Ghrelin antagonizes MPTP-induced neurotoxicity to the dopaminergic neurons in mouse substantia nigra

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ABSTRACT

Ghrelin, a stomach-derived hormone which induces growth hormone release and promotes positive energy balance, has been reported to inhibit cell apoptosis in endotheliocytes, osteoblasts and cardiocytes. Recent evidence has shown that ghrelin can also inhibit neuronal apoptosis of the hypothalamus and the hippocampus. However, little is known about the effects of ghrelin on the substantia nigra pars compacta (SNpc) neurons in which ghrelin's receptor, growth hormone secretagogue receptor (GHSR)-1a, is highly expressed. In the present study, we investigated whether ghrelin could protect nigral dopaminergic neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in mice. We observed that ghrelin, acting through GHS-R 1a, inhibited MPTP-induced dopaminergic neuronal loss in the SNpc as well as dopamine depletion in the striatum. Ghrelin could also reverse the down-regulated expression of Bcl-2, up-regulated the expression of Bax, and caspase-3 activation caused by MPTP. This study demonstrated that ghrelin might be a potential protector of dopaminergic neurons in a therapeutic strategy for Parkinson’s disease.

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Introduction

Parkinson’s disease (PD) is clinically characterized by resting tremor, rigidity and bradykinesia. The major neuropathological feature of PD is degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Several mechanisms are involved in the pathogenesis of dopaminergic nigrostriatal degeneration of PD patients, such as apoptosis, oxidative stress, increased iron content and mitochondrial dysfunction. (Bredesen et al., 2006; Jenner and Olanow, 2006; Jiang et al., 2006; Xie et al., 2003;). These interrelated events form a complex cascade and finally lead to neuronal death by apoptosis (Dauer and Przedborski, 2003; Hunot and Hirsch, 2003; Barnham et al., 2004; Bredesen et al., 2006; Jenner and Olanow, 2006). Anti-apoptosis strategies could, in principle, prevent or delay the progression of PD.

Ghrelin, a 28-amino-acid stomach-derived hormone, has been identified as a solely endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). It is produced and secreted mainly by the stomach, and acts on the pituitary and the hypothalamus to stimulate the release of growth hormone and promote adiposity and appetite (Kojima et al., 1999; Date et al., 2000; Peino et al., 2000; Tschop et al., 2000; Nakazato et al., 2001). Recent studies revealed that ghrelin stimulates proliferation and inhibits apoptosis in several cell types via its fully functional receptor, GHS-R 1a. (Baldanzi et al., 2002; Kim et al., 2004, 2005; Nanzer et al., 2004; Chung et al., 2007). Besides the hypothalamus (Bennett et al., 1997; Guan et al., 1997), GHS-R 1a is also expressed abundantly in the SNpc (Zigman et al., 2006). Therefore, the function of ghrelin on the SNpc needs to be studied. In the present study, we investigated the neuroprotective effect of ghrelin on a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced subacute PD mouse model (Tatton and Kish, 1997; Vila et al., 2000). Additionally, d-Lys3-GHRP-6, the antagonist for GHS-R 1a, (Chung et al., 2007) was employed to study the mechanism of ghrelin’s neuroprotective effect.

Materials and methods

Animals and treatment

All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of Qingdao University. 129 healthy male C57BL/6 mice at the age of 10 weeks were housed with a 12 h light–dark cycle at room temperature (19±2 °C) and with free access to food and water. Mice were randomly divided into 6 groups: (1) control mice: mice received saline intracerebral-ventricularly (i.c.v.) only; (2) ghrelin solely-treated group: to exclude the effect of ghrelin itself on dopaminergic neurons, mice received ghrelin (Sigma, St. Louis, MO, USA, 200 ng, i.c.v.) injections once per day for 8 consecutive days; (3) d-Lys3-GHRP-6 solely-treated group: to exclude the effect of ghrelin receptor’s antagonist, d-Lys3-GHRP-6, on dopaminergic neurons in the SN, mice received 60μg d-Lys3-GHRP-6 injections once per day for 8 consecutive days; (4) D-Lys3-GHRP-6 + ghrelin group: to exclude the effect of ghrelin receptor’s antagonist, d-Lys3-GHRP-6, and dopaminergic neurons in the SN, mice received ghrelin (Sigma, St. Louis, MO, USA, 200 ng, i.c.v.) injections once per day for 8 consecutive days; (5) MPTP group: mice received 60μg d-Lys3-GHRP-6 injections once per day for 8 consecutive days; (6) MPTP + ghrelin group: mice received 60μg d-Lys3-GHRP-6 injections once per day for 8 consecutive days.
Double immuno-fluorescent labeling of GHS-R 1a with tyrosine hydroxylase (TH) in mouse SNpc

Three normal mice were anesthetized with sodium pentobarbital (5 mg/100 g body weight, i.p.) and then perfused transcardially with 0.01% heparin/saline followed by 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.4 (PFA/PBS). The brains were removed and post-fixed in above paraformaldehyde solution for 6 h, then immersed in 20% sucrose solution overnight for dehydration. Each brain block containing the SN was cut into 20-µm-thick coronal sections on a freezing microtome and mounted on polylysine-coated slides. Non-specific binding was blocked with 10% goat serum containing 0.3% Triton X-100 in PBS for 1 h. The sections were then incubated overnight with primary antibodies consisting of mouse anti-rat TH (Sigma, St. Louis, MO, USA, 1:2,000) and rabbit anti-human GHS-R 1a (Phoenix Pharmaceutical, Belmont, California, USA, 1:20) at 4 °C in a humidified chamber. Fluorescent isothiocyanate-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG (1:200) were used as secondary antibodies. Finally, the sections were examined using a Fluoview FV500 laser confocal scanning microscope (Olympus, Japan). Negative control tests were run without primary or secondary antibodies.

TH and caspase-3 immunohistochemistry staining

Six mice from each group were used. Of every six 20-µm-thick sections throughout the SN, the first and the second were selected for TH staining and caspase-3 staining individually. Sections were incubated in 99.7% methanol containing 0.3% hydrogen peroxide for 30 min to eliminate the endogenous peroxidase. Non-specific binding was blocked with 10% goat serum for 15 min at 37 °C. The sections were incubated with the primary antibodies overnight at 4 °C in humidity chambers at the following dilutions: mouse anti-rat TH (1:5,000) and rabbit anti-rat caspase-3 (Santa Cruz, California, USA, 1:250). The sections were then washed in PBS and incubated with biotinylated goat anti-rabbit IgG for 30 min at 37 °C, followed by amplification with streptavidin peroxidase for 30 min at 37 °C. Finally, diaminobenzidine-hydrogen peroxide (0.01%) was used as the chromogen. Negative control tests were run without primary or secondary antibodies. TH-ir neurons and caspase-3-ir cells were counted in totally six sections throughout the entire rostrocaudal extent of the SNpc (interaural 0.88 to 0.16 mm; Franklin and Paxinos, 2001). All sections were coded and examined blind. The survival rate of dopaminergic neurons in the SNpc was determined by comparing the number of TH-ir neurons of the tested group to that of the normal control group. (Hong et al., 2007).

High-performance liquid chromatography with electrochemical detection (HPLC)

Six mice from each group were killed by cervical vertebra dislocation. The striatum and SN were carefully isolated for HPLC and RNA extraction respectively. The levels of the striatal dopamine (DA) and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were determined using a previously described method by high-performance liquid chromatography equipped with a 2465 electrochemical detector (Waters, USA) (Jiang et al., 2006).

Total RNA extraction and reverse transcriptase-polymerase chain reaction

Total RNA was isolated from the SN 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 Bcl-2 cDNA fragment (amplified products were 409 bp length) with the primers (forward: 5′- GTC CCG CCT CTT CAC CCT -3′; reverse: 5′- CCC ACT CGT AGC CCC TCT -3′), Bax cDNA fragment (amplified products were 307 bp length) with the primers (forward: 5′- GCC GAA TTG ATG AAT AAC -3′; reverse: 5′- CCG AAG TAG GAG AGG AGG -3′) and GAPDH cDNA fragment (amplified products were 340 bp length) with the primers (forward: 5′- GAC CAC AGT CCA TGC CAT CAC -3′; reverse: 5′- GCT GCA TCA CTC CAT CAC -3′). The expression of house-keeping gene, GAPDH mRNA, was used as an internal standard. PCRs were run for 30 cycles in an Eppendorf Mastercycler. Denaturing, annealing, and extension reactions were performed at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. The PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide, and detected by UV irradiation. The levels of Bcl-2 and Bax mRNA were expressed as their respective ratios to GAPDH mRNA.

Statistical analysis

Statistical analysis was conducted using software of SPSS 13.0 (SPSS Inc., Chicago, IL). Student—Newman—Keuls test was used after one-way ANOVA to determine statistical differences among all the groups. In addition, t-test was conducted to test for the significance of the difference between 200 ng ghrelin pretreated and D-Lys3-GHRP-6 pretreated groups. Data are presented as mean±SEM and differences were considered significant at P<0.05 and highly significant at P<0.01.

Results

Localization of GHS-R 1a on TH-ir neurons in the SNpc

Double-labeling immunofluorescence showed the colocalization of TH (green, Fig. 1A) and GHS-R 1a (red, Fig. 1B) in the SNpc. The result demonstrated that GHS-R 1a was expressed in the dopaminergic neurons of the SNpc (Fig. 1C).

Ghrelin protected dopaminergic neurons against MPTP neurotoxicity

After MPTP treatment, the animals showed a significant loss of TH-ir neurons; the survival ratio of TH-ir neurons in the SNpc was only 67% compared with the normal controls (Figs. 2B, b, E). Ghrelin or D-Lys3-GHRP-6 themselves had no effect on the number of TH-ir neurons (Fig. 2E). However, ghrelin showed a marked dose-dependent protective effect on dopaminergic neurons against MPTP neurotoxicity. Lower doses of ghrelin (50 ng and 100 ng/mouse) had no neuroprotective effect (Fig. 2E), while 200 ng/mouse ghrelin preserved as many as 82% of TH-ir neurons compared with that of the control, and the survival ratio of TH-ir neurons increased to 87% with the treatment of 400 ng/mouse ghrelin, which was significantly different from that of the MPTP solely-treated group (P<0.01; Fig. 2E). Pre-injection of the GHS-R 1a receptor specific antagonist D-Lys3-GHRP-6, completely abolished the neuroprotective effect of ghrelin (Figs. 2D, d, E).
Ghrelin attenuated MPTP-induced depletion of DA and its metabolites in the striatum

HPLC revealed that the systemic injection of MPTP resulted in significant depletion of DA and its metabolites in the striatum. MPTP treatment caused a 72% depletion of DA compared with that of the control (Fig. 3A). Ghrelin at a dose of 50 ng/mouse did not influence the contents of DA, DOPAC or HVA in the striatum. The DA levels in 100 ng, 200 ng and 400 ng ghrelin pretreated mice were 171%, 288% and 292% of that in MPTP solely-treated group, respectively. Both 200 ng and 400 ng ghrelin could also increase the striatal DOPAC and HVA levels. Although 100 ng ghrelin had some tendency to increase the levels of DOPAC and HVA, it had no statistical significance (Figs. 3B, C). Pretreatment with d-Lys³-GHRP-6 completely abolished ghrelin-mediated attenuation of MPTP-induced depletion of striatal DA, DOPAC and HVA (Fig. 3).

Ghrelin increased Bcl-2/Bax ratio

Bcl-2, as an anti-apoptotic member of the Bcl-2 family, can bind to Bax to form Bcl-2:Bax heterodimers, thereby attenuating the pro-apoptotic effect of Bax (Oltvai et al., 1993). In the present study, we detected Bcl-2 and Bax mRNA expression levels by RT-PCR. MPTP...
resulted in a dramatic down-regulation of Bcl-2 mRNA and up-regulation of Bax mRNA in the SN (Figs. 4A and B). Pretreatment with ghrelin at the doses of 100 ng, 200 ng and 400 ng/mouse could inhibit the decrease of Bcl-2 caused by MPTP. Bax mRNA expression seemed to be more sensitive to ghrelin; even 50 ng/mouse ghrelin could inhibit the increase of Bax in MPTP mice (Fig. 4B). Therefore, Bcl-2/Bax ratio in the SNpc of MPTP mice significantly increased due to the ghrelin pretreatment (Fig. 4C). Ghrelin’s effects on Bcl-2 and Bax were completely inhibited by D-Lys3-GHRP-6.

Ghrelin inhibited caspase-3 activation by MPTP

Caspase-3 plays a key role in the process of cell apoptosis. Thus, in this study, we used immunoreactivity of caspase-3 to represent the

Fig. 3. Ghrelin attenuated MPTP-induced depletion of DA and its metabolites in the striatum (n=6). A: Striatal DA levels in the mice of the control, ghrelin solely-treated (200 ng/mouse), MPTP solely-treated, MPTP+ghrelin (50 ng, 100 ng, 200 ng and 400 ng/mouse) and MPTP+ghrelin (200 ng/mouse)+D-Lys3-GHRP-6 (60 μg/mouse) groups. B: Striatal DOPAC levels in the different groups. C: Striatal HVA levels in the different groups. Each value represented the mean±SEM. *P<0.01, compared with the MPTP solely-treated group; #P<0.01, compared with the MPTP+ghrelin (200 ng/mouse) group.

Fig. 4. Ghrelin increased Bcl-2 and decreased Bax mRNA level, thus increased Bcl-2/Bax ratio in MPTP-treated mice (n=6). A: Effect of ghrelin on Bcl-2 mRNA level in the mice of the control, ghrelin (200 ng/mouse) solely-treated, MPTP solely-treated, MPTP+ghrelin (200 ng/mouse)+D-Lys3-GHRP-6 (60 μg/mouse) groups. B: Effect of ghrelin on Bax mRNA level in different groups. Each value represented the mean±SEM. *P<0.01, compared with the MPTP solely-treated group; #P<0.01, compared with the MPTP+ghrelin (200 ng/mouse) group.
injection. The effect of ghrelin on inducing adiposity and increasing body weight was ignored here because the effective dosage of ghrelin to induce adiposity was 2.4 μmol/kg for mice (Tschop et al., 2000). In addition, we noticed that the body weights of the animals had no marked change (data not shown). Although ghrelin can pass through the blood-brain barrier, we still chose i.c.v. for administration to focus on the effect of ghrelin on the brain neurons.

MPTP produces clinical, biochemical and pathological features similar to those observed in idiopathic PD. The MPTP-induced PD mouse is one of the most commonly used animal models for studying the apoptosis of dopaminergic neurons in SNpc (Heikila et al., 1984; Langston and Ballard, 1984). In the present study, the TH-ir neurons in the SNpc, and DA content in the striatum, dramatically decreased while the activation of caspase-3 increased significantly in MPTP PD mice. The results are consistent with the study which showed that subacute MPTP administration could induce a cell apoptosis PD model (Przedborski et al., 2000). Via its active MPP+ form, MPTP is taken up by dopaminergic neurons and leads to an impairment in mitochondrial function. The subsequent energy failure with ATP depletion increases formation of free radicals (Schmidt and Ferger, 2001) and cytochrome c release (Gomez et al., 2001; Singh and Dixit, 2007). Hence, caspase-3 is activated and causes hydrolysis of numerous cellular proteins and the activation of endonucleases, finally leading to cell death (Hartmann et al., 2000).

The mechanism of the neuroprotective effect of ghrelin in the present study may be related to attenuating the activity of caspase-3 via regulating Bcl-2 and Bax gene expression in the SNpc. The Bcl-2 family of proteins play an important role in intracellular apoptotic signal transduction by regulating the permeability of the mitochondrial membrane (Kane et al., 1993; Offen et al., 1998). Both Bcl-2 and Bax are involved in the regulation of caspase-3 mediated apoptosis (Reed, 1997). Bcl-2 can inhibit apoptosis by binding to the pro-apoptotic Bax, Bcl-xs, and Bad proteins, it is believed that the Bcl-2/Bax ratio is a determining factor for the cell’s fate (Korsmeyer, 1995; Vila et al., 2001). MPTP is known to decrease Bcl-2 and increase Bax in the striatum (Youdim and Arraf, 2004), thereby tilting the balance towards apoptosis. Primary cultured neurons which over-express Bcl-2 could be resistant to the toxicity of MPTP (Offen et al., 1998); our ghrelin-treated cells showed an increased Bcl-2/Bax ratio, prevention of cytochrome c release, and inhibition of caspase-3 activation, rescuing SNpc neurons from MPTP-induced toxicity. Recent research also showed that ghrelin can increase Bcl-2/Bax ratio and inhibit caspase-3 activation, hence protecting neurons from oxygen–glucose deprivation insult. Moreover, an in vivo administration of ghrelin significantly reduced infarct volume in an ischemia induced animal apoptosis model (Chung et al., 2007).

Along with altering the status of Bcl-2 family of proteins, ghrelin also suppresses oxygen–glucose deprivation-induced reactive oxygen species production. It is well known that MPTP is capable of producing highly toxic hydroxyl radicals (Schmidt and Ferger, 2001), which also involves high levels of iron (Poirier et al., 1985; Temlett et al., 1994; Lan and Jiang, 1997). Our previous study showed increased iron levels in the SNpc in mice after MPTP administration (Jiang et al., 2003), and we also found that ghrelin could antagonize MPTP-induced iron accumulation in the SNpc (data not shown). Whether this peptide could also act as an iron chelator to exert its effect still needs further investigation.

In conclusion, we demonstrate that ghrelin can antagonize MPTP-induced neurotoxicity to nigral dopaminergic neurons in vivo mediated by GHS-R 1a via an anti-apoptotic effect. It remains for future basic and clinical research to determine the potential utility of ghrelin in PD. If ghrelin could induce regeneration or prevent degeneration of dopaminergic neurons, it might be a potential important therapeutic tool in a therapeutic strategy for PD.

Acknowledgments

This study is supported by grants from the National Program of Basic Research sponsored by the Ministry of Science and Technology of...
China (2006CB500704, 2007CB516700) and the National Foundation of Natural Science of China (No.30770757).

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