Effect of α -synuclein on the promoter activity of tyrosine hydroxylase gene

Nan GAO^{1,2,3}, Yao-Hua LI¹, Xin LI¹, Shun YU¹, Gui-Lian FU³, Biao CHEN^{1,2}

¹Department of Neurobiology, Beijing Institute of Geriatrics, Xuanwu Hospital of Capital University of Medical Sciences, Beijing 100053, China

²Chinese National Human Genome Center, Beijing 100176, China

³Department of Molecular Biology, Beihua University Medical College, Jilin 132013, China

Abstract: Objective To approach the associated mechanism by which α -synuclein (α -Syn) might regulate the metabolism of dopamine. **Methods** A DNA fragment, located at -495 to +25 of the human tyrosine hydroxylase (TH) gene, was amplified by PCR and inserted into the pGL₃-Basic luciferase reporter vector. The recombinant plasmid pGL₃-THprom was transfected into a dopaminergic cell line MES23.5 or a α -Syn over-expressed MES23.5 (named MES23.5/h α -Syn⁺). The promoter activity was detected by the Dual Luciferase Assay System. **Results** The luciferase activities in the MES23.5 cells transfected with pGL₃-Basic, pGL₃-THprom, and pGL₃-Control vectors were 5.60 ± 0.67 , 26.80 ± 4.11 , and 32.90 ± 4.75 , respectively. On the other hand, the luciferase activity of pGL₃-THprom in the MES23.5 (26.80 ± 4.11) was significantly higher than that in the MES23.5/h α -Syn⁺ (14.40 ± 0.61) ($P < 0.01$). **Conclusion** These results indicate that the -495 to +25 region in the TH gene possesses promoter activity for controlling the gene expression, and that α -Syn may negatively regulate the metabolism of dopamine by affecting the function of TH promoter as a *trans*-acting factor.

Keywords: α -synuclein; tyrosine hydroxylase; gene expression; dopamine

1 Introduction

α -Synuclein (α -Syn) is a 140-amino acid protein that is richly expressed in the central nervous system^[1]. α -Syn is implicated in the pathogenesis of Parkinson's disease (PD): researches indicate that α -Syn is abundant in Lewy bodies^[5,6], point mutations of α -Syn was found in familial autosomal dominant PD (A53T, A30P, E46K)^[2-4], and multiple copies of wild-type α -Syn were identified in some families with the disease^[14-16].

The role for α -Syn in PD is well established. However, its functions, especially the functions with regard to dopamine neurons, are still unfolding. α -Syn binds to tyrosine hydroxylase (TH), a rate-limiting enzyme in dopamine biosynthesis, and influences its activity^[7]. The TH expression was greatly decreased in α -Syn-transfected dopaminergic cells^[8]. These results revealed a close correlation between the function of α -Syn and dopamine metabolism.

The latest evidence that oxidative stress could induce nuclear translocation of α -Syn implied that α -Syn might participate in regulating some genes' expression^[9]. Here, we construct a luciferase reporter vector with a partial region of TH promoter to explore the impact of wild-type α -Syn on TH gene expression. We discovered that the -495 to +25 region of the TH gene possessed the promoter activity for controlling the gene expression, and α -Syn may act as a *trans*-acting factor to inhibit the expression of TH gene in MES23.5 dopaminergic cell lines.

2 Materials and methods

2.1 Materials A hybrid line MES23.5, produced by fusing rat embryonic mesencephalic dopaminergic neurons with mouse neuroblastoma-glioma cell line N18TG2. MES23.5/h α -Syn⁺ was obtained by stably transfected with wild-type human α -Syn gene. The two cell lines were kindly provided by the Institute of Parkinson's Disease in USA^[10]. Plasmid pMD18-T, PCR Fragment Recovery Kit, DNA polymerase LA Taq, restriction enzyme *Sma*I and *Bgl*II were purchased from TaKaRa (Japan); pGL₃ luciferase reporter vector, phRL-TK synthetic *Renilla* luciferase reporter vector, and Dual-Luciferase Reporter Assay System were purchased from

Corresponding author: Yao-Hua LI
Tel: 86-10-83198271
E-mail: liyao-hua1962@yahoo.com.cn
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Promega; plasmid pEGFP-C1 was purchased from Clontech; Ligation High was purchased from Toyobo (Japan); Lipofectamine 2000 was purchased from Invitrogen; DMEM/F12 medium was purchased from GIBCO; G418 and Sato's ingredients were purchased from Sigma. Others were analytical reagent.

2.2 Plasmid vector The pGL₃-Basic vector lacks eukaryotic promoter and enhancer sequences, allowing maximum flexibility in cloning putative regulatory sequence. The positive control pGL₃-Control vector contains SV40 promoter and enhancer sequences, resulting in strong expression of luc⁺ in many types of mammalian cells. The phRL-TK synthetic *Renilla* luciferase reporter vector contains a synthetic *Renilla* gene sequence.

2.3 Human genome DNA extraction Whole blood 5–10 mL was obtained from normal human, and anticoagulated with 1.0 mmol/L EDTA-Na₂. Erythrocytes in the blood were lysed by 10 mmol/L Tris-HCl (pH 7.5), containing 0.32 mol/L sucrose, 5 mmol/L MgCl₂, and 1% (V/V) Triton X-100. Leucocyte fractions obtained by centrifugation (16 000 g, 30 s) were lysed by 10 mmol/L Tris-HCl (pH 8.3), containing 50 mmol/L KCl, 25 mmol/L MgCl₂, 0.1 mg/mL gelatin, 0.45% (V/V) Nonidet P40 and 0.45% (V/V) Tween 20, and then incubated with 20 mg SDS and 60 g/mL protease K at 55 °C overnight. After saturated NaCl solution was added, the genome DNA was isolated by centrifugation, and purified with ethanol precipitation.

2.4 Isolation of TH Gene promoter fragment The upstream and downstream primers were designed according to the human TH promoter region (M23597, GenBank) sequence: 5'-CAG ATG GCA CTC CTA GGA ACC AC-3' and 5'-gaagatct TCA GTG TGG AGG TCC GGG CT-3', respectively; the later was added a *Bg*/II enzyme site. Human genome DNA was used as a templet for the PCR amplification under the following conditions: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, and 30 cycles. The PCR products were separated by agarose gel electrophoresis. An approximately 500 bp DNA fragment was purified and cloned to pMD18-T vector.

2.5 Construction of Reporter Vector Recombinant pMD18-T vector was incised by *Sma*I and *Bg*/II. An approximately 500 bp DNA fragment was purified and inserted into plasmid pGL₃-Basic vector. The recombinant vector pGL₃-THprom was identified by sequencing, and used to the subsequent experiments.

2.6 Cell culture MES23.5 cell was resuscitated from

liquid nitrogen, suspended with 5% heat inactivated fetal bovine serum and Sato's ingredients, inoculated in 25 cm² flask and then cultured at 37 °C with 5% (V/V) CO₂. The culture of MES23.5/hα-Syn⁺ cell was similar to that of MES23.5 cell except to add 200 µg/mL of G418 into the medium.

2.7 Gene transfection and dual-luciferase reporter assay

Lipofectamine 2000 reagent was used for gene transfection according to the protocol provided. The cells were seeded in 96-well plates at the density of 1×10⁴ cells/100 µL each well. Twenty-four hours later, the pGL₃-Basic, pGL₃-THprom or pGL₃-Control vectors in the Lipofectamine 2000 (W/V, 1:4) was added. As an internal control to reflect the difference of cell quantity and the efficiency of transfection, phRL-TK was co-transfected (WphRL/WpGL₃, 1:25) with the pGL₃ vectors. Five hours later, the cells were changed with fresh medium, then cultured for another 24 h, and were disrupted. The cell lysates were treated according to the protocol of Dual-Luciferase Reporter Assay System. Luciferase activities of firefly and *Renilla* were measured using a GENios Pro reader (Tecan, Mannedorf, Zurich, Switzerland).

2.8 Statistics Data were analyzed by one-factor analysis of variance and independent samples test with SPSS 11.5 statistical software. Data were expressed as mean±SEM, representing the average value over 6 independently repeated experiments.

3 Results

3.1 Objective gene cloning The -495 to +25 region in human TH gene was amplified by PCR. An approximately 500 bp DNA fragment on agarose gel (Fig. 1) was purified, then cloned to pMD18-T vector, and subcloned to pGL₃-Basic vector. The recombinant luciferase reporter vector

Fig. 1 Agarose electrophoresis of PCR products. Lane 1: DNA marker; 2: PCR product.

Fig 2 Transfection efficiency of the pEGFP-C1 vector in the MES23.5 cells (A–C) and the MES23.5/h α -Syn⁺ cells (D–F). Before transfection, both cells were seeded in 96-well plates (A, D). Cells transfected with plasmids turned to round under the light microscope (B, E). Observed under the inverted fluorescence microscope, green fluorescence could be seen in both cells, indicating that they were transfected with pEGFP-C1 (C, F). Scale bar, 100 μ m.

Fig. 3 Promoter activity assay for the human DNA fragment in MES23.5 cells. * $P < 0.01$ vs pGL₃ Basic.

Fig. 4 Dual-luciferase activities assay for pGL₃-THprom in MES23.5 and MES23.5/h α -Syn⁺. * $P < 0.01$.

pGL₃-THprom was identified by sequencing in Shanghai Biotechnology and Service Corporation, Ltd.

3.2 Cell transfection Twenty-four hours after the pEGFP-C1 was transfected, green fluorescence was observed in about 70%–80% of cells under the inverted fluorescence microscope (Fig. 2).

3.3 Promoter activity assay of the pGL₃-THprom The dual luciferase activities in the MES23.5 cells transfected with pGL₃-Basic, pGL₃-THprom and pGL₃-Control plasmids were 5.60 ± 0.67 , 26.80 ± 4.11 and 32.90 ± 4.75 , respectively (Fig. 3). On the other hand, the dual luciferase activity of pGL₃-THprom in the MES23.5 (26.80 ± 4.11) was significantly higher than that in the MES23.5/h α -Syn⁺ (14.40 ± 0.61) ($P <$

0.01, Fig. 4).

4 Discussion

α -Syn is richly expressed in the brain. It is strongly implicated in the pathogenesis of PD, though its physiologic function is still unclear^[11]. Recent studies suggest that α -Syn is implicated in the dopamine biosynthesis. For example, α -Syn binding to TH^[7] could inhibit TH gene expression in dopaminergic cells^[8]. The finding of nuclear translocation indicated that α -Syn might regulate the gene expression^[9]. In this study, we established a reporter assay system with the –495 to +25 region of TH gene, proving that α -Syn inhibits promoter activity of TH gene in dopam-

inergic cell line MES23.5.

The amplified DNA fragment was approximately 500 bp (Fig 1). Its sequence was in accordance with the -495 to +25 region in the TH gene. Though cAMP protein binding site (-33 to -40) and TATA signal (-23 to -29) present in this region, we do not know whether this region has the promoter activity. In our research, the TH gene fragment was inserted into pGL₃-Basic to construct a luciferase reporter vector pGL₃-THprom. The dual luciferase activities in the MES23.5 cells transfected with pGL₃-THprom, pGL₃-Basic and pGL₃-Control plasmids were 26.80±4.11, 5.60±0.67 and 32.90±4.75 respectively, indicating that the -495 to +25 of TH gene has the promoter activity as one of *cis*-acting elements. On the other hand, the dual luciferase activity of pGL₃-THprom in the MES23.5 cells was 26.80±4.11, but in MES23.5/α-Syn⁺ cells was 14.40±0.61, differed significantly ($P<0.01$). This result suggests that α-Syn might exert the *trans*-acting function to inhibit TH gene promoter activity and down-regulate the anabolism of dopamine. After pEGFP-C1 was transfected into the MES23.5 and the MES23.5/α-Syn⁺ cells, the green fluorescence could be observed in about 70%–80% of cells. This result indicated that the transient transfection efficiency was satisfying. However, it was lower in another dopaminergic cell line, SH-SY5Y (data not shown). After transfection, the cells had morphological changes but survived, indicating that the cells might be damaged during the transfection.

There are 7 836 bp in TH gene, including 13 exons and 12 introns. In prior study, a low degree of homology was observed in the sequence of TH promoters among human, mouse, and rat species^[12].

At present, the regulatory mechanism of TH gene has been investigated, though still unclear. It is reported that the CpG-rich sequence located outside the 5' promoter region of the human TH gene. Since the methylation status of CpG dinucleotides located in or near regulatory elements affects gene expression, TH gene may be expressed according to its methylation pattern^[13].

In conclusion, we confirmed that the upstream approximately 500 bp region of TH gene has the promoter activity by transfected it into MES23.5. The intimate function of other regions of TH promoter remains to be confirmed. In addition, α-Syn is richly expressed in the brain. Besides striatum and substantia nigra, cerebral cortex, hippocampus and amygdale are also rich of α-Syn. Therefore, α-Syn may also regulate other genes' expression besides TH gene.

This problem will be further studied.

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α - 突触核蛋白对酪氨酸羟化酶基因启动子的影响

高楠^{1,2,3}, 李尧华¹, 李昕¹, 于顺¹, 傅桂莲³, 陈彪^{1,2}

¹首都医科大学宣武医院, 北京老年医学研究所, 北京 100053

²国家人类基因组北方研究中心, 北京 100176

³北华大学医学院分子生物学教研室, 吉林市 132013

摘要: **目的** 探讨 α -突触核蛋白对多巴胺代谢的调节机制。**方法** 以人基因组 DNA 为模板, PCR 法扩增酪氨酸羟化酶(Tyrosine Hydroxylase, TH)基因区的部分 DNA 片段(-495 ~ +25), 将其插入质粒 pGL₃-Basic, 构建荧光素酶报告基因重组质粒 pGL₃-THprom, 转染多巴胺能神经元细胞系 MES23.5 和 MES23.5/h α -Syn⁺。**结果** 双荧光素酶活性分析表明, pGL₃-Basic、pGL₃-THprom 和 pGL₃-Control 在 MES23.5 细胞中表达的荧光素酶活性分别为 5.60 ± 0.67 , 26.80 ± 4.11 和 32.90 ± 4.75 , 而 pGL₃-THprom 在 MES23.5 和 MES23.5/h α -Syn⁺ 中表达的荧光素酶活性分别为 26.80 ± 4.11 和 14.40 ± 0.61 , 差异极显著($P < 0.01$)。**结论** 这些结果提示扩增的 DNA 片段具有启动子活性, 而 α -Syn 作为反式作用因子通过影响 TH 基因启动子的功能发挥对多巴胺代谢的负性调控作用。

关键词: α - 突触核蛋白; 酪氨酸羟化酶; 基因表达; 多巴胺