

RESEARCH

IGF-1 protects SH-SY5Y cells against MPP⁺-induced apoptosis via PI3K/PDK-1/Akt pathway

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Abstract

Insulin-like growth factor (IGF)-1 is a well-known anti-apoptotic pro-survival factor and phosphatidylinositol-3-kinase (PI3K)/Akt pathway is linked to cell survival induced by IGF-1. It is also reported that Akt signaling is modulated by 3-phosphoinositide-dependent kinase-1 (PDK1). In the current study, we investigated whether the anti-apoptotic effect of IGF-1 in SH-SY5Y cells exposed to 1-methyl-4-phenylpyridinium (MPP⁺) is associated with the activity of PI3K/PDK1/Akt pathway. Treatment of cells with IGF-1 inhibited MPP⁺-induced apoptotic cell death. IGF-1-induced activation of Akt and the protective effect of IGF-1 on MPP⁺-induced apoptosis were abolished by chemical inhibition of PDK1 (GSK2334470) or PI3K (LY294002). The phosphorylated levels of Akt and PDK1 were significantly suppressed after MPP⁺ exposure, while IGF-1 treatment completely restored MPP⁺-induced reductions in phosphorylation. IGF-1 protected cells from MPP⁺ insult by suppressing intracellular reactive oxygen species (ROS) production and malondialdehyde levels and increasing superoxide dismutase activity. Mitochondrial ROS levels were also increased during MPP⁺ exposure, which were attenuated by IGF-1 treatment. In addition, IGF-1-treated cells showed increased activities of succinate dehydrogenase and citrate synthase, stabilization of mitochondrial transmembrane potential, increased ratio of Bcl-2 to Bax, prevention of cytochrome c release and inhibition of caspase-3 activation with PARP cleavage. Furthermore, the protective effects of IGF-1 on oxidative stress and mitochondrial dysfunction were attenuated when cells were preincubated with GSK2334470 or LY294002. Our data suggest that IGF-1 protects SH-SY5Y cells against MPP⁺-associated oxidative stress by preserving mitochondrial integrity and inhibiting mitochondrial apoptotic cascades via the activation of PI3K/PDK1/Akt pathway.

Key Words

- ▶ IGF-1
- ▶ apoptosis
- ▶ PDK1
- ▶ Akt
- ▶ MPP⁺

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. PD affects 1% of the population worldwide after the age of 65 years (1). The disease is characterized by a progressive dopaminergic neurodegeneration in the substantia nigra

pars compacta (SNpc) and the loss of projecting nerve fibers in the striatum (2), which results in dysfunction of extrapyramidal motor system. Although the precise molecular mechanisms underlying dopaminergic neurodegeneration are not still elucidated, numerous

lines of evidence indicate that mitochondrial dysfunction and oxidative stress-mediated apoptosis play an important role in the pathogenesis of PD (3, 4). Some neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), paraquat and rotenone, are used to model PD and most of these agents are associated with enhanced apoptotic cell death (2). MPTP is the most widely studied neurotoxin in this class, because it selectively destroys dopaminergic neurons in mouse SN. After systemic administration, MPTP is metabolized to the active toxic molecule 1-methyl-4-phenylpyridinium ion (MPP⁺), which inhibits the mitochondrial electron transport chain complex I, leading to impaired mitochondrial energy metabolism and enhanced ROS production (5). The resultant oxidative stress and damaged mitochondrial energy metabolism cause broad dopaminergic neurodegeneration in the SNpc (4). Furthermore, it has been shown that MPP⁺ decreases mitochondrial DNA content (6), resulting in mitochondrial dysfunction and further aggravation of the oxidative stress and subsequent apoptotic cell death (7). Oxidative stress also plays a key role in the depolarization of mitochondrial membrane potential ($\Delta\psi_M$) and the release of cytochrome c from the mitochondria into the cytoplasm (4). Upon entry into the cytosol, cytochrome c complexes with the caspase-activating protein Apaf-1, leading to the activation of apoptotic caspase cascade (8). It has been shown that MPP⁺ produces oxidative stress, mitochondrial dysfunction and apoptosis in human neuroblastoma cell line SH-SY5Y (9, 10, 11). Therefore, the molecules targeting pharmacological intervention of mitochondrial dysfunction and oxidative stress-dependent apoptosis induced by MPP⁺ may be promising candidates for neuroprotective agents in PD.

Insulin-like growth factor (IGF)-1, a small peptide composed of 70 amino acids, is a well-known neurotrophic and anti-apoptotic pro-survival factor and plays an important role in the regulation of metabolism, cellular function, growth and differentiation (12). The biologic actions of IGF-1 are mediated via the IGF-1 receptor (IGF-1R), a tyrosine kinase receptor (13). When IGF-1 binds to its corresponding receptor, it triggers downstream signaling pathways, of which the most important is the phosphatidylinositol-3-kinase (PI3K)/Akt pathway (14). The PI3K/Akt pathway has been implicated in the regulation of cell survival (15) and dysregulation of Akt signaling has been observed in PD *in vivo* and *in vitro* models (16, 17, 18). It is known that 3-phosphoinositide-dependent kinase-1 (PDK1) phosphorylates the activation loop of Akt at Thr308 by

docking to phosphatidylinositol-3,4,5-triphosphate at the plasma membrane (19, 20). However, to the best of our knowledge, the role of PI3K/PDK1 pathway in IGF-1-mediated activation of Akt has never been investigated.

IGF-1 could become a potential beneficial therapeutic strategy by improving mitochondrial function, decreasing oxidative stress and preventing apoptosis in a PI3K/Akt-dependent manner (21, 22). High expression of IGF-1R in dopaminergic neurons of the SN (23) and increased loss of SN dopaminergic neurons after MPTP injection in IGF-1R^{+/-} mice (24) suggest that IGF-1 may act as a neuroprotective factor in PD. Indeed, IGF-1 has been shown to act as a survival factor and inhibit apoptosis in PC12 cells (25) and SH-EP1 cells (26) against MPP⁺ insult. IGF-1 has also been known to successfully reduce the damage after 6-OHDA-induced toxicity in rodent neuronal cultures (27). Based on these observations, it is likely that survival-promoting effect of IGF-1 via the Akt pathway may be at least partly regulated by the activation of PDK1.

In the current study, we hypothesized that the activities and functions of PI3K/PDK1 pathway, upstream of Akt, would be important in the anti-apoptotic effects of IGF-1 against MPP⁺-induced cell injury. Therefore, to test this hypothesis, we examined the effect of IGF-1 on the survival of SH-SY5Y cells exposed to MPP⁺ insult. SH-SY5Y cells, a cell line from a human neuroblastoma, have many characteristics of dopaminergic neurons, and these cells have been widely used as a model of studying PD-related neurotoxicity, including MPP⁺ (28). To determine the mechanism of IGF-1-induced anti-apoptotic effect, selective inhibitors of PDK1 and PI3K were employed. We also investigated the role of PI3K/PDK1/Akt pathway in the inhibitory effect of IGF-1 on MPP⁺-induced oxidative stress-mediated apoptosis and mitochondrial dysfunction.

Materials and methods

Materials

Human recombinant IGF-1 was obtained from Sigma Chemical. Dulbecco's modified Eagle's medium (DMEM)/F12 was from Gibco/Invitrogen. Primary antibodies to caspase-3, cleaved poly(ADP-ribose) polymerase (PARP), Bcl-2, Bax, cytochrome c, PDK1, Akt and were obtained from Cell Signaling Technology. Bax was purchased from Abcam and β -actin was from Santa Cruz Biotechnology. LY294002 was obtained from Sigma and GSK2334470 was procured from Tocris (Ellisville, MO, USA). All tissue culture reagents were obtained from

Gibco/Invitrogen, and all other reagents were obtained from Sigma unless otherwise indicated.

Cell cultures and treatments

SH-SY5Y human neuroblastoma cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum, 100U/mL penicillin and 100mg/mL streptomycin in a humidified atmosphere of 5% CO₂. Cells were serum starved for 1 h before treatment with IGF-1. To determine if IGF-1 protects SH-SY5Y cells from MPP⁺-induced insult, cells were pretreated with IGF-1 (10 nM) or vehicle (saline) for 1 h. Then, cells were exposed to 1 mM MPP⁺ or vehicle for 24 h. Experiments were also performed by adding the following pharmacological inhibitors to culture media, GSK2334470 (2 μM) or LY294002 (4 μM). To investigate the effect of IGF-1 on the PI3K/PDK1/Akt pathway, cells were treated with IGF-1 or vehicle for 1 h in the absence or presence of pharmacological inhibitors and assayed by Western blotting described below.

Assessment of cell death and apoptosis

Cells viability was measured by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell death was assessed by the measurement of lactate dehydrogenase (LDH) released from damaged cells in the extracellular fluid using a Cytotoxicity Detection Kit (LDH) (Roche Diagnostics). Histone-complexed DNA fragments were quantified by the Cell Death Detection ELISA (Roche) according to the manufacturer's protocol.

Detection of intracellular and mitochondrial ROS production

Intracellular ROS levels were assessed using the ROS-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFDA) (Sigma-Aldrich). SH-SY5Y cells were incubated with 2.5 μM DCFDA for 15 min. For the detection of mitochondrial ROS production, we used the mitochondrial-sensitive dye MitoSOX Red (Molecular Probes). The cells were incubated with 3 μM MitoSOX Red for 10 min. Fluorescence was captured using a 630× objective lens on a Carl Zeiss LSM 700 Meta confocal microscope (485-nm excitation and 535-nm emission for DCFDA; 510-nm excitation and 580-nm emission for MitoSOX Red). DCFDA and MitoSOX Red fluorescences were quantified from cells of interest using the measurement functions on the Carl Zeiss confocal software.

Detection of oxidative stress markers malondialdehyde (MDA) and superoxide dismutase (SOD)

The lipid peroxidation was determined by measuring the levels of malondialdehyde (MDA) using a Lipid Peroxidation (MDA) Colorimetric/Fluorometric Assay Kit (BioVision), according to the manufacturer's instructions. Total superoxide dismutase (SOD) activities were assessed using a SOD assay kit – WST (Dojindo, Kumamoto, Japan) following the manufacturer's instructions.

Assay of mitochondrial enzyme activities

Quantitative assays of the activities of specific mitochondrial enzymes, succinate dehydrogenase (SDH) and citrate synthase (CS) were performed in cell homogenates using Succinate Dehydrogenase Activity Colorimetric Assay Kit (BioVision) and Citrate Synthase Activity Colorimetric Assay Kit (BioVision), respectively, according to the manufacturer's instructions.

Assessment of mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential was assessed with the fluorescent dye JC-1 Mitochondrial Membrane Potential Detection kit (Stratagene) and confocal microscopy following the manufacturer's instructions. In brief, cells were incubated with 1×JC-1 reagent solution at 37°C for 15 min. Culture slides were washed and mounted with PBS, and confocal images were acquired by the Carl Zeiss LSM 700 Meta confocal microscope. The red-to-green fluorescence ratio was quantified from cells of interest using the measurement functions on the confocal microscopy software.

Western blotting

Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 140 mM NaCl, 1% (w/v) Nonidet P-40, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF and 10 μg/mL aprotinin. Cell lysates were separated by 8 or 12% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Millipore). For the detection of Bax, Bcl-2 and cytochrome c, cells were fractionated into cytosol and mitochondria using a Mitochondria Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The membranes were soaked in blocking buffer

(1× Tris-buffered saline, 0.1% Tween 20, 5% nonfat dry milk) for 1 h and incubated overnight at 4°C with the primary antibody. Blots were developed using a peroxidase-conjugated anti-rabbit and anti-mouse IgG and a chemiluminescent detection system (Santa Cruz Biotechnology). The bands were visualized using a ChemiDoc XRS system (Bio-Rad) and quantified using Quantity One imaging software (Bio-Rad). The p-PDK1 (Ser241) and p-Akt (Thr 308 and Ser473) band intensities were normalized to PDK1 and Akt band intensities, respectively. The intensities of cleaved PARP were by the β-actin band intensity, the intensities of Bax were by COX IV intensities and the intensities of Bcl-2 and cytochrome c were adjusted by the intensities of α-tubulin intensities.

Statistical analysis

Data are presented as the mean ± s.e.m. (n=4/treatment). Each experiment was repeated three times, giving essentially identical results. Statistical analysis between groups was performed using 1-way ANOVA and the Holm–Sidak method for multiple comparisons using SigmaStat for Windows, version 3.10 (Systat Software, Inc., Point Richmond, CA, USA). P<0.05 was considered statistically significant.

Results

Effect of IGF-1 on MPP⁺-induced cytotoxicity

To examine the effect of MPP⁺ on SH-SY5Y cell viability, we treated cells with increasing concentrations of MPP⁺ for 24 h. MPP⁺ caused significant reduction in MTT values (Fig. 1A) and remarkable increase in LDH activity (Fig. 1C) in a concentration-dependent manner. A final concentration of 1 mM MPP⁺ was considered as an optimal concentration for the induction of cytotoxic effect on SH-SY5Y cells, and this dose was used for the rest of the experiments. In order to investigate whether IGF-1 may act as a survival factor for SH-SY5Y cells, we assayed the effect of IGF-1 on cell death induced by MPP⁺ insult. As shown in Fig. 1B, MPP⁺-induced cell death was partially but significantly attenuated by pretreatment of cells with 10 nM IGF-1. The LDH activity assay showed that pretreatment of cells with IGF-1 suppressed MPP⁺-induced release of LDH (Fig. 1D). SH-SY5Y cells exposed to MPP⁺ have been observed to undergo apoptosis (9, 10, 11). Therefore, we investigated the effects of IGF-1 on MPP⁺-induced apoptosis in SH-SY5Y cells. The percentage of DNA fragmentation was significantly increased MPP⁺ in a concentration-dependent manner (Fig. 1E). Pretreatment of cells with IGF-1 partially but

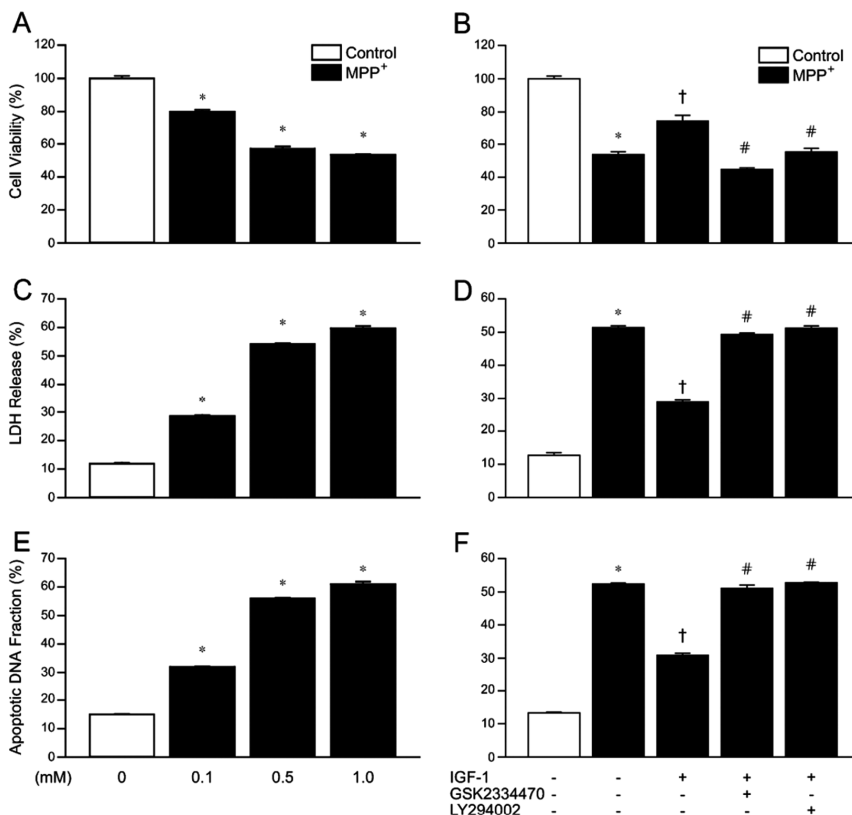


Figure 1 Effect of IGF-1 on MPP⁺-induced cytotoxicity. (A, C and E) SH-SY5Y cells were treated with increasing concentration of MPP⁺ for 24 h. (B, D and F) Cells were pretreated with IGF-1 (10 nM) for 1 h and then cells were exposed to 1 mM MPP⁺ for 24 h. (A and B) Cell viability measured by the MTT assay. (C and D) LDH release was assessed by the LDH assay kit. (E and F) DNA fragmentation, a marker of apoptosis, measured by ELISA. Values are mean ± s.e.m. (n=4). Each experiment was repeated twice. *P<0.05 vs vehicle-treated control and †P<0.05 vs MPP⁺-treated cells.

significantly prevented apoptosis induced by MPP⁺ (Fig. 1F). The PI3K/PDK1/Akt pathway may be involved in the IGF-1-induced survival-promoting and anti-apoptotic effects in SH-SY5Y cells exposed to MPP⁺ insult. To further verify this hypothesis, we tested whether pretreatment of cells with the PDK1 inhibitor GSK2334470 or the PI3K inhibitor LY294002 impaired the effect of IGF-1 against MPP⁺. We found that all these inhibitors significantly blocked the survival-promoting (MTT assay and LDH release; Fig. 1B and D) and anti-apoptotic effects (DNA fragmentation; Fig. 1F) of IGF-1 on SH-SY5Y cells. These data suggest that IGF-1 inhibits apoptotic cell death induced by MPP⁺ via the activation of PI3K/PDK1/Akt pathway.

Effect of IGF-1 on Akt phosphorylation

Next, we examined the effect of IGF-1 on Akt phosphorylation in SH-SY5Y cells. Treatment of cells with 10 nM IGF-1 significantly increased phosphorylation of Akt at both Thr308 (Fig. 2A) and Ser478 (Fig. 2B), as previously reported (29). Since Akt is activated via the PI3K/PDK1 pathway (30), we further determined this signaling pathway was involved in IGF-1-induced Akt activation. We found that pretreatment of cells with the PDK1 inhibitor GSK2334470 or the PI3K inhibitor LY294002 significantly attenuated IGF-1-induced phosphorylation of Akt at both sites, suggesting that PI3K/PDK1 pathway contributes to IGF-1-induced activation of Akt.

Effect of IGF-1 on MPP⁺-induced suppression of PDK1 and Akt phosphorylation

MPP⁺ is known to suppress the phosphorylation of Akt in SH-SY5Y cells (18, 31). Similar to this finding, the treatment of cells with MPP⁺ decreased the levels of phosphorylated Akt at both residues after 24-h exposure of MPP⁺ (Fig. 3A). In addition, we found that phosphorylated levels of PDK1 were also decreased by MPP⁺ (Fig. 3B). In contrast, phosphorylated levels of Akt and PDK1 were significantly increased when cells were pretreated with IGF-1 (Fig. 3A and B). Equal amounts of protein were confirmed by immunodetection of β -actin.

Effect of IGF-1 on MPP⁺-induced oxidative stress

To determine whether IGF-1 may inhibit MPP⁺-induced toxicity by suppression of ROS generation, we examined

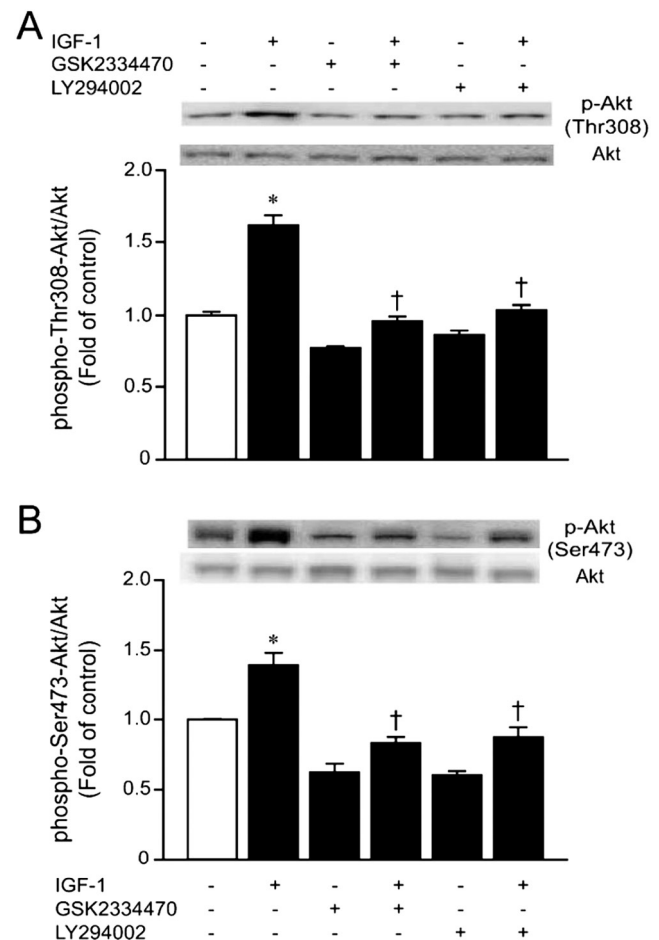


Figure 2

Effect of IGF-1 on Akt phosphorylation at Thr308 (A) and Ser478 (B). PI3K/PDK1 pathway mediates IGF-1-induced phosphorylation of Akt at both Thr308 and Ser478. Cells were preincubated with 2 μ M GSK2334470 or 4 μ M LY294002 for 30 min, then treated with 10 nM IGF-1 for 1 h and assayed by Western blotting as above. The phospho-Akt band intensity was normalized to Akt intensity and expressed as relative band intensity. Values are mean \pm s.e.m. ($n=4$). Each experiment was repeated twice. * $P<0.05$ vs control and † $P<0.05$ vs IGF-1-treated cells.

the changes in intracellular ROS levels. As shown in Fig. 4A, compared with saline-treated controls, cells treated with MPP⁺ showed increased cytosolic ROS production. In contrast, pretreatment of cells with IGF-1 significantly reduced the increase in DCF fluorescence induced by MPP⁺. MDA, one of the terminal products of polyunsaturated fatty acid peroxidation in cells, is used for lipid peroxidation. As shown in Fig. 4B, the levels of MDA in MPP⁺-treated cells were significantly increased than those of the vehicle-treated cells, indicating that MPP⁺ induced an elevated oxidative stress in SH-SY5Y cells. By contrast, IGF-1 treatment significantly attenuated the increase in MDA levels induced by MPP⁺. To further examine the underlying mechanisms of the antioxidant

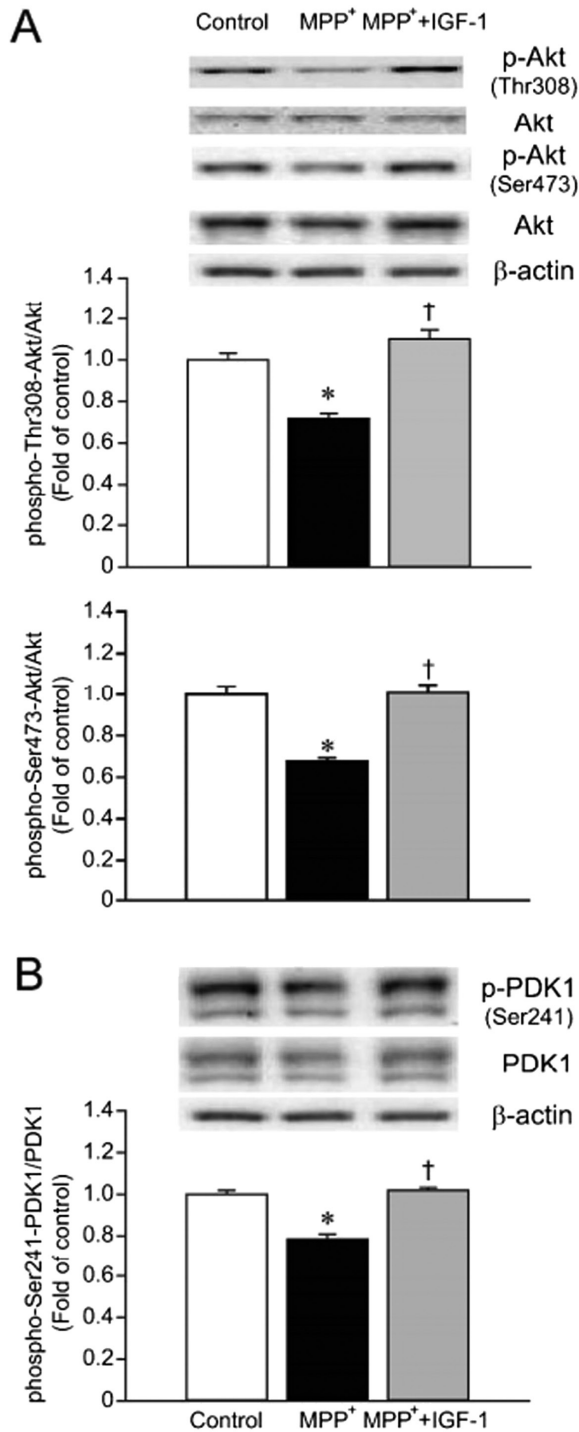


Figure 3
Effect of IGF-1 on MPP⁺-induced suppression of Akt and PDK1 phosphorylation. SH-SY5Y cells were pretreated with 10 nM IGF-1 for 1 h. Then cells were exposed to 1 mM MPP⁺ for 24 h. Protein lysates were prepared and assayed by Western blotting using specific anti-phospho-Akt (Thr308 and Ser473) and anti-Akt antibodies (A) or anti-phospho-PDK1 (Ser241) and anti-PDK1 antibodies (B). The band intensities of phospho-forms were normalized to the band intensities of total-forms respectively, and they were expressed as fold of control. Values are mean \pm s.e.m. ($n=4$). Each experiment was repeated twice. * $P<0.05$ vs vehicle-treated control and † $P<0.05$ vs MPP⁺-insulted, vehicle-treated cells.

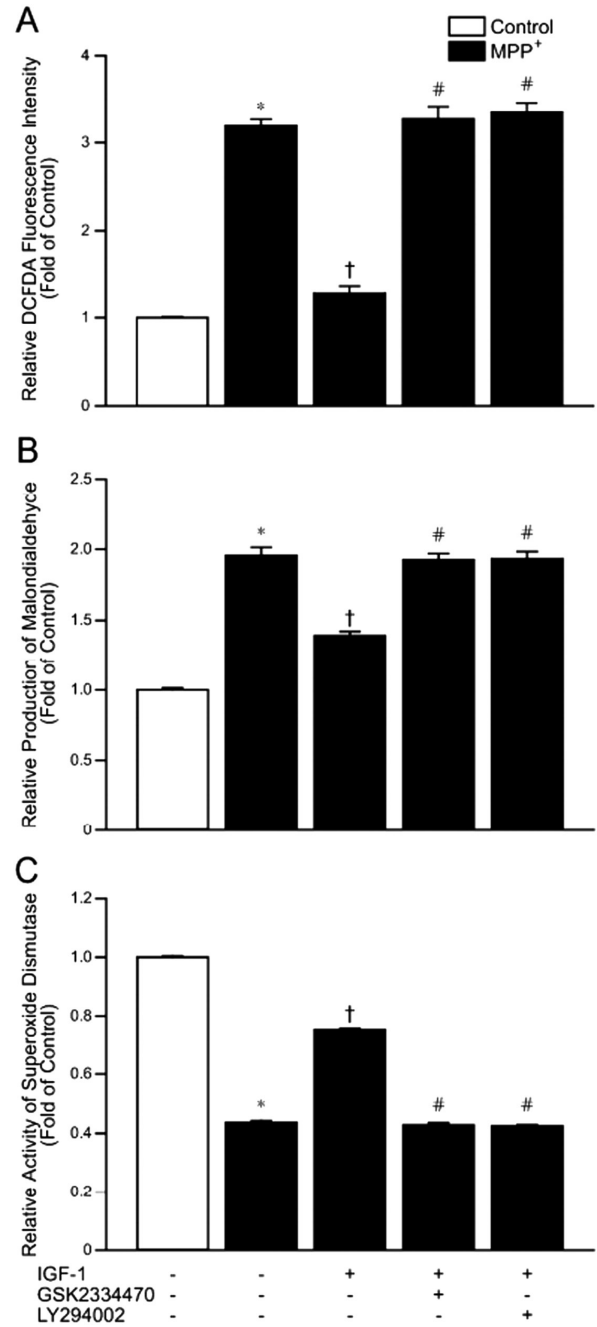


Figure 4
Effect of IGF-1 on MPP⁺-induced oxidative stress. SH-SY5Y cells were preincubated with 2 μ M GSK2334470 or 4 μ M LY294002 for 30 min and then treated with 10 nM IGF-1 for 1 h. Then, cells were exposed to 1 mM MPP⁺ for 24 h. (A) ROS levels were determined using confocal microscopy on cells stained with the ROS-sensitive fluorescent dye 2', 7'-dichlorofluorescein diacetate (DCF-DA). DCF fluorescence was quantified from cells of interest using the measurement functions on the Carl Zeiss LSM 700 Meta software. The results are representatives of three independent experiments. (B) Malondialdehyde (MDA) levels were measured by thiobarbituric acid method using a MDA assay kit. (C) Superoxide dismutase (SOD) activities were assessed using a SOD assay kit. * $P<0.05$ vs vehicle-treated control, † $P<0.05$ vs MPP⁺-insulted, vehicle-treated cells and # $P<0.05$ vs MPP⁺-insulted, IGF-1-treated cells.

properties of IGF-1 against MPP⁺ insult, we assessed the activity of antioxidant enzyme SOD. We found that exposure of cells to MPP⁺ resulted in a significant reduction of SOD activity, whereas IGF-1 prevented MPP⁺-induced decrease in SOD activity (Fig. 4C). These results suggest that IGF-1 attenuated MPP⁺-induced oxidative stress by decreasing ROS production and MDA levels and increasing SOD activity. Moreover, pretreatment of cells with GSK2334470 or LY294002 significantly attenuated IGF-1-induced inhibition of intracellular ROS production and MDA levels in SH-SY5Y cells subjected to MPP⁺ insult (Fig. 4A and B), suggesting the PI3K/PDK1/Akt pathway contributes to IGF-1-induced suppression of ROS levels and MDA contents. IGF-1-induced augmentation of SOD activity was also blocked when cells were pretreated with the PDK1 inhibitor or the PI3K inhibitor (Fig. 4C).

Effect of IGF-1 on MPP⁺-induced mitochondrial dysfunction

Next, we assessed mitochondrial ROS levels using MitoSOX Red as a mitochondrial superoxide indicator. Compared with vehicle-treated control, MPP⁺ increased mitochondrial ROS levels, whereas IGF-1 significantly reduced the increase in MitoSOX Red fluorescence induced by MPP⁺ (Fig. 5A). IGF-1 failed to inhibit MPP⁺-

induced increase in mitochondrial ROS levels when cells were pretreated with GSK2334470 or LY294002. Next, we measured the activities of key enzymes in the Krebs cycle pathway, SDH and CS. We found a robust reduction in the activity of SDH and modest diminution in the CS activity in the MPP⁺-treated cells (Fig. 5B and C). In contrast, IGF-1 treatment significantly attenuated the inhibitory effect of MPP⁺ on citric acid cycle enzymes. The attenuating effect of IGF-1 on MPP⁺-induced suppression of SDH and CS activities was abolished when cells were pretreated with GSK 2334470 or LY294002 (Fig. 5C and D). It has been reported that MPP⁺ destabilizes $\Delta\psi_M$ due to the opening of the mitochondrial permeability transition (PT) pore (32, 33), and IGF-1 attenuates apoptosis by preventing mitochondrial depolarization (34, 35). Therefore, we examined the effect of IGF-1 on $\Delta\psi_M$ using JC-1, which exhibits potential-dependent accumulation in the mitochondria. As shown in Fig. 5D, it was observed that $\Delta\psi_M$ was significantly decreased as indicated by the marked decreases in red/green fluorescence intensity ratio when cells were exposed to MPP⁺. In contrast, pretreatment of cells with IGF-1 partially but significantly attenuated the MPP⁺-induced decrease in the ratio (Fig. 5D). Moreover, the mitochondrial membrane-stabilizing effect of IGF-1 was inhibited by the pretreatment of cells with GSK2334470 or LY294002 (Fig. 5D).

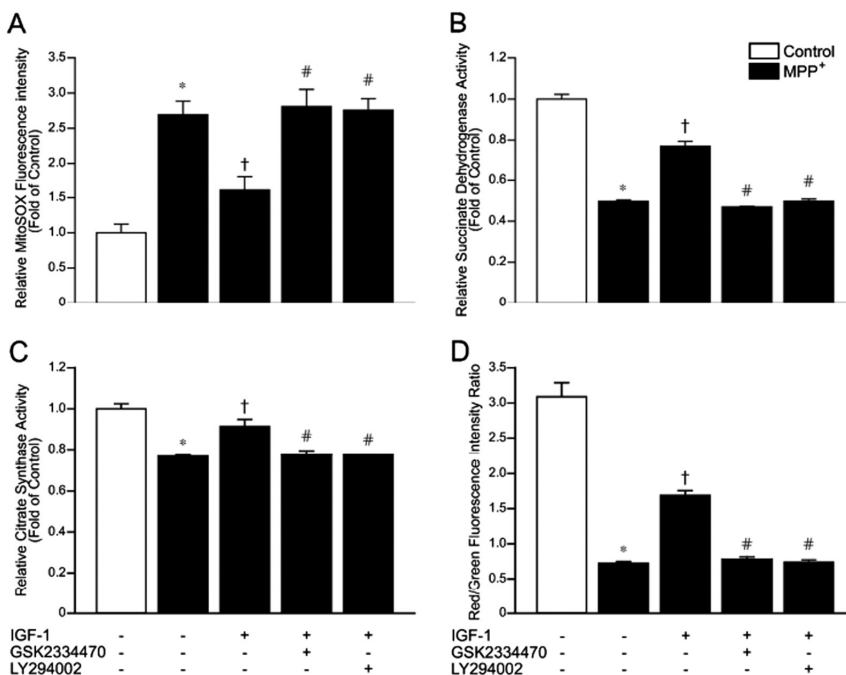


Figure 5

Effect of IGF-1 on MPP⁺-induced mitochondrial dysfunction. SH-SY5Y cells were preincubated with 2 μ M GSK2334470 or 4 μ M LY294002 for 30 min and then treated with 10 nM IGF-1 for 1 h. Then, cells were exposed to 1 mM MPP⁺ for 24 h. (A) Mitochondrial ROS levels were determined using confocal microscopy on cells stained with MitoSOX Red, a mitochondrial superoxide indicator. MitoSOX Red fluorescence was quantified from cells of interest using the measurement functions on the Carl Zeiss LSM 700 Meta software. (B) Succinate dehydrogenase (SDH) activity was assessed by measuring 2,6-dichlorophenol-indophenol-dependent reduction by succinate using a SDH assay kit. (C) Citrate synthase (CS) activity was assessed by measuring the conversion rate of 5,5'-dithiobis-(2-nitrobenzoic acid) to 2-nitro-5-thiobenzoate in the presence of oxaloacetate and acetyl-CoA using a CS assay kit. (D) The $\Delta\psi_M$ was determined by confocal microscopy using JC-1 dye. The ratio of red-to-green fluorescence was quantified in cells of interest using the measurement functions on the Carl Zeiss LSM 700 Meta software. * $P < 0.05$ vs vehicle-treated control, † $P < 0.05$ vs MPP⁺-insulted, vehicle-treated cells and # $P < 0.05$ vs MPP⁺-insulted, IGF-1-treated cells.

Effect of IGF-1 on MPP⁺-induced apoptosis at the mitochondrial level

To examine changes in the protein levels of Bax, we performed Western blots using cytosolic and mitochondrial fractions and found that MPP⁺ increased Bax protein in the mitochondrial fraction of cells (Fig. 6A), suggesting a translocation from the cytosol to the mitochondria. IGF-1 significantly inhibited the MPP⁺-induced increase in Bax protein in the mitochondria (Fig. 6A). We also investigated the levels of Bcl-2 protein in the cytosol and found that Bcl-2 protein levels were decreased by MPP⁺, whereas IGF-1 treatment increased Bcl-2 (Fig. 6A), thereby significantly increasing the Bcl-2/Bax ratio (Fig. 6B). Additionally, IGF-1 failed to increase the ratio of Bcl-2/Bax when cells were pretreated with the PDK1 inhibitor or PI3K inhibitor (Fig. 6B). To investigate whether IGF-1 inhibits MPP⁺-induced release of cytochrome c, Western blots were carried out on cytosolic and mitochondrial fractions of SH-SY5Y cells. As shown in Fig. 6A and C, exposure of cells to MPP⁺ resulted in the release of cytochrome c from the mitochondria to the cytosol, which was significantly reduced by pretreatment with IGF-1. COX IV was detected in mitochondrial fractions but not in the cytosolic fractions (Fig. 6A). Then, we examined whether MPP⁺ induces activation of caspase-3 and found that MPP⁺

exposure resulted in an increase in protein levels of active caspase-3 (Fig. 6D). By contrast, IGF-1 significantly reduced MPP⁺-induced augmentation in active caspase-3 protein levels (Fig. 6D). We further investigated the effect of IGF-1 on MPP⁺-induced apoptosis by the evidence of cleavage of PARP and found that IGF-1 significantly attenuated PARP cleavage when cells were exposed to MPP⁺ (Fig. 6E). In addition, the pathways mediating the IGF-1-induced inhibition of cytochrome c release, caspase-3 activation and PARP cleavage were examined by exposure of cells to GSK2334470 or LY294002. As shown in Fig. 6C, D and E, these inhibitors attenuated the release of cytochrome c, activation of caspase-3 and cleavage of PARP.

Discussion

IGF-1 is a well-known anti-apoptotic/pro-survival factor for neuronal cells in various *in vitro* models of neurodegenerative diseases. In the current study, our results demonstrate that IGF-1 treatment reduces apoptotic cell death caused by MPP⁺ insult in SH-SY5Y cells as evidenced by the enhanced cell viability and the decreased apoptosis. Phosphorylated levels of PDK1 and Akt were significantly reduced after exposure to MPP⁺, while pretreatment of

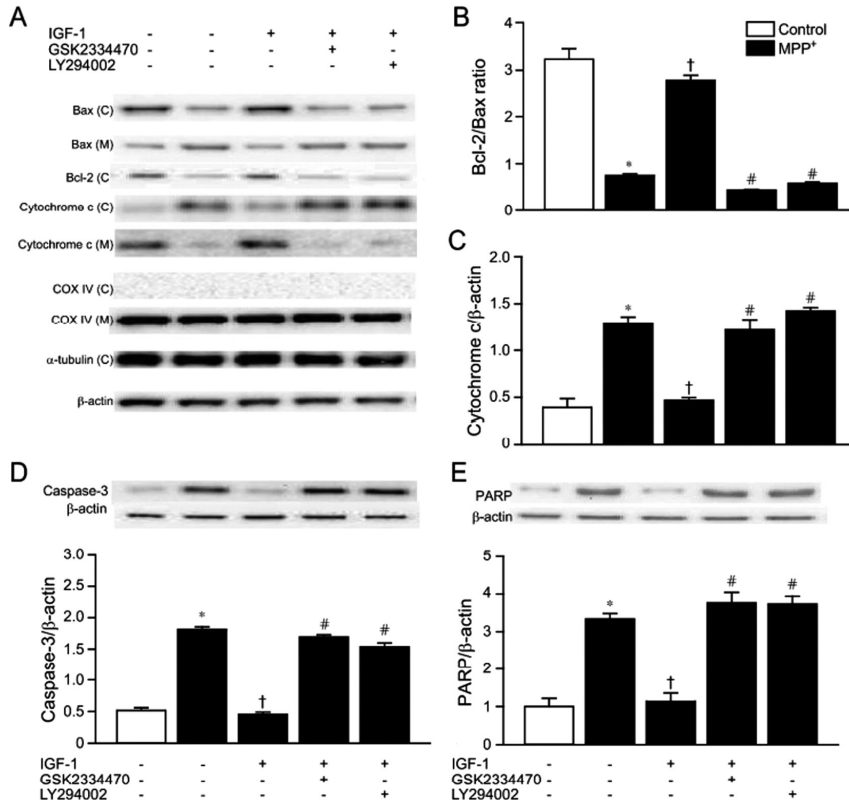


Figure 6
Effect of IGF-1 on protein levels of Bax, Bcl-2, cytochrome c, active caspase-3 and PARP in cells exposed to MPP⁺. SH-SY5Y cells were preincubated with 2 μM GSK2334470 or 4 μM LY294002 for 30 min and then treated with 10 nM IGF-1 for 1 h. Then, cells were exposed to 1 mM MPP⁺ for 24 h. (A) Representative Western blot images of Bax in the cytosolic and mitochondrial fraction, Bcl-2 in the cytosolic fraction, cytochrome c in the cytosolic and mitochondrial fraction, COX IV in the cytosolic and mitochondrial fraction, α-tubulin in the cytosolic fraction and β-actin are shown. (B and C) Bar graphs show the ratio of protein levels of Bcl-2 to Bax (B) and cytochrome c protein levels in the cytosolic fraction (C). (D) Representative Western blot images of active caspase-3 are shown in the upper insets. Bar graphs show the protein levels of active caspase-3. (E) Representative Western blot images of PARP are shown in the upper insets. Bar graphs show the protein levels of PARP. *P < 0.05 vs vehicle-treated control, †P < 0.05 vs MPP⁺-insulted, vehicle-treated cells and #P < 0.05 vs MPP⁺-insulted, IGF-1-treated cells.

cells with IGF-1 completely prevented MPP⁺-induced reductions in phosphorylation. We showed that IGF-1 suppresses oxidative stress and attenuates mitochondrial dysfunction induced by MPP⁺. In addition, we present evidence that IGF-1 alters the status of the Bcl-2 family of proteins, inhibiting cytochrome c release, caspase-3 activity, PARP cleavage and promoting the survival of SH-SY5Y cells exposed to MPP⁺. The protective effect of IGF-1 against MPP⁺ insult appears to be mediated through the activation of PI3K/PDK1/Akt signaling pathway.

It has been demonstrated that IGF-1 promotes differentiation and proliferation and sustains survival, preventing apoptosis of neuronal cells (36). The ability of IGF-1 to augment neuronal survival is associated with its apoptosis-inhibiting effect. In the current study, we found that IGF-1 prevented MPP⁺-induced cell death by inhibiting apoptosis. This observation is similar to the report of Sun *et al.* (25) and Wang *et al.* (26), where IGF-1 rescued PC12 and SH-EP1 cells from MPP⁺-induced apoptosis via the activation of Akt/glycogen synthase kinase-3 pathway. Binding of IGF-1 to its corresponding receptor results in the activation of the PI3K/Akt pathway, one of the main IGF-1R signaling cascades (14). We have shown that the protective effect of IGF-1 is mediated by the activation of the PI3K/Akt pathway because the PI3K inhibitor LY294002 blocked IGF-1-induced phosphorylation of Akt and the protective effect of IGF-1 against MPP⁺ insult, similar to previous papers demonstrating that IGF-1 inhibits apoptosis through the activation of this signaling pathway (37, 38, 39). In order to determine the upstream signaling event leading to Akt activation in the IGF-1-treated SH-SY5Y cells, we examined the phosphorylation of Akt and found that IGF-1 led to a potent and sustained activation of Akt. Upstream kinases, such as PI3K, mammalian target of rapamycin complex, and PDK1 modulate Akt signaling. Previous studies suggested that PDK1 is required for Akt-induced inhibition of apoptosis (40, 41). In the current study, the highly potent and selective PDK1 inhibitor GSK2334470 significantly suppressed IGF-1-induced phosphorylation of Akt and the anti-apoptotic effect of IGF-1. Furthermore, IGF-1-induced attenuation of oxidative stress and restoration of mitochondrial function were inhibited when cells were pretreated with GSK2334470 or LY294002. These data suggest that PI3K/PDK1 pathway is involved in IGF-1-induced Akt activation, and the survival-promoting effect of IGF-1 is mediated via PI3K/PDK1/Akt signaling pathway.

It has been reported that Akt and phosphorylated Akt-containing SNpc dopaminergic neurons are severely

depleted in the brain in PD obtained at autopsy (17). The selective loss of Akt activity was also observed in ventral midbrain of mice treated with MPTP (16). Moreover, Akt phosphorylation was decreased when SH-SY5Y cells were exposed to MPP⁺ (18, 31). Considering that Akt is a critical molecule for neuronal survival and Akt signaling is impaired in PD, the molecules activating PI3K/Akt pathway may have pro-survival potential. In the present study, we have shown that exposure of cells to MPP⁺ significantly reduced the levels of Akt phosphorylation, similar to the previous reports (18, 31), suggesting a role of impaired Akt phosphorylation in MPP⁺-induced apoptosis in SH-SY5Y cells. IGF-1 completely prevented this reduction of Akt phosphorylation induced by MPP⁺ insult. The stimulatory effect of IGF-1 on Akt phosphorylation may contribute to maintaining the PI3K/Akt signaling pathway and resulting in the promotion of cell survival. Next, we further examined the levels of phosphorylation of PDK1 and found significantly reduced levels of phosphorylation in MPP⁺-treated cells. This finding suggests that the mechanisms underlying MPP⁺-induced apoptosis may include impairment in the activity of the PI3K/PDK1 pathway. To the best of our knowledge, this is the first report demonstrating the downregulation of PDK1 phosphorylation induced by MPP⁺. In this study, we also found that IGF-1 completely restored MPP⁺-induced reduction of PDK1 phosphorylation, suggesting that PI3K/PDK1 pathway could be linked to anti-apoptotic effect of IGF-1. In this regard, we propose that the ability of IGF-1 to prevent MPP⁺-induced reduction in PI3K/PDK1/Akt signaling pathway plays an important role in its protective effect in this experimental PD model.

Oxidative stress is known to play an important role in apoptosis after MPP⁺ exposure (32). In order to investigate the balance between oxidation and antioxidation in MPP⁺-treated cells, we measured MDA levels and SOD activity. In agreement with previous reports (42, 43), MPP⁺ caused an increase in MDA levels and a decrease in SOD activity suggesting the disbalance of lipid peroxidation and antioxidant protection system. However, this MPP⁺-induced imbalance was attenuated by IGF-1 treatment. Our data suggest that IGF-1 mitigated oxidative damage system and augmented antioxidative defense system by decreasing MDA contents and increasing the activity of SOD. In the present study, we have shown that IGF-1 treatment prevents MPP⁺-induced ROS generation. Because ROS play a role in early and late steps of the regulation of apoptosis (44), the capability of IGF-1 to decrease ROS production seems to be important for its protective mechanisms against MPP⁺ cytotoxicity. Indeed,

IGF-1 is shown to reduce oxidative stress (39, 45, 46, 47). The anti-apoptotic protein Bcl-2 is a reasonable target for the putative antioxidant capacity of IGF-1 because IGF-1 induces Bcl-2 (48), and it has been shown that Bcl-2 can protect cells from apoptosis by preventing ROS accumulation (49). Therefore, we consider that the increased Bcl-2 protein levels in IGF-1-treated cells may both promote cell survival and protect against MPP⁺-induced oxidative stress.

It has been suggested that mitochondria are an important source of cytosolic ROS (50, 51) and increases in mitochondrial ROS generation induce cellular oxidative damage and tissue dysfunction (52). MPP⁺ can be concentrated in mitochondria of dopaminergic neurons, where it blocks the mitochondrial electron transport chain complex I, resulting in the enhanced ROS generation, decreased ATP synthesis and subsequent cell death (5). We therefore examined if MPP⁺ exposure increased mitochondrial ROS levels and found that mitochondria-derived superoxide production was significantly increased during MPP⁺ exposure, as previously reported (53), and this accumulation of mitochondrial ROS was significantly attenuated by IGF-1 treatment. Similar findings were observed in striatal cells, in which IGF-1 decreased mitochondrial ROS induced by mutant huntingtin (22). Next, we wanted to identify the effects of IGF-1 on mitochondrial bioenergetic function and mitochondrial integrity by investigating the activity of key mitochondrial enzymes in the citric acid cycle, SDH and CS. SDH, also known as mitochondrial complex II, plays a critical role in cellular oxidative phosphorylation and is associated with oxidative stress. Given that the activity of SDH was decreased in PD patients (54) and dysfunction or inhibition of the SDH can cause mitochondrial dysfunction and interruption of ATP production (55), SDH could be regarded as one of the main regulators in neuroprotection in PD. As one of the gatekeepers of the Krebs cycle, CS plays an important role in regulating the energy flux and metabolic rate of the cell. Impaired activity of CS is known to interfere mitochondrial function and aggravate age-related hearing loss (56) and IGF-1 maintains CS activity in myocytes following hypoxia/reoxygenation stress (47). In this study, the activities of SDH and CS were also decreased by MPP⁺. In contrast, in IGF-1-treated SH-SY5Y cells, the activities of SDH and CS were increased, indicating that IGF-1 is capable of preventing dysfunction of the citric acid cycle and mitochondrial oxidative system in SH-SY5Y cells exposed to MPP⁺.

MPP⁺-induced oxidative stress leads to the opening of mitochondrial PT pore, through which cytochrome c is released from mitochondrial inter-membrane space prior to caspase activation (57). The mitochondrial PT pore is regulated by the Bcl-2 family proteins, such as Bcl-2 and Bax (58, 59, 60). The Bcl-2 protein prevents apoptosis by maintaining the $\Delta\psi_M$ and blocking the release of cytochrome c, whereas the Bax protein stimulates apoptosis by collapsing the $\Delta\psi_M$, leading to increased cytochrome c release. Indeed, we found that MPP⁺ caused a significant decrease in the level of Bcl-2 and a significant increase in the level of Bax, thereby decreasing the ratio of Bcl-2 to Bax. In contrast, IGF-1 increased Bcl-2 and decreased Bax levels in MPP⁺-treated cells. Bax is mainly distributed in the cytoplasm in healthy cells but activated Bax is translocated to the mitochondrial outer membrane under apoptotic stimuli, such as MPP⁺ (9, 42, 43). Our results showed that Bax was moved from the cytoplasm to the mitochondria by MPP⁺ insult while IGF-1 treatment completely restored the MPP⁺-induced translocation of Bax. In this study, we also observed that IGF-1 prevented MPP⁺-induced mitochondrial membrane depolarization, suggesting that IGF-1 may inhibit mitochondrial PT pore opening in SH-SY5Y cells exposed to MPP⁺. Taken together, these results suggest that IGF-1 stabilizes the $\Delta\psi_M$ by regulating Bcl-2 family proteins during MPP⁺ exposure and by that, preventing the activation of mitochondrial apoptotic cascades.

In this study, translocation of cytochrome c from mitochondria into the cytoplasm was observed when cells were exposed to MPP⁺, as previously reported (61). Apoptosis can be prevented by suppressing cytochrome c release because transport of cytochrome c to the cytoplasm is needed for the initiation of mitochondrial apoptotic cascades (58, 59). Upon released, cytochrome c forms the apoptosome, a complex composed of apoptosis-activating factor Apaf-1, procaspase-9 and adenosine triphosphate, leading to the activation of caspase-9 and caspase-3 with PARP cleavage and resulting in apoptosis (58, 59). It has been reported that IGF-1 exerts its anti-apoptotic effects on SH-SY5Y cells exposed to proteasome inhibitor epoxomicin by preventing cytochrome c release, inhibiting caspase-3 activation and suppressing PARP cleavage (62). In agreement with these findings, in the current study, IGF-1 prevented the MPP⁺-induced release of cytochrome c and subsequent activation of caspase-3 with PARP cleavage, thus inhibiting activation of the apoptotic pathway.

In conclusion, our findings indicate that IGF-1 is a powerful survival factor that protects SH-SY5Y cells from oxidative stress-mediated apoptosis induced by MPP⁺ by reducing mitochondrial dysfunction. Specifically, the protective mechanism of IGF-1 involves preserving the activity of the key enzymes in the Krebs cycle, stabilizing the $\Delta\psi_M$, increasing the ratio of Bcl-2 to Bax, maintaining the mitochondrial retention of cytochrome c and reducing activation of caspase-3 with PARP cleavage. Our data also show that the effects of IGF-1 seem to be mediated by the activation of PI3K/PDK1/Akt pathway. IGF-1 is likely to represent a plausible therapeutic target for the treatment of neurodegenerative disorders including PD.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Chanyang Kim carried out the laboratory experiments. Seungjoon Park designed the study, analyzed and interpreted the data and wrote the manuscript.

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