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Compensatory mechanisms of neuroprotection by PKD signaling against oxidative damage in experimental models of Parkinson's disease (PD): Relevance to PD drug discovery strategies

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**Compensatory mechanisms of neuroprotection by PKD signaling
against oxidative damage in experimental models of Parkinson's
disease (PD): Relevance to PD drug discovery strategies**

by

Arunkumar Asaithambi

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Toxicology

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Iowa State University

Ames, Iowa

2011

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*This dissertation is dedicated to the memory of my father Dr. N. Asaithambi.
His unexpected and tragic loss due to leukemia motivated me to pursue doctoral
research in the areas of drug discovery with a hope that my research can contribute
to humanity's defense against life threatening diseases in some way.*

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ABSTRACT

Oxidative stress is a key pathophysiological mechanism contributing to the selective degeneration of dopaminergic neurons in Parkinson's disease. Unraveling the molecular mechanisms underlying various stages of oxidative neuronal damage is critical to better understanding the diseases and developing new treatment modalities. We previously showed that protein kinase C delta (PKC δ) proteolytic activation during the late stages of oxidative stress is a key proapoptotic signaling mechanism that contributes to oxidative damage in Parkinson's disease (PD) models. The time course studies revealed that PKC δ activation precedes apoptotic cell death and that cells resisted early insults of oxidative damage, suggesting that some intrinsic compensatory response protects neurons from early oxidative insult. In this study, we identified that protein kinase D1 (PKD1) functions as a key anti-apoptotic kinase to protect neuronal cells against early stages of oxidative stress. Exposure of dopaminergic neuronal cells to H₂O₂ and 6-OHDA induced PKD1 activation loop (PKD1S744/748) phosphorylation long before induction of neuronal cell death. Blockade of PKC δ cleavage, PKC δ knockdown or overexpression of a cleavage-resistant PKC δ mutant effectively attenuated PKD1 activation, indicating that PKC δ proteolytic activation regulates PKD1 phosphorylation. Furthermore, the PKC δ catalytic fragment, but not the regulatory fragment, increased PKD1 activation, confirming PKC δ activity modulates PKD1 activation. We also identified that phosphorylation of S916 at the C-terminal is a preceding event required for PKD1 activation loop phosphorylation. Importantly, negative modulation of PKD1 by the RNAi knockdown or overexpression of PKD1S916A phospho-defective mutants augmented oxidative stress-induced apoptosis, while positive modulation of PKD1 by the overexpression of full length PKD1 or

constitutively active PKD1 plasmids attenuated oxidative stress-induced apoptosis, suggesting an anti-apoptotic role for PKD1 during oxidative neuronal injury.

Since we showed the importance of PKD1 activation in cell culture models of Parkinson's disease, we further proceeded to characterize PKD1 signal transduction involved in a pre-clinical model of Parkinson's disease. Exposure of dopaminergic neuronal cells or primary mesencephalic neurons to MPP+ induced PKD1 activation loop (PKD1S744/748) phosphorylation and activation. PKD1 is activated and translocates to the nucleus in the nigral dopaminergic neurons of mice exposed to acute MPTP treatment. Inhibition of PKC δ activation by siRNA knockdown or DEVD-fmk prevented PKD1 activation during MPP+ treatment in dopaminergic neuronal cells. To further validate this finding, we used the PKC δ knock-out (PKC δ -/-) mouse model. PKD1 was not activated in the nigral dopaminergic neurons of PKC δ knock-out (PKC δ -/-) mice exposed to acute MPTP treatment. Earlier we reported that Fyn kinase regulates PKC δ in dopaminergic cells. So we used a Fyn knockout (Fyn -/-) mouse model to test the hypothesis that Fyn kinase acts as an upstream regulator of PKC δ -PKD1 signaling in PD. PKD1 was not activated in nigral dopaminergic neurons of Fyn knockout (Fyn -/-) mice exposed to acute MPTP treatment. We also identified that phosphorylation of S916 at the C-terminal occurs in nigral dopaminergic neurons of mice exposed to MPTP and S916 phosphorylation does not occur in (PKC δ -/-) mice, confirming PKC δ 's tight control over PKD1 activation. Further, dopaminergic neurons co-treated with the PKD1 inhibitor kbNB 142-70 and exposed to MPP+ exacerbated neuronal death, confirming the survival role of PKD1. Consistent with other results, PKD1 was activated in the nigral dopaminergic neurons of human post-mortem PD brains. Having confirmed

that positive modulation of PKD1 can be a novel neuroprotective strategy, we took a translational approach by developing PKD1 activator and characterizing the protective function in pre-clinical models of Parkinson's disease. Positive genetic modulation of PKD1 by overexpression of constitutively active PKD1 protected against MPP⁺-induced toxicity. Pharmacological activation by rosiglitazone protected against while inhibition by kbNB 142-70 exacerbated MPP⁺ and 6-OHDA toxicity in cell culture PD models. Peptides were rationally designed and screened for their ability to activate PKD1 using various screening methods. Peptide AK-P4 was identified to activate PKD1 specifically and protect against MPP⁺ and 6-OHDA in both N27 cells and primary mesencephalic neurons. Further, AK-P4 tagged with the TAT sequence (AK-P4T) delivered using intravenous injections activated PKD1 in mice. The neuroprotective effects of AK-P4T were tested using the subchronic MPTP mouse model. Co-treatment with AK-P4T restored the neurotransmitter levels and the behavioral and locomotor activities of the MPTP treated mice significantly.

Collectively, we show that PKD1 is regulated in a Fyn- PKC δ dependent manner and represents a novel intrinsic protective response in counteracting early stage oxidative damage in PD. Further, we demonstrate that the rationally designed PKD1 activator peptide AK-P4T positively modulated PKD1 and protected against neurodegeneration in the pre-clinical models of PD. Overall, that positive modulation of PKD1 by AK-P4T suggests its promise as a potential therapeutic agent against PD.

CHAPTER 1: GENERAL INTRODUCTION

Dissertation organization

An Alternative thesis format is used for this thesis, which includes modified versions of manuscripts either published or to be submitted for publication. The thesis contains a general introduction, four research papers, a general conclusion, and an acknowledgement. References for each individual section are listed at the end of corresponding chapters, except for the background and literature review: references for these are listed following the general conclusion. The general introduction (Chapter I) outlines the current knowledge of the Parkinson's disease etiology and therapies and covers the overview of the research objective. Background and literature review I provide background information on Parkinson's disease, current available symptomatic / protective strategies and the novel signal transduction therapy approaches. Background and literature review II of the chapter summarizes the role of Protein Kinase D1 (PKD1) in different cell signaling process, functions and various disease pathologies. Chapter II "Protein Kinase D1 (PKD1) activation mediates a compensatory protective response during early stages of oxidative stress-induced neuronal degeneration" is a research paper published in the journal *Molecular Neurodegeneration* (6:43, 2011). Chapter III "Protein Kinase D1 (PKD1) activation protects against dopaminergic neuronal degeneration induced by Parkinsonian specific toxicant 6-OHDA" has been submitted to the journal *BBA-Biochimica et Biophysica Acta*. Chapter IV "Fyn-PKC δ Signaling Regulates the Anti-Apoptotic PKD1 in Experimental Models of Parkinson's disease" is a research paper to be submitted to *PLOS ONE* and chapter V "Rationally designed peptide based PKD1 activator protects against neurodegeneration in pre-clinical models of Parkinson's disease" will be submitted for publication in

Science Translational Medicine. 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 is a major neurodegenerative disorder affecting over a million Americans with an annual cost of several billion dollars. Current treatment approaches available for PD are symptomatic and fail to prevent the progression of the neurodegenerative process. The currently available drugs are limited in their effectiveness to either slow or stop the progressive neurodegenerative processes in PD, largely due to the lack of mechanistic insights into the selective dopaminergic degenerative process. Experimental findings from cell cultures, animal models, and humans indicate that oxidative stress and apoptosis may contribute to the pathophysiological processes underlying many neurodegenerative diseases including Parkinson's disease (2) (3) (4) (5) (6). Our understanding of oxidative damage induced neurodegeneration has increased over recent years. Oxidative stress induced apoptosis is a continual cell death process involving multiple stages and signaling molecules and generally can be divided into two distinct phases; the early stage where ROS initiation occur and the late stages where irreversible oxidative damage leads to cell death (7) (8).

Most current investigations of signaling in neurodegenerative disorders have primarily focused on signaling pathways that are activated only during cell death. We have earlier explored one such pro-apoptotic pathway mediated by PKC δ cleavage in dopaminergic neurons (8-10). But the compensatory responses against oxidative damage remain largely unexplored. Recently,

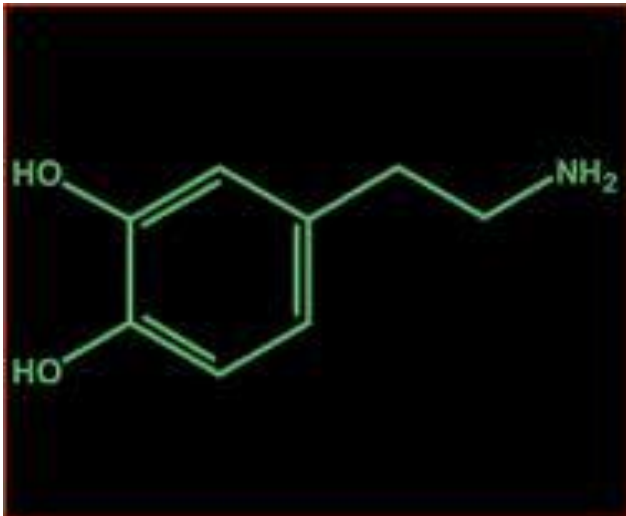
we unravelled a novel compensatory protective mechanism in dopaminergic neurons mediated by protein kinase D1 (PKD1). This review will provide an overview about PKD1, its mode of activation and functional significance during oxidative damage as this might lead to a novel and alternative translational approaches to treat neurodegeneration.

Background and Literature Review 1

Parkinson's disease and therapeutic options

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) region of the mid-brain. Cardinal PD symptoms range from resting tremors, bradykinesia (slowness of movements), akinesia (difficulty in initiating movements) rigidity and facial palsy (11). Non-Motor symptoms include speech abnormalities, sleep abnormalities, constipation, olfactory dysfunction, depression/ cognitive impairment (12). Parkinson's disease process occurs through numerous stages of , as described by Braak *et al.*, (13, 14). In stages 1-2 the presence of PD related inclusions are mainly confined to the IX/X motor nucleus or the reticular zone (medulla oblongata). In stages 3-4 the pathology spreads to the upper and lower brain stem but is not co-existent with cortical inclusion bodies and finally in the stages 5-6 there is severe involvement of neo-cortex along with frontal and temporal lobes. Some non-motor symptoms like speech sleep disorder, and olfactory dysfunctions, might antedate the appearance of motor symptoms while cognitive impairment can follow the motor symptoms during the late stages. But the progression is not linear and can be faster with mild impairment and slower with marked impairment. Two subtypes have been recently noted; one tremor dominant form occurring in younger patients of age around 45 years

and another form dominant with rigidity, abnormal gait and postural instability occurring in older patients of age greater than 55 (15). Parkinson's disease is idiopathic and can be multifactorial with both genetic and environmental causes can contribute to the disease progression but the exact cause of this pathology remains unclear (16). Although numerous genetic mutations have been associated with familial Parkinsonism (17) (18), these mutations are only unique to the familial cases and account for less than 5-10% of the PD occurrences. Mutations have been found in some important genes like α -synuclein, parkin and DJ1. 2- 3% of early onset and 50 % of late onset shows mutation in these genes. Some of the other genes which are associated with PD include PINK1, LRRK2, EIF4G1 etc. Environmental



causes also have been proposed. The

environmental chemical toxicants like maneb, paraquat, rotenone and MPTP have been shown to lead to

neurodegenerative pathology not only in animal and cellular models but also in human subjects (19) (20) (21). Basal ganglia in the mid brain, comprises of the striatum (sub divided into the caudate nucleus/putamen or neostriatum and the ventral striatum), the external (GPe) and internal (GPi) pallidal segments, the subthalamic nucleus and the substantia nigra (made up of the pars compacta-SNpc and the pars reticulata-SNpr). Striatal nuclei and the output basal ganglia connections are sub-divided into two pathways referred to as the direct pathway made up of GPi, SNpr and the indirect pathway (GABAergic neurons- Enkephalin) made up of GPe segment , further

connecting the GPi/SNpr via the subthalamic nucleus (STN) (22). The direct and the indirect pathways collectively function to exert an inhibitory effect on the basal ganglia inhibitory output to the thalamo cortical pathway. Dopamine acts via a net inhibitory effect (D2 receptors) over the indirect pathway and a excitatory effect on the direct pathway to maintain the smoothness of motion (23) (24). Dopamine (DA) is a neurotransmitter that is synthesized from the amino acid tyrosine by the rate limiting tyrosine hydroxylase (TH). Tyrosine is first converted to L-3, 4-dihydroxyphenylalanine (L-DOPA) by TH and decarboxylated to DA by the aromatic L-amino acid decarboxylase (AADC) (25). Dopamine can be converted to Noradrenaline (NE) by dopamine- β -hydroxylase or it can be sequestered into synaptic vesicles by vesicular monoamine transporter (VMAT). Vesicles fuse with the synaptic membrane and release DA into the synaptic cleft, where it acts on various dopamine receptors and initiate different signaling mechanisms. Excess DA is removed from the synaptic cleft through re-uptake process by the dopamine transporter (DAT). Dopamine (DA) homeostasis is critically dependent on the dopamine transporter (DAT) located in the nerve terminals (26). Chemical compounds that are structurally similar to DA exhibit similar uptake V_{max} via the transporter system into the dopaminergic system.

There are four major brain dopaminergic pathways in the mid-brain; nigrostriatal, mesolimbic, mesocortical and the tuberoinfundibular (27). Nigrostriatal pathways arise from the dopamine producing neuronal cell bodies of the SNpc and act to inhibit the release of excitatory cholinergic neurotransmitters from the striatum via the Gs linked D2 receptors. The extreme loss of the dopaminergic neurons in the substantia nigra (>50%) and the striatum (>70%) in turn leads to the development of motor symptoms seen in Parkinson's disease. In addition to the loss of dopaminergic neurons the major pathological features of PD are the dystrophic neuritis termed

The history of PD therapy – from J. Parkinson to the present	
1817	– J. Parkinson proposes to take blood from the upper part of the neck to relieve congestion in the medulla oblongata
1860s	– Ordenstein introduces the use of belladonna extracts
1940s	– Introduction of synthetic anticholinergics
1950s	– Introduction of stereotactic surgery for PD
	– Largely unnoticed reports on the antiparkinsonian effects of apomorphine by Struppler and Schwab
1960s	– Reports by Birkmayer + Hornykiewicz and Cotzias et al on efficacy of levodopa
	– Report on antiparkinsonian effects of amantadine
1970s	– Start of development of DA agonists
1980s	– Development of the concept of neuroprotection via MAO-B inhibition
	– First proof-of-concept studies with continuous intravenous delivery of L-dopa to treat motor complications
1990s	– Introduction of COMT inhibitors
	– Introduction of nonergoline DA agonists
	– First reports on STN-DBS for advanced PD
	– First reports of successful striatal fetal cell transplantation in PD
2000–2010	– Trials establishing delay of dyskinesia when initiating therapy with DA agonists
	– Antipsychotic efficacy of clozapine in PD psychoses
	– Efficacy of rivastigmine to improve PD dementia
	– Controlled trials of fetal cell transplantation
	– Proof-of-concept studies of gene therapy

“Lewy neuritis” (LN) and cytoplasmic inclusion bodies referred to as “Lewy bodies”.

Many environmental factors are linked to neurodegenerative but none of these factors have been proved to be the cause of the disease. 1,2,3,6-methyl-phenyl-tetrahydropyridine (MPTP) a potent neurotoxin was identified to induce Parkinsonian like pathology (28) MPTP is a byproduct of a synthetic meperidine derivative and can get oxidized to pyridinium ion (MPP⁺) which is neurotoxic as it acts as an inhibitor of the complex I of the mitochondrial respiratory chain and mediating oxidative damage and apoptosis (29). A majority of the toxins like rotenone, paraquat and MPTP which induce the clinical and pathological features similar to PD have been demonstrated to be a potent mitochondrial complex I enzyme inhibitors (20) (30) (31). Furthermore oxidative stress induced mitochondrial dysfunction and bioenergetic crisis was demonstrated in PD post mortem brain studies (32) (33). Current treatment options are mostly symptomatic and do not slow down the progression of PD.

Parkinson's disease therapy

The therapeutic options for medical management of PD have increased through the years. The treatment is mostly symptomatic and can be dopaminergic and non-dopaminergic based. Current successful pharmacological therapeutic measures have been the administration of the dopamine precursor drug levodopa {Eadie, 1995 #4}. Eventhough Levodopa administration has been successful in treating Parkinsonian symptoms, the prolonged administration can cause some motor system side-effects like aggravated forms of dyskinesias on the disease (34) (35). Promising results from preclinical studies showed that dopamine agonists can be a treatment for PD. Patients treated with dopamine agonists pramipexole and ropinirole for example showed significantly lower reduction of F-DOPA uptake compared to levodopa treatment. Eventhough, there is reduced decline of radiotracer, it did not translate clinically as motor function of levodopa- treated patients were better than those treated with dopamine agonists.

Glutamate antagonists

Research on pathophysiology of PD has shown dysfunctional mitochondria and reduced complex I stimulating increased glutamatergic activity and making the dopaminergic neurons vulnerable in neurodegeneration. Pre-clinical studies have shown that *N*-methyl-d-aspartate (NMDA) receptor antagonists blocking the activation of NMDA receptors by glutamate have antiparkinsonian effects and potentiate the effect of dopamine on striatal function. Studies on riluzole, a glutamate antagonists show neuroprotective effects in animal models of PD. In clincial trials on PD patients, the authors concluded that riluzole does not show superiority over placebo on disease progression or to alleviate the symptoms (36) (37) (38) (1)

Monoamine oxidase inhibitors

Oxidative stress was considered an important contributor to neurodegeneration in PD. Use of MPTP toxicity model and its blockade by MAO-B inhibitors in pre-clinical models, MAO-B inhibitor was probed for its neuroprotective effects. For example one of the selective inhibitor of MAO-B rasagiline was tested in PD patients. Early treatment of rasagiline proved to be efficacious in PD patients even though there is a criticism about the methodology of the trial (36) (37) (38) (1)

Neurotrophic factors

Neurotrophic factors which help survival of dopaminergic neurons in substantia nigra, has been proposed as a potential treatment for PD. Glial cell line-derived neurotrophic factor (GDNF) has been demonstrated to have neuroprotective effects on nigral dopaminergic neurons. A randomized trial compared the effects of monthly intracerebroventricular administration of placebo and recombinant GDNF in patients suffering from advanced PD. The study was not effective and possible reason being that did not reach its intended targets. A randomized, trial in PD patients receiving bilateral continuous intraputamenal infusion of GDNF or placebo via intraparenchymatous catheters stereotactically targeted to the posterior dorsal putamen showed some beneficial effect of but did not translate into a significant clinical improvement of motor function (36) (37) (38) (1).

Antioxidants and Bioenergetics

Mitochondrial dysfunction and oxidative stress have long been implicated as pathophysiological mechanisms of dopaminergic neuronal loss in PD. First evidence emerged from observations that abuse of 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), whose active metabolite 1-methyl-4-

phenyl-pyridine (MPP⁺) is an inhibitor of the mitochondrial complex I can induce irreversible parkinsonian condition. Further studies in postmortem brain of patients suffering from idiopathic PD have shown reduction in mitochondrial complex I activity indicating a role of mitochondrial dysfunction in the pathogenesis of PD.

Coenzyme Q₁₀ (CoQ₁₀) is an antioxidant and an essential cofactor in the mitochondrial respiratory chain. Previous Studies have shown reduced levels of CoQ₁₀ in PD patients. In the Coenzyme Q₁₀ Evaluation-2 (QE2) study, treatment with CoQ₁₀ showed significant benefit for PD patients. Based on this observation a randomized trial examining CoQ₁₀ in phase III trials to evaluate the putative disease-modifying effects were performed. The study indicated that CoQ₁₀ could be considered for evaluation in future trials. Future studies with larger cohorts and longer treatment periods are required to examine the neuroprotective effects of CoQ₁₀ (36) (37) (38) (1).

Another pharmacological approach to increase bioenergetics in PD was using dietary supplement creatine, which plays an important role in mitochondrial energy production. Creatine is converted to phosphocreatine to transfer phosphoryl groups in mitochondrial ATP synthesis and buffering intracellular energy stores. In two separate clinical trials the results were conflicting and require a well designed trial to probe creatines's protective effects.

Alpha-tocopherol or vitamin E, another antioxidant, has also been investigated, but showed conflicting results like creatine. Current drugs active in clinical trials are given below in the table.

Based on the success of kinase modulators in cancer along with more light on the cellular and molecular pathways, focus to develop a 'signal

Compound or intervention	Mechanisms of action	Phase	Primary (and secondary) outcome measures	ClinicalTrials.gov identifier
Motor symptoms/wearing-off				
AAV-hAADC-2	Intraputaminal infusion of AADC	II	Safety	NCT00229736
Caffeine	Nonselective adenosine receptor agonist	II/III	Tolerability (UPDRS II and III) Change in ESS (UPDRS)	NCT01190735 NCT00459420
Nicotine	Nicotine receptor agonist	II	UPDRS III score in defined OFF condition	NCT00873392
		IV	Stop signal task (inhibitory control)	NCT01216904
GAD (NLX-P120)	Reduction of STN hyperactivity	II	Outcomes not specified	NCT00643890
Istradefylline	Adenosine A _{2A} receptor antagonist	III	Adverse events (reduction in OFF time)	NCT00957203
IPX066	Levodopa/carbidopa extended release	III	UPDRS I-IV (Open label extension)	NCT01096186
		III	Motor fluctuations based on home diaries	NCT01130493
Preladenant (SCH-420814)	Adenosine A _{2A} receptor antagonist	III	Change from baseline in UPDRS II and III	NCT01155479
		III	Change from baseline in mean OFF time	NCT01155466
		III	Change from baseline in mean OFF time	NCT01227265
		III	Safety	NCT01215227
SYN-115	Adenosine A _{2A} receptor antagonist	II/III	Change from baseline in mean OFF time (dyskinesia)	NCT01283594
ProSavin	Intraputaminal injection of the three main enzymes of dopamine synthesis	I/II	Safety (UPDRS)	NCT00627588
Safinamide	MAOB inhibitor, glutamate release inhibitor and dopamine reuptake inhibitor	II	Reduction in PD-CRS versus baseline	NCT01211587
		III	Time from baseline to first intervention	NCT01028586
		III	Change from baseline in UPDRS III	NCT00605683
		III	Change from baseline in daily ON time	NCT00627640
DBS in the STN	Modulation of STN activity	IV	Difference in PDQ-39 summary index	NCT00354133
XP-21279	Transported prodrug of levodopa	II	Change in OFF time	NCT01171313
Dyskinesia				
AFQ-056	mGluR5 antagonist	II	Change from baseline in modified AIMS	NCT00986414
Eltoprazine	5-HT _{1A} /5-HT _{1B} receptor agonist	II	Outcomes not specified	The Michael J. Fox Foundation for Parkinson's Research
Ortopidine (ACR-325)	Dopaminergic stabilizer	I	Safety and tolerability (dyskinesia)	NCT01023282
Safinamide	MAOB inhibitor, glutamate release inhibitor and dopamine reuptake inhibitor	II	Reduction in UDysRS compared to baseline (new MDS-UPDRS)	NCT01113320
Gait disorders				
Memantine	NMDA antagonist	IV	Stride length	NCT01108029
Methylphenidate	Dopamine and noradrenaline enhancer	IV	Number of steps on the stand-walk-sit test	NCT00914095
		IV	Gait, attention and orthostatic hypotension	NCT01244269
DBS in the PPN	Drive gait and locomotion	I/II	Optimal parameters of DBS in the PPN	NCT00821743
Hallucinations and psychosis				
Pimavanserin (ACP-103)	5-HT _{2A} receptor antagonist	III	Antipsychotic efficacy based on SAPS	NCT01174004
		III	Safety	NCT00550238
Depression and anxiety				
SAM	Co-substrate of methyl group transfer	II/III	Change in Hamilton Depression Scale	NCT00070941
Sleep disorders				
Caffeine	Nonselective adenosine receptor agonist	II	Tolerability (ESS and UPDRS)	NCT01190735
		II/III	Change in ESS scores (UPDRS)	NCT00459420
Pitolisant (BF2.649)	Histamine H ₃ inverse receptor agonist	III	Change in ESS	NCT01036139
		III	Change in ESS	NCT01066442
Autonomic failure				
Droxidopa	Noradrenaline precursor	III	Mean change in the Orthostatic Hypotension Questionnaire composite score	NCT01176240
Lubiprostone	Prostaglandin E1 derivative	IV	Spontaneous bowel movement frequency	NCT00908076
		IV	Change in bowel movement frequency	NCT00849784
Solifenacin	Muscarinic receptor antagonist	IV	Reduction in the mean number of micturitions	NCT01018264
Disease modification or neuroprotection				
Coenzyme Q10	Modulator of mitochondrial function	III	Change in UPDRS total score	NCT00740714
Creatine	Modulator of mitochondrial function	III	Disease progression over 5 years	NCT00449865
Deferiprone	Iron chelator	II/III	Decrease in substantia nigra iron overload (UPDRS I-IV)	NCT00943748
Inosine	Urate precursor	II	Tolerability and safety	NCT00833690
Isradipine CR	Calcium antagonist	II	Tolerability (UPDRS II and III)	NCT00909545
		II	Tolerability (UPDRS)	NCT00753636
G-CSF	Haematopoietic growth factor	II	UPDRS III	NCT01227681
Green tea polyphenols	Antioxidant	II	Delay of progression of motor dysfunction	NCT00461942
AAV2-Neurturin (CERE-120)	Neurotrophic growth factor; intraputaminal and intranigral injection	I/II	Change from baseline in UPDRS III in OFF condition	NCT00985517
PDGF (sNN0031)	Intracerebroventricular injection of PDGF	I/II	Safety and tolerability (UPDRS)	NCT00866502
Cogane (PYM-50028)	Oral neurotrophic factor modulator	II	Change from baseline in UPDRS II and III	NCT01060878

transduction therapy' has increased for PD to offer neuroprotection. The following sections will focus on i) recent advances in the mitochondrial apoptotic process and ii) current advances in signal transduction therapy

targeting the apoptotic process to prevent dopaminergic degeneration for neuroprotection.

Oxidative damage induced apoptosis in neurodegeneration

Studies in human PD brain tissues indicate the role of apoptosis in neurodegeneration. TUNEL (terminal dUTP nick-end labeling) staining a measurement for DNA fragmentation gives some evidence for the occurrence of apoptosis in post-mortem tissues. Studies done in cellular and animal models also conclusively show that neurodegeneration occurs through apoptosis.

Apoptosis can be divided into two groups as the extrinsic or death ligand mediated pathway and the intrinsic or the mitochondrial pathway of apoptosis (Vila and Przedborski 2003). The extrinsic pathway utilizes the recruitment of cell-surface death receptors like Fas/CD95 which has Fas-Ligand as a ligand and the tumor necrosis factor receptor-1 (TNFR-1) having TNF- α as a ligand (Wallach et al. 1999). Caspase family of enzymes potentiates and regulates apoptotic signaling. These enzymes have specificity for aspartic acid on the N-terminal side of the substrate containing a specific pentapeptide sequence (QACXG). Caspase activation involves the proteolytic cleavage of the enzyme to a large subunit (p17-p20) and a small sub-unit (p10-p12) which form self heterodimers and leading to active form of the enzyme. Based on type of activity in cells the caspases can be broadly classified into two main groups; initiator caspases (that regulate effector caspases as caspases 1, 2, 4, 5, 8, 9, and 10) and the effector caspases (that execute apoptosis as caspases 3, 6, 7, and 14). Once the extrinsic pathway is activated, adaptor proteins will recruit caspase-8 and then in turn activate effector caspase-3 directly. Caspase-8 can also cleave Bid (a BH3-only member of the B-cell leukemia/lymphoma 2 [Bcl-2] family of proteins) ((39) (40). Since mediation

of apoptosis through mitochondrial dysfunction has been described as one of the major causes of neurodegenerative disorders (41) (42) , this section will focus on mitochondrial apoptosis.

During the early stage, the oxidative stress leads to an oxidant/ anti-oxidant homeostatic imbalance and mitochondrial dysfunction resulting in the formation of mitochondrial permeability transition pore (mtTP), cytochrome C release and caspase-3 activation. In the late stage, considerable fraction of mitochondria is affected resulting in bioenergetic crisis, overgeneration of ROS, DNA fragmentation and neuronal death.

The inner membrane of the mitochondrion forms Ca^{++} channels between the mitochondrial matrix and the cytosol which are known as permeability transition pores (PTPs) (43). Cytochrome c is released from the mitochondria through permeability transition pores (PTPs). Once cytochrome c is released, it forms a large dATP/ATP dependent complex with an adapter protein Apaf-1 and the pro-caspase-9 which is referred to as the apoptosome and is a primary initiator factor of downstream apoptotic cascade. Caspase-9 further activates caspase-3 through proteolytic cleavage (44).

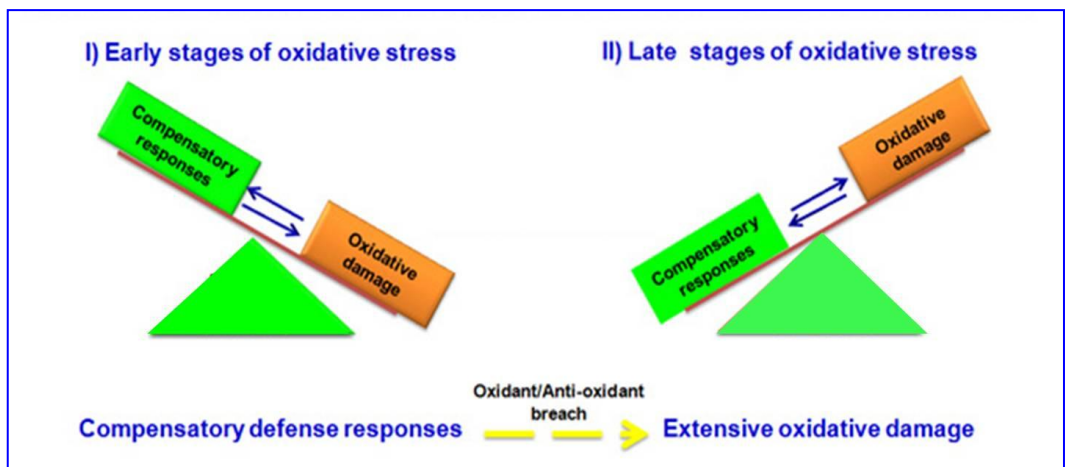
Release of proteins and enzymes from the mitochondria is closely monitored by Bcl-2 family of proteins (McDonnell et al. 1996; Reed 1996; Blandino and Strano 1997). Bcl2 family can be divided into the pro-apoptotic members and the anti-apoptotic members. Bak and Bax regulate cytochrome c release from the inner mitochondrial membrane (Lutter et al. 2000; Kuwana et al. 2002). Bcl2 has been shown to affect protection by inhibiting intracellular free radicals, and increasing buffering ability of excess Ca^{++} in the mitochondria (Kane et al. 1993; Ellerby et al. 1996; Zhu et al. 2000).

Many protein kinases that signal for both neuronal survival and death are activated at both stages.

Signal transduction therapy

Cell death kinases

Recent advances in drug discovery have led to novel signal transduction therapies for many diseases and disorders. Current signal transduction therapies in PD mainly focus on developing inhibitors against cell death (2) (3) (4) (5) (6). The cell survival kinases mediate compensatory responses by activating many anti-oxidant, anti-apoptotic signaling cascades and try to maintain the oxidant/ anti-oxidant homeostatic balance. But the failure to counteract the oxidative damage and the persistent generation of ROS results in apoptosis mediated by cell death kinases.



Some of the cell death kinases involved in the execution of apoptosis includes PKC δ , JNK, MLK, ASK1, MAPK etc. Sustained or delayed activation of ERK promoted cell death and neuronal degeneration (45-48). Activation of JNK almost exclusively leads to cell death (49). ASK1 activation sensitizes the neurons to cell death (50). MLK family of kinases acts as an upstream activator of the pro-apoptotic JNK in the apoptotic cascade (51). PKC δ cleavage sensitizes the neurons to death in pe-clinical models of

neurodegeneration (6). These cell death kinases have also been shown to relay specifically the ROS generated during mitochondrial dysfunction.

Mutations in kinases like DJ, LRRK2, Parkin, PINK1, α -synuclein have also been associated with Parkinson's disease. Parkin, PINK1, DJ1 has been linked to mitochondrial biogenesis, energy production and neuroprotection.

Therapeutic interventions developed against some of these major cell death kinases including JNK, GSK, MLK, MAPK, havenot progressed to the treatment stage (45-48). For example, the mixed lineage kinase inhibitor CEP-1347 failed to stop or slow down PD progression in clinical trials (52). One of the hypothesis given for the failure was that the therapeutic strategy of inhibiting kinases involved in cell death execution might be too late to stop neurodegeneration in addition to the complex signal transduction process (53). This has resulted in reevaluating the scientific thinking to develop alternative approaches through better understanding of the neurodegeneration signaling mechanisms.

Compensatory protective responses

One of the alternative hypotheses is neuroprotection through modulating the survival factors. Some of the key compensatory responses are the anti-oxidant factors GSH, α -tocopherol, SOD, GPX which resist the early stage ROS generation; anti-apoptotic Bcl2 family of proteins that resist the formation of permeability transition pore is the second layer of defense against cell death during the intermediate stage; the late stage anti-apoptotic defenses are mediated by anti-apoptotic proteins like IAP family members and chaperones like HSPs. Several HSPs (for example, HSP27, HSP70 and HSP90) act as specific anti-apoptotic factors during the late stages after caspase-3 activation. This occurs through multiple mechanisms, including:

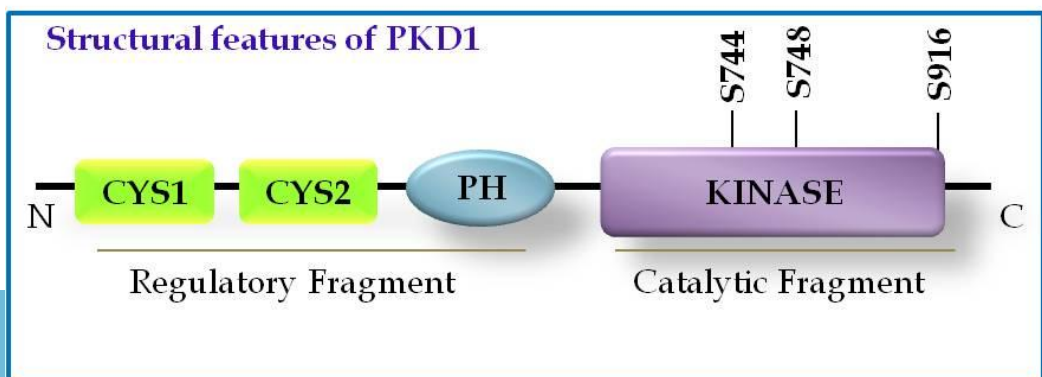
apoptosome inhibition, blockage of endonucleases from mitochondria release, cytosolic endonucleases sequestration, preventing protein aggregation and inhibition of mitochondrial DNase activity. In line with these observations, brain-targeted overexpression of HSPs conferred neuroprotection. The members of the inhibitor of apoptosis protein (IAP) family act as an endogenous defense against excessive caspase activation. In-vivo overexpression of the IAPs including NAIP, IAPs 1 and 2 had reduced CNS tissue damage (54).

Unlike the cell survival kinases the key protein kinases that mediate protective compensatory responses to counteract oxidative damage in Parkinson's disease are still largely unexplored. So far only AKT is known to offer neuroprotection is (54). Recently, we identified an oxidant/anti-oxidant signal transduction circuit in the progression of PD. PKD1 activation acts as a cell survival switch in the circuit and protect against early oxidative damage but deactivated during the late stages which co-relates with dopaminergic degeneration (55). Our studies in PD models have shown that positive modulation of PKD1 protects against dopaminergic degeneration (55).

Background and Literature Review 2

Different Roles of Protein Kinase D1 (PKD1)

Protein kinase D1 (PKD) belongs to the CAMK family of kinases and has a molecular weight of 110kDa. PKD family consists of three related serine/threonine kinases PKD1, PKD2 and PKD3. PKD1 has a regulatory fragment and a catalytic fragment. The regulatory fragment consists of two cysteine rich domains (cys I, cys II) and the pleckstrin homology domain (PH). The regulatory domain has an inhibitory effect on the catalytic (56, 57). PKD was first identified in both mouse and human by two different labs (58) (59) and can be activated by phosphorylation at the dual serine residues (ser 744/748) in the kinase domain by different PKCs depending upon the cellular type and stimuli (59, 60). For example, *PKCtheta* and *PKCepsilon* can increase PKD1 activation loop phosphorylation and activation in kidney cell lines (61) (62). *PKCeta* and *PKCdelta* can bind to the PH domain and phosphorylate the activation loop of PKD1 in COS-7 and HeLa cells (63, 64). PKD1 activation has recently been shown to play a role in multiple signaling functions including proliferation, cytoskeletal reorganization, Golgi function, immune function, and cell survival in many non-neuronal cell models (65-69). PKD1 has been shown to regulate various cell signaling molecules and pathways including ERK1/2, JNK pathways (70-72), effector enzymes like MnSOD that scavenge ROS (68) (64), transcriptional regulators including NF- κ B and MEF2 (73) (74),



stress responsive chaperones like HSP27 (75), and some members of the HDAC family (68, 76, 77). Recently, PKD1 was recognized as an important mitochondrial ROS sensor that translocates to the nucleus to switch on cell survival mechanisms (64).

PKD are distributed intracellularly in the cytosol, Golgi and mitochondria. The PKD1 translocation and activation requires involvement of different domains. Cysteine rich domain 1 (C2) helps in translocation of PKD1 to the Golgi apparatus. Cysteine rich domain 2 (C2) is required for diacyl glycerol binding and Gαq binding. Once activated by PKCs through activation loop phosphorylation in the cytosol or mitochondria, PKD translocates to nucleus and activates many transcription factors (**). Cysteine rich domain 2 (C2) is required for nuclear import whereas PH domain is required for nuclear export (65-69, 78).

PKD1 mode of activation

PKD1 gets activated in response to various stimuli including growth factors, phorbol esters, G-protein coupled receptors, genotoxic stress and oxidative stress. The mode of activation also depends on the context of PKD1's multiple signaling functions including proliferation, cell survival, cell death, cytoskeletal reorganization, golgi function and immune function in many non-neuronal cell models (65-69).

PKD1 activation methods can be classified into four major ones:

GPCR Signaling

Multiple Stimuli including mitogenic neuropeptides and growth factors such as bombesin, vasopressin, endothelin, bradykinin, and platelet-derived growth factor activate PKD1. Rozengurt et al showed that DAG production during

external stimuli causes PKC activation which can further interact with PKD1 PH domain to relieve PH domain autoinhibition and transphosphorylate at the activation loop serine residue S744 and S748 resulting in PKD1 activation. All four novel PKC isoforms have been implicated in activation of PKD1. PKC ϵ and PKC η strongly induced PKD1 activation in COS-7 cells. Classical PKC member PKC α was also implicated in PKD1 activation. PKD cysteine rich domains bind with DAG; C2 is specific to plasma membrane translocation while C1 is specific to translocation to golgi outer membrane (64, 78-81)

Oxidative stress specific Src-Ab1-PKC pathway

Storz et al showed that during oxidative stress in HeLa cells PKD1 act as a mitochondrial specific oxidative stress sensor. PKD1 and PKC δ are recruited to mitochondrial membrane through PLD1 mediated DAG signaling. Further, the C-abl kinase phosphorylates at PKD1 Y 469 residue opening up the kinase through a conformational change; this creates a binding site at PKD1 C1a domain for the PKC δ C2 domain to bind and phosphorylate the activation loop S744/S748 residues and C-src is found to be the upstream regulator of this process. Activated PKD1 cause nuclear translocation of NF- κ B and further induce expression of mitochondrial MnSOD which scavenge ROS (64, 78-81).

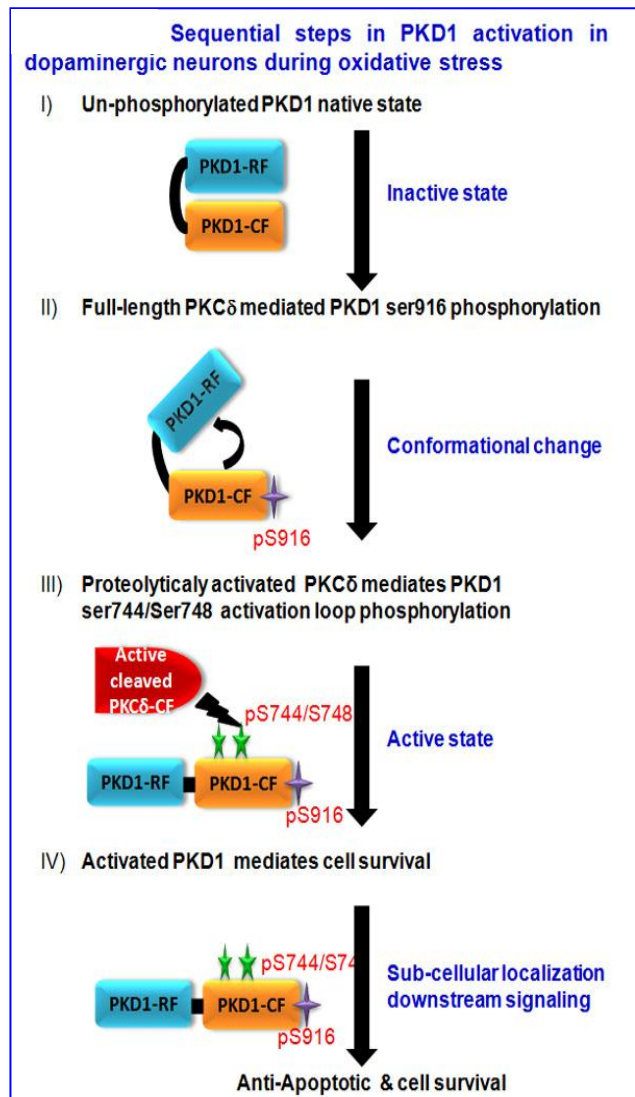
Proteolytic activation of PKD1

Häussermanna et al showed that PKD1 can generate a 62 kD fragment persistently active catalytic fragment when exposed to stimuli like TNF alpha, beta-D-arabinofuranosylcytosine (ara-C), 1 2-O-tetradecanoylphorbol-13-acetate (TPA), bryostatin 1 or cycloheximide. Further PKD1 was cleaved by calpain in response to arachidonic acid treatment (82).

Together, PKD1 can persistently get activated in response to proteolytic cleavage.

PKD1 activation by proteolytically activated PKC δ catalytic fragment (PKC δ -CF)

We identified that proteolytically activated PKC δ (PKC δ - CF) activates protein kinase D1 (PKD1) by phosphorylating the activation loop (PKD1S744/748) in dopaminergic neuronal cells exposed to oxidative stress. We identified that phosphorylation of S916 at the C-terminal and not Y469 is a preceding event required for PKD1 activation loop phosphorylation. We also found that phosphorylation of S916 is



dependent on PKC δ . Previously we have shown that proteolytically activated PKC δ (PKC δ - CF) act as an oxidative stress sensor. This unique method of activation is specific in neuronal injury suggesting a protective compensatory role for PKD1 during oxidative stress (55).

PKD1 Signal transduction

Cytoskeletal organization and cellular motility

Lamellipodia are believed to be the motor required for polarized cellular migration and movement in mammalian cells (Yamaguchi & Condeelis, 2007). Actin depolymerization contributes to this cellular movement. Cofilin is a family of proteins that can depolymerize actin filaments. Phosphorylation of cofilin suppresses cell motility whereas cofilin dephosphorylation by protein phosphatase, SSH1L, restores cell motility. SSH1L phosphorylation by PKD1 contributes eventually to the disruption of SSH1L from phospho-cofilin and thereby regulating Lamellipodia cell movement and migration. PKD1 phosphorylates the Ras effector RIN1 to inhibit F-actin remodelling and cell motility (81) (83)

In many cancers including prostate, breast and stomach there is a downregulation of PKD1 and E-cadherin (84). PKD1 phosphorylation of the E-cadherin results in the inhibition of motility. PKD1 also regulate E-cadherin gene expression and downregulate mesenchymal protein expression. Therefore, Reduced PKD1 function decreases E-cadherin function and expression but promotes mesenchymal gene expression contributing to cancer (81).

PKD1 phosphorylates the transcription factor SNAIL, leading to the de-repression of E-cadherin in prostate tumours and cell lines. Further, PKD1 overexpression decreased tumour development in a xenograft model (81).

Golgi Function:

PKD kinases especially PKD2 and PKD3 regulate the fission of vesicles that carry protein and lipid cargo from the trans-golgi network (TGN) to the plasma membrane in HeLa cells. The enzyme PI4KIII β located in the golgi apparatus

is activated by PKD. PI4KIII β phosphorylates phosphatidyl inositol to generate PI4P which is an important lipid mediator for vesicular trafficking. For example PI4P act as a docking site for some lipid and sterol transfer proteins that delivers ceramide (CERT) and cholesterol (OSBP) to Golgi membranes. Both ceramide and cholesterol are required for further downstream signaling events and for packaging/budding in TGN (85, 86)

G β 1 γ 2 subunits of heterotrimeric G-proteins bind Golgi membranes and further activate PKD, which is a downstream target which further promotes golgi vesicle fission and delivery of secreted proteins to the plasma membrane (65).

PKD1 regulates insulin secretion by increasing Golgi fission in β -cells (87). Active, Golgi-associated PKDs are detected in hippocampal neurons (88).

Transcriptional regulation:

Histone deacetylases regulate gene transcription by repression through chromatin remodelling, recruitment of co-repressors, deacetylation etc. PKD1 phosphorylates HDAC5 and promote nuclear export of HDACs thus de-repressing MEF2-mediated transcription. Phosphorylation of HDAC5 by PKD1 also de-represses Nkx2-5-mediated.

In endothelial cells PKD activation by VEGF stimulates PLC γ and HDAC7 phosphorylation leading to MEF2 dependent transcriptional regulation and angiogenesis.

PKD1 phosphorylates HDAC7 and promote nuclear export of HDAC7 thus de-repressing RUNX-mediated transcription and bone formation when stimulated by bone morphogenetic proteins.

Immune Functions:

C. elegans lacking DKF-2A, a PKD1 homologue are vulnerable to human and *C. elegans* bacterial pathogens. TPA-1, a PKC δ homologue regulates DKF-2A activation. DKF-2A activates PMK-1, a p38 α MAPK homologue which is essential for induction of most of the immune function genes. Signaling through T cell receptor activates PKD1 and increases SAPK/JNK/NF-kappa B activation through hematopoietic progenitor kinase (HPK1) activation in Jurkat T cells. PKD1 may augments T cell adhesion. Also during T-cell receptor stimulation, CD4 and CD8 co-receptors expression occurs in response to RhoA induced PKD1 activation (89). On the other hand; T-cell-receptor β -chain expression is downregulated by cytoplasmic PKD1. B cell receptor stimulation activates PKD1 through CD40 and TRAF2 binding. PKC is activated through COS-7 cells and lymphocytes. Mast cell IgE receptor stimulation also activates PKD1 (84).

PKD is a major oxidative stress sensor and the role of PKD1 in oxidative signaling will be detailed in the following section.

PKD1 and oxidative stress signaling

PKD predominantly act as a cell survival kinase during oxidative stress. During oxidative stress in HeLa cells PKD1 and PKC δ are recruited to mitochondrial membrane through PLD1 mediated DAG signaling. Further, the C-abl kinase phosphorylates at PKD1 Y 469 residue opening up the kinase through a conformational change; this creates a binding site at PKD1 C1a domain for the PKC δ C2 domain to bind and phosphorylate the activation loop S744/S748 residues and C-src is found to be the upstream regulator of this process. Activated PKD1 cause nuclear translocation of NF- κ B and further induce expression of mitochondrial MnSOD which scavenge

ROS. This C-Src/ C-Abl/ PKC δ / PKD1 signalling module leads to NF- κ B-mediated transcription of pro-survival genes in HeLa cells (64, 68).

In HeLa cells PKD might affect apoptosis through regulation of JNK. In response to apoptotic signals, mitochondria release hydrogen peroxide and nitric oxide (NO). During oxidative stress NO is produced by mitochondrial NO synthase. Which inhibit complex III electron transfer to increase superoxide and hydrogen peroxide production. JNK is activated during this process and downregulate the antiapoptotic proteins Bcl-2 and Bcl-XL and mediates opening of the membrane transition pore and the voltage-dependent anion channel (VDAC) through phosphorylation which results in apoptotic signaling through Ca²⁺ release, cytochrome C release and caspases activation. PKD can form a complex with JNK and suppresses JNK activation in some cancer cells (70)

PKD can mediate NF- κ B activation and inactivates p38 MAPK and regulates cell survival in oxidative stress-induced intestinal epithelial cell injury. Further, PKD1^{WT} attenuated MKK3/6 phosphorylation to downregulate p38 MAPK in this cell lines. ROS also induce cytoskeletal reorganization that induce RhoA and Rho kinase (ROCK) activation. ROCK further activates Src and PKC signaling resulting in activation of PKD1–NF- κ B signalling. In pancreatic β cells, early induction of the antiapoptotic and anti-inflammatory gene TNFAIP3/A20 during stress is controlled by PKD1 activation. PKD1 induces A20 promoter activity and causes A20's negative feedback regulation of NF- κ B signaling (81).

Role of PKD1 in various disease pathologies

Decreased function of PKD1 as a cause in disease pathologies

Breast cancer

PKD1 protein decreased in human breast-cancer tissue arrays in invasive and metastatic ductal carcinomas (90, 91). PKD1 over expression in invasive breast-cancer cells decreased migration and invasion. Activating PKD1 downregulates matrix metalloproteases (MMPs) and decreases cell migration and invasion . Thus, restoring PKD1 expression or activation can reduce migration and metastasis of breast tumour cells.

Prostate cancer

Analysis of human prostate tumour samples revealed correlations between decreased PKD1 and E-cadherin mRNA levels. PKD1 phosphorylation of the E-cadherin results in the inhibition of motility (84). PKD1 also regulate E-cadherin gene expression and downregulate mesenchymal protein expression. Therefore, Reduced PKD1 function decreases E-cadherin function and expression but promotes mesenchymal gene expression contributing to cancer. PKD1 phosphorylates the transcription factor SNAIL, leading to the de-repression of E-cadherin in prostate tumours and cell lines. Further, PKD1 overexpression decreased tumour development in a xenograft model. PKD1 downregulates androgen receptor (AR) function and prostate cancer cell proliferation.

Together, PKDs can decrease cell motility by phosphorylating SSH1L, cortactin, E-cadherin, SNAIL and RIN1, or decreasing MMP expression.

Stomach cancer

PKD1 levels are decreased in primary gastric tumors cell lines due to the hypermethylation of the PKD1 promoter region. Methylation of PKD1 promoter increases with ageing. Knockdown of PKD1 increased migration and invasion in gastric cancer cells.

The following view has been put forth; the presence of PKD1 may enhance survival and proliferation and necessary for early stage tumor promotion events. But silencing of PKD1 may allow tumor progression into invasive stages and metastasis.

Diabetes

PKD1 enhances glucose-dependent insulin secretion by increasing Golgi fission in β -cells. This PKD1 activation is negatively modulated by p38 δ MAP kinase by phosphorylation. Disruption of the p38 δ gene in mice persistently activates PKD1 which regulates insulin and glucose levels (87).

The M3 acetylcholine (ACh) receptor promotes insulin release from pancreatic β -cells and PKD1 act as a key downstream effector to enhance glucose-dependent insulin secretion. PKD1 activation and ACh-augmented insulin secretion are suppressed in cells overexpressing phosphorylation-deficient M3 receptors.

PKD1 regulates insulin release and homeostatic regulation of glucose metabolism (81).

Increased function of PKD1 as a cause in disease pathologies

Pancreatic cancer

PKD1 might play a role in pancreatic cancer cells. There is an increase in expression of activated PKD1/2 in pancreatic cancer tissue microarrays.

In pancreatic cancer cell lines, PKD1 overexpression led to increased cell proliferation rate through upregulation of survivin and FLICE-inhibitory protein (c-FLIPL). PKD activation stimulated DNA synthesis and mitogenic signaling in human pancreatic cancer. PKD1 phosphorylated Hsp27 which is linked to cytoskeleton rearrangement, cell mobility, cell survival, and proliferation and

has increased expression in many types of cancer. PKD inhibitor CRT0066101 reduced tumor (92).

Skin cancer

PKD has been found to promote hyperproliferative disorders of the skin (92).

Lung cancer and lymphoma

Very little is known about the role of PKD in small cell lung cancer (SCLC). One study has shown that the PKC/PKD signaling pathway exists in the SCLC cell lines H69, H345, and H510 and that PKD can be activated by phorbol esters and bombesin in these cells. To date, there have been no reports on PKD expression and distribution in actual human tumor samples (92).

Angiogenesis

In endothelial cells PKD activation by VEGF stimulates PLC γ and HDAC7 phosphorylation leading to MEF2 dependent transcriptional regulation and

Tumor Type	Expression
Breast	↓
Pancreatic	↑
Prostate	↑↓
Basal Cell Carcinoma	↑
Gastric	↓

angiogenesis. HDAC7-regulates endothelial-cell migration, tube formation and genesis of capillaries (92).

Cardiac hypertrophy

Cardiac hypertrophy is the response to conditions like hypertension, heart muscle injury (myocardial infarction), or neurohormones. PKD1 activates

MEF2. MEF2 recruits co-activators increases Ca^{2+} release and energy metabolism. This results in hypertrophy, and eventually heart failure. Further activated CAMTA2–Nkx2-5 complex drives cardiac gene transcription, promoting hypertrophy along with MEF2. (Fielitz et al, 2008)

Skeletal muscles

Muscle fatigue is caused by a decline of muscle performance with activity due to reasons including failure of excitation-contraction, coupling, metabolism alteration and reactive oxygen species. PKD1 induces expression of slow-twitch contractile proteins that mediate muscle endurance through HDAC5 phosphorylation and MEF2 activation (93).

PKD1 mediates myocardial excitation–contraction coupling and phosphorylates troponin I reducing the Ca^{2+} sensitivity of myofibres and diminishes twitch amplitude. Bone morphogenetic proteins promote bone formation and improve skeletal function. HDAC7 repression of RUNX binding is released by PKD1 phosphorylation of HDAC7 (81).

PKD1 in CNS

The PKD is involved in protein sorting and packaging in TGN vesicles of dendrites (94) (88). Neuronal polarity and post-synaptic functions are regulated by this function. Any change to PKD activity also causes change in dendritic branching. Reduction in PKD levels increases the endocytosis of dendritic-membrane proteins (94). Together the study show that PKDs regulates cell polarity and dendritic specialization by playing an essential part in protein sorting, packaging and targeting in the TGN and suppress endocytosis of dendritic membrane proteins.

PKD1 phosphorylates KI220, a transmembrane scaffold protein at the plasma membrane which regulates neuronal development, morphogenesis and

polarity. It also modulates phosphorylation of proteins like MAP1 and stathmin. Autophosphorylation of PKD1 Ser 916 is shown to be important for transporting KI220 from the TGN to the plasma membrane .

PKD plays a very important role in associative learning in *C.elegans*. *C. elegans* detects and chemotactic towards Na⁺ but pre-incubation with Na⁺ during starvation results in Na⁺ avoidance. *Caenorhabditis elegans* gene, *dkf-2* (PKD homolog), encodes DKF-2A which is present mainly in intestine, and DKF-2B which is mainly found in neurons. DKF-2B is expressed in neurons responsible for Na⁺ chemotaxis and learning (92). *dkf-2* gene disruption has no effect on Na⁺ chemotaxis, but suppresses Na⁺-dependent learning. Restoring *Dkf-2* function brings back learning in *dkf-2* null animals. Interestingly, EGL-8, a PLC β homologue and TPA-1, a PKC δ homologue act as upstream regulators of both DKF-2B and DKF-2A in *C.Elegans* (92). The study demonstrate that PKD help enable learning and behavioral plasticity by transducing and integrating signals from environment to both neurons and intestine and thereby regulating a crucial neuronal function.

Metabotropic glutamate receptors (mGluRs) regulate strength and plasticity of synaptic connections in the brain. In hippocampal cultures, mGluR5 stimulation results in PKD phosphorylation at Ser-916 residue.

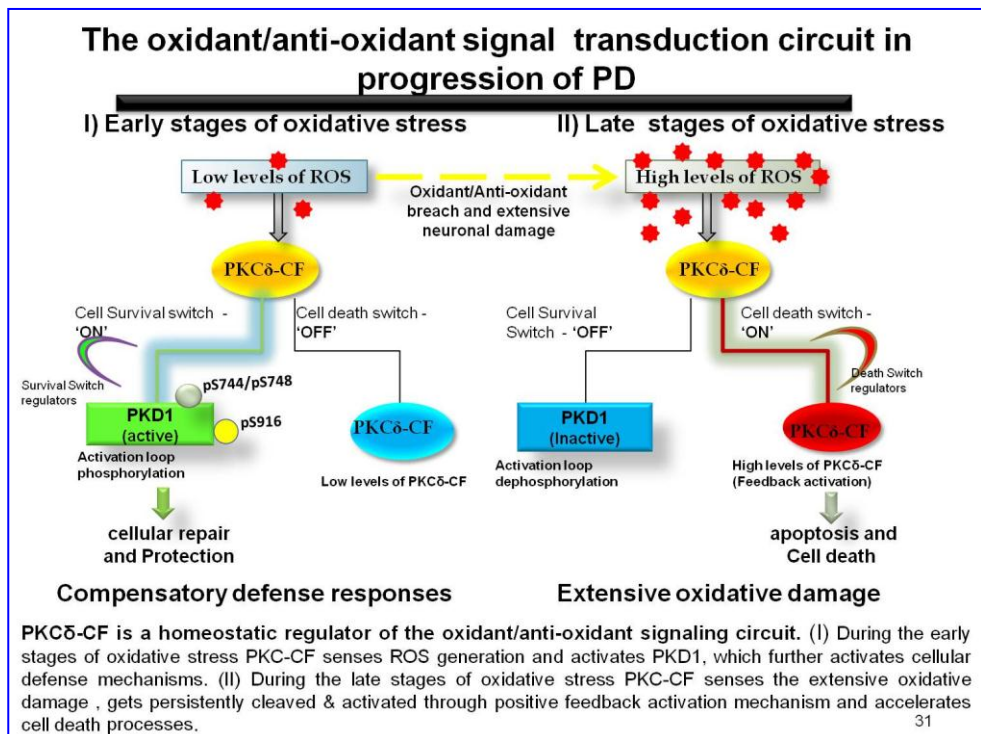
Protease-activated receptor 2 is activated during inflammation and injury. PKD isoforms are expressed in primary sensory neurons that are responsible for mediating inflammation and pain transmission function. PAR(2) agonists activate PKD isoforms in HEK293 cells and DRG neurons. And PKD activation is considered to be an important signaling cascade in protease-induced activation of nociceptive neurons and can be an important new target for antiinflammatory and analgesic therapies.

Tachykinins stimulate NK(3) receptors and contribute to excitatory transmission in primary afferent neurons (IPANs) in small intestine. Protein kinase C (PKC), - Protein kinase D (PKD) signal transduction are found to propagate the signal.

In *Drosophila*, PKD1 disruption resulted in tissue loss, wing defects and degeneration of the retina. This confirms that PKD is involved in developmental function in *Drosophila*.

PKD1 plays a prominent role in neuronal polarity, learning signal transmission etc. But PKD1's role in neurodegeneration remains relatively unknown. Recently it was reported that protein kinase D (PKD) act as early marker of neuronal DNA damage. PKD-activation was detected during genotoxic stress and ataxia telangiectasia-mutated (ATM) activation. The study also showed that PKD stimulation was very specific to DNA. These results were also replicated in- vivo, in rats where injection of DNA-toxin etoposide showed increased activation of PKD.

Our in-vitro and in-vivo results demonstrate that the PKD1-mediated protective mechanism is a constituent of a novel signal transduction circuit that regulates cell survival and cell death during various stages of oxidative stress in dopaminergic neuronal system. A detailed comparative analysis of PKC δ proteolytic activation, PKD1 activation loop phosphorylation and the extent of cell death during oxidative stress revealed an interesting functional relationship between activation of kinases and regulation of cell death. Comparison of PKD1 activation and cytotoxicity shows that PKD1 activation is maximal during the early oxidative stress stage when no measurable



cytotoxicity is noted. Interestingly, when PKD1 activation begins to decline at the end of the early stage, cell death begins to occur. Also, the level of PKC δ proteolytic activation directly correlates with the extent of cell death at the later stage of oxidative stress. As depicted in the scheme, in the early stages of oxidative insult, PKC δ acts as an oxidative stress sensor/regulator and activates the survival switch PKD1 against oxidative damage. However, prolonged oxidative insult creates a homeostatic imbalance, causing deactivation / switching OFF of PKD1 and persistent proteolytic activation of

PKC δ , the death switch contributes to extensive neuronal damage. We previously showed a parallel proapoptotic mechanism involving translocation of PKC δ catalytic fragments to the mitochondria, resulting in a persistent increase in caspase-3 dependent PKC δ proteolytic cleavage via a positive feedback loop mechanism. Our results suggest that positive modulation of the PKD1-mediated protective mechanism against oxidative damage in dopaminergic neurons may provide novel neuroprotective strategies for treatment of PD (55).

Chapter II: Protein Kinase D1 (PKD1) activation mediates a compensatory protective response during early stages of oxidative stress-induced neuronal degeneration

A paper published in *Molecular Neurodegeneration* 2011 6:43.

Arunkumar Asaithambi, Arthi Kanthasamy, Hariharan Saminathan, Vellareddy Anantharam and Anumantha G. Kanthasamy

Abstract

Background

Oxidative stress is a key pathophysiological mechanism contributing to degenerative processes in many neurodegenerative diseases and therefore, unraveling molecular mechanisms underlying various stages of oxidative neuronal damage is critical to better understanding the diseases and developing new treatment modalities. We previously showed that protein kinase C delta (PKC δ) proteolytic activation during the late stages of oxidative stress is a key proapoptotic signaling mechanism that contributes to oxidative damage in Parkinson's disease (PD) models. The time course studies revealed that PKC δ activation precedes apoptotic cell death and that cells resisted early insults of oxidative damage, suggesting that some intrinsic compensatory response protects neurons from early oxidative insult. Therefore, the purpose of the present study was to characterize protective signaling pathways in dopaminergic neurons during early stages of oxidative stress.

Results

Herein, we identify that protein kinase D1 (PKD1) functions as a key anti-apoptotic kinase to protect neuronal cells against early stages of oxidative stress. Exposure of dopaminergic neuronal cells to H₂O₂ or 6-OHDA induced PKD1 activation loop (PKD1S744/748) phosphorylation long before induction of neuronal cell death. Blockade of PKC δ cleavage, PKC δ knockdown or overexpression of a cleavage-resistant PKC δ mutant effectively attenuated PKD1 activation, indicating that PKC δ proteolytic activation regulates PKD1 phosphorylation. Furthermore, the PKC δ catalytic fragment, but not the regulatory fragment, increased PKD1 activation, confirming PKC δ activity modulates PKD1 activation. We also identified that phosphorylation of S916 at the C-terminal is a preceding event required for PKD1 activation loop phosphorylation. Importantly, negative modulation of PKD1 by the RNAi knockdown or overexpression of PKD1^{S916A} phospho-defective mutants augmented oxidative stress-induced apoptosis, while positive modulation of PKD1 by the overexpression of full length PKD1 or constitutively active PKD1 plasmids attenuated oxidative stress-induced apoptosis, suggesting an anti-apoptotic role for PKD1 during oxidative neuronal injury.

Conclusion

Collectively, our results demonstrate that PKC δ -dependent activation of PKD1 represents a novel intrinsic protective response in counteracting early stage oxidative damage in neuronal cells. Our results suggest that positive modulation of the PKD1-mediated compensatory protective mechanism against oxidative damage in dopaminergic neurons may provide novel neuroprotective strategies for treatment of PD.

Introduction

Oxidative stress-induced neuronal damage has been implicated in many neurodegenerative disorders including Parkinson's disease (PD), Alzheimer's diseases, ALS, Huntington's diseases and stroke (1), (2), (3) (4) (5) (6) (7). Neuronal cells maintain an oxidant/antioxidant homeostatic balance, and any breach in redox homeostasis causes excessive ROS production, resulting in oxidative damage (8) (9) (10). Oxidative stress triggers apoptosis through activation of many signaling molecules including kinases and proteases (11) (12) (13) (14) (15), and the roles of these signaling molecules in dopaminergic cell death are being actively investigated. Recently, we demonstrated that the proteolytic activation of PKC δ , a novel PKC family member, mediates apoptosis in cell culture and animal models of PD (15) (16) (17) (18) (19).

PKC δ can be activated by membrane translocation, phosphorylation, or proteolytic cleavage by caspase-3, leading to persistently active catalytic fragments. We previously showed that various oxidative stressors like H₂O₂, MPP⁺ and 6-OHDA induce PKC δ cleavage to increase the kinase activity and apoptosis in dopaminergic cells (20) (15) (21) (16). The time course studies revealed that the pro-apoptotic proteolytic activation of PKC δ occurs well before apoptotic cell death, and that cells resist early oxidative damage, suggesting that some key intrinsic compensatory responses protect neurons from the initial oxidative insult. Therefore, we speculated that the persistently active catalytic fragment of PKC δ might have other functions during the early stages of oxidative stress, and so we further explored downstream signaling mechanisms.

Protein kinase D1 (PKD1) is a calcium/calmodulin-dependent member of the CAMK kinase family and can be activated by dual phosphorylation of serine residues (Ser 744/748) in the catalytic domain by different PKCs, depending upon the cellular type and stimuli (22-24). PKD1 is activated in response to multiple stimuli including growth factors, phorbol esters, G-protein coupled receptors, genotoxic stress and oxidative stress (25-28). In non-neuronal cells, PKD1 activation has been shown to play a role in diverse cellular functions including proliferation, cytoskeletal reorganization, golgi function and immune response (27, 29-32). PKD1 has been shown to regulate various cell signaling molecules and pathways including ERK1/2, JNK pathways (33-35), effector enzymes like MnSOD that scavenge ROS (31) (36), transcriptional regulators including NF- κ B and MEF2 (37) (38), stress responsive chaperones like HSP27 (39), and some members of the HDAC family (26, 31, 40). Recently, PKD1 was recognized as an important mitochondrial ROS sensor that translocates to the nucleus to switch on cell survival mechanisms (36). Also, PKD1 activation loop phosphorylation has been shown as an early marker of sympathetic neuronal DNA damage (41). In neuronal models, PKD1 regulates trafficking of dendritic membrane proteins to maintain neuronal polarity and synaptic plasticity (42).

While many biological functions of PKD1 are beginning to emerge, the role of PKD1 in the brain, specifically in the nigral dopaminergic system, remains unknown. The relationship between PKD1 signaling and neurodegeneration has not yet been examined in a single study. Herein, we demonstrate that PKD1 closely interacts with PKC δ and serves as a key compensatory protective mechanism in dopaminergic neuronal cells during the early stages of oxidative insult.

Materials and Methods:

Cell Culture

The immortalized rat mesencephalic dopaminergic neuronal cell line (N27) was a kind gift from 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 of penicillin, and 50 µg/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C, as described previously (16). N27 cells are used widely as a cell culture model for PD (20) (15) (21) (16, 43).

Primary mesencephalic neuronal culture

Primary mesencephalic neuronal cultures were prepared from the ventral mesencephalon of gestational 16- to 18-day-old mouse embryos, as described earlier (44). Tissues were dissected from E16 to E18 mouse embryos maintained in ice cold Ca²⁺-free Hanks' balanced salt solution and then dissociated in Hanks' balanced salt solution containing trypsin-0.25% EDTA for 30 min at 37°C. The dissociated cells were then plated at equal density (0.5×10^6 cells) on 12 mm coverslips precoated with 0.1 mg/ml poly-D-lysine. Cultures were maintained in neurobasal medium fortified with B-27 supplements, 500 µM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). The cells were maintained in a humidified CO₂ incubator (5% CO₂ and 37°C). Half of the culture medium was replaced every 2 days. Approximately 6- to 7-day-old cultures were used for experiments. Primary mesencephalic dopaminergic neuronal cells were exposed to 10 µM for 1 h.

Treatment Paradigm

N27 cells were exposed to H₂O₂ (100 μM) for 0–4 h at 37°C. Primary neurons were exposed to H₂O₂ (10 μM) for 1 h. Cell lysates were used for Western blotting and immunoprecipitation studies. Untreated cells were grown in the complete medium and used as the experimental control.

Cytotoxicity Assays

Cell death was determined using the Sytox green cytotoxicity assay, after exposing the N27 cells to H₂O₂ (100 μM), as described previously. This cytotoxicity assay was optimized for a multiwell format, which is more efficient and sensitive than other cytotoxicity measurements (45) (46). Briefly, N27 cells were grown in 24-well cell culture plates at 100,000 cells per well and treated with H₂O₂ (100 μM) and 1 μM Sytox green fluorescent dye. The Sytox green assay allows dead cells to be viewed directly under a fluorescence microscope, as well as quantitatively measured with a fluorescence microplate reader (excitation 485 nm; emission 538 nm) (Biotek). Phase contrast and fluorescent images were taken after H₂O₂ exposure with a NIKON TE2000 microscope, and pictures were captured with a SPOT digital camera.

ROS Generation Assay

ROS generation was monitored by CM-DCFDA dye, as described previously (15, 47). This is a non-fluorescent dye in its reduced form, but after oxidation in the cells, the acetate group is removed by cellular esterases, resulting in fluorescence. N27 cells were seeded in 48-well plates at a confluence of 40,000 cells / well for 24 h. First, cells were loaded with 10μM CM-DCFDA dye (Invitrogen) at 37°C for 1 h in the dark. Cells were then treated with H₂O₂ in Hanks' balanced salt solution (HBSS) and the fluorescence of the cells was

measured using the synergy 2 fluorescence plate reader (Biotek) at various time points (excitation 485 nm; emission 538 nm).

Immunocytochemistry

The primary mesencephalic neurons or N27 cells after H₂O₂ treatment were fixed with 4% paraformaldehyde and processed for immunocytochemical staining. First, nonspecific sites were blocked with 2% bovine serum albumin, 0.5% Triton and 0.05% Tween-20 in phosphate-buffered saline (PBS) for 20 min. The cells then were incubated with antibodies directed against TH, native PKD1 and PKD1-pS744/S748 in PBS containing 1% BSA at 4°C overnight, followed by incubation with Alexa 488 and Alexa 568 conjugated secondary antibodies in PBS containing 1% BSA. Secondary antibody treatments were followed by incubation with Hoechst 33342 dye for 5 min at room temperature to stain the nucleus. The coverslips containing stained cells were washed with PBS, mounted on slides, and viewed under a Nikon inverted fluorescence microscope (model TE-2000U; Nikon, Tokyo, Japan). Both fluorescence and confocal images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Western Blot Analysis

Cells were lysed in either modified RIPA buffer or M-PER buffer (Thermo Scientific) for Western blot, immunoprecipitation and kinase assays. Lysates containing equal amounts of protein were loaded in each lane and separated on 10-12% SDS-PAGE, as described previously (Kaul et al., 2003). PKD1 polyclonal (1:1000), PKC δ polyclonal (1: 1000), PKD1-pS744/S748 (1:1000), PKD1-pS916 (1:1000), PKD1-pY469 (1:1000), - and β -actin (1:10000) antibodies were used to blot the membranes. IR dye-800 conjugated anti-rabbit (1:5000) and Alexa Fluor 680 conjugated anti-mouse (1:10000) were

used for antibody detection with the Odyssey IR Imaging system (LICOR), as previously described.

PKC δ Kinase Assay

Immunoprecipitation and PKC δ kinase assay were performed as described earlier (15). After cell lysis, cell were immunoprecipitated using a polyclonal PKC δ rabbit antibody and protein A Sepharose, and washed three times with PKC δ kinase buffer (40 mM Tris (pH 7.4), 20 mM MgCl₂, 20 μ M ATP, 2.5 mM CaCl₂). The reaction was started by adding 20 μ l of buffer containing 0.4 mg histone and 5 μ Ci of [γ -³²P]ATP (4,500 Ci/mM). After incubation for 10 min at 30°C, SDS loading buffer (2X) was added to the samples to terminate the reaction. The reaction products were separated on SDS-PAGE (12%), and the H1-phosphorylated bands were detected using a phosphoimager (Fujifilm FLA-5100) and quantified with MultiGauge V3.0 software.

Protein Kinase D1 Kinase Assay

The cells were exposed to H₂O₂ (100 μ M) for 1 h and cell lysates were immunoprecipitated, as previously reported, with native PKD1 antibody (Santa Cruz). The kinase reaction was carried out at room temperature for 20 min after adding 10 μ l of kinase substrate mix (0.1 mM ATP + 10 μ ci [γ -³²P] ATP + 2 μ g Syntide 2 peptide substrate in kinase buffer). Kinase buffer contains 20 mM Tris pH 7.5, 10 mM MgCl₂, and 1 mM DTT. The samples were centrifuged to terminate the kinase reaction, and the supernatants containing the phosphorylated peptide were applied as spots to P81 phosphocellulose squares (Whatmann). The papers were washed four times with 0.75% phosphoric acid and once with acetone and dried, and activity was determined by liquid scintillation counting. The samples were also

loaded on a SDS-PAGE and probed for native PKD1 to determine equal loading.

DNA Fragmentation Assay

DNA fragmentation was measured using the Cell Death Detection ELISA Plus assay kit (Roche), for the detection of early apoptotic death, as described previously (15, 47). After 100 μ m H₂O₂ treatment, the cells were spun down at 200 \times g for 5 min and washed once with PBS. Then cells were lysed with lysis buffer provided with the kit. After lysis, the samples were spun down at 10,000 rpm for 10 min to collect the supernatant that was used to measure DNA fragmentation. The supernatants were further dispensed into the microtiter plates coated with streptavidin containing HRP-conjugated antibody cocktail that can detect the nucleosomes. After 2 h incubation, the HRP substrate provided in the kit was added. Measurements were taken in a Synergy 2 multiwell plate reader at 405 nm, with 490 nm as a reference reading.

Transient and Stable Transfections

cDNA encoding PKC δ catalytic fragment (PKC δ -CF), PKC δ regulatory fragment (PKC δ -RF) and PKC δ caspase-resistant mutant (PKC δ -CRM) (PKC δ ^{D327A}) from the pEGFPN1 vector were subcloned into the lentiviral expression vector plenti6/V5-d-TOPO in our lab (herein referred to as V5-PKC δ -CF, V5-PKC δ -RF, V5-PKC δ -CRM) (15, 48). ViraPower Lentiviral Expression System (Invitrogen) was used to establish stable transfections of a caspase-resistant mutant of PKC δ ^{D327A} (48). Full-length human PKD1 plasmid (PKD1-FL), PKD1 activation loop, active PKD1^{S744E/S748E} (PKD1-CA) and PKD1^{S916A} mutants were obtained from Addgene, Inc. (49) (50) (37). Electroporation was carried out with an Amaxa Nucleofector transfecto-

instrument, as per the manufacturer's protocol. The transfected cells were then transferred to T-75 flasks or 6-well plates as desired and allowed to grow for a 24 h period before the treatment.

RNAi

PKC δ -siRNA was prepared by an in vitro transcription method, as described previously (20). PKC δ -siRNA effectively suppressed > 80% of PKC δ protein expression levels within 24 h post-transfection. Predesigned PKD1-siRNA was purchased from IDT, Inc. PKD1-siRNA effectively suppressed > 80% of PKD1 protein expression levels after 36 h post-transfection. N27 cells (50–70% confluence) were transfected with siRNA duplexes using an Amaxa Nucleofector kit (Amaxa), as described in our previous study (20).

Statistical Analysis

Data analysis was performed using Prism 3.0 software (GraphPad Software, San Diego, CA). Bonferroni's multiple comparison testing was used to find the significant differences between treatment and control groups. Differences with $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered significantly different from $n \geq 6$ from two or more independent experiments, and are indicated in the figures.

Results

Role of PKC δ cleavage in the early stages of H₂O₂-induced oxidative stress in dopaminergic neuronal cells

H₂O₂ is a common oxidative insult used to probe various cellular signaling pathways in different cell types including neuronal cells (15, 51, 52). We have already demonstrated that H₂O₂ causes dose- and time-dependent

cytotoxicity, DNA fragmentation and cell death in the dopaminergic neuronal N27 cell model (15). In order to determine the interrelationship between PKC δ proteolytic cleavage and oxidative stress-induced cell death, we examined the time course of PKC δ proteolytic activation and cell death. As shown in Fig 1A, generation of ROS occurs as early as 1 h after H₂O₂ treatment. Cytotoxicity begins between 90 and 120 min (Fig 1B). PKC δ proteolytic cleavage and the kinase activity increases during the early stage of H₂O₂ exposure at 60 min (Fig 1C, D). Comparison of PKC δ proteolytic activation and cytotoxicity at 60 min revealed that PKC δ proteolytic activation occurs during the early stages of oxidative stress preceding cell death (Fig 1E). There was no cell death during this intermediate period between ROS generation and PKC δ proteolytic activation. PKC δ knockdown by RNAi almost completely blocked the cell death induced by H₂O₂ at 120 min of treatment, demonstrating the pro-apoptotic function performed by PKC δ in N27 cells (Fig 1F). Importantly, a significant lag period between induction of cell death and PKC δ proteolytic activation was observed. This interesting observation prompted a search for cell signaling mechanisms associated with a compensatory protective response involving PKC δ proteolytic activation during early stages of oxidative stress.

Oxidative stress induces phosphorylation and activation of PKD1 in a time-dependent manner

We hypothesized that the proteolytically cleaved PKC δ might activate other downstream cell survival signaling molecules to counteract the early stage of oxidative insult. To test this hypothesis, we first used a bioinformatic approach to search for a key pro-survival molecule that interacts with PKC δ . Scansite Motif Scanner software (53) predicted that out of all the PKCs, only

PKC δ can phosphorylate the protein kinase D1 (PKD1) activation loop ser residue at high stringency search (additional file 1). Further literature review indicated that PKD1 is an oxidative stress-responsive kinase that can be activated by phosphorylation at the activation loop (S744/S748) in non-neuronal models (22-25, 54). This encouraged us to look further for the expression and activation of PKD1 in dopaminergic neuronal cells. Immunocytochemical staining showed abundant expression of PKD1 in N27 dopaminergic cells, as visualized by confocal and fluorescence microscopy (Fig 2A), which is similar to the expression pattern of PKC δ in this cell type. Importantly, PKD1 is also highly expressed in primary tyrosine hydroxylase (TH) positive dopaminergic neurons obtained from mouse substantia nigra in the cytosolic region (Fig 2B).

To determine if oxidative stress can induce PKD1 Ser 744/Ser 748 phosphorylation in the activation loop, we examined the ability of H₂O₂ to induce time-dependent PKD1 activation loop phosphorylation in N27 dopaminergic cells. As shown in Fig 2C, 100 μ M H₂O₂ induced transient PKD1 activation loop phosphorylation corresponding to the 120 kDa band starting at around 30 min, peaking at 60 min and returning to control levels after 90 min, with native protein levels remaining the same. We also observed a second band around 100 kDa, which might correspond to the other isoform, PKD2. According to the manufacturers (Cell Signaling Technology), the phospho-specific antibody can also detect PKD2 Ser 706/Ser 710 phosphorylation because of the conserved activation loop residues between PKD isoforms. However, the PKD2 activation loop phosphorylation does not follow the transient pattern of activation observed with PKD1 (Fig 2C). Furthermore, the activation loop phosphorylation of PKD1 increased the PKD1 kinase activity, as measured by a [³²P] kinase

assay using Syntide 2 substrate (Fig 2D). Collectively, these results demonstrate that oxidative stress activates PKD1 at early stages through phosphorylation of the dual phospho sites pS744/pS748.

Oxidative stress-induced PKD1 activation depends on PKC δ

To further determine whether PKC δ regulates PKD1, we used both pharmacological and genetic approaches to suppress the PKC δ and then measured the level of PKD1 activation. Treatment of N27 cells with the PKC δ inhibitor rottlerin (1 μ M) completely suppressed H₂O₂ -induced PKD1 activation (Fig 3A), suggesting a potential role of PKC δ in PKD1 activation. Next, we used the PKC δ -specific siRNA to knockdown PKC δ and then probed for PKC δ expression and PKD1 phosphorylation. PKC δ knockdown completely attenuated PKD1 activation loop phosphorylation except for the bottom band, corresponding to 100 kDa PKD2 (Fig 3B). Furthermore, PKD1 activity was measured by [³²P] kinase assay in PKC δ knockdown samples. The suppression in PKC δ expression completely attenuated PKD1 activation during H₂O₂ -mediated oxidative stress, confirming that PKC δ is indeed involved in PKD1 activation (Fig 3C). To show the specificity of PKC δ in PKD1 activation in neuronal models, we used PKC α siRNA and then measured PKD1 activation loop phosphorylation (additional file 2). Knockdown of PKC α did not attenuate PKD1 activation loop phosphorylation, further demonstrating the specificity of PKC δ -mediated PKD1 phosphorylation in dopaminergic neuronal models.

The constitutively active catalytic fragment of PKC δ mediates PKD1 activation

Time course analysis of PKC δ proteolytic cleavage and PKD1 activation revealed that the onset of proteolytic activation of PKC δ coincides with the maximal activation of PKD1 at 60 min following H₂O₂-induced oxidative stress (Fig 4A). Previously, we had shown that caspase-3 inhibitor z-DEVD-fmk and pan caspase inhibitor ZVAD-fmk attenuate H₂O₂-induced proteolytic activation of PKC δ . Therefore, we examined whether the proteolytically activated PKC δ contributes to PKD1 activation. As shown in Fig 4B, co-treatment with z-DEVD-fmk and ZVAD-fmk for 1 h significantly blocked H₂O₂-induced activation loop phosphorylation of PKD1 as well as proteolytic cleavage of PKC δ . Coincidentally, H₂O₂-induced activation loop phosphorylation of PKD2 was not affected in the presence of these caspase inhibitors. To further confirm the role of proteolytically activated PKC δ in PKD1 activation, we used N27 cells stably expressing the PKC δ cleavage-resistant mutant PKC δ^{D327A} (PKC δ -CRM). H₂O₂ treatment in PKC δ -CRM cells significantly attenuated PKD1 phosphorylation, confirming that the proteolytically cleaved PKC δ contributes to PKD1 phosphorylation during early stages of oxidative stress (Fig 4C). To further support this observation, we separately over-expressed the PKC δ catalytic fragment (V5-PKC δ -CF) and PKC δ regulatory fragment (V5-PKC δ -RF) using a lentiviral vector (plenti6/V5-D-TOPO) in N27 cells and then evaluated for activation of PKD1. Interestingly, V5-PKC δ -CF over-expressing cells had increased PKD1 phosphorylation, while V5-PKC δ -RF over-expressing cells did not increase phospho-PKD1 levels (Fig 4D). Collectively, these results demonstrate that the constitutively active catalytic fragment of PKC δ mediates PKD1 activation loop phosphorylation.

PKD1 activation functions as an anti-apoptotic protective mechanism against oxidative stress

We previously reported that proteolytically activated PKC δ (PKC δ -CF) mediates apoptosis in neurotoxicity models (15, 19, 55) and therefore, we initially hypothesized that PKC δ proteolytic cleavage-dependent activation of PKD1 may have a proapoptotic function. Surprisingly, PKD1 knockdown siRNA significantly augmented H₂O₂-induced cell death at 2 h, as measured by Sytox cell death assay (Fig 5A). PKD1 knockdown increased cell death by nearly twofold compared to control groups during H₂O₂ treatment, indicating a pro-survival role for PKD1 against oxidative stress (Fig 5B). Visualization of PKD1 knockdown cells by phase contrast and fluorescence microscopy further confirmed that PKD1 knockdown cells are more sensitive than non-specific control cells to H₂O₂-induced cytotoxicity (Fig 5C and D). We further measured the H₂O₂-induced apoptosis in PKD1 knockdown cells by DNA fragmentation ELISA assay at 3 h. The results showed an increase in DNA fragmentation in the PKD1 knockdown group, as compared to the non-specific siRNA control group, further confirming the anti-apoptotic role of PKD1 in dopaminergic cells (Fig 5E).

PKD1 C-terminal Ser 916 phosphorylation precedes PKD1 Ser 744/Ser 748 activation loop phosphorylation during oxidative stress

We also characterized the sequential mechanisms of PKD1 activation in dopaminergic neuronal cells. We investigated the other two key phosphorylation sites of PKD1, Tyr 469 and Ser 916. As shown in Fig 6A H₂O₂-induces transient phosphorylation of PKD1 Ser 916 in a time-dependent manner. The data suggests that PKD1 C-terminal Ser 916 was rapidly

phosphorylated as early as 10 min after H₂O₂ treatment continues to increase up to 90 min and decreases at 150 min (Fig 6A). The phospho PKD1-pS916 antibody detects a doublet PKD1 band. On the other hand, H₂O₂ failed to induce PKD1-tyr 469 phosphorylation in N27 dopaminergic cells (Fig 6A). Our results are consistent with a previous study suggesting that PKD1 tyr 469 phosphorylation does not occur in H₂O₂-treated Swiss 3T3 cells (56). Similarly, it was shown that Vasopressin a GPCR agonist can activates PKD1 in Swiss 3T3 cells [30]. Since H₂O₂ did not phosphorylate tyr 469, we used desmopressin, a synthetic analogue of vasopressin to induce tyr 469 phosphorylation. Exposure of N27 dopaminergic cells to the positive control desmopressin induced tyr 469 phosphorylation (Fig 6B). To further evaluate the involvement of PKD1 Ser 916 phosphorylation in PKD1 activation, we overexpressed the PKD1^{S916A} mutant and PKD1^{WT} plasmids in dopaminergic cells and then stimulated the cells with H₂O₂. Overexpression of PKD1^{S916A} mutant blocked PKD1 activation loop phosphorylation (Fig 6C), suggesting that Ser 916 phosphorylation is an early event that has to occur prior to PKD1 activation loop phosphorylation. Furthermore, knockdown of PKC δ attenuated PKD1^{S916} phosphorylation, demonstrating that this is PKC δ dependent (Fig 6D). Together, these results suggest that PKD1 Ser 744/Ser 748 activation loop phosphorylation is intrinsically regulated by PKC δ via C-terminal phosphorylation of PKD1 Ser916.

PKD1 activation acts as a protective compensatory mechanism during early stages of oxidative stress

To understand the relationship between PKC δ /PKD1 activation and neuronal cell death during oxidative stress, we quantified the PKC δ /PKD1 activation profile from the Western blots and then compared them with the profile of neuronal cell death during H₂O₂-induced oxidative stress. As shown in Fig

7A, PKD1 was activated at the early stages of oxidative insult and no measurable cell death was observed until PKD1 activation started declining to basal levels. Alternatively, PKC δ activation was concomitantly increased along with H₂O₂-induced cytotoxic cell death at the later stage of oxidative stress. Thus, the inverse correlation of PKD1 activation with cytotoxicity suggests that PKD1 activation may act as a compensatory protective response during early stages of oxidative insult. To test this hypothesis, we first overexpressed the full-length human PKD1 (PKD1^{WT}) in N27 cells and then examined the H₂O₂-induced cytotoxicity. As anticipated, PKD1^{WT} overexpression protected the dopaminergic cells against oxidative stress-induced cytotoxicity at 2 h (Fig 7B). In order to establish the pro-survival function of PKD1 activation, we examined whether overexpression of the PKD1^{S916A} phosphorylation defective mutant exacerbated H₂O₂-induced neuronal cell death. As shown in Fig 7C, overexpression of the PKD1^{S916A} phosphorylation defective mutant exacerbated H₂O₂-induced cytotoxic cell death as early as 90 minutes, compared to vector overexpressing cells. To further confirm our hypothesis that PKD1 activation loop phosphorylation acts as an early protective compensatory response, we overexpressed the activation loop active plasmid (PKD1^{S744E/S748E}), where the replacement of serine with glutamate makes the kinase constitutively active (50), and cytotoxicity was monitored for up to 3h following H₂O₂ treatment (Fig 7D). The constitutively active PKD1 mutants tremendously suppressed the cytotoxicity, even during late stages of oxidative insult, indicating that PKD1 activation is a very significant early protective compensatory mechanism in dopaminergic cells. Collectively, these results demonstrate that PKD1 is a cell survival kinase that is activated during the early stages of oxidative stress to protect against cytotoxicity in dopaminergic cell models.

Activated PKD1 translocates to nucleus during oxidative stress in cell culture models of neurodegeneration

We also performed immunocytochemical staining to examine the subcellular localization of activated PKD1 during oxidative stress. Activated PKD1 (PKD1pS744/pS748) (green) co-localized with the nuclear Hoechst stain (blue) at 1 h during H₂O₂-induced oxidative stress in N27 cells, as visualized by fluorescence microscopy (Fig 8A). These results indicate that activated PKD1 translocates to the nucleus of dopaminergic neuronal cells to carry out cell repair and survival functions.

Furthermore, we examined oxidative stress-induced PKD1 activation in primary mesencephalic dopaminergic neurons. Mouse primary mesencephalic neuronal cultures were treated with a low dose of 10 μM H₂O₂ to induce oxidative stress and then subcellular localization of PKD1 activation was monitored by TH/PKD1 double immunolabeling. Activated PKD1 (red) (PKD1 pS744/pS748) co-localized (pink) with the nuclear Hoechst stain (blue) following H₂O₂ treatment in primary mesencephalic neurons staining for TH (green), as visualized by fluorescence microscopy (Fig 8B). We also examined the activation profile of PKD1 pS916 C-terminal phosphorylation. PKD1pS916 (red) was localized (pink/yellow) in both cytosol and nucleus of TH +ve primary mesencephalic neurons staining for green during H₂O₂-induced oxidative stress (Fig 8C).

Parkinsonian-specific toxicant causes PKD1 activation

Activation of this signaling pathway was also tested using the parkinsonian-specific toxicant 6-OHDA. We treated N27 cells with 100 μM 6-OHDA and performed a time-course analysis for 1, 3, and 6 h. PKD1 activation started as early as 1 h and continued until 3 h before reaching control levels at

around 6 h (Fig. 9). This further confirmed the involvement of PKD1 signaling during parkinsonian-specific oxidative insult.

Discussion

The present study reveals a novel protective compensatory signaling mechanism via PKC δ -PKD1 molecular interaction in dopaminergic neuronal cells. Through our collective results, we report for the first time four key findings in a dopaminergic neuronal model pertinent to oxidative stress-mediated neurodegenerative processes: (i) A proteolytically activated catalytic PKC δ fragment (PKC δ -CF) phosphorylates and activates protein kinase D1 (PKD1); (ii) PKD1 activation counteracts early stage oxidative damage and protects dopaminergic neuronal cells from cytotoxicity; (iii) PKC δ -dependent phosphorylation of ser 916 residue precedes ser 744/ser 748; (iv) PKC δ - PKD1 crosstalk tightly regulates cell survival and cell death to maintain cellular homeostasis in response to oxidative damage. The elucidation of this compensatory signal transduction mechanism in neurodegenerative diseases may enhance understanding of degenerative processes and lead to development of novel treatment modalities.

H₂O₂-induced cytotoxicity causes apoptosis in neuronal and non-neuronal cells (15, 51, 52, 57). Generally, oxidative stress-induced apoptosis can be classified into early and late stages. DNA fragmentation occurs in the late stage of apoptosis and is preceded by ROS generation, mitochondrial dysfunction and caspase-3 activation and membrane phosphatidyl exposure (10) (55). In neurodegenerative disorders, especially PD, the signaling mechanisms that contribute to increased vulnerability of dopaminergic neurons to oxidative damage are still under investigation. Most current research focuses on cell death mechanisms in dopaminergic neurons. Some

of the signaling kinases responsible for cell death mechanisms in PD include JNK, MLK, MAPK, LRRK2, etc. (58-61). Earlier, the involvement of a novel biochemical mechanism for cell death in dopaminergic neurons through caspase-mediated proteolytic activation of PKC δ was demonstrated (15) (16) (17) (18) (19). The high levels of persistently active PKC δ catalytic fragment mediate apoptosis during oxidative stress in both cell culture and animal models of PD (15) (16) (17) (18) (19). We also have shown in our earlier study that a positive feedback loop exists during the late stages of oxidative stress, where the persistently active PKC δ catalytic fragment translocates to the mitochondria to promote cytochrome C release and apoptosis (16, 17, 62).

We previously demonstrated proteolytic activation of PKC δ occurs during the early stages of oxidative stress, even before cell death can occur, and coincides with the initiation of mitochondrial ROS generation/caspase-3 activation in dopaminergic neurons (15) (17). Thus, we speculated that proteolytically activated PKC δ might play a regulatory role during the early stages of apoptosis. Previous research suggests the presence of a variety of protective compensatory mechanisms that counteract the early oxidative insult (8) (9) (10). Since we observed in our present study a significant lag time before induction of cell death during the early stages of oxidative stress (Fig 1B), we hypothesize that proteolytically activated PKC δ might sense the extent of oxidative damage and act as a homeostatic regulator in response to oxidative stress, modulating cell survival and cell death mechanisms through interactions with protective signaling molecules.

Protein kinase D1 (PKD1) is emerging as an important signaling molecule associated with oxidative stress in non-neuronal cell lines (31) (35, 36). Studies have shown that oxidative stress increases PKD1 activation loop

phosphorylation (pS744/pS748) via full length PKCs, including PKC δ , in non-neuronal models (37, 63-66). However, the functions of PKD1 during oxidative stress-induced neurodegeneration have not been studied previously. In the present study, we report that cleaved active PKC δ phosphorylates the activation loop of PKD1 and activates the kinase during the early stages of H₂O₂-induced oxidative stress in dopaminergic neuronal cells. We also observed a similar activation pattern for PKD1 and PKC δ during oxidative stress caused by the parkinsonian-specific toxicant 6-OHDA (Fig. 9). To our knowledge, this is the first report of a novel cell survival/cell death signal regulation by the cleaved catalytic fragment of PKC δ at two different stages of apoptosis based on the extent of oxidative damage.

PKD1 is mainly activated by a diacylglycerol-dependent PKCs mechanism (22, 67) or by PKD1 cleavage (68-70). A recent study shows that PKD1 auto-inhibition is released through phosphorylation at the Y463 site in the regulatory domain, leading to the activation loop phosphorylation by PKC δ full length (PKC δ -FL) in Hela cells (24). PKD1 is in a closed conformation during the resting stage, with the regulatory fragment having an autoinhibitory effect on the catalytic fragment (54, 71). Multiple phosphorylation sites on PKD1 seem to be important for its activation loop phosphorylation, depending on the cell types and stimuli. In human cancer cell lines, PKD1 can be phosphorylated at multiple sites including Y463, S910 (corresponding to murine Y469, S916) (24, 72). Phosphorylation of Ser 916 (murine) autophosphorylation site correlated with PKD1 activation loop phosphorylation (65, 73). During oxidative stress in non-neuronal models, Tyr 469 is phosphorylated by upstream kinases, which results in release of the Pleckstrin homology (PH) domain autoinhibition prior to activation loop

phosphorylation; this mechanism does not involve C-terminus Ser 916 phosphorylation (24, 37). In our dopaminergic neuronal models, oxidative stress failed to induce PKD1 Tyr 469 phosphorylation (Fig. 6A), whereas PKD1 Tyr 469 phosphorylation was induced by the positive control desmopressin (Fig. 6B). Our results demonstrate that the mechanism of PKD1 activation in dopaminergic neurons is distinct from the mechanisms in other non-neuronal models. We demonstrate that S916 phosphorylation, but not Tyr 469 phosphorylation, is a preceding event that occurs and is required for PKD1ser744/Ser748 activation loop phosphorylation (Fig. 6). Our data suggest that Ser 916 phosphorylation on the C-terminal of PKD1 may open the conformation for full activation of the kinase through activation loop phosphorylation during oxidative stress in dopaminergic neurons. A detailed comparative analysis of PKC δ proteolytic activation, PKD1 activation loop phosphorylation and the extent of cell death during oxidative stress revealed an interesting functional relationship between activation of kinases and regulation of cell death. Comparison of PKD1 activation and cytotoxicity shows that PKD1 activation is maximal during the early oxidative stress stage when no measurable cytotoxicity is noted (Fig 7A). Interestingly, when PKD1 activation begins to decline at the end of the early stage, cell death begins to occur. Also, the level of PKC δ proteolytic activation directly correlates with the extent of cell death at the later stage of oxidative stress. When the constitutively active PKD1 mutant (PKD1^{S744E/S748E}) is overexpressed, dopaminergic cells are resistant to H₂O₂ -induced neurotoxicity, even during the late stages of oxidative stress (Fig 7D), which is consistent with our hypothesis that PKD1 activation protects against oxidative damage. The downstream signaling mechanisms of PKD1 activation in dopaminergic neuronal cells are not known. PKD1 translocates to the nucleus and regulates phosphorylation of HDACs and various transcription factors in

various non-neuronal cell lines including B cell, cardiomyocytes & oestoblasts (31) (23) (40, 74). PKD1 translocation to the nucleus after activation in dopaminergic neurons is also noted in the present study (Fig. 8), suggesting that nuclear translocation of PKD1 may activate key cell survival transcription factors and genes. Thus, we suggest that PKD1 functions as a cell survival switch and turns 'ON' a protective compensatory mechanism in dopaminergic neurons. Studies are underway to characterize the downstream protective response of PKD1 signaling in nigral dopaminergic neurons.

Conclusions

Our results demonstrate that the PKD1-mediated protective mechanism is a novel signal transduction pathway that regulates cell survival and cell death during various stages of oxidative stress in dopaminergic neuronal cells. As depicted in Fig. 10, in the early stages of oxidative insult, PKC δ acts as an oxidative stress sensor/regulator and activates PKD1, which serves as a key compensatory protective mechanism against oxidative damage. However, prolonged oxidative insult creates a homeostatic imbalance, causing deactivation of PKD1 and persistent proteolytic activation of PKC δ that contribute to extensive neuronal damage. We previously showed a parallel proapoptotic mechanism involving translocation of PKC δ catalytic fragments to the mitochondria, resulting in a persistent increase in caspase-3 dependent PKC δ proteolytic cleavage via a positive feedback loop mechanism (16, 17, 62). Our results suggest that positive modulation of the PKD1-mediated protective mechanism against oxidative damage in dopaminergic neurons may provide novel neuroprotective strategies for treatment of PD.

Abbreviations

PD, Parkinson's disease; PKD1, Protein kinase D1; MAPK, Mitogen-activated protein kinases PKC δ , Protein kinase C delta; CAMK, Ca²⁺/Calmodulin-Dependent Protein Kinase II; JNK, c-Jun N-terminal kinases; LRRK2, Leucine-rich repeat kinase 2 (LRRK2); MLK, Mixed-lineage kinase; ROS, Reactive oxygen species; MnSOD, Manganese superoxide dismutase; WB, Western Blot; PKC, Protein kinase C; PKC α , Protein kinase C alpha.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

All authors read and approved the final manuscript.

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Figure 1

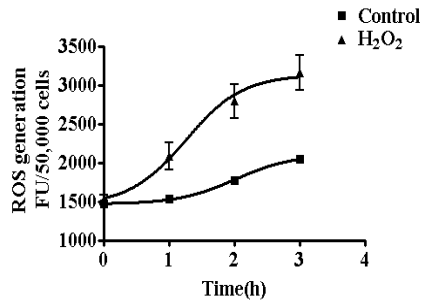
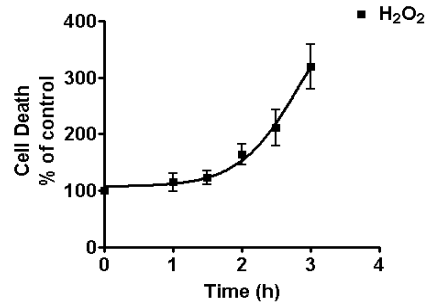
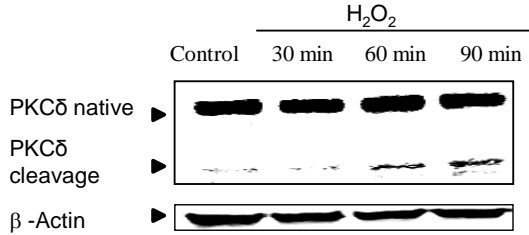
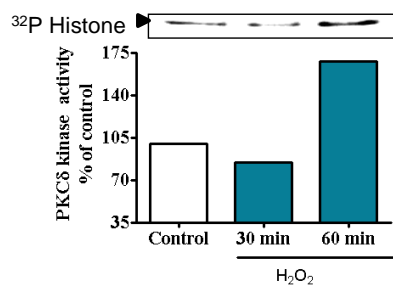
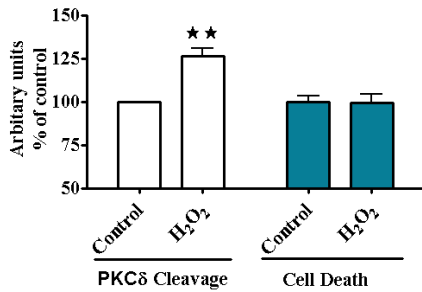
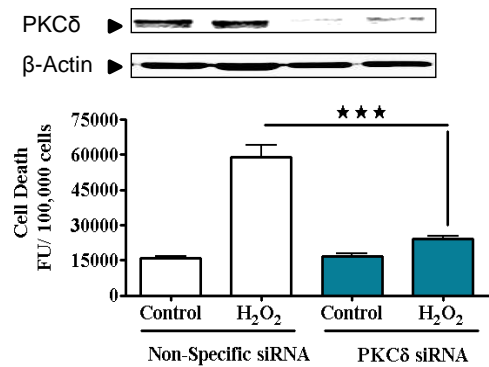
A. H₂O₂-induced ROS generationB. H₂O₂-induced cell deathC. PKC δ cleavageD. PKC δ kinase assayE. PKC δ cleavage and cell death at early stageF. PKC δ siRNA knockdown and cell death at late stage

Figure 2

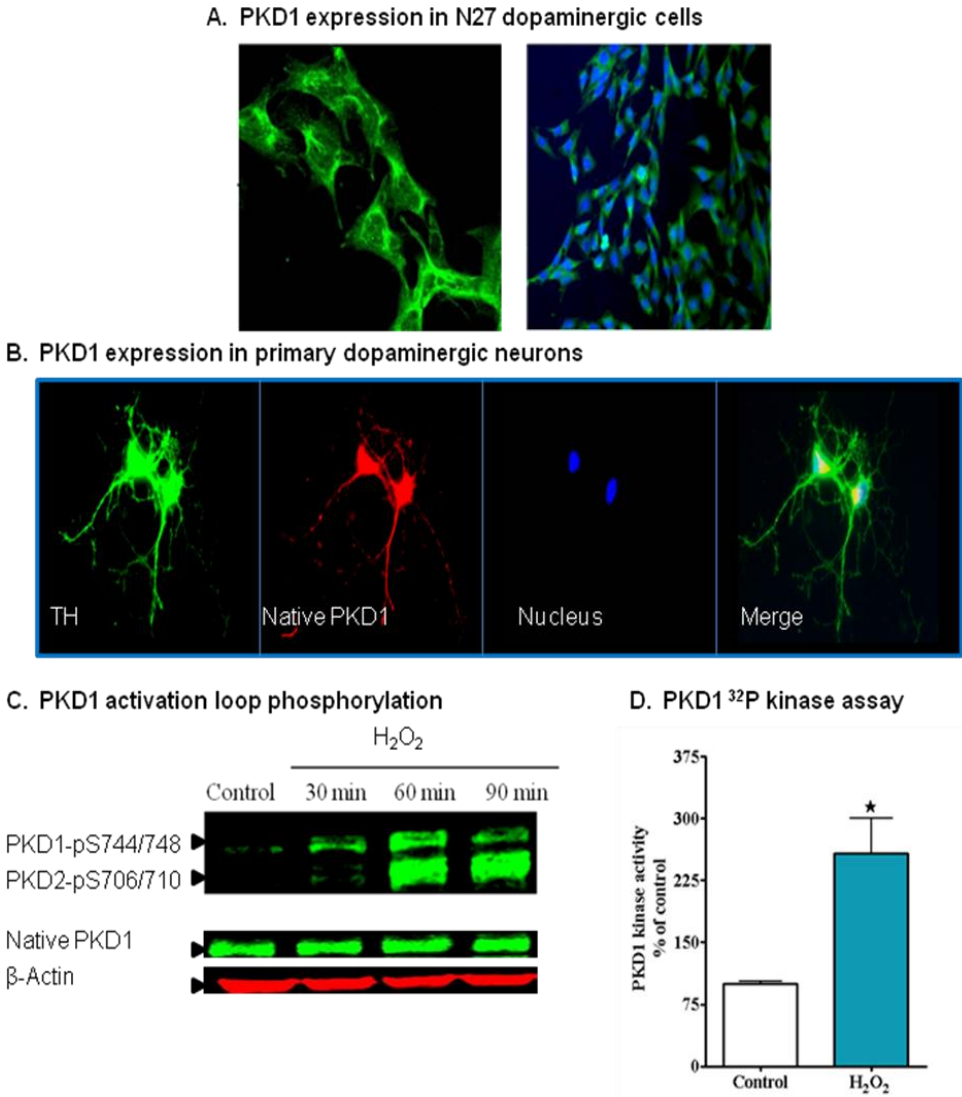


Figure 3

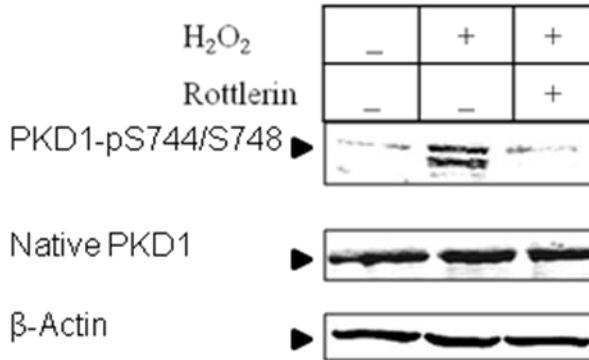
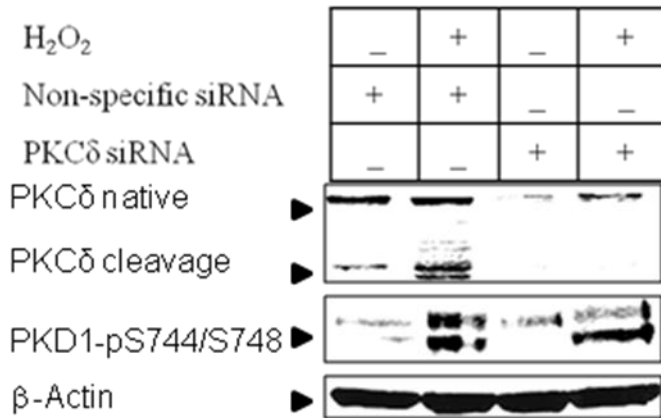
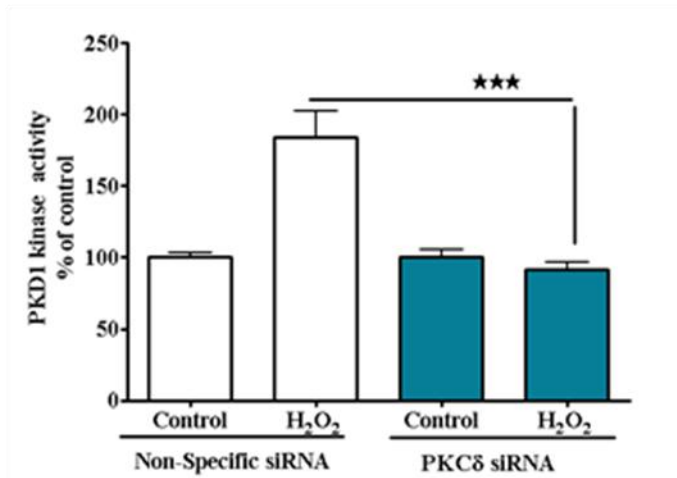
A. PKC δ dependent PKD1 activationB. PKC δ siRNA knockdown and PKD1 activationC. PKD1 ³²P kinase assay

Figure 4

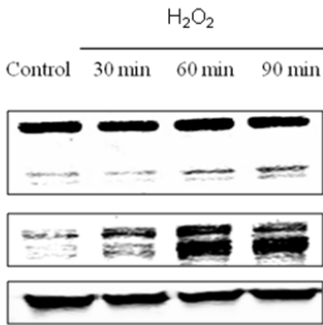
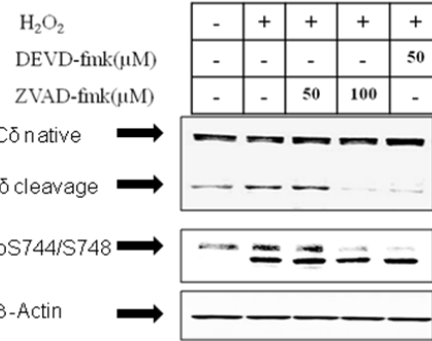
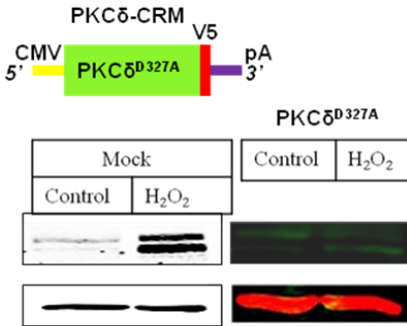
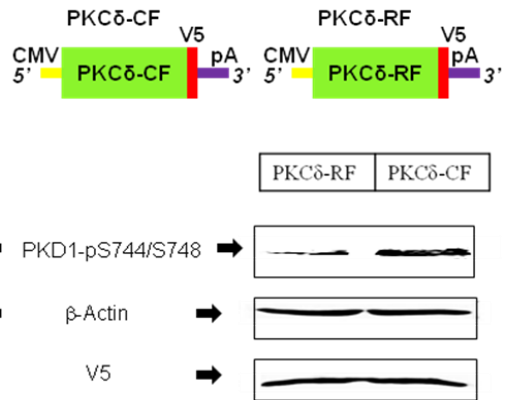
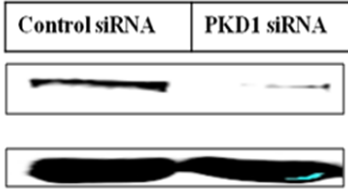
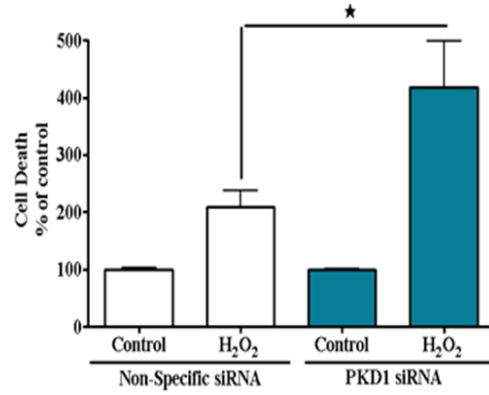
A. PKC δ cleavage and PKD1 activationB. Inhibition of PKC δ proteolytic cleavage and PKD1 activationC. PKC δ cleavage resistant mutant and PKD1 activationD. PKC δ functional mutants and PKD1 activation

Figure 5

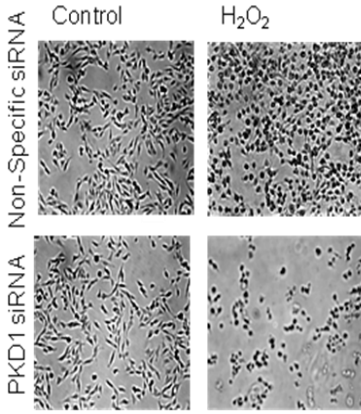
A. PKD1 siRNA knockdown



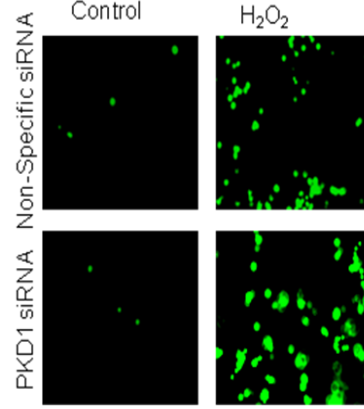
B. PKD1 siRNA knockdown and cell death



C. Phase-Contrast image



D. Flourescence image



E. PKD1 siRNA knockdown and DNA fragmentation assay

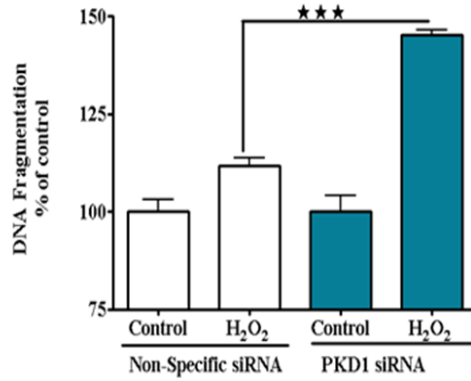
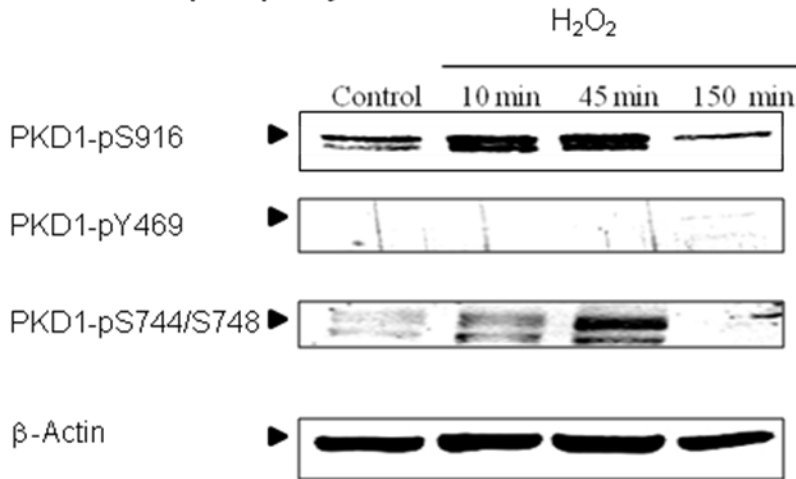


Figure 6

A. PKD1-S916 phosphorylation



B. PKD1-Y469 phosphorylation

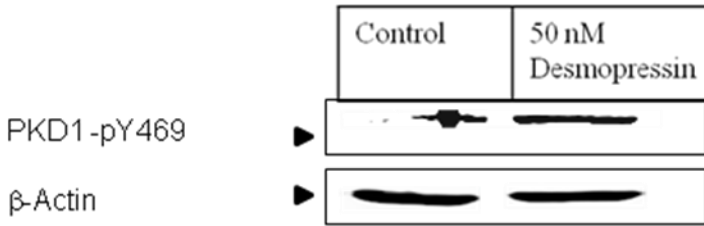
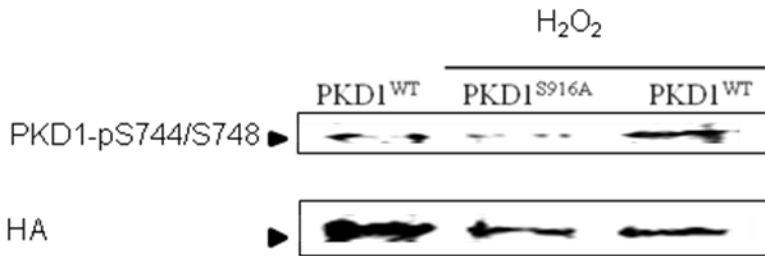
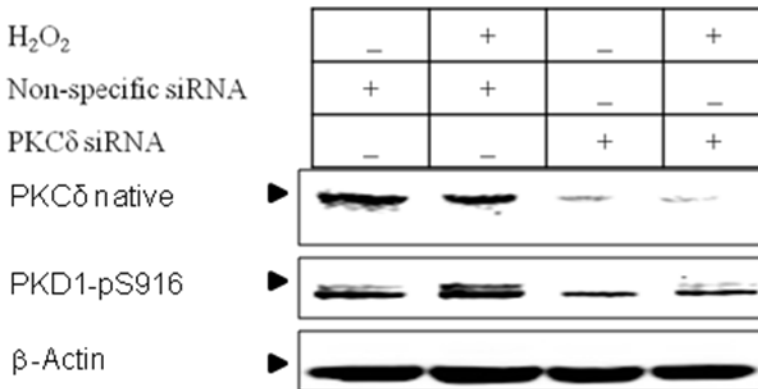
C. PKD1^{S916A} mutant and PKD1 activationD. PKC δ siRNA and PKD1-S916 phosphorylation

Figure 7

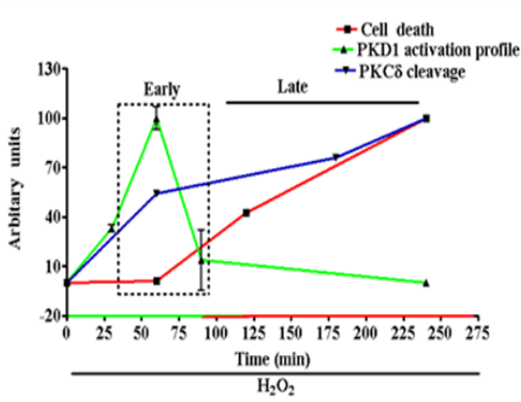
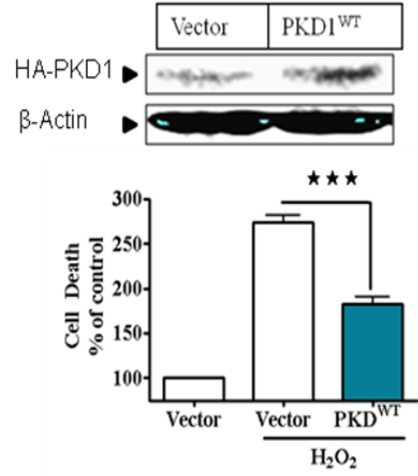
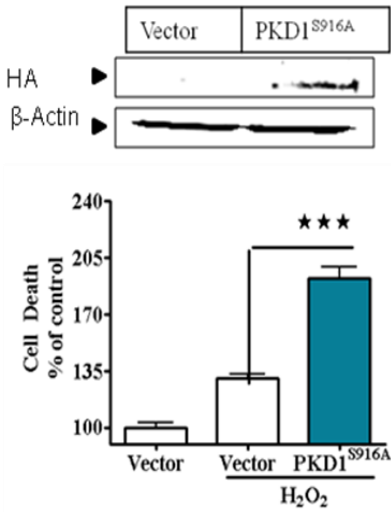
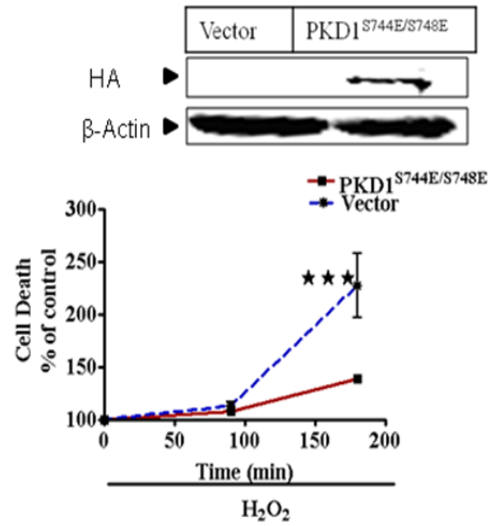
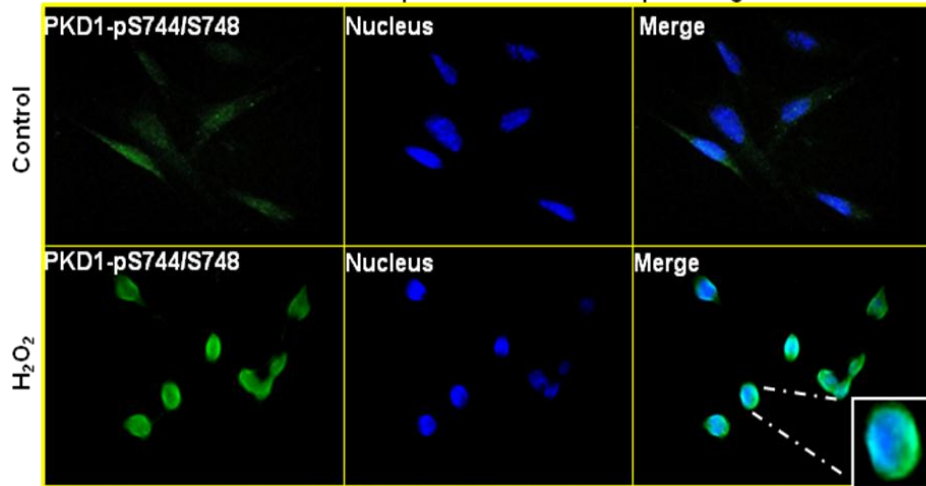
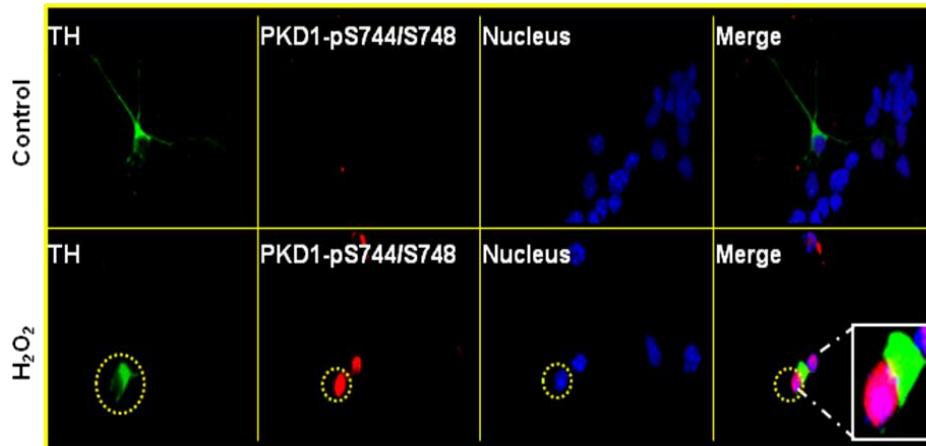
A. Activation profiles of PKD1, PKC δ and cell deathB. Cell survival in PKD1^{WT} expressing cellsC. Cell death in PKD1^{S916A} mutantD. Cell survival in PKD1^{S744E/S748E} mutant

Figure 8.

A. Subcellular localization of PKD1-pS744/S748 in N27 dopaminergic cells



B. Subcellular localization of PKD1-pS744/S748 in primary dopaminergic neurons



C. Subcellular localization of PKD1-pS916 in primary dopaminergic neurons

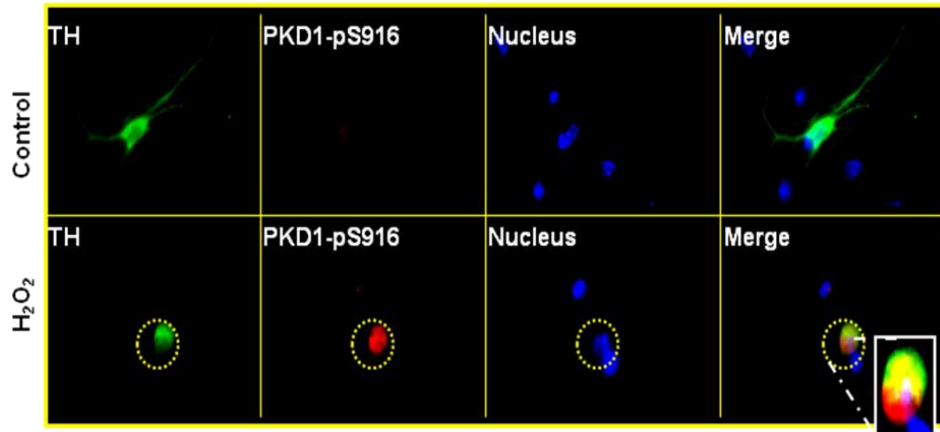


Figure 9

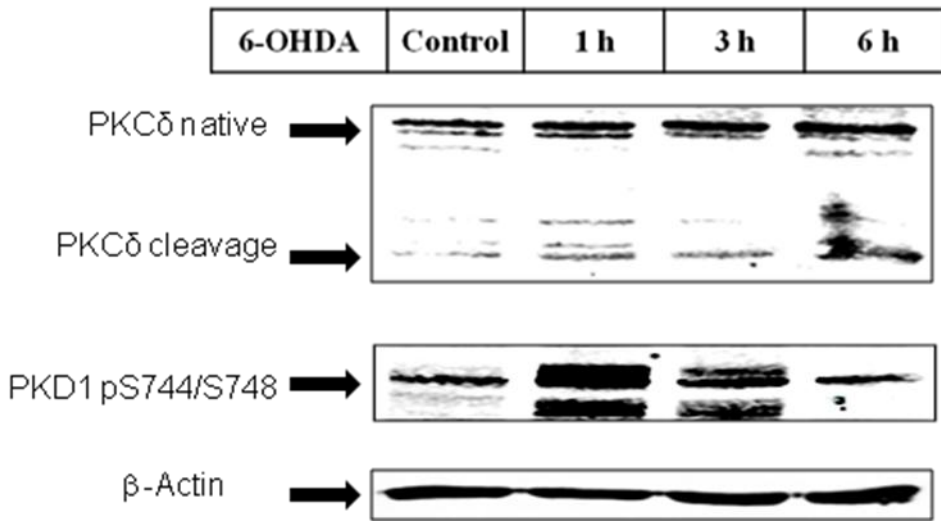


Figure 10

Schematic of PKD1 signal transduction

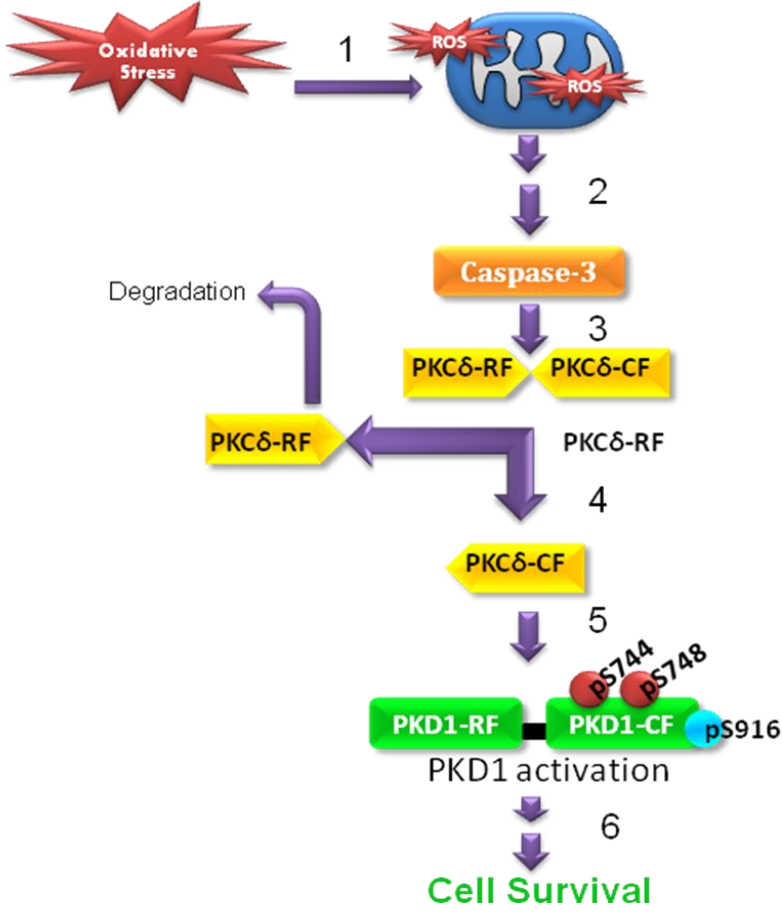


Figure legends

Figure 1. Relationship between PKC δ proteolytic activation and cell death during initial stages of H₂O₂ -induced oxidative stress in N27 dopaminergic neuronal cell model.

N27 dopaminergic cells were treated with H₂O₂ (100 μ M) for 0-180 min and assayed for ROS generation using DCFDA dye (A) and for cytotoxicity using Sytox green dye (B). Non-linear regression graph from two or more independent experiments (n=6-8). PKC δ cleavage was monitored by Western blot in a time dependent manner for 0 - 90 min in N27 dopaminergic cells treated with H₂O₂ (100 μ M) (C). N27 dopaminergic cells were treated with H₂O₂ (100 μ M) for 0, 30 or 60 minutes and PKC δ kinase activity was measured using [³²P] kinase assay; the bands were quantified for the graph (D). Data quantified from B and C were used to generate a bar graph and were compared with cytotoxicity and PKC δ cleavage following H₂O₂ exposure for 60 min. **, p<0.01 as indicated by two-way ANOVA analysis using Bonferroni post test (E). N27 cells were transfected with 1 μ M PKC δ siRNA and non-specific siRNA for 24 h and treated with 100 μ M H₂O₂ and monitored for cytotoxicity using Sytox green dye, which showed significant protection from oxidative stress. ***, p<0.001 denotes significant difference between non-specific siRNA- H₂O₂ and PKC δ siRNA H₂O₂-treated groups from two or more independent experiments (n= 6-8). Statistics were performed by one-way ANOVA analysis using Bonferroni post test (F).

Figure 2. PKD1 is highly expressed in dopaminergic neurons and activated during initial stages of H₂O₂ -induced oxidative stress

Immunofluorescence analysis of N27 dopaminergic cells stained for native PKD1 using fluorescence and confocal microscopy. Nuclei were stained with

Hoechst dye (A). Primary dopaminergic neurons staining for tyrosine hydroxylase (TH) obtained from the mouse substantia nigral region show co-localization of native PKD1 with TH. TH – Green, PKD1 native – Red, Nucleus – Blue, Yellow -Merge. Nuclei were stained with Hoechst dye (B). N27 dopaminergic neuronal cells were treated with or without H₂O₂ (100 μM) for 30, 60 or 90 min and probed for PKD1 activation loop phosphorylation pS744/pS748 and native PKD1 expression (C). N27 cells were treated with or without H₂O₂ (100 μM) and PKD1 kinase activity was measured by [³²P] kinase assay using syntide 2 substrate at 60 min (D). *, p<0.05 denotes significant difference between untreated and H₂O₂-treated groups.

Figure 3. PKCδ dependent phosphorylation of PKD1 activation loop.

N27 dopaminergic cells were treated with H₂O₂ (100 μM) with or without 1 μM rottlerin, and the lysates were probed for PKD1 activation phosphorylation (A). N27 dopaminergic cells were transfected with 1 μM PKCδ siRNA and non-specific siRNA and monitored for PKCδ protein expression and PKD1 activation loop phosphorylation after H₂O₂ treatment (B) and PKD1 kinase activity assay was performed. **, p<0.01 denotes significant difference between NS-siRNA-H₂O₂ and PKCδ-siRNA-H₂O₂ groups (C).

Figure 4. Proteolytically activated PKCδ-CF contributes to PKD1 phosphorylation.

N27 dopaminergic cells treated with H₂O₂ (100 μM) for 30, 60 or 90 minutes were monitored for PKCδ-CF (A). N27 dopaminergic cells were treated with H₂O₂ ± DEVD-fmk (50 μM) and ± ZVAD-fmk (50 μM and 100 μM) for 60 min and monitored for PKD1 activation and PKCδ cleavage (B). N27 dopaminergic cells stably expressing the cleavage-resistant mutant of PKCδ (PKCδ^{D327A}) were treated with H₂O₂ and monitored for PKD1 activation (C).

PKD1 activation was monitored in N27 dopaminergic cells transfected with the catalytic fragment of PKC δ (PKC δ -CF) and the regulatory fragment of PKC δ (PKC δ -RF). Additionally, the mock transfection group treated with or without H₂O₂ was also monitored for PKD1 activation (D).

Figure 5. PKD1 has a cell survival function during oxidative stress in dopaminergic neuronal cells

N27 dopaminergic cells were transfected with 1 μ M PKD1 siRNA and non-specific siRNA (A) and treated with 100 μ M H₂O₂ for 120 minutes and monitored for cytotoxicity using Sytox green dye. Fluorescence measurements for the incorporation of Sytox green read using a fluorescence plate reader (B) and visualised by phase contrast and fluorescence microscopy (C&D). DNA fragmentation assay (E) showed increased cytotoxicity and apoptosis in PKD1 knocked down samples exposed to H₂O₂. *, p<0.05 and ***, p<0.001 denote significant difference between Non-Specific siRNA- H₂O₂ and PKD1 siRNA-H₂O₂ treated groups.

Figure 6. PKC δ -dependent phosphorylation of PKD1 at S916 site precedes PKD1 S744/S748 active loop phosphorylation

N27 dopaminergic cells were treated with 100 μ M H₂O₂ for 10-90 min and monitored for PKD1Y469, PKD1 S916 and PKD1 S744/S748 phosphorylation (A). N27 dopaminergic cells were treated with or without 50 nM desmopressin for 1 h and monitored for PKD1 Y469 phosphorylation. The cells show PKD1 Y469 phosphorylation when exposed to desmopressin, while carbachol does not cause PKD1 Y469 phosphorylation (B). N27 cells expressing PKD1^{S916A} mutant blocked PKD1 activation during oxidative stress, as seen by Western blotting for PKD1 S744/S748 phosphorylation and HA expression (C). N27 dopaminergic cells were transfected with 1 μ M

PKC δ siRNA and non-specific siRNA and monitored for PKC δ protein expression and PKD1 S916 phosphorylation after treatment with or without H₂O₂ (D).

Figure 7. PKD1 activation acts as an early protective compensatory mechanism.

A comparative time course graph based on quantifying PKD1 activation profile, PKC δ cleavage profile and cytotoxicity during H₂O₂ exposure (A). N27 dopaminergic cells transiently transfected with 5 μ M full length PKD1 plasmid (PKD1^{WT}) and 5 μ M vector plasmid were treated with or without 100 μ M H₂O₂ for 150 minutes and monitored for cytotoxicity using sytox green; PKD1 protected against cytotoxicity (B). N27 dopaminergic cells transiently transfected with 5 μ M PKD1^{S916A} plasmid and 5 μ M vector plasmid were treated with or without 100 μ M H₂O₂ for 150 minutes and monitored for cytotoxicity using sytox green; increased cytotoxicity was observed in the cells (C). N27 dopaminergic cells transiently transfected with 5 μ M PKD1^{S744E/S748E} and 5 μ M vector plasmid (D) were treated with or without 100 μ M H₂O₂ and monitored for cytotoxicity at various time points using sytox green. ***, $p < 0.001$ denotes significant difference between treatment groups from $n \geq 6$.

Figure 8. Activated PKD1 translocates to nucleus during H₂O₂ -induced oxidative stress.

Immunofluorescence analysis of N27 dopaminergic cells stained for activated PKD1 using fluorescence microscopy during H₂O₂ exposure show translocation to nucleus. PKD1pS744/S748 – Green, Nucleus – Blue. Nuclei were stained with Hoechst dye (A). Immunofluorescence analysis of primary dopaminergic neurons staining for TH obtained from the mouse substantia

nigral region shows translocation of activated PKD1 to the nucleus during H_2O_2 exposure. TH-Green, PKD1pS744/S748 – Red, Nucleus – Blue, Merge - Pink. Nuclei were stained with Hoechst dye (B). Primary dopaminergic neurons staining for TH show presence of PKD1pS916 in both cytosol and nucleus during H_2O_2 exposure. TH – Green, PKD1pS916– Red, Nucleus – Blue, Pink – Merge in nucleus, yellow –Merge in cytosol. Nuclei were stained with Hoechst dye (C).

Figure 9. PKD1 is activated by 6-OHDA induced oxidative stress

N27 dopaminergic neuronal cells were treated with or without 6-OHDA (100 μ M) for 1, 3, and 6 h and probed for PKD1 activation loop phosphorylation pS744/pS748 and PKC δ cleavage.

Figure 10. Schematic of PKC δ -PKD1 signal transduction mechanism during oxidative stress in dopaminergic neuronal cells.

1) Oxidative stress causes mitochondrial impairment; 2) activation of caspase cascade; 3) caspase-3 mediates proteolytic cleavage of PKC δ ; 4) proteolytically cleaved PKC δ -catalytic fragment (CF) is active; 5) PKC δ -CF activates PKD1 by activation loop phosphorylation during the early stage of oxidative stress; 6) Fully active PKD1 regulates cell survival function in N27 dopaminergic cells.

CHAPTER III: Protein Kinase D1 (PKD1) Activation Protects Against Dopaminergic Neuronal Degeneration Induced by Parkinsonian Specific Toxicant 6-OHDA

Manuscript will be communicated to *Biochimica et Biophysica Acta*

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Anantharam and Anumantha G. Kanthasamy[§]

Abstract

Oxidative stress has been shown to contribute to the specific dopaminergic degenerative process that occurs in Parkinson's disease. Protein kinases are involved in mediating both neuronal survival and death during this process. Several studies have implicated protein kinases that mediate apoptotic cell death, however, very little is available about protein kinases that regulate the cell survival to counteract the oxidative damage and neuronal degeneration. In the present study, we characterized the protective PKD1 signaling pathway in cell culture models of Parkinson's disease. We studied 6-hydroxy dopamine (6-OHDA) induced cell death in N27 dopaminergic cell model and in primary mesencephalic neurons. Our results indicated that 6-OHDA-induced induced PKD1 activation loop (PKD1S744/S748) phosphorylation during early stages of oxidative stress induced dopaminergic cell death. Our results also indicated that 6-OHDA induced phosphorylation of C-terminally located S916 in PKD1. PKD1 S916 phosphorylation precedes and is required for PKD1 activation loop (PKD1S744/748) phosphorylation. Importantly, negative modulation of PKD1 activation by RNAi knockdown or by pharmacological inhibitor kbNB-14270 augmented 6-OHDA induced

apoptosis, while positive modulation of PKD1 by the overexpression of full length PKD1 (PKD1^{WT}) or constitutively active PKD1 (PKD1^{S744E/S748E}), attenuated 6-OHDA induced apoptosis, suggesting an anti-apoptotic role for PKD1 during oxidative neuronal injury. Collectively, our results suggest that positive modulation of the PKD1 mediated signal transduction can provide a novel neuroprotective strategy against PD.

Keywords: 6-OHDA, Protein Kinase D1, Parkinson's disease, Oxidative stress, Neuronal survival, Apoptosis

1. Introduction

Parkinson's disease is a major neurodegenerative disorder affecting over a million Americans with an annual cost of several billion dollars. The incidence of PD cases is projected to dramatically increase with the advancing median age of the U.S. population. This creates a scientific imperative to improve the understanding of the causes and medical management of PD. Several studies have shown that environmental toxicants and oxidative stress are the major factors for the etiology of age-related neurodegeneration. Experimental findings from cell cultures, animal models, and humans indicate that oxidative stress and apoptosis may contribute to the pathophysiological processes in PD [1, 2]. Oxidative stress induced apoptosis is a continual cell death process involving multiple signaling molecules and therefore, identification of key molecules that contribute to the apoptotic cell death process in dopaminergic neurons might provide novel therapeutic targets [1-7]. To study the key signaling molecules involved in oxidative stress induced neuronal apoptosis in PD, we used the specific neurotoxicant 6-hydroxydopamine (2,4,5-trihydroxyphenylethylamine;

6-OHDA 6-OHDA has been shown to induce mitochondrial dysfunction and oxidative stress [8, 9]. Further 6-OHDA is oxidized in dopaminergic neurons to produce reactive oxygen species that damage various macro-molecules and organelles. However, key intrinsic signaling molecules that contribute to increased vulnerability of dopaminergic neurons to oxidative damage are not well understood. We have previously shown that oxidative stress and mitochondrial dysfunction induce caspase-3 mediate proteolytic cleavage of PKC δ , which then mediates apoptotic cell death in cell culture and animal models of PD [10-14]. We recently discovered that PKD1 is activated during the early stages as a compensatory mechanism against oxidative damage [15]. PKD1 is a calcium/calmodulin-dependent kinase and is activated in response to multiple stimuli in different disease models and activation has been shown to play a role in diverse cellular functions [16-23]. PKD1 can be activated by dual phosphorylation of serine residues at the activation loop (Ser 744/ Ser 748) in the catalytic domain [24-26]. Here, we demonstrate 6-OHDA activated PKD1 signaling pathway serves as a key compensatory protective mechanism in dopaminergic neurons during the early stages of oxidative insult and dopaminergic degeneration in cell culture models of PD.

2. Materials and Methods:

2.1 Cell Culture

The immortalized dopaminergic neuronal cell line obtained from the rat mesencephalon (N27 cells) was a kind gift from Dr. Kedar N. Prasad (University of Colorado Health Sciences Center, Denver, CO). N27 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 2 mm l-glutamine, 50 units of penicillin, and 50 μ g/ml streptomycin. Cells kept

in a humidified atmosphere of 5% CO₂ at 37 °C, as described previously [11]. Various groups use N27 cells as a cell culture model for PD [10,11] [27-29].

2.2 Primary mesencephalic neuronal culture

Ventral mesencephalon of gestational 16- to 18-day-old mouse embryos were used to prepare primary mesencephalic neuronal cultures [30]. Tissues obtained from E16 to E18 mouse embryos were dissociated in Hanks' balanced salt solution containing trypsin-0.25% EDTA for 30 min at 37°C. The dissociated cells were then plated at equal density (0.5×10^6 cells) on 0.1 mg/ml poly-D-lysine precoated 12 mm coverslips. Neurobasal medium fortified with B-27 supplements, 500 μ M l-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) were used to grow the cells. The cells were maintained in a humidified CO₂ incubator (5% CO₂ and 37°C). Approximately 5- to 6-day-old cultures were used for experiments. Primary mesencephalic dopaminergic neuronal cells were exposed to 10 μ M 6-OHDA for 1 h.

2.3 Treatment Paradigm

N27 cells were exposed to 6-OHDA (100 μ M) for 0–9 h at 37°C. Primary neurons were exposed to 6-OHDA (10 μ M) for 1-3 h. Cell lysates were used for Western blotting and immunoprecipitation studies. Untreated cells were grown in the complete medium and used as the experimental control.

2.4 Cytotoxicity Assays

Cytotoxicity of N27 cells against 6-OHDA (100 μ M) was determined using the Sytox green cytotoxicity assay, as described previously. This cytotoxicity assay was optimized for a multiwell high-throughput format, which is more efficient and sensitive than other cytotoxicity measurements [31,32]. Briefly,

N27 cells were grown in 24-well cell culture plates at 100,000 cells per well and treated with 6-OHDA (100 μ m) along with 1 μ m Sytox green fluorescent dye. The Sytox green assay allows dead cells to be viewed directly under a fluorescence microscope. Further, the fluorescence can be quantitatively measured using a fluorescence microplate reader (excitation 485 nm; emission 538 nm) (Biotek). Phase contrast and fluorescent images were taken after 6-OHDA exposure using a NIKON TE2000 microscope, and captured with a SPOT digital camera.

2.5 Immunocytochemistry

The primary mesencephalic neurons or N27 cells after H₂O₂ treatment were fixed using 4% paraformaldehyde. Non-specific sites were blocked with 2% bovine serum albumin, 0.5% Triton and 0.05% Tween-20 in phosphate-buffered saline (PBS). The cells then were incubated with anti- TH, anti-native PKD1 and anti-PKD1-pS744/S748 primary antibodies in PBS containing 1% BSA at 4°C overnight. This was followed by incubation with Alexa 488 and Alexa 568 conjugated secondary antibodies in PBS containing 1% BSA. Further incubation was done with nuclear Hoechst 33342 dye to stain the nucleus. After further washing, the coverslips were mounted on slides, and viewed under a Nikon inverted fluorescence microscope (model TE-2000U; Nikon, Tokyo, Japan). The images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

2.6 Western Blot Analysis

Cells were lysed in either modified RIPA buffer or M-PER buffer (Thermo Scientific) for Western blot, immunoprecipitation and kinase assays. PKD1 polyclonal (1:1000), PKD1-pS744/S748 (1:1000), PKD1-pS916 (1:1000), and β -actin (1:10000) antibodies were used to blot the membranes. IR dye-800

conjugated anti-rabbit (1:5000) and Alexa Fluor 680 conjugated anti-mouse (1:10000) were used for antibody detection with the Odyssey IR Imaging system (LICOR), as previously described.

2.7 Transient Transfections

Full-length human PKD1 plasmids (PKD1-FL) were obtained from Addgene, Inc. [33]. Electroporation was carried out with an Amaxa Nucleofector transfecter instrument, as per the manufacturer's protocol. The transfected cells were then transferred to T-75 flasks or 6-well plates as desired and allowed to grow for a 24 h period before the treatment.

2.8 RNAi

Predesigned PKD1-siRNA was purchased from IDT, Inc. PKD1-siRNA effectively suppressed > 80% of PKD1 protein expression levels after 36 h post-transfection. N27 cells (50–70% confluence) were transfected with siRNA duplexes using an Amaxa Nucleofector kit (Amaxa), as described in our previous study [27].

2.9 Statistical Analysis

Data analysis was done using Prism 3.0 software (GraphPad Software, San Diego, CA). Bonferroni's multiple comparison testing was applied to find if the differences are significant between treatment and control groups. Differences with $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered significantly different from two or more independent experiments ($n \geq 6$), and are indicated in the figures.

3. Results

3.1 6-OHDA induces cytotoxic cell death of N27 dopaminergic cells

We first determined the vulnerability of N27 dopaminergic neuronal cells to 6-OHDA induced cytotoxicity using Sytox cell death assay. The N27 cells were treated with 100 μ M 6-OHDA for upto 7 hrs. As shown in Fig 1A, 6-OHDA induced cytotoxic cell death in a time-dependent manner beginning at 3 hr. 6-OHDA induced more than 3-fold increase in cell death at 9h. Visualization of Sytox stained N27 cells treated with fluorescence microscopy further confirmed the sensitivity of cells to 6-OHDA induced cytotoxicity (Fig 1B). These results suggested that 6-OHDA induced cytotoxic cell death in a time-dependent manner.

3.2 6-OHDA induces activation loop phosphorylation of PKD1.

Phosphorylation of ser744/S748 located in the activation loop of PKD1 results in its activation. Therefore, we determined if 6-OHDA induces activation loop phosphorylation of PKD1 in N27 dopaminergic cells. We used the antibody directed against phosphorylated Ser744/Ser748 in PKD1. As shown in Fig 2A, PKD1 activation occurs in a time dependent manner from 0 - 7 hr as determined by western blot analysis of phosphorylated Ser744/Ser748. The phosphorylated Ser744/Ser748 PKD1, 110 kDa band peaks 1 hr and goes back to basal levels over 7 hrs. Densitometry analysis revealed that 6-OHDA induced an increase in phosphorylated Ser744/Ser748 PKD1 band by many folds in 1 hr and decreased to control levels in 3 hr (Fig 2B). Thus, 6-OHDA induced phosphorylation of PKD1 Ser744/Ser748 occurred at early stages of oxidative insult and preceded cytotoxic cell death (Fig 2B).

Immunohistochemical staining with phospho-specific antibody revealed that 100 μ M 6-OHDA-induced nuclear translocation of activation loop

phosphorylated PKD1 (pS744/pS748, green) staining as evidenced by co-localized with the nuclear Hoechst stain (blue) as visualized by fluorescence microscopy (Fig 2C).

3.3 6-OHDA induces activation loop phosphorylation of PKD1 in primary mesencephalic neurons

Next, we examined if PKD1 activation occurs in primary mesencephalic neurons exposed to 6-OHDA. Immunocytochemical staining was performed to examine the subcellular localization of PKD1 in primary mesencephalic neurons. We were excited to see high level expression of PKD1 in the cytosolic region of tyrosine hydroxylase (TH) (green) positive primary dopaminergic neurons obtained from mouse substantia nigra (Fig 3A). Further, we performed immunocytochemical staining to examine the localization pattern of activated PKD1 in a parkinsonian specific cell culture model. Our immunocytochemical staining for activated PKD1 showed that the activated PKD1 (pS744/pS748) (red) co-localized with the nuclear Hoechst stain (blue) during 10 μ M 6-OHDA-induced oxidative stress in primary mesencephalic neurons, as visualized by fluorescence microscopy (Fig 3B). Unlike phosphorylated PKD1, 6-OHDA does not induce nuclear translocation native PKD1 (data not shown). These results indicate that only activated PKD1 translocates to the nucleus of primary dopaminergic neurons when treated with a parkinsonian specific toxicant like 6-OHDA.

3.4 Ser 916 phosphorylation precedes activation loop phosphorylation PKD1

In addition at activation loop phosphorylation of PKD1 (pS744/pS748), oxidative stress has been shown to also induce PKD1 Ser 916 phosphorylation. Further, we and others have observed that ser916

phosphorylation occurs prior to and is prerequisite for activation loop PKD1 (pS744/pS748) phosphorylation (unpublished observations) [34]. Here, we investigated the role of PKD1 C-terminal Ser 916 phosphorylation during 6-OHDA treatment. As shown in Fig 4A, exposure to 100 μ M 6-OHDA induced PKD1 Ser 916 phosphorylation occurs as early as 30 min and precedes activation loop PKD1 (pS744/pS748), phosphorylation (Fig 4A). To further, confirm the role of PKD1 Ser 916 phosphorylation in 6-OHDA induced PKD1 activation, we overexpressed the phosphorylation defective mutant PKD1^{S916A} mutant and native wild type PKD1^{WT} in N27 dopaminergic cells by transient transfection and then exposed the cells to 100 μ M 6-OHDA for 1 hr. Overexpression of PKD1^{S916A} mutant blocked 6-OHDA induced PKD1 activation loop phosphorylation, suggesting that Ser 916 phosphorylation precedes and is required for PKD1 activation loop phosphorylation (Fig 4B). Furthermore, immunocytochemical staining for Ser 916 phosphorylation showed that PKD1pS916 (red) was localized (pink/yellow) in both cytosol and nucleus of TH +ve (green) primary mesencephalic neurons during 10 μ M 6-OHDA treatment (Fig 4C). Together, these results suggest that C-terminal PKD1 Ser916 phosphorylation regulates PKD1 activation during 6-OHDA treatment in N27 dopaminergic cells and in primary mesencephalic cultures.

3.5 Pharmacological inhibition and RNAi suppression of PKD1 expression and activation exacerbates 6-OHDA induced dopaminergic cell death

Previous reports have shown that PKD1 act as a cell survival kinase in non-neuronal models [22] [35-38]. To determine if PKD1's plays a survival role in 6-OHDA induced dopaminergic cell death, we used an allosteric PKD inhibitor kbNB 142-70. Briefly, N27 dopaminergic cells were exposed to 100 μ M 6-OHDA in the presence or absence of 50 μ M PKD inhibitor kbNB 142-

70 and determined cytotoxic cell death using Sytox green assay. As shown in Fig 5A, co-treatment with 50 μ M kbNB 142-70 exacerbated 6-OHDA-induced cytotoxic cell death by more than 50% compared to 6-OHDA alone treated cells. To further confirm cell survival role of PKD1, we used siRNA directed against the coding region of PKD1 to suppress its expression and monitored cell death using Sytox cell death assay. Briefly, N27 dopaminergic cells were transfected with PKD1 siRNA and non-specific siRNA, 24 hrs post-transfection cells were exposed to 100 μ M 6-OHDA. PKD1 expression was almost completely suppressed in PKD1 siRNA transfected compared to non-specific siRNA-transfected N27 dopaminergic cells (Fig 5B). As shown in Fig 5C, in Sytox assays, 6-OHDA induced cytotoxic cell death increased by 10-fold in PKD1 siRNA transfected cells compared to 5-fold in non-specific siRNA transfected dopaminergic cells at 9 hr. Together these results suggest that negative modulation of PKD1 using pharmacological and siRNA approaches results in exacerbated cell death in the presence of 6-OHDA and the cell survival role for PKD1.

3.6 Overexpression of wild-type and constitutively active PKD1 protects against 6-OHDA induced cytotoxic cell death

To unequivocally confirm the cell survival role of PKD1 we overexpressed the full length wild-type human PKD1 plasmid (PKD1^{WT}) and constitutively active PKD1^{S744E/S748E} in N27 cells and then exposed to 6-OHDA. Mutation of serine residues at position 744 and 748 results in constitutively active PKD1 [39]. As shown in Fig. 6A, overexpression of PKD1^{WT} significantly attenuated 6-OHDA induced cytotoxic cell death compared to vector transfected dopaminergic cells in a time-dependent manner. Similarly, overexpression of constitutively active PKD1^{S744E/S748E} also significantly attenuated 6-OHDA induced cytotoxic cell death compared to vector transfected dopaminergic

cells in a time-dependent manner (Fig. 6B). These results unequivocally demonstrate cell survival role as well as protective compensatory role of PKD1 during 6-OHDA induced dopaminergic cell death.

4. Discussion

This study reveals the existence of a novel defensive signaling mechanism in cell culture models of PD. Here, we report for the first time three key findings in a dopaminergic degeneration model using 6-OHDA: (i) PKD1 activation counteracts early stage oxidative damage in cell culture models of PD (ii) Phosphorylation of Ser 916 residue regulates activation loop phosphorylation (PKD1- pS744/S748); (ii) Modulation of PKD1 signaling has protective effects against 6-OHDA induced dopaminergic cell death.

Oxidative stress triggers apoptosis and causes neurodegeneration through activation of multiple signaling molecules including kinases and proteases [10][40-43]. Even though previous studies have shown the presence of cellular compensatory mechanisms that counteract the early oxidative insult, the protein kinases that are involved in this mechanism are still being investigated [44-46]. PKD1 is an important survival signaling transduction kinase associated with oxidative stress in non-neuronal cell lines [22] [35-38] [47]. Studies have shown that oxidative stress induces PKD1 activation via activation loop phosphorylation pS744/pS748. Several PKCs including PKC α have been shown to mediate PKD1 activation loop phosphorylation in non-neuronal systems [47-51]. The role of PKD1 in the brain, especially in dopaminergic degeneration remains unknown. The relationship between

PKD1 signaling and neurodegeneration has not yet been examined before in detail.

In the current study, we explored the relationship between PKD1 signaling and dopaminergic degeneration in cell culture models of PD. Several studies have demonstrated DNA fragmentation during late stages of 6-OHDA induced apoptotic cell death. DNA fragmentation is preceded by ROS generation, mitochondrial dysfunction, and caspase-3 activation [52-54]. We have recently shown ROS generation, mitochondrial dysfunction and caspase-3-mediated proteolytic activation play a critical role 6-OHDA induced dopaminergic degeneration in cell culture and animal models of PD (Kanthasamy 2006 and unpublished observations). In the present study, we report the activation of PKD1 during the early stages of 6-OHDA induced oxidative stress in cell culture models of PD. Multiple phosphorylation sites have been implicated in PKD1 activation loop phosphorylation, depending on the cell types and stimuli [26] [55]. Previous groups have shown the involvement of Ser 916 phosphorylation in PKD1 activation in non-neuronal models [34, 50, 56]. We demonstrate that S916 phosphorylation is a preceding event required for PKD1ser744/Ser748 activation loop phosphorylation [15]. Our data suggest phosphorylation of C-terminally located Ser 916 phosphorylation results in unmasking of the ser744/Ser748 residues located in the activation loop for subsequent phosphorylation and PKD1 activation during 6-OHDA induced cytotoxicity. Further both the pharmacological and genetic inhibition exacerbated 6-OHDA induced dopaminergic cell death suggesting that PKD1 is a cell survival kinase. Comparison of PKD1 activation and 6-OHDA induced cytotoxicity shows that PKD1 activation is maximal during the early oxidative stress stage when no measurable cytotoxicity is occurs. Interestingly, when PKD1 activation begins to decline at the end of the early stage oxidative stress, cell death

begins to occur. When the constitutively active PKD1 mutant (PKD1^{S744E/S748E}) or wild-type PKD1 plasmid (PKD1^{WT}) is overexpressed, dopaminergic cells are resistant to 6-OHDA -induced cytotoxicity, even during the late stages of oxidative stress, which is consistent with our hypothesis that PKD1 activation protects against oxidative damage in dopaminergic cells. Furthermore, we also observed 6-OHDA treatment induces nuclear translocation of phosphorylated PKD1 to possibly regulate transcription of cell survival transcription factors and genes in dopaminergic cell death. Our results are consistent with recent studies demonstrating nuclear translocation of PKD1 in B cells, cardiomyocytes & oestoblasts [22] [25] [57, 58].

In conclusion, our results demonstrate the existence of a novel protective signaling mechanism mediated by PKD1 in dopaminergic neurons. During the early stage of 6-OHDA induced oxidative stress, PKD1 is activated and has protective function against oxidative damage. However, prolonged oxidative insult causes deactivation of PKD1 which might contribute to neuronal death. Further negative modulation of PKD1 increases cell death, while positive modulation decreases cell death. Our results suggest that PKD1 may function as a cell survival switch in dopaminergic neurons and its modulation can be a novel neuroprotective strategy against oxidative damage in PD.

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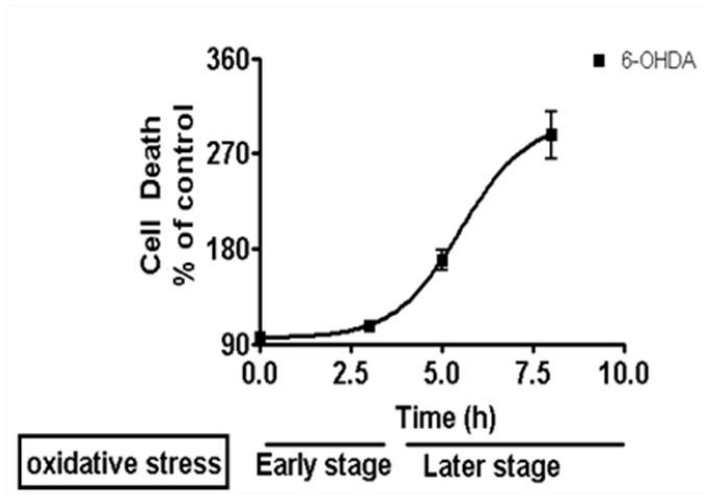
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Figure 1.

A



B



FIGURE 2.

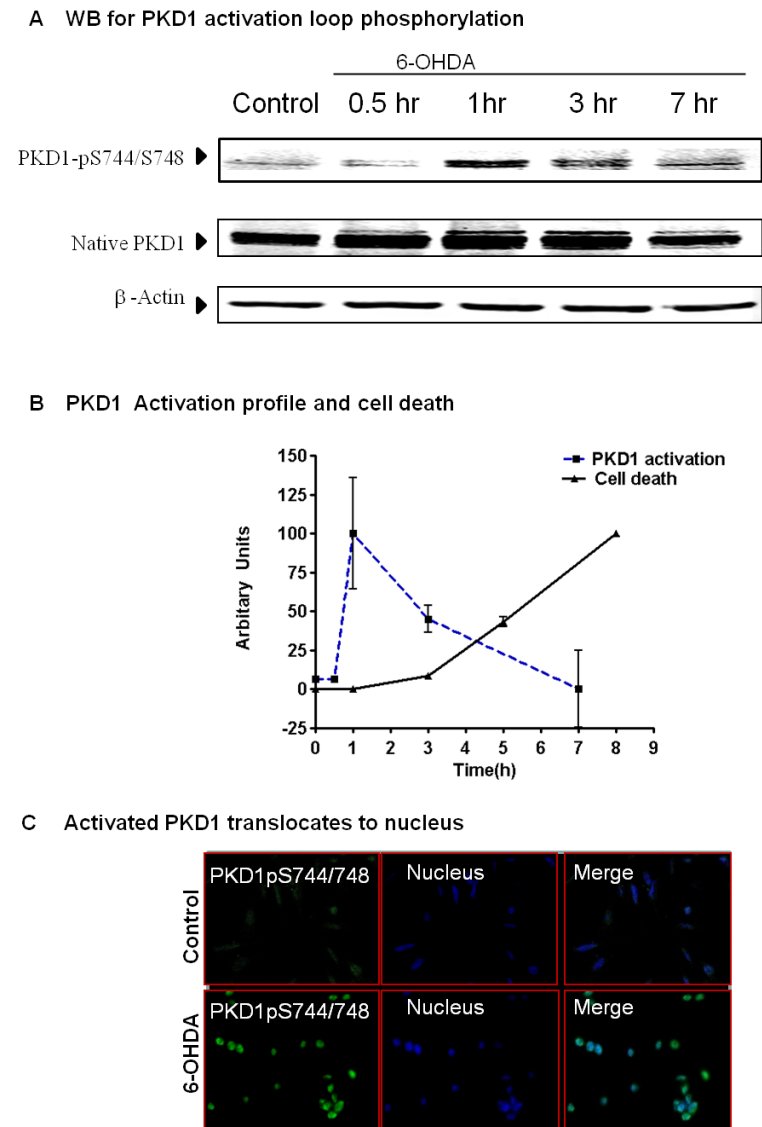
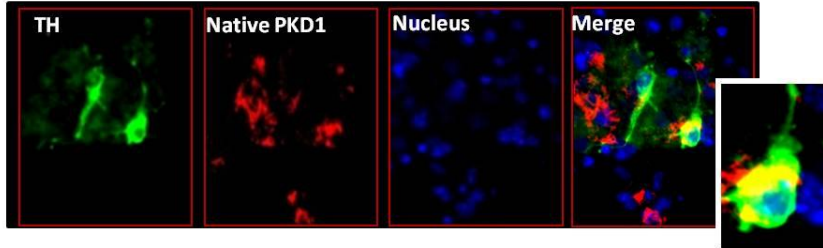


Fig 3.

A. Native PKD1 expression in primary mesencephalic neurons



B. Activated PKD1 translocates to nucleus in mesencephalic neurons

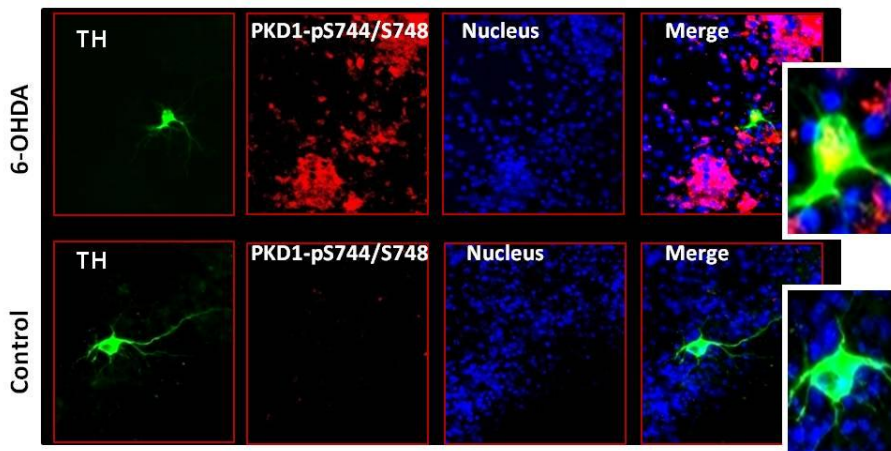
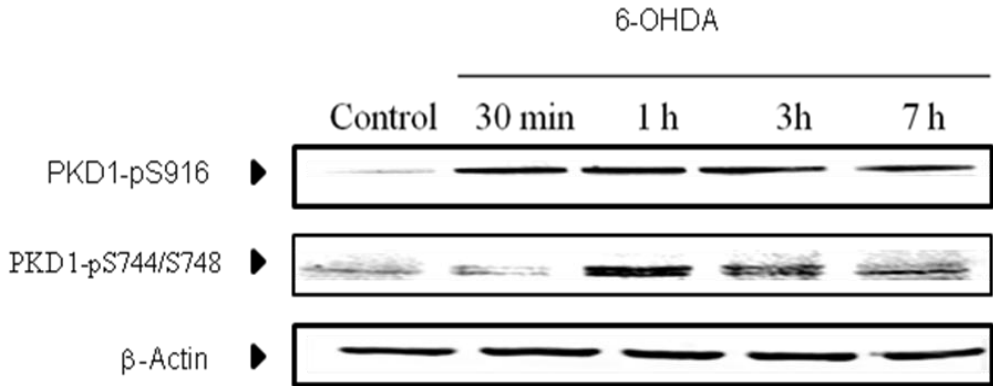
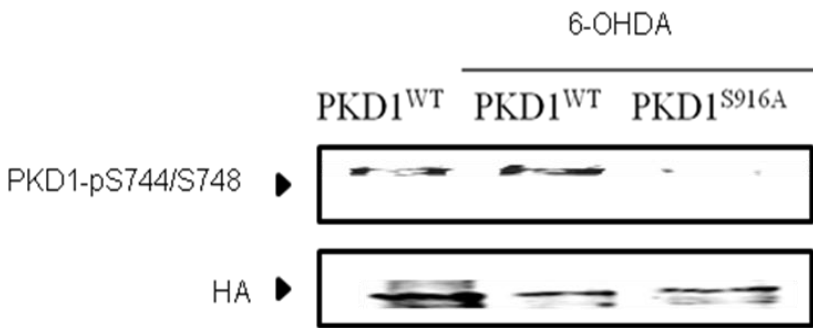


Fig 4.

A. PKD1-S916 phosphorylation in 6-OHDA treatment

B. PKD1-pS744/S748 phosphorylation in PKD1^{S916A} mutant

C. PKD1-S916 phosphorylation in primary neurons

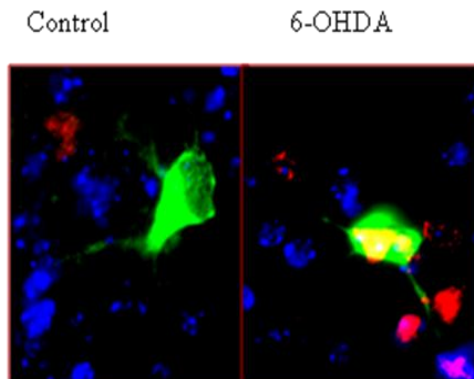
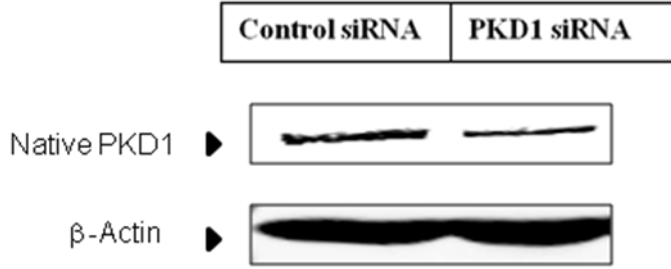
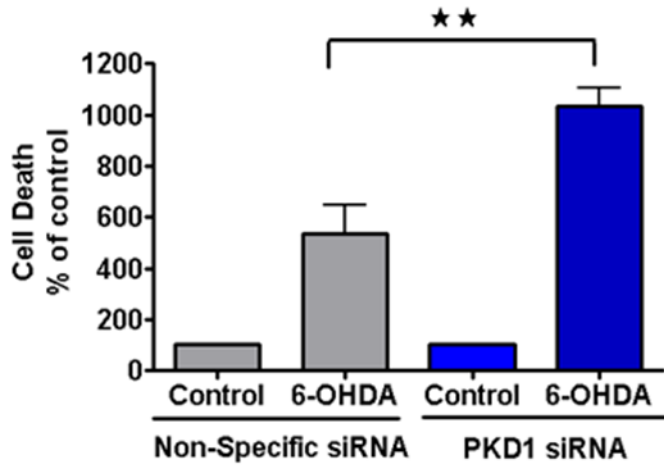


Fig 5.

A. PKD1 knockdown in N27 cells



B. PKD1 knockdown and cell death



C. PKD1 inhibitor and cell death

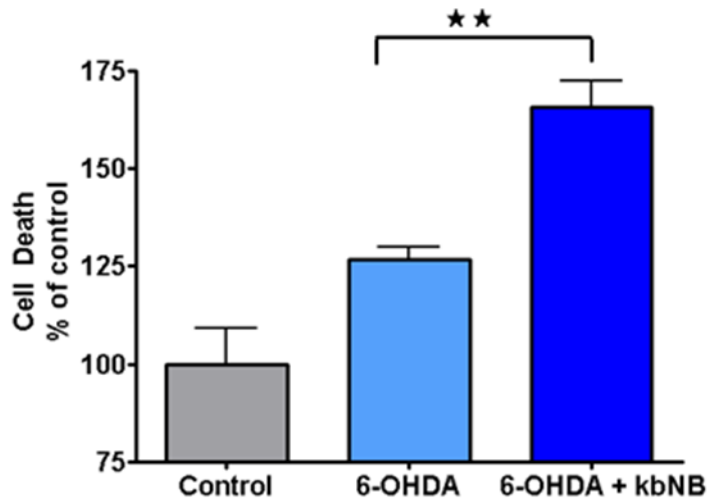


Fig6.

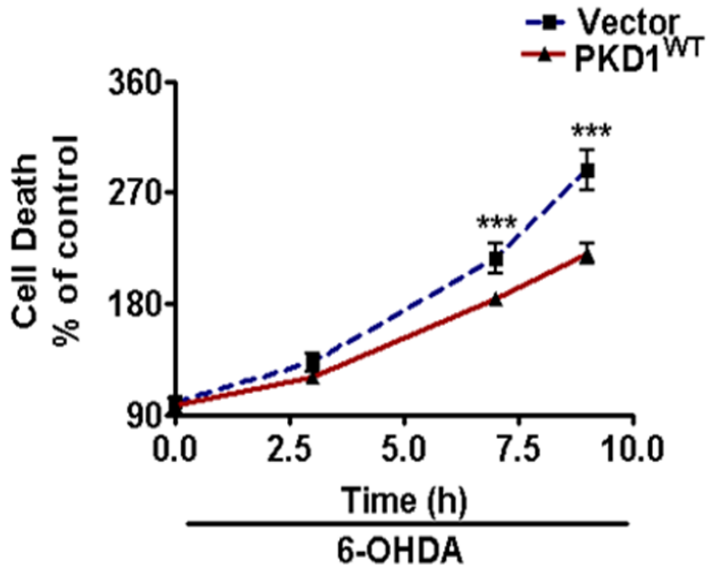
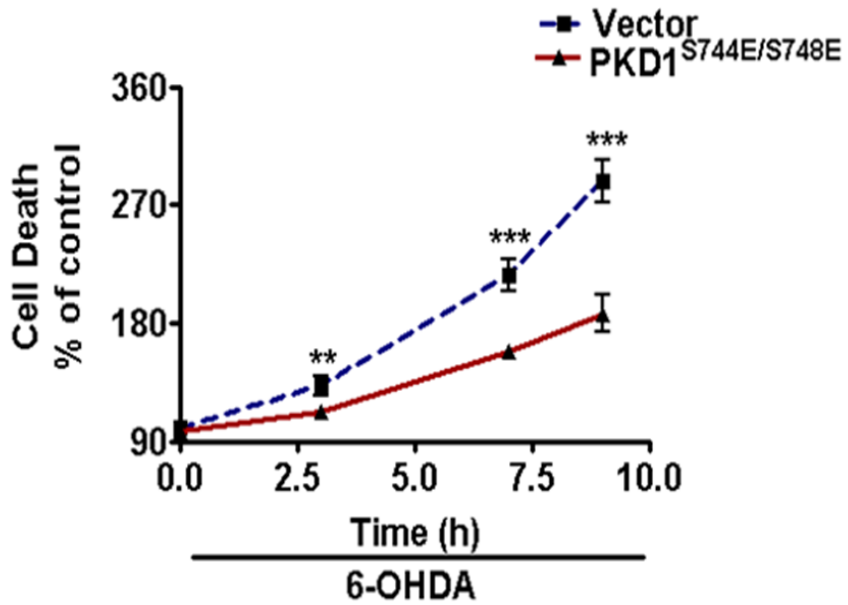
A. PKD1^{WT} over expression protects from cell deathB. PKD1^{S744E/S748E} over expression protects from cell death

Figure legends

Figure 1. 6-OHDA -induced oxidative stress causes cell death in N27 dopaminergic neuronal cell models.

N27 dopaminergic cells were treated with 6-OHDA (100 μ M) for 0-9 hr and assayed for cytotoxicity using Sytox green dye. Fluorescence measurements for the incorporation of Sytox green read using a fluorescence plate reader. Non-linear regression graph was performed from two or more independent experiments (n=6-8) (A) and visualised by fluorescence microscopy (B).

Figure 2. PKD1 is activated in N27 cells during 6-OHDA -induced oxidative stress

N27 dopaminergic neuronal cells were treated with or without 6-OHDA (100 μ M) for 1- 7 hr and probed for PKD1 activation loop phosphorylation pS744/pS748 and native PKD1 expression (A). A comparative time course graph based on quantifying PKD1 activation profile, and cytotoxicity during 6-OHDA exposure (B). Immunofluorescence analysis of N27 dopaminergic cells stained for activated PKD1 using fluorescence microscopy during 6-OHDA exposure show translocation to nucleus. PKD1pS744/S748 – Green, Nucleus – Blue. Nuclei were stained with Hoechst dye (C).

Figure 3. PKD1 is highly expressed in dopaminergic neurons and activated during 6-OHDA-induced oxidative stress

Primary dopaminergic neurons staining for tyrosine hydroxylase (TH) obtained from the mouse substantia nigral region show co-localization of native PKD1 with TH in the cytosol. TH – Green, PKD1 native – Red, Nucleus – Blue, Yellow -Merge. Nuclei were stained with Hoechst dye (A). Immunofluorescence analysis of primary dopaminergic neurons staining for

TH obtained from the mouse substantia nigral region shows translocation of activated PKD1 to the nucleus during 6-OHDA exposure. TH-Green, PKD1pS744/S748 – Red, Nucleus – Blue, Merge - Pink. Nuclei were stained with Hoechst dye (B).

Figure 4. PKC δ -dependent phosphorylation of PKD1 at S916 site precedes PKD1 S744/S748 active loop phosphorylation

N27 dopaminergic cells were treated with 100 μ M 6-OHDA for 1-7h and monitored for PKD1 S916 and PKD1 S744/S748 phosphorylation (A). N27 cells transiently expressing PKD1^{S916A} mutant prevent PKD1 activation during oxidative stress, as seen by Western blotting for PKD1 S744/S748 phosphorylation and HA expression (B). Primary dopaminergic neurons staining for TH show presence of PKD1pS916 in both cytosol and nucleus during 6-OHDA exposure. TH – Green, PKD1pS916– Red, red/yellow – Merge (C).

Figure 5. PKD1 activation is essential for cell survival function during 6-OHDA induced oxidative stress in dopaminergic neurons

N27 dopaminergic cells were transfected with 1 μ M PKD1 siRNA and non-specific siRNA (A) and treated with 100 μ M 6-OHDA for 9h and monitored for cytotoxicity using Sytox green dye. Fluorescence measurements for the incorporation of Sytox green read using a fluorescence plate reader. **, $p < 0.01$ denote significant difference between Non-Specific siRNA-6-OHDA and PKD1 siRNA-6-OHDA treated groups (B). N27 cells were co-treated with or without PKD1 inhibitor kb-NB 14270 and monitored for cell death during 6-OHDA treatment for 4h (C).

Figure 6. Modulation of PKD1 offers neuroprotection in dopaminergic neuronal cells

N27 dopaminergic cells transiently transfected with 5 μ M full length PKD1 plasmid (PKD1^{WT}) and 5 μ M vector plasmid were treated with or without 100 μ M 6-OHDA and monitored for cytotoxicity at various time points using sytox green. ***, $p < 0.001$ denotes significant difference between treatment groups from $n \geq 6$ (A). N27 dopaminergic cells transiently transfected with 5 μ M PKD1 constitutively active PKD1^{S744E/S748E} mutant and 5 μ M vector plasmid were treated with or without 100 μ M 6-OHDA and monitored for cytotoxicity at various time points using sytox green. ***, $p < 0.001$ denotes significant difference between treatment groups from $n \geq 6$ (B).

Chapter III: Fyn-PKC δ Signaling Regulates the Anti-Apoptotic PKD1 in Experimental Models of Parkinson's disease

Manuscript will be communicated to PLOS ONE

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Abstract

Background

Protein Kinase C δ (PKC δ) is a major pro-apoptotic kinase that contributes to neurodegeneration in Parkinson's disease (PD). While studying the key pro-apoptotic signaling events down stream of PKC δ , we unravelled the novel Protein Kinase D1 (PKD1) signaling mechanism which acted as a key compensatory protective response against early oxidative damage in dopaminergic system. Therefore, the purpose of the present study was to extensively characterize the key protein kinases involved in this survival signaling pathway in experimental models of Parkinson's disease.

Results

Exposure of dopaminergic neurons to MPP⁺ / MPTP in both cell culture and animal models induced PKD1 activation loop (PKD1S744/748) phosphorylation, activation and nuclear translocation in the nigral dopaminergic neurons. Inhibition of PKC δ prevented PKD1 activation during MPP⁺ treatment. PKD1 was also not activated in the nigral dopaminergic neurons of PKC δ knock-out (PKC δ ^{-/-}) mice exposed to acute MPTP treatment. Earlier we reported that Fyn kinase regulates PKC δ in

dopaminergic cells and the Fyn knock-out (Fyn $-/-$) mice exposed to acute MPTP treatment showed no PKD1 activation. Also, the C-terminus S916 phosphorylation occurs only in MPTP treated naïve mice but not in (PKC δ $-/-$) mice, confirming PKC δ 's tight control over PKD1 activation. Further Dopaminergic neurons co-treated with PKD1 inhibitor kbNB 142-70 and exposed to MPP $^{+}$ exacerbated neuronal death confirming the survival role of PKD1. Consistent with other results PKD1 was found activated in the surviving nigral dopaminergic neurons of human post-mortem PD brains

Conclusion

Collectively, our results demonstrate that PKD1 is regulated in a Fyn- PKC δ dependent manner and represents a novel intrinsic protective response in counteracting early stage oxidative damage in PD.

Introduction

The incidence of Parkinson's disease (PD) cases is dramatically increasing with in the U.S. population, with more than 80% of the cases being idiopathic. Current PD treatment options available are only symptomatic, creating a scientific imperative to understand the causes and medical management of PD (1, 2). Oxidative stress is a major factor that contributes to neurodegeneration in PD ((3) (4) (5) (6) (7). Better understanding of the key signaling molecules involved during the degenerative process caused by oxidative damage will open up novel neuroprotective avenues in Parkinson's disease (8), (1), (9) (10) (2) (11) (12). Our laboratory elucidated a novel proapoptotic signaling mechanism in dopaminergic system; we showed that PKC δ is an oxidative stress sensor that is persistently activated by caspase-3 dependent proteolytic cleavage (7) (13) (14) (15) (16).

PKD1 is a CAMK family member and activated in response to various stimuli in different disease models and activation has been shown to play a role in diverse cellular functions including proliferation, cytoskeletal reorganization, golgi function and immune response (17-20) (19, 21-24). PKD1 can be activated by dual phosphorylation of serine residues at the activation loop (Ser 744/ Ser 748) in the catalytic domain in response to multiple stimuli including growth factors, phorbol esters, G-protein coupled receptors, genotoxic stress and oxidative stress (25-27). The role of PKD1 signaling in neurodegenerative diseases remains unexplored.

While studying the downstream targets of PKC δ , our initial in-silico and in-vitro analyses indicated that PKC δ can phosphorylate the activation loop serine residue of PKD1. Surprisingly, PKD1 was found to be a PKC δ -dependent cell survival kinase rather than a cell death kinase in dopaminergic models. This led us to further investigate the signaling mechanisms

underlying the neuroprotective function mediated by PKD1 in experimental models of PD. In this study, we used the classic MPP⁺/MPTP toxicant based model to understand PKD1 signaling in Parkinson's disease. MPTP becomes toxic after it is metabolized into 1-methyl-4-phenylpyridinium (MPP⁺) in glial cells and interferes with the functioning of complex I of the electron transport chain in neurons leading to generation of free radicals and causes cell death (28, 29). Herein, we report a novel functional interplay between protein kinases in which the PKD1 survival signaling pathway is regulated in a Fyn-PKC δ dependent fashion in MPP⁺/MPTP induced neurodegeneration in both cell culture and animal models of PD.

Materials and Methods:

Cell Culture

The immortalized rat mesencephalic dopaminergic neuronal cell line (N27) was a kind gift from 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 of penicillin, and 50 μ g/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C, as described previously (13). N27 cells are used widely as a cell culture model for PD (45) (7) (33) (13, 34).

Primary mesencephalic neuronal culture

Primary mesencephalic neuronal cultures were prepared from the ventral mesencephalon of gestational 16- to 18-day-old mouse embryos, as described earlier (46). Tissues were dissected from E16 to E18 mouse embryos maintained in ice cold Ca²⁺-free Hanks' balanced salt solution and then dissociated in Hanks' balanced salt solution containing trypsin-0.25%

EDTA for 30 min at 37°C. The dissociated cells were then plated at equal density (0.5×10^6 cells) on 12 mm coverslips precoated with 0.1 mg/ml poly-D-lysine. Cultures were maintained in neurobasal medium fortified with B-27 supplements, 500 μ M l-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). The cells were maintained in a humidified CO₂ incubator (5% CO₂ and 37°C). Half of the culture medium was replaced every 2 days. Approximately 6- to 7-day-old cultures were used for experiments. Primary mesencephalic dopaminergic neuronal cells were exposed to 10 μ M for 1 h.

Treatment Paradigm

N27 cells were exposed to MPP⁺ (300 μ M) for 0– 24h at 37°C. Cell lysates were used for Western blotting and immunoprecipitation studies. Primary neurons were exposed to MPP⁺ (5 μ M /10 μ M) for 24/48 h for immunochemical staining and dopamine uptake assays. Untreated cells were grown in the complete medium and used as the experimental control.

Cytotoxicity Assays

Cell death was determined using the Sytox green cytotoxicity assay, after exposing the N27 cells to MPP⁺ (300 μ M), as described previously. This cytotoxicity assay was optimized for a multiwell format, which is more efficient and sensitive than other cytotoxicity measurements (47) (48). Briefly, N27 cells were grown in 24-well cell culture plates at 100,000 cells per well and treated with MPP⁺ (300 μ M) and 1 μ M Sytox green fluorescent dye. The Sytox green assay allows dead cells to be viewed directly under a fluorescence microscope, as well as quantitatively measured with a fluorescence microplate reader (excitation 485 nm; emission 538 nm) (Biotek). Phase contrast and fluorescent images were taken after MPP⁺

exposure with a NIKON TE2000 microscope, and pictures were captured with a SPOT digital camera.

Human Post-mortem PD brain slices

Human tissues are obtained from the VA Medical Center at the University of Iowa. 40 μ M free floating sections were used for immunostaining with anti-phospho antibodies specific for TH, and PKD1. Very recently, we have optimized the condition for immunohistochemical analysis of human brain tissues in our laboratory. Biochemical and histological data will be compared between control and PD post-mortem brains.

Immunocytochemistry

The primary mesencephalic neurons or N27 cells after MPP⁺ treatment were fixed with 4% paraformaldehyde and processed for immunocytochemical staining. First, nonspecific sites were blocked with 2% bovine serum albumin, 0.5% Triton and 0.05% Tween-20 in phosphate-buffered saline (PBS) for 20 min. The cells then were incubated with antibodies directed against TH, native PKD1 and PKD1-pS744/S748 in PBS containing 1% BSA at 4°C overnight, followed by incubation with Alexa 488 and Alexa 568 conjugated secondary antibodies in PBS containing 1% BSA. Secondary antibody treatments were followed by incubation with Hoechst 33342 dye for 5 min at room temperature to stain the nucleus. The coverslips containing stained cells were washed with PBS, mounted on slides, and viewed under a Nikon inverted fluorescence microscope (model TE-2000U; Nikon, Tokyo, Japan). Both fluorescence and confocal images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Immunohistochemical staining of brain slices.

Mice were perfused with 4% paraformaldehyde and then the brain slices were cut on a microtome into 20 μm sections. Brain sections were blocked with PBS containing 1% BSA and 0.1% Triton X-100 in for 20 min and then incubated with antibodies directed against PKD1, PKD1-pS744/S748 and TH overnight at 4°C followed by incubation with either Alexa 488-conjugated (green, 1:1000) or Alexa 568-conjugated (red, 1:1000) secondary antibody for 1 h at RT. Secondary antibody treatments were followed by incubation with Hoechst 33342 (10 $\mu\text{g}/\text{ml}$) for 3 min at RT to stain the nucleus. Then the slices were mounted on a slide and viewed under a Nikon inverted fluorescence microscope (model TE-2000U); images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Western Blot Analysis

Cells were lysed in either modified RIPA buffer or M-PER buffer (Thermo Scientific) for Western blot, immunoprecipitation and kinase assays. Lysates containing equal amounts of protein were loaded in each lane and separated on 10-12% SDS-PAGE, as described previously (Kaul et al., 2003). PKD1 polyclonal (1:1000), PKC δ polyclonal (1: 1000), PKD1-pS744/S748 (1:1000), PKD1-pS916 (1:1000), Fyn (1:1000), - and β -actin (1:10000) antibodies were used to blot the membranes. IR dye-800 conjugated anti-rabbit (1:5000) and Alexa Fluor 680 conjugated anti-mouse (1:10000) were used for antibody detection with the Odyssey IR Imaging system (LICOR), as previously described.

PKC δ Kinase Assay

Immunoprecipitation and PKC δ kinase assay were performed as described earlier (7). After cell lysis, cell were immunoprecipitated using a polyclonal

PKC δ rabbit antibody and protein A Sepharose, and washed three times with PKC δ kinase buffer (40 mM Tris (pH 7.4), 20 mM MgCl₂, 20 μ M ATP, 2.5 mM CaCl₂). The reaction was started by adding 20 μ l of buffer containing 0.4 mg histone and 5 μ Ci of [γ -³²P]ATP (4,500 Ci/mM). After incubation for 10 min at 30°C, SDS loading buffer (2X) was added to the samples to terminate the reaction. The reaction products were separated on SDS-PAGE (12%), and the H1-phosphorylated bands were detected using a phosphoimager (Fujifilm FLA-5100) and quantified with MultiGauge V3.0 software.

Protein Kinase D1 Kinase Assay

The cells were exposed to MPP⁺ (300 μ M) for 1 h and cell lysates were immunoprecipitated, as previously reported, with native PKD1 antibody (Santa Cruz). The kinase reaction was carried out at room temperature for 20 min after adding 10 μ l of kinase substrate mix (0.1 mM ATP + 10 μ ci [γ -³²P]ATP + 2 μ g Syntide 2 peptide substrate in kinase buffer). Kinase buffer contains 20 mM Tris pH 7.5, 10 mM MgCl₂, and 1 mM DTT. The samples were centrifuged to terminate the kinase reaction, and the supernatants containing the phosphorylated peptide were applied as spots to P81 phosphocellulose squares (Whatmann). The papers were washed four times with 0.75% phosphoric acid and once with acetone and dried, and activity was determined by liquid scintillation counting. The samples were also loaded on a SDS-PAGE and probed for native PKD1 to determine equal loading.

Uptake of [³H] Dopamine

The effects of PKD1 inhibitor Kb-NB142-70 on the uptake of dopamine were assessed in fetal mouse mesencephalic cultures using [³H]dopamine (DA), as described previously (Afeseh et al., 2009). In brief, after incubation for 48 h with 5 μ M MPP⁺, with or without Kb-NB142-70, medium with the treatment

was removed and cells were then washed once by assay incubation (Krebs-Ringer) buffer (5.6 mM glucose, 1.3 mM EDTA, 1.2 mM magnesium sulfate, 1.8 mM calcium chloride, 4.7 mM potassium chloride, 120 mM sodium chloride, and 16 mM sodium phosphate). Cells were incubated with 10 μ M [3 H] DA (30 Ci/mol) for 20 min at 37°C. The uptake was stopped by removing the reaction mixture and followed by three washes with fresh Krebs-Ringer buffer. Cells were then collected with the use of 1 N NaOH, and the radioactivity was measured by liquid scintillation counting.

RNAi

PKC δ -siRNA was prepared by an in vitro transcription method, as described previously (45). PKC δ -siRNA effectively suppressed > 80% of PKC δ protein expression levels within 24 h post-transfection.

Statistical Analysis

Data analysis was performed using Prism 3.0 software (GraphPad Software, San Diego, CA). Bonferroni's multiple comparison testing was used to find the significant differences between treatment and control groups. Differences with $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered significantly different from $n \geq 6$ from two or more independent experiments, and are indicated in the figures.

RESULTS

Parkinsonian specific toxicant MPP $^+$ induces PKD1 activation in cell culture models of PD

We examined the activation loop phosphorylation (Ser744/Ser748) of PKD1. MPP $^+$ (300 μ M) induced PKD1 activation loop phosphorylation and activation in N27 dopaminergic cells. As shown in Fig 1A, PKD1 activation loop

phosphorylation occurs in a time dependent manner from 0 - 24 hr as determined by western blot analysis. PKD1 phosphorylation at Ser744/Ser748, peaks at 12 hr over a 24 hr period. Furthermore, the Phosphorylation of PKD1 Ser744/Ser748 residues increased PKD1 kinase activity, as measured by a [³²P] kinase assay using Syntide 2 substrate at 12 h (Fig 1B).

Next, we examined PKD1 signaling in primary mesencephalic neurons exposed to 10 μ M MPP⁺ for 24 h. Immunocytochemical staining was performed to examine the subcellular localization of native PKD1 in primary mesencephalic neurons. We saw high level expression of PKD1 in the cytosolic region of MPP⁺ treated or untreated tyrosine hydroxylase (TH) (green) positive primary dopaminergic neurons obtained from mouse substantia nigra (Fig 1C). Further, we performed immunocytochemical staining to examine the localization pattern of activated PKD1 in MPP⁺ treated neurons. Our immunocytochemical staining for activated PKD1 showed that the activated PKD1 (pS744/pS748) (red) co-localized with the nuclear Hoechst stain (blue) during 10 μ M MPP⁺-induced oxidative stress in primary mesencephalic neurons , as visualized by fluorescence microscopy (Fig 1D). These results indicate that only activated PKD1 translocates to the nucleus of primary dopaminergic neurons when treated with a parkinsonian specific toxicant like MPP⁺.

MPTP induces PKD1 activation in mouse substantia nigra

Extension of the PKD1 oxidative signaling pathway from cell culture models to animal models is the next logical step in understanding the role of PKD1 signaling in PD. Therefore, we verified PKD1 expression pattern in mouse nigral tissues using immunostaining. As shown in Fig 2A cytosolic region of

tyrosine hydroxylase (TH) (green) positive dopaminergic neurons showed high expression of native PKD1.

Since PKD1 is highly expressed in the nigral tissue, we examined the activation pattern of PKD1 (pSer744/pSer748). As shown in Fig 2B time course studies of PKD1 activation in mice subjected to acute MPTP treatment paradigm shows that PKD1 is activated at 24 hours in the substantia nigral region as monitored by PKD1 activation loop phosphorylation (pS744/pS748). Further immunochemical staining of activated PKD1 (PKD1-pS744/pS748) at 24 hrs after acute MPTP treatment shows co-localization of activated PKD1 (red) in the nucleus of TH positive dopaminergic neurons (green) in mouse substantia nigra supporting the results obtained using MPP⁺ in cell culture models of PD (Fig 2C). These results suggest that PKD1 is activated in the dopaminergic neurons present in the substantia nigra.

PKC δ mediates PKD1 activation in cell culture models

The activation loop serine residue was predicted to be phosphorylated by PKC δ at a high stringency in-silico analysis (Asaithambi et al. 2011). So we tested the involvement of PKC δ in PKD1 activation in specific dopaminergic degeneration induced by MPP⁺ / MPTP. Knockdown of PKC δ by specific siRNA (Fig 3A) greatly reduced PKC δ kinase activity as measured by ability of immunoprecipitated PKC δ to phosphorylate the substrate histone at 15 hr MPP⁺ treatment using [³²P] ATP (Fig S1). Further, PKC δ knockdown greatly reduced PKC δ kinase activity as measured by a [³²P] kinase assay using Syntide 2 substrate during 15h MPP⁺ treatment suggesting the involvement of PKC δ in PKD1 regulation (Fig 3B). We also have shown that the caspase-3 inhibitor DEVD-fmk and src family kinases inhibitor TSKI can inhibit PKC δ kinase activity during oxidative stress (Kaul et al. 2003). Therefore, in this

study we used these inhibitors to confirm if PKC δ inhibition can block PKD1 activation. DEVD-fmk and TSKI treatment significantly attenuated PKC δ kinase activity (Fig S 2) and PKD1 kinase activity as measured by [32 P] kinase assay using Syntide 2 substrate (Fig 3C). Collectively, these results confirm the role of PKC δ in regulating PKD1 activation in N27 cells using MPP $^{+}$.

Attenuation of PKD1 activation in PKC δ knock out (-/-) mice

After establishing the involvement of PKC δ in PKD1 signal transduction in cell culture models, these findings were extended to animal models using PKC δ knock out (PKC δ (-/-)) mice. We used the primary mesencephalic dopaminergic neurons obtained from embryos of naïve and PKC δ knockout mice. We treated the primary dopaminergic neurons with 10 μ M MPP $^{+}$ for 24 hours and compared the activation of PKD1 using PKD1-pS744/pS748 antibody between PKC δ (+/+) and PKC δ (-/-) mice. As shown in Fig 4A, Activated PKD1 (PKD1pS744/pS748) (red) co-localized with the nuclear Hoechst stain (blue) during 10 μ M MPP $^{+}$ -induced oxidative stress in primary mesencephalic neurons obtained from PKC δ (+/+) but not in PKC δ (-/-), as visualized by fluorescence microscopy. Additional validation of this study was done using substantia nigra brain tissues obtained from adult PKC δ (-/-) and PKC δ (+/+) mice exposed to acute MPTP treatment paradigm. Mice were treated with 4 doses of MPTP (18 mg/kg) in two hour intervals and sacrificed 24 hours after the last injection. As shown in Fig 4B, PKD1 pS744/pS748 levels were completely attenuated in the brain lysates of PKC δ (-/-) mice compared with PKC δ (+/+) mice. These results confirm that PKC δ is the upstream regulator of PKD1 in both cell culture and animal models of PD.

Fyn Kinase is the upstream regulator of PKC δ -PKD1 signaling in dopaminergic neurons

Recently, we showed that Fyn kinase regulates PKC δ during oxidative stress (30). In this study, we examined if Fyn kinase acts as an upstream regulator of PKC δ -PKD1 signaling in dopaminergic neurons. We used the primary mesencephalic dopaminergic neurons obtained from embryos of naïve and Fyn knock out (Fyn (-/-)) mice. We treated the cultured primary neurons with 10 μ M MPP⁺ for 24 hours and compared the activation of PKD1 using PKD1-pS744/pS748 antibody between the two groups. As shown in Fig 5A, Activated PKD1 (PKD1pS744/pS748) (red) co-localized with the nuclear Hoechst stain (blue) during MPP⁺ -induced oxidative stress in primary mesencephalic neurons obtained from Fyn (+/+) but not in Fyn (-/-), as visualized by fluorescence microscopy. Further these results obtained in cell culture studies were extended to animal models of PD. Fyn (-/-) and Fyn (+/+) mice were treated with 4 doses of MPTP (18 mg/kg) in two hour intervals and sacrificed 24 hours after the last injection. As shown in Fig 5B, PKD1 pS744/pS748 levels were completely attenuated in the brain lysates of Fyn (-/-) mice compared with Fyn (+/+) mice. These results confirm that Fyn kinase is the upstream regulator of PKC δ -PKD1 signal transduction in both cell culture and animal models of PD.

PKC δ dependent PKD1 S916 phosphorylation in dopaminergic neurons

In addition to the activation loop phosphorylation of PKD1 (pS744/pS748), oxidative stress has been shown to cause PKD1 Ser 916 phosphorylation (31, 32). In this study, we investigated PKD1 C-terminal Ser 916 phosphorylation in specific dopaminergic degeneration. We used the primary mesencephalic neurons obtained from E16-E18 mouse embryos and treated

with 10 μ M MPP⁺. As shown in Fig 6A, PKD1pS916 (red) was localized (pink/yellow) in both cytosol and nucleus of TH +ve primary mesencephalic neurons staining for green during MPP⁺ treatment. Next, we examined the primary dopaminergic neurons obtained from embryos of PKC δ (-/-) and PKC δ (+/+) for PKD1-pS744/pS748 levels. We treated the primary neurons with 10 μ M MPP⁺ for 24 hours and compared the activation of PKD1 using PKD1-pS744/pS748 antibody between the two groups. As shown in Fig 6B, Activated PKD1 (PKD1pS744/pS748) (red) co-localized with both cytosol and nucleus in dopaminergic neurons obtained from PKC δ (+/+) animals but not in PKC δ (-/-) animals, as visualized by fluorescence microscopy. Together, these results suggest that a PKC δ dependent C-terminal PKD1 Ser916 phosphorylation occurs in dopaminergic neurons.

PKD1 activation acts as a novel compensatory mechanism in PD models

The role of PKD1 was tested in this model using a specific PKD1 allosteric inhibitor (kbNB 142-70). kbNB 142-70 inhibited PKD1 by completely attenuating PKD1-S744/S748 and PKD1-S916 phosphorylation levels (Fig 7A). Surprisingly, the co-treatment of N27 cells with 50 μ M kbNB 142-70 exacerbated cell death nearly two folds when exposed to MPP⁺ and monitored cell death using Sytox cell death assay indicating a cell survival role for PKD1 (Fig 7B). Next, we checked the effects of PKD1 inhibition on the functional efficiency of dopaminergic neurons using H³ dopamine uptake assay. We co-treated primary mesencephalic neurons obtained from E16-E18 mouse embryos with 50 μ M PKD1 inhibitor kbNB 142-70 and performed [H³] dopamine uptake assay during 5 μ M MPP⁺ treatments for 48 hr. As shown in Fig 8C, PKD1 inhibition reduced the dopamine uptake of the primary neurons nearly two folds compared to MPP⁺ only treatment.

Together, these results confirm that PKD1 is anti-apoptotic in dopaminergic neurons exposed to MPP⁺ toxicity.

PKD1 activation in human PD patients

The biomedical relevance of these findings in cell culture and animal models were tested in humans using the tissues obtained from post-mortem PD cases. We performed immunohistochemical staining to examine PKD1 activation in human PD brain. Activated PKD1 (PKD1pS744/pS748) (green) co-localized with the nuclear Hoechst stain (blue) in TH +ve (green) dopaminergic neurons of PD patients, as visualized by fluorescence microscopy. These results indicate that activated PKD1 translocates to the nucleus of intact surviving dopaminergic neurons possibly to carry out neuronal survival functions. These results indicate that PKD1 plays a very important role in survival functions in PD patients.

Discussion

This study systematically elucidates the presence of a novel neuronal survival signaling mechanism in PD models. Using the classical MPTP model to induce dopaminergic degeneration, we demonstrate the following: (i) The anti-apoptotic protein kinase D1 (PKD1) is activated during early stage oxidative damage caused by MPP⁺/MPTP in cell culture and animal models of PD (ii) PKC δ regulates PKD1 activation (iii) Fyn Kinase acts as an upstream regulator of PKC δ – PKD1 signaling. The elucidation of this novel survival signaling pathway in dopaminergic degenerative processes may lead to the development of novel treatment modalities.

Oxidative damage induced apoptosis through activation of many pro-apoptotic signaling kinases and proteases in neurodegeneration (3) (4) (5) (6)

(7). We earlier reported a novel pro-apoptotic signaling mechanism mediated by PKC δ in dopaminergic system (7) (33) (13, 34). Caspase-3-mediated proteolytic activation of PKC δ plays a critical role in causing neurodegeneration in various cell culture and animal models including 6-OHDA and MPTP/MPP⁺ induced dopaminergic degeneration (7) (33) (13, 34). While studying the downstream pro-apoptotic signal transducers of PKC δ , preliminary in-silico analyses predicted that PKC δ can phosphorylate the activation loop serine residue of PKD1(31). Studies have previously shown that protein kinase D1 (PKD1) was a key signal transduction kinase associated with oxidative stress, so we hypothesized that PKC δ might cause cell death through activating PKD1 (23) (35, 36). Unexpectedly, we found out that PKD1 is a cell survival kinase that protects against early oxidative damage in parkinsonian specific MPP⁺/MPTP models. Our results show that MPP⁺ induced PKD1 activation in a PKC δ dependent mechanism. Our findings were strengthened by using PKC δ (-/-) (PKC δ knock out) mice subjected to acute MPTP treatment. These results confirm the presence of the novel protective PKC δ -PKD1 pathway in dopaminergic neurons in Parkinsonian condition. This is the first study to extensively delineate the role of PKD1 in a parkinsonian specific neurodegenerative model and supports our earlier results using a general oxidant like H₂O₂ (31).

Following this observation, we probed for the key upstream regulator of PKC δ -PKD1 signaling. Fyn kinase, a non-receptor tyrosine kinase from Src family has been shown to regulate PKC δ proteolytic activation in experimental models of PD (31). Dopaminergic neurons from Fyn (-/-) (Fyn kinase knock out) mice treated with MPTP do not show PKD1 activation which is in agreement with the results obtained in cell culture models treated with MPP⁺. Our results indicate that Fyn kinase regulates PKC δ -PKD1 signaling pathway in PD experimental models. Multiple phosphorylation sites have

been implicated in PKD1 activation loop phosphorylation, depending on the cell types and stimuli (27) (37). Previous groups have shown the involvement of Ser 916 phosphorylation in PKD1 activation in non-neuronal models (32, 38, 39). Ser 916 phosphorylation is a preceding event required for PKD1ser744/Ser748 activation loop phosphorylation (31). Our current data suggest that phosphorylation of C-terminally located Ser 916 phosphorylation occurs in primary neurons treated with MPP⁺. Further, Dopaminergic neurons from PKC δ (-/-) mice exposed to MPTP/MPP⁺ treatment do not show PKD1 Ser 916 phosphorylation, confirming a PKC δ dependent Ser 916 phosphorylation in PD models.

Eventhough our current study has confirmed the activation of anti-apoptotic PKD1 in pre-clinical models of PD. The findings will be more relevant if demonstrated in human Parkinsonian patients. Our examination of samples obtained from post-mortem human PD patients showed high levels of PKD1 activation compared to age matched controls. Put together we suggest that the anti-apoptotic PKD1 signaling controlled by Fyn-PKC δ is activated as an early protective compensatory mechanism in Parkinson's disease.

Some of the key pro-apoptotic signaling kinases known to cause dopaminergic degeneration include JNK, MLK, MAPK, LRRK2, etc. (40-43). Therapeutic intervention by inhibiting the pro-apoptotic MLK family of kinases failed to stop PD progression in clinical trials (44). An alternative approach for neuroprotection against PD could be through positively modulating the anti-apoptotic signaling mechanisms in neurons. But so far not much is known about kinases having survival function in dopaminergic system. Our elucidation of a novel early protective signal transduction mechanism in dopaminergic system could open up a new chapter in neuroprotection.

Studies are currently underway to functionally modulate PKD1 signaling and see if it can be a possible neuroprotective strategy in PD.

In conclusion, our results demonstrate the activation of the novel protective signaling mechanism mediated by PKD1 in MPTP induced dopaminergic degeneration. PKD1 activation is tightly regulated by Fyn – PKC δ signaling pathway. Our results suggest that positive modulation of the anti-apoptotic PKD1 pathway can be a novel neuroprotective strategy against oxidative damage in PD.

Abbreviations

PD, Parkinson's disease; PKD1, Protein kinase D1; MAPK, Mitogen-activated protein kinases PKC δ , Protein kinase C delta; CAMK, Ca²⁺/Calmodulin-Dependent Protein Kinase II; JNK, c-Jun N-terminal kinases; LRRK2, Leucine-rich repeat kinase 2 (LRRK2); MLK, Mixed-lineage kinase; ROS, Reactive oxygen species; MnSOD, Manganese superoxide dismutase; WB, Western Blot; PKC, Protein kinase C; PKC α , Protein kinase C alpha.

Competing interests

The authors declare the have no competing interests.

Authors' contributions

All authors read and approved the final manuscript.

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FIGURE 1:

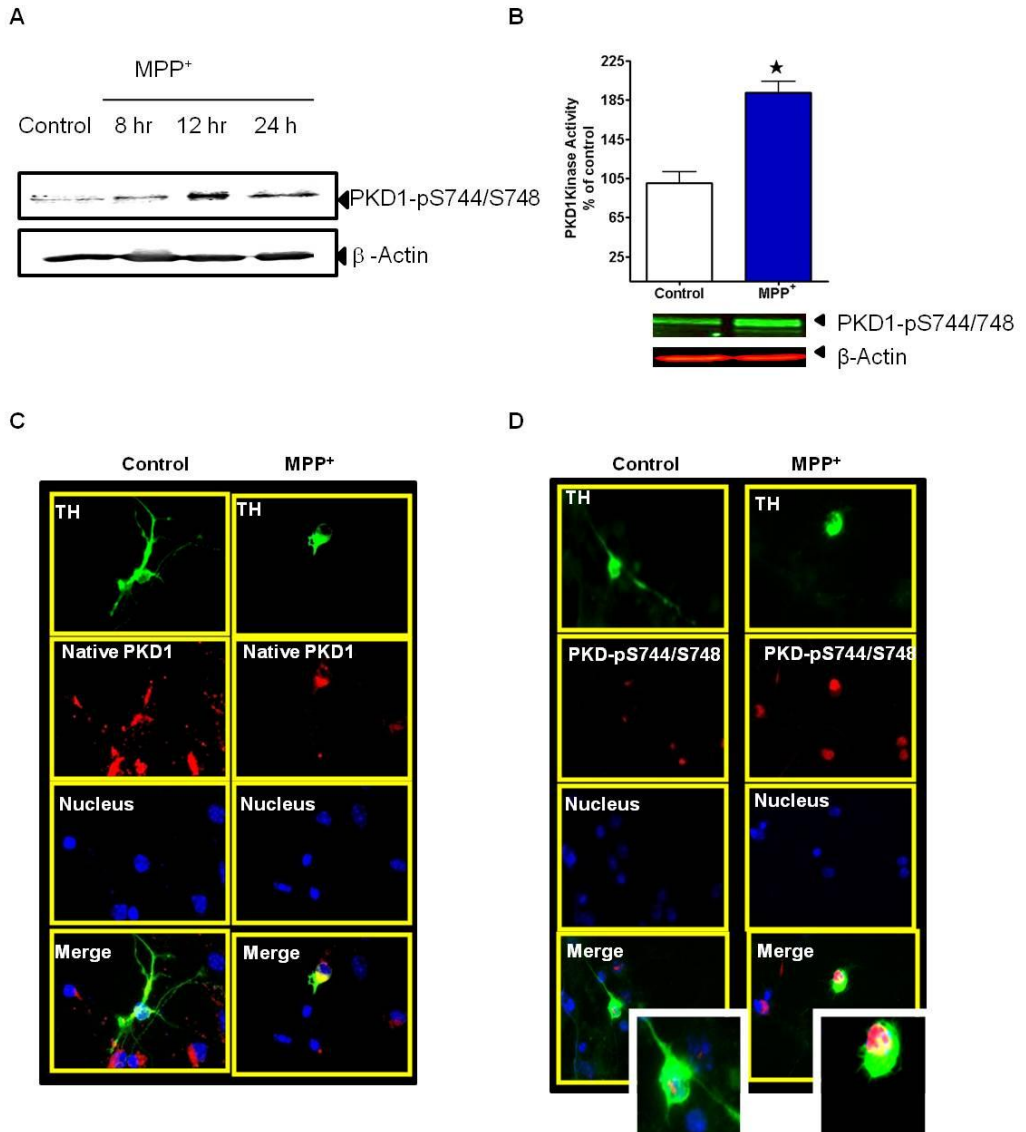


FIGURE 2:

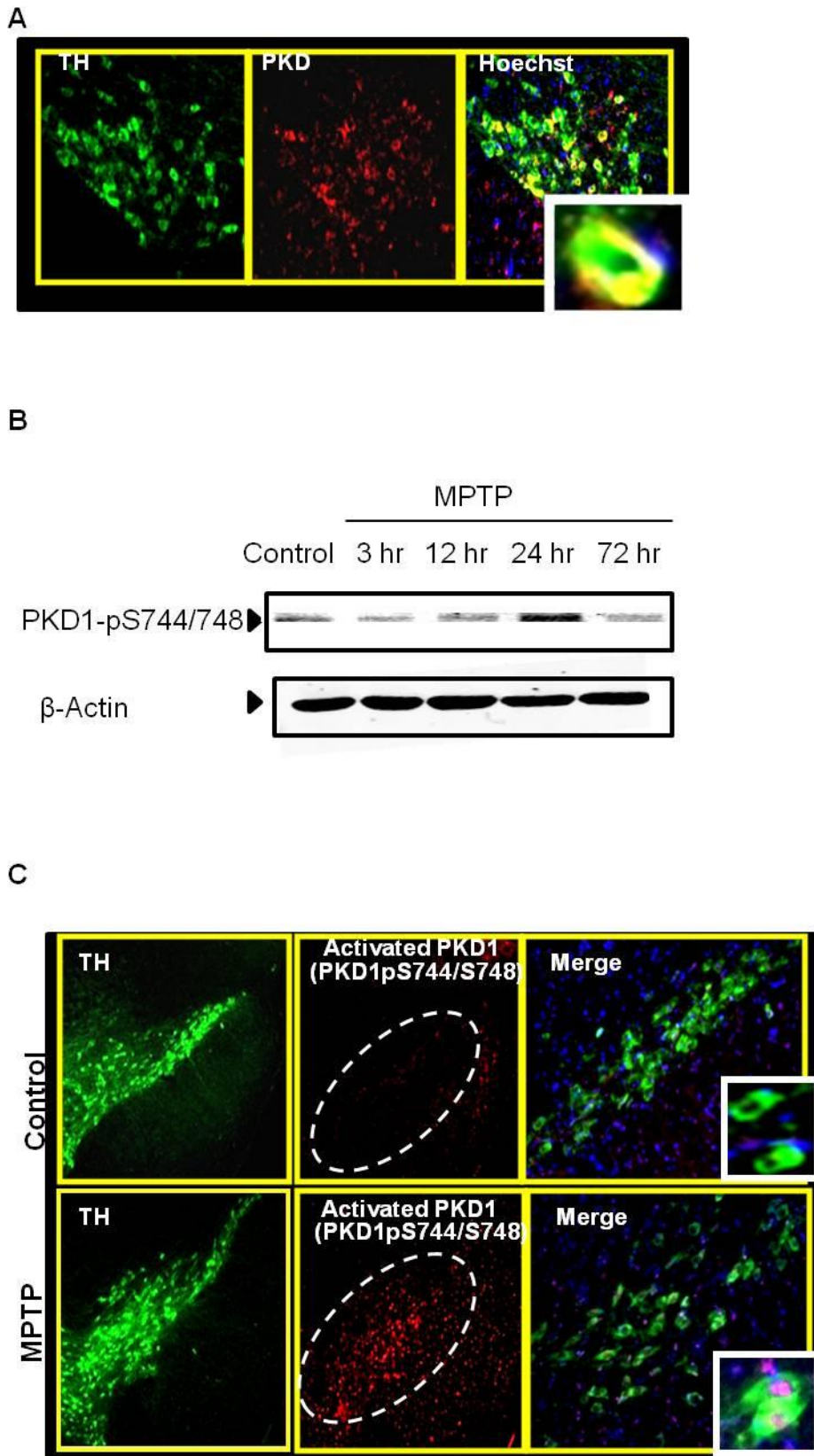


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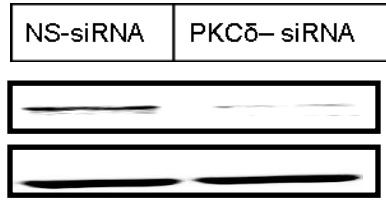
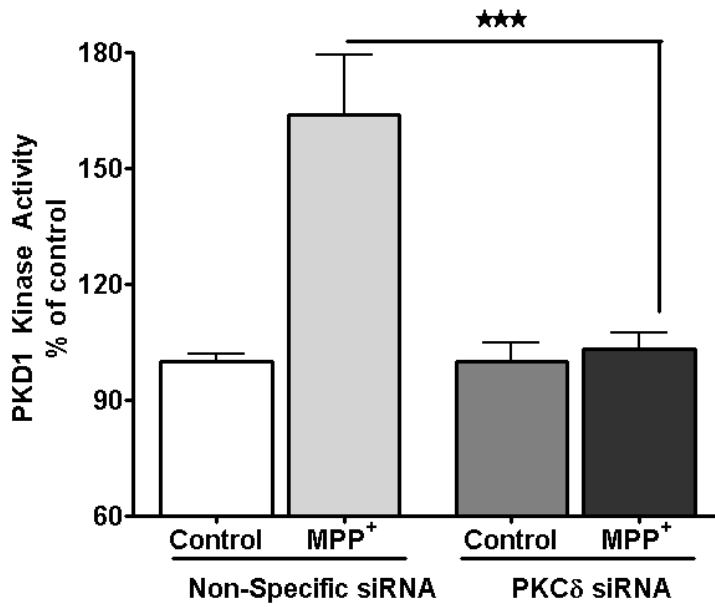
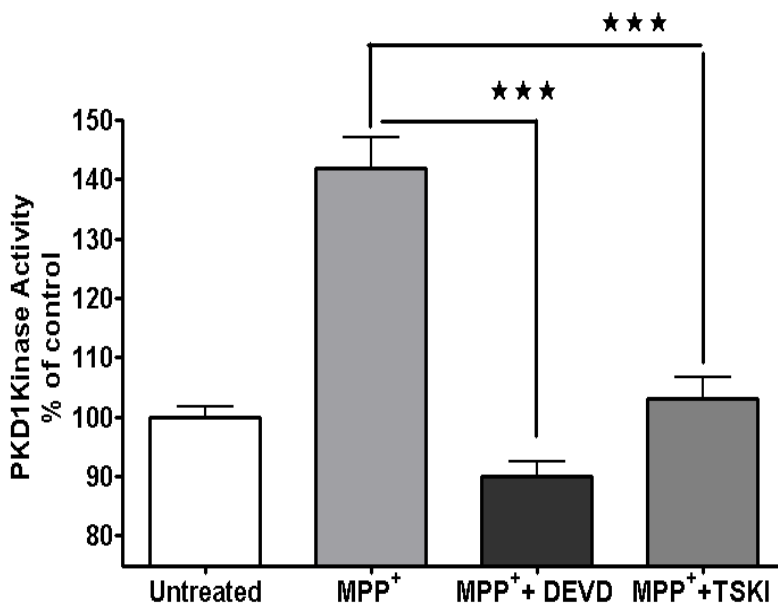
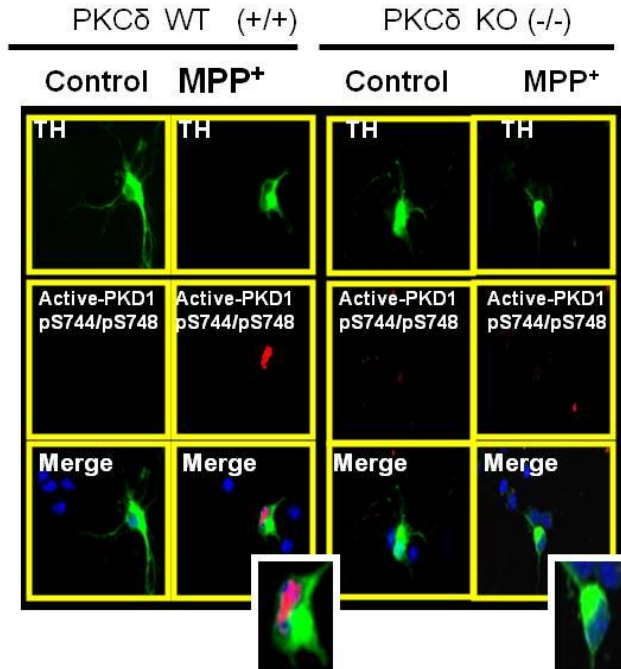
A PKC δ siRNA knockdownB PKC δ siRNA and PKD1 kinase assayC PKC δ inhibitors and PKD1 kinase assay

FIGURE 4:

A



B

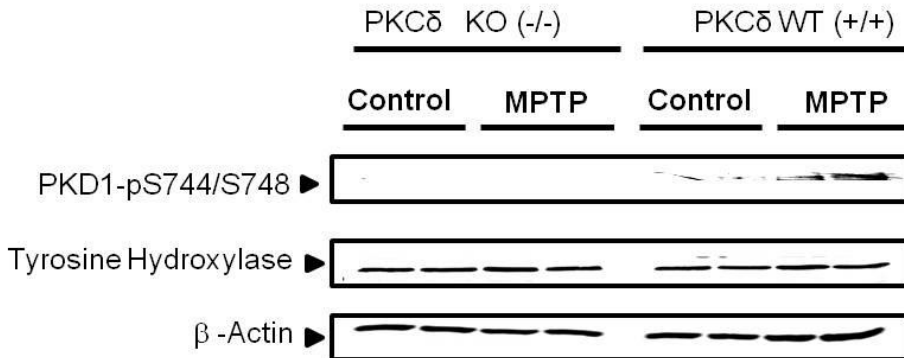
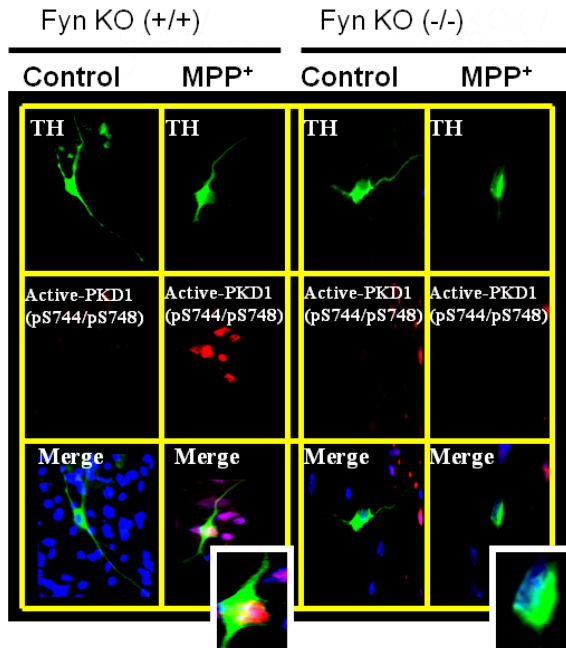


FIGURE 5:

A



B

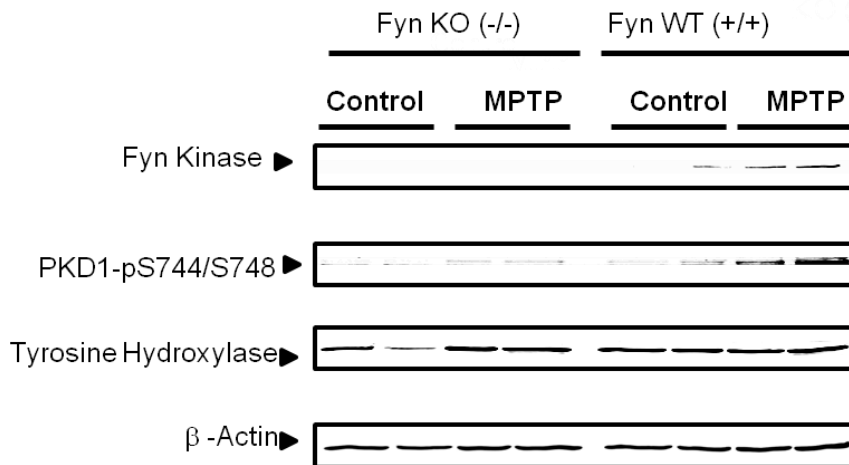
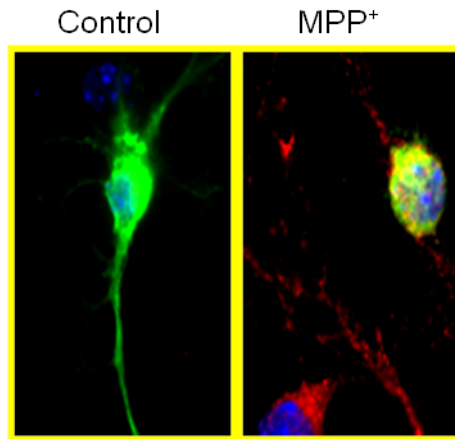


FIGURE 6:

A



B

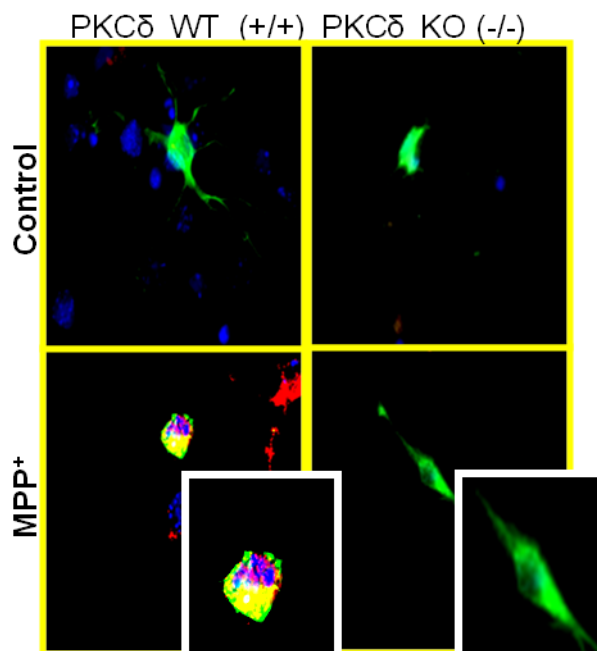
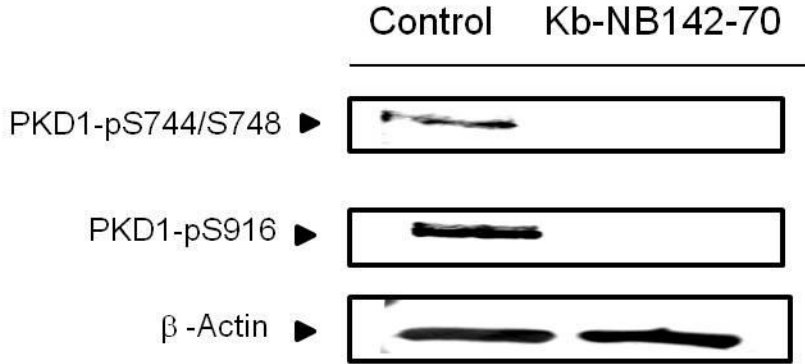
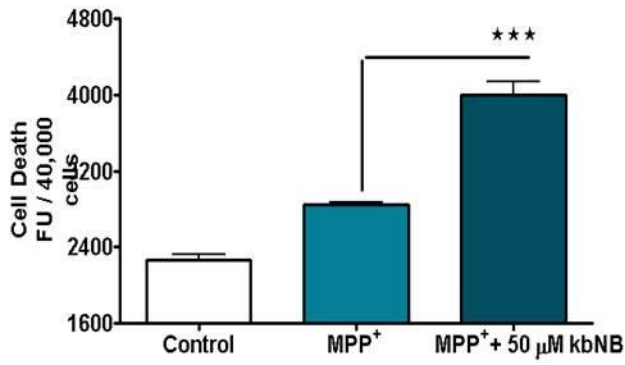


FIGURE 7:

A



B



C

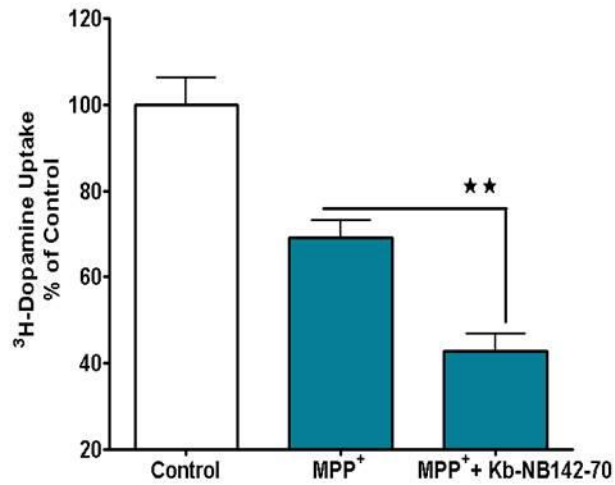


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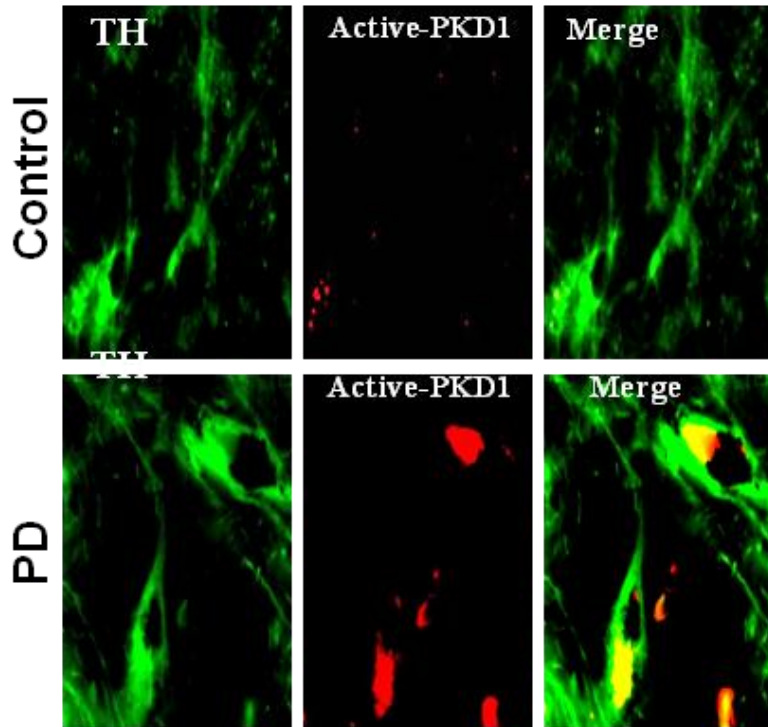


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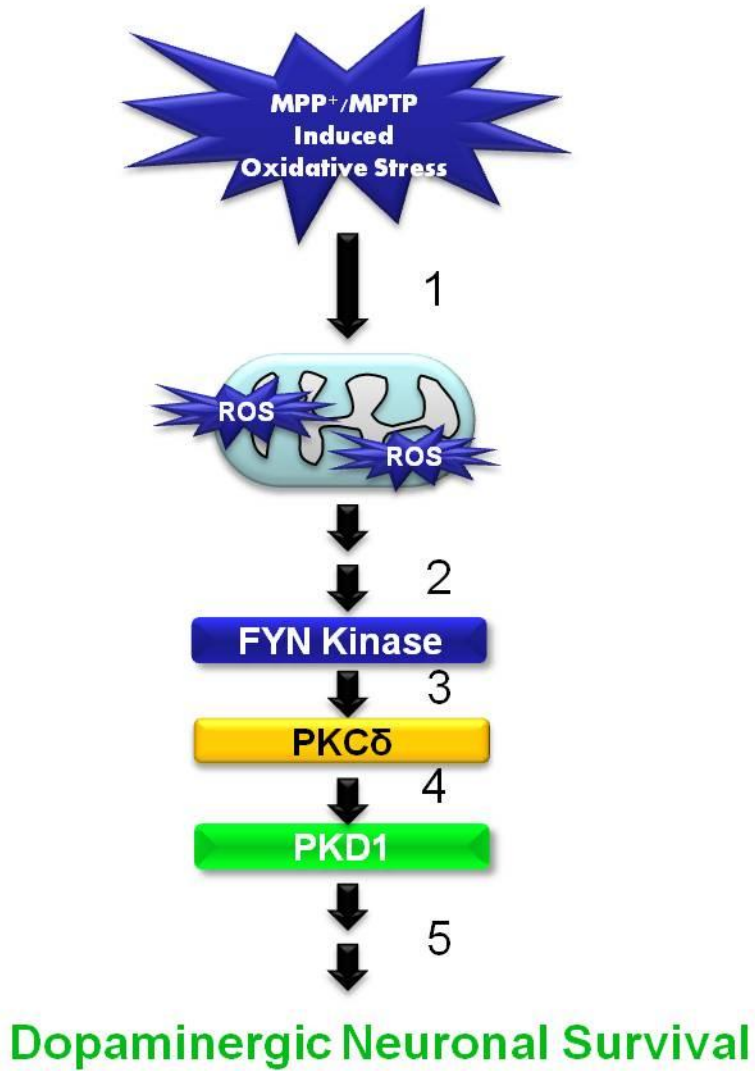


Figure legends

Figure 1. PKD1 is activated during MPP⁺ -treatment in cell culture models of PD

N27 dopaminergic neuronal cells were treated with or without MPP⁺ (300 μ M) for 8, 12 or 24 h and probed for PKD1 activation loop phosphorylation pS744/pS748 (A). N27 cells were treated with or without MPP⁺ (300 μ M) and PKD1 kinase activity was measured by [³²P] kinase assay using syntide 2 substrate at 12 h. *, p<0.05 denotes significant difference between untreated and MPP⁺ -treated groups (B). Primary dopaminergic neurons staining for tyrosine hydroxylase (TH) obtained from the mouse substantia nigral region show co-localization of native PKD1 with TH. TH – Green, PKD1 native – Red, Nucleus – Blue, Yellow -Merge. Nuclei were stained with Hoechst dye (C). Primary dopaminergic neurons staining for tyrosine hydroxylase (TH) obtained from the mouse substantia nigral region show co-localization of activated PKD1 (pS744/pS748) with nucleus. TH – Green, PKD1 (pS744/pS748)– Red, Nucleus – Blue, Pink -Merge. Nuclei were stained with Hoechst dye (D).

Figure 2. PKD1 is activated during MPTP treatment in animal models of PD

Immunohistochemical analysis of Mouse brain slices cut at the level of the substantia nigra staining for tyrosine hydroxylase (TH) show co-localization of native PKD1 with TH. TH – Green, PKD1 native – Red, Nucleus – Blue, Yellow -Merge. Nuclei were stained with Hoechst dye (A). C57Bl/6 mice were exposed to acute MPTP treatment and sacrificed at 3, 12, 24, 72h and probed for PKD1 activation loop phosphorylation (pS744/pS748) (B). Immunohistochemical analysis of dopaminergic neurons staining for tyrosine

hydroxylase (TH) from Mouse brain slices cut at the level of the substantia nigra after acute MPTP treatment show co-localization of active PKD1 (pS744/pS748) with nucleus. TH – Green, PKD1 (pS744/pS748) – Red, Nucleus – Blue, Pink -Merge. Nuclei were stained with Hoechst dye (C).

Figure 3. PKD1 activation is regulated by PKC δ in cell culture models of PD

N27 dopaminergic cells were transfected with 1 μ M PKC δ siRNA and non-specific siRNA and monitored for PKC δ protein expression (A), and PKC δ kinase activity assay (B) and PKD1 kinase activity assay was performed (C). ***, $p < 0.01$ denotes significant difference between NS-siRNA-MPP $^+$ and PKC δ -siRNA-MPP $^+$ groups. N27 dopaminergic cells were treated with MPP $^+$ (300 μ M) with or without 50 μ M DEVD and 5 μ M TSKI, and further PKC δ kinase activity assay (D) and PKD1 kinase activity assay was performed (E). ***, $p < 0.01$ denotes significant difference between MPP $^+$ and DEVD/TSKI co-treated groups.

Figure 4. PKD1 activation is regulated by PKC δ in animal models of PD.

Primary mesencephalic cultures obtained from PKC δ WT (+/+) and PKC δ KO (-/-) mice were treated with 10 μ M MPP $^+$ and immunochemical staining was performed. Activated PKD1 (pS744/pS748) co-localized with the nucleus of dopaminergic neurons obtained only from PKC δ WT (+/+) staining for tyrosine hydroxylase (TH). TH – Green, PKD1 (pS744/pS748) – Red, Nucleus – Blue, Pink -Merge. Nuclei were stained with Hoechst dye (D). PKC δ WT (+/+) and PKC δ KO (-/-) adult mice were exposed to acute MPTP treatment and sacrificed at 24h after the last injection and the brain homogenates from the substantia nigral region were blotted for active PKD1 (pS744/pS748) (B).

Figure 5. Fynkinase acts as an upstream regulator of PKC δ -PKD1 signaling

Primary mesencephalic cultures obtained from Fyn WT (+/+) and Fyn KO (-/-) mice were treated with 10 μ M MPP⁺ and immunochemical staining was performed. Activated PKD1 (pS744/pS748) co-localized with the nucleus of dopaminergic neurons obtained only from Fyn WT (+/+) staining for tyrosine hydroxylase (TH). (A). Fyn WT (+/+) and Fyn KO (-/-) adult mice were exposed to acute MPTP treatment and sacrificed at 24h after the last injection and the brain homogenates from the substantia nigral region were blotted for active PKD1 (pS744/pS748) (B). TH – Green, PKD1 (pS744/pS748) – Red, Nucleus – Blue, Pink -Merge. Nuclei were stained with Hoechst dye

Figure 6. PKD1 S916 phosphorylation is dependent on PKC δ

Primary dopaminergic neurons staining for TH show presence of PKD1pS916 in both cytosol and nucleus during 10 μ M MPP⁺ exposure. (A). Primary mesencephalic cultures obtained from PKC δ WT (+/+) and PKC δ KO (-/-) mice were treated with 10 μ M MPP⁺ and immunochemical staining was performed. PKD1 pS916 co-localized with the nucleus and cytosol of dopaminergic neurons obtained only from PKC δ WT (+/+) staining for tyrosine hydroxylase (TH). TH – Green, PKD1 (pS744/pS748) – Red, Nucleus – Blue, Pink -Merge. Nuclei were stained with Hoechst dye (D).

Figure 7. PKD1 protects against MPP⁺ induced oxidative stress in dopaminergic neurons

N27 cells were co-treated with or without 50 μ M PKD1 inhibitor kb-NB 14270 (A) and monitored for cell death during MPP⁺ treatment (B). Mouse primary mesencephalic neurons were co-treated with or without 50 μ M PKD1 inhibitor kb-NB 14270 and monitored for viability during MPP⁺ treatment using ³H dopamine uptake assay (C). ***, $p < 0.001$ and **, $p < 0.01$ denotes significant difference between treatment groups from $n \geq 6$.

Figure 8. PKD1 activation occurs in human PD patients

Human post-mortem PD brain sections were blotted for active PKD1 (pS744/pS748) (A). TH – Green, PKD1 (pS744/pS748) – Red, Nucleus – Blue, Pink -Merge. Nuclei were stained with Hoechst dye

Figure 9. Schematic of Fyn-PKC δ -PKD1 signal transduction mechanism in pre-clinical models

1) Oxidative stress causes mitochondrial impairment; 2) Activation of Fynkinase; 3) Fyn kinase regulates the proteolytic activation of PKC δ ; 4) Proteolytically activated PKC δ activates PKD1; 5) PKD1 activation protects stress during the early stages of oxidative stress in dopaminergic neurons

Chapter V: Rationally designed PKD1 activator protects against neurodegeneration in pre-clinical models of Parkinson's disease

Planned communication to Science Translational Medicine

Arunkumar Asaithambi, Anamitra Ghosh*, Muhammet Ay*, Arthi Kanthasamy, Katherine Mullin, Vellareddy Anantharam and Anumantha G. Kanthasamy[§]

Abstract

Oxidative stress leads to degeneration in Parkinson's disease (PD). The key signal transduction and regulatory networks that are involved during this degenerative process in PD are currently being investigated for novel neuro-protective strategies. We recently discovered that the activation of Protein Kinase D1 (PKD1) acts as a novel compensatory mechanism in Parkinson's disease models and positive modulation of PKD1 can be a therapeutic strategy. Therefore, the purpose of the present study was to take a translational approach by developing a PKD1 activator and characterizing the protective function in pre-clinical models of Parkinson's disease.

Results

Positive genetic modulation of PKD1 by overexpression of constitutively active PKD1 protected against MPP⁺ induced toxicity. Pharmacological activation by Rosiglitazone protected, whereas inhibition by kbNB 142-70 exacerbated against MPP⁺ and 6-OHDA toxicity in cell culture PD models. Peptides were rationally designed and screened for their ability to activate PKD1 using various screening methods. Peptide AK-P4 was identified to activate PKD1 specifically and protect against MPP⁺ and 6-OHDA in both

N27 cells and primary mesencephalic neurons. Further AK-P4 tagged with TAT sequence (AK-P4T) delivered using intra-venous injections activated PKD1 in mice. The neuro-protective effects of AK-P4T were tested using the sub-chronic MPTP mice model. Co-treatment with AK-P4T restored the neurotransmitter levels and the behavioral and locomotory activities of the MPTP treated mice significantly.

Collectively, our results demonstrate that rationally designed PKD1 activator peptide AK-P4T positively modulated PKD1 and protected against neurodegeneration in the pre-clinical models of PD. Our results suggest that positive modulation of the PKD1 using AK-P4T shows promise as a potential therapeutic agent against PD.

Introduction

Parkinson's disease (PD) is the second common neurodegenerative disorder affecting over a million Americans. Current treatment approaches available for Parkinson's disease are symptomatic and fail to prevent the progression of the neurodegenerative process. The currently available drugs are limited in their effectiveness to either slow or stop the progressive neurodegenerative processes in PD, largely due to the lack of mechanistic insights into the selective dopaminergic degenerative process (1, 2).

Experimental findings from cell cultures, animal models, and humans indicate that oxidative stress and apoptosis may contribute to the pathophysiological processes in PD. Neuronal cells maintain an oxidant / antioxidant homeostatic balance. Any breach in this homeostasis causes excessive ROS production and oxidative damage, which might lead to neurodegenerative disorders (3), (1), (4) (5) (2) (6) (7).

Most investigations of signaling in neurodegenerative disorders including ours have primarily focused on signaling pathways that is involved in execution of cell death during this oxidant / antioxidant homeostatic breach (8) (9) (10) (11) (12). None of the therapeutic interventions developed against the major cell death kinases have progressed to the treatment stage, probably due to the lack of understanding of the complex signal transduction process (13). This resulted in an imperative to find an alternative approach to prevent PD progression.

The protective compensatory mechanisms to counteract the oxidative damage in Parkinson's disease are still largely unexplored. Recently, we reported that protein kinase D1 (PKD1) an oxidative stress sensor is activated during the early stages of oxidative stress to maintain the homeostatic balance and promote cell survival in PD cell culture and animal models (14). PKD1 get fully activated through phosphorylation at activation loop serine sites (S744/S748). Further phosphorylation at PKD1 S916 preceeds and regulates the activation loop phosphorylation (14)

We hypothesized that positive modulation of PKD1 .can ba a novel therapeutic approach against PD. So, we rationally designed peptide modulators to activate PKD1. The PKD1 activator peptide AK-P4T protected against neurodegeneration in both cell culture and animal models of PD. Herein, we describe the design, development and characterization of the peptide based drug AK-P4T as a novel therapeutic agent in pre-clinical models of Parkinson's disease.

Materials and Methods:

Cell Culture

The immortalized rat mesencephalic dopaminergic neuronal cell line (N27) was a kind gift from 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 of penicillin, and 50 µg/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C, as described previously (28). N27 cells are used widely as a cell culture model for PD (29) (12) (30) (28, 31).

Primary mesencephalic neuronal culture

Primary mesencephalic neuronal cultures were prepared from the ventral mesencephalon of gestational 16- to 18-day-old mouse embryos, as described earlier (32). Tissues were dissected from E16 to E18 mouse embryos maintained in ice cold Ca²⁺-free Hanks' balanced salt solution and then dissociated in Hanks' balanced salt solution containing trypsin-0.25% EDTA for 30 min at 37°C. The dissociated cells were then plated at equal density (0.5×10^6 cells) on 12 mm coverslips precoated with 0.1 mg/ml poly-D-lysine. Cultures were maintained in neurobasal medium fortified with B-27 supplements, 500 µM l-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). The cells were maintained in a humidified CO₂ incubator (5% CO₂ and 37°C). Half of the culture medium was replaced every 2 days. Approximately 6- to 7-day-old cultures were used for experiments. Primary mesencephalic dopaminergic neuronal cells were exposed to 10 µM for 1 h.

Cytotoxicity Assays

Cell death was determined using the Sytox green cytotoxicity assay, after exposing the N27 cells to H₂O₂ (100 μm), as described previously. This cytotoxicity assay was optimized for a multiwell format, which is more efficient and sensitive than other cytotoxicity measurements (33) (34). Briefly, N27 cells were grown in 24-well cell culture plates at 100,000 cells per well and treated with H₂O₂ (100 μm) and 1 μm Sytox green fluorescent dye. The Sytox green assay allows dead cells to be viewed directly under a fluorescence microscope, as well as quantitatively measured with a fluorescence microplate reader (excitation 485 nm; emission 538 nm) (Biotek). Phase contrast and fluorescent images were taken after H₂O₂ exposure with a NIKON TE2000 microscope, and pictures were captured with a SPOT digital camera.

Immunocytochemistry

The primary mesencephalic neurons or N27 cells after H₂O₂ treatment were fixed with 4% paraformaldehyde and processed for immunocytochemical staining. First, nonspecific sites were blocked with 2% bovine serum albumin, 0.5% Triton and 0.05% Tween-20 in phosphate-buffered saline (PBS) for 20 min. The cells then were incubated with antibodies directed against TH, native PKD1 and PKD1-pS744/S748 in PBS containing 1% BSA at 4°C overnight, followed by incubation with Alexa 488 and Alexa 568 conjugated secondary antibodies in PBS containing 1% BSA. Secondary antibody treatments were followed by incubation with Hoechst 33342 dye for 5 min at room temperature to stain the nucleus. The coverslips containing stained cells were washed with PBS, mounted on slides, and viewed under a Nikon inverted fluorescence microscope (model TE-2000U; Nikon, Tokyo, Japan).

Both fluorescence and confocal images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Immunohistochemical staining of brain slices.

Mice were perfused with 4% paraformaldehyde and then the brain slices were cut on a microtome into 20 μm sections. Brain sections were blocked with PBS containing 1% BSA and 0.1% Triton X-100 in for 20 min and then incubated with antibodies directed against PKD1, PKD1-pS744/S748 and TH overnight at 4°C followed by incubation with either Alexa 488-conjugated (green, 1:1000) or Alexa 568-conjugated (red, 1:1000) secondary antibody for 1 h at RT. Secondary antibody treatments were followed by incubation with Hoechst 33342 (10 $\mu\text{g}/\text{ml}$) for 3 min at RT to stain the nucleus. Then the slices were mounted on a slide and viewed under a Nikon inverted fluorescence microscope (model TE-2000U); images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Diaminobenzidine immunostaining and stereological counting of TH-positive neurons

Tyrosine hydroxylase–diaminobenzidine (DAB) immunostaining was performed in striatal and substantia nigral sections as described previously. Mice brains after sacrifice were perfused with 4% paraformaldehyde (PFA) and postfixed with PFA and 30% sucrose. The fixed brains were subsequently cut using previous term cryostat into 30- μm coronal sections and kept in 30% sucrose–ethylene glycol solution at – 20 °C. On the day of staining, sections were rinsed with PBS and incubated with the anti-TH antibody (Calbiochem rabbit anti-mouse, 1:1600) overnight at 4 °C. Biotinylated anti-rabbit secondary antibody was used for 1 h at room temperature. The sections were then incubated with avidin peroxidase

(Vectastatin ABC Elite kit) for 30 min at room temperature. Immunolabeling was observed using DAB, which yielded previous term brown stain. Total numbers of TH-stained neurons in SNpc were counted stereologically with Stereo Investigator software (MicroBrightField, Williston, VT, USA), using an optical fractionators.

Western Blot Analysis

Cells were lysed in either modified RIPA buffer or M-PER buffer (Thermo Scientific) for Western blot, immunoprecipitation and kinase assays. Lysates containing equal amounts of protein were loaded in each lane and separated on 10-12% SDS-PAGE, as described previously (Kaul et al., 2003). PKD1 polyclonal (1:1000), PKC δ polyclonal (1: 1000), PKD1-pS744/S748 (1:1000), PKD1-pS916 (1:1000), PKD1-pY469 (1:1000), - and β -actin (1:10000) antibodies were used to blot the membranes. IR dye-800 conjugated anti-rabbit (1:5000) and Alexa Fluor 680 conjugated anti-mouse (1:10000) were used for antibody detection with the Odyssey IR Imaging system (LICOR), as previously described.

PKC δ Kinase Assay

Immunoprecipitation and PKC δ kinase assay were performed as described earlier (12). After cell lysis, cell were immunoprecipitated using a polyclonal PKC δ rabbit antibody and protein A Sepharose, and washed three times with PKC δ kinase buffer (40 mM Tris (pH 7.4), 20 mM MgCl₂, 20 μ M ATP, 2.5 mM CaCl₂). The reaction was started by adding 20 μ l of buffer containing 0.4 mg histone and 5 μ Ci of [γ -³²P]ATP (4,500 Ci/mM). After incubation for 10 min at 30°C, SDS loading buffer (2X) was added to the samples to terminate the reaction. The reaction products were separated on SDS-PAGE (12%), and

the H1-phosphorylated bands were detected using a phosphoimager (Fujifilm FLA-5100) and quantified with MultiGauge V3.0 software.

Protein Kinase D1 Kinase Assay

The cells were exposed to H₂O₂ (100 μM) for 1 h and cell lysates were immunoprecipitated, as previously reported, with native PKD1 antibody (Santa Cruz). The kinase reaction was carried out at room temperature for 20 min after adding 10 μl of kinase substrate mix (0.1 mM ATP + 10 μCi [γ-³²P] ATP + 2 μg Syntide 2 peptide substrate in kinase buffer). Kinase buffer contains 20 mM Tris pH 7.5, 10 mM MgCl₂, and 1 mM DTT. Kinase assay performed using recombinant PKD1 protein in cell free system contained 90 μg/ml Phosphatidyl serine and 175 nM PMA in addition in the assay buffer. The samples were centrifuged to terminate the kinase reaction, and the supernatants containing the phosphorylated peptide were applied as spots to P81 phosphocellulose squares (Whatmann). The papers were washed four times with 0.75% phosphoric acid and once with acetone and dried, and activity was determined by liquid scintillation counting. The samples were also loaded on a SDS-PAGE and probed for native PKD1 to determine equal loading.

Transient and Stable Transfections

PKD1 activation loop, active PKD1S744E/S748E (PKD1-CA) were obtained from Addgene, Inc. (15). Electroporation was carried out with an Amaxa Nucleofector instrument, as per the manufacturer's protocol. The transfected cells were then transferred to T-75 flasks or 6-well plates as desired and allowed to grow for a 24 h period before the treatment.

Uptake of [³H] Dopamine

The effects of PKD1 inhibitor AK-P4T on the uptake of dopamine were assessed in fetal mouse mesencephalic cultures using [³H]dopamine (DA), as described previously (Afeseh et al., 2009). In brief, after incubation for 48 h with 5 μM MPP⁺, with or without Kb-NB142-70, medium with the treatment was removed and cells were then washed once by assay incubation (Krebs-Ringer) buffer (5.6 mM glucose, 1.3 mM EDTA, 1.2 mM magnesium sulfate, 1.8 mM calcium chloride, 4.7 mM potassium chloride, 120 mM sodium chloride, and 16 mM sodium phosphate). Cells were incubated with 10 μM [³H] DA (30 Ci/mol) for 20 min at 37°C. Positive controls were obtained by incubating the cells with 10 μM [³H] DA together with 1 nM mazindol (potent dopamine reuptake inhibitor). The uptake was stopped by removing the reaction mixture and followed by three washes with fresh Krebs-Ringer buffer. Cells were then collected with the use of 1 N NaOH, and the radioactivity was measured by liquid scintillation counting.

HPLC analysis of striatal dopamine and its metabolite levels

High-performance liquid chromatography (HPLC) with electrochemical detection were used to quantify striatal dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) levels. Samples briefly, after 7 days of MPTP injection, mice were sacrificed and striata were collected and stored at - 80 °C. On the day of analysis, neurotransmitters from striatal tissues were extracted in 0.1 M perchloric acid solutions containing 0.05% Na₂EDTA and 0.1% Na₂S₂O₅ and isoproterenol (as an internal standard). The extracts were filtered in 0.22-μm spin tubes, and 20 μl of the sample was loaded for analysis. DA, DOPAC, and HVA were separated isocratically in a reversed-phase column using a flow rate of 0.7 ml/min. An HPLC system (ESA, Bedford, MA, USA) with an automatic sampler equipped with a refrigerated temperature control

(Model 542; ESA) was used in these experiments. The electrochemical detection system consisted of a Coulochem Model 5100A with a microanalysis cell

(Model 5014A) and a guard cell (Model 5020; ESA). Standard stock solutions of catecholamines (1 mg/ml) were prepared in perchloric acid solution and further diluted to a final working concentration of 50 pg/ μ l before injection. The data acquisition and analysis were performed using the EZStart HPLC software (ESA).

Animals and treatment

Six- to 8-week-old male C57BL/6 mice weighing 24 to 28 g were housed under standard conditions: constant temperature (22 ± 1 °C) and humidity (relative, 30%) and a 12-h light/dark cycle. Mice were allowed free access to food and water. Use of the animals and protocol procedures were approved and supervised by the Committee on Animal Care at Iowa State University (Ames, IA, USA). Mice received AK-P4T (5 mg/kg dose) and the control peptide intravenous injections for 1 day (pretreatment before MPTP administration), 5 days (co-treatment with MPTP), and 7 days (post-treatment with MPTP). In the subchronic MPTP regimens, MPTP (25 mg/kg) was injected intraperitoneally via a single dose daily starting on day 2 for 5 days. The control mice received saline at the same dose.

Behavioral measurements

An open-field experiment for testing locomotor activities after MPTP and AK-P4T treatments. An automated device (AccuScan, Model RXYZCM-16; Columbus, OH, USA) was used to measure the spontaneous activity of the mice. The activity chamber was 40 × 40 × 30.5 cm, made of clear Plexiglas, and covered with a Plexiglas lid with holes for ventilation. The infrared

monitoring sensors were located every 2.54 cm along the perimeter (16 infrared beams along each side) and 2.5 cm above the floor. Two additional sets of 16 sensors were located 8.0 cm above the floor on opposite sides. Data were collected and analyzed by a VersaMax analyzer (AccuScan, Model CDA-8). Before any treatment, mice were placed inside the infrared monitor for 10 min daily for 3 consecutive days to train them. Five days after the last MPTP injection, both open-field and rotarod experiments were conducted.

Statistical Analysis

Data analysis was performed using Prism 3.0 software (GraphPad Software, San Diego, CA). Bonferroni's multiple comparison testing was used to find the significant differences between treatment and control groups. Differences with $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered significantly different from $n \geq 6$ from two or more independent experiments, and are indicated in the figures.

Results

Genetic and Pharmacological modulation of PKD1 activation protects against cell death in PD cell culture model

We used the two major parkinsonian toxicants 6-OHDA and MPP⁺ in the current study. We have previously shown that PKD1 is activated in 6-OHDA and MPP⁺ induced oxidative stress (14). As shown in Fig 1A 100 μ M 6-OHDA and 300 μ M MPP⁺ induces PKD1 activation loop phosphorylation (pS744/pS748) in a time dependent manner in N27 dopaminergic cells. Further, positive and negative genetic manipulation of PKD1 confirms the cell survival role of PKD1. We overexpressed the constitutively active human

PKD1 plasmid (PKD1^{-S744E/S748E}) and phospho-defective PKD1^{S916A} in N27 cells and then exposed to 6-OHDA and MPP⁺. Mutation of serine residues at position 744 and 748 results in constitutively active PKD1 (15). As shown in Fig. 1C, overexpression of PKD1^{S744E/S748E} significantly attenuated 6-OHDA and MPP⁺ induced cytotoxic cell death compared to vector transfected dopaminergic cells, whereas overexpression of phospho-defective PKD1^{S916A} significantly attenuated 6-OHDA and MPP⁺ induced cytotoxic cell death compared to vector transfected dopaminergic cells.

Since genetic modulation of PKD1 can protect neuronal cells, we hypothesized that pharmacological modulation of PKD1 should influence cell survival and death. N27 dopaminergic cells were exposed to 300 μ M MPP⁺ in the presence or absence of 50 μ M PKD inhibitor kbNB 142-70 and cytotoxic cell death was determined using Sytox green assay. As shown in Fig 1D, co-treatment with 50 μ M kbNB 142-70 exacerbated MPP⁺-induced cytotoxic cell death compared to MPP⁺ alone treated cells.

Next, as a proof of concept we examined if positive pharmacological modulation of PKD1 can protect against oxidative damage and cell death. Since there are no PKD1 activators available, we screened for PKD1 activators. We have previously shown that in dopaminergic neurons proteolytically activated PKC δ phosphorylates PKD1 during the early stage, so we looked for drugs that can cause persistent activation of PKD1 independent of PKC δ cleavage. After carefully reviewing different PKD1 activation mechanisms in various other models, we handpicked some drugs and screened them for their potential to activate PKD1 through these mechanisms. As shown in Fig 1E primary screening of 10 μ M Carbachol, 50 μ M Desmopressin, 100 μ M Rosiglitazone, 100 ng/ml BMP2, and 25mM Glucose were performed using western blot to screen for their potential to phosphorylate activation loop of PKD1 (pS744E/pS748E). Eventhough both

Rosiglitazone and Desmopressin induced PKD1 activation loop phosphorylation, only Rosiglitazone's mechanism of activation was independent of PKC δ cleavage. Next, we used a genetic based FRET reporter optimized in a plate reader format as a secondary screening method to report PKD1 activity. Previously this FRET reporter has been used to monitor PKD1 activity directly in cells (16-18). As shown in Fig 1F 10 μ M Rosiglitazone increased PKD1 activity as shown by the FRET change (CFP/YFP) read out. Further, only 10 μ M Rosiglitazone protected against 100 μ M 6-OHDA induced cell death in N27 cells. Next, we examined if Rosiglitazone can protect against mouse primary dopaminergic neurons. As showed in Fig 1G Immunofluorescence pictures of Tyrosine Hydroxylase (TH) positive dopaminergic neurons showed protection against 10 μ M 6-OHDA when co-treated with 10 μ M Rosiglitazone. These results unequivocally demonstrate that positive pharmacological modulation of PKD1 has protective compensatory role against oxidative damage.

Rational drug design

Eventhough we showed that positive modulation of PKD1 through Rosiglitazone has protective function in cell culture models of PD. We wanted to develop specific activators of PKD1, as rosiglitazone is not specific and has other known functions (16, 17). Previously it has been shown that specific peptide based regulators targeting protein-protein interactions of PKCs have been successfully developed and advanced to clinical trials in cardiovascular diseases (19) (20). PKD1 has a very different structure compared to PKCs; it has two cysteine rich domains and a pleckstrin homology (PH) domain in the regulatory fragment. It was shown that the regulatory fragment importantly the PH domain has an auto-inhibitory role by interacting with the catalytic fragment but lacks a defined pseudo-substrate

domain like PKCs (21, 22). After careful analysis of PKD1 primary and secondary structure, we hypothesized that PKD1 region containing the consensus substrate-like motif (LXRXXS/T) and pseudosubstrate-like motif (LXRXX#, LXR##, #- serine or threonine residue replaced mostly by a hydrophobic/aliphatic residue) can regulate PKD1 intra-protein interactions. Peptides mimicking these regions can be highly specific modulators of PKD1 activity. As shown in Fig 2A, Alignment of distantly related PKD1 sequences from murine and *C.elegans* show the presence of highly conserved regions around the substrate-like and pseudosubstrate-like sequences only in the regulatory fragment. Further, the regions containing the pseudo-substrate like sequence are highly conserved between the three PKD isoforms, whereas the substrate-like sequence is present only in PKD1 (Fig 2B). We chose four of these regions from each domain; the highly conserved 6 aa peptide AK-P1 (6-11 amino acids), and 11 aa peptide AK-P2 (36-46 amino acids) were synthesized from the cysteine rich domain 1, 10 aa peptide AK-P3 (295-395 amino acids) were synthesized from the cysteine rich domain 2, 12 aa peptide AK-P4 (405-416 amino acids) were synthesized from the pleckstrin homology (PH) domain. Additionally, Peptides disrupting protein-protein interactions have also been shown to regulate kinase activity. Mochly-Rosen group reported that there are regions in PKC enzymes that are homologous to the PKC carrier protein RACK (termed pseudo-RACKS) and the peptides synthesized mimicking this region can facilitate PKC translocation and activation (23). Using this rational, we detected similar regions in PKD1 that are homologous to PKD1 interaction proteins 14-3-3 γ and G β γ . As shown in Fig 2C, a 5 aa peptide AK-P5 (54-58 amino acids) from the cysteine rich domain 1 (pseudo-14-3-3 γ), a 9 aa peptide AK-P6 (79-87 amino acids) from the pleckstrin homology (PH) domain (pseudo- G β γ) and a 5 aa control peptide AK-P7 (135-139 amino acids) were synthesized.

Primary screening of the synthesized peptides were done using the phospho-specific anti-bodies to detect PKD1 S744/S748 and S916 phosphorylation. As shown in Fig 2D, 50 μ M of AK-P3 and AK-P4 induced PKD1 S744/S748 and S916 phosphorylation in N27 cells. Secondary screening was performed by monitoring PKD1 kinase activity as measured by a [32 P] kinase assay using Syntide 2 substrate (Fig 2E). Peptides AK-P3 and AK-P4 significantly increased PKD1 activity validating the results obtained from primary screening whereas AK-P1 significantly decreased kinase activity. The specificity of the peptides in activating PKD1 was tested in a cell free system using a human recombinant PKD1 enzyme. As shown in Fig 2C, AK-P4 increase PKD1 kinase activity and directly activates PKD1. Together these results confirm that peptides mimicking PKD1's substrate-like region acts an inhibitor whereas peptides mimicking highly conserved PKD1's pseudosubstrate-like region acts an activator.

PKD1 activation by AK-P4 TAT in cell culture models of PD

The effect of PKD1 peptide activators on cell survival was tested in N27 cells using toxins MPP⁺ and 6-OHDA. Fig 3A shows that N27 cells treated with 50 μ M AK-P3 and AK-P4 protected against 50 μ M 6-OHDA induced toxicity. But only 50 μ M AK-P4 not AK-P3 protected against N27 cells treated with 300 μ M MPP⁺ (Fig 3B). Additional validation of the role of AK-P4 was sought by extending these studies to animal models. To facilitate efficient delivery of AK-P4 into the brain, we tagged the AK-P4 peptide with a 13 aa HIV-TAT transporter peptide through diglycine conjugation. Neuroprotective effect of the TAT conjugated AK-P4 peptide (AK-P4T) was tested initially in N27 cells and mouse primary mesencephalic neuronal cultures. As shown in Fig 3C AK-P4T protected against 100 μ M 6-OHDA induced toxicity at a concentration of 100 nM. Further, primary mesencephalic neurons were co-treated with 50

nM AK-P4T to determine its protective effect against 5 μ M MPP⁺ treatment for 48 hours. As shown in fig 3D, the number of surviving tyrosine hydroxylase (TH) positive neurons were almost equal to control levels whereas MPP⁺ treatment group only showed 30%. Next, effects of AK-P4T on the functional efficiency of dopaminergic neurons were studied using H³ dopamine uptake assay. TH neurite length in AK-P4T co-treatment group was equal to control levels whereas TH neurite length was reduced 3-fold in MPP⁺ treated neurons (Fig 3E). Further, As shown in fig 3E, AK-P4T co-treatment brought back the dopamine uptake of MPP⁺ treated neurons to 80% of control, whereas MPP⁺ treatment group only showed 50% dopamine uptake. to 80% of control, whereas MPP⁺ treatment group only showed 50% dopamine uptake. Visualisation of TH neuron morphology through fluorescence microscopy shows clear neuroprotective effect of AK-P4T (Fig 3G).

Together, the results demonstrate the protective effect of AK-P4T convincingly in PD cell culture models.

Intravenous Delivery of AK-P4T effectively activates PKD1 in animal models

After characterizing the neuroprotective effect of the activator peptide (AK-P4T) in cell culture models of PD, we examined the ability of AK-P4T to activate PKD1 in the brain. First, we tested different methods to deliver the activator peptide effectively into the brain. Different doses of AK-P4T peptide were delivered intra-peritoneally using different administration paradigms. The intra-peritoneal Ak-P4T delivery of 5mg/kg activated PKD1 in mice after 4 h of injections but the fold increase of PKD1 in the substantia nigra was not significant as monitored by the activation loop phosphorylation (PKD1pS744/pS748) (Fig 4A). So we pursued different routes to deliver AK-P4T. We used nanopolymers to deliver the peptides. As detailed in the

materials and methods section, we used polymer (50:50 CPTEG:CPH) to coat the peptides and delivered them intra-nasally. But the polymer conjugated Ak-P4T peptide through this delivery route in mice did not activate PKD1 in the substantia nigral region (Fig 4B). So we tried the intra-venous injections to deliver the peptides. We administered 5mg/kg peptide through the tail vein of the mice and sacrificed at 4 and 8 hr. As shown in Fig 4C, the intra-venous Ak-P4T peptide delivery activated PKD1 in the substantia-nigra starting at 4 h. We see a similar trend of PKD1 activation in striatum starting at 4h, with a (Fig 4D). Quantification of the activation loop phosphorylation (PKD1pS744/pS748) at 8 h shows a 1.4 fold increase in PKD1 activation in the substantia nigra and a 2 fold increase in PKD1 activation in the striatum (Fig 4c and D). Further, intra-venous administration of Ak-P4T also increased PKD1 S916 phosphorylation starting at 4h confirming the Ak-P4T allosteric activation of PKD1(Fig 4E).

Collectively, these results show that intra-venous delivery of the Ak-P4T peptide can activate PKD1 effectively.

AK-P4T protects against dopaminergic degeneration in PD animal model

Next, we examined the neuroprotective efficacy of AK-P4T in a sub-acute MPTP mouse model of PD. Since AK-P4T activated PKD1 through intra-venous administration, we chose this delivery route. Animals were administered AK-P4T (5 mg/kg body weight) 24 h before MPTP administration. Animals were then cotreated with AK-P4T and MPTP (25 mg/kg i.p.) daily for 5 days and the AK-P4T treatment was continued for 7 more days and the animals were sacrificed. Saline- and vehicle-injected animals were used as controls. To assess the protective effect of Ak-P4T, we first examined whether AK-P4T could block MPTP-induced loss of striatal

dopamine and its metabolites. As shown in Fig. 5A, MPTP treatment induced loss of dopamine (>75%) (A), DOPAC (>80%) (B), in mouse striatum. The dopamine levels were determined to be 72.21 ± 16.67 , 18.56 ± 2.18 and 38.95 ± 5.365 pg/mg protein in control-, MPTP-, and MPTP + 5 mg/kg AK-P4T treated animals, respectively. Pretreatment with 5 mg/kg AK-P4T afforded more than 50% protection against MPTP-induced striatal dopamine loss. Similar results were found for dopamine metabolites DOPAC and HVA (Fig. 5B and C). Further, the western blotting of striatal lysates for Tyrosine Hydroxylase (TH) shows TH levels rescued in the AK-P4T group (Fig. 5D). Further, immunochemical DAB staining shows the rescue of TH levels in AK-P4T co-treatment group compared to MPTP group in both striatal and substantia nigra sections (Fig. 5E). Western blotting of substantia nigra lysates shows that PKD1 activation is preserved in AK-P4T co-treated groups (Fig. 5E). Collectively, these data suggest that AK-P4T treatment could afford protection against MPTP-induced nigro-striatal dopamine and dopamine metabolite loss in animal PD models by persistent activation of PKD1.

AK-P4T attenuates motor deficits

With evidence suggesting that AK-P4T protects against MPTP-induced dopamine and TH neuronal loss, we next determined whether AK-P4T treatment could also afford protection against MPTP-induced motor deficits. We compared the motor activity of vehicle plus control peptide, MPTP plus control peptide, and MPTP plus AK-P4T-treated animals, using a VersaMax computerized activity monitoring system (Accuscan, Columbus, OH). This system uses infrared sensors to measure repetitive movements both in the horizontal and vertical planes in real time, and it provides color-coded output. Representative activity maps of the animals are presented in Fig. 6A. The

cumulative behavioral activities are monitored and expressed as percentage of the vehicle-treated group, and they were obtained at day 9 of the sub-acute MPTP treatment paradigm. Statistical analyses of both raw data and percentage of control showed significant differences in various behavioral activities. The data indicate that the horizontal motor activity (>20% reduction), number of movements (>20% reduction), vertical motor activity (>40% reduction), vertical movement time (>50% reduction) total distance travelled (>20% reduction), , vertical number of movements (>40% reduction), movement time (>20% reduction), stereotypy count (>20% reduction), margin distance (>20% reduction), margin time (>20% reduction), stereotypy time (>20% reduction), rearing activity (>40% reduction), rearing time (>50% reduction), were significantly reduced in MPTP-treated animals compared with the vehicle-treated group (Fig 6B-N). However, administration of AK-P4T almost completely restored all the behavioral and locomotor patterns of MPTP-treated animals to the levels observed in control animals (Fig 6B-N). These results demonstrate AK-P4T treatment attenuates MPTP-induced locomotor deficits in pre-clinical models of mice.

Discussion

The present study demonstrates that a rationally designed allosteric activator peptide of PKD1 protects against dopaminergic neurodegeneration in pre-clinical models of Parkinson's disease. Extensive characterization of PKD1 signal transduction in pre-clinical models of PD showed that, positive modulation of PKD1 can be a novel therapeutic strategy against PD. Herein, we report for the first time some key findings pertinent to neuroprotection against Parkinson's disease: (i) rationally designed peptide modulators based on the substrate-like and pseudo-substrate like sequences present in the

regulatory fragment activate or inhibit PKD1 allosterically by mimicking and disrupting the native intra-protein interactions (ii) one of the peptide activators AK-P4 protected against both 6-OHDA and MPP⁺ induced toxicity in N27 dopaminergic cells (iii) TAT₄₇₋₅₇ peptide conjugated AK-P4 (AK-P4T) rescued TH⁺ neurons against MPP⁺ and prevented dopaminergic neurodegeneration in primary mesencephalic cultures (iv) effective delivery of AK-P4T through intravenous administration activated PKD1 and protected against MPTP-induced motor deficits, striatal dopamine depletion, and nigral dopaminergic neuronal loss in a sub-acute MPTP animal model. To our knowledge, this is the first demonstration of a novel neuroprotective strategy against PD by positively modulating an anti-apoptotic kinase PKD1 using a rationally designed allosteric activator.

Based on these findings we hypothesized that prolonging the activation of the survival switch PKD1 through a specific activator can prevent or delay PD progression. Recently, Peptide based drugs have successfully entered clinical trials or approved as a treatment for many disorders. Additionally, they are specific with fewer side effects compared to small molecule drugs (24). We utilised the vast protein databases and developed a new approach to find PKD1 modulators. We began with our hypothesis that interfering with intra-protein interactions of PKD1 can modulate PKD1. PKD1 in native state is auto-inhibited by its regulatory fragment consisting of the cystine rich domains and pleckstin homology (PH) domain but do not have a pseudo-substrate region like some PKC family members. Careful analysis of PKD1 protein sequence revealed highly conserved regions similar to the consensus substrate sequence. Our results suggest that peptides synthesized mimicking the pseudo-substrate like region activates the kinase, while peptide mimicking the substrate like region inhibits the kinase. Particularly,

the peptide AK-P4 mimicking the highly conserved pseudo-substrate like region in the PH domain protected against both 6-OHDA and MPP⁺ mediated toxicity.

Previous studies have shown that the cell penetrating peptides derived from the trans-activating transcriptional activator (TAT) from HIV can be used to deliver therapeutic peptides (25) (26). AK-P4 Peptide was conjugated with the TAT₄₇₋₅₇ (AK-P4T) to facilitate effective delivery across the blood-brain barrier. Even though, progress has been made to deliver therapeutic peptides for neurodegenerative disorders, effective peptide drug delivery remains as an area for improvement (27). We tested different routes and methods to effectively deliver the peptide; intra-peritoneal administration, intra-nasal administration of peptides coated with nanopolymers, intra-venous administration. Our results indicate that only AK-P4T peptide administered through intra-venous route can activate PKD1 in the nigro-striatal pathway after effectively crossing the blood-brain barrier. We used intra-venous route for all our studies, even though exploration of other methods are underway.

Our findings in pre-clinical models using MPTP demonstrate that AK-P4T delivered through intra-venous route activates PKD1, and rescues dopaminergic neurons from succumbing to cell death. Further the nigro-striatal dopaminergic neuronal loss is reduced improving the neurotransmission function of dopaminergic neurons. This considerably improves the motor function as the mice behave like the control group.

In conclusion, we demonstrate that activation of the novel protective signaling mechanism mediated by PKD1 in dopaminergic neurons by a peptide based PKD1 activator offers neuroprotection in pre-clinical models of PD by slowing down or stopping dopaminergic degeneration. This drug offers therapeutic promise for PD.

Abbreviations

PD, Parkinson's disease; PKD1, Protein kinase D1; MAPK, Mitogen-activated protein kinases PKC δ , Protein kinase C delta; CAMK, Ca²⁺ /Calmodulin-Dependent Protein Kinase II; JNK, c-Jun N-terminal kinases; LRRK2, Leucine-rich repeat kinase 2 (LRRK2); MLK, Mixed-lineage kinase; ROS, Reactive oxygen species; MnSOD, Manganese superoxide dismutase; WB, Western Blot; PKC, Protein kinase C; PKC α , Protein kinase C alpha.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

All authors read and approved the final manuscript.

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Fig 1:

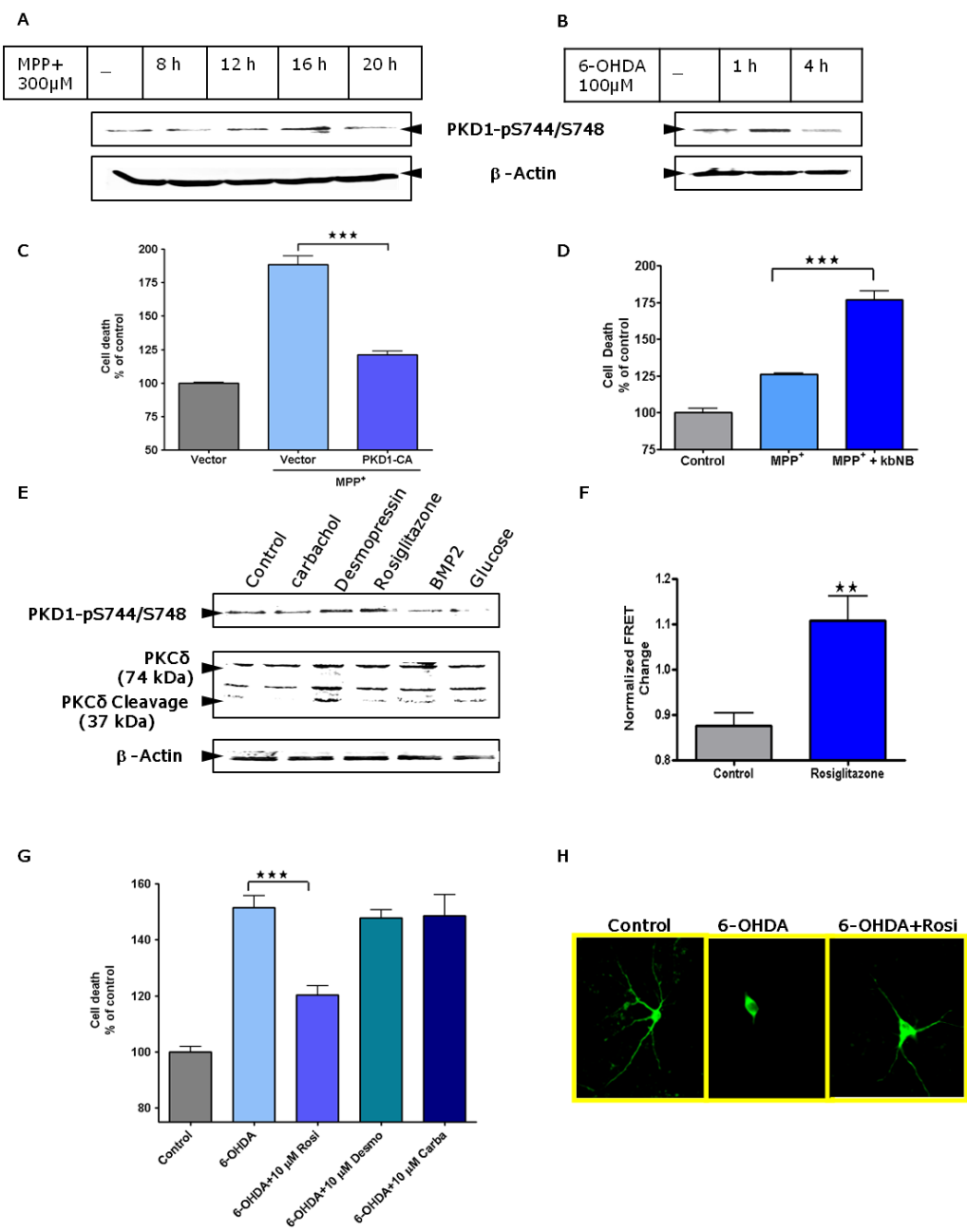


Fig 2:
A

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LXXEX#
RAT  HS APL LKPP SPLPAAAMAAAALALUFGSGPAPFPAPCAAPAGGISTHLLGLKSPV 60
MOUSE HSUPLKPP SPLPAAAMAAAALALUFGSGPAPFPAPCAAPAGGISTHLLGLKSPV 60
CELEGANS -----HDGSGGSDTVGDHVLVYVCTHRLV 65
LXXEX#
RAT  LL LQDS SGGVSLAHVEMACSIUDKFPCCGTVGLVDK LLL FPHDP ASEM ILGLUKIASD 120
MOUSE LL LQDS SGGVSLAHVEMACSIUDKFPCCGTVGLVDK LLL FPHDP ASEM ILGLUKIASD 120
CELEGANS PL IR---HDQHLMLAERARQIVQG-FQMLDTRHWY---L FPHDP YRFTLLYP ITSAHQ 77
LXXEX#
RAT  IQEGDL IDVVLAS AT FET FQ IEPHAL FOKSVRAP AFDRC DRLMGLVQGLKCEGGL 180
MOUSE IQEGDL IDVVLAS AT FET FQ IEPHAL FOKSVRAP AFDRC DRLMGLVQGLKCEGGL 180
CELEGANS ITSGILLIHLQRT---EAMIFRQVREKSNRPFPCFCGMLTGLMGLVQGLKCEGGL 194
RAT  WYRKC AKRIFMNCSDRRELSMUSLITGLGTVRTASAEPTSTADPELLSPUSPFEQR 240
MOUSE WYRKC AKRIFMNCSDRRELSMUSLITGLGTVRTASAEPTSTADPELLSPUSPFEQR 240
CELEGANS WYRKC SWAASWNC-----APGAPGAPSSPFLLEPTPTTPTPVA 197
LXXEX#
RAT  SPSEY IGRKESNSQSYVGGP IQDLKLLMSEKQVPHY FVINSYRPTVC QFCKMLGL 300
MOUSE SPSEY IGRKESNSQSYVGGP IQDLKLLMSEKQVPHY FVINSYRPTVC QFCKMLGL 300
CELEGANS ALD-----TPTGLPHTLIEHSYRQPTVCKMVDKMLGL 210
LXXEX#
RAT  FKGLQ CEDCRFMCHGCAPKVFPHKLEVT INGELLSPGAE SDVQHEGSDSDSDSENS 360
MOUSE FKGLQ CEDCRFMCHGCAPKVFPHKLEVT INGELLSPGAE SDVQHEGSDSDSDSENS 360
CELEGANS VKGLKCEDCGQVHCHC SMLASRCV-----LLEKFLIPNASQAESKVFYLK 237
LXXEX#
RAT  GLHDHDEHWVDTETALADGQSDGAEHQDPADQEDSEFTISPTSNVILPHLQVQVUR 420
MOUSE GLHDHDEHWVDTETALADGQSDGAEHQDPADQEDSEFTISPTSNVILPHLQVQVUR 420
CELEGANS -----LEMAIISVNTDEGPE-----GSSSDMIFLPLGQV 272

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hydrophobic residue

1. AK-P1-YDKILLFRHDP --Only present in MURINE. 2. AK-P1-YDKILLFRHDP --Score: 6/11 matches --64.5% identical between MURINE and C.ELEGANS. 3. AK-P3-LLKGLFRQGL --Score: 6/10 matches --80.0% identical between MURINE and C.ELEGANS. 4. AK-P4-STSNIPLMRVY --Score: 11/12 matches --91.8% identical between MURINE and C.ELEGANS

B

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Peptide1: AK-P1
PKD2  -----MAAASHPAGLPSPPGSGPPPP--GGLELQS--PPLLPQIPAPGSQV 45
pk41  -----HSUPLKPPSPPLPAAAAA---AAALVPGSFPAPFPAPCAAPAGG 47
PKD3  HSAMNSPFAQKSVFMTVSAULPAPSPCS SPTKGLSARLSMGSFSAPSLTNSRGSVHTU 60

Peptide2: AK-P2
PKD2  SFHIQGLTRFPQLFAAS---ELARVQLACS IUDKFPCCGTVGLVDK LLL FPHDP 101
pk41  SFELQIGLREPVALLQDSGGVSLAHVEMACSIUDKFPCCGTVGLVDK LLL FPHDP 107
PKD3  SFILQIGLREPVTEAQL---SLASVQVQVYVQKFPCCGTVGLVDK LLL FPHDP 117

Peptide3: AK-P3
PKD2  TVCQACEKLLK LFRQGLQEDCRFMCHGCAPKVFPHKLEVT INGELLSPGAE SDVQ 327
pk41  TVCQACEKLLK LFRQGLQEDCRFMCHGCAPKVFPHKLEVT INGELLSPGAE SDVQ 347
PKD3  TICVQCEKLLK LFRQGLQEDCRFMCHGCAPKVFPHKLEVT INGELLSPGAE SDVQ 342

Peptide4: AK-P4
PKD2  EEAAVYSEADKSS- ISELEDSGVIF-----GSHSE SALSMS--EEEEGGHKAQSL 379
pk41  EECSDDNDSEMSGLHDHDEHWVDTETALADGQSDGAEHQDPADQEDSEFTISPTSN 373
PKD3  S I DSDVNSG GSGGLDSESPPEP-----KHTLDTVDLQVREKSTQCT 358

PKD2  FVILPHLQVQVURSTSNVILPHLQVQVURSTSNVILPHLQVQVURSTSNVILPHLQV 437
pk41  FVILPHLQVQVURSTSNVILPHLQVQVURSTSNVILPHLQVQVURSTSNVILPHLQV 467
PKD3  FVILPHLQVQVURSTSNVILPHLQVQVURSTSNVILPHLQVQVURSTSNVILPHLQV 455

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C

Peptide 5: AK-p5--REKRS

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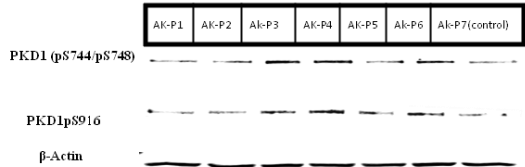
pkd1- LSTSAPE-PLLQKSPSEFTIGREK-RSNSQSYIGRPIH--LDKILMSKVVPHPTVHSY 81
14-3-3 γ VVSSIEQKTEGAEKKQOHAREYREKITELELRLD ICNDVLSLLEKFLIPNASQAESKVFYLK 120

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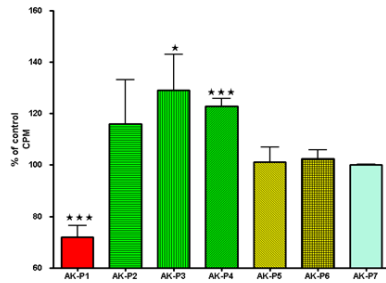
Peptide 6: AK-P6--QSVKHTKRR

Peptide 7: AK-P7--ATFED (Control Peptide)

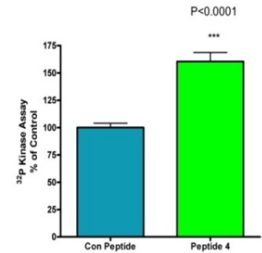
D



E



F



G

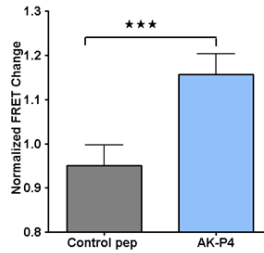


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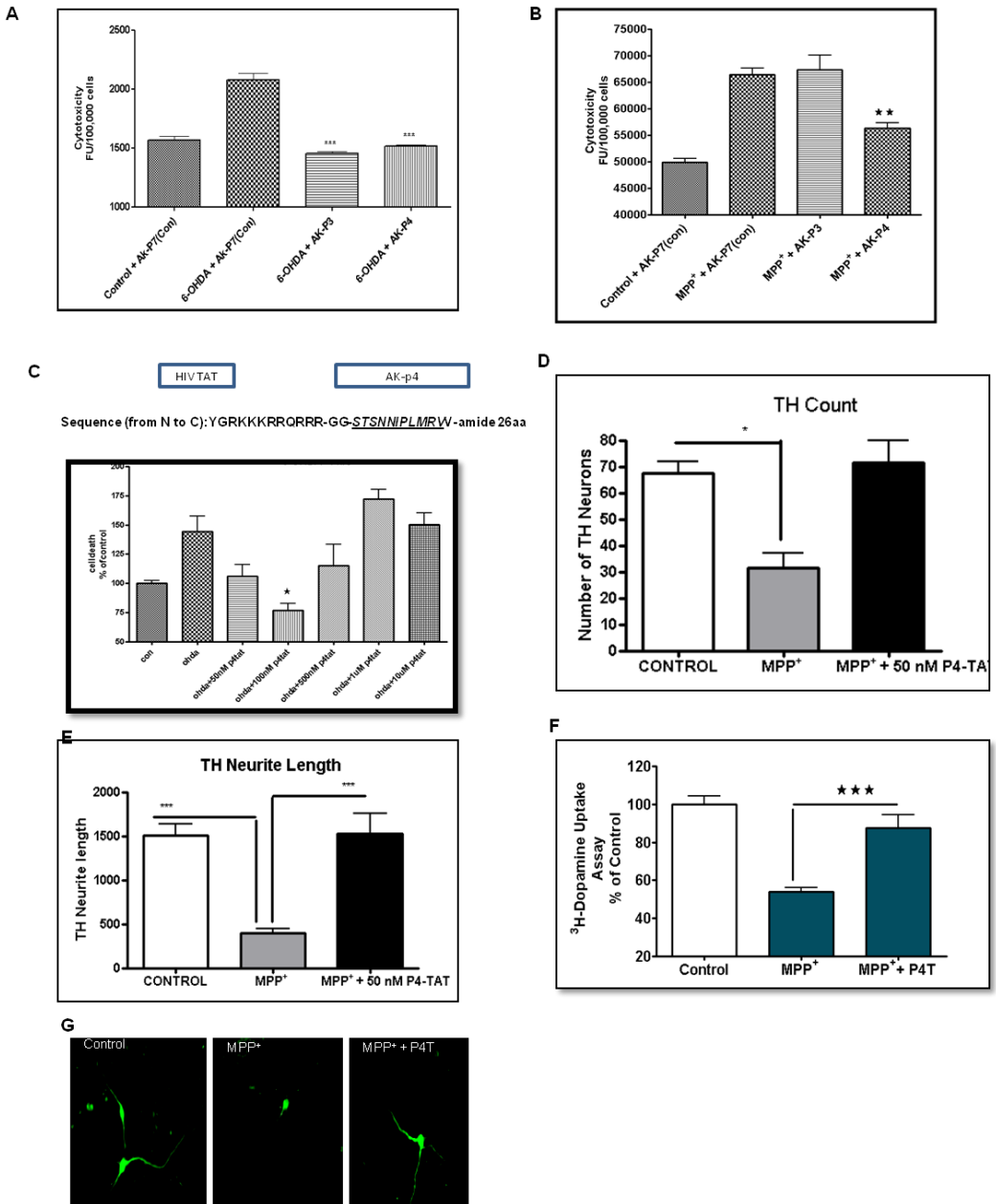


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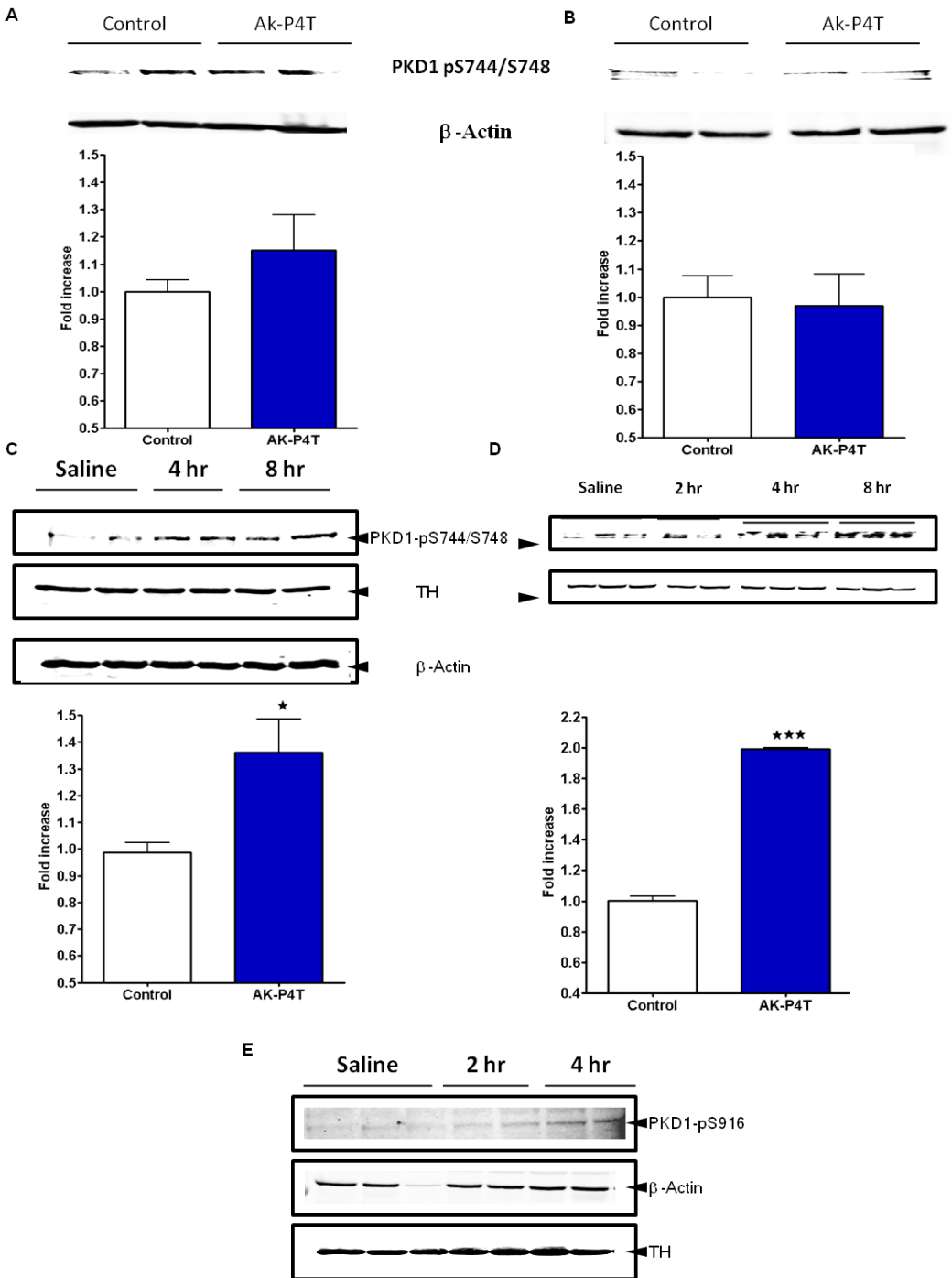


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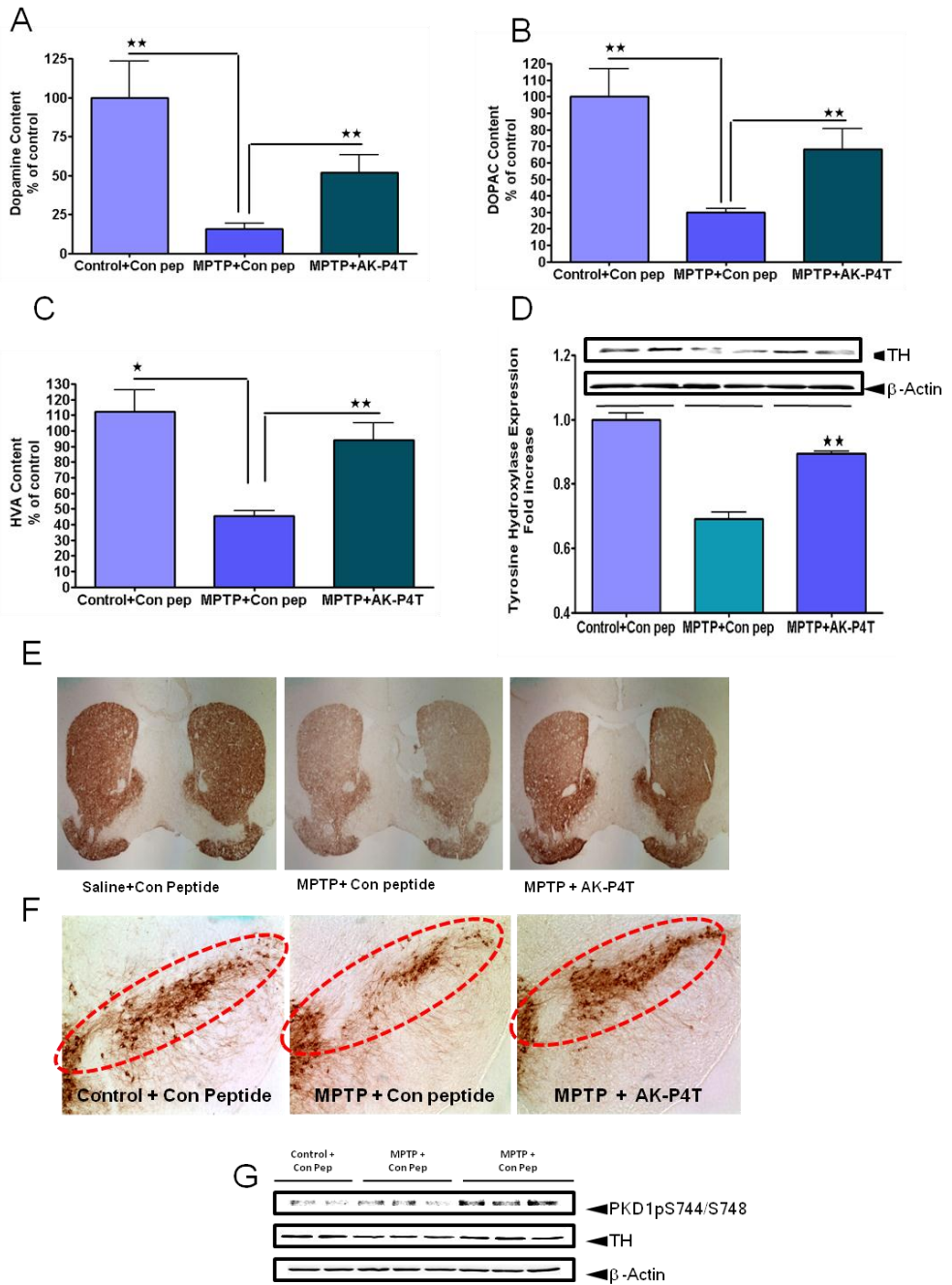


Fig 6

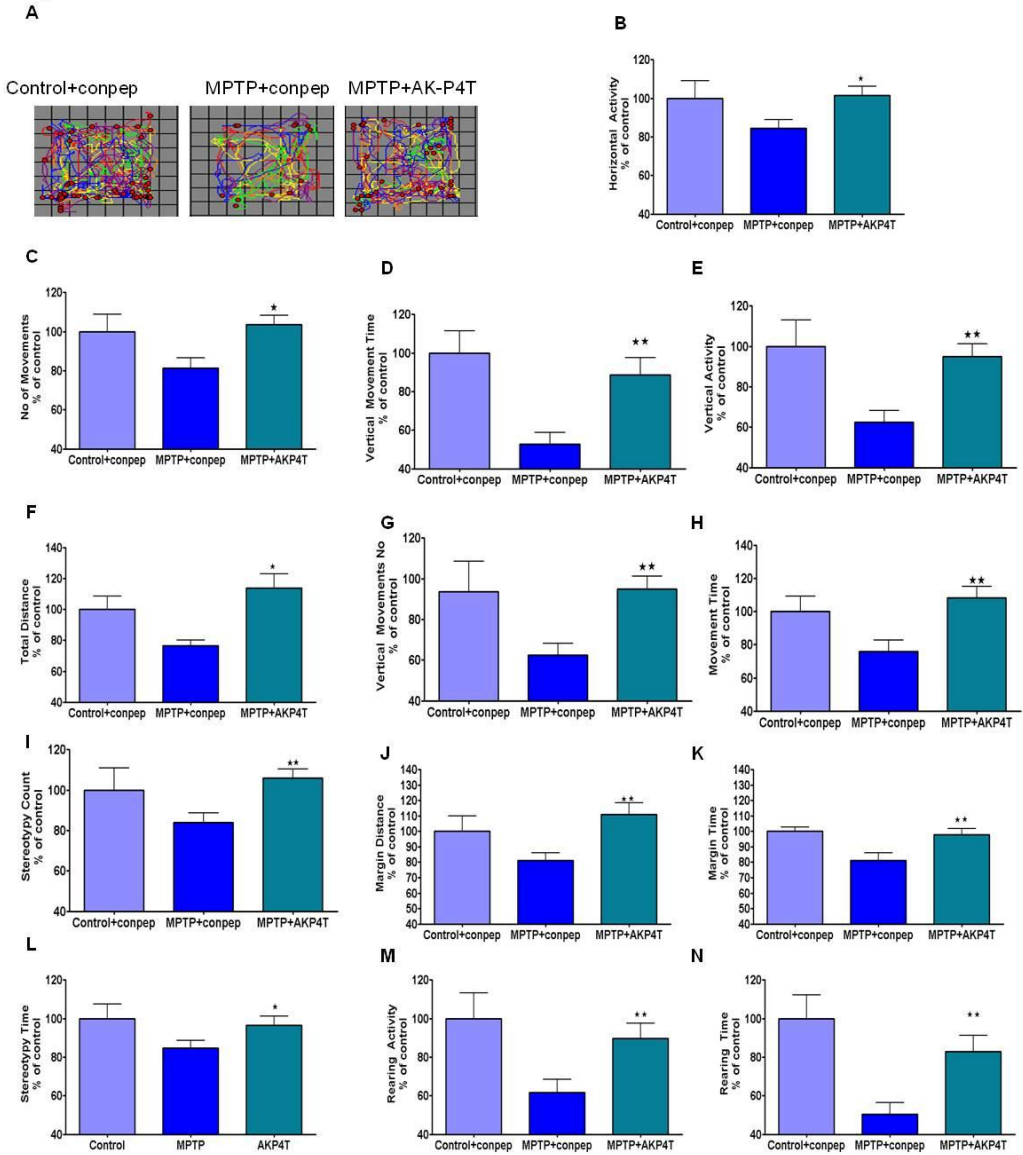


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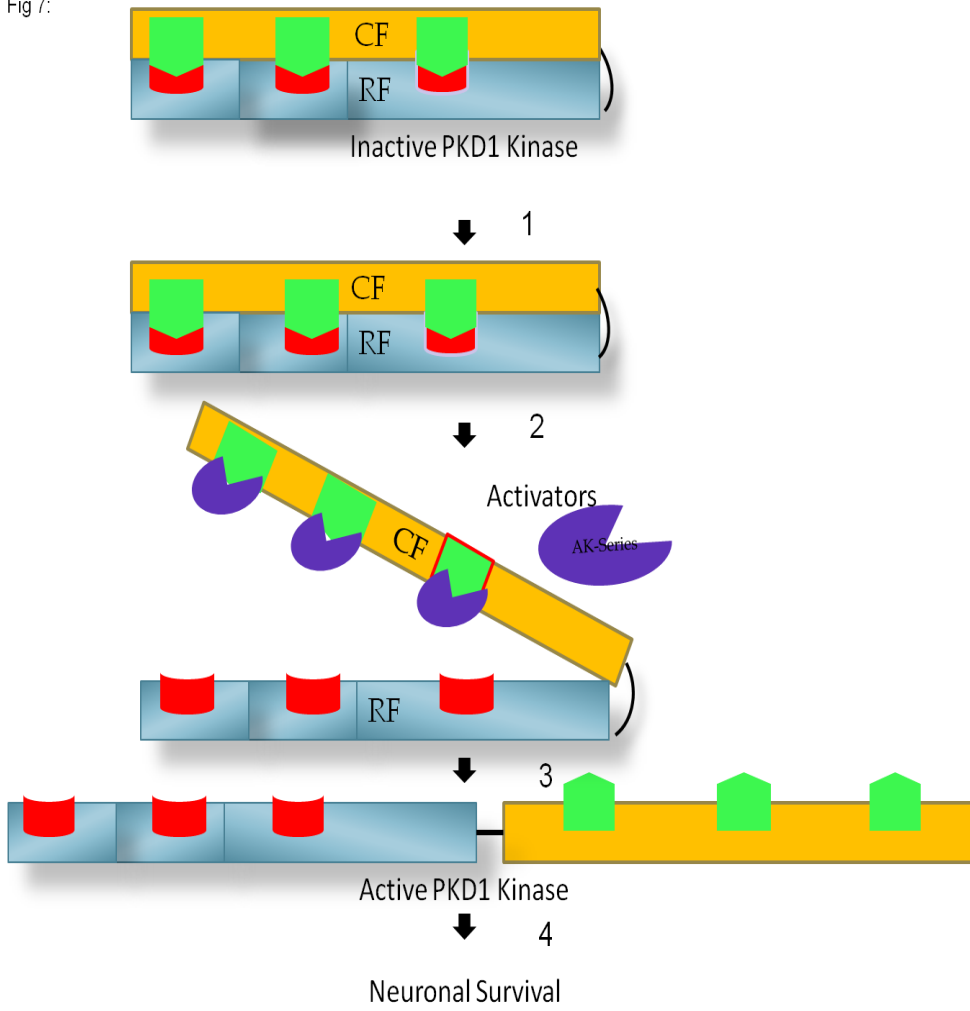


Figure legends

Figure1. Pharmacological activation of PKD1 by Rosiglitazone shows neuro-protection in N27 cells and primary neurons

N27 dopaminergic cells were treated with MPP⁺ (300 μ M) for 0-20 h and 6-OHDA (100 μ M) for 0-4 h and blotted for PKD1 activation loop phosphorylation pS744/pS748 (A & B). N27 dopaminergic cells transiently transfected with 5 μ M PKD1^{S744E/S748E} and 5 μ M vector plasmid were treated with or without 300 μ M MPP⁺ and monitored for cytotoxicity at various time points using sytox green. (C). N27 cells were co-treated with or without 50 μ M PKD1 inhibitor kb-NB 14270 and monitored for cell death during MPP⁺ treatment (D). N27 cells were treated with 10 μ M Carbachol, 50 μ M Desmopressin, 100 μ M Rosiglitazone, 100 ng/ml BMP2, and 25mM Glucose and probed for PKD1 (pS744E/pS748E) and PKC δ cleavage (E). N27 cells transfected with 5 μ M PKD1 kinase activity reporter (PKD1-KAR) plasmid and 5 μ M vector plasmid were treated with Rosiglitazone and monitored for CFP/YFP FRET change in a high-throughput format (F). N27 cells were co-treated with or without 10 μ M Rosiglitazone and monitored for cell death during 6-OHDA treatment (G). Tyrosine Hydroxylase (TH) positive primary mesencephalic neurons treated with co-treated with or without 10 μ M Rosiglitazone were monitored for changes in neuronal morphology and neuroprotection using immune-flouescence (H). $p < 0.001$ denotes significant difference between treatment groups from $n \geq 6$. ($n = 6-8$).

Figure 2. Rationally designed peptide activates PKD1

Alignment of amino acid sequences of PKD1 regulatory fragment between distantly related different organisms rat, mouse and C.elegans, shows the presence of the consensus substrate-like motif (LXRXXS/T) and

pseudosubstrate-like motif (LXRXX#, LXR##, #- serine or threonine residue replaced mostly by a hydrophobic/aliphatic residue) (A). Alignment of mouse PKD isoforms PKD1, PKD2, PKD3 amino acid sequences containing the four (AK-P1, AK-P2, AK-P3, AK-P4) substrate-like (LXRXXS/T) and pseudosubstrate-like region shows high level of conservation (B). 5 aa peptide AK-P5 (54-58 amino acids) from the cysteine rich domain 1 (pseudo-14-3-3 γ), a 9 aa peptide AK-P6 (79-87 amino acids) from the pleckstrin homology (PH) domain (pseudo-G $\beta\gamma$) and a 5 aa scramble control peptide AK-P7 (135-139 amino acids) were synthesized (C). Primary screening: N27 cells were treated with the peptides were probed for PKD1 (pS744E/pS748E) at 60 min (D). Secondary screening: N27 cells were treated with the peptides and PKD1 kinase activity was measured using [³²P] kinase assay; the bands were quantified for the graph *, p<0.05, **, p<0.01, ***, p<0.001 denotes significant difference between the control peptide and activator/inhibitor peptide groups (E). [³²P] kinase assay was performed using recombinant human PKD1 protein in a cell-free system to test the specific and direct role of AK-P4 peptide (F). N27 cells transfected with 5 μ M PKD1 kinase activity reporter (PKD1-KAR) plasmid and 5 μ M vector plasmid were treated with AK-P4 and monitored for CFP/YFP FRET change (G).

Figure 3. AK-P4 protects against neuronal death in dopaminergic neurons

N27 dopaminergic cells were treated with 6-OHDA (100 μ M) and MPP⁺ (300 μ M) with or without control peptide, AK-P3, AK-P4 and probed for cytotoxicity (A, B). N27 cells were treated with 6-OHDA (100 μ M) and co-treated with different doses (0-10 μ M) AK-P4T (AK-P4 was tagged with aa HIV-TAT transporter peptide) and probed for cytotoxicity (C). Mouse primary mesencephalic neurons were co-treated with or without 50 nM AK-P4T and

monitored for number of protected TH neurons (D), TH neurite length (E), neurotransmission function (F), length viability during MPP⁺ treatment using ³H dopamine uptake assay (G), TH neuronal morphology (G). *, p<0.05, **, p<0.01, ***, p<0.001 denotes significant difference between the control and treatment groups.

Figure 4. In-vivo delivery of AK-P4T

C57Bl/6 mice was administered AK-P4T through intra-peritoneal injections (A), intra-nasal administration of peptides coated with nanopolymers (B), intra-venous administration (C) and monitored for PKD1pS744/pS748 in the substantia nigra and striatum (D). C57Bl/6 mice was administered AK-P4T through intra-venous injections and monitored for PKD1pS916 in the substantia nigra.

Figure 5. AK-P4T mediated PKD1 activation rescues neurotransmitter levels and dopaminergic function in pr-clinical model of PD

Neuroprotective efficacy of AK-P4T was examined in a sub-acute MPTP mouse model of PD. Animals pre-treated and co-treated with AK-P4T and control peptide (5 mg/kg body weight) were sacrificed at the end of the study and the following analyses were performed: Striatal brain lysates were analysed for Dopamine (A), DOPAC (B), HVA (C) levels were measured using HPLC. Striatal lysates were blotted for Tyrosine Hydroxylase (TH) (D). Immunohistochemical analysis of dopaminergic neurons staining for tyrosine hydroxylase (TH) DAB staining were performed from Mouse brain slices cut at the level of the striatum (E) and substantia nigra (F). Substantia nigral lysates were blotted for Tyrosine Hydroxylase (TH) and PKD1 pS744/S748 (G).

Figure 6. AK-P4T completely attenuates behavioral and locomotory deficits in pre-clinical PD model

Behavioral and locomotory changes were measured after AK-P4T and control peptide (5 mg/kg body weight) co-treatment in the sub-chronic MPTP mouse model of PD. Following parameters were measured using versamex: horizontal motor activity, number of movements, vertical motor activity, vertical movement time, total distance travelled, vertical number of movements, movement time, stereotypy count, margin distance, margin time, stereotypy time, rearing activity, rearing time, were significantly reduced in MPTP-treated animals compared with the vehicle-treated group (Fig 6B-N).

Figure 7. Working Model for AK-P4T activation mechanism

1. Regulatory fragment auto-inhibits catalytic fragment through inhibitory pockets containing the pseudo-substrate like sequences.
2. Peptide regulators (AK-Series) mimics the inhibitory pockets, interacts with the catalytic fragment and release the auto-inhibition.
3. Persistent activation of PKD1 promotes neuronal survival and neurodegeneration

Chapter VI: GENERAL CONCLUSIONS

The major findings of each research chapter included in this dissertation have been described and the implications for dopaminergic degeneration have been interpreted in the discussion section of each individual chapter. This section presents an overview of the results and findings of this dissertation, with special emphasis on the role of the survival switch, protein kinase D1 signal transduction, with respect to dopaminergic degeneration and development of a PKD1 activator for neuroprotection.

A novel oxidative signal transduction circuit controlling neuronal survival and death during oxidative stress in the dopaminergic system.

Our results demonstrate that the PKD1-mediated protective mechanism is a novel signal transduction pathway that regulates cell survival and cell death during various stages of oxidative stress in dopaminergic neuronal cells. In the early stages of oxidative insult, PKC δ acts as an oxidative stress sensor/regulator and activates PKD1, which serves as a key compensatory protective mechanism against oxidative damage. However, prolonged oxidative insult creates a homeostatic imbalance, causing deactivation of PKD1 and persistent proteolytic activation of PKC δ that contribute to extensive neuronal damage. We report that cleaved active PKC δ (PKC δ -CF) phosphorylates the activation loop (Ser744/Ser748) of PKD1 and activates the kinase during the early stages of H₂O₂- and 6-OHDA-induced oxidative stress in dopaminergic neuronal cells. Multiple phosphorylation sites on PKD1 seem to be important for its activation loop phosphorylation, depending on the cell types and stimuli. Our data suggest that Ser 916 phosphorylation in the C-terminal of PKD1 may open the conformation for full activation of the kinase through activation loop phosphorylation during oxidative stress in dopaminergic neurons. When the constitutively active

PKD1 mutant (PKD1S744E/S748E) is overexpressed, dopaminergic cells are resistant to H₂O₂- and 6-OHDA-induced neurotoxicity, even during the late stages of oxidative stress, which demonstrates that PKD1 activation protects against oxidative damage.

The anti-apoptotic PKD1 signal transduction is regulated by Fyn kinase-PKC δ in in vitro and in vivo models of PD.

Using the classic MPTP disease model to induce dopaminergic degeneration and the powerful transgenic mouse models as tools, we further investigated the biomedical importance of this novel survival signaling pathway in dopaminergic degenerative processes. Our current results show that the parkinsonian specific toxicant MPP⁺ induced PKD1 activation in a PKC δ dependent mechanism. Results from N27 cells and primary mesencephalic neurons show that PKC δ inhibition prevents PKD1 activation during MPP⁺-induced oxidative damage. Our findings were strengthened by using PKC δ (-/-) (PKC δ knockout) mice subjected to acute MPTP treatment. Dopaminergic neurons from PKC δ (-/-) mice exposed to MPTP/MPP⁺ treatment do not show PKD1 activation. Following this observation, we also looked at the key upstream regulator of PKC δ -PKD1 signaling. Fyn kinase has recently been shown to regulate PKC δ proteolytic activation in both cell culture and animal models of PD(95). We examined whether Fyn kinase acts as an upstream regulator of PKD1 activation via PKC δ . Dopaminergic neurons from Fyn (-/-) (Fyn kinase knockout) mice treated with MPTP do not show PKD1 activation, which is in agreement with the results obtained in cell culture models treated with MPP⁺. Our examination of samples obtained from post-mortem human PD patients showed high levels of PKD1 activation compared to age matched controls. Put together, we suggest that the anti-apoptotic PKD1 signaling

controlled by Fyn-PKC δ is activated as an early protective compensatory mechanism in Parkinson's disease.

Rationally designed peptide activates PKD1, protects against dopaminergic degeneration and rescues normal neurochemical and behavioral patterns in pre-clinical models of PD.

Based on these findings, we hypothesized that prolonging the activation of the survival switch PKD1 through a specific activator can prevent or delay PD progression. PKD1 in its native state is auto-inhibited by its regulatory fragment consisting of the cysteine rich domains and pleckstin homology (PH) domain. We rationally designed peptide mimics to interfere with intra-protein interactions and modulate PKD1. We screened the peptides to activate PKD1 using different strategies, including the radioactive kinase assay and the genetic reporter based assay. We developed the genetic reporter assays in high throughput format for monitoring PKD1 kinase activity. The peptide AK-P4, mimicking the highly conserved pseudo-substrate like region in the PH domain, activated and protected against both 6-OHDA and MPP+ mediated toxicity.

Previous studies have shown that the cell penetrating peptides derived from the trans-activating transcriptional activator (TAT) from HIV can be used to deliver therapeutic peptides (96). AK-P4 Peptide was conjugated with the TAT47-57 (AK-P4T) to facilitate effective delivery across the blood-brain barrier. Although progress has been made in delivering therapeutic peptides for neurodegenerative disorders, effective peptide drug delivery needs to be improved. We tested different routes and methods to effectively deliver the peptide; our results indicate that only AK-P4T peptide administered through the intravenous route can activate PKD1 in the nigrostriatal pathway after crossing the blood-brain barrier. Our findings in the subchronic MPTP model

demonstrate that AK-P4T delivered intravenously activates PKD1, and rescues dopaminergic neurons from death. Further, the nigrostriatal dopaminergic neuronal loss is reduced, and neurotransmission and motor function are considerably improved to control levels.

In summary, proteolytically activated catalytic PKC δ fragment (PKC δ -CF) phosphorylates protein kinase D1 (PKD1), resulting in PKD1 activation, which further counteracts early stage oxidative damage and protects dopaminergic neurons. Positive modulation of PKD1 using genetic or pharmacological methods protects against oxidative damage, whereas negative modulation of PKD1 exacerbates oxidative damage. PKD1 signaling is biomedically important for survival, as shown in MPTP models. Fyn Kinase acts as an upstream regulator of PKC δ – PKD1 signaling both in vitro and in vivo. The activation of the PKD1 protective signaling mechanism mediated by a rationally designed peptide activator offers neuroprotection in preclinical models of PD. The development of our translational strategy shows therapeutic promise for PD.

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