Apelin-13 attenuates ER stress-associated apoptosis induced by MPP+ in SH-SY5Y cells

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Abstract. Apelin-13, a neuropeptide that acts as a ligand for a putative receptor related to the angiotensin II type receptor, elicits neuroprotective effects in numerous neurological conditions, such as Huntington's disease and cerebral ischemia. Parkinson's disease (PD), one of the most prevalent neurodegenerative diseases, is caused by damage to neurons in the brain; however, the underlying mechanism remains unclear. The present study explored the effects of apelin-13 on SH-SY5Y human neuroblastoma cells treated with 1-methyl-4-phenylpyridine (MPP^+). Cell growth, cell viability, and apoptosis were measured by real-time cell analysis, the Cell Counting Kit-8 assay, and flow cytometry, respectively. In addition, the expression levels of extracellular signal-regulated kinase (ERK) 1/2, p38 mitogen-activated protein kinase (MAPK), glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), and cleaved caspase-12 were assessed by western blotting. MPP^+ treatment decreased the viability of SH-SY5Y cells and increased their apoptosis; however, these changes were attenuated by pretreatment with apelin-13. Treatment with MPP^+ for 24 h significantly increased the expression levels of phospho-ERK1/2, phospho-p38, GRP78, CHOP, and cleaved caspase-12 in SH-SY5Y cells. Pretreatment with apelin-13 significantly attenuated the upregulation of GRP78, CHOP and cleaved caspase-12 in MPP^+-treated SH-SY5Y cells, and significantly enhanced the expression levels of phospho-ERK1/2. Taken together, the present results support a model in which apelin-13 inhibits MPP^+-induced apoptosis of SH-SY5Y cells by decreasing the expression of GRP78, CHOP, and cleaved caspase-12, and by increasing the expression of phospho-ERK1/2. The present findings suggest that apelin-13 may be useful for the treatment of PD.

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Abbreviations: AD, Alzheimer's disease; CCK-8, Cell Counting Kit-8; ER, endoplasmic reticulum; ERK1/2, extracellular signal-regulated kinase 1/2; GRP78, glucose-regulated protein 78; I/R, ischemia-reperfusion; MAPK, mitogen-activated protein kinase; MPP^+, 1-methyl-4-phenylpyridine; NMDA, N-methyl-D-aspartic acid; PI3K, phosphoinositide 3-kinase; Akt, AKT serine/threonine kinase 1; PD, Parkinson's disease; RTCA, real-time cell analysis

Key words: apelin-13, Parkinson's disease, glucose-regulated protein 78, neuroprotection

Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease worldwide and is characterized by bradykinesia, muscle rigidity, and reduced independence (1-3). Oxidative stress, aging, infection, genetic and environmental factors are considered major causes of PD (4-7). However, the pathogenesis of the disease is not fully understood.

The endoplasmic reticulum (ER) is a major subcellular organelle and serves an important role in various cellular functions, including protein folding, protein trafficking and steroid and lipid metabolism (8-10). Multiple factors, such as oxidative stress and ischemia-reperfusion (I/R) injury, can induce the release of inflammatory cytokines and lead to ER stress (11). ER stress is a widely used marker of PD and is also closely related to other neurological disorders, such as Huntington's disease and Alzheimer's disease (AD) (10). Investigation of the mechanism underlying ER stress in PD may lead to the development of new therapeutic strategies.

Apelin, a neuropeptide that acts as a ligand for the orphan G protein-coupled apelin receptor (APJ), has been extracted from bovine stomach tissue and undergoes cleavage to generate apelin-13, -17 and -36 (12-14). Apelin peptides are associated with neuroprotection and cytoprotection. Apelin-13 reduces the cerebral infarct volume in a middle cerebral artery occlusion rat model by downregulating expression of inflammatory factors (15). In addition, the activity of apelin-13 is higher compared with apelin-17 and -36 (14-16). Apelin-36, a long apelin peptide, protects against ischemic brain injury by decreasing the levels of caspase-3 and BCL2 associated X (Bax), which are well-established apoptotic markers, thereby reducing neurological deficits (17). Furthermore, treatment with apelin-13 markedly decreases brain edema and the

total infarct volume and suppresses apoptosis by reducing caspase-3 activation (18). Our previous study has demonstrated that apelin-13 protects against cerebral I/R injury (15), consistent with other literature. However, the mechanism by which apelin protects neurons against 1-methyl-4-phenylpyridine (MPP^+)-induced apoptosis in a cellular model of PD remain unclear. The present study investigated the effects of apelin-13 on MPP^+-treated SH-SY5Y cells, a cellular model of PD, and the underlying molecular mechanism.

Materials and methods

Reagents and cell culture. Human apelin-13 [half maximal effective concentration (EC50)=0.37 nM (19)] was obtained from Phoenix Pharmaceuticals (St. Joseph, MO, USA). Its amino acid sequence is Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro -Met-Pro-Phe (20). MPP^+ iodide and the Cell Counting Kit-8 (CCK-8) were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Antibodies against p38 mitogen-activated protein kinase (MAPK; cat. no. 8690), phosphorylated (phospho)-p38 MAPK (cat. no. 4511), extracellular signal-regulated kinase (ERK) 1/2 (cat. no. 4696), phospho-ERK1/2 (cat. no. 9101), glucose-regulated protein 78 (GRP78, also known as BiP; cat. no. 3183), C/EBP homologous protein (CHOP; cat. no. 2895), and caspase-12 (cat. no. 2202) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-β-actin primary antibody (cat. no. TA-09), polyclonal goat anti-mouse (cat. no. ZB-2305) and anti-rabbit secondary antibodies (cat. no. ZB-2301) were obtained from OriGene Technologies, Inc. (Beijing, China) (21). The SH-SY5Y cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) (22-24) and routinely cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 U/ml penicillin/streptomycin in the presence of 5% CO_2 at 37˚C.

Real-time cell analysis (RTCA). Real-time cell analysis (RTCA) (25) was performed to study the effects of MPP^+ on SH-SY5Y cells. Each well of an E-Plate 16 was incubated with 100 μl of culture media for 30 min at 37˚C to ensure substrate equilibrium; this background step was crucial. Next, SH-SY5Y cells were digested with 0.25% trypsin/0.02% EDTA solution, and 100 μl of culture media containing $5x10^4$ cells was added to each well of the E-Plate 16 and incubated overnight at 37˚C. Finally, cells were treated with 0, 100, 250, 500, 750 or 1,000 μM MPP^+ with or without 100 nM apelin-13 and monitored for 36 h.

CCK-8 assay. The CCK-8 assay was used to measure the viability of SH-SY5Y cells treated with MPP^+ with or without apelin-13. SH-SY5Y cells were digested as aforementioned and then transferred to a 96-well transparent microplate in 100 μl of DMEM containing 10% FBS ($5x10^4$ cells/well). Following incubation overnight, the medium was replaced by fresh complete medium containing the indicated drugs and cells were cultured for ~24 h. Thereafter, 10 μl of 5 mg/ml CCK-8 solution was added to each well and plates were incubated in the presence of 5% CO_2 at 37˚C for 0.5-4 h to allow dehydrogenases to reduce WST-8. Absorbance of each well at 450 nm was measured using a microplate reader. Cell viability was calculated using the following formula: Cell viability (%)=(A-B)/(A'-B) x100, where A is the average absorbance of wells containing the indicated drugs, SH-SY5Y cells, and CCK-8 solution; A' is the average absorbance of wells containing SH-SY5Y cells and CCK-8 solution; and B is the average absorbance of wells containing medium and CCK-8 solution.

Flow cytometry. SH-SY5Y cells ($5x10^5$ cells/well) were collected by centrifugation (800 x g for 5 min at room temperature), treated with MPP^+ for 24 h, and washed with PBS. 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 for 5 min. 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), using an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Data are representative of at least three independent experiments. The results were analyzed using BD CellQuest Pro software (version 5.1; BD Biosciences).

Western blotting. SH-SY5Y cells were transferred to 6-well plates and incubated overnight in the presence of 5% CO_2 at 37˚C. Thereafter, cells were treated with 0, 100, 250, 500, 750 or 1,000 μM MPP^+ for 24 h with or without 100 nM apelin-13, washed with ice-cold PBS, and lysed in RIPA buffer. Cells were collected in clean 1.5 ml centrifuge tubes and cleared by centrifugation at 1,200 x g for 35 min at 4˚C. The protein concentration was determined by the bicinchoninic acid assay, using bovine serum albumin as a standard. Equal amounts (35 μg) of cell extracts were analyzed by 10% SDS-PAGE. Separated proteins were transferred to polyvinylidene fluoride membranes at 300 mA for 40-80 min. Membranes were blocked with TBS/0.1% Tween-20 (TBST) containing 5% milk at room temperature for 1 h, rinsed thrice with TBST for 10 min, and incubated overnight at 4˚C with rabbit anti-phospho-p38 (1:1,000), rabbit anti-phospho-ERK1/2 (1:2,000), rabbit anti-GRP78 (1:1,000), mouse anti-CHOP (1:1,000), and rabbit anti-caspase-12 (1:1,000) antibodies. Thereafter, membranes were washed with TBST and incubated with appropriate secondary antibodies [goat anti-rabbit-IgG (1:5,000) for phospho-ERK1/2, phospho-p38, GRP78, and caspase-12, and goat anti-mouse-IgG (1:5,000) for CHOP] at room temperature for 1 h. Protein bands were visualized using an enhanced chemiluminescence detection system, and their intensities were measured by densitometry, which was analyzed using ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA). To analyze differences in protein loading between the lanes, blots initially probed with anti-phospho-p38, anti-phospho-ERK1/2, and anti-GRP78 antibodies were stripped and re-probed with mouse anti-p38 (1:1,000), anti-ERK1/2 (1:2,000), and anti-actin (1:1,000) antibodies. Subsequently, blots were incubated with goat anti-mouse-IgG (1:5,000) antibodies at room temperature for 1 h and then visualized as aforementioned.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA,

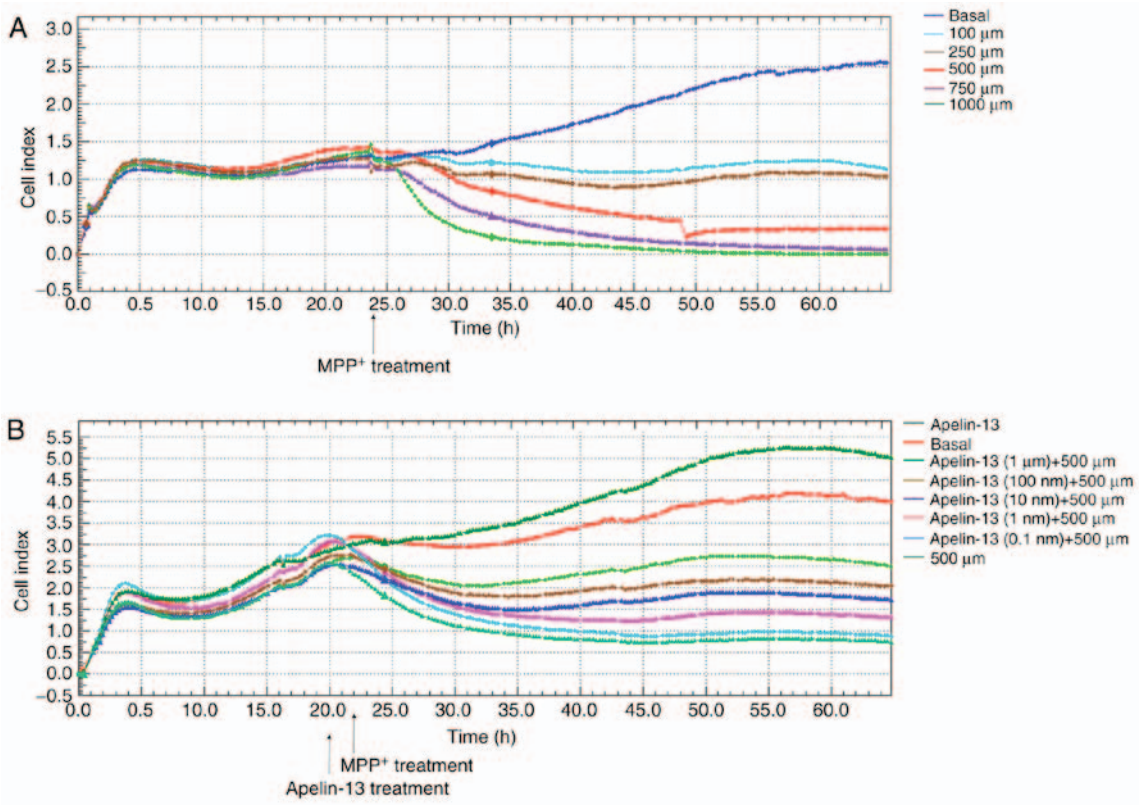

Figure 1. Effects of different concentrations of MPP+ and apelin-13 on the survival index of SH-SY5Y cells determined by RTCA. (A) SH-SY5Y cells were grown for 24 h and then treated with 0, 100, 250, 500, 750 and 1,000 μM MPP+ for 36 h. The survival index was determined by RTCA. (B) Cells were pretreated with 0, 0.1, 1, 10, 100 and 1,000 nM apelin-13 for 2 h and then treated with 500 μM MPP+ for 36 h. MPP+, 1-methyl-4-phenylpyridine; RTCA, real-time cell analysis.

USA). Data are presented as mean ± standard deviation of at least three independent experiments. The Student's paired t-test was used to assess differences between two groups. One-way analysis of variance followed by Tukey's test was used to assess differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Apelin-13 attenuates the MPP+-induced decrease in the survival index of SH-SY5Y cells. SH-SY5Y cells were incubated with 0, 100, 250, 500, 750, and 1,000 μM MPP+ for 36 h, and their growth was monitored by RTCA. The results demonstrated that the cell index of SH-SY5Y cells without MPP+ treatment (basal) was gradually increased to ~2.5 at 36 h, while the cell index of SH-SY5Y cells with MPP+ treatment for 36 h were ~1.25, 1.0, 0.35, 0.1 and 0.02 for 100, 250, 500, 750 and 1,000 μM MPP+, respectively (Fig. 1A). The results indicated that MPP+ treatment decreased the survival index of SH-SY5Y cells in a dose-dependent manner. Next, the neuroprotective effect of apelin-13 was investigated in SH-SY5Y cells treated with 500 μM MPP+. Pretreatment with different concentrations of apelin-13 (0.1, 1, 10, 100 and 1,000 nM) for 2 h dramatically increased the survival of MPP+-treated SH-SY5Y cells in a dose-dependent manner. The cell index of SH-SY5Y cells without MPP+ or apelin-13 (basal) for 36 h was increased to ~4.1, while the cell index of SH-SY5Y cells with 100 nM apelin-13 treatment for 36 h was markedly increased to ~5.2 (Fig. 1B). The cell index of SH-SY5Y cells with 500 μM MPP+ treatment for 36 h was reduced to ~0.8 (Fig. 1B). However, the cell indexes of SH-SY5Y cells with the pretreatment of 0.1, 1, 10, 100, and 1,000 nM apelin-13 for 2 h and then co-cultured with 500 μM MPP+ for 36 h were ~1.0, 1.49, 1.75, 2.1, and 2.52, respectively (Fig. 1B). These data indicate that apelin-13 protected SH-SY5Y cells against MPP+-induced neurotoxicity.

Apelin-13 attenuates the MPP+-induced decrease in the viability of SH-SY5Y cells. To determine the optimal concentration of MPP+ for subsequent experiments, SH-SY5Y cells were treated with 0-1,000 μM MPP+ and their viability was measured using the CCK-8 assay. MPP+ treatment markedly decreased the viability of SH-SY5Y cells in a dose-dependent manner, and treatment with 500 μM MPP+ killed ~50% of the cells (Fig. 2A and B). Thus, the dose of 500 μM MPP+ was selected as the optimal concentration for subsequent experiments. To evaluate the neuroprotective effect of apelin-13, SH-SY5Y cells were pretreated with 0-1,000 nM apelin-13 for 2 h, then treated with 500 μM MPP+, and finally evaluated for viability with the CCK-8 assay. Apelin-13 dose-dependently attenuated the MPP+-induced decrease in the viability of SH-SY5Y cells, and this effect plateaued at 100 nM apelin-13 (Fig. 2C). Thus, the dose of 100 nM apelin-13 was selected as the optimal concentration for further experiments.

Next, the effect of pretreatment with 100 nM apelin-13 was evaluated on SH-SY5Y cells treated with 500 μM MPP+. Apelin-13 significantly protected SH-SY5Y cells against MPP+-induced neurotoxicity compared with the control (Fig. 2D). The ratio of viable cells was 16% higher in the group

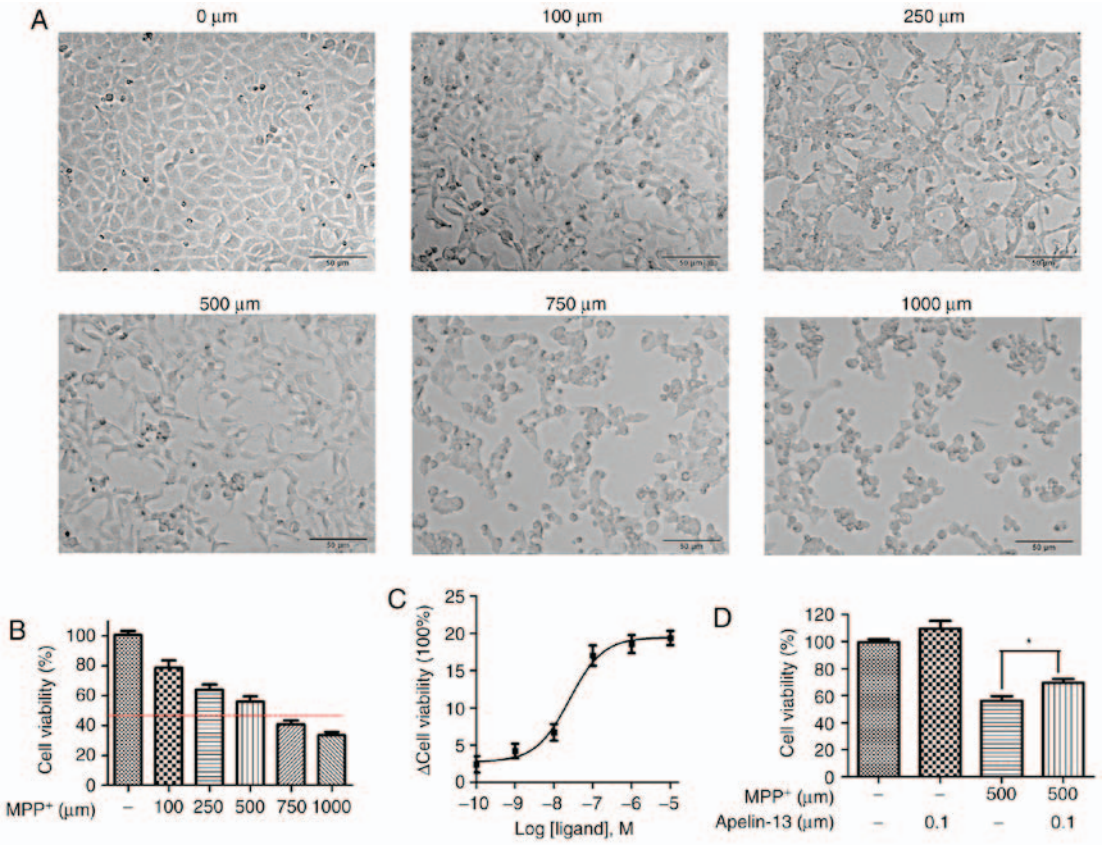

Figure 2. Apelin-13 protects SH-SY5Y cells against MPP+-induced damage. (A) Representative images from light microscopy analysis of SH-SY5Y cells treated with the indicated concentrations of MPP+ for 24 h. (B) Viability of SH-SY5Y cells treated with 0-1,000 μM MPP+ for 24 h was assessed by the CCK-8 assay. (C) Dose-response curve of the effects of apelin-13 on the death of SH-SY5Y cells induced by treatment with 500 μM MPP+. (D) Effects of apelin-13 on MPP+-induced SH-SY5Y cell death assessed by the CCK-8 assay. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. MPP+-treated cells. MPP+, 1-methyl-4-phenylpyridine; CCK-8, Cell Counting Kit-8.

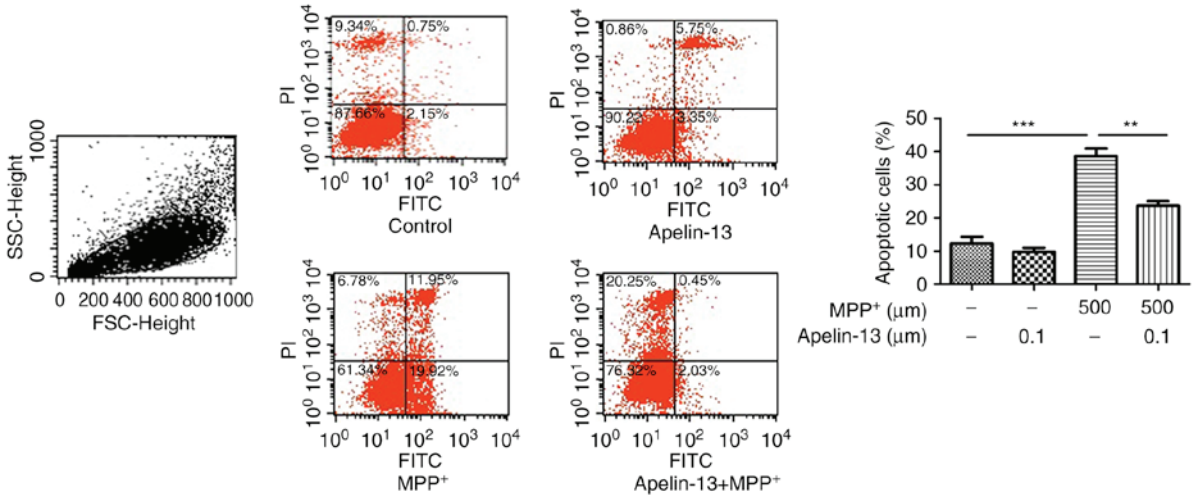

Figure 3. Apelin-13 inhibits MPP+-induced apoptosis of SH-SY5Y cells. Apoptosis of SH-SY5Y cells was evaluated by flow cytometry. Cells were treated with 100 nM apelin-13 for 2 h and then with 500 μM MPP+ for 24 h. Values are presented as the mean ± standard deviation of three independent experiments. **P<0.01 and ***P<0.001, with comparisons indicated by lines. MPP+, 1-methyl-4-phenylpyridine; FITC, fluorescein isothiocyanate; PI, propidium iodide.

pretreated with apelin-13 compared with the group treated with MPP+ alone (P<0.05; Fig. 2D). These data indicate that apelin-13 protected SH-SY5Y cells against MPP+-induced neurotoxicity.

Apelin-13 inhibits MPP+-induced apoptosis of SH-SY5Y cells. To further explore the effect of apelin-13 on neuronal cell survival, apoptosis of SH-SY5Y cells was evaluated by flow cytometry. The % of apoptotic SH-SY5Y cells was ~26% higher in the group treated with 500 μM MPP+ compared with the control group (P<0.001; Fig. 3), while only ~23% of SH-SY5Y cells were apoptotic in the group pretreated with 100 nM apelin-13 (P<0.01 vs. MPP+-treated alone group; Fig. 3).

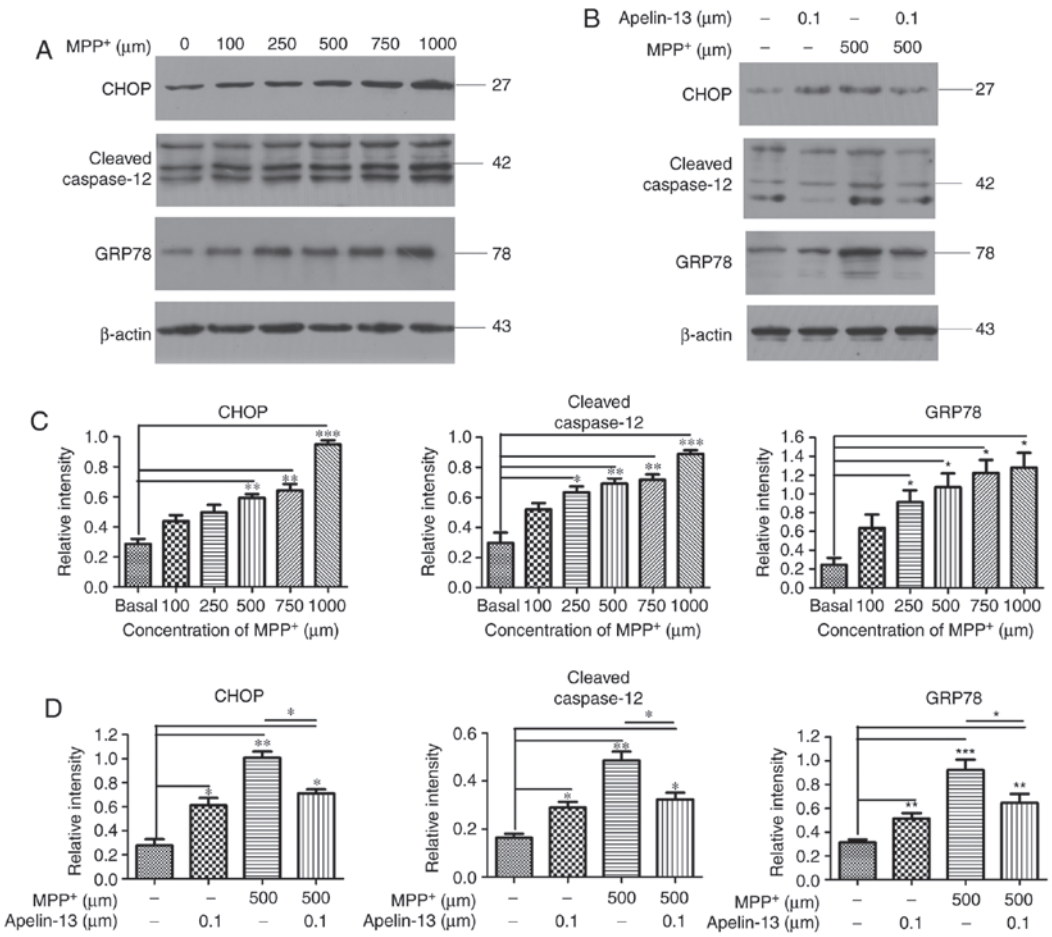

Figure 4. Apelin-13 attenuates the MPP+-induced increases in CHOP, cleaved caspase-12 and GRP78 expression in SH-SY5Y cells. (A) Representative images from western blot analysis for CHOP, cleaved caspase-12 and GRP78 protein expression levels in SH-SY5Y cells treated with various concentrations of MPP+ for 24 h, and (B) in SH-SY5Y cells treated with 100 nM apelin-13 for 2 h and then with 500 μM MPP+ for 24 h. β-actin was detected as an internal control. (C) Quantification of relative protein expression levels for blots in panel A. (D) Quantification of relative protein expression levels for blots in panel B. Data are presented as mean ± standard error of the mean from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001, with comparisons indicated by lines. MPP+, 1-methyl-4-phenylpyridine; CHOP, C/EBP homologous protein; GRP78, glucose-regulated protein 78.

Apelin-13 attenuates GRP78, CHOP, and cleaved caspase-12 upregulation in MPP+-treated SH-SY5Y cells. GRP78 has a key role in multiple neurodegenerative diseases, including AD and PD. To investigate whether GRP78 is involved in the neurotoxic effect of MPP+, SH-SY5Y cells were treated with various concentrations of MPP+ for 24 h (Fig. 4A). Western blot analysis demonstrated that treatment with 100, 250, 500, 750 and 1,000 μM MPP+ increased the expression levels of GRP78 in a dose-dependent manner (0.64±0.13, 0.91±0.12, 1.07±0.14, 1.22±0.13, and 1.27±0.16, respectively; Fig. 4C). To further investigate the molecular mechanism by which apelin-13 elicits neuroprotective effects in SH-SY5Y cells, cells were pretreated with 100 nM apelin-13 for 2 h and then exposed to 500 μM MPP+ for 24 h (Fig. 4B). Pretreatment with apelin-13 attenuated the MPP+-induced increase in GRP78 expression (Fig. 4D).

Activated GRP78 increases the level of CHOP, which causes ER stress and promotes apoptosis. To determine whether CHOP is involved in the neuroprotective effect of apelin-13, the expression levels of the CHOP protein were examined. Treatment with 100, 250, 500, 750 and 1,000 μM MPP+ increased CHOP expression in a dose-dependent manner (0.44±0.03, 0.49±0.04, 0.59±0.03, 0.64±0.03, and 0.95±0.04, respectively; Fig. 4C). Pretreatment with apelin-13 attenuated the increase in CHOP expression induced by treatment with 500 μM MPP+ (Fig. 4D).

Caspase-12 is activated during ER stress-induced apoptosis, and it has an important role in neurodegenerative diseases. To investigate whether apelin-13 treatment affected caspase-12 activity, the expression levels of cleaved caspase-12 were examined by western blotting. Treatment with 100, 250, 500, 750 and 1,000 μM MPP+ increased cleaved caspase-12 expression in a dose-dependent manner (0.52±0.03, 0.63±0.03, 0.69±0.04, 0.72±0.05 and 0.89±0.06, respectively; Fig. 4C). Pretreatment with apelin-13 attenuated the increase in cleaved caspase-12 expression induced by treatment with 500 μM MPP+ (Fig. 4D). These results indicate that apelin-13 prevented MPP+-induced apoptosis of SH-SY5Y cells by reducing the expression of GRP78, CHOP and cleaved caspase-12.

Apelin-13 upregulates phospho-ERK1/2 expression in MPP+-treated SH-SY5Y cells. MAPK family members p38 and ERK1/2 have important roles in maintaining normal cellular functions. To investigate the mechanism by which apelin-13 inhibited MPP+-induced apoptosis of SH-SY5Y cells, immunoblotting for ERK1/2 and p38 was performed. Treatment

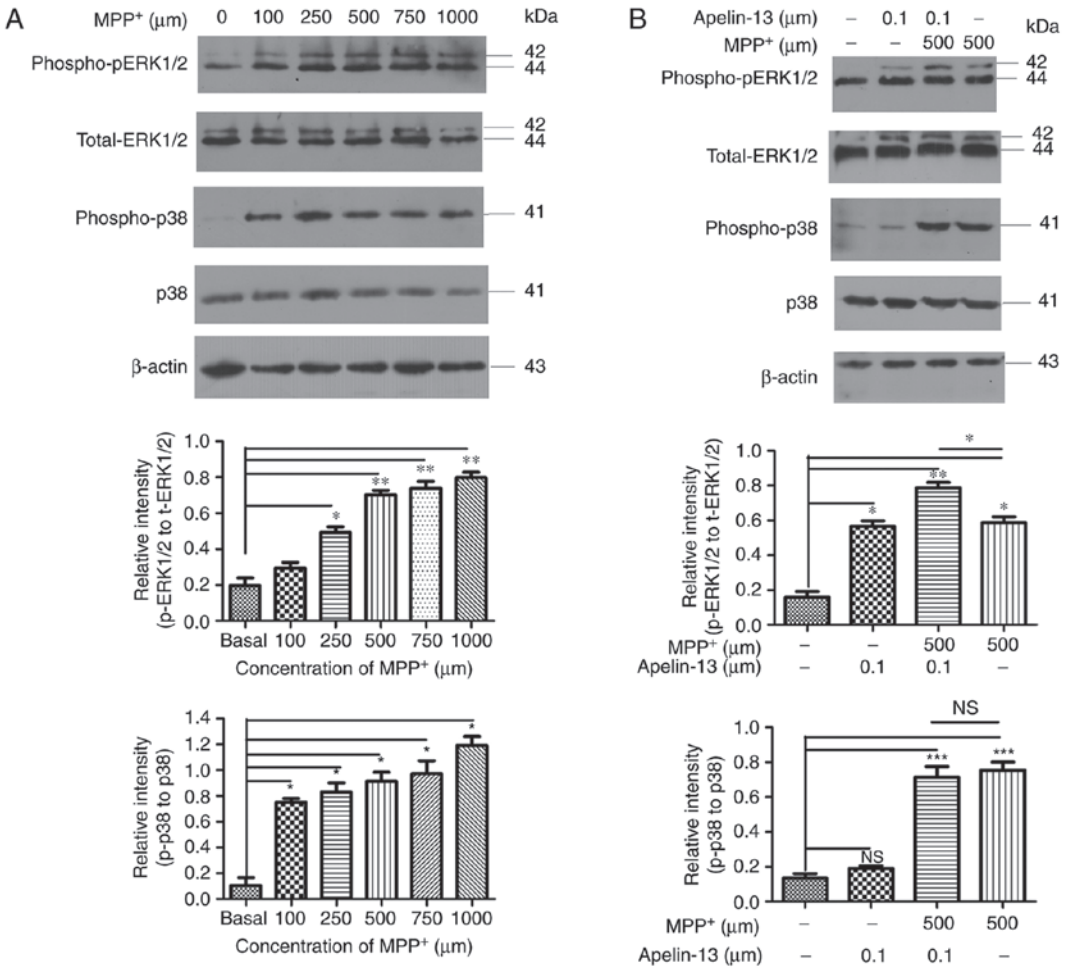

Figure 5. Apelin-13 increases the levels of phospho-ERK1/2 in MPP+-treated SH-SY5Y cells. (A) SH-SY5Y cells were treated with various concentrations of MPP+ for 24 h. (B) SH-SY5Y cells were treated with 100 nM apelin-13 for 2 h and then with 500 μM MPP+ for 24 h. The protein expression levels of total ERK1/2, phospho-pERK1/2, total p38 and phospho-p38 were examined by western blot analysis. β-actin was detected as an internal control. Data are presented as mean ± standard error of the mean from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001, with comparisons indicated by lines. ERK1/2, extracellular signal-regulated kinase 1/2; MPP+, 1-methyl-4-phenylpyridine; NS, not significant.

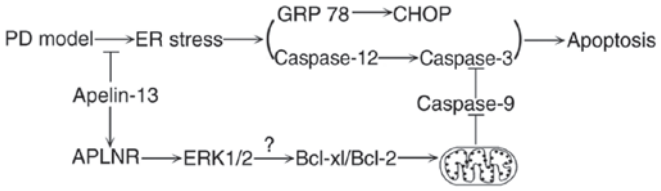

Figure 6. Schematic diagram of the mechanism by which apelin-13 may attenuate ER stress-induced apoptosis in an *in vitro* PD model. Apelin-13 protects SH-SY5Y cells against apoptosis by inhibiting MPP+-induced ER stress and by blocking the subsequent upregulation of GRP78, CHOP and cleaved caspase-12. ER, endoplasmic reticulum; MPP+, 1-methyl-4-phenylpyridine; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; APLNR, apelin receptor; ERK1/2, extracellular signal-regulated kinase 1/2; Bcl-2, BCL2 apoptosis regulator; Bcl-xl, B-cell lymphoma-extra large.

with 100, 250, 500, 750 and 1,000 μM MPP+ increased expression of phospho-ERK1/2 (0.29±0.03, 0.49±0.03, 0.70±0.02, 0.73±0.04, and 0.80±0.05, respectively; Fig. 5A) and phospho-p38 (0.7±0.02, 0.83±0.07, 0.91±0.05, 0.97±0.10, and 1.19±0.06, respectively; Fig. 5A) in a dose-dependent manner. Pretreatment with apelin-13 significantly increased expression of phospho-ERK1/2 (Fig. 5B), but did not affect the expression of phospho-p38 (Fig. 5B).

Discussion

Since apelin was discovered in 1998 (19), a number of studies have demonstrated that the apelin/APJ signaling pathway has a crucial role in the function and dysfunction of the cardiovascular system (heart failure and hypertension), the urinary tract system (fluid balance, diuresis, and vasopressin synthesis), and the gastrointestinal and endocrine systems (obesity and insulin resistance) (17,26,27). APJ and its endogenous ligand apelin are widely distributed in numerous organs and tissues, including the lung, adipose tissue, endothelium, heart, stomach, pancreatic islands, and central nervous system (28). Recent studies have demonstrated that apelin is neuroprotective in various neurological diseases, such as insomnia, AD and PD; however, the underlying molecular mechanism remains unknown (14). Apelin-13, -17, -36, and -77 protect hippocampal neurons against excitotoxic injury by inducing phosphorylation of AKT serine/threonine (Akt) kinases and Raf-1 proto-oncogene/ERK1/2. In addition, apelin protects

hippocampal neurons against excitotoxic injury induced by the N-methyl-D-aspartic acid (NMDA) receptor (29).

Recent studies have demonstrated that, in addition to its protective effect in hippocampal neurons, apelin may also directly elicit protective effects in cultured mouse cortical neurons by reducing NMDA-induced intracellular Ca^{2+} accumulation, cytochrome *c* release, mitochondrial depolarization, caspase-3 activation, and reactive oxygen species generation (30). Furthermore, apelin protects human vascular smooth muscle cells and cultured rat bone marrow mesenchymal stem cells against apoptosis by inhibiting the phosphoinositide 3-kinase (PI3K)/Akt and MAPK/ERK1/2 signaling pathways (31,32). In addition, apelin-13 and -36 protect against myocardial I/R injury by activating the reperfusion injury salvage kinase pathway, which involves PI3K/Akt and p44/42 MAPK (33). These neuroprotective effects of apelin are consistent with the present findings. In the present study, the growth of control SH-SY5Y cells and cells treated with MPP^+ alone, or apelin-13 plus MPP^+ was monitored over 36 h by RTCA. Apelin-13 significantly attenuated MPP^+-induced neurotoxicity and improved the cell survival index. The CCK-8 assay demonstrated that apelin-13 markedly increased the viability of MPP^+-treated SH-SY5Y cells, while flow cytometry revealed that apelin-13 reduced the % of apoptotic SH-SY5Y cells following MPP treatment. These results suggest that apelin-13 elicited neuroprotective effects in human SH-SY5Y cells, an *in vitro* model of PD.

GRP78, a Hsp70 family ATPase, was discovered in chicken embryo fibroblasts in 1997 (34-36). This protein is an important molecular chaperone in the ER and is involved in multiple processes, including assembly, folding, and translocation of nascent polypeptides, calcium homeostasis, and targeting of misfolded proteins to the ER-associated protein degradation pathway (37-39). A series of studies indicate that GRP78 is not only involved in various aspects of tumor development (such as cancer cell survival, proliferation, and metastasis, angiogenesis, and chemoresistance), but also in many age-related neurodegenerative disorders, including amyotrophic lateral sclerosis, prion-related disorders, ischemic stroke, PD and AD (40,41). Expression of GRP78 is significantly higher in 3xTg-AD mice, a triple transgenic AD model, compared with control mice (42). In addition, GRP78 is overexpressed in post-mortem human AD samples and in *in vitro* models of AD, and ER stress increases its expression in several tissues by ~3-fold (43,44). Recent studies indicate that expression of GRP78 is increased and that this protein interacts with α-synuclein in glucose-deprived SH-SY5Y cells, and similar phenomena are observed in HEK293 cells transfected with SYN120 (amino acids 1-120 of α-synuclein) (45). Furthermore, there is evidence that pretreatment with apelin reduces ER stress in diabetic Akita mice by decreasing expression of GRP78 and other proteins, including endoplasmic reticulum to nucleus signaling 1 (ERN1, also known as IRE1α), protein kinase R-like endoplasmic reticulum kinase (PERK), heat shock protein (Hsp) 70, and calnexin (46). CHOP and caspase-12 also have important roles in ER stress. Apelin was reported to significantly attenuate ER stress-induced expression of CHOP, cleaved caspase-12, and GRP78 in the heart and thereby protect cells against I/R injury-induced apoptosis (47). A recent study indicates that apelin-36 protects neurons against cerebral I/R injury by attenuating ER stress-induced elevations in GRP78 and CHOP expression in rats following ischemic stroke (48). There is evidence that telmisartan elicits neuroprotective effects by suppressing ER stress-induced increases in GRP78 and cleaved caspase-12 expression in a rat model of PD (49). However, the effect of apelin-13 on ER stress-induced upregulation in GRP78, CHOP, and cleaved caspase-12 in a cellular model of PD had not been explored to date. In the present study, MPP^+ treatment increased the GRP78, CHOP and cleaved caspase-12 expression levels in human SH-SY5Y cells in a dose-dependent manner, and this effect was attenuated by pretreatment with apelin-13, suggesting that apelin-13 prevented ER stress. As previously described, increased expression of GRP78, CHOP, and cleaved caspase-12 is closely related to apoptosis (47-49). Apelin-13 may mediate an anti-apoptotic effect in the cellular model of PD by inhibiting ER stress (Fig. 6). This is consistent with the present findings that pretreatment with apelin-13 markedly inhibited MPP^+-induced apoptosis of SH-SY5Y cells.

ERK1 and ERK2, which have molecular weights of 44 and 42 kDa respectively, transmit signals from the surface receptors to the nucleus. Activated ERK1/2 serve important roles in cell proliferation, cell differentiation, and maintenance of cell morphology via modulating their targets, including ELK1, activating transcription factor (ATF) and activator protein (AP)-1. MAPKs also have important roles in cell proliferation (50,51). Several experiments demonstrate that ERK1/2 are activated in APJ-transfected cells, including HEK293T cells, mouse enteroendocrine cells, and CHO cells, following treatment with apelin (26,52,53). However, apelin was reported to promote the proliferation of human osteoblasts by activating the Akt signaling pathway, not the MAPK pathway (p38, c-Jun N-terminal kinase, and ERK1/2) (54). In the present study, although MPP^+ treatment dose-dependently increased phosphorylation of ERK1/2 and p38 in SH-SY5Y cells, apelin-13 significantly increased expression of pERK1/2, but did not affect phopsho-p38. These results are consistent with our previous finding that apelin-13 activates ERK1/2, but not phopsho-p38, via coupling of the apelin receptor to Gi2-protein (20). A recent study demonstrated that apelin-13 protects the brain against I/R injury by activating the ERK1/2 signaling pathway, and that PD98059, an ERK1/2 inhibitor, markedly attenuates the elevation of BCL2 apoptosis regulator (Bcl-2) expression in the brain (55). These results indicate that apelin-13 protects neurons against I/R injury-induced apoptosis partly by activating the ERK1/2 signaling pathway and thereby increasing Bcl-2 expression (55). In the present study, apelin-13 was demonstrated to significantly upregulate phospho-pERK12 expression and to inhibit GRP78/CHOP/cleaved-caspase-12 activation, resulting in a decrease of MPP^+-induced neuronal apoptosis in SH-SY5Y cells. Expression of phosho-ERK1/2 is activated by Gi2-protein and upregulated by β-arrestin protein, which may explain why apelin-13 only increased the expression of phospho-ERK1/2 and not phospho-p38. Apelin-13 may elicit neuroprotective effects in the cellular model of PD via this mechanism (Fig. 6), although further studies will be required to confirm this.

In summary, the present study demonstrated that apelin-13 protected neurons against ER stress-associated apoptosis in

an *in vitro* model of PD by downregulating GRP78, CHOP and cleaved caspase-12 and by upregulating phospho-ERK1/2. However, the precise mechanism needs to be further elucidated. Further experiments, investigating the neuroprotective effects of apelin-13 in animal models of PD, are underway.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

YJ, BC and BB designed the experiments. YJ, HL, BJ, ZW and CY performed the experiments. YJ, BJ, CW and YP analyzed the data. YJ, HL, BC and BB wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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