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Ghrelin prevents 1-methyl-4-phenylpyridinium ion-induced cytotoxicity through antioxidation and NF-κB modulation in MES23.5 cells

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ABSTRACT

Ghrelin, a 28-amino acid peptide, is an endogenous ligand for the growth hormone secretagogue (GHS) receptor. Our previous data showed that ghrelin could inhibit apoptosis in Parkinson's disease (PD) models both *in vitro* and *in vivo*. There is now growing evidence that oxidative stress has a critical role in the etiology of PD. And ghrelin was reported to possess anti-inflammatory, antioxidant effects. Dose ghrelin protect dopaminergic neurons by its antioxidant effect? In the present study, 1-methyl-4-phenylpyridinium (MPP^+) was used to evaluate the possible antioxidant effects of ghrelin on MPP^+-induced neurotoxicity in MES23.5 cells and the underlying mechanisms. Our results showed that MPP^+ significantly increased malonaldehyde (MDA) level and Bax/Bcl2 ratio, reduced the level of Cu–Zn superoxide dismutase (SOD) and catalase (CAT). Ghrelin protected MES23.5 cells against MPP^+-induced neurotoxicity by reversing these changes. Furthermore, ghrelin pretreatment significantly inhibited MPP^+-induced nuclear factor-kappaB translocation. These results suggest that the protective effects of ghrelin on MPP^+-induced cytotoxicity may be ascribed to its antioxidative properties, and the modulation of nuclear factor-kappaB.

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that stems from the loss of dopaminergic neurons of the substantia nigra (SN). Accumulating evidence supported that among the proposed mechanisms of dopaminergic degeneration, oxidative stress played an important role in cell death in PD (Burkhardt and Weber, 1994; Drechsel and Patel, 2009; Hosamani and Muralidhara, 2009; Schapira, 2009; Skolimowski et al., 2003; Yokoyama et al., 2008). Some enzymic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), can remove the reactive oxygen species (ROS) to defend the oxidative stress. It was reported that SOD can convert superoxide radicals to hydrogen peroxide and subsequently convert to water by CAT and GPx (Wang et al., 2007). Therefore, drugs that exhibit properties of an antioxidant, regulating the levels of enzymic antioxidants (such as SOD, CAT) may serve as potential candidates for the treatment of PD.

Ghrelin is a newly discovered hormone, produced principally in the stomach, and has been identified as the endogenous ligand for the growth hormone secretagogue receptor (GHSR) (Kojima et al., 1999). In our previous study, we have observed the neuroprotective effects of ghrelin on dopaminergic neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -induced neurotoxicity *in vivo* and 1-methyl-4-phenylpyridinium (MPP^+)-induced apoptosis in MES23.5 cells *in vitro* via its fully functional receptor, GHS-R 1a (Dong et al., 2009; Jiang et al., 2008). Many researches have revealed that ghrelin could mitigate proinflammatory cytokine production and diminish oxidative stress (Ding et al., 2008; El Eter et al., 2007; Hou et al., 2009; Iseri et al., 2008; Li et al., 2004; Sakane et al., 2008). Also some studies have reported that ghrelin mediated its anti-inflammatory and antioxidant effects by modulation of several important molecular targets, including transcription factors (e.g., NF-κB), enzymes (e.g., SOD and COX-2), and cytokines (e.g., TNFα, IL-1β, IL-8, and IL-6) (Hou et al., 2009; Li et al., 2004; Waseem et al., 2008; Xu et al., 2008; Zhou and Xue, 2009).

In the present study, MPP^+, as a neurotoxin used in cellular models of Parkinson's disease, was used to elucidate whether ghrelin could protect dopaminergic cells from MPP^+ neurotoxicity by its anti-oxidation and the underlying mechanisms. We demonstrated that the neuroprotective effect of ghrelin on MPP^+-treated MES23.5 cells is due to its anti-oxidation properties and regulating the malonaldehyde (MDA) levels, Cu/Zn-SOD and CAT levels, Bax/Bcl2 ratio, as well as the translocation of NF-κB.

Materials and methods

Materials

Rat ghrelin was purchased from Phoenix Pharmaceuticals, Inc (Phoenix, TX, USA). Dulbecco's modified Eagle's medium Nutrient

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Mixture-F12 (DMEM/F12) were from Gibco (Grand Island, NY, USA). Reverse transcriptase was purchased from Promega (Madison, WI, USA). MDA Assay Kits were purchased from Nanjing Institute of Jiancheng Biological Engineering (Nanjing, China). The other chemicals and regents of the highest grade available were from local commercial sources.

Cell culture

MES23.5 cells were offered by Dr. Wei-Dong Le (Baylor College of Medicine, TX, USA). It is a dopaminergic cell line hybridized from murine neuroblastoma-glioma N18TG2 cells with rat mesencephalic neurons, which exhibits several properties similar to the primary neurons originated in the substantia nigra (Crawford et al., 1992). Cells were cultured in DMEM/F12 containing Sato's components growth medium supplemented with 5% FBS, 100 units/mL of penicillin and 100 mg/mL of streptomycin at 37 °C, in a humid 5% CO_2, 95% air environment. For experiments, cells were seeded at a density of $1\times10^5/cm^2$ in the plastic flasks or plates. Our previous study showed that ghrelin (10^{-9} mol/L) pretreatment for 20 min could significantly inhibit LDH leakage outside the cells induced by MPP^+ (Dong et al., 2009). Ghrelin (10^{-9} mol/L) was chosen to do the following experiments. To study the protective effects of ghrelin, cells were pretreated with ghrelin (10^{-9} mol/L) or vehicle (saline) for 20 min, and then incubated with MPP^+ (200 μmol/L) for 24 h.

Measurement of MDA levels

For the lipid peroxidation measurements, cells were lysed by ultrasound and protein concentration was measured by the Bradford method. The levels of the terminal product of lipid peroxidation MDA were measured as the indication of lipid peroxidation, using a commercially available kit (Nanjing Institute of Jiancheng Biological Engineering, China). The thiobarbituric acid-reactive substrates were quantified using 1, 1, 3, 3-tetraethoxypropane as the standard. The absorbance at 532 nm was measured with a colorimetry.

Total RNA extraction and reverse transcriptase-polymerase chain reaction

Total RNA was isolated from MES23.5 cells by using TRIzol reagent according to the manufacturer's instructions. Reverse transcription was performed using the AMV reverse transcription system (Promega Corporation, Madison, WI, USA). We amplified CAT cDNA fragment (372 bp) with the primers (forward: 5'-CTA TCC TGA CAC TCA CCG CCA T-3'; reverse: 5'-TTC TTG ACC GCT TTC TTC TGG A-3') and GAPDH (575 bp) with the primers (forward: 5'-TGTTCCAGTATGATTCTACCCA-3'; reverse: 5'-GGTAGGAACACGGAAGGC-3'). DNA was amplified immediately with a single cycle at 94 °C for 5 min and 30 cycles at 94 °C for 30 s and 58 °C for 30 s and 72 °C for 30 s for CAT; and a final extension step was taken at 72 °C for 10 min. Ethidium bromide stained gels were scanned and qualified using Tanon Image Software. The intensity of each band was normalized against the intensity of GAPDH.

Western blot analysis

Cells were lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL aprotinin. The extraction and isolation of nuclear and cytoplasmic protein were performed according to the method of Wang et al., using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China) (Wang et al., 2009). The protein concentration was determined by the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA). Sixty microgram total proteins was separated by SDS-polyacrylamide gel and then transferred to PVDF membrane. After blocking with 5% non-fat milk at room temperature for 2 h, the membranes were incubated with rabbit-anti-rat bcl-2 antibody (Santa Cruz Biotechnology, USA, 1:400), rabbit-anti-rat Bax antibody (Santa Cruz Biotechnology, USA, 1:400), rabbit-anti-rat CAT antibody (Abcam Biotechnology, Hong Kong, 1:2,000), rabbit-anti-rat NF-κB antibody (Santa Cruz Biotechnology, USA, 1:200), rabbit-anti-rat IκBα antibody (Beverly, MA, USA, 1:2,000), rabbit anti-rat Cu/Zn-SOD antibody (BIOS, China, 1:100) for overnight at 4 °C; Rabbit anti-rat β-actin (BIOS, China, 1:500) and mouse anti-rat PCNA (Santa Cruz Biotechnology, USA, 1:200) antibody for 2 h at room temperature. Anti-rabbit and anti-mouse secondary antibody conjugated to horseradish peroxidase were used at 1:10,000 (Santa Cruz Biotechnology, USA). Cross-reactivity was visualized using ECL western blotting detection reagents and then analyzed through scanning densitometry by a Tanon Image System.

Statistical analysis

Results are presented as mean ± SEM. One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test was used to compare differences between means in more than two groups. A probability value of $P<0.05$ was considered to be statistically significant.

Results

Ghrelin antagonized MPP^+-induced intracellular ROS production and lipid peroxidation

Our previous study demonstrated that cells pretreated with ghrelin showed a less ROS production than that of the cells treated with MPP^+ (Dong et al., 2009). This indicated ghrelin could inhibit the MPP^+-induced ROS production. MDA is an index of lipid peroxidation and is a well-known marker in the pathologic molecular process in oxidative stress. To further elucidate the antioxidant effect of ghrelin, in the present study, we observed MDA levels in MES23.5 cells. As shown in Fig. 1, the levels of MDA in MPP^+-treated MES23.5 cells were significantly higher than that of the control ($P<0.01$), indicating an elevated oxidative stress in the MES23.5 cells after MPP^+ treatment. While, ghrelin pretreatment could significantly attenuate the MPP^+ -induced increase in MDA levels ($P<0.01$).

Ghrelin reversed MPP^+-induced CAT and Cu/ Zn- SOD down-regulation in MES23.5 cells

To further investigate the underlying mechanisms of the antioxidant effect of ghrelin against MPP^+, we examined antioxidant enzyme including CAT and Cu/Zn-SOD expression, which are highly potent

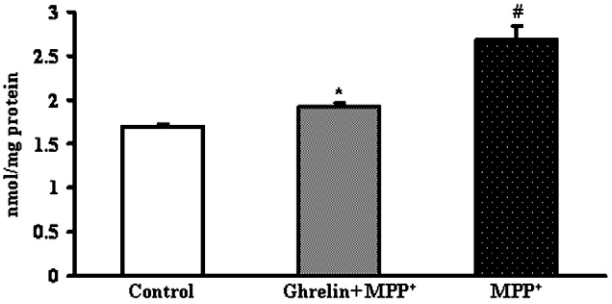

Fig. 1. Ghrelin antagonized MPP^+-induced increase in MDA levels. Cells were treated with vehicle, MPP^+ (200 μmol/L) or ghrelin(10^{-9} mol/L) prior to MPP^+ for 24 h, then MDA levels were measured by MDA Assay Kit. MPP^+ treatment caused increase in MDA levels in MES23.5 cells and ghrelin pretreatment partly prevented this increase. Data were shown as means ± SEM. $^{\#}P<0.01$, compared to the control group; $^{*}P<0.01$, compared to MPP^+ group.

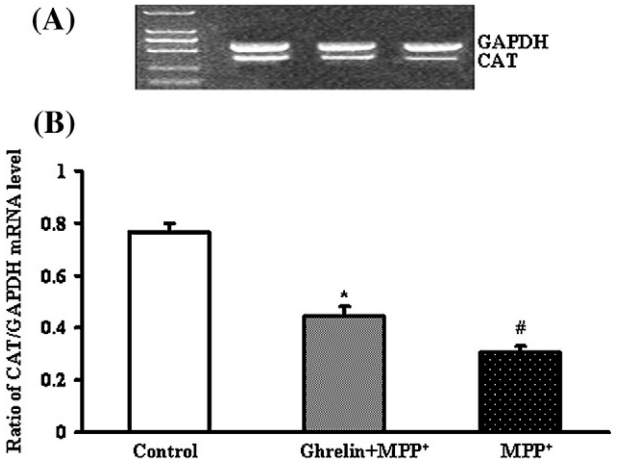

Fig. 2. Effect of ghrelin on MPP^+-induced CAT mRNA expression in MES23.5 cells. Cells were treated with vehicle, MPP^+ (200 μmol/L) or ghrelin(10^{-9} mol/L) prior to MPP^+ for 24 h. (A) RT-PCR was applied to detect CAT mRNA levels. Decreased mRNA expression of CAT was observed in MPP^+-treated cells. Ghrelin pretreatment prevented the downregulation of CAT partly. GAPDH was used as a loading control. (B) Statistical analysis. CAT band intensities were normalized with GAPDH band intensity. Data were presented as the ratio of CAT to GAPDH. Each value represented as mean ± SEM ($n=3$). $^{\#}P<0.01$, compared to the control group; $^{*}P<0.01$, compared to MPP^+ group.

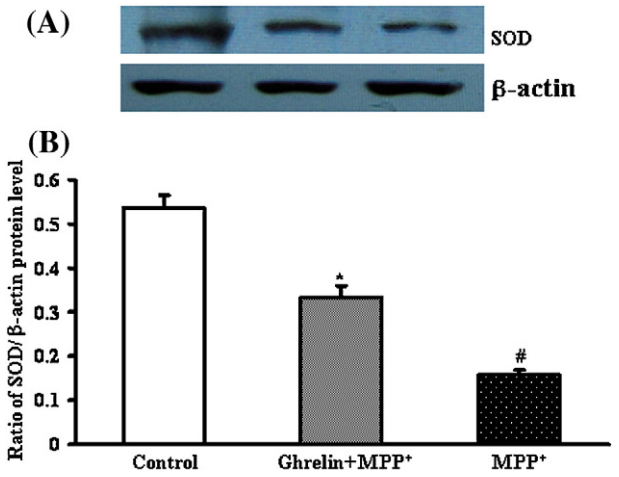

Fig. 4. Effect of ghrelin on MPP^+-induced Cu/Zn-SOD protein expression in MES23.5 cells. Cells were treated with vehicle, MPP^+ (200 μmol/L) or ghrelin (10^{-9} mol/L) prior to MPP^+ for 24 h, and then the expression of Cu/Zn-SOD was detected by western blots. (A) Decreased protein expression of Cu/Zn-SOD was observed in MPP^+-treated cells. Ghrelin pretreatment prevented the downregulation of Cu/Zn-SOD partly. β-actin was used as a loading control. (B) Statistical analysis. Data were presented as the ratio of Cu/Zn-SOD toβ-actin. Each value represented the mean ± SEM ($n=3$). $^{\#}P<0.01$, compared to the control; $^{*}P<0.01$, compared to MPP^+ group.

protective agent against cell injury during oxidative stress in the following study. Results demonstrated that MPP^+ treatment resulted in reduction of CAT in both mRNA and protein levels after 24 h compared with the control, whereas pretreatment with ghrelin enhanced the mRNA expression and protein expression of CAT as shown in Figs. 2 and 3. Meanwhile, we also examined MPP^+ significantly decreased the Cu/Zn-SOD protein levels, while pretreatment with ghrelin strongly prevented the depletion of Cu/Zn-SOD as shown in Fig. 4.

Ghrelin inhibits MPP^+-induced changes in the protein levels of Bax and Bcl-2

Studies have revealed that oxidative stress could cause cell apoptosis. Several protein families are considered to be specifically involved in regulating programmed apoptotic cell death, such as Bcl-2 and Bax (Oltvai et al., 1993). In this study, the Bax to Bcl-2 expression ratio was used to determine whether cells have undergone apoptosis. The protein levels of Bax increased and Bcl-2 decreased in MPP^+-treated cells compared with that of the control. However, these were reversed upon ghrelin pretreatment. And the Bax/Bcl-2 ratio increased to 2.84-fold of control upon treatment with MPP^+, while ghrelin attenuated these changes (Fig. 5). These results suggested a notion that ghrelin pretreatment shifted the balance between positive and negative regulators of apoptosis towards cell survival.

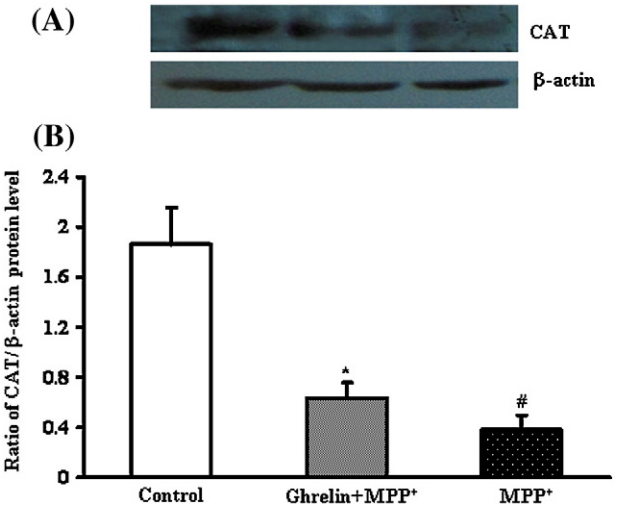

Fig. 3. Effect of ghrelin on MPP^+-induced CAT protein expression in MES23.5 cells. Cells were treated with vehicle, MPP^+ (200 μmol/L) or ghrelin (10^{-9} mol/L) prior to MPP^+ for 24 h, and the expression of CAT was detected by western blots. (A) Decreased protein expression of CAT was observed in MPP^+-treated cells. Ghrelin pretreatment prevented the downregulation of CAT partly. β-actin was used as a loading control. (B) Statistical analysis. Data were presented as the ratio of CAT to β-actin. Each value represented as mean ± SEM ($n=3$). $^{\#}P<0.01$, compared to the control; $^{*}P<0.01$, compared to MPP^+ group.

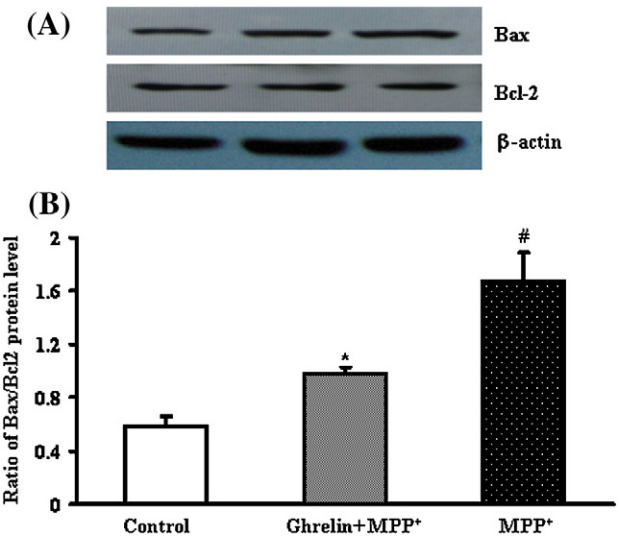

Fig. 5. Effect of ghrelin on MPP^+-induced Bcl-2 and Bax protein expression in MES23.5 cells. Cells were treated with vehicle, MPP^+ (200 μmol/L) or ghrelin (10^{-9} mol/L) prior to MPP^+ for 24 h, and then the protein expressions of Bcl-2 and Bax were detected by western blots. (A) Decreased protein expression of Bcl-2 and increased protein expression of Bax were observed in MPP^+-treated cells. Ghrelin pretreatment attenuated the change. β-actin was used as a loading control and (B) statistical analysis. Data were presented as the ratio of Bax/Bcl-2 to β-actin. Each value represented the mean ± SEM. ($n=3$). $^{\#}P<0.01$, compared to the control group; $^{*}P<0.01$, compared to MPP^+ group.

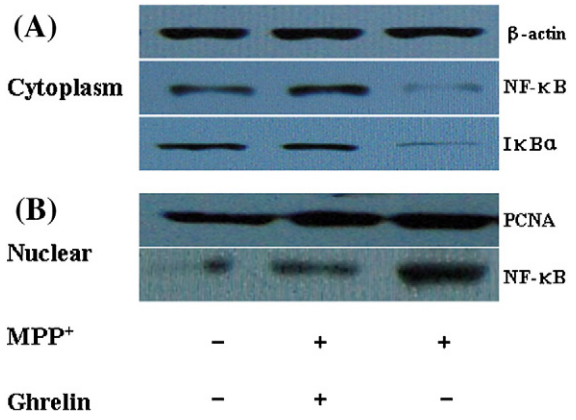

Fig. 6. Effect of ghrelin on MPP^+-induced NF-κB translocation in MES23.5 cells. Cells were treated with vehicle, MPP^+ (200 μmol/L) or ghrelin (10^{-9} mol/L) prior to MPP^+ for 24 h, and then the protein expressions of NF-κB and IκBα were detected by western blots. (A) Decreased expressions of NF-κB and IκBα were observed in MPP^+-treated cells in the cytoplasm. Ghrelin pretreatment prevented the downregulation of NF-κB and IκBα induced by MPP^+ partly. β-actin was used as a loading control. (B) Increased expression of NF-κB was observed in the nucleus of MPP^+-treated cells. Ghrelin pretreatment considerably prevented the upregulation of NF-κB in the nucleus. PCNA was used as a loading control.

Ghrelin inhibits MPP^+-induced NF-κB translocation in MES23.5 cells

The mechanism underlying the antioxidant effect of ghrelin against MPP^+ was also investigated by examining NF-κB translocation in MES23.5 cells, using polyclonal antibody of NF-κB. As demonstrated in Fig. 6, MPP^+ induced a clear NF-κB nuclear translocation, which was inhibited by ghrelin pretreatment. As IκB degradation regulated the translocation of NF-κB, we then investigated the degradation of IκBα in this study. Results showed the protein levels of IκBα decreased in MPP^+-treated cells, while pretreatment with ghrelin could increase the protein levels of IκBα. This indicates an IκBα-dependent regulation is involved in this process and ghrelin could prevent the translocation of NF-κB in an IκBα-dependent manner.

Discussion

In this study, we demonstrated that ghrelin could attenuate MPP^+-induced neurotoxicity by anti-oxidation in MES23.5 cells, and this effect of ghrelin was achieved by the regulation of the MDA levels, Cu/Zn-SOD and CAT levels, Bax/Bcl2 ratio, as well as the translocation of NF-κB.

The etiology of PD is still not fully understood. Evidence proves that oxidative stress plays an important role in the onset and/or progression of PD, which could be blocked or delayed by a wide variety of antioxidants (Chaudhuri et al., 2006; de Vries et al., 2008; Kamat et al., 2008). MPP^+, a neurotoxin to induce PD model, is the active metabolite of MPTP (Arora et al., 1990). It could inhibit complex I of the mitochondrial respiratory chain, and may induce oxidative stress and dopaminergic neurodegeneration (Kitamura et al., 2000; Tipton and Singer, 1993). Therefore, the possible neuroprotective effect of ghrelin on MPP^+-induced oxidative stress was first investigated by detecting the levels of MDA in this study. MDA is an index of lipid peroxidation and is a well-known marker indicating the extent of oxidative stress. The present study showed that MPP^+-treatment significantly increased MDA formation, which could be inhibited by ghrelin. This is consistent with other studies that the protection of ghrelin was possibly accomplished through the antioxidant activity observed *in vivo* and *in vitro* (El Eter et al., 2007; Kui et al., 2009; Liu et al., 2009).

Evidence also proved that antioxidant enzymes, such as SOD and CAT, can protect cellular components from damage by ROS, which represents the primary line of defense. SOD can convert superoxide radicals to hydrogen peroxide and subsequently convert to water by CAT and GPx (Wang et al., 2007). While a high degree of oxidative stress could overwhelm any endogenous protective antioxidant system including SOD and CAT (Han and Zhao, 2009; Jung et al., 2006; Tsai and Yin, 2008). The present study showed that MPP^+-treatment significantly decreased the levels of SOD and CAT in MES23.5 cells. Ghrelin pretreatment caused a significant increase in SOD, CAT levels in MPP^+-treated MES23.5 cells. This is in accordance with previous study, showing the regulation of ghrelin on SOD and CAT expression (Iseri et al., 2008; Obay et al., 2008; Yang et al., 2007; Zwirska-Korczala et al., 2007).

Studies have revealed that ROS could cause cell death via apoptosis. In this study, we found that treatment of MPP^+ results in an increase in Bax and a decrease in Bcl-2 expression in MES23.5 cells. This could be reversed by pretreatment with ghrelin in MPP^+-treated MES23.5 cells. These results provide evidence for the anti-apoptosis role of ghrelin in MPP^+-treated MES23.5 cells via mitochondrial pathway.

Oxidative stress-induced ROS generation has been implicated in NF-κB activation (Schreck et al., 1991), though the exact target is still unknown. A number of studies have demonstrated that a free radical scavenging activity may participate in the inhibition of NF-κB activation (Blackwell and Christman, 1997; Bonizzi et al., 2000; Levites et al., 2002). This further confirmed the role of ROS in NF-κB activation. In the inactive state, NF-κB is detained in the cytosol through complexation with IκB proteins. IκB is degraded against stimulus, and NF-κB is then released from the cytosolic complex and translocates to the nucleus, where it activates the transcription of genes that contain an NF-κB binding site (Gloire et al., 2006). Some researchers suggest that neurotoxins such as MPTP, rotenone and 6-hydroxydopamine (6-OHDA) used in an experimental model of PD could activate NF-κB (Aoki et al., 2009; Blum et al., 2001; Ghribi et al., 2003). Our observation that ghrelin pretreatment attenuated MPP^+-induced degradation of IκBα and NF-κB activity suggests ghrelin inhibited NF-κB translocation by decreasing ROS generation at least. However, the exact mechanisms of the NF-κB translocation were still not fully elucidated. Some study reported that there are crosstalks between NF-κB and nuclear factor E2-related factor 2 (Nrf2) (Jin et al., 2008; Liu et al., 2008). So, we supposed that Nrf2 may take part in the modulation of NF-κB. Moreover, the substances regulated by Nrf2, such as products derived from Heme Oxygenase-1 (HO-1) may play a role in inhibition of NF-κB activation (Ferris et al., 1999; Rushworth et al., 2005; Satoh et al., 2006). This provides the evidence for the possible regulation of Nrf2 on NF-κB translocation. Further investigation should be conducted to examine the underlying mechanisms.

In summary, the present study has demonstrated that ghrelin exerted antioxidant effect against MPP^+-induced cell death, by a mechanism, believed to increase the levels of SOD, CAT and decrease the levels of MDA, and inhibit the translocation of NF-κB. These results add further evidence to explore the therapeutic potential of ghrelin in treating neurodegenerative diseases.

Acknowledgments

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