et al., 2002; Rideout et al., 2005). However, underlying cell death mechanisms during UPS dysfunction remains to be determined. In the present study, we showed substantial reduction of proteasomal activity shortly after exposure to 5.0 μ M MG-132 (70%, Fig 1A), which was followed by progressive dissipation of mitochondrial membrane potential (Fig 1B). Mitochondrial depolarization has been extensively observed during apoptosis, concurrent with mitochondrial release of proapoptotic molecules in some apoptosis models (Ly et al., 2003). Following MG-132 treatment, cytosolic cytochrome c and Smac levels progressively increased in N27 cells (Fig. 2A). It appears that mitochondrial release of cytochrome c occurred as a consequence of proteasome inhibition by MG-132, but not due to a direct stimulatory effect of MG-132 on mitochondria, since incubation of isolated mitochondria with MG-132 failed to trigger mitochondrial release of cytochrome c (Fig. 2B). Association of cytosolic cytochrome c with Apaf-1 and dATP/ATP as the apoptosome complex is essential for the activation of initiator and effector caspases. Following MG-132 treatment, significant activation of caspase-9 and -3 was observed for 90 min (Fig. 2C-D). Unexpectedly, caspase-8 and -9 activities were significantly lower following MG-132 treatment within 60 min (Fig. 2C), presumably due to accumulation of anti-apoptotic proteins such as IAPs or Mcl-1 upon proteasome inhibition (Yang et al., 2000; Nijhawan et al., 2003).

Slight activation of caspase-8 at late timepoints (150 and 180 min) agrees with previous reports demonstrating caspase-8 activation as the result of caspase-9 and -3 activation (Viswanath et al., 2001); whereas caspase-8 appears to play a negligible role in caspase-3 activation. Notably, apoptosis proceeded exclusively through the mitochondria-mediated apoptotic pathway, since caspase-3 activation was completely suppressed by the caspase-9 inhibitor LEHD-FMK (Fig 2E), indicative of caspase-9 as the major upstream initiator caspase.

Proteolytic cleavage of PKC δ , as an endogenous substrate of caspases-3, physically dissociates the auto-inhibitory regulatory fragment from its catalytic fragment, thus permanently activating its kinase activity. Tyr-311 phosphorylation of PKC δ is critical for its caspase-3-mediated proteolytic cleavage (Kaul et al., 2005). The proteolytically activated PKC δ has been previously shown to be the key mediator for oxidative stress-induced apoptosis in dopaminergic neuronal cells, possibly through positive feedback activation of caspase-3 (Anantharam et al., 2002; Kaul et al., 2003; Yang et al., 2004; D'Costa and Denning, 2005). Phosphorylation of caspase-3 by full-length PKC δ has been shown to increase enzymatic activity of caspase-3 in monocytes (Voss et al., 2005). However, PKC δ amplifies caspase-3 activation via distinct mechanisms in dopaminergic neuronal cells. Proteolytic activation of PKC δ in N27 cells appears to depend on caspase-3 activation in N27 cells (Fig. 3A-B); whereas caspase-3 activation is preceded by PKC δ activation in monocytes (Voss et al., 2005). The direct proapoptotic effect of PKC δ -CF is manifested by the elevation of caspase-3 activity in PKC δ -CF transfected cells (Fig. 5B), consistent with apoptotic features observed previously in the cells expressing PKC δ -CF (Denning et al., 2002). In addition, PKC δ likely enhances activation of caspase-9, which further activates the

downstream effector caspase-3 activation, since the PKC δ -specific inhibitor rottlerin attenuates activation of caspase-3 and upstream initiator caspase-9 in this experimental setting (Fig. 5C-D).

Induction of ROS generation following prolonged exposure to proteasome inhibitors Bortezomib (Ling et al., 2003), MG-132, lactaystin (Wu et al., 2002), and PS-341 (Fribley et al., 2004) has been implicated as a key mediator for some downstream cellular events, including apoptosis in several cell lines. Oxidative stress has been demonstrated to activate caspase-3 and PKC δ in N27 cells (Kaul et al., 2003; Kitazawa et al., 2003). In an attempt to determine whether dopaminergic apoptosis following MG-132 exposure involves oxidative stress, ROS generation was measured; no significant increase in ROS generation was noted (Fig. 4A). The antioxidant MnTBAP, which has been previously shown to effectively inhibit caspase-3 activation during oxidative stress in the N27 cells (Kaul et al., 2003), failed to attenuated caspase-3 activation induced by MG-132 (Fig. 4B). Our data suggest that ROS generation plays negligible role in apoptotic cell death following proteasome inhibition in mesencephalic dopaminergic neuronal cells.

The mitochondria-dependent proapoptotic capacity of active PKC δ , as indicated by suppression of caspase-9 activation by rottlerin, was accompanied by mitochondrial translocation of PKC δ . The proteolytically activated PKC δ appears to be readily available for mitochondrial translocation in the mesencephalic dopaminergic neuronal cells, consistent with mitochondria as a target organelle as reported previously (Denning et al., 2002) in addition to nuclei (Cross et al., 2000), Golgi (Kajimoto et al., 2004) and endoplasmic reticulum (Zrachia et al., 2002). In an attempt to determine whether mitochondrial translocation of proteolytically activated PKC δ underlies its proapoptotic effect, marked

activation of caspase-3 was noted in the N27 cells expressing mitochondria targeted PKC δ -CF (Fig.6B), but not PKC δ -RF or LacZ. This indicates that mitochondrial translocation of PKC δ -CF possibly underlies its feedback amplification of caspase activation extensively observed (Anantharam et al., 2002; Kaul et al., 2003; Kitazawa et al., 2003). Considering that multiple ways lead to PKC δ activation, we conducted additional experiments to verify that PKC δ proteolytic activation mediates its mitochondria-dependent proapoptotic effect. Expression of a caspase-3 cleavage-resistant mutant of PKC δ (PKC δ^{D327A}), which effectively inhibited the proteolytic cleavage of endogenous PKC δ (Fig. 7B), significantly attenuated the activation of mitochondrial apoptosis triggered by MG-132 (Fig. 7C- E), consistent with a recent study showing that PKC δ -CRM reduces the mitochondrial release of cytochrome c in UV-challenged keratinocytes (D'Costa and Denning, 2005). Phosphorylation of mitochondrial resident proteins by active PKC δ likely underlies its effect on mitochondrial apoptosis. Several mitochondrial proteins have been characterized as candidate substrates of PKC δ , including phospholipid scramblase (He et al., 2007) and pyruvate dehydrogenase kinase (Churchill et al., 2005).

In summary, the present study demonstrates that proteolytic activation and mitochondrial translocation of PKC δ underlies its feedback activation of mitochondrial apoptosis during proteasome dysfunction in mesencephalic dopaminergic neuronal cells. This likely explains the role of PKC δ as a key mediator in oxidative stress-induced dopaminergic apoptosis as shown in previous studies. Taken together, PKC δ could function as a common mediator promoting dopaminergic degeneration during UPS dysfunction or oxidative stress in nigral dopamine neurons, in which PKC δ is highly expressed. This knowledge advances

our understanding of the pathogenesis of nigrostriatal degeneration and validates PKC δ as potential target for therapeutic manipulation of PD.

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FIGURE 1.

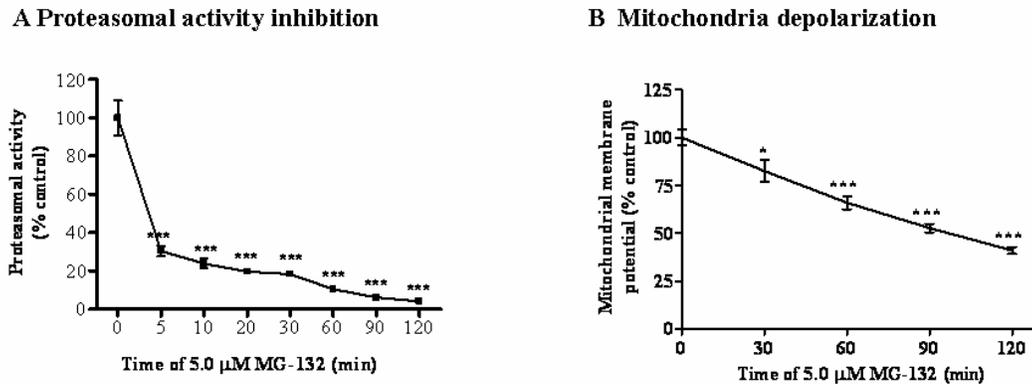
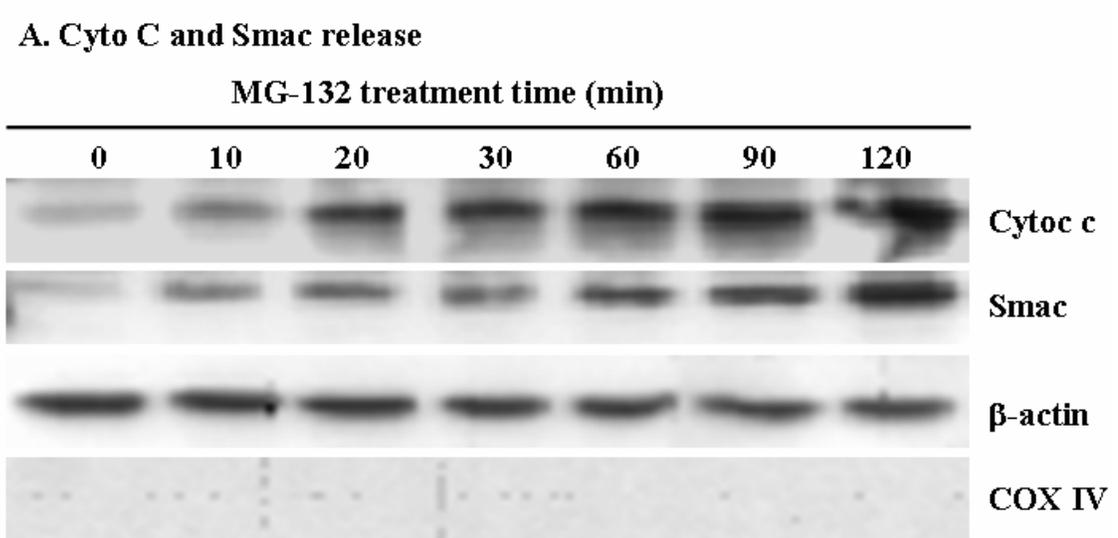


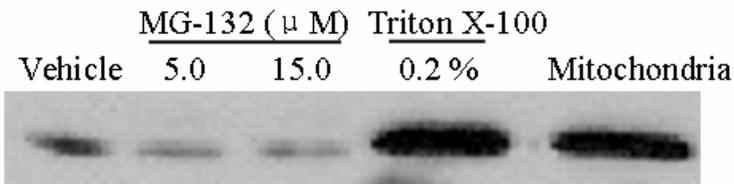
Fig.1 Proteasome inhibition by MG-132 precedes mitochondria depolarization.

A. Determination of proteasomal activity. N27 cells were treated with 5.0 μ M MG-132 for indicated duration before cell collection and assessment of chymotrypsin-like proteasomal activity using Suc-LLVY-AMC. Enzymatic activity is presented as percentage over vehicle-treated control group. Values represent mean \pm S.E.M for 6 samples in each group. **B. Flow cytometric determination of mitochondrial membrane potential.** N27 cells were treated with 5.0 μ M MG-132 for indicated duration. The intensity of red fluorescence for aggregated JC-1 and green fluorescence for monomer JC-1 were determined using flow cytometry, and the red/green ratio was used as the measurement of membrane potential. Values presented as mean \pm S.E.M represent results of 2 experiments with N=6 and 4 respectively. * p <0.05, *** p <0.001 comparing with control group (One-way ANOVA followed by Dunnett's-post test).

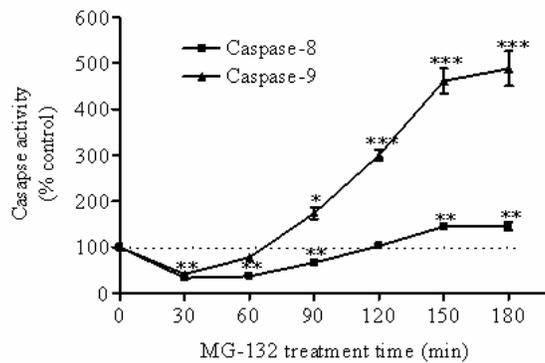
FIGURE 2.



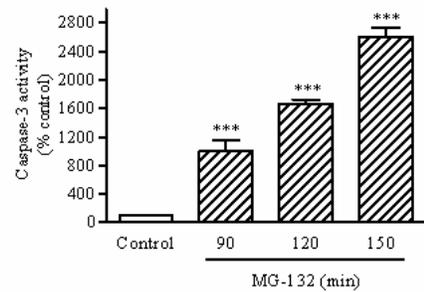
B. No cytochrome C release in vitro



C: Activation of caspase-8, 9



D: Activation of caspase-3



E. Blockage of caspase-3 activation by caspase-9 inhibitor

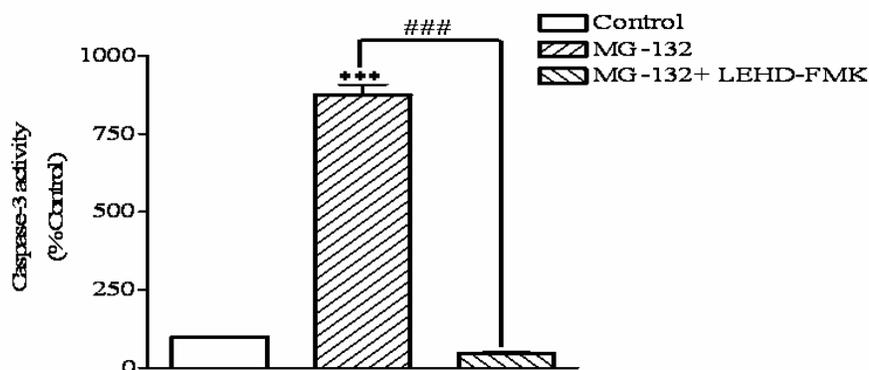
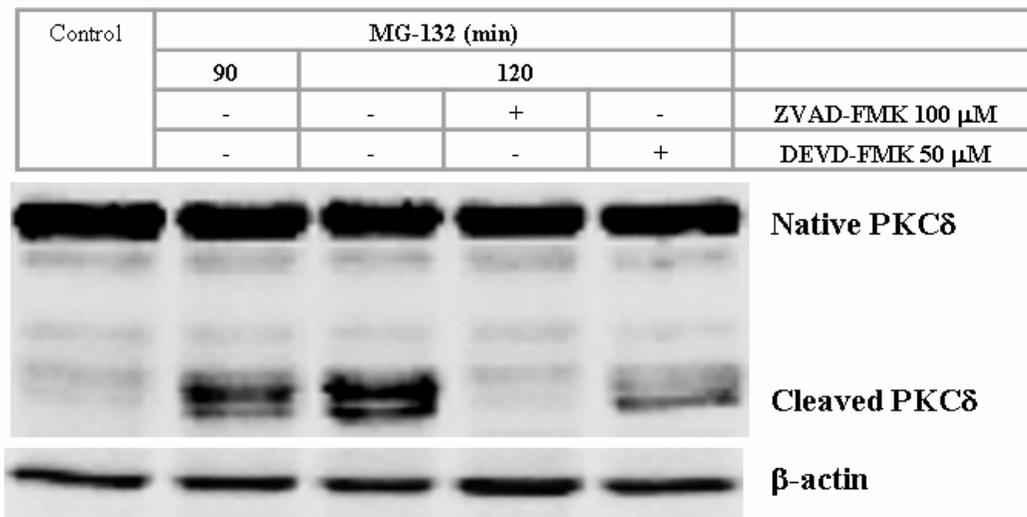


Fig 2. Proteasome inhibition by MG-132 triggers mitochondria-mediated apoptosis.

A. Mitochondrial release of cytochrome c and Smac. N27 cells were treated with 5.0 μ M MG-132 for the indicated duration. The cytosolic fractions prepared from the treated cells were resolved on 15% SDS-PAGE and blotted with antibodies against cytochrome c (Cyto c), Smac, β -actin or COX IV. **B. In vitro mitochondrial release.** Mitochondria were isolated from N27 cells and resuspended in the isolation buffer at 2.0 mg/ml. The equal amount of mitochondrial suspension were incubated with 5.0 (lane 2) or 15.0 μ M (lane 3) MG-132 for 1 hr, with 0.2% Triton X-100 incubation as positive control to release cyto c (lane 4). Lane 5 is input of isolated mitochondria. **C and D. Activation of caspase-8, -9 and -3.** Cells were treated with 5.0 μ M MG-132 for 30, 60, 90, 120 or 180 min. The caspase-8, -9 and -3 activities were determined using fluoregenic substrates as described in the materials and methods. Data is presented as mean \pm S.E.M for 8 samples derived from two experiments. **E. Inhibition of caspase-3 activation by caspase-9 inhibitor LEHD-FMK.** N27 cells were preincubated with LEHD-FMK (50 μ M) for 40 min before treated with 5.0 μ M MG-132 for additional 120 min. The cells were collected for caspase-9 assay. Values represent mean \pm

S.E.M from 6 individual samples. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs vehicle treated control group (One-way ANOVA followed by Dunnett's-post test).

FIGURE 3.

A. Proteolytic cleavage of PKC δ 

B. Activation of Kinase activity

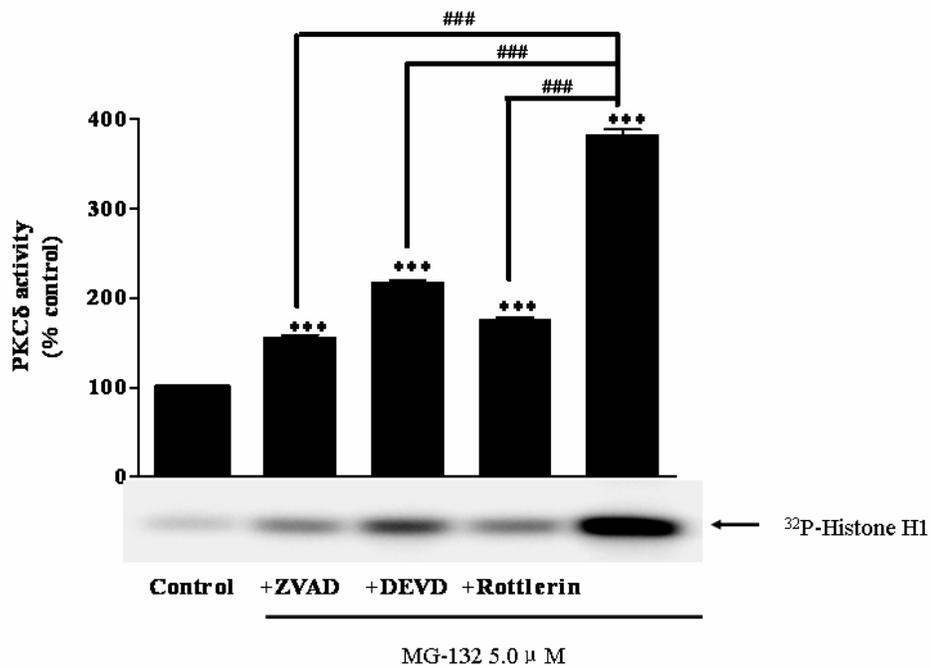
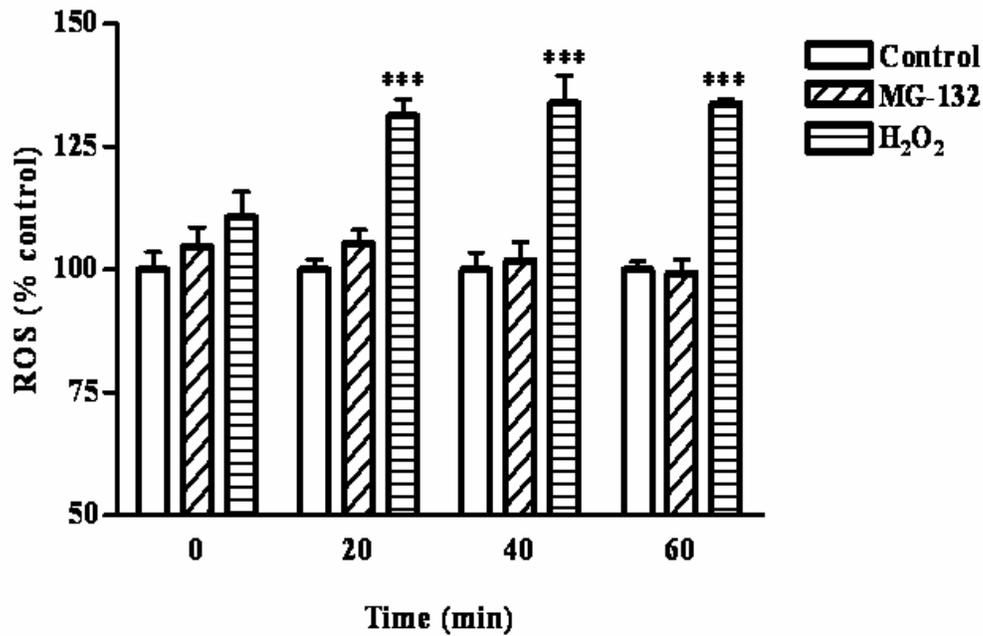


Fig.3 Proteasomal inhibition by MG-132 leads to caspase-3 mediated proteolytic activation of PKC. A. Proteolytic cleavage of PKC δ .

N27 cells were treated with 5.0 μ M MG-132 for 90 or 120 min. For inhibitor study, cells were preincubated with 100 μ M ZVAD or 50 μ M DEVD-FMK for 40 min before 120 min MG-132 treatment. Equal amount of protein from individual samples were separated in SDA-PAGE and immunoblotted with antibody for PKC δ . Reprobing membrane with β -actin antibody to ensure equal protein loading. **B. Activation of PKC δ kinase.** N27 cells were exposed to 5.0 μ M MG-132 for 120 min. For inhibitor study, cells were pretreated with with 100.0 μ M ZVAD-fmk (+ZVAD), 50.0 μ M DEVE-fmk (+DEVD) or 2.5 μ M rottlerin (+Rottlerin) for 40 min. The cell lysates were prepared for PKC δ immunoprecipitation, and the kinase activity associated with immunoprecipitates was assayed by determining the intensity of the 32 P-labeled H1. The arrow indicates the radioactively labeled H1. Densitometric analysis for the intensity of H1 bands is presented as percent of control. The data represents the mean \pm S.E.M. from 4 separate experiments. ***P<0.001 comparing with vehicle-treated groups (One-way ANOVA followed by Dunnett's-post test), and ####P<0.001 comparing with MG-132 treatment group (One-way ANOVA followed by Bonferroni-post test)..

FIGURE 4.

A. MG-132 does not induce ROS generation



B. Effect of MnTBAP on MG-132-induced caspase-3 activation

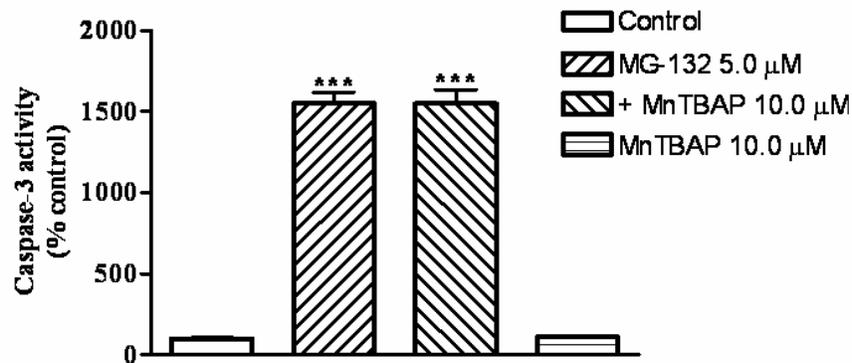


Fig.4 Caspase-3 and PKC δ activation following MG-132 exposure is independent of ROS generation.

Fig.4 Caspase-3 and PKC δ activation following MG-132 exposure is independent of ROS generation.

A. ROS assay. Intracellular superoxide was quantified by cytometric determination of fluorescence intensity of oxidized dihydroethidine in N27 following exposure to 5.0 μ M MG-132 for 0, 20, 40 or 60 min, as described in the material and methods section. Data represent the mean \pm S.E.M. for two separate experiments with 2 or 5 samples respectively. Treatment with 200 μ M H₂O₂, which promotes superoxide production, was used as positive control. **(B) Effect of MnTBAP on MG-132-induced caspase-3 activation.** Cells were treated with either with 2.5 μ M MG-132 alone or pretreated with 10.0 μ M MnTBAP 30 min prior to MG-132 treatment. The caspase-3 activity was determined as described above. Data are presented as mean \pm S.E.M. from 6 samples in each group. ***p<0.001 comparing with vehicle-treated control cells (One-way ANOVA followed by Dunnett's-post test).

FIGURE 5.

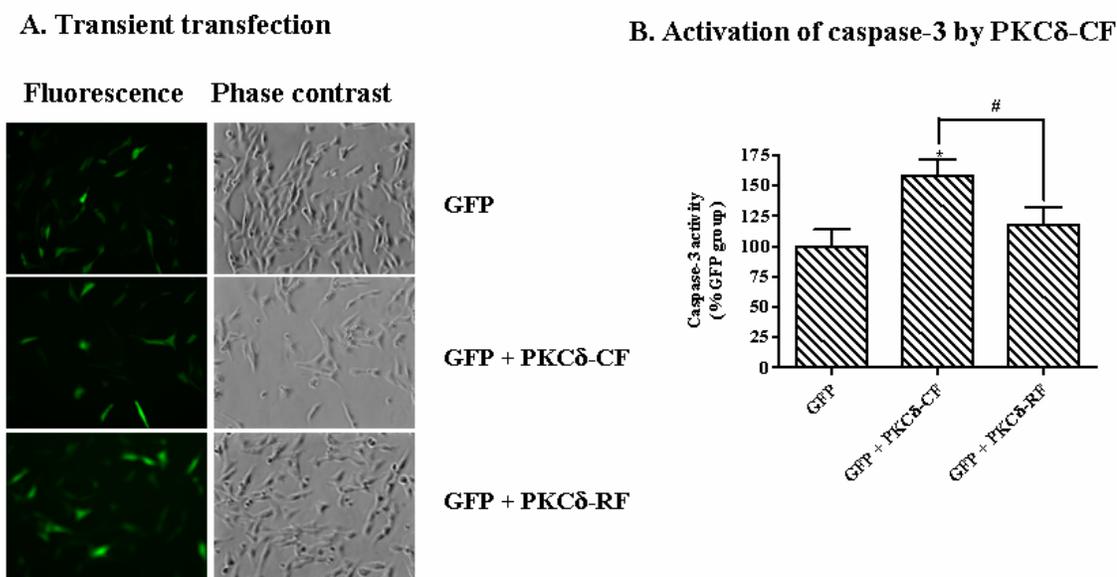
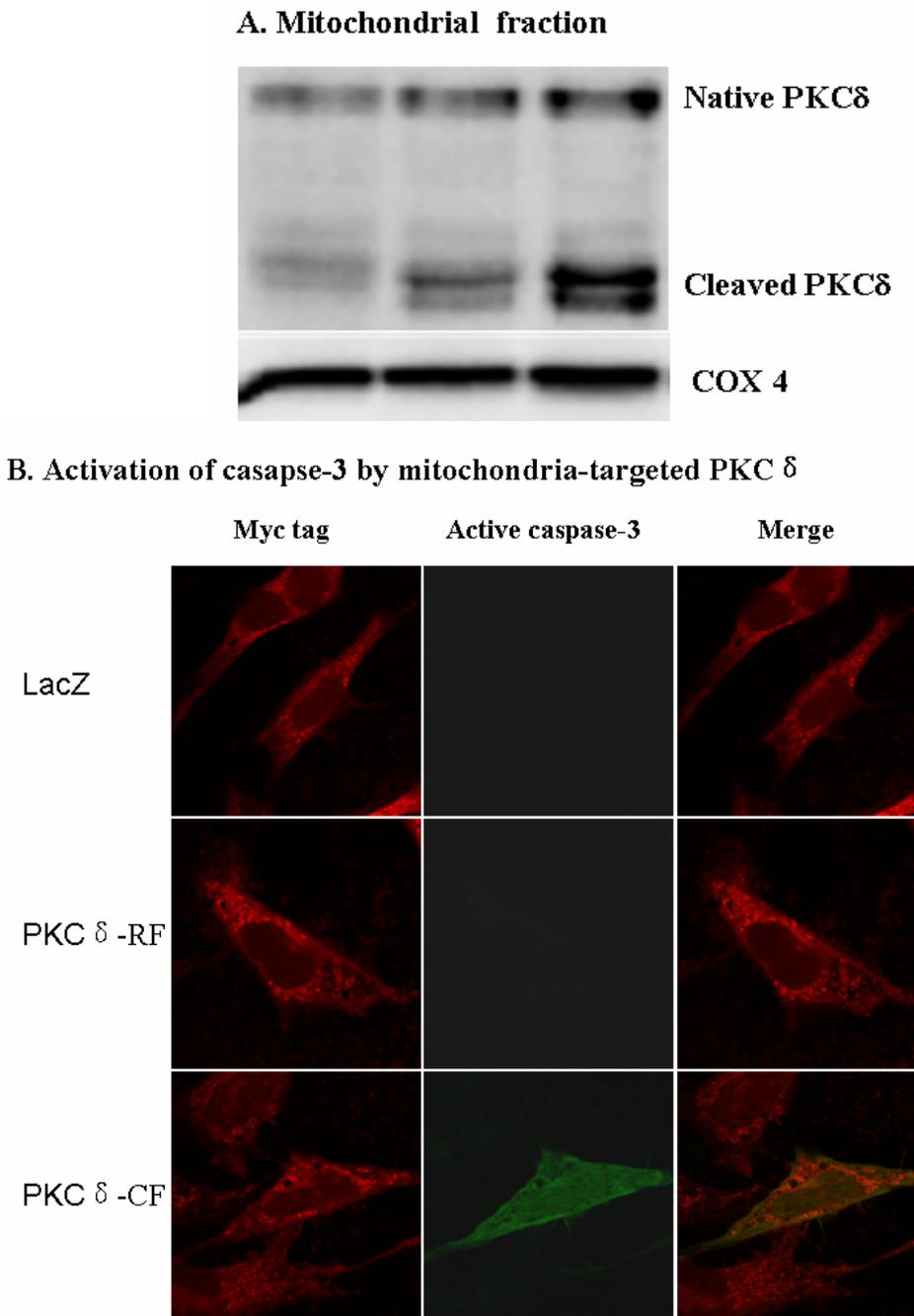


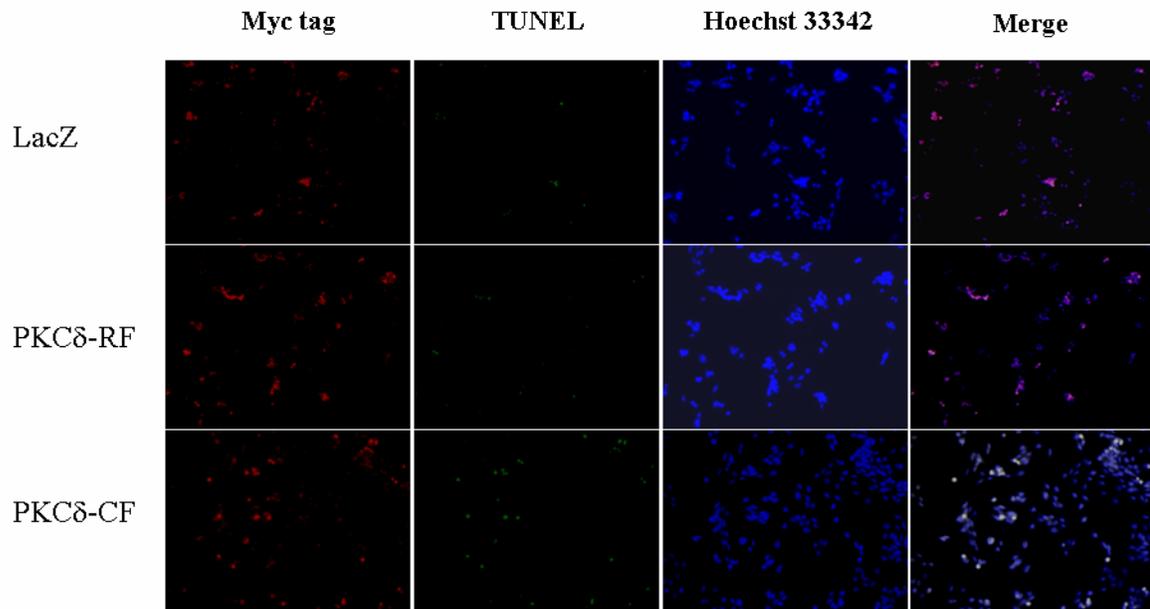
Fig.5 Activation of caspase-3 by PKCδ-CF.

Twenty-four hour after transfection, phase contrast images and fluorescence images were taken to determine transfection efficiency (A). The transfected cells were collected for caspase-3 assay (B). Values represent mean \pm S.E.M. from 6 samples in each group. * $p < 0.05$ vs cells transfected with pmaxGFP alone; # $p < 0.05$ comparing the indicated groups (One-way ANOVA followed by Bonferroni-post test). C. Inhibition of mitochondria apoptosis by rottlerin. N27 cells were treated with 5.0 μ M MG-132 for 120 min with or without 40 min rottlerin (2.5 μ M) pretreatment. Treatment with rottlerin alone was included in the experiment. Caspase-9 (C) and -3 activities (D) were assayed for the treated cells as described above. Data are presented as mean \pm S.E.M. from 6 samples in each group. *** $p < 0.001$ comparing with vehicle-treated control cells (One-way ANOVA followed by Dunnett's-post test). ### $p < 0.001$, comparison between the indicated group (One-way ANOVA followed by Bonferroni-post test).

FIGURE 6.



C. TUNEL staining



A. Mitochondria-localized active PKCδ on apoptosis.

Mitochondria fraction was prepared from cells exposed to 5.0 μM MG-132 for 90 or 120 min. Mitochondrial lysates were separated on SDS-PAGE and immunoblotted with PKCδ antibody, and the membrane was reprobed COX IV to show equal protein loading. **B. Mitochondria-localized active PKCδ activates caspase-3.** After N27 cells were transfected with pCMV/myc/mito containing coding sequence for LacZ, PKCδ-RF or PKCδ-CF, double immunostaining was conducted using mouse Myc tag primary antibody and rabbit active caspase-3 antibody. The Myc tag and active caspase-3 were visualized using Cy3 conjugated anti-mouse (red) and Alexa-488 conjugated anti-rabbit (green) secondary antibodies. **C. TUNEL staining in the transfected cells.** After transfection for 24 hr, cells were subjected to TUNEL staining (green) and immunostaining with Myc tag antibody (red). The images were analyzed with fluorescence microscopy under 10× objective len.

FIGURE 7.

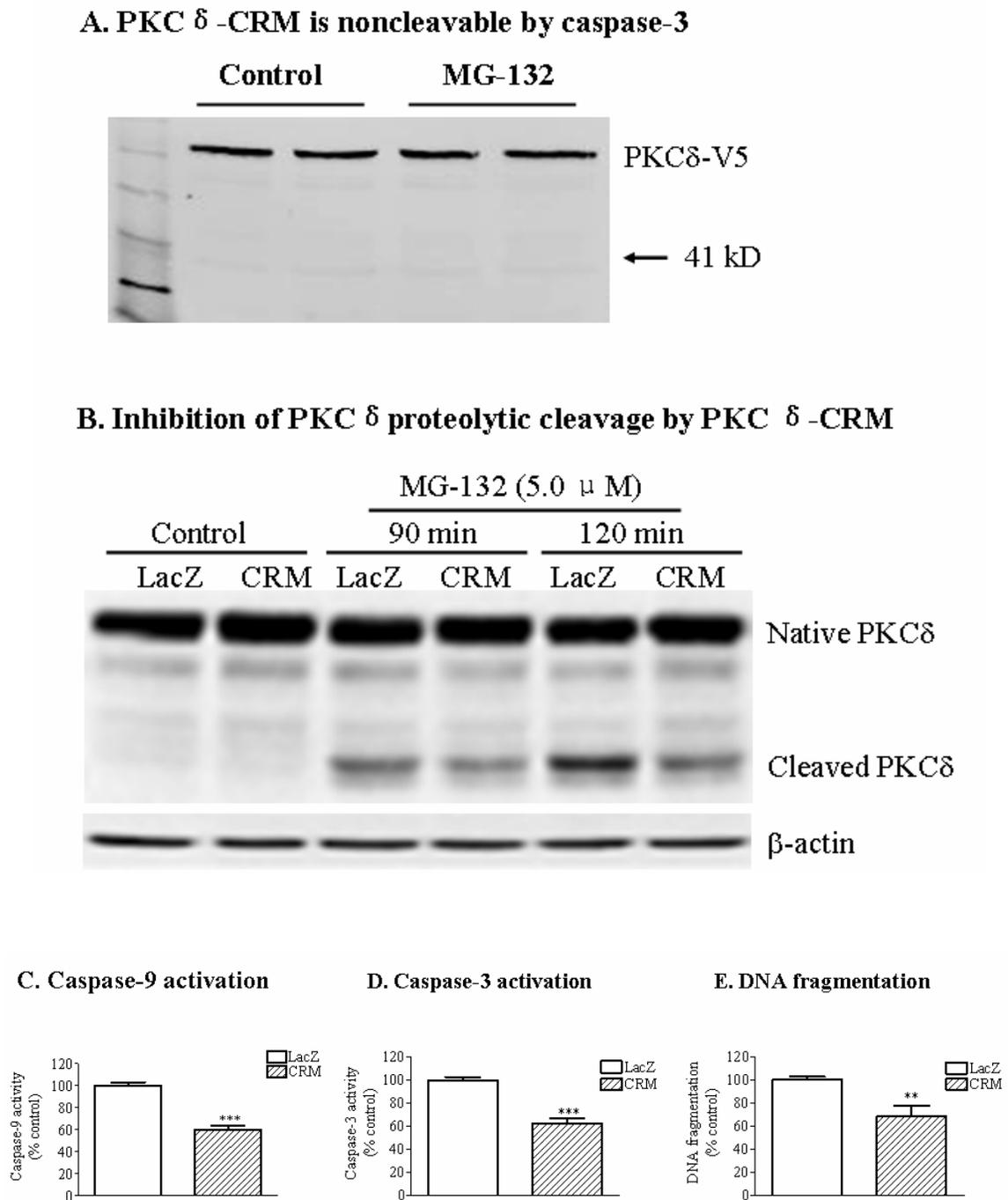


Fig.7 Suppression of PKC δ proteolytic activation protects cells from mitochondria mediated apoptosis during proteasome inhibition.

A) PKC δ -CRM is noncleavable. N27 cells stably expressing PKC δ -CRM were treated with 5.0 μ M MG-132 for 120 min. The cell lysate prepared from the treated cells were resolved on SDS-PAGE and immunoblotted with V5 antibody. The arrow indicates the position for the V5- tagged cleaved PKC δ , if any, at 41 kD. **B) Suppression of PKC δ proteolytic cleavage by PKC δ -CRM.** N27 cells stably transfected with LacZ (as control) and PKC δ -CRM were treated with 5.0 μ M MG-132. Equal amount of protein from the LacZ and CRM cells were separated in SDS-PAGE and transferred into nitrocellulose membrane for immunoblotting with PKC δ antibodies. The membrane was reprobed and blotted with β -actin antibody. **C) D) and E)** Suppression of mitochondria-mediated apoptosis by PKC δ -CRM. **Caspase-9 activity (C), caspase-3 activity (D)** and DNA fragmentation (E) were determined for LacZ and CRM cells exposed to MG-132 for 120 min. The values are expressed as the percentage of the vehicle treated control cells. Results represent mean \pm S.E.M from 2 separate experiments in quadruplet. * $p < 0.05$, and *** $p < 0.001$ (Student's t-test).

**CHAPTER VI: NOVEL NEUROPROTECTIVE ROLE OF LYSINE48 MUTANT
UBIQUITIN DURING NEUROTOXICANTS-INDUCED UBIQUITIN-
PROTEASOME DYSFUNCTION IN PARKINSON'S DISEASE MODELS**

Faneng Sun, Qinglin Li, Anumantha G Kanthasamy, Daqing Huang, Vellareddy
Anantharam, Arthi Kanthasamy

ABSTRACT

Impaired proteolysis by ubiquitin proteasome system (UPS) has been linked to the pathogenesis of Parkinson's disease (PD). Studies showed that excessive cytosolic dopamine and exposure to MPP⁺ and dieldrin compromise UPS-mediated protein degradation, whereas the contributory role of UPS impairment in dopaminergic neurotoxin-induced neurodegeneration remains unsolved. In the present study, we characterized the effects of dopamine (200 μ M for 12, 18 or 24 h), MPP⁺ (200 μ M for 12, 18 or 24 h), and dieldrin (60 μ M for 20, 40 or 60 min) on UPS function, and determined the role of polyubiquitin sites in dopaminergic neuron survival or death. Enzymatic analysis indicates that proteasomal activities were differentially affected by neurotoxin exposure. Reduction of peptidyl-glutamyl peptide hydrolase (PGPH) and chymotrypsin-like activity (C-L) were observed in cells following exposure to dopamine, MPP⁺ and dieldrin; whereas trypsin-like activity (T-L) increases significantly following exposure to dopamine (18 and 24 h), but not MPP⁺ or dieldrin. Furthermore, exposure to dopamine, MPP⁺ or dieldrin elevated ubiquitinated proteins in N27 cells. To determine whether the accumulation of polyubiquitinated proteins is involved in the cell death process, we established cell lines stably expressing wild-type or mutant ubiquitin (ubiquitin^{K48R} or ubiquitin^{K63R}). Results show that ubiquitin^{K48R} significantly suppresses caspase-3 activation and cell death following exposure to dopamine, MPP⁺ or dieldrin, indicating a critical role of accumulation of K48 polyubiquitin in

dopaminergic degeneration. Interestingly, mutation at lysine 63 (K63R), which is not required for extension of polyubiquitin chains destined for UPS degradation, significantly sensitized N27 cells to neurotoxicity. Collectively, these novel findings suggest that UPS dysfunction actively contributes to dopaminergic degeneration during neurotoxin exposure through accumulation of K48 polyubiquitin.

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder. The cardinal motor symptoms of PD include bradykinesia, resting tremors, and rigidity, and a high proportion of PD cases eventually develop cognitive impairment and other symptoms. Pathologically, the disease is characterized by prominent and preferential loss of nigral dopamine neurons and presence of Lewy bodies in the remaining dopamine neurons (1). The underlying mechanisms for selective dopamine neuron death remain poorly understood. Dopamine homeostasis misregulation has been proposed as the underlying mechanism for the vulnerability of nigral dopamine neurons (2), since excessive cytosolic dopamine is detrimental to dopamine neurons. A recent study by Mosharov and colleagues demonstrated that α -synuclein mutants dissipate the proton gradient across the vesicle membrane and elevated cytosolic dopamine levels (3), indicative of the pathological relevance of dopamine neurotoxicity in selective dopamine degeneration. Excessive cytosolic dopamine likely also contributes to the neurotoxicity provoked by methamphetamine (4, 5). MPTP and its active metabolite MPP⁺ are commonly used Parkinsonian neurotoxins for PD modeling, due to their selective neurotoxicity to dopaminergic neurons. Extensive studies exploring MPTP/MPP⁺ have shown that exposure to MPTP/MPP⁺ causes dopaminergic neuron death by

mitochondria inhibition and oxidative stress (6). Epidemiological, experimental and postmortem studies have linked dieldrin, an organochlorine pesticide, to PD development (7). A study by Kitazawa and coworkers showed that dopamine release and ROS generation are early responses during dieldrin exposure, and that dieldrin elicits gross neurotoxicity in dopaminergic neuronal cells (8).

Intracellular proteolysis by ubiquitin-proteasome system (UPS) plays an important role in maintenance of cellular homeostasis by getting rid of unwanted, damaged and misfolded proteins. Impairment of UPS-mediated proteolysis has been linked to the pathogenesis of Parkinson's disease (PD), since PD-related genes Parkin and Ucl-L1 function as important components of UPS degradation, and the presence of intracellular protein aggregations indicates proteolytic stress in dopaminergic neurons during the degeneration process (1, 9). Studies have shown that exposure to MPP⁺ (10), dopamine (11) or dieldrin reduces proteasome activity in dopaminergic neuronal cells. Additionally, chronic treatment with dopaminergic neurotoxin MPTP impairs UPS function, as demonstrated by the reduction of proteasome subunits and activity (12, 13). Currently, the contributory roles of impaired UPS degradation in dopaminergic degeneration are poorly understood, since UPS dysfunction could occur as a response secondary to other cell events such as mitochondria inhibition, oxidative stress or cell death. The direct consequence of proteasome inhibition is the accumulation of K48 polyubiquitinated proteins. In this study, we demonstrated for the first time that dominant negative ubiquitin^{K48R}, which terminates K48 polyubiquitin chain extension, effectively rescues dopaminergic neurons from neurodegeneration *in vitro*.

MATERIAL AND METHODS

Cell culture. As previously described (14), immortalized mesencephalic dopaminergic clonal cells (N27 cells) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

Enzymatic assay for proteasomal peptidase activities. The assay was conducted as described previously (15) with modification. Briefly, cells were resuspended in hypotonic buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA) supplemented with 10 µM digitonin, and incubated for 20 min at 37 °C. The cell lysates were then centrifuged at 16,000 x g for 5 min, and the supernatant was collected and incubated with fluorogenic substrates Suc-LLVY-AMC (75 µM), Boc-LRR-AMC (100 µM) or Z-LLE-AMC (100 µM) at 37°C for chymotrypsin-like (C-L), trypsin-like (T-L) and peptidyl-glutamyl peptide hydrolase (PGPH) proteasomal activities, respectively. After incubation for 30 min, fluorescence intensity of the enzymatic products was determined using a fluorescence plate reader with Ex/Em at 380/460 nm (Gemini Plate Reader, Molecular Devices Corporation). Enzymatic activities were expressed as fluorescence unit per milligram protein.

Assay for caspase activity. caspase-3 activity was determined as described previously (15). After treatment, cells were collected and washed with PBS. The cells were then incubated in hypotonic Tris buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA) with 10 µM digitonin for 30 min to obtain the cell lysate, which was subjected to centrifugation at 16,000 x g for 5 min. The resulting supernatant was incubated with the fluorogenic substrate

DEVD-AFC (Biomol International, Plymouth Meeting, PA) to measure caspase-3 activity using a fluorescence plate reader (Ex/Em: 400/505 nm).

Plasmid construction and cell transfection. The plasmids for the His₆-tagged wild-type human ubiquitin-EGFP fusion, ubiquitin^{K48R} or ubiquitin^{K63R} counterparts were kind gifts from Dr. Douglas A. Gray (Ottawa Health Research Institute, Ontario, Canada). The coding sequence for His₆-ubiquitin-EGFP in the original vectors was amplified using PCR, and then cloned into pCEP4 vector at Xho I and Hind III restriction sites by following standard cloning procedure. The constructed plasmids were confirmed by DNA sequencing. AMAXA was used for electroporation transfection of the constructs. To verify stable expression, single clones were selected and screened by hygromycin exposure (200 µM).

DNA fragmentation assay. Cell Death Detection ELISA Plus Assay Kit purchased from Roche Applied Science, (Indianapolis, IN), was used for the DNA fragmentation assay as previously described (14). The ELISA method measures the amount of histone-associated low molecular weight DNA in cell lysates. After treatment, cells were lysed with lysis buffer provided with the kit, and the cell lysate was centrifuged at 200 x g. The supernatant was transferred into streptavidin-coated wells in a microplate, and incubated with a mixture of biotin-labeled anti-histone antibody and HRP-conjugated anti-DNA antibody. After several washings, the immunoreaction complex was measured colorimetrically with ABTS as the substrate for HRP at 405 nm with 490 nm as reference wavelength. Protein concentration was determined by the Bradford protein assay.

Western blot. Standard procedure was followed for Western blot. After cell treatment, cells were lysed with PBS containing protease inhibitors and Triton X-100 (0.2%, v/v). The lysates were centrifuged at 16,000 x g for 40 min to obtain the supernatant. Equal amount of proteins from each sample was resolved on SDS-PAGE and immunoblotted with ubiquitin (Dako, rabbit polyclonal, 1:1000) and β -actin (Sigma, mouse monoclonal, 1:10000).

Quantification of cell death using trypan blue exclusion test. Following treatment, both the floating cells and the attached cells were collected, and the cells were then resuspended in PBS with 0.4% trypan. After 5 min incubation, the cell suspension was used to count the dead and living cells under a light microscope. Cell death was expressed as the percentage of dead cells over the total number of cells.

Data analysis

Data analysis was conducted with Prism 4.0 software (GraphPad software, San Diego, CA). One-way ANOVA was followed by Dunnett's test or Bonferroni's test for multiple comparisons, and Student's t-test was used for single comparisons. A statistically significant difference was accepted if $p < 0.05$.

RESULTS

1. Effect of dopamine, MPP⁺ and dieldrin on proteasomal activity. Analysis of proteasomal activities indicated that exposure to dopamine (12, 18 or 24 h), MPP⁺ (12 or 24 h) or dieldrin (60 min) significantly reduced PGPH activity (Panel a of Fig. 1A, B and C). The C-L peptidase activity (Panel b of Fig. 1A, B and C) was altered in a pattern similar to

PDGH activity, with significant inhibition observed during exposure to dieldrin (20 to 60 min), dopamine (18 h) and MPP⁺ (12, 24 h); whereas no significant change in the T-L activity was noted except a significant elevation at later timepoints following dopamine exposure (18 and 24 hr) (Panel c of Fig. 1A, B and C).

2. Accumulation of ubiquitinated proteins following exposure to dopamine, MPP⁺ or dieldrin. Tagging of target proteins with lysine 48 (K48)-linked polyubiquitin chains is the prerequisite for their UPS degradation. Incomplete removal of the polyubiquitinated proteins results in their accumulation during proteasome inhibition. Determination of the cellular ubiquitinated proteins using Western blot indicated that exposure to dopamine or MPP⁺ (Fig. 2A), or dieldrin (Fig. 2B) substantially elevated intracellular ubiquitinated proteins in time dependent manner.

3. Suppression of apoptosis by ubiquitin^{K48R} following exposure to dopamine or dieldrin. To examine whether the accumulation of K48 polyubiquitins contributes to neurotoxicity, we established cell lines stably expressing wild-type (wt) ubiquitin, ubiquitin^{K48R} or ubiquitin^{K63R}. An assay for the caspase-3 activation showed significantly less caspase-3 activation in N27 cells stably expressing ubiquitin^{K48R} following exposure to dopamine, MPP⁺ and dieldrin ($p < 0.001$), whereas caspase-3 activation was more prominent in N27 cells expressing ubiquitin^{K63R}.

DNA fragmentation is the endpoint event of apoptosis; this was measured by quantifying the cytoplasmic histone-complexed DNA fragments. As shown in Fig. 3B,

exposure to dopamine, MPP⁺, or dieldrin significantly increased DNA fragmentation; those increases were significantly suppressed by ubiquitin^{K48R}.

4. Rescue of dopaminergic neuronal cells from cytotoxicity of dopamine, MPP⁺ and dieldrin. Finally, we compared cell death in cells expressing wt ubiquitin or ubiquitin^{K48R} following exposure to dopamine, MPP⁺ or dieldrin. As shown in Fig. 4A, following 200 μ M dopamine treatment for 30 h, the dead cells accounted for more than 41% of cells expressing wt ubiquitin, while the percentage of the dead cells was 22% in ubiquitin^{K48R} transfected cells ($p < 0.001$ vs dopamine-treated wt ubiquitin cells). With 300 μ M MPP⁺ treatment for 30 h (Fig. 4A, panel b), approximately 30% of wt ubiquitin cells and 18% of ubiquitin^{K48R} transfected cells were dead ($p < 0.001$ vs MPP⁺-treated wt ubiquitin cells), respectively. Analysis of dieldrin-treated (60 μ M) cells showed that exposure to dieldrin for 5 hr resulted in 23% in wt ubiquitin-expressing cells and 15% cell death in cells transfected with ubiquitin^{K48R} cells ($p < 0.05$ vs dieldrin treated wt ubiquitin cells; Fig.4A, panel c).

DISCUSSION

The present study clearly demonstrates impaired UPS function following exposure to dopamine, MPP⁺ and dieldrin, manifested by altered proteasomal activities and abnormal accumulation of ubiquitinated protein. We showed that expression of a dominant negative ubiquitin^{K48R} effectively protected the N27 cells from apoptotic cell death following exposure to dopamine, MPP⁺ and or dieldrin. Overall the data indicate that accumulation of K48

polyubiquitin chains plays a key role in dopaminergic degeneration triggered by neurotoxic challenges such as exposure to elevated levels of dopamine, MPP⁺ or dieldrin.

Genetic and biochemical analysis of PD have implicated defective UPS degradation as a pathogenic factor for PD (9, 16). Extensive studies *in vivo* and *in vitro* using proteasome inhibitors have provided convincing experimental data supporting the etiopathological roles of UPS dysfunction in PD (17-20). Studies also showed impaired UPS function as the result of exposure to dopamine neurotoxins including excessive dopamine (11), MPP⁺ (10) or organochlorine pesticide dieldrin (21). However, the contributory role and cell death mechanisms of UPS dysfunction during neurotoxin-induced dopaminergic degeneration remain poorly understood. In the present study, we showed that exposure to dopamine, MPP⁺ and dieldrin affects proteasomal activities. Interestingly, the PGDH and C-L proteasomal activities appear to be significantly suppressed by these toxins in a similar pattern. However, no significant reduction of T-L proteasomal activity was observed, in fact, a significant increase was detected during treatment with dopamine (18, 24 hr). Though it is known that PGDH, C-L and T-L proteasomal activities are associated with different β -subunits present in the inner ring of the 20S proteasome (22, 23), how these activities are differentially affected remains unknown. Previous studies suggested that several factors contribute to the negative effect of dopamine on proteasomal activities, such as dopamine-related ROS generation (11), inhibitory effect of its metabolite aminochrome (24) or its neuromelanin derivative (25). Significant reduction of C-L and PGDH proteasomal activities following exposure to MPP⁺ and dieldrin is presumably the result of ATP depletion, but not ROS generation (10). Pretreatment with the antioxidants Trolox or MnTBAP is incapable of restoring the loss of proteasomal activity during dieldrin treatment (unpublished

observation). Likely, T-L activity elevated at the later timepoint of dopamine treatment (18, 24 hr) is an adaptive response to remove accumulated ubiquitinated proteins (Fig. 2), since this activity appears to be more tolerant of the neuronal challenge by dopamine, MPP⁺ and dieldrin.

Several environmental neurotoxins have been shown to compromise proteasomal function (1). However, the role of UPS impairment in dopaminergic degeneration following exposure to environmental neurotoxins requires further investigation. The direct consequence of proteasome inhibition is the incomplete degradation and resulting accumulation of K48 polyubiquitin chain-tagged target proteins. Here we showed that expression of a dominant negative ubiquitin^{K48R} in the dopaminergic neuronal cells effectively rescued dopaminergic neurons from apoptotic cell death caused by dopamine, MPP⁺ or dieldrin. This evidence suggests that UPS dysfunction actively contributes to the environmental neurotoxins-induced dopaminergic degeneration through accumulated K48 polyubiquitin chains. In addition to Lys 48, other internal lysine residues (Lys 6, 11, 29, 63) could be used as sites for polyubiquitin chain extension (26). K63 polyubiquitin chains, which are not involved in proteasomal degradation, are the second most abundant type of polyubiquitin chain. The same Lys to Arg mutation at Lys 63 of ubiquitin elicits profound neurotoxicity in the cells, rather than neuroprotection, which is indicative of the specificity of the K48 polyubiquitination site in neuroprotection.

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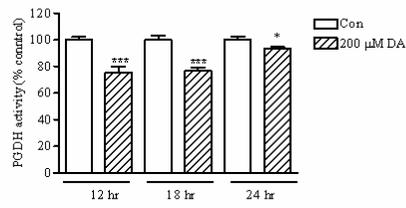
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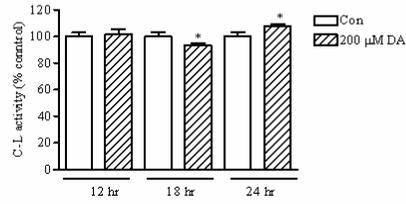
FIGURE 1.

A. Dopamine treatment

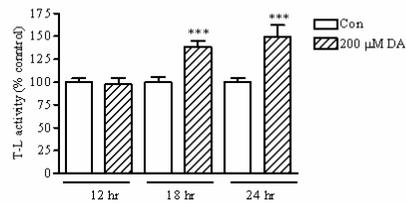
a. PGDH activity



b. C-L activity

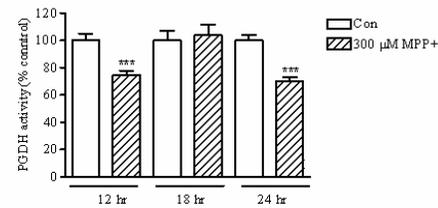


c. T-L activity

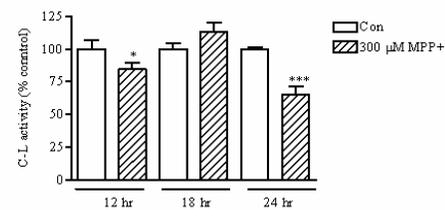


B. MPP+ treatment

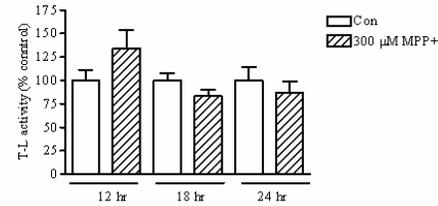
a. PGDH activity



b. C-L activity



c. T-L activity



C. Dieldrin treatment

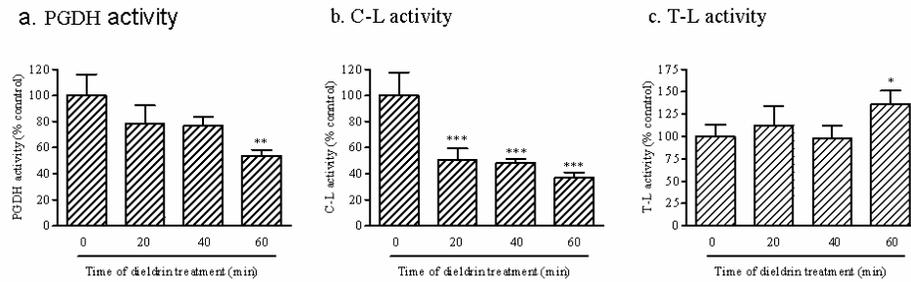


Fig. 1 Effect of dopamine, MPP⁺ and dieldrin on the proteasomal activities.

N27 cells were treated with 200 μ M dopamine (A) or 300 μ M MPP⁺ (B) for 12, 18 or 24 hr. In the case of dieldrin (C), cells were exposed to 60 μ M dieldrin for 20, 40 and 60 min. The treated cells were collected for proteasomal activity assay using Z-LLE-AMC, Suc-LLVY-AMC, or Boc-LRR-AMC as substrates for PGDH (panel a), C-L (panel b) or T-L (panel c) proteasomal activities, respectively. Values represent mean \pm S.E.M. from six individual samples. * p <0.05, *** p <0.001 (Student's t-test) in comparison with time-matched control groups.

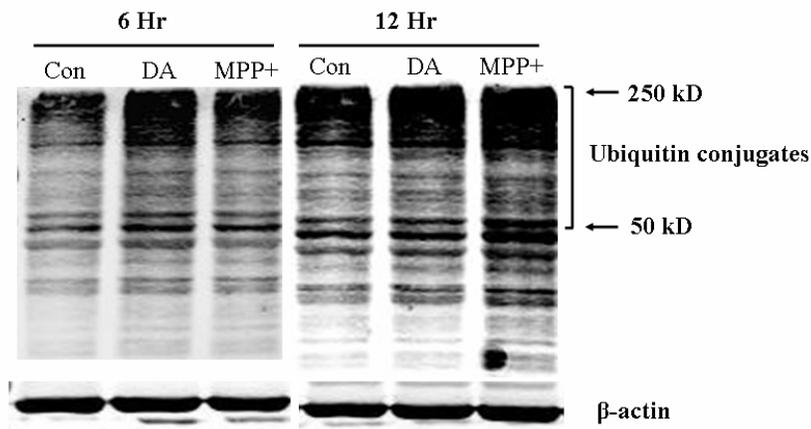
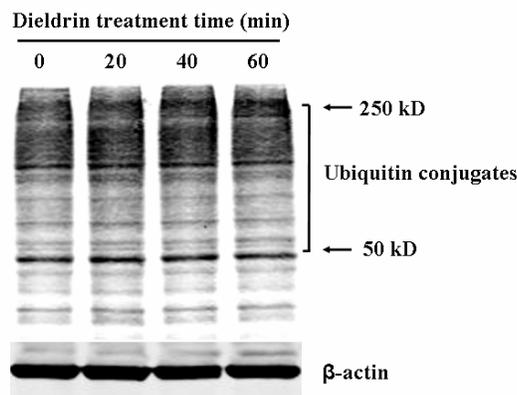
FIGURE.2**A. Accumulation of ubiquitinated proteins (Dopamine and MPP+ treatment)****B. Accumulation of ubiquitinated proteins (Dieldrin treatment)**

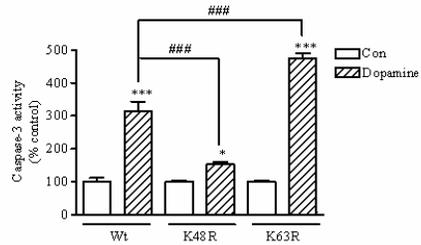
Fig. 2 Accumulation of ubiquitinated proteins following exposure to dopamine, MPP+ and dieldrin.

Following treatment with 200 μ M dopamine, or 300 μ M MPP⁺ (A) for 12 or 18 hr, cells were collected by trypsinization. For dieldrin (60 μ M) treatment, cells were collected 20, 40 or 60 min after exposure (B). The whole cell lysates prepared from the treated cells were resolved on SDS-PAGE and immunoblotted with antibodies against ubiquitin and β -actin.

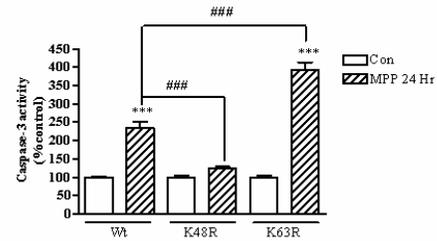
FIGURE.3

A. Caspase-3 activation

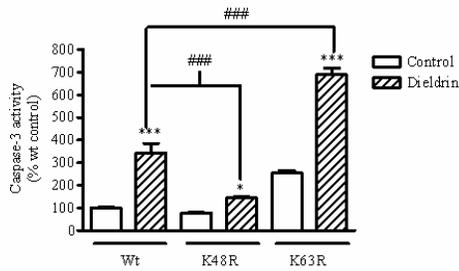
a. Dopamine treatment



b. MPP+ treatment

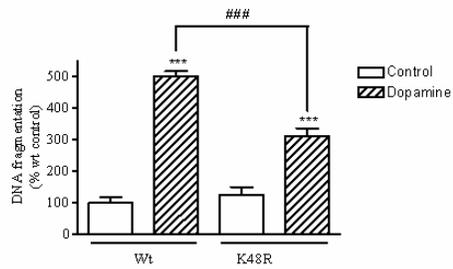


c. Dieldrin treatment

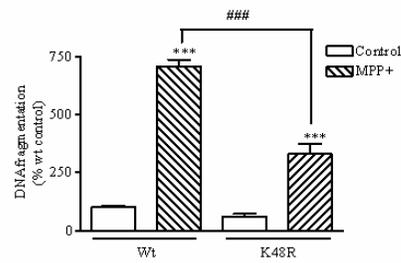


B. DNA fragmentation

a. Dopamine treatment



b. MPP+ treatment



c. Dieldrin treatment

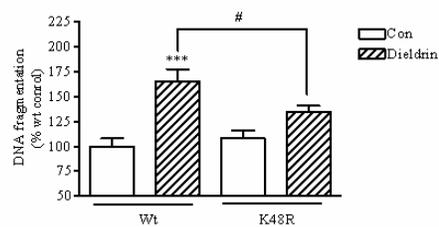


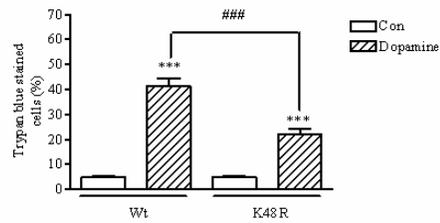
Fig. 3 Suppression of apoptosis by ubiquitin^{K48R} following exposure to dopamine or dieldrin.

Cell lines stably expressing linear fusion of His₆-tagged wild-type (wt) ubiquitin-GFP (His6-ubiquitin-GFP) or its mutant counterparts His6-ubiquitin^{K48R}-GFP or His6-ubiquitin^{K63R}-GFP were subjected to 24 hr treatment with 200 μ M dopamine, 300 μ M MPP⁺ for 24 hr or 4 hr treatment with 60 μ M dieldrin. **A. Caspase-3 activation.** Enzymatic activity of caspase-3 was assessed with DEVD-AFC as substrate for dopamine- (panel a), MPP⁺- (panel b) and dieldrin-treated (panel c) cells. Values represent mean \pm S.E.M. N=6 for dopamine and MPP⁺ experiment; whereas the data were derived from 3 individual experiments with 6, 6, and 5 samples respectively in the dieldrin treatment. **B. DNA fragmentation.** DNA fragmentation was determined using ELISA method for cells receiving 24-hour with dopamine (panel a) or MPP⁺ (panel b) treatment, or 4-hour dieldrin (panel c) treatment as described in the Materials and Methods. Data are presented as mean \pm S.E.M., with 5 samples in each group. Statistical significance was determined by one-way ANOVA followed by Bonferroni's Multiple Comparison Test. *p<0.05, **p<0.01, *** p<0.001, comparing with individual control group; ###p<0.001, in comparison with indicated group.

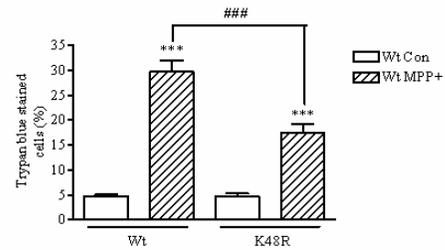
FIGURE.4

A. Trypan blue exclusion for cell death

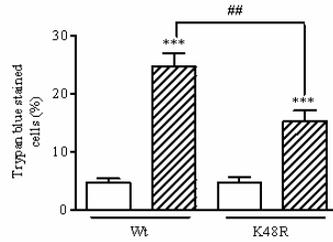
a. Dopamine treatment



b. MPP+ treatment



c. Dieldrin treatment



B. Phase contrast images

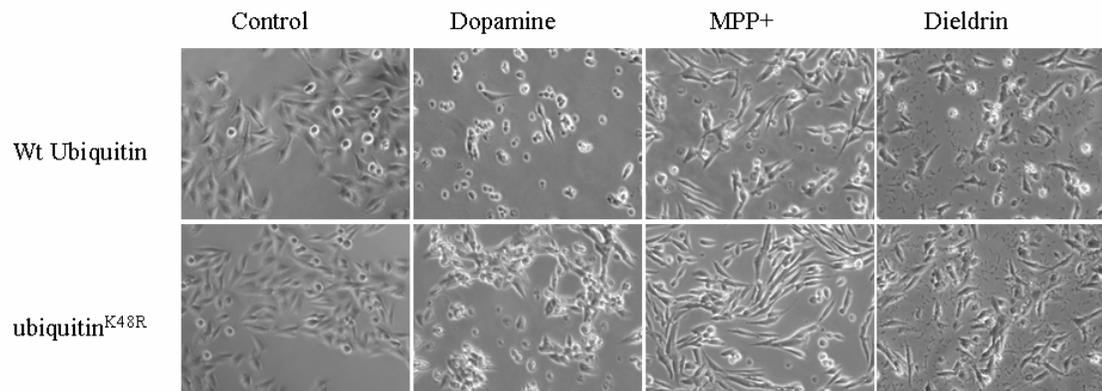


Fig. 4 Rescue of dopaminergic neuronal cells from the cytotoxicity of dopamine, MPP⁺ and dieldrin.

Three lines of stable cells (His₆-tagged wt, K48R or K63R ubiquitin-GFP) were treated with 200 μ M dopamine or 300 μ M MPP⁺ for up to 30 hr. Treatment with 60 μ M dieldrin lasted 5 hr. Phase contrast images were captured after treatment (B). All the floating and attached cells in each sample were pooled together for the trypan blue cell death assay as described in the Materials and Methods (A). Panel a: dopamine treatment; panel b: MPP⁺ treatment; panel c: dieldrin treatment. Data are present as mean \pm S.E.M. for the percentage of trypan blue stained cells. N=6, Statistic comparisons were conducted using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. *p<0.05, **p<0.01, *** p<0.001, comparing with individual control group; ###p<0.001, in comparison with indicated group.

Chapter VII: GENERAL CONCLUSION

The major findings of each research chapter included in this thesis have been described, and implications of the findings to dopaminergic degeneration have been interpreted in the discussion section of each individual chapter. This section presents an overview of the results and findings in the thesis herein, with special emphasis on the contributory role of impaired ubiquitin-proteasome degradation in environmental neurotoxins-induced apoptosis, and its relevance to the pathogenesis of Parkinson's disease.

Gene-environment interaction of dieldrin and α -synuclein converges to impair ubiquitin-proteasome mediated proteolysis and induces dopaminergic degeneration.

Ubiquitin-proteasome and lysosome are two primary cellular degradation systems. Many proteins including short-live signal proteins, misfolded and oxidatively damaged proteins are subjected to proteolysis by UPS (Glickman and Ciechanover, 2002; Jung et al., 2007). The extensive presence of protein aggregates in the form of inclusions known as Lewy bodies in nigral dopaminergic neurons (McNaught et al., 2002a; McNaught et al., 2002b) and involvement of several PD genes in the UPS degradation pathway (Wood-Kaczmar et al., 2006), strongly support a role for proteasomal dysfunction in pathogenesis of PD.

Currently, the etiopathogenesis of PD remains poorly understood, although both genetic and environmental factors are believed to contribute to PD development. Epidemiological studies consistently implicate exposure to pesticides as a potential risk factor for PD (Di Monte et al., 2002; Lai et al., 2002; Kanthasamy et al., 2005). Indeed, these studies provide important clues for determining the environmental risk factor for PD;

additional laboratory studies of rotenone and paraquat exposure showed both pesticides effectively induce dopaminergic degeneration *in vivo* and *in vitro* (Dauer and Przedborski, 2003; von Bohlen und Halbach et al., 2004; Dinis-Oliveira et al., 2006). The organochlorine pesticide dieldrin has also been implicated as a PD-related neurotoxin in epidemiological (Kanthasamy et al., 2005) and postmortem analyses (Fleming et al., 1994; Corrigan et al., 2000; Kanthasamy et al., 2005). The studies presented in this thesis showed that exposure to subtoxic concentrations of dieldrin impairs UPS-mediated protein aggregation, as demonstrated by a reduction in proteasome activity and elevation of ubiquitinated proteins in dopaminergic neuronal cells. However, more severe proteasomal inhibition and neurotoxicity was exhibited in the dopaminergic neuronal cells overexpressing wild-type human α -synuclein, indicating that dieldrin and α -synuclein converge to impair UPS function, thus triggering dopaminergic degeneration. Overproduction of α -synuclein, as the result of gene locus multiplication in familial PD (Singleton et al., 2003; Chartier-Harlin et al., 2004; Ibanez et al., 2004), supports the idea that genetic mutation predisposes dopaminergic neurons to neurotoxicity of environmental neurotoxins. Consistent with the proteasomal inhibition by dieldrin observed in the present studies, a broad range of pesticides including dieldrin and rotenone have been shown to inhibit proteasomal activity in a different cell line (Wang et al., 2006). Defective proteasome function could result from oxidative stress when oxidatively damaged proteins overwhelm the degradation capacity of the proteasome (Okada et al., 1999; Shamoto-Nagai et al., 2003); whereas several antioxidants including Trolox, MnTBAP and CoQ failed to preserve UPS function following dieldrin exposure. Though experimental data is absent, inhibition of ATP production by dieldrin due to inhibition of

mitochondrial function (Bergen, 1971; Pardini et al., 1971), possibly accounts for the observed UPS impairment, similarly to MPP⁺ (Hoglinger et al., 2003).

The pathophysiological roles of α -synuclein in dopaminergic degeneration remain uncertain. Examination of the direct effect of α -synuclein on neuronal viability or neuronal vulnerability to dopaminergic neurotoxins using overexpression models has yielded inconsistent results. Various factors, including expression level, have been hypothesized to account for the opposing roles of α -synuclein on neuronal viability (Seo et al., 2002; Xu et al., 2002; Zourlidou et al., 2003). Extremely low levels of α -synuclein were expressed in the undifferentiated N27 dopaminergic neuronal cells. Exogenously introduced human α -synuclein expressed at physiological levels comparable to rat substantia nigra appears to be protective initially, then substantially potentiates neurotoxicity of dieldrin, suggesting that α -synuclein possibly protects dopaminergic neurons from the toxicity of an acute neuronal challenge, but not from chronic stress. A recent study by Chandra and colleagues revealed unexpected roles of α -synuclein in preventing neurodegeneration caused by CSP α inactivation *in vivo* (Chandra et al., 2005). However, in reality, α -synuclein could be detrimental to dopaminergic neurons, with chronic exposure to environmental factors as the dominant risk factor for PD. This idea is consistent with slow progression of neurodegeneration in α -synuclein transgenic mice (Fernagut and Chesselet, 2004).

Proteasome inhibitor MG-132-induced mitochondrial apoptosis involves mitochondrial accumulation of ubiquitinated proteins----rescue of dopaminergic neurons from apoptosis by dominant negative mutant K48R ubiquitin. Mitochondrial dysfunction and UPS impairment are the two major hypotheses for PD pathogenesis. Normal

UPS function plays an essential role in maintenance of physiology and structural integrity of mitochondria, though the underlying mechanisms remain elusive (Abou-Sleiman et al., 2006). The results from our studies show that proteasome inhibition by MG-132 diminishes mitochondria membrane potential and activates mitochondria-mediated apoptosis, as demonstrated by caspase-9 and -3 activation. A study by Sullivan and coworkers showed chronic exposure to proteasome inhibitors compromises maximum mitochondrial respiration and slowed mitochondria turnover (Sullivan et al., 2004). The novel finding of our study demonstrates that profound and preferential accumulation of ubiquitinated proteins in mitochondria may likely mediate mitochondria dysfunction and activation of mitochondrial apoptosis. Expression of dominant negative mutant ubiquitin^{K48R} using different vectors significantly abolishes the MG-132-induced activation of mitochondrial apoptosis, suggesting that mitochondrial accumulation of ubiquitin conjugates actively contributes to mitochondrial apoptosis. The potent anti-apoptotic effect of the ubiquitin^{K48R} probably explains the marked neuroprotection of the ubiquitin^{K48R} observed in spinocerebellar ataxia type 1 mouse models (Tsirigotis et al., 2006). Further study of neuroprotective mechanisms will focus on identification of mitochondrial molecules linking polyubiquitination and apoptosis, thus delineating the apoptosis pathway in dopaminergic degeneration.

Mitochondria-dependent positive feedback amplification of apoptosis by PKC δ promotes proteasome inhibitor MG-132-induced dopaminergic degeneration. PKC δ activation could occur as the results of its membrane translocation, phosphorylation or proteolytic cleavage. Phosphorylation of caspase-3 by active full-length PKC δ has been shown to increase the enzymatic activity of caspase-3, and amplify the apoptotic signal in

monocytes (Voss et al., 2005). Studies have shown that proteolytic activation of PKC δ is a key mediator for apoptosis during oxidative stress in dopaminergic neuronal cells. In the MG-132-induced apoptosis model, proteasomal inhibition precedes the caspase-3-dependent mitochondrial apoptotic cascade. Proteolytic activation of PKC δ by caspase-3 is required for the full activation of caspase-3, suggesting that positive feedback amplification of caspase-3 by PKC δ is mitochondria-dependent in dopaminergic neuronal cells. Additionally, mitochondrial translocation of proteolytically activated PKC δ appears to mediate its proapoptotic effect, since mitochondria-targeted expression of the catalytic fragment of PKC δ results in caspase-3 activation. Though we do not have data regarding mechanisms of how mitochondria-localized PKC δ activates mitochondrial apoptosis, other studies have shown phosphorylation and regulation by PKC δ of scramblase (He et al., 2007) and pyruvate dehydrogenase kinase (Churchill et al., 2005) as likely mitochondrial targets. Future studies may need to focus on identifying the primary mitochondrial target of PKC δ that contributes to amplification of caspase-3 activity during UPS dysfunction.

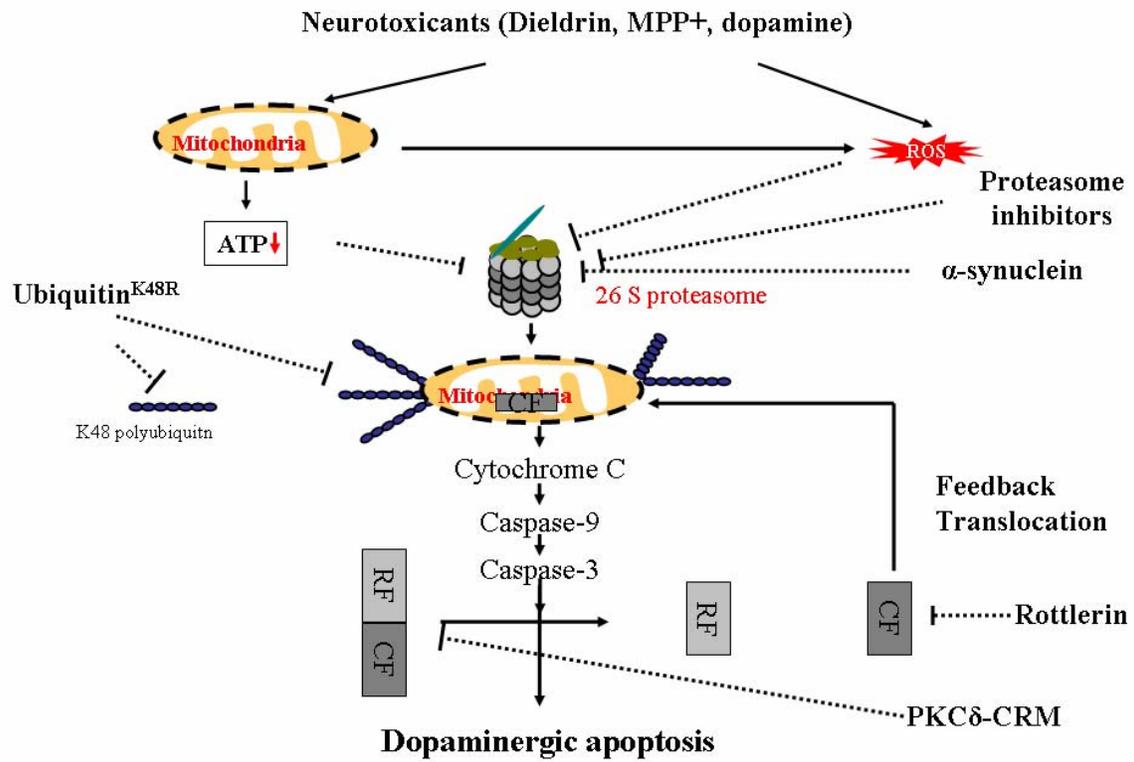
Functional impairment of the ubiquitin-proteasome system during exposure to neurotoxins dieldrin, MPP⁺ and dopamine contributes to dopaminergic degeneration.

Studies showed that exposure to MPP⁺ (Hoglinger et al., 2003), dopamine (Keller et al., 2000) and dieldrin reduces proteasome activities in dopaminergic neuronal cells. Chronic treatment with dopaminergic neurotoxin MPTP impairs UPS function, as demonstrated by the reduction of proteasomal subunits and activity (Fornai et al., 2005; Zeng et al., 2006). Currently, the contributory roles of impaired UPS degradation in dopaminergic degeneration upon neurotoxin exposure are still unknown; impaired UPS could occur as a response

secondary to other cell events such as mitochondria inhibition, oxidative stress or cell death. The direct consequence of proteasome inhibition is the accumulation of K48 polyubiquitinated proteins. In an attempt to determine whether the accumulation of polyubiquitinated proteins following MPP⁺, dopamine or dieldrin treatment is involved in the cell death process, we found that N27 cells stably expressing K48R mutant ubiquitin confers neuronal resistance against neurotoxicity of MPP⁺, dopamine or dieldrin. This work demonstrates for the first time the effective rescue of dopaminergic neurons from neurodegeneration *in vitro* by dominant negative ubiquitin^{K48R}, which suppresses K48 polyubiquitin chain extension. These results provide a promising entry point to dissect out the inter-relationship between two proposed major pathogenic factors for PD: mitochondria dysfunction and impairment of ubiquitin proteasome protein degradation.

In summary, exposure to environmental neurotoxins and gene-environment interactions could converge to compromise the proteolytic capacity of the ubiquitin proteasome system, by promoting ROS generation, depleting cellular ATP or a direct inhibitory effect on the proteasome (Scheme-1). Impairment in proteasome function leads to preferential accumulation of ubiquitinated proteins in the mitochondria, which induces mitochondrial dysfunction and release of proapoptotic molecules; these events subsequently activate mitochondria-mediated apoptosis. Proteolytic activation of PKC δ during the process contributes to the full activation of caspase-3 through mitochondrial translocation and possibly phosphorylating mitochondrial target proteins by activated PKC δ . Thus, the interplay between UPS impairment and mitochondrial dysfunction promotes the degenerative processes in dopaminergic neurons.

Scheme-1



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