The identification of rare monogenic forms of Parkinson's disease (PD) has provided tremendous insights into the molecular pathogenesis of the disorder. Mitochondrial dysfunction and oxidative stress are thought to play a prominent role in the pathogenesis of PD, but how the monogenic mutation gene causes the disease onset or progression is largely unknown. In this study we investigated the effects of wild-type and R492X mutation in the PTEN-induced putative kinase 1 (PINK1). Cell cultures show that R492X PINK1 mutation induces the generation of cellular reactive oxidative species (ROS), degrades cell membrane potential, causes cytochrome C (Cyt.C) release from mitochondrial to cytoplasm, attenuates mitochondrial complex I activity, and lastly, causes changes in mitochondrial numbers and morphology; especially when cells are treated with 1-Methyl-4-phenylpyridinium ion (MPP+). Our results suggest that the R492X mutation can cause mitochondrial dysfunction and oxidative stress and can associate with MPP+ to induce mitochondrial dysfunction and oxidative stress.

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with familial forms of Parkinson’s disease (FPD) (Belin and Westerlund, 2008; Klein and Lohmann-Hedrich, 2007). Understanding the molecular lesions associated with these FPD genes promises to shed light on the pathogenesis of the more common forms of disease. Interestingly, studies using animal models and cell culture models have linked mutations of FPD genes to impairments of mitochondrial structure and function, oxidative stress (Schapira, 2008; Dodson and Guo, 2007; Zhang et al., 2005), reinforcing the general involvement of mitochondrial dysfunction and oxidative stress in PD pathogenesis.

PINK1 is located in the PARK6 locus on chromosome 1p36 and encodes a 581 amino-acid protein with a predicted N-terminal mitochondrial targeting sequence and a conserved serine/threonine kinase domain (Valente et al., 2004). The PINK1 mutation is the second most common cause of autosomal recessive PD after parkin; more than 40 pathogenic mutations have been reported (Belin and Westerlund, 2008; Klein and Lohmann-Hedrich, 2007; Bonifati et al., 2005). Functional studies have shown the wild-type PINK1 protein may protect cells against stress-induced death and PINK1-deficient cells were more susceptible to apoptosis when exposure to mitochondrial toxins (Valente et al., 2004; Bonifati et al., 2005; Silvestri et al., 2005; Deng et al., 2005; Gandhi et al., 2006; Gautier et al., 2008; Wood-Kaczmar et al., 2008; Morais et al., 2009). Reduced complex I activity and increased levels of oxidative stress were also found in patient fibroblasts and immortalized lymphoblasts from individuals carrying G309D PINK1 mutation (Hoepken et al., 2007). Our recent study also shows that P399L PINK1 mutation was susceptible to MPP+-induced cell death (Tang et al., 2006).

Recently we found a homozygous R492X PINK1 mutation in a Chinese family with autosomal recessive early-onset parkinsonism (Guo et al., 2008). To date, no detailed report has been found in the literature describing how R492X causes PD. To investigate the role of the PINK1 mutation in the pathogenesis of PD, we established SH-SY5Y neuroblastoma cell lines. These cell lines are stable and reliably express wild-type and R492X mutated PINK1 proteins, respectively (Fig. 1).

2. Results

2.1. The establishment of stable transfected SH-SY5Y cell lines

After the transfection with Lipofectamine 2000 and screened with G418, we established stable transfected empty-vector, wild-type and R492X mutated PINK1 SH-SY5Y neuroblastoma cell lines. Using western blots, we found that the SH-SY5Y empty-vector cell line has no corresponding band. Two protein bands, 62 kD and 52 kD in size protein band can be seen in wild-type PINK1 and R492X mutated cell line. This suggests the SH-SY5Y neuroblastoma cell lines can express stable wild-type and R492X mutated PINK1 proteins, respectively (Fig. 1).

2.2. R492X mutated PINK1 protein decreases the mitochondrial membrane potential, and more severity in the induction of MPP+

Under basal conditions, compared to the SH-SY5Y cell as control, the mitochondrial membrane potentials of the vector, wild PINK1 and R492X PINK1 groups were 93.25±6.63, 95.98±1.68 and 60.13±1.50, respectively. The R492X mutated PINK1 group significantly decreases the mitochondrial membrane potential compared to the vector and wild-type PINK1 groups.

When cells were treated with 200 μM MPP+ for 24 h, the mitochondrial membrane potentials of the control, vector, wild PINK1 and R492X PINK1 groups were 69.44±3.87, 66.83±2.78, 71.17±4.17 and 45.89±6.55, respectively. The mitochondrial membrane potential was decreased in each group, especially the R492X PINK1 group, which was significantly different from the vector and wild-type PINK1 groups (Fig. 2). The results suggest that R492X PINK1 mutation causes the mitochondrial membrane potential to drawdown, especially in the induction of MPP+.
2.3. R492X mutated PINK1 protein causes the mitochondrial complex I deficiency and more severity in the induction of MPP⁺

Using 2,6-dichloroindophenol (DCIP) as the terminal electron receptor, the reduction of DCIP can be followed with spectrophotometric analysis at 600 nm. As the electrons produced by other NADH-dehydrogenases are not accepted by decylubiquinone, reduction of DCIP is primarily caused by complex I activity, resulting in very high rotenone-sensitive activity. Under basal conditions, compared to the SH-SYSY cell as control, the mitochondrial respiratory complex I activity of the vector, wild PINK1 and R492X PINK1 groups were 101.67±7.53, 110.35±5.47 and 87.33±11.64, respectively. This result shows that under the basal conditions, R492X mutation causes the decrease of mitochondrial respiratory complex I activity. When cells were treated with 200 μM MPP⁺ for 24 h, the mitochondrial respiratory complex I activity decreased in each group when treated with 200 μM MPP⁺, especially the R492X PINK1 mutation (Fig. 3). This suggests that the R492X mutation causes mitochondrial respiratory complex I deficiency, especially under neurotoxic condition.

2.4. R492X mutated PINK1 protein increases mitochondrial reactive oxidative species generation under the induction of MPP⁺

Under basal conditions, compared to the SH-SYSY cell as control, the ROS generation of the vector, wild PINK1 and R492X PINK1 groups were 109.897±6.604, 116.613±4.290 and 121.719±3.885. There is no significant ROS generation difference between each group. When cells were treated with 200 μM MPP⁺ for 24 h, the ROS generation of the control, vector, wild PINK1 and R492X PINK1 groups were 160.39±4.894, 166.251±6.609, 183.081±1.799 and 213.386±2.199. This result shows generation of ROS increased with the treatment of 200 μM MPP⁺, especially in the R492X PINK1 mutation group, which was significantly different compared to the MPP⁺-induced control group (Fig. 4).

2.5. R492X mutated PINK1 protein causes mitochondrial cytochrome C release and increases under the induction of MPP⁺

We isolated cytoplasm and mitochondrial proteins then used western blots to detect the cyt.C translocation. The results show that under basal condition, the R492X mutation causes an increase of cyt.C in the cytosol, and the decrease of cyt.C in the mitochondria. The induction by MPP⁺ leads to an increase of cytosolic cytochrome C in SH-SYSY cells in each group: cytoplasm and mitochondrial proteins. There was a significant induction in the MPP⁺-induced translocation of cyt.C into the cytosol of cells over-expressing the R492X mutation (Figs. 5 and 6).

2.6. R492X mutated PINK1 protein induces apoptosis, and enhances apoptosis under the induction of MPP⁺

We used Annexin V and PI staining, and cell analysis by flow cytometry to detect the apoptosis rate. Under basal conditions, the apoptosis rate of the control, vector, wild PINK1 and R492X PINK1 were 1.69±0.14, 1.88±0.14, 2.11±0.34 and 3.23±0.178.
This result shows that under the basal conditions, R492X mutation causes the increased apoptosis. When cells were treated with 200 μM MPP+ for 24 h, the apoptosis rate of the control, vector, wild PINK1 and R492X were 4.14±0.24, 4.62±0.42, 4.44±0.44 and 6.17±0.62. This result shows apoptotic cells increased with the treatment of 200 μM MPP+, especially in the R492X PINK1 mutation group, which was significantly different compared to the MPP+-induced control group (Fig. 7).

2.7. R492X mutated PINK1 protein causes mitochondrial morphological changes, and these changes increase upon induction of MPP+

The results show that mitochondria in SH-SY5Y control cells, empty-vector and wild-type PINK1 have normal morphology and confluency. Mitochondria in R492X mutation appear elliptical; swelling in mitochondrial crista is common, but the number of cells does not decrease significantly. When treated with 200 μM MPP+, mitochondria in each group displayed different degrees of swelling. Mitochondrial cristae became densely stained and the number of mitochondria has no significant change, suggesting that mitochondria are in a compensation stage. However, there are great changes in mitochondria in the R492X mutation group, including severe swelling, spherical in shape. Severe swelling, breakage and a decrease in mitochondrial cristae represent severe mitochondrial morphological changes (Figs. 8 and 9).

Fig. 5 – Cytoplasmic cyt.C contents of each group in media with or without MPP+. a: western blot, 1: control (SH-SY5Y cell); 2: empty-vector; 3: wild-type PINK1; 4: R492X mutation; b: The histogram represents three experimental groups and the control group. In the presence or absence of 200 μM MPP+ for 24 h, cytoplasmic protein was isolated. Cytoplasmic cyt.C contents were determined by western blot. Data are expressed as the mean ± SD (n = 3), ratio over actin. *P < 0.05, compared with the control absence of MPP+, †P < 0.05, compared with the control presence of MPP+.

Fig. 6 – Mitochondrial cyt.C contents of each group in media with or without MPP+. a: western blot, 1: control (SH-SY5Y cell); 2: empty-vector; 3: wild-type PINK1; 4: R492X mutation; b: The histogram represents three experimental groups and the control group. In the presence or absence of 200 μM MPP+ for 24 h, cytoplasmic protein was isolated. Cytoplasmic cyt.C contents were determined by western blot. Data are expressed as the mean ± SD (n = 3), ratio over actin. *P < 0.05, compared with the control absence of MPP+, †P < 0.05, compared with the control presence of MPP+, ‡P < 0.05, compared with wild-type PINK1 presence of MPP+.

Fig. 7 – Cell apoptosis of each group in media with or without MPP+. The histogram represents three experimental groups and the control group. SH-SY5Y cell lines were used for controls; The empty-vector, wild-type, R492X mutation PINK1 cell line was used for the stable transfection. Under the presence or absence of 200 μmol MPP+ for 24 h, cellular apoptosis was determined by flow cytometry. Data are expressed as the mean ± SD (n = 3). *P < 0.05, compared with the control absence of MPP+, †P < 0.05, compared with the control presence of MPP+.
3. Discussion

The molecular mechanisms underlying neurodegeneration in PD remain unclear. The recent realization that acute and chronic stressors to the cells lead to structural and functional impairments of mitochondria has redefined the role of mitochondria in disease etiology. Physiologically, mitochondria perform a variety of key cellular regulatory processes, including ATP production, ROS generation and detoxification, most important, mitochondrial dysfunction triggers signaling cascades for cellular necrosis and apoptosis of cells (Knott et al., 2008). There is some substantial evidence that suggests that mitochondrial dysfunction is a major contributor. Several mitochondrial toxins induce PD-like symptoms in humans and animal models (Sherer et al., 2002; Bové et al., 2005) and systemic mitochondrial dysfunction appears to be a feature of a large proportion of PD sufferers (Schapira, 2007). Several genes involved in rare heritable forms of Parkinsonism have been implicated in mitochondrial biology, including the PINK1 and parkin genes (Valente et al., 2004; Bonifati et al., 2005; Silvestri et al., 2005; Deng et al., 2005; Gandhi et al., 2006; Abou-Sleiman et al., 2006; Yang et al., 2006, 2008; Gautier et al., 2008; Wood-Kaczmar et al., 2008; Morais et al., 2009).

PINK1 is the first mitochondrial localized AREP associated protein kinase, and the putative role suggests that it might account for the mechanism related to mitochondrial function in PD (Valente et al., 2004). PINK1 may have a modulating effect on mitochondrial-dependent cell death pathways. In the present study, we investigated the role of PINK1 in basal and MPP⁺-induced neuronal apoptosis, oxidative stress and mitochondrial dysfunction. Our results established neuronal cells which can stably express wild-type and R492X mutated PINK1 protein. Under basal conditions, stable expression of the R492X mutated PINK1 protein, unlike the wild-type PINK1 protein, causes mitochondrial cyt.C release and cellular apoptosis, induce a decrease in mitochondrial membrane potential and mitochondrial respiratory complex I activity. When treated

Fig. 8 – Mitochondrial ultramicrostructure of each group in media without MPP⁺. a: control (SH-SY5Y cell); b: empty-vector; c: wild-type PINK1; d: R492X mutation. Panels a, b and c show both normal numbers and structure of mitochondria. Mitochondria in panel d appear to be deformed, swollen, and show a decrease in the mitochondrial cristae. However the number of mitochondria shows no obvious decrease. The arrows indicated mitochondria.
with 200 μM MPP⁺ for 24 h, R492X mutated PINK1 protein, unlike the wild-type PINK1 protein, augments in mitochondrial cyt.C release and cellular apoptosis rate, increases cellular ROS production, deteriorates in mitochondrial membrane potential and Complex I activity, causes severe mitochondrial swelling and attenuates mitochondrial cristae. From our results, it seems the R492X mutation is a dominant-negative or gain-of-function dominant mutation, and can induce cellular mitochondrial dysfunction and oxidative stress, especially with the environmental neurotoxin (MPP⁺). Our results also show that genetic defect and environmental neurotoxin can associate and collaborate to induce cellular mitochondrial dysfunction and oxidative stress.

One possible mechanism by which PINK1 mutations may lead to dopaminergic cell loss and Parkinsonism is by directly disrupting PINK1 enzymatic function. Initial biochemical analyses suggest that a subset of PD-associated missense mutations reduce kinase activity (Beilina et al., 2005). Kinase activity appears necessary for PINK1’s protective function, as inactivation of the kinase by mutation of the ATP-binding lysine in β-strand 3 is sufficient to prevent protection from apoptosis of staurosporine-treated SH-SY5Y neuroblastoma cells (Petit et al., 2005). R492X mutation affects conserved functional residues crucial for kinase activity according to bioinformatics, so it is probable that R492X mutation affects the mitochondrial dysfunction through its abnormal kinase activity.

4. Experimental procedures

4.1. Plasmid construction

Wild-type human PINK1 cDNA was amplified from fetus brain cDNA library by PCR with primers sense 5′-GAATTCCAC-CATGGCGGTGCAGACA-3’ and antisense 5′-GGATCC-GCCACAGGGCTGCCCTCA-3’. The R492X PINK1 cDNA was amplified from wild-type human PINK1 cDNA by PCR with primers sense 5′-GAATTCCACCATGGCGG-TGC-3’ and
antisense 5′-CGGGGATCTGGAGCAGTGGCCTGACC-3′. The PCR products were cloned into pcDNA3.1-myc-his-(−) B, flanked by EcoRI and BamHI restriction enzyme sites. The integrity and precision of the constructs were confirmed by sequencing analysis.

4.2. Cell culture conditions and establishment of stable transfected SH-SYSY cell lines

Human dopaminergic neuroblastoma SH-SYSY cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FBS) (GIBCO), 100 U/ml penicillin, and 200 μg/ml streptomycin at 37 °C under an atmosphere of 95% O₂ and 5% CO₂. The plasmids of empty-vector, wild-type and R492X mutated PINK1 were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After screening in G418 (800 μg/ml) for two weeks, transfected cells were used. Western blots were used to test for the expression of transfected genes. The primary antibodies used were mouse anti-myc monoclonal antibody (1:1000, Santa Cruz Biotechnology, USA). The verified cell lines were cultured with 1-Methyl-4-phenylpyridinium ion (MPP+) (200 μmol/l) for 24 h.

4.3. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured by the incorporation of a cationic fluorescent dye rhodamine 123 as previously described (Moriya et al., 2000). Briefly, after a 30 min incubation in DMEM with 10% FBS, the cells were changed to serum-free medium containing 10 μmol rhodamine 123 and incubated at 37 °C for 15 min. The cells were then collected and the fluorescence intensity was analyzed with a Hitachi F-4500 spectrophotofluorimeter (490 nm excitation and 515 nm emission) with 15 min after incubation.

4.4. Isolation of mitochondria and cytoplasmic protein

The mitochondrial isolation procedures were conducted using established methodologies as published (Sullivan et al., 2003). Briefly, the cells were precipitated with a centrifuge at 300 g 4 °C for 5 min, then resuspended in mitochondrial isolation buffer (0.5 ml) (215 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin, 1 mM EGTA, 20 mM HEPES, pH 7.2). The mitochondria were purified by differential centrifugation at 1300 g for 5 min to pellet nuclei and unbroken cells. The supernatant was then centrifuged at 13,000 g for 10 min to pellet the mitochondria. The pellet was resuspended in EGTA-free isolation buffer and centrifuged at 10,000 g for 10 min. The resulting pellet was isolated mitochondria and the supernatant contained protein in the cytoplasm. The pellet was resuspended in EGTA-free isolation buffer (10 mg/ml).

4.5. Measurement of mitochondrial complex I

We measured complex I with a spectrophotometer at 600 nm as previously described (Janssen et al., 2007). An incubation volume of 1 mL contains potassium phosphate (25 mmol/L), BSA (3.5 g/L), DCIP (60 mol/L), decylubiquinone (70 mol/L), antimycin-A (1.0 mol/L), and NADH (0.2 mmol/L), pH 7.8. Decylubiquinone and antimycin-A were dissolved in dimethyl sulfoxide (17.5 mmol/L and 1.0 mmol/L, respectively). We prepared a stock solution of 80 g/L BSA in potassium phosphate buffer (5 mmol/L, pH 7.4; then diluted to 70 g/L and stored in 1 mL aliquots at 30 °C. Of this solution, 50 μL was added to a final reaction volume of 1 mL. We preincubated an aliquot of 2.5 to 10 μL mitochondrial suspension from cultured cells at 37 °C in 960 μl incubation mixture without NADH. After three min, we added 20 μL NADH (10 mmol/L) and measured the absorbance at 30 s intervals for three min at 37 °C. Then we added 1 mmol/L rotenone 1.0 μL with dimethyl sulfoxide (DMSO) and measured the absorbance again at 30 s intervals for three min. Complex I activity was expressed as U/g protein, in which 1 U complex I activity equals a reduction of 1 μmol DCIP per minute.

4.6. Measurement of intracellular reactive oxidative species

When cells were grown to approximately 90% confluence, cells were incubated at 37 °C and CO₂ for 30 min. Fresh reconstituted, cell-permeable nonfluorescent 2′,7′-dichloro-dihydrofluorescein diacetate (DCFH-DA) (Invitrogen) was loaded directly into the growth plate to equal a final concentration of 10 μM solution in DMEM. After incubation, cells were washed with PBS then resuspended in PBS alone. Samples were then analyzed immediately on a FACSCalibur flow cytometer utilizing excitation at 488 nm and fluorescein isothiocyanate (FITC) filter detection parameters. Cell Quest Pro software (BD Biosciences) was used for both data acquisition and analysis to produce histogram plots and median peak values.

4.7. Western blotting for mitochondrial and cytoplasmic cytochrome C contents

The protein was quantified by Pierce Biotechnology-BCA Protein Assay Reagent (PIERCE). An aliquot (50 μg as protein) of the supernatant was loaded onto a 12% SDS gel and transferred to a PVDF membrane (Bio-Rad). After the PVDF membrane was incubated in 10 mM TBS with 1.0% Tween 20 and 10% dehydrated skim milk to block nonspecific protein binding, the membrane was incubated with primary antibodies overnight at 4 °C in the rocking bed. The primary antibodies used were mouse anti-cytochrome monoclonal antibody (1:1000, Santa Cruz Biotechnology, USA), and mouse anti-actin monoclonal antibody (1:5000, Santa Cruz Biotechnology, USA), and mouse antimyacin-A (1.0 mol/L). We added 1 mmol/L rotenone 1.0 μL and measured the absorbance at 30 s intervals for three min at 37 °C. Then we added 1 mmol/L rotenone 1.0 μL with dimethyl sulfoxide (DMSO) and measured the absorbance again at 30 s intervals for three min. Complex I activity was expressed as U/g protein, in which 1 U complex I activity equals a reduction of 1 μmol DCIP per minute.

4.8. Flow cytometric analysis using Annexin V and PI

Approximately 1×10⁶ cells were centrifuged (120 g, 5 min) to remove the medium, washed three times with binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂) and stained with 5 μl of 25 μg/ml Annexin V-FITC. After 15 min of incubation, cells were washed with binding buffer, incubated with PI (final concentration, 3.7 μM) for 10 min, and then kept on ice without exposure to light prior to analysis by flow cytometry analysis. Fifteen thousand events were analyzed per test in list.
mode. Annexin V and PI emissions were detected in the FL1-H and FL2-H channels of a FACS Calibur flow cytometer (BD Biosciences), using emission filters of 525 nm (FL1-H) and 575 nm (FL2-H).

4.9. Electron microscope

Cells were fixed in freshly prepared 2.5% glutaraldehyde 4–6 h. Cells were washed with PBS and immersed in 1% osmic acid for 2 h. Cells were then washed with PBS, dehydrated gradually with alcohol, infiltrated with epoxypropane and araldite, mounted on pure araldite and left overnight. Sections (60–70 nm) were stained in 2% uranyl acetate and lead citrate, and analyzed by Hitachi H-7500 electron microscope.

4.10. Statistical analysis

Each experiment above has been repeated at least three times. The results are presented as Mean±SD. Significant changes were assessed using Student’s t-test and one-way ANOVA, and p-values <0.05 were considered statistically significant.

Acknowledgments

This work was supported by grant 2006cb500700 from the Major State Basic Research Development Program of China (973 Program)(to Dr. Beisha Tang), grant 2006AA02A408 from the National “863” High-Tech Research and Development Program of China (to Dr. Beisha Tang), grants 30570638, 30900469 from the National Natural Science Foundation of China (to Drs. Jifeng Guo).}

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