Neurosci Bull February 1, 2014, 30(1): 118–124. http://www.neurosci.cn
DOI: 10.1007/s12264-013-1405-0

·Original Article·

Protection of MES23.5 dopaminergic cells by obestatin is mediated by proliferative rather than anti-apoptotic action

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ABSTRACT

Obestatin is an endogenous peptide sharing a precursor with ghrelin. This study aims to investigate whether and how obestatin protects MES23.5 dopaminergic cells against 1-methyl-4-phenylpyridinium (MPP^+)-induced neurotoxicity. MES23.5 cells were pretreated with obestatin (10^{-13}–10^{-6} mol/L) for 20 min prior to incubation with 200 μmol/L MPP^+ for 12 or 24 h, or treated with obestatin alone (10^{-13} to 10^{-6} mol/L) for 0, 6, 12, and 24 h. The methyl thiazolyl tetrazolium (MTT) assay was used to measure cell viability. Flow cytometry was used to measure the caspase-3 activity and the mitochondrial transmembrane potential**.** Proliferating cell nuclear antigen (PCNA) protein levels were determined by Western blotting. Obestatin (10^{-13} to 10^{-7} mol/L) pretreatment blocked or even reversed the MPP^+-induced reduction of viability in MES23.5 cells, but had no effect on MPP^+-induced mitochondrial transmembrane potential collapse and caspase-3 activation. When applied alone, obestatin increased viability. Elevated PCNA levels occurred with 10^{-7}, 10^{-9}, 10^{-11} and 10^{-13} mol/L obestatin treatment for 12 h. The results suggest that the protective effects of obestatin against MPP^+ in MES23.5 cells are due to its proliferation-promoting rather than anti-apoptotic effects.

Keywords: obestatin; MPP^+; proliferation; apoptosis; dopaminergic cells

INTRODUCTION

Obestatin, a 23-amino-acid peptide hormone, was first discovered in 2005[1]. It was originally extracted from rat stomach, and is present in the duodenum, gastric mucosa, colon, jejunum, spleen, pancreas, breast milk, mammary glands, plasma, saliva, and testis[2]. By binding to receptors that have yet to be defined, obestatin has both central and peripheral actions, including regulation of energy homeostasis, hormone secretion, improving memory, regulating sleep, and inhibiting water drinking[3-10]. To date, there are conflicting opinions on the relationship between obestatin and its possible receptors, G protein-coupled receptor-39 (GPR39) and glucagon-like peptide-1 receptor (GLP-1R)[1, 11, 12]. It has been reported that both GPR39 and GLP-1R might mediate the proliferative effects of obestatin through the phosphorylation of ERK1/2[12, 13].

Obestatin is derived from the post-translational cleavage of preproghrelin, the same peptide precursor as ghrelin. We previously reported that the neuroprotective effects of ghrelin are mediated by its anti-apoptotic action on dopaminergic neurons in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice *in vivo*, and 1-methyl-4-phenylpyridinium (MPP^+)-treated MES23.5 cells *in vitro*[14, 15]. In the present study, we tested whether obestatin had similar neuroprotective effects on MES23.5 dopaminergic cells, which express GPR39 (data not shown). The MES23.5 cell line is a neuroblastoma derived from somatic cell fusion[16, 17], and expresses three characteristics of dopaminergic neurons, tyrosine hydroxylase positivity, expression of the omega-conotoxin

receptor, and synthesis of dopamine. So the results from MES23.5 cells could provide reliable evidence related to dopaminergic neurons.

MATERIALS AND METHODS

Materials

The primary antibody against proliferating cell nuclear antigen (PCNA) was from Cell Signaling Technology (Boston, MA). Dulbecco's modified Eagle's medium nutrient mixture-F12 (DMEM/F12) was from Gibco (Grand Island, NY). The phycoerythrin (PE)-conjugated monoclonal active caspase-3 antibody apoptosis kit was from BD Bioscience (San Diego, CA).

Cell Culture

MES23.5 cells were provided by Professor Wei-Dong Le (Baylor College of Medicine, Houston, TX). Cells were cultured at 37°C in a 95% air/5% CO_2 humidified atmosphere in DMEM/F12 with Sato components and 5% heat-inactivated FBS[14, 18-21].

Methyl Thiazolyl Tetrazolium (MTT) Assay

To assess the neuroprotective effects of obestatin, MES23.5 cells were seeded in 96-well plates at 2×10^4 cells/well in 100 μL culture medium. After 24 h, cells were pre-incubated with obestatin (10^{-6} to 10^{-13} mol/L) dissolved in DMEM/F12 without serum supplement for 20 min, and then treated with MPP^+ (final concentration 200 μmol/L) for another 24 h. Then MTT was added (5 mg/mL), incubated for 4 h at 37°C, and then cell viability was assessed at 494 nm and 630 nm with a spectrophotometer (Tecan, Grodig, Austria).

To evaluate the proliferative properties of obestatin alone, 10^{-6} to 10^{-13} mol/L obestatin was applied to the cells and incubated for 24 h. All the subsequent procedures were as above.

Measurement of Mitochondrial Transmembrane Potential (ΔΨm)

After pretreatment with different concentrations of obestatin (10^{-7} to 10^{-13} mol/L) for 20 min, cells were treated with MPP^+ (final concentration 200 μmol/L) without serum for a further 24 h, and then incubated with rhodamine123 at a final concentration of 5 μmol/L for 30 min at 37°C. Fluorescence intensity was recorded at 488 nm excitation and 525 nm emission by flow cytometry[14, 19, 22-24].

Active Caspase-3 Assay

Caspase-3 activity was measured by flow cytometry using a PE-conjugated monoclonal active caspase-3 antibody apoptosis kit (BD Bioscience)[14, 19, 20]. After washing twice with cold phosphate-buffered saline, cells were resuspended in Citofix/Cytoperm™ (0.5 mL) at a density of 1×10^6 cells/0.5 mL and incubated on ice for 20 min. Cells were then washed twice with Perm/Wash buffer and incubated with antibody (100 μL Perm/Wash buffer plus 20 μL antibody per sample) for 30 min. After one wash with Perm/Wash buffer, cells were resuspended in 0.5 mL Perm/Wash buffer and analyzed by flow cytometry at 523 nm excitation and 658 nm emission. Apoptosis was evaluated as the percentage of caspase-3-immunoreactive cells in the total number of cells using Cellquest Software (BD Bioscience).

Western Blotting

Cells were lysed in buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Nonidet-40, 0.5% sodium deoxycholate, 1 mmol/L EDTA, and a protease inhibitor cocktail. The protein concentrations were determined using BCA kits (Beyotime, Shanghai, China). After separation on 10% SDS-polyacrylamide gels, proteins were transferred to PVDF membranes. After blocking with 10% non-fat milk for 2 h, the membranes were incubated with primary antibody against PCNA (1:20 000, CST, Boston, MA), or rabbit anti-β-actin monoclonal antibody (1:10 000, Sigma, St Louis, MO), and then with secondary antibodies coupled to horseradish peroxidase (Santa Cruz, Dallas, TX). Protein bands were developed by immobilon western chemiluminescent HRP substrate (Millipore, Billerica, MA). Images were catched and results were analyzed on a UVP image system (UVP, Cambridge, UK).

Statistical Analysis

SPSS 17.0 was used to analyze the data, and each experiment was performed in triplicate. The results are presented as mean ± SEM. One-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test was used to compare group differences. P <0.05 was considered to be statistically significant.

RESULTS

Obestatin Antagonized MPP^+-induced Cytotoxicity in MES23.5 Cells

Obestatin alone (10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} mol/L) promoted the viability of MES23.5 cells (135.61%, 138.23%, 131.60%, 147.02%, 134.68%, 134.28%, and 116.00%, respectively) compared with the control (Fig. 1A). A significant reduction in viability was found after 200 μmol/L MPP^+ treatment. Obestatin (10^{-13}–10^{-7} mol/L) pretreatment, however, significantly restored viability. Compared with the 63.40% viability in the MPP^+-treated group, the viability was 113.40%, 122.52%, 106.82%, 100.74%, 93.35%, 92.21% and 90.56% in the 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} mol/L obestatin-pretreated groups, respectively (Fig. 1B). Among the concentrations, 10^{-13} and 10^{-12} mol/L obestatin had the greatest protective effect.

Obestatin Did Not Antagonize MPP^+-induced Mitochondrial Transmembrane Potential (ΔΨm) Collapse in MES23.5 Cells

ΔΨm changes reflect mitochondrial function, which is involved in apoptosis. There was an apparent collapse in ΔΨm in MPP^+-treated cells. No recovery of ΔΨm was detected in cells with 10^{-7}, 10^{-9}, 10^{-11}, or 10^{-13} mol/L obestatin pretreatment (Fig. 2).

Obestatin Did Not Antagonize MPP^+-induced Caspase-3 Activation in MES23.5 Cells

Caspase-3 activation is associated with apoptosis. MPP^+ treatment induced a significant increase in caspase-3 activity in MES23.5 cells, which was, however, not

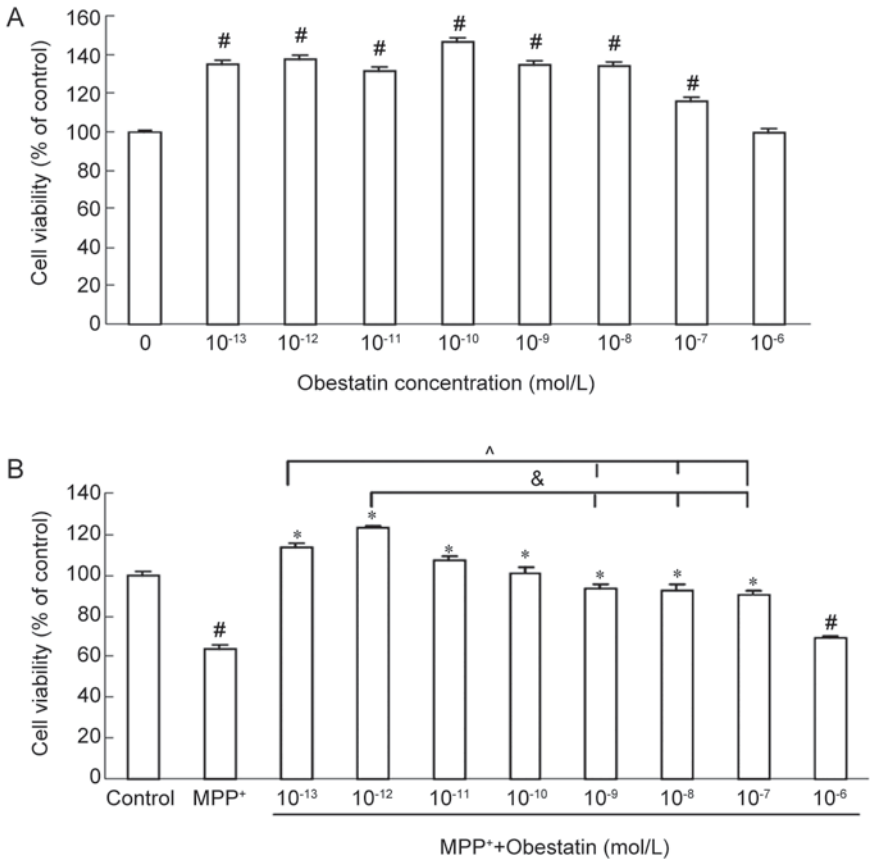

Fig. 1. Cell viability with obestatin incubation determined by MTT assay. A: Obestatin (10^{-13} to 10^{-7} mol/L) treatment alone significantly increased the viability of MES23.5 cells. B: Viability with obestatin treatment prior to MPP^+ treatment. Restoration of viability was found when cells were pre-incubated with obestatin (10^{-13} to 10^{-7} mol/L). $^{\#}P$ <0.05 vs control; $^{*}P$ <0.05 vs MPP^+-treated group; $^{\wedge}P$ <0.05 *vs* 10^{-13} mol/L obestatin group; $^{\&}P$ <0.05 *vs* 10^{-12} mol/L obestatin-treated group.

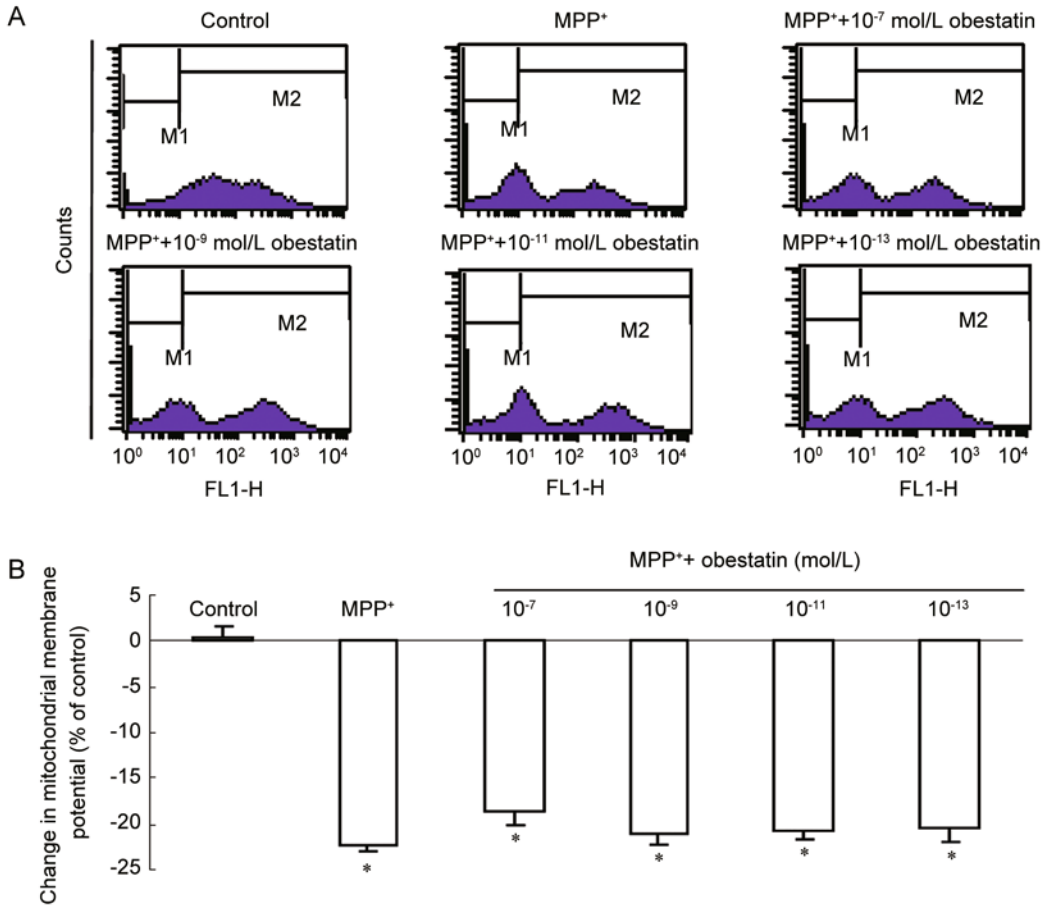

Fig. 2. No changes were found in mitochondrial transmembrane potential (ΔΨm) with obestatin pretreatment. MPP^+ (200 μmol/L) treatment resulted in a ΔΨm decrease assessed by flow cytometry after 24 h exposure. Pre-incubation with obestatin (10^{-13} to 10^{-7} mol/L) did not abolish these changes. A: Fluorometric assays of ΔΨm. B: Statistical analysis. Data are presented as percentage of control (set at 100%). *P <0.05 compared with the control. FL1-H, the pulse height of channel 1 (488 nm excitation and 525 nm emission); M1, the gate of damaged cells; M2, the gate of normal cells.

influenced by obestatin pretreatment (10^{-7}, 10^{-9}, 10^{-11}, and 10^{-13} mol/L) (Fig. 3).

Obestatin Induced PCNA Up-regulation

The levels of PCNA, one of the markers of cell proliferation[25, 26], were increased with 10^{-9} mol/L obestatin treatment for 6 h, and peaked at 12 h. Other concentrations (10^{-7}, 10^{-11}, and 10^{-13} mol/L) had the same effect. MPP^+ (200 μmol/L) down-regulated the PCNA protein level at 12 h, which was, however, restored and even reversed by obestatin pretreatment (10^{-7}, 10^{-9}, 10^{-11}, and 10^{-13} mol/L) for 20 min. These results indicated that obestatin at 10^{-13} to 10^{-7} mol/L has proliferative effects on MES23.5 cells (Fig. 4).

DISCUSSION

This is the first report on the neuroprotective effects of obestatin in dopaminergic cells. Our results demonstrated that obestatin, when applied alone at 10^{-13}–10^{-7} mol/L, had proliferative, but not dose-dependent effects, and it effectively protected MES23.5 cells from MPP^+-induced cytotoxicity by its proliferative rather than its anti-apoptotic effects.

Since obestatin has the same peptide precursor as ghrelin, we first hypothesized that the protective effect of obestatin was anti-apoptotic, as we had shown for ghrelin[14, 15]. The neurotoxin that creates the classical cell

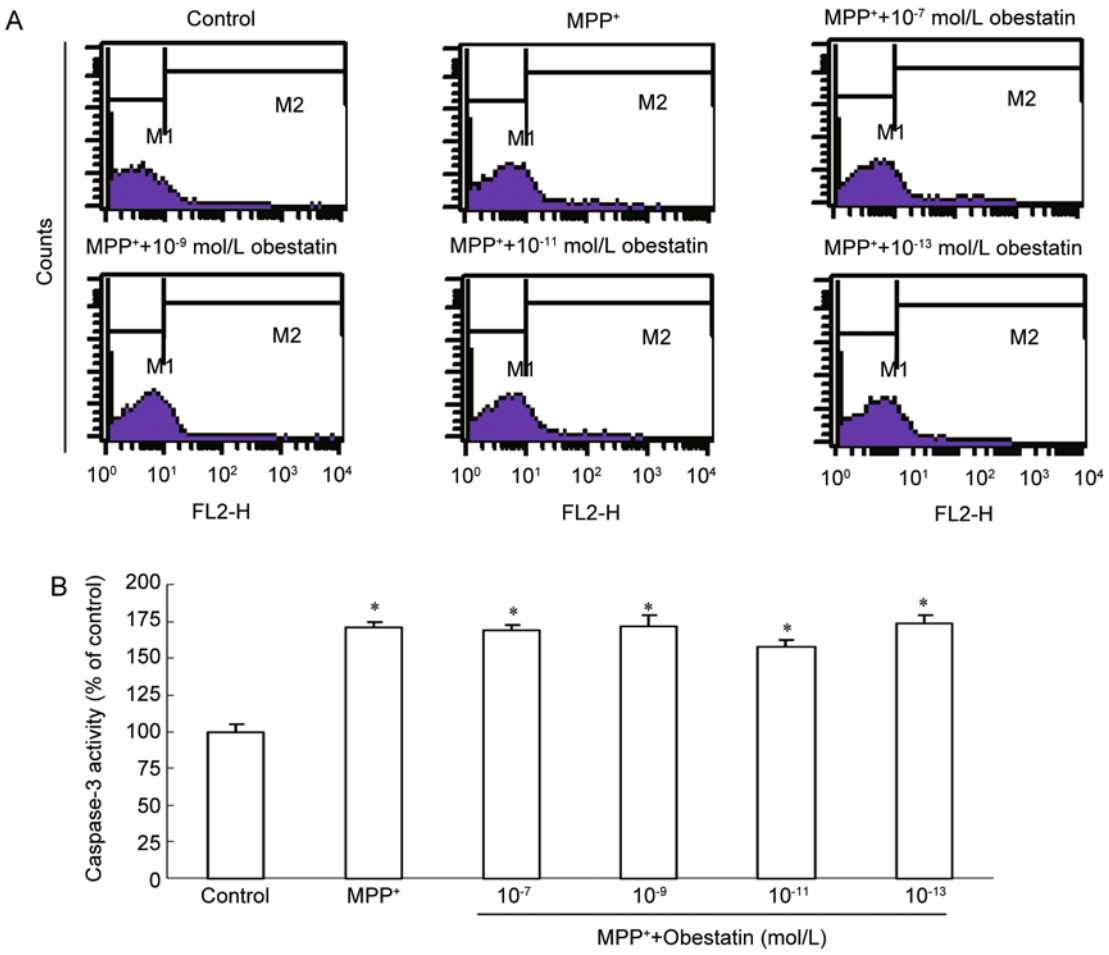

Fig. 3. MPP$^+$-induced caspase-3 activation was not abolished by obestatin pretreatment, as assessed by flow cytometry. A: Fluorometric assay of caspase-3 activity from cells treated with vehicle or obestatin (10^{-13} to 10^{-7} mol/L) 20 min prior to MPP$^+$ treatment for 24 h. Caspase-3 activity was increased after MPP$^+$ treatment; however, this effect was not attenuated by obestatin pretreatment. B: Statistical analysis. *P <0.05 compared with control. FL2-H, the pulse height of channel 2 (523 nm excitation and 658 nm emission); M1, the gate of normal cells; M2, the gate of damaged cells.

model of Parkinson's disease[27, 28], MPP$^+$, is well-known to induce apoptosis by selectively damaging mitochondria[29]. Mitochondrial dysfunctions play important roles in the pathogenic cascades of apoptosis, and are involved in the pathogenesis of Parkinson's disease[14, 30, 31]. However, we showed that obestatin neither inhibited caspase-3 activation nor reversed the ΔΨm collapse induced by MPP$^+$. This is in accord with studies showing that obestatin does not prevent the apoptosis induced by cytosine arabinoside in HL-1 cardiomyocytes[32], and even aggravates apoptosis in pig ovarian cells[33].

Moreover, we found positive results for PCNA expression, which was strongly suggestive of an effect of obestatin on proliferation in MES23.5 dopaminergic cells, in accord with other studies showing that obestatin stimulates proliferation in primary cultured human retinal epithelial cells[13], human gastric cancer cells[34], and pig ovarian granulosa cells[33]. Obestatin has been reported to induce the up-regulation of several proliferation markers, such as PCNA, cyclin B1, and mitogen-activated protein kinase[35]. The underlying mechanisms could be related to its binding with its receptor and the subsequent activation of PI3K, PKCε, and Src for ERK1/2 induction *in vitro*[12,13,34]. Activation of this signaling pathway might

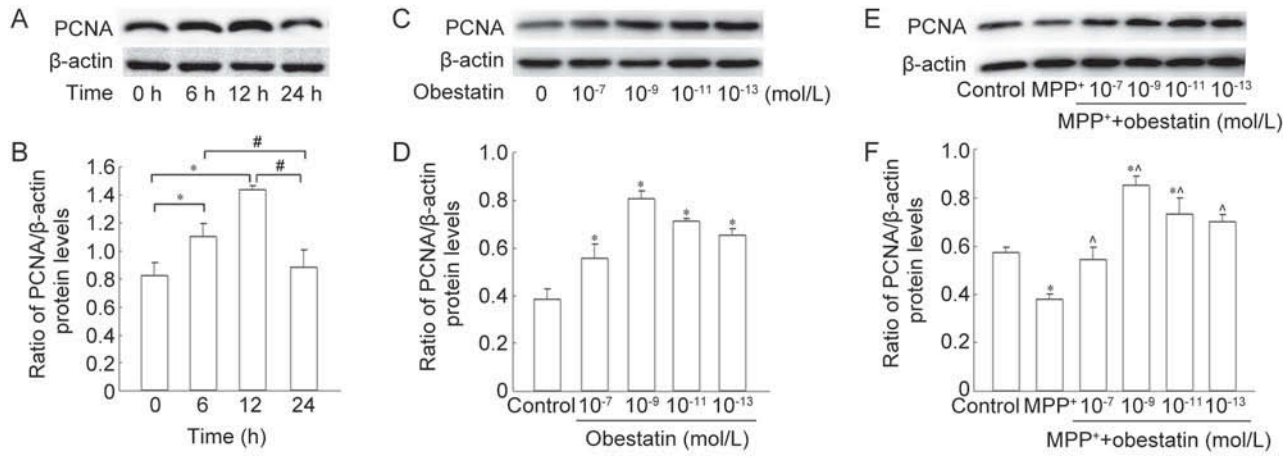

Fig. 4. Effects of obestatin on PCNA expression determined by Western blotting. A: PCNA levels were up-regulated in MES23.5 cells with 10^{-9} mol/L obestatin treatment for 6 h and peaked at 12 h. C: Obestatin at other concentrations (10^{-7}, 10^{-9}, 10^{-11}, and 10^{-13} mol/L) also induced PCNA up-regulation at 12 h. E: PCNA expression was decreased by MPP^+ treatment, but the decrease was fully blocked by obestatin pretreatment (10^{-7}, 10^{-9}, 10^{-11}, and 10^{-13} mol/L). B, D, and F are quantitative data of A, C, and E respectively. Data are presented as mean ± SEM of six independent experiments. *P <0.05 *vs* control; #P <0.05; ^P <0.05 *vs* MPP^+-treated group.

account for the proliferative effects on MES23.5 cells. However, there is still controversy on the proliferative effect of obestatin. Some studies have revealed no effect of obestatin on cell viability in the mouse cardiomyocyte cell line HL-1 or 3T3-L1 preadipocytes[32, 36]. These results indicate that the proliferative effects of obestatin are tissue-specific.

In summary, we provide direct evidence that the protective effects of obestatin are due to its proliferative but not anti-apoptotic action in MES23.5 dopaminergic cells. For dopaminergic neurons in the central nervous system that cannot proliferate, obestatin might not act as a neuroprotective agent for Parkinson's disease.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Basic Research Development Program (973 Program) of China (2012CB526703, 2011CB504102), the National Natural Science Foundation of China (81171207), the Ministry of Education of China (20103706110002), the Municipal Science and Technology Bureau of Qingdao (12-1-4-2-(19)-jch), China, and the Program for Scientific Research Innovation Team in Colleges and Universities of Shandong Province, China.

Received date: 2013-02-26; Accepted date: 2013-09-24

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