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Activation of AMPK and inactivation of Akt result in suppression of mTOR-mediated S6K1 and 4E-BP1 pathways leading to neuronal cell death in *in vitro* models of Parkinson's disease

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder characterized by loss of dopaminergic neurons. Dysregulation of mammalian target of rapamycin (mTOR) has been implicated in the pathogenesis of PD. However, the underlying mechanism is incompletely elucidated. Here, we show that PD mimetics (6-hydroxydopamine, N-methyl-4-phenylpyridine or rotenone) suppressed phosphorylation of mTOR, S6K1 and 4E-BP1, reduced cell viability, and activated caspase-3 and PARP in PC12 cells and primary neurons. Overexpression of wild-type mTOR or constitutively active S6K1, or downregulation of 4E-BP1 in PC12 cells partially prevented cell death in response to the PD toxins, revealing that mTOR-mediated S6K1 and 4E-BP1 pathways due to the PD toxins were inhibited, leading to neuronal cell death. Furthermore, we found that the inhibition of mTOR signaling contributing to neuronal cell death was attributed to suppression of Akt and activation of AMPK. This is supported by the findings that ectopic expression of constitutively active Akt or dominant negative AMPK α , or inhibition of AMPK α with compound C partially attenuated inhibition of phosphorylation of mTOR, S6K1 and 4E-BP1, activation of caspase-3, and neuronal cell death triggered by the PD toxins. The results indicate that PD stresses activate AMPK and

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inactivate Akt, causing neuronal cell death via inhibiting mTOR-mediated S6K1 and 4E-BP1 pathways. Our findings suggest that proper co-manipulation of AMPK/Akt/mTOR signaling may be a potential strategy for prevention and treatment of PD.

Keywords

AMPK; Akt; mTOR; neuronal cells; Parkinson's disease

1. Introduction

Parkinson's disease (PD) is a neurodegenerative movement disorder characterized by progressive loss of central and peripheral neurons, and especially the substantia nigra pars compacta (SNc) dopaminergic neurons [1-3]. To understand the mechanism of neuronal cell death and develop neuroprotective therapies in PD, numerous studies have been carried out in postmortem brains as well as experimental cell and animal models of PD [3-7]. Although the precise etiology of PD is unknown, increasing evidence has pointed to association of neuronal loss with apoptosis, which can be triggered by oxidative stress, impairment of mitochondrial respiration, and abnormal protein aggregation [4, 5, 8]. Especially, recent data have shown that dysregulation of mammalian target of rapamycin (mTOR) is implicated in the pathogenesis of PD [9, 10]. However, the underlying mechanism is incompletely elucidated.

mTOR, a serine/threonine (Ser/Thr) protein kinase, is a central controller of cell proliferation, growth and survival [11, 12]. mTOR functions at least as two complexes, mTORC1 and mTORC2, with distinct substrate specificities [12]. mTORC1 regulates phosphorylation of p70 S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) [11-13], whereas mTORC2 phosphorylates Akt on Ser473 [14]. In addition to phosphorylation at Ser473, Akt activity is also positively regulated by phosphorylation at Thr308, which is mediated by phosphoinositide-dependent kinase 1 (PDK1) and requires activation of phosphatidylinositol 3'-kinase (PI3K) signaling [11, 12, 14-17]. Activated PI3K catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate (PIP3). Increased PIP3 binds to the pleckstrin homology domain of Akt and, in combination with additional Ser/Thr phosphorylation of Akt by PDK1 and mTORC2, results in full activation of Akt. Subsequently, activated PI3K or Akt may positively regulate mTOR, leading to increased phosphorylation of S6K1 and 4E-BP1, the two best-characterized downstream effector molecules of mTOR [11-13]. Numerous studies have demonstrated that mTOR regulates differentiation and survival in neurons, and plays an important role in synaptic plasticity, learning and memory, and food uptake in adult brain [18, 19]. mTOR activity is modified in various pathologic states of the nervous system, including brain tumors, tuberous sclerosis, cortical dysplasia and neurodegenerative disorders such as PD, Alzheimer's disease (AD), and Huntington's disease (HD) [19]. Active Akt, as a major regulator of neuronal cell survival [20], is negatively associated with dopaminergic neurodegeneration in PD [2, 21]. Additionally, AMP-activated protein kinase (AMPK) is activated in response to oxidative stress [22], and also plays a critical role in neurodegenerative diseases [23, 24]. Multiple studies have

demonstrated that Akt can be inhibited by 6-OHDA or hydrogen peroxide (H₂O₂) in neuronal cells [2, 9, 21, 25]. H₂O₂ induces neuronal apoptosis by activation of AMPK, leading to suppression of mTOR pathway [25]. This prompts us to study how both AMPK and Akt activity influence mTOR signaling pathway contributing to neuronal cell death in the context of PD.

Here, we show both activation of AMPK and inactivation of Akt in *in vitro* PD models, which contributes to suppression of mTOR-mediated S6K1 and 4E-BP1 pathways and induction of neuronal cell death. Our findings suggest that proper co-manipulation of AMPK/Akt/mTOR signaling may be a potential strategy for prevention and treatment of PD.

2. Materials and methods

2.1. Materials

6-Hydroxydopamine (6-OHDA), bovine serum albumin (BSA), poly-D-lysine (PDL), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), and protease inhibitor cocktail were purchased from Sigma (St Louis, MO, USA), whereas compound C and 1-methyl-4-phenylpyridin-1-ium (MPP⁺) were provided by Calbiochem (San Diego, CA, USA). Dulbecco's modified Eagle medium (DMEM), 0.05% Trypsin-EDTA, NEUROBASAL™ Media, and B27 Supplement were purchased from Invitrogen (Grand Island, NY, USA). Horse serum and fetal bovine serum (FBS) were supplied by Hyclone (Logan, UT, USA). Enhanced chemiluminescence solution was from Millipore (Billerica, MA, USA), whereas normal goat serum from Chemicon International Inc (Temecula, CA, USA). The following antibodies were used: phospho-S6K1 (Thr389), phospho-4E-BP1 (Thr70), 4E-BP1, phospho-S6 ribosomal protein (Ser235/236), S6 ribosomal protein, PDK1, phospho-Akt (Ser473), phospho-Akt (Thr308), caspase-3, cleaved-caspase-3, PARP (all from Cell Signaling Technology, Beverly, MA, USA); phospho-PDK1 (Ser241), β -tubulin, phospho-mTOR (Ser2448), mTOR, phospho-AMPK α (Thr172), AMPK α , acetyl-CoA carboxylase (ACC), phospho-ACC (Ser79), HA, FLAG (all from Sigma); Akt, S6K1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Goat anti-rabbit IgG-horseradish peroxidase (HRP) and Goat anti-mouse IgG-HRP (Pierce); Goat anti-rabbit IgG (H+L)-FITC (Invitrogen). Other chemicals were provided by local commercial sources and were of analytical grade quality.

2.2. Cell culture

Rat pheochromocytoma (PC12) cell line was from American Type Culture Collection (ATCC) (Manassas, VA, USA), which was used for no more than 10 passages. Cells, seeded in a 6-well or 96-well plate coated with 0.2 μ g/ml PDL, were cultured in antibiotic-free DMEM supplemented with 10% horse serum and 5% FBS. Cells were incubated at 37°C in a humidified incubator containing 5% CO₂.

To isolate primary neurons, fetal mice at 16-18 days of gestation were chosen and primary cortical neurons were isolated and cultured as described [25]. After that, cells were seeded in a 6-well (2×10^6 cells/well) or 96-well (1×10^4 cells/well) plate coated with 10 μ g/ml PDL in NEUROBASAL™ Media (Invitrogen) supplemented with 2% B27 Supplement.

(Invitrogen), 2 mM glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 5 µg/ml insulin (Sigma), and 40 µg/ml of gentamicin (Invitrogen), and grown in a humid incubator (37°C, 5% CO₂). Fresh medium was replaced every 3 days. The primary neurons were used for experiments after 6 days of culture.

2.3. Recombinant adenoviral constructs and infection of cells

The recombinant adenoviral vectors encoding FLAG-tagged wild-type mTOR (Ad-mTOR-wt), hemagglutinin (HA)-tagged constitutively active S6K1 (Ad-S6K1-ca), HA-tagged myristoylated, constitutively active Akt (Ad-myr-Akt), HA-tagged dominant negative AMPK α 1 (Ad-dn-AMPK α), and green fluorescence protein (Ad-GFP) were described previously [25-27]. The viruses were amplified, titrated and used as described [25, 28]. For experiments, PC12 cells were cultured in the growth medium, and infected with the individual adenovirus for 24 h at 5 of multiplicity of infection (MOI = 5). Subsequently, cells were used for experiments. Cells infected with Ad-GFP alone served as a control. Expression of FLAG-tagged mTOR-wt and HA-tagged S6K1-ca, myr-Akt or dn-AMPK α were determined by Western blotting with antibodies to FLAG and HA, respectively.

2.4. Lentiviral shRNA cloning, production, and infection

Lentiviral shRNAs to GFP, and 4E-BP1 were described previously [25]. The lentivirus-expressing GFP-target shRNA was used as control. Monolayer PC12 cells, when grown to about 70% confluence, were infected with above lentivirus-containing supernatant in the presence of 8 µg/ml polybrene and, exposed to 2 µg/ml puromycin after 24 h of infection. In 5 days, cells were used for experiments.

2.5. Analysis for cell viability and morphology

PC12 cells and/or primary neurons, seeded in a 96-well plate (1×10^4 cells/well), were treated with different concentration of 6-OHDA (0-240 µM) for 24 h, with/without MPP⁺ (1 mM) or rotenone (1 µM) for 24, or with/without 6-OHDA (120 µM), MPP⁺ (1 mM) or rotenone (1 µM) for 24 h following pre-incubation with/without compound C (20 µM) for 2 h with 5 replicates of each treatment. In some cases, PC12 cells, infected with Ad-mTOR, Ad-S6K1-ca, Ad-myr-Akt, Ad-dn-AMPK α , or Ad-GFP (control), or infected with lentiviral shRNA to 4E-BP1 or GFP, respectively, were seeded at a density of 1×10^4 cells/well in a 96-well plate or 5×10^5 cells/well in a 6-well plate. Next day, cells were exposed to 6-OHDA (120 and/or 240 µM), MPP⁺ (1 mM) or rotenone (1 µM) for 24 h. Subsequently, cell viability was evaluated using an MTT assay by measuring the optical density at 570 nm under an ELx800 Microplate Reader (Bio-Tek Instruments, Inc. Winooski, VT, USA). The images for morphological analysis were taken with a Nikon Eclipse TE2000-U inverted phase-contrast microscope (Nikon, Tokyo, Japan) (200 $\times$) equipped with a digital camera.

2.6. Assay for cell immunofluorescence and DAPI staining

PC12 cells and primary neurons were seeded at a density of 5×10^5 cells/well in a 6-well plate containing a PDL-coated glass coverslip per well. Next day, after treatment with or without 6-OHDA (120 µM), MPP⁺ (1 mM) or rotenone (1 µM) for 24 h, cells were fixed with 4% paraformaldehyde prepared in 0.1 M PBS (pH 7.4) for 2 h at 4°C, and then washed

three times with PBS. Thereafter coverslips containing cells were incubated with 3% normal goat serum (diluted in PBS containing 1% BSA) for 1 h at room temperature to block non-specific binding. The double staining was done by firstly adding primary antibodies to phospho-Akt (Ser473) or phospho-Akt (Thr308) (1:100, diluted in PBS containing 1% BSA) for overnight incubation at 4°C, followed by washing three times in PBS and then incubating with secondary antibody to FITC-conjugated anti-rabbit (1:500, diluted in PBS containing 1% BSA) for 1 h at room temperature. The cells were then washed in PBS, and further three times with 0.1 % Triton X-100 in PBS for 5 min per time. The following staining was done by adding DAPI (4 µg/ml in deionized water) as described [29]. Finally, slides were mounted in glycerol/PBS (1 : 1, v/v) containing 2.5% 1,4-diazabicyclo-(2,2,2)octane. Cells were observed under a fluorescence microscope (Nikon 80i, Tokyo, Japan) and the images were taken with a digital camera.

2.7. Western blot analysis

Brain tissues homogenized in 3 ml of ice-cold RIPA buffer [50 mM Tris, pH 7.2; 150 mM NaCl; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 1% Triton X-100; 10 mM NaF; 1 mM Na₃VO₄; protease inhibitor cocktail (1:1000)]. For *in vitro* PC12 cells or murine primary neurons, after treatment, cells were briefly washed with cold PBS, and then on ice, lysed in RIPA buffer. Homogenates or lysates were sonicated for 10 s and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants were collected. Protein concentration was determined by bicinchoninic acid assay with bovine serum albumin as a standard (Pierce). After that, Western blotting was performed as described previously [25].

2.8. Statistical analysis

All data were presented as mean ± SEM. Student's *t*-test for non-paired replicates was used to identify statistically significant differences between treatment means. Group variability and interaction were compared using either one-way or two-way ANOVA followed by Bonferroni's post-tests to compare replicate means. Significance was accepted at *P* < 0.05.

3. Results

3.1. PD toxin-induced neuronal cell death is related to inhibition of mTOR-mediated S6K1 and 4E-BP1 pathways

mTOR is a central controller of cell proliferation, growth and survival [11, 12]. To elucidate how mTOR signaling contributes to PD pathogenesis, PC12 cells and primary neurons were used. We found that treatment with 6-OHDA (0-240 µM) for 12 h decreased phosphorylation of mTOR, S6K1, and 4E-BP1 concentration-dependently, and at concentrations of > 120 µM, 6-OHDA dramatically reduced phosphorylation of S6K1/4E-BP1 (Fig. 1A). Also, 6-OHDA inhibited phosphorylation of S6K1/4E-BP1 time-dependently. As shown in Fig. 1B, at > 4 h post-treatment, 6-OHDA obviously reduced phosphorylation of S6K1/4E-BP1, and the effect was sustained for >24 h. 6-OHDA did not markedly alter those total protein levels (Fig. 1A and B). Similar results were also observed in PC12 cells and primary neurons exposed to MPP⁺ (1 mM) or rotenone (1 µM) for 12 and 24 h (Fig. 1C). It should be mentioned that phosphorylation status of 4E-BP1 was also detected with an antibody to 4E-BP1. Phosphorylation of 4E-BP1 decreases its

electrophoretic mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis [29]. As demonstrated in Fig. 1A, 6-OHDA decreased phosphorylation of 4E-BP1 in a concentration-dependent manner, as indicated by the decrease in the intensity of the uppermost band γ and by the increase in the higher mobility band α and β that corresponds to a less phosphorylated form of 4E-BP1. These findings indicate that PD toxins (6-OHDA, MPP⁺ or rotenone) inhibit mTOR signaling pathway in the neuronal cells.

In addition, we found that 6-OHDA, MPP⁺ or rotenone coincidentally reduced cell viability in PC12 cells and primary neurons (Fig. 1D and E). Furthermore, treatment with these compounds resulted in robust cleavages of caspase-3/PARP in the cells (Fig. 1F). The data suggest that inactivation of mTOR signaling pathway may contribute to neuronal cell death in PD.

To demonstrate the importance of mTOR-mediated S6K1 and 4E-BP1 pathways in the pathogenesis of PD, first of all, we tested whether overexpression of mTOR has any protective effect on PD toxins-induced cell death. PC12 cells, infected with Ad-mTOR or Ad-GFP (control), were exposed to 6-OHDA (120 and 240 μ M) for 12 or 24 h, followed by Western blotting and morphological analysis. The results showed that ectopic expression of FLAG-tagged wt-mTOR conferred substantial resistance to 6-OHDA-induced inhibition of S6K1/4E-BP1 phosphorylation (Fig. 2A), as well as cell death (Fig. 2B). Similar results were also seen in the cells exposed to MPP⁺ (1 mM) or rotenone (1 μ M) (data not shown). Consistently, 6-OHDA-, MPP⁺- or rotenone-activated caspase-3 was apparently attenuated by overexpression of mTOR (Fig. 2C), indicating that neuronal cell death due to the PD toxins is related to inhibition of mTOR.

To evaluate the role of S6K1 pathway in PD toxin-induced neuronal cell death, a recombinant adenovirus expressing HA-tagged constitutively active S6K1 (Ad-S6K1-ca) was employed. As shown in Fig. 3A, a high level of S6K1-ca was observed in PC12 cells infected with Ad-S6K1-ca, but not in those infected with Ad-GFP (as control), as detected by immunoblotting with antibodies to HA and S6K1. The basal level of phosphorylation of S6 ribosomal protein, a substrate of S6K1, was substantially high in PC12 cells, which was not obviously enhanced by expression of S6K1-ca. However, when the cells were exposed to 6-OHDA (120 and 240 μ M) for 12 h, expression of S6K1-ca rendered a high resistance to 6-OHDA inhibition of phosphorylation of S6K1 and S6. Interestingly, expression of S6K1-ca also partially rescued the cells from 6-OHDA-induced cell death (Fig. 3B and C). Similar results were seen in the cells exposed to MPP⁺ (1 mM) or rotenone (1 μ M) (data not shown). Furthermore, cells expressing S6K1-ca, but not GFP, were also resistant to the PD toxins-induced cleavages of caspase-3 (Fig. 3D), suggesting that inhibition of mTOR-mediated S6K1 pathway at least, in part, contributes to neuronal cell death in the PD models.

Next, we assessed the role of 4E-BP1 pathway in PD toxin-induced neuronal cell death. As 4E-BP1 functions as a suppressor of eIF4E [30], downregulation of 4E-BP1 would lead to loss of suppression of eIF4E. In this study, 4E-BP1 was downregulated by > 90% in PC12 cells infected with lentiviral shRNA to 4E-BP1 compared to control cells infected with lentiviral shRNA to GFP (Fig. 4A). Of note, silencing 4E-BP1 significantly attenuated the cytotoxicity induced by 6-OHDA (120-240 μ M) (Fig. 4B and C), MPP⁺ (1 mM) or rotenone

(1 μ M) (data not shown). Further, silencing 4E-BP1 markedly blocked cleavages of caspase-3 induced by the PD toxins (Fig. 4D), suggesting that inhibition of 4E-BP1 pathway is also in part responsible for neuronal cell death in PD. Collectively, our findings support the notion that inhibition of mTOR-mediated S6K1 and 4E-BP1 pathways are involved in neuronal cell death in the context of PD.

3.2. PD toxins inactivates Akt, resulting in inhibition of mTOR-mediated S6K1 and 4E-BP1 pathways, as well as cell death

Akt is phosphorylated at Ser473 by mTORC2 and at Thr308 by PDK1 [11, 12], and Akt is a major regulator of neuronal cell survival [20]. To substantiate the role of Akt in the regulation of neuronal cell death in PD, PC12 cells and primary neurons were exposed to 6-OHDA (0-240 μ M) for 12 h or 6-OHDA (120 μ M) for 0-24 h, followed by Western blotting. We found that 6-OHDA reduced phosphorylation of Akt (Ser473 and Thr308) and PDK1 (Ser241) concentration- and time-dependently, although total cellular protein levels of Akt and PDK1 were not altered (Fig. 5A and B). This was also recapitulated in the cells treated with MPP⁺ (1 mM) or rotenone (1 μ M) (Fig. 5C). This was further supported by our immunostaining result that treatment of PC12 cells with 6-OHDA (120 μ M), MPP⁺ (1 mM) or rotenone (1 μ M) for 24 h remarkably inhibited phosphorylation of (Ser473 and Thr308) (in green) (Fig. 5D and E). Similar results were also observed in primary neurons (data not shown). Noticeably, the PD toxins also significantly increased number of the cells with nuclear fragmentation and condensation, as detected by DAPI staining (in blue) (Fig. 5D and E) as well. The results suggest that the inactivation of Akt may be implicated in neuronal cell death in PD.

mTOR can be positively regulated by Akt [11, 12]. To confirm the role of Akt in the regulation of mTOR signaling and neuronal cell death in PD, recombinant adenovirus encoding HA-tagged constitutively active Akt (Ad-myr-Akt) was utilized. As shown in Fig. 6A, a high level of HA-tagged Akt mutant was seen in PC12 cells infected with Ad-myr-Akt, but not in the cells infected with Ad-GFP (control virus).. Expression of myr-Akt led to robust Akt protein expression and Akt phosphorylation at Ser473, indicating that the Akt mutant functioned in the cells as expected. Of importance, overexpression of myr-Akt remarkably attenuated 6-OHDA-, MPP⁺- or rotenone-induced loss of phosphorylation of Akt, mTOR, S6K1 and 4E-BP1 in the cells (Fig. 6A). Additionally, overexpression of myr-Akt also potently blocked activation of caspase-3 and in part attenuated the cytotoxicity of 6-OHDA, MPP⁺ or rotenone (24 h) in PC12 cells (Fig. 6B). These findings indicate that inactivation of Akt due to the PD toxins results in neuronal cell death via inhibition of mTOR-mediated S6K1 and 4E-BP1 pathways.

3.3. PD toxins activates AMPK, leading to inhibition of mTOR-mediated S6K1 and 4E-BP1 pathways, as well as cell death

AMPK is a major negative regulator of mTOR pathway [31]. Therefore, we further studied whether AMPK is activated, leading to inhibition of mTOR signaling and neuronal cell death in PD. For this, PC12 cells were exposed to 6-OHDA (0-240 μ M) for 12 h, to 6-OHDA (120 μ M) for indicated time (0-24 h), or to MPP⁺ (1 mM) and rotenone (1 μ M) for 12 and 24 h. We found that 6-OHDA, MPP⁺ or rotenone strongly increased phosphorylation

of AMPK α and its substrate ACC concentration- and time-dependently, despite no effect on total cellular protein level of AMPK α (Fig. 7A-C). Similar data were observed in primary neurons (Fig. 7A-C). These findings suggest activation of AMPK in neuronal cells induced by the PD mimetics.

To determine association of activated AMPK with inhibition of mTOR signaling and consequential neuronal cell death in PD, compound C (AMPK inhibitor) were employed. When PC12 cells were pre-treated with/without compound C (10 μ M) for 2 h, and then exposed to 6-OHDA (120 μ M), MPP⁺ (1 mM), or rotenone (1 μ M) for 12 h or 24 h, we found that compound C potently attenuated the PD toxins-induced phosphorylation of AMPK α , inhibition of phosphorylation of mTOR, S6K1 and 4E-BP1, as well as reduction of pro-caspase-3 and cell viability (Fig. 8A and B). Similar results were also seen in primary neurons (data not shown).

To further verify the role of AMPK in inhibition of mTOR signaling and induction of neuronal cell death in PD, PC12 cells, infected with Ad-dn-AMPK α and Ad-GFP (as control), were exposed to 6-OHDA (120 μ M), MPP⁺ (1 mM), or rotenone (1 μ M) for 12 h or 24 h. Western blotting revealed that ectopic expression of dn-AMPK α obviously inhibited the PD toxins-induced phosphorylation of AMPK α (Fig. 8C). Consistently, inhibition of phosphorylation of mTOR, S6K1 and 4E-BP1, as well as activation of caspase-3 due to 6-OHDA, MPP⁺ or rotenone was markedly attenuated by expression of dn-AMPK α (Fig. 8C). Interestingly, expression of dn-AMPK α also partially protected PC12 cells from cell death induced by the PD toxins (Fig. 8D). The results underscore that activation of AMPK by the PD toxins contributes to neuronal cell death through inhibition of mTOR-mediated S6K1 and 4E-BP1 pathways.

4. Discussion

The findings from postmortem brains and experimental cell and animal models of PD have highlighted the role of apoptosis in the process of dopaminergic neuron death [1-3]. However, it is unclear what signaling molecules are critical for determining the mode of neuronal cell death. Currently, oral administration of levodopa is a gold standard therapy for the early stage of PD, but levodopa only ameliorates clinical manifestations of the disease rather than prevents neuronal cell death [9, 32]. Therefore, it is of great importance to identify a novel therapeutic target and strategy to intervene neuronal cell death related to PD. Here, we provide evidence that mTOR signaling pathway was inhibited, leading to neuronal cell death via co-operative activation of AMPK and inactivation of Akt in *in vitro* (cell culture) PD models by using PD mimetics (6-OHDA, MPP⁺ and rotenone).

mTOR is a central controller for cell growth, proliferation and survival [11, 12]. However, the role of mTOR in the regulation of neuronal cell survival remains mysterious. Both activation and inactivation of mTOR signaling have been found to contribute to AD and PD progression [10, 33]. Malagelada's group shows that 6-OHDA inhibits mTOR kinase activity involved in neuronal cell death [9]. Recently we have observed that on one hand, cadmium, a heavy metal polluted in the environment, induces oxidative stress and neuronal cell death by activating mTOR, which can be prevented by pre-treatment with rapamycin, a

specific mTOR inhibitor [34, 35]; on the other hand, hydrogen peroxide induces neuronal cell death by inhibiting mTOR, which can be attenuated by overexpression of mTOR [25]. From the above findings, we deduce that a certain level of mTOR activity may be essential for neuronal cell survival and normal function, but too low/high level of mTOR activity may be detrimental to neurons. However, unfortunately, so far we have no clue what level of mTOR activity is optimal for neuronal cell survival and function.

As for how aberrant mTOR activity causes neuronal cell death, it has been speculated that neurons, unlike tumor cells, are non-mitotic, hyperactive mTOR signaling may activate the cell cycle machinery, but the cells do not replicate, ultimately committing to suicide (apoptosis) [10]. Also, given that mTOR is a master kinase regulating protein and lipid synthesis and metabolism [11, 12], hyperactive mTOR signaling may not only consume more energy (ATP), but also produce more by-products, such as reactive oxygen species (ROS), which may also contribute to neuronal cell death. In contrast, hypoactive mTOR signaling may inhibit synthesis of some proteins that are necessary for neuronal survival, synaptic transmission, etc. [10]. It has been described that loss of mTOR activity and the persistent activation of 4E-BP1 due to dominant mutations of leucine-rich repeat kinase 2 (LRRK2) inhibits translation initiation, indeed causing the loss of dopaminergic neurons [36].

To gain insights into the molecular mechanism by which mTOR regulates neuronal cell survival, we carried out studies in the cellular PD models using various PD toxins, including 6-OHDA, MPP⁺ and rotenone, which are frequently used in the field [2, 5, 37, 38]. We found that these PD toxins very potently inhibited phosphorylation of mTOR, S6K1 and 4E-BP1, which contributed to neuronal cell death in PC12 cells and primary neurons. This is strongly supported by the findings that overexpression of wild-type mTOR, constitutively active S6K1 or downregulation of 4E-BP1 partially prevented neuronal cell death induced by 6-OHDA, MPP⁺ and rotenone, respectively. Therefore, we propose that suppression of mTOR-mediated S6K1 and 4E-BP1 pathways plays a crucial role in the pathogenesis of PD.

It has been described that 6-OHDA-induced neuronal cell death is associated with its inhibition of Akt activity [2]. Also, AMPK is activated in PD models mediated by MPP⁺ and MPTP [39], and activation of AMPK following ischemia promotes damage of hippocampal and cortical neurons [40]. Akt and AMPK are well known to regulate mTOR pathway positively and negatively, respectively [11, 12]. After we observed activation of AMPK and inactivation of Akt in our *in vitro* PD models, we reasoned that inhibition of mTOR signaling in PD might be a consequence of Akt inhibition and/or AMPK activation. To this end, genetic and pharmacological approaches were employed. We found that ectopic expression of constitutively active Akt or dominant negative AMPK α , or inhibition of AMPK α with compound C partially prevented inhibition of mTOR-mediated S6K1 and 4E-BP1 pathways and neuronal cell death triggered by 6-OHDA, MPP⁺ or rotenone. Therefore, our findings support the notion that PD toxins-induced activation of AMPK and inactivation of Akt may cooperatively result in suppression of mTOR-mediated S6K1 and 4E-BP1 pathways, thereby leading to neuronal cell death in the context of PD.

A new question that arises from this work is how PD-associated stress triggers the aberrant AMPK/Akt activity and consequential inhibition of mTOR signaling. It is known that oxidative stress is at the center of various neurodegenerative diseases [41], and documented as a key pathogenic event of PD [42-46]. A series of recent reports have pointed to double-faced role of oxidative stress, e.g. ROS, in activation or inhibition of related signaling pathways [25, 34, 47]. For example, cadmium- or tetrahydrobiopterin-induced ROS, or H₂O₂, a well-known oxidant, activates MAPKs pathway including JNK, Erk1/2 and/or p38 pathways, and inhibits protein phosphatase 2A and 5, leading to neuronal cell death [34, 35, 47-50]. Cadmium induction of ROS mediates the activation of mTOR pathway contributing to neuronal cell death by activating Akt and inhibiting AMPK [34], whereas H₂O₂ blocks mTOR signaling in part through activation of AMPK and inhibition of Akt [25]. Currently, we do not know whether ROS and what kinds of ROS affect the activity of AMPK/Akt as well as their effects on mTOR signaling in the development of PD. Undoubtedly, more studies are needed to address these issues.

In conclusion, our results show a paralleled event in which AMPK is activated and Akt is inactivated in *in vitro* PD models. Both activation of AMPK and inactivation of Akt contribute to suppression of mTOR-mediated S6K1 and 4E-BP1 pathways, thereby leading to neuronal cell death in the context of PD. Our findings suggest that proper co-manipulation of AMPK/Akt/mTOR signaling may be a potential therapeutic strategy for prevention and treatment of PD.

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Abbreviations

4E-BP1	eukaryotic initiation factor 4E binding protein 1
6-OHDA	6-hydroxydopamine
ACC	acetyl-CoA carboxylase
AD	Alzheimer disease
AMPK	AMP-dependent/activated protein kinase
DAB	3,3'-diaminobenzidine tetrachloride
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
HA	hemagglutinin

HD	Huntington's disease
MPP⁺	1-Methyl-4-phenylpyridin-1-ium
mTOR	mammalian target of rapamycin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
PARP	poly ADP-ribose polymerase
PBS	phosphate buffered saline
PD	Parkinson disease
PDK1/2	3-Phosphoinositide-dependent protein kinase-1/2
PDL	poly-D-lysine
PI3K	phosphatidylinositol 3'-kinase
Akt (PKB)	protein kinase B
S6K1	ribosomal p70 S6 kinase 1
Ser/Thr	serine/threonine

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Highlights

- PD toxin-induced neuronal cell death is related to inhibition of mTOR-mediated S6K1 and 4E-BP1 pathways.
- PD toxins induce activation of AMPK and inactivation of Akt in neuronal cells.
- Activation of AMPK and inactivation of Akt contribute to suppression of mTOR-mediated S6K1 and 4E-BP1 pathways, thereby leading to neuronal cell death in the context of PD.
- Co-manipulation of AMPK/Akt/mTOR signaling may be a potential strategy for prevention of PD

Fig. 1. Inhibition of mTOR signaling pathway is associated with neuronal cell death in cellular models of PD. PC12 cells and primary neurons were treated with 6-OHDA (0-240 μM or 120 μM), MPP⁺ (1 mM) or rotenone (1 μM) for 12 and/or 24 h, as described in Materials and Methods. Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. Cell viability was evaluated using an MTT assay. (A and B) 6-OHDA (0-240 μM for 12 h or 120 μM for 0-24 h) obviously inhibited phosphorylation of mTOR, S6K1, and 4E-BP1 in a concentration- and time-dependent manner, and (C) treatment with MPP⁺ (1 mM) or rotenone (1 μM) for 12 and 24 h also inhibited mTORC1 signaling in PC12 cells and primary neurons. (D) Treatment with 6-OHDA (0-240 μM) for 24 h concentration-dependently reduced cell viability, and (E) treatment with MPP⁺ (1 mM) or rotenone (1 μM) for 24 h significantly decreased cell viability as well. (F) 6-OHDA (120 μM), MPP⁺ (1 mM) or rotenone (1 μM) increased cleavages of caspase-3 and PARP in PC12 cells and primary neurons. Results are presented as mean ± SEM, n = 5. ***P* < 0.01, difference with control group.

Fig. 2. Expression of wt-mTOR partially prevents neuronal cell death in cellular models of PD. PC12 cells, infected with replication-defective adenoviral recombinant expressing FLAG-tagged wt-mTOR (Ad-mTOR) or the control adenovirus (Ad-GFP), were exposed to 6-OHDA (120 and 240 μ M) for 12 h or 24 h, or to 6-OHDA (120 μ M), MPP⁺ (1 mM) and rotenone (1 μ M) for 12 h, followed by Western blotting using indicated antibodies and morphological analysis using a Nikon Eclipse TE2000-U inverted phase-contrast microscope (200 $\times$) equipped with a digital camera. Scale bar: 100 μ m. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. (A) Ectopic expression of FLAG-tagged wt-mTOR conferred substantial resistance to 6-OHDA inhibition of mTOR-mediated phosphorylation of S6K1 and 4E-BP1. (B) Overexpression of mTOR partially prevented 6-OHDA-induced cell death. (C) 6-OHDA, MPP⁺ or rotenone activation of caspase-3 was apparently attenuated by overexpression of mTOR.

Fig. 3.

Expression of constitutively active S6K1 partially attenuates neuronal cell death in cellular models of PD. PC12 cells, infected with replication-defective adenoviral recombinant expressing constitutively active S6K1 (Ad-S6K1-ca) or Ad-GFP (as control), were exposed to 6-OHDA (120 and 240 μ M) for 12 h or 24 h, or to 6-OHDA (120 μ M), MPP⁺ (1 mM) and rotenone (1 μ M) for 12 h, followed by Western blotting using indicated antibodies and analysis for cell viability and morphology, as described in Materials and Methods. Scale bar: 100 μ m. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. (A) Expression of HA-tagged S6K1-ca potently blocked 6-OHDA inhibition of phosphorylation of S6K1 and S6 ribosomal protein. (B and C) Expression of S6K1-ca partially rescued the cells from 6-OHDA-induced cell death. (D) Cell expressing S6K1-ca, but not GFP, prevented 6-OHDA-, MPP⁺- or rotenone-induced activation of caspase-3. Results are presented as mean $\pm$ SEM, $n = 5$. ^a $P < 0.05$, difference with control group; ^b $P < 0.05$, Ad-S6K1-ca group vs Ad-GFP group.

Fig. 4. Downregulation of 4E-BP1 partially rescues neuronal cell death in cellular models of PD. PC12 cells, infected with lentiviral shRNA to 4E-BP1 or GFP (as control), were exposed to 6-OHDA (120 and 240 μ M) for 12 h or 24 h, or to 6-OHDA (120 μ M), MPP⁺ (1 mM) and rotenone (1 μ M) for 12 h, followed by Western blotting using indicated antibodies and analysis for cell viability and morphology, as described in Materials and Methods. Scale bar: 100 μ m. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. (A) Expression of cellular 4E-BP1 protein was downregulated by ~90% by lentiviral shRNA to 4E-BP1. (B and C) 4E-BP1 shRNA markedly rescued morphology and cell viability of PC12 cells exposed to 6-OHDA (120 and 240 μ M) for 24 h, respectively. (D) Cleavages of caspase-3 induced by 6-OHDA, MPP⁺ or rotenone were obviously blocked by downregulation of 4E-BP1 in PC12 cells. Results are presented as mean $\pm$ SEM, n = 5. ^a P < 0.05, difference with control group; ^b P < 0.05, 4E-BP1 shRNA vs GFP shRNA group.

Fig. 5. Loss of Akt phosphorylation is associated with neuronal cell death in cellular models of PD. PC12 cells and/or primary neurons were treated with 6-OHDA (0-240 μ M or 120 μ M), MPP⁺ (1 mM) or rotenone (1 μ M) for 12 and/or 24 h, followed by Western blotting as well as immunofluorescence/DAPI staining, as described in Materials and Methods. Scale bar: 20 μ m. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. (A and B) 6-OHDA (0-240 μ M for 12 h or 120 μ M for 0-24 h) reduced phosphorylation of PDK1 and Akt in a concentration- and time-dependent manner, and (C) exposure to MPP⁺ (1 mM) or rotenone (1 μ M) for 12 and 24 h also inhibited phosphorylation of PDK1 and Akt in PC12 cells and primary neurons. (D and E) Treatment of PC12 cells with 6-OHDA (120 μ M), MPP⁺ (1 mM), or rotenone (1 μ M) for 24 h decreased expression of p-Akt (Ser473 and Thr308, both immunostained in green), and increased nuclear fragmentation and condensation in the cells (DAPI staining in blue).

Fig. 6. Expression of constitutively active Akt partially prevents inhibition of mTOR signaling and neuronal cell death in cellular models of PD. PC12 cells, infected with Ad-myr-Akt or Ad-GFP (for control), were exposed to 6-OHDA (120 μ M), MPP⁺ (1 mM) or rotenone (1 μ M) for 12 or 24 h, respectively, followed by Western blotting using indicated antibodies and cell viability analysis as described in Methods and Materials. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. (A and B) Overexpression of myr-Akt dramatically reduced 6-OHDA-, MPP⁺- or rotenone-induced loss of phosphorylation of Akt, mTOR, S6K1 and 4E-BP1, and increase of cleaved-caspase-3, as well as cell death in the cells. Results are presented as mean $\pm$ SEM, n = 5. ^a*P* < 0.05, difference with control group; ^b*P* < 0.05, Ad-myr-Akt group vs Ad-GFP group.

Fig. 7.

AMPK is activated in cellular models of PD. PC12 cells and primary neurons were treated with 6-OHDA (0-240 μM or 120 μM), MPP⁺ (1 mM) or rotenone (1 μM) for 12 and/or 24 h, followed by Western blotting using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (A and B) 6-OHDA (0-240 μM for 12 h or 120 μM for 0-24 h) induced phosphorylation of AMPKα and its substrate ACC in a concentration- and time-dependent manner, and (C) treatment with MPP⁺ (1 mM) or rotenone (1 μM) for 12 and 24 h also induced phosphorylation of AMPKα and its substrate ACC in PC12 cells and primary neurons.

Fig. 8. Pharmacological inhibition of AMPK or expression of dominant negative AMPK α partially prevents inhibition of mTOR signaling and neuronal cell death in cellular models of PD. PC12 cells pretreated with compound C (20 μ M) for 2 h, or PC12 cells infected with Ad-dn-AMPK α and Ad-GFP (as control), were exposed to 6-OHDA (120 μ M), MPP⁺ (1 mM) or rotenone (1 μ M) for 12 or 24 h, respectively, followed by Western blotting using indicated antibodies and cell viability analysis as described in Methods and Materials. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. (A-D) Compound C or ectopic expression of dn-AMPK α obviously blocked phosphorylation of AMPK α and its substrate ACC, inhibition of phosphorylation of mTOR, S6K1 and 4E-BP1, and decrease of pro-caspase-3, as well as cell death in PC12 cells induced by 6-OHDA, MPP⁺ or rotenone. Results are presented as mean $\pm$ SEM, $n = 5$. ^a $P < 0.05$, difference with control group; ^b $P < 0.05$, - Compound C group vs + Compound C group; ^c $P < 0.05$, Ad-dn-AMPK α group vs Ad-GFP group.