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Myricitrin blocks activation of NF-κB and MAPK signaling pathways to protect nigrostriatum neuron in LPS-stimulated mice

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ABSTRACT

Myricitrin, a bioactive and natural flavonoids, is well known for its anti-inflammatory and antioxidant properties. However, the anti-neuroinflammation and possible mechanism has not been fully elucidated. Therefore, the present study was to investigate the possible mechanism of its neuroprotection and anti-neuroinflammation in the nigrostriatum of LPS-stimulated mice. The results showed that myricitrin improved neuron injury and raised the expressions of PSD-95 protein and TH protein in the nigrostriatum of LPS-stimulated mice. In addition, myricitrin decreased the production of pro-inflammatory factors including IL-1β, IL-6 and TNFα, decreased the level of chemokine MCP-1, and suppressed the expressions of COX-2 and iNOS. Meanwhile, myricitrin suppressed HMGB1, TLR4, and MyD88 expression in the nigrostriatum of LPS-stimulated mice. Furthermore, myricitrin inhibited NF-κB and MAPK signaling pathways activated by LPS. In conclusion, our studies suggest that myricitrin blocks activation of protects NF-κB and MAPK signaling pathways to nigrostiatum neuron from injury in LPS-stimulated mice and is beneficial to treatment nigrostriatum inflammation of PD.

1. Introduction

Recent studies have shown that the pathogenesis of neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease(HD), is closely associated with glial cells activation and peripheral immune cells infiltration, producing and releasing a large number of pro-inflammatory cytokines and chemokines (Ransohoff et al., 2015). PD is associated with low levels of production of dopamine (DA) caused by a progressive loss of neurons in the midbrain, resulting in changes in neural conduction within the nigrostriatum (Patil et al., 2014). Postsynaptic dense area (PSD) is the structural basis for postsynaptic signal transduction and integration. PSD-95 contributes to the normal function of the nerve cells, and it would decrease after synapses losing and can be seen in PD animal model (Fourie et al., 2014; Nash et al., 2005). Tyrosine hydroxylase (TH), an important enzyme in the dopamine generation pathway, is associated with the occurrence of many neurogenic diseases, such as PD, schizophrenia, bipolar disorder and so on (Haavik and Toska, 1998; Kunugi et al., 1998).

Toll-like receptors (TLRs), a classic receptor for microglial activation, can recognize and bind with lipopolysaccharide (LPS) to activate microglia and initiate neuroinflammatory response by accelerating the production of pro-inflammatory cytokines (Qin et al., 2005, 2007). In general sense, during the inflammatory progression, any endogenous factors, microbes or microbial products like LPS selectively bind with the upstream signaling molecules like TLR4, which further triggers the activation of another upstream adaptor protein molecule known as MyD88. Subsequently, MyD88 triggers the interleukin 1 receptor-associated kinases those involving the activation of several inflammatory mediated signaling events, specifically, NF-κB and mitogen-activated protein kinases (MAPK) signaling pathways, and contribute to the release of numerous inflammatory mediators like PGE_2, COX-2, NO, iNOS, TNF-α, IL-1β, IL-6 and so on (Haque et al., 2018; Harikrishnan et al., 2018; Lu et al., 2008). Therefore, NF-κB and MAPK signaling pathways play important roles in inflammatory response.

Myricitrin (Fig. 1), a flavonoid compound isolated from the dry bark of *Myrica rubra* (Lour.) Siebold & Zucc, has been reported various bioactivities, such as anti-inflammatory activtity, anti-oxidative

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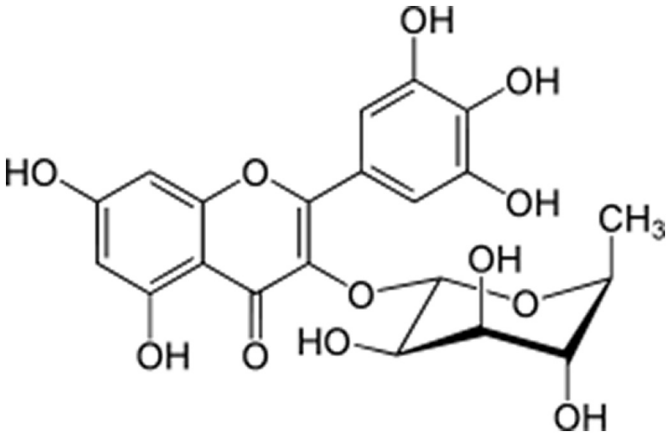

Fig. 1. Chemical structure of myricitrin.

activity, and anti-fibrotic activity (Sun et al., 2013; Domitrović et al., 2015). Our previous investigation have found myricitrin attenuated 6-OHDA-induced mitochondrial dysfunction and alleviated methylglyoxal-induced mitochondrial dysfunction (Wang et al., 2013, 2014). It is concluded that myricitrin might had a potential in treatment of PD. However, the neuroprotection and anti-neuroinflammation of myricitrin in the nigrostriatum are not very clear. Therefore, in the present study, its anti-neuroinflammation and possible mechanism in the nigrostriatum of LPS-stimulated mice were investigated.

2. Materials and methods

2.1. Reagents

LPS was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). IL-1β, IL-6, TNFα, and MCP-1 ELISA kits were purchased from Genetimes Technology Inc. (Shanghai, China). Anti-TH antibody, anti-PSD95 antibody, anti-COX-2 antibody, anti-iNOS antibody, anti-HMGB1 antibody, anti-TLR4 antibody, anti-MyD88 antibody, and anti-β-actin antibody were all the products of Abcam (Cambridge, United Kingdom). Anti-NF-κB p65 antibody, anti-phospho-NF-κB p65 antibody, anti-IκBα antibody, anti-phospho-IκBα, anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-p38, anti-phospho-p38 were all the products of Cell Signaling Technology.

2.2. Animals and treatment

Adult male BALB/c mice weighing 18–22 g were purchased from Laboratory Animal Centre of Beijing Hua-Fu-Kang Bioscience Co, LTD (Beijing, China; Animal certification number was SCXK (Jing) 2014-0004). All animals were kept under controlled temperature of 23 ± 2 °C and humidity of 55 ± 5% with a regular 12 h light-dark cycle. The food and water were available ad libitum. After being acclimatized for 3 days, BALB/c mice were randomly divided into the following groups: Control group (i.g. administration of saline once a day for 7 days); LPS model group (i.g. administration of saline once a day for 7 days and i.p. LPS 5 mg/kg on the 7th day); LPS + MYR-20 group (i.g. administration of myricitrin 20 mg/kg once a day for 7 days and i.p. LPS 5 mg/kg on the 7th day); LPS + MYR-50 group (i.g. administration of myricitrin 50 mg/kg once a day for 7 days and i.p. LPS 5 mg/kg on the 7th day). After LPS stimulation 6 h, the nigrostriatum tissues of mice were harvested for further research. All animal care and experimental procedures regarding the animals were approved by the Animal Experimental Center, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College.

2.3. NeuN staining by immunohistochemistry

After LPS administration, the mice were perfused with 0.1 M PBS and followed by 4% paraformaldehyde for immunofluorescence staining (Yang et al., 2019). Briefly, brain sections were mounted onto gelatin-coated coverslips and allowed to air dry. Subsequently, after blocked and permeabilized, brain sections were incubated with anti-NeuN primary antibody (1:200) for overnight at 4 °C. After washing with PBS, the brain sections were incubated with Cy3-conjugated secondary antibody for 2 h at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Finally, cover-slips were mounted onto microscope slides and digital images were collected.

2.4. ELISA assay

The nigrostriatum tissues of mice were homogenized in cool saline and centrifugated at 4500 rpm for 10 min at 4 °C. The protein concentration of supernatant was quantified by BCA assay and the levels of IL-1β, IL-6, TNF-α, and MCP-1 in the supernatant were detected by ELISA kits according to manufacturer's instructions. Briefly, 100 μL of the supernatant samples and standards were added into each well of ELISA plate. Meantime, the biotinylated anti-mouse antibody solution was added to each well except for the blank control well, and incubated for 2 h at 37 °C. Then the plates were washed with wash buffer for 5 times. After washing, 100 μL of the HRP-streptavidin conjugated secondary antibody was added to each well and incubated for 60 min at 37 °C. Subsequently, TMB solution was added and incubated at room temperature for 15 min. At last, stop solution was added to each well and absorbance at 450 nm was measured using a microplate reader. The cytokine concentration was determined using a standard curve. Cytokines level are expressed as pg of cytokines/mg total protein.

2.5. Western blot analysis

The nigrostriatum tissues of mice were homogenized in cool RIPA buffer with the cocktail protease inhibitor and lysed on the ice for 30 min. Afterwards, the supernatant was collected and quantified by BCA assay. Proteins were separated by 10% SDS-PAGE gel and transferred on the PVDF membranes. The membranes were incubated in 5% BSA solution in order to occupy the nonspecific sites on the membranes at 37 °C. Next, the membranes were incubated with anti-COX-2 antibody (Rt, 1:2000), anti-iNOS antibody (Rt, 1:1000), anti-PSD95 antibody (Rt, 1:2000), anti-TH antibody (Rt, 1:1000), anti-HMGB1 antibody (Rt, 1:1000), anti-TLR4 antibody (Rt, 1:1000), anti-MyD88 antibody (Rt, 1:1000), anti-NF-κB p65 antibody (Rt, 1:1000), anti-Phospho-NF-κB p65 antibody (Rt, 1:1000), anti-IκBα antibody (Ms, 1:1000), anti-Phospho-IκBα (Rt, 1:1000), anti-ERK (Rt, 1:1000), anti-Phospho-ERK (Rt, 1:1000), anti-JNK (Rt, 1:1000), anti-Phospho-JNK (Rt, 1:1000), anti-p38 (Ms, 1:1000), anti-Phospho-p38 (Rt, 1:1000) and anti-β-actin antibody respectively at 4 °C overnight. After horseradish peroxidase-conjugated secondary antibody incubation, the signal densities on the blots were measured using the enhanced ECL system and normalized using an internal control. The results from animals under various experiment conditions then were normalized by value of the corresponding control animal (fold change relative to control).

2.6. Real-time PCR analysis

TRIzol method was used to extract RNA as described previously (Cheng et al., 2018). The nigrostriatum tissues of mice were prepared by homogenizer in cool Trizol. Then, the supernatants were added 200 μL Chloroform and vortexed dramatically. After centrifuging at 12,000*g* for 15 min at 4 °C, the supernatants were collected and added 500 μL Isopropanol. After standing at room temperature for 30 min, the samples were centrifuged at 12,000*g* for 15 min at 4 °C. After removing

the supernatant, the precipitate was washed by 75% ethanol. Centrifuging at 12,000*g* for 5 min at 4 °C, the supernatant was removed completely. Adding RNase free water into the pipes to fully dissolve RNA, the RNA was quantified by measuring the absorbance at 260 nm and 280 nm. Use the formula A260 × dilution × 40 = μg RNA/mL to determine concentration. RNA was reverse transcribed into cDNA by using the PrimeScript RT reagent kit. After RNA was reverse transcribed to cDNA, the cDNA was used to real-time PCR by using TransStart Top Green qPCR Super Mix. The Primers included HMGB1 (forward, GGC TGACAAGGCTCGTTATG; reverse, GGGCGGTACTCAGAACAGAA), TLR4 (forward, CTCTGGGGAGGCACATCTTC; reverse, CAGGTCCAAG TTGCCGTTTC), MyD88 (forward, CCGCCTATCGCTGTTCTTGA; reverse, CCAGGCATCCAACAAACTGC) and β-actin (forward, AGGCCAA CCGTGAAAAGATG; reverse, TGGCGTGAGGGAGAGCATAG). Relative quantitative analysis was determined using real-time SYBR green fluorescence and then calculated by the relative quantification of $2^{-\Delta\Delta Ct}$ method and using β-actin as internal reference.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.02 (GraphPad Software Inc., CA, USA) to compare the differences between each group. Data are expressed as the mean ± SD. Differences between the various groups were compared using one-way ANOVA and the Student-Newman-Keuls test, and $p < .05$ was considered as statistically significant.

3. Results

3.1. Myricitrin improved neuron morphology in nigrostriatum of LPS-stimulated mice

NeuN is a widely used marker for mature neurons expressed in nucleus and cell body of neurons. As compared to normal control, a clear decrease in NeuN staining was observed in LPS-stimulated mice, which may reflect of stunned or damaged cells were increased stimulated by LPS. However, after treatment with myricitrin 50 mg/kg, there was a increase in NeuN staining of nigrostriatum as compared to LPS-stimulated mice (Fig. 2A and B).

3.2. Myricitrin upregulated the expression of TH and PSD-95 in nigrostriatum of LPS-stimulated mice

To further explore the protective effect of myricitrin on nigrostriatum, the levels of TH and PSD-95 in nigrostriatum were detected by WB assay. In LPS-stimulated group, the expression of TH and PSD-95 were both lower than that of in control group (both $p < .01$). However, myricitrin treatment at the doses of 20 mg/kg and 50 mg/kg both significantly improved the expression of TH (Fig. 3A) and PSD-95 (Fig. 3B) decreased by LPS injury. These data suggested that myricitrin could protect against nigrastriatum neuron injury by LPS.

3.3. Myricitrin decreased inflammatory factors and mediators in nigrostriatum of LPS-stimulated mice

To evaluate the anti-inflammationary effects of myricitrin, the levels of IL-1β, IL-6, TNF-α and MCP-1 were assayed by ELISA. In LPS-stimulated group, the levels of IL-1β, IL-6, TNF-α, and MCP-1 were all increased in nigrostriatum tissues (all $p < .01$). However, myricitrin at the doses of 25 and 50 mg/kg both significantly decreased the levels of IL-1β (Fig. 4A), IL-6 (Fig. 4B), TNF-α (Fig. 4C), and MCP-1 (Fig. 4D) in nigrostriatum tissues. In addition, the levels of COX-2 and iNOS were determined by WB. The results showed that the expression of COX-2 and iNOS were increased in the LPS-stimulated group (both $p < .01$). Whereas, myricitrin at the doses of 20 mg/kg and 50 mg/kg treatment both significantly decreased the expressions of COX-2 (Fig. 5A) and iNOS (Fig. 5B).

3.4. Myricitrin down-regulated TLR4-MyD88 signaling pathway in nigrostriatum of LPS-stimulated mice

Whether myricitrin exerted anti-neuroinflammatory effects associate with regulating HMGB1, TLR4, MyD88? To answer this question, the expression of HMGB1, TLR4 and MyD88 were determined both at at protein level and mRNA level. Fig. 6A, B, and C showed that protein levels of HMGB1, TLR4 and MyD88 in nigrostriatum of LPS-stimulated mice were all higher than that of in normal control group. Then we detected the mRNA level of HMGB1, TLR4 and MyD88 in nigrostriatum of LPS-stimulated mice were all higher than that of in normal control group (Fig. 6D, E and F). However, myricitrin treatment at the doses of 20 mg/kg and 50 mg/kg significantly suppressed HMGB1, TLR4 and MyD88 expression both at protein level and at mRNA level. These results indicate that myricitrin is likely to exert anti-neuroinflammatory effects via down-regulating TLR4/MyD88 pathway.

3.5. Myricitrin inhibited the phosphorylation of NF-κB signaling pathway in nigrostriatum of LPS-stimulated mice

The activation of NF-κB signaling pathway is involved in LPS-stimulated inflammation process. Next, we detected the expression of NF-κB p65, phospho-NF-κB p65, IκBα and phospho-IκBα by western blot. Results indicated that the expression of phospho-NF-κB p65 (Fig. 7A, $p < .05$) and phospho-IκBα (Fig. 7C, $p < .01$) were increased after LPS application. And the ratios of p-p65/p65 (Fig. 7B, $p < .01$) and p-IκBα/IκBα (Fig. 7D, $p < .01$) were significantly higher than that of control group. While myricitrin treatment inhibited the expression of phospho-NF-κB p65 and phospho-IκBα, which suggested that myricitrin alleviated LPS-induced neuroinflammation in nigrostriatum of mice by inhibiting the activation of NF-κB signaling pathway.

3.6. Myricitrin suppressed translocation of NF-κB in nigrostriatum of LPS-stimulated mice

In addition to the phosphorylation of NF-κB signaling pathway, the NF-κB nuclear translocation was further investigated. The results showed that the level of NF-κB p65 in nucleus was significantly increased in nigrostriatum of LPS-stimulated mice (Fig. 8A, $p < .01$), but there was no significant difference in cytoplasm (Fig. 8B). However, myricitrin treatment at doses of 20 mg/kg and 50 mg/kg both suppressed NF-κB nuclear translocation to nucleus (Fig. 8A, both $p < .01$). These results indicates that myricitrin is a negative mediator of LPS-activated NF-κB nuclear translocation in nigrostriatum of LPS-stimulated mice.

3.7. Myricitrin down-regulated phosphorylation of p38, ERK and JNK in nigrostriatum of LPS-stimulated mice

Activation of MAPK signaling pathways leads to the expression of inflammatory factors and promotes inflammatory processes. Therefore, MAPK signaling pathways are considered to be crucial mechanisms in controlling inflammatory activity. To elucidate the mechanism of anti-inflammatory action of myricitrin in MAPK signaling pathway, the expression of ERK, p-ERK, JNK, p-JNK, p38, and p-p38 were further determined by western blot assay. As shown in Fig. 9A, C, and E, LPS upregulated the phosphorylation of the p38, JNK and ERK in the nigrostriatum of LSP-stimulated mice ($p < .01$, $p < .01$, $p < .05$, respectively). In addition, the ratios of p-p38 to p38 (Fig. 9B), p-ERK to ERK (Fig. 9D), and p-JNK to JNK (Fig. 9F) were all significantly increased in nigrostriatum of LPS-stimulated mice compared to control group ($p < .01$, $p < .01$, $p < .05$, respectively). However, myricitrin treatment at the doses of 20 and 50 mg/kg significantly suppressed the phosphorylation of p38, ERK and JNK.

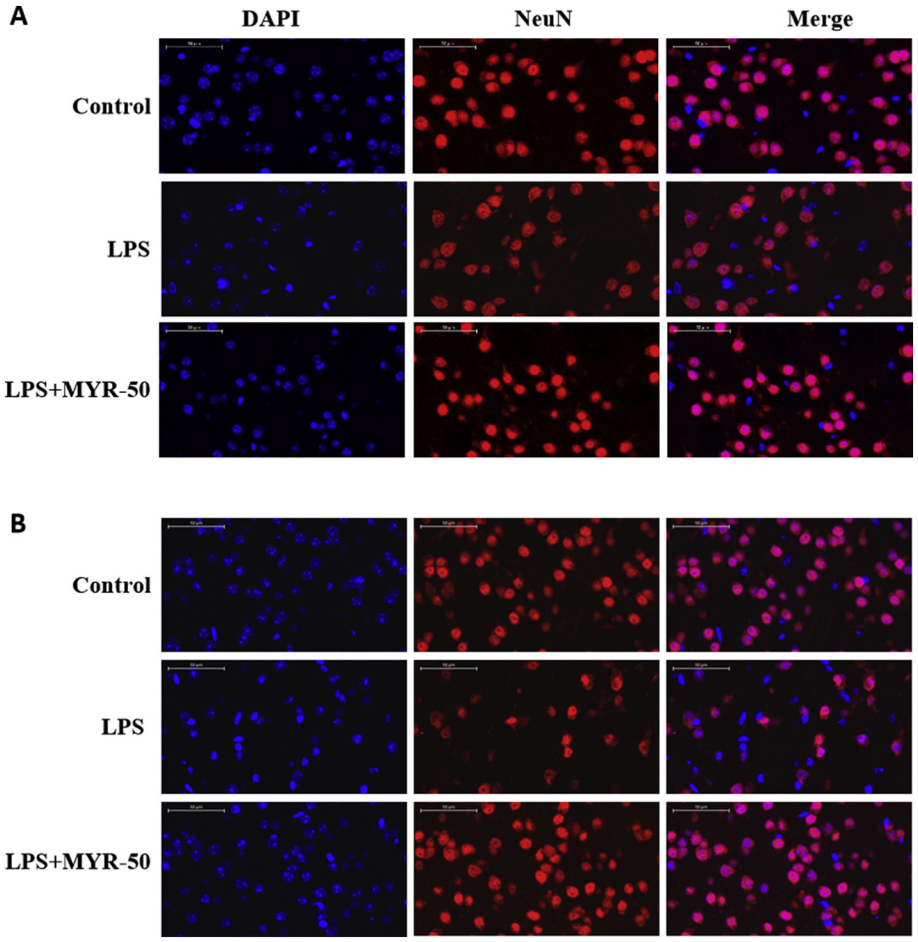

Fig. 2. Representative fluorescent micrographs of immunofluorescent staining with the neuronal antibody NeuN. Scale bars: 50 μm.

4. Discussion

In the present study, we investigated the anti-neuroinflammation of myricitrin and explored the mechanism. Our study found that myricitrin has a potential anti-neuroinflammation in the nigrostriatum of LPS-stimulated mice. Meanwhile, we firstly found that myricitrin exerts anti-neuroinflammation associated with inhibiting TLR4/MyD88 pathway, blocking activation of NF-κB and phosphorylation of MAPKs.

NeuN is expressed in nucleus and cell body of most neurons and not in glial cells, oligodendrocytes, astrocytes, or microglial cells (Wolf et al., 1996). Immunoreactivity for NeuN has been reported to decrease dramatically following CNS injury (Igarashi et al., 2001; Davoli et al., 2002; Sugawara et al., 2002). Previous studies have demonstrated that loss of NeuN immunoreactivity occurs with CNS injury (Bendel et al., 2005; Ajmo Jr. et al., 2006). Similar to previous studies, we demonstrated a clear decrease in NeuN staining following LPS injury as

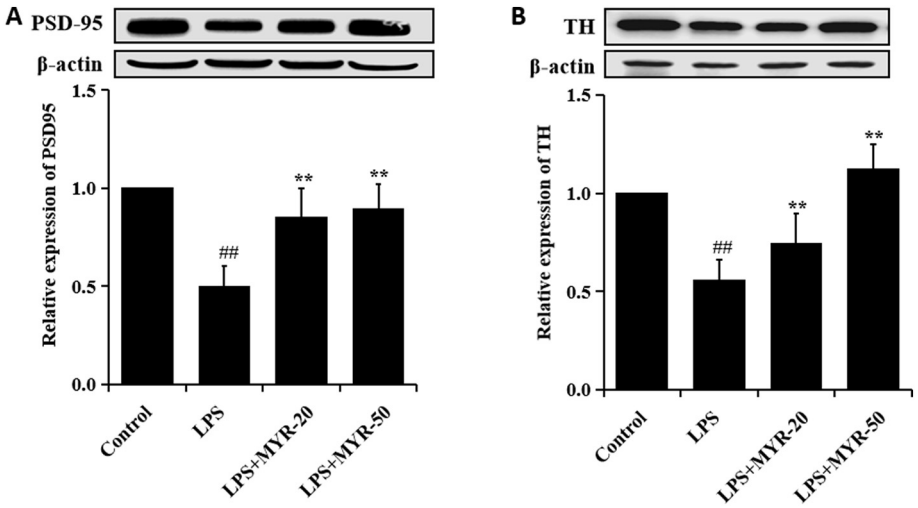

Fig. 3. Effect of myricitrin on the expressions of TH and PSD-95 in nigrostriatum of LPS-stimulated mice. Values are mean ± SD of four experiments. $^{\#\#}p < .01$ vs. Control group; $^{**}p < .01$ vs. LPS group.

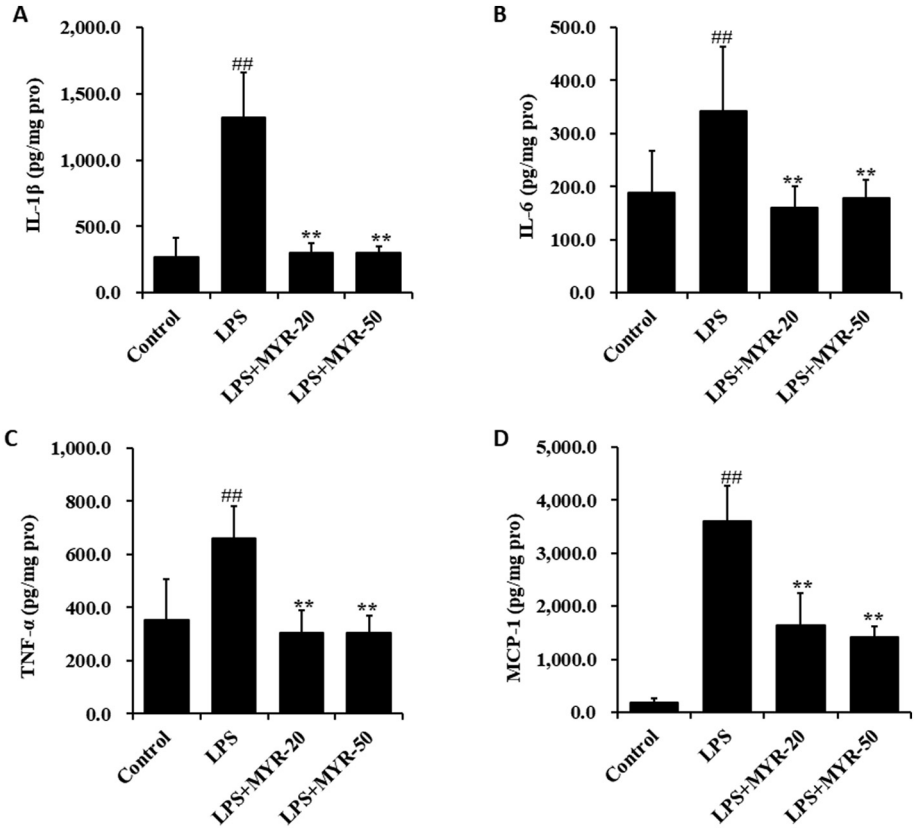

Fig. 4. Effect of myricitrin on inflammatory factors in nigrostriatum of LPS-stimulated mice. Values are mean ± SD of four experiments. $^{\#\#}p < .01$ vs. Control group; $^{**}p < .01$ vs. LPS group.

compared to normal group. Whereas, there was clear increase in NeuN staining after myricitrin treatment, which indicated that myricitrin treatment improved stunned or damaged neuron injured by LPS in nigrostriatum of mice.

PD is a common central nervous system degenerative disease in the elderly, with pathological changes in the nigrostriatal DA neurons (Schneider et al., 2017). TH participates in the synthesis of dopamine and catalyzes the conversion of tyrosine to the precursor of DA (Tekin et al., 2014). The reduction of TH in the nigrostriatal DA region is a characteristic change in PD (Johnson et al., 2019). Recent studies have confirmed that the expression of TH in mouse brain is significantly reduced after stimulation with LPS in mice during puberty, while mice show Parkinson-like behavior (Wang et al., 2010). Consistent with previous study, we also found LPS decreased the expression of TH in this study. Furthermore, myricitrin treatment improved TH expression in nigrostriatum of LPS-stimulated mice. This indicated that myricitrin has a protective effect on nigrostriatum and improves the decrease of DA after brain injury. Therefore, it is speculated that myricitrin may play a role in the treatment of PD, but the effect of myricitrin on the level of DA and its metabolites still need to be further explored in future research.

PSD-95 is a member of the membrane-associated guanylate kinase family and is involved in pathophysiological processes through interaction with membrane proteins. In recent years, more and more studies

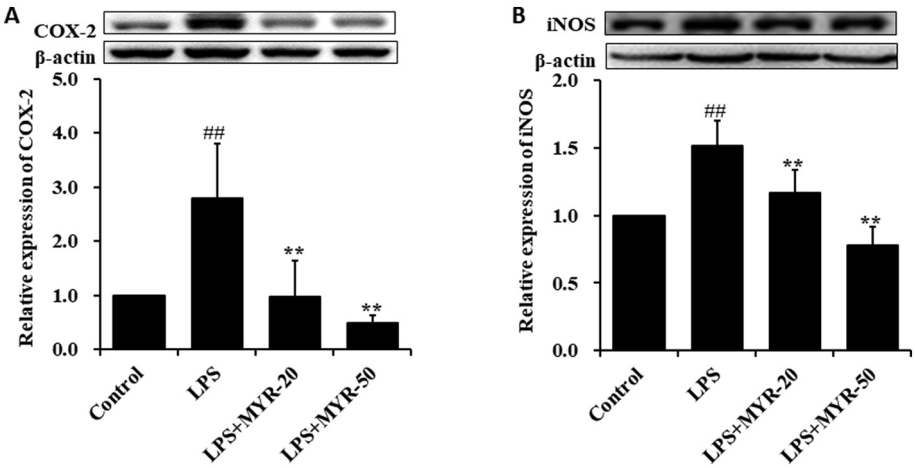

Fig. 5. Effect of myricitrin on the expression of COX2 and iNOS in nigrostriatum of LPS-stimulated mice. Values are mean ± SD of four experiments. $^{\#\#}p < .01$ vs. Control group; $^{**}p < .01$ vs. LPS group.

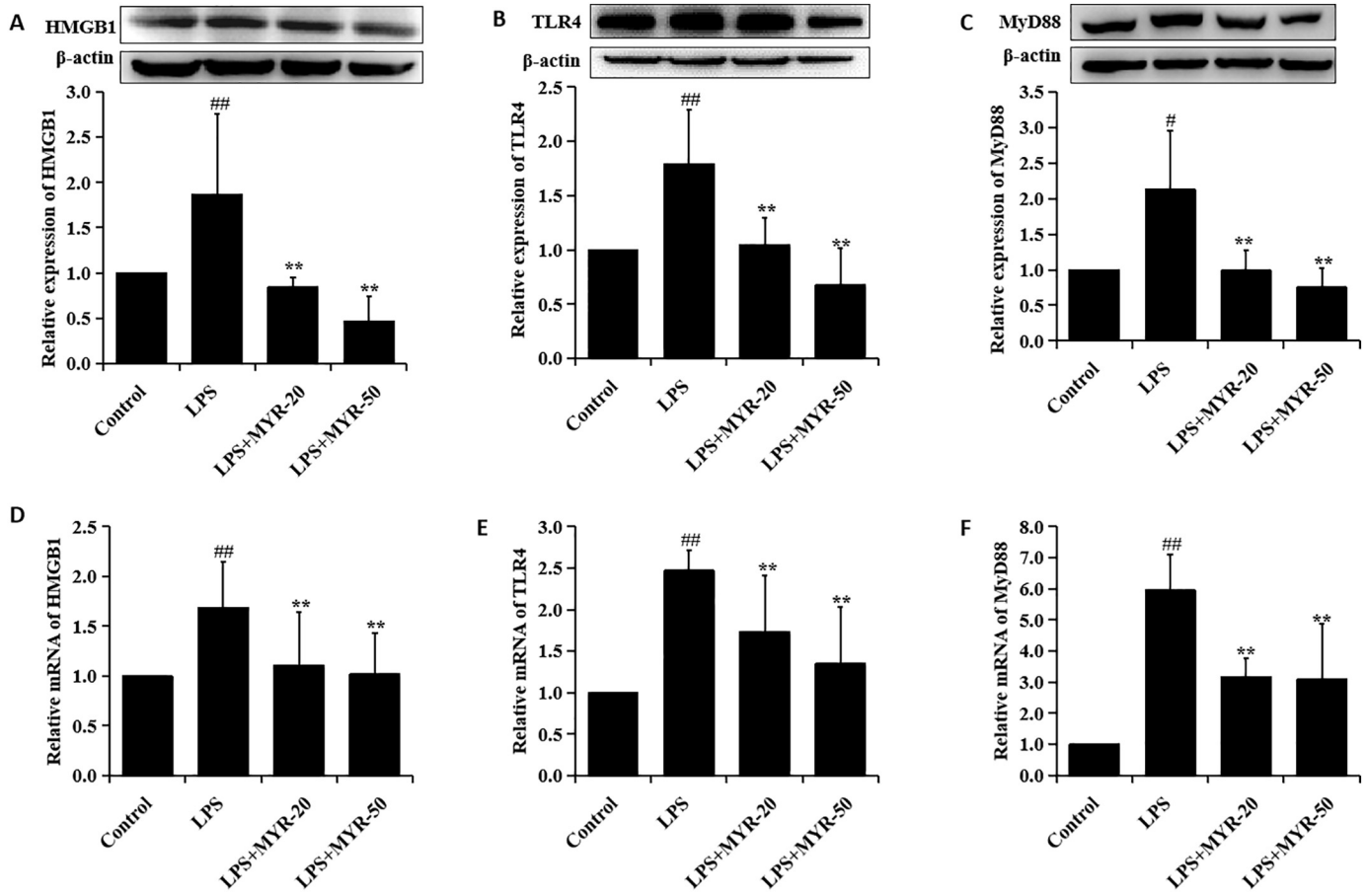

Fig. 6. Effect of myricitrin on the protein expression and mRNA level of HMGB1, TLR4 and MyD88. (A) Relative expression of HMGB1 protein; (B) Relative expression of TLR4 protein; (C) Relative expression of MyD88 protein; (D) Relative mRNA level of HMGB1; (E) Relative mRNA level of TLR4; (F) Relative mRNA level of MyD88. Values are mean ± SD of four experiments. $^{\#\#}p < .01$ vs. Control group; $^{**}p < .01$ vs. LPS group.

have found that PSD-95 is closely related to neurodegenerative diseases and cerebral ischemia injury (Wang et al., 2010). Recent studies have shown that synaptic proteins are closely related to learning and memory functions, and excessive loss of synaptic proteins may cause cognitive impairment (Zong et al., 2019). Our results showed that the expression of PSD-95 in the nigrostriatum was significantly decreased in nigrostriatum of LPS-stimulated mice, whereas myricitrin treatment improved PSD-95 expression. These results indicated that myricitrin exerts neuroprotetion by improving synaptic density in nigrostriatum of LPS-stimulated mice.

Accumulating evidence has demonstrated that neuroinflammation plays an important role in the progression of neurodegenerative diseases (Niranjan, 2018). It is currently believed that characteristic of neuroinflammation are glial cell activation, destruction of the blood-brain barrier, and peripheral immune cells entering the brain parenchyma (Tsui et al., 2018). Our data showed that LPS promoted the release of inflammatory cytokines, such as IL-1β, IL-6, TNF-α, MCP-1, COX-2 and iNOS, in the nigrostriatum of LPS-stimulated mice. It is to be noted that from our experiment myricitrin could suppress the levels of these inflammatory factors in nigrostriatum of LPS-stimulated mice. These data indicated that myricitrin can protect the nigrostriatum from neuroinflammation injury.

HMGB1, a non-histone chromatin-binding protein, is widely involved in various inflammatory reactions and is related to many CNS diseases, including stroke, traumatic brain injury, PD, AD and epilepsy (Nishibori, 2018; Nishibori et al., 2019). Studies have demonstrated that inhibition of HMGB1 helps to reduce neuroinflammation and improve BBB destruction in PD (Sasaki et al., 2016). It is reported that LPS increased expression of HMGB1 in brain tissue, and participated in LPS-induced neuroinflammatory processes (Cheng et al., 2018; Li et al., 2013, 2018). Consistent with previous studies, our results also showed that the expression of HMGB1 in the nigrostriatum of mice was increased after LPS challenge. And as expected, myricitrin downregulated the level of HMGB1. HMGB1 and LPS can bind to TLR4 with the dimerization and upregulating of MyD88, which can trigger and promote the downstream signal transduction, such as NF-κB, MAPKs pathway (Sasimol et al., 2018; Wu et al., 2018). In our results, we also found that LPS increased the level of TLR4 and MyD88 and myricitrin can downregulate the expressions of TLR4 and MyD88.

NF-κB, a family of transcription factors, have the effect on regulation of inflammatory response (Vallabhapurapu and Karin, 2009). In general, NF-κB is sequestered by inhibitors of κB (IκB) in the cytoplasm in resting cells. Once receiving stimuli, such as LPS, NF-κB was release from IκB and translocated from cytoplasm into nucleus to promote the transcription of downstream genes (Nepali et al., 2017). NF-κB is known to play a vital role in the mediation of immune and inflammatory responses (Zhang and Ghosh, 2001). Under normal conditions, the NF-κB dimers p50 and p65 exist in the cytoplasm in a complex with the inhibitor protein IκB. When IκB is activated, it is phosphorylated and then ubiquitinated, leading to its degradation. Subsequently, the free NF-κB dimer translocates to the nucleus and promoting the transcription of various pro-inflammatory enzymes (Mercurio and Manning, 1999; Ghosh and Hayden, 2008; Lappas et al., 2002; Ren et al., 2019; Cai et al., 2018). Many studies have found that inhibition of NF-κB pathway can effectively reduce LPS-induced inflammation (Fu et al., 2018; Ding et al., 2018). In this study, we also found that the expression of phosphorylated IκB and NF-κB were remarkably upregulated in LPS-stimulated group, which are consistent with previous

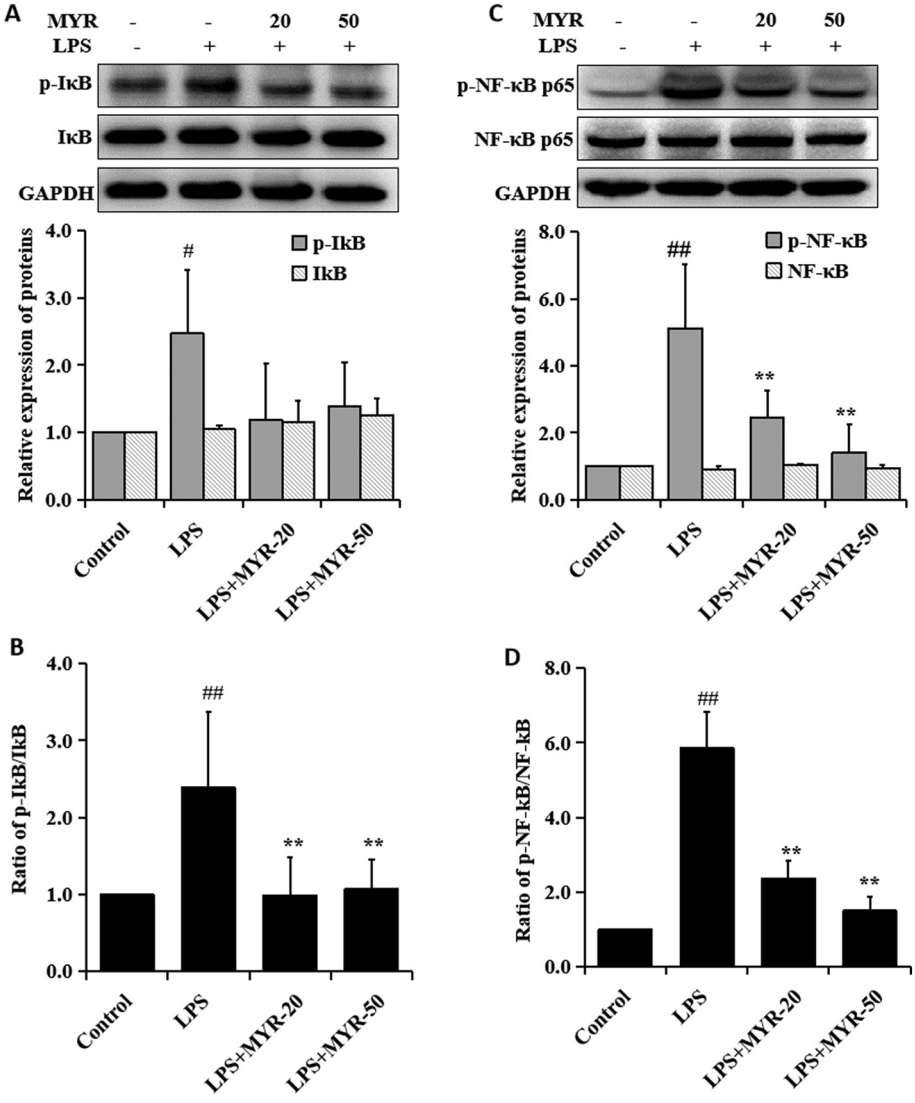

Fig. 7. Effect of myricitrin on phosphorylation of IκB and NFκB-p65. (A) Representative images and relative expression of p-IκB and IκB; (B) Ratio of p-IκB to IκB. (C) Representative images and relative expression of p-NF-κB p65 and NF-κB p65; (D) Ratio of p-NF-κB p65 to NF-κB p65. Values are mean ± SD of four experiments. $^{\#}p < .01$, $^{\#\#}p < .01$ vs. Control group; $^{**}p < .01$ vs. LPS group.

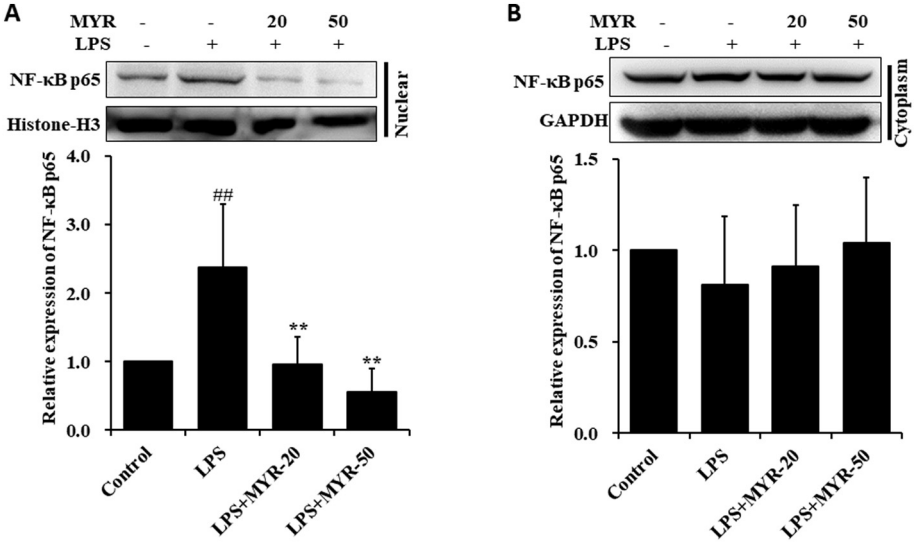

Fig. 8. Effect of myricitrin on nuclear transposition of NF-κB p65. (A) Representative images and relative expression of NF-κB p65 in nuclear fraction. (B) Representative images and relative expression of NF-κB p65 in cytoplasmic fraction. Values are mean ± SD of four experiments. $^{\#\#}p < .01$ vs. Control group; $^{**}p < .01$ vs. LPS group.

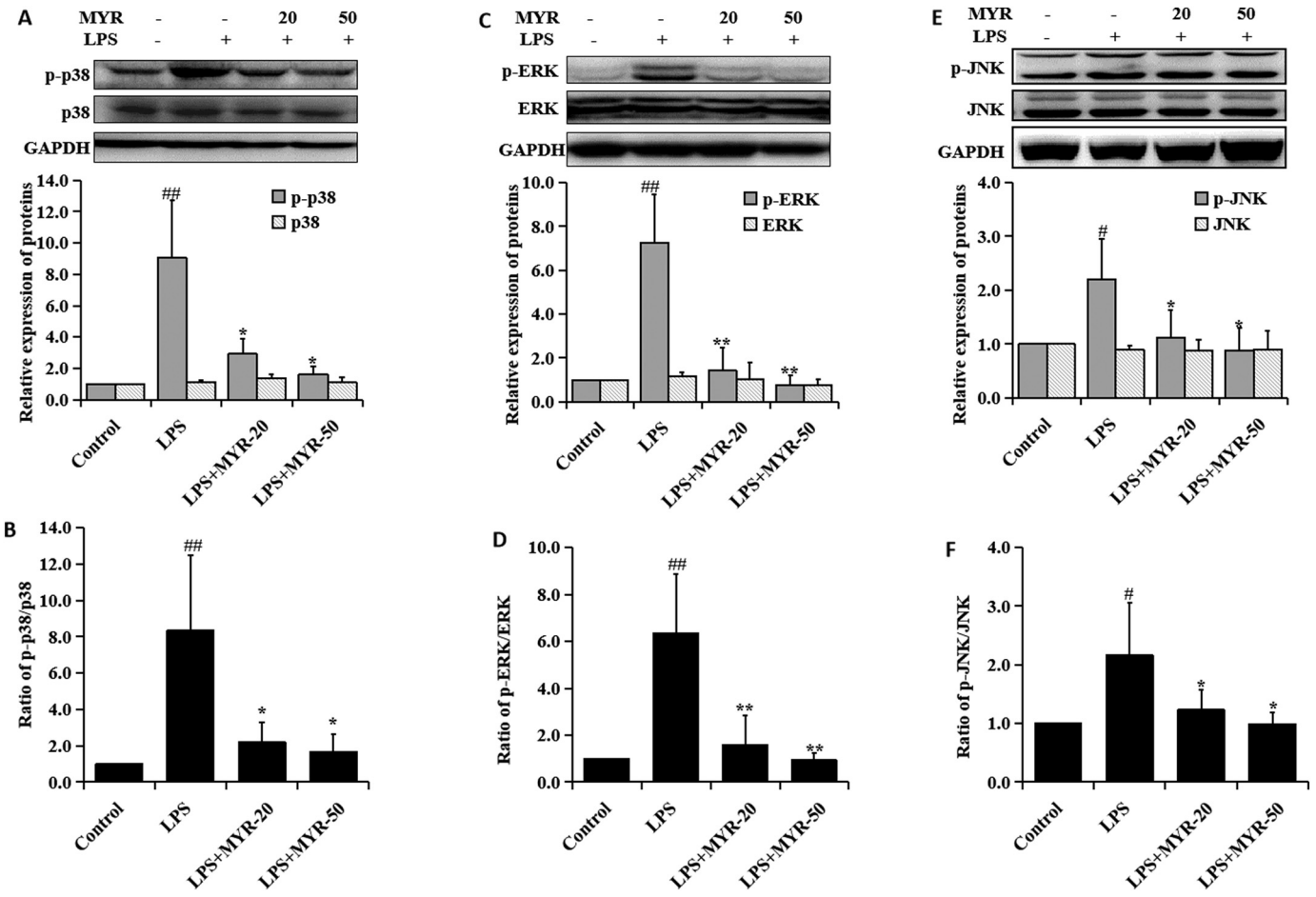

Fig. 9. Effect of myricitrin on the phosphorylation of p38, ERK and JNK in the stratum tissues of mice stimulated by LPS. (A) Representative images and relative expression of p-p38 and p38; (B) Ratio of p-p38 to p38; (C) Representative images and relative expression of p-ERK and ERK; (D) Ratio of p-ERK to ERK; (E) Representative images and relative expression of p-JNK and JNK; (F) Ratio of p-JNK to JNK. Values are mean ± SD of four experiments. $^{\#}p < .01$, $^{\#\#}p < .01$ vs. Control group; $^{*}p < .05$, $^{**}p < .01$ vs. LPS group.

studies. However, myricitrin revealed concentration-dependent suppression of phosphorylation of IκB and NF-κB. And we found that myricitrin exerts anti-inflammatory effects mainly by affecting the translocation and phosphorylation of NF-κB pathway. In a conclusion, the anti-inflammatory mechanism of myricitrin is likely related to inhibition of NF-κB pathway.

MAPKs are intracellular serine/threonine protein kinases that consist of extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun NH2-terminal kinase (JNK) (Reibman et al., 2000). MAPKs are a major family of kinases associated with the inflammation process (Pearson et al., 2001; Yu et al., 2018). Many stress stimuli can activate MAPKs like ionizing radiation, cytokines and growth factors (Zhang et al., 2017). Moreover, lipopolysaccharide (LPS) can activate MAPKs, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. These proteins then modulate cytokine production and the expression of pro-inflammatory enzymes, such as NF-κB, iNOS, COX-2, TNF-α, and IL-1β (Rao, 2001; Rajapakse et al., 2008). In this study, our results showed that LPS activated the MAPKs proteins, such as p38, ERK, and JNK. However, myricitrin can reversed the activation of p38, ERK and JNK induced by LPS. But there are many downstream targets of the MAPK pathways, and the role of myricitrin for its downstream targets still needs further exploration and confirmation.

In conclusion, our studies found myricitrin protects nigrostriatum neuron in LPS-stimulated mice, which is associated with its anti-neuroinflammtion via blocking the activation of NF-κB and MAPKs signaling pathways. It is speculated that myricitrin is beneficial to treatment of PD for its anti-neuroinflammation in nigrostriatum. Our investigation will enrich the pharmacological effects of myricitrin, expand the scope of clinical application, and provide new idea for neuroprotion and anti-neuroinflammation treatment in PD. However, in this experiment, we preliminarily observed whether myricetin plays roles in the NF-κB and MAPKs signaling pathways and there is a lack of in-depth study about these signaling pathways. In-depth study in the future, different specific inhibitors (e.g., p38, JNK, ERK, NF-κB inhibitors) will be used to further explore the mechanism of myricitin.

Declaration of Competing Interests

The authors declare no conflict of interest.

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References

Ajmo Jr., C.T., Vernon, D.O., Collier, L., Pennypacker, K.R., Cuevas, J., 2006. Sigma

receptor activation reduces infarct size at 24 hours after permanent middle cerebral artery occlusion in rats. Curr. Neurovasc. Res. 3, 89–98.
Bendel, O., Alkass, K., Bueters, T., von Euler, M., von Euler, G., 2005. Reproducible loss of CA1 neurons following carotid artery occlusion combined with halothane-induced hypotension. Brain Res. 1033, 135–142.
Cai, B., Seong, K.J., Bae, S.W., Chun, C., Kim, W.J., Jung, J.Y., 2018. A synthetic diosgenin primary amine derivative attenuates LPS-stimulated inflammation via inhibition of NF-κB and JNK MAPK signaling in microglial BV2 cells. Int. Immunopharmacol. 61, 204–214.
Cheng, X., Yang, Y.L., Yang, H., Wang, Y.H., Du, G.H., 2018. Kaempferol alleviates LPS-induced neuroinflammation and BBB dysfunction in mice via inhibiting HMGB1 release and down-regulating TLR4/MyD88 pathway. Int. Immunopharmacol. 56, 29–35.
Davoli, M.A., Fourtounis, J., Tam, J., Xanthoudakis, S., Nicholson, D., Robertson, G.S., Ng, G.Y., Xu, D., 2002. Immunohistochemical and biochemical assessment of caspase-3 activation and DNA fragmentation following transient focal ischemia in the rat. Neuroscience 115, 125–136.
Ding, R.R., Chen, W., Guo, C.Y., Liao, W.T., Yang, X., Liao, F.E., Lin, J.M., Mei, H.F., Zeng, Y., 2018. Dangguishaoyao-San attenuates LPS-induced neuroinflammation via the TLRs/NF-κB signaling pathway. Biomed. Pharmacother. 105, 187–194.
Domitrović, R., Rashed, K., Cvijanović, O., Vladimir-Knežević, S., Škoda, M., Višnić, A., 2015. Myricitrin exhibits antioxidant, anti-inflammatory and antifibrotic activity in carbon tetrachloride-intoxicated mice. Chem. Biol. Interact. 230, 21–29.
Fourie, C., Kim, E., Waldvogel, H., Wong, J.M., McGregor, A., Faull, R.L., Montgomery, J.M., 2014. Differential changes in postsynaptic density proteins in postmortem Huntington's disease and Parkinson's disease human brains. J. Neurodegener. Dis. 2014, 938530.
Fu, L., Zhu, P., Qi, S., Li, C., Zhao, K., 2018. MicroRNA-92a antagonism attenuates lipopolysaccharide (LPS)-induced pulmonary inflammation and injury in mice through suppressing the PTEN/AKT/NF-κB signaling pathway. Biomed. Pharmacother. 107, 703–711.
Ghosh, S., Hayden, M.S., 2008. New regulators of NF-kappaB in inflammation. Nat. Rev. Immunol. 8, 837–848.
Haavik, J., Toska, K., 1998. Tyrosine hydroxylase and Parkinson's disease. Mol. Neurobiol. 16 (3), 285–309.
Haque, M.A., Jantan, I., Harikrishnan, H., 2018. Zerumbone suppresses the activation of inflammatory mediators in LPS-stimulated U937 macrophages through MyD88-dependent NF-κB/MAPK/PI3K-Akt signaling pathways. Int. Immunopharmacol. 55, 312–322.
Harikrishnan, H., Jantan, I., Haque, M.A., Kumolosasi, E., 2018. Phyllanthin from *Phyllanthus amarus* inhibits LPS-induced proinflammatory responses in U937 macrophages via downregulation of NF-κB/MAPK/PI3K-Akt signaling pathways. Phytother. Res. 32, 2510–2519.
Igarashi, T., Huang, T.T., Noble, L.J., 2001. Regional vulnerability after traumatic brain injury: gender differences in mice that overexpress human copper, zinc superoxide dismutase. Exp. Neurol. 172, 332–341.
Johnson, M.E., Zhou, X.F., Bobrovskaya, L., 2019. The effects of rotenone on TH, BDNF and BDNF-related proteins in the brain and periphery: relevance to early Parkinson's disease. J. Chem. Neuroanat. 97, 23–32.
Kunugi, H., Kawada, Y., Hattori, M., Ueki, A., Otsuka, M., Nanko, S., 1998. Association study of structural mutations of the tyrosine hydroxylase gene with schizophrenia and Parkinson's disease. Am. J. Med. Genet. 81 (2), 131.
Lappas, M., Permezel, M., Georgiou, H.M., Rice, G.E., 2002. Nuclear factor kappa B regulation of proinflammatory cytokines in human gestational tissues in vitro. Biol. Reprod. 67, 668–673.
Li, R.L., Zhang, Z.Z., Peng, M., Wu, Y., Zhang, J.J., Wang, C.Y., Wang, Y.L., 2013. Postoperative impairment of cognitive function in old mice: a possible role for neuroinflammation mediated by HMGB1, S100B, and RAGE. J. Surg. Res. 185, 815–824.
Li, N., Liu, X.X., Hong, M., Huang, X.Z., Chen, H., Xu, J.H., Wang, C., Zhang, Y.X., Zhong, J.X., Nie, H., Gong, Q., 2018. Sodium butyrate alleviates LPS-induced acute lung injury in mice via inhibiting HMGB1 release. Int. Immunopharmacol. 56, 242–248.
Lu, Y.C., Yeh, W.C., Ohashi, P.S., 2008. LPS/TLR4 signal transduction pathway. Cytokine 42 (2), 145.
Mercurio, F., Manning, A.M., 1999. Multiple signals converging on NF-κB. Curr. Opin. Cell Biol. 11, 226–232.
Nash, J.E., Johnston, T.H., Collingridge, G.L., Garner, C.C., Brotchie, J.M., 2005. Subcellular redistribution of the synapse-associated proteins PSD-95 and SAP97 in animal models of Parkinson's disease and L-DOPA-induced dyskinesia. FASEB J. 19 (6), 583–585.
Nepali, S., Ki, H.H., Lee, J.H., Lee, H.Y., Kim, D.K., Lee, Y.M., 2017. Wheatgrass-derived polysaccharide has antiinflammatory, anti-oxidative and anti-apoptotic effects on LPS-induced hepatic injury in mice. Phytother. Res. 31 (7), 1107–1116.
Niranjan, R., 2018. Recent advances in the mechanisms of neuroinflammation and their roles in neurodegeneration. Neurochem. Int. 120, 13–20.
Nishibori, M., 2018. HMGB1 as a representative DAMP and anti-HMGB1 antibody therapy. Nihon Yakurigaku Zasshi. 151, 4–8.
Nishibori, M., Mori, S., Takahashi, H.K., 2019. Anti-HMGB1 monoclonal antibody therapy for a wide range of CNS and PNS diseases. J. Pharmacol. Sci. 140, 94–101.
Patil, D.A., Patil, V.A., Bari, S.B., Surana, S.J., Patil, P.O., 2014. Animal models for Parkinson's disease. CNS Neurol. Disord. Drug Targets 13 (9), 1580–1594.
Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K., Cobb, M.H., 2001. Mitogen activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr. Rev. 22, 153–183.
Qin, L., Li, G., Qian, X., Liu, Y., Wu, X., Liu, B., Hong, J.S., Block, M.L., 2005. Interactive role of the toll-like receptor 4 and reactive oxygen species in LPS-induced microglia activation. Glia 52 (1), 78–84.
Qin, L., Wu, X., Block, M.L., Liu, Y., Breese, G.R., Hong, J.S., Knapp, D.J., Crews, F.T., 2007. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 55 (5), 453–462.
Rajapakse, N., Kim, M.M., Mendis, E., Kim, S.K., 2008. Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-stimulated RAW264.7 cells by carboxybutyrylated glucosamine takes place via down-regulation of mitogen-activated protein kinase-mediated nuclear factor-kappaB signaling. Immunology 123, 348–357.
Ransohoff, R.M., Schafer, D., Vincent, A., Blachère, N.E., Bar-Or, A., 2015. Neuroinflammation: ways in which the immune system affects the brain. Neurotherapeutics 12 (4), 896–909.
Rao, K.M., 2001. Map kinase activation in macrophages. J. Leukoc. Biol. 69, 3–10.
Reibman, J., Talbot, A.T., Hsu, Y., Ou, G., Jover, J., Nilsen, D., 2000. Regulation of expression of granulocyte-macrophage colony-stimulating factor in human bronchial epithelial cells: roles of protein kinase C and mitogen-activated protein kinases. J. Immunol. 165, 1618–1625.
Ren, J., Li, L., Wang, Y., Zhai, J., Chen, G., Hu, K., 2019. Gambogic acid induces heme oxygenase-1 through Nrf2 signaling pathway and inhibits NF-κB and MAPK activation to reduce inflammation in LPS-activated RAW264.7 cells. Biomed. Pharmacother. 109, 555–562.
Sasaki, T., Liu, K., Agari, T., Yasuhara, T., Morimoto, J., Okazaki, M., Takeuchi, H., Toyoshima, A., Sasada, S., Shinko, A., Kondo, A., Kameda, M., Miyazaki, I., Asanuma, M., Borlongan, C.V., Nishibori, M., Date, I., 2016. Anti-high mobility group box 1 antibody exerts neuroprotection in a rat model of Parkinson's disease. Exp. Neurol. 1, 220–231 275 Pt.
Sasimol, U., Chayanut, K., Peraphan, P., Thanyaluck, P., Prachya, K., 2018. Sesamin suppresses LPS-induced microglial activation via regulation of TLR4 expression. J. Funct. Foods 49, 32–43.
Schneider, R.B., Iourinets, J., Richard, I.H., 2017. Parkinson's disease psychosis: presentation, diagnosis and management. Neurodegener. Dis. Manag. 7, 365–376.
Sugawara, T., Lewen, A., Noshita, N., Gasche, Y., Chan, P.H., 2002. Effects of global ischemia duration on neuronal, astroglial, oligodendroglial, and microglial reactions in the vulnerable hippocampal CA1 subregion in rats. J. Neurotrauma 19, 85–98.
Sun, G.B., Qin, M., Ye, J.X., Pan, R.L., Meng, X.B., Wang, M., Luo, Y., Li, Z.Y., Wang, H.W., Sun, X.B., 2013. Inhibitory effects of Myricitrin on oxidative stress-induced endothelial damage and early atherosclerosis in ApoE−/− mice. Toxicol. Appl. Pharmacol. 271 (1), 114.
Tekin, I., Roskoski, R.J., Carkaci-Salli, N., Vrana, K.E., 2014. Complex molecular regulation of tyrosine hydroxylase. J. Neural Transm. (Vienna) 121, 1451–1481.
Tsui, C., Koss, K., Churchward, M.A., Todd, K.G., 2018. Biomaterials and glia: Progress on designs to modulate neuroinflammation. Acta Biomater. 11, 7.
Vallabhapurapu, S., Karin, M., 2009. Regulation and function of NF-kappaB transcription factors in the immune system. Annu. Rev. Immunol. 27, 693–733.
Wang, W.W., Hu, S.Q., Li, C., Zhou, C., Qi, S.H., Zhang, G.Y., 2010. Transduced PDZ1 domain of PSD-95 decreases Src phosphorylation and increases nNOS (Ser847) phosphorylation contributing to neuroprotection after cerebral ischemia. Brain Res. 1328, 162–170.
Wang, Y.H., Xuan, Z.H., Tian, S., He, G.R., Du, G.H., 2013. Myricitrin attenuates 6-hydroxydopamine-induced mitochondrial damage and apoptosis in PC12 cells via inhibition of mitochondrial oxidation. J. Funct. Foods 5, 337–345.
Wang, Y.H., Yu, H.T., Pu, X.P., Du, G.H., 2014. Myricitrin alleviates methylglyoxal-induced mitochondrial dysfunction and AGEs/RAGE/NFκB pathway activation in SH-SY5Y cells. J. Mol. Neurosci. 53, 562–572.
Wolf, H.K., Buslei, R., Schmidt-Kastner, R., Schmidt-Kastner, P.K., Pietsch, T., Wiestler, O.D., Blumcke, I., 1996. NeuN: a useful neuronal marker for diagnostic histopathology. J. Histochem. Cytochem. 44, 1167–1171.
Wu, X., Gao, H., Hou, Y., Yu, J., Sun, W., Wang, Y., Chen, X., Feng, Y., Xu, Q.M., Chen, X., 2018. Dihydronortanshinone, a natural product, alleviates LPS-induced inflammatory response through NF-κB, mitochondrial ROS, and MAPK pathways. Toxicol. Appl. Pharmacol. 355, 1–8.
Yang, Y.L., Cheng, X., Li, W.H., Liu, M., Wang, Y.H., Du, G.H., 2019. Kaempferol attenuates LPS-induced striatum injury in mice involving anti-neuroinflammation, maintaining BBB integrity, and down-regulating the HMGB1/TLR4 pathway. Int. J. Mol. Sci. 20, 491.
Yu, X., Quan, J., Long, W., Chen, H., Wang, R., Guo, J., Lin, X., Mai, S., 2018. LL-37 inhibits LPS-induced inflammation and stimulates the osteogenic differentiation of BMSCs via P2X7 receptor and MAPK signaling pathway. Exp. Cell Res. 372, 178–187.
Zhang, G., Ghosh, S., 2001. Toll-like receptor-mediated NF-kappaB activation: a phylogenetically conserved paradigm in innate immunity. J. Clin. Investig. 107, 13–19.
Zhang, X., Wang, Y., Xiao, C., Wei, Z., Wang, J., Yang, Z., Fu, Y., 2017. Resveratrol inhibits LPS-induced mice mastitis through attenuating the MAPK and NF-κB signaling pathway. Microb. Pathog. 107, 462–467.
Zong, M.M., Zhou, Z.Q., Ji, M.H., Jia, M., Tang, H., Yang, J.J., 2019. Activation of beta2-adrenoceptor attenuates sepsis-induced hippocampus-dependent cognitive impairments by reversing neuroinflammation and synaptic abnormalities. Front. Cell. Neurosci. 13, 293.