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Apelin-36 exerts the cytoprotective effect against MPP^+-induced cytotoxicity in SH-SY5Y cells through PI3K/Akt/mTOR autophagy pathway

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ABSTRACT

Aims: Parkinson's disease (PD) is a common neurodegenerative disease typically associated with the accumulation of α-synuclein. Autophagy impairment is thought to be involved in the dopaminergic neurodegeneration in PD. We investigate the effect of Apelin-36 on the activated phosphatidylinositol 3-kinase (PI3K)/protein kinase B(Akt)/the mammalian target of rapamycin (mTOR) autophagy pathway in 1-methyl-4-phenylpyridinium (MPP^+)-treated SH-SY5Y cells, which is involved in the cytoprotective effect of Apelin-36.

Main methods: SH-SY5Y cells were treated with 1-Methyl-4-phenylpyridine (MPP^+) with or without Apelin-36. The cell viability, apoptotic ratio, the form of autophagic vacuoles, the expression of tyrosine hydroxylase (TH), α-synuclein, phosphorylation of PI3K, AKT, mTOR, microtubule-associated protein 1 Light Chain 3 II/I (LC3II/I) and p62 were detected to investigate the neuroprotective effect of Apelin-36.

Key findings: The results indicate that Apelin-36 significantly improved the cell viability and decreased the apoptosis in MPP^+-treated SH-SY5Y cells. The decreased expression of tyrosine hydroxylase (TH) induced by MPP^+ was significantly increased by Apelin36 pretreatment. Moreover, Apelin36 significantly increased the autophagic vacuoles. The ratio of LC3II/I was significantly increased by Apelin36, as well as the decreased p62 expression. In addition, the activated PI3K/AKT/mTOR pathway induced by MPP^+ was significantly inhibited by Apelin36. Additionally, Apelin36 significantly decreased the α-synuclein expression. Furthermore, the cytoprotective effect of Apelin-36 was weakened by pretreatment with Insulin-like Growth Factor-1 (IGF-1), an activator of PI3K/Akt, and MHY1485, an mTOR activator.

Significance: Our results demonstrated that Apelin-36 protects against MPP^+-induced cytotoxicity through PI3K/Akt/mTOR autophagy pathway in PD model in vitro, which provides a new theoretical basis for the treatment of PD.

1. Introduction

Parkinson's disease (PD) is a common degenerative disease of the central nervous system characterized by a progressive degeneration of dopaminergic neurons within the substantia nigra compacta (SNpc) [1,2], resulting in motor disturbances such as resting tremor, bradykinesia, and rigidity [3,4]. The formation of intraneuronal protein inclusions (Lewy bodies) is deemed as the neuropathological hallmark of PD, and α-synuclein is the major constituent of Lewy bodies [5]. Mounting evidence indicated that mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress, autophagy disfunction and calcium overload may be involved in the pathogenesis of PD [6–8].

Autophagy [9] is a lysosomal-dependent degradation pathway widely present in eukaryotic cells. It is the process by which cells recycle nutrients in cytoplasm and dispose of unfolded proteins and damaged organelles. mTOR is a key molecule in the initiation of autophagy. It is a serine/threonine kinase that can sense intracellular amino acid levels or the number of ATPs to regulate autophagy. Unc-51 like autophagy activating kinase1 (ULK1), autophagy-related protein 13 (Atg13) and RB1-inducible coiled-coil 1 (RB1CC1/FIP200) are present in a complex regulated by the autophagy regulatory molecule rapamycin target protein mTOR [10]. When nutrient is deficient, mTOR is isolated from the Atg13-FIP200-Atg101 complex, which activates ULK1 and phosphorylates Atg13-FIP200-Atg101 complex, thereby induces autophagy. After the initiation of autophagy, autophagic vesicle begin extend and encapsulates degradation substrate, followed by the

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autophagosome formed. LC3-II is a marker of autophagosome, we can detect the expression level of LC3-II to detect the development of autophagy [11]. Finally, autophagosomes are fused with lysosomes to form autolysosome, and their inclusions are degraded by lysosomal hydrolase to complete the autophagy process. Autophagy disfunction is significant in neurological diseases, including cerebral ischemia-reperfusion injury [12,13], PD [14,15], Alzheimer's disease (AD) [16–18], and lateral sclerosis [19,20]. It has been reported that the autophagy in dopaminergic neurons could be influenced by PD-related genes (SNCA, LRRK2, UCH-L1, GBA, DJ-1) [21–25] and the neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone and 6-hydroxydopa (6-OHDA) [26–28], leading to the abnormal aggregation of α-synuclein. As autophagy is one of the main systems involved in the degradation of α-synuclein, up-regulation of autophagy may be an attractive strategy to degrade α-synuclein aggregation in PD [29].

PI3K and Akt are two important factors upstream of mTOR. PI3K/Akt/mTOR is an important signaling pathway of mTOR-dependent autophagy [30]. The tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2) proteins are the downstream of the PI3K/Akt pathway, and the phosphorylated TSC1/2 activates the small G protein Ras Homologue Enriched in Brain (Rheb), thereby enhancing the activity of mTOR kinase and inhibiting the occurrence of autophagy [31]. It has been shown that the degradation of toxic α-synuclein was increased by the inhibition of mTOR in Rotenone-treated SH-SY5Y cells [32]. Furthermore, mTOR inhibitor alleviates the toxicity of PC12 cells induced by 6-OHDA [33]. Moreover, the impaired autophagy for the activation of the PI3K/Akt/mTOR pathway triggers the form of inflammasome in MPTP-treated PD mouse model [34]. So, inhibition of PI3K/Akt/mTOR autophagic pathway may be a promising strategy for PD therapy.

Apelin is an endocrine peptide that has been shown to be neuroprotective [35]. The 77-amino acid Apelin pro-peptide can be hydrolyzed into fragments of different lengths [36,37]. Apelin-13 and Apelin-36 are the more biologically active Apelin receptor agonists. It has been shown that Apelin-13 reduces the apoptosis caused by serum deprivation in primary cortical neurons [35]. Lateral ventricle injection of Apelin-13 has neuroprotective effect on the increased cerebral infarction volume in ischemic stroke of rats [38,39]. A recent report showed that the apoptosis is attenuated by Apelin-36 in Rats with ischemic stroke, and ERS is involved in the neoroprotective effect of Apelin-36 [40]. In the study of PD, Apelin-13 improves the cognitive impairment in a rat model of PD and protects dopaminergic neurons through the inhibition of oxidative stress and apoptosis [41,42].

However, the underlying molecular mechanism of Apelin remains poorly understood. For the autophagy is proved to be important in the pathogenesis of PD, we established a hypothesis that autophagy may be regulated by Apelin-36 in a PD model in vitro. In the present work, we investigated the effect of Apein-36 on the increased apoptosis and decreased autophagy in MPP^+-treated SH-SY5Y cells, for confirming that Apelin-36 is cytoprotective in PD, and autophagy is involved in the cytoprotective mechanisms of Apelin-36.

2. Materials and methods

2.1. Cell culture

Human neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection (Manassas, VA, USA). SH-SY5Y cells were grown with Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Inc. Waltham, MA, USA), penicillin (100U/mL), and streptomycin (100 U/mL) and maintained at 37 °C in 5% CO_2 atmosphere.

2.2. CCK-8 assay

The purpose of CCK-8 assay was to measure the cell viability of SH-SY5Y cells with different treatments. SH-SY5Y cells were seeded in 96-well plates (5×10^4 cells/well), and then exposed to MPP^+ with or without the pretreatment with Apelin-36 (PHOENIX PHARMACEUTICALS, Inc. Burlingame, U.S.A), IGF-1(R&D Systems, Inc. Minneapolis, U.S.A) or MHY1485(Med Chem Express, U.S.A). After 24h, CCK-8 solution (10μL/well) was added and incubated at 37 °C in a 5% CO_2 atmosphere for 2h in a humidified incubator. The absorbance value was measured at 450nm wavelength on a Biotek plate reader (Bio-Rad, USA). The experiments were repeated three times. And the cell viability was expressed as the percentage of the control group.

2.3. Hoechst 33342 staining

Cell apoptosis was evaluated by Hoechst 33342 staining. Briefly, SH-SY5Y cells were evenly seeded into 24-well culture plates at an approximate density of 2×10^4 cells/well. After treated with MPP^+ for 24h with or without Apelin-36 pretreatment for 2h, SH-SY5Y cells were washed with PBS twice and immobilized with 4% paraformaldehyde for 20 min at room temperature. Then cells were washed with PBS and stained with 1ml of Hoechst 33342 (5 mg/mL in PBS) at 37 °C in a 5% CO_2 atmosphere for 20 min. Stained cells were washed twice with PBS

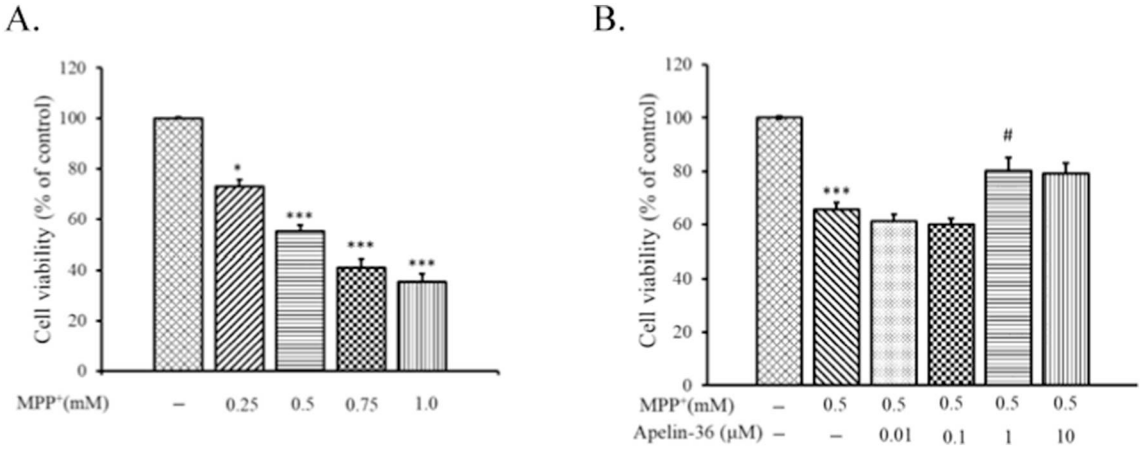

Fig. 1. Apelin-36 improved the cell viability in MPP^+-treated SH-SY5Y cells.
(A) SH-SY5Y cells were treated with MPP^+ for different concentrations (0.25mmol/L, 0.5 mmol/L, 0.75mmol/L, 1.0mmol/L) for 24h. Cell viability was measured by CCK-8 assay. (B) Apein-36(0.01μmmol/L, 0.1μmmol/L, 1μmmol/L, 10μmmol/L) were pretreated for 2h before MPP^+ (0.5 mmol/L) treatment for 24h. Cell viability was measured by CCK-8 assay. Values were presented as mean ± SEM of three independent experiments and the cell viability was expressed as the percentage of the control group. $^*p < 0.05$ and $^{***}p < 0.001$ vs control group cells. $^{\#}p < 0.05$ vs MPP^+ group cells.

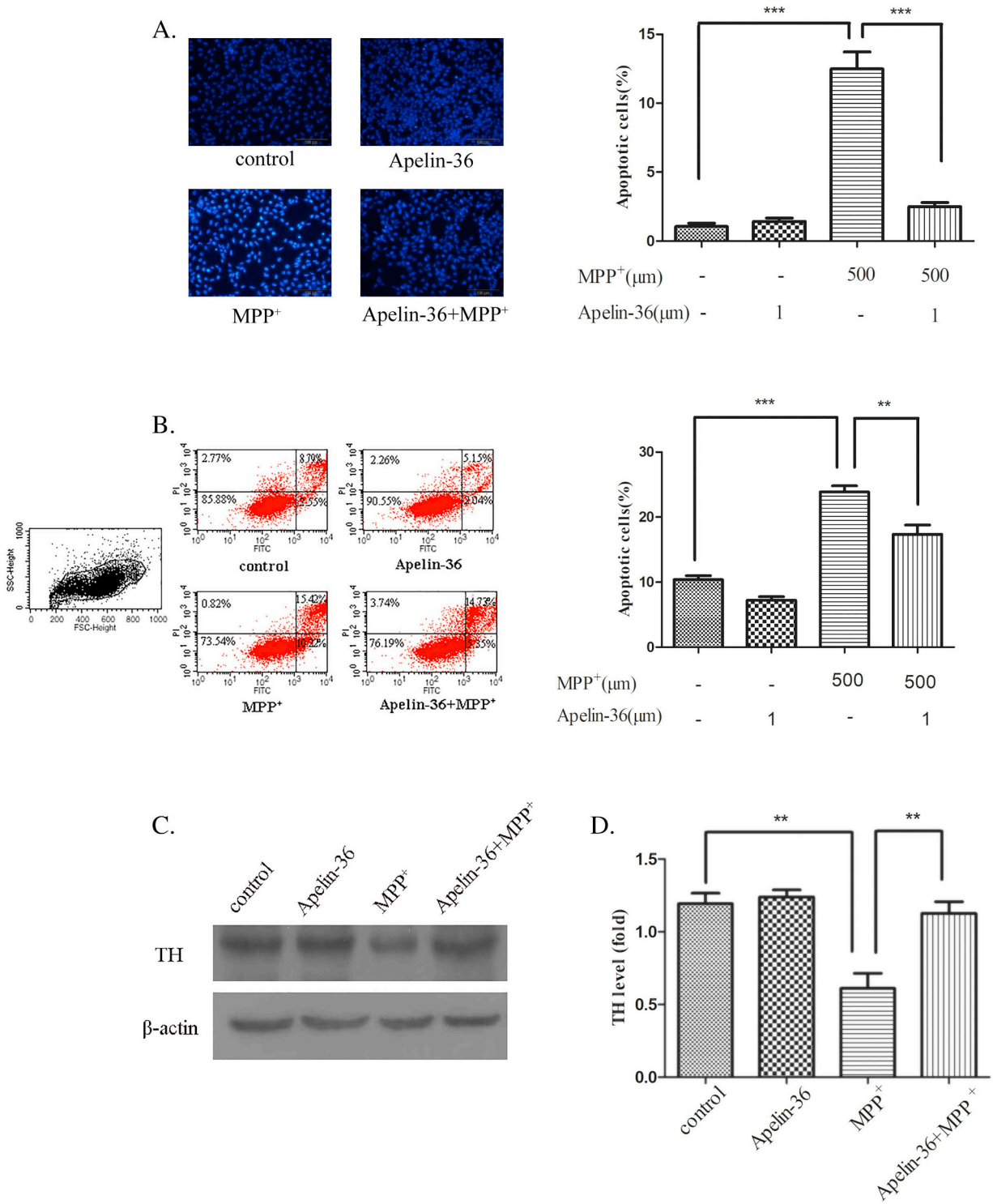

Fig. 2. Apelin-36 attenuated the apoptosis induced by MPP^+ in SH-SY5Y cells.
(A) The apoptosis of SH-SY5Y cells was evaluated by Hoechst 33342 staining. The apoptotic cells were identified morphologically by the strong nuclear staining of Hoechst 33342 because of DNA fragmentation and nuclear shrinkage. The fluorescence was observed with microscopy (Bar = 100 μm). Apoptotic rate (%) = number of apoptotic cells/number of total cells × 100%. (B) The apoptotic rates of SH-SY5Y cells were evaluated by flow cytometry assay. SH-SY5Y cells were pretreated with 1μmmol/L Apelin-36 for 2 h and then co-cultured with 500μM MPP^+ for 24 h. (C and D) Western blotting and quantitative analysis for TH. Values were presented as the mean ± SEM of three independent experiments.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

and visualized under Olympus microscope. Apoptotic cells were identified morphologically by the strong nuclear staining of Hoechst 33342 because of DNA fragmentation and nuclear shrinkage. Apoptotic rate was calculated as follows: Apoptotic rate (%) = number of apoptotic cells / number of total cells × 100%.

2.4. Flow cytometry assay

The flow cytometry was used to measure the apoptotic rate of SH-SY5Y cells treated with MPP^+ with or without Apelin-36, IGF-1 or MHY1485 pretreatment. SH-SY5Y cells were seeded into 6-well plates

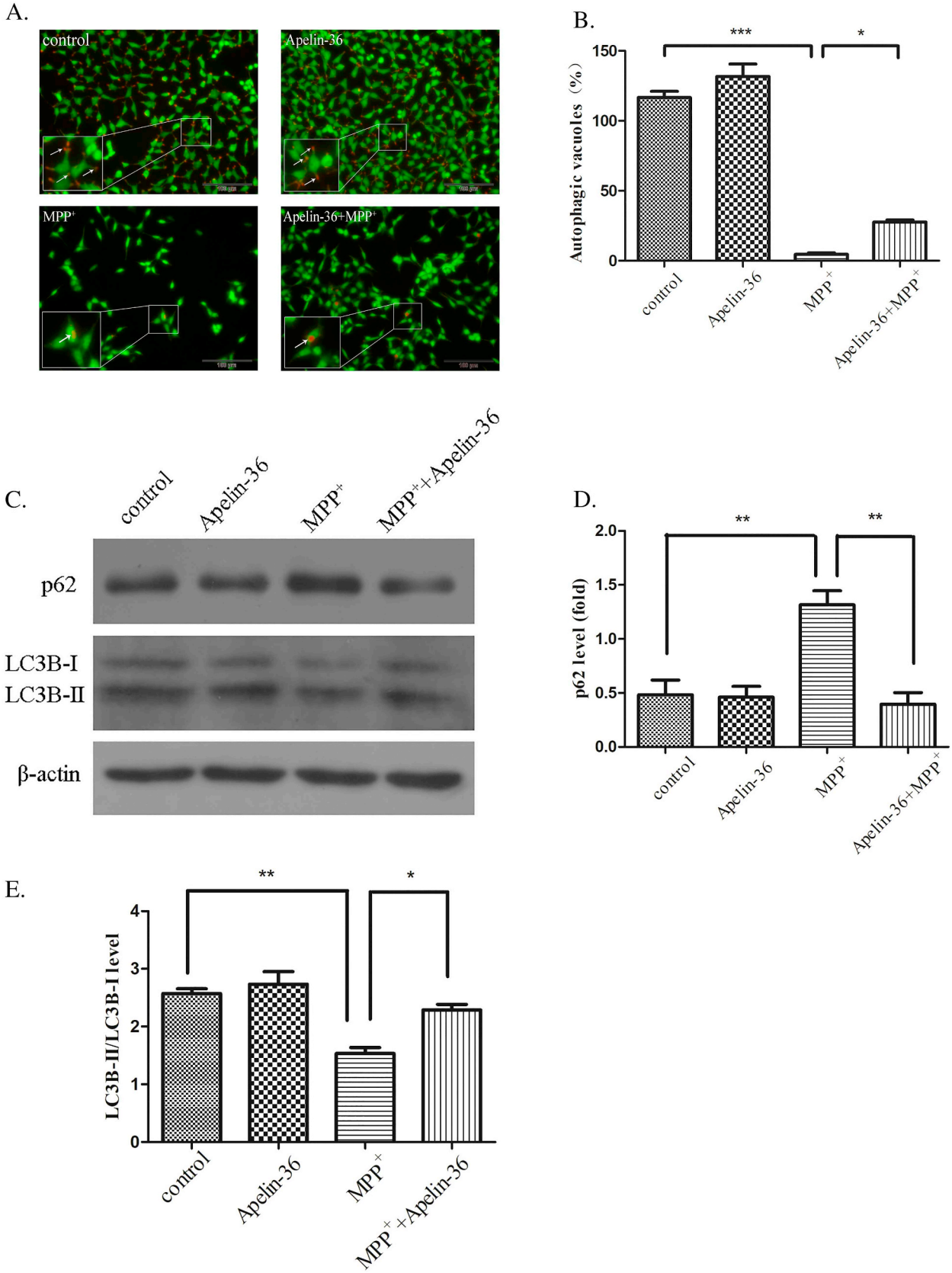

Fig. 3. The effect of Apelin-36 on the decreased autophagy and the activated PI3K/Akt/mTOR pathway in MPP^+-treated SH-SY5Y cells.
(A and B) The autophagic vacuoles in the harvested SH-SY5Y cells were stained red by AO staining under a fluorescence microscope (Bar = 100μm). (C–E) Western blotting and quantitative analysis for p62 and LC3B in SH-SY5Y cells. (F–I) Western blotting and quantitative analysis for p-mTOR, mTOR, p-PI3K, PI3K, p-Akt and Akt in SH-SY5Y cells. Values were presented as mean ± SEM of three independent experiments. *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

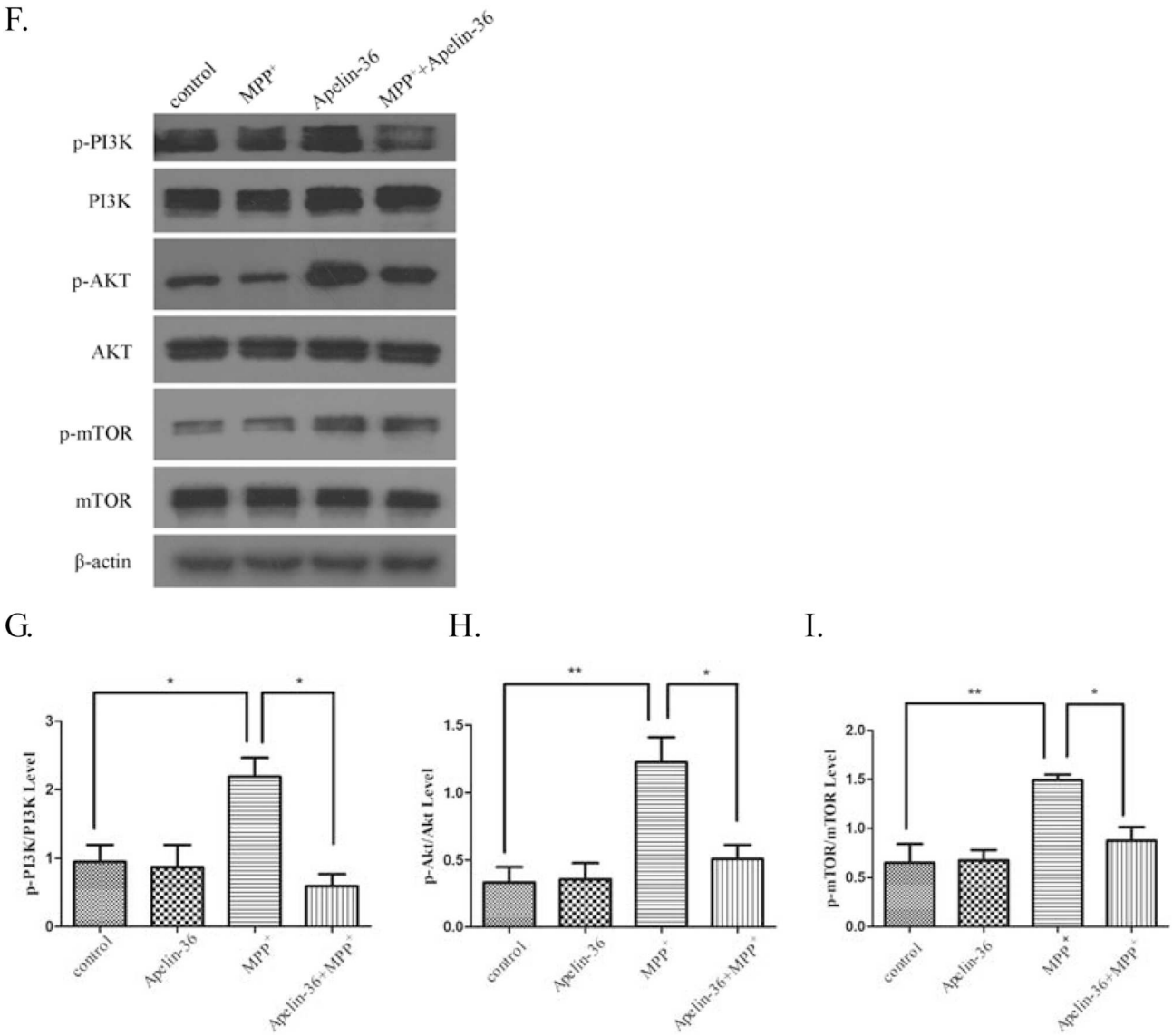

Fig. 3. (*continued*)

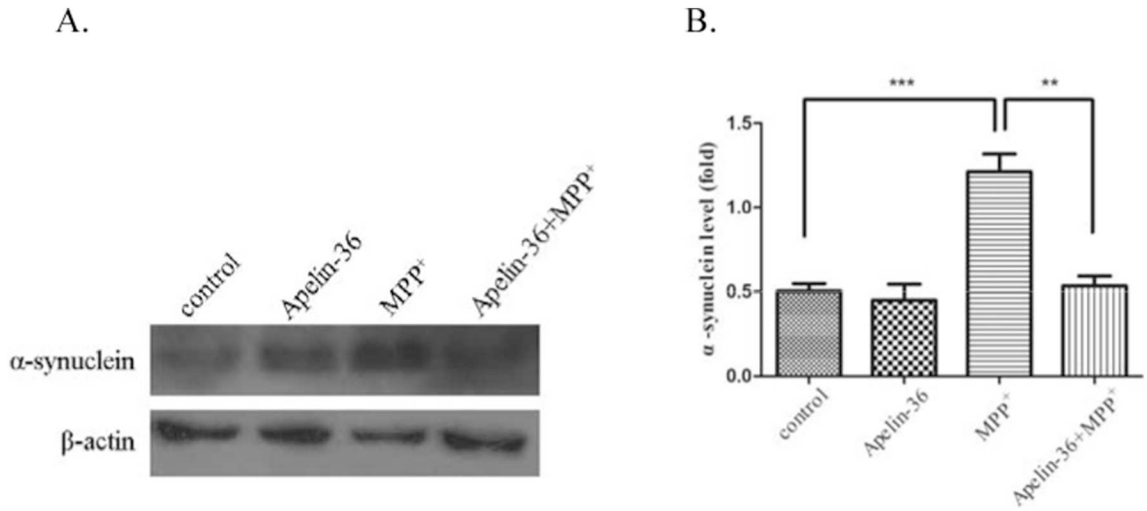

Fig. 4. The effect of Apelin-36 on MPP^+-induced α-synuclein expression in SH-SY5Y cells.
(A and B) Western blotting and quantitative analysis for α-synuclein in SH-SY5Y cells. Values were presented as mean ± SEM of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$.

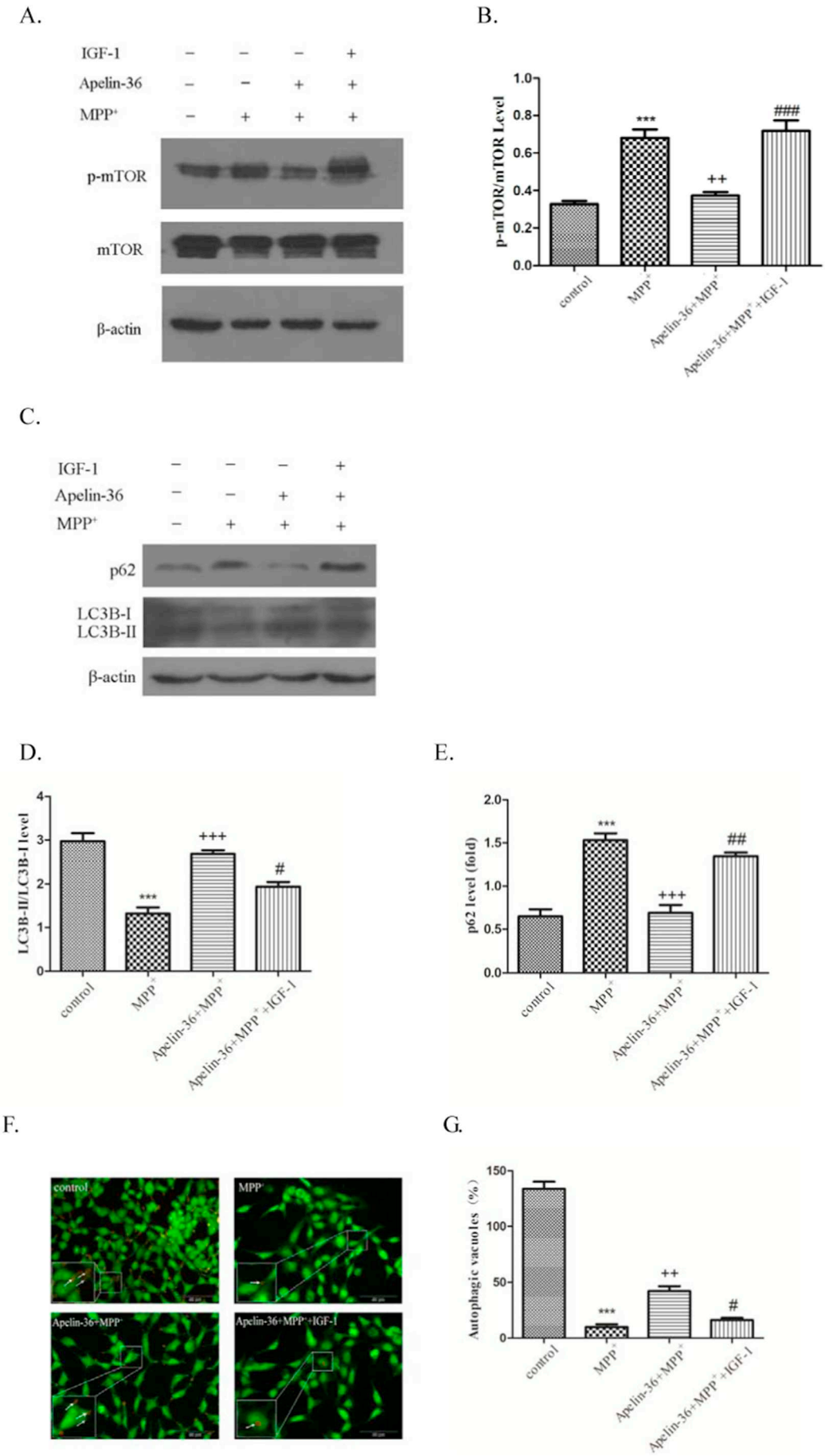

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Fig. 5. IGF-1 pretreatment inhibited mTOR-mediated autophagy in SH-SY5Y cells. (A–E) Western blotting and quantitative analysis for p-mTOR/mTOR, LC3B-I/LC3B-II and p62 in SH-SY5Y cells. (F and G) The autophagic vacuoles in the harvested SH-SY5Y cells were stained red by acridine orange staining under a fluorescence microscope (Bar = 50μm). Values were presented as mean ± SEM of three independent experiments. $^{***}p < 0.001$ vs control group. $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs MPP^+ group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs MPP^+ + Apelin-36 group.

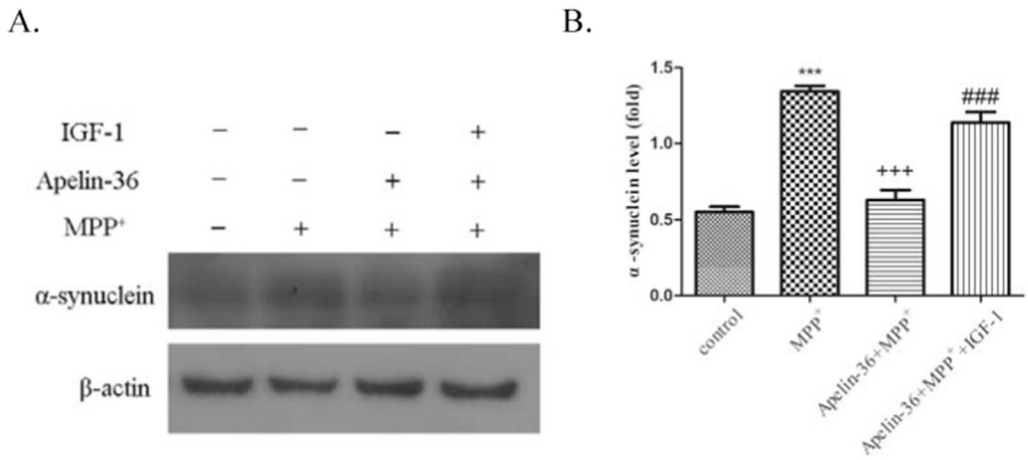

Fig. 6. IGF-1 pretreatment aggravated the abnormal aggregation of α-synuclein in SH-SY5Y cells.
(A and B) Western blotting and quantitative analysis for α-synuclein in SH-SY5Y cells. Values were presented as mean ± SEM of three independent experiments. $^{***}p < 0.001$ vs control group. $^{+++}p < 0.001$ vs MPP^+ group. $^{\#\#\#}p < 0.001$ vs MPP^+ + Apelin-36 group.

treated with drugs treatments as described above. Then SH-SY5Y cells were washed with PBS twice and digested with trypsin without ethylene diamine tetraacetic acid (EDTA). Next, SH-SY5Y cells (5×10^5 cells/well) were collected by centrifugation (900rpm for 3 min at room temperature). Thereafter, 500 μL of Annexin V binding buffer (Biouniquer Technology, Beijing, China) was added to each cell suspension followed by 5 μL of Annexin V-fluorescein isothiocyanate (FITC) and 5 μL of propidium iodide(PI) for 10 min at room temperature protect form light. A total of 10,000 cells were analyzed per sample by flow cytometry, which was performed using a BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Data were representative of at least three independent experiments. The results were analyzed using BD CellQuest Prosoftware (version 5.1, BD Biosciences).

2.5. Acridine orange (AO) staining for autophagy

Autophagy is characterized by the formation and promotion of acidic vesicular organelles. Red stained vesicular organelles can be detected by Acridine orange (AO) staining. SH-SY5Y cells were cultured in 24-well plates by drugs treatments as described above. The cells were stained with 2ml AO (5 mg/mL, Sigma-Aldrich) each well for 25 min at 37 °C in a 5% CO_2 atmosphere. Images were obtained by using Olympus microscope.

2.6. Western blot analysis

SH-SY5Y cells were lysed with the RIPA buffer supplemented with PMSF. The lysed samples were kept in ice for 30 min and shaken every 5 min, and then centrifuged at 12,000 for 30min at 4 °C. The supernatants were used for Western blot analysis. The lysates were resolved on 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membrane at 4 °C. The membranes were blocked with 5% skim milk in TBS/0.1% Tween-20 (TBST) for 1h at room temperature and then incubated overnight at 4 °C with the following primary antibodies: anti-α-synuclein (1:1000, NUVOS), anti-TH (1:1000, Abcam), anti-p62 (1:1000, Abcam), anti-LC3B (1:2500, Abcam), anti-mTOR(1:1000, Cell Signaling Technology), anti-p-mTOR(1:1000, Cell Signaling Technology), anti-PI3K(1:1000, Cell Signaling Technology), anti-p-PI3K (1:1000, Abcam), anti-Akt(1:1000, Cell Signaling Technology), anti-p-Akt (1:1000, Cell Signaling Technology), and anti-β-actin (1:1000, Zhongshan Golden Bridge Biotechnology). Then the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibodies (1:5000, Zhongshan Golden Bridge Biotechnology) for 1h at room temperature followed by chemiluminescence assay. The optical density was quantified by Image-Pro Plus VERSion 6.0 (Media Cybernetics Inc., Bethesda, MD, USA).

2.7. Statistical analysis

Values were represented mean ± SEM. One-way ANOVA followed by Tukey test was performed by using GraphPad Prism. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Apelin-36 attenuated MPP^+-induced cytotoxicity in SH-SY5Y cells

SH-SY5Y cells were treated with different concentrations of MPP^+ (250μM, 500μM, 750μM and 1000μM) for 24h and the cell viability was measured by CCK-8 assay (Fig. 1A). We found that MPP^+ significantly reduced the cell viability of SH-SY5Y cells compared to control group. With the concentration of MPP^+ increasing, the cell viability declined in a dose-dependent manner. When the cells were treated with 500μM MPP^+ for 24h, the cell viability significantly decreased by about 50%. And we found that 1 μM of Apelin-36 significantly attenuated the decreased cell viability induced by MPP^+ (Fig. 1B). The data indicated that Apelin-36 could attenuate MPP^+-induced cytotoxicity.

To further investigate the effect of Apelin-36 on the apoptosis of SH-SY5Y cells induced by MPP^+, SH-SY5Y cells were stained with hoechest33442 (Fig. 2A). The results showed that the apoptotic cells was significantly increased in MPP^+-treated SH-SY5Y cells compared to the control group. And Apelin-36 pretreatment significantly decreased the apoptotic ratio induced by MPP^+. Furthermore, the apoptotic rate of SH-SY5Y cells was analyzed by flow cytometry (Fig. 2B). Compared with the control group, the apoptotic rate of MPP^+-treated group was significantly elevated. And the apoptosis induced by MPP^+ was significantly reduced by Apelin-36 pretreatment. It was indicated that Apelin-36 was cytoprotective against the apoptosis induced by MPP^+. TH is an enzyme for dopamine synthesis, the decreased expression of TH will contribute to PD [43]. We found that Apelin-36 pretreatment significantly improved the expression of TH, which further proved the cytoprotective effect of Apelin-36 in PD (Fig. 2C).

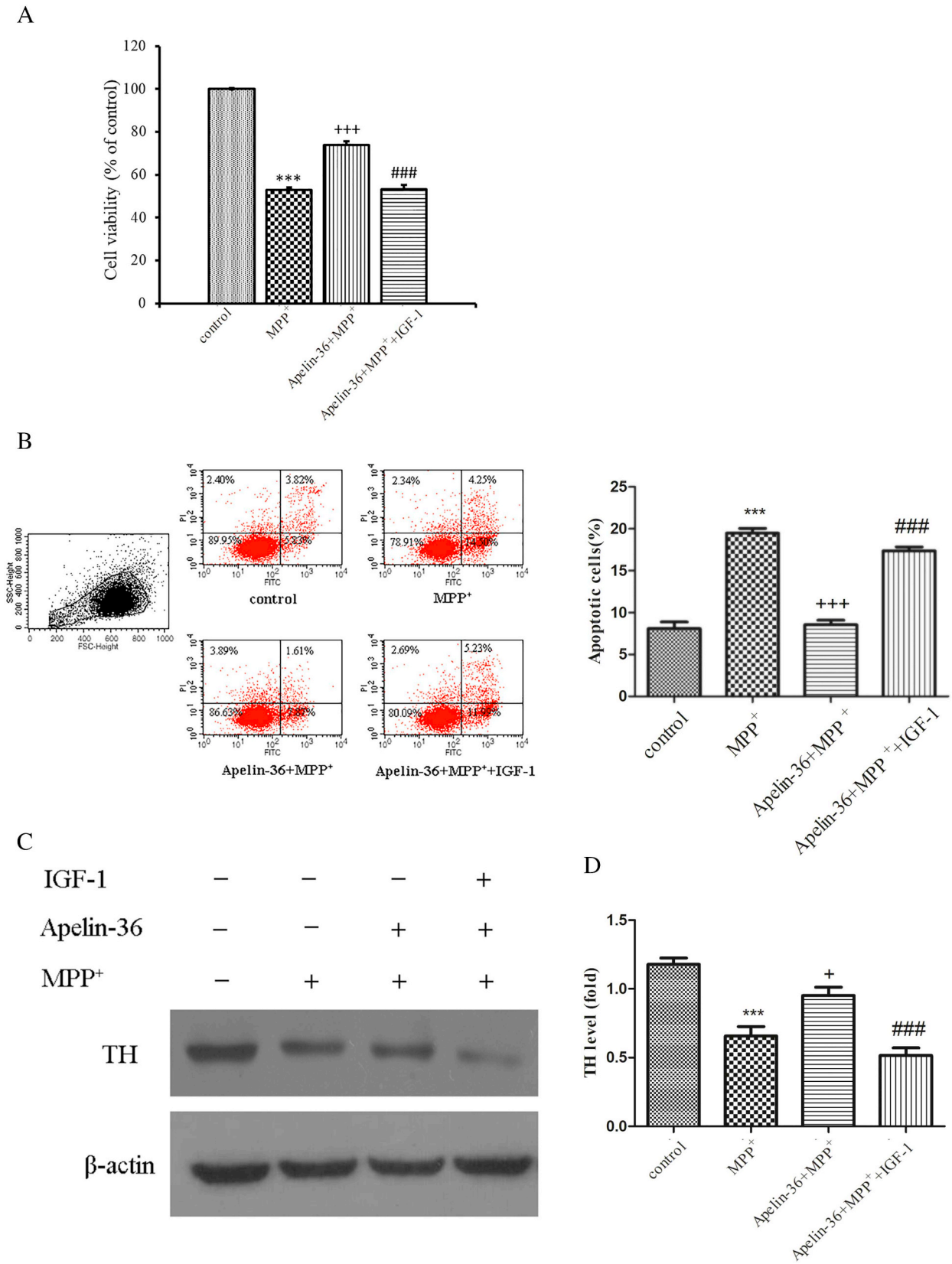

Fig. 7. The cytoprotective effect of Apelin-36 in MPP^+-treated SH-SY5Y cells was weakened by pretreatment with IGF-1.
(A) Cell viability was measured by CCK-8 assay. (B) The apoptotic ratio of SH-SY5Y cells were evaluated by flow cytometry assay. (C and D) Western blotting and quantitative analysis for TH in SH-SY5Y cells. Values were presented as mean ± SEM of three independent experiments. $^{***}p < 0.001$ vs control group. $^{+}p < 0.05$ and $^{+++}p < 0.001$ vs MPP^+ group. $^{###}p < 0.001$ vs MPP^+ + Apelin-36 group.

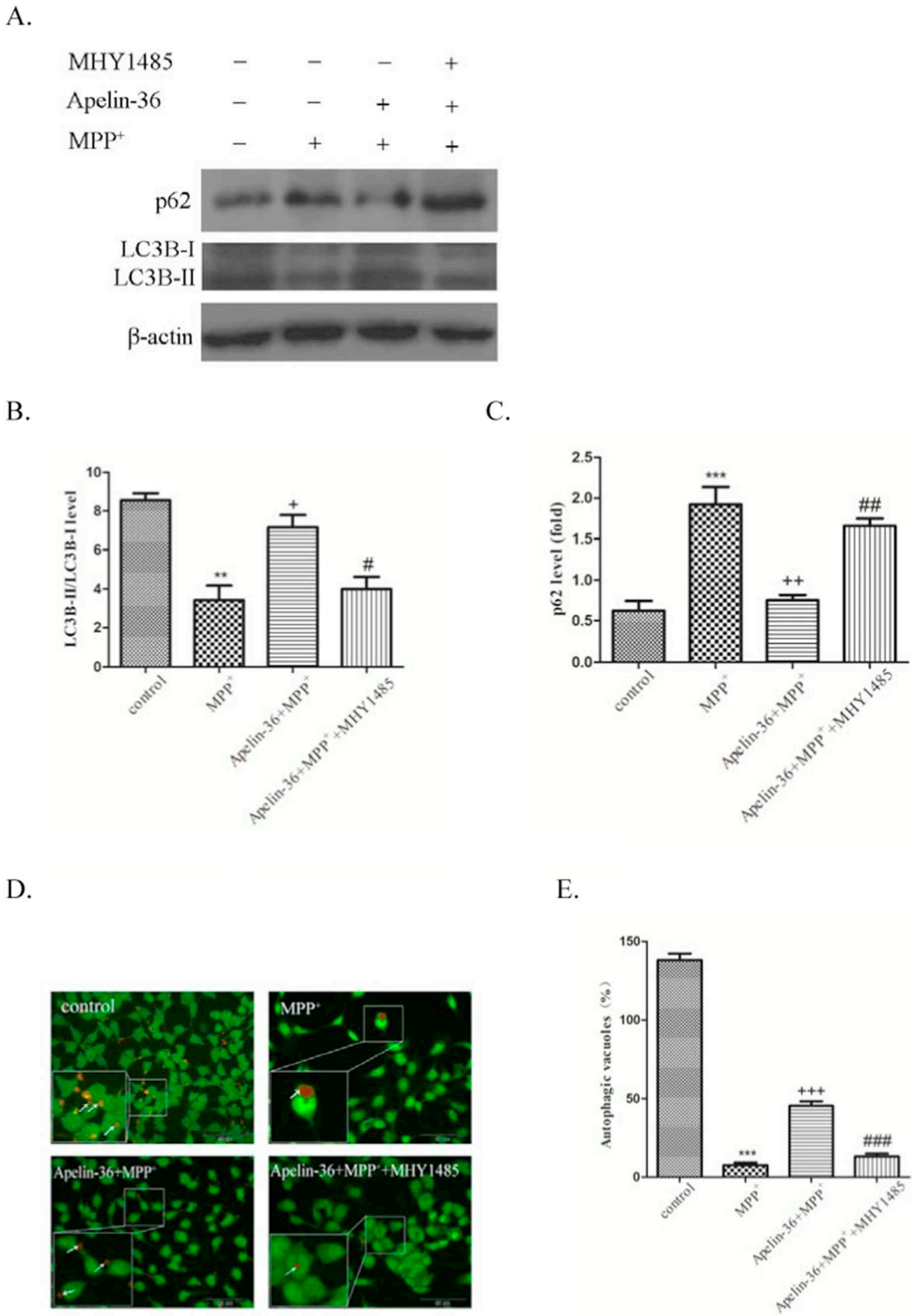

Fig. 8. MHY1485 pretreatment inhibited autophagy in SH-SY5Y cells.
(A–C) Western blotting and quantitative analysis for LC3B-I/LC3B-II and p62 in SH-SY5Y cells. (D and E) The autophagic vacuoles in the harvested SH-SY5Y cells were stained red by acridine orange staining under a fluorescence microscope (Bar = 50μm). Values were presented as mean ± SEM of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs control group. $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs MPP^+ group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs MPP^+ + Apelin-36 group.

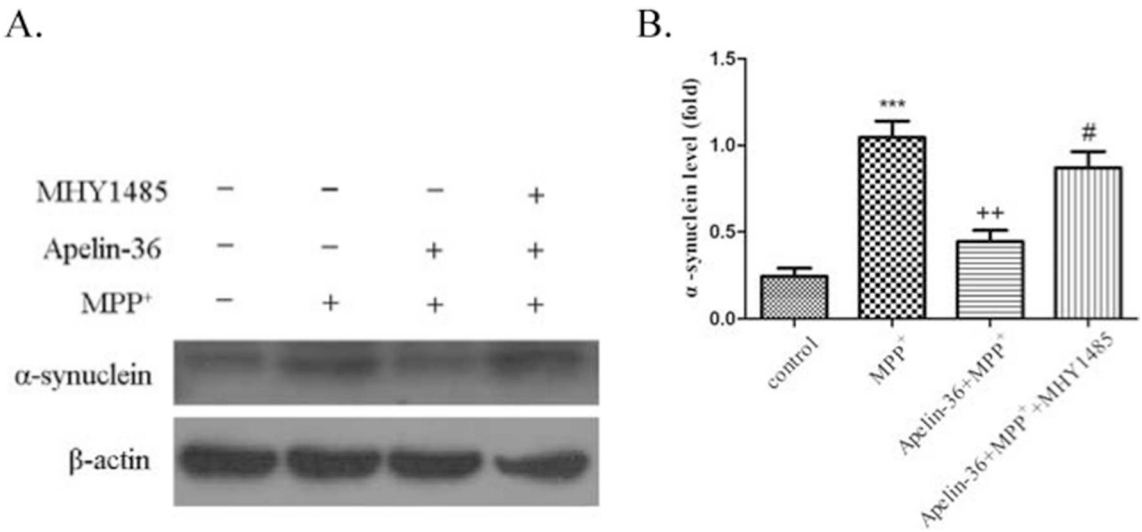

Fig. 9. MHY1485 pretreatment aggravated the abnormal aggregation of α-synuclein in SH-SY5Y cells.
(A and B) Western blotting and quantitative analysis for α-synuclein in SH-SY5Y cells. Values were presented as mean ± SEM of three independent experiments. $^{***}p < 0.001$ vs control group. $^{+++}p < 0.001$ vs MPP^+ group. $^{###}p < 0.001$ vs MPP^+ + Apelin-36 group.

3.2. Apein-36 enhanced autophagy in MPP^+-treated SH-SY5Y cells

To investigate the effect of Apelin-36 on autophagy, we determined the autophagic vacuoles in SH-SY5Y cells by AO staining. The number of red-stained autophagic vacuoles in MPP^+-treated SH-SY5Y cells was significantly reduced compared with the control group (Fig. 3A). While Apelin-36 pretreatment significantly increased the autophagic vacuoles in MPP^+-treated SH-SY5Y cells (Fig. 3A). Furthermore, we found that the pretreatment of Apelin-36 significantly increased the ratio of LC3B-II/LC3B-I and reduced the expression of p62 in MPP^+-treated SH-SY5Y cells (Fig. 3C), indicating that Apelin-36 promotes autophagy in MPP^+-treated SH-SY5Y cells.

PI3K/Akt/mTOR pathway is a classical autophagic pathway [44]. In our experiment, we explore the effect of Apelin-36 on this autophagic pathway in MPP^+-treated SH-SY5Y cells. mTOR is a negative regulator of autophagy which is regulated by the phosphorylation of PI3K and Akt [44]. The results showed that the ratio of p-mTOR/mTOR, p-PI3K/PI3K and p-Akt/Akt were significantly increased in MPP^+-treated SH-SY5Y cells compared with the control group (Fig. 3F). Apelin-36 pretreatment significantly decreased the ratio of p-mTOR/mTOR, p-PI3K/PI3K and p-Akt/Akt compared with MPP^+ group (Fig. 3F). These suggested that Apelin-36 inhibits the PI3K/Akt/mTOR autophagic pathway.

3.3. Apelin-36 reversed MPP^+-induced α-synuclein expression in SH-SY5Y cells

Compelling evidence has been shown that abnormally accumulation of α-synuclein is responsible for the DA neuronal death in PD [45], and the clearance of α-synuclein is strongly dependent on autophagy [29]. In the present work, we investigated the effect of Apelin-36 on MPP^+-induced α-synuclein expression in SH-SY5Y cells by western blot (Fig. 4A). The result showed that α-synuclein expression was significantly increased by MPP^+ administration in SH-SY5Y cells. Interestingly, Apelin-36 pretreatment significantly reversed α-synuclein expression compared with MPP^+ group cells.

3.4. PI3K/AKT/mTOR autophagic pathway was involved in the cytoprotective effect of Apelin-36 in MPP^+-treated SH-SY5Y cells

We investigated the effect of Apelin-36 on PI3K/AKT/mTOR autophagy pathway. IGF-1 is known to bind to the IGF-1 receptor, which can activate the PI3K/AKT signaling pathway [46]. We found that the ratio of p-mTOR/mTOR was significantly decreased by pretreatment with Apelin-36 in MPP^+-treated SH-SY5Y cells (Fig. 5A). Interestingly, we found that the pretreatment of IGF-1 for 30min before the addition of Apelin-36 dramatically increased the ratio of p-mTOR/mTOR in MPP^+-treated SH-SY5Y cells (Fig. 5A). As shown in Fig. 5C, the ratio of LC3B-II/LC3B-I was significantly decreased and the expression of p62 was significantly increased in Apelin-36 + MPP^+ + IGF-1 group compared with Apelin-36 + MPP^+ group. Also, the autophagic vacuoles was significantly decreased in Apelin-36 + MPP^+ + IGF-1 group compared with Apelin-36 + MPP^+ group (Fig. 5F), indicating that autophagy was inhibited by pretreatment of IGF-1.

Subsequently, we found that the expression of α-synuclein was significantly increased by pretreatment of IGF-1(Fig. 5A). The result indicates that IGF-1 aggravates the abnormal accumulation of α-synuclein.

In addition, we found that the cell viability was decreased and the apoptotic ratio was increased in Apelin-36 + MPP^+ + IGF-1 group compared with Apelin-36 + MPP^+ group (Fig. 6A and B). Moreover, IGF-1 pretreatment significantly decreased the expression of TH (Fig. 6C), indicating that the cytoprotective effect of Apelin-36 was weakened by pretreatment with IGF-1. These findings reveal that Apelin-36 protects against MPP^+-induced cytotoxicity by inhibiting PI3K/AKT pathway (Fig. 7).

We further investigated whether Apelin-36 protected SH-SY5Y cells against MPP^+ induced cytotoxicity by inhibiting PI3K/AKT/mTOR autophagy pathway. It has been reported that MHY1485 is an activator of mTOR, and MHY1485 is known to down-regulating the autophagy by inhibiting the fusion of autophagosomes and lysosomes [47,48]. As shown in Fig. 8A, the ratio of LC3B-II/LC3B-I was dramatically decreased and the expression of p62 was significantly increased by the pretreatment of MHY1485 for 30min before the addition of Apelin-36 in MPP^+-treated SH-SY5Y cells. Moreover, we found that the autophagic vacuoles were significantly decreased in SH-SY5Y cells by pretreatment with MHY1485 (Fig. 8D).

Subsequently, we found that the expression of α-synuclein was significantly increased by pretreatment of MHY1485 (Fig. 9A). The result indicates that MHY1485 aggravates the abnormal accumulation of α-synuclein.

Furthermore, the result showed that MHY1485 pretreatment significantly decreased the cell viability and increased the apoptotic ratio in SH-SY5Y cells (Fig. 10A and B). Additionally, the expression of TH

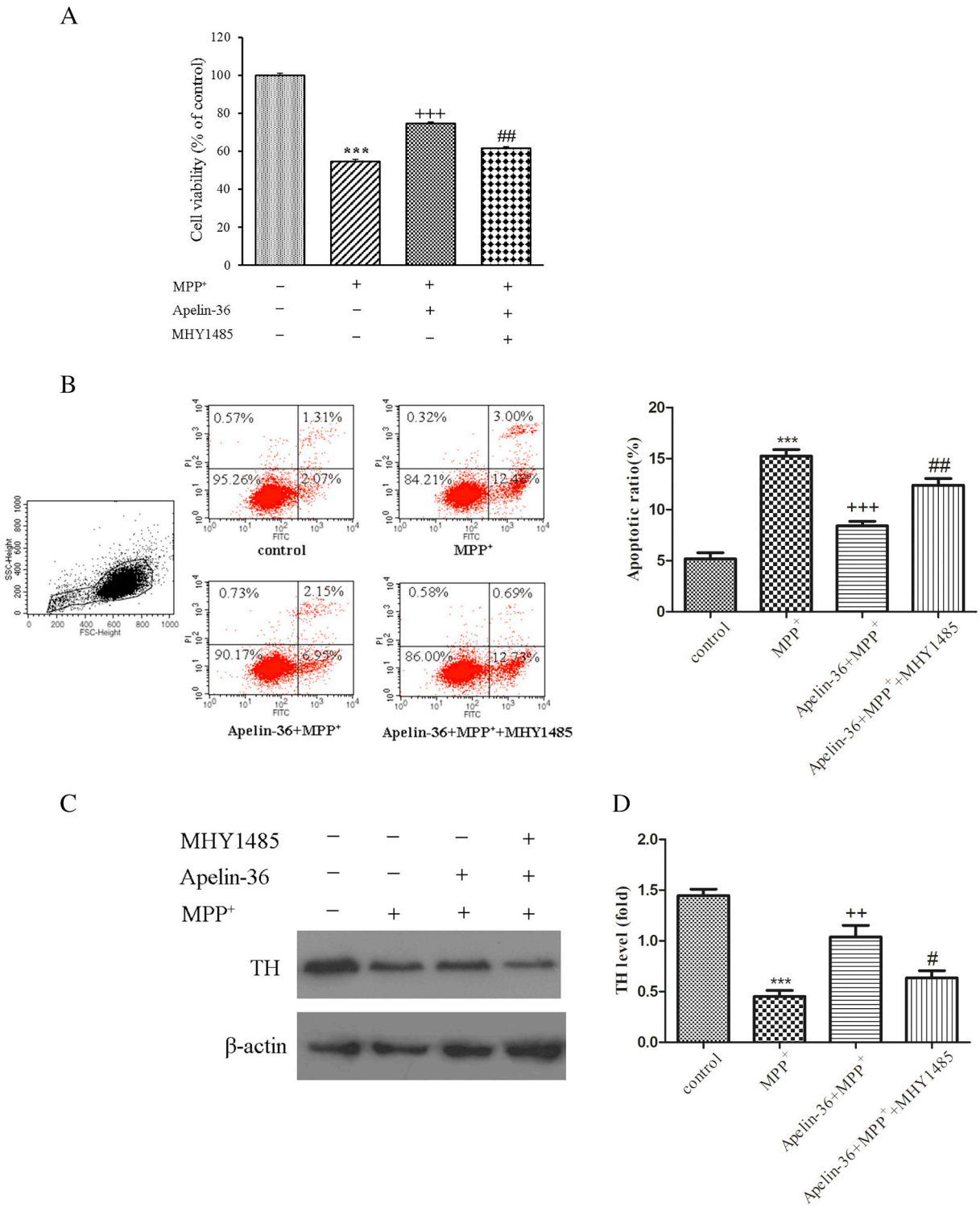

Fig. 10. The cytoprotective effect of Apelin-36 in MPP^+-treated SH-SY5Y cells was weakened by pretreatment with MHY1485.
(A) Cell viability was measured by CCK-8 assay. (B) The apoptotic ratio of SH-SY5Y cells were evaluated by flow cytometry assay. (C and D) Western blotting and quantitative analysis for TH in SH-SY5Y cells. Values were presented as mean ± SEM of three independent experiments. $^{***}p < 0.001$ vs control group. $^{+}p < 0.05$ and $^{+++}p < 0.001$ vs MPP^+ group. $^{\#\#\#}p < 0.001$ vs MPP^+ + Apelin-36 group.

was significantly decreased by pretreatment with MHY1485 (Fig. 10C), indicating that the cytoprotective effect of Apelin-36 was weakened by pretreatment with MHY1485. These results suggest that Apelin-36 protects against MPP^+-induced cytotoxicity by inhibition of PI3K/AKT/mTOR autophagy pathway.

4. Discussion

In our present experiment, human neuroblastoma SH-SY5Y cells with dopamine neuron properties were studied for PD [49]. We investigated the possible protective effect of the neuropeptide Apelin-36

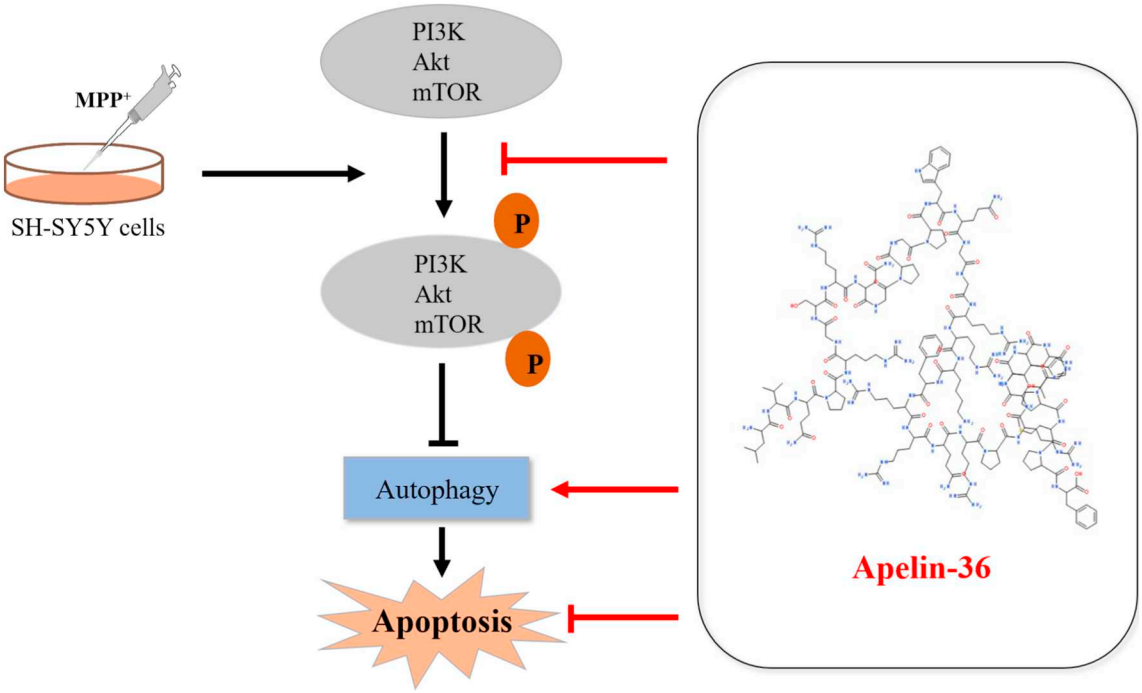

Fig. 11. Working model.
Apelin-36 attenuates MPP$^+$-induced cytotoxicity by inhibiting PI3K/Akt/mTOR pathway and promoting autophagy in a PD model in vitro.

in SH-SY5Y cells. The results showed that Apelin-36 significantly improved the cell viability of MPP$^+$-treated SH-SY5Y cells and decreased the apoptotic ratio. Furthermore, we found that PI3K/Akt/mTOR autophagic pathway was involved in the cytoprotective effect of Apelin-36 in the cellular PD model. Taken together, we conclude that Apelin-36 may be used for the prevention and treatment of PD (Fig. 11).

Autophagy has been documented to play a key role in the pathogenesis of PD. It is reported that α-synuclein is predominantly degraded by autophagy [50]. Studies have found that the autophagy could be damaged by the mutation in PD-related genes and neurotoxin, which promotes the development of PD [21,27]. Previous study has been shown that A30P mutant α-synuclein causes autophagy dysfunction in Ventral mesencephalic neurons [51]. In addition, in a rotenone-induced PD mice model, autophagy was dramatically inhibited, leading to the abnormal aggregation of α-synuclein [52]. The autophagy also decreased in MPP$^+$-treated SH-SY5Y cells, as well as in MPTP-treated mice, which induces apoptosis and degeneration of dopaminergic neurons [53]. In the present work, we showed that the ratio of LC3B-II/LC3B-I was significantly decreased in MPP$^+$-treated SH-SY5Y cells, as well as the increased p62 expression, which is consistent with the results of mounting studies mentioned above. Therefore, searching therapeutic intervention in autophagy is impending for PD.

It has been reported that dysregulation in the PI3K/Akt/mTOR pathway is contributed to the loss of dopaminergic neurons in PD [54]. In both nondifferentiated and differentiated SH-SY5Y dopaminergic cells, the mRNA of PI3K, Akt and mTOR were up-regulated by MPP$^+$ [55]. mTOR is a major regulator of the autophagic process. PI3K and Akt are two important factors upstream of mTOR. The proteins of TSC1 and TSC2 are the downstream of the PI3K/Akt pathway, and the phosphorylated TSC1/2 activates the small G protein Ras Homologue Enriched in Brain (Rheb), then, mTOR is activated followed by the inhibition of autophagy [31]. PI3K/Akt/mTOR is an important signaling pathway in the process of mTOR-dependent autophagy, and it is involved in the clearance of abnormal aggregated protein, which plays a very important role in neurodegenerative diseases such as PD for anti-apoptosis [30,56,57]. It has been shown that the degradation of toxic α-synuclein was increased by inhibition of mTOR in Rotenone-treated SH-SY5Y cells [32]. Similarly, Manganese-induced autophagic dysregulation and abnormal aggregation of α-synuclein in SH-SY5Y cells are ameliorated by mTOR inhibition [58]. Furthermore, mTOR inhibitor alleviates the toxicity of PC12 cells induced by 6-OHDA [33]. Moreover, the impaired autophagy for the activation of the PI3K/Akt/mTOR pathway triggers the form of inflammasome in MPTP-treated PD mouse model [34]. In addition, moxibustion, a traditional Chinese medicine, could inhibit mTOR and promote autophagy, which increases the survival of dopaminergic neurons in the SNpc of PD rats induced by rotenone [59]. Consistent with the previous studies, our data showed that the PI3K/Akt/mTOR pathway was activated in MPP$^+$-treated SH-SY5Y cells, following by the autophagy inhibition. And the expression of α-synuclein was increased, as well as the decreased TH expression.

Apelin and its receptor APJ are widely distributed in the body and has high expression levels in the brain, adipose tissue, skeletal muscle, and cardiovascular system, which exerts corresponding physiological functions [60]. Previous studies demonstrated positive effects of Apelin on central nervous system disease. In 6-OHDA-treated SH-SY5Y cells, Apelin-13 alleviates 6-OHDA-induced cytotoxicity by antioxidant and anti-apoptotic [42]. In addition, in 6-OHDA-induced PD models in vivo, Apelin-13 has been proved to ameliorate cognitive impairments in rats [41]. A recent report showed that the apoptosis is attenuated by Apelin-36 in Rats with ischemic stroke, and ERS is involved in the neoroprotective effect of Apelin-36 [40]. However, the underlying molecular mechanism of Apelin remains poorly understood. For the autophagy is proved to be important in the pathogenesis of PD, we established a hypothesis that autophagy may be regulated by Apelin-36 in a PD model in vitro. Our results showed that Apelin-36 improved the cell viability and inhibited apoptosis in MPP$^+$-treated SH-SY5Y cells. Furthermore, Apelin-36 reversed the increased expression of p-PI3K, p-Akt and p-mTOR by MPP$^+$, following by the ratio of LC3B-II/LC3B-I improvement and p62 decline. Then, the expression of α-synuclein was decreased, as well as the increased TH expression by Apelin-36 pretreatment. Interestingly, we demonstrated that the effect of Apelin-36 could be weakened by PI3K/Akt activator IGF-1 and mTOR activator MHY1485, which suggested that PI3K/Akt/mTOR autophagic pathway may contribute to the cytoprotective effect of Apelin-36 in PD.

5. Conclusion

We conclude that Apelin-36 reduces MPP^+-induced cytotoxicity through PI3K/Akt/mTOR autophagic pathway in SH-SY5Y cells, suggesting that Apelin-36 may be used for the treatment of PD in the future. Moreover, we will further explore the role of Apelin-36 in primary dopaminergic neurons and in vivo models of PD.

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