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Berberine ameliorates lipopolysaccharide-induced astrocyte activation and migration via NDUFC2

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ABSTRACT: Astrocytes are an abundant cell type in central nervous system (CNS) and play a critical role in CNS homeostasis. Abnormal astrocytes have been consistently observed in diverse CNS diseases, which lead to enlarged inflammatory reactions and glial scars. As a naturally extracted monomer with anti-inflammatory properties, berberine shows a potential therapeutic effect on abnormal astrocytes. However, the mechanism of berberine in the regulation of abnormal astrocytes is still unclear. Here, we have shown that berberine restored lipopolysaccharide (LPS)-induced mitochondrial abnormalities by upregulating NDUFC2, a subunit of ubiquinone oxidoreductase, expression resulting in astrocyte rehabilitation. Pretreatment of 10 μg/ml berberine inhibited LPS-induced astrocyte activation and migration. The knockdown of NDUFC2 repressed the inhibitory action of berberine on LPS-induced astrocyte activation and migration; and, as a result, mitochondria injury was increased. These findings prove a new mechanism in astrocyte reaction and provide a potential curative target of berberine for inflammatory diseases.

KEYWORDS: astrocytes, cell activation, cell migration, berberine, lipopolysaccharide

INTRODUCTION

Characterized as the most abundant cells in the entire central nervous system (CNS), astrocytes are capable of multiple participatory functions in physiological and pathological processes [1]. While inflammation is a common reactive phenomenon in CNS diseases, previous studies have highlighted the correlation between astrocytes and inflammation [2, 3]. Inflammation induced by tissue injury, infection or other exogenous stresses, in spite of the initial aims to attenuate the inflammatory aggression in CNS, leads to astrocyte activation and accelerates astrocyte migration to form of glial scars [4]. Glial scars can separate neural tissue from non-neural tissue along perivascular spaces, meninges, and tissue lesions, which serves as functional barriers restricting the entry of inflammatory cells into CNS [5, 6]. However, excessive astrocyte activation and migration, as another injury patten, result in the release of inflammatory cytokines and reactive oxygen species, which then aggravate the neurological dysfunctions [7]. The double-edged sword makes great confusion to the treatment of CNS diseases. Thus, it is vital to investigate the molecular mechanism of astrocyte activation and migration so that appropriate intervention could be applied to prevent their adverse effects in CNS.

Berberine is a natural monomer extracted from *Coptis chinensis* with anti-inflammatory, anti-hyperglycemic, anti-hyperlipidemic, and anti-neoplastic physiological activities. Berberine exhibits a wide antibacterial spectrum and antibacterial effect on both Gram-positive and Gram-negative bacteria *in vitro* [8]. In CNS diseases, berberine remains to show exciting biological therapeutic effects including ischemic stroke [9], Alzheimer's disease [10], and Parkinson's disease [11]. Existing researches indicate that berberine for oral administration could suppress astrocyte activation, elevate superoxide dismutase activity, and downregulate lipid peroxidation and nitrite levels in the hippocampus of streptozotocin-induced diabetic mice [12, 13]. Nonetheless, the mechanism of berberine in astrocyte activation and migration is still unclear.

NDUFC2, a subunit of ubiquinone oxidoreductase, was previously demonstrated its association with reactive oxygen species and mitochondrial damages [14]. Existing reports on NDUFC2 mainly focus on cardiovascular diseases; therefore, it is hardly facile to elucidate the effects of NDUFC2 in CNS diseases. Here, we aimed to investigate the role of NDUFC2 in astrocyte activation and migration induced by lipopolysaccharide (LPS) *in vitro*.

MATERIALS AND METHODS

Animals

C57BL/6 mice were housed at a constant temperature and humidity with a 12/12 h light/dark cycle. Food and water were available ad libitum. Animal experiments were conducted according to the ethical guidelines set by the Ethics Committee of Laboratory Animal Care and Welfare, Nantong University. Mice were injected intraperitoneally with 20 mg/kg LPS (Solarbio, L8880) once and gavaged administration with 100 mg/kg berberine for 3 days. Cortex samples were collected after narcosis.

Cell cultures

According to a previous study, primary mouse astrocytes were obtained from C57BL/6N mice within 3 days after birth, and the general steps were described below [15]. This work was sanctioned by the animal use and care committee of Nantong University. After dissociation of blood vessels, whole brains were chopped, and tissue pieces were digested by trypsin containing ethylene diamine tetraacetic acid. Culture medium supplemented with fetal bovine serum (FBS, 10% v/v) and penicillin-streptomycin solution (PSS, 1% v/v) was used to terminate the digestion. The suspension was filtered using 40 μm cell filter to remove impurities and, then, centrifuged. Cell pellets collected after centrifugation were resuspended and planted in cell culture flask.

C8-D1A astrocytes were purchased from China Center for Type Culture Collection and maintained in a humidified chamber at 37 °C under 5% CO_2 and culture medium containing FBS (10% v/v) and PSS (1% v/v).

Green fluorescent protein (GFP) lentivirus and small interfering RNA (siRNA) transfection

C8-D1A astrocytes were pretreated with polybrene and, then, transfected with GFP lentivirus in multiplicity of infection of 5 in the culture medium without FBS for 24 h. FBS-free medium was replaced with normal culture medium after lentivirus transfection, and puromycin of 2 μg/ml was applied to acquire pure C8-D1A-GFP astrocytes.

C8-D1A astrocytes were transfected with 0.1 μM siRNA and 1 μl transfection reagent in serum-free culture medium for 24 h, and subsequently the medium was replaced with normal culture medium. The sequences of NDUFC2 siRNA1, NDUFC2 siRNA2, and NDUFC2 siRNA3 were 5′-ATGTTTGGTTATATAAAATTA dTdT -3′, 5′- CCUGCCUCAAGAUGAUGAAdTdT -3′, and 5′- AAGATTTTCCTGAAAAAGAGAdTdT-3′, respectively.

Western blot analysis

Cell samples were lysed in radio-immunoprecipitation assay buffer, and protein concentration was measured by bicinchoninic acid assay. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and, then, transferred onto polyvinylidene fluoride membranes. The membranes were incubated with first antibody at 4 °C overnight after blocking in 5% nonfat dry milk for 1 h at room temperature. Second antibody labeled with horseradish peroxidase was used to combine the first antibody. Membranes were soaked with an electrochemiluminescence solution, and detection was conducted using digital image scanner. Quantification was performed by densitometry using Image J software.

Real-time polymerase chain reaction (PCR)

Total RNAs extracted from astrocytes were reversely transcribed to cDNA. Then, real-time PCR was performed to determine NDUFC2 mRNA level following the manufacturer's instructions (Q121-02, Vazyme). The following primers were employed: mouse NDUFC2 (forward: 5′-CCCTTAAGAGGGATGCTGCC-3′, reverse: 5′-TACGGCCAAATCCGTTCACA-3′); mouse GAPDH (forward: 5′-AGGTCGGTGTGAACGGATTTG-3′, reverse: 5′-TGTAGACCATGTAGTTGAGGTCA-3′).

Migration assay

Capability of cell migration was evaluated by wound-healing assay. C8-D1A-GFP astrocytes were planted in a 24-well plate until 70–80% confluence. In the center of well bottom, straight line was scraped by sterile 200-μl pipette tips. C8-D1A-GFP astrocytes were treated with berberine and/or LPS, and digital images of the cell gap were captured at 0-h and 24-h points. The migration length was evaluated by Image J software.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

Astrocytes were cultured in culture medium containing 10% FBS and 1% penicillin-streptomycin with/without berberine treatment for 24 h. MTT of 5 mg/ml was blended with culture medium in 96-well plates at 37 °C for 2 h until purple precipitate was visible. The supernatant was discarded, and the precipitate was dissolved in 200 μl dimethyl sulfoxide. The obtained solution's absorbance at 490 nm was examined by using automatic microplate reader.

Transmission electron microscopy

The treated cell pellets were washed with cold phosphate buffered saline and fixed in a mixture solution of 2% glutaraldehyde and formaldehyde in 0.1 M sodium cacodylate buffer at 4 °C overnight. Then, cell pellets were washed with 264 mM sucrose solution in 0.1 M sodium cacodylate buffer before being transferred to 1% osmium tetroxide in 0.1 M sodium cacodylate for 2 h. The pellets were washed 3 times with H_2O, stained with 2% aqueous uranyl acetate for 2 h, dehydrated in an ethanol series, and infiltrated in propylene oxide 2 times before being embedding in Epon 812. Ultrathin sections (90-nm thick) were cut using an ultramicrotome with a diamond knife. Grids were poststained with 2% saturated uranyl acetate in 50% ethanol and 1% lead citrate (pH 12) and examined using an H-7650 electron microscope, and images were captured with a Ganton 830 digital camera.

Statistical analysis

All experiments were repeated 3 times. Data were analyzed by GraphPad Prism 8 software and represented as means ± SEM. Comparisons between two groups and

between multiple groups were done using Student's *t*-test and one-way analysis of variance, respectively. Statistical results were considered significant at a $p < 0.05$.

RESULTS

LPS induced astrocyte activation and migration

To investigate the mechanism of astrocyte activation and migration, an activated astrocyte model was successfully developed in primary astrocytes and C8-D1A astrocytes with different concentrations of LPS *in vitro*. Treatment of 100 ng/ml LPS was considered as the appropriate concentration because it significantly activated glial fibrillary acidic protein (GFAP) expression (Fig. 1A,B), and the concentration was applied in the subsequent experiments. In migration assay, C8-D1A astrocytes treated with 100 ng/ml LPS for 24 h notably elevated the migrated distance as shown in Fig. 1C.

Berberine suppressed astrocyte viability in dose dependent

As berberine is known for its extensive anti-inflammatory effects, we hypothesized that it played a critical role in astrocyte activation and migration induced by LPS. To avoid excessive dose administration, MTT assay was performed with multiple concentrations of berberine. As shown in Fig. 2A,B, berberine of low concentrations (5 μg/ml and 10 μg/ml) did not affect the cell viability, while the high concentrations (20 μg/ml and 30 μg/ml) notably restrained cell viability, indicating that the 10 μg/ml berberine concentration was the applicable dose for abnormal astrocyte treatment.

Berberine ameliorated astrocyte activation and migration induced by LPS

To examine whether berberine could relieve the unhealthy changes of astrocytes, 10 μg/ml berberine was used to interfere astrocytes in advance for 1 h. As shown in Fig. 3A,B, pretreatment with 10 μg/ml berberine inhibited the upregulated GFAP expression induced by LPS in both primary astrocytes and C8-D1A astrocytes. In migration assay, LPS treatment accelerated the C8-D1A astrocyte migration in comparison to the control group, and berberine suppressed the cell locomotion as expected (Fig. 3C). These results indicated that berberine had an inhibitory role in LPS-induced reactive astrocytes.

Berberine ameliorated astrocyte activation and migration via NDUFC2

As shown by a previous study, NDUFC2 involved in reactive oxygen species and mitochondrial damages [14], suggesting the possible involvement of NDUFC2 in reactive astrocyte process induced by LPS. The expression of NDUFC2 in both LPS treated primary and C8-D1A astrocytes was lessened (Fig. 4A,B). However, the lessened expression was inhibited by the pretreatment of 10 μg/ml berberine (Fig. 4C,D). Also, pretreatment of berberine inhibited the lessened expression of NDUFC2 mRNA induced by LPS (Fig. 4E,F).

To verify whether berberine having the anti-reactive function via NDUFC, we designed 3 siRNAs targeting NDUFC2 mRNA. In the presence of NDUFC2 siRNA2, NDUFC2 expression was significantly decreased (Fig. 5A,B). Hence, NDUFC2 siRNA2 was used in the subsequent experiments. The inhibitory effect of berberine on elevated GFAP expression was diminished by NDUFC2 siRNA (Fig. 5C,D). Moreover, in the migration assay, administration of NDUFC2 siRNA increased the migrated distance in comparison to the berberine group with LPS treatment (Fig. 5E). These results demonstrated that berberine restrained astrocyte activation and migration via NDUFC2.

Berberine suppressed mitochondrial abnormalities

Having shown the regulation of berberine in NDUFC2, we sought to investigate whether berberine affected the mitochondrial structure and morphology via mediating NDUFC2 expression. Transmission electron microscope was applied to observe mitochondrial structure and morphology of primary astrocytes. Compared with the control group (Fig. 6A), LPS treatment induced the volume expansion and mitochondrial cristae disappearance (Fig. 6B). Berberine rescued the destruction of mitochondria by LPS treatment (Fig. 6C). However, administration of NDUFC2 siRNA increased the mitochondria injury in comparison to the berberine group with LPS treatment (Fig. 6D). These results indicated that berberine repaired LPS-induced mitochondrial abnormalities by upregulating NDUFC2 expression.

Berberine inhibited the astrocyte activation *in vivo*

To further investigate the effect of berberine on astrocyte activation, we established an inflammatory model induced by intraperitoneal injection of LPS [15]. The result showed that gavage administration of berberine significantly inhibited the astrocyte activation by testing GFAP expression (Fig. 7). Furthermore, gavage administration of berberine rescued the decreased expression of NDUFC2 induced by LPS treatment (Fig. 7).

DISCUSSION

In the last decades, astrocytes have been cogitated as the supportive cells in CNS. However, along with the deepening researches on CNS, astrocytes obtained much more attention due to their diverse function. Under normal conditions, neurons take in trophic factors, glutamic acid, and adenosine 5′-triphosphate (ATP) released from astrocytes to maintain their metabolism [16]. In CNS injury, a large number of astrocytes translate themselves into reactive state;

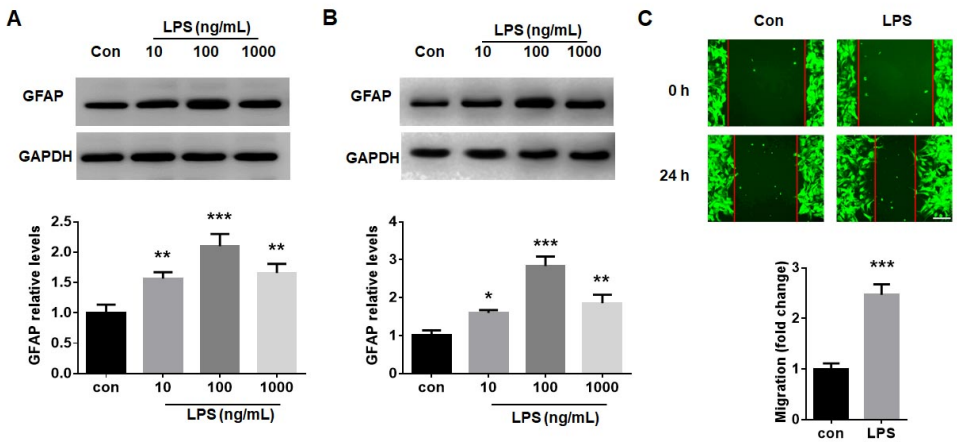

Fig. 1 GFAP expression in astrocytes in the presence of LPS. (A–B), representative blots showing the GFAP expression in primary astrocytes (A) and in C8-D1A astrocytes (B) subjected to LPS treatment for 24 h; (C), effect of LPS on the migration of C8-D1A astrocytes evaluated by scratch assay. Scale bar: 200 μm; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control group; Con = control.

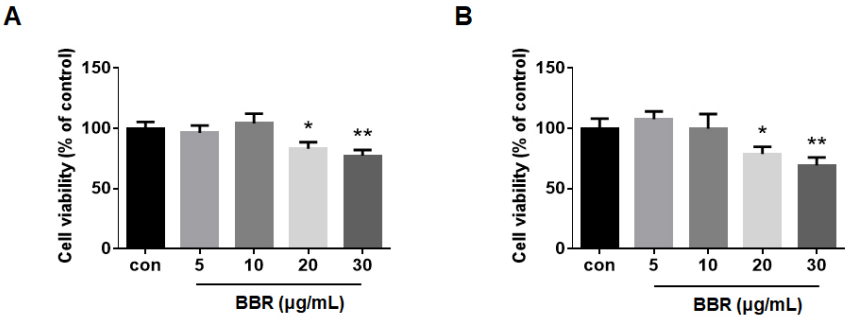

Fig. 2 Cell viability of astrocytes in the presence of berberine. (A–B), MTT assay evaluating the cell viability of primary astrocytes (A) and C8-D1A astrocytes (B) subjected to multiple doses of berberine treatment. * $p < 0.05$ and ** $p < 0.01$ versus control group. Con = control; BBR = berberine.

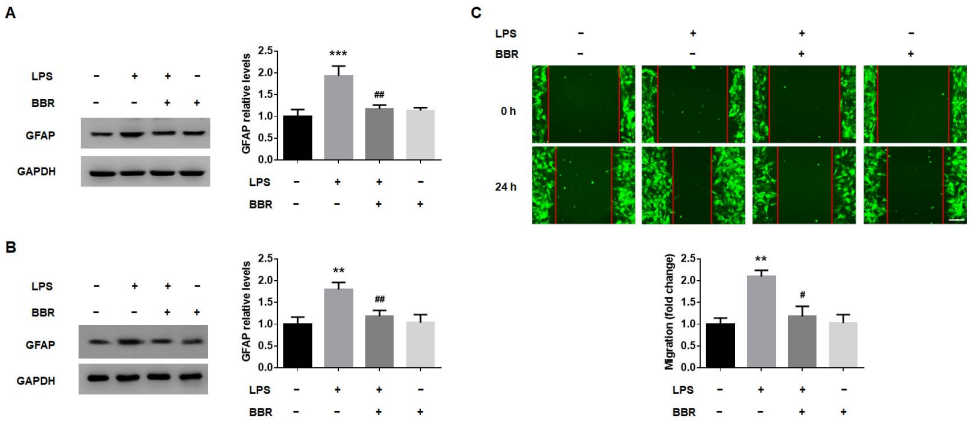

Fig. 3 Inhibition by berberine of astrocyte activation and migration induced by LPS. (A–B), effect of berberine on LPS-induced astrocyte activation evaluated by western blot with representative blots showing the GFAP expression in primary astrocytes (A) and C8-D1A astrocytes (B) subjected to LPS and/or berberine treatment; (C), effect of berberine on LPS-induced migration of C8-D1A astrocytes evaluated by scratch assay. Scale bar: 200 μm. ** $p < 0.01$ and *** $p < 0.001$ versus control group. # $p < 0.05$ and ## $p < 0.01$ versus LPS group. Con = control; BBR = berberine.

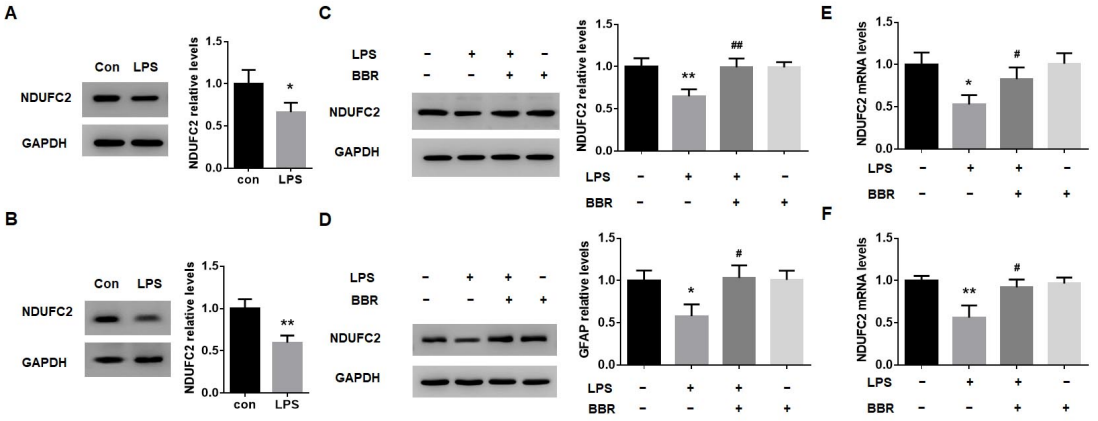

Fig. 4 Inhibition by berberine of the LPS-induced reduction of NDUFC2 expression. (A–B), representative blots showing the NDUFC2 expression in primary astrocytes (A) and C8-D1A astrocytes (B) subjected to LPS treatment for 24 h; (C–D), effect of berberine on the LPS-induced increase in NDUFC2 expression evaluated by western blot with representative blots showing the NDUFC2 expression in primary astrocytes (C) and C8-D1A astrocytes (D) subjected to LPS and/or berberine treatment; (E–F), effect of berberine on the LPS-induced increase in the expression of NDUFC2 mRNA evaluated by real-time PCR. * $p < 0.05$ and ** $p < 0.01$ versus control group. $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ versus LPS group. Con = control; BBR = berberine.

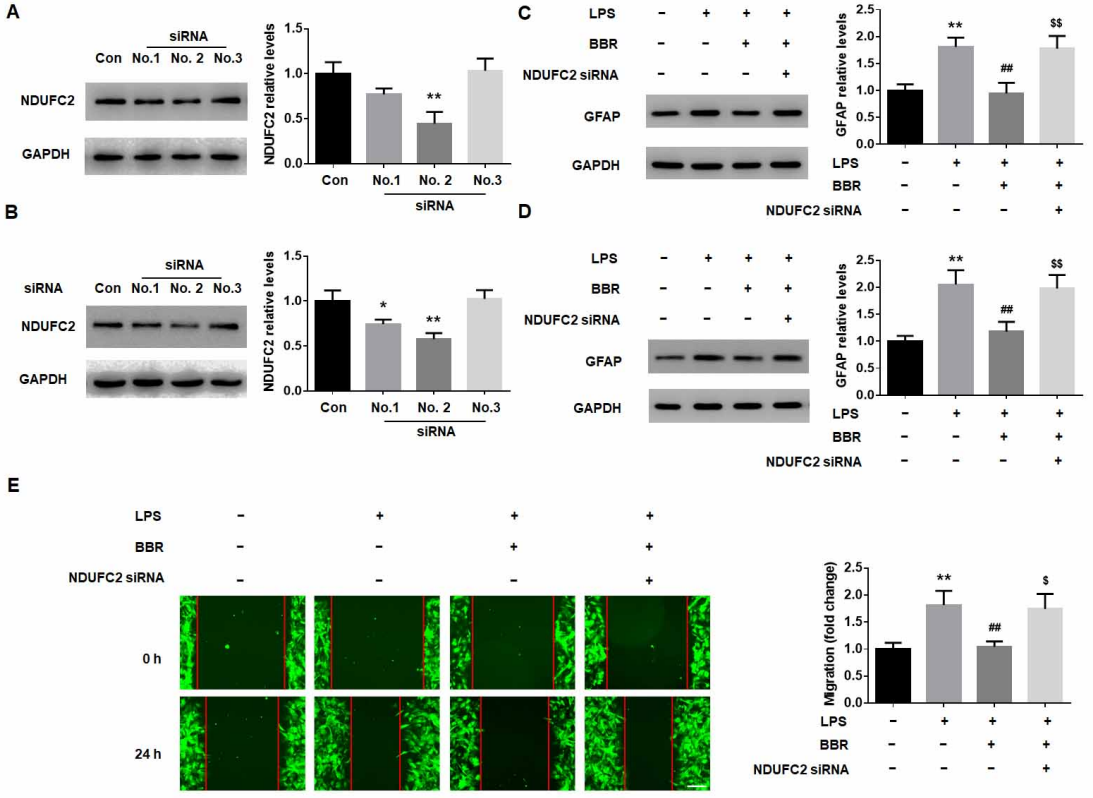

Fig. 5 Inhibition by berberine of astrocyte activation and migration via NDUFC2. (A–B), efficiency of NDUFC2 siRNA verified in primary astrocytes (A) and C8-D1A astrocytes (B) using western blot. ** $p < 0.01$ versus control group. (C–D), role of NDUFC2 in the berberine-mediated suppression of astrocyte activation evaluated by western blot with representative blots showing the GFAP expression in primary astrocytes (C) and C8-D1A astrocytes (D) subjected to LPS and/or berberine treatment before NDUFC2 siRNA transfection. (E), role of NDUFC2 in the berberine-mediated suppression of astrocyte migration evaluated by scratch assay. Scale bar: 200 μm. ** $p < 0.01$ versus control group. $^{\#\#}$ $p < 0.01$ versus LPS group. $^{\$}$ $p < 0.05$ and $^{\$\$}$ $p < 0.01$ versus LPS+BBR group. Con = control; No. =number; BBR = berberine.

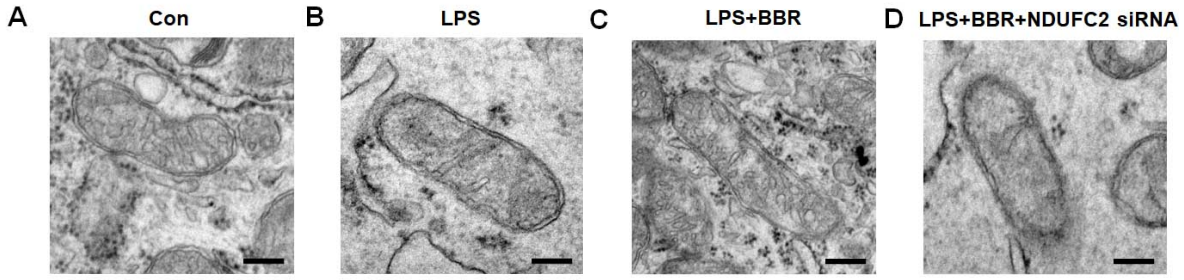

Fig. 6 Inhibition by berberine of mitochondrial abnormalities via NDUFC2. (A–D), transmission electron microscopic images showing the mitochondrial structural features after multiple types of treatment. Scale bar: 0.2 μm.

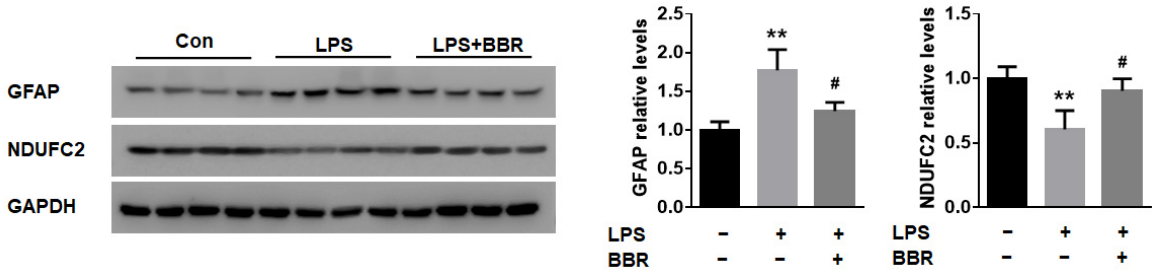

Fig. 7 Inhibition by berberine of astrocyte activation and NDUFC2 decrease. Representative blots showed the GFAP and NDUFC2 expression in mouse cortex samples with the treatments of LPS and berberine. $n = 4$/each group. ** $p < 0.01$ versus control group. $^{\#}$ $p < 0.05$ versus LPS group. Con = control; BBR = berberine.

and the expression of GFAP, the astrocyte activation marker, is extremely upregulated. Reactive astrocytes migrate to the injured region and proliferate to form glial scars [17]. Recent advanced researches showed that reactive astrocyte proliferation was not an all-or-nothing phenomenon, but a fine-graded succession of changes ranging from reversible transition in cell status and gene expression [18]. In our study, LPS-induced astrocyte activation was accompanied with migration, which is consistent with previous reports. Compared with primary astrocytes, C8-D1A astrocytes have higher migration efficiency. Thus, LPS-induced migration assay was performed on C8-D1A astrocytes.

Anecdotal evidence suggests that berberine is capable of anti-inflammatory action and could be used as a potential treatment of neuroinflammation-associated diseases. Previous studies showed that berberine induced the upregulation of heme oxygenase-1 via phosphatidylinositol 3-kinase/protein kinase B and NFE2 like bZIP transcription factor 2 pathway in astrocytes [19]; meanwhile, berberine was involved in the regulation of reactive microglia in multiple animal models [20–22]. *In vitro* experiments showed that berberine inhibited the increased expression of inflammatory mediators as tumor necrosis factor-α and interleukin-1β in BV2 cells [22]. Although excessive berberine inhibited tumor cell proliferation and further resulted in tumor cell death [23, 24], an appropriate dose of berberine functioned as anti-inflammatory drug without affecting cell survival [25]. In our study, 20 μg/ml and 30 μg/ml berberine concentrations resulted in a decreased cell viability; therefore, berberine of 10 μg/ml was chosen to verify its beneficial role in astrocyte activation and migration. Based on the aforementioned fact, it was important for us to apply an appropriate dose in pharmaceutical research to avoid cytotoxic effect. Moreover, berberine of 10 μg/ml did not show any significant effect on GFAP expression and migration distance compared with the control group, which provided additional evidence supporting the harmlessness of 10 μg/ml berberine.

NDUFC2 is a subunit of ubiquinone oxidoreductase and reported as a critical element in regulation of mitochondrial function, especially the ATP synthesis and respiratory electron transport. According to human protein atlas, NDUFC2 is mainly distributed in mitochondria and fully justify in the regulation of mitochondrial function. Growing number of studied results have demonstrated that inflammatory reaction originates from mitochondrial dysfunction, which leads to elevated reactive oxygen species and subsequent activation of inflammatory pathway [26]. There is a fact that NDUFC2 deficiency represents an emerging mechanism of cardiovascular diseases [27, 28]. Clinical trial data showed that a significant reduction of NDUFC2 expression is detected in peripheral blood mononuclear cell of patients with acute cardiovascular disease. NDUFC2 silencing affects vascular cell viabil-

ity and angiogenesis while stimulating the expression of markers of plaque rupture. It is easy to understand that abnormal oxygen levels in the blood induced by acute cardiovascular disease lead to a decrease in NDUFC2 expression, triggering the impairment of mitochondrial function. Our results clearly showed that NDUFC2 could suppress astrocyte activation and migration, probably governing reactive astrocyte via mitochondrial dysfunction. Therefore, anti-inflammatory action of berberine might be involved in mitochondrial function to restrain astrocyte activation and migration. This study proved a new mechanism in astrocyte reaction and provided a potential curative target of berberine for inflammatory diseases.

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