Introduction

Glucagon-like peptide-2 (GLP-2) is a hormone with 33-amino acid derived from proglucagon in the gut and central nervous system (CNS). In 2012, the first GLP-2 peptide analogue, teduglutide, was approved for the treatment of short bowel syndrome (SBS) and following this, an increasing number of GLP-2 peptide analogues were developed for clinical use [1]. GLP-2 is a hormone that regulates food intake [2], inhibits growth of osteosarcoma cells and promotes directed differentiation of osteoblasts [3], and furthermore shows cytoprotective effects in combat lung injury [4]. However, the role of GLP-2 in the CNS had only recently been studied. It has been previously shown that analogues of the sister hormone Glucagon-like peptide-1 (GLP-1) showed neuroprotective effects. Here we investigated the effect of a GLP-2 agonist in a cell model of Parkinson’s disease (PD) created by treating SH-SY5Y or Neuro-2a cells with 1-Methyl-4-phenyl-pyridine ion (MPP+). Cell viability and cell cytotoxicity was detected by MTT and LDH assays, respectively. The protein expression levels of mitochondrial, autophagy and apoptotic biomarkers including PGC-1α, Mfn2, IRE1, ATG7, LC3B, Beclin1 and Bcl-2 were detected by western blot. Mitochondrial superoxide was detected by MitoSOX Red. In addition, mitochondrial morphology, autophagosome and apoptotic corpuscles were observed by transmission electron microscope (TEM). We found that the GLP-1 and the GLP-2 agonists both protect cells against mitochondrial damage, autophagy impairments and apoptosis induced by MPP+ both in SH-SY5Y and Neuro-2a cells. Cell signaling for mitogenesis was enhanced, and oxidative stress levels much reduced by the drugs. This demonstrates for the first time the neuroprotective effects of a GLP-2 analogue in PD cellular models, in which oxidative stress, autophagy and apoptosis play crucial roles. The protective effects were comparable to those seen with the GLP-1 analogue liraglutide. The results suggest that not only GLP-1, but also GLP-2 has neuroprotective properties and may be useful as a novel treatment of PD.
GLP-1, GLP-2, and glicentin in the intestine and brain by the hormone proto-invertase. In the brain, GLP-2 is found in discrete clusters of neurons in the brain stem and hypothalamus [14]. GLP-2 receptor (GLP-2R) expressing neurons have been identified in the Cortex, the hippocampus, the ventromedial hypothalamic nucleus, the nucleus of the solitary tract, the parabrachial nucleus, the supramammillary nucleus, and the substantia nigra. Moreover, GLP-2 showed cytoprotective properties in cells derived from the CNS [15].

The biological activity of GLP-2 is mediated by GLP-2R, a member of the G-protein coupled receptor superfamily. GLP-2R is highly expressed in the intestinal tract [16] and exists in the CNS also [2, 15, 17, 18]. In mouse hippocampal and cortical cells, GLP-2 stimulates cAMP production and reduces glutamate-induced neuronal death and cell death induced by apoptosis [15]. Similarly in mouse hippocampal neurons, GLP-2 activated mouse hippocampal neurons through PKA signaling, and increased glucose uptake in a PKA-dependent manner [19]. In addition to the cAMP pathway, the GLP-2 receptor activates the PLC-PI3k-Akt-mTOR pathway which is the same second messenger pathway that the insulin receptor activates [20]. A new study suggested that GLP2R and the AKT-mTOR-p70S6K pathway in the hippocampus are promising targets to treat cognition deficits in a chronic cerebral hypoperfusion animal model [9]. Another pathway is ERK signaling through which GLP-2 can regulate cognitive function [8]. It appears that GLP-2R signaling pathways act in energy utilization, block apoptosis, induce insulin re-sensitization, enhance protein synthesis, reduce inflammation and glia activation, and enhance cell repair and growth.

We have previously shown that activating the GLP-1 pathway has neuroprotective effects in animal models of Alzheimer’s and Parkinson’s disease [21–23]. A phase II clinical trial in PD patients showed that a GLP-1 receptor agonist is effective in treating PD patients [24]. GLP-1 is co-released with GLP-2 during the processing of proglucagon [25]. However, the protective effects and the mechanisms of GLP-2 signaling in models of Parkinson’s disease (PD) have not been investigated. PD is the second major neurodegenerative disease in the world that no drugs can stop or even reverse disease progression in it. In this study, we investigated the effect of a protease-resistant GLP-2 analogue in cell models of PD induced by 1-Methyl-4-phenyl-pyridine (MPTP). MPP+ was diluted in culture medium at concentrations of 0.5 mM, 1 mM, 2 mM and 4 mM, respectively, to confirm a lowest concentration of 100 nM which was selected on the basis of previous preparations were diluted in culture medium to a final working concentration of 100 nM. Liraglutide is HAEGTFTSDVSSYLEGQAAK[(γE)-(Pal)]EFIAWLVRGRG; (Pal= palmitoyl acid). The purity of the peptides is 95 %.

**Materials and Methods**

**Materials**

Lactate dehydrogenase (LDH) kit was purchased from Roche Diagnostics Ltd (West Sussex, UK). PCG-1a antibody (ab54481), Mitofusin 2 antibody (ab124773), IRE1 antibody (ab48187), ATG7 antibody (ab133528), LC3B antibody (ab48394), Beclin1 antibody (ab207612) and ECL Western Blotting Substrate were purchased from Abcam (MA, USA). Bcl-2 antibody and horseradish peroxidase (HRP)-linked secondary antibodies against rabbit and mouse IgG of the primary antibodies were from BOSTER (Wuhan, China). Bovine serum albumin (BSA) and MPP+ were obtained from Sigma-Aldrich Company Ltd (Dorset, UK). DMEM/F-12 and fetal bovine serum (FBS) were purchased from Gibco. DMEM high glucose was from Biological Industries Ltd (HaZafon, Israel). Penicillin-Streptomycin solution, 0.25 % trypsin-EDTA solution without phenol red, NP-40 lysis buffer, MITT, BCA protein concentration determination kit, paraffomaldehyde and dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS; pH 7.4), Tween 20, 5 × SDS-PAGE sample loading buffer and other reagents for cell culture and western blotting were obtained from Solarbio (Beijing, China). MitoSOX Red mitochondrial superoxide indicator (M36008) was purchased from Invitrogen. The peptides of GLP-2 agonist and Liraglutide were synthesized by China Peptides (Shanghai, China). The amino acid sequence of the GLP-2 analogue is HGDSFSDEMSTLDNLATRDFINWIQIKTID. The amino acid sequence of Liraglutide is HAEHTFTSDVSSYLEGQAAK[(γE)-(Pal)]EFIAWLVRGRG; (Pal= palmitoyl acid).

**Cell culture**

The human neuroblastoma SH-SY5Y cell line (ATCC® CRL-2266) and Neuro-2a cell line (ATCC® CCL-131) were obtained from Cell Resource Center, Shanghai Institute of life sciences, Chinese Academy of Sciences (Shanghai, China). SH-SY5Y cells were cultivated in Dulbecco’s modified eagle medium/nutrient mixture F-12 (DMEM/F-12, 1:1; 1X) Glutamax supplemented with 10 % FBS, 100 IU ml−1 of Penicillin and 100 μg · ml−1 of Streptomycin. Neuro-2a Cells were cultivated in Dulbecco’s modified eagle medium (DMEM, 1 ×, high glucose) Glutamax supplemented with 10 % FBS, 100 IU ml−1 of Penicillin and 100 μg · ml−1 of Streptomycin. Cells were maintained at 37 °C in a humidified incubator with 5 % CO2 and 95 % air. Cells were subcultured when 80–90 % confluent and seeded at 1:4 ratio. Culture medium was renewed every 3 to 4 days.

**Cell treatment**

MPP+ was diluted in PBS at a concentration of 100 mM, aliquoted and stored at −20 °C until used. GLP-2 agonist and Liraglutide were diluted in sterile water at a concentration of 1 mM, aliquoted and stored at −20 °C until used as well. For the experiments, firstly, MPP+ was diluted in culture medium at concentrations of 0.5 mM, 1 mM, 2 mM and 4 mM, respectively, to confirm a lowest concentration that decreased the cell viability significantly and to be applied in subsequent experiments. GLP-2 agonist was diluted in culture medium at concentrations of 1 nM, 10 nM, 100 nM and 200 nM, to select a concentration that reversed the cell damage significantly and to be applied in subsequent experiments. Liraglutide stock preparations were diluted in culture medium to a final working concentration of 100 nM which was selected on the basis of previous experiments [26–28].

**Cell viability and cytotoxicity assessment**

Cell viability and cytotoxicity were determined using MITT and LDH assay respectively. The assays were performed in 96-well plates. Both of SH-SY5Y cells and Neuro-2a cells were seeded at a density of 3 × 104 cells in 100 μL per well for 24 h for use. In order to confirm the concentration of MPP+ for the cell model of PD, the cells were stressed with different concentrations of MPP+ (0.5 mM, 1 mM, 2 mM and 4 mM) for 16 h, then the cell viability and cytotoxicity were determined. For the drug test, cells were pretreated with 0 nM, 1 nM, 10 nM, 100 nM and 200 nM of GLP-2 agonist for 12 h, respectively, MPP+ was added subsequently for another 16 h, a
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concentration that reversed the cell damage significantly was determined. Following cell treatments, 50 μL of the supernatant was collected and the cell cytotoxicity was assessed according to the manufacturer’s instructions: the collected cell supernatant was incubated with the reaction mixture for 30 min at room temperature, then the reaction was terminated by adding the stop solution from the kit and absorbance was measured at 490 nm using a microplate reader. The absorbance values are proportional to the cytotoxicity of the cells damage. Meanwhile, 20 μL of MTT (5 mg/mL) was added to each well and maintained at 37 °C in a humidified incubator, after incubating for 4 h, the supernatants were removed and 100 μL of DMSO was added to each well. After the formazan crystals dissolved completely, the absorbance was measured at 570 nm using a microplate reader. The absorbance values are proportional to the number of viable cells. All cell treatments were performed in sextuplicate per plate per experiment.

Western blotting
2 × 10⁶ cells were seeded at 6 cm² dish for 24 h. Cells were pretreated with 100 nM of GLP-2 agonist or Liraglutide for 12 h before stressed with 2 mM of MPP + for another 16 h. Thereafter, cells were washed once with ice-cold 1× PBS and harvested by 0.25 % trypsin and centrifugation at 1 000 rpm at 4 °C for 5 min. Cells were resuspended in 1× cell lysis buffer containing protease/phosphatase inhibitor (1×) and kept on ice for 30 min. Total protein was extracted by centrifugation at 12 000 rpm at 4 °C for 10 min. BCA protein concentration determination kit was conducted to estimate the protein concentration of the samples, according to the manufacturer’s instructions. Protein in lysate (10 μg) was boiled with 5× SDS-PAGE instructions. Protein in lysate (10 μg) was boiled with 5× SDS-PAGE sample loading buffer at 99 °C for 10 min to be denatured. Total protein was extracted (1:2 500), ATG7 (1:10 000), LC3B (1:1 000), Beclin1 (1:2 000), Bcl-2 (1:300) and β-actin (1:400) in 5 % (w/v) skimmed milk overnight at 4 °C. Blots were washed three times in PBST for 10 min each and incubated with the HRP-linked secondary antibodies against the corresponding species IgG (1:5 000) for 1 h at room temperature. Blots were blocked in 5 % (w/v) skimmed milk in 1× PBS with 0.05 % Tween 20 (PBST) for 1 h at room temperature, and the blots were cut apart based on the protein molecular weight and incubated with the primary antibodies against PGC-1α (1:2 000), Mfn2 (1:1 000), IRE1 (1:2 500), ATG7 (1:10 000), LC3B (1:1 000), Beclin1 (1:2 000), Bcl-2 (1:300) and β-actin (1:400) in 5 % (w/v) skimmed milk overnight at 4 °C. Blots were washed three times in PBST for 10 min each and incubated with the HRP-linked secondary antibodies against the corresponding species IgG (1:5 000) for 1 h at room temperature. Blots were developed using ECL western blotting detection reagent kit as per manufacturer’s instructions. Bio-Rad Imaging System was used to image chemiluminescent bands. Image J and Prism 7 software programs were used to perform the densitometric analysis. β-Actin protein was served as loading control, to which relative peak intensities of the examined proteins were normalized.

Mitochondrial superoxide detection
4.2 × 10⁶ cells were seeded at 12-well plate for 24 h. Cells were pretreated with 100 nM of GLP-2 agonist or Liraglutide for 12 h before stressed with 2 mM of MPP + for another 16 h. MitosOX Red mitochondrial superoxide indicator was performed to detect the mitochondrial superoxide. 5 μM of MitosOX reagent working solution diluted in cell culture medium was applied to cover cells. Cells were incubated at 37 °C for 10 min, protected from light. Cells were washed gently for three times with warm culture medium. Cells were stained with 100 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) which was diluted in cell culture medium at 37 °C for 20 min. After washing three times with warm culture medium, cells in culture medium were observed using fluorescence microscope. Images were taken and analyzed using the Image J graphic analysis program (freeware form the NIH, USA). Dead cells were not counted in the quantification of fluorescence. Results were statistically analyzed using the program Prism 7 (GraphPad software, USA).

Transmission electron microscope (TEM)
To observe mitochondrial morphology, autophagosome and apoptotic corpuscle of the cells in different groups, 2 × 10⁶ cells were seeded in cell culture dish with a diameter of 6 cm for 24 h. Cells were treated with different concentrations of MPP + for 16 h. Cells in each group were stained with 100 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) which was diluted in cell culture medium at 37 °C for 20 min. After washing three times with warm culture medium, cells in culture medium were observed using fluorescence microscope. Images were taken and analyzed using the Image J graphic analysis program (freeware form the NIH, USA). Dead cells were not counted in the quantification of fluorescence. Results were statistically analyzed using the program Prism 7 (GraphPad software, USA).
were pretreated with 100 nM GLP-2 agonist or Liraglutide for 12 h before stressed with 2 mM MPP+ for another 16 h. Each group was performed in triple. Cells were harvested using cell scrapers and centrifugation at 2000 rpm at 4 °C for 15 min. After fixed with 2.5% glutaraldehyde for 4 h, the solution was refreshed and rinsed with 0.1 M PBS, then fixed with 1% osmium acid solution for 2 h, rinsed again with 0.1 M PBS, dehydrated with ethanol and acetone and embedded with epoxy resin 812 finally. After sliced, slices were stained with saturated uranium acetate solution and lead citrate solution. Three slices were observed in each sample after dried by projection electron microscope (JEM-1400, Japan). Mitochondrial morphology, autophagosomes and apoptotic corpuscles were observed. TEM was performed by TEM Center of Henan University of Chinese Medicine.

Statistics
Statistical analysis in this study was performed using EXCEL and GraphPad Prism 7 software (GraphPad software, USA). The exper-
Experimental data were analyzed by one-way ANOVA with post-hoc tests or Student’s t-tests. Data are shown as Mean ± SD, and a $P < 0.05$ was considered as significant.

Results

2 mM MPP+ decreases cell viability and increases LDH levels, the GLP-1 and GLP-2 analogues protect cell against damage from MPP+ both in SH-SY5Y and Neuro-2a cells

The effective concentration of MPP+ for the cell model of PD was estimated in a dose-response test. The cells were stressed with different concentrations of MPP+ (0.5 mM, 1 mM, 2 mM and 4 mM) for 16 h, then the cell viability and cytotoxicity were determined.

$\text{Fig. 1a,c}$ showed at list 2 mM MPP+ decreases cell viability significantly comparing to control group in both two cell lines ($P < 0.05$). During the injury of nerve cells, LDH is released into the culture supernatant, and the leakage rate of LDH in the supernatant reflects the degree of cell injury or death. $\text{Fig. 1b,d}$ show that at least 1 mM MPP+ increases LDH levels significantly comparing to control group in both two cell lines ($P < 0.05$). The cell viability and cytotoxicity had a dose-effect relationship with the concentration of MPP+. Therefore, 2 mM MPP+ was selected to test the drugs subsequently. For the drug test, cells were pretreated with 0 nM, 1 nM, 10 nM, 100 nM and 200 nM of GLP-2 agonist for 12 h, respectively, with 100 nM Liraglutide used as a positive control. MPP+ was added subsequently for another 16 h. $\text{Fig. 1e-h}$ showed 100 nM GLP-2 agonist and 100 nM Liraglutide both increased cell survival and reduced cytotoxicity significantly in both two cell lines ($P < 0.05$). GLP-2 agonist alone did not cause a significant change in cell viability and cytotoxicity in both SH-SY5Y and Neuro-2a cells (data not shown).

The drugs protect cells against mitochondrial damage, autophagy impairments and apoptotic signaling induced by MPP+

PGC-1α is a key factor that mediates mitochondrial biosynthesis [29]. Mitofusin2 (Mfn2) regulates mitochondrial fusion, transport and autophagy. As shown in $\text{Fig. 2}$, 2 mM MPP+ decreased PGC-1α and Mfn2 levels significantly ($P < 0.05$), 100 nM GLP-2 agonist and 100 nM Liraglutide both protected cells against the changes of the protein levels induced by MPP+ significantly ($P < 0.05$). IRE1 is the core receptor of ER stress, which can sense the accumulation of unfolded proteins in the endoplasmic reticulum lumen and transmit this stimulation to other areas of the cell. As shown in $\text{Fig. 2}$, the expression levels of IRE1 was decreased by MPP+ and 100 nM GLP-2 agonist and 100 nM Liraglutide both inhibited the decrease of IRE1 significantly ($P < 0.05$). MPP+ has been shown to perturb the autophagy flux [30]. In this light, we investigated the expression of autophagy-related ATG7 and LC3B (II) proteins following GLP-2 agonist or 100 nM Liraglutide pretreatment and MPP+ stress. ATG7 is used to assemble autophagosomes and LC3B is a marker of autophagy, the content of which is proportional to the degree of autophagy. As shown in $\text{Fig. 2}$, the two protein levels induced by MPP+ significantly ($P < 0.05$), 100 nM GLP-2 agonist and 100 nM Liraglutide both inhibited the increase of the two protein levels induced by MPP+ significantly ($P < 0.05$). Bcl-2 exists in the outer membrane of mitochondria, nuclear membrane and endoplasmic reticulum which has the effect of inhibiting apoptosis. 2 mM MPP+ decreased the expression of Bcl-2 in both cell lines significantly ($P < 0.05$). GLP-2 agonist and 100 nM Liraglutide both inhibited the decrease of Bcl-2 expression induced by MPP+ ($P < 0.05$).

GLP-2 agonist protects cells from the increase of mitochondrial superoxide induced by MPP+

MitoSOX red was used to detect the mitochondrial superoxide in cells. As shown in $\text{Fig. 3}$, mitochondrial superoxide was stained with red which represented positive cells, 2 mM MPP+ increased the positive rate and GLP-2 agonist and 100 nM Liraglutide both in-
hibited the increase of mitochondrial superoxide significantly \( (P < 0.05) \).

**GLP-2 agonist protects cells against the increases of autophagy and apoptosis from MPP +**

TEM is the “gold standard” for the observation of autophagy. The ultrastructure of autophagy is characterized by vesicular bodies of different sizes wrapped in a bilayer or multilayer membrane, also known as autophagy vesicles. As shown in ▶ Fig. 4, a large number of autophagy vesicles appeared in the cells of MPP + -treated group, with contents visible in the vesicles. The control group showed clear cell structure and normal organelle morphology. In the GLP-2 agonist and 100 nM Liraglutide-pretreated group, multiple autophagosomes could be seen in both SH-SYSY and N2a cells. Apoptosis was also observed using TEM, as shown in ▶ Fig. 4. In the MPP + group, the cell volume was reduced and the nucleus cleaved into fragments (SH-SYSY) or had collapsed and shrank (Neuro-2a). In the GLP-2 agonist and 100 nM Liraglutide-pretreated groups, the apoptosis in SH-SYSY cells we reduced as nucleus collapsed and shrank less, and in Neuro-2a cells they shrank only slightly.

**Discussion**

Previous studies demonstrated that GLP-2 has protective effects on neurons. *In vitro* studies have shown that GLP-2 could protect neurons from excitotoxic damage [14] and reduce glutamate-induced cell death [15]. In *in vivo* studies, GLP-2 treatment showed some positive effects in the brain. It was reported that the GLP-2 long-acting analogue (teduglutide) can improve obesity-related neuroinflammation and central stress conditions, and reduce the death of nerve cells via apoptosis by reducing inflammation and oxidative stress in the brain [6]. Recent studies reported that GLP-2 can rescue memory impairments and neuropathological changes in mouse models of sporadic AD induced by the intracerebroventricular administration of streptozotocin, this is due to its potential to reduce oxidative stress [5]. Importantly, GLP-2 signaling had clear neuroprotective effects in a re-perfusion stroke model in rats [9] and improved learning, memory and cognitive ability in a cerebral ischemia mouse model of vascular dementia [8]. GLP-2 decreased oxidative damage in the cerebral ischemia/reperfusion model, in which GLP-2 increased the MDA (malondialdehyde) levels and MPO (myeloperoxidase) activity, and reduced the number of apoptotic hippocampal neurons [7]. In addition, the protective effect of GLP-2 on memory impairment in lipopolysaccharide (LPS)-treated mice were investigated, and it was found that GLP-2 can reduce the inflammation response and protect and improve memory function [12]. GLP-2 can induce antidepressant-like effects on adrenocorticotropic hormone (ACTH)-treated mice [10, 11].

In our study, the viability and cytotoxicity of MPP + on SH-SHSY and Neuro-2a cells were investigated. MPP + is a toxic radical that is produced in neurons when metabolizing MPTP, which can induce PD in humans [31]. The experimental results show that the cytotoxicity of MPP + was dose-dependent, and 2 mM MPP + was selected for subsequent experiments. In the MPP + cell damage model, 100 nM of the GLP-2 agonist or Liraglutide increased cell viability significantly. At the same time, the GLP-2 agonist and Liraglutide both reduced MPP + induced LDH release. Our study demonstrates that the GLP-2 agonist protects neuronal cells through ways of protecting mitochondria, enhancing autophagy, and reducing apoptotic signaling. On the one hand, the GLP-2 analogue
reversed the MPP + induced decrease of PGC-1α, Mfn2, and IRE1 levels, and decreased mitochondrial superoxide levels, thus reducing oxidative stress in mitochondria and endoplasmic reticulum stress. On the other hand, the GLP-2 agonist normalized autophagy and restored the function of mitochondria, which was also confirmed by electron microscopy. Finally, the GLP-2 analogue inhibited apoptosis by promoting the expression of Bcl-2, and TEM images also confirmed that the GLP-2 analogue decreased apoptosis. Our results show that MPP + induced cell damage, activated mitochondrial autophagy and disrupted autophagy homeostasis, leading to mitochondrial oxidative stress injury and mitochondrial dysfunctions. Thus, like the GLP-1 agonist Liraglutide, GLP-2 has protective effect on neuronal cells. As GLP-1 receptor agonists show neuroprotective effects in different animal models and have shown good protective effects in first clinical trials in AD and PD patients [23, 24], the observation that the GLP-2 analogue displays comparable neuroprotective effects in this study is of great interest and establishes the GLP-2 signaling pathway as a promising drug target to develop novel treatments for PD.

Conclusion

In conclusion, GLP-2 agonist protects neuronal cells against mitochondrial damage, autophagy impairments and apoptosis induced by MPP + both in SH-SYSY and Neuro-2a cells. GLP-2 receptor agonists have the potential to act as a novel treatment for PD, perhaps in unison with GLP-1 analogues.

Conflict of Interest

The authors declare that they have no conflict of interest.

References


