IOWA STATE UNIVERSITY
Digital Repository

Graduate Theses and Dissertations

2016

Translational drug discovery approaches targeting the PKD1 signaling in Parkinson's disease

Muhammet Ay
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/etd

Part of the Genetics Commons, and the Neuroscience and Neurobiology Commons

Translational drug discovery approaches targeting the PKD1 signaling in

Parkinson's disease

by

Muhammet Ay

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:

Anumantha G. Kanthasamy, Major Professor
Qijing Zhang
Arthi Kanthasamy
Edward Yu
Cathy Miller
Joshua Selsby

Iowa State University

Ames, Iowa

2016

To my father Mustafa Ay

TABLE OF CONTENTS

ABSTRACT

Parkinson's disease (PD) is a chronic neurodegenerative disorder that affects about 1% of people over the age of 60. PD is characterized pathologically by the progressive loss of dopaminergic neurons and the presence of Lewy bodies in the substantia nigra, dramatic depletion of dopamine in striatum, and activation of glial cells. Although the etiology of PD is not fully understood, evidence points strongly to the involvement of oxidative stress and mitochondrial dysfunction resulting from impaired mitochondrial biogenesis. Therefore, identifying cell signaling mechanisms regulating mitochondrial biogenesis is critically important to the development of new treatment strategies for PD. We have recently shown that activation of protein kinase D1 (PKD1) is neuroprotective and that positive modulation of PKD1 protects against neuronal cell death in cell culture models of PD. The goals of my Ph.D. thesis work were to understand the molecular mechanisms of PKD1-mediated neuroprotection using cell culture and animal models of PD and to test the efficacy of positive modulators of PKD1 signaling as potential neuroprotective agents in preclinical models of PD. Herein, we demonstrate for the first time that PKD1 activation positively regulates PGC-1α transcriptional activity. Overexpression of constitutively active PKD1 increased PGC-1α promoter activity, mRNA and protein expression in MN9D dopaminergic neuronal cells. Moreover, treatment of MN9D cells with rationally designed PKD1 activator peptide (AKP4T) enhanced expression of PGC-1α and other markers of mitochondrial biogenesis. Interestingly, treatment of cells with a PKD1 inhibitor (kbNB-14270) strongly suppressed mRNA expression of PGC-1α and TFAM. Importantly, AKP4T treatment protected against dopaminergic neurotoxicity in human dopaminergic neurons. Next, we adopted a rationale-based pharmacological screening

approach to identify natural compounds that activate PKD1 and found that quercetin effectively activated PKD1-mediated neuroprotective signaling and promoted mitochondrial biogenesis and bioenergetics capacity in dopaminergic cells. Furthermore, quercetin protected against dopaminergic neurodegeneration by reversing striatal dopamine depletion, TH neuronal cell loss and behavioral deficits in the MitoPark transgenic mouse model of PD. Finally, we synthesized Mito-Met, a mitochondria-targeted analog of the anti-diabetic drug metformin. We demonstrate that Mito-Met induces activation of pro-survival PKD1 kinase more potently than its parent compound metformin in dopaminergic neuronal cells. Interestingly, Mito-Met stimulated mitochondrial biogenesis and bioenergetics capacity and also protected against MPP^+-induced neurotoxicity. More importantly, Mito-Met treatment significantly reduced motor deficits and striatal dopamine depletion in MitoPark mice. Collectively, the research described herein provides evidence that PKD1 activation promotes mitochondrial biogenesis in dopaminergic neurons. We also show that positive modulation of PKD1 signaling affords neuroprotection against dopaminergic neurodegeneration in preclinical models of PD. Our results suggest that PKD1 is a promising druggable target that merits further preclinical investigations for the treatment of PD.

CHAPTER I: GENERAL INTRODUCTION

Dissertation Organization

This dissertation is written in an alternative thesis format and consists of manuscripts that are being prepared for submission. The dissertation contains a general introduction, three research papers, a general conclusion that discusses the overall findings from all the chapters, and acknowledgments. The references for each individual section are listed at the end of the corresponding chapters, except for the general introduction whose references are listed following the general conclusions. The introduction under Chapter I provides a brief overview of Parkinson's disease (PD) and the research objective. The Background and Literature Review I provides background information on PD and the role of oxidative stress and mitochondrial dysfunction in its pathogenesis. The Background and Literature Review II summarizes the role of protein kinase D1 (PKD1) in different cellular functions and in the pathology of various diseases. The Background and Literature Review III is a book chapter that was recently published in *Nutraceuticals: Efficacy, Safety, and Toxicity.* Chapter II, III, and IV will be shortly communicated to the *Journal of Neurochemistry*, *Molecular Neurodegeneration*, and *Molecular Pharmacology*, respectively.

This dissertation contains the experimental results obtained by the author during his Ph.D. study under the supervision of his major professor Dr. Anumantha G. Kanthasamy at Iowa State University.

Introduction

Parkinson's disease (PD) is a chronic, progressive, neurodegenerative disorder that affects about 1 million people in the United States and the frequency of PD is predicted to double by 2040 as the average age of population increases. PD was first described by James Parkinson in 1817 as the "shaking palsy", and it is recognized today as the second most prevalent neurodegenerative disorder after Alzheimer's disease (Shulman et al., 2011). The average age of onset is 60 years and the average duration of the disease from diagnosis to death is 15 years (Lees et al., 2009). Studies have suggested that men are more prone to develop PD than women (Baldereschi et al., 2000, Van Den Eeden et al., 2003, Wooten et al., 2004). Pathological hallmarks of PD are the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of Lewy bodies containing aggregated α-synuclein and other degradation products (Dauer and Przedborski, 2003). When the symptoms of PD begin to appear, around 80% of striatal dopamine is depleted and around 60% of dopaminergic neurons in the SNpc have already been lost. The main clinical feature of PD is severe impairment in motor function such as tremors, bradykinesia, rigidity, gait difficulty, and postural instability (Thomas and Beal, 2007). Recently, non-motor symptoms have also been associated with PD. Non-motor symptoms of PD include speech abnormalities, sleep difficulties, constipation, olfactory dysfunction, depression, and cognitive impairment (Chaudhuri and Schapira, 2009). Current treatment options for PD provide only symptomatic improvement and fail to halt the progression of the neurodegenerative process due to the lack of mechanistic insights into the selective degeneration of dopaminergic neurons in the SNpc. Although the pathological mechanisms underlying the initiation of dopaminergic degeneration

remain elusive, oxidative stress, mitochondrial dysfunction, proteosomal dysfunction, and apoptosis are recognized as key elements in the pathogenesis of PD (Levy et al., 2009). Epidemiological studies suggest that both genetic and environmental factors play a role in the pathogenesis of PD (Di Monte, 2003). Several causative genes have been linked to PD development, such as α-synuclein, DJ-1, Parkin, LRRK2, PINK1 and Uch-L1. However, over 90% of PD cases are sporadic, in which environmental factors play a dominant etiologic role (Lai et al., 2002). These environmental factors include exposure to pesticides, herbicides, heavy metals, well-water drinking, and head trauma (Lai et al., 2002, Goldman, 2014, Elbaz et al., 2015).

Mitochondrial dysfunction and oxidative stress have been well-established in the pathogenesis of neurodegenerative diseases including PD (Beal, 2003, Chaturvedi and Flint Beal, 2013, Subramaniam and Chesselet, 2013). Parkinsonian neurotoxicants, such as 1-methyl-4-phenylpyridinium (MPP^+) or rotenone, inhibit mitochondrial complex I activity, and reduced complex I activity has been found in the substantia nigra of PD patients (Schapira et al., 1990). Accumulating evidence suggests that impaired mitochondrial biogenesis is a major mechanism of mitochondrial dysfunction in PD (Zheng et al., 2010, Youdim and Oh, 2013). Hence, identifying the key signaling mechanisms regulating mitochondrial biogenesis is essential for the development of new treatment strategies for PD. We have recently shown that PKD1 is an oxidative stress-sensitive kinase that plays a compensatory survival role in dopaminergic neurons (Asaithambi et al., 2011, Asaithambi et al., 2014, Ay et al., 2015). The present study seeks to understand the molecular mechanisms of PKD1-mediated neuroprotection and the potential role of PKD1 in regulating mitochondrial biogenesis.

Background and Literature Review I

This section of the review will cover the genetics of Parkinson's disease (PD), the role of mitochondrial dysfunction and oxidative stress in PD, transcriptional regulation of mitochondrial biogenesis, the molecular links between PD and Type 2 Diabetes (T2DM), the MitoPark transgenic mouse model of PD, and current neuroprotective strategies for PD.

The Genetics of PD

Although the great majority of PD cases is sporadic, around 5% to 10% of PD cases are due to Mendelian mutations. Our understanding of the genetic basis of PD has greatly advanced in the last two decades. The genes identified to cause familial forms of PD have been assigned to PARK loci and currently 18 PARK loci have been associated with PD (Table 1) (Fujioka et al., 2014). Better understanding of the genetic components of PD will also help in the identification of the molecular meshanisms underlying the pathogenesis of disease and provide a basis for the development of new therapeutic strategies for PD. Among the PARK genes whose mutations are associated with PD, *SNCA* (PARK1/PARK4), *LRRK2* (PARK8), *HTRA2* (PARK13), *VPS35* (PARK17), *EIF4G1* (PARK18), and an unknown gene PARK3 have been shown to cause dominantly inherited PD (Gasser et al., 1998, Singleton et al., 2003, Paisan-Ruiz et al., 2004, Strauss et al., 2005, Chartier-Harlin et al., 2011, Vilarino-Guell et al., 2011). On the other hand, *Parkin* (PARK2), *PINK1* (PARK6), *DJ-1* (PARK7), *ATP13A2* (PARK9), *PLA2G6* (PARK14), and *FBXO7* (PARK15) have been shown to cause recessively inherited PD (Kitada et al., 1998, Bonifati et al., 2003, Valente et al., 2004, Ramirez et al., 2006, Di Fonzo et al., 2009, Paisan-Ruiz et al., 2009).

Table 1: Summary of PD-associated genes

Locus	Chromosome	Gene	Inheritance and phenotype
PARK1	4q21-q23	*SNCA*	Dominant, DLB features
PARK2	6q25.2-q27	*Parkin*	Recessive, EO, no LB
PARK3	2p13	Unknown	Dominant, Classical PD
PARK4	4q21	*SNCA* (triplication)	Dominant, EO with DLB features
PARK5	4p13	*UCH-L1*	Classical PD
PARK6	1p36.2	*PINK1*	Recessive, EO, slow progression with LB
PARK7	1p36	*DJ1*	Recessive, EO, slow progression
PARK8	12q12	*LRRK2*	Dominant, Classical PD
PARK9	1p36	*ATP13A2*	Recessive, juvenile parkinsonism
PARK10	1p32	Unknown	Classical PD
PARK11	2q37	Unknown	Classical PD
PARK12	Xq21-q25	Unknown	X-linked, Classical PD
PARK13	2p12	*HTRA2*	Dominant or risk factor, Classical PD
PARK14	22q12-q13	*PLAG26*	Recessive, EO
PARK15	22q12-q13	*FBXO7*	Recessive, juvenile parkinsonism
PARK16	1q32	Unknown	Risk factor, Classical PD
PARK17	16q11.2	*VPS35*	Dominant, Classical PD
PARK18	3q27.1	*EIF4G1*	Dominant, Classical PD

EO: early onset, DLB: Dementia with Lewy bodies, LB: Lewy bodies. Classic PD refers to the late-onset clinical idiopathic PD phenotype.

The *SNCA* gene encoding the protein alpha-synuclein (α-synuclein) was the first gene implicated in the familial forms of PD (Polymeropoulos et al., 1997). Alpha-synuclein is a major component of Lewy bodies in patients with both familial and sporadic PD (Spillantini et al., 1997). Three missense mutations were detected in the *SNCA* gene at A53T, A30P, and E46K in Greek, German, and Spanish familial PD patients, respectively (Polymeropoulos et al., 1997, Kruger et al., 1998, Zarranz et al., 2004). Duplication of the *SNCA* gene is present in 1-2% of patients with autosomal dominant PD, whereas point mutations and triplications of

the *SNCA* gene is very rare (Ibanez et al., 2009). Mutations in the *SNCA* gene are recognized as the second most common cause of dominant PD. Most of the PD patients with *SNCA* mutations had clinical and pathological features similar to those observed in sporadic PD and responded to levodopa treatment. Moreover, inclusions of α-synuclein were also detected in other neurodegenerative diseases, including Lewy body dementia (LBD), Alzheimer disease (AD), and multiple system atrophy (MSA) (Spillantini et al., 1998, Mukaetova-Ladinska et al., 2000, Burn and Jaros, 2001).

LRRK2 is the second gene associated with autosomal dominant PD and the mutations in the *LRRK2 gene* are the most common cause of dominant PD that accounts for 5-40% of patients with sporadic or familial PD, depending on the population (Deng et al., 2005, Orr-Urtreger et al., 2007, Hulihan et al., 2008). More than 100 *LRRK2* mutations have been reported both in familial and sporadic cases of PD, but only seven of them are truly pathogenic (Nuytemans et al., 2010). In particular, the *LRRK2* G2019S mutation is the most frequent substitution in the Caucasian population, accounting for approximately 1-2% of sporadic and 5-6% of familial PD cases (Deng et al., 2005). G2019S mutation has been shown to increase the kinase activity of LRRK2 (Greggio and Cookson, 2009).

Mutations in the *parkin* gene cause autosomal recessive juvenile-onset parkinsonism (AR-JP) (Kitada et al., 1998). *Parkin* mutations are the most common cause of autosomal recessive early-onset PD. More than 100 *parkin* mutations, including point mutations, exonic rearrangements, deletions, and duplications, have been identified in PD families and these *parkin* mutations account for 50% of the familial and 20% of the sporadic early-onset PD cases (Lucking et al., 2000, Tan and Skipper, 2007). LB pathology is not observed in many patients

with pathogenic *parkin* mutations (Hattori et al., 2000). Parkin has been shown to function as an E3 ubiquitin ligase that ubiquitinates unnecessary, damaged or misfolded proteins for their degradation by the ubiquitin-proteosomal system (Leroy et al., 1998, Shimura et al., 2000). Parkin has been shown to play a role in the maintenance of mitochondria, repair of mitochondrial DNA damage, and mitophagy of dysfunctional mitochondria (Deng et al., 2008, Narendra et al., 2008, Rothfuss et al., 2009).

Mutations in the *PINK1* gene are the most common cause of autosomal recessive early-onset PD and include include point mutations, as well as insertions and deletions that result in frameshift and truncation of the protein (Kumazawa et al., 2008). Over 60 different pathogenic *PINK1* mutations were found in PD patients and *PINK1* mutations account for 1-8% of familial PD with recessive inheritance, depending on the ethnic background (Klein and Schlossmacher, 2007, Hatano et al., 2009). *PINK1* gene encodes a 581amino acid protein kinase that contains an N-terminal mitochondrial targeting motif, a catalytic serine/threonine kinase domain, and a C-terminal autoregulatory domain (Valente et al., 2004). The majority of the reported *PINK1* mutations are loss-of-function mutations that affect the kinase domain of the protein, indicating the important role of the enzymatic activity of PINK1 in the pathogenesis of PD (Abou-Sleiman et al., 2006). Recent studies have shown that PINK1 functions upstream of parkin in a common pathway which regulates the selective elimination of damaged mitochondria through mitophagy (Figure 1) (Youle and Narendra, 2011). PINK1 localizes to outer membrane of depolarized mitochondria and recruits parkin to damaged mitochondria from cytosol to initiate the mitophagy process. PINK1 has been shown to protect against oxidative stress-induced apoptosis (Petit et al., 2005).
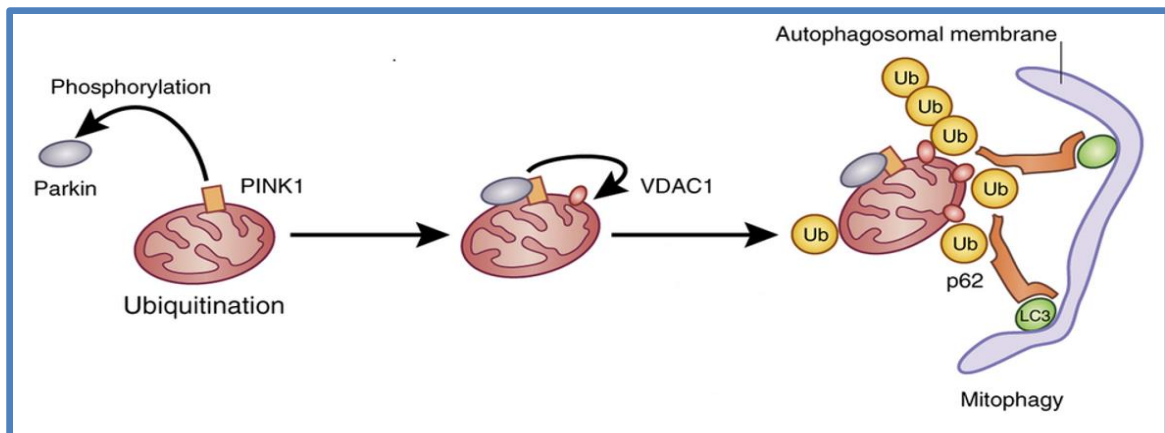
Figure 1: Regulation of mitophagy by PINK1 and Parkin. This figure is adapted and modified from (Popovic et al., 2014).

DJ-1 mutations cause autosomal recessive early-onset PD and account for 1% of early-onset PD cases (Abou-Sleiman et al., 2003). In general, patients with *DJ-1* mutations exhibit similar clinical presentation with *parkin* or *PINK1* mutations-associated parkinsonism (Hatano et al., 2009). The *DJ-1* gene encodes a ubiquitously expressed 189 amino acid protein that functions as an oxidative stress sensor (Canet-Aviles et al., 2004). It is noteworthy that disease-causing mutations in the *DJ-1* gene may lead to formation of heterodimers between wild type and mutant DJ-1 with reduced neuroprotective function and antioxidant activity (Malgieri and Eliezer, 2008). There is growing evidence suggests that DJ-1 functions in parallel with PINK1/parkin pathway to regulate mitochondrial function and mitophagy in response to oxidative stress (McCoy and Cookson, 2011, Thomas et al., 2011). Importantly, increased expression of DJ-1 and irreversibly oxidized DJ-1 protein were observed in human PD brain (Choi et al., 2006, Saito et al., 2014).

Mitochondrial dysfunction and oxidative stress in PD

Oxidative stress has been strongly implicated in the process of neurodegeneration in PD pathogenesis. Oxidative stress can result from either excessive production of ROS or

insufficient antioxidant defense and can potentially damage cellular lipids, proteins, and DNA (Miller et al., 2009). Postmortem studies have consistently revealed high levels of oxidation of lipids, proteins, and nucleic acids in the SNpc of sporadic PD brains (Alam et al., 1997, Jenner, 2003, Tsang and Chung, 2009). Mitochondrial respiratory chain is the main source of ROS, in particular the hydrogen peroxide and superoxide anions. These ROS can be converted to even more potent ROS, such as the hydroxyl radical and hydroxyl anion in the presence of ferrous iron (Winterbourn, 2008). It is worth mentioning that the level of iron is significantly increased in the SNpc of PD brains (Dexter et al., 1989). In addition to mitochondria, auto-oxidation of dopamine, a reaction that generates superoxide and hydrogen peroxide, as well as reactive dopamine quinones, specifically contributes to the cellular ROS in dopaminergic neurons (Hastings, 2009). This dopamine-dependent oxidative stress could at least partially explain the selective vulnerability of dopaminergic neurons in PD. Decreased GSH levels were also observed in the substantia nigra of PD patients (Sian et al., 1994). Nrf2 is a transcription factor that regulates the expression of antioxidant and anti-inflammatory proteins (Lee and Johnson, 2004). Interestingly, increased nuclear localization of Nrf2 was observed in human PD brain, suggesting the compensatory role of Nrf2 signaling to protect dopaminergic neurons (Ramsey et al., 2007). Studies have also demonstrated that overexpression of Nrf2 protected against dopaminergic neurodegeneration in a mouse model of PD (Chen et al., 2009) and that disruption of Nrf2 increased the vulnerability of neurons to MPTP-induced neurotoxicity (Burton et al., 2006). Another important contributor of oxidative stress is nitric oxide (NO), which is generated by nitric oxide synthase (NOS) (Jenner, 2003). Reaction of ROS with NO produces highly toxic reactive nitrogen species (RNS), such as the peroxynitrite and nitro-tyrosyl radicals (Zhang et al., 2000).

Overwhelming evidence suggests that mitochondrial dysfunction is an important contributor to the neurodegenerative process observed in PD (Thomas and Beal, 2007, Onyango, 2008). Impairment of complex I activity of the mitochondrial electron transport chain has been detected in the SNpc, skeletal muscle, lymphocytes, and platelets of patients with PD (Mizuno et al., 1989, Schapira et al., 1989, Barroso et al., 1993, Haas et al., 1995). Moreover, increased oxidation of complex I subunits and reduced rates of electron transfer through complex I, as well as misassembly of complex I, were also demonstrated in PD brains (Keeney et al., 2006). It is also noteworthy that increased mtDNA deletions were detected in nigral neurons in human PD brains (Bender et al., 2006).

MPTP is an environmental toxin that can cause an acute and irreversible parkinsonism in human and non-human primates (Langston et al., 1983). MPTP is a lipophilic molecule that can easily cross the blood-brain barrier and be metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP) in a reaction catalyzed by the monoamine oxidase B (MAOB) in glial cells. However, MPDP$^+$ is an unstable molecule which is immediately converted to 1-methyl-4-phenylpyridinium (MPP$^+$), the actual neurotoxin (Langston et al., 1984). MPP$^+$ is selectively taken up by the dopamine neurons via the dopamine transporter (DAT), where it is concentrated in mitochondaria, causes the complex I defect and in turn produces ROS and ultimately leads to cell death (Javitch et al., 1985, Przedborski and Vila, 2003). MPP$^+$ has been shown to activate apoptotic events including the activation of caspase-3 and caspase-9 and release of cytochrome c (Viswanath et al., 2001). In addition to MPTP, a variety of pesticides with related properties, such as rotenone, paraquat, and dieldrin have been reported to inhibit mitochondrial respiratory chain and induce oxidative stress (Brown et al., 2006).

Mitochondrial fusion and fission dynamics

Mitochondria are highly dynamic organelles which undergo continuous fusion and fission to maintain functional mitochondria. The fusion and fission processes are essential for cell growth, mitochondrial quality control, respiration, and the distribution of mitochondria within the cells, especially in neurons (Van Laar and Berman, 2009). Mitochondrial fusion process is governed by three different GTPases, mitofusion 1 (Mfn1), mitofusion 2 (Mfn2), and optic atrophy 1 (OPA1). Mfn1 and Mfn2 regulate outer membrane fusion, while OPA1 regulates the fusion of the inner mitochondrial membrane (Gaweda-Walerych and Zekanowski, 2013). Mitochondrial fission process is regulated by another GTPase, dynamin-related protein (Drp1) and mitochondrial fission 1 protein (Fis1) that is localized on the OMM and recruits Drp1 to sites of fission on the mitochondrial surface (Figure 2) (Suen et al., 2008).
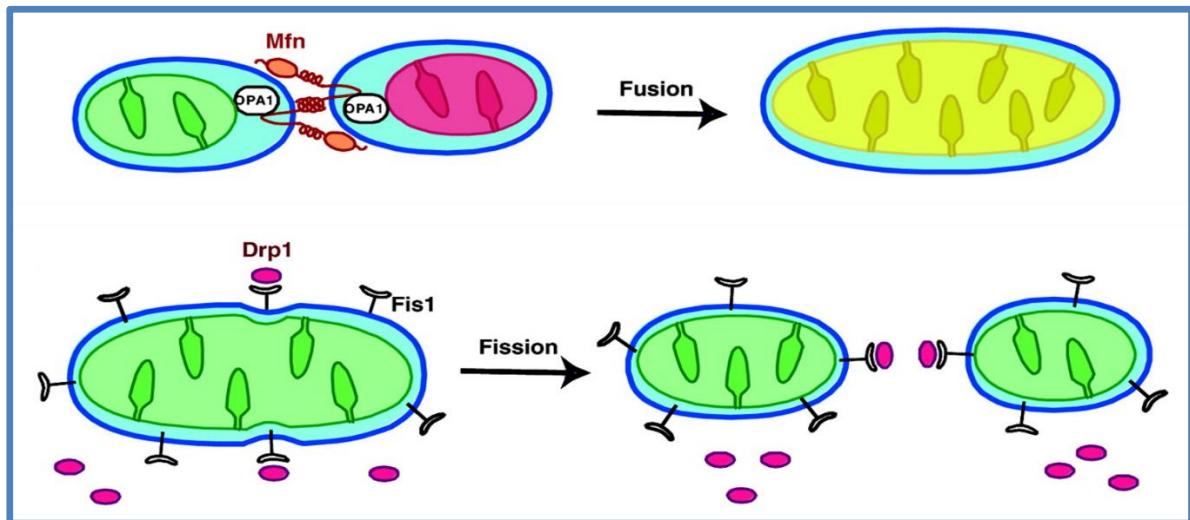

Figure 2: Regulation of mitochondrial fusion and fission processes. This figure is adapted and modified from (Chen and Chan, 2005).

Mitochondrial fusion process facilitates the complementation of damaged mtDNA, proteins, and lipids (Detmer and Chan, 2007). On the other hand, mitochondrial fission plays

a role in the transport of mitochondria to nerve terminals, apoptosis, and mitophagy (Perier and Vila, 2012). It was reported that knockdown of Drp1 protected against 6-OHDA-induced neurotoxicity in dopaminergic neuronal cells (Gomez-Lazaro et al., 2008). Moreover, Mfn1 overexpression was shown to attenuate nitric oxide-induced neuronal cell death (Barsoum et al., 2006). Importantly, treatment with a Drp1 inhibitor (mdivi-1) can significantly reverse the mitochondrial defects induced by the overexpression of mutant PINK1 (Cui et al., 2010). It was also shown that overexpression of Mfn2 or OPA1 protected against mitochondrial defects in parkin- or PINK1-deficient cells (Lutz et al., 2009). Recently, it has been shown that pharmacological or genetic inhibition of Drp1 significantly protects against dopaminergic neurotoxicity in animal models of PD (Rappold et al., 2014).

Transcriptional control of mitochondrial biogenesis

Mitochondrial dysfunction play a central role in the pathogenesis of PD (Exner et al., 2012). There is now growing evidence of impaired mitochondrial biogenesis as a major mechanism of mitochondrial dysfunction in PD (Zheng et al., 2010, Youdim and Oh, 2013). Mitochondrial biogenesis is a complex process that involves the growth and division of pre-existing mitochondria. The number of total functioning mitochondria in a cell is balanced by mitochondrial biogenesis and mitophagy processes. Mitochondria possess their own double-stranded circular DNA, which is ~16.5 kb in vertebrates and encodes 13 subunits of the respiratory chain, 22 tRNAs, and 2rRNAs (Perier and Vila, 2012). Since mitochondrial DNA (mtDNA) has a limited coding capacity, most of the proteins required for mitochondrial functions and biogenesis are encoded by nuclear DNA (Wiseman et al., 1977). Mitochondrial biogenesis is a highly regulated process and can be activated by different stimuli such as

exercise, cold exposure, caloric restriction, oxidative stress, cell division and differentiation (Ventura-Clapier et al., 2008). Transcriptional regulation of mitochondrial biogenesis includes peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α), the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2), and the mitochondrial transcription factor A (TFAM) (Scarpulla, 2008). PGC-1α is a transcriptional coactivator that stimulates mitochondrial biogenesis by activating NRF-1 and NRF-2 transcription factors, which increase the expression of TFAM. Accumulating evidence indicates that PGC-1α serves as a master regulator mitochondrial biogenesis in mammals (Ventura-Clapier et al., 2008, Jornayvaz and Shulman, 2010). The PGC-1 family of transcription coactivators consists of three proteins, PGC-1α, PGC-1β, and PGC-1-related coactivator (PRC) (Kelly and Scarpulla, 2004). The PGC-1 family members share similar molecular structure and functions. PGC-1α expression is inducible in response to physiological stimuli; however, PRC and PGC-1β expression are less inducible (Rowe et al., 2010). PGC-1α lacks DNA binding activity but interacts with different transcription factors including NRFs on the TFAM gene promoter (Liang and Ward, 2006). TFAM is required for mtDNA replication and transcription and binds to upstream of the light-strand promoter (LSP) and heavy-strand promoter (HSP) to regulate the expression of the 13 mtDNA gene products (Ngo et al., 2011). TFAM also binds to random sites on mtDNA and is involved in mtDNA maintenance and integrity (Scarpulla, 2008).

Molecular links between Type 2 Diabetes (T2DM) and PD

Type 2 diabetes (T2DM) is one of the most common chronic disorders and the most common form of diabetes, affecting approximately 90% of people with diabetes (Santiago and Potashkin, 2014). There is growing literature suggesting that T2DM is a risk factor for

neurodegenerative diseases including PD (Sun et al., 2012, Santiago and Potashkin, 2013a). Although the potential links between PD and T2DM have just begun to emerge, both diseases share some similar pathogenic pathways, such as mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress, and inflammation (Sandyk, 1993, Santiago and Potashkin, 2013b). There are also some shared genetic connections between PD and T2DM; expressions of two PD-associated genes, PINK1 and DJ1, were altered in patients with diabetes (Scheele et al., 2007, Jain et al., 2012). Moreover, PGC-1α expression was reported to be reduced in both T2DM and PD patients (Mootha et al., 2003, Zheng et al., 2010). Recently, it was reported that high fat-fed rats (an animal model for T2DM) showed increased vulnerability to a Parkinsonian specific toxicant 6-OHDA and exhibited enhanced nigrostriatal dopamine (DA) depletion (Morris et al., 2010). Another study demonstrated that treatment of T2DM mice with MPTP increased the loss of dopaminergic neurons and the activation of glial cells in the substantia nigra of db/db mice (Wang et al., 2014a). Furthermore, some agents used in the treatment of T2DM have been shown to have neuroprotective effects in animal models of PD (Dehmer et al., 2004, Li et al., 2010). Metformin is one the most commonly used anti-diabetic drug with a high safety profile (Dunn and Peters, 1995). Recently, metformin has been demonstrated to protect against dopaminergic neurodegeneration in the MPTP mouse model of PD (Patil et al., 2014).

The MitoPark mouse model of PD

The use of animal models to gain insight into human diseases and the potential treatment is extremely valuable. As opposed to isolated cell culture (*in vitro*) models, the use of a living animal as a model system is more reliable for studying multifactorial diseases like

PD. Although toxin-based models, such as rotenone, paraquat, 6-OHDA, and MPTP, have been widely used as animal models of PD, they don't fully recapitulate the human PD pathology. The acute nature of toxin-based models of PD limits their usefulness in PD drug development and to study the compensatory cell survival mechanisms which might occur in PD patients during the course of the disease. The MitoPark model has been recently developed as an animal model of PD by inactivation of TFAM in dopaminergic neurons (Ekstrand et al., 2007). TFAM is required for mtDNA replication and transcription, and also plays an important role for mtDNA maintenance and integrity (Scarpulla, 2008). The *TFAM* gene was disrupted by using the cre-loxP conditional knockout strategy. Dopamine transporter (DAT) promoter was used to drive the expression of *cre* recombinase. DAT-cre mice were crossed with loxP-flanked TFAM mice to selectively disrupt the *TFAM* gene in dopaminergic neurons (Figure 3). Given that mitochondrial dysfunction is an important factor in PD pathogenesis, MitoPark mouse was created to mimic mitochondrial dysfunction. The MitoPark transgenic mouse recapitulates several aspects of human PD, such as adult-onset, progressive degeneration of dopaminergic neurons, protein inclusion in nigral tissues, and responsiveness to levodopa (Galter et al., 2010). MitoPark motor performance does not differ from age-matched controls before the age of 12 weeks. However, at 12 weeks of age, these mice begin to display significantly reduced locomotor activity and these deficits become more

Figure 3: MitoPark Breeding Scheme

severe at 24 weeks of age. As previously reported, the progressive motor function is accompanied by a progressive loss of dopaminergic neurons in the substantia nigra and a reduction of striatal dopamine (Ekstrand et al., 2007).

Neuroprotective strategies for PD

Unfortunately, there is no cure for PD. Current treatment options for PD are only symptomatic and none of them slow or prevent the progression of PD (Obeso et al., 2010). Current standard treatment is based on levodopa, which is a dopamine precursor and processed to dopamine in the brain (Clarke, 2004). Although the dopamine replacement therapy with levodopa is initially effective for most patients to improve PD symptoms, the prolonged administration of levodopa can cause some side effects such as wearing-off, dyskinesias, and dystonia. Moreover, the clinical efficacy of levodopa often declines as the disease advances (Lewitt, 2008). Some of the PD medications and therapies in clinical trials will be summarized below.

Besides dopamine replacement therapies, dopamine agonists are also used to treat PD. Dopamine agonists mimick the effect of dopamine in the brain. Even though treatment with dopamine agonists, such as pramipexole and ropinerole, results in a lower incidence of dyskinesia than levodopa, they are not as effective as levodopa for treating all symptoms of PD and also cause some other side effects including hallucinations, gambling, and sexual obsessions (Kelley et al., 2012). It has been shown that glutamate can play a role in the pathophysiology of PD by acting as a neurotoxin when mitochondrial function is impaired in the dopaminergic neurons (Blandini et al., 1996). Studies have shown that riluzole, a glutamate antagonist, has neuroprotective effects in animal models of PD (Araki et al., 2001, Obinu et

al., 2002). However, no symptomatic effect of riluzole was observed in clinical trials in PD (Jankovic and Hunter, 2002). Monoamine oxidase (MAO) inhibitors inhibit degradation of dopamine and have potential as disease modifying agents for PD. MAO inhibitors can prolong the action of levodopa when used in combination with levodopa. The neuroprotective effects of rasagiline, a widely used MAO inhibitor, have been demonstrated in experimental models of PD (Kupsch et al., 2001, Mandel et al., 2005). Clinical studies have shown that rasagiline can alleviate symptoms of PD in patients when used as a monotherapy or in combination with levodopa (Parkinson Study, 2002, 2005, Rascol et al., 2005).   Catechol O-methyltransferase (COMT) inhibitors slow the metabolism of levodopa and allow more levodopa to reach the brain. Tolcapone, a COMT inhibitor, has been tested in PD patients as an adjunct to levodopa therapy. Clinical trials revealed that adjunctive therapy with tolcapone reduces the amount of levodopa needed and can improve motor symptoms of PD (Factor et al., 2001, Suchowersky et al., 2001). Interestingly, tolcapone has been recently shown to improve nonmotor symptoms of PD in levodopa-treated patients (Muller and Investigators, 2014). Although tolcapone was reported to cause liver toxicity in some patients (Watkins, 2000), tolcapone can be considered safe and well-tolerated in patients with PD (Borges, 2005, Lees et al., 2007).

Neurotrophic factors help survival of dopaminergic neurons in the substantia nigra and have been proposed as potential therapeutic agents for PD. Glial cell derived neurotrophic factor (GDNF) has been shown to have neuroprotective effects on dopaminergic neurons in experimental models of PD (Cass, 1996, Zeng et al., 2006, Schober et al., 2007). However, no benefit of GDNF was observed in clinical trials for PD (Nutt et al., 2003, Patel and Gill, 2007). Recently, neurturin gene therapy failed to show any significant efficacy for PD in a clinical trial (Warren Olanow et al., 2015).

Since mitochondrial dysfunction and oxidative stress have long been implicated as pathophysiological mechanisms of dopaminergic neurodegeneration, various bioenergetics and antioxidants are being tested in clinical trials of PD. Coenzyme Q10 (CoQ10) is an antioxidant and a component of the electron transport chain (ETC), where it accepts electrons from complexes I and II (Yang et al., 2009). Neuroprotective effects of CoQ10 have been reported in experimental models of PD (Beal et al., 1998, Moon et al., 2005, Somayajulu et al., 2005). Altough a previous phase II clinical trial showed a modest but nonsignificant benefit (Shults et al., 2002), a recently completed phase III clinical trial of CoQ10 failed to show any clinical benefit for PD patients (Parkinson Study Group et al., 2014). Creatine is a nitrogenous guanidine compound which forms high energy phosphate bonds and plays an important role in mitochondrial energy production. Studies have shown that creatine protected against neurodegeneration in cell culture and animal models of PD (Matthews et al., 1999, Andres et al., 2005). Unfortunately, a recently completed clinical trial revealed that creatine treatment did not improve clinical outcomes in PD patients (Writing Group for the et al., 2015).

In addition to the above-mentioned studies, several clinical trials are underway using antioxidants (inosine and glutathione), anti-inflammatory compounds (pioglitazone), compounds that increase the removal of alpha-synuclein from brain (phenylbutyrate), and repurposed antidiabetic drug (exenatide) and hypertension drug (isradipine) for patients with PD.

Background and Literature Review II

This section of the review will cover the classification and domain structure of protein kinase D1 (PKD1), the biological functions of PKD1, the mechanisms of PKD1 activation, the role of PKD1 in various disease pathologies, PKCδ-mediated PKD1 activation during oxidative stress, and the transcriptional regulation of PKD1 expression.

Protein kinase D1 (PKD1)

Protein kinase D1 (PKD1) was originally classified as an atypical member of the PKC family and called PKCμ (Johannes et al., 1994). However, later studies revealed that PKD1 has additional protein modules including a pleckstrin homology (PH) domain and a hydrophobic region that are not found in PKCs (Iglesias and Rozengurt, 1998). Moreover, the catalytic domain of PKD1 demonstrates high sequence similarity to the kinase domain of the calcium/calmodulin-dependent kinase (CAMK) family and PKD1 was therefore classified as a member of the CAMK family of protein kinases (Manning et al., 2002). PKD1 is a serine/threonine kinase and the founding member of the PKD family that also includes PKD2 and PKD3. PKD1 was first identified in both human and mouse in 1994 by two different laboratories and is the most studied member of the PKD family (Johannes et al., 1994, Valverde et al., 1994). PKD3 and PKD2 were identified in 1999 and 2001, respectively (Hayashi et al., 1999, Sturany et al., 2001).

PKD1 consists of an N-terminal regulatory fragment and a C-terminal catalytic fragment. The regulatory fragment contains two cysteine-rich zinc finger regions (C1a and C1b domains) and a pleckstrin homology (PH) domain (Figure 4). The PH domain has an inhibitory effect on the catalytic activity of PKD1, as deletion of the entire PH domain results

in constitutive activation of PKD1 (Iglesias and Rozengurt, 1998). The cysteine-rich domain (CRD) binds to both diacylglycerol (DAG) and phorbol esters and mediates PKD1 membrane translocation (Rozengurt et al., 1995). It was also reported that C1a domains shows a higher DAG affinity than the C1b domain while the C1b domain shows a higher affinity than C1a for phorbol esters (Chen et al., 2008a). Moreover, several phosphorylation sites (Y93, S208, S223, S255, Y469, S744, S748, S916) have been identified in PKD1 (Zhang et al., 2009).
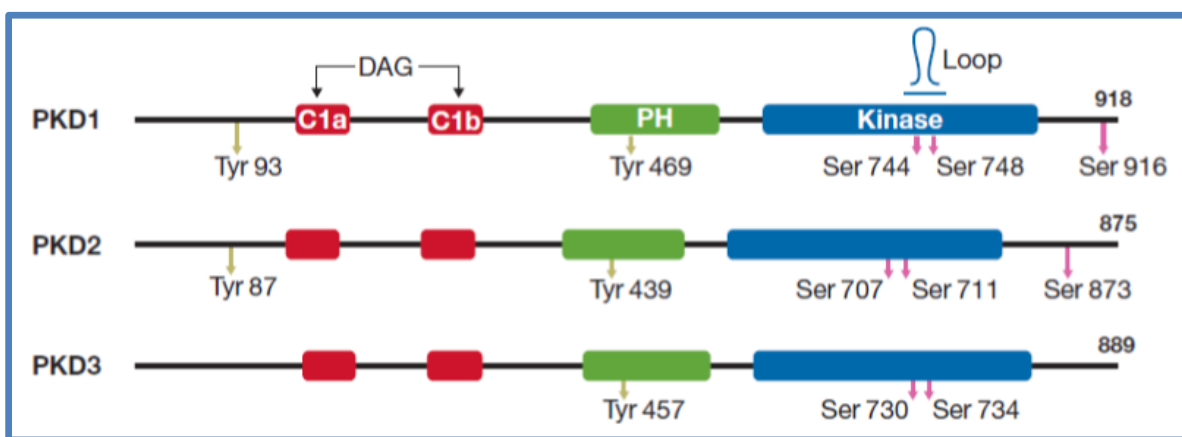
Figure 4: Domain structure of PKD isoforms. This figure is adapted and modified from (Fu and Rubin, 2011)

Biological functions of PKD1

PKD1 is mainly localized in cytosol and is also found in Golgi apparatus and mitochondria in small quantities (Van Lint et al., 2002). We and others have shown that PKD1 can also translocate to nucleus upon activation (Rey et al., 2001, Asaithambi et al., 2014). PKD1 carries out different cellular functions depending on its subcellular localization and has a quite distinct substrate specificity (Table 2). Under resting conditions, PKD1 localizes in cytosol and has very low kinase activity because of the inhibitory effect of CRD and PH domains on the catalytic domain (Iglesias and Rozengurt, 1998, 1999).

Table 2: The list of known substrates of the PKD family

PKD Substrate	Residue	Target Sequence
	−5 −30	
CERT (human)	S132	S L R R H G S M V S
Cortactin (human)	S298	K L A K H E S Q Q D
CREB (human)	S133	I L S R R P S Y R K
DLC-1 (human)	S327	P V T R T R S L S A
HDAC5 (human)	S259	P L R K T A S E P N
HDAC5 (human)	S498	P L S R T Q S S P L
HDAC7 (human)	S155	P L R K T V S E P N
HDAC7 (human)	S358	P L S R T R S E P L
HDAC7 (human)	S486	P L S R A Q S S P A
HPK1 (human)	S171	T L A R R L S F I G
HSP27 (human)	S82	A L S R Q L S S G V
Kidins22 (rat)	S918	T I T R Q M S F D L
MYBPC3 (human)	S304	S L L K K S S F R T
Par-1 (human)	S400	K V Q R S V S A N P
Rin1 (human)	S351	P L L R S M S A A F
SSH-1 (human)	S978	P L K R S H S L A K
TNNI (rat)	S24	P V R R R S S A N Y
TLR5 (human)	S805	Q L M K H Q S I R G
TRPV1 (rat)	S116	R L Y D R R S I F D

This table is adapted and modified from (Rozengurt, 2011).

At the Golgi apparatus, PKD1 plays a role in vesicle formation and transport of these vesicles to the plasma membrane after the Golgi (Hausser et al., 2005). It has been shown that binding of PKD1 to the trans-Golgi network (TGN) is mediated by the C1a domain of PKD1 (Maeda et al., 2001). PKD1 has been shown to phosphorylate phosphatidylinositol-4-kinase III beta (PI4KIIIβ) in the Golgi (Hausser et al., 2005). PI4KIIIβ is known to regulate vesicle fission and transport of cargo proteins (Graham and Burd, 2011). Moreover, PKD1 regulates insulin secretion by increasing Golgi fission in β-cells (Sumara et al., 2009). Interestingly, PKD1 was reported to regulate the integrity of Golgi and dendritic arborization in hippocampal neurons (Czondor et al., 2009).

PKD1 has been shown to be involved in cell survival, proliferation, migration, and invasion processes that are vital to cancer progression (Wong and Jin, 2005, Guha et al., 2010,

LaValle et al., 2010). PKD1 overexpression resulted in increased DNA synthesis upon treatment with bombesin, vasopressin, and phorbol esters in Swiss 3T3 cells (Zhukova et al., 2001). PKD1 has been reported to positively modulate cell signaling pathways involved in cell proliferation, including the Ras-Raf-MEK-ERK signaling (Van Lint et al., 2002, Sinnett-Smith et al., 2004). PKD1 was also reported to mediate downregulation of the JNK signaling pathway, which has been implicated in the regulation of apoptotic pathways (Hurd and Rozengurt, 2001).

PKD1 signaling has been demonstrated to be involved in immune function. The activity of β1 integrins in T cells was shown to be regulated by PKD1 signaling (Medeiros et al., 2005). It was shown that PKD1 plays a role in the proliferation and differentiation of T cell progenitors in the tymus (Marklund et al., 2003). PKD1 has also been implicated in toll-like receptor (TLR) function. In macrophages, PKD1 has been shown to function as downstream target in TLR9 signaling (Park et al., 2008), while it was reported to be a downstream target in TLR2 signaling in mast cells (Murphy et al., 2007). In addition, it has been reported that PKD1 phosphorylates TLR5 on Ser805, resulting in increased p38 MAPK activation and production of inflammatory cytokines (Ivison et al., 2007).

The function of PKD1 in the brain still remains poorly understood. PKD1 has been shown to play a role in neuronal polarity and protein sorting and packaging in TGN (Bisbal et al., 2008). Bisbal et al showed that PKD1 suppression resulted in mispackaging and increased endocytosis of dendritic membrane proteins, suggesting that PKD1 regulates protein sorting, packaging, and targeting in the TGN in hippocampal neurons. PKD1 has also been shown to phosphorylate KI220, a transmembrane scaffold protein at the plasma membrane which

regulates neuronal development and polarity (Sanchez-Ruiloba et al., 2006). It was also reported that PKD1 activation serves as an early marker of neuronal DNA damage (Besirli and Johnson, 2006). Recently, PKD1 has been shown to phosphorylate neuronal nitric oxide synthase (nNOS) on Ser1412, resulting in increased nNOS activity (Sanchez-Ruiloba et al., 2014). Moreover, Wang et al reported that PKD1 can exert cocaine-induced behavioral responses by directly phosphorylating the dopamine D1 receptor (D1R) on Ser421 (Wang et al., 2014b). The role of PKD1 in dopaminergic neurons will be discussed later in this chapter.

PKD1 activation mechanisms

Previous studies have revealed that PKD1 can be activated by at least three different mechanisms, including the phospholipase C/protein kinase C (PLC/PKC) signaling pathway, nonreceptor tyrosine kinases Src/Abl signaling pathway, and caspase 3-dependent proteolytic cleavage (Steinberg, 2012). The PLC/PKC signaling pathway is the best-understood mechanism of PKD1 activation (Rozengurt et al., 2005). The PLC signaling is activated by different stimuli, including G-protein coupled receptor (GPCR) agonists, neuropeptides, and growth factors. The PLC activation results in DAG production from the membrane lipid phosphatidylinositol 4, 5-bisphosphate (PIP2). DAG can directly interact with the C1b domain of PKD1, resulting in the activation and recruitment of PKD1 to the plasma membrane. DAG can also trigger PKD1 activation indirectly through activation of PKCs at the membrane. Following the membrane translocation of PKD1, PKD1 gets phosphorylated in the activation loop at Ser744 and Ser748 residues by novel PKCs (Waldron et al., 1999, Rykx et al., 2003). This activation loop phosphorylation of PKD1 releases the autoinhibitory effect of PH domain on the catalytic domain and results in PKD1 activation (Rozengurt et al., 2005). It is

noteworthy that treatment with PKC inhibitors completely blocked DAG-induced PKD1 activation and that mutation of both serine residues to alanine (Ser744/748Ala) suppressed PKD1 activation by DAG (Van Lint et al., 2002, Rykx et al., 2003).

Src and c-Abl kinases have been shown to phosphorylate PKD1 at Tyr463 (in the PH domain) during oxidative stress conditions (Storz and Toker, 2003). Tyr463 phosphorylation of PKD1 opens up the kinase through a conformational change, releases the intramolecular autoinhibition, and leads to PKD1 activation by PKCδ (Storz, 2007). Storz et al showed that PKD1 functions as a mitochondrial specific oxidative stress sensor (Storz et al., 2005). It has been demonstrated that mitochondrial ROS increases DAG formation catalyzed by phospholipase D1 (PLD1) and binding of DAG to the C1a and C1b domains of PKD1 is essential for the activation and mitochondrial translocation of PKD1 (Cowell et al., 2009). Moreover, Cowell et al showed that overexpression of active Src led to increased mitochondrial localization of PKD1, while treatment with Src family kinase (SFK) inhibitor blocked mitochondrial localization of PKD1.

PKD1 can also be activated by caspase-3 cleavage (Endo et al., 2000, Vantus et al., 2004). PKD1 cleavage was observed in U937 cells treated with apoptotic agents, including TNFα and beta-D-arabinofuranosylcytosine (ara-C) (Haussermann et al., 1999). Caspase-3 cleaves PKD1 between the cysteine-rich and PH domains and generates F62 and F59 PKD1 fragments (Vantus et al., 2004). Vantus et al reported that PKD1 cleavage product showed higher kinase activity compared with the full length PKD1. Moreover, overexpression of the 62 kDa fragment of PKD1 led to increased susceptibility of cells to DNA damage-induced apoptosis (Endo et al., 2000).

The role of PKD1 in various disease pathologies

Given that PKD1 plays an important role in a number of cellular processes, including cell proliferation, survival, and migration and immune function, dysregulation of PKD1 expression has been linked to the development of various diseases, including cancers and cardiovascular hypertrophy. PKD1 has gained substantial interest in recent years as a potential druggable target for cancer treatment. Studies have revealed that expression of PKD isoforms is either upregulated or downregulated in various cancer types (LaValle et al., 2010, Sundram et al., 2011).

PKD1 is downregulated in breast tumors compared to normal breast tissues (Eiseler et al., 2009). Eiseler et al demonstrated that PKD1 negatively modulated the expression of matrix metalloproteinases (MMPs) and that PKD1 expression was downregulated through DNA methylation in breast cancer cells. It is noteworthy that there was no detectable change in PKD2 and PKD3 expression in breast cancer tissues. Moreover, PKD1 and PKD2 have been shown to phosphorylate the cofilin phosphatase slingshot 1 like (SSH1L) and inhibit cell migration in breast cancer cell lines (Peterburs et al., 2009).

PKD1 expression is also downregulated in gastric tumors and gastric cancer cell lines via promoter hypermethylation (Kim et al., 2008). Their results revealed that promoter methylation of PKD1 gradually increased with aging in normal mucosal tissues, indicating that PKD1 methylation could be an early event that predisposes people to gastric cancer. In addition, RNAi-mediated knockdown of PKD1 significantly increased migration and invasion in gastric cancer cells.

PKD1 has been shown to inhibit androgen receptor (AR) function and migration and proliferation of prostate cancer cells, suggesting PKD1 plays a tumor suppressor role in prostate cancer (Mak et al., 2008). Decreased expression of PKD1 was observed in androgen-independent human prostate cancer specimens (Jaggi et al., 2003). Jaggi et al demonstrated that the expression of PKD1 was gradually decreased during the progression of prostate cancer from androgen-dependent to androgen-independent state. PKD1 has been reported to phosphorylate E-cadherin, resulting in enhanced cell-cell adhesion and decreased cell motility in prostate cancer (Jaggi et al., 2005). In contrast, increased expression and nuclear localization of PKD3 was observed in prostate tumor tissues (Chen et al., 2008b). They also showed that knockdown of PKD3 significantly attenuated cell proliferation in PC3 cells, suggesting the isoform-specific functions of PKD1 and PKD3 in prostate cancer.

PKD1 has been shown to be upregulated in pancreatic tumor tissues compared to normal pancreatic tissues (Trauzold et al., 2003). Their results showed that pharmacological inhibition of PKD1 increased the susceptibility of pancreatic adenocarcinoma cells to apoptosis, while overexpression of PKD1 increased cell growth and telomerase activity. Furthermore, Harikumar et al reported that treatment with a pharmacological inhibitor of PKD1 significantly reduced pancreatic cancer growth and decreased the expression of cyclin D1, survivin, and cIAP-1 proteins in a xenograft mouse model of pancreatic cancer (Harikumar et al., 2010).

Increased expression of PKD1 was also observed in basal cell carcinoma (BCC) (Ristich et al., 2006). Moreover, exposure of mouse keratinocytes to ultraviolet B (UVB) irradiation led to PKD1 activation mediated by Src family tyrosine kinases (Arun et al., 2011).

They also demonstrated that PKD1 overexpression resulted in a significant decrease in UVB-induced apoptosis.

Besides its role in cancer development, PKD1 has also been shown to function as a mediator of cardiac hypertrophy (Vega et al., 2004, Harrison et al., 2006, Fielitz et al., 2008). Previous studies have suggested that class II histone deacetylases (HDACs) are negative regulators of cardiac hypertrophy by virtue of its inhibitory effect on the myocyte enhancer factor 2 (MEF2) transcription factor (Figure 5) (McKinsey e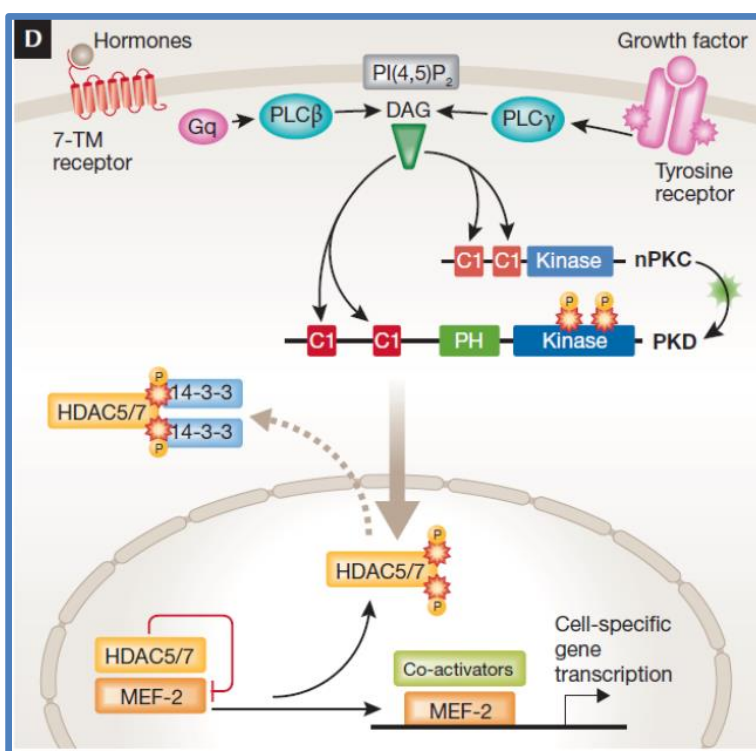t al., 2000, Zhang et al., 2002). MEF2 regulates the expression of genes involved in pathological cardiac remodeling and myocyte hypertrophy (McKinsey et al., 2000, Backs and Olson, 2006). It has been shown that PKD1 can directly phosphorylate HDAC5, a member of class IIa HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) (Huynh and McKinsey, 2006). Following

Figure 5: The role of PKD1 signaling in pathological cardiac remodeling. This figure is adapted and modified from (Fu and Rubin, 2011)

HDAC5 phosphorylation by PKD1, 14-3-3 proteins bind to HDAC5 and induces the nuclear export of HDAC5 (McKinsey et al., 2000, Vega et al., 2004). Harrison et al demonstrated that PKD1 is activated during the the pathological cardiac hypertrophy and that overexpression of the PKD1-CA in mouse heart resulted in dilated cardiomyopathy. They also found that PKD1

knockdown significantly attenuated agonist-induced HDAC5 nuclear export. Fielitz et al have shown that cardiac-specific PKD1 knockout mice displayed a reduced hypertrophy and fetal gene activation in response to pressure overload (Fielitz et al., 2008). Interestingly, another study have reported that HDAC9 knockout mice showed increased sensitivity to hypertrophic stimuli (Zhang et al., 2002). These results suggest that PKD1 functions as a class IIa HDAC kinase and plays an important role in cardiac hypertrophy.

PKCδ-mediated PKD1 activation during oxidative stress

Protein kinase C-delta (PKCδ), a member of the novel PKC family, is ubiquitously expressed in most tissues including brain, spleen, ovary, lung, and uterus, as well as many cell types (Leibersperger et al., 1991). A number of stimuli including ROS, chemicals, ultraviolet radiation, growth factors, and cytokines have been shown to activate PKCδ signaling (Denning et al., 1996, Konishi et al., 2001, Anantharam et al., 2002, Carpenter et al., 2002). PKCδ is activated by at least three different mechanisms, such as membrane translocation, proteolytic cleavage, and tyrosine phosphorylation.

Previous studies from our laboratory reported that PKCδ is an oxidative stress-sensitive kinase and that a proteolytically activated form of PKCδ has an important role in the process of dopaminergic neuronal cell death (Anantharam et al., 2002, Kaul et al., 2003, Kanthasamy et al., 2010). Caspase-3 cleaves PKCδ to yield a 41-kDa catalytically active and a 38-kDa regulatory PKCδ fragments, resulting in permanent dissociation of the regulatory subunit for persistent kinase activity. We have demonstrated that oxidative stress promotes caspase-3 dependent proteolytic activation of PKCδ and proteolytically activated PKCδ mediates apoptotic cell death induced by dopaminergic neurotoxins (Mn, dieldrin, MPP+, and 6-OHDA)

in cell culture and animal models of PD (Kaul et al., 2003, Kitazawa et al., 2003, Zhang et al., 2007). Furthermore, we have also shown that inhibition of PKCδ by treatment with a novel peptide inhibitor targeted the caspase-3 recognition site in PKCδ, or by RNAi-mediated knockdown of PKCδ is sufficient to prevent dopaminergic neuronal degeneration (Anantharam et al., 2004, Kanthasamy et al., 2006).

While studying the downstream signaling of PKCδ, we unexpectedly found that PKD1 is rapidly activated during the initial stage of oxidative stress. Our immunocytochemistry and Western blotting analyses revealed that PKD1 is highly expressed in dopaminergic neurons.
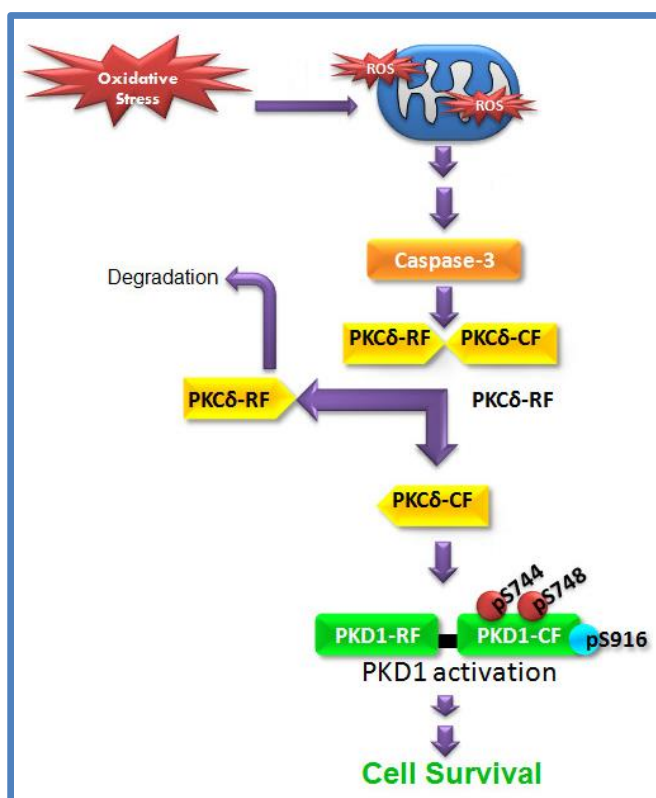

Figure 6: PKCδ-dependent PKD1 activation in dopaminergic neuronal cells. This figure is adapted and modified from (Asaithambi et al., 2011)

Next, we determined whether PKCδ plays any role in PKD1 activation during the early stage of oxidative stress. To test the role of PKCδ in PKD1 activation, we used both genetic and pharmacological approaches. Our results showed that treatment of cells with PKCδ inhibitor rottlerin and RNAi-mediated knockdown of PKCδ almost completely blocked PKD1 activation loop phosphorylation (Ser744/748) and kinase activity during oxidative stress (Asaithambi et al., 2011). Moreover, overexpression of the PKCδ catalytic fragment (PKCδ-CF) significantly induced PKD1 activation loop phosphorylation. After observing the PKCδ-dependent PKD1 activation, we
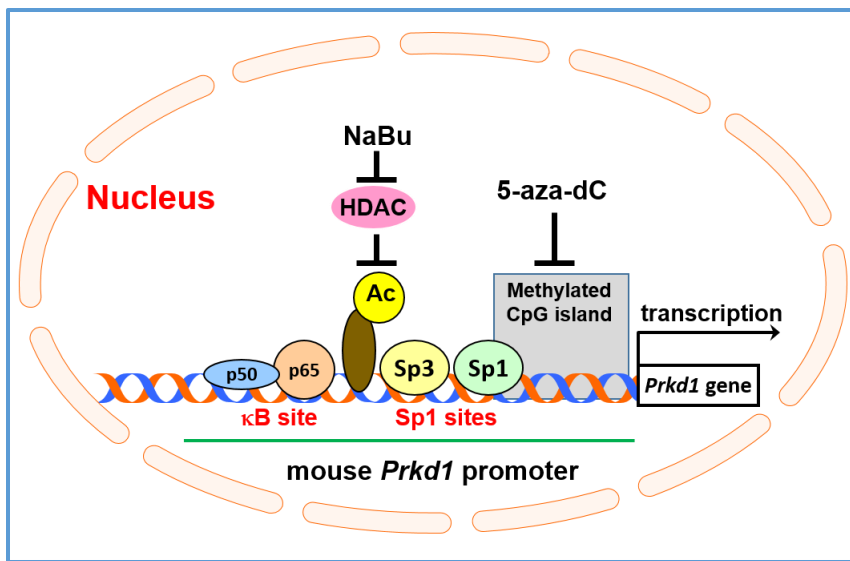
wanted to determine the functional role of PKD1 during oxidative stress. Surprisingly, RNAi-mediated knockdown of PKD1 increased susceptibility of dopaminergic cells to oxidative stress, while overexpression of the constitutively active mutant of PKD1 (PKD1-CA) significantly attenuated oxidative stress-induced cell death in dopaminergic cells. These results suggest that PKD1 plays a compensatory cell survival role during the initial stage of oxidative stress in dopaminergic neurons. Nevertheless, prolonged oxidative damage results in deactivation of PKD1 and persistent proteolytic activation of PKCδ, which leads to degeneration of dopaminergic neurons. Finally, PKCδ-dependent PKD1 activation is illustrated in Figure 6.

Transcriptional regulation of PKD1

The *Prkd1* gene encodes murine protein kinase D1 (PKD1) that is a ubiquitously expressed serine/threonine kinase. Although the mechanisms of PKD1 activation have been investigated in many model systems, the molecular mechanisms responsible for transcriptional regulation of the *Prkd1* gene in mammalian systems are largely unknown. This prompted us to investigate the structure of the mouse *Prkd1* promoter. We recently reported the molecular cloning, epigenetic regulation, and characterization of the *Prkd1* gene promoter in dopaminergic cells (Ay et al., 2015). To our knowledge, our study is the first to characterize the mouse *Prkd1* promoter and its activity.

In this study, we cloned a 1.6 kb fragment of the *Prkd1* gene promoter into a luciferase reporter vector. Our results revealed the presence of strong positive and negative regulatory elements within the non-coding part of exon 1, suggesting that non-coding part of exon 1 contributes to the transcriptional regulation of *Prkd1* gene promoter in neuronal cells.

Progressive 5' and 3' deletion analyses indicated that the promoter region -250/+113 had the strongest promoter activity. *In silico* analysis of the *Prkd1* promoter uncovered binding sites for key redox transcription factors including Sp1 and NF-κB. Overexpression of Sp1, Sp3, and NF-κB-p65 proteins stimulated *Prkd1* promoter activity. Binding of Sp3 and NF-κB-p65 to the *Prkd1* promoter was confirmed using chromatin immunoprecipitation. Moreover, treatment with the Sp inhibitor mithramycin-A significantly attenuated *Prkd1* promoter activity and PKD1 mRNA and protein expression, suggesting the regulatory roles of Sp1 and Sp3 on *Prkd1* promoter activity.

Figure 7: Transcriptional regulation of the *Prkd1* gene promoter

Further mechanistic studies revealed that pharmacologically inhibiting histone acetylation and DNA methylation upregulates PKD1 mRNA expression. Our results showed that *Prkd1* promoter is partially methylated in dopaminergic cells. Taken together, we demonstrated that Sp1, Sp3 and NF-κB-p65 can transactivate the mouse *Prkd1* promoter and that DNA methylation and histone modification are key regulatory events controlling the expression of pro-survival kinase PKD1 in dopaminergic neuronal cells (Figure 7).

Background and Literature Review III

Quercetin

Muhammet Ay, Adhithiya Charli, Huajun Jin, Vellareddy Anantharam, Arthi Kanthasamy, and Anumantha G. Kanthasamy‡

Parkinson's Disorder Research Laboratory, Iowa Center for Advanced Neurotoxicology, Department of Biomedical Sciences, Iowa State University, Ames, Iowa 50011, USA

‡ To whom correspondence should be addressed: Dept. of Biomedical Sciences, Iowa State University, 2062 College of Veterinary Medicine Building, Ames, IA 50011. Tel: 515-294-2516; Fax: 515-294-2315; Email: akanthas@iastate.edu

Abstract

Quercetin is a natural flavonoid found abundantly in vegetables and fruits. There is growing evidence suggesting that quercetin has therapeutic potential for the prevention and treatment of different diseases, including cardiovascular disease, cancer, and neurodegenerative disease. Mechanistically, quercetin has been shown to exert antioxidant, anti-inflammatory and anticancer activities in a number of cellular and animal models, as well as in humans through modulating the signaling pathways and gene expression involved in these processes. This book chapter focuses on experimental studies supporting the anticancer, cardioprotective, and neuroprotective effects of quercetin.

Introduction

Dietary flavonoids were first discovered by Nobel Prize laureate Albert Szent Gyorgyi in 1936, and they are widely recognized for their potential beneficial effects on human health. Quercetin is the major flavonoid in our daily diet (Table 1) (see further details: http://www.ars.usda.gov/SP2UserFiles/Place/80400525/Data/Flav/Flav_R03-1.pdf), and its estimated daily intake is between 5 and 40 mg (Hertog et al., 1995). After absorption, quercetin is mainly metabolized in the intestine and liver. The plasma concentration of quercetin is normally in the nanomolar range, but can reach the micromolar range after consumption of quercetin-rich foods (Russo et al., 2012). The table-1 summarizes foods with high quercetin levels.
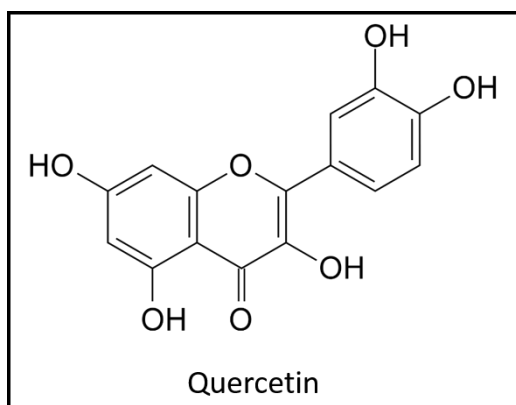
Quercetin contains three rings and five hydroxyl groups (Fig. 1). Quercetin mainly occurs as glycosides, ethers, and, to a lesser extent, sulfates (Williams and Grayer, 2004), and glycosylation of quercetin increases its hydrophilicity, stability, and bioavailability (He et al., 2006). So far, quercetin is the most extensively studied flavonoid that has been shown to exhibit antioxidant, antiviral, antibacterial, anti-inflammatory, and anticarcinogenic properties (Duthie and Dobson, 1999, Ramos et al., 2006, Boots et al., 2008, Zandi et al., 2011).

Table 3: Dietary sources and concentrations of quercetin

Food Source (*ref. USDA 2014*)	Quercetin Content (*mg/100g*)	Food Source (*ref. USDA 2014*)	Quercetin Content (*mg/100g*)
Capers, raw	234	Red chard leaves, raw	7
Dill weed, fresh	55	Chicory greens, raw	6
Oregano, fresh	7	Chives, raw	5
Apples with skin	4	Cilantro leaves, raw	53
Bayberries, raw	4	Corn poppy, leaves	26
Blackberries, raw	4	Dock, raw	86
Blueberries, raw	4	Drumstick leaves, raw	17
Cherries, sweet, raw	2	Fennel leaves, raw	49
Chokeberry, raw	19	Hartwort leaves	29
Cranberries, raw	15	Kale, raw	23
Crowberries, raw	5	Lettuce, red leaf, raw	8
Currants, black, raw	4	Lovage, leaves, raw	170
Elderberries, raw	27	Mizuna	9
Figs, raw	5	Mustard greens, raw	9
Goji berry, dried	14	Nalta jute, raw	24
Grapes, black	2	Okra, raw	21
Juniper berries, ripe	47	Onions, red, raw	39
Lingonberries, raw	13	Peppers, yellow wax, raw	51
Pitanga, raw	6	Radicchio, raw	32
Plums, purple, raw	2	Radish leaves, raw	70
Rowanberries, raw	7	Rocket, wild, raw	66
Saskatoon berries	17	Sweet potato leaves, raw	17
Arugula, raw	8	Watercress, raw	30
Asparagus, raw	14	Chia seeds, raw	18
Broccoli, raw	3	Carob fiber	58

Quercetin and cancer

Quercetin has been considered a potential anti-cancer agent because it can inhibit cell proliferation and angiogenesis and induce apoptosis and cellular senescence (Russo et al., 1999, Lamson and Brignall, 2000, Zamin et al., 2009). Quercetin can induce cell death and cell cycle arrest in cancer cells through the down-regulation of oncogenes including Mcl-1, Bcl-2,

Ras, MEK, and PI3K or the up-regulation of tumor suppressor genes including p53 and p21 (Iwao and Tsukamoto, 1999, Sharma et al., 2005, Lim et al., 2007, Spagnuolo et al., 2011). In MDA-MB-231 human breast cancer cells, quercetin was reported to suppress cell proliferation through the up-regulation of Connexin 43 (Cx43) (Conklin et al., 2007), whose expression correlates inversely with the aggressiveness of the tumor (Laird et al., 1999). Similarly, another study demonstrated that quercetin inhibited the growth of MCF-7 breast cancer cells and also down-regulated the expression of survivin, which is highly expressed in most cancers (Deng et al., 2013).

Quercetin has been reported to induce apoptosis in a human prostate cell line (LNCaP) by inducing the dissociation of Bax from Bcl-xL, activating caspases and inhibiting phosphorylation of Akt (Kim and Lee, 2007, Lee et al., 2008). Urokinase-type plasminogen activator (uPA) has been associated with cancer invasion including prostate cancer. Quercetin significantly decreased the expressions of uPA, MMP-2, MMP-9, EGF, β-catenin, and NF-κB and thus suppressed the proliferation, migration, and invasion of prostate cancer cells (Vijayababu et al., 2005, Senthilkumar et al., 2011). Recently, the chemopreventive effects of quercetin have been studied in an *in vivo* model of prostate cancer (Sharmila et al., 2014). The results showed that quercetin significantly reversed the reduction in SOD, catalase, and GPx activities and the increase in Akt phosphorylation in prostate cancer-induced rats.

Activation of β-catenin/Tcf signaling plays a central role in colorectal carcinogenesis (Park et al., 2005). Interestingly, quercetin has been shown to inhibit cell proliferation through suppression of β-catenin/Tcf signaling and survivin expression in SW480 colon cancer cells (Shan et al., 2009). Quercetin has also been demonstrated to suppress cell proliferation in HT-

29 colon cancer cells by activating AMPK and decreasing the expression of COX-2, which is known to be overexpressed in colon cancer (Lee et al., 2009). Moreover, quercetin inhibited colorectal carcinogenesis and reduced the size of colorectal tumors in azoxymethane (AOM)-treated rats (Dihal et al., 2006).

Quercetin has been reported to induce apoptosis in A549 lung cancer cells via caspase-3 activation, PARP cleavage, ERK activation, and c-Jun phosphorylation (Nguyen et al., 2004). The chemopreventive efficacy of quercetin was investigated using benzo(a)pyrene (B(a)P)-treated mice as an *in vivo* model of lung carcinogenesis, wherein pre-treatment with quercetin protected against B(a)P-induced cytotoxicity by increasing the activity of antioxidants (SOD, catalase, GPx, GST) (Kamaraj et al., 2007). More importantly, epidemiological studies reported an inverse association of quercetin and lung cancer (Knekt et al., 2002, Cui et al., 2008).

Quercetin has also been shown to reduce chromosomal aberration during 7, 12-dimethylbenz(a)anthracene (DMBA)-induced mouse skin carcinogenesis (Sengupta et al., 2001). Similarly, quercetin treatment inhibited the DMBA-TPA-induced tumor formation in the BK5.IGF-1 transgenic mouse model for skin carcinogenesis and it suppressed activation of IGF-1 signaling (Jung et al., 2013).

In addition to the above-mentioned studies, anti-cancer effects of quercetin have also been investigated in other types of cancer, including pancreatic, ovarian, and gastric cancer (Angst et al., 2013, Maciejczyk and Surowiak, 2013, Ramachandran et al., 2013). Taken together, quercetin can act as an anti-cancer agent by virtue of its ability to modulate pro- and anti-apoptotic proteins, cell cycle progression, angiogenesis, autophagy, inflammation, protein

kinases involved in cell proliferation, and telomerase activity (Lee et al., 2004, Psahoulia et al., 2007, Boly et al., 2011, Cosan et al., 2011, Russo et al., 2012).

Quercetin and cardiovascular diseases

Quercetin is a well-known inhibitor of cellular proliferation and also an effective minimizer of DNA damage (Boyle et al., 2000, Choi et al., 2008). Natural source of quercetin from plants has been shown to increase the bioavailability of this flavonoid (Miean and Mohamed, 2001). Several studies have demonstrated a protective response by quercetin against cardiovascular disorders (Larson et al., 2012).

Quercetin exhibits a number of properties that lead to cardiovascular protection, such as being anti-oxidative (da Silva et al., 2000, Chaillou and Nazareno, 2006), antiplatelet, anti-smooth muscle cell proliferation and migration, an enhancer of cardiac cell mitochondrial function and an inhibitor of NF-κB (Gryglewski et al., 1987, Duarte et al., 1993a, Duarte et al., 1993b, Hajra et al., 2000, Alcocer et al., 2002, Moon et al., 2003).

Many animal studies involving diet supplementation with quercetin have shown to be protective against cardiac problems. For example, studies conducted by Han et al., have demonstrated that a quercetin-supplemented diet attenuated the development of cardiac hypertrophy in a rat model of pressure overloads (Han et al., 2009, Gao et al., 2014). Moreover, a recent study conducted by Jimenez et al., also showed a promising effect of quercetin. Their study revealed that quercetin and its plasma metabolites effectively lowered levels of vascular superoxide by inhibiting NADPH activity in the vascular smooth muscle cells (Jimenez et al., 2015). On the contrary, a few studies have shown that the risk and severity of cardiovascular

problems were not lessened in a spontaneously hypertensive rat model receiving a quercetin-supplemented diet (Carlstrom et al., 2007).

Epidemiological evidence also indicates that quercetin is protective against cardiac diseases. One of the most famous studies was the Zutphen Elderly Study, which demonstrated that increasing flavonoid (high quercetin content) intake by dietary means decreased the risk of heart disease mortality (Hertog et al., 1993). One other study showing a promising protective effect of quercetin against cardiovascular disease risk was performed by Sarah Egert et al (Egert et al., 2009). The study involved overweight subjects between ages 25-65 years with a high cardiovascular disease risk phenotype. The subjects were randomised in a double-blind, placebo-controlled cross-over trial, with the quercetin treatment group receiving about 150 mg per day for a period of 6 weeks followed by a 5-week washout period. The results of this study stated that quercetin lowered the systolic blood pressure and plasma levels of oxidised LDL in subjects with a high cardiovascular disease risk phenotype (Egert et al., 2009). During the past couple of decades, a number of consolidated studies have highlighted the efficiency of quercetin in preventing coronary heart disease (Formica and Regelson, 1995, Pace-Asciak et al., 1995, Saleem and Basha, 2010). Moreover, these studies also provided us novel insights into understanding the effects of dietary quercetin on body metabolism. Apart from the field of cardiovascular diseases, quercetin has also proved to be a useful supplement for athletes. Many studies have shown that intake of quercetin supplements by athletes improved their performance and generally facilitated healthy maintenance of the body (Nieman et al., 2010, Askari et al., 2013). In line with these data, several recent reports demonstrated that quercetin supplements also strengthened the athletes' development of immunity (Walsh et al., 2011, Gleeson, 2013).

Quercetin and neurodegenerative diseases

Oxidative stress is recognized as an important factor in the pathogenesis of several neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD). The brain is highly susceptible to oxidative stress, given its high content of polyunsaturated fatty acids, high oxygen consumption, and lower antioxidant capacity (Sies, 1993, Bellissimo et al., 2001, Freitas et al., 2004). Therefore, increasing the brain's antioxidant capacity may provide protection against oxidative stress-induced neurodegeneration.

Recently, different groups of polyphenols have been tested for their disease-modifying potential for neurodegenerative diseases. For quercetin, there is now compelling evidence of its neuroprotective role in various neurodegenerative diseases (Ansari et al., 2009, Haleagrahara et al., 2013, Karuppagounder et al., 2013, Sabogal-Guaqueta et al., 2015).

Quercetin has been explored as a promising disease-modifying neuroprotective agent for AD in various experimental models. A glucoside form of quercetin, quercetin-3'-glucoside, reduced H_2O_2-induced ROS generation and also protected against Aβ-induced cell death in PC12 cells (Zhu et al., 2007). Similarly, another study investigated whether quercetin protects against oxidative stress and Aβ-induced cell death in primary neurons. It found that lower concentrations of quercetin (5 and 10 µM) reduced protein carbonyl, 3-NT, and HNE levels, as wells as cell death in Aβ-treated primary cortical neuron cultures, whereas higher concentrations of quercetin (20 and 40 µM) showed opposite effects (Ansari et al., 2009). Moreover, the role of ginkgo flavonols (mainly composed of quercetin, kaempferol, and isorhamnetin) in a depression-related signaling pathway mediated by BDNF/pCREB was

tested in double-transgenic (Tg APP/PS1) mice (Hou et al., 2010). They found that gingko flavonols stimulated the CREB-BDNF signaling pathway, reduced Aβ production in the hippocampus and also reversed the spatial learning deficit in transgenic mice as evidenced by improved performance in the Morris water maze. The potential neuroprotective effects of quercetin have also been investigated in a triple-transgenic mouse model of AD (3xTg-AD) (Sabogal-Guaqueta et al., 2015). Their results revealed that quercetin treatment significantly reversed β-amyloidosis and tauopathy and reduced astrogliosis and microgliosis in 3xTg-AD mice. Furthermore, in comparison with vehicle-treated 3xTg-AD mice, quercetin-treated 3xTg-AD mice performed better in learning and memory tasks. Since acetylcholine-esterase (AchE) inhibitors that are widely used in the treatment of AD may cause notable side effects, the possible AchE inhibitory effect of quercetin was tested and compared with conventional drugs by an *in silico* analysis (Islam et al., 2013). Their analysis revealed that quercetin's binding strength to the active site of the enzyme was better than conventional drugs and that a 4-OH methylated form of quercetin (azaleatin) had an even better binding affinity than quercetin, suggesting the natural compound quercetin has significant AchE inhibitory effect as compared to widely used conventional drugs.

Owing to its antioxidant and anti-inflammatory properties, quercetin has been gaining interest as a neuroprotective agent for PD. Isoquercitrin, a glycoside form of quercetin, has been shown to play a neuroprotective role against 6-OHDA-induced neurotoxicity in PC12 cells (Magalingam et al., 2014). In this study, they showed that pre-treatment with isoquercitrin increased levels of ROS-scavenging enzymes (SOD, catalase, and GPx) and reduced lipid peroxidation in 6-OHDA-treated PC12 cells. Similarly, quercetin was shown to reduce protein carbonyl content and lipid hydroperoxide (LPO) levels in the striatum of 6-OHDA-treated rats

(Haleagrahara et al., 2011). They also found that quercetin treatment significantly reduced striatal dopamine depletion and total glutathione (GSH) depletion and improved neuronal survival in 6-OHDA-treated rats. Interestingly, quercetin has also been shown to enhance cognitive function in 6-OHDA-treated rats as evidenced by improved performance in the Morris water maze (Sriraksa et al., 2012). Additionally, their results demonstrated that quercetin treatment decreased hippocampal acetylcholinesterase (AChE) activity and protected against neurodegeneration in all sub-regions of the hippocampus. Likewise, quercetin was also reported to protect against 1-methyl-4-phenylpyridinium ion (MPP^+)-induced neurotoxicity in PC12 cells (Bournival et al., 2009). They found that pre-treatment with quercetin significantly reduced MPP^+-induced cell death as evidenced by decreases in both caspase-3 activation and DNA fragmentation. Furthermore, decreased nuclear translocation of apoptosis-inducing factor (AIF) and decreased cytochrome c release into cytosol were also observed in this study. Another study explored the potential protective effect of quercetin in a 1-methyl-4- phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) mouse model of PD (Lv et al., 2012). Their results revealed that quercetin treatment reduced motor deficits and striatal dopamine depletion and also increased SOD and GPx activities in MPTP-treated mice. Furthermore, quercetin treatment reduced lipid peroxidation in MPTP-treated mice as evidenced by reduced immunoreactivity of 4-HNE in striatal neurons. Quercetin has also been shown to protect against rotenone-induced nigral neurodegeneration (Karuppagounder et al., 2013). Their results showed that quercetin treatment improved mitochondrial complex I activity and reduced striatal dopamine and nigral GSH depletion in rotenone-lesioned rats. Finally, it has recently been shown that quercetin acts as an anti-inflammatory agent that restricts MPP^+-induced microglial activation (Bournival et al., 2012). They found that pretreatment with quercetin reduced MPP^+-induced

IL-6, IL-1β, iNOS, and TNFα expression in N9 microglial cells. They further demonstrated that pretreatment with quercetin protected against cell death induced by microglial activation, when PC12 cells were co-cultured with N9 cells treated with MPP^{+}.

The protective effects of quercetin have also been observed in other neurodegenerative diseases, including HD, and Amyotrophic lateral sclerosis (ALS) (Said Ahmed et al., 2000, Sandhir and Mehrotra, 2013, Chakraborty et al., 2014). Collectively, all these studies suggest that quercetin is a promising neuroprotective agent for the treatment of neurodegenerative diseases.

Conclusion

This chapter discusses the potential use of quercetin in the prevention of cancer, cardiovascular diseases, and neurodegenerative diseases. As described above, there is strong evidence that quercetin can modulate several cellular signaling pathways involved in regulating the antioxidant response, cell survival, apoptosis, and inflammation. More large-scale epidemiological studies as well as well-designed clinical trials are needed to further clarify the potential benefits and to substantiate the efficacy of quercetin for the prevention and treatment of human diseases.

CHAPTER II: ACTIVATION OF PKD1 POSITIVELY REGULATES PGC-1α TRANSCRIPTIONAL ACTIVITY AND PROTECTS AGAINST DOPAMINERGIC NEUROTOXICITY

Muhammet Ay, Arunkumar Asaithambi, Dilshan Harischandra, Arthi Kanthasamy, Huajun Jin, Vellareddy Anantharam and Anumantha G. Kanthasamy‡

Parkinson's Disorder Research Laboratory, Iowa Center for Advanced Neurotoxicology, Department of Biomedical Sciences, Iowa State University, Ames, Iowa 50011, USA

‡ To whom correspondence should be addressed: Dept. of Biomedical Sciences, Iowa State University, 2062 College of Veterinary Medicine Building, Ames, IA 50011. Tel: 515-294-2516; Fax: 515-294-2315; Email: akanthas@iastate.edu

Keywords: PKD1, Parkinson's disease, PGC-1α, mitochondrial biogenesis, MPTP, neurotoxicity.

The abbreviations used are: PKD1, protein kinase D1; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1-alpha; 6-OHDA, 6-hydroxydopamine; LDH, lactate dehydrogenase; LUHMES, Lund human mesencephalic; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; CREB, cAMP response-element binding protein; BDNF, brain-derived neurotrophic factor; CYTB, cytochrome B; mtDNA, mitochondrial DNA; SN, substantia nigra; HDACs, histone deacetylases; MEF2, myocyte enhancer factor 2; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Abstract

Mitochondrial dysfunction has been associated with many neurological diseases including Parkinson's disease (PD). Impaired mitochondrial biogenesis is recognized as a contributing factor to mitochondrial dysfunction in PD. Therefore, identifying cell signaling mechanisms regulating mitochondrial biogenesis is critically important to the development of new treatment strategies for PD. We have recently shown that activation of protein kinase D1 (PKD1) plays a neuroprotective role and that positive modulation of PKD1 can offer neuroprotection against neuronal cell death in PD models. In this study, we attempted to identify downstream effectors of the neuroprotective PKD1 signaling and found that PKD1 activation positively regulates PGC-1α transcriptional activity. Overexpression of constitutively active PKD1 increased PGC-1α promoter activity and mRNA and protein expression in MN9D dopaminergic neuronal cells; however, PGC-1β and PRC mRNA expression were not affected by PKD1 overexpression. Moreover, treatment of MN9D cells with rationally designed PKD1 activator peptide (AKP4T) enhanced mRNA and protein expression of PGC-1α and also mRNA expression of TFAM, CYTB, and Cox III. Interestingly, treatment of cells with PKD1 inhibitor (kbNB-14270) strongly suppressed mRNA expression of PGC-1α and TFAM. Additionally, PKD1 overexpression also stimulated BDNF expression and MEF2 activity in MN9D cells. More importantly, pretreatment with AKP4T significantly protected against 6-OHDA-induced neurotoxicity in a human mesencephalic neuronal cell model. Collectively, our results suggest that positive modulation of PKD1 induces PGC-1α transcription and may offer neuroprotection to dopaminergic neurons.

Introduction

Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α) is the founding member of the PGC-1 family of coactivators that consists of PGC-1α, PGC-1β, and PGC-related coactivator (PRC) and is highly expressed in tissues with high energy demands, including skeletal muscle, brown fat, heart, and brain (Esterbauer et al., 1999, Tritos et al., 2003). PGC-1α functions as key regulator of mitochondrial biogenesis (Wu et al., 1999) and stimulates mitochondrial biogenesis by activating the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2), which increase the expression of mitochondrial transcription factor A (TFAM) (Scarpulla, 2008). Even though PGC-1α doesn't possess histone-modifying enzymatic activity, it can interact with various coactivators including CBP/p300 through its N-terminal activation domain (Puigserver et al., 1999). Previous studies have demonstrated that PGC-1α transcription is mainly controlled by CREB and MEF2 transcription factors (Akimoto et al., 2008). PGC-1α activity is also regulated by post-translational modifications including phosphorylation and acetylation. AMP-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase (MAPK) can directly phosphorylate and activate PGC-1α in skeletal muscle (Puigserver et al., 2001, Jager et al., 2007). On the other hand, Sirt1-mediated deacetylation increases PGC-1α coactivation (Rodgers et al., 2005), while GCN5 acetylates and inhibits PGC-1α activity (Lerin et al., 2006).

Mitochondrial dysfunction has long been associated with the pathogenesis of Parkinson's disease (PD) (Schapira et al., 1990, Olanow and Tatton, 1999). There is now growing evidence that impaired mitochondrial biogenesis is one the key mechanisms of mitochondrial dysfunction in PD (Zheng et al., 2010, Youdim and Oh, 2013) and regulation of

mitochondrial biogenesis is critically important to maintain a healthy pool of mitochondria in neurons (Wareski et al., 2009). Compelling evidence suggest that decreased PGC-1α expression may play an important role in the pathogenesis of PD. Genes regulated by PGC-1α are underexpressed in PD patients (Zheng et al., 2010). Studies have reported that PGC-1α knockout mice are more sensitive to MPTP-induced neurotoxicity (St-Pierre et al., 2006), and that PGC-1α overexpressing mice are more resistant to dopaminergic neurodegeneration induced by MPTP (Mudo et al., 2012). It has also been shown that PGC-1α null mice are more vulnerable to α-synuclein toxicity (Ciron et al., 2015). It is thus of therapeutic interest to study the signaling events controlling PGC-1α expression in dopaminergic neurons.

We have recently demonstrated that protein kinase D1 (PKD1) becomes activated in response to neurotoxic insults and plays a neuroprotective role in dopaminergic cells (Asaithambi et al., 2014, Ay et al., 2015). This led us to study downstream effectors of PKD1-mediated neuroprotective signaling. The present study aimed to investigate whether PKD1 activation contributes to the transcriptional regulation of PGC-1α in dopaminergic neuronal cells and protects against dopaminergic neurotoxicity. Here we show that PKD1 activation increases PGC-1α transcriptional activity in a dopaminergic cell model, possibly through CREB phosphorylation and MEF2 activation. We also demonstrate that positive modulation of PKD1 signaling significantly attenuates 6-OHDA-induced neuronal damage in human dopaminergic neurons.

Materials and Methods

Chemicals and Reagents. MPTP-HCl and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Lois, MO). The Bradford protein assay kit was purchased from Bio-Rad. PKD1

inhibitor kbNB-14270 was obtained from Tocris Bioscience (Ellisville, MO). Antibodies against α-tubulin and BDNF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho PKD1 (p-S744/748), total CREB, and phosho-CREB (p-S133) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-PGC-1α antibody was purchased from Millipore (Billerica, MA). Lipofectamine 2000 reagent, Alexa 680-conjugated anti-mouse secondary antibody and all cell culture reagents were obtained from Invitrogen. IRDye800-conjugated anti-rabbit secondary was purchased from Rockland Labs (Gilbertsville, PA). Cell Titer 96 Aqueous One Solution proliferation assay kit was obtained from Promega (Madison, WI). [^{3}H] Dopamine was obtained from PerkinElmer Life Sciences (Boston, MA). In Vitro Toxicology Assay Kit (lactic dehydrogenase based) was purchased from Sigma-Aldrich (St. Lois, MO).

Cell Cultures. The mouse dopaminergic MN9D cell line was generously provided by Dr. Syed Ali (National Center for Toxicological Research/Food and Drug Administration, Jefferson, AR) and cultured as described previously (Jin et al., 2011, Ay et al., 2015). Briefly, MN9D cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 units penicillin, and 50 μg/ml streptomycin and maintained at 37°C in a 5% CO_2 atmosphere. The Lund human mesencephalic (LUHMES) cells were grown and differentiated, as described previously (Jin et al., 2014).

Transient Transfections and Reporter Gene Assays. Transient transfections of MN9D cells were performed using Lipofectamine 2000 reagent according to the manufacturer's protocol, as described previously (Ay et al. 2015). The 3.1 kb mouse PGC-1α promoter construct and HDAC5 (S2A) mutant were generously provided by Dr. Zhen Yan (University of Virginia

School of Medicine). Full-length human PKD1 plasmid (PKD1-WT) (Addgene plasmid # 10808), constitutively active PKD1 (PKD1-CA) (Addgene plasmid # 10810), the PH domain-deleted PKD1 (PKD1ΔPH) (Addgene plasmid # 10811), and 3X-MEF2 luciferase reporter construct (Addgene plasmid # 32967) were obtained from Addgene Inc. (Cambridge, MA). Cells were seeded into 12-well plates at a density of 15×10^4 cells/well one day before transfection. Each transfection was performed with 1 μg of reporter constructs and the indicated amounts of expression plasmids and pcDNA3-LacZ (0.2 μg) was included in each transfection to normalize transfection efficiencies. Cells were harvested 48 hours after transfection, lysed in 150 μl of Reporter Lysis Buffer (Promega), and assayed for luciferase activity. Luciferase activity was measured on a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT) using the luciferase assay system (Promega). Luciferase activities were normalized to $β$-galactosidase activity.

Quantitative Real-Time RT-PCR. Total RNA was isolated from cells using the Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA), and 1-2 μg RNA was used to generate cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed on an Mx300P QPCR system (Stratagene) using the RT^2 SYBR Green qPCR Mastermix kit (Qiagen) and QuantiTect Primer Assay kit (Qiagen). All RT-qPCR reactions were performed in triplicate and normalized with 18S rRNA. Primers for all genes were purchased from Qiagen. PCR conditions are available upon request. Dissociation curves were run to verify the singularity of the PCR product. The data were analyzed using the comparative threshold cycle method.

Western Blot Analysis. After treatments, cells or brain tissues were collected and resuspended in a modified RIPA buffer containing protease and phosphatase inhibitors. Briefly, lysates containing equal amounts of protein were loaded in each lane and separated on a 10-12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad). The membranes were blocked in LI-COR blocking buffer for 1 hour at room temperature and then incubated overnight at 4°C with the corresponding primary antibodies. Western blot was performed using IRDye 800 anti-rabbit and Alexa Fluor 680 goat anti-mouse. Western blot images were captured and analyzed with an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Cell Viability Assays. MTS cell viability assay was performed, as described previously (Jin et al., 2014). Briefly, differentiated LUHMES cells were seeded onto 96-well plates and treated at differentiation day 5. After treatment, 20 µl of MTS solution was added to each well, and the plates were incubated at 37°C and 5% CO_2 for 2 h. Measurements were made at 490 nm using a fluorescence microplate reader (SpectraMax Gemini XS, Molecular Devices).

Lactate Dehydrogenase Assays. Lactate dehydrogenase (LDH) assay was performed using the In Vitro Toxicology Assay Kit (lactic dehydrogenase based) from Sigma, according to the manufacturer's instructions with minor modifications. Briefly, pre-differentiated LUHMES cells were plated in 96-well plate. On day 5 of differentiation, cells were pre-treated with AKP4T (20 nM) for 1 h and then co-treated with 6-OHDA (30 µM) and AKP4T for 4 h. After the treatment, 10 µl of the extracellular supernatant was added to the LDH assay mixture and incubated at room temperature in the dark for 30 minutes. LDH activity was measured at 490 nm using a microplate reader (SpectraMax Gemini XS, Molecular Devices). The background absorbance was measured at 690 nm and subtracted from the measurements at 490 nm.

High Affinity [³H] Dopamine Uptake Assays. Dopamine uptake assay was performed, as described previously (Ghosh et al., 2013). Briefly, differentiated LUHMES cells were pre-treated with AKP4T (20 nM) for 1 h and then co-treated with 6-OHDA (30 µM) and AKP4T for 4 h. After the treatments, cells were washed with Krebs Ringer buffer (16 mM Na_3PO_4, 5.6 mM glucose, 1.8 mM $CaCl_2$, 1.3 mM EDTA, 1.2 mM $MgSO_4$, 4.7 mM KCl, 120 mM NaCl) and incubated with 10 µM ^{3}H-DA for 30 min at 37°C in Krebs Ringer buffer. Nonspecific dopamine uptake was determined by adding 1 nM Mazindol, a dopamine reuptake blocker. The uptake was stopped by washing the cultures 3X with fresh ice-cold Krebs Ringer buffer. Afterwards, cells were lysed with 1 N NaOH. Radioactivity was measured by liquid scintillation counter (Tri-Carb 4000, Packard Instrument Co.) after adding a 5-ml scintillation cocktail to each vial.

Animals and Treatment. Eight to ten week-old C57BL/6 mice were housed under standard conditions: constant temperature (22±1 °C), humidity (relative, 30%), and a 12 h light/dark cycle. Mice were given free access to food and water. Animal care and protocol procedures were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University (Ames, IA). Mice were treated with MPTP-HCl (25 mg/kg/day) intraperitoneally for five consecutive days. Control peptide and PKD1 activator peptide (AKP4T) (5 mg/kg/day) were dissolved in saline and administered intravenously. The drug treatment started 1 day before the MPTP insult, continued throughout the MPTP treatment period, and extended for another 7 days of post-MPTP treatment. After the treatments, mice were subjected to behavioral, neurochemical, and biochemical measurements.

Statistical Analysis. Data analysis was performed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). Data were first analyzed by using one-way ANOVA and then the Tukey multiple comparison test was used for statistical comparisons. Differences with $P < 0.05$ were considered significant.

Results

PKD1 overexpression induces PGC-1α expression in MN9D cells

PKD1 has been shown to act as a mitochondrial ROS sensor that relays the signal to nucleus to activate cell survival mechanisms in non-neuronal cells (Storz, 2007). Recent studies from our group have shown that PKD1 gets activated during the early stages of oxidative stress and plays a neuroprotective role in dopaminergic neuronal cells (Asaithambi et al., 2011, Asaithambi et al., 2014, Ay et al., 2015). It has been shown that PGC-1α expression is also increased during oxidative stress to protect cells against oxidative damage (St-Pierre et al., 2006). However, the signaling mechanisms regulating PGC-1α transcription during oxidative stress remain largely unknown. Here, we wanted to investigate if oxidative stress-sensitive kinase PKD1 has any role in transcriptional activation of PGC-1α. To determine the role of PKD1 in regulating PGC-1α transcriptional activity, MN9D cells were cotransfected with PGC-1α promoter-reporter construct and the full length PKD1 (PKD1-WT) and constitutively active PKD1 (PKD1-CA and PKD1ΔPH) expression vectors. Overexpression of PKD1 constructs significantly increased PGC-1α promoter activity (Fig. 1A-C). PGC-1α mRNA expression was also significantly increased in MN9D cells overexpressing PKD1-CA (Fig. 2A). Interestingly, overexpression of PKD1ΔPH (PH domain deleted, constitutively active PKD1) resulted in a dose-dependent increase in mRNA expression of PGC-1α (Fig. 2B).

After observing increased PGC-1α transcriptional activity by overexpression of PKD1, we wanted to examine if PKD1 overexpression also leads to upregulation of PGC-1β and PRC, other members of PGC-1 family. As shown in Fig. 3A and 3B, mRNA expression of PGC-1β and PRC were not affected in PKD1ΔPH-overexpressing cells. Moreover, increased expression of PGC-1α protein was observed in MN9D cells overexpressing PKD1-WT and PKD1ΔPH (Fig. 3D). Collectively, these results suggest that PKD1 could be involved in transcriptional regulation of PGC-1α in dopaminergic cells.

Modulation of PKD1 signaling affects PGC-1α expression

To further explore the role of PKD1 in transcriptional activation of PGC-1α, we used a rationally designed PKD1 peptide activator (AKP4T) that we recently synthesized in our laboratory. We have shown that AKP4T disrupts the interaction between the inhibitory pleckstrin homology (PH) domain and the catalytic domain of PKD1 and significantly increases PKD1 kinase activity (unpublished data). First, we investigated the effect of AKP4T on PGC-1α expression in MN9D cells. Treatment of cells with 100 nM of AKP4T for 2 h increased PGC-1α protein (Fig. 4A) and mRNA expression (Fig. 4B). Since CREB is known to enhance PGC-1α transcription (Herzig et al., 2001), we also checked phospho CREB levels and observed an increase in CREB phosphorylation (Fig. 4A), suggesting PKD1 may regulate PGC-1α transcription through CREB. Given that PGC-1α functions as an important regulator of mitochondrial biogenesis, we wanted to examine mRNA levels of some marker genes of mitochondrial biogenesis. Interestingly, AKP4T treatment resulted in significant increases in mRNA expression of TFAM (Fig. 4C), CYTB (Fig. 4D), and Cox III (Fig. 4E). To further substantiate the role of PKD1 signaling in PGC-1α transcription, we used kbNB-14270, a

pharmacological inhibitor of PKD1. Treatment of MN9D cells with 10-50 µM of kbNB-14270 significantly suppressed PGC-1α mRNA expression (Fig. 5A). Reduced mRNA expression of TFAM was also observed in kbNB-14270-treated MN9D cells (Fig. 5B). These data indicate that positive modulation of PKD1 signaling can enhance PGC-1α transcription.

PKD1 overexpression stimulates MEF2 activity in MN9D cells

Myocyte enhancer factor 2 (MEF2) has been shown to bind to the PGC-1α promoter and transactivate the PGC-1α gene (Handschin et al., 2003) and mutation of MEF2 binding sites strongly reduced PGC-1α promoter activity (Akimoto et al., 2008). Class IIa HDACs, including HDAC4 and HDAC5 are known to repress MEF2 function and phosphorylation of those HDACs releases MEF2 from the inhibitory effect of HDACs (Lu et al., 2000). It has been shown that PKD1 can directly phosphorylate class IIa HDACs in non-neuronal cells (Parra et al., 2005, Sinnett-Smith et al., 2014). Since MEF2 activation can lead to transcriptional activation of PGC-1α, we wanted to examine if PKD1 overexpression increases MEF2 activity in dopaminergic neuronal cells. For this study, MN9D cells were cotransfected with 3X-MEF2 luciferase reporter construct and the PKD1ΔPH or kinase-dead PKD1 (PKD1 kd) expression vectors. Overexpression of PKD1ΔPH, but not PKD1 kd, strongly induced MEF2 activity in MN9D cells (Fig. 6A). However, overexpression of nonphosphorylatable mutant of HDAC5 (HDAC5 S259A and S498A) completely blocked the activation of MEF2 induced by PKD1ΔPH overexpression (Fig. 6B), suggesting that MEF2 transcriptional activity is mainly controlled by HDAC5 in dopaminergic neuronal cells. These findings indicate that MEF2 might be involved in PKD1-mediated activation of PGC-1α transcription.

Positive modulation of PKD1 increases BDNF expression

We have previously reported that positive modulation of PKD1 signaling protects against oxidative stress-induced cell death in dopaminergic cells (Asaithambi et al., 2014, Ay et al., 2015). In an effort to identify downstream effectors of PKD1 signaling, we wanted to examine if PKD1 can regulate expression of brain-derived neurotrophic factor (BDNF). Previously, studies have shown that BDNF increases survival of dopaminergic neurons in vitro (Hyman et al., 1991) and that BDNF protects against 6-OHDA-induced dopaminergic neurodegeneration (Levivier et al., 1995). In the previous experiments, we showed that PKD1 activation increases CREB phosphorylation (Fig. 4A). CREB is known to upregulate BDNF transcription by binding to the CRE site in the BDNF promoter IV (Tao et al., 1998, Pruunsild et al., 2011). To determine the role of PKD1 in regulating BDNF expression, MN9D cells were transfected with the PKD1-WT or PKD1-CA. Overexpression of either PKD1-WT or PKD1-CA induced BDNF mRNA expression (Fig. 7A). Increased BDNF protein expression was also observed in MN9D cells overexpressing the PKD1-CA (Fig. 7B). Next, MN9D cells were treated with 100 nM AKP4T for 2 h and AKP4T treatment significantly increased mRNA expression of BDNF (Fig. 7C). Moreover, treatment of MN9D cells with 10-50 µM kbNB-14270, a pharmacological inhibitor of PKD1, resulted in a significant decrease in BDNF mRNA expression (Fig. 7D). These results suggest that PKD1 signaling can regulate BDNF expression possibly through CREB phosphorylation.

AKP4T protects against dopaminergic neurotoxicity in human dopaminergic neurons

Our experiments revealed that PKD1 activator peptide AKP4T can increase expression of PGC-1α and some other marker genes of mitochondrial biogenesis and also upregulates

BDNF expression. For this study, we wanted to test the potential neuroprotective effect of AKP4T in a human mesencephalic neuronal cell model (LUHMES). Differentiated LUHMES cells were pre-treated with 20 nM AKP4T for 1 h and then co-treated with 30 µM 6-OHDA for 4 h. We first measured the 6-OHDA-induced neurotoxicity by MTS cell viability and LDH release assays. As evident from Fig. 8, A and B, 6-OHDA treatment markedly increased cell death and LDH release in LUHMES cells. In contrast, pre- and co-treatment with AKP4T significantly attenuated 6-OHDA-induced neurotoxicity in both assays. Next, integrity of dopaminergic neurons was assessed by dopamine uptake assay. As expected, 6-OHDA treatment strongly decreased dopamine uptake activity (Fig. 8C). Importantly, pre- and co-treatment with AKP4T significantly reduced 6-OHDA-induced loss of dopamine uptake activity. Collectively, these results suggest that PKD1 activator AKP4T has neuroprotective effects against 6-OHDA-induced neurotoxicity in dopaminergic neurons.

AKP4T increases PGC-1α expression in vivo

Our in vitro experiments showed that AKP4T treatment increases PGC-1α expression in dopaminergic neuronal cells (Fig. 4, A and B). Here, we wanted to examine whether this stimulatory effect of AKP4T on PGC-1α expression can be replicated in vivo. Mice were pre-treated intravenously with either AKP4T or saline 1 day before MPTP administration and then co-treated with MPTP (25 mg/kg i.p.) and AKP4T (5 mg/kg) for 5 days and AKP4T treatment continued for another 7 days. After the treatment, mice were sacrificed and brain tissues were collected and processed for immunoblot analysis. Protein levels of PGC-1α were evaluated in substantia nigra tissue samples and Western blots analysis showed that PGC-1α expression significantly increased in AKP4T-pre-treated mice compared to mice treated with MPTP alone

(Fig. 9, A and B), suggesting that positive modulation of PKD1 increases PGC-1α expression in vivo.

Discussion

The results of the present study provide novel evidence that PKD1 activation can enhance PGC-1α transcriptional activity in dopaminergic neurons. We also show that positive modulation of PKD1 pro-survival signaling can provide significant neuroprotection against MPP[+]-induced neurotoxicity. Although previous studies, including our own, have reported that PKD1 can translocate to the nucleus in different cell types, including dopaminergic cells (Rey et al., 2003, Parra et al., 2005, Storz et al., 2005, Jensen et al., 2009, Asaithambi et al., 2014), the nuclear targets of PKD1 remain largely unknown. Our findings suggest that PGC-1α can serve as a downstream target of PKD1 signaling to promote dopaminergic neuron survival.

PGC-1α has been shown to be upregulated following oxidative stress and to induce expression of genes involved in ROS defense system (St-Pierre et al., 2006). It is also noteworthy that the neuroprotective effect of PGC-1α has been documented in PD patients and experimental models of PD (Zheng et al., 2010, Mudo et al., 2012, Ciron et al., 2015). It is thus worthwhile to explore the upstream signals that regulate PGC-1α expression in dopaminergic cells. We recently reported that PKD1 is rapidly activated during the initial stage of oxidative stress and play a neuroprotective role against oxidative insults in cell culture models of PD (Asaithambi et al., 2011, Asaithambi et al., 2014). Our current study sought to investigate whether PGC-1α plays a role as a downstream effector of this PKD1-mediated neuroprotective signaling. In this study, we first investigated whether PKD1 plays any role in regulating PGC-1α transcriptional activity, and our results demonstrated that overexpression of constitutively

active forms of PKD1 (PKD1-CA and PKD1ΔPH) significantly enhances PGC-1α promoter

activity (Fig. 1, B and C), mRNA (Fig. 2, A and B) and protein expression (Fig. 3D). However,

PKD1 overexpression did not affect PGC-1β and PRC mRNA expression (Fig. 3, A and B).

We also found that PKD1 peptide activator (AKP4T) can increase PGC-1α mRNA and protein

expression in MN9D dopaminergic neuronal cells (Fig. 4, A and B). Increased mRNA

expression of TFAM, CYTB, and Cox III were also observed in AKP4T-treated MN9D cells

(Fig. 4C-E). Moreover, treatment of MN9D cells with a pharmacological inhibitor of PKD1

(kbNB-14270) almost completely suppressed basal PGC-1α mRNA expression (Fig. 5A) and

significantly decreased mRNA expression of TFAM (Fig. 5B). It is also noteworthy that

AKP4T treatment increased PGC-1α expression in the substantia nigra of MPTP-treated mice

(Fig. 9, A and B). Interestingly, these results suggest that genetic or pharmacological

modulation of PKD1 activity can positively regulate PGC-1α expression. After observing a

link between PKD1 signaling and PGC-1α expression, we wanted to investigate the possible

molecular mechanisms involved in PKD1-mediated upregulation of PGC-1α in dopaminergic

neurons. Since CREB is a well-known substrate of PKD1 (Johannessen et al., 2007) and can

transactivate the PGC-1α promoter (Herzig et al., 2001), we measured the levels of CREB

phosphorylation in AKP4T-treated MN9D cells and found a significant increase in CREB

phosphorylation at Ser133, suggesting positive modulation of PKD1 signaling can induce

PGC-1α transcription via CREB phosphorylation. MEF2 is another well-known transcription

factor that can induce PGC-1α promoter activity (Handschin et al., 2003). Studies have also

reported that MEF2 can promote neuronal survival against toxicity in the MPTP animal model

of PD (Smith et al., 2006, Mount et al., 2013). Our results revealed that PKD1 overexpression

can strongly induce MEF2 transcriptional activity in MN9D cells (Fig. 6A). However,

coexpression of a nonphosphorylatable mutant of HDAC5 completely inhibited PKD1-induced MEF2 activation (Fig. 6B). These data are consistent with previous studies that have demonstrated PKD1 can increase MEF2 activity by phosphorylating HDACs in non-neuronal cells (Xu et al., 2007, Kim et al., 2008). Our results suggest that MEF2 activation might serve as another possible mechanism for PKD1-mediated PGC-1α upregulation. However, more mechanistic studies are needed to elucidate the precise role of PKD1 in transcriptional regulation of PGC-1α gene in dopaminergic neurons. Additionally, after observing increased CREB phosphorylation in AKP4T-treated MN9D cells, we were interested to determine if PKD1 plays any role in regulating BDNF expression in dopaminergic cells. Our data show that PKD1 overexpression resulted in increased BDNF mRNA (Fig. 7A) and protein expression (Fig. 7B). Moreover, treatment of MN9D cells with AKP4T increased BDNF mRNA level (Fig. 7C), while treatment with kbNB-14270 significantly decreased mRNA expression of BDNF (Fig. 7D). It is likely that BDNF may also be involved in PKD1-mediated neuroprotection in dopaminergic neurons. Taken together, PKD1 seems to promote dopaminergic neuron survival by activating different protective mechanisms.

Another interesting finding of this study is that positive modulation of PKD1 can effectively protect against dopaminergic neurotoxicity in a human mesencephalic neuronal cell model of PD. Here, we wanted to investigate whether positive modulation of PKD1 signaling by AKP4T can protect against dopaminergic neurotoxicity. Our results revealed that AKP4T treatment significantly ameliorates 6-OHDA-induced neurotoxicity, as evidenced by the MTS cell viability, LDH release, and dopamine uptake assays (Fig. 8A-C), suggesting that positive modulation of PKD1 signaling can offer neuroprotection against dopaminergic neurotoxicity.

In summary, our results show for the first time that PKD1 can mediate transcriptional upregulation of PGC-1α in dopaminergic neurons and MEF2 activation and CREB phosphorylation can account for this PKD1-mediated PGC-1α upregulation. However, more studies are required to delineate the relationship between PKD1 activation and PGC-1α upregulation in dopaminergic neurons. Moreover, we also report that PKD1 activation can enhance BDNF expression, suggesting that PKD1 can function as an upstream kinase that activates various protective mechanisms to promote neuronal survival. Finally, we found that positive modulation of PKD1 signaling can effectively protect against dopaminergic neurotoxicity. Overall, our results provide novel evidence that targeting PKD1 signaling has a therapeutic value for the development of novel neuroprotective strategies against dopaminergic neurodegeneration.

REFERENCES

Akimoto T, Li P, Yan Z (2008) Functional interaction of regulatory factors with the Pgc-1alpha promoter in response to exercise by in vivo imaging. American journal of physiology Cell physiology 295:C288-292.

Asaithambi A, Ay M, Jin H, Gosh A, Anantharam V, Kanthasamy A, Kanthasamy AG (2014) Protein kinase D1 (PKD1) phosphorylation promotes dopaminergic neuronal survival during 6-OHDA-induced oxidative stress. PloS one 9:e96947.

Asaithambi A, Kanthasamy A, Saminathan H, Anantharam V, Kanthasamy AG (2011) Protein kinase D1 (PKD1) activation mediates a compensatory protective response during early stages of oxidative stress-induced neuronal degeneration. Molecular neurodegeneration 6:43.

Ay M, Jin H, Harischandra DS, Asaithambi A, Kanthasamy A, Anantharam V, Kanthasamy AG (2015) Molecular cloning, epigenetic regulation, and functional characterization of Prkd1 gene promoter in dopaminergic cell culture models of Parkinson's disease. Journal of neurochemistry 135:402-415.

Ciron C, Zheng L, Bobela W, Knott GW, Leone TC, Kelly DP, Schneider BL (2015) PGC-1alpha activity in nigral dopamine neurons determines vulnerability to alpha-synuclein. Acta neuropathologica communications 3:16.

Esterbauer H, Oberkofler H, Krempler F, Patsch W (1999) Human peroxisome proliferator activated receptor gamma coactivator 1 (PPARGC1) gene: cDNA sequence, genomic organization, chromosomal localization, and tissue expression. Genomics 62:98-102.

Ghosh A, Saminathan H, Kanthasamy A, Anantharam V, Jin H, Sondarva G, Harischandra DS, Qian Z, Rana A, Kanthasamy AG (2013) The peptidyl-prolyl isomerase Pin1 up-regulation and proapoptotic function in dopaminergic neurons: relevance to the pathogenesis of Parkinson disease. The Journal of biological chemistry 288:21955-21971.

Handschin C, Rhee J, Lin J, Tarr PT, Spiegelman BM (2003) An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. Proceedings of the National Academy of Sciences of the United States of America 100:7111-7116.

Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B, Montminy M (2001) CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. Nature 413:179-183.

Hyman C, Hofer M, Barde YA, Juhasz M, Yancopoulos GD, Squinto SP, Lindsay RM (1991) BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature 350:230-232.

Jager S, Handschin C, St-Pierre J, Spiegelman BM (2007) AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha.

Proceedings of the National Academy of Sciences of the United States of America 104:12017-12022.

Jensen ED, Gopalakrishnan R, Westendorf JJ (2009) Bone morphogenic protein 2 activates protein kinase D to regulate histone deacetylase 7 localization and repression of Runx2. The Journal of biological chemistry 284:2225-2234.

Jin H, Kanthasamy A, Harischandra DS, Kondru N, Ghosh A, Panicker N, Anantharam V, Rana A, Kanthasamy AG (2014) Histone Hyperacetylation Upregulates PKCdelta in Dopaminergic Neurons to Induce Cell Death: Relevance to Epigenetic Mechanisms of Neurodegeneration in Parkinson's Disease. The Journal of biological chemistry.

Johannessen M, Delghandi MP, Rykx A, Dragset M, Vandenheede JR, Van Lint J, Moens U (2007) Protein kinase D induces transcription through direct phosphorylation of the cAMP-response element-binding protein. The Journal of biological chemistry 282:14777-14787.

Kim MS, Fielitz J, McAnally J, Shelton JM, Lemon DD, McKinsey TA, Richardson JA, Bassel-Duby R, Olson EN (2008) Protein kinase D1 stimulates MEF2 activity in skeletal muscle and enhances muscle performance. Molecular and cellular biology 28:3600-3609.

Lerin C, Rodgers JT, Kalume DE, Kim SH, Pandey A, Puigserver P (2006) GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1alpha. Cell metabolism 3:429-438.

Levivier M, Przedborski S, Bencsics C, Kang UJ (1995) Intrastriatal implantation of fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevents degeneration of dopaminergic neurons in a rat model of Parkinson's disease. J Neurosci 15:7810-7820.

Lu J, McKinsey TA, Nicol RL, Olson EN (2000) Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. Proceedings of the National Academy of Sciences of the United States of America 97:4070-4075.

Mount MP, Zhang Y, Amini M, Callaghan S, Kulczycki J, Mao Z, Slack RS, Anisman H, Park DS (2013) Perturbation of transcription factor Nur77 expression mediated by myocyte enhancer factor 2D (MEF2D) regulates dopaminergic neuron loss in response to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The Journal of biological chemistry 288:14362-14371.

Mudo G, Makela J, Di Liberto V, Tselykh TV, Olivieri M, Piepponen P, Eriksson O, Malkia A, Bonomo A, Kairisalo M, Aguirre JA, Korhonen L, Belluardo N, Lindholm D (2012) Transgenic expression and activation of PGC-1alpha protect dopaminergic neurons in the MPTP mouse model of Parkinson's disease. Cellular and molecular life sciences : CMLS 69:1153-1165.

Olanow CW, Tatton WG (1999) Etiology and pathogenesis of Parkinson's disease. Annual review of neuroscience 22:123-144.

Parra M, Kasler H, McKinsey TA, Olson EN, Verdin E (2005) Protein kinase D1 phosphorylates HDAC7 and induces its nuclear export after T-cell receptor activation. The Journal of biological chemistry 280:13762-13770.

Pruunsild P, Sepp M, Orav E, Koppel I, Timmusk T (2011) Identification of cis-elements and transcription factors regulating neuronal activity-dependent transcription of human BDNF gene. J Neurosci 31:3295-3308.

Puigserver P, Adelmant G, Wu Z, Fan M, Xu J, O'Malley B, Spiegelman BM (1999) Activation of PPARgamma coactivator-1 through transcription factor docking. Science (New York, NY 286:1368-1371.

Puigserver P, Rhee J, Lin J, Wu Z, Yoon JC, Zhang CY, Krauss S, Mootha VK, Lowell BB, Spiegelman BM (2001) Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1. Molecular cell 8:971-982.

Rey O, Zhukova E, Sinnett-Smith J, Rozengurt E (2003) Vasopressin-induced intracellular redistribution of protein kinase D in intestinal epithelial cells. Journal of cellular physiology 196:483-492.

Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P (2005) Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature 434:113-118.

Scarpulla RC (2008) Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiological reviews 88:611-638.

Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD (1990) Mitochondrial complex I deficiency in Parkinson's disease. Journal of neurochemistry 54:823-827.

Sinnett-Smith J, Ni Y, Wang J, Ming M, Young SH, Rozengurt E (2014) Protein kinase D1 mediates class IIa histone deacetylase phosphorylation and nuclear extrusion in intestinal epithelial cells: role in mitogenic signaling. American journal of physiology Cell physiology 306:C961-971.

Smith PD, Mount MP, Shree R, Callaghan S, Slack RS, Anisman H, Vincent I, Wang X, Mao Z, Park DS (2006) Calpain-regulated p35/cdk5 plays a central role in dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2. J Neurosci 26:440-447.

St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, Spiegelman BM (2006) Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. Cell 127:397-408.

Storz P (2007) Mitochondrial ROS--radical detoxification, mediated by protein kinase D. Trends in cell biology 17:13-18.

Storz P, Doppler H, Toker A (2005) Protein kinase D mediates mitochondrion-to-nucleus signaling and detoxification from mitochondrial reactive oxygen species. Molecular and cellular biology 25:8520-8530.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20:709-726.

Tritos NA, Mastaitis JW, Kokkotou EG, Puigserver P, Spiegelman BM, Maratos-Flier E (2003) Characterization of the peroxisome proliferator activated receptor coactivator 1 alpha (PGC 1alpha) expression in the murine brain. Brain research 961:255-260.

Wareski P, Vaarmann A, Choubey V, Safiulina D, Liiv J, Kuum M, Kaasik A (2009) PGC-1{alpha} and PGC-1{beta} regulate mitochondrial density in neurons. The Journal of biological chemistry 284:21379-21385.

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98:115-124.

Xu X, Ha CH, Wong C, Wang W, Hausser A, Pfizenmaier K, Olson EN, McKinsey TA, Jin ZG (2007) Angiotensin II stimulates protein kinase D-dependent histone deacetylase 5 phosphorylation and nuclear export leading to vascular smooth muscle cell hypertrophy. Arteriosclerosis, thrombosis, and vascular biology 27:2355-2362.

Youdim MB, Oh YJ (2013) Promise of neurorestoration and mitochondrial biogenesis in Parkinson's disease with multi target drugs: an alternative to stem cell therapy. Experimental neurobiology 22:167-172.

Zheng B, Liao Z, Locascio JJ, Lesniak KA, Roderick SS, Watt ML, Eklund AC, Zhang-James Y, Kim PD, Hauser MA, Grunblatt E, Moran LB, Mandel SA, Riederer P, Miller RM, Federoff HJ, Wullner U, Papapetropoulos S, Youdim MB, Cantuti-Castelvetri I, Young AB, Vance JM, Davis RL, Hedreen JC, Adler CH, Beach TG, Graeber MB, Middleton FA, Rochet JC, Scherzer CR (2010) PGC-1alpha, a potential therapeutic target for early intervention in Parkinson's disease. Science translational medicine 2:52ra73.
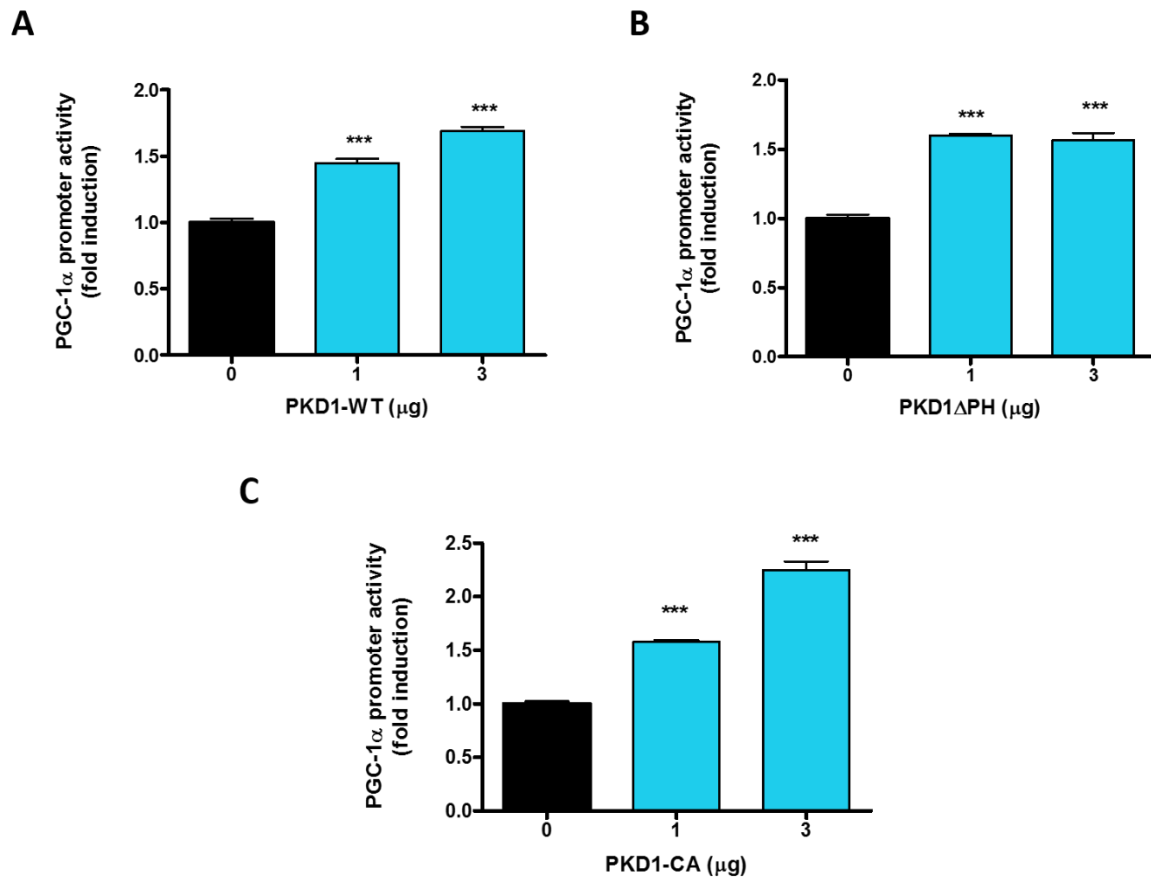
Figure 1. Overexpression of PKD1-WT, PKD1ΔPH, and PKD1–CA increases PGC-1α promoter activity. *A-C,* Variable amounts (µg) of PKD1-WT (*A*), PKD1ΔPH (*B*), PKD1–CA (*C*) or empty vector (pcDNA3) were cotransfected with the PGC-1α promoter construct into MN9D cells. Luciferase activity was measured 48 h after transfection and normalized to *β*-galactosidase activity. Values are expressed as fold induction over empty vector control and represent the mean ± SEM of three replicates (***$p < 0.001$).
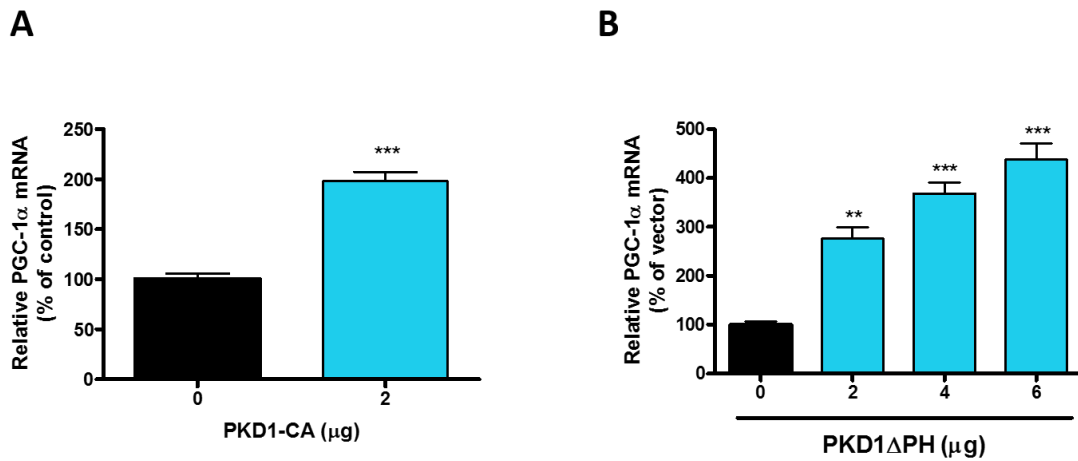
A

B

Figure 2. Overexpression of PKD1-CA and PKD1ΔPH increases PGC-1α mRNA expression. *A,* MN9D cells were transfected with either 2 µg of the PKD1–CA construct or empty vector for 48 h. Real-time RT-PCR analysis of PGC-1α mRNA levels were performed. 18S rRNA served as internal control. *B,* MN9D cells were transfected with variable amounts (2-6 µg) of the PKD1ΔPH construct for 48 h. Real-time RT-PCR analysis of PGC-1α mRNA levels was performed. 18S rRNA served as internal control. Values are expressed as a percentage of the activity of empty vector-transfected cells and represent the mean ± SEM of three replicates (** $p < 0.01$; *** $p < 0.001$).
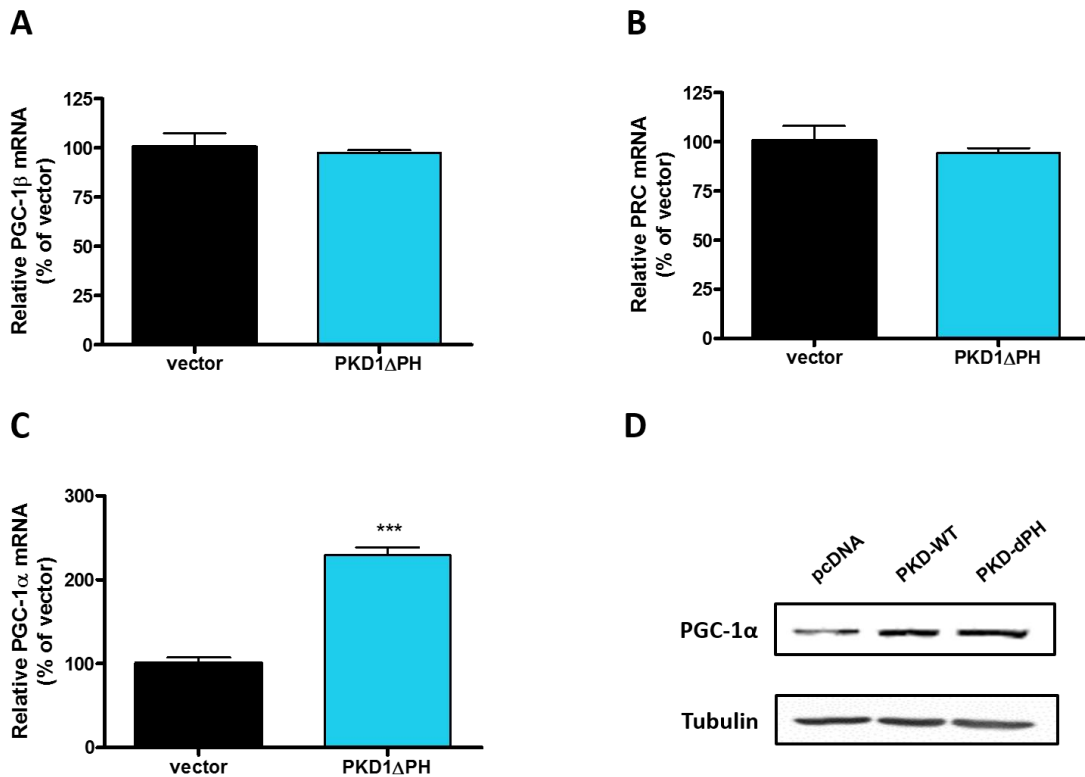
Figure 3. Overexpression of PKD1ΔPH increases PGC-1α, but not PGC-1β and PRC, mRNA and protein expression. *A-C,* MN9D cells were transfected with either 2 µg of the PKD1ΔPH construct or empty vector for 48 h. Real-time RT-PCR analyses of PGC-1β (*A*), PRC (*B*), and PGC-1α (*C*) mRNA levels were performed. 18S rRNA served as internal control. Values are expressed as a percentage of the activity of empty vector-transfected cells and represent the mean ± SEM of three replicates (*** $p < 0.001$). *D,* MN9D cells were transfected with either an empty, PKD1–WT or PKD1ΔPH expression vector for 48 h and PGC-1α protein expression was analyzed by Western blot.
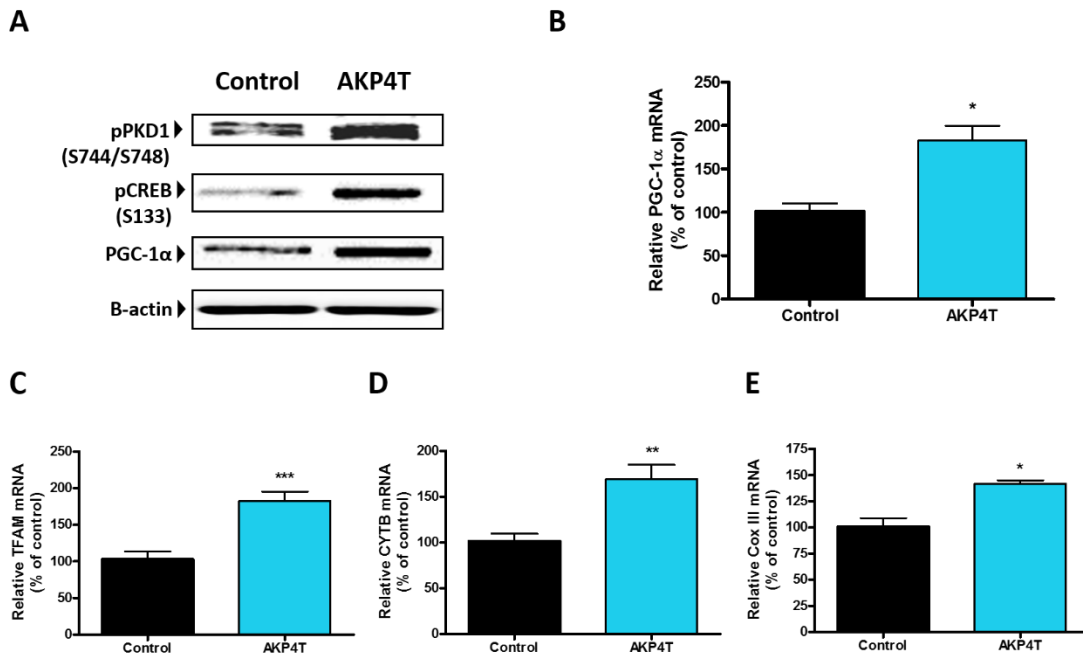
Figure 4. PKD1 activator peptide (AKP4T) increased PGC-1α expression and expression of marker genes of mitochondrial biogenesis. *A,* MN9D cells were treated with 100 nM AKP4T for 2 h and cell lysates were prepared and subjected to Western blot analysis. Representative immunoblots of phospho-PKD1 (S744/748), phospho-CREB (S133), and PGC-1α are shown. *B-E,* MN9D cells were treated with 100 nM AKP4T for 2 h and real-time RT-PCR analyses of PGC-1α (*B*), TFAM (*C*), cytochrome B (CYTB) (*D*), and Cox III (*E*) mRNA levels were performed. 18S rRNA served as internal control. Values are expressed as a percentage of the activity of control and represent the mean ± SEM of three independent experiments performed in triplicate (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; between the control and AKP4T-treated samples).
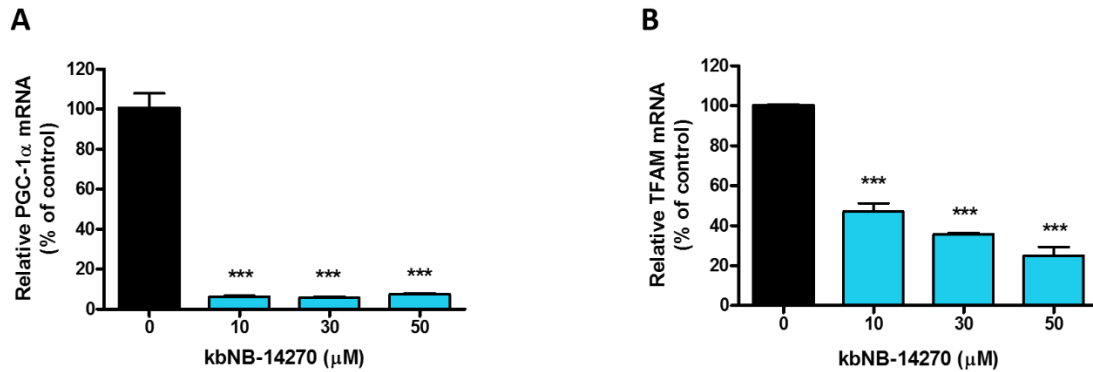
Figure 5. PKD1 inhibitor kbNB-14270 suppresses PGC-1α and TFAM mRNA expression. *A* and *B,* MN9D cells were treated with varying concentrations of kbNB-14270 (10-50 µM) for 2 h. Real-time RT-PCR analyses of PGC-1α (*A*) and TFAM (*B*) mRNA levels were performed. 18S rRNA served as internal control. Values are expressed as a percentage of the activity of control and represent the mean ± SEM of three replicates (*** $p < 0.001$; between the control and kbNB-14270-treated samples).
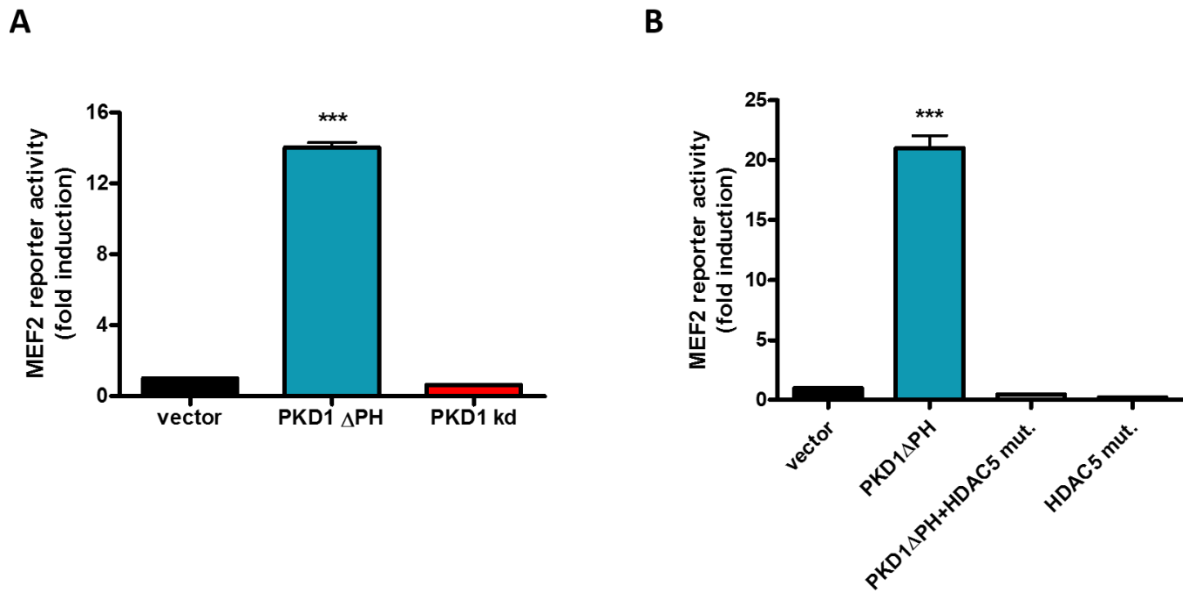
A

B

Figure 6. Overexpression of PKD1ΔPH induces MEF2 activity in MN9D cells. *A,* MN9D cells were cotransfected with 3X-MEF2 luciferase reporter construct and the PKD1ΔPH or kinase-dead PKD1 (PKD1 kd) expression vectors. *B,* MN9D cells were cotransfected with 3X-MEF2 luciferase reporter construct and PKD1ΔPH or HDAC5 mutant either separately or together. Luciferase activity was measured 48 h after transfection and normalized to *β*-galactosidase activity. Values are expressed as fold induction over empty vector control and represent the mean ± SEM of three replicates (***$p < 0.001$).
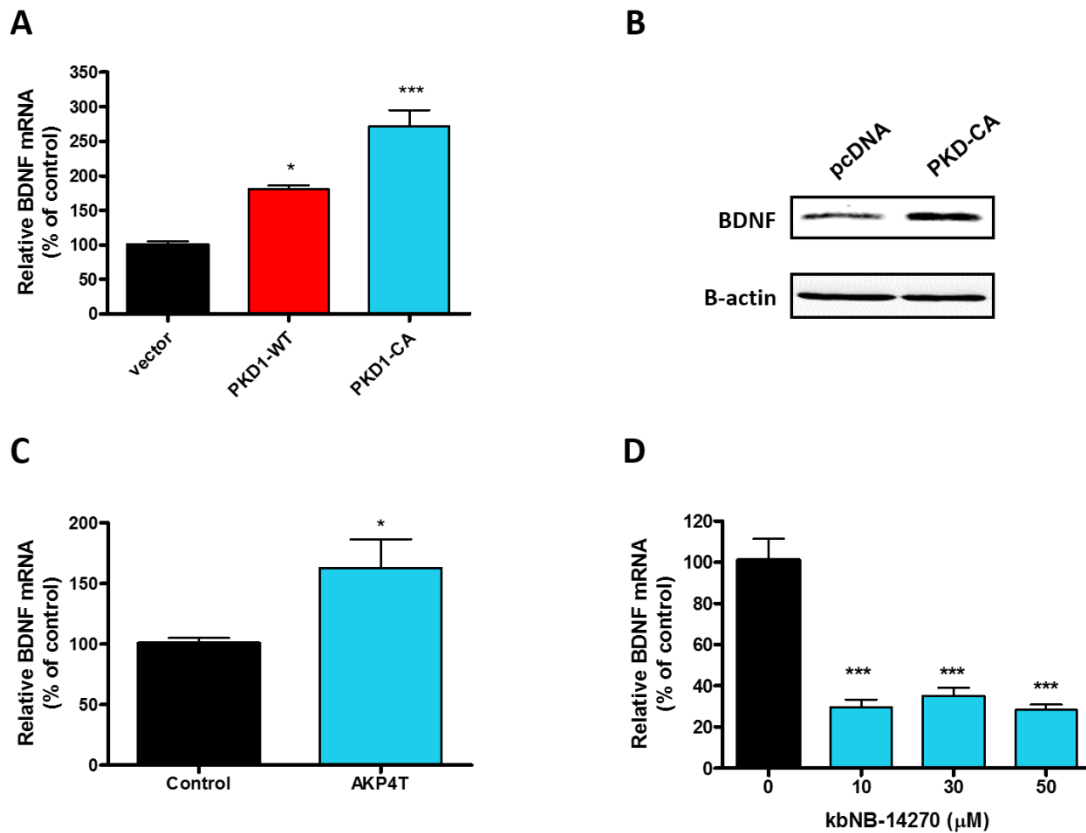
Figure 7. Positive modulation of PKD1 increases BDNF mRNA and protein expression.

A, MN9D cells were transfected with either an empty, PKD1–WT or PKD1-CA expression vector for 48 h and real-time RT-PCR analysis of BDNF was performed. 18S rRNA served as internal control. *B,* MN9D cells transfected with either empty vector or PKD1-CA expression vector for 48 h and BDNF protein expression was analyzed by Western blot. MN9D cells were treated with either 100 nM AKP4T (*C*) or varying concentrations of kbNB-14270 (10-50 µM) (*D*) for 2 h and real-time RT-PCR analysis of BDNF was performed. 18S rRNA served as internal control. Values represent the mean ± SEM of three replicates (* $p < 0.05$; *** $p < 0.001$; between the control and AKP4T- or kbNB-14270-treated samples).
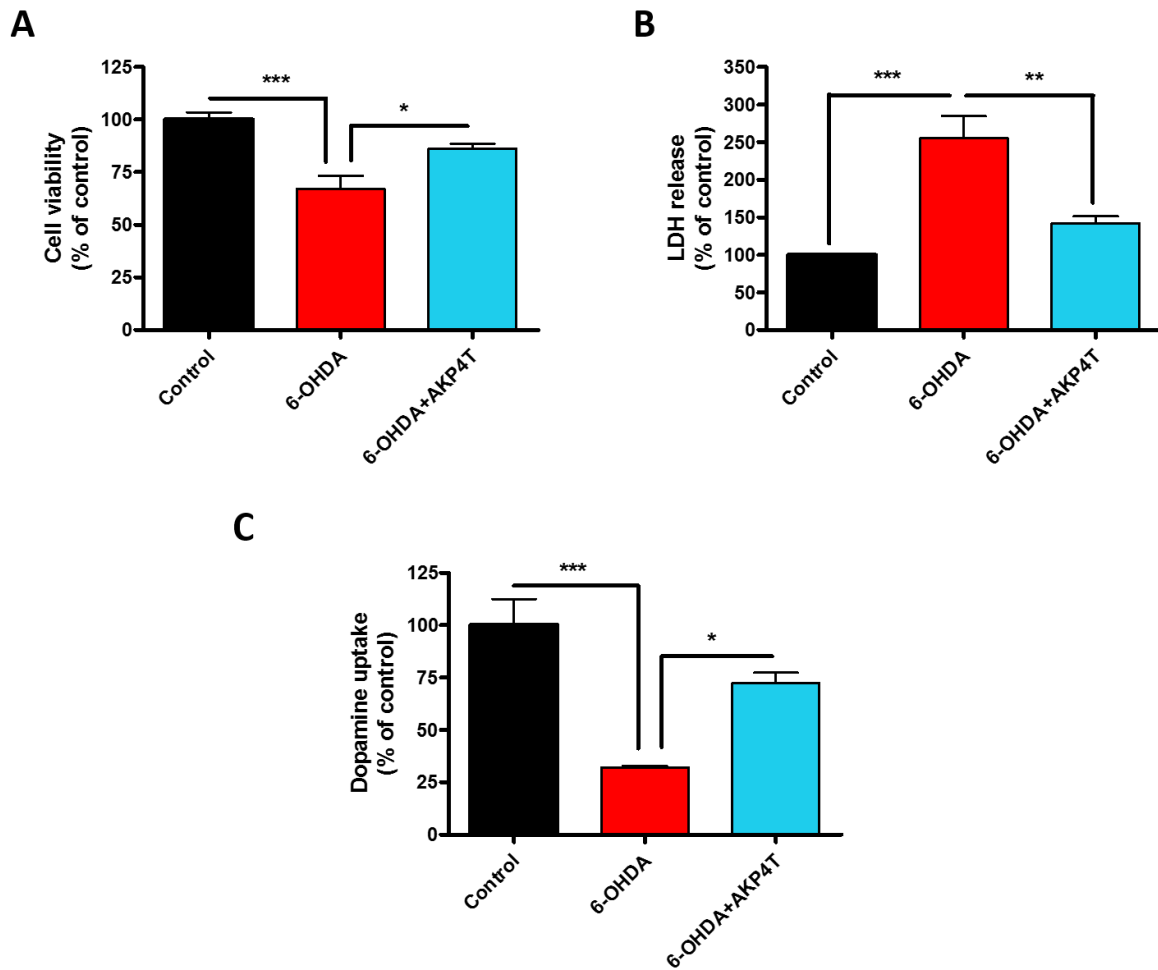
Figure 8. AKP4T protects against 6-OHDA-induced neurotoxicity in LUHMES cells. *A-C,* Differentiated LUHMES cells were pre-treated with 20 nM AKP4T for 1 h and then co-treated with 30 µM 6-OHDA and 20 nM AKP4T for 4 h. 6-OHDA-induced neurotoxicity was measured using the MTS assay (*A*), LDH assay (*B*), and dopamine uptake assay (*C*). Values are expressed as a percentage of the activity of control and represent the mean ± SEM of at least three independent experiments (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).
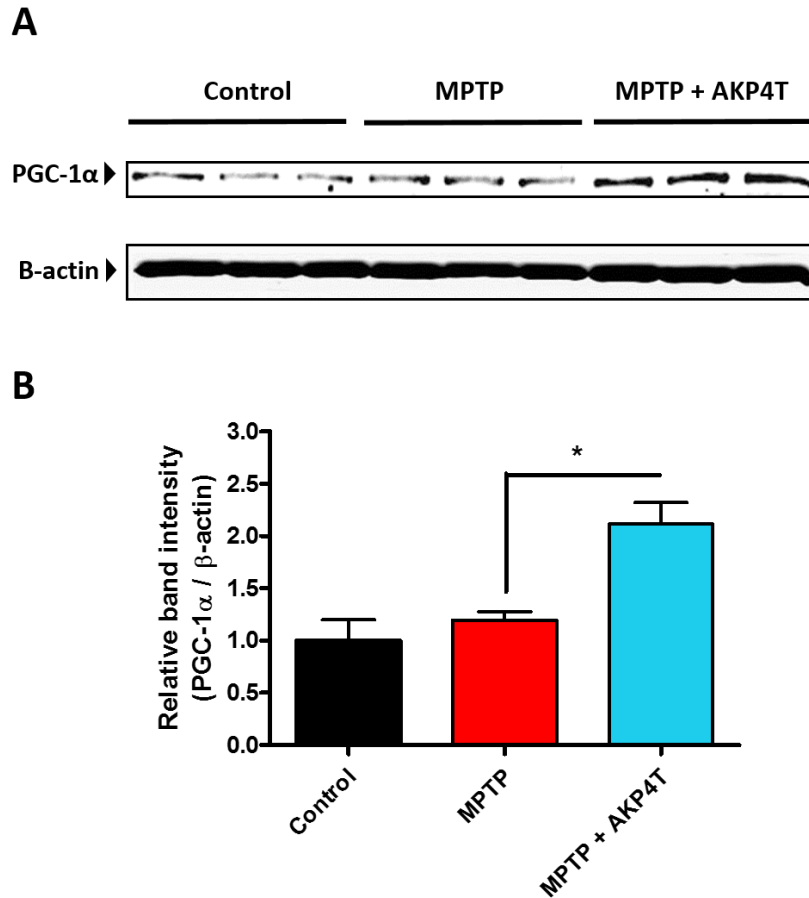
A

B

Figure 9. AKP4T increases PGC-1α expression in the substantia nigra of MPTP-treated mice. *A* and *B,* Mice were pre-treated with AKP4T or control peptide (CP) (5 mg/kg) 1 day before MPTP administration and then co-treated with MPTP (25 mg/kg) and AKP4T for 5 days and AKP4T treatment continued for another 7 days. Substantia nigra (SN) tissues were harvested, prepared, and analyzed for PGC-1α expression. Representative Western blot shows the expression of PGC-1α in SN (*A*) and the graph represents densitometric analysis of PGC-1α protein levels normalized to β-actin in the SN (*B*). Results are the mean ± SEM of five mice per group (* $p < 0.05$).

CHAPTER III: QUERCETIN PROTECTS AGAINST PROGRESSIVE DOPAMINERGIC NEURODEGENERATION IN CELL CULTURE AND MITOPARK ANIMAL MODELS OF PARKINSON'S DISEASE

Muhammet Ay, Jie Luo, Monica Langley, Arthi Kanthasamy, Huajun Jin, Vellareddy Anantharam and Anumantha G. Kanthasamy‡

Parkinson's Disorder Research Laboratory, Iowa Center for Advanced Neurotoxicology, Department of Biomedical Sciences, Iowa State University, Ames, Iowa 50011, USA

‡ To whom correspondence should be addressed: Dept. of Biomedical Sciences, Iowa State University, 2062 College of Veterinary Medicine Building, Ames, IA 50011. Tel: 515-294-2516; Fax: 515-294-2315; Email: akanthas@iastate.edu

Keywords: MitoPark, quercetin, Parkinson disease, PGC-1α, mitochondrial biogenesis, PKD1, neurodegeneration.

The abbreviations used are: PKD1, protein kinase D1; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1-alpha; 6-OHDA, 6-hydroxydopamine; OCR, oxygen consumption rate; HPLC, high-performance liquid chromatography; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; DAB, 3,3'-diaminobenzidine; CREB, cAMP response-element binding protein; BDNF, brain-derived neurotrophic factor; CYTB, cytochrome B; mtDNA, mitochondrial DNA; SN, substantia nigra; CBP, CREB binding protein; CRE, cAMP response element

Abstract

We have recently shown that protein kinase D1 (PKD1) plays a major compensatory survival role in dopaminergic neurons. To identify natural compounds that activate PKD1, we adopted a rationale based pharmacological screening approach using dopaminergic neuronal cell model. Herein, we identified quercetin, a natural flavonoid widely found in vegetables and fruits can effectively activate PKD1 protective signaling. This study aimed to evaluate the effect of quercetin on protective mechanisms in dopaminergic neurons and to test the efficacy of quercetin in MitoPark mouse model of Parkinson's disease (PD). Western blotting analysis revealed that quercetin treatment significantly induced the phosphorylation and activation of PKD1 and Akt in MN9D dopaminergic neuronal cells. Activation of Akt, however, was inhibited by pharmacological inhibition or siRNA knockdown of PKD1, suggesting that Akt acts as a downstream target of PKD1 signaling during quercetin treatment. Quercetin treatment also enhanced CREB phosphorylation and expression of CREB target gene BDNF. Results from qRT-PCR, Western blot analysis, mtDNA content analysis, and Mitotracker assay experiments revealed that quercetin can induce mitochondrial biogenesis in MN9D cells. Furthermore, quercetin increased mitochondrial bioenergetics capacity in dopaminergic cells. Importantly, quercetin treatment protected against 6-OHDA-induced neurotoxicity in MN9D cells. Finally, we evaluated the neuroprotective effects of quercetin against the progressive neurodegenerative process by using MitoPark mouse model of PD. Administering quercetin (25 mg/kg) once daily to 12-week-old MitoPark mice via oral gavage for 6 weeks significantly reversed behavioral deficits, striatal dopamine depletion, and TH neuronal cell loss in MitoPark mice. Our findings suggest that quercetin activates cell survival mechanisms in

dopaminergic neurons and is a promising neuroprotective drug candidate for the treatment of PD.

Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disorder that affects 1% of people over the age of 60 (Lees et al., 2009). PD is characterized pathologically by the progressive loss of dopaminergic neurons and the presence of Lewy bodies in the substantia nigra, dramatic depletion of dopamine in striatum, and activation of glial cells (Dauer and Przedborski, 2003, Kalia and Lang, 2015). The main clinical features of PD include tremor, rigidity, bradykinesia, gait difficulty, and postural instability (Marjama-Lyons and Koller, 2000). Recently, some non-motor symptoms including depression, sleep difficulty, and olfactory dysfunction have also been recognized in PD pathogenesis (Chaudhuri and Schapira, 2009). Although the etiology of PD is not fully understood, there is strong evidence for the involvement of oxidative stress and mitochondrial dysfunction in the pathogenesis of PD (Cardoso et al., 2005, Schapira, 2008). Recent studies have shown that impaired mitochondrial biogenesis contributes to mitochondrial dysfunction in PD (Zheng et al., 2010, Youdim and Oh, 2013). Therefore, stimulation of mitochondrial biogenesis can provide neuroprotection against dopaminergic neurodegeneration in PD.

Although toxin-based models have been widely used as animal models of PD, they don't fully recapitulate the human PD pathology. The acute nature of toxin-based models of PD limits their usefulness in PD drug development and to study the compensatory cell survival mechanisms which might occur in PD patients during the course of the disease. The MitoPark model has been recently developed as an animal model of PD by inactivation of TFAM in

dopaminergic neurons (Ekstrand et al., 2007). MitoPark mouse recapitulates several aspects of human PD, such as adult-onset, progressive degeneration of dopaminergic neurons, protein inclusion in nigral tissues, and responsiveness to levodopa (Galter et al., 2010). Thus, the MitoPark mouse represents a useful model for testing potential neuroprotective agents for the treatment of PD.

Quercetin is a natural polyphenol, which is abundantly found in vegetables and fruits and is the main flavonoid in our daily diet (Hertog et al., 1995). Quercetin has therapeutic potential for the prevention and treatment of neurodegenerative diseases such as Alzheimer's disease (AD) and PD due to its antioxidant and anti-inflammatory properties and its ability to cross the blood-brain barrier (Ansari et al., 2009, Haleagrahara et al., 2011, Ishisaka et al., 2011). Quercetin has been shown to have neuroprotective effects in toxin-based models of PD (Haleagrahara et al., 2011, Lv et al., 2012). However, the molecular mechanisms of quercetin-induced neuroprotection have not been well clarified. Recent studies from our group showed that protein kinase D1 (PKD1) plays a major compensatory survival role in dopaminergic neurons exposed to oxidative insults (Asaithambi et al., 2014, Ay et al., 2015) and also regulates the transcriptional activity of peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α) that stimulates mitochondrial biogenesis (unpublished data). The aim of our present study was to unravel the molecular mechanisms underlying the neuroprotective effect of quercetin and to evaluate the neuroprotective efficacy of quercetin against the progressive neurodegenerative process by using MitoPark mouse model of PD. Our results demonstrate that quercetin activates PKD1 and Akt pro-survival kinases, stimulates mitochondrial biogenesis, and increases mitochondrial bioenergetics capacity in dopaminergic

neuronal cells. Notably, quercetin protects against dopaminergic neurodegeneration in MitoPark mouse model of PD. Overall, our results show that quercetin is a promising neuroprotective drug candidate for the treatment of PD.

Materials and Methods

*Chemicals and Reagents***.** 6-hydroxydopamine (6-OHDA) and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Lois, MO). Quercetin was provided by Dr. Joshua Selsby (Iowa State University, Department of Animal Sciences). The Bradford protein assay kit was purchased from Bio-Rad. Antibodies against PKD1, α-tubulin, BDNF, and mtTFA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho PKD1 (p-S744/748 and p-S916), phospho-Akt (p-S473), total Akt, total CREB, phosho-CREB (p-S133) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-PGC-1α antibody was purchased from Millipore (Billerica, MA). Lipofectamine 2000 reagent, Alexa 680-conjugated anti-mouse secondary antibody and all cell culture reagents were obtained from Invitrogen. IRDye800-conjugated anti-rabbit secondary was purchased from Rockland Labs (Gilbertsville, PA).

Cell Cultures. The mouse dopaminergic MN9D cell line was a kind gift from Dr. Syed Ali (National Center for Toxicological Research/Food and Drug Administration, Jefferson, AR) and cultured as described previously (Jin et al., 2011, Jin et al., 2014). The human dopaminergic neuroblastoma SH-SY5Y cell line was obtained from the American Type Culture Collection. Briefly, MN9D and SH-SY5Y cells were grown in Dulbecco's modified

Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 units penicillin, and 50 μg/ml streptomycin and maintained at 37°C in a 5% CO_2 atmosphere. The rat mesencephalic dopaminergic cell line, referred to as N27 cells, was kindly provided by 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 penicillin, and 50 μg/ml streptomycin and maintained at 37°C in a 5% CO_2 atmosphere, as described previously (Anantharam et al., 2002, Asaithambi et al., 2014).

Transient Transfections, Reporter Gene Assays, and RNAi. Transient transfections of MN9D cells were performed using Lipofectamine 2000 reagent according to the manufacturer's protocol, as described previously (Jin et al., 2011). The 3.1 kb mouse PGC-1α promoter construct was a generous gift from Dr. Zhen Yan (University of Virginia School of Medicine). Cells were seeded into 12-well plates at a density of 15×10^4 cells/well one day before transfection. Each transfection was performed with 1 μg of reporter constructs along with 0.2 μg of pcDNA3-LacZ to normalize transfection efficiencies. In quercetin treatment experiments, indicated doses of quercetin were added 24 h after transfection. Cells were harvested 24 hours after treatment, lysed in 150 μl of Reporter Lysis Buffer (Promega), and assayed for luciferase activity. Luciferase activity was measured on a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT) using the luciferase assay system (Promega). Luciferase activities were normalized to *β*-galactosidase activity.

To silence PKD1 expression, SH-SY5Y cells were transfected with pSUPER-PKD1-RNAi (Addgene #10815) or pSUPER (Oligoengine) using the Lipofectamine 2000 reagent. Forty-

eight hours after transfection, cells were treated with 10µM quercetin for 24 h. Western blot was used to confirm the extent of PKD1 expression.

Quantitative Real-Time RT-PCR. Total RNA was isolated from cells using the Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA), and 1-2 µg RNA was used to generate cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed on an Mx300P QPCR system (Stratagene) using the RT^2 SYBR Green qPCR Mastermix kit (Qiagen) and QuantiTect Primer Assay kit (Qiagen). All RT-qPCR reactions were performed in triplicate and normalized to β-actin housekeeping gene. The primer sequences and PCR conditions are available upon request. Dissociation curves were run to verify the singularity of the PCR product. The data were analyzed using the comparative threshold cycle (Ct) method.

Quantitative Real-Time PCR Analysis of mtDNA content. MN9D cells were plated in 6-well plates at a density of 3×10^5 cells/well. Twenty-four hours later, cells were treated with 10 and 30 µM quercetin for 24 h. Genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. mtDNA content was determined by quantitative PCR with SYBR green. 10 ng nuclear DNA and 1 ng mitochondrial DNA were amplified using the primers for mitochondrial cytochrome b and nuclear β-actin genes. Reactions were performed using an Mx300P QPCR system (Stratagene).

MitoTracker Assay. MN9D cells were plated in 96-well plates at a density of 1×10^4 cells/well. After 24 hours, cells were treated with 10 and 30 µM quercetin for 24 h. Briefly, cells were washed with serum-free medium and stained with 200 nM MitoTracker Green FM (Invitrogen)

for 30 min. Cells were washed three times with PBS and staining was detected on a fluorescence microplate reader (excitation 485 nm, emission 520 nm).

Western Blot Analysis. After treatments, cells or brain tissues were collected and resuspended in a modified RIPA buffer containing protease and phosphatase inhibitors. Briefly, lysates containing equal amounts of protein were loaded in each lane and separated on a 10-12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad). The membranes were blocked in LI-COR blocking buffer for 1 hour at room temperature and then incubated overnight at 4°C with the corresponding primary antibodies. Western blot was performed using IRDye 800 anti-rabbit, Alexa Fluor 680 goat anti-mouse, and Alexa Fluor 680 donkey anti-goat secondary antibodies. Western blot images were captured and analyzed with an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Cell Viability Assay. MN9D cells were plated in 6-well plates at a density of 3×10^5 cells/well. Twenty-four hours later, cells were pre-treated with 10 µM quercetin for 1 h and then co-treated with 50 µM 6-OHDA for 24 h. After the treatment, cells were collected and resuspended in culture medium. 100 µl of Muse annexin V and dead cell reagent (Millipore) and 100 µl of culture medium containing 3×10^4 cells were mixed and incubated for 20 min in the dark. Viable and apoptotic cells were analyzed using the Muse Cell Analyzer.

Measurement of Mitochondrial Oxygen Consumption. The Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) was used to measure oxygen consumption rate (OCR) in N27 cells. Briefly, N27 cells were seeded at 10,000 cells/well in a Seahorse Bioscience polystyrene microplate. Twenty-four hours later, cells were treated with 1 and 10 µM quercetin for 24 h. After the treatment, cells were changed to assay media and

the N27 cells-containing culture plates were loaded into Seahorse instrument to collect baseline OCR measurements. The experimental agents (oligomycin (1 µg/ml), FCCP (3 µM), and antimycin A (10 µM)) were injected using the included ports on the XF96 cartridges and OCR measurements were collected. Basal OCR, ATP-linked OCR, maximal OCR, and spare respiratory capacity were calculated, as described previously (Dranka et al. 2010).

Animals and Treatment. 12-week-old MitoPark and C57BL/6 mice were housed under standard conditions: constant temperature (22±1 °C), humidity (relative, 30%), and a 12 h light/dark cycle. Mice were given free access to food and water. Animal care and protocol procedures were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University (Ames, IA). Mice were treated with either vehicle (5% DMSO in corn oil) or quercetin (25 mg/kg/day) via oral gavage for 6 weeks. After the treatments, mice were subjected to behavioral, neurochemical, biochemical, and histological measurements.

High-Performance Liquid Chromatography (HPLC) Analysis. The striatal dopamine and dihydrophenylacetic acid (DOPAC), and homovanillic acid (HVA) levels were quantified using high performance liquid chromatography (HPLC) with electrochemical detection. Samples were prepared and quantified, as described previously (Ghosh et al., 2013). Briefly, neurotransmitters from striatal tissues were extracted in 0.1 M perchloric acid containing 0.05% Na_2EDTA and 0.1% $Na_2S_2O_5$ and isoproterenol (internal standard). Dopamine, DOPAC, and HVA were separated isocratically using a C-18 reversed phase column with a flow rate 0.6 ml/min. An HPLC system (ESA, Bedford, MA) with an automatic sampler equipped with a refrigerated temperature control was used for these experiments.

3,3'-Diaminobenzidine (DAB) Immunostaining. Tyrosine hydroxylase-diaminobenzidine (DAB) immunostaining was performed in striatal and substantia nigral sections, as described previously (Ghosh et al., 2010). Briefly, mice were sacrificed and perfused with 4% paraformaldehyde and postfixed with paraformaldehyde and 30% sucrose. The fixed brains were cut into 30 µm coronal sections and kept in 30% sucrose-ethylene glycol solution at -20 °C. On the day of staining, 30 µm sections were washed with PBS and incubated with anti-TH antibody (1:1600, Calbiochem) overnight at 4 °C. Biotinylated anti-rabbit secondary antibody was used for 1 h at room temperature followed by incubation with avidin peroxidase (Vectastatin ABC Elite Kit) for 30 min at room temperature. Immunolabeling was observed using DAB, which yielded a brown-colored stain.

Behavioral Measurements. An open-field experiment was performed to measure the locomotor activity of mice using an automated device (AccuScan, Columbus, OH). The rotarod test was performed to evaluate the motor coordination and balance of mice using a constant 20-rpm speed. Before the treatments, mice were trained two consecutive days. One day prior to sacrifice, open-field and rotarod experiments were conducted. Locomotor activities were recorded for 10 min. Data were collected and analyzed using the Versaplot and Versadat softwares.

Statistical Analysis. Data analysis was performed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). Data were first analyzed by using one-way ANOVA and then the Tukey multiple comparison test was used for statistical comparisons. Differences with $P < 0.05$ were considered significant.

Results

Quercetin stimulates the phosphorylation of pro-survival kinases PKD1 and Akt

Quercetin has been shown to have neuroprotective effects in neurotoxicant-induced animal models of PD (Lv et al., 2012, Haleagrahara et al., 2013, Karuppagounder et al., 2013). However, the molecular mechanisms of quercetin-induced neuroprotection have not been well clarified. We recently reported that activation of protein kinase D1 (PKD1) protects dopaminergic cells from oxidative stress-induced cell death (Asaithambi et al., 2011, Asaithambi et al., 2014). Therefore, we wanted to test whether quercetin can activate the pro-survival kinase PKD1. We treated mouse dopaminergic MN9D cells with different concentrations of quercetin (0.3-30 µM) for 24 h and found that quercetin treatment significantly induced the phosphorylation of both activation loop (S744/748) and autophosphorylation (S916) sites of PKD1 (Fig. 1A). Next, we wanted to examine the effect of quercetin on another pro-survival kinase Akt. The neuroprotective role of Akt against dopaminergic neurodegeneration has been reported previously (Ries et al., 2006). As seen in Fig. 1B, treatment of MN9D cells with quercetin for 24 h induced a dramatic increase in Akt phosphorylation. Next, we wanted to explore if there is any relationship between PKD1 and Akt pro-survival kinases during quercetin treatment. Pretreatment of MN9D cells with a pharmacological inhibitor of PKD1 (CID755673) significantly suppressed the phosphorylation of Akt induced by quercetin (Fig. 1C). Furthermore, we transfected SH-SY5Y cells with PKD1-RNAi plasmid for 48 h and then treated with 10 µM quercetin for 24 h. As evident from Fig. 1D, knockdown of PKD1 inhibited quercetin-induced Akt phosphorylation in SH-SY5Y cells. Taken together, these results suggest that quercetin can activate PKD1 and Akt pro-

survival kinases in dopaminergic cells, and that Akt appears to act downstream of PKD1 during quercetin treatment.

Quercetin induces the expression of CREB target gene BDNF

The cAMP response-element binding protein (CREB) is known to be a transcriptional activator of BDNF (Tao et al., 1998), and the activity of CREB is regulated by the phosphorylation of S133 (Johannessen et al., 2004). First, we wanted to examine whether quercetin can induce CREB phosphorylation and found that quercetin significantly enhanced CREB phosphorylation at serine 133 (Fig. 2A). After observing increased phosphorylation of CREB by quercetin treatment, we determined the protein expression of BDNF and found that treatment with quercetin for 24 h significantly increased BDNF protein levels. To determine whether quercetin upregulated BDNF expression at the transcriptional level, we measured BDNF mRNA expression. As evident from Fig. 2B, treatment with 10 and 30 µM quercetin for 24 h significantly induced BDNF mRNA expression. Together, these results suggest that quercetin upregulates BDNF gene expression possibly through CREB phosphorylation in dopaminergic neuronal cells.

Quercetin stimulates mitochondrial biogenesis in MN9D dopaminergic neuronal cells

To further explore the effect of quercetin on dopaminergic neuronal cells, we wanted to determine whether quercetin can induce mitochondrial biogenesis in MN9D cells. First, we checked the mRNA expressions of markers of mitochondrial biogenesis and found that treatment of MN9D cells with 10 and 30 µM quercetin significantly increased mRNA expressions of PGC-1α, TFAM, and cytochrome B (CYTB) (Fig. 3A). Next, we measured

whether quercetin can induce PGC-1α promoter activity. We transfected MN9D cells with PGC-1α promoter-reporter construct, and then 24 h post-transfection, we treated cells with quercetin for 24 h. As shown in Fig. 3B, quercetin significantly increased the PGC-1α promoter activity. Since quercetin significantly increased PGC-1α and TFAM mRNA expression (Fig. 3A), we wanted to examine PGC-1α and TFAM protein expression and found that PGC-1α and TFAM protein levels were significantly upregulated in quercetin-exposed MN9D cells (Fig. 3C). To further confirm that quercetin can stimulate mitochondrial biogenesis, we assessed mitochondrial content of quercetin-treated cells by MitoTracker assay and found that 10 and 30 µM quercetin treatment significantly increased mitochondrial mass in MN9D cells (Fig. 3D). Furthermore, mitochondrial DNA (mtDNA) content was also significantly increased in MN9D cells treated with 10 and 30 µM quercetin for 24 h (Fig. 3E). Collectively, these results clearly demonstrate that quercetin can stimulate mitochondrial biogenesis in dopaminergic neuronal cells.

Quercetin increases mitochondrial bioenergetics capacity in N27 dopaminergic cells

After establishing that quercetin induces mitochondrial biogenesis, we wanted to examine the effect of quercetin on cellular bioenergetics in N27 dopaminergic neuronal cells. We treated N27 cells with 1 and 10 µM quercetin for 24 h and measured the oxygen consumption rates (OCR) using the Seahorse technology. Importantly, quercetin treatment significantly enhanced mitochondrial oxygen consumption, as evidenced by increases in basal OCR, ATP-linked respiration, maximal OCR, and spare respiratory capacity (Fig. 4A-E). These data suggest that quercetin treatment increases oxidative phosphorylation in dopaminergic neuronal cells.

Quercetin reduces 6-OHDA-induced neurotoxicity in MN9D cells

As described above, our results demonstrated that quercetin can effectively induce activation of prosurvival kinases PKD1 and Akt and mitochondrial biogenesis. To further confirm the neuroprotective effect of quercetin, MN9D cells were pre-treated with 10 µM quercetin for 1 h and then co-treated with the dopaminergic neurotoxicant 6-OHDA (50 µM) for 24 h. Apoptosis and cell viability were measured by flow cytometry (Muse Cell Analyser). As shown in Fig. 5A-C, 6-OHDA treatment led to a substantial reduction in cell viability. However, pretreatment with quercetin significantly reduced the total number of apoptotic cells when compared to cells treated with 6-OHDA alone. These results demonstrated that quercetin can protect MN9D cells against 6-OHDA-induced cell death.

Quercetin protects against dopaminergic neurodegeneration in MitoPark mice

Since our in vitro experiments revealed that quercetin has a neuroprotective effect on dopaminergic neuronal cells, we wanted to extend our in vitro observations into an in vivo model of Parkinson's disease. As depicted in Fig. 6A, 12-week-old MitoPark and control mice were treated with quercetin (25 mg/kg) daily via oral gavage for 6 weeks. Expectedly, a significant loss of TH-positive dopaminergic neurons in the substantia nigra (SN) and TH-positive terminals in striatum was observed in MitoPark mice compared with control mice (Fig. 6B). Importantly, quercetin treatment significantly reduced nigrostriatal degeneration in MitoPark mice. Consistent with these data, western blot analysis of TH in substantia nigra confirmed that quercetin treatment significantly prevented loss of TH levels in the substantia nigra of MitoPark mice (Fig. 6D and E). Furthermore, we determined whether quercetin could reduce striatal dopamine depletion in MitoPark mice. As shown in Fig. 6C, quercetin treatment

also significantly restored striatal dopamine levels in MitoPark mice. Collectively, these results demonstrate that quercetin can slow down the degeneration of dopaminergic neurons in the MitoPark mouse model of PD.

Quercetin ameliorates motor deficits in MitoPark mice

After observing the neuroprotective effect of quercetin against nigrostriatal degeneration, we evaluated whether quercetin treatment improves motor deficits in MitoPark mice. MitoPark mice begin to show motor deficits from 12 weeks of age onwards compared to age-matched control mice (Ekstrand et al., 2007). Six weeks after quercetin treatment, one day prior to sacrifice, locomotor activity and rotarod performance of MitoPark mice were measured using a Versamax activity monitor and a rotarod instrument. As shown in the representative maps (Versa-Plot), the total movement of MitoPark mice significantly decreased (Fig. 7A). On the other hand, quercetin treatment significantly improved locomotion in MitoPark mice. We found that quercetin treatment significantly reversed the decreases in horizontal activity (Fig. 7B), vertical activity (Fig. 7C), observed stereotypies (Fig. 7E), and rearing activity (Fig. 7F) of MitoPark mice. Moreover, MitoPark mice-treated with quercetin spent significantly more time on the rotarod than the MitoPark mice-treated with vehicle (Fig. 7G). Collectively, these findings suggest that quercetin improves motor dysfunction in the MitoPark mouse model of PD.

Discussion

In the present study, we have attempted to elucidate the molecular mechanisms underlying quercetin-mediated neuroprotection and to test the efficacy of quercetin in the MitoPark mouse model of PD. Although some studies have shown that quercetin is protective against neurodegenerative processes, yet the molecular mechanisms and signaling events governing the protective effect of quercetin remain elusive. Our study demonstrates that quercetin can activate the pro-survival kinases PKD1 and Akt and induces the expression of BDNF in dopaminergic neuronal cells. Furthermore, we show that quercetin increases mitochondrial biogenesis and bioenergetics capacity in cell culture models of PD. More importantly, we observed that quercetin protects against progressive nigrostriatal degeneration, striatal dopamine depletion and behavioral deficits in MitoPark mice.

Quercetin is one of the main flavonoids in our daily diet and has been recognized for having antioxidant, antiviral, antibacterial, anti-inflammatory, and anticarcinogenic properties (Duthie and Dobson, 1999, Ramos et al., 2006, Boots et al., 2008, Zandi et al., 2011). Several lines of evidence suggest that quercetin has therapeutic potential for the prevention and treatment of different human diseases, including neurodegenerative diseases (Ansari et al., 2009, Egert et al., 2009, Haleagrahara et al., 2011, Chakraborty et al., 2014, Sabogal-Guaqueta et al., 2015). Although recent studies have documented the neuroprotective effect of quercetin in toxin-based models of PD, the molecular mechanisms behind the protective effect of quercetin are not well-clarified (Haleagrahara et al., 2011, Lv et al., 2012). In this study, we first investigated the effect of quercetin on the pro-survival kinases PKD1 and Akt in dopaminergic neuronal cells. We have recently reported that PKD1 plays an anti-apoptotic role

in dopaminergic neurons exposed to oxidative stress (Asaithambi et al., 2011, Ay et al., 2015). The protective role of Akt has been documented for neurodegenerative diseases, including PD (Humbert et al., 2002, Ries et al., 2006, Zhang et al., 2010). Ries et al showed that viral overexpression of Akt in the substantia nigra significantly protected against 6-OHDA-induced neurodegeneration. In addition, it has been reported that treatment of mice with the Parkinsonian neurotoxicant MPTP led to significant reduction in phospho-Akt levels (Durgadoss et al., 2012), pointing to the pro-survival role of Akt in dopaminergic neurons. Our results showed that quercetin can effectively activate PKD1 and Akt kinase in dopaminergic neuronal cells (Fig. 1, A and B). Next, we wanted to investigate the downstream and upstream relationships between PKD1 and Akt. Previously, it has been shown that PKD3, another isoform of the PKD family, regulates Akt signaling in prostate cancer cells (Chen et al., 2008). In our experiments, we found that pharmacological inhibition or genetic knockdown of PKD1 reduces quercetin-induced Akt phosphorylation (Fig. 1, C and D), suggesting that Akt kinase might function downstream of PKD1 during quercetin treatment in dopaminergic cells. After observing a significant increase in PKD1 phosphorylation by quercetin treatment, we wanted to check the phosphorylation status of CREB because PKD1 can directly phosphorylate CREB at Ser133 (Johannessen et al., 2007). CREB has been shown to regulate the expression of genes involved in neuroprotection (Shieh et al., 1998, Meller et al., 2005, Barneda-Zahonero et al., 2012). Once CREB gets phosphorylated at Ser133, the CREB binding protein (CBP) binds to CREB and facilitates transcription of the cAMP response element (CRE)-dependent genes including BDNF (Tao et al., 1998, Lonze and Ginty, 2002). In our experiments, we show that quercetin can increase CREB phosphorylation (Fig. 2A) and also BDNF protein and mRNA expression in MN9D dopaminergic neuronal cells (Fig. 2, A and B). Moreover, we also

observed that pre-treatment with quercetin significantly attenuates 6-OHDA-induced neurotoxicity (Fig. 5A-C). Together, these data suggest that quercetin can protect dopaminergic cells against neurotoxic insults and that PKD1/CREB/BDNF axis might mediate the protective effect of quercetin in dopaminergic neuronal cells.

Mitochondria play a pivotal role in the survival or death of neurons and mitochondrial dysfunction has been recognized as an important contributor to the neurodegenerative process observed in PD (Olanow and Tatton, 1999, Onyango, 2008). Previous studies have also shown that impaired mitochondrial biogenesis contributes to mitochondrial dysfunction in PD (Zheng et al., 2010, Youdim and Oh, 2013). PGC-1α is a transcriptional coactivator that regulates mitochondrial biogenesis and antioxidant defenses (Clark and Simon, 2009). Importantly, genes regulated by PGC-1α are underexpressed in PD patients (Zheng et al., 2010) and PGC-1α activity has been shown to decrease by loss of parkin function and binding of alpha synuclein to its promoter (Shin et al., 2011, Siddiqui et al., 2012), suggesting that decreased PGC-1α expression may play a role in the pathogenesis of PD. Therefore, we wanted to check if quercetin can enhance mitochondrial biogenesis in dopaminergic cells. We found that quercetin increased mRNA expressions of PGC-1α, TFAM, and CYTB, marker genes for mitochondrial biogenesis, in MN9D cells (Fig. 3A). TFAM is required for mtDNA replication and transcription and also known to play a role in mtDNA maintenance and integrity (Scarpulla, 2008, Ngo et al., 2011). We also observed increased PGC-1α promoter activity (Fig. 3B) and protein expression of PGC-1α and TFAM (Fig. 3C) with the treatment of quercetin. Furthermore, we found increases in MitoTracker staining (Fig. 3D) and mtDNA content (Fig. 3E) in MN9D cells treated with quercetin, suggesting quercetin can effectively

enhance mitochondrial biogenesis in dopaminergic cells. Next, we measured the effect of quercetin on cellular bioenergetics and found that quercetin can significantly increase mitochondrial oxygen consumption rates in dopaminergic cells (Fig. 4A-E). Our results demonstrate that quercetin can serve as an effective natural compound to improve mitochondrial function and biogenesis in dopaminergic cells.

To further substantiate the neuroprotective effects of quercetin, we used the MitoPark transgenic animal model of PD. The MitoPark model was recently developed by specific inactivation of TFAM in dopaminergic neurons (Ekstrand et al., 2007). MitoPark mice show similar motor performance to their age-matched controls until the age of 12 weeks. However, from 12 weeks of age onwards, MitoPark mice begin to display impaired locomotor activity accompanied by a progressive loss of dopaminergic neurons in the substantia nigra and a reduction of striatal dopamine. The MitoPark mouse provides a useful tool for testing the efficacy of candidate neuroprotective drugs since it has a chronic and progressive nature and also recapitulates several features of PD in humans. In our study, 12-week-old MitoPark mice were treated with quercetin for six weeks and our results show that quercetin can protect against neurodegeneration in MitoPark mice. We show that quercetin reversed behavioral deficits, striatal dopamine depletion, and TH neuronal cell loss in MitoPark mice (Fig. 6 and 7), suggesting that quercetin has potential to slow down the progression of disease.

In summary, we demonstrate for the first time that quercetin can effectively activate the pro-survival kinase PKD1 in dopaminergic cells. Quercetin also induces Akt and CREB phosphorylation and BDNF expression. Our results suggest that PKD1/CREB/BDNF axis may at least partially mediate the neuroprotective effects of quercetin. Moreover, quercetin

treatment increases mitochondrial biogenesis in dopaminergic neurons. Importantly, quercetin protects against dopaminergic neurodegeneration in the MitoPark transgenic mouse model of PD. Our findings clearly suggest that quercetin is a promising neuroprotective agent and merits further investigation for the prevention and treatment of neurodegenerative diseases, including PD.

REFERENCES

Anantharam V, Kitazawa M, Wagner J, Kaul S, Kanthasamy AG (2002) Caspase-3-dependent proteolytic cleavage of protein kinase Cdelta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese tricarbonyl. J Neurosci 22:1738-1751.

Ansari MA, Abdul HM, Joshi G, Opii WO, Butterfield DA (2009) Protective effect of quercetin in primary neurons against Abeta(1-42): relevance to Alzheimer's disease. The Journal of nutritional biochemistry 20:269-275.

Asaithambi A, Ay M, Jin H, Gosh A, Anantharam V, Kanthasamy A, Kanthasamy AG (2014) Protein kinase D1 (PKD1) phosphorylation promotes dopaminergic neuronal survival during 6-OHDA-induced oxidative stress. PloS one 9:e96947.

Asaithambi A, Kanthasamy A, Saminathan H, Anantharam V, Kanthasamy AG (2011) Protein kinase D1 (PKD1) activation mediates a compensatory protective response during early stages of oxidative stress-induced neuronal degeneration. Molecular neurodegeneration 6:43.

Ay M, Jin H, Harischandra DS, Asaithambi A, Kanthasamy A, Anantharam V, Kanthasamy AG (2015) Molecular cloning, epigenetic regulation, and functional characterization of Prkd1 gene promoter in dopaminergic cell culture models of Parkinson's disease. Journal of neurochemistry 135:402-415.

Barneda-Zahonero B, Servitja JM, Badiola N, Minano-Molina AJ, Fado R, Saura CA, Rodriguez-Alvarez J (2012) Nurr1 protein is required for N-methyl-D-aspartic acid (NMDA) receptor-mediated neuronal survival. The Journal of biological chemistry 287:11351-11362.

Boots AW, Wilms LC, Swennen EL, Kleinjans JC, Bast A, Haenen GR (2008) In vitro and ex vivo anti-inflammatory activity of quercetin in healthy volunteers. Nutrition (Burbank, Los Angeles County, Calif 24:703-710.

Cardoso SM, Moreira PI, Agostinho P, Pereira C, Oliveira CR (2005) Neurodegenerative pathways in Parkinson's disease: therapeutic strategies. Current drug targets 4:405-419.

Chakraborty J, Singh R, Dutta D, Naskar A, Rajamma U, Mohanakumar KP (2014) Quercetin improves behavioral deficiencies, restores astrocytes and microglia, and reduces serotonin metabolism in 3-nitropropionic acid-induced rat model of Huntington's Disease. CNS neuroscience & therapeutics 20:10-19.

Chaudhuri KR, Schapira AH (2009) Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. Lancet neurology 8:464-474.

Chen J, Deng F, Singh SV, Wang QJ (2008) Protein kinase D3 (PKD3) contributes to prostate cancer cell growth and survival through a PKCepsilon/PKD3 pathway downstream of Akt and ERK 1/2. Cancer research 68:3844-3853.

Clark J, Simon DK (2009) Transcribe to survive: transcriptional control of antioxidant defense programs for neuroprotection in Parkinson's disease. Antioxidants & redox signaling 11:509-528.

Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889-909.

Durgadoss L, Nidadavolu P, Valli RK, Saeed U, Mishra M, Seth P, Ravindranath V (2012) Redox modification of Akt mediated by the dopaminergic neurotoxin MPTP, in mouse midbrain, leads to down-regulation of pAkt. FASEB J 26:1473-1483.

Duthie SJ, Dobson VL (1999) Dietary flavonoids protect human colonocyte DNA from oxidative attack in vitro. European journal of nutrition 38:28-34.

Egert S, Bosy-Westphal A, Seiberl J, Kurbitz C, Settler U, Plachta-Danielzik S, Wagner AE, Frank J, Schrezenmeir J, Rimbach G, Wolffram S, Muller MJ (2009) Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: a double-blinded, placebo-controlled cross-over study. The British journal of nutrition 102:1065-1074.

Ekstrand MI, Terzioglu M, Galter D, Zhu S, Hofstetter C, Lindqvist E, Thams S, Bergstrand A, Hansson FS, Trifunovic A, Hoffer B, Cullheim S, Mohammed AH, Olson L, Larsson NG (2007) Progressive parkinsonism in mice with respiratory-chain-deficient dopamine neurons. Proceedings of the National Academy of Sciences of the United States of America 104:1325-1330.

Galter D, Pernold K, Yoshitake T, Lindqvist E, Hoffer B, Kehr J, Larsson NG, Olson L (2010) MitoPark mice mirror the slow progression of key symptoms and L-DOPA response in Parkinson's disease. Genes, brain, and behavior 9:173-181.

Ghosh A, Chandran K, Kalivendi SV, Joseph J, Antholine WE, Hillard CJ, Kanthasamy A, Kanthasamy A, Kalyanaraman B (2010) Neuroprotection by a mitochondria-targeted drug in a Parkinson's disease model. Free radical biology & medicine 49:1674-1684.

Ghosh A, Saminathan H, Kanthasamy A, Anantharam V, Jin H, Sondarva G, Harischandra DS, Qian Z, Rana A, Kanthasamy AG (2013) The peptidyl-prolyl isomerase Pin1 up-regulation and proapoptotic function in dopaminergic neurons: relevance to the pathogenesis of Parkinson disease. The Journal of biological chemistry 288:21955-21971.

Haleagrahara N, Siew CJ, Mitra NK, Kumari M (2011) Neuroprotective effect of bioflavonoid quercetin in 6-hydroxydopamine-induced oxidative stress biomarkers in the rat striatum. Neuroscience letters 500:139-143.

Haleagrahara N, Siew CJ, Ponnusamy K (2013) Effect of quercetin and desferrioxamine on 6-hydroxydopamine (6-OHDA) induced neurotoxicity in striatum of rats. The Journal of toxicological sciences 38:25-33.

Hertog MG, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S, et al. (1995) Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Archives of internal medicine 155:381-386.

Humbert S, Bryson EA, Cordelieres FP, Connors NC, Datta SR, Finkbeiner S, Greenberg ME, Saudou F (2002) The IGF-1/Akt pathway is neuroprotective in Huntington's disease and involves Huntingtin phosphorylation by Akt. Developmental cell 2:831-837.

Ishisaka A, Ichikawa S, Sakakibara H, Piskula MK, Nakamura T, Kato Y, Ito M, Miyamoto K, Tsuji A, Kawai Y, Terao J (2011) Accumulation of orally administered quercetin in brain tissue and its antioxidative effects in rats. Free radical biology & medicine 51:1329-1336.

Jin H, Kanthasamy A, Anantharam V, Rana A, Kanthasamy AG (2011) Transcriptional regulation of pro-apoptotic protein kinase Cdelta: implications for oxidative stress-induced neuronal cell death. The Journal of biological chemistry 286:19840-19859.

Jin H, Kanthasamy A, Harischandra DS, Kondru N, Ghosh A, Panicker N, Anantharam V, Rana A, Kanthasamy AG (2014) Histone Hyperacetylation Up-regulates Protein Kinase Cdelta in Dopaminergic Neurons to Induce Cell Death: RELEVANCE TO EPIGENETIC MECHANISMS OF NEURODEGENERATION IN PARKINSON DISEASE. The Journal of biological chemistry 289:34743-34767.

Johannessen M, Delghandi MP, Moens U (2004) What turns CREB on? Cellular signalling 16:1211-1227.

Johannessen M, Delghandi MP, Rykx A, Dragset M, Vandenheede JR, Van Lint J, Moens U (2007) Protein kinase D induces transcription through direct phosphorylation of the cAMP-response element-binding protein. The Journal of biological chemistry 282:14777-14787.

Kalia LV, Lang AE (2015) Parkinson's disease. Lancet.

Karuppagounder SS, Madathil SK, Pandey M, Haobam R, Rajamma U, Mohanakumar KP (2013) Quercetin up-regulates mitochondrial complex-I activity to protect against programmed cell death in rotenone model of Parkinson's disease in rats. Neuroscience 236:136-148.

Lees AJ, Hardy J, Revesz T (2009) Parkinson's disease. Lancet 373:2055-2066.

Lonze BE, Ginty DD (2002) Function and regulation of CREB family transcription factors in the nervous system. Neuron 35:605-623.

Lv C, Hong T, Yang Z, Zhang Y, Wang L, Dong M, Zhao J, Mu J, Meng Y (2012) Effect of Quercetin in the 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-Induced Mouse Model of Parkinson's Disease. Evid Based Complement Alternat Med 2012:928643.

Marjama-Lyons J, Koller W (2000) Tremor-predominant Parkinson's disease. Approaches to treatment. Drugs & aging 16:273-278.

Meller R, Minami M, Cameron JA, Impey S, Chen D, Lan JQ, Henshall DC, Simon RP (2005) CREB-mediated Bcl-2 protein expression after ischemic preconditioning. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 25:234-246.

Ngo HB, Kaiser JT, Chan DC (2011) The mitochondrial transcription and packaging factor Tfam imposes a U-turn on mitochondrial DNA. Nature structural & molecular biology 18:1290-1296.

Olanow CW, Tatton WG (1999) Etiology and pathogenesis of Parkinson's disease. Annual review of neuroscience 22:123-144.

Onyango IG (2008) Mitochondrial dysfunction and oxidative stress in Parkinson's disease. Neurochemical research 33:589-597.

Ramos FA, Takaishi Y, Shirotori M, Kawaguchi Y, Tsuchiya K, Shibata H, Higuti T, Tadokoro T, Takeuchi M (2006) Antibacterial and antioxidant activities of quercetin oxidation products from yellow onion (Allium cepa) skin. Journal of agricultural and food chemistry 54:3551-3557.

Ries V, Henchcliffe C, Kareva T, Rzhetskaya M, Bland R, During MJ, Kholodilov N, Burke RE (2006) Oncoprotein Akt/PKB induces trophic effects in murine models of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America 103:18757-18762.

Sabogal-Guaqueta AM, Munoz-Manco JI, Ramirez-Pineda JR, Lamprea-Rodriguez M, Osorio E, Cardona-Gomez GP (2015) The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice. Neuropharmacology 93C:134-145.

Scarpulla RC (2008) Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiological reviews 88:611-638.

Schapira AH (2008) Mitochondria in the aetiology and pathogenesis of Parkinson's disease. Lancet neurology 7:97-109.

Shieh PB, Hu SC, Bobb K, Timmusk T, Ghosh A (1998) Identification of a signaling pathway involved in calcium regulation of BDNF expression. Neuron 20:727-740.

Shin JH, Ko HS, Kang H, Lee Y, Lee YI, Pletinkova O, Troconso JC, Dawson VL, Dawson TM (2011) PARIS (ZNF746) repression of PGC-1alpha contributes to neurodegeneration in Parkinson's disease. Cell 144:689-702.

Siddiqui A, Chinta SJ, Mallajosyula JK, Rajagopolan S, Hanson I, Rane A, Melov S, Andersen JK (2012) Selective binding of nuclear alpha-synuclein to the PGC1alpha promoter under conditions of oxidative stress may contribute to losses in mitochondrial function: implications for Parkinson's disease. Free radical biology & medicine 53:993-1003.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20:709-726.

Youdim MB, Oh YJ (2013) Promise of neurorestoration and mitochondrial biogenesis in Parkinson's disease with multi target drugs: an alternative to stem cell therapy. Experimental neurobiology 22:167-172.

Zandi K, Teoh BT, Sam SS, Wong PF, Mustafa MR, Abubakar S (2011) Antiviral activity of four types of bioflavonoid against dengue virus type-2. Virology journal 8:560.

Zhang L, Qu Y, Tang J, Chen D, Fu X, Mao M, Mu D (2010) PI3K/Akt signaling pathway is required for neuroprotection of thalidomide on hypoxic-ischemic cortical neurons in vitro. Brain research 1357:157-165.

Zheng B, Liao Z, Locascio JJ, Lesniak KA, Roderick SS, Watt ML, Eklund AC, Zhang-James Y, Kim PD, Hauser MA, Grunblatt E, Moran LB, Mandel SA, Riederer P, Miller RM, Federoff HJ, Wullner U, Papapetropoulos S, Youdim MB, Cantuti-Castelvetri I, Young AB, Vance JM, Davis RL, Hedreen JC, Adler CH, Beach TG, Graeber MB, Middleton FA, Rochet JC, Scherzer CR (2010) PGC-1alpha, a potential therapeutic target for early intervention in Parkinson's disease. Science translational medicine 2:52ra73.
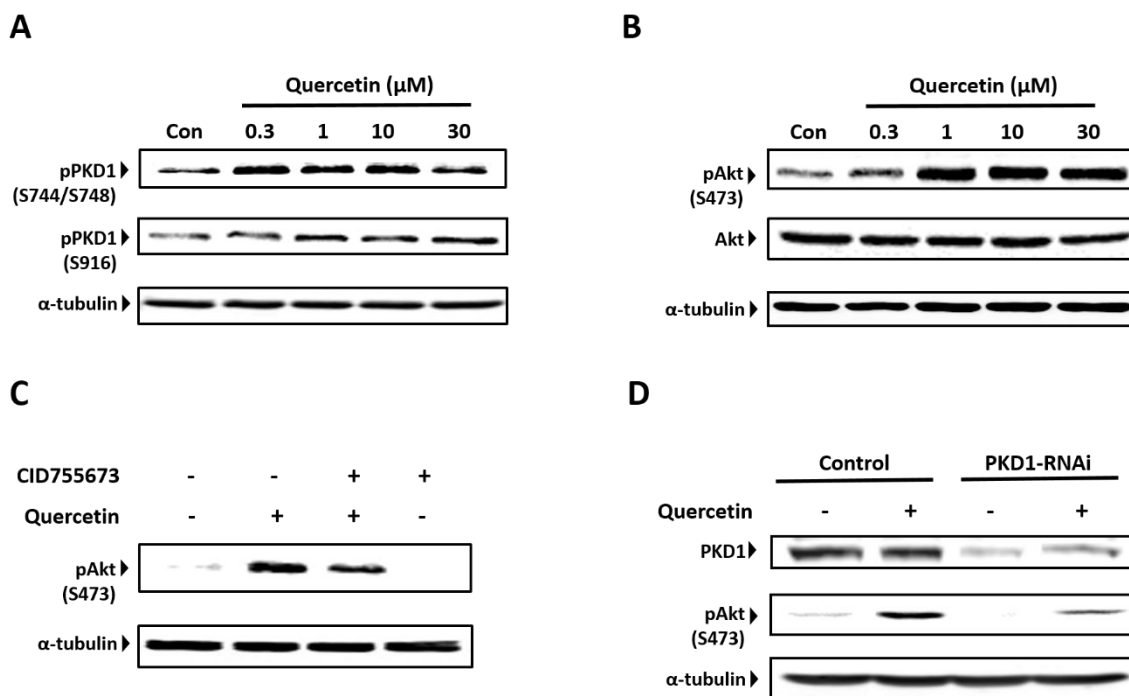
Figure 1. Activation of pro-survival PKD1 and Akt kinase by quercetin. *A* and *B,* MN9D dopaminergic neuronal cells were treated with varying concentrations of quercetin (0.3-30 µM) for 24 h and cell lysates were prepared and subjected to Western blot analysis. Representative immunoblots of PKD1 S744/748 and S916 phosphorylation (*A*) and total Akt and Akt S473 phosphorylation (*B*) are shown. *C*, MN9D cells were pre-treated with 50 µM PKD1 inhibitor CID 755673 for 1 h and then co-treated with 10 µM quercetin for 24 h and phospho-Akt (S473) levels were determined by Western blot analysis. *D*, SH-SY5Y cells were transfected with pSUPER-PKD1-RNAi or pSUPER plasmids. Forty-eight hours after transfection, cells were treated with 10 µM quercetin for 24 h and total PKD1 and phospho-Akt (S473) levels were analyzed by Western blot.
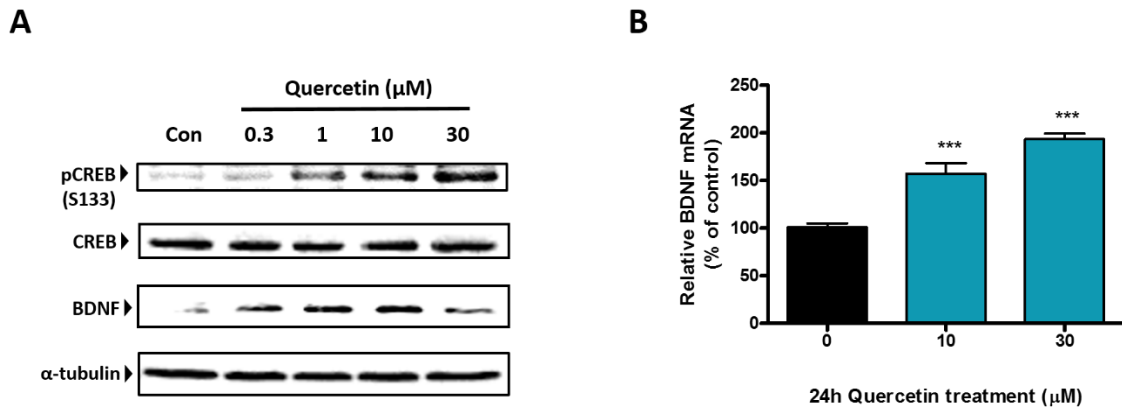
Figure 2. Quercetin induces the expression of CREB target gene BDNF. *A,* MN9D cells were treated with varying concentrations of quercetin (0.3-30 µM) for 24 h and cell lysates were prepared and subjected to Western blot analysis. Representative immunoblots of phospho-CREB (S133), total CREB, and BDNF are shown. *B,* MN9D cells were treated with 10 and 30 µM quercetin for 24 h and real-time RT-PCR analysis of BDNF mRNA level was performed. 18S rRNA served as internal control. Values are expressed as a percentage of the activity of control and represent the means ± SEM of three replicates (***, p<0.001; between the control and quercetin-treated samples).
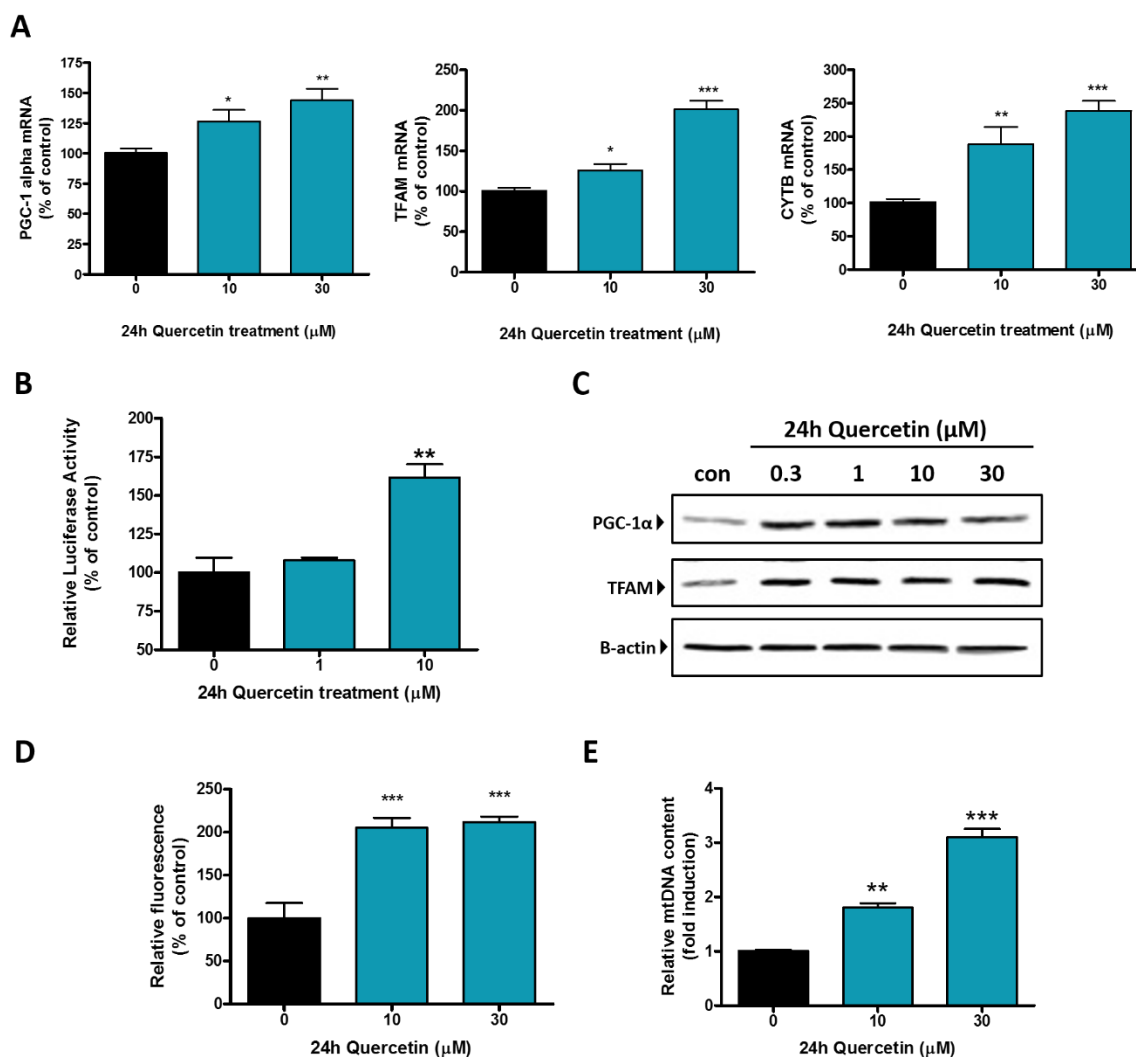
Figure 3. Quercetin stimulates mitochondrial biogenesis in MN9D cells. *A,* MN9D cells were treated with 10 and 30 µM quercetin for 24 h and real-time RT-PCR analyses of PGC-1α, TFAM, and cytochrome B (CYTB) mRNA levels were performed. 18S rRNA served as internal control. Values are expressed as a percentage of the activity of control and represent the means ± SEM of three independent experiments performed in triplicate (*, p<0.05; **, p<0.01; ***, p<0.001; between the control and quercetin-treated samples). *B,* MN9D cells

were transfected with PGC-1α promoter-reporter construct, and then 24 h post-transfection, treated with quercetin 1 and 10 µM quercetin for 24 h. Luciferase activities were measured and normalized to *β*-galactosidase activity. Values are expressed as a percentage of the activity of control and represent the means ± SEM of three replicates (**, $p<0.01$; between the control and quercetin-treated samples). *C,* MN9D cells were treated with varying concentrations of quercetin for 24 h and cell lysates were prepared and subjected to Western blot analysis. Representative immunoblots of PGC-1α and TFAM are shown. *D,* MN9D cells were treated with 10 and 30 µM quercetin for 24 h and stained with 200 nM MitoTracker Green FM. MitoTracker staining was detected on a fluorescence microplate reader. Values are expressed as a percentage of the activity of control and represent the means ± SEM of four replicates (***, $p<0.001$; between the control and quercetin-treated samples). *E,* MN9D cells were treated with 10 and 30 µM quercetin for 24 h. Genomic DNA was isolated and mtDNA content was determined by quantitative PCR with SYBR green. 10 ng nuclear DNA and 1 ng mitochondrial DNA were amplified using the primers for mitochondrial cytochrome b and nuclear β-actin genes. Values are expressed as a percentage of the activity of control and represent the means ± SEM of three replicates (**, $p<0.01$; ***, $p<0.001$; between the control and quercetin-treated samples).
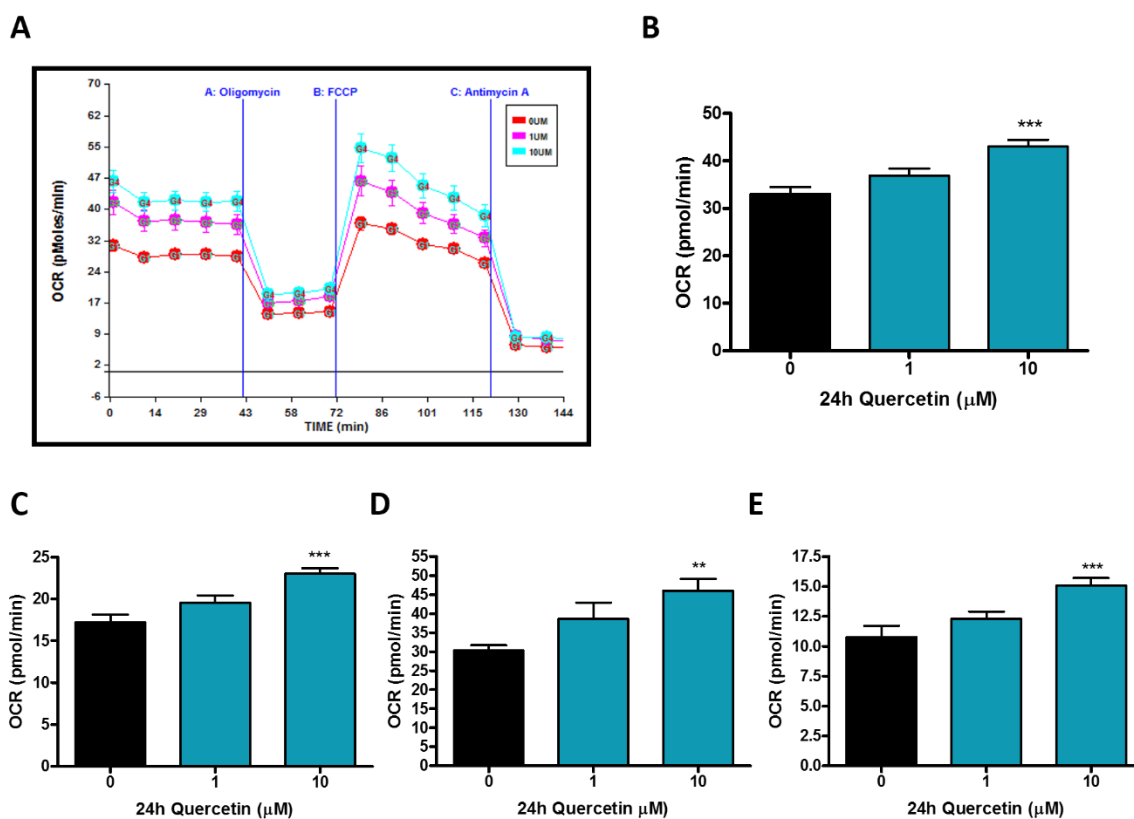
Figure 4. Effect of quercetin on mitochondrial respiration rate in N27 dopaminergic cells.

A-E, N27 dopaminergic neuronal cells were treated with 1 and 10 µM quercetin for 24 h and the N27 cells-containing culture plates were loaded into the Seahorse XF96 analyzer for the OCR measurement. Mitochondrial dynamics were measured using the sequential injection of oligomycin A (1 µg/ml), FCCP (1 µM), and antimycin A (10 µM) (*A*). Basal OCR (*B*), ATP-linked respiration (*C*), maximal OCR (*D*), and spare respiratory capacity (*E*) were calculated from the output OCR values. Values represent the means ± SEM of four replicates (**, $p < 0.01$; ***, $p < 0.001$; between the control and quercetin-treated samples).
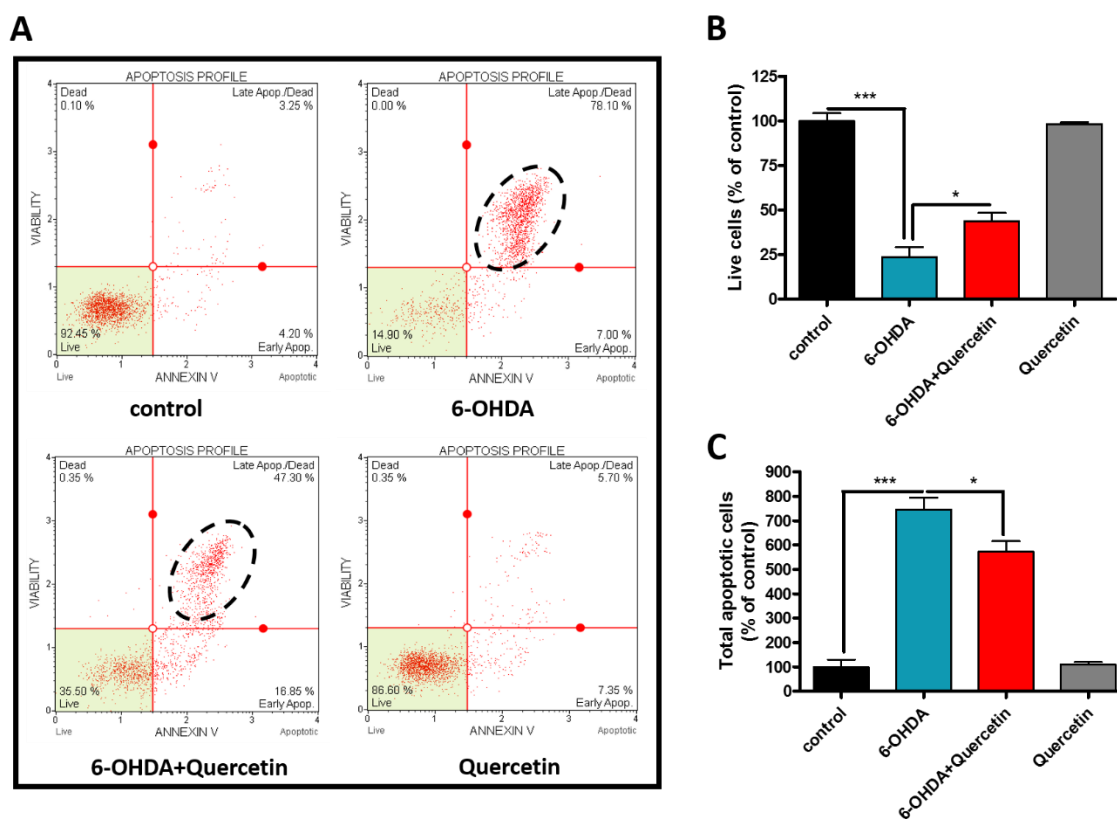
Figure 5. Quercetin protects against 6-OHDA-induced neurotoxicity. *A-C*, MN9D dopaminergic cells were pre-treated with 10 µM quercetin for 1 h and then co-treated with 6-OHDA (50 µM) for 24 h and assayed for cytotoxicity using the Muse cell analyzer (*A*). The percentage of live cells (*B*) and total apoptotic cells (*C*) was quantified by flow cytometry. Values are expressed as a percentage of the activity of control and represent the means ± SEM of four independent experiments (*, $p < 0.05$; ***, $p < 0.001$).
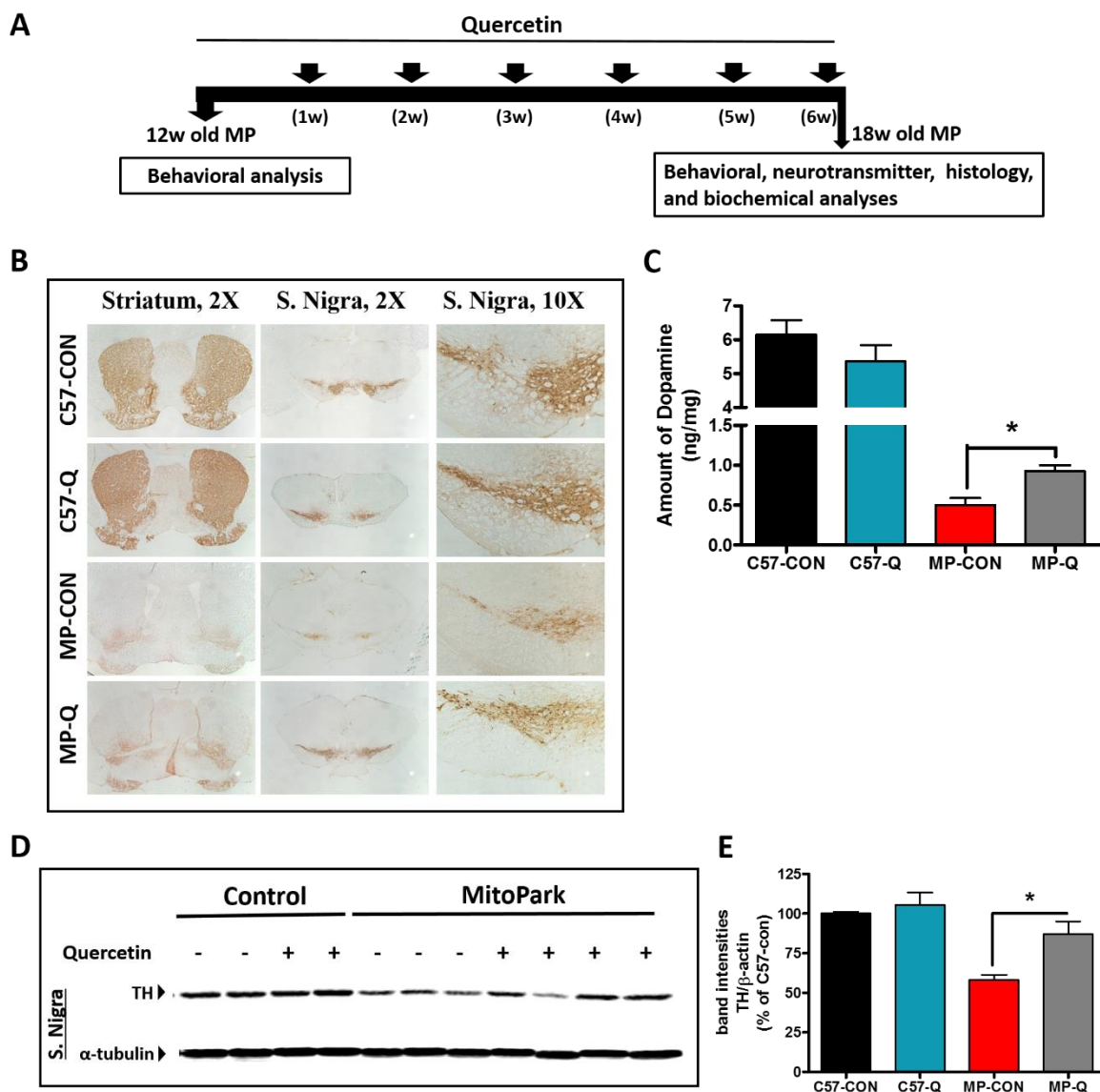
Figure 6. Quercetin reduces TH expression loss and striatal dopamine depletion in MitoPark mice. *A*, Treatment schedule of MitoPark mice with quercetin. 12-week-old MitoPark mice were treated with quercetin (25 mg/kg/day) via oral gavage for 6 weeks. TH-DAB immunostaining was performed in the striatum and substantia nigra (*B*). Striatal dopamine levels were measured by HPLC (*C*). Representative immunoblots showing the

expression of TH in the substantia nigra (*D*). The graph represents the band intensity analysis

of TH protein levels (*E*). Values are expressed as a percentage of the activity of control mice

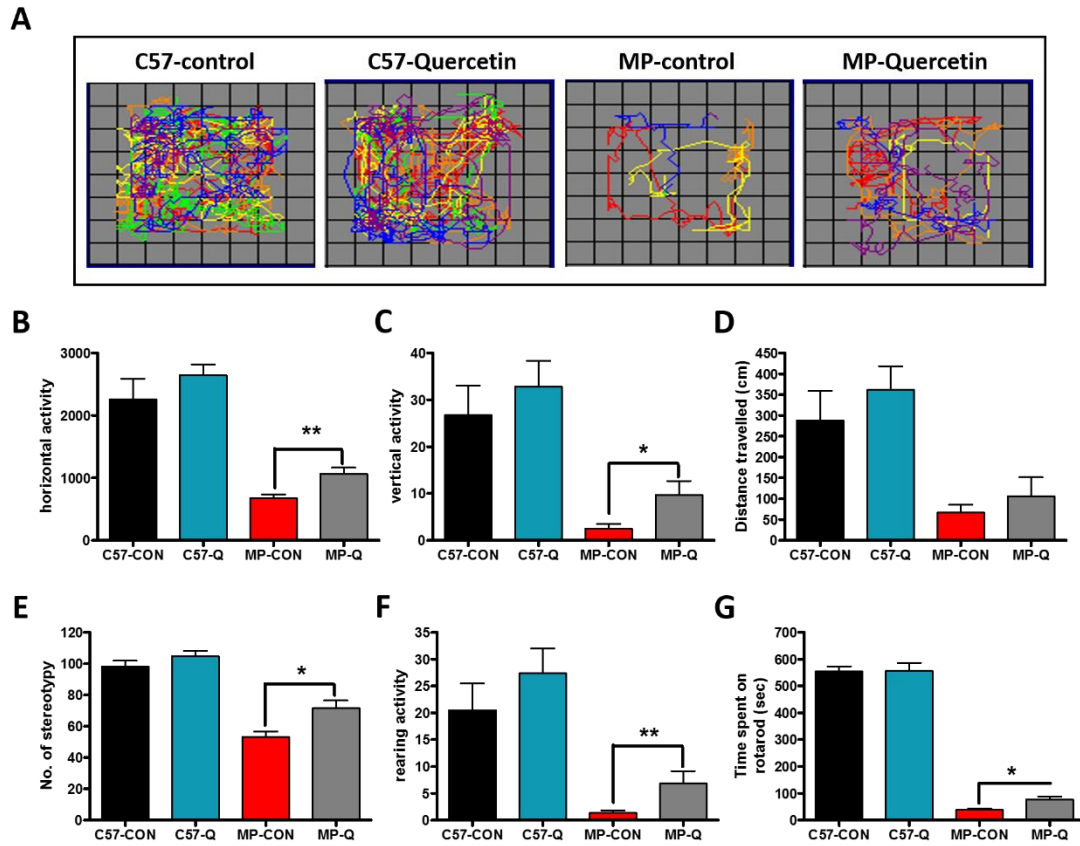and represent the means ± SEM of five mice per group (*, $p < 0.05$).

Figure 7. Quercetin improves motor functions in MitoPark mice. 12-week-old MitoPark mice were treated with quercetin (25 mg/kg/day) via oral gavage for 6 weeks. The locomotor activities were measured using a VersaMax system and a rotarod instrument one day prior to sacrifice. Moving track of mice (*A*), horizontal activity (*B*), vertical activity (*C*), total distance travelled (*D*), number of stereotypies (*E*), rearing activity (*F*), and time spent on rotarod at 20 rpm (*G*). Values represent the means ± SEM of eight mice per group (*, $p<0.05$; **, $p<0.01$).

CHAPTER IV: A NOVEL COMPOUND MITO-METFORMIN INDUCES PKD1 ACTIVATION AND PROTECTS DOPAMINERGIC NEURONS IN CELL CULTURE AND MITOPARK ANIMAL MODELS OF PARKINSON'S DISEASE

Muhammet Ay[1], Adhithiya Charli[1], Monica Langley[1], Arthi Kanthasamy[1], Huajun Jin[1], Vellareddy Anantharam[1], Balaraman Kalyanaraman[2], and Anumantha G. Kanthasamy[1]‡

1. Parkinson's Disorder Research Laboratory, Iowa Center for Advanced Neurotoxicology, Department of Biomedical Sciences, Iowa State University, Ames, Iowa, 50011, USA

2. Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI, 53226, USA

‡ To whom correspondence should be addressed: Dept. of Biomedical Sciences, Iowa State University, 2062 College of Veterinary Medicine Building, Ames, IA 50011. Tel: 515-294-2516; Fax: 515-294-2315; Email: akanthas@iastate.edu

Keywords: Metformin, PKD1, Parkinson's disease, mitochondria, mitochondrial biogenesis, MitoPark, neuroprotection.

The abbreviations used are: PKD1, protein kinase D1; CAMK, calcium/calmodulin-dependent protein kinase; T2DM, type 2 diabetes mellitus; 6-OHDA, 6-hydroxydopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TPP, triphenylphosphonium; AMPK, the AMP-activated protein kinase; mtDNA, mitochondrial DNA; OCR, oxygen consumption rate; HPLC, high-performance liquid chromatography; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; DAB, 3,3'-diaminobenzidine; TH; tyrosine hydroxylase.

Abstract

Impaired mitochondrial function and biogenesis contribute to the pathogenesis of Parkinson's disease (PD). Thus, identifying the key signaling mechanisms regulating mitochondrial biogenesis is crucial to developing new treatment strategies for PD. We have recently reported that PKD1 activation protects against neuronal cell death in PD models, and possibly regulates mitochondrial biogenesis. To improve the translational potential of our mechanistic studies for preclinical drug discovery, we synthesized Mito-Met, a mitochondria-targeted analog derived from conjugating the anti-diabetic drug metformin with a triphenylphosphonium functional group and then evaluated the preclinical efficacy of Mito-Met in cell culture and MitoPark animal models of PD. Treatment with Mito-Met (100-300 nM) significantly induced PKD1 phosphorylation, as well as Akt and AMPKα phosphorylations, more potently than metformin in N27 dopaminergic neuronal cells. Furthermore, Mito-Met increased the mRNA and protein expression of TFAM, implying that Mito-Met can promote mitochondrial biogenesis. Interestingly, Mito-Met significantly increased mitochondrial bioenergetics capacity in N27 cells. Mito-Met also reduced mitochondrial fragmentation induced by the Parkinsonian neurotoxicant MPP^+ in N27 cells and protected against MPP^+-induced TH-positive neurite loss in primary neurons. More importantly, Mito-Met treatment (10 mg/kg, oral gavage for 8 weeks) significantly improved motor deficits and reduced striatal dopamine depletion in MitoPark mice. Taken together, our results demonstrate that Mito-Met possesses profound neuroprotective effects in both in vitro and in vivo models of PD, suggesting that organelle-specific drug targeting could eventually yield a neuroprotective strategy for treating PD.

Introduction

Parkinson's disease (PD) is the most common aging-related neurodegenerative movement disorder that affects more than one million Americans and costs several billion dollars to the nation every year. PD is mainly characterized by the progressive loss of dopaminergic neurons in the substantia nigra of the midbrain that is involved in controlling movement and motor coordination (Shulman et al., 2011). Although several gene mutations and environmental factors have been linked to the pathogenesis of PD, the etiology of the disease is not yet fully understood (Lai et al., 2002, Schapira and Jenner, 2011, Trinh and Farrer, 2013). However, compelling evidence from both experimental studies and postmortem human brain tissues suggests that oxidative stress and mitochondrial dysfunction play important roles in the pathogenesis of PD (Sanders and Greenamyre, 2013, Subramaniam and Chesselet, 2013, Ryan et al., 2015).

Protein kinase D1 (PKD1) is the founding member of the PKD family that belongs to the calcium/calmodulin-dependent protein kinase (CAMK) family (Manning et al., 2002). PKD1 is ubiquitously expressed and plays an important role in regulating several cellular functions, including Golgi function, membrane trafficking, and cell survival (Jamora et al., 1999, Prigozhina and Waterman-Storer, 2004, Storz et al., 2005). We have recently demonstrated that PKD1 is rapidly activated during oxidative stress and that constitutive overexpression of PKD1 protected against oxidative stress-induced cell death (Asaithambi et al., 2011, Asaithambi et al., 2014), while negative modulation of PKD1 signaling increased the susceptibility of dopaminergic neurons to oxidative stress (Ay et al., 2015). Moreover, we

observed that the activation of PKD1 signaling can promote mitochondrial biogenesis in dopaminergic cells (unpublished data).

Type 2 diabetes (T2DM) is one of the most common chronic disorders and the most common form of diabetes, affecting approximately 90% of people with diabetes (Santiago and Potashkin, 2014). There is growing literature suggesting that T2DM is a risk factor for neurodegenerative diseases including PD (Sun et al., 2012, Santiago and Potashkin, 2013b). Although the mechanism behind the association between PD and T2DM is currently unknown, both diseases share some similar pathogenic pathways, such as mitochondrial dysfunction, oxidative stress, endoplasmic reticulum (ER) stress, and inflammation (Sandyk, 1993, Santiago and Potashkin, 2013a). Studies have also reported that diabetic animals showed increased vulnerability to Parkinsonian specific toxicants, including 6-OHDA and MPTP (Morris et al., 2010, Wang et al., 2014). Metformin is one of the most commonly used anti-diabetic drugs with a high safety profile (Dunn and Peters, 1995). Metformin has been recently shown to protect against dopaminergic neurodegeneration in the MPTP mouse model of PD (Patil et al., 2014). More importantly, a cohort study revealed that the combination therapy of metformin and sulfonylurea reduced the risk of developing PD in patients with type 2 diabetes in a Taiwanese population (Wahlqvist et al., 2012).

Given that mitochondrial dysfunction is an important factor in PD pathogenesis, targeting compounds into mitochondria may lead to the discovery of more specific, less toxic, and more effective neuroprotective agents for PD. Attaching a lipophilic cation triphenylphosphonium (TPP^+) to compounds has been shown to facilitate the increased accumulation of compounds inside the mitochondria (Smith et al., 2003). In this study, we

synthesized Mito-Met, a mitochondria-targeted metformin analog in conjugation with a TPP$^+$ group, and then tested the neuroprotective efficacy of Mito-Met in cell culture and MitoPark animal models of PD. Our results show that Mito-Met activates PKD1 signaling, enhances mitochondrial function, and protects against neurotoxicity in a cell model of PD. Our results also reveal that Mito-Met has neuroprotective effects in the MitoPark mouse model of PD.

Materials and Methods

Chemicals and Reagents. 1-Methyl-4-phenyl tetrahydropteridine (MPP$^+$ iodide) and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Lois, MO). The Bradford protein assay kit was purchased from Bio-Rad. Antibodies against PKD1, α-tubulin, and mtTFA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho PKD1 (p-S744/748 and p-S916), phospho-Akt (p-S473), total Akt, phosho-AMPKα (p-Thr172) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-TH antibody was purchased from Millipore (Billerica, MA). PKD1 inhibitor CID755673 was purchased from Tocris Bioscience (Ellisville, MO). Alexa 680-conjugated anti-mouse secondary antibody, MitoTracker Red FM, and all cell culture reagents were obtained from Invitrogen (Carlsbad, CA). IRDye800-conjugated anti-rabbit secondary was purchased from Rockland Labs (Gilbertsville, PA).

Cell Cultures. The rat dopaminergic N27 cell line was a kind gift from Dr. Kedar N. Prasad (University of Colorado Health Sciences Center, Denver, CO) and cultured as described previously (Kaul et al., 2003). Briefly, N27 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 μg/ml streptomycin and maintained at 37°C in a 5% CO$_2$ atmosphere. We also used the primary

mesencephalic neuronal culture to assess the protective effect of Mito-Met against MPP$^+$-induced TH-positive neurite loss. The primary mesencephalic culture was prepared from the ventral mesencephalon of gestational 14-day-old mouse embryos as described previously (Zhang et al., 2007). Briefly, the mesencephalic tissues from E14 mouse embryos were dissected and maintained in ice cold Ca^{2+}-free Hank's balanced salt solution, and then dissociated in Hank's balanced salt solution containing trypsin-0.25% EDTA for 30 min at 37°C. Cultures were maintained in Neurobasal medium supplemented with B27. Half of the culture medium was replaced every 2 days. Approximately 6- to 7-day-old cultures were used for experiments. Primary mesencephalic neuronal cells were treated with 10 µM MPP$^+$ in the presence or absence of Mito-Met (100-300 nM) for 24 h.

Quantitative Real-Time RT-PCR. Total RNA was isolated from cells using the Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA), and 1-2 µg RNA was used to generate cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed on an Mx300P QPCR system (Stratagene) using the RT^2 SYBR Green qPCR Mastermix kit (Qiagen) and QuantiTect Primer Assay kit (Qiagen). All RT-qPCR reactions were performed in triplicate and normalized to β-actin housekeeping gene. The primer sequences and PCR conditions are available upon request. Dissociation curves were run to verify the singularity of the PCR product. The data were analyzed using the comparative threshold cycle (Ct) method.

Quantitative Real-Time PCR Analysis of mtDNA content. N27 cells were plated in 6-well plates at a density of 2×10^5 cells/well. Twenty-four hours later, cells were treated with 0.1-1 µM Mito-Met for 24 h. Genomic DNA was isolated using the DNeasy Blood and Tissue kit

(Qiagen, Valencia, CA) according to the manufacturer's protocol and mtDNA content was determined by quantitative PCR with SYBR green. 10 ng nuclear DNA and 1 ng mitochondrial DNA were amplified using the primers for mitochondrial ND1 and nuclear β-actin genes. Reactions were performed using an Mx300P QPCR system (Stratagene).

Western Blot Analysis. After treatments, cells or brain tissues were collected and resuspended in a modified RIPA buffer containing protease and phosphatase inhibitors. Briefly, lysates containing equal amounts of protein were loaded in each lane and separated on a 10-15% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad). The membranes were blocked in LI-COR blocking buffer for 1 hour at room temperature and then incubated overnight at 4°C with the corresponding primary antibodies. Western blot was performed using IRDye 800 anti-rabbit, Alexa Fluor 680 goat anti-mouse, and Alexa Fluor 680 donkey anti-goat secondary antibodies. Western blot images were captured and analyzed with an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Immunocytochemistry and quantification of TH-positive neuronal processes. Primary mesencephalic dopaminergic neurons were fixed with 4% paraformaldehyde for 30 min at room temperature. After washing, cells were permeabilized with blocking agent (2% bovine serum albumin, 0.5% Triton X-100, and 0.05% Tween-20 in PBS) for 1 h. Cells were then incubated with anti-TH antibody (1:1200, Millipore) at 4°C overnight. Fluorescently-conjugated secondary antibody (Alexa Fluor 488-conjugated anti-mouse antibody, 1:2000) was used followed by incubation with 10 µg/ml Hoechst 33342 for 5 min at room temperature to stain the nucleus. Cover slips were mounted on glass slides and cells were visualized by Z-Stack imaging with the Leica confocal microscope. 3D image reconstruction was performed

using the IMARIS software. The length of TH-positive neuronal processes in primary dopaminergic neurons from each coverslip was measured using MetaMorph software (Molecular Devices) as described previously (Ghosh et al., 2013, Charli et al., 2015). For quantification of neuronal processes, pictures were taken at 60X magnification. TH-positive neuronal processes were counted in six individual cultures for each treatment.

MitoTracker staining and analysis of mitochondrial morphology. N27 cells were stained with MitoTracker Red FM (200 nM) for 30 min at 37°C, and then fixed with 4% paraformaldehyde for 30 min at room temperature. After washing, cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature, as described previously (Charli et al., 2015). Cover slips were mounted on glass slides and imaged through a Nikon TE2000 microscope with a SPOT color digital camera (Diagnostic Instruments, Sterling Heights, MI). Quantification of mitochondrial parameters such as mitochondrial length and degree of circularity was performed using a macro text file plug-in for ImageJ (Dagda et al., 2009).

Measurement of Mitochondrial Oxygen Consumption. The Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) was used to measure oxygen consumption rates (OCR) in N27 cells. Briefly, N27 cells were seeded at 10,000 cells/well in a Seahorse Bioscience polystyrene microplate. Twenty-four hours later, cells were treated with 100 and 300 nM Mito-Met for 3 h. After the treatment, cells were changed to assay media and the N27 cells-containing culture plates were loaded into Seahorse instrument to collect baseline OCR measurements. The experimental agents (oligomycin (1 µg/ml), FCCP (3 µM), and antimycin A (10 µM)) were injected using the included ports on the XF96 cartridges and OCR

measurements were collected. Basal OCR, ATP-linked OCR, maximal OCR, and spare respiratory capacity were calculated, as described previously (Dranka et al. 2010).

Animals and Treatment. 12-week-old MitoPark and C57BL/6 mice were housed under standard conditions: constant temperature (22±1 °C), humidity (relative, 30%), and a 12 h light/dark cycle. Mice were given free access to food and water. Animal care and protocol procedures were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University (Ames, IA). Mice were treated with either saline or Mito-Met (10 mg/kg) via oral gavage three times per week for 8 weeks. After the treatments, mice were subjected to behavioral, neurochemical, and biochemical measurements.

High-Performance Liquid Chromatography (HPLC) Analysis. The striatal dopamine and dihydrophenylacetic acid (DOPAC), and homovanillic acid (HVA) levels were quantified using high performance liquid chromatography (HPLC) with electrochemical detection. Samples were prepared and quantified, as described previously (Ghosh et al., 2013). Briefly, neurotransmitters from striatal tissues were extracted in 0.1 M perchloric acid containing 0.05% Na_2EDTA and 0.1% $Na_2S_2O_5$ and isoproterenol (internal standard). Dopamine, DOPAC, and HVA were separated isocratically using a C-18 reversed phase column with a flow rate 0.6 ml/min. An HPLC system (ESA, Bedford, MA) with an automatic sampler equipped with a refrigerated temperature control was used for these experiments.

Behavioral Measurements. An open-field experiment was performed to measure the locomotor activity of mice using an automated device (AccuScan, Columbus, OH). Before the treatments, mice were trained two consecutive days. One day prior to sacrifice, open-field

experiments were conducted. Locomotor activities were recorded for 10 min. Data were collected and analyzed using the Versaplot and Versadat softwares.

Statistical Analysis. Data analysis was performed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). Data were first analyzed by using one-way ANOVA and then the Tukey multiple comparison test was used for statistical comparisons. Differences with $P < 0.05$ were considered significant.

Results

Mito-Met induces activation of PKD1 and its potential downstream targets Akt and AMPK in a cell model of PD

To evaluate our hypothesis that Mito-Met is more potent than its parent compound metformin in providing neuroprotection, we wanted to examine the effects of metformin and Mito-Met on the activation of pro-survival kinase PKD1, recently reported by our group to have neuroprotective effects against oxidative stress-induced cell death in dopaminergic neurons (Asaithambi et al., 2011, Asaithambi et al., 2014). We first treated N27 dopaminergic neuronal cells with metformin (100 and 1000 µM) and Mito-Met (100 and 300 nM) for 3 h and checked the phosphorylation of PKD1. As evident from Fig. 1, A and B, although both metformin and Mito-Met induced the phosphorylation of PKD1, Mito-Met was more than 300 fold more efficacious than metformin in activating PKD1. Next, we treated N27 cells with 100 and 300 nM Mito-Met for 3 h and 6 h and measured the PKD1 activation loop (S744/748) and C-terminal phosphorylation (S916). As shown in Fig. 1C, Mito-Met significantly increased the

phosphorylation of both S744/748 and S916 of PKD1 at both 3 h and 6 h time points. However, expression of total PKD1 was not affected by Mito-Met treatment (Fig. 1C). Following the observation that Mito-Met enhances PKD1 activation, we wanted to determine whether Mito-Met can also increase the activation of Akt. Akt has been previously shown to have neuroprotective effects in cell culture and animal models of PD (Ries et al., 2006, Gunjima et al., 2014). Immunoblot analysis revealed that treatment of N27 cells with Mito-Met (100 nM and 300 nM) for 3 h and 6 h resulted in increased Akt phosphorylation (Fig. 2A). To further explore the potential neuroprotective effect of Mito-Met, we examined whether Mito-Met can activate AMPK in dopaminergic neuronal cells. Metformin, the parent compound of Mito-Met, is known to activate AMPK in different cell types, including neurons (Ouyang et al., 2011, Stephenne et al., 2011, Hou et al., 2015). AMPK has also been reported to exert neuroprotective effect against dopaminergic neurodegeneration (Ng et al., 2012). To determine AMPK activation, we used an antibody directed against phosphorylated Thr172 within the catalytic subunit (alpha) of AMPK. As shown in Fig. 2A, Mito-Met treatment increased AMPK Thr172 phosphorylation in N27 cells. Next, we wanted to address whether PKD1 lies upstream of Akt and AMPK kinase during Mito-Met treatment. N27 cells were pretreated with a pharmacological inhibitor of PKD1 (CID755673) for 1 h and then cotreated with CID755673 (50 µM) and Mito-Met (100 nM) for 3 hours. Interestingly, pretreatment with CID755673 blocked the Mito-Met-induced activation of both Akt and AMPK kinase (Fig. 2B). Collectively, these data suggest that Mito-Met can effectively activate PKD1 and its potential downstream effectors Akt and AMPK kinase in dopaminergic cells.

Mito-Met stimulates mitochondrial biogenesis and bioenergetics capacity in N27 cells

After observing that Mito-Met can enhance the activation PKD1, we investigated whether Mito-Met stimulates mitochondrial biogenesis. Because we recently observed that positive modulation of PKD1 can induce mitochondrial biogenesis in dopaminergic neurons (unpublished data). We first investigated the effect of Mito-Met on TFAM expression. TFAM is transcription factor that is required for mtDNA replication and transcription to regulate mitochondrial biogenesis (Virbasius and Scarpulla, 1994, Scarpulla, 2008). N27 cells were treated with 100 and 300 nM Mito-Met for 3 h and 6 h and TFAM levels were determined by Western blot analysis. As seen in Fig. 3, A and B, the expression of TFAM protein was significantly increased in N27 cells treated with Mito-Met. Next, we measured TFAM mRNA expression using real-time RT-PCR at 3 h time point. Exposure of N27 cells to Mito-Met (100-1000 nM) significantly increased TFAM mRNA expression (Fig. 3C). We also determined relative mtDNA copy number using mitochondrial ND1 and nuclear β-actin genes and found that Mito-Met treatment (1000 nM) significantly increased mtDNA content in N27 cells (Fig. 3D). Furthermore, we wanted to evaluate the effect of Mito-Met on cellular bioenergetics in dopaminergic neuronal cells. N27 cells were treated with 100 and 300 nM Mito-Met for 3 h and the oxygen consumption rates (OCR) were measured using a Seahorse XF96 analyzer. Interestingly, we observed significant increases in basal OCR and ATP-linked respiration in Mito-Met-treated N27 cells, while rotenone, a well-known mitochondrial complex I inhibitor, almost completely blocked mitochondrial respiration (Fig. 4A-C). These results suggest that Mito-Met can stimulate mitochondrial biogenesis and oxidative phosphorylation in dopaminergic cells.

Mito-Met protects against MPP⁺-induced mitochondrial damage and neurotoxicity

To further explore the potential neuroprotective effect of Mito-Met, we first examined whether Mito-Met can reduce MPP⁺-induced mitochondrial damage. N27 cells were pretreated with 300 nM Mito-Met for 1 h and then cotreated with Mito-Met and MPP⁺ (300 µM) for 16 h and stained with MitoTracker to visualize mitochondrial morphology. Expectedly, MPP⁺ treatment strongly induced mitochondrial fragmentation (Fig. 5A); however, Mito-Met pretreatment significantly ameliorated mitochondrial morphology in MPP⁺-treated cells as evidenced by improved mitochondrial length and circularity (Fig. 5, B and C). Next, we tested the protective effect of Mito-Met in primary dopaminergic neurons from E14 mouse embryos. The primary nigral neurons were treated with 10 µM MPP⁺ in the presence or absence of Mito-Met (100 nM) for 24 h and immunostained with TH antibody. To evaluate whether Mito-Met treatment protects against MPP⁺-induced dopaminergic neurodegeneration, we measured the length of dopaminergic neurites. The immunocytochemical analysis revealed that Mito-Met treatment significantly protected against MPP⁺-induced TH-positive neurite loss, as evidenced by a significant increase in the length of neuronal processes in primary nigral cultures cotreated with MPP⁺ and Mito-Met compared with cultures treated with MPP⁺ alone (Fig. 6, A and B). Together, these data demonstrate that Mito-Met can effectively protect dopaminergic neurons from MPP⁺-induced toxicity.

Mito-Met improves motor deficits and attenuates striatal dopamine depletion in MitoPark mice

The results of our *in vitro* experiments demonstrated that Mito-Met can afford neuroprotection against dopaminergic neurotoxicity. Here, we wanted to test the efficacy of

Mito-Met against dopaminergic neurodegeneration in the MitoPark mouse model of PD. As seen in Fig. 7A, MitoPark and control mice were treated with either saline or Mito-Met (10 mg/kg) three times per week by oral gavage for 8 weeks. One day prior to sacrifice, mice were analyzed for their locomotor activities. MitoPark mice develop progressive motor deficits from 12 weeks of age onwards (Ekstrand et al., 2007). As expected, 20-week-old MitoPark mice had a dramatic reduction in total movement (Fig. 7B). Notably, improved locomotion was observed in the MitoPark mice-treated with Mito-Met. Mito-Met treatment reversed the reduction in horizontal activity (Fig. 7C), total distance travelled (Fig. 7D), number of movements (Fig. 7E), and stereotypy counts (Fig. 7F) of MitoPark mice. Moreover, we also determined whether Mito-Met treatment attenuates striatal dopamine depletion in MitoPark mice. Mice were sacrificed one day after the last Mito-Met treatment and striatal tissues were processed for HPLC analysis of dopamine. Neurochemical analysis revealed that the dopamine and DOPAC levels were significantly higher in the MitoPark mice-treated with Mito-Met than the MitoPark mice-treated with saline (Fig. 8, A and B). Collectively, these results suggest that Mito-Met treatment can improve motor deficits and at least partially prevent striatal dopamine depletion in MitoPark mice.

Discussion

The findings of this study provide evidence that a mitochondria targeted analog of metformin, Mito-Met, exerts neuroprotective effects in cell culture and MitoPark mouse model of PD. Our results revealed that Mito-Met can effectively activate the PKD1-mediated neuroprotective signaling. We also demonstrate that Mito-Met enhances mitochondrial

biogenesis and bioenergetics capacity. In addition, Mito-Met protects against MPP$^+$ neurotoxicity in a cell model of PD. Finally, and perhaps most importantly, Mito-Met treatment has neuroprotective effects against behavioral deficits and striatal dopamine depletion in the MitoPark transgenic mouse model of PD.

Metformin has been used for several decades to treat diabetic patients. Besides its anti-diabetic effect, metformin has been recently shown to have neuroprotective effects in experimental stroke (Li et al., 2010), against ethanol-induced neuronal apoptosis (Ullah et al., 2012), alpha-synuclein neurotoxicity (Dulovic et al., 2014) and MPTP-induced dopaminergic neurodegeneration (Patil et al., 2014). In this study, we synthesized Mito-Met, a mitochondria-targeted (TPP$^+$ conjugated) analog of metformin. Mitochondria-targeted compounds have been reported to show neuroprotective effects in experimental models of PD by virtue of their reduced toxicity and increased accumulation inside the mitochondria (Yang et al., 2009, Ghosh et al., 2010, Solesio et al., 2013). Here, we first examined whether Mito-Met can activate the PKD1 pro-survival signaling in dopaminergic neuronal cells. Recent studies from our group suggest that positive modulators of PKD1 signaling could serve as potential neuroprotective agents for the treatment of PD (Asaithambi et al., 2011, Asaithambi et al., 2014, Ay et al., 2015). Our results demonstrated that Mito-Met at nanomolar concentrations can activate PKD1, whereas micromolar concentrations of metformin are needed to activate PKD1 in dopaminergic neuronal cells (Fig. 1, A and B), suggesting Mito-Met is much more potent than its parent compound metformin. In an effort to identify molecular mechanisms governing the potential neuroprotective effect of Mito-Met, we also checked Akt and AMPK activation and observed increased phosphorylation of Akt and AMPK in Mito-Met-treated cells (Fig. 2A).

Akt has been previously implicated in the survival of dopaminergic neurons (Ries et al., 2006, Gunjima et al., 2014). AMPK is a well-known target of metformin in different cell types, including non-neuronal and neuronal cells (Ouyang et al., 2011, Stephenne et al., 2011, Hou et al., 2015) and has been reported to protect against dopaminergic neurodegeneration in cell culture and Drosophila models of PD (Ng et al., 2012, Dulovic et al., 2014). Moreover, our studies using a pharmacological inhibitor of PKD1 revealed that PKD1 appears to act upstream of Akt and AMPK kinases during the Mito-Met treatment of dopaminergic cells (Fig. 2B). However, more mechanistic studies using both genetic and pharmacological approaches are required to fully identify the relationship between these kinases in dopaminergic neurons.

Metformin has been previously shown to inhibit mitochondrial complex I activity in both hepatoma and cancer cells (Owen et al., 2000, Andrzejewski et al., 2014) and the anti-cancer effect of Metformin is attributed to its inhibitory effect on mitochondrial complex I (Wheaton et al., 2014). However, the notion that metformin inhibits complex I is controversial because preservation of complex I activity has been reported in metformin-treated mice (Martin-Montalvo et al., 2013) and in the plasma of metformin-treated diabetic patients (Larsen et al., 2012). Because of these conflicting reports, we wanted to determine whether Mito-Met has any inhibitory effect on mitochondrial function in dopaminergic cells. Our results revealed that Mito-Met, even at higher concentrations (1 and 10 µM), does not adversely affect mitochondrial function as evidenced by the MitoSOX and aconitase activity assays (Supp. Fig. 1). Since we previously observed that positive modulation of PKD1 signaling can promote mitochondrial biogenesis (unpublished data), we evaluated the effect of Mito-Met on mitochondrial biogenesis. Our data show that Mito-Met can increase TFAM

expression (Fig. 3A-C) and mtDNA copy number (Fig. 3D), suggesting Mito-Met can promote mitochondrial biogenesis in dopaminergic cells. Next, we determined the effect of Mito-Met on mitochondrial respiration. Interestingly, Mito-Met treatment resulted in significant increases in basal OCR (Fig. 4B) and ATP-linked respiration (Fig. 4C) in dopaminergic neuronal cells. Together, our results indicate that our mitochondria-targeted metformin analog (Mito-Met) can improve mitochondrial biogenesis and function in dopaminergic cells.

After observing enhanced activation of survival kinases and mitochondrial function in Mito-Met-treated cells, we evaluated the potential neuroprotective effect of Mito-Met against MPP^+-induced neurotoxicity. We first assessed the effect of Mito-Met pretreatment on mitochondrial morphology and integrity in MPP^+-treated N27 cells and observed that Mito-Met significantly reduced mitochondrial fragmentation induced by MPP^+ (Fig. 5, A and B). Of note, there was no change in mitochondrial morphology in N27 cells treated with Mito-Met alone. Next, we determined the protective effect of Mito-Met against MPP^+-induced dopaminergic neurodegeneration in primary mesencephalic cultures. Importantly, cotreatment of primary mesencephalic neurons with Mito-Met significantly protected against MPP^+-induced TH-positive neurite loss (Fig. 6, A and B), further supporting the neuroprotective effect of Mito-Met in dopaminergic cells.

To test the neuroprotective efficacy of Mito-Met in vivo, we used the MitoPark transgenic mouse model of PD. This transgenic mouse model of mitochondrial dysfunction recapitulates several features of human PD, including adult onset, progressive dopaminergic neurodegeneration and motor deficits (Ekstrand et al., 2007, Galter et al., 2010). Since MitoPark mice begin to show motor deficits from 12 weeks of age onwards, we treated 12-

week-old MitoPark mice with either saline or Mito-Met for eight weeks. Our results revealed that Mito-Met significantly reversed behavioral deficits in MitoPark mice (Fig. 7B-F). Furthermore, higher levels of dopamine and DOPAC were observed in the Mito-Met-treated MitoPark mice compared with the MitoPark mice-treated with saline, suggesting Mito-Met can protect against dopaminergic neurodegeneration in MitoPark mice. Additionally, blood chemistry analysis revealed no adverse effects in Mito-Met-treated mice (Table 1).

In conclusion, our data demonstrate that mitochondria-targeted metformin analog (Mito-Met) is able to activate PKD1-mediated neuroprotective signaling and to enhance mitochondrial function. Importantly, Mito-Met can protect against MPP^+-induced neurotoxicity in cell culture models of PD. Additionally, Mito-Met treatment can reverse behavioral deficits and attenuate dopamine depletion in the MitoPark animal model of PD. Overall, our results suggest that Mito-Met is a potential neuroprotective agent that deserves further preclinical evaluations for the treatment of PD.

REFERENCES

Andrzejewski S, Gravel SP, Pollak M, St-Pierre J (2014) Metformin directly acts on mitochondria to alter cellular bioenergetics. Cancer & metabolism 2:12.

Asaithambi A, Ay M, Jin H, Gosh A, Anantharam V, Kanthasamy A, Kanthasamy AG (2014) Protein kinase D1 (PKD1) phosphorylation promotes dopaminergic neuronal survival during 6-OHDA-induced oxidative stress. PloS one 9:e96947.

Asaithambi A, Kanthasamy A, Saminathan H, Anantharam V, Kanthasamy AG (2011) Protein kinase D1 (PKD1) activation mediates a compensatory protective response during early stages of oxidative stress-induced neuronal degeneration. Molecular neurodegeneration 6:43.

Ay M, Jin H, Harischandra DS, Asaithambi A, Kanthasamy A, Anantharam V, Kanthasamy AG (2015) Molecular cloning, epigenetic regulation, and functional characterization of Prkd1 gene promoter in dopaminergic cell culture models of Parkinson's disease. Journal of neurochemistry 135:402-415.

Charli A, Jin H, Anantharam V, Kanthasamy A, Kanthasamy AG (2015) Alterations in mitochondrial dynamics induced by tebufenpyrad and pyridaben in a dopaminergic neuronal cell culture model. Neurotoxicology.

Dagda RK, Cherra SJ, 3rd, Kulich SM, Tandon A, Park D, Chu CT (2009) Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. The Journal of biological chemistry 284:13843-13855.

Dulovic M, Jovanovic M, Xilouri M, Stefanis L, Harhaji-Trajkovic L, Kravic-Stevovic T, Paunovic V, Ardah MT, El-Agnaf OM, Kostic V, Markovic I, Trajkovic V (2014) The protective role of AMP-activated protein kinase in alpha-synuclein neurotoxicity in vitro. Neurobiology of disease 63:1-11.

Dunn CJ, Peters DH (1995) Metformin. A review of its pharmacological properties and therapeutic use in non-insulin-dependent diabetes mellitus. Drugs 49:721-749.

Ekstrand MI, Terzioglu M, Galter D, Zhu S, Hofstetter C, Lindqvist E, Thams S, Bergstrand A, Hansson FS, Trifunovic A, Hoffer B, Cullheim S, Mohammed AH, Olson L, Larsson NG (2007) Progressive parkinsonism in mice with respiratory-chain-deficient dopamine neurons. Proceedings of the National Academy of Sciences of the United States of America 104:1325-1330.

Galter D, Pernold K, Yoshitake T, Lindqvist E, Hoffer B, Kehr J, Larsson NG, Olson L (2010) MitoPark mice mirror the slow progression of key symptoms and L-DOPA response in Parkinson's disease. Genes, brain, and behavior 9:173-181.

Ghosh A, Chandran K, Kalivendi SV, Joseph J, Antholine WE, Hillard CJ, Kanthasamy A, Kanthasamy A, Kalyanaraman B (2010) Neuroprotection by a mitochondria-targeted drug in a Parkinson's disease model. Free radical biology & medicine 49:1674-1684.

Ghosh A, Saminathan H, Kanthasamy A, Anantharam V, Jin H, Sondarva G, Harischandra DS, Qian Z, Rana A, Kanthasamy AG (2013) The peptidyl-prolyl isomerase Pin1 up-regulation and proapoptotic function in dopaminergic neurons: relevance to the

pathogenesis of Parkinson disease. The Journal of biological chemistry 288:21955-21971.

Gunjima K, Tomiyama R, Takakura K, Yamada T, Hashida K, Nakamura Y, Konishi T, Matsugo S, Hori O (2014) 3,4-dihydroxybenzalacetone protects against Parkinson's disease-related neurotoxin 6-OHDA through Akt/Nrf2/glutathione pathway. Journal of cellular biochemistry 115:151-160.

Hou YS, Guan JJ, Xu HD, Wu F, Sheng R, Qin ZH (2015) Sestrin2 Protects Dopaminergic Cells against Rotenone Toxicity through AMPK-Dependent Autophagy Activation. Molecular and cellular biology 35:2740-2751.

Jamora C, Yamanouye N, Van Lint J, Laudenslager J, Vandenheede JR, Faulkner DJ, Malhotra V (1999) Gbetagamma-mediated regulation of Golgi organization is through the direct activation of protein kinase D. Cell 98:59-68.

Kaul S, Kanthasamy A, Kitazawa M, Anantharam V, Kanthasamy AG (2003) Caspase-3 dependent proteolytic activation of protein kinase C delta mediates and regulates 1-methyl-4-phenylpyridinium (MPP+)-induced apoptotic cell death in dopaminergic cells: relevance to oxidative stress in dopaminergic degeneration. The European journal of neuroscience 18:1387-1401.

Lai BC, Marion SA, Teschke K, Tsui JK (2002) Occupational and environmental risk factors for Parkinson's disease. Parkinsonism & related disorders 8:297-309.

Larsen S, Rabol R, Hansen CN, Madsbad S, Helge JW, Dela F (2012) Metformin-treated patients with type 2 diabetes have normal mitochondrial complex I respiration. Diabetologia 55:443-449.

Li J, Benashski SE, Venna VR, McCullough LD (2010) Effects of metformin in experimental stroke. Stroke; a journal of cerebral circulation 41:2645-2652.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. Science (New York, NY 298:1912-1934.

Martin-Montalvo A, Mercken EM, Mitchell SJ, Palacios HH, Mote PL, Scheibye-Knudsen M, Gomes AP, Ward TM, Minor RK, Blouin MJ, Schwab M, Pollak M, Zhang Y, Yu Y, Becker KG, Bohr VA, Ingram DK, Sinclair DA, Wolf NS, Spindler SR, Bernier M, de Cabo R (2013) Metformin improves healthspan and lifespan in mice. Nature communications 4:2192.

Morris JK, Bomhoff GL, Stanford JA, Geiger PC (2010) Neurodegeneration in an animal model of Parkinson's disease is exacerbated by a high-fat diet. American journal of physiology 299:R1082-1090.

Ng CH, Guan MS, Koh C, Ouyang X, Yu F, Tan EK, O'Neill SP, Zhang X, Chung J, Lim KL (2012) AMP kinase activation mitigates dopaminergic dysfunction and mitochondrial abnormalities in Drosophila models of Parkinson's disease. J Neurosci 32:14311-14317.

Ouyang J, Parakhia RA, Ochs RS (2011) Metformin activates AMP kinase through inhibition of AMP deaminase. The Journal of biological chemistry 286:1-11.

Owen MR, Doran E, Halestrap AP (2000) Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. The Biochemical journal 348 Pt 3:607-614.

Patil SP, Jain PD, Ghumatkar PJ, Tambe R, Sathaye S (2014) Neuroprotective effect of metformin in MPTP-induced Parkinson's disease in mice. Neuroscience 277:747-754.

Prigozhina NL, Waterman-Storer CM (2004) Protein kinase D-mediated anterograde membrane trafficking is required for fibroblast motility. Curr Biol 14:88-98.

Ries V, Henchcliffe C, Kareva T, Rzhetskaya M, Bland R, During MJ, Kholodilov N, Burke RE (2006) Oncoprotein Akt/PKB induces trophic effects in murine models of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America 103:18757-18762.

Ryan BJ, Hoek S, Fon EA, Wade-Martins R (2015) Mitochondrial dysfunction and mitophagy in Parkinson's: from familial to sporadic disease. Trends in biochemical sciences 40:200-210.

Sanders LH, Greenamyre JT (2013) Oxidative damage to macromolecules in human Parkinson disease and the rotenone model. Free radical biology & medicine 62:111-120.

Sandyk R (1993) The relationship between diabetes mellitus and Parkinson's disease. The International journal of neuroscience 69:125-130.

Santiago JA, Potashkin JA (2013a) Integrative network analysis unveils convergent molecular pathways in Parkinson's disease and diabetes. PloS one 8:e83940.

Santiago JA, Potashkin JA (2013b) Shared dysregulated pathways lead to Parkinson's disease and diabetes. Trends in molecular medicine 19:176-186.

Santiago JA, Potashkin JA (2014) System-based approaches to decode the molecular links in Parkinson's disease and diabetes. Neurobiology of disease.

Scarpulla RC (2008) Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiological reviews 88:611-638.

Schapira AH, Jenner P (2011) Etiology and pathogenesis of Parkinson's disease. Mov Disord 26:1049-1055.

Shulman JM, De Jager PL, Feany MB (2011) Parkinson's disease: genetics and pathogenesis. Annual review of pathology 6:193-222.

Smith RA, Porteous CM, Gane AM, Murphy MP (2003) Delivery of bioactive molecules to mitochondria in vivo. Proceedings of the National Academy of Sciences of the United States of America 100:5407-5412.

Solesio ME, Prime TA, Logan A, Murphy MP, Del Mar Arroyo-Jimenez M, Jordan J, Galindo MF (2013) The mitochondria-targeted anti-oxidant MitoQ reduces aspects of mitochondrial fission in the 6-OHDA cell model of Parkinson's disease. Biochimica et biophysica acta 1832:174-182.

Stephenne X, Foretz M, Taleux N, van der Zon GC, Sokal E, Hue L, Viollet B, Guigas B (2011) Metformin activates AMP-activated protein kinase in primary human hepatocytes by decreasing cellular energy status. Diabetologia 54:3101-3110.

Storz P, Doppler H, Toker A (2005) Protein kinase D mediates mitochondrion-to-nucleus signaling and detoxification from mitochondrial reactive oxygen species. Molecular and cellular biology 25:8520-8530.

Subramaniam SR, Chesselet MF (2013) Mitochondrial dysfunction and oxidative stress in Parkinson's disease. Progress in neurobiology 106-107:17-32.

Sun Y, Chang YH, Chen HF, Su YH, Su HF, Li CY (2012) Risk of Parkinson disease onset in patients with diabetes: a 9-year population-based cohort study with age and sex stratifications. Diabetes care 35:1047-1049.

Trinh J, Farrer M (2013) Advances in the genetics of Parkinson disease. Nature reviews Neurology 9:445-454.

Ullah I, Ullah N, Naseer MI, Lee HY, Kim MO (2012) Neuroprotection with metformin and thymoquinone against ethanol-induced apoptotic neurodegeneration in prenatal rat cortical neurons. BMC neuroscience 13:11.

Virbasius JV, Scarpulla RC (1994) Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. Proceedings of the National Academy of Sciences of the United States of America 91:1309-1313.

Wahlqvist ML, Lee MS, Hsu CC, Chuang SY, Lee JT, Tsai HN (2012) Metformin-inclusive sulfonylurea therapy reduces the risk of Parkinson's disease occurring with Type 2 diabetes in a Taiwanese population cohort. Parkinsonism & related disorders 18:753-758.

Wang L, Zhai YQ, Xu LL, Qiao C, Sun XL, Ding JH, Lu M, Hu G (2014) Metabolic inflammation exacerbates dopaminergic neuronal degeneration in response to acute MPTP challenge in type 2 diabetes mice. Experimental neurology 251:22-29.

Wheaton WW, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E, Glasauer A, Dufour E, Mutlu GM, Budigner GS, Chandel NS (2014) Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. eLife 3:e02242.

Yang L, Zhao K, Calingasan NY, Luo G, Szeto HH, Beal MF (2009) Mitochondria targeted peptides protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. Antioxidants & redox signaling 11:2095-2104.

Zhang D, Anantharam V, Kanthasamy A, Kanthasamy AG (2007) Neuroprotective effect of protein kinase C delta inhibitor rottlerin in cell culture and animal models of Parkinson's disease. The Journal of pharmacology and experimental therapeutics 322:913-922.
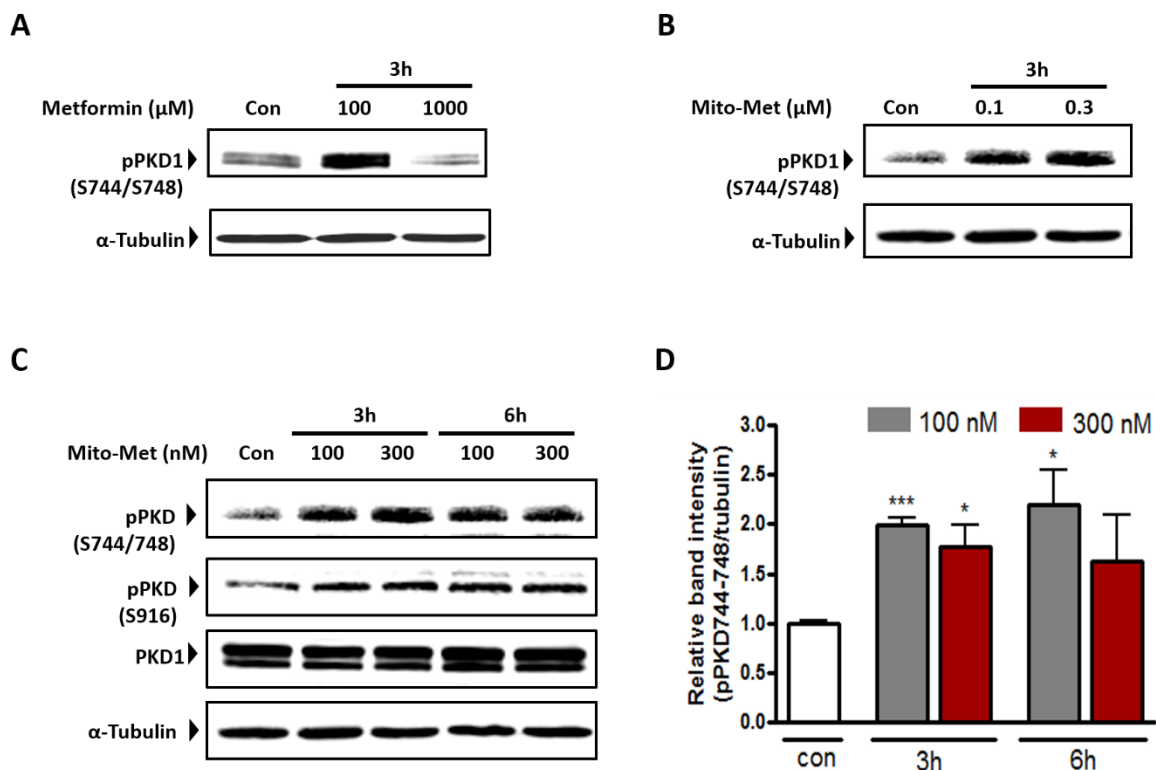
Figure 1. Activation of PKD1 by Mito-Met in N27 cells. *A,* N27 dopaminergic neuronal cells were treated with metformin (100 and 1000 µM) for 3 h. *B,* N27 cells were treated with Mito-Met (100 and 300 nM) for 3 h. Cell lysates were prepared and subjected to Western blot analysis. Representative immunoblots of PKD1 S744/748 phosphorylation are shown. *C,* N27 cells were treated with 100 and 300 nM Mito-Met for 3 h and 6 h. Cell lysates were prepared and subjected to Western blot analysis. Representative immunoblots of total PKD1, PKD1 S744/748 and S916 phosphorylation are shown. *D,* the graph represents the densitometric analysis of phospho-PKD1 S744/748 levels. Results are the mean ± SEM of at least three independent experiments (* $p < 0.05$; *** $p < 0.001$).
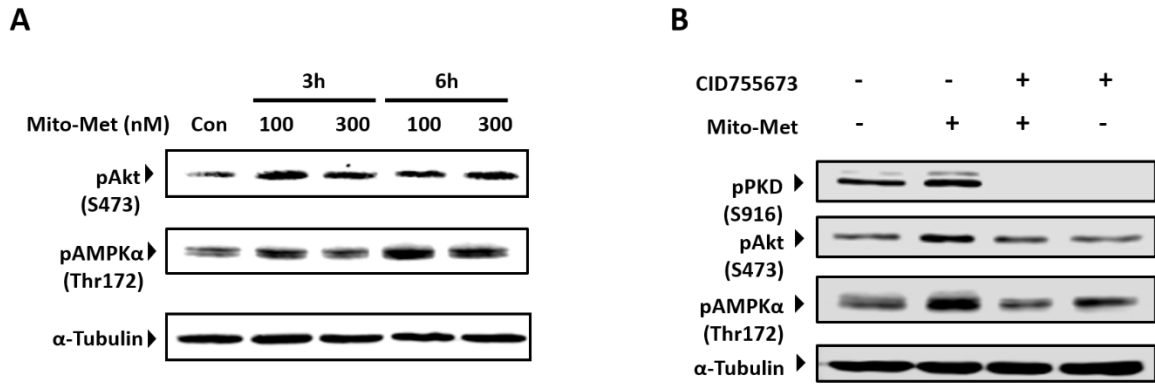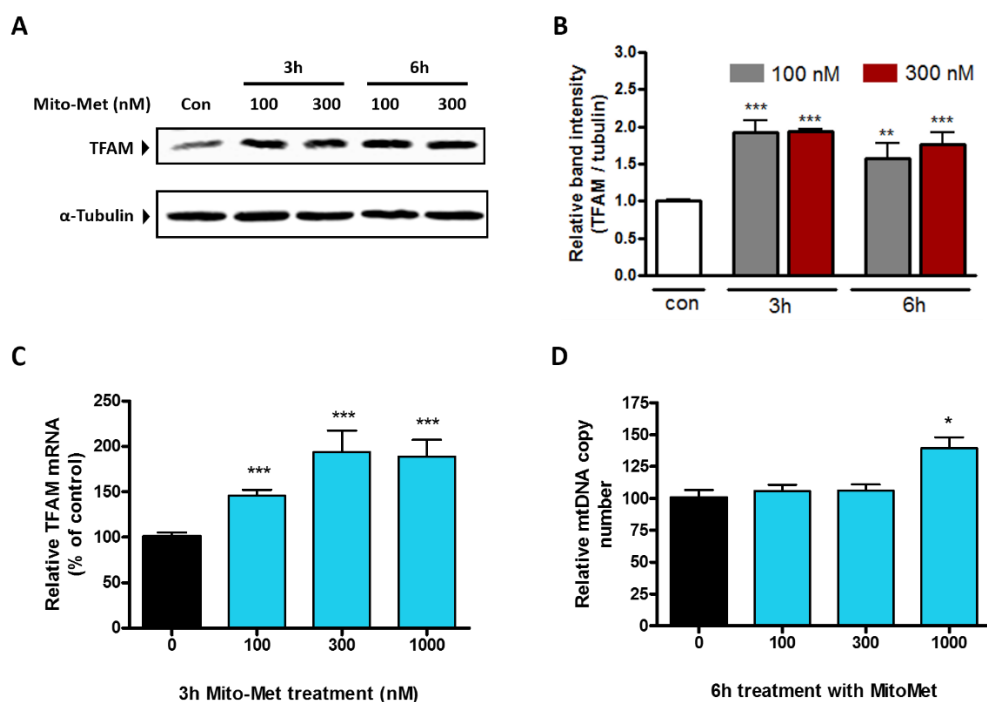
Figure 2. Activation of Akt and AMPK by Mito-Met in N27 cells. *A,* N27 cells were treated with 100 and 300 nM Mito-Met for 3 h and 6 h. Cell lysates were prepared and phospho-Akt (S473) and phospho-AMPKα (Thr172) levels were determined by Western blot analysis. *B,* N27 cells were pretreated with 50 µM PKD1 inhibitor CID755673 for 1 h and then cotreated with 100 nM Mito-Met for 3 h. Cell lysates were prepared and subjected to Western blot analyses of phospho-PKD1 (S916), phospho-Akt (S473), and phospho-AMPKα (Thr172).

Figure 3. Mito-Met increases TFAM expression in N27 cells. *A,* N27 cells were treated with 100 and 300 nM Mito-Met for 3 h and 6 h. Cell lysates were prepared and TFAM levels were determined by Western blot analysis. *B,* the graph represents the densitometric analysis of TFAM protein levels normalized to α-tubulin. *C,* N27 cells were treated with Mito-Met (100-1000 nM) for 3 h. Real-time RT-PCR analysis of TFAM mRNA level was performed. 18S rRNA served as internal control. *D,* N27 cells were treated with Mito-Met (100-1000 nM) for 6 h. Genomic DNA was isolated and mtDNA content was determined by quantitative PCR with SYBR green. 10 ng nuclear DNA and 1 ng mitochondrial DNA were amplified using the primers for mitochondrial ND1 and nuclear β-actin genes. Results are the mean ± SEM of three independent experiments (* $p < 0.05$; ** $p < 0.01$; ***, $p<0.001$; between the control and Mito-Met-treated samples).

A

B

Basal OCR

C

ATP-linked respiration
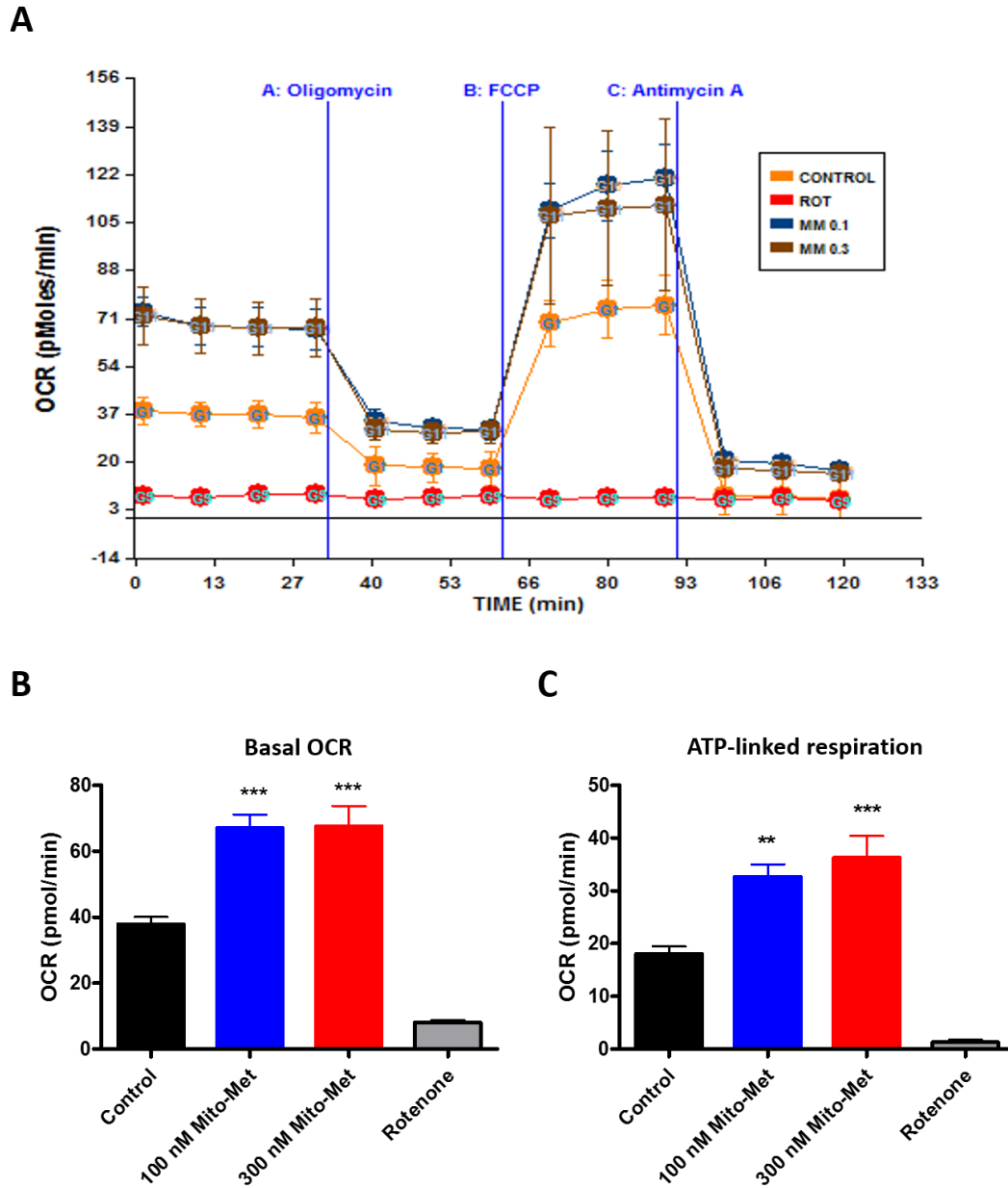
Figure 4. Mito-Met increases mitochondrial bioenergetics capacity. *A-C*, N27 dopaminergic neuronal cells were treated with 100 and 300 nM Mito-Met for 3 h. Rotenone (1 μM) was included as a positive control. N27 cells-containing culture plates were loaded into

the Seahorse XF96 analyzer for the OCR measurement. Mitochondrial dynamics were measured using the sequential injection of oligomycin A (1 µg/ml), FCCP (1 µM), and antimycin A (10 µM) (*A*). Basal OCR (*B*) and ATP-linked respiration (*C*) were calculated from the output OCR values. Values represent the means ± SEM of four replicates (**, $p<0.01$; ***, $p<0.001$; between the control and Mito-Met-treated samples).
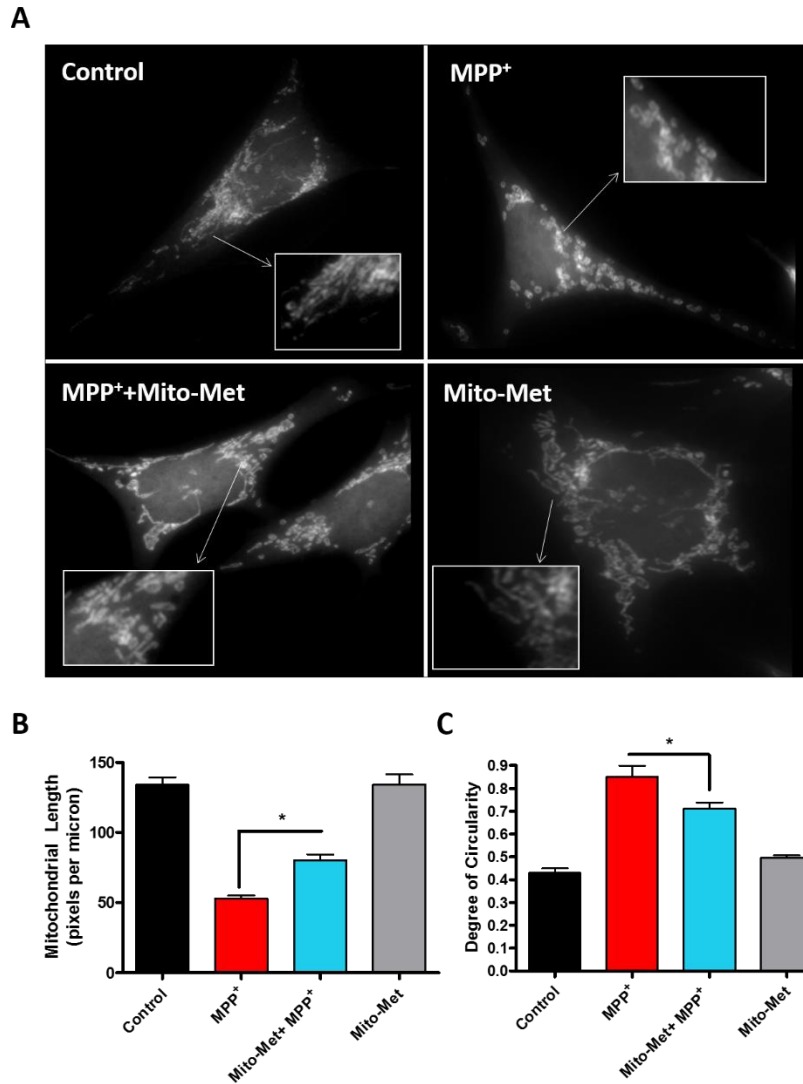
Figure 5. Mito-Met reduces MPP$^+$-induced mitochondrial fragmentation. *A-C,* N27 cells were pretreated with 300 nM Mito-Met for 1 h, and then cotreated with 300 µM MPP$^+$ for 16 h. Cells were stained with the MitoTracker red dye. Images were taken at a magnification of 60X (*A*). Mitochondrial length (*B*) and degree of circularity (*C*) were quantified using ImageJ. Values represent the means ± SEM of two independent experiments performed in hexaplicate (*, p<0.05; between the Mito-Met-pretreated and MPP$^+$ (alone)-treated groups).
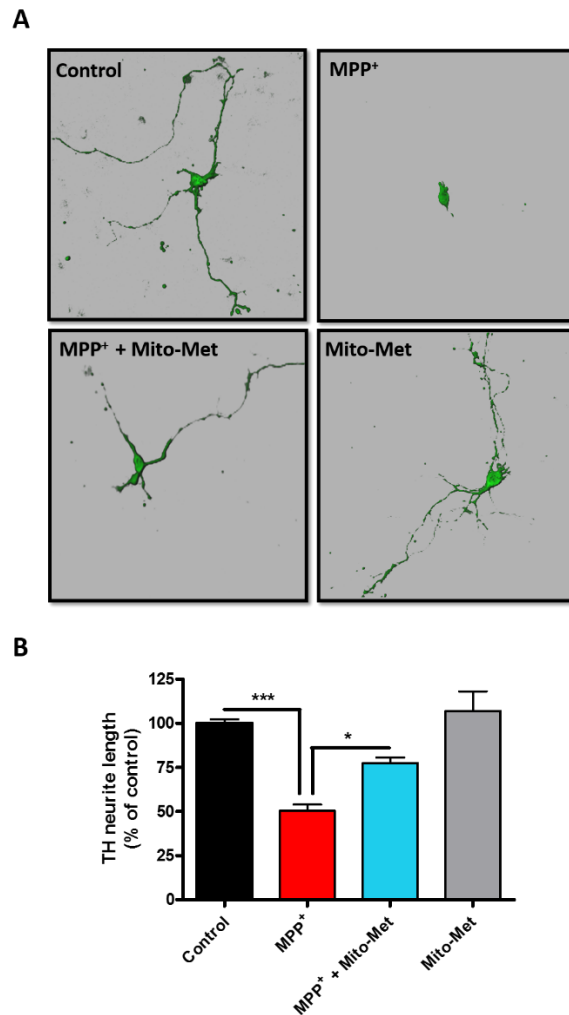
Figure 6. Mito-Met protects against MPP+-induced neurotoxicity in primary neurons. *A-B,* the primary mesencephalic neurons were treated with 10 µM MPP+ in the presence or absence of Mito-Met (100 nM) for 24 h and immunostained with TH antibody. Images were taken at 60X magnification using the Leica confocal microscope and 3D image reconstruction was performed using the IMARIS software (*A*). The length of TH-positive neuronal processes in primary dopaminergic neurons from each coverslip was measured using the MetaMorph software (*B*). The experiments were performed in hexaplicate (*, p<0.05; ***, p<0.001).
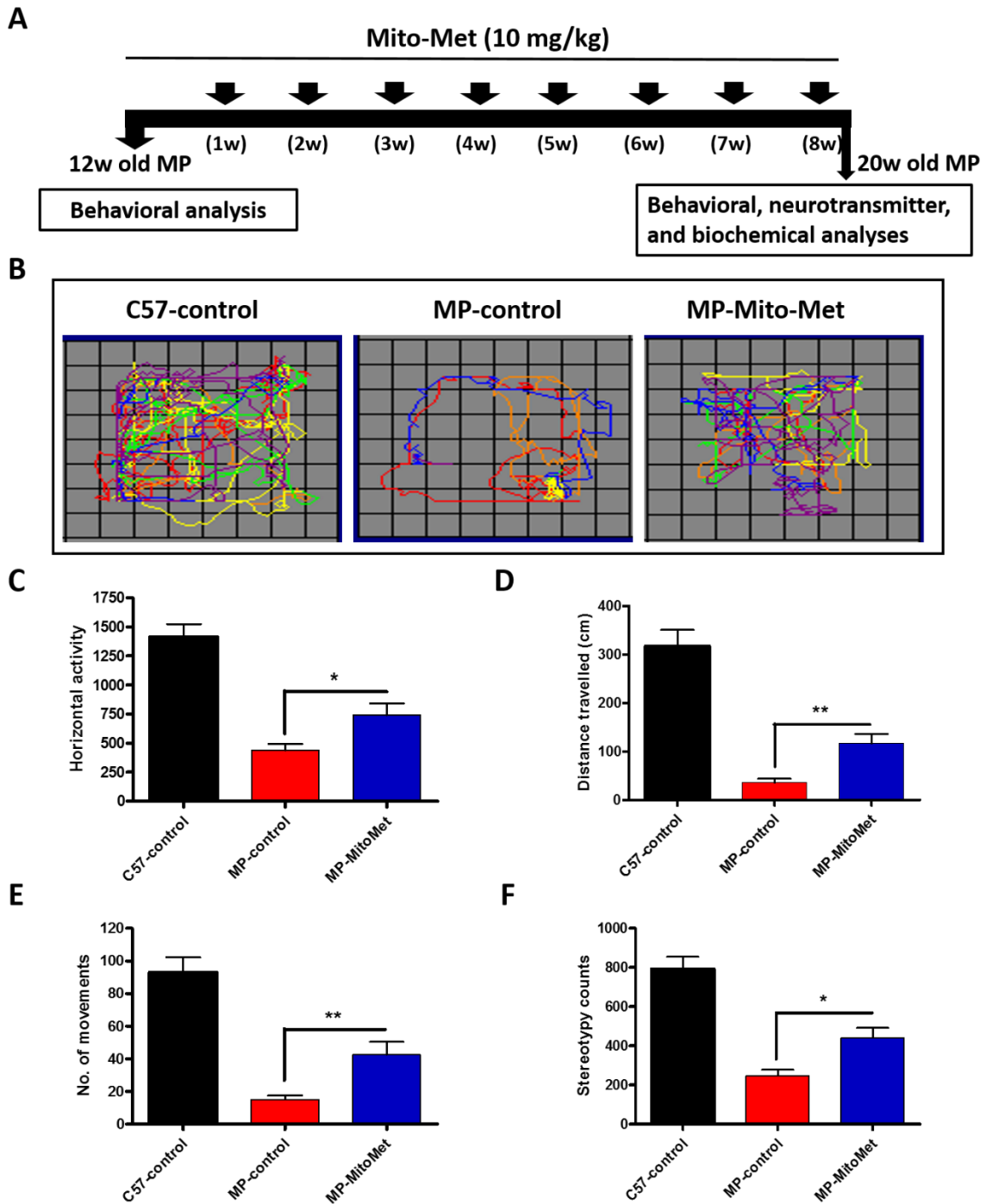
Figure 7. Mito-Met improves motor deficits in MitoPark mice. *A*, Treatment schedule of

MitoPark mice with Mito-Met. 12-week-old MitoPark mice were treated with either saline or

Mito-Met (10 mg/kg) via oral gavage three times per week for 8 weeks. The locomotor

activities were measured using a VersaMax system one day prior to sacrifice. Moving track of mice (*B*), horizontal activity (*C*), total distance travelled (*D*), number of movements (*E*), and stereotypy counts (*F*). Values represent the means ± SEM of six mice per group (*, $p < 0.05$; **, $p < 0.01$).
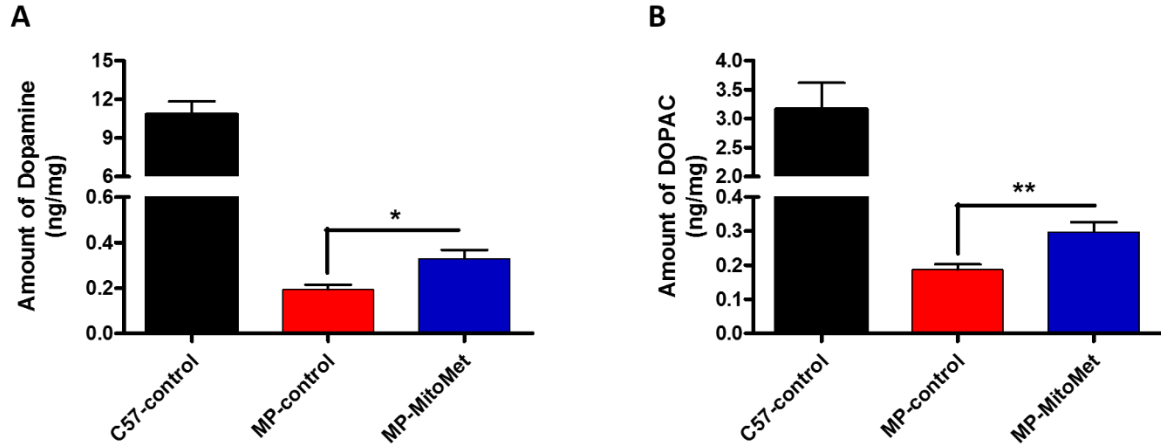
Figure 8. Mito-Met attenuates striatal dopamine depletion in MitoPark mice. *A-B,* 12-week-old MitoPark mice were treated with either saline or Mito-Met (10 mg/kg) via oral gavage three times per week for 8 weeks. Mice were sacrificed one day after the last Mito-Met treatment and dopamine (*A*) and DOPAC (*B*) levels were measured from striatal tissues by HPLC analysis. Results are the means ± SEM of five mice per group (*, p<0.05; **, p<0.01).
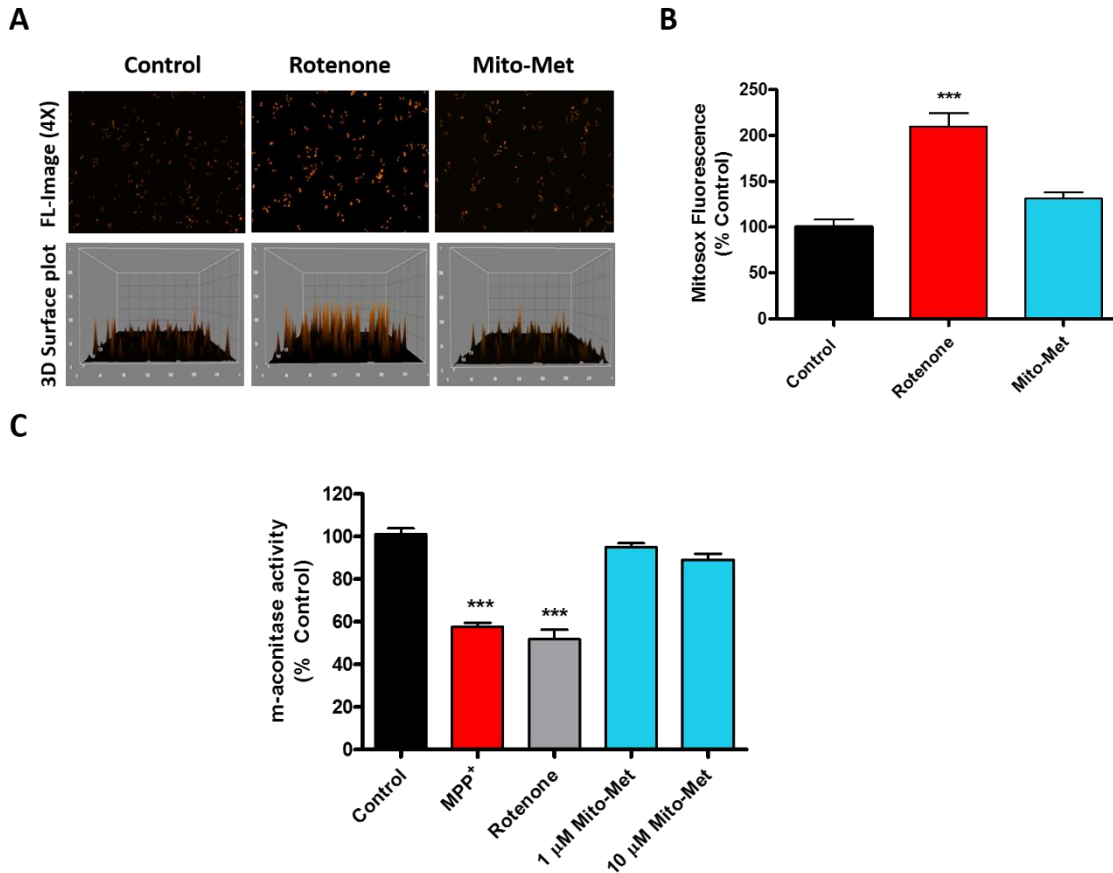
Supp. Figure 1. Mito-Met doesn't adversely affect mitochondrial function. *A-B,* N27 cells were treated with 1 µM rotenone and 1 µM Mito-Met for 6 h. Mitochondrial ROS generation was measured using the MitoSOX staining and fluorescence images were taken at 4X magnification (*A*). MitoSOX fluorescence was quantified using the Cytation 3 microplate reader (*B*). *C,* N27 cells were treated with 300 µM MPP$^+$, 1 µM rotenone, and 1 and 10 µM Mito-Met for 6 h and mitochondrial aconitase activity was measured.

Table 1. Changes in the blood chemistry of mice-treated with Mito-Met

Parameters	Control (3h)	MitoMet (3h)		Control (48h)	MitoMet (48h)		
	Mean ± SEM	Mean ± SEM	p value	Mean ± SEM	Mean ± SEM	p value	Ref. value
Albumin	3.23 ± 0.44	3.93 ± 0.12	0.20	4.07 ± 0.20	4.30 ± 0.10	0.36	2.5 - 4.8 gm/dl
Alk phos	70.67 ± 9.33	94.00 ± 5.57	0.10	134.0 ± 12.58	98.00 ± 10.12	0.09	62 - 209 IU/L
ALT	65.67 ± 14.08	104.3 ± 22.58	0.22	60.33 ± 9.26	67.33 ± 10.73	0.65	28 - 132 IU/L
Amylase	1061 ± 127.4	1149 ± 8.41	0.53	858.3 ± 38.40	819.0 ± 26.66	0.45	
Total bilirubin	0.30 ± 0.00	0.30 ± 0.00	-	0.367 ± 0.03	0.30 ± 0.00	0.18	0.1 - 0.9 mg/dl
BUN	15.33 ± 2.85	16.67 ± 1.45	0.70	14.67 ± 1.67	19.67 ± 1.33	0.08	18 - 29 mg/dl
Calcium	11.73 ± 0.23	10.87 ± 0.09	0.03	10.73 ± 0.15	11.33 ± 0.18	0.06	5.9 - 9.4 mg/dl
Phosphorus	10.20 ± 1.08	9.87 ± 0.30	0.78	8.30 ± 0.12	9.70 ± 0.67	0.11	6.1 - 10.1 mg/dl
Creatinine	< 0.2	< 0.2	-	< 0.2	< 0.2	-	0.2 - 0.8 mg/dl
Glucose	292.3 ± 12.81	285.0 ± 17.52	0.75	227.7 ± 16.27	268.7 ± 23.68	0.23	230 - 304 mg/dl
Sodium	159.0 ± 1.53	160.3 ± 2.33	0.66	159.0 ± 1.53	160.3 ± 1.76	0.60	124 - 174 mEq/L
Potassium	> 8.5	> 8.5	-	> 8.5	> 8.5	-	4.6 - 8 mEq/L
Total protein	5.50 ± 0.06	5.73 ± 0.13	0.18	5.17 ± 0.18	5.43 ± 0.19	0.36	3.6 - 6.6 gm/dl

CHAPTER V: GENERAL CONCLUSIONS

This section presents an overview of the results and findings of this dissertation, with special emphasis on the overall implications of these findings for developing novel neuroprotective strategies for Parkinson's disease. The major findings of each research chapter included in this dissertation are covered in the Discussion of each chapter.

PKD1 activation positively regulates PGC-1α and BDNF expression in dopaminergic neuronal cells

We have recently shown that PKD1 plays a compensatory cell survival role during the initial stage of oxidative stress in cell culture models of PD (Asaithambi et al., 2011, Asaithambi et al., 2014). In the present study, we investigated the molecular mechanisms of PKD1-mediated neuroprotection. Since PGC-1α was previously reported to serve as a compensatory response to mitochondrial stress (Rohas et al., 2007), we investigated PKD1's role in regulating PGC-1α expression in dopaminergic cells. Interestingly, our results show that PKD1 overexpression increased PGC-1α promoter activity and mRNA/protein expression. Treatment of cells with a PKD1 peptide activator (AKP4T) also increased PGC-1α mRNA and protein expression, while treatment of cells with a pharmacological inhibitor of PKD1 (kbNB-14270) almost completely suppressed basal PGC-1α mRNA expression, suggesting that PKD1 activation can positively regulate PGC-1α transcriptional activity. Our results also demonstrate that activation of PKD1 signaling increased CREB phosphorylation and MEF2 activity, which could account for PKD1-mediated upregulation of PGC-1α. However, more mechanistic studies are needed to elucidate the precise role of PKD1 in transcriptional regulation of the PGC-1α gene in dopaminergic neurons. Given that impaired mitochondrial biogenesis and

reduced expression of PGC-1α-responsive genes play an important role in PD pathogenesis (Zheng et al., 2010), identifying the regulatory role of PKD1 in PGC-1α expression is important for the development of novel neuroprotective agents for PD.

Our results show dramatically increased MEF2 activity in dopaminergic neuronal cells overexpressing the constitutively active form of PKD1. Interestingly, overexpressing the non-phosphorylatable mutant of HDAC5 completely blocked the PKD1-induced MEF2 activation, suggesting that PKD1 can phosphorylate HDAC5, mediating the nuclear export of HDAC5 and thus positively regulating MEF2 transcriptional activity. MEF2D is known to protect against dopaminergic neurodegeneration in the MPTP mouse model of PD (Yao et al., 2012, Mount et al., 2013), and also to localize to mitochondria and regulate the expression of ND6, an essential subunit of mitochondrial complex I, in dopaminergic cells (She et al., 2011). She et al. demonstrated that mitochondrial MEF2D levels were also significantly reduced in PD patients, indicating an important role of MEF2D in the survival of dopaminergic neurons. We are currently performing experiments to identify potential MEF2 targets involved in PKD1-mediated neuroprotection.

After observing increased CREB phosphorylation in dopaminergic cells treated with AKP4T, we determined PKD1's role in regulating BDNF expression. Our data show that PKD1 overexpression and AKP4T treatment significantly increases mRNA and protein expression of BDNF, indicating that BDNF may also be involved in PKD1-mediated neuroprotection in dopaminergic neurons. Importantly, treatment with AKP4T significantly protected against dopaminergic neurotoxicity in a human mesencephalic neuronal cell model of PD, suggesting that positive modulation of PKD1 signaling protects dopaminergic neurons.

Quercetin, a natural flavonoid, activates pro-survival PKD1 signaling and protects against neurodegeneration in a transgenic mouse model of PD

Following the observation that positive modulation of PKD1 offers neuroprotection, we adopted a rationale-based pharmacological screening approach to identify natural compounds that activate PKD1 and found that quercetin strongly induces PKD1 activation in dopaminergic cells. Although some studies have shown that quercetin protects against neurodegenerative processes, the molecular mechanisms and signaling events governing the protective effect of quercetin remain elusive. Our results demonstrate that quercetin activates the pro-survival kinases PKD1 and Akt and that quercetin stimulates mitochondrial biogenesis and bioenergetics capacity in dopaminergic neurons. Moreover, quercetin (25 mg/kg/day) protected against motor deficits, nigrostriatal neurodegeneration, and striatal dopamine loss in the transgenic MitoPark mouse model of PD. We recognize that a number of potentially neuroprotective agents showing apparent anti-Parkinsonian effects in preclinical studies have failed to show any neuroprotective effect in humans. One reason for their failure in clinical trials is the lack of good animal models of PD for translational research. We tested the neuroprotective efficacy of quercetin in MitoPark mice because they represent the most severe mouse model for recapitulating several key features of PD in humans, such as adult-onset degeneration of nigrostriatal dopamine circuitry and the progressive course of phenotypic manifestations and neurodegeneration (Ekstrand et al., 2007, Gubellini and Kachidian, 2015). Moreover, the chronic and progressive nature of the MitoPark model enables studying compensatory mechanisms which might occur in patients during the course of the disease. Our

findings clearly suggest that quercetin deserves further preclinical and clinical investigations for the prevention and treatment of neurodegenerative diseases, including PD.

Mito-metformin (Mito-Met) induces PKD1 activation and protects dopaminergic neurons in cell culture and animal models of PD

Emerging evidence suggests that type 2 diabetes (T2DM) is a risk factor for neurodegenerative diseases, including PD (Sun et al., 2012, Santiago and Potashkin, 2013b), but the mechanism behind the association between PD and T2DM is currently unknown. Metformin, one of the most commonly used anti-diabetic drugs with a high safety profile, was recently shown to have neuroprotective effects in a toxin-based mouse model of PD (Patil et al., 2014). More importantly, a cohort study revealed that the combination therapy of metformin and sulfonylurea reduced the risk of developing PD in patients with T2DM in a Taiwanese population (Wahlqvist et al., 2012). Another interesting study demonstrated that metformin promotes lifespan in mice by reducing chronic inflammation and increasing antioxidant defense mechanisms (Martin-Montalvo et al., 2013).

Mitochondrial dysfunction plays a central role in the neurodegenerative process observed in PD (Schapira and Jenner, 2011, Subramaniam and Chesselet, 2013). Therefore, targeting compounds into mitochondria may lead to the discovery of more specific, less toxic, and more effective neuroprotective agents for PD. In our study, we conjugated metformin with a TPP$^+$ group to synthesize Mito-Met, a mitochondria-targeted metformin analog. Our data show that the novel compound Mito-Met effectively activated PKD1-mediated neuroprotective signaling and enhanced mitochondrial biogenesis and bioenergetics capacity in a cell model of PD. Moreover, Mito-Met treatment has neuroprotective effects against

behavioral deficits and striatal dopamine depletion in the MitoPark transgenic mouse model of PD. Studies are currently underway to determine the pharmacokinetic profile of Mito-Met. The neuroprotective efficacy of Mito-Met will also be tested in toxin-based models of PD.
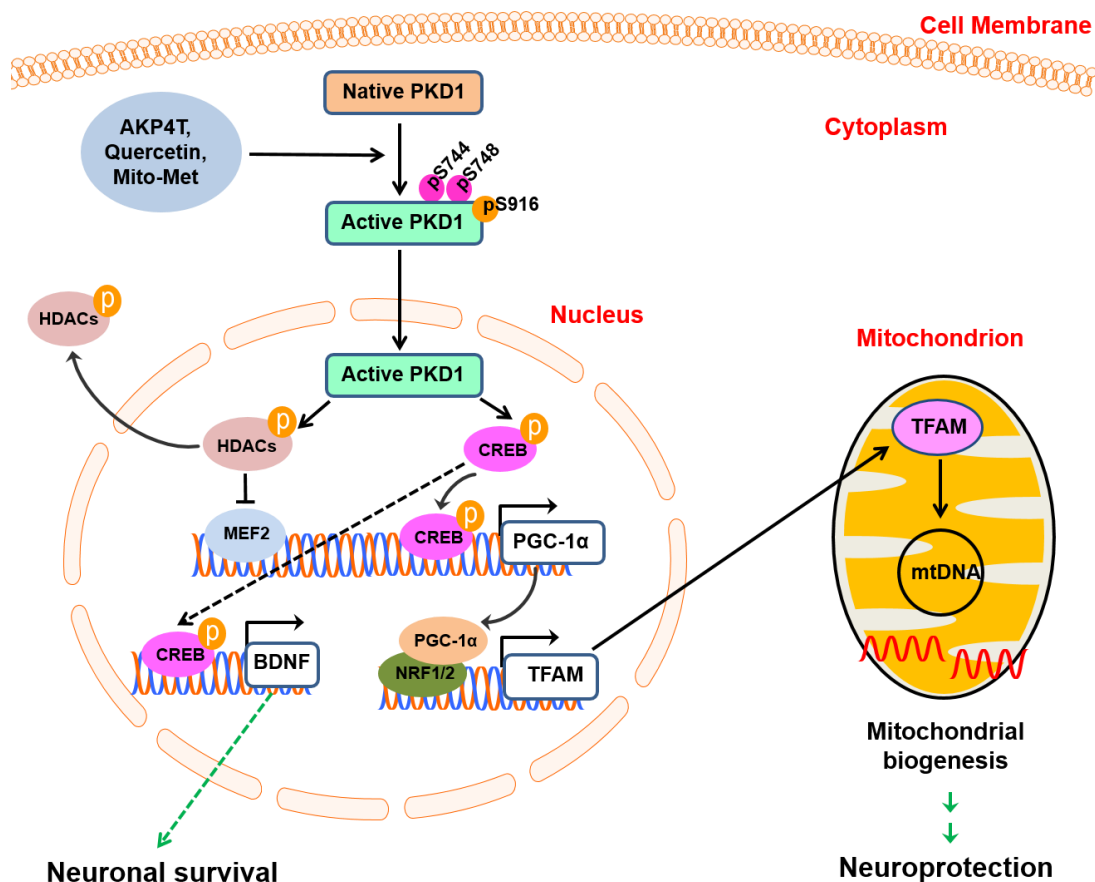
Scheme 1: Integrated mechanisms of PKD1-mediated neuroprotective signaling in dopaminergic cells

In summary, our findings suggest that PKD1 serves as an upstream kinase that regulates key neuroprotective mechanisms to promote dopaminergic neuron survival (Scheme 1) and that targeting PKD1 signaling has therapeutic value for the development of novel neuroprotective strategies against dopaminergic neurodegeneration.

REFERENCES

Abou-Sleiman PM, Healy DG, Quinn N, Lees AJ, Wood NW (2003) The role of pathogenic DJ-1 mutations in Parkinson's disease. Annals of neurology 54:283-286.

Abou-Sleiman PM, Muqit MM, Wood NW (2006) Expanding insights of mitochondrial dysfunction in Parkinson's disease. Nature reviews 7:207-219.

Alam ZI, Jenner A, Daniel SE, Lees AJ, Cairns N, Marsden CD, Jenner P, Halliwell B (1997) Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. Journal of neurochemistry 69:1196-1203.

Alcocer F, Whitley D, Salazar-Gonzalez JF, Jordan WD, Sellers MT, Eckhoff DE, Suzuki K, Macrae C, Bland KI (2002) Quercetin inhibits human vascular smooth muscle cell proliferation and migration. Surgery 131:198-204.

Anantharam V, Kitazawa M, Latchoumycandane C, Kanthasamy A, Kanthasamy AG (2004) Blockade of PKCdelta proteolytic activation by loss of function mutants rescues mesencephalic dopaminergic neurons from methylcyclopentadienyl manganese tricarbonyl (MMT)-induced apoptotic cell death. Annals of the New York Academy of Sciences 1035:271-289.

Anantharam V, Kitazawa M, Wagner J, Kaul S, Kanthasamy AG (2002) Caspase-3-dependent proteolytic cleavage of protein kinase Cdelta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese tricarbonyl. J Neurosci 22:1738-1751.

Andres RH, Huber AW, Schlattner U, Perez-Bouza A, Krebs SH, Seiler RW, Wallimann T, Widmer HR (2005) Effects of creatine treatment on the survival of dopaminergic neurons in cultured fetal ventral mesencephalic tissue. Neuroscience 133:701-713.

Angst E, Park JL, Moro A, Lu QY, Lu X, Li G, King J, Chen M, Reber HA, Go VL, Eibl G, Hines OJ (2013) The flavonoid quercetin inhibits pancreatic cancer growth in vitro and in vivo. Pancreas 42:223-229.

Ansari MA, Abdul HM, Joshi G, Opii WO, Butterfield DA (2009) Protective effect of quercetin in primary neurons against Abeta(1-42): relevance to Alzheimer's disease. The Journal of nutritional biochemistry 20:269-275.

Araki T, Kumagai T, Tanaka K, Matsubara M, Kato H, Itoyama Y, Imai Y (2001) Neuroprotective effect of riluzole in MPTP-treated mice. Brain research 918:176-181.

Arun SN, Kaddour-Djebbar I, Shapiro BA, Bollag WB (2011) Ultraviolet B irradiation and activation of protein kinase D in primary mouse epidermal keratinocytes. Oncogene 30:1586-1596.

Asaithambi A, Ay M, Jin H, Gosh A, Anantharam V, Kanthasamy A, Kanthasamy AG (2014) Protein kinase D1 (PKD1) phosphorylation promotes dopaminergic neuronal survival during 6-OHDA-induced oxidative stress. PloS one 9:e96947.

Asaithambi A, Kanthasamy A, Saminathan H, Anantharam V, Kanthasamy AG (2011) Protein kinase D1 (PKD1) activation mediates a compensatory protective response during early

stages of oxidative stress-induced neuronal degeneration. Molecular neurodegeneration 6:43.

Askari G, Ghiasvand R, Paknahad Z, Karimian J, Rabiee K, Sharifirad G, Feizi A (2013) The effects of quercetin supplementation on body composition, exercise performance and muscle damage indices in athletes. International journal of preventive medicine 4:21-26.

Ay M, Jin H, Harischandra DS, Asaithambi A, Kanthasamy A, Anantharam V, Kanthasamy AG (2015) Molecular cloning, epigenetic regulation, and functional characterization of Prkd1 gene promoter in dopaminergic cell culture models of Parkinson's disease. Journal of neurochemistry 135:402-415.

Backs J, Olson EN (2006) Control of cardiac growth by histone acetylation/deacetylation. Circulation research 98:15-24.

Baldereschi M, Di Carlo A, Rocca WA, Vanni P, Maggi S, Perissinotto E, Grigoletto F, Amaducci L, Inzitari D (2000) Parkinson's disease and parkinsonism in a longitudinal study: two-fold higher incidence in men. ILSA Working Group. Italian Longitudinal Study on Aging. Neurology 55:1358-1363.

Barroso N, Campos Y, Huertas R, Esteban J, Molina JA, Alonso A, Gutierrez-Rivas E, Arenas J (1993) Respiratory chain enzyme activities in lymphocytes from untreated patients with Parkinson disease. Clinical chemistry 39:667-669.

Barsoum MJ, Yuan H, Gerencser AA, Liot G, Kushnareva Y, Graber S, Kovacs I, Lee WD, Waggoner J, Cui J, White AD, Bossy B, Martinou JC, Youle RJ, Lipton SA, Ellisman MH, Perkins GA, Bossy-Wetzel E (2006) Nitric oxide-induced mitochondrial fission is regulated by dynamin-related GTPases in neurons. The EMBO journal 25:3900-3911.

Beal MF (2003) Mitochondria, oxidative damage, and inflammation in Parkinson's disease. Annals of the New York Academy of Sciences 991:120-131.

Beal MF, Matthews RT, Tieleman A, Shults CW (1998) Coenzyme Q10 attenuates the 1-methyl-4-phenyl-1,2,3,tetrahydropyridine (MPTP) induced loss of striatal dopamine and dopaminergic axons in aged mice. Brain research 783:109-114.

Bellissimo MI, Amado D, Abdalla DS, Ferreira EC, Cavalheiro EA, Naffah-Mazzacoratti MG (2001) Superoxide dismutase, glutathione peroxidase activities and the hydroperoxide concentration are modified in the hippocampus of epileptic rats. Epilepsy research 46:121-128.

Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, Jaros E, Hersheson JS, Betts J, Klopstock T, Taylor RW, Turnbull DM (2006) High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nature genetics 38:515-517.

Besirli CG, Johnson EM, Jr. (2006) The activation loop phosphorylation of protein kinase D is an early marker of neuronal DNA damage. Journal of neurochemistry 99:218-225.

Bisbal M, Conde C, Donoso M, Bollati F, Sesma J, Quiroga S, Diaz Anel A, Malhotra V, Marzolo MP, Caceres A (2008) Protein kinase d regulates trafficking of dendritic membrane proteins in developing neurons. J Neurosci 28:9297-9308.

Blandini F, Porter RH, Greenamyre JT (1996) Glutamate and Parkinson's disease. Molecular neurobiology 12:73-94.

Boly R, Gras T, Lamkami T, Guissou P, Serteyn D, Kiss R, Dubois J (2011) Quercetin inhibits a large panel of kinases implicated in cancer cell biology. International journal of oncology 38:833-842.

Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P (2003) Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science (New York, NY 299:256-259.

Boots AW, Wilms LC, Swennen EL, Kleinjans JC, Bast A, Haenen GR (2008) In vitro and ex vivo anti-inflammatory activity of quercetin in healthy volunteers. Nutrition (Burbank, Los Angeles County, Calif 24:703-710.

Borges N (2005) Tolcapone in Parkinson's disease: liver toxicity and clinical efficacy. Expert opinion on drug safety 4:69-73.

Bournival J, Plouffe M, Renaud J, Provencher C, Martinoli MG (2012) Quercetin and sesamin protect dopaminergic cells from MPP+-induced neuroinflammation in a microglial

(N9)-neuronal (PC12) coculture system. Oxidative medicine and cellular longevity 2012:921941.

Bournival J, Quessy P, Martinoli MG (2009) Protective effects of resveratrol and quercetin against MPP+ -induced oxidative stress act by modulating markers of apoptotic death in dopaminergic neurons. Cellular and molecular neurobiology 29:1169-1180.

Boyle SP, Dobson VL, Duthie SJ, Kyle JA, Collins AR (2000) Absorption and DNA protective effects of flavonoid glycosides from an onion meal. European journal of nutrition 39:213-223.

Brown TP, Rumsby PC, Capleton AC, Rushton L, Levy LS (2006) Pesticides and Parkinson's disease--is there a link? Environmental health perspectives 114:156-164.

Burn DJ, Jaros E (2001) Multiple system atrophy: cellular and molecular pathology. Molecular pathology : MP 54:419-426.

Burton NC, Kensler TW, Guilarte TR (2006) In vivo modulation of the Parkinsonian phenotype by Nrf2. Neurotoxicology 27:1094-1100.

Canet-Aviles RM, Wilson MA, Miller DW, Ahmad R, McLendon C, Bandyopadhyay S, Baptista MJ, Ringe D, Petsko GA, Cookson MR (2004) The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. Proceedings of the National Academy of Sciences of the United States of America 101:9103-9108.

Carlstrom J, Symons JD, Wu TC, Bruno RS, Litwin SE, Jalili T (2007) A quercetin supplemented diet does not prevent cardiovascular complications in spontaneously hypertensive rats. The Journal of nutrition 137:628-633.

Carpenter L, Cordery D, Biden TJ (2002) Inhibition of protein kinase C delta protects rat INS-1 cells against interleukin-1beta and streptozotocin-induced apoptosis. Diabetes 51:317-324.

Cass WA (1996) GDNF selectively protects dopamine neurons over serotonin neurons against the neurotoxic effects of methamphetamine. J Neurosci 16:8132-8139.

Chaillou LL, Nazareno MA (2006) New method to determine antioxidant activity of polyphenols. Journal of agricultural and food chemistry 54:8397-8402.

Chakraborty J, Singh R, Dutta D, Naskar A, Rajamma U, Mohanakumar KP (2014) Quercetin improves behavioral deficiencies, restores astrocytes and microglia, and reduces serotonin metabolism in 3-nitropropionic acid-induced rat model of Huntington's Disease. CNS neuroscience & therapeutics 20:10-19.

Chartier-Harlin MC, Dachsel JC, Vilarino-Guell C, Lincoln SJ, Lepretre F, Hulihan MM, Kachergus J, Milnerwood AJ, Tapia L, Song MS, Le Rhun E, Mutez E, Larvor L, Duflot A, Vanbesien-Mailliot C, Kreisler A, Ross OA, Nishioka K, Soto-Ortolaza AI, Cobb SA, Melrose HL, Behrouz B, Keeling BH, Bacon JA, Hentati E, Williams L, Yanagiya A, Sonenberg N, Lockhart PJ, Zubair AC, Uitti RJ, Aasly JO, Krygowska-Wajs A, Opala G, Wszolek ZK, Frigerio R, Maraganore DM, Gosal D, Lynch T, Hutchinson M, Bentivoglio AR, Valente EM, Nichols WC, Pankratz N, Foroud T,

Gibson RA, Hentati F, Dickson DW, Destee A, Farrer MJ (2011) Translation initiator EIF4G1 mutations in familial Parkinson disease. American journal of human genetics 89:398-406.

Chaturvedi RK, Flint Beal M (2013) Mitochondrial diseases of the brain. Free radical biology & medicine 63:1-29.

Chaudhuri KR, Schapira AH (2009) Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. Lancet neurology 8:464-474.

Chen H, Chan DC (2005) Emerging functions of mammalian mitochondrial fusion and fission. Human molecular genetics 14 Spec No. 2:R283-289.

Chen J, Deng F, Li J, Wang QJ (2008a) Selective binding of phorbol esters and diacylglycerol by individual C1 domains of the PKD family. The Biochemical journal 411:333-342.

Chen J, Deng F, Singh SV, Wang QJ (2008b) Protein kinase D3 (PKD3) contributes to prostate cancer cell growth and survival through a PKCepsilon/PKD3 pathway downstream of Akt and ERK 1/2. Cancer research 68:3844-3853.

Chen PC, Vargas MR, Pani AK, Smeyne RJ, Johnson DA, Kan YW, Johnson JA (2009) Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: Critical role for the astrocyte. Proceedings of the National Academy of Sciences of the United States of America 106:2933-2938.

Choi EJ, Bae SM, Ahn WS (2008) Antiproliferative effects of quercetin through cell cycle arrest and apoptosis in human breast cancer MDA-MB-453 cells. Archives of pharmacal research 31:1281-1285.

Choi J, Sullards MC, Olzmann JA, Rees HD, Weintraub ST, Bostwick DE, Gearing M, Levey AI, Chin LS, Li L (2006) Oxidative damage of DJ-1 is linked to sporadic Parkinson and Alzheimer diseases. The Journal of biological chemistry 281:10816-10824.

Clarke CE (2004) Neuroprotection and pharmacotherapy for motor symptoms in Parkinson's disease. Lancet neurology 3:466-474.

Conklin CM, Bechberger JF, MacFabe D, Guthrie N, Kurowska EM, Naus CC (2007) Genistein and quercetin increase connexin43 and suppress growth of breast cancer cells. Carcinogenesis 28:93-100.

Cosan DT, Soyocak A, Basaran A, Degirmenci I, Gunes HV, Sahin FM (2011) Effects of various agents on DNA fragmentation and telomerase enzyme activities in adenocarcinoma cell lines. Molecular biology reports 38:2463-2469.

Cowell CF, Doppler H, Yan IK, Hausser A, Umezawa Y, Storz P (2009) Mitochondrial diacylglycerol initiates protein-kinase D1-mediated ROS signaling. Journal of cell science 122:919-928.

Cui M, Tang X, Christian WV, Yoon Y, Tieu K (2010) Perturbations in mitochondrial dynamics induced by human mutant PINK1 can be rescued by the mitochondrial division inhibitor mdivi-1. The Journal of biological chemistry 285:11740-11752.

Cui Y, Morgenstern H, Greenland S, Tashkin DP, Mao JT, Cai L, Cozen W, Mack TM, Lu QY, Zhang ZF (2008) Dietary flavonoid intake and lung cancer--a population-based case-control study. Cancer 112:2241-2248.

Czondor K, Ellwanger K, Fuchs YF, Lutz S, Gulyas M, Mansuy IM, Hausser A, Pfizenmaier K, Schlett K (2009) Protein kinase D controls the integrity of Golgi apparatus and the maintenance of dendritic arborization in hippocampal neurons. Molecular biology of the cell 20:2108-2120.

da Silva EL, Abdalla DS, Terao J (2000) Inhibitory effect of flavonoids on low-density lipoprotein peroxidation catalyzed by mammalian 15-lipoxygenase. IUBMB life 49:289-295.

Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889-909.

Dehmer T, Heneka MT, Sastre M, Dichgans J, Schulz JB (2004) Protection by pioglitazone in the MPTP model of Parkinson's disease correlates with I kappa B alpha induction and block of NF kappa B and iNOS activation. Journal of neurochemistry 88:494-501.

Deng H, Dodson MW, Huang H, Guo M (2008) The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in Drosophila. Proceedings of the National Academy of Sciences of the United States of America 105:14503-14508.

Deng H, Le W, Guo Y, Hunter CB, Xie W, Jankovic J (2005) Genetic and clinical identification of Parkinson's disease patients with LRRK2 G2019S mutation. Annals of neurology 57:933-934.

Deng XH, Song HY, Zhou YF, Yuan GY, Zheng FJ (2013) Effects of quercetin on the proliferation of breast cancer cells and expression of survivin. Experimental and therapeutic medicine 6:1155-1158.

Denning MF, Dlugosz AA, Threadgill DW, Magnuson T, Yuspa SH (1996) Activation of the epidermal growth factor receptor signal transduction pathway stimulates tyrosine phosphorylation of protein kinase C delta. The Journal of biological chemistry 271:5325-5331.

Detmer SA, Chan DC (2007) Functions and dysfunctions of mitochondrial dynamics. Nature reviews 8:870-879.

Dexter DT, Wells FR, Lees AJ, Agid F, Agid Y, Jenner P, Marsden CD (1989) Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease. Journal of neurochemistry 52:1830-1836.

Di Fonzo A, Dekker MC, Montagna P, Baruzzi A, Yonova EH, Correia Guedes L, Szczerbinska A, Zhao T, Dubbel-Hulsman LO, Wouters CH, de Graaff E, Oyen WJ, Simons EJ, Breedveld GJ, Oostra BA, Horstink MW, Bonifati V (2009) FBXO7 mutations cause autosomal recessive, early-onset parkinsonian-pyramidal syndrome. Neurology 72:240-245.

Di Monte DA (2003) The environment and Parkinson's disease: is the nigrostriatal system preferentially targeted by neurotoxins? Lancet neurology 2:531-538.

Dihal AA, de Boer VC, van der Woude H, Tilburgs C, Bruijntjes JP, Alink GM, Rietjens IM, Woutersen RA, Stierum RH (2006) Quercetin, but not its glycosidated conjugate rutin, inhibits azoxymethane-induced colorectal carcinogenesis in F344 rats. The Journal of nutrition 136:2862-2867.

Duarte J, Perez-Vizcaino F, Zarzuelo A, Jimenez J, Tamargo J (1993a) Vasodilator effects of quercetin in isolated rat vascular smooth muscle. European journal of pharmacology 239:1-7.

Duarte J, Perez Vizcaino F, Utrilla P, Jimenez J, Tamargo J, Zarzuelo A (1993b) Vasodilatory effects of flavonoids in rat aortic smooth muscle. Structure-activity relationships. General pharmacology 24:857-862.

Dunn CJ, Peters DH (1995) Metformin. A review of its pharmacological properties and therapeutic use in non-insulin-dependent diabetes mellitus. Drugs 49:721-749.

Duthie SJ, Dobson VL (1999) Dietary flavonoids protect human colonocyte DNA from oxidative attack in vitro. European journal of nutrition 38:28-34.

Egert S, Bosy-Westphal A, Seiberl J, Kurbitz C, Settler U, Plachta-Danielzik S, Wagner AE, Frank J, Schrezenmeir J, Rimbach G, Wolffram S, Muller MJ (2009) Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: a double-

blinded, placebo-controlled cross-over study. The British journal of nutrition 102:1065-1074.

Eiseler T, Doppler H, Yan IK, Goodison S, Storz P (2009) Protein kinase D1 regulates matrix metalloproteinase expression and inhibits breast cancer cell invasion. Breast Cancer Res 11:R13.

Ekstrand MI, Terzioglu M, Galter D, Zhu S, Hofstetter C, Lindqvist E, Thams S, Bergstrand A, Hansson FS, Trifunovic A, Hoffer B, Cullheim S, Mohammed AH, Olson L, Larsson NG (2007) Progressive parkinsonism in mice with respiratory-chain-deficient dopamine neurons. Proceedings of the National Academy of Sciences of the United States of America 104:1325-1330.

Elbaz A, Carcaillon L, Kab S, Moisan F (2015) Epidemiology of Parkinson's disease. Revue neurologique.

Endo K, Oki E, Biedermann V, Kojima H, Yoshida K, Johannes FJ, Kufe D, Datta R (2000) Proteolytic cleavage and activation of protein kinase C [micro] by caspase-3 in the apoptotic response of cells to 1-beta -D-arabinofuranosylcytosine and other genotoxic agents. The Journal of biological chemistry 275:18476-18481.

Exner N, Lutz AK, Haass C, Winklhofer KF (2012) Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences. The EMBO journal 31:3038-3062.

Factor SA, Molho ES, Feustel PJ, Brown DL, Evans SM (2001) Long-term comparative experience with tolcapone and entacapone in advanced Parkinson's disease. Clinical neuropharmacology 24:295-299.

Fielitz J, Kim MS, Shelton JM, Qi X, Hill JA, Richardson JA, Bassel-Duby R, Olson EN (2008) Requirement of protein kinase D1 for pathological cardiac remodeling. Proceedings of the National Academy of Sciences of the United States of America 105:3059-3063.

Formica JV, Regelson W (1995) Review of the biology of Quercetin and related bioflavonoids. Food Chem Toxicol 33:1061-1080.

Freitas RM, Nascimento VS, Vasconcelos SM, Sousa FC, Viana GS, Fonteles MM (2004) Catalase activity in cerebellum, hippocampus, frontal cortex and striatum after status epilepticus induced by pilocarpine in Wistar rats. Neuroscience letters 365:102-105.

Fu Y, Rubin CS (2011) Protein kinase D: coupling extracellular stimuli to the regulation of cell physiology. EMBO reports 12:785-796.

Fujioka S, Ogaki K, Tacik PM, Uitti RJ, Ross OA, Wszolek ZK (2014) Update on novel familial forms of Parkinson's disease and multiple system atrophy. Parkinsonism & related disorders 20 Suppl 1:S29-34.

Galter D, Pernold K, Yoshitake T, Lindqvist E, Hoffer B, Kehr J, Larsson NG, Olson L (2010) MitoPark mice mirror the slow progression of key symptoms and L-DOPA response in Parkinson's disease. Genes, brain, and behavior 9:173-181.

Gao C, Chen X, Li J, Li Y, Tang Y, Liu L, Chen S, Yu H, Liu L, Yao P (2014) Myocardial mitochondrial oxidative stress and dysfunction in intense exercise: regulatory effects of quercetin. European journal of applied physiology 114:695-705.

Gasser T, Muller-Myhsok B, Wszolek ZK, Oehlmann R, Calne DB, Bonifati V, Bereznai B, Fabrizio E, Vieregge P, Horstmann RD (1998) A susceptibility locus for Parkinson's disease maps to chromosome 2p13. Nature genetics 18:262-265.

Gaweda-Walerych K, Zekanowski C (2013) Integrated pathways of parkin control over mitochondrial maintenance - relevance to Parkinson's disease pathogenesis. Acta neurobiologiae experimentalis 73:199-224.

Gleeson M (2013) Nutritional support to maintain proper immune status during intense training. Nestle Nutrition Institute workshop series 75:85-97.

Goldman SM (2014) Environmental toxins and Parkinson's disease. Annual review of pharmacology and toxicology 54:141-164.

Gomez-Lazaro M, Bonekamp NA, Galindo MF, Jordan J, Schrader M (2008) 6-Hydroxydopamine (6-OHDA) induces Drp1-dependent mitochondrial fragmentation in SH-SY5Y cells. Free radical biology & medicine 44:1960-1969.

Graham TR, Burd CG (2011) Coordination of Golgi functions by phosphatidylinositol 4-kinases. Trends in cell biology 21:113-121.

Greggio E, Cookson MR (2009) Leucine-rich repeat kinase 2 mutations and Parkinson's disease: three questions. ASN neuro 1.

Gryglewski RJ, Korbut R, Robak J, Swies J (1987) On the mechanism of antithrombotic action of flavonoids. Biochemical pharmacology 36:317-322.

Gubellini P, Kachidian P (2015) Animal models of Parkinson's disease: An updated overview. Revue neurologique 171:750-761.

Guha S, Tanasanvimon S, Sinnett-Smith J, Rozengurt E (2010) Role of protein kinase D signaling in pancreatic cancer. Biochemical pharmacology 80:1946-1954.

Haas RH, Nasirian F, Nakano K, Ward D, Pay M, Hill R, Shults CW (1995) Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease. Annals of neurology 37:714-722.

Hajra L, Evans AI, Chen M, Hyduk SJ, Collins T, Cybulsky MI (2000) The NF-kappa B signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation. Proceedings of the National Academy of Sciences of the United States of America 97:9052-9057.

Haleagrahara N, Siew CJ, Mitra NK, Kumari M (2011) Neuroprotective effect of bioflavonoid quercetin in 6-hydroxydopamine-induced oxidative stress biomarkers in the rat striatum. Neuroscience letters 500:139-143.

Haleagrahara N, Siew CJ, Ponnusamy K (2013) Effect of quercetin and desferrioxamine on 6-hydroxydopamine (6-OHDA) induced neurotoxicity in striatum of rats. The Journal of toxicological sciences 38:25-33.

Han JJ, Hao J, Kim CH, Hong JS, Ahn HY, Lee YS (2009) Quercetin prevents cardiac hypertrophy induced by pressure overload in rats. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 71:737-743.

Harikumar KB, Kunnumakkara AB, Ochi N, Tong Z, Deorukhkar A, Sung B, Kelland L, Jamieson S, Sutherland R, Raynham T, Charles M, Bagherzadeh A, Foxton C, Boakes A, Farooq M, Maru D, Diagaradjane P, Matsuo Y, Sinnett-Smith J, Gelovani J, Krishnan S, Aggarwal BB, Rozengurt E, Ireson CR, Guha S (2010) A novel small-molecule inhibitor of protein kinase D blocks pancreatic cancer growth in vitro and in vivo. Molecular cancer therapeutics 9:1136-1146.

Harrison BC, Kim MS, van Rooij E, Plato CF, Papst PJ, Vega RB, McAnally JA, Richardson JA, Bassel-Duby R, Olson EN, McKinsey TA (2006) Regulation of cardiac stress signaling by protein kinase d1. Molecular and cellular biology 26:3875-3888.

Hastings TG (2009) The role of dopamine oxidation in mitochondrial dysfunction: implications for Parkinson's disease. Journal of bioenergetics and biomembranes 41:469-472.

Hatano T, Kubo S, Sato S, Hattori N (2009) Pathogenesis of familial Parkinson's disease: new insights based on monogenic forms of Parkinson's disease. Journal of neurochemistry 111:1075-1093.

Hattori N, Shimura H, Kubo S, Kitada T, Wang M, Asakawa S, Minashima S, Shimizu N, Suzuki T, Tanaka K, Mizuno Y (2000) Autosomal recessive juvenile parkinsonism: a key to understanding nigral degeneration in sporadic Parkinson's disease.

Neuropathology : official journal of the Japanese Society of Neuropathology 20 Suppl:S85-90.

Hausser A, Storz P, Martens S, Link G, Toker A, Pfizenmaier K (2005) Protein kinase D regulates vesicular transport by phosphorylating and activating phosphatidylinositol-4 kinase IIIbeta at the Golgi complex. Nature cell biology 7:880-886.

Haussermann S, Kittstein W, Rincke G, Johannes FJ, Marks F, Gschwendt M (1999) Proteolytic cleavage of protein kinase Cmu upon induction of apoptosis in U937 cells. FEBS letters 462:442-446.

Hayashi A, Seki N, Hattori A, Kozuma S, Saito T (1999) PKCnu, a new member of the protein kinase C family, composes a fourth subfamily with PKCmu. Biochimica et biophysica acta 1450:99-106.

He XZ, Wang X, Dixon RA (2006) Mutational analysis of the Medicago glycosyltransferase UGT71G1 reveals residues that control regioselectivity for (iso)flavonoid glycosylation. The Journal of biological chemistry 281:34441-34447.

Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342:1007-1011.

Hertog MG, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S, et al. (1995) Flavonoid intake and long-term risk of

coronary heart disease and cancer in the seven countries study. Archives of internal medicine 155:381-386.

Hou Y, Aboukhatwa MA, Lei DL, Manaye K, Khan I, Luo Y (2010) Anti-depressant natural flavonols modulate BDNF and beta amyloid in neurons and hippocampus of double TgAD mice. Neuropharmacology 58:911-920.

Hulihan MM, Ishihara-Paul L, Kachergus J, Warren L, Amouri R, Elango R, Prinjha RK, Upmanyu R, Kefi M, Zouari M, Sassi SB, Yahmed SB, El Euch-Fayeche G, Matthews PM, Middleton LT, Gibson RA, Hentati F, Farrer MJ (2008) LRRK2 Gly2019Ser penetrance in Arab-Berber patients from Tunisia: a case-control genetic study. Lancet neurology 7:591-594.

Hurd C, Rozengurt E (2001) Protein kinase D is sufficient to suppress EGF-induced c-Jun Ser 63 phosphorylation. Biochemical and biophysical research communications 282:404-408.

Huynh QK, McKinsey TA (2006) Protein kinase D directly phosphorylates histone deacetylase 5 via a random sequential kinetic mechanism. Archives of biochemistry and biophysics 450:141-148.

Ibanez P, Lesage S, Janin S, Lohmann E, Durif F, Destee A, Bonnet AM, Brefel-Courbon C, Heath S, Zelenika D, Agid Y, Durr A, Brice A, French Parkinson's Disease Genetics Study G (2009) Alpha-synuclein gene rearrangements in dominantly inherited parkinsonism: frequency, phenotype, and mechanisms. Archives of neurology 66:102-108.

Iglesias T, Rozengurt E (1998) Protein kinase D activation by mutations within its pleckstrin homology domain. The Journal of biological chemistry 273:410-416.

Iglesias T, Rozengurt E (1999) Protein kinase D activation by deletion of its cysteine-rich motifs. FEBS letters 454:53-56.

Islam MR, Zaman A, Jahan I, Chakravorty R, Chakraborty S (2013) In silico QSAR analysis of quercetin reveals its potential as therapeutic drug for Alzheimer's disease. J Young Pharm 5:173-179.

Ivison SM, Graham NR, Bernales CQ, Kifayet A, Ng N, Shobab LA, Steiner TS (2007) Protein kinase D interaction with TLR5 is required for inflammatory signaling in response to bacterial flagellin. Journal of immunology 178:5735-5743.

Iwao K, Tsukamoto I (1999) Quercetin inhibited DNA synthesis and induced apoptosis associated with increase in c-fos mRNA level and the upregulation of p21WAF1CIP1 mRNA and protein expression during liver regeneration after partial hepatectomy. Biochimica et biophysica acta 1427:112-120.

Jaggi M, Rao PS, Smith DJ, Hemstreet GP, Balaji KC (2003) Protein kinase C mu is down-regulated in androgen-independent prostate cancer. Biochemical and biophysical research communications 307:254-260.

Jaggi M, Rao PS, Smith DJ, Wheelock MJ, Johnson KR, Hemstreet GP, Balaji KC (2005) E-cadherin phosphorylation by protein kinase D1/protein kinase C{mu} is associated

with altered cellular aggregation and motility in prostate cancer. Cancer research 65:483-492.

Jain S, van Kesteren RE, Heutink P (2012) High content screening in neurodegenerative diseases. J Vis Exp e3452.

Jankovic J, Hunter C (2002) A double-blind, placebo-controlled and longitudinal study of riluzole in early Parkinson's disease. Parkinsonism & related disorders 8:271-276.

Javitch JA, D'Amato RJ, Strittmatter SM, Snyder SH (1985) Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6 -tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. Proceedings of the National Academy of Sciences of the United States of America 82:2173-2177.

Jenner P (2003) Oxidative stress in Parkinson's disease. Annals of neurology 53 Suppl 3:S26-36; discussion S36-28.

Jimenez R, Lopez-Sepulveda R, Romero M, Toral M, Cogolludo A, Perez-Vizcaino F, Duarte J (2015) Quercetin and its metabolites inhibit the membrane NADPH oxidase activity in vascular smooth muscle cells from normotensive and spontaneously hypertensive rats. Food & function 6:409-414.

Johannes FJ, Prestle J, Eis S, Oberhagemann P, Pfizenmaier K (1994) PKCu is a novel, atypical member of the protein kinase C family. The Journal of biological chemistry 269:6140-6148.

Jornayvaz FR, Shulman GI (2010) Regulation of mitochondrial biogenesis. Essays in biochemistry 47:69-84.

Jung M, Bu SY, Tak KH, Park JE, Kim E (2013) Anticarcinogenic effect of quercetin by inhibition of insulin-like growth factor (IGF)-1 signaling in mouse skin cancer. Nutrition research and practice 7:439-445.

Kamaraj S, Vinodhkumar R, Anandakumar P, Jagan S, Ramakrishnan G, Devaki T (2007) The effects of quercetin on antioxidant status and tumor markers in the lung and serum of mice treated with benzo(a)pyrene. Biological & pharmaceutical bulletin 30:2268-2273.

Kanthasamy A, Jin H, Mehrotra S, Mishra R, Kanthasamy A, Rana A (2010) Novel cell death signaling pathways in neurotoxicity models of dopaminergic degeneration: relevance to oxidative stress and neuroinflammation in Parkinson's disease. Neurotoxicology 31:555-561.

Kanthasamy AG, Anantharam V, Zhang D, Latchoumycandane C, Jin H, Kaul S, Kanthasamy A (2006) A novel peptide inhibitor targeted to caspase-3 cleavage site of a proapoptotic kinase protein kinase C delta (PKCdelta) protects against dopaminergic neuronal degeneration in Parkinson's disease models. Free radical biology & medicine 41:1578-1589.

Karuppagounder SS, Madathil SK, Pandey M, Haobam R, Rajamma U, Mohanakumar KP (2013) Quercetin up-regulates mitochondrial complex-I activity to protect against programmed cell death in rotenone model of Parkinson's disease in rats. Neuroscience 236:136-148.

Kaul S, Kanthasamy A, Kitazawa M, Anantharam V, Kanthasamy AG (2003) Caspase-3 dependent proteolytic activation of protein kinase C delta mediates and regulates 1-methyl-4-phenylpyridinium (MPP+)-induced apoptotic cell death in dopaminergic cells: relevance to oxidative stress in dopaminergic degeneration. The European journal of neuroscience 18:1387-1401.

Keeney PM, Xie J, Capaldi RA, Bennett JP, Jr. (2006) Parkinson's disease brain mitochondrial complex I has oxidatively damaged subunits and is functionally impaired and misassembled. J Neurosci 26:5256-5264.

Kelley BJ, Duker AP, Chiu P (2012) Dopamine agonists and pathologic behaviors. Parkinson's disease 2012:603631.

Kelly DP, Scarpulla RC (2004) Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes & development 18:357-368.

Kim M, Jang HR, Kim JH, Noh SM, Song KS, Cho JS, Jeong HY, Norman JC, Caswell PT, Kang GH, Kim SY, Yoo HS, Kim YS (2008) Epigenetic inactivation of protein kinase D1 in gastric cancer and its role in gastric cancer cell migration and invasion. Carcinogenesis 29:629-637.

Kim YH, Lee YJ (2007) TRAIL apoptosis is enhanced by quercetin through Akt dephosphorylation. Journal of cellular biochemistry 100:998-1009.

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 392:605-608.

Kitazawa M, Anantharam V, Kanthasamy AG (2003) Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase Cdelta in dopaminergic cells: relevance to oxidative stress and dopaminergic degeneration. Neuroscience 119:945-964.

Klein C, Schlossmacher MG (2007) Parkinson disease, 10 years after its genetic revolution: multiple clues to a complex disorder. Neurology 69:2093-2104.

Knekt P, Kumpulainen J, Jarvinen R, Rissanen H, Heliovaara M, Reunanen A, Hakulinen T, Aromaa A (2002) Flavonoid intake and risk of chronic diseases. The American journal of clinical nutrition 76:560-568.

Konishi H, Yamauchi E, Taniguchi H, Yamamoto T, Matsuzaki H, Takemura Y, Ohmae K, Kikkawa U, Nishizuka Y (2001) Phosphorylation sites of protein kinase C delta in H2O2-treated cells and its activation by tyrosine kinase in vitro. Proceedings of the National Academy of Sciences of the United States of America 98:6587-6592.

Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Riess O (1998) Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nature genetics 18:106-108.

Kumazawa R, Tomiyama H, Li Y, Imamichi Y, Funayama M, Yoshino H, Yokochi F, Fukusako T, Takehisa Y, Kashihara K, Kondo T, Elibol B, Bostantjopoulou S, Toda T, Takahashi H, Yoshii F, Mizuno Y, Hattori N (2008) Mutation analysis of the PINK1 gene in 391 patients with Parkinson disease. Archives of neurology 65:802-808.

Kupsch A, Sautter J, Gotz ME, Breithaupt W, Schwarz J, Youdim MB, Riederer P, Gerlach M, Oertel WH (2001) Monoamine oxidase-inhibition and MPTP-induced neurotoxicity in the non-human primate: comparison of rasagiline (TVP 1012) with selegiline. J Neural Transm (Vienna) 108:985-1009.

Lai BC, Marion SA, Teschke K, Tsui JK (2002) Occupational and environmental risk factors for Parkinson's disease. Parkinsonism & related disorders 8:297-309.

Laird DW, Fistouris P, Batist G, Alpert L, Huynh HT, Carystinos GD, Alaoui-Jamali MA (1999) Deficiency of connexin43 gap junctions is an independent marker for breast tumors. Cancer research 59:4104-4110.

Lamson DW, Brignall MS (2000) Antioxidants and cancer, part 3: quercetin. Altern Med Rev 5:196-208.

Langston JW, Ballard P, Tetrud JW, Irwin I (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science (New York, NY 219:979-980.

Langston JW, Irwin I, Langston EB, Forno LS (1984) 1-Methyl-4-phenylpyridinium ion (MPP+): identification of a metabolite of MPTP, a toxin selective to the substantia nigra. Neuroscience letters 48:87-92.

Larson AJ, Symons JD, Jalili T (2012) Therapeutic potential of quercetin to decrease blood pressure: review of efficacy and mechanisms. Advances in nutrition (Bethesda, Md 3:39-46.

LaValle CR, George KM, Sharlow ER, Lazo JS, Wipf P, Wang QJ (2010) Protein kinase D as a potential new target for cancer therapy. Biochimica et biophysica acta 1806:183-192.

Lee DH, Szczepanski M, Lee YJ (2008) Role of Bax in quercetin-induced apoptosis in human prostate cancer cells. Biochemical pharmacology 75:2345-2355.

Lee JM, Johnson JA (2004) An important role of Nrf2-ARE pathway in the cellular defense mechanism. Journal of biochemistry and molecular biology 37:139-143.

Lee LT, Huang YT, Hwang JJ, Lee AY, Ke FC, Huang CJ, Kandaswami C, Lee PP, Lee MT (2004) Transinactivation of the epidermal growth factor receptor tyrosine kinase and focal adhesion kinase phosphorylation by dietary flavonoids: effect on invasive potential of human carcinoma cells. Biochemical pharmacology 67:2103-2114.

Lee YK, Park SY, Kim YM, Lee WS, Park OJ (2009) AMP kinase/cyclooxygenase-2 pathway regulates proliferation and apoptosis of cancer cells treated with quercetin. Experimental & molecular medicine 41:201-207.

Lees AJ, Hardy J, Revesz T (2009) Parkinson's disease. Lancet 373:2055-2066.

Lees AJ, Ratziu V, Tolosa E, Oertel WH (2007) Safety and tolerability of adjunctive tolcapone treatment in patients with early Parkinson's disease. Journal of neurology, neurosurgery, and psychiatry 78:944-948.

Leibersperger H, Gschwendt M, Gernold M, Marks F (1991) Immunological demonstration of a calcium-unresponsive protein kinase C of the delta-type in different species and murine tissues. Predominance in epidermis. The Journal of biological chemistry 266:14778-14784.

Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, Harta G, Brownstein MJ, Jonnalagada S, Chernova T, Dehejia A, Lavedan C, Gasser T, Steinbach PJ, Wilkinson KD, Polymeropoulos MH (1998) The ubiquitin pathway in Parkinson's disease. Nature 395:451-452.

Levy OA, Malagelada C, Greene LA (2009) Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis 14:478-500.

Lewitt PA (2008) Levodopa for the treatment of Parkinson's disease. The New England journal of medicine 359:2468-2476.

Li J, Benashski SE, Venna VR, McCullough LD (2010) Effects of metformin in experimental stroke. Stroke; a journal of cerebral circulation 41:2645-2652.

Liang H, Ward WF (2006) PGC-1alpha: a key regulator of energy metabolism. Advances in physiology education 30:145-151.

Lim JH, Park JW, Min DS, Chang JS, Lee YH, Park YB, Choi KS, Kwon TK (2007) NAG-1 up-regulation mediated by EGR-1 and p53 is critical for quercetin-induced apoptosis in HCT116 colon carcinoma cells. Apoptosis 12:411-421.

Lucking CB, Durr A, Bonifati V, Vaughan J, De Michele G, Gasser T, Harhangi BS, Meco G, Denefle P, Wood NW, Agid Y, Brice A, French Parkinson's Disease Genetics Study G, European Consortium on Genetic Susceptibility in Parkinson's D (2000) Association between early-onset Parkinson's disease and mutations in the parkin gene. The New England journal of medicine 342:1560-1567.

Lutz AK, Exner N, Fett ME, Schlehe JS, Kloos K, Lammermann K, Brunner B, Kurz-Drexler A, Vogel F, Reichert AS, Bouman L, Vogt-Weisenhorn D, Wurst W, Tatzelt J, Haass C, Winklhofer KF (2009) Loss of parkin or PINK1 function increases Drp1-dependent mitochondrial fragmentation. The Journal of biological chemistry 284:22938-22951.

Lv C, Hong T, Yang Z, Zhang Y, Wang L, Dong M, Zhao J, Mu J, Meng Y (2012) Effect of Quercetin in the 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-Induced Mouse Model of Parkinson's Disease. Evid Based Complement Alternat Med 2012:928643.

Maciejczyk A, Surowiak P (2013) Quercetin inhibits proliferation and increases sensitivity of ovarian cancer cells to cisplatin and paclitaxel. Ginekologia polska 84:590-595.

Maeda Y, Beznoussenko GV, Van Lint J, Mironov AA, Malhotra V (2001) Recruitment of protein kinase D to the trans-Golgi network via the first cysteine-rich domain. The EMBO journal 20:5982-5990.

Magalingam KB, Radhakrishnan A, Haleagrahara N (2014) Protective effects of flavonol isoquercitrin, against 6-hydroxy dopamine (6-OHDA)-induced toxicity in PC12 cells. BMC research notes 7:49.

Mak P, Jaggi M, Syed V, Chauhan SC, Hassan S, Biswas H, Balaji KC (2008) Protein kinase D1 (PKD1) influences androgen receptor (AR) function in prostate cancer cells. Biochemical and biophysical research communications 373:618-623.

Malgieri G, Eliezer D (2008) Structural effects of Parkinson's disease linked DJ-1 mutations. Protein science : a publication of the Protein Society 17:855-868.

Mandel S, Weinreb O, Amit T, Youdim MB (2005) Mechanism of neuroprotective action of the anti-Parkinson drug rasagiline and its derivatives. Brain research Brain research reviews 48:379-387.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. Science (New York, NY 298:1912-1934.

Marklund U, Lightfoot K, Cantrell D (2003) Intracellular location and cell context-dependent function of protein kinase D. Immunity 19:491-501.

Martin-Montalvo A, Mercken EM, Mitchell SJ, Palacios HH, Mote PL, Scheibye-Knudsen M, Gomes AP, Ward TM, Minor RK, Blouin MJ, Schwab M, Pollak M, Zhang Y, Yu Y, Becker KG, Bohr VA, Ingram DK, Sinclair DA, Wolf NS, Spindler SR, Bernier M, de Cabo R (2013) Metformin improves healthspan and lifespan in mice. Nature communications 4:2192.

Matthews RT, Ferrante RJ, Klivenyi P, Yang L, Klein AM, Mueller G, Kaddurah-Daouk R, Beal MF (1999) Creatine and cyclocreatine attenuate MPTP neurotoxicity. Experimental neurology 157:142-149.

McCoy MK, Cookson MR (2011) DJ-1 regulation of mitochondrial function and autophagy through oxidative stress. Autophagy 7:531-532.

McKinsey TA, Zhang CL, Lu J, Olson EN (2000) Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature 408:106-111.

Medeiros RB, Dickey DM, Chung H, Quale AC, Nagarajan LR, Billadeau DD, Shimizu Y (2005) Protein kinase D1 and the beta 1 integrin cytoplasmic domain control beta 1 integrin function via regulation of Rap1 activation. Immunity 23:213-226.

Miean KH, Mohamed S (2001) Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. Journal of agricultural and food chemistry 49:3106-3112.

Miller RL, James-Kracke M, Sun GY, Sun AY (2009) Oxidative and inflammatory pathways in Parkinson's disease. Neurochemical research 34:55-65.

Mizuno Y, Ohta S, Tanaka M, Takamiya S, Suzuki K, Sato T, Oya H, Ozawa T, Kagawa Y (1989) Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. Biochemical and biophysical research communications 163:1450-1455.

Moon SK, Cho GO, Jung SY, Gal SW, Kwon TK, Lee YC, Madamanchi NR, Kim CH (2003) Quercetin exerts multiple inhibitory effects on vascular smooth muscle cells: role of ERK1/2, cell-cycle regulation, and matrix metalloproteinase-9. Biochemical and biophysical research communications 301:1069-1078.

Moon Y, Lee KH, Park JH, Geum D, Kim K (2005) Mitochondrial membrane depolarization and the selective death of dopaminergic neurons by rotenone: protective effect of coenzyme Q10. Journal of neurochemistry 93:1199-1208.

Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nature genetics 34:267-273.

Morris JK, Bomhoff GL, Stanford JA, Geiger PC (2010) Neurodegeneration in an animal model of Parkinson's disease is exacerbated by a high-fat diet. American journal of physiology 299:R1082-1090.

Mount MP, Zhang Y, Amini M, Callaghan S, Kulczycki J, Mao Z, Slack RS, Anisman H, Park DS (2013) Perturbation of transcription factor Nur77 expression mediated by myocyte enhancer factor 2D (MEF2D) regulates dopaminergic neuron loss in response to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The Journal of biological chemistry 288:14362-14371.

Mukaetova-Ladinska EB, Hurt J, Jakes R, Xuereb J, Honer WG, Wischik CM (2000) Alpha-synuclein inclusions in Alzheimer and Lewy body diseases. Journal of neuropathology and experimental neurology 59:408-417.

Muller T, Investigators TS (2014) Tolcapone addition improves Parkinson's disease associated nonmotor symptoms. Therapeutic advances in neurological disorders 7:77-82.

Murphy TR, Legere HJ, 3rd, Katz HR (2007) Activation of protein kinase D1 in mast cells in response to innate, adaptive, and growth factor signals. Journal of immunology 179:7876-7882.

Narendra D, Tanaka A, Suen DF, Youle RJ (2008) Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. The Journal of cell biology 183:795-803.

Ngo HB, Kaiser JT, Chan DC (2011) The mitochondrial transcription and packaging factor Tfam imposes a U-turn on mitochondrial DNA. Nature structural & molecular biology 18:1290-1296.

Nguyen TT, Tran E, Nguyen TH, Do PT, Huynh TH, Huynh H (2004) The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. Carcinogenesis 25:647-659.

Nieman DC, Williams AS, Shanely RA, Jin F, McAnulty SR, Triplett NT, Austin MD, Henson DA (2010) Quercetin's influence on exercise performance and muscle mitochondrial biogenesis. Medicine and science in sports and exercise 42:338-345.

Nutt JG, Burchiel KJ, Comella CL, Jankovic J, Lang AE, Laws ER, Jr., Lozano AM, Penn RD, Simpson RK, Jr., Stacy M, Wooten GF, factor IGSGIiGcl-dn (2003) Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. Neurology 60:69-73.

Nuytemans K, Theuns J, Cruts M, Van Broeckhoven C (2010) Genetic etiology of Parkinson disease associated with mutations in the SNCA, PARK2, PINK1, PARK7, and LRRK2 genes: a mutation update. Human mutation 31:763-780.

Obeso JA, Rodriguez-Oroz MC, Goetz CG, Marin C, Kordower JH, Rodriguez M, Hirsch EC, Farrer M, Schapira AH, Halliday G (2010) Missing pieces in the Parkinson's disease puzzle. Nature medicine 16:653-661.

Obinu MC, Reibaud M, Blanchard V, Moussaoui S, Imperato A (2002) Neuroprotective effect of riluzole in a primate model of Parkinson's disease: behavioral and histological evidence. Mov Disord 17:13-19.

Onyango IG (2008) Mitochondrial dysfunction and oxidative stress in Parkinson's disease. Neurochemical research 33:589-597.

Orr-Urtreger A, Shifrin C, Rozovski U, Rosner S, Bercovich D, Gurevich T, Yagev-More H, Bar-Shira A, Giladi N (2007) The LRRK2 G2019S mutation in Ashkenazi Jews with Parkinson disease: is there a gender effect? Neurology 69:1595-1602.

Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G, Goldberg DM (1995) The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. Clinica chimica acta; international journal of clinical chemistry 235:207-219.

Paisan-Ruiz C, Bhatia KP, Li A, Hernandez D, Davis M, Wood NW, Hardy J, Houlden H, Singleton A, Schneider SA (2009) Characterization of PLA2G6 as a locus for dystonia-parkinsonism. Annals of neurology 65:19-23.

Paisan-Ruiz C, Jain S, Evans EW, Gilks WP, Simon J, van der Brug M, Lopez de Munain A, Aparicio S, Gil AM, Khan N, Johnson J, Martinez JR, Nicholl D, Carrera IM, Pena AS, de Silva R, Lees A, Marti-Masso JF, Perez-Tur J, Wood NW, Singleton AB (2004) Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron 44:595-600.

Park CH, Chang JY, Hahm ER, Park S, Kim HK, Yang CH (2005) Quercetin, a potent inhibitor against beta-catenin/Tcf signaling in SW480 colon cancer cells. Biochemical and biophysical research communications 328:227-234.

Park JE, Kim YI, Yi AK (2008) Protein kinase D1: a new component in TLR9 signaling. Journal of immunology 181:2044-2055.

Parkinson Study G (2002) A controlled trial of rasagiline in early Parkinson disease: the TEMPO Study. Archives of neurology 59:1937-1943.

Parkinson Study G (2005) A randomized placebo-controlled trial of rasagiline in levodopa-treated patients with Parkinson disease and motor fluctuations: the PRESTO study. Archives of neurology 62:241-248.

Parkinson Study Group QEI, Beal MF, Oakes D, Shoulson I, Henchcliffe C, Galpern WR, Haas R, Juncos JL, Nutt JG, Voss TS, Ravina B, Shults CM, Helles K, Snively V, Lew

MF, Griebner B, Watts A, Gao S, Pourcher E, Bond L, Kompoliti K, Agarwal P, Sia C, Jog M, Cole L, Sultana M, Kurlan R, Richard I, Deeley C, Waters CH, Figueroa A, Arkun A, Brodsky M, Ondo WG, Hunter CB, Jimenez-Shahed J, Palao A, Miyasaki JM, So J, Tetrud J, Reys L, Smith K, Singer C, Blenke A, Russell DS, Cotto C, Friedman JH, Lannon M, Zhang L, Drasby E, Kumar R, Subramanian T, Ford DS, Grimes DA, Cote D, Conway J, Siderowf AD, Evatt ML, Sommerfeld B, Lieberman AN, Okun MS, Rodriguez RL, Merritt S, Swartz CL, Martin WR, King P, Stover N, Guthrie S, Watts RL, Ahmed A, Fernandez HH, Winters A, Mari Z, Dawson TM, Dunlop B, Feigin AS, Shannon B, Nirenberg MJ, Ogg M, Ellias SA, Thomas CA, Frei K, Bodis-Wollner I, Glazman S, Mayer T, Hauser RA, Pahwa R, Langhammer A, Ranawaya R, Derwent L, Sethi KD, Farrow B, Prakash R, Litvan I, Robinson A, Sahay A, Gartner M, Hinson VK, Markind S, Pelikan M, Perlmutter JS, Hartlein J, Molho E, Evans S, Adler CH, Duffy A, Lind M, Elmer L, Davis K, Spears J, Wilson S, Leehey MA, Hermanowicz N, Niswonger S, Shill HA, Obradov S, Rajput A, Cowper M, Lessig S, Song D, Fontaine D, Zadikoff C, Williams K, Blindauer KA, Bergholte J, Propsom CS, Stacy MA, Field J, Mihaila D, Chilton M, Uc EY, Sieren J, Simon DK, Kraics L, Silver A, Boyd JT, Hamill RW, Ingvoldstad C, Young J, Thomas K, Kostyk SK, Wojcieszek J, Pfeiffer RF, Panisset M, Beland M, Reich SG, Cines M, Zappala N, Rivest J, Zweig R, Lumina LP, Hilliard CL, Grill S, Kellermann M, Tuite P, Rolandelli S, Kang UJ, Young J, Rao J, Cook MM, Severt L, Boyar K (2014) A randomized clinical trial of high-dosage coenzyme Q10 in early Parkinson disease: no evidence of benefit. JAMA neurology 71:543-552.

Patel NK, Gill SS (2007) GDNF delivery for Parkinson's disease. Acta neurochirurgica Supplement 97:135-154.

Patil SP, Jain PD, Ghumatkar PJ, Tambe R, Sathaye S (2014) Neuroprotective effect of metformin in MPTP-induced Parkinson's disease in mice. Neuroscience 277:747-754.

Perier C, Vila M (2012) Mitochondrial biology and Parkinson's disease. Cold Spring Harbor perspectives in medicine 2:a009332.

Peterburs P, Heering J, Link G, Pfizenmaier K, Olayioye MA, Hausser A (2009) Protein kinase D regulates cell migration by direct phosphorylation of the cofilin phosphatase slingshot 1 like. Cancer research 69:5634-5638.

Petit A, Kawarai T, Paitel E, Sanjo N, Maj M, Scheid M, Chen F, Gu Y, Hasegawa H, Salehi-Rad S, Wang L, Rogaeva E, Fraser P, Robinson B, St George-Hyslop P, Tandon A (2005) Wild-type PINK1 prevents basal and induced neuronal apoptosis, a protective effect abrogated by Parkinson disease-related mutations. The Journal of biological chemistry 280:34025-34032.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science (New York, NY 276:2045-2047.

Popovic D, Vucic D, Dikic I (2014) Ubiquitination in disease pathogenesis and treatment. Nature medicine 20:1242-1253.

Przedborski S, Vila M (2003) The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model: a tool to explore the pathogenesis of Parkinson's disease. Annals of the New York Academy of Sciences 991:189-198.

Psahoulia FH, Moumtzi S, Roberts ML, Sasazuki T, Shirasawa S, Pintzas A (2007) Quercetin mediates preferential degradation of oncogenic Ras and causes autophagy in Ha-RAS-transformed human colon cells. Carcinogenesis 28:1021-1031.

Ramachandran L, Manu KA, Shanmugam MK, Li F, Siveen KS, Vali S, Kapoor S, Abbasi T, Surana R, Smoot DT, Ashktorab H, Tan P, Ahn KS, Yap CW, Kumar AP, Sethi G (2013) Isorhamnetin inhibits proliferation and invasion and induces apoptosis through the modulation of peroxisome proliferator-activated receptor gamma activation pathway in gastric cancer. The Journal of biological chemistry 287:38028-38040.

Ramirez A, Heimbach A, Grundemann J, Stiller B, Hampshire D, Cid LP, Goebel I, Mubaidin AF, Wriekat AL, Roeper J, Al-Din A, Hillmer AM, Karsak M, Liss B, Woods CG, Behrens MI, Kubisch C (2006) Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. Nature genetics 38:1184-1191.

Ramos FA, Takaishi Y, Shirotori M, Kawaguchi Y, Tsuchiya K, Shibata H, Higuti T, Tadokoro T, Takeuchi M (2006) Antibacterial and antioxidant activities of quercetin oxidation

products from yellow onion (Allium cepa) skin. Journal of agricultural and food chemistry 54:3551-3557.

Ramsey CP, Glass CA, Montgomery MB, Lindl KA, Ritson GP, Chia LA, Hamilton RL, Chu CT, Jordan-Sciutto KL (2007) Expression of Nrf2 in neurodegenerative diseases. Journal of neuropathology and experimental neurology 66:75-85.

Rappold PM, Cui M, Grima JC, Fan RZ, de Mesy-Bentley KL, Chen L, Zhuang X, Bowers WJ, Tieu K (2014) Drp1 inhibition attenuates neurotoxicity and dopamine release deficits in vivo. Nature communications 5:5244.

Rascol O, Brooks DJ, Melamed E, Oertel W, Poewe W, Stocchi F, Tolosa E, group Ls (2005) Rasagiline as an adjunct to levodopa in patients with Parkinson's disease and motor fluctuations (LARGO, Lasting effect in Adjunct therapy with Rasagiline Given Once daily, study): a randomised, double-blind, parallel-group trial. Lancet 365:947-954.

Rey O, Sinnett-Smith J, Zhukova E, Rozengurt E (2001) Regulated nucleocytoplasmic transport of protein kinase D in response to G protein-coupled receptor activation. The Journal of biological chemistry 276:49228-49235.

Ristich VL, Bowman PH, Dodd ME, Bollag WB (2006) Protein kinase D distribution in normal human epidermis, basal cell carcinoma and psoriasis. The British journal of dermatology 154:586-593.

Rohas LM, St-Pierre J, Uldry M, Jager S, Handschin C, Spiegelman BM (2007) A fundamental system of cellular energy homeostasis regulated by PGC-1alpha. Proceedings of the National Academy of Sciences of the United States of America 104:7933-7938.

Rothfuss O, Fischer H, Hasegawa T, Maisel M, Leitner P, Miesel F, Sharma M, Bornemann A, Berg D, Gasser T, Patenge N (2009) Parkin protects mitochondrial genome integrity and supports mitochondrial DNA repair. Human molecular genetics 18:3832-3850.

Rowe GC, Jiang A, Arany Z (2010) PGC-1 coactivators in cardiac development and disease. Circulation research 107:825-838.

Rozengurt E (2011) Protein kinase D signaling: multiple biological functions in health and disease. Physiology 26:23-33.

Rozengurt E, Rey O, Waldron RT (2005) Protein kinase D signaling. The Journal of biological chemistry 280:13205-13208.

Rozengurt E, Sinnett-Smith J, Van Lint J, Valverde AM (1995) Protein kinase D (PKD): a novel target for diacylglycerol and phorbol esters. Mutation research 333:153-160.

Russo M, Palumbo R, Tedesco I, Mazzarella G, Russo P, Iacomino G, Russo GL (1999) Quercetin and anti-CD95(Fas/Apo1) enhance apoptosis in HPB-ALL cell line. FEBS letters 462:322-328.

Russo M, Spagnuolo C, Tedesco I, Bilotto S, Russo GL (2012) The flavonoid quercetin in disease prevention and therapy: facts and fancies. Biochemical pharmacology 83:6-15.

Rykx A, De Kimpe L, Mikhalap S, Vantus T, Seufferlein T, Vandenheede JR, Van Lint J (2003) Protein kinase D: a family affair. FEBS letters 546:81-86.

Sabogal-Guaqueta AM, Munoz-Manco JI, Ramirez-Pineda JR, Lamprea-Rodriguez M, Osorio E, Cardona-Gomez GP (2015) The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice. Neuropharmacology 93C:134-145.

Said Ahmed M, Hung WY, Zu JS, Hockberger P, Siddique T (2000) Increased reactive oxygen species in familial amyotrophic lateral sclerosis with mutations in SOD1. Journal of the neurological sciences 176:88-94.

Saito Y, Miyasaka T, Hatsuta H, Takahashi-Niki K, Hayashi K, Mita Y, Kusano-Arai O, Iwanari H, Ariga H, Hamakubo T, Yoshida Y, Niki E, Murayama S, Ihara Y, Noguchi N (2014) Immunostaining of oxidized DJ-1 in human and mouse brains. Journal of neuropathology and experimental neurology 73:714-728.

Saleem TS, Basha SD (2010) Red wine: A drink to your heart. Journal of cardiovascular disease research 1:171-176.

Sanchez-Ruiloba L, Aicart-Ramos C, Garcia-Guerra L, Pose-Utrilla J, Rodriguez-Crespo I, Iglesias T (2014) Protein kinase D interacts with neuronal nitric oxide synthase and phosphorylates the activatory residue serine 1412. PloS one 9:e95191.

Sanchez-Ruiloba L, Cabrera-Poch N, Rodriguez-Martinez M, Lopez-Menendez C, Jean-Mairet RM, Higuero AM, Iglesias T (2006) Protein kinase D intracellular localization

and activity control kinase D-interacting substrate of 220-kDa traffic through a postsynaptic density-95/discs large/zonula occludens-1-binding motif. The Journal of biological chemistry 281:18888-18900.

Sandhir R, Mehrotra A (2013) Quercetin supplementation is effective in improving mitochondrial dysfunctions induced by 3-nitropropionic acid: implications in Huntington's disease. Biochimica et biophysica acta 1832:421-430.

Sandyk R (1993) The relationship between diabetes mellitus and Parkinson's disease. The International journal of neuroscience 69:125-130.

Santiago JA, Potashkin JA (2013a) Integrative network analysis unveils convergent molecular pathways in Parkinson's disease and diabetes. PloS one 8:e83940.

Santiago JA, Potashkin JA (2013b) Shared dysregulated pathways lead to Parkinson's disease and diabetes. Trends in molecular medicine 19:176-186.

Santiago JA, Potashkin JA (2014) System-based approaches to decode the molecular links in Parkinson's disease and diabetes. Neurobiology of disease.

Scarpulla RC (2008) Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiological reviews 88:611-638.

Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD (1990) Mitochondrial complex I deficiency in Parkinson's disease. Journal of neurochemistry 54:823-827.

Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD (1989) Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1:1269.

Schapira AH, Jenner P (2011) Etiology and pathogenesis of Parkinson's disease. Mov Disord 26:1049-1055.

Scheele C, Nielsen AR, Walden TB, Sewell DA, Fischer CP, Brogan RJ, Petrovic N, Larsson O, Tesch PA, Wennmalm K, Hutchinson DS, Cannon B, Wahlestedt C, Pedersen BK, Timmons JA (2007) Altered regulation of the PINK1 locus: a link between type 2 diabetes and neurodegeneration? Faseb J 21:3653-3665.

Schober A, Peterziel H, von Bartheld CS, Simon H, Krieglstein K, Unsicker K (2007) GDNF applied to the MPTP-lesioned nigrostriatal system requires TGF-beta for its neuroprotective action. Neurobiology of disease 25:378-391.

Sengupta A, Ghosh S, Das S (2001) Modulation of DMBA induced genotoxicity in bone marrow by quercetin during skin carcinogenesis. J Exp Clin Cancer Res 20:131-134.

Senthilkumar K, Arunkumar R, Elumalai P, Sharmila G, Gunadharini DN, Banudevi S, Krishnamoorthy G, Benson CS, Arunakaran J (2011) Quercetin inhibits invasion, migration and signalling molecules involved in cell survival and proliferation of prostate cancer cell line (PC-3). Cell biochemistry and function 29:87-95.

Shan BE, Wang MX, Li RQ (2009) Quercetin inhibit human SW480 colon cancer growth in association with inhibition of cyclin D1 and survivin expression through Wnt/beta-catenin signaling pathway. Cancer investigation 27:604-612.

Sharma H, Sen S, Singh N (2005) Molecular pathways in the chemosensitization of cisplatin by quercetin in human head and neck cancer. Cancer biology & therapy 4:949-955.

Sharmila G, Bhat FA, Arunkumar R, Elumalai P, Raja Singh P, Senthilkumar K, Arunakaran J (2014) Chemopreventive effect of quercetin, a natural dietary flavonoid on prostate cancer in in vivo model. Clinical nutrition (Edinburgh, Scotland) 33:718-726.

She H, Yang Q, Shepherd K, Smith Y, Miller G, Testa C, Mao Z (2011) Direct regulation of complex I by mitochondrial MEF2D is disrupted in a mouse model of Parkinson disease and in human patients. The Journal of clinical investigation 121:930-940.

Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, Shimizu N, Iwai K, Chiba T, Tanaka K, Suzuki T (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nature genetics 25:302-305.

Shulman JM, De Jager PL, Feany MB (2011) Parkinson's disease: genetics and pathogenesis. Annual review of pathology 6:193-222.

Shults CW, Oakes D, Kieburtz K, Beal MF, Haas R, Plumb S, Juncos JL, Nutt J, Shoulson I, Carter J, Kompoliti K, Perlmutter JS, Reich S, Stern M, Watts RL, Kurlan R, Molho E, Harrison M, Lew M, Parkinson Study G (2002) Effects of coenzyme Q10 in early Parkinson disease: evidence of slowing of the functional decline. Archives of neurology 59:1541-1550.

Sian J, Dexter DT, Lees AJ, Daniel S, Agid Y, Javoy-Agid F, Jenner P, Marsden CD (1994) Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. Annals of neurology 36:348-355.

Sies H (1993) Strategies of antioxidant defense. European journal of biochemistry / FEBS 215:213-219.

Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D, Adler C, Cookson MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K (2003) alpha-Synuclein locus triplication causes Parkinson's disease. Science (New York, NY 302:841.

Sinnett-Smith J, Zhukova E, Hsieh N, Jiang X, Rozengurt E (2004) Protein kinase D potentiates DNA synthesis induced by Gq-coupled receptors by increasing the duration of ERK signaling in swiss 3T3 cells. The Journal of biological chemistry 279:16883-16893.

Somayajulu M, McCarthy S, Hung M, Sikorska M, Borowy-Borowski H, Pandey S (2005) Role of mitochondria in neuronal cell death induced by oxidative stress; neuroprotection by Coenzyme Q10. Neurobiology of disease 18:618-627.

Spagnuolo C, Cerella C, Russo M, Chateauvieux S, Diederich M, Russo GL (2011) Quercetin downregulates Mcl-1 by acting on mRNA stability and protein degradation. British journal of cancer 105:221-230.

Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M (1998) alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proceedings of the National Academy of Sciences of the United States of America 95:6469-6473.

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997) Alpha-synuclein in Lewy bodies. Nature 388:839-840.

Sriraksa N, Wattanathorn J, Muchimapura S, Tiamkao S, Brown K, Chaisiwamongkol K (2012) Cognitive-enhancing effect of quercetin in a rat model of Parkinson's disease induced by 6-hydroxydopamine. Evid Based Complement Alternat Med 2012:823206.

Steinberg SF (2012) Regulation of protein kinase D1 activity. Molecular pharmacology 81:284-291.

Storz P (2007) Mitochondrial ROS--radical detoxification, mediated by protein kinase D. Trends in cell biology 17:13-18.

Storz P, Doppler H, Toker A (2005) Protein kinase D mediates mitochondrion-to-nucleus signaling and detoxification from mitochondrial reactive oxygen species. Molecular and cellular biology 25:8520-8530.

Storz P, Toker A (2003) Protein kinase D mediates a stress-induced NF-kappaB activation and survival pathway. The EMBO journal 22:109-120.

Strauss KM, Martins LM, Plun-Favreau H, Marx FP, Kautzmann S, Berg D, Gasser T, Wszolek Z, Muller T, Bornemann A, Wolburg H, Downward J, Riess O, Schulz JB,

Kruger R (2005) Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. Human molecular genetics 14:2099-2111.

Sturany S, Van Lint J, Muller F, Wilda M, Hameister H, Hocker M, Brey A, Gern U, Vandenheede J, Gress T, Adler G, Seufferlein T (2001) Molecular cloning and characterization of the human protein kinase D2. A novel member of the protein kinase D family of serine threonine kinases. The Journal of biological chemistry 276:3310-3318.

Subramaniam SR, Chesselet MF (2013) Mitochondrial dysfunction and oxidative stress in Parkinson's disease. Progress in neurobiology 106-107:17-32.

Suchowersky O, Bailey P, Pourcher E, Bulger L, Facciponte G (2001) Comparison of two dosages of tolcapone added to levodopa in nonfluctuating patients with PD. Clinical neuropharmacology 24:214-220.

Suen DF, Norris KL, Youle RJ (2008) Mitochondrial dynamics and apoptosis. Genes & development 22:1577-1590.

Sumara G, Formentini I, Collins S, Sumara I, Windak R, Bodenmiller B, Ramracheya R, Caille D, Jiang H, Platt KA, Meda P, Aebersold R, Rorsman P, Ricci R (2009) Regulation of PKD by the MAPK p38delta in insulin secretion and glucose homeostasis. Cell 136:235-248.

Sun Y, Chang YH, Chen HF, Su YH, Su HF, Li CY (2012) Risk of Parkinson disease onset in patients with diabetes: a 9-year population-based cohort study with age and sex stratifications. Diabetes care 35:1047-1049.

Sundram V, Chauhan SC, Jaggi M (2011) Emerging roles of protein kinase D1 in cancer. Molecular cancer research : MCR 9:985-996.

Tan EK, Skipper LM (2007) Pathogenic mutations in Parkinson disease. Human mutation 28:641-653.

Thomas B, Beal MF (2007) Parkinson's disease. Human molecular genetics 16 Spec No. 2:R183-194.

Thomas KJ, McCoy MK, Blackinton J, Beilina A, van der Brug M, Sandebring A, Miller D, Maric D, Cedazo-Minguez A, Cookson MR (2011) DJ-1 acts in parallel to the PINK1/parkin pathway to control mitochondrial function and autophagy. Human molecular genetics 20:40-50.

Trauzold A, Schmiedel S, Sipos B, Wermann H, Westphal S, Roder C, Klapper W, Arlt A, Lehnert L, Ungefroren H, Johannes FJ, Kalthoff H (2003) PKCmu prevents CD95-mediated apoptosis and enhances proliferation in pancreatic tumour cells. Oncogene 22:8939-8947.

Tsang AH, Chung KK (2009) Oxidative and nitrosative stress in Parkinson's disease. Biochimica et biophysica acta 1792:643-650.

Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del Turco D, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, Gonzalez-Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G, Wood NW (2004) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science (New York, NY 304:1158-1160.

Valverde AM, Sinnett-Smith J, Van Lint J, Rozengurt E (1994) Molecular cloning and characterization of protein kinase D: a target for diacylglycerol and phorbol esters with a distinctive catalytic domain. Proceedings of the National Academy of Sciences of the United States of America 91:8572-8576.

Van Den Eeden SK, Tanner CM, Bernstein AL, Fross RD, Leimpeter A, Bloch DA, Nelson LM (2003) Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity. American journal of epidemiology 157:1015-1022.

Van Laar VS, Berman SB (2009) Mitochondrial dynamics in Parkinson's disease. Experimental neurology 218:247-256.

Van Lint J, Rykx A, Maeda Y, Vantus T, Sturany S, Malhotra V, Vandenheede JR, Seufferlein T (2002) Protein kinase D: an intracellular traffic regulator on the move. Trends in cell biology 12:193-200.

Vantus T, Vertommen D, Saelens X, Rykx A, De Kimpe L, Vancauwenbergh S, Mikhalap S, Waelkens E, Keri G, Seufferlein T, Vandenabeele P, Rider MH, Vandenheede JR, Van Lint J (2004) Doxorubicin-induced activation of protein kinase D1 through caspase-

mediated proteolytic cleavage: identification of two cleavage sites by microsequencing. Cellular signalling 16:703-709.

Vega RB, Harrison BC, Meadows E, Roberts CR, Papst PJ, Olson EN, McKinsey TA (2004) Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. Molecular and cellular biology 24:8374-8385.

Ventura-Clapier R, Garnier A, Veksler V (2008) Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. Cardiovascular research 79:208-217.

Vijayababu MR, Kanagaraj P, Arunkumar A, Ilangovan R, Aruldhas MM, Arunakaran J (2005) Quercetin-induced growth inhibition and cell death in prostatic carcinoma cells (PC-3) are associated with increase in p21 and hypophosphorylated retinoblastoma proteins expression. Journal of cancer research and clinical oncology 131:765-771.

Vilarino-Guell C, Wider C, Ross OA, Dachsel JC, Kachergus JM, Lincoln SJ, Soto-Ortolaza AI, Cobb SA, Wilhoite GJ, Bacon JA, Behrouz B, Melrose HL, Hentati E, Puschmann A, Evans DM, Conibear E, Wasserman WW, Aasly JO, Burkhard PR, Djaldetti R, Ghika J, Hentati F, Krygowska-Wajs A, Lynch T, Melamed E, Rajput A, Rajput AH, Solida A, Wu RM, Uitti RJ, Wszolek ZK, Vingerhoets F, Farrer MJ (2011) VPS35 mutations in Parkinson disease. American journal of human genetics 89:162-167.

Viswanath V, Wu Y, Boonplueang R, Chen S, Stevenson FF, Yantiri F, Yang L, Beal MF, Andersen JK (2001) Caspase-9 activation results in downstream caspase-8 activation and bid cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease. J Neurosci 21:9519-9528.

Wahlqvist ML, Lee MS, Hsu CC, Chuang SY, Lee JT, Tsai HN (2012) Metformin-inclusive sulfonylurea therapy reduces the risk of Parkinson's disease occurring with Type 2 diabetes in a Taiwanese population cohort. Parkinsonism & related disorders 18:753-758.

Waldron RT, Iglesias T, Rozengurt E (1999) The pleckstrin homology domain of protein kinase D interacts preferentially with the eta isoform of protein kinase C. The Journal of biological chemistry 274:9224-9230.

Walsh NP, Gleeson M, Pyne DB, Nieman DC, Dhabhar FS, Shephard RJ, Oliver SJ, Bermon S, Kajeniene A (2011) Position statement. Part two: Maintaining immune health. Exercise immunology review 17:64-103.

Wang L, Zhai YQ, Xu LL, Qiao C, Sun XL, Ding JH, Lu M, Hu G (2014a) Metabolic inflammation exacerbates dopaminergic neuronal degeneration in response to acute MPTP challenge in type 2 diabetes mice. Experimental neurology 251:22-29.

Wang N, Su P, Zhang Y, Lu J, Xing B, Kang K, Li W, Wang Y (2014b) Protein kinase D1-dependent phosphorylation of dopamine D1 receptor regulates cocaine-induced behavioral responses. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 39:1290-1301.

Warren Olanow C, Bartus RT, Baumann TL, Factor S, Boulis N, Stacy M, Turner DA, Marks W, Larson P, Starr PA, Jankovic J, Simpson R, Watts R, Guthrie B, Poston K, Henderson JM, Stern M, Baltuch G, Goetz CG, Herzog C, Kordower JH, Alterman R, Lozano AM, Lang AE (2015) Gene delivery of neurturin to putamen and substantia

nigra in Parkinson disease: A double-blind, randomized, controlled trial. Annals of neurology 78:248-257.

Watkins P (2000) COMT inhibitors and liver toxicity. Neurology 55:S51-52; discussion S53-56.

Williams CA, Grayer RJ (2004) Anthocyanins and other flavonoids. Natural product reports 21:539-573.

Winterbourn CC (2008) Reconciling the chemistry and biology of reactive oxygen species. Nature chemical biology 4:278-286.

Wiseman A, Gillham NW, Boynton JE (1977) Nuclear mutations affecting mitochondrial structure and function in Chlamydomonas. The Journal of cell biology 73:56-77.

Wong C, Jin ZG (2005) Protein kinase C-dependent protein kinase D activation modulates ERK signal pathway and endothelial cell proliferation by vascular endothelial growth factor. The Journal of biological chemistry 280:33262-33269.

Wooten GF, Currie LJ, Bovbjerg VE, Lee JK, Patrie J (2004) Are men at greater risk for Parkinson's disease than women? Journal of neurology, neurosurgery, and psychiatry 75:637-639.

Writing Group for the NETiPDI, Kieburtz K, Tilley BC, Elm JJ, Babcock D, Hauser R, Ross GW, Augustine AH, Augustine EU, Aminoff MJ, Bodis-Wollner IG, Boyd J, Cambi F, Chou K, Christine CW, Cines M, Dahodwala N, Derwent L, Dewey RB, Jr., Hawthorne K, Houghton DJ, Kamp C, Leehey M, Lew MF, Liang GS, Luo ST, Mari

Z, Morgan JC, Parashos S, Perez A, Petrovitch H, Rajan S, Reichwein S, Roth JT, Schneider JS, Shannon KM, Simon DK, Simuni T, Singer C, Sudarsky L, Tanner CM, Umeh CC, Williams K, Wills AM (2015) Effect of creatine monohydrate on clinical progression in patients with Parkinson disease: a randomized clinical trial. Jama 313:584-593.

Yang L, Calingasan NY, Wille EJ, Cormier K, Smith K, Ferrante RJ, Beal MF (2009) Combination therapy with coenzyme Q10 and creatine produces additive neuroprotective effects in models of Parkinson's and Huntington's diseases. Journal of neurochemistry 109:1427-1439.

Yao L, Li W, She H, Dou J, Jia L, He Y, Yang Q, Zhu J, Capiro NL, Walker DI, Pennell KD, Pang Y, Liu Y, Han Y, Mao Z (2012) Activation of transcription factor MEF2D by bis(3)-cognitin protects dopaminergic neurons and ameliorates Parkinsonian motor defects. The Journal of biological chemistry 287:34246-34255.

Youdim MB, Oh YJ (2013) Promise of neurorestoration and mitochondrial biogenesis in Parkinson's disease with multi target drugs: an alternative to stem cell therapy. Experimental neurobiology 22:167-172.

Youle RJ, Narendra DP (2011) Mechanisms of mitophagy. Nature reviews 12:9-14.

Zamin LL, Filippi-Chiela EC, Dillenburg-Pilla P, Horn F, Salbego C, Lenz G (2009) Resveratrol and quercetin cooperate to induce senescence-like growth arrest in C6 rat glioma cells. Cancer science 100:1655-1662.

Zandi K, Teoh BT, Sam SS, Wong PF, Mustafa MR, Abubakar S (2011) Antiviral activity of four types of bioflavonoid against dengue virus type-2. Virology journal 8:560.

Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atares B, Llorens V, Gomez Tortosa E, del Ser T, Munoz DG, de Yebenes JG (2004) The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Annals of neurology 55:164-173.

Zeng X, Chen J, Deng X, Liu Y, Rao MS, Cadet JL, Freed WJ (2006) An in vitro model of human dopaminergic neurons derived from embryonic stem cells: MPP+ toxicity and GDNF neuroprotection. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 31:2708-2715.

Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN (2002) Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell 110:479-488.

Zhang D, Anantharam V, Kanthasamy A, Kanthasamy AG (2007) Neuroprotective effect of protein kinase C delta inhibitor rottlerin in cell culture and animal models of Parkinson's disease. The Journal of pharmacology and experimental therapeutics 322:913-922.

Zhang W, Xu J, Jia CK, Zheng SS (2009) Modulating protein kinase D1 signal transduction. Chinese medical journal 122:1816-1820.

Zhang Y, Dawson VL, Dawson TM (2000) Oxidative stress and genetics in the pathogenesis of Parkinson's disease. Neurobiology of disease 7:240-250.

Zheng B, Liao Z, Locascio JJ, Lesniak KA, Roderick SS, Watt ML, Eklund AC, Zhang-James Y, Kim PD, Hauser MA, Grunblatt E, Moran LB, Mandel SA, Riederer P, Miller RM, Federoff HJ, Wullner U, Papapetropoulos S, Youdim MB, Cantuti-Castelvetri I, Young AB, Vance JM, Davis RL, Hedreen JC, Adler CH, Beach TG, Graeber MB, Middleton FA, Rochet JC, Scherzer CR (2010) PGC-1alpha, a potential therapeutic target for early intervention in Parkinson's disease. Science translational medicine 2:52ra73.

Zhu JT, Choi RC, Chu GK, Cheung AW, Gao QT, Li J, Jiang ZY, Dong TT, Tsim KW (2007) Flavonoids possess neuroprotective effects on cultured pheochromocytoma PC12 cells: a comparison of different flavonoids in activating estrogenic effect and in preventing beta-amyloid-induced cell death. Journal of agricultural and food chemistry 55:2438-2445.

Zhukova E, Sinnett-Smith J, Rozengurt E (2001) Protein kinase D potentiates DNA synthesis and cell proliferation induced by bombesin, vasopressin, or phorbol esters in Swiss 3T3 cells. The Journal of biological chemistry 276:40298-40305.

ACKNOWLEDGMENTS