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ORIGINAL PAPER

Insulin-like growth factor 1 protects human neuroblastoma cells SH-EP1 against MPP^+-induced apoptosis by AKT/GSK-3β/JNK signaling

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Abstract Parkinson's disease (PD) is primarily caused by severe degeneration and loss of dopamine neurons in the substantia nigra pars compacta. Thus, preventing the death of dopaminergic neurons is thought to be a potential strategy to interfere with the development of PD. In the present work, we studied the effect of insulin-like growth factor-1 (IGF-1) on 1-methyl-4-phenylpyridinium (MPP^+)-induced apoptosis in human neuroblastoma SH-EP1 cells. We found that the PI3K/AKT pathway plays a central role in IGF-mediated cell survival against MPP^+ neurotoxicity. Furthermore, we demonstrated that the protective effect of AKT is largely dependent on the inactivation of GSK-3β, since inhibition of GSK-3β by its inhibitor, BIO, could mimic the protective effect of IGF-1 on MPP^+-induced cell death in SH-EP1 cells. Interestingly, the IGF-1 potentiated PI3K/AKT activity is found to negatively regulate the JNK related apoptotic pathway and this negative regulation is further shown to be mediated by AKT-dependent GSK-3β inactivation. Thus, our results demonstrated that IGF-1 protects SH-EP1 cells from MPP^+-induced apoptotic cell death via PI3K/AKT/GSK-3β pathway, which in turn inhibits MPP^+-induced JNK activation.

Keywords IGF-1 · MPP^+ · SH-EP1 · Apoptosis · AKT · GSK-3β · JNK

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Introduction

Parkinson's disease (PD), the second most common neurodegenerative disease after Alzheimer's disease, affects about 1% of the "over 65" population [1]. Clinical symptoms of PD include tremor, muscle rigidity, slowness of voluntary movement and postural instability. PD is mainly caused by the decrease in number of dopaminergic neurons located in the substantia nigra pars compacta (SNpc) [2]. Apoptosis is involved in the loss of dopaminergic neurons in SNpc based on the presence of DNA fragmentation, characteristic of apoptosis, in SNpc of PD patients as shown by TUNEL staining [3, 4] and the observation of morphological signs of apoptosis in degenerating SNpc [3, 5, 6]. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a potent and selective nigrostriatal dopaminergic neurotoxin, is a useful model to study the mechanisms of PD because it causes a syndrome that mimics most of the clinical features of idiopathic PD in humans, nonhuman primates, and mice [7]. MPTP induces the loss of pigmented neurons in the SNpc of the baboons [8] and mice [9] in vivo. Its active metabolite, 1-methyl-4-phenylpyridine ion (MPP^+), produces morphological features of apoptosis in many dopaminergic cells, such as PC12 [10] and SH-SY5Y cells [11] in vitro. Thus, preventing apoptosis induced by MPP^+ in vitro may provide some useful clues for the effective therapy of PD in clinical practices.

Insulin-like growth factor (IGF-1) is a multifunctional peptide that is structurally similar to insulin, and is essential for normal fetal and postnatal growth, development, metabolism and apoptosis in mammals [12]. The biological effects of IGF-1 are mainly mediated by IGF-1 receptor (IGF-1R), which phosphorylates several downstream substrates and activates the MAPK/ERK pathway

and PI3K/AKT pathway [13]. IGF-1 may have a neuroprotective potential in PD considering that its receptors are relatively highly expressed in the SN of human brain [14] and IGF-1R$^{+/-}$ mice show increased loss of dopamine neurons in SNpc after MPTP treatment compared to WT mice [15]. It has also been demonstrated that IGF-1 increases the survival of neurons in animal model from apoptosis induced by neuronal injury [16, 17]. In a cellular PD model, IGF-1 rescues cultured granule cells from dopamine-induced cell death [18]. IGF-1 also prevents cultured motor neurons from cell death induced by glutamate [19, 20] and cortical neurons from apoptosis induced by serum deprivation [21]. Hence, studies on its neuroprotective mechanism against MPP^+ neurotoxicity could possibly provide a potential therapeutic strategy for PD.

SH-EP1 cells are derived from the human neuroblastoma cell line SK-N-SH and have the same origin as SH-SY5Y, and moreover, SH-EP1 and SH-SY5Y cells can interconvert into each other morphologically and biochemically [22]. Therefore, SH-EP1 cell line is an acceptable candidate for being used as a PD model. The involvement of IGF-1 signaling pathways in cell survival has been identified in many cell types, but its downstream targets are frequently cell type-specific. Thus, it is worthwhile to determine the protective mechanism against MPP^+ neurotoxicity in SH-EP1 PD model.

In the present study, we aimed to determine the downstream signaling pathways of IGF-1 that antagonizes MPP^+-induced apoptosis in SH-EP1 cells. Here we show that IGF-1 effectively protects SH-EP1 cells against MPP^+-induced apoptotic cell death. Moreover, we demonstrate that the activation of the PI3K/AKT and subsequent reduction of c-Jun N-terminal kinases (JNK) activation by AKT-dependent GSK-3β inhibition are important for the survival of SH-EP1 cells against MPP^+ neurotoxicity.

Materials and methods

Cell culture and drug treatment

Human neuroblastoma cell lines SH-EP1 (gifts from Dr. Evelyne Goillot, Laboratoire d'Immunologie, Centre Leon Berard, France and Eva Feldman, University of Michigan, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 in air at 37°C. The culture medium was changed to DMEM without serum 16 h before experimental treatments, to reduce constitutive activity of kinases [23]. The selected concentration of MPP^+ (Sigma, St. Louis, MO, USA) was based on our previous study, which was the most effective concentration in killing SH-EP1 cells [24]. LY294002, a specific inhibitor of PI3K, Akti, a specific inhibitor of AKT, 6-bromoindirubin-3′-oxime (BIO), a specific inhibitor of GSK-3β and SP600125, a specific inhibitor of JNK1/2 were purchased from Sigma. Cells were incubated with inhibitors for 1 h prior to MPP^+ or MPP^+ plus IGF-1 (Sigma, St. Louis, MO, USA) treatment.

Cell viability assay

Cell survival was examined using crystal violet staining as described previously [25]. In brief, 2×10^4 cells were seeded onto 96-well plates. Following overnight incubation, cells were washed with fresh medium without serum and treated with reagents. All drugs were diluted in the same medium. After 24 h treatment, the plate was stained with 0.5% crystal violet in 20% methanol for 20 min at room temperature and then washed with tap water. Crystal violet in stained cells was dissolved with 20% acidic acid, and measured at a wavelength of 570 nm with Tecan (Männedorf, Switzerland). Absolute reading values were normalized by scaling to the mean of SH-EP1 culture grown in DMEM alone (defined as 100%). At least three independent experiments were performed in triplicate.

DAPI staining of SH-EP1 cells

SH-EP1 cells were plated on coverslips coated with 0.1% poly-L-lysine (PLL) in 12-well plates. After being treated with MPP^+ or MPP^+ plus IGF-1 for 24 h, cells were washed with PBS and fixed with 4% cold paraformaldehyde for 15 min. SH-EP1 cells were washed with PBS for 3 times, and then incubated in 4,6 diamidine-2-phenyllindole dihydrochloride (DAPI) (Sigma, St. Louis, MO, USA) solution (5 μg/ml) for 15 min. After that, the cells were washed with PBS for 3 times before examining by the fluorescent light microscope (Carl Zeiss, Thornwood, NY, USA).

Western blot

After treatment, cells in 60-cm dishes were washed twice with cold PBS (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 140 mM NaCl, pH 7.4) and then lysed in ice-cold lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mM PMSF, and Roche's complete protease inhibitors) and centrifuged at 14,000 *g* for 20 min at 4°C. The proteins in the supernatant were assessed using a Protein Assay Kit II (BioRad, Hercules, USA). Forty μg of protein samples was resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, CA, USA). The membranes were blocked with PBS-T (0.1%

Tween-20 in PBS) containing 5% non-fat milk, and then incubated with different primary antibodies (anti-β-actin from Sigma, anti-cleaved caspase 9, cleaved caspase-3, cleaved PARP, phospho-ERK1/2, phospho-AKT, AKT, phospho-GSK-3β, GSK-3β and phospho-JNK1/2 from Cell Signaling Technology (Beverly, MA, USA), anti-ERK2 and JNK1 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After washing with PBS for 30 min, the membranes were further incubated with horse radish peroxidase-conjugated secondary antibodies (Sigma) and developed using Pierce's West Pico Chemiluminescence substrate (Pierce, IL, USA). In some cases, we quantified immunoblots by measuring the immunoreactive protein band density with the software ImageJ 1.41.

Statistical analysis

Data were expressed as mean ± S.E. values. The group means were compared by analysis of variance, and the significance of differences was determined by post hoc testing using Bonferroni's method. Differences were considered significant at $P < 0.05$.

Results

IGF-1 protects SH-EP1 cells from MPP^+-induced apoptotic cell death

SH-EP1 cells were treated with MPP^+ to induce cell death [24]. The protective effect of IGF-1 was assessed by cell viability assay. In cells treated with MPP^+, the cell viability was about 34% of the control, whereas IGF-1 greatly increased the survival of SH-EP1 cells in a dose-dependent manner. The maximum protection occurred at 200 ng/ml of IGF-1 (Fig. 1a). The protective effect of IGF-1 was observed at a concentration as low as 10 ng/ml, while 200 ng/ml IGF-1 increased the cell survival to 59% ($P < 0.01$, compared to treatment with MPP^+ alone) of the control and further increasing IGF-1 concentration to 400 ng/ml only slightly increased cell survival, but the increase was not significant compared with the survival at 200 ng/ml of IGF-1 ($P > 0.05$) (Fig. 1a). Consistent with the cell survival data, DAPI staining also showed that IGF-1 could markedly reduce the number of MPP^+-induced apoptotic nuclei in SH-EP1 cells (Fig. 1c, d).

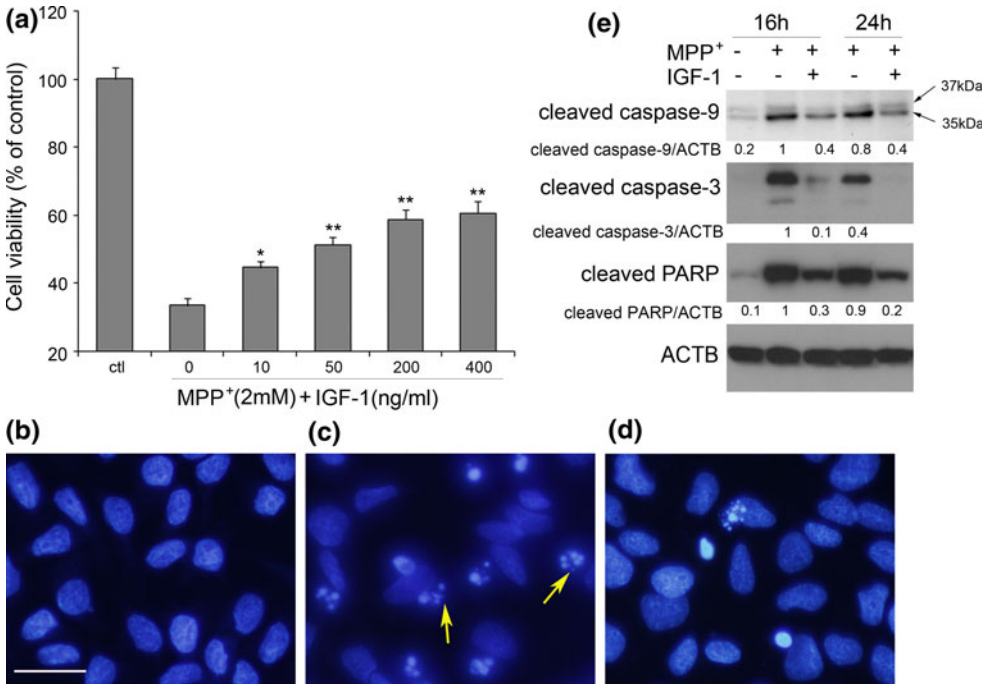

Fig. 1 IGF-1 promotes SH-EP1 cells survival against MPP^+ neurotoxicity. **a** IGF-1 protects SH-EP1 cells from MPP^+-induced cell death in a concentration-dependent manner. Cells were treated with MPP^+ or MPP^+ plus IGF-1 for 24 h. Cell viability was assessed by cell survival assay. Data are expressed as percent of values in untreated control cultures, and represent the mean ± S.E. of three experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$, compared with MPP^+-treated cells. Apoptosis was detected by DAPI staining, **b** control, **c** 2 mM MPP^+, **d** 2 mM MPP^+ plus 200 ng IGF-1. *Bar*: 50 μm, *arrows* indicate apoptotic bodies. **e** IGF-1 reduced the activation of caspase-9, caspase-3 and cleavage of PARP. Cells were treated with MPP^+ or MPP^+ plus IGF-1 for 16 and 24 h. Samples were assessed by western blot with antibody against the cleaved caspase-9, caspase-3 and PARP. Numbers indicated densitometrically determined cleaved caspase-9, -3, and PARP protein level relative to β-actin. Three independent experiments were done which yield similar results, and a representative blot is shown. In all bolts, expression of β-actin (ACTB) is shown as a protein loading control

Since MPP^+ induces mitochondria dysfunction and release of cytochrome C to trigger apoptosis [26, 27] and the activation of caspase-9 and caspase-3 are the major downstream events of the mitochondrial apoptotic pathway, we determined their activation in MPP^+ treated SH-EP1 cells in the presence or absence of IGF-1 (200 ng/ml). As shown in Fig. 1b, MPP^+ caused a significant activation of caspase-9, caspase-3 16 or 24 h post-treatment and these activations were greatly blocked by IGF-1. Moreover, cleavage of the caspase substrate poly (ADP-ribose) polymerase (PARP), a biochemical feature of apoptosis, was also assessed. Cleavage of PARP was also observed within 16 and 24 h of treatment with MPP^+, but the cleavage was significantly inhibited by IGF-1 (Fig. 1e). These results suggest that IGF-1 promotes SH-EP1 survival by preventing apoptosis induced by MPP^+.

IGF-1-promoted cell survival is mediated by the PI3K/AKT pathway, not MAPK/ERK pathway

It has been reported that the protective effects of IGF-1 are mainly mediated by the activation of its two important downstream signaling pathways: the PI3K/AKT pathway and the MAPK/ERK pathway [13]. To determine whether IGF-1 protects SH-EP1 cells from MPP^+-induced apoptosis through these pathways, we examined the activation of AKT and ERK in SH-EP1 cells upon IGF-1 treatment. Cells were treated with MPP^+ or IGF-1 plus MPP^+ for 15 min, 1, 4, and 24 h, and cell extracts were subjected to western blot analysis to detect phosphorylated AKT (active, pAKT). AKT was phosphorylated at Ser473 when cells were treated with IGF-1 and MPP^+, whereas treatment with MPP^+ alone did not induce the phosphorylation of AKT. The activation of AKT quickly reached the peak level in 15 min and sustained to 24 h after IGF-1 treatment (Fig. 2a). To further assess the contribution of PI3K/AKT pathway in IGF-1-promoted cell survival, we preincubated SH-EP1 cells with LY294002 or Akti for 1 h before the treatments. Cell survival was assessed 24 h after treatment. LY294002, a specific PI3K inhibitor that acts on the ATP-binding site of the enzyme, or Akti, a specific inhibitor of AKT, acts on the pleckstrin homology (PH) domain of AKT, suppressed the activation of AKT (Fig. 2c, d) and partially, but significantly, abolished the protective effect of IGF-1 against cell death induced by MPP^+ (Fig. 2b). As control, LY294002 or Akti alone did not significantly affect the cell survival of untreated SH-EP1 cells (data not shown). In addition, these two inhibitors also abolished the

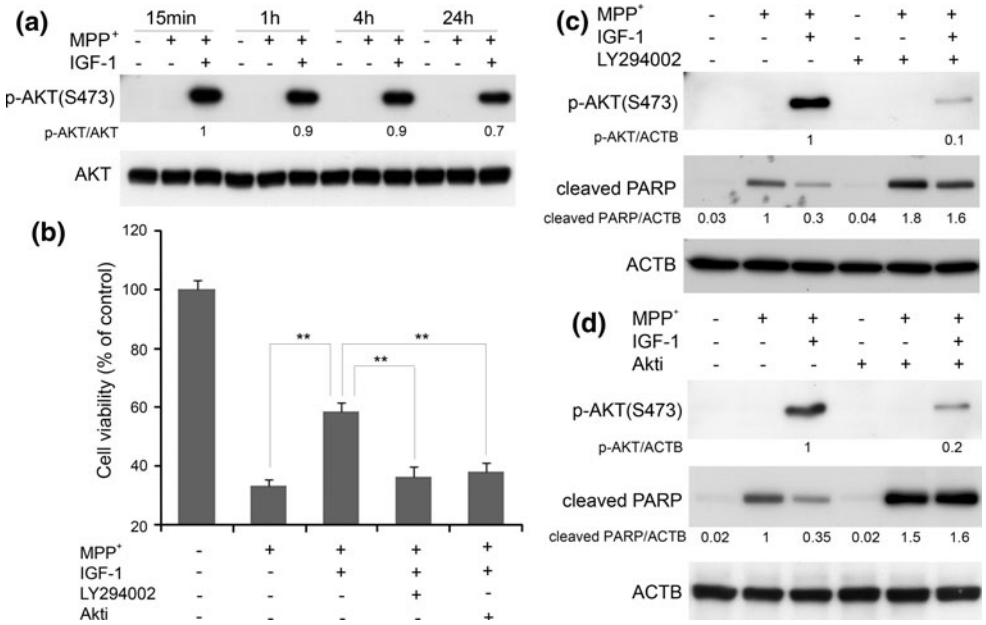

Fig. 2 IGF-1-dependent inhibition of apoptosis induced by MPP^+ in SH-EP1 cells is through the PI3K/Akt pathway. **a** Treatment with MPP^+ or MPP^+ plus IGF-1 induced a rapid and sustained AKT activation in SH-EP1 cells. Cells were incubated with MPP^+ or MPP^+ plus IGF-1 for a time course as indicated, samples were examined by western blot with antibody against phosphorylated AKT (S473). **b** In the presence of LY294002 (20 μM) or Akti (5 μM), cells were cultured with MPP^+ or MPP^+ plus IGF-1 for 24 h. Cell survivals were determined by cell survival assay. Data are from three repeated experiments. ** $P < 0.01$, compared to cells treated with MPP^+ plus IGF-1. In the presence of LY294002 (**c**) or Akti (**d**), cells were incubated with MPP^+ or MPP^+ plus IGF-1 for 16 h, protein extracts were analyzed by western blot with antibodies against phosphorylated AKT (S473) or cleaved PARP. *Numbers* indicated densitometrically determined phosphorylation of AKT relative to AKT or cleaved PARP protein level relative to β-actin. Three independent experiments were done which yield similar results, and a representative blot is shown. In all bolts, expression of AKT or β-actin (ACTB) is shown as a protein loading control

reduced PARP cleavage mediated by IGF-1 as shown in Fig. 2c, d. These results indicate that IGF-1-mediated cell survival against MPP^+ neurotoxicity is dependent on the PI3K/AKT pathway.

As MAPK/ERK pathway is another important downstream signaling pathway involved in IGF-1-mediated cell protection, we tested the effect of IGF-1 on ERK activation and its involvement in IGF-1-promoted cells survival against MPP^+-induced neurotoxicity. As shown in Fig. 3a, ERK was activated by MPP^+, and the peak phosphorylation of ERK occurred within 15 min after MPP^+ treatment. Phosphorylation level then dropped to baseline at 1 h, followed by significant increase at 24 h. The same pattern of ERK phosphorylation was also observed in SH-EP1 cells treated with IGF-1 plus MPP^+, indicating that IGF-1 had no significant effect on the activation of ERK. To further check the possible involvement of ERK in IGF-1-enhanced cell survival, we pretreated SH-EP1 cells with U0126, a specific inhibitor of MEK1 which is the upstream effector of ERK1/2, for 1 h before the treatment with MPP^+ or IGF-1 plus MPP^+, and cell survival was determined after 24 h. As expected, U0126 completely blocked ERK1/2 activation. However, the inhibitor had no effect on IGF-1-enhanced cell survival ($P > 0.05$, compared with SH-EP1 cells treated with MPP^+ plus IGF-1) (Fig. 3b). Furthermore, it had no influence on the reduced PARP cleavage mediated by IGF-1 as shown in Fig. 3c, suggesting that activation of ERK is unlikely to be involved in IGF-1-enhanced cell survival in SH-EP1 cells.

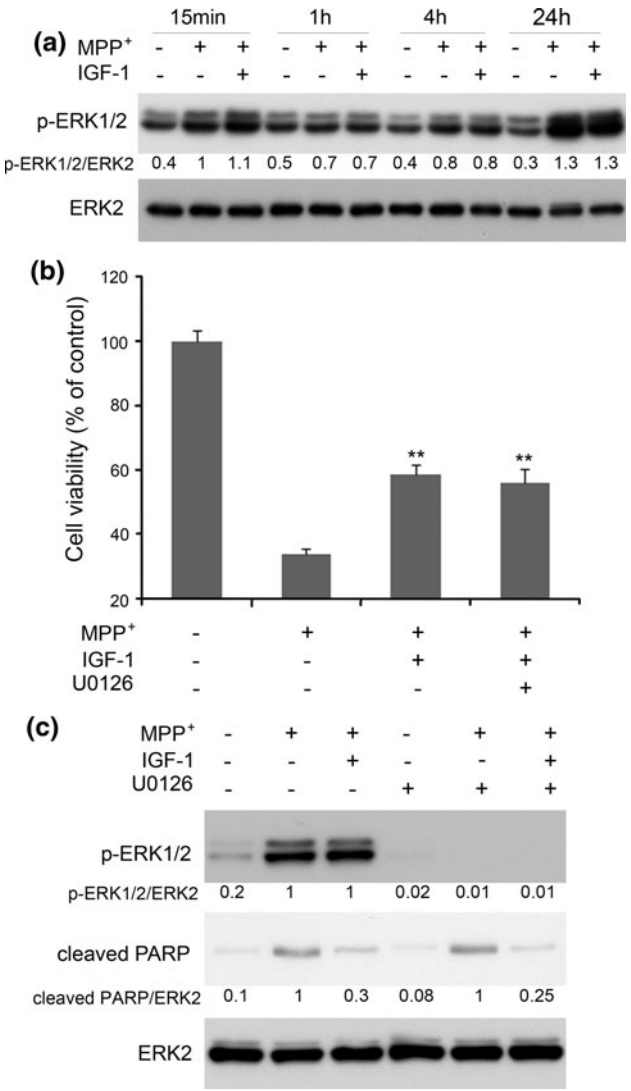

Fig. 3 MAPK/ERK pathway is not involved in IGF-1-mediated protection against MPP^+ neurotoxicity in SH-EP1 cells. **a** SH-EP1 cells were treated with MPP^+ or MPP^+ plus IGF-1 for a time course as indicated, samples were assessed by western blotting with antibody against p-ERK1/2. **b** In the presence of MEK1 inhibitor, U0126, cells were cultured with MPP^+ or MPP^+ plus IGF-1 for 24 h. Cell survivals were determined by cell survival assay. Data are from three repeated experiments. ** $P < 0.01$, compared to MPP^+-treated SH-EP1 cells. **c** In the presence of U0126, cells were incubated with MPP^+ or MPP^+ plus IGF-1 for 16 h, protein extracts were analyzed by western blot with antibodies against p-ERK1/2 and cleaved PARP. Numbers indicated densitometrically determined phosphorylation level of ERK or cleaved PARP protein level relative to ERK2. Three independent experiments were done which yield similar results, and a representative blot is shown. In all bolts, level of total ERK2 is shown as a protein loading control

IGF-1-mediated cell protection is dependent on the inhibition of GSK-3β by PI3K/AKT pathway

GSK-3β is constitutively active in cells and can be inactivated through the phosphorylation of its serine residues (S9) by AKT [28, 29]. To test the possibility that IGF-1 could phosphorylate and inactivate GSK-3β in SH-EP1 cells, we examined the phosphorylation of GSK-3β by western blot in SH-EP1 cells after incubation with MPP^+ or MPP^+ plus IGF-1. Compared with MPP^+-treated cells, MPP^+ plus IGF-1-treated cells had a significant increase in phosphorylation of GSK-3β (Fig. 4a). To check whether IGF-1-induced inactivation of GSK-3β is mediated by PI3K/AKT pathway, we preincubated SH-EP1 cells with LY294002 or Akti for 1 h before treatments. As shown in Fig. 4b, c, the phosphorylation of GSK-3β induced by IGF-1 was counteracted by LY294002 or Akti, indicating that IGF -1-induced inhibition on GSK-3β was mediated by the PI3K/AKT pathway.

GSK-3β has pro-apoptotic roles in PC12, Rat-1 cells and cerebellar granule neurons, as its inhibition protects cells against apoptotic stimuli [30, 31]. To further determine whether inactivation of GSK-3β by PI3K/AKT pathway is important for survival of SH-EP1 cells against MPP^+ neurotoxicity, we pretreated SH-EP1 cells with the GSK-3β inhibitor BIO prior to the addition of MPP^+. Cell survival was examined 24 h later. As expected, BIO could confer cell protection against MPP^+ insults in SH-EP1

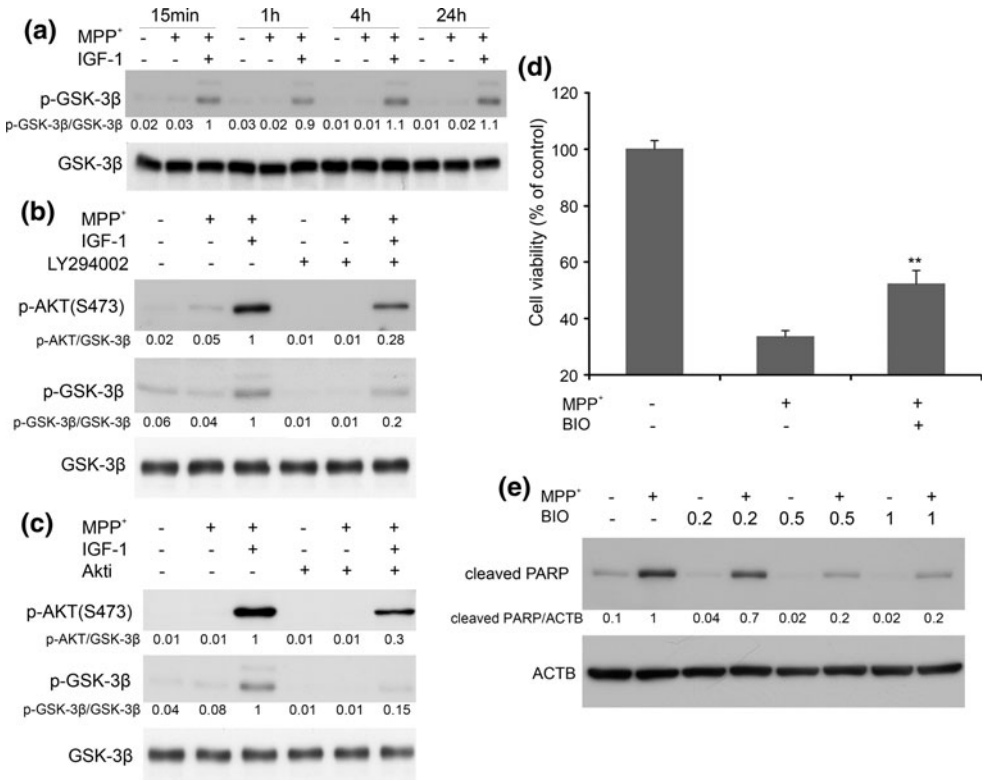

Fig. 4 AKT-mediated survival of SH-EP cells is dependent on the inhibition of GSK-3β. **a** cells were treated with MPP^+ or MPP^+ plus IGF-1 for a time course as indicated, samples were assessed by western blotting with antibody against phosphorylated GSK-3β (S9). In the presence of LY294002 (**b**) and Akti (**c**), cells were treated with MPP^+ or IGF-1 plus MPP^+ for 4 h. Cell extracts were analyzed by western blot with antibodies against p-GSK-3β. **d** In the presence of GSK-3β inhibitor, BIO (0.5 μM), cells were incubated with MPP^+ for 24 h. Cell survival was examined with cell survival assay. Data are means ± S.E of three replicate values in 3 separate experiments. ** $P < 0.01$, compared to MPP^+-treated SH-EP1 cells. **e** In the presence of BIO (0.2, 0.5 and 1 μM), SH-EP1 cells were incubated with MPP^+ for 16 h, and then samples were examined by western blot with antibody against cleaved PARP. Numbers indicated densitometrically determined p-AKT(S473) or p-GSK-3β protein level relative to GSK-3β or cleaved PARP protein level relative to β-actin. Three independent experiments were done which yield similar results, and a representative blot is shown. In all blots, staining for total GSK-3β or β-actin (ACTB) was used as a loading control

cells ($P < 0.01$, compared to SH-EP1 cells treated with MPP^+ alone) (Fig. 4d). As control, BIO alone did not significantly influence the cell survival of untreated SH-EP1 cells (data not shown). Consistently, MPP^+-induced cleavage of PARP was also inhibited by BIO (Fig. 4e).

IGF-1-promoted cell protection is mediated by the inhibition of JNK activation induced by MPP^+ via GSK-3β inhibition by AKT

We have recently reported that MPP^+ induces an activation of JNK and inhibition of such activation by a JNK specific inhibitor, SP600125, significantly reduces MPP^+-induced cell death in SH-EP1 cells [24]. IGF-1 has been showed to block the activation of JNK and thereafter preventing apoptosis in many cell types, such as human embryonic kidney 293, L929 cells, vascular smooth muscle cells, and isolated human islets [32–34]. On the other hand, in mouse embryonic fibroblast, IGF-1 was found to activates JNK and then promote cell proliferation [35]. Thus, it is interesting to determine whether there was any crosstalk between PI3K/AKT pathway and JNK pathway in SH-EP1 cells. We compared the activation of JNK1/2 in response to MPP^+ or MPP^+ plus IGF-1 and found that MPP^+-induced JNK activation was strongly inhibited by IGF-1 (Fig. 5a, b). Meanwhile, we found that the inhibition of JNK by IGF-1 was dependent on PI3K/AKT pathway, because either LY292004 or Akti could reverse such inhibition. GSK-3β has been reported to function as a natural activator of mitogen-activated protein kinase kinase kinase 1 (MEKK1), an upstream activator of JNK [36]. To evaluate the contribution of GSK-3β inactivation by AKT in JNK inhibition, we examined the effect of BIO on JNK activation. As expected, we found that BIO reproduced the effect of IGF-1 and strongly inhibited MPP^+-induced JNK activation (Fig. 5c), indicating that AKT-dependent JNK inhibition is mediated by GSK-3β. Lastly, as an additional evidence to support the role of JNK activation in cell

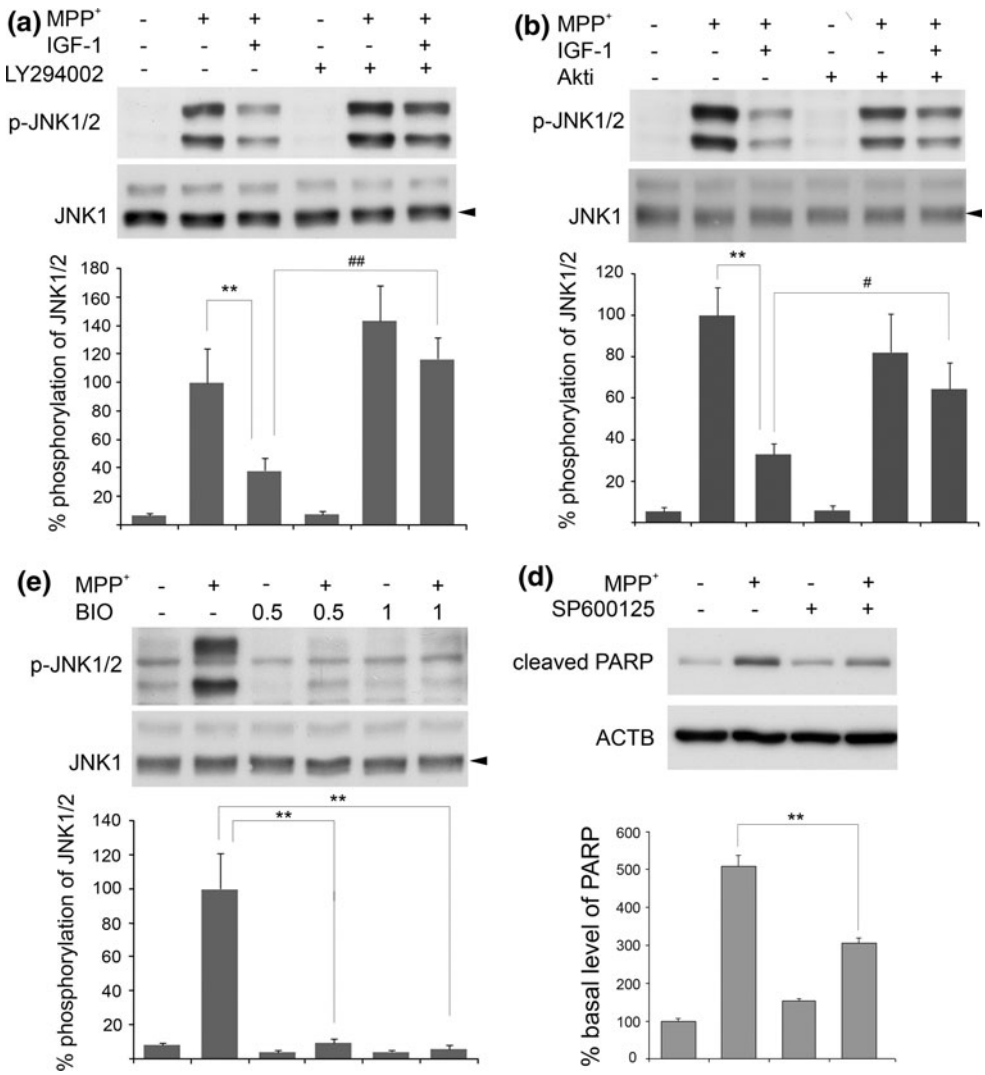

Fig. 5 AKT-mediated survival of SH-EP cells is dependent on the inhibition of JNK. In the presence of LY294002 (**a**) and Akti (**b**), cells were treated with MPP^+ or IGF-1 plus MPP^+ for 4 h. Cell extracts were analyzed by western blot with antibodies against phosphorylated JNK. **c** cells were pretreated with BIO for 1 h before treatment with MPP for 4 h, samples were assessed by western blot with antibodies against phosphorylated JNK. **d** In the presence of JNK inhibitor SP600125(30 μM), cells were incubated with MPP^+ for 16 h, samples were examined by western blot with antibody against cleaved PARP. Densitometry is shown below as the ratio of p-JNK1/2/JNK1 or cleaved PARP/β-actin. Data are from three independent experiments with similar results and are means ± S.E. ** $P < 0.01$, compared to SH-EP1 cells treated with MPP^+ alone. # $P < 0.05$, ## $P < 0.01$, compared to SH-EP1 cells treated with MPP^+ plus IGF-1. In all blots, staining for total JNK1 or β-actin (ACTB) was used as a loading control

apoptotic death, we found that JNK inhibitor (SP600125) could block the cleavage of apoptotic marker PARP (Fig. 5d).

Discussion

Apoptosis is thought to be involved in the loss of dopaminergic neurons in SNpc of patients with PD. MPTP is a neurotoxin that induces a syndrome that mimics the core neurological symptoms of PD in humans and causes dopamine neuronal apoptosis in SNpc of PD patients and mice [2]. Thus, rescuing neuronal apoptosis induced by MPP^+ neurotoxin with pro-survival factors might present a potential therapeutic strategy for PD. IGF-1 is a potent neural survival factor and widely used to protect neurons from apoptosis. For instance, IGF-I protects rat hippocampal neurons from apoptosis induced by amyloid-derived peptides, in an in vitro model of neurodegenerative disease [37]. IGF-I also prevents apoptosis in cortical neurons caused by serum deprivation [21]. Moreover, IGF-1 rescues human neuroblastoma cells SH-SY5Y from osmotic stress-induced apoptosis [38]. In the present study, we demonstrated that IGF-1 protects SH-EP1 cells from MPP^+-induced cell death through the activation of PI3K/AKT signaling pathway. Treatment of SH-EP1 cells with IGF-1 activates AKT, leading to the inhibition of GSK-3β and subsequent inactivation of JNK. As a result, the inhibition of JNK by AKT-dependent GSK-3β inactivation leads to the attenuation of MPP^+-induced apoptosis. Our results may provide a new insight into signaling mechanisms mediated by IGF-1 in cell survival against MPP^+-induced apoptosis and may have significance in the future PD therapy development.

The protective effects of IGF-1 are mediated by binding to its receptor, IGF-1R. Once IGF-1 binds to IGF-1R, IGF-1R initiates its downstream signaling pathways, such as MAPK/ERK pathway or PI/3K/AKT pathway (13). Many

studies have implicated the PI3K/AKT pathway in cell protection under various stresses [39–41]. Activation of PI3K/AKT pathway by nerve growth factor (NGF) produces an anti-apoptotic signal in SH-SY5Y cells against MPP^+-induced apoptosis [42]. PI3K/AKT pathway also prevents hippocampal neurons from apoptosis caused by corticosterone [43] or glutamate toxicity [44] and PC12 cells from apoptosis induced by serum withdrawal or UV irradiation [45]. In the attempt to determine the downstream signaling events underlying the neuroprotective mechanism of IGF-1, we assessed the activation of AKT and found that IGF-1 induced a potent and sustained activation of AKT in SH-EP1 cells. The PI3K inhibitor, LY294002, or AKT inhibitor, Akti, abolished the activation of AKT as well as the anti-apoptotic effects of IGF-1. Interestingly, in the presence of LY294002 or Akti, MPP^+-induced cell apoptosis were significantly enhanced in SH-EP1 cells treated with or without IGF-1. This suggests that inhibitors strongly reduced the neuroprotection resulting from basal level and IGF-1 induced PI3K/AKT activities. Taken together, our results indicated that the PI3K/AKT signaling pathway is responsible for the protective effect of IGF-1 against MPP^+-induced apoptosis in SH-EP1 cells.

Several AKT substrates have been reported in recent years, such as Bad, caspase-9 and GSK-3β [46, 47]. GSK-3β is a constitutively active kinase and can be inactivated through phosphorylation by AKT [28]. GSK-3β has been shown to be involved in MPP^+-induced mitochondrial dysfunction, and blockage of GSK-3β activation protects dopaminergic neurons from MPP^+-mediated neurotoxicity [48–50]. Moreover, treatment with a GSK-3β inhibitor, lithium, could produce neuroprotective effects against neurotoxicity induced by MPP^+ in PC12 cells [51] or in MPTP-induced striatal dopaminergic neurodegeneration and dopamine depletion in a mouse model of PD [52]. Consistent with these reports, we showed here that IGF-1-mediated inactivation of GSK-3β protects SH-EP1 cells from MPP^+-induced cell death. This conclusion was supported by two evidences. First, GSK-3β was phosphorylated and inactivated in response to IGF-1 stimulation and the inactivation is reversed when SH-EP1 cells were pretreated with LY294002 or Akti. Second, BIO, a GSK-3β inhibitor, mimicked the protective effect of IGF-1 against MPP^+ cytotoxicity and blocked MPP^+-induced PARP cleavage. Our findings further confirmed the conclusion that inactivation of GSK-3β plays an important role in reducing MPP^+-induced neurotoxicity.

JNK, an established mediator of stress-induced apoptosis, is involved in the neurodegenerative processes in PD pathogenesis [53–55], and it represents a potential therapeutic target for blockage of apoptosis induced by MPP^+. In vivo, JNK inhibitor, SP600125, or CEP-1347/KT-7515, attenuates MPTP-mediated JNK activation and the loss of nigrostriatal dopaminergic neurons [7, 56, 57]. Furthermore, overexpression of JNK binding domain (JBD) of JNK-interacting protein-1 (JIP-1, a scaffold protein that inhibits JNK) blocks JNK activation and protects dopaminergic neurons from death [55]. Recently, one of our studies showed that SP600125 attenuates MPP^+-induced apoptotic cell death in SH-EP1 and SH-SY5Y cells [24]. In the present study, we found that IGF-1-promoted cells survival is executed by inhibition of JNK activation, as suggested by that MPP^+-induced JNK activation was suppressed by IGF-1 and such suppression could be reversed by LY294002 or Akti. Moreover, cleaved PARP, an apoptotic marker, was significantly reduced by SP600125 in MPP^+-treated cells, providing a solid evidence to support the involvement of JNK signaling in MPP^+-induced apoptosis.

Although many reports have shown that inhibition of GSK-3β through PI3K/AKT suppresses MPP^+-induced apoptosis in several cell types [23, 30, 48, 49], the mechanism about how GSK-3β inactivation facilities cell survival is still not fully understood. It has been demonstrated that GSK-3β inhibition suppresses MPP^+-induced activation of caspase-3 and p53, a pro-apoptotic tumor suppressor protein, thereafter contributes to cell survival [48]. GSK-3β inhibition has also been reported to block MPP^+-induced apoptosis by reducing hyperphosphorylation of Tau, along with decreased levels of accumulated α-Syn [58]. In the present study, we found that GSK-3β inhibition can promote cell survival via inhibiting JNK activation, as supported by the observation that BIO reproduced the inhibitory effect of IGF-1 on JNK activation induced by MPP^+. Similarly, previous studies have demonstrated that activation of GSK-3β preceded the activation of JNK and that this effect contributed to apoptotic signaling [36, 59]. Taken together, these data suggest that the inhibition of MPP^+-induced JNK activation is a downstream event of AKT/GSK-3β signaling and may add more weight to GSK-3β blockage in the treatment of PD.

IGF-1 could also activate the MAPK/ERK pathway, but the role of ERK activation in neuronal cell survival is still controversial. MAPK/ERK pathway can either enhance the neuronal cell survival or induce apoptotic cell death, depending on the specific cell types and insults. ERK has been reported to protect neurons against apoptosis induced by trophic factor deprivation [60], DNA damage-inducing drugs [61] or hypoxia–ischemia [62]. ERK activation has also been demonstrated to promote neuronal cell death caused by glutamate [30, 63, 64] or okadaic acid [65]. In our present study, we demonstrated that complete inhibition of ERK did not attenuate the protective effect of IGF-1. This result indicates that MAPK/ERK may not be involved in the anti-apoptotic effects of IGF-1 against MPP^+ neurotoxicity. The biological significance of

MAPK/ERK activation by IGF-1 in SH-EP1 cells is not clear, and further study is needed to clarify this issue.

In summary, our data show that IGF-1 provides a strong protection against MPP^+-mediated SH-EP1 cells death through the PI3K/AKT pathway, not the MAPK/ERK pathway. Moreover, PI3K/AKT pathway-dependent SH-EP1 cells survival is mediated by JNK inactivation via AKT-dependent GSK-3β inhibition. Thus, our results may provide a new insight in the treatment of PD by targeting GSK-3β to block the pro-apoptotic JNK signaling.

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