Dose- and time-dependent α -synuclein aggregation induced by ferric iron in SK-N-SH cells

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Abstract: Objective Intracellular formation of Lewy body (LB) is one of the hallmarks of Parkinson's disease. The main component of LB is aggregated α -synuclein, present in the substantia nigra where iron accumulation also occurs. The present study was aimed to study the relationship between iron and α -synuclein aggregation. **Methods** SK-N-SH cells were treated with different concentrations of ferric iron for 24 h or 48 h. MTT assay was conducted to determine the cell viability. Thioflavine S staining was used to detect α -synuclein aggregation. **Results** With the increase of iron concentration, the cell viability decreased significantly. At the concentrations of 5 mmol/L and 10 mmol/L, iron induced α -synuclein aggregation more severely than at the concentration of 1 mmol/L. Besides, 48-h treatment-induced aggregation was more severe than that induced by 24-h treatment, at the corresponding iron concentrations. **Conclusion** Ferric iron can induce α -synuclein aggregation, which is toxic to the cells, in a dose- and time-dependent way.

Keywords: Parkinson's disease; α -synuclein; ferric ammonium citrate

1 Introduction

Parkinson's disease (PD), first described by James Parkinson in 1817 as the "shaking palsy", is a central nervous system disorder characterized by the degeneration of nigrostriatal dopaminergic neurons in the substantia nigra (SN) and the presence of intracytoplasmic inclusions called Lewy bodies (LBs)^[1]. In LBs, a large number of molecules have been identified^[2], among which the most prominent components are ubiquitin and fibrillar α -synuclein^[3].

α -Synuclein is a neuronal protein that abundantly exists in both soluble and membrane-bound forms in non-pathological conditions^[4,5]. A predicted iron responsive element

(IRE) is located in its 5'-untranslated region (UTR)^[6]. α -Synuclein is likely to undergo misfolding when exposed to a number of environmental and genetic factors. Further evidence for the fundamental role of α -synuclein in the pathogenesis of PD comes from the findings of α -synuclein over-expression and 3 point mutations in its gene in family PD^[7-9]. Duplication or triplication of α -synuclein gene is sufficient to trigger the onset of PD^[10]. These findings suggest that aggregation of α -synuclein may be a key event in PD.

Interruption in iron homeostasis appears to become more severe with PD progression. Iron accumulation has been found in the SN of PD patients and animal models^[11-19], and α -synuclein aggregation also exists in this area. The high concentration of ferric iron has been proved to promote the aggregation of A53T mutant α -synuclein in dopaminergic cells^[20]. *In vitro* studies also show that when incubated with ferrous^[21] or ferric iron^[22], α -synuclein folding, oligomerization,

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Article ID: 1673-7067(2010)03-0205-06
Received date: 2010-01-17; Accepted date: 2010-02-25

and aggregation would be promoted.

The present study was aimed to elucidate the relationship between iron and α -synuclein aggregation in SK-N-SH cells, a human neuroblastoma cell line.

2 Materials and methods

2.1 Materials 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ammonium iron (III) citrate (ferric ammonium citrate) and thioflavin S were from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM; Ham) was purchased from Gibco (Grand Island, NY, USA). All other chemicals and reagents were commercially available and of the highest grade.

2.2 Cell culture SK-N-SH cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin, at 37 °C in a humidified environment with 5% CO₂ and 95% O₂. Cells were digested with 0.25% trypsin plus 0.02% EDTA, prepared in DMEM with 10% FBS supplement, and then seeded at a density of 1×10^5 /mL on coverslips or 96-well plates. Cells were pretreated with different concentrations of ferric iron (prepared in DMEM without serum) for 24 h or 48 h, following which MTT assay and thioflavine S staining were conducted, respectively.

2.3 MTT assay After cell adhesion to the plate, different concentrations of ferric iron were added, to a final concentration of 0–10 mmol/L, and incubated for 24 h. Then 20 μ L MTT (5 mg/mL) was added to each well, and incubated at 37 °C for 4 h. After that, the medium was replaced by 100 μ L DMSO, and absorbance was read at 570 nm using a colorimetric assay (Tecan, Austria). Data were calculated as the average value from 6 replicates, and each experiment was repeated 4 times.

2.4 Thioflavine S staining After the treatment with ferric iron for 24 h or 48 h, cells were washed once with 0.01% PBS. Then aggregation of α -synuclein was observed using thioflavin S staining, according to the previous description^[23]. Briefly, cells grown on coverslips were fixed in 4% paraformaldehyde for 30 min. After being washed 2 $\times$ 5 min, cells were incubated with 0.05% thioflavine S for 8 min, and washed three times in 80% ethanol, followed by once in distilled water.

Then the cells were mounted with 70% glycerol. The fluorescence signals were observed with an Olympus confocal laser scanning microscope (Olympus, Japan) at a magnification of 200 $\times$, and then measured at an excitation wavelength of 488 nm and at an emission wavelength of 525 nm.

2.5 Data analysis Data were presented as mean $\pm$ SEM and analyzed by One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Ferric iron induced cell loss in SK-N-SH cells As shown in Fig. 1, after treatment with ferric iron at the final concentration of 1 mmol/L, 5 mmol/L or 10 mmol/L for 24 h, cell viability was significantly reduced, as revealed by MTT assay ($P < 0.05$).

3.2 Iron induced α -synuclein aggregation in a dose- and time-dependent way α -Synuclein aggregation in the SK-N-SH cells was examined by thioflavin S staining after ferric iron treatment. As shown in Fig. 2, the aggregation of α -synuclein was enhanced after treatment with freshly prepared ferric iron, compared with that of control. The aggregation induced by 5 mmol/L and 10 mmol/L iron treatment was more severe than that induced by 1 mmol/L iron. Moreover, 48-h treatment-induced aggregation was more severe than that induced by 24-h treatment, at the corresponding iron concentrations. These findings indicated that ferric iron-

Fig. 1 SK-N-SH cell viability after iron treatment, revealed by MTT assay. The cell viability was significantly reduced after treatment with 1–10 mmol/L ferric iron for 24 h. Data were from 4 independent experiments. * $P < 0.05$ vs Control.

Fig. 2 Identification of α -synuclein aggregates by thioflavine S staining in SK-N-SH cells. Treatment with ferric iron (1-10 mmol/L) for 24 h (B-D) or 48 h (b-d) could induce α -synuclein aggregation, compared with the control group (A/a). The aggregation induced by 5 mmol/L (C/c) and 10 mmol/L (D/d) iron treatment was more severe than that induced by 1 mmol/L (B/b) iron. Moreover, 48-h treatment-induced aggregation was more severe than that induced by 24-h treatment, at the corresponding iron concentrations. Arrows indicated the thioflavine S-positive aggregates. Scale bar: 100 μ m.

induced α -synuclein aggregation was dose- and time-dependent.

4 Discussion

In the present study, we demonstrate that with the increase in iron concentration, the SK-N-SH cell viability decreased significantly. Among the 3 doses, 5 mmol/L and 10 mmol/L iron induced more severe α -synuclein aggregation in SK-N-SH cells than 1 mmol/L iron did. Besides, 48-h treatment-induced aggregation was more severe than that induced by 24-h treatment, at the corresponding iron concentrations. This result suggests that iron-induced α -synuclein aggregation is dose- and time-dependent. The aggregated α -synuclein or the process of aggregation may contribute to iron-induced neurotoxicity in SK-N-SH cells.

Multiple factors are involved in the pathogenesis of PD, such as apoptosis, oxidative stress, increased iron content, and mitochondrial dysfunction^[18,24-27]. A high level of iron has been found in the SN of parkinsonian^[28] and PD mouse models^[17,19,29-35], where α -synuclein aggregation also exists. Synucleinopathies are generally associated with iron accumulation, which is consistent with the pathological link between iron and α -synuclein aggregation^[36]. Our studies further confirm that high concentrations of ferric iron can induce α -synuclein aggregation in dopaminergic cell lines, in a dose- and time-dependent way, which is toxic to the cells. This is consistent with other reports that ferrous^[21] or ferric iron^[22] could promote purified α -synuclein monomer folding, oligomerization, and aggregation *in vitro*. Co-treatment with iron and other free radical generators, such as dopamine and hydrogen peroxide, can also induce α -synuclein aggregation^[20]. In the present study, we observed that cell viability decreased significantly after iron treatment, probably due to the α -synuclein oligomer, which could form ion-permeable pores in a planar lipid bilayer, thus mediating α -synuclein-dependent toxicity in neuronal cells^[37].

However, the mechanisms underlying the iron-induced α -synuclein aggregation remain still unclear. Oxidative stress is one of the widely accepted mechanisms. Ferric iron can react with superoxide in the Haber-Weiss reaction^[38] to produce ferrous again, thereby affecting redox cycling. The ex-

posure of α -synuclein to oxidative agents induces the formation of high-order oligomers^[39]. Recently, a putative IRE within the 5'-UTR of α -synuclein mRNA^[6] has been computerized, suggesting that iron may induce α -synuclein aggregation by regulating its expression.

In summary, the present study shows that iron induces α -synuclein aggregation in a dose- and time-dependent way. The aggregated α -synuclein or the process of aggregation may contribute to iron-induced neurotoxicity in SK-N-SH cells. However, further studies are needed to elucidate the mechanism underlying iron-induced α -synuclein aggregation.

Acknowledgements: This work was supported by the grants from the National Basic Research Development Program of China (No. 2006CB500704), the National Natural Science Foundation of China (No. 30930036, 30870858) and the Natural Science Fund for Distinguished Young Scholars of Shandong Province, China (No. JQ200807).

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铁离子对 α -突触核蛋白聚集的诱发作用具有剂量和时间依赖性

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摘要: **目的** 帕金森氏病的一个重要标志是胞浆内路易小体的形成。路易小体的主要成分是 α -突触核蛋白的聚集体, 它和铁的积聚一同存在于黑质区。本实验旨在研究铁和 α -突触核蛋白聚集的关系。**方法** SK-N-SH细胞在不同浓度的三价铁作用下, 分别孵育24 h或48 h, 用MTT法和硫磺素S染色法分别检测细胞存活率、观察 α -突触核蛋白的聚集情况。**结果** 随着铁离子浓度的升高, 细胞存活率显著下降。其中5 mmol/L和10 mmol/L三价铁引起的 α -突触核蛋白的聚集相较于1 mmol/L的三价铁引起的聚集更为严重。此外, 在同一浓度的三价铁作用下, 孵育48 h引起的聚集比孵育24 h引起的聚集更严重。**结论** 铁离子诱发的 α -突触核蛋白的聚集具有剂量和时间的依赖性, 并且 α -突触核蛋白的聚集介导了铁的毒性作用。

关键词: 帕金森氏病; α -突触核蛋白; 枸橼酸铁胺