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Rosmarinic acid attenuates inflammatory responses through inhibiting HMGB1/TLR4/NF-κB signaling pathway in a mouse model of Parkinson's disease

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ABSTRACT

Inflammation contributes to the pathological processes in patients and animal models of PD. Rosmarinic acid (RA) has been demonstrated to protect neurons in PD models. The present study aimed to evaluate the anti-inflammatory effect of RA on PD and reveal possible pharmacological mechanisms. 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) was injected to mice to establish PD model in vivo. BV-2 cells were exposed to 1-methyl-4-phenylpyridinium (MPP^+) and α-synuclein to establish PD model in vitro. Results showed that treatment with RA dose-dependently improved motor function of PD mice, increased the number of tyrosine hydroxylase-positive cells, reduced production of pro-inflammatory cytokines, and inhibited microglia activation in ventral midbrain. In cell study, RA also decreased MPP^+ or α-synuclein-induced secretion of pro-inflammatory cytokines. Furthermore, RA treatment downregulated the expression levels of HMGB1, TLR4 and Myd88 and inhibited NF-κB nuclear expression both in PD animal and cell models. These findings indicated that RA could attenuate inflammatory responses through suppressing HMGB1/TLR4/NF-κB signaling pathway, which may contribute to its anti-PD activity.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder. > 4 million people suffer from this disease but no curable treatment is available [1]. PD is pathologically characterized by loss of dopaminergic neurons in substantia nigra and striatum and clinically characterized by shaking, muscle rigidity, and bradykinesia [2,3]. Although the pathological mechanisms of PD are currently unclear, inflammation has been shown to contribute to the pathological processes in patients and animal models of PD. In human post-mortem studies, various typical pro-inflammatory cytokines were found to be elevated in the brain of PD patients, including tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, and IL-6 [4–6]. Treatment with non-steroidal anti-inflammatory drugs can reduce the risk of PD development [7–9]. In basic studies, activated microglia was observed in both 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA)-induced PD animals [10–12]. The high mobility group box 1 (HMGB-1)/toll-like receptor 4 (TLR4) axis is an important signaling pathway in the process of inflammatory responses. Previous study has shown that HMGB1 and TLR4 expression levels were higher in the peripheral blood of patients with PD than in healthy volunteers [13]. Importantly, high expression of the HMGB1/TLR4 is closely associated with PD development, drug treatment effectiveness, and disease duration [13]. Therefore, HMGB1/TLR4 axis plays an essential role in PD pathogenesis.

Alpha-synuclein spreading is now considered to mediate the progressive neurodegeneration in PD [14,15]. Alpha-synuclein is a 14 kDa neuronal pre-synaptic protein [16]. It also localized to nucleus, cytosol and some membrane structures [17]. Although the mechanism is still unclear, evidence shows that α-synuclein accumulates into protofibrils or higher-order oligomers causes neuronal toxicity [18,19]. Inhibiting α-synuclein protein expression may be beneficial for the recovery of PD [20,21].

Rosmarinic acid (α-o-caffeoyl-3, 4-dihydroxyphenyl lactic acid; RA) is a natural hydroxylated polyphenolic compound which is widely found in plants of Boraginaceae and subfamily Nepetoideae of the Labiatae [22]. It has a broad range of applications due to its wide spectrum of biological activities. In the study of PD treatment, RA was

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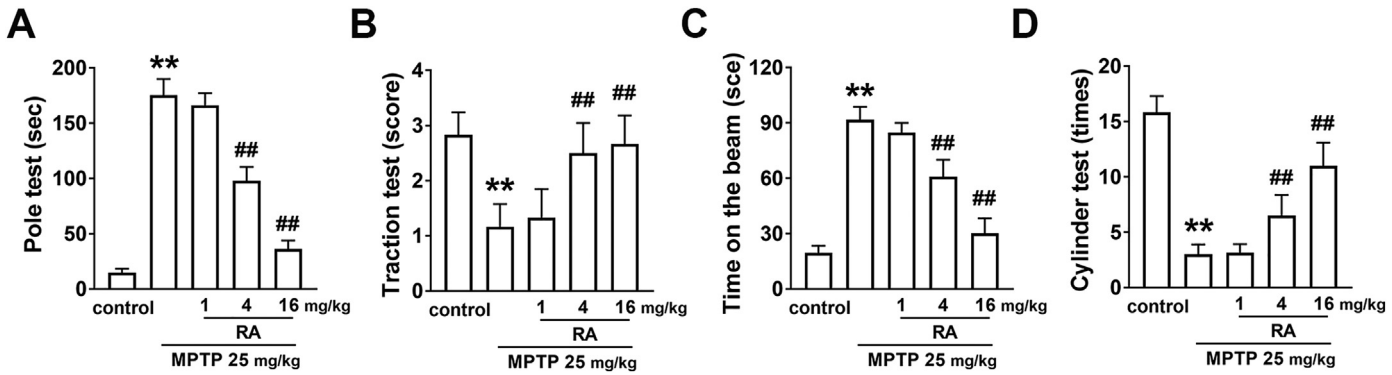

Fig. 1. RA improved the dyskinesia in MPTP-induced PD mice. Effect of RA on pole test (A), traction test (B), beam-crossing task (C), and cylinder test (D) at 8 or 9 days after the first RA treatment. **P < 0.01 vs. control; ##P < 0.01 vs. MPTP, n = 6 mice in each group.

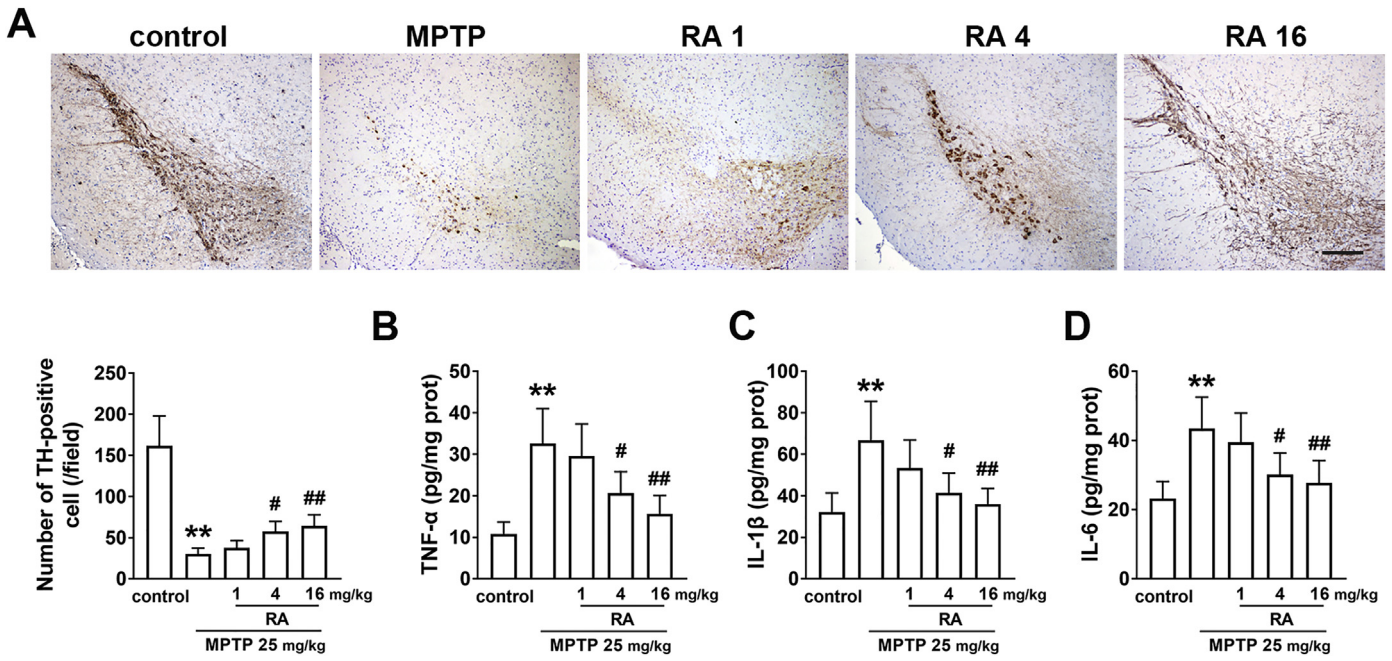

Fig. 2. RA increased TH expression and reduced pro-inflammatory cytokines secretion in the ventral midbrain of MPTP-induced PD mice. (A) Immunohistochemical staining of TH in the ventral midbrain of mice. Scale bar: 200 μm. The contents of TNF-α (B), IL-1β (C), and IL-6 (D) in the ventral midbrain. **P < 0.01 vs. control; ##P < 0.01 vs. MPTP, n = 6 mice in each group.

found to antagonize 1-methyl-4-phenylpyridinium (MPP^+)-induced neurotoxicity in MES23.5 cells [23], attenuate 6-nigrostriatal dopaminergic degeneration in ODHA-induced PD mice [24], and reduce depletion of dopamine (DA) in MPTP-induced PD mice [25]. In addition, RA has been demonstrated to prevent the synaptic dysfunction by interfering with α-synuclein aggregation [26]. These studies indicate that RA is a potential compound for PD treatment. However, the pharmacological mechanisms of RA on anti-PD have not been fully revealed. In previous studies, the findings were focused on the neuron-protection of RA. In the present study, the effect of RA on inflammatory responses and microglia activation was evaluated in MPTP-induced PD mice in vivo and in MPP^+ and α-synuclein-induced BV-2 cells in vitro. The possible regulatory action on the HMGB1/TLR4 signaling pathway was also assessed.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (10-week old) obtained from Liaoning Changsheng Biotechnology Co. Ltd. (Benxi, China) were maintained in a standard experimental animal room with constant temperature (22 ± 1 °C), relative humidity (60%), a strict 12 h/12 h light–dark cycle, and free access to water and food. All experimental procedure was approved by Ethic Committee of China Medical University and performed according to the guidelines for the Care and Use of Laboratory Animals.

The mice were randomly divided into 5 groups: sham, MPTP, MPTP + RA (1 mg/kg), MPTP + RA (4 mg/kg) and MPTP + RA (16 mg/kg) (n = 30 in each group). Mice in the three MPTP groups received an intraperitoneal injection of MPTP (M132847, Aladdin regents Co. Ltd., Shanghai, China) at 25 mg/kg daily for 8 days followed by RA treatment until the mice were sacrificed. RA (R4033, Sigma-Aldrich, St Louis, MO, USA) was dissolved in saline and intraperitoneally injected daily at indicated concentrations. Mice in the sham group received the same volume of saline throughout the study. The mice in the MPTP group received an intraperitoneal injection of MPTP at 25 mg/kg daily for 8 days not followed by RA treatment. Then mice in the MPTP group received the same volume of saline during the treatment days. The mice were euthanized at the end of the behavioral tests and the tissues were collected for further examinations. Six mice in each group were randomly selected for behavioral test and histological

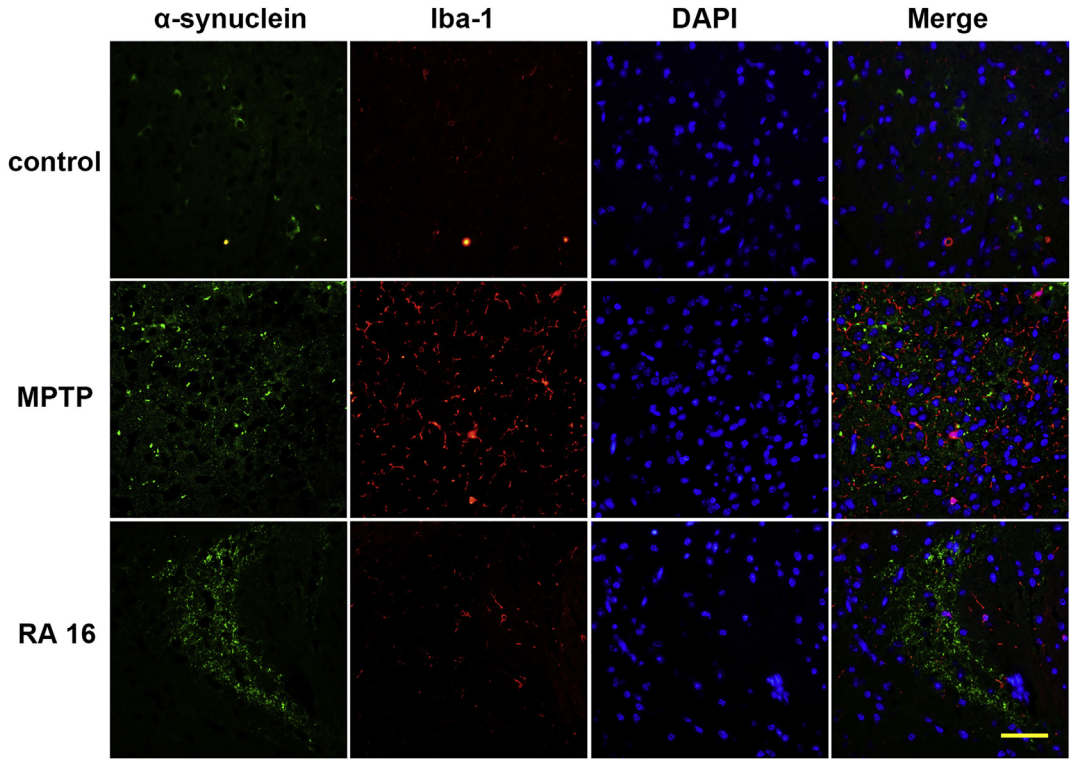

Fig. 3. RA reduced microglia activation and α-synuclein accumulation in the ventral midbrain of MPTP-induced PD mice. Scale bar: 50 μm. n = 6 mice in each group.

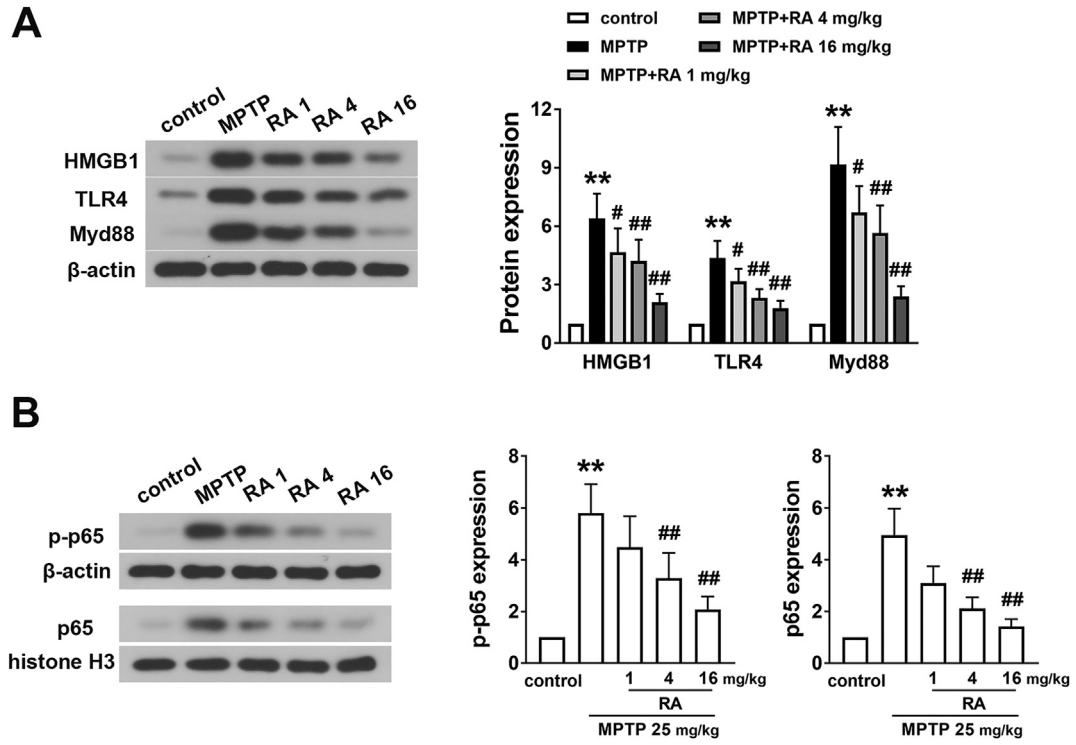

Fig. 4. RA suppressed HMGB1/TLR4/NF-κB activation in the ventral midbrain of MPTP-induced PD mice. Western blot analysis of HMGB1, TLR4 and Myd88 expression levels (A) and p-p65 whole cell expression and p65 nuclear expression (B). **P < 0.01 vs. control; ##P < 0.01 vs. MPTP, n = 6 mice in each group.

examination. Twelve mice in each group were randomly selected for ELISA, and the other 12 mice were used for western blot analysis. For ELISA and western blot analysis, the tissue samples from two mice were mixed because the tissue from one mouse was too tiny to assess.

2.2. Behavioral test

Pole test was performed at day 8 of RA treatment according to the previous describe [27]. Each mouse was positioned head downwards on top of a wood pole and the time taken to climb down the pole was recorded. The pole was 1 cm in diameter, 50 cm in height and rough-

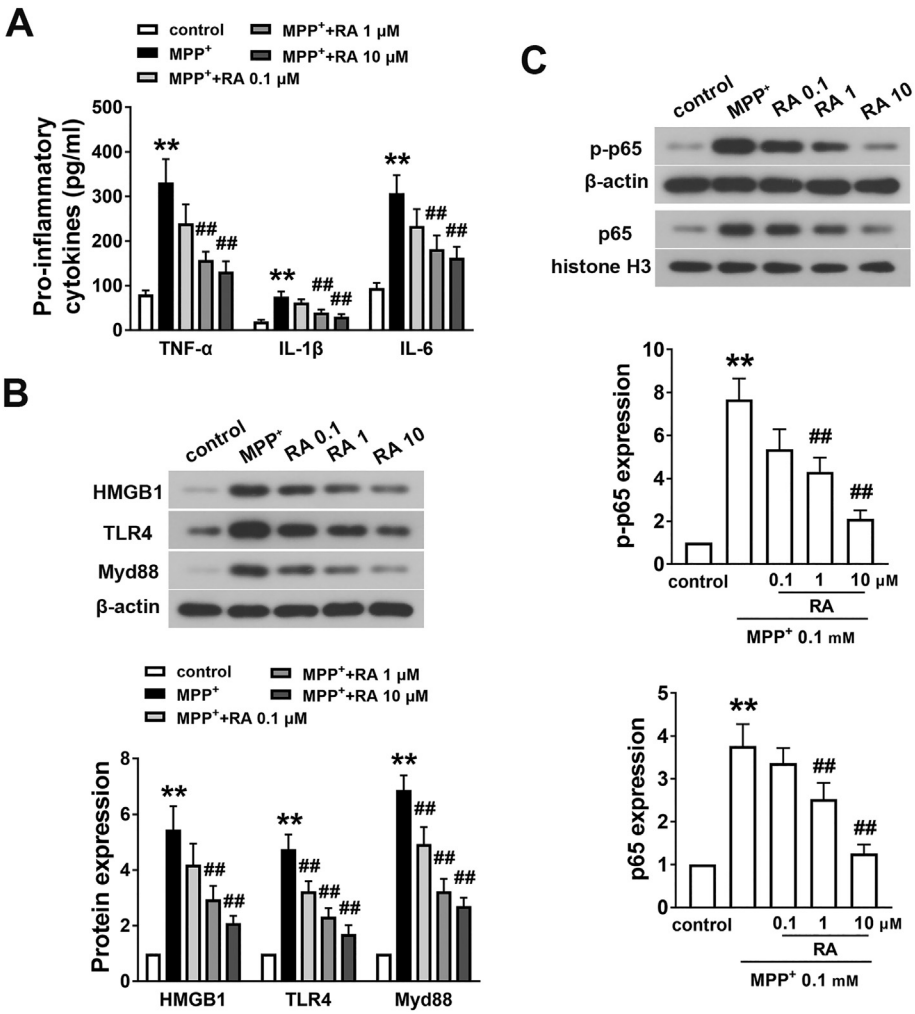

Fig. 5. RA reduced pro-inflammatory cytokines secretion and suppressed HMGB1/TLR4/NF-κB activation in MPP^+-exposed BV-2 cells. (A): Levels of TNF-α (A), IL-1β (B), and IL-6 (C) in the medium. (B): Western blot analysis of HMGB1, TLR4 and Myd88 expression levels. (C): Western blot analysis of p-p65 whole cell expression and p65 nuclear expression. $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. MPP^+, n = 3 (biological repeat).

surfaced. The test was repeated three times and the average of the descending times was calculated.

Traction test was performed on a horizontal wire (1.5 mm in diameter) 1 h after the pole test. The forepaws of mouse were placed on the wire and the placements were scored as following standard: 3: both hind limbs seized the wire; 2: only one hind limb seized the wire; 1: no hind limb seized the wire [27].

Beam-crossing test was performed at day 9 of RA treatment. Each mouse was placed at one end of an elevated beam with 100-cm long and 2-cm wide. The time it took to cross the beam was recorded [28].

Cylinder test was carried out 1 h after the beam-crossing test. Each mouse was placed into a transparent cylinder and recorded for 5 min. The number of times that the forepaws touched the wall was counted [29].

2.3. Cell culture and treatment

Mouse microglia cell line BV-2 was obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China). The cells were seeded in 6-well plates and cultured in MEM medium (PM150410, Procell) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) in 37 °C. After 24 h, 0.1 mM MPP^+ or 5 μM α-synuclein were added to the medium and incubated for 6 h. Then, 0.1 μM, 1 μM, or 10 μM RA were added. The cells and supernatant were collected after 24 h for further assays.

2.4. Measurement of proinflammatory cytokines

The brain was removed immediately after the mice were sacrificed. The ventral midbrain, which contained the substantia nigra pars compacta (SNpc), was isolated and homogenized in cooled PBS on ice. The homogenate was centrifuged at 430*g* for 10 min and the supernatant was collected for proinflammatory cytokines measurement. The cell medium was centrifuged at 1000*g* for 20 min. The supernatant was collected for proinflammatory cytokines measurement. The levels of TNF-α, IL-1β and IL-6 in the ventral midbrain or cell medium were determined using commercial ELISA kits (USCN Life Science, Wuhan, China) according to manufacturer's protocols.

2.5. Immunostaining

The midbrain tissues were fixed in 4% paraformaldehyde at 4 °C for 24 h, embedded in paraffin, and cut into 5-μm-thick sections. The sections were deparaffinized in xylene, hydrated using a series of ethanol and boiled in sodium citrate antigen retrieval solution for 10 min. Then, the sections were blocked with goat serum (SL038, Solarbio Science & Technology, Co., Ltd., Beijing, China) for 15 min at room temperature. For immunohistochemical staining, the sections were incubated in anti-tyrosine hydroxylase (TH) antibody (1:200, rabbit polyclonal, ab112, Abcam, Cambridge, UK) at 4 °C overnight. After a wash stage, the sections were incubated in horseradish peroxidase-conjugated

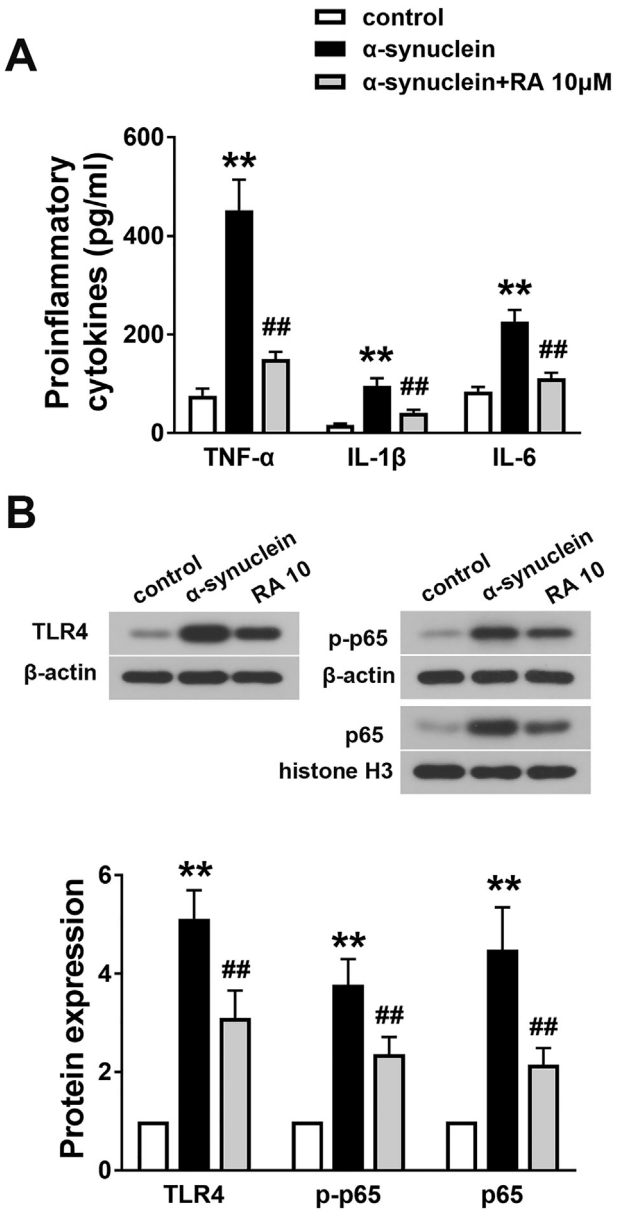

Fig. 6. RA attenuated inflammatory responses in α-synuclein-exposed BV-2 cells. (A): Levels of TNF-α (A), IL-1β (B), and IL-6 (C) in the medium. (B): Western blot analysis of TLR4 and p-p65expression levels and p65 nuclear expression. **P < 0.01 vs. control; ##P < 0.01 vs. MPP^+, n = 3 (biological repeat).

secondary immunoglobulin G (1:5000, Beyotime) at 37 °C for 1 h. The staining was visualized using 3,3′-diaminobenzidine (Solarbio Science & Technology) and co-stained using hematoxylin. For immunofluorescence staining, the sections were incubated in anti-Iba-1 antibody (1:200, rabbit monoclonal, ab178847, Abcam, Cambridge, UK) and anti-α-synuclein antibody (1:100, mouse monoclonal, NBP1-05194, Novus Biologicals, Littleton, CO, USA) at 4 °C overnight. After a wash stage, the sections were incubated in Cy3-labeled goat anti rabbit IgG and FITC-labeled goat anti mouse IgG (all Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 90 min. The sections were washed using PBS and co-stained with 4′,6-diamidino-2-phenylindole (DAPI, C1002, Beyotime). The stain was observed under a fluorescence microscope (DP73; Olympus, Tokyo, Japan).

2.6. Western blot analysis

The midbrain tissues or cells were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 1% phenylmethanesulfonyl fluoride (all Beyotime) on ice. The homogenate was centrifuged at 12,000*g* at 4 °C for 15 min and the supernatant was collected. Nuclear protein was extracted using a nuclear and cytoplasmic protein extraction kit (Beyotime) following the manufacture's protocol. The concentration of the protein was determined using a BCA protein assay kit (Beyotime). Equal amounts of protein samples (each 40 μg) were separated on 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking in 5% BSA, the membranes were incubated with primary antibodies at 4 °C overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG (Proteintech Group, Inc. Rosemont, IL, USA) at 37 °C for 40 min. Protein blots were visualized with enhanced chemiluminescence (7 Sea Pharmtech, Shanghai, China) and quantified using Gel-Pro-Analyzer software (Media Cybernetics, Bethesda, MD). β-Actin and histone H3 were used as internal control for whole protein and nuclear protein, respectively. The primary antibodies used were as follows: anti-HMGB1 antibody (1:1000, 10829-1-AP), anti-Myd88 antibody (1:500, 23230-1-AP), anti-TLR-4 antibody (1:500, 19811-1-AP), anti-p65 antibody (1:500, 10745-1-AP), anti-Histone H3 antibody (1:500, 17168-1-AP), anti-β-actin antibody (1:2000, 60008-1-Ig) (all Proteintech), and anti-p-p65 antibody (1:1000, #3033, Cell Signaling Technology, Danvers, MA, USA).

2.7. Statistical analysis

Data are presented as the mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Tukey's test for data of equal variances and Dunnett T3 test for data of unequal variances. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. RA improved MPTP-induced dyskinesia in mice

To investigate the effect of RA treatment on motor function, pole test, traction test, beam-crossing and cylinder test were conducted in the present study. The results of pole and traction tests showed that MPTP injection significantly increased the descending time and decreased limb movements score as compared to the control group ($P < 0.01$, Fig. 1A and B). However, 4 mg/kg and 16 mg/kg RA treatment significantly decreased descending time and increased the score in a dose-dependent manner, which indicated an improvement in total locomotor activity and limb movements. In addition, in beam-crossing test, MPTP injection markedly increased the time that the mouse spent to cross the beam ($P < 0.01$ vs. the control, Fig. 1C), whereas 4 mg/kg and 16 mg/kg RA treatment significantly decreased the time of the mouse on the beam ($P < 0.01$ vs. the MPTP group). Furthermore, the results of cylinder test demonstrated that front limb use was significantly reduced in MPTP-injected mice compared with the control mice, which indicated front limb use asymmetry in PD (Fig. 2D). Treatment with RA (4 mg/kg and 16 mg/kg) increased the times of front limb use, too.

3.2. RA increased TH expression and attenuated inflammatory responses and microglia activation in ventral midbrain

As shown in Fig. 2, a marked loss of TH-positive cells was observed in substantia nigra of the MPTP-injected mice ($P < 0.01$ vs. the control mice, Fig. 2A). RA treatment (4 mg/kg and 16 mg/kg) provided a significant protection from MPTP-induced TH cell death in midbrain. In

addition, MPTP induced significant over-production of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 in the ventral midbrain, while 4 mg/kg and 16 mg/kg RA treatment, except for 1 mg/kg, decreased the production of these cytokines. In addition, immunofluorescence staining showed that Iba-1 and α-synuclein expressions were increased in the ventral midbrain of MPTP group compared with that in the control group. After RA (16 mg/kg) treatment, the number of Iba-1-positive cells was decreased and α-synuclein accumulated around the microglia was reduced (Fig. 3).

3.3. RA inhibited MPTP-induced HMGB1/TLR4/nuclear factor kappa-B (NF-κB) activation

Activation of the HMGB1/TLR4/NF-κB signaling pathway in the ventral midbrain was evaluated in the present study. From the western blot analysis, we found that protein expression levels of HMGB1, TLR4 and its downstream protein Myd88 were upregulated after MPTP injection (Fig. 4A). In addition, phosphorylation level of p65 and nuclear p65 level were also significantly elevated in the ventral midbrain of MPTP-induced mice (Fig. 4B). Treatment with RA (4 mg/kg and 16 mg/kg) markedly downregulated the expression levels of HMGB1, TLR4 and Myd88 and inhibited p65 phosphorylation and nuclear translocation.

3.4. RA attenuates MPP^+-induced inflammatory responses in BV-2 cells

The anti-inflammatory effect of RA was further confirmed in cultured BV-2 cells in vitro. As shown in Fig. 5, MPP^+ addition significantly increased the secretion of pro-inflammatory cytokines TNF-α, IL-1β and IL-6, while treatment with RA markedly suppressed the secretion of these cytokines. In the investigation of signaling pathway, we also found that the HMGB1/TLR4/NF-κB signaling pathway was activated after MPP^+ stimulation, as evidenced by the upregulated protein expressions of HMGB1, TLR4 and Myd88 and enhanced p65 phosphorylation and nuclear p65 expression. In line with the in vivo study, RA (1 μM and 10 μM) inhibited MPP^+-induced over-production of these pro-inflammatory cytokines, reduced protein expression levels of HMGB1, TLR4 and Myd88, and inhibited p65 phosphorylation and nuclear expression.

3.5. RA attenuated α-synuclein-induced inflammatory responses in BV-2 cells

To further investigate the mechanisms of the anti-PD effect of RA, we stimulated BV-2 cells with α-synuclein. Results demonstrated that α-synuclein also stimulated increased secretion of TNF-α, IL-1β and IL-6, upregulation of TLR4, and phosphorylation and nuclear translocation of p65 (Fig. 6). Similar as the effect of RA on MPP^+-induced inflammatory responses in BV-2 cells, RA (10 μM) suppressed these changes stimulated by α-synuclein.

4. Discussion

In the present study, we investigated the mechanisms of the anti-PD effect of RA from the perspective of inflammation and microglia activation. We found that treatment of RA dose-dependently improved motor function of PD mice. In addition, RA could reduce production of pro-inflammatory cytokines and microglia activation in ventral midbrain. Furthermore, RA treatment could suppress activation of the HMGB1/TLR4 signaling pathway and inhibit NF-κB nuclear translocation.

TH is a rate-limiting enzyme that converts tyrosine to L-DOPA. Downregulation of TH expression leads to reduction of dopamine production and causes PD [30,31]. In the present study, we demonstrated that treatment with RA at 4 mg/kg and 16 mg/kg increased TH-positive cells in the substantia nigra of PD mice, which indicated that RA treatment may protect against MPTP induced loss of TH-positive cells. Because of the dopaminergic dysfunction in the brain, MPTP also causes behavior disorder in animals. In the present study, RA treatment counteracted MPTP-induced reduction of locomotor activity and dysfunction of limb movements. Our findings suggested that RA exhibited neuroprotective effect against MPTP-induced PD in mice.

Inflammation is an important process in various neurodegenerative disorders including PD [32]. Inflammatory responses under control should be a compensatory mechanism which protects organ from injury, while uncontrolled inflammation causes cellular toxicity and exacerbates diseases. In 1988, activated microglia in the substantia nigra was observed in PD patients, which is the first report of CNS inflammation [33]. Although its role in the development of PD has not been fully revealed, increasing evidence shows that inflammation is implicated in PD. Microglia activation is a presentative feature in CNS inflammation. PET studies have demonstrated the widespread activation of microglia in the brain of PD patients [34,35]. Importantly, activation of microglia in the midbrain was found to be closely correlated with the motor severity of parkinsonism and dopaminergic terminal loss [36]. In addition, high expressions of genes encoding typical pro-inflammatory cytokines, such as TNF-α and IL-1β, are also associated with the onset and development of PD [37,38]. Although some studies came out negative results [39,40], more investigations found treatment of non-steroidal anti-inflammatory drugs (NSAIDs) could reduce the risk of developing PD [41–44]. The anti-inflammatory action of RA has been well studied in previous studies [45,46]. Furthermore, RA was found to inhibit lipopolysaccharide-induced activation of microglia [46,47]. In the present study, we found that RA improved motor function of PD mice. Meanwhile, the inflammatory responses were suppressed and activation of microglia was inhibited in the ventral midbrain of PD mice. In addition, RA could also reduce MPP^+-induced secretion of pro-inflammatory cytokines in a dose-dependent manner. MPP^+ is the metabolite of MPTP [48]. The in vitro study further confirmed that microglia might be a target cell of RA in PD treatment. These data indicated that the anti-inflammatory action of RA might be associated with its anti-PD activity.

HMGB1 is a non-histone chromosome-binding protein which express in many tissues including brain. Studies have found it is associated with TLR4-mediated inflammatory response and plays an important role in a variety of inflammatory diseases [49]. Nowadays, HMGB1/TLR4 axis is a key pro-inflammatory signaling pathway, and this signaling also plays a major role in promoting neuritis. Intracerebroventricular injection of HMGB1 was found to increase the productions of pro-inflammatory cytokines in mouse brain [50]. In PD, clinical study found that the expression of the protein in HMGB1/TLR4 axis was significantly upregulated in the PD patients than in the healthy volunteers, as well as the downstream factors MyD88, NF-κB, and TNF-α [13]. Mechanically, activated astrocytes and microglia in the brain stimulate the release of HMGB1, and HMGB1 itself promotes the activation of glia to form a vicious circle, which accelerates the inflammatory responses in brain [51]. Thus, inhibition of this signaling pathway should be beneficial for the treatment of PD. The effect of RA on HMGB1/TLR4 axis has been demonstrated in the studies on other tissues and cultured cells [52–54]. In line with these studies, results in the present study also found that treatment of RA markedly downregulated the protein expression levels in HMGB1/TLR4 axis, including HMGB1, TLR4 and Myd88. In addition, the downstream NF-κB signaling was also suppressed, as evidenced by the lowered p65 phosphorylation and nuclear translocation. These results suggested that suppressing the activation of HMGB1 HMGB1/TLR4/NF-κB axis might participate in the neuroprotection of RA against PD.

Alpha-synuclein is the first gene to be associated with PD [55]. Although the pathological mechanisms of α-synuclein have not been fully understood, it is acknowledged to be an important factor in the development of PD. Alvarez-Erviti et al. found that α-synuclein could induced microglia activation [56]. This report indicates that α-

synuclein is also involved in inflammatory responses in PD brain. Given the essential role of neuroinflammation in the development of PD and the importance of α-synuclein in PD pathology, we investigate the effect of RA on α-synuclein accumulation in the midbrain of PD mice and α-synuclein-exposed BV-2 cells. As expected, α-synuclein induced overproduction of pro-inflammatory cytokines and led to NF-κB p65 nuclear translocation in BV-2 cells. Treatment with RA reduced α-synuclein accumulation around microglia in the midbrain, inhibited inflammatory responses and suppressed inflammatory signaling activation in the BV-2 cells. Previous studies have demonstrated that RA prevents α-synuclein oligomer-induced synaptic dysfunction by interfering with α-synuclein oligomerization [26]. Here we provide the new evidence that RA may exhibit the neuroprotective function through inhibited α-synuclein-induced inflammation.

It needs to note that in Alvarez-Erviti's study, MPP^+ failed to induce inflammatory responses in BV-2 cells. However, in our and others' studies, MPP^+ caused increased secretion of pro-inflammatory cytokines [57,58]. This discrepancy may due to the different experimental environments, but exact explanation needs to be confirmed in further studies. In addition, the mechanisms of RA on α-synuclein in PD have not been fully revealed in the present study. We focused on anti-inflammatory effect of RA and only reported that RA could inhibit α-synuclein accumulation in the midbrain and attenuate α-synuclein-induced inflammation in cultured glia cells. The possible effects of RA on oligomeric and phosphorylated α-synuclein have not been included, which will be further investigated in our future study.

In conclusion, our findings demonstrate that RA prevents the neurodegeneration in PD mice by reducing pro-inflammatory cytokine release and attenuating microglial activation in the ventral midbrain. This anti-neuroinflammatory effect of RA may be associated to its abilities to inhibit the activation of HMGB1/TLR4/NF-κB signaling pathway.

Acknowledgements

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