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Astragaloside IV ameliorates motor deficits and dopaminergic neuron degeneration via inhibiting neuroinflammation and oxidative stress in a Parkinson's disease mouse model

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ABSTRACT

Oxidative stress and neuroinflammation are the key and early events during the pathological process of Parkinson's disease (PD). Thus, therapeutic intervention to regulate oxidative stress and neuroinflammation would be an effective strategy to alleviate the progression of PD. Astragaloside IV, the main active component isolated from *Astragalus membranaceus*, has been shown to possess anti-inflammatory and anti-oxidant properties in neurodegeneration diseases, however, the molecular mechanisms of Astragaloside IV in the pathology of PD are still unclear. In this study, we explored the mechanisms of Astragaloside IV of PD on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mice model and lipopolysaccharide (LPS)-induced BV2 microglia cells. Our results showed Astragaloside IV significantly alleviated behavioral impairments and dopaminergic neuron degeneration induced by MPTP. Also, Astragaloside IV inhibited microglia activation and reduced the oxidative stress of MPTP mouse model. In addition, Astragaloside IV significantly inhibited NFκB mediated NLRP3 inflammasome activation and activated Nrf2 both in vivo and in vitro. Furthermore, Astragaloside IV lessened reactive oxygen species (ROS) generation in LPS-induced BV2 microglia cells remarkably. These findings demonstrate that Astragaloside IV protects dopaminergic neuron from neuroinflammation and oxidative stress which are largely dependent upon activation of the Nrf2 pathways and suppression of NFκB/NLRP3 inflammasome signaling pathway. Therefore, Astragaloside IV is a promising neuroprotective agent that should be further developed for neurodegeneration diseases.

1. Introduction

Parkinson's disease (PD) is a common progressive neurodegeneration disease which occurs with genetic and environmental onset. It characterize by extra pyramidal motor dysfunction including tremor, rigidity, bradykinesia and several non-motor symptoms such as sleep and cognitive problems [1]. The hallmark of the diseases is the progressive loss of dopaminergic neurons in substantia nigra compacta (SNpc) and striatum. Several studies assume that mitochondrial dysfunction, oxidative stress, neuroinflammation and apoptosis are involved with the pathological process of PD [2–5]. Recently, oxidative stress and neuroinflammation are supported as the early and key pathological feature of PD [6,7]. Therefore, therapeutic intervention to inhibit oxidative stress and neuroinflammation would be an effective strategy to alleviate the progression of PD.

Neuroinflammation is a potent contributor to promote the progression of PD [2,8,9]. Recent studies showed that inflammasome activation is implicated in inflammatory neurodegenerative disorders such as AD, traumatic brain injury (TBI) and PD [3,10,11]. Nucleotide binding and oligomerization domain-like (Nod) receptor family pyrin domain-containing 3 (NLRP3) inflammasome is the most widely studied member of the NLR family, which is composed of nod-like receptor

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protein NLRP3, adaptor protein ASC and pro-cascapse-1. It can be activated to trigger a pro-inflammatory response under the stimulation of the pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular pattern (DAMPs) and ROS [12]. Activation of NLRP3 inflammasome demands two signals: The first signal is to activate the NFκB pathway to facilitate pro-IL-1β assembly and promote NLRP3 transcription and translation, which is the priming step of NLRP3 inflammasome activation. The second signal is to form the NLRP3 inflammasome complex including NLRP3, ASC and caspsase-1 and then in turn cleaves pro-IL-1β into IL-1β. The activation of NLRP3 inflammasome will promote the maturity and release of IL-1β, which is detrimental to the dopaminergic neuronal survival. Strategies to inhibition NLRP3 inflammasome activation are effective to ameliorate the neuroinflammation and dopaminergic neuronal degeneration of PD [13–16].

Oxidative stress is often considered as a common feature of inflammatory responses, which is companied with an increased intracellular overproduction of ROS and reduced antioxidant capacity. ROS, on the other hand, exerts an upstream signal of NLRP3 inflammasome activation, which activates NLRP3 to trigger an inflammatory response [17,18]. Nuclear factor E2-related factor 2 (Nrf2) is a key endogenous regulator in anti-oxidant defense mechanisms, which plays a vital role in the amelioration of various inflammatory and oxidative stress-induced diseases [19–22]. Recently studies showed that activating Nrf2 could negatively regulate NLRP3 inflammasome activity via inhibiting ROS production [17]. Another studies also demonstrated that Nrf2-activating compounds such as tertiary butylhydroquinone and sulforaphane is protective to the injured dopaminergic neuron [23–28]. Therefore, activating Nrf2 may contribute to anti-oxidative stress and anti-inflammation, which is beneficial for attenuating the severity of PD.

Astragaloside IV is a saponin and serving as the predominant constituent of Astragalus membranaceus, which has been suggested to have anti-inflammatory, anti-oxidative and immuno-regulatory activities in various diseases [29–32]. Recently, studies demonstrated that Astragaloside IV could alleviate neuroinflammation via inhibiting NFκB/NLRP3 inflammasome axis of depression mice [33]. There are also reports showing that Astragaloside IV could activate Nrf2-mediated antioxidant pathway to alleviate acute kidney injury and brain injury [34–36]. Although researchers also found Astragaloside IV is effective to against the motor injury induced by MPTP, however, the molecular mechanisms remain unclear [37]. In this study, we seek to investigate the potential mechanisms of Astragaloside IV in a MPTP-induced mouse model and LPS-induced BV2 microglia cells model of PD.

2. Materials and methods

2.1. Antibodies and reagents

The primary antibodies including Rabbit monoclonal anti-NFκB, Rabbit monoclonal anti-p-NFκBp65, Rabbit monoclonal anti-NLRP3, Rabbit monoclonal anti-ASC, Rabbit monoclonal anti-Caspase-1, Rabbit monoclonal anti-IL-1β and Rabbit monoclonal anti-LaminB were purchased form Cell Signaling Technology, Inc. Rabbit monoclonal anti-TH, Rabbit monoclonal anti-Nrf2 and Rat monoclonal anti-CD68 were purchased form Abcam, Inc. Rabbit monoclonal anti- Iba-1 antibody were purchased from Woko, Inc. Mouse anti-β-actin was purchased from Sigma (St. Louis, MO, USA). All secondary antibodies (Goat anti-mouse, Goat anti-rabbit IgG, Alexa Fluor 488 anti-Rabbit IgG, Alexa Fluor 594 anti-Rat IgG and Alexa Fluor 594 anti-Rabbit IgG) were purchased form Cell Signaling Technology, Inc. Astragaloside IV (98% of purity) was obtained from Chengdu Herbpurify Co., Ltd. (China). MPTP was purchased from Sigma (USA).

2.2. Animals and drug treatment

Eight-week adult male C57BL/6 mice (weight 25–30 g) were obtained from Experimental Animal Center of the Guangzhou University of Chinese Medicine (Guangzhou, China). Animals were group-housed under a 12 h light/dark cycle with free access to water and food. All animal care and experimentation were approved by the principles and guidelines of Ministry of Health, Peoples Republic of China for the Care and Use of Laboratory Animals and the ethical standards for laboratory animals of Guangzhou University of Chinese Medicine.

After one week of acclimation, mice were randomly divided into five groups: (1) Control group; (2) Control + AS (Astragaloside IV, 40 mg/kg) group; (3) MPTP group; (4) MPTP + AS (Astragaloside IV, 10 mg/kg) group; (5) MPTP + AS (Astragaloside IV, 40 mg/kg) group. Mice had intraperitoneal injection with MPTP (18 mg/kg) four times at 2 h intervals to establish PD acute models. Mice were pretreated twice daily with Astragaloside IV by oral gavage as described above for 7 days before MPTP injection. Animals in the control and model group were injected with an equivalent volume of 0.9% saline. After MPTP administration, mice were treated with Astragaloside IV for 7 consecutive days, accompanied with the behavioral test.

2.3. Behavioral test

The rotarod test and pole-climbing test were used to evaluate the motor dysfunctions of MPTP-induced mice in this study. Briefly, the mice were placed on the rotating rod for 2 min at the speed of 20 rounds per minute as described previously [38]. The latency to fall was defined by the duration of the mice on the rod until their first drop.

For the pole-climbing test, the procedures were carried out as previously described [39]. Briefly, a wooden pole, 50 cm in length and 0.5 cm in diameter, was placed on the table and wrapped in gauze to prevent slippage. The mice were placed on the top of the pole and allowed to climb down without interference for three successive times with a 5-min interval. The time of each animal to climb down the pole was recorded. The average of time spent in the three trials was used for statistical analyses.

2.4. Tissue preparations

After behavioral tests, the mice were sacrificed under sodium pentobarbital anesthesia. The midbrain region from the whole brain was isolated immediately and stored at −80 °C for western blot. In each group, four brain samples were collected and postfixed overnight in 4% PFA at room temperature. They were transferred to sucrose solution for dehydration according to a previously method for immunohistochemical stain analysis [15].

2.5. Cell culture and drug treatment

BV-2 microglia cells were obtained from the Chinese Academia Sinica (Shanghai, China) and maintained in DMEM media supplemented with 10% fetal bovine serum and antibiotics at 37 °C in a humidified incubator supplied with 5% CO_2. Activation of inflammasome induced in vitro was performed according to a previously described method with few modifications [15]. For inducing inflammasome activation, the cells were seeded (1×10^6 cells/well) in a 6-well plate, incubated for 24 h, and then treated with or without Astragaloside IV (10 μM or 40 μM) for another 6 h. After that, the cells were stimulated with LPS (500 ng/ml) for 3 h, and then stimulated with ATP (2.5 mM) for 45 min.

2.6. Immunofluorescence

The immunofluorescence detection was conducted as described previously with few modifications [15]. Coronal sections were prepared

by a freezing microtome (Leica, Germany) and stored in an antifreeze solution. The sections were blocked with 10% goat serum (with 0.5% Triton X-100) in Tris-buffered saline for 20 min and then labeled with TH (dilution 1:2000), Iba-1 antibody (dilution 1:200) and CD68 (dilution 1:200), in blocking buffer for 48 h at 4 °C. After being washed three times with PBS, the sections were incubated with anti-rabbit secondary antibodies conjugated to Alexa Fluor 594 (1:1000) or Alexa Fluor 488 (1:1000) for 1 h. After being washed with PBS three times, the sections were added to a few drops of DAPI. Finally, the sections were covered with coverslips and mounted with anti-fade fluorescence mounting medium (Beyotime Biotechnology). The slides were then observed under a fluorescence microscope (Model DMi8, Leica, Germany). The total number of TH positive neurons and Iba-1/CD68 double positive microglia in the entire SNpc range was counted from the brains of each group of four mice. Each brain contains 6 consecutive slices of 3 intervals. The final average of the positive cells in the four animals was taken as the data of the samples. Quantitative data is expressed as the percentage of vehicle group of TH-positive neurons or Iba-1 and CD68 positive microglia.

2.7. β-Galactosidase staining

β -Galactosidase staining was used to determine the senescence of dopaminergic neurons cells in SNpc according to the manufacturer's instruction (Beyotime). Briefly, sections were incubated with fixed solution for 20 min at room temperature. After being washed three times with PBS, the sections were incubated with dyeing working solution at 37 °C overnight. After natural drying, the sections were mounted with neutral balsam and observed under the microscope. The β-galactosidase positive cells in SNpc area were counted by the same methods as the TH positive cells. The quantitative data is expressed as the percentage of β-galactosidase positive cells in SNpc area.

2.8. Nuclear protein extraction

The midbrain tissue was cut into very small pieces as much as possible and then the nuclear proteins were extracted using a nuclear and cytoplasmic protein extraction kit (Beyotime, Shanghai, China) according to the manufacturer's instructions.

2.9. Western blot analysis

Western blot assays for the target proteins were performed according to our previous study [38]. The tissue and cells protein concentrations were measured by BCA Protein Assay protein assay (Millipore, American). Equal amounts of sample (20 μg or 40 μg) were separated by 10%–15% SDS PAGE and the resolved protein was transferred to polyvinylidene difluoride membranes (Millipore, American). The membranes were soaked with 7% (w/v) skim milk for 1.5 h at room temperature, and then incubated with primary antibodies overnight at 4 °C. The membranes were washed 3 times with TBST with Tween 20 for 10 min each, followed by incubation with secondary antibodies: the peroxidase-conjugated anti-mouse (1:4000) or anti-rabbit IgG (1:4000) for 1.5 h at room temperature. The blots were visualized by using an ECL western blot detection kit (Millipore, WBKLS0500). Image J (National Institutes of Health, Bethesda, Maryland, USA) was used to evaluate the densitometry.

2.10. Determination of ROS production

Intracellular ROS levels were measured by using the fluorescent probe DCFH-DA (Sigma-Aldrich) or DHE (Beyotime), according to the manufacturer's protocol. After drug treatment as described above, BV2 cells were rinsed with phosphate-buffered saline (PBS), incubated with 10 μM DCFH-DA or DHE and diluted in serum-free DMEM in dark at 37 °C for 30 min. After incubation, cells were rinsed with PBS and then observed under a fluorescence microscope (Model DMi8, Leica, Germany). The quantitative data is expressed as the fluorescence intensity in relative to control group.

2.11. SOD, GSHpx, total glutathione and GSSG activity assays

The activities of SOD, GSH-Px, the concentration of total Glutathione and GSSG in mouse serum were tested using commercial kits, as specified by the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

2.12. Statistical analysis

All data were expressed as mean ± standard error. Statistical results were obtained by using the statistical software SPSS 17.0. One-way analysis of variance (ANOVA) was used to analyze statistical differences between groups under different conditions and the Student's *t*-test was performed. $P < 0.05$ was considered significant difference.

3. Results

3.1. Astragaloside IV alleviates motor impairment in MPTP-induced PD mice

In this study, we evaluated the effect of Astragaloside IV on PD by rotarod test and pole-climbing test. As shown in Fig. 1A, MPTP-treated mice exhibited a marked decrease in latency to fall compared with those in the control group on day 1, day 3 and day 5 after MPTP injection in rotarod test. However, Astragaloside IV treatment (40 mg/kg) significantly increased the latency to fall in rotarod test on day 3 and day 5 after MPTP injection. Similarly, mice in MPTP group showed a

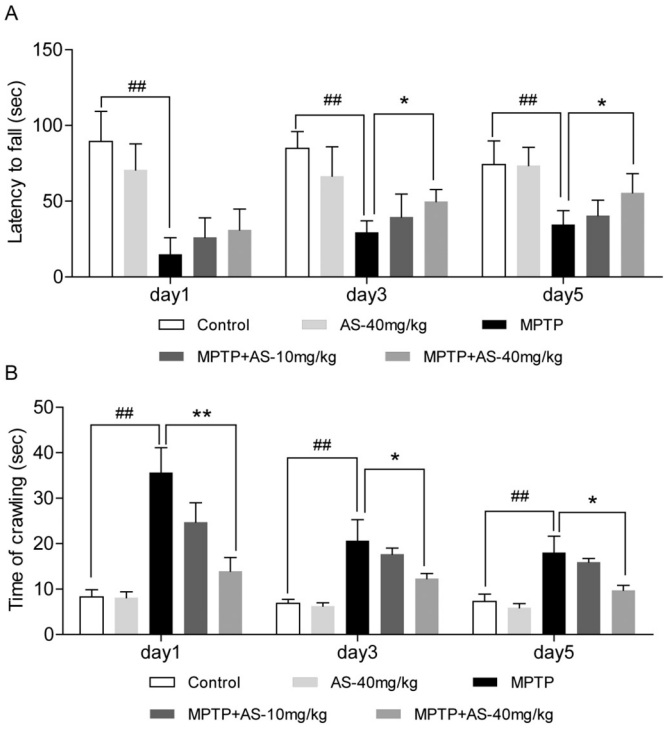


Fig. 1. Astragaloside IV alleviates motor impairment in MPTP-induced PD mice.
Lantency to fall in Rotarod test on the day 1, day 3 and day 5 after MPTP injection (A). The time of crawing in the pole-climbing test on the day 1, day 3 and day 5 after MPTP injection (B). Experimental values were expressed as means ± SEM, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, vs. Control group, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. MPTP-treated group. (n = 10–12 in each group).

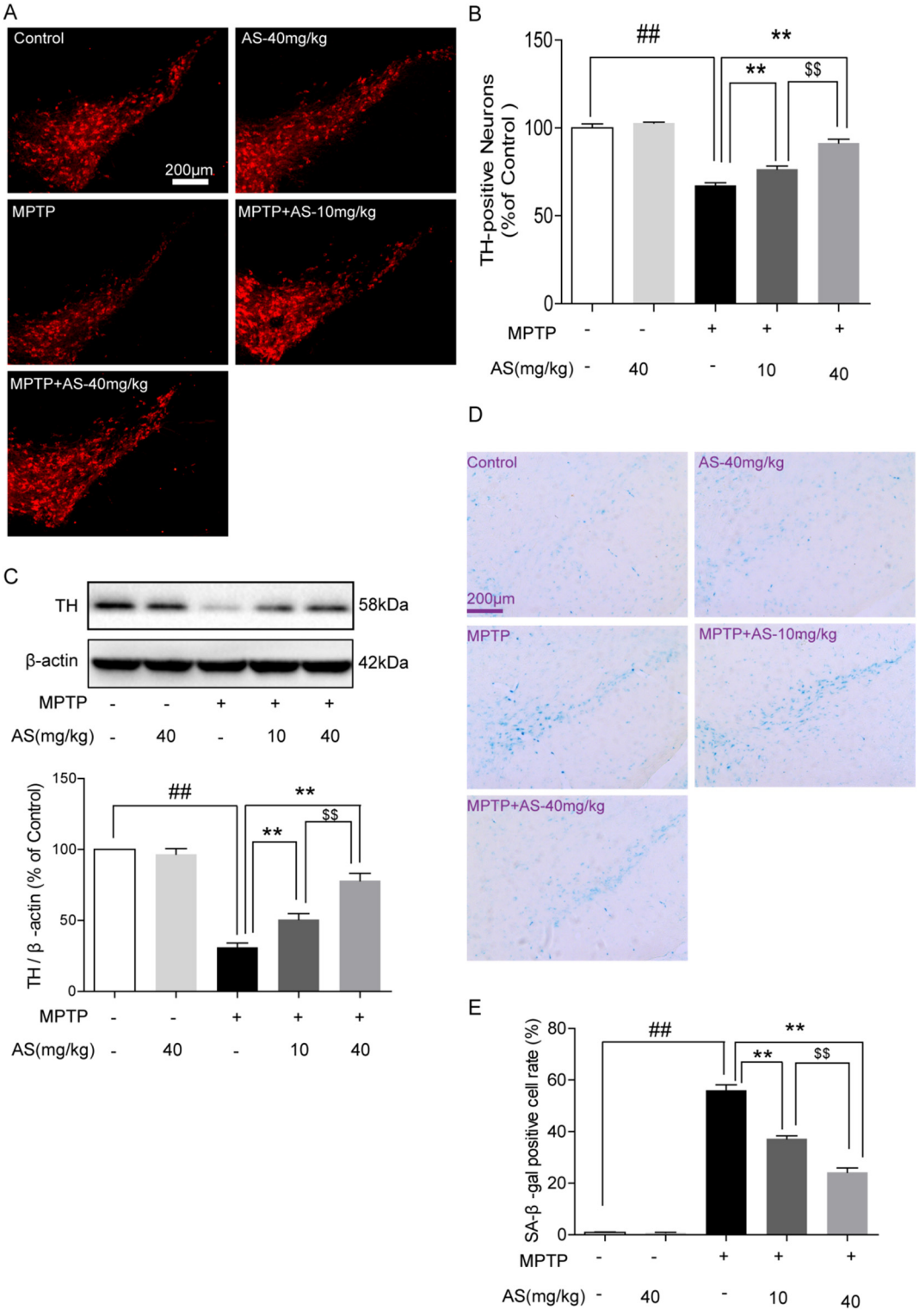
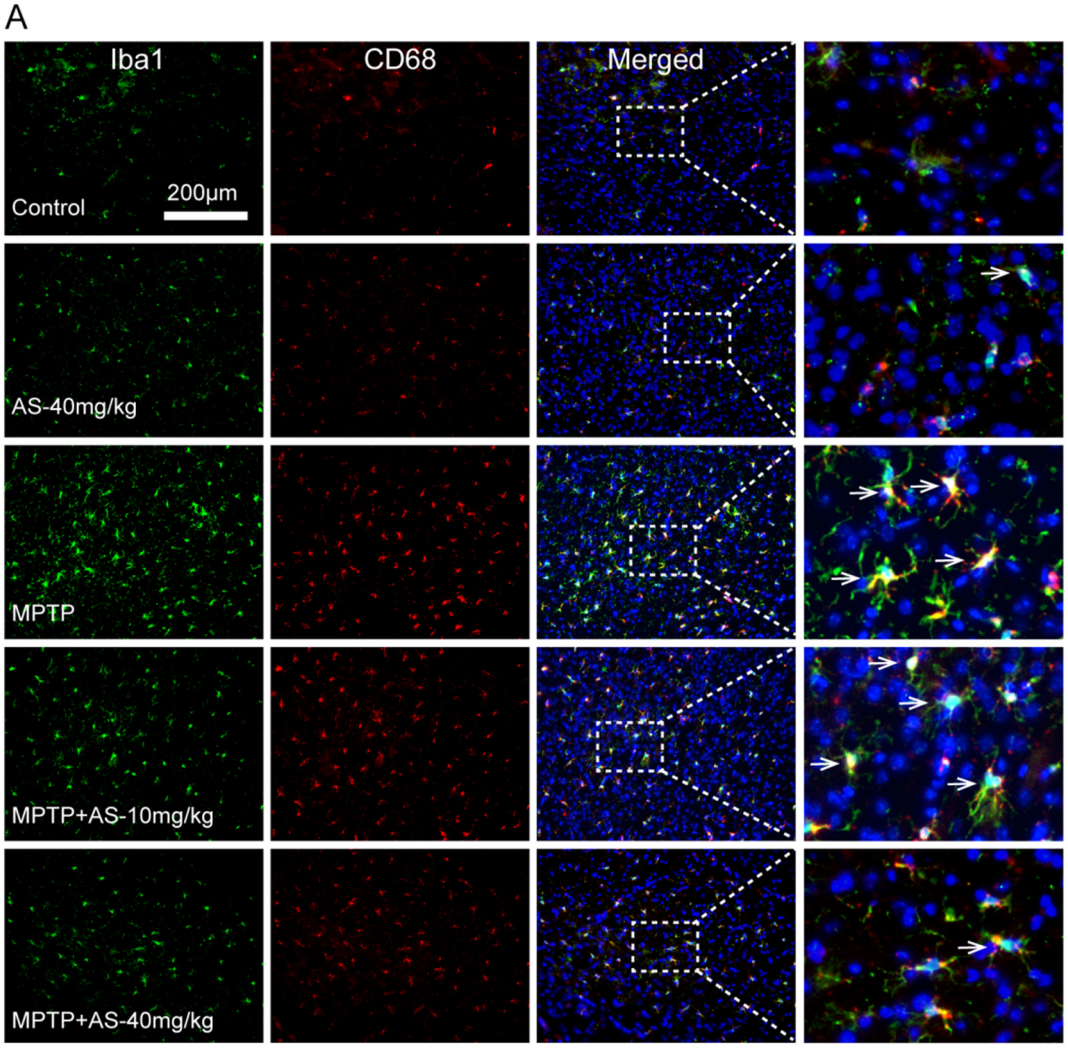
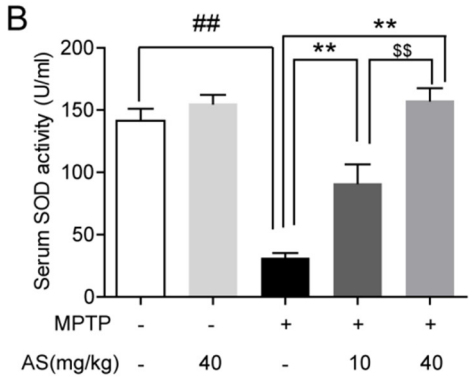
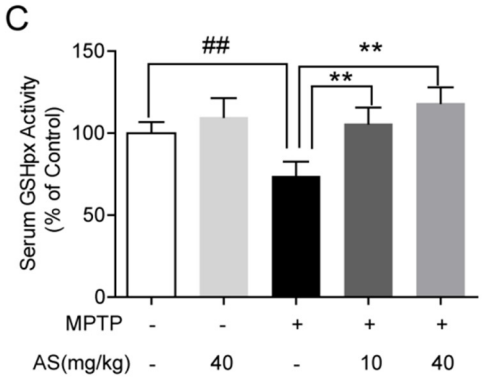


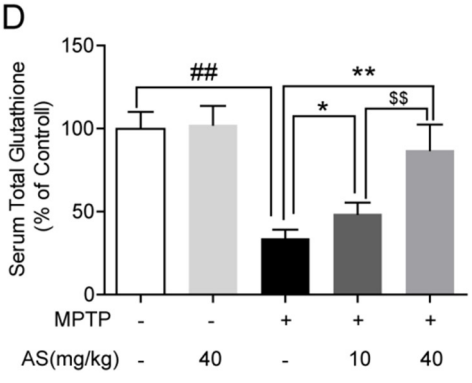
Fig. 2. Astragaloside IV protects against MPTP-induced dopaminergic neurons degeneration in substantia nigra.
Representive immunofluorescence images (A) and the statistical results (B) of TH-positive neurons in the SNpc area of each group. The TH protein expression and the statistic results of midbrain (C) were shown by western blotting ($n = 3$ per group). The representive images and the statistical results for the senescence of dopaminergic neuron (E) in the SNpc area ($n = 4$ per group). Experimental values were expressed as means ± SEM, $^{\#}p < 0.05$and $^{\#\#}p < 0.01$, vs. Control group, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. MPTP-treated group. $^{\$\$}p < 0.01$ represented the statistical analysis of the two groups.

longer crawling time in contrasted to those in the control group on day 1, day 3 and day 5 after MPTP injection in pole-climbing test (Fig. 1B). However, high dose of Astragaloside IV (40 mg/kg) treatment decreased the crawling time on day 1, 3 and 5 after MPTP injection. These results indicate that Astragaloside IV is effective to alleviate MPTP-induced motor impairment.









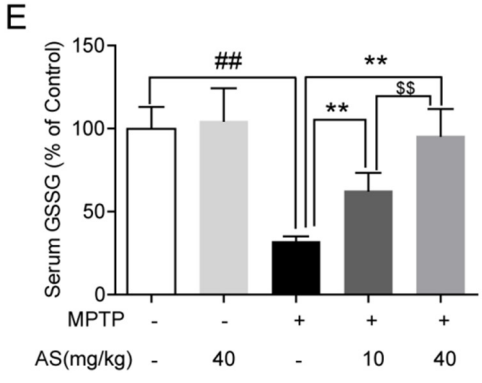


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Fig. 3. Astragaloside IV inhibits microglia activation and oxidative stress in MPTP-induced PD mice.
Representive immunofluorescence images of microglia activation with the specific anti-Iba-1 antibody (green) and CD68 (red) in SNpc area (n = 4 per group) (A). The activity of SOD (B), GSHpx (C), Total Glutathione (D) and GSSG (E) in the serum of the mice ($n = 6$ per group). Experimental values were expressed as means ± SEM, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, vs. Control group, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. MPTP-treated group. $^{\$\$}p < 0.01$ represented the statistical analysis of the two groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Astragaloside IV protects against MPTP-induced dopaminergic neurons degeneration

The selective loss of dopaminergic neurons in the substantia nigra (SN) is the main pathological feature of PD. To verify whether Astragaloside IV have effect on the pathological damage induced by MPTP, we detected the level of tyrosine hydroxylase (TH) by immunofluorescence staining and western blotting. As shown in Fig. 2A and B, MPTP administration caused an obvious loss of TH positive neurons in SNpc in comparison with that in control group. Correspondingly, MPTP treatment also induced a remarkable reduction of TH protein expression of midbrain (Fig. 2C). In addition, the dopaminergic neuron senescence induced by MPTP was also examined by β-Galactosidase staining. As shown in Fig. 2D and E, MPTP administration induced an increasing percentage of senescence neurons in SNpc in comparison with that in the control group. Interestingly, Astragaloside IV treatment significantly increased both the number of TH positive neurons in SNpc and the TH protein expression in midbrain. Moreover, Astragaloside IV ameliorated the dopaminergic neurons senescence remarkably. These results indicate that Astragaloside IV is effective to alleviate the MPTP-induced dopaminergic neuronal degeneration.

3.3. Astragaloside IV inhibits neuroinflammation and oxidative stress in MPTP-induced PD mice

Neuroinflammation and oxidative stress are the early and key events of PD. The activated microglia is the characteristic hallmarks of neuroinflammation of central nervous system (CNS) [40]. Thus, we labeled the microglia with Iba-1 and CD68 in the SNpc of MPTP-induced mice to investigate whether Astragaloside IV have an impact on neuroinflammation of PD. As shown in Fig. 3A, more microglia with large somas and numerous short branches were found after MPTP injection, in addition, increased number of Iba-1 and CD68 double positive microglia (indicated with white arrows) were also observed in the SNpc of MPTP group, which are indicators of microglia activation. Interestingly, Astragaloside IV suppressed the microglia activation by both changing the morphology (Fig. 3A) and decreasing the number of Iba-1 and CD68 double positive microglia cells (Fig. S1).

Next, we detected the enzyme ability of SOD, GSHpx and the concentration of total Glutathione and GSSG in the serum of MPTP mice to confirm the anti-oxidative effect of Astragaloside IV on PD. As shown in Fig. 3B to E, MPTP administration induced a significantly depletion of the anti-oxidant enzyme activities, including those of SOD, GSH-Px, total Glutathione and GSSG in relative to control group, and these effects were markedly reversed by Astragaloside IV treatment. These results indicate Astragaloside IV could inhibit oxidative stress of PD mice induced by MPTP injury.

3.4. Astragaloside IV inhibits NLRP3 inflammasome activation in the MPTP-induced PD mice and LPS-induced BV2 microglia

As NFκB/NLRP3 pathway is considered a key signaling pathway in mediating neuroinflammation, we explored the effect of Astragaloside IV on NFκB and NLRP3 inflammasome activation of PD both in vivo and in vitro. We performed immunofluorescence staining to observe the translocation of NFκB into the nucleus in SNpc area with the treatment of Astragaloside IV after MPTP injury. As shown in Fig. 4A and Fig. S2A, we observed a reduction in NFκB nuclear accumulation of SNpc area in treated with Astragaloside IV when compared with those treated with MPTP alone. We also found a significant decrease in phosphorylated nucleus NFκBp65 following treatment with Astragaloside IV (Fig. 4B). Similarly, Astragaloside IV treatment also caused a decline in NLRP3 inflammasome protein expression (including NLRP3, ASC, Pro-Caspase-1, Caspase-1, Pro-IL-1β and IL-1β) in midbrain of MPTP-induced mice (Fig. 4C).

Microglia NLRP3 inflammasome activation is confirmed to play a key role in the development of neuroinflammation of PD. So, the effect of Astragaloside IV on NLRP3 inflammasome activation in vitro was assessed in BV2 microglia cells. Here, we treated with LPS plus ATP to activate NLRP3 inflammasome of BV2 microglia cells rapidly [15,17]. The results showed that LPS plus ATP induced an increase of NFκB nuclear accumulation (Fig. 4D and Fig. S2B) and an increase of nucleus NFκBp65 phosphorylation in BV2 microglia cells (Fig. 4E). In addition, it also induced an increase of NLRP3, Caspase-1, ASC and IL-1β protein expression ($P < 0.01$, Fig. 4F). In contrast, Astragaloside IV decreased NFκB and NLRP3 inflammasome levels. These results indicated Astragaloside IV inhibits NLRP3 inflammasome activation of PD both in vivo and in vitro.

3.5. Astragaloside IV activates Nrf2 in MPTP-induced mice and LPS-induced BV2 microglia cells

Activate Nrf2 could scavenge different sources of ROS to protect neuron from inflammation and oxidative stress-induced damage. So, we detected and analyzed the protein levels of Nrf2 by immunofluorescence staining and western blotting assays. As shown in Fig. 5A to 5D, MPTP injection resulted to a decrease of Nrf2 nuclear transcription and protein expression as compared with control mice. Similarly, stimulation with LPS plus ATP also led to a remarkable decrease of Nrf2 nuclear translocation and Nrf2 protein expression of BV2 microglia cells (Fig. 5E to H). In contrast, Astragaloside IV treatment increased Nrf-2 levels remarkably both in vivo and in vitro.

Then, we measured the ROS production by the fluorescence of DCFDA and DHE. As shown in Fig. 5I to K, LPS plus ATP caused higher fluorescence intensity of DCFDA and DHE compared with the control group, which is the reflection of the overproduction of ROS. However, Astragaloside IV decreased the production of ROS significantly, evidenced by the decreased the fluorescence intensity of DCFDA and DHE. These results suggest that Astragaloside IV could activate Nrf2 and reduce the ROS production of PD.

4. Discussion

In present study, we evaluated the effect and mechanisms of Astragaloside IV on oxidative stress and neuroinflammation of PD both in vivo and in vitro. We found that Astragaloside IV is effective to alleviate the motor dysfunction and dopaminergic neuron degeneration of MPTP mice. In addition, we determined Astragaloside IV inhibited the oxidative stress and neuroinflammation induced by MPTP. Furthermore, we found that Astragaloside IV suppressed NLRP3 inflammasome activation and activated Nrf2 in MPTP-induced PD mice and LPS-induced BV2 microglia. Besides, we also found Astragaloside IV lessened ROS production in BV2 microglia cells. These findings demonstrate that Astragaloside IV mediated neuroprotection in MPTP mouse model against neuroinflammation and oxidative stress probably via promoting Nrf2 antioxidant pathways activation and suppressing NLRP3 inflammasome activation. Therefore, Astragaloside IV might be the therapeutic drug for PD treatment.

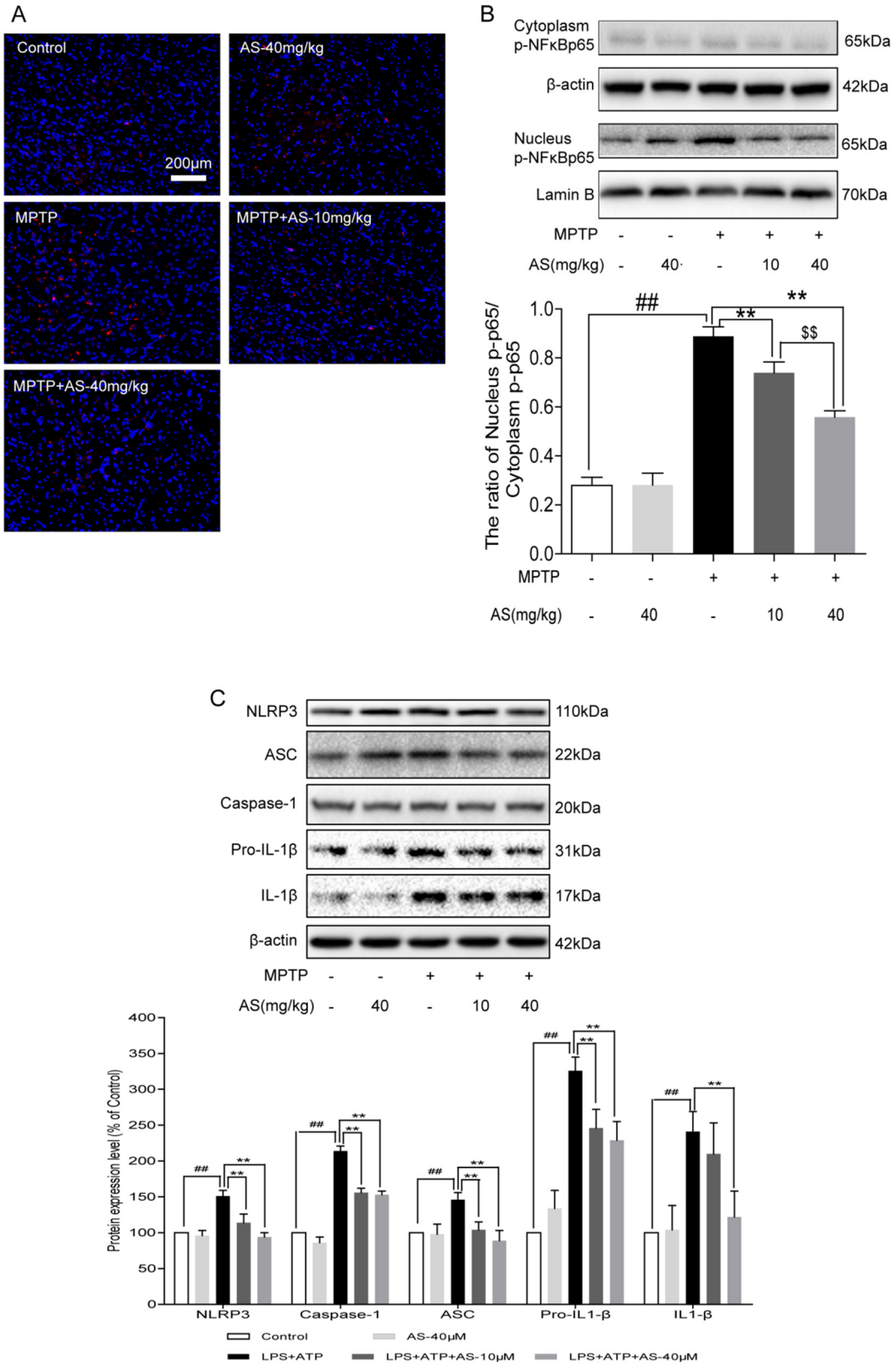

Fig. 4. Astragaloside IV inhibits NFκB-mediated NLRP3 inflammasome activation in MPTP-induced PD mice and LPS-induced BV2 microglia cells. Representive immunofluorescence images of NFκB nuclear accumulation in SNpc area (A) and BV2 microglia (n = 4 per group) (D). The protein bands of cytoplasm and nucleus p-NFκBp65 and the statistic results of the ratio of nucleus p-NFκBp65/cytoplasm p-NFκBp65 in midbrain (B) and BV2 microglia (E) were shown by western blotting (n = 3 per group). NLRP3 inflammasome components (including NLRP3, ASC, Caspase-1 and pro-IL-1β as well as IL-1β) in midbrain (C) and BV2 cells (F) were measured by western blotting and β-actin was detected as an internal control. Experimental values were expressed as means ± SEM, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, vs. Control group, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. MPTP-treated or LPS + ATP group. $^{\$\$}p < 0.01$ represented the statistical analysis of the two groups.

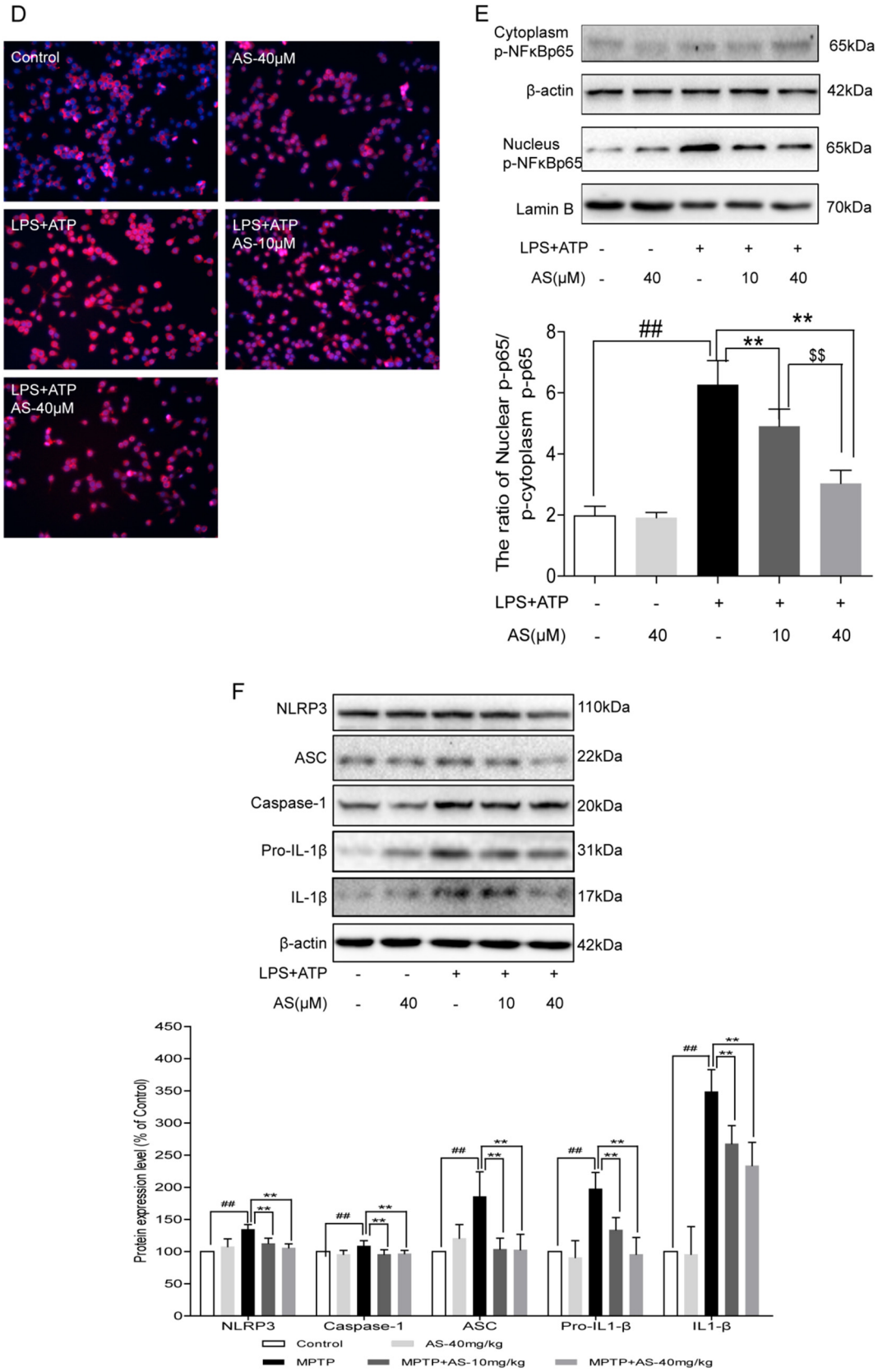

Fig. 4. (*continued*)

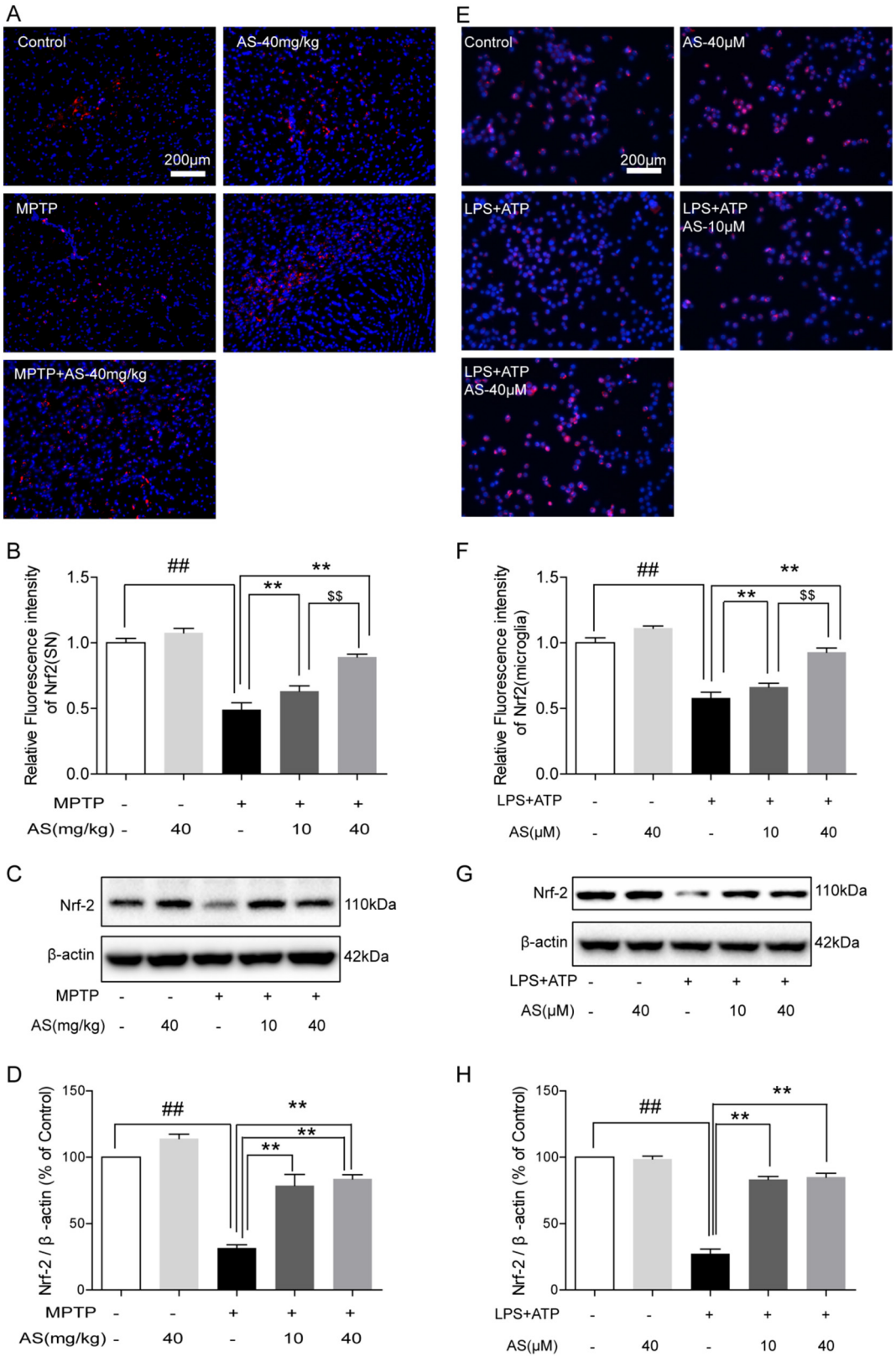

Fig. 5. Astragaloside IV activates Nrf2 in MPTP-induced PD mice and LPS-induced BV2 microglia cells.
Representive immunofluorescence images of Nrf2 nuclear translocation in MPTP mice (A) and BV2 microglia (E). The relative fluorescence intensity of Nrf2 in MPTP mice (B) and BV2 microglia (n = 4 per group) (F). The protein bands and statistic results of Nrf2 in midbrain (C-D) and BV2 microglia (G-H) was measured by western blotting and β-actin was detected as an internal control (n = 3 per group). The ROS generation in BV2 microglia was revealed by the DCFH-DA (I-J) and DHE assays (K-L). Experimental values were expressed as means ± SEM, $^{\#}p < 0.05$and $^{\#\#}p < 0.01$, vs. Control group, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. MPTP-treated or LPS + ATP group, $^{\$\$}p < 0.01$ represented the statistical analysis of the two groups.

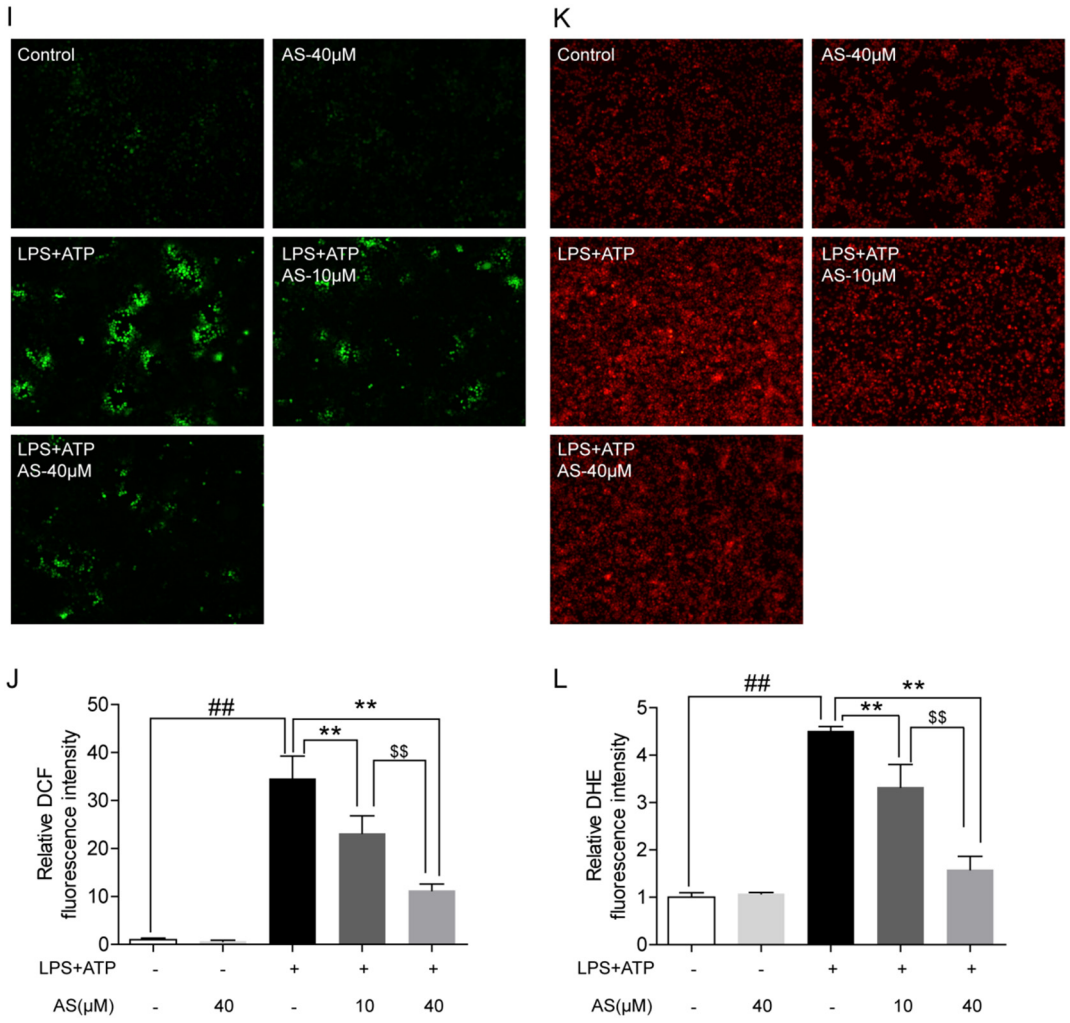

Fig. 5. (*continued*)

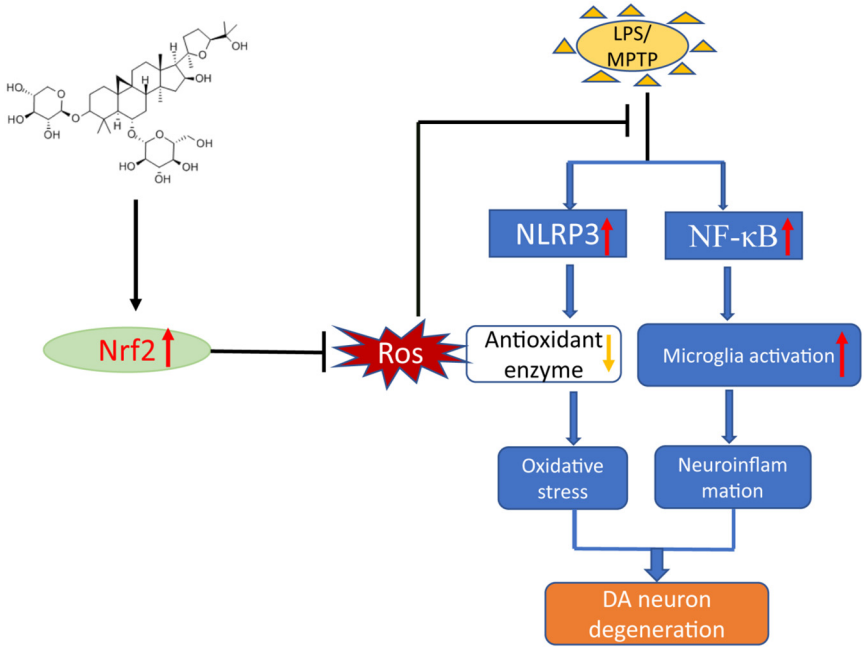

Fig. 6. Mechanisms that Astragaloside IV protects dopaminergic neurons form neuroinflammation and oxidative stress.

The MPTP mouse model is a classical experimental model to mimic the clinical changes, biochemical and neuropathological changes in human PD [41,42]. Therefore, in this investigation, we established an acute PD animal model to investigate the effect of Astragaloside IV on motor deficits and neuropathological changes. We determined Astragaloside IV is effective to alleviate the motor impairment induced by MPTP, which is accordance with previous study [37]. Moreover, our data also demonstrated that amelioration of Astragaloside IV treatment in selective loss and senesce of dopaminergic neurons, which further support the hypothesis that Astragaloside IV might be the potential drug for PD therapy.

Increasing evidence suggests neuroinflammation and oxidative stress are the early and key events of PD, which might be the leading factor to cause dopaminergic degeneration. In recent years, the antioxidants and anti-inflammatory drugs has become increasingly popular for the treatment of PD [43]. So, we evaluated the effect of Astragaloside IV on neuroinflammation and oxidative stress in MPTP-induced mice. The microglia play a vital role in the development of neuroinflammation of PD. The activated microglia will release inflammatory cytokines such as TNFα and IL-1β, which is detrimental to the dopaminergic neuron survival. Thus, we explored the effect of Astragaloside IV on neuroinflammation via microglia activation. We observed Astragaloside IV suppressed the microglia activation both via changing the morphology and decreasing the number of Iba-1 and CD68 double positive microglia cells. We also determined Astragaloside IV is effective to against oxidative stress induced by MPTP injury evidenced by enhancing the activities of anti-oxidative enzyme including of SOD, GSH-Px, total Glutathione and GSSG. These data indicate Astragaloside IV is effective to against the oxidative stress and neuroinflammation induced by MPTP injury, which might be the key mechanism of Astragaloside IV treatment on PD.

The NLRP3 inflammasome, especially the NLRP3 inflammasome in microglia, is thought to be the key molecular in mediating the development of the neuroinflammation of PD [15,44,45]. Increasing evidence indicated that the activation of NFκB is a prerequisite step (priming) for NLRP3 inflammasome activation. Therefore, we detected and analyzed the level of NFκB and NLRP3 inflammasome of PD both in vivo *and* in vitro. We found a decrease of NFκB nuclear accumulation and NLRP3 inflammasome components protein expression of MPTP mice following with Astragaloside IV treatment. We next stimulated the BV2 microglia with LPS plus ATP (specific NLRP3 activator) to induce an rapidly activation of NFκB and NLRP3 inflammasome. Data in vitro further revealed that Astragaloside IV treatment is effectively to inhibit the NFκB and NLRP3 inflamamsome activation of microglia. These findings indicate that Astragaloside IV could inhibit NLRP3 inflammasome activation of PD, which might be the potential molecular mechanism that Astragaloside IV exert the inhibition effect on neuroinflammation of PD.

Nrf2 is a major regulator of redox homeostasis and activation of Nrf2 could negative regulate NLRP3 by inhibiting ROS-induced NLRP3 priming [17]. Previous studies have showed Astragaloside IV could active Nrf2 on acute kidney and brain injury [34,46]. To measure the effect of Astragaloside IV on Nrf2 activation of PD, we used immunofluorescence and western blotting assays to test the nuclear accumulation and protein expression of Nrf2 both in vivo and in vitro. We found an increase of nuclear accumulation of Nrf2 and Nrf2 level after Astragaloside IV treatment, both in MPTP-induced mice and LPS-induced BV2 microglia cells. Importantly, Astragaloside IV decreased the ROS production induced by LPS and ATP in BV2 microglia. These data indicate that Astragaloside IV could activate Nrf2 and decrease the production of ROS of PD. These results indicate activation of Nrf2 might be the potential therapeutic target for some uncontrolled inflammasome activation–associated disease including PD.

Emerging evidence showed oxidative stress and neuroinflammation are the extremely related events of inflammatory diseases. Oxidative stress can exaggerate pro-inflammatory factors expression and inflammatory cells can similarly trigger overproduction of ROS, which creates a vicious cycle to provoke the development of various inflammatory diseases [47]. In this study, we found that Astragaloside IV is neuroprotective to against the motor deficits and dopaminergic neurodegeneration from neuroinflammation and oxidative stress induced by MPTP. Intriguingly, Astragaloside IV suppresses the NLRP3 inflammasome activation, in the meanwhile, promotes the Nrf2 activation and decreases the ROS production. Taken together, the molecular mechanism of the neuroprotective effect of Astragaloside IV on oxidative stress and neuroinflammation of MPTP-induced PD might be largely depending on activating of Nrf2 (Fig. 6).

There are several potential limitations deserving attention in our experiments. Even though we demonstrated that Astragaloside IV could inhibit the NLRP3 inflammasome signaling, while activates Nrf2 of PD both in vivo and in vitro, whether Astragaloside IV inhibits the NLPRP3 inflammasome directly via activating Nrf2 remains obscure due to the lack of Nrf2 overexpress or NLRP3 knockout mice. Second, the levels of keep1, the company molecular of Nrf2, were not examined in the present study, which might lessen the convincing of the effect of Astragaloside IV on oxidative stress. Therefore, further experiments are needed to settle these issues.

5. Conclusions

In conclusion, our findings demonstrated that Astragaloside IV effectively protected motor deficits and dopaminergic neuron degeneration from oxidative stress and inflammation damage. This study provides beneficial evidence for the application of Astragaloside IV in the prevention of inflammasome and oxidative stress-associated diseases, especially PD.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Authors' contributions

Cong Yang and Yousheng Mo contributed equally to this work.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.05.036.

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