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Original Research Article

Regulatory mechanism of miR-146a on MPTP-induced neuroinflammation in mice with Parkinson's disease

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Abstract

Purpose: *To study the regulatory influence of microRNA-146a (miR-146a) on 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine (MPTP)-induced neuroinflammation in mice with Parkinson's disease (PD).*
Methods: *Forty specific pathogen-free (SPF) male C57BL/6 mice were divided into 2 groups: normal control and PD groups. The 2 groups were each divided into 2 subgroups: miR-146a inhibitor and inhibitor control groups. The mRNA and protein expressions of miR-146a, interleukin-1 receptor-associated kinase-1 (IRAK-1) and P65-NF-κB were determined by quantitative real-time polymerase chain reaction (qRT-PCR) and immunoblot assay, respectively. Levels of interleukin (IL)-1, IL-6 and TNF-α were assayed by enzyme-linked immunosorbent assay (ELISA).*
Results: *The level of expression of miR-146a was significantly and time-dependently increased in PD mice, relative to control ($p < 0.05$). In PD group, mRNA and protein expressions of IRAK-1 were markedly higher in miR-146a inhibitor group than in inhibitor control ($p < 0.05$). Protein expression of P65-NF-κB was significantly upregulated in brains of PD mice, relative to normal mice ($p < 0.05$). Moreover, IL-1, IL-6 and TNF-α levels were significantly higher in brains of PD mice than in control*
Conclusion: *These results show the involvement of miR-146a in the etiology of PD, and that its regulation of neuroinflammation occurs via inhibition of IRAK1 gene expression. This finding may be useful in the development of new anti-PD drugs based on miR-146a.*

Keywords: *Inflammatory cytokines, IRAKI-1, MicroRNA-146a, Neuroinflammation, Parkinson's disease*

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INTRODUCTION

Parkinson's disease (PD), a degenerative disease of the CNS, is prevalent among older adults. It is characterized by tremor, myotonia and bradykinesia (reduced movement). The main pathological changes in PD are degeneration and death of dopaminergic neurons in substantia

nigra of the brain. With a total incidence of 0.3 %, the disease ranks second only to Alzheimer's disease (AD) [1]. Parkinson's disease (PD) has a strong positive correlation with age [1]. The rise in its incidence places serious burden on the family of sufferers and society. Till date, the pathogenesis of PD is largely unknown, although it may entail environmental and genetic factors [2]. Studies have shown that damage to dopaminergic neurons in the substantia nigra results in mitochondrial dysfunction, neuronal immune response, inflammatory response, oxidative stress, apoptosis and autophagy. Moreover, defective mitochondrial function leads to apoptosis of dopaminergic neurons in dense area of substantia nigra. At present, there is a dearth of clinical biomarkers for early diagnosis of PD.

Only recently, the role of miRNA in the immune system received attention. A non-coding RNA with regulatory functions, miRNA participates in physiological processes of the cell via regulation of genes after transcription [4]. A role exists for miRNAs in the regulation of the progression of neurodegenerative diseases, including PD [5]. MicroRNA-146a (miR-146a) is highly expressed in the CNS. Its expression has been reported to be significantly downregulated in serum of patients with AD. It has also been demonstrated to promote inflammatory response of cells via increased expression of TLR2 gene [6]. The aim of this research was to study the regulatory mechanism of miR-146a on MPTP-induced neuroinflammation in PD mice.

EXPERIMENTAL

Materials

Ham's F-12 medium and DMEM were bought from Shanghai Fuze Trading Co. Ltd, while BCA protein kit was product of Beijing Kangjiahongyuan Biotechnology Co. Ltd. Antibodies for (IRAK-1) and P65-NF-κB were products of Wuhan IpU Biotechnology Co. Ltd. Alkaline phosphatase development kit and constant temperature horizontal shaker were obtained from Shanghai Caiyou Industrial Co. Ltd. Lipofectamine 2000 transfection reagent was bought from Shanghai Beinuo Biotechnology Co. Ltd. Protein pre-dyeing marker was product of Beijing Baoxentai Biotechnology Co. Ltd.

Enzyme-linked immunosorbent assay (ELISA) kit was obtained from Beijing Baiolaibo Technology Co. Ltd. Deionic formamide was bought from Shanghai Rongweida Industrial Co. Ltd. AxyPrep DNA gel recovery kit was product of Beijing Jiehuibo Biotech. Cryogenic centrifuge was bought from Sichuan Shuke Instrument Co. Ltd. Light microscope was purchased from Guangzhou Kexite Scientific Instruments Ltd. Constant temperature incubator was product of Hangzhou Nuoding Scientific Equipment Co. Ltd. Autoclave was bought from Nanjing Baden Medical Co. Ltd. Electrophoresis apparatus and tank were obtained from Hangzhou Bigo Flying Sequence Biotechnology Co. Ltd. Thermostatic water bath was bought from Shanghai Tibose Biotechnology Co. Ltd. Spectrophotometer was product of Beijing Jinda Sunshine Technology Co. Ltd. Quantitative PCR machine was bought from Shanghai Aiyan Biotechnology Co. Ltd., while brain stereo locator was obtained from Nanjing Calvin Biotechnology Co. Ltd.

Mice

Specific-pathogen-free (SPF) male C57BL/6 mice (n = 40) aged 8 - 10 weeks and weighing 20 – 26 g (mean age = 9 ± 1 weeks; mean weight = 23 ± 3 g) were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co. Ltd. The mice were accommodated in plastic cages under equal light/dark photoperiod, and were permitted unlimited access to water and feed. The study protocol received approval from the Institutional Animal Ethics Committee of People's Hospital of Ningxia Hui Autonomous Region (Approval No.200903041), according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [7].

Two groups of mice were used, each with 20 animals: normal control and PD groups. The 2 groups were each divided into 2 subgroups (5 mice/group): miR-146a inhibitor and inhibitor control groups. Parkinson's disease was induced with single *i.p.* administration of MPTP (30 mg/kg). Normal control mice received equivalent volume of normal saline. The mice were euthanized at different time points: day 0, 1, 5, 10 and 20 post-induction. Complete brain tissue of each mouse was excised and the midbrain removed and used for biochemical analysis. Moreover, lateral ventricles of mice in the subgroups were injected with miR-146a inhibitor or inhibitor control. Two days after induction of PD, the mice were anesthetized and dissected to obtain brain tissues which were stored at -80 ℃ prior to analysis.

qRT-PCR assay

After thawing, frozen mouse brain was homogenized with TRizol reagent. Total RNA was also obtained with TRIzol extraction, and its quality and concentration were measured colorimetrically. The total RNA was then

converted to cDNA according to the instructions of reverse transcription kit. Reverse transcription was carried out on PCR machine at 42 ºC for 50 min, with incubation for 5 min at 95 ºC. The synthesized cDNA was amplified via PCR for determination of the expression levels of miR-146a, IRAK1 and P65-NF-κB under standard assay conditions. The relative mRNA levels were estimated using $2^{-\Delta\Delta Ct}$ procedure, with β-actin as standard gene.

Table 1: The primers used in PCR

Gene	Primer sequence
IRAK1	F: TGACTTCAGCCGCTACTTCC R: GTTCAGCTGTAGCCGAGAGT
U6	F: GCAGTCAACGGATTTGGT R: GTGATGGGATTTCCATTGAT

Immunoblotting

Mice brain tissues were homogenized with PBS and lysed with cold RIPA buffer in the presence of inhibitors of protease and phosphatase (PMSF). The protein content was measured with BCA method. Then, 30-μg protein portions were resolved via SDS-PAGE and transferred to PVDF membrane. Subsequently, the membrane was incubated with 5 % fat-free milk solution to achieve sealing. Thereafter, the membrane was incubated for 12 h at 4 ºC with 1:1000 diluted 1º antibodies for miR-146a, IRAK1, P65-NF-κB and β-actin, followed by incubation with HRP-linked 2º antibody for 60 min at laboratory temperature. Blot development was performed with X-ray film, while ImageJ Launcher software was used for Grayscale analysis. Protein expression levels were calculated relative to β-actin which served as standard.

ELISA

The levels of IL-1, IL-6 and TNF-α were determined using ELISA. Mid-brain tissues of mice were homogenized with ice-cold normal saline and centrifuged at 3500 rpm for 10 min to obtain clear supernatant which was stored overnight at 4 ºC. After washing with PBS for 1 min, 200 μL of blocking solution was added, and after 1 h, washing was done thrice with PBS within 3 min. Blank control, standard and sample were added to their respective wells and incubated for 1 h at 37 ºC. The mixture was rinsed with PBS for 3 min. This was followed by the addition of enzyme-labeled secondary antibody, and after 30 min, rinsing was done with PBS for 3 min. Exactly 50 μL of substrate A/B was put in well and incubated for 20 min at 37 ºC. After a 10-min agitation, the optical density of each well was determined at 450 nm.

Statistics

Measurement results are presented as mean ± SEM, and were statistically analyzed with SPSS (21.0). Group comparisons were done with *t*-test, with $p < 0.05$ indicating statistically significant differences.

RESULTS

Expression level of miR-146a in brain tissues of mice with PD

The level of expression of miR-146a was significantly and time-dependently increased in PD mice, relative to normal control group (Figure 1).

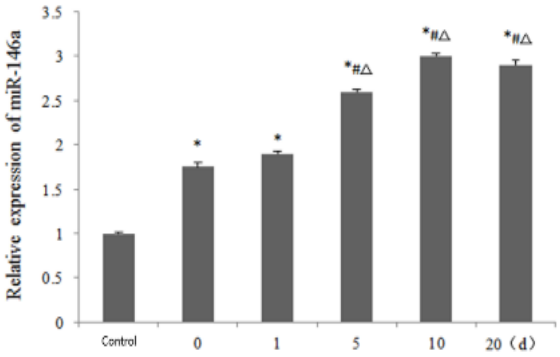

Figure 1: Expression level of miR-146a in mice brain tissues. $^{*}P < 0.05$, vs control; $^{\#}p < 0.05$, vs day 0; $^{*\triangle}p < 0.05$, vs day 1; $^{\#\triangle}p < 0.05$, vs day 5

Expression levels of IRAK-1 mRNA and protein in mice brain tissues

These were markedly lower in brain tissues of PD mice than in normal control mice ($p < 0.05$). In PD group, IRAK-I mRNA and protein were markedly higher in miR-146a inhibitor subgroup than in inhibitor control subgroup (Table 2).

Table 2: Comparison of IRAK-1 mRNA and protein expressions in mice brain tissues

Group		IRAK-1 mRNA	IRAK-1 protein
Normal control	MiR-146a inhibitor	1.08 ± 0.12	1.11 ± 0.17
	Inhibitor control	1.02 ± 0.01	1.00 ± 0.01
Parkinson's disease	MiR-146a inhibitor	0.90 ± 0.06$^{*\#}$	0.95 ± 0.12$^{*\#}$
	Inhibitor control	0.71 ± 0.08*	0.60 ± 0.07*

$^{*}P < 0.05$, vs normal mice; $^{\#}p < 0.05$, vs miR-146a inhibitor control in PD mice

Expression levels of NF-κB protein in brains of mice with PD

As shown in Figure 2 and Figure 3, protein expression of P65-NF-κB was markedly increased in brains of mice with PD, relative to normal control group. In contrast, in PD group, there was no marked difference in P65-NF-κB protein expression between miR-146a inhibitor and inhibitor control subgroups.

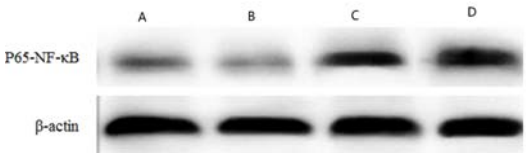

Figure 2: Levels of P65-NF-κB protein in each group, as measured using Western blotting. **A.** Normal control - miR-146a inhibitor; B. Normal control - Inhibitor control; C. Parkinson's disease - miR-146a inhibitor; D. Parkinson's disease -Inhibitor control.

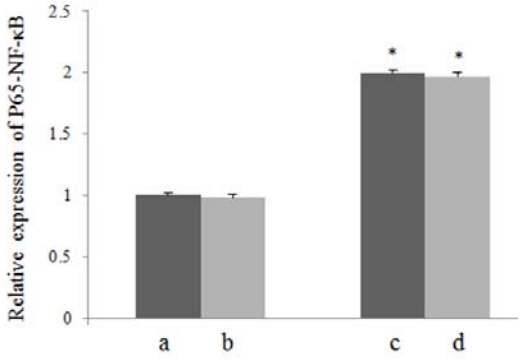

Figure 3: Expression level of P65-NF-κB protein in brain tissues of mice with PD. a. Normal control-Inhibitor control; b. Normal control-miR-146a inhibitor; c. Parkinson's disease -Inhibitor control. d. Parkinson's disease-miR-146a inhibitor; $^{*}p < 0.05$, vs normal control group

Levels of inflammatory cytokines in brain tissues of PD mice

Table 3 and Figure 4 show that inflammatory cytokines were markedly higher in brains of PD mice than in normal control group, but the levels of these inflammatory cytokines were comparable between miR-146a inhibitor and inhibitor control subgroups in mice with PD.

DISCUSSION

Parkinsonism is a neurodegenerative ailment marked by a sustained loss of dopaminergic neurons from brain stem. The pathology of the disease is based on degeneration of dopaminergic neurons and attendant decrease in dopamine levels in dense region of substantia nigra [8]. Statistics show that PD is prevalent among older adults (age ≥ 60 years), with total incidence of 1 - 2 %. The pathogenesis of PD is complex, and its diagnosis is based mainly on clinical manifestations presented.

Table 3: Levels of inflammatory factors in brain tissues of mice with PD (μg/L)

Group		IL-1	IL-6	TNF-α
Normal control	*MiR-146a inhibitor*	0.31 ± 0.02	1.26 ± 0.02	2.60 ± 0.04
	Inhibitor control	0.32 ± 0.01	1.29 ± 0.03	2.62 ± 0.05
Parkinson's disease	*MiR-146a inhibitor*	0.40 ± 0.01*	1.45 ± 0.03*	3.20 ± 0.06*
	Inhibitor control	0.41 ± 0.01*	1.47 ± 0.02*	3.24 ± 0.03*

*$P < 0.05$, vs normal control

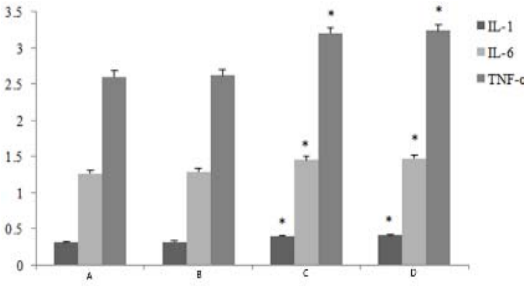

Figure 4: Levels of inflammatory cytokines in brain tissues of mice with PD. **A.** Normal control - miR-146a inhibitor; B. Normal control - Inhibitor control; C. Parkinson's disease - miR-146a inhibitor; D. Parkinson's disease -Inhibitor control. $^{*}P < 0.05$, compared with normal control group

In recent times, pathogenic genes have been associated with PD [9]. The involvement of miRNAs in the pathogenesis of PD has been reported [9]. MicroRNAs (miRNAs) are non-coding RNAs which possess regulatory function. These miRNAs which contain approximately 20 – 24 nucleotides, exist freely in cells. MicroRNAs (miRNAs) participate in the regulation of gene transcription via complementary binding to 3' untranslated region (3' UTR) of the gene. Their interactions with genes inhibit or induce mRNA degradation, thereby affecting the stability of mRNA.

Studies have shown that miRNA genes make up one-third of the human genome [10,11]. While a single miRNA can regulate multiple target genes, multiple miRNAs may combine to regulate a single target gene. MicroRNA-mediated gene expression are vital in the maintenance of normal cell division cycle, apoptosis and intermediary metabolism [12]. It has been reported that

multiple miRNAs participate in the pathogenesis of PD [13]. The mechanism involved in post-transcriptional regulation by miRNA has been demonstrated to be involved in the pathogenesis of PD, and it is thought to serve as sensitive index for diagnosis of PD as well as evaluation of disease progression and prognosis [14].

MicroRNA-146a (miR-146a) is an NF-κB-dependent molecule located on chromosome 5 in humans. Indeed, miR-146a is an immune-modulatory molecule implicated in tumorigenesis and inflammatory response, and it is abnormally expressed in a variety of nervous system tumors. A study has reported that miR-146a was markedly lower in cerebrospinal fluid (CSF) of PD subjects than in normal healthy patients [15].

Interleukin-1 receptor-associated kinase-1 (IRAK-1) gene which encodes 712 amino acids, is located on chromosome Xq28. Its 3' UTR and exon region with two polymorphism loci (rs3027898 and rs1059703), promote NF-κB activation. Polymorphism in IRAK1 is associated with increased susceptibility to certain diseases such as whooping cough and systemic lupus erythematosus (SLE) [16]. Studies have shown that miR-146a exerts its role in disease via regulation of IRAK-1 gene. In addition, miR-146a suppresses inflammation via mechanisms involving inhibition of NF-κB pathway and downregulation of IRAK-1 protein expression [17].

The present study has demonstrated that miR-146a expression level was significantly and time-dependently increased in PD mice, relative to normal control group, an indication that miR-146a may be implicated in the etiology of PD. In PD mice, IRAK-1 mRNA and protein expression levels were markedly higher in miR-146a inhibitor group than in inhibitor control group. Similarly, P65-NF-κB protein, IL-1, TNF-α and IL-6 in brains of mice with PD were markedly higher than those in normal control mice, an indication that miR-146a may negatively regulate IRAK-1 gene and activate NF-κB pathway downstream via regulation of expression of related inflammatory cytokines. Results of previous studies show that the promoter region of miR-146a contains a binding site for NF-κB, which enhances the transcription of miR-146a, while downregulating IRAK1 protein expression [17-19].

CONCLUSION

The results obtained in this study show that miR-146a is implicated in the etiology of PD, and its regulation of neuroinflammation occurs via inhibition of IRAK-1 gene expression. This finding may be useful in the quest for new anti-PD drugs based on miR-146a.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jiangtao Guo designed the study, supervised the data collection, and analyzed the data. Xuqing Cao interpreted the data and prepared the manuscript for publication. Hidek Mochizuki, Zhimin Shi, Peilan Zhang, Zhimei Liu, Tao Zhang, Jinxi Qi and Dong Xu supervised the data collection, analyzed the data and reviewed the draft of the manuscript. Xuqing Cao, Jiangtao Guo contributed equally to this work as co-first author.

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