Barbaloin Alleviates Cerebral Ischemia and Reperfusion Injury in Rats and Inhibits Lipopolysaccharide-stimulated Inflammatory Responses in BV2 Microglial Cells

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Research

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Abstract

Background: Barbaloin (BAR) is a bioactive anthracycline derived from the leaf exudates of aloe plants with a variety of biological and pharmacological properties. The present study was performed to investigate the neuroprotective effects of BAR against cerebral ischemia and reperfusion (I/R) injury in rats as well as the possible underlying mechanisms. Middle cerebral artery occlusion followed by reperfusion was used to induce cerebral I/R injury in rats, and BAR was administered intraperitoneally after the onset of ischemia.

Results: We found that BAR remarkably improved neurological scores, reduced brain infarct volume, and inhibited neuronal apoptosis in cerebral I/R rats. Furthermore, BAR up-regulated Bcl-2 protein levels and down-regulated Bax, active caspase-3, and inducible nitric oxide synthase (iNOS) in ischemic cortex. I/R injury-induced increases in malondialdehyde content and decreases in glutathione peroxidase, glutathione, and superoxide dismutase activities were significantly attenuated by BAR administration. *In vitro*, BAR pretreatment inhibited the contents of proinflammatory cytokines (tumor necrosis factor- α , iNOS, and interleukin-6) and reduced protein levels of iNOS and nuclear expression of nuclear factor- κ B p65 in lipopolysaccharide-stimulated BV-2 microglial cells.

Conclusion: Taken together, our data suggest that BAR possesses neuroprotective effects against cerebral I/R injury through anti-oxidative, anti-apoptotic, and anti-inflammatory activities.

Background

Stroke represents the second common cause of death among elderly people over 60 years old and the most common cause of permanent disability worldwide (1). It is estimated that ischemic stroke is consisted of approximately 80% of all stroke patients (2, 3). Ischemic stroke causes irreversible brain damages because of the sudden blockage of blood flow followed by blood supply restoration and simultaneous reoxygenation (4). Despite the pathogenesis of ischemic stroke remains largely unknown, several processes including inflammation, oxidative stress, excitotoxicity, calcium overload, and apoptosis are associated with ischemic injuries (5, 6). Currently, intravenous tissue-plasminogen activator (tPA) administration is the only approved medical therapy available for ischemic stroke patients, but the limited therapeutic time window for tPA treatment and an increased risk for intracranial hemorrhage severely limit its clinical use (7, 8). Thus, development of new and effective therapies for ischemic stroke is urgently required.

Barbaloin (BAR) is the major bioactive anthraquinone-C-glycoside derived from the Chinese traditional medicine aloe vera (9). Recent evidence indicates that BAR has various pharmacological activities, such as anti-inflammatory (10), antioxidant (11), antitumor (12), and anti-arrhythmic properties (13). Several reports have documented that BAR attenuates myocardial ischemia/reperfusion (I/R) injury, and these effects are linked to regulation of the adenosine monophosphate-activated protein kinase and the

CNPY2-PERK signaling pathways (14, 15). However, whether BAR can protect against cerebral I/R injury remains to be investigated.

Herein, we evaluated if BAR shows neuroprotective effects against middle cerebral artery occlusion (MCAO)-induced brain injuries in rats and in BV-2 microglial cells stimulated with lipopolysaccharide (LPS).

Methods

Animals

Male Sprague-Dawley rats (weighing 220 ~ 240 g) were obtained from Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, Liaoning) and housed in specific pathogen-free environments (12 h light/dark cycles at 24–26 °C) in plastic cages. The animal experiment protocols were reviewed and approved by the Animal Care and Use Committee of Shengjing Hospital (No. 2018-56). The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

Establishment of cerebral I/R injury model

The middle cerebral artery occlusion (MCAO) method was used to establish cerebral I/R injury model as previously reported. In brief, rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (350 mg/kg) prior to the surgical procedures. A midline neck incision was made to expose and isolate the internal, external, and right common carotid arteries. A 4 – 0 silicon rubber-coated nylon monofilament was inserted from the external carotid artery lumen to the internal carotid artery and advanced to the root of the right middle cerebral artery, where the rounded tip blocked blood flow. After 90 min occlusion, the reperfusion was initiated via withdrawal of the monofilament. Sham-operated rats underwent an identical surgery procedure, but the filament was not inserted.

Treatment of rats with BAR

A total of 96 rats were randomly divided into the following four groups (n = 24): (1) Sham: rats received an identical surgery procedure, without filament insertion; (2) ischemia and reperfusion (I/R): rats underwent MCAO; (3 and 4) rats underwent MCAO and were intraperitoneally injected with BAR (50 and 100 mg/kg; purity ≥ 98%, Yuanye Biotechnology, Shanghai, China) immediately and at 8 and 16 h after reperfusion. An equal volume of DMSO was simultaneously given to the sham and I/R rats. After 24 h of reperfusion, all rats were killed with lethal doses of chloral hydrate, and the brain tissues were harvested for further use. The doses for BAR used in this study was on the basis of our preliminary experiments and literature reports (11, 14)

Neurologic deficits score

At 24 h after reperfusion, neurologic functions were assessed with a blind method using a 5-point scale (16): 0, normal behavior; 1, failure to stretch left front legs fully; 2, turn around circles; 3, falling to the left;

4, inability to walk spontaneously and showed a depressed level of consciousness.

Measurement of infarct volume

After neurological deficit evaluation, the brain tissues were collected and coronally cut into sections, followed by staining with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich). After fixed in 10% formalin overnight, the sections were photographed, and the percentage of infarction was defined as the infarct size relative to ipsilateral hemisphere size in each coronal slices.

Malondialdehyde (MDA) and glutathione (GSH) contents and superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in cerebral cortex tissues

The ipsilateral cerebral cortex tissues were dissected out, homogenized, and centrifuged to collect the supernatants. Thereafter, the supernatants were used to evaluate MDA and GSH contents and GSH-Px and SOD activities (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

TUNEL staining was carried out using a commercially available One Step TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to supplier's instructions. Roughly, the brains were immersed in 10% buffered formalin and embedded in paraffin wax. Deparaffinized sections (5- μ m thickness) were stained with fluorescein-5-isothiocyanate (FITC)-labelled TUNEL at 37 °C for 1 h. Thereafter, 4',6-diamidino-2-phenylindole (DAPI) staining was performed to visualize the nuclei, and the TUNEL-positive cells were imaged and quantified in each observation field ($\times$ 400).

Immunohistochemistry

Deparaffinized sections (5- μ m thickness) were heated at 100 °C in 10 mM citrate buffer (pH 6.0). Then, 3% hydrogen peroxide and 10% normal goat serum were used to inhibit hydrogen peroxidase activity and non-specific protein binding, respectively. The sections were reacted with an anti-inducible nitric oxide (iNOS) rabbit polyclonal antibody (1:50 diluted; Biosynthesis, Beijing, China) overnight at 4 °C, followed by incubation with a biotinylated immunoglobulin G (IgG, goat anti-rabbit, 1:200 diluted, Zhongshan Goldenbridge, Beijing, China). The staining was detected using horseradish peroxidase-conjugated streptavidin and detected with 3,3'-diaminobenzidine, followed by counterstained with hematoxylin. The ImageJ software (1.37 version, National Institute of Health, Bethesda, MD, USA) was used to quantify the intensities of the immuno-labels.

Cell culture and treatment

Mouse microglia BV2 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). BV2 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit-Haemek, Israel) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) at 37 °C in a 5% CO₂ incubator. BV2 cells were exposed to BAR (50 and 100 µM) for 30 min and then exposed to LPS (100 ng/mL; Sigma-Aldrich, St. Louis, MO, USA) for 6 h or 24 h.

Cytotoxicity

To investigate the cytotoxicity of BAR, BV-2 cells (at a density of 4.0×10^3 cells/well) were seeded into 96-well plates and treated with different concentrations of BAR (0, 25, 50, 100, and 200 µM) for 24 h. Cell growth was assayed with Cell Counting Kit 8 (Beyotime Institute of Biotechnology) in accordance with the supplier's protocols.

Enzyme-linked immunosorbent assay (ELISA)

After pretreatment with BAR for 30 min, BV2 cells (1×10^5) were exposed to LPS (100 ng/mL) for another 6 h. The cell culture medium was centrifuged at 4 °C at $1000 \times g$ for 15 min to collect the supernatant. The contents of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were evaluated using ELISA kits (R&D Systems, Minneapolis, MN, USA) following the recommended protocols.

Western blot analysis

Total protein and nuclear protein extracts were prepared using available kits (Beyotime Institute of Biotechnology) according to standard protocols. Equivalent amounts of proteins were boiled at 100 °C, separated by sodium dodecyl sulfate polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocked with 5% (w/v) nonfat milk, the membranes were immunoblotted overnight at 4 °C with anti-Bax, anti-Bcl-2, anti-nuclear factor (NF)-κB p65 (1:400 diluted; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-iNOS (1:400 diluted), anti-cleaved caspase-3 (1:1000 diluted; Biosynthesis Biotechnology) rabbit polyclonal antibodies. Afterward, a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was applied, and the specific binding of primary antibody was visualized with an enhanced chemiluminescence assay. β-actin was detected in parallel and served as a loading control. Densitometric analysis was carried out using the Gel Pro 3.0 software.

Statistics

Raw data are presented as mean ± standard deviation. Statistical differences were analyzed by one-way analysis of variance with Tukey's post hoc test, and a P value less than 0.05 was statistically significant. Statistical analyses were performed using GraphPad Prism 5.0 statistical software (GraphPad Software, La Jolla, CA, USA).

Results

BAR protects rats against cerebral I/R injury

At 24 h after reperfusion, neurological deficits were evaluated using a 5-point scale, and infarct size was determined with TTC staining. TTC staining showed that sham operated-rats exhibited no infarct, but MCAO surgery and 24 h of reperfusion resulted in obvious infarct volume. Interestingly, infarct volumes of the brain were significantly reduced in BAR treatment rats compared with the cerebral I/R injury rats (Fig. 1A and 1B). As shown in Fig. 1C, no neurological deficits were observed in sham rats, but a significant increase in neurological deficit score was found in the cerebral I/R injury rats at 24 h after reperfusion. When pretreated with BAR, the score was significantly reduced with that in I/R rats ($P < 0.01$). Our data demonstrate that BAR exerts protective effects on cerebral I/R injury in rats.

BAR reduces I/R-induced neuronal apoptosis in rats

Next, TUNEL staining was conducted to detect apoptotic status in the brains. TUNEL-positive cells were rarely observed in the sham rats, but they were abundant in MCAO rats at 24 h after reperfusion. In contrast, the numbers of TUNEL-positive cells were remarkably decreased in BAR treatment rats, as compared to the I/R rats (Fig. 2A and 2B). In addition, the protein levels of several apoptotic regulators (Bcl-2, Bax and active caspase-3) in the ischemic brain were measured by Western blot analysis. We found MCAO surgery and reperfusion caused sharp increases in active caspase-3, Bax and Bax/Bcl-2 levels and decreases in Bcl-2 levels in I/R brains in comparison to sham brains. These protein levels were remarkably reversed by BAR administration (Fig. 2C and 2D). Our data suggest that BAR alleviates cerebral I/R injury probably through suppressing cell apoptosis.

BAR reduces oxidative stress in brains of I/R rats

GSH-Px and SOD activities and MDA and GSH contents in the rat brains were assessed after 24 h of reperfusion. MDA contents were significantly increased in I/R rats; however, BAR administration remarkably attenuated I/R-induced rise in MDA contents (Fig. 3A). Moreover, GSH levels and GSH-Px and SOD activities were remarkably decreased after 24 h of reperfusion, but BAR administration significantly restored these decreases (Fig. 3B-D). Our findings indicate that BAR reduces I/R-induced neuronal oxidative stress in rats.

BAR down-regulates iNOS protein levels in the ischemic brains

Elevated expression of iNOS is linked to pathophysiology of cerebral I/R injury (17). Thus, immunohistochemistry and Western blot analysis were done to evaluate iNOS protein levels in the ischemic brains. The images of the immunohistochemical staining of iNOS are presented in Fig. 4A. The I/R brains showed a significant rise in iNOS reactivity compared with the sham-operated brains. However, BAR administration dampened these alterations (Fig. 4B). Moreover, Western blot analysis found a dramatical rise in iNOS protein levels in I/R ischemic brains in comparison to sham brains. The increase of iNOS protein levels in the ischemic cortex of rats after reperfusion was attenuated by BAR administration in a dose-dependent fashion (Fig. 4C and 4D). These results indicate that BAR suppresses iNOS expression in ischemic cortex of rats.

BAR inhibits LPS-induced inflammatory mediators in BV2 cells

Before the assessment of the anti-inflammatory effects of BAR, the cytotoxic effects of BAR on BV-2 cells were detected by CCK-8 assay. As illustrated in Fig. 5A, treatment with BAR less than 200 μ M did not affect the growth of BV-2 cells. Next, the effects of BAR on inflammatory mediators in LPS-treated BV-2 cells were investigated. We observed that LPS treatment dramatically increased the contents of proinflammatory mediators (TNF- α and IL-6), while BAR pretreatment significantly reversed these effects in a dose-dependent manner (Fig. 5B). Furthermore, Western blot analysis was carried out to detect iNOS and nuclear NF- κ B p65 levels in BV-2 cells upon LPS stimulation. As illustrated in Fig. 5B and 5C, iNOS and nuclear NF- κ B p65 levels were remarkably increased by LPS. However, treatment with BAR dose-dependently inhibited the increases in iNOS and nuclear NF- κ B p65 protein levels ($P < 0.01$). These observations reveal that BAR pretreatment inhibits inflammatory responses in BV2 cells upon LPS stimulation.

Discussion

BAR is a naturally occurring bioactive anthracycline derived from the leaf exudates of aloe plants, and aloe vera has been used as an ingredient in a wide range of dietary and cosmetic products (18). Mounting evidence indicates that aloe exerts a variety of pharmacological activities, including neuroprotective effects (19). For instance, Rathor and colleagues have documented that the extract of aloe vera leaf has anticonvulsant and anti-oxidant activities against acute and chronic epilepsy in mice (20). However, the neuroprotective effects of BAR, the most abundant anthracycline present in aloe vera, have not been reported. In this study, intraperitoneal injection of BAR significantly attenuated MCAO-induced cerebral I/R injury in rats, as shown by improvements in neurological scores and decreases in brain infarct volume. Meanwhile, BAR reduced oxidative stress and iNOS expression and inhibited neuronal apoptosis in cerebral I/R rats. In addition, pretreatment with BAR suppressed LPS-stimulated inflammatory responses, iNOS expression, and NF- κ B activation in BV-2 microglial cells.

Aberrant neuronal apoptosis represents a pathological hallmark of many human neurological diseases, such as diabetic encephalopathy, stroke, Alzheimer's disease, and amyotrophic lateral sclerosis (21, 22). It has been demonstrated that apoptosis is responsible for neuronal death in cortex following cerebral ischemia (23). In this study, the number of TUNEL-positive cells were increased in the ischemic cortex of I/R rats compared with that in the sham rats, and this increase in TUNEL-positive cells was remarkably reversed by BAR treatment. Our findings agree with a previous study showing that BAR inhibits apoptosis in cardiomyocytes and attenuates myocardial I/R injury in rats (15). Caspase-3, an important executioner of programmed cell death, is activated to initiate apoptotic DNA fragmentation in MCAO-induced focal cerebral I/R injury, thereby leading to expansion of infarct volume (24). The Bcl-2 family is consisted of anti-apoptotic (Bcl-2, Bcl-xl, and Bcl-w) and pro-apoptotic members (Bax, Bak, and Bok) (25). Herein, we observed that BAR administration suppressed activated caspase-3 and Bax protein levels, but increased

Bcl-2 protein levels in the ischemic brains, suggesting the mechanisms by which BAR inhibits neuronal apoptosis. However, the mechanisms underlying regulation of Bcl-2 and Bax by BAR need to be further investigated.

Oxidative stress, also termed as excessive reactive oxygen species (ROS) production overwhelming intrinsic antioxidant defense, is linked to the pathophysiology of brain I/R injury (26). High levels of ROS can react with membrane lipids and oxidize membrane proteins to initiate lipid peroxidation, resulting in neuronal apoptosis (27). MDA, a stable final product in the lipid peroxidation process, is commonly determined to indirectly measure ROS content (28). In addition, antioxidant enzymes, such as GSH, GSH-Px and SOD, are a group of ROS scavengers that detoxify ROS to protect against ROS-induced damages (29). Therefore, assessment of these enzyme activities is used to evaluate the antioxidant capacities. Herein, we found that I/R injury resulted in obvious increases in MDA contents and decreases in GSH levels and SOD and GSH-Px activities in the ischemic brains, and BAR administration significantly reversed these changes in brain tissues. Similarly, BAR has also been shown to be potent antioxidant in cardiomyocytes (13, 14). In addition to the antioxidant actions, its ability to restore antioxidant enzymes may also contribute to the therapeutic benefits of BAR. Collectively, our data indicate that BAR protects brains from I/R-induced damages by limiting oxidative stress.

Neuroinflammation is known as a major contributor that results in neuron damage and death following cerebral ischemia (30). NF- κ B is a central regulator in inflammatory process (31). In response to ROS, NF- κ B is activated in I/R injury to induce excessive release of proinflammatory mediators (e.g. iNOS, TNF- α and IL-6), thereby aggravating brain damages (32). NF- κ B inactivation or inhibition of iNOS expression attenuates brain ischemic injury (32, 33). In this study, BAR suppressed I/R-induced iNOS expression in the ischemic cortex and inhibited LPS-stimulated production of proinflammatory cytokines (IL-6, TNF- α and iNOS) and nuclear NF- κ B p65 in BV2 cells. Our results are consistent with published literatures reporting that BAR attenuated ulcerative colitis-induced and LPS-induced acute lung injury *via* suppressing the release of pro-inflammatory cytokines (10, 11). Altogether, it is plausible that BAR exerts the neuroprotective effects in cerebral I/R injury *via* its anti-inflammatory properties.

There are several limitations to acknowledge. Firstly, the underlying mechanisms by which BAR exerts the anti-oxidative, anti-apoptotic, and anti-inflammatory activities are not investigated. Secondly, the present study treated I/R rats after reperfusion, whether pretreatment of BAR before I/R injury shows similar beneficial effects has not been determined. Thirdly, the animal number in this study is relatively small.

Conclusions

In conclusion, we demonstrated that intraperitoneal injection of BAR after initial cerebral ischemia obviously restricted brain infarct size and improved neurological outcome in MCAO-induced cerebral ischemia rats. BAR exerts the neuroprotective effects, at least in part, by inhibiting apoptosis, oxidative stress, and inflammatory response. Our data imply that BAR is a useful candidate to prevent and treat ischemic stroke.

Abbreviations

BAR, Barbaloin; I/R, ischemia and reperfusion; iNOS, inducible nitric oxide synthase; tPA, tissue-plasminogen activator; MCAO, middle cerebral artery occlusion; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; TTC, 2,3,5-triphenyltetrazolium chloride; MDA, malondialdehyde; GSH, glutathione; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; FITC, fluorescein-5-isothiocyanate; ELISA, enzyme-linked immunosorbent assay; TNF- α , tumor necrosis factor; IL-6, interleukin-6; NF- κ B, nuclear factor; ROS, reactive oxygen species.

Declarations

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Contributions

LW performed the experiments and analyzed and interpreted the data. LZ wrote the manuscript. All authors read and approved the final manuscript.

Ethical approval

The animal procedures used in this study were approved by the Animal Care and Use Committee of Shengjing Hospital (No. 2018-56). The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

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focal cerebral ischemia protects brain and reduces inflammation in a rat model of experimental

Figures

Figure 1

Effects of BAR on infarct volumes and neurological deficit scores in rats after 90 min of MCAO and 24 h of reperfusion. (A) Representative images of coronal brain sections stained with 2, 3, 5-triphenyltetrazolium chloride. (B) Quantitative analysis of brain infarct volume. (C) Neurologic deficit scores. The data are expressed as mean $\pm$ SD (n = 8). #P<0.05 and ##P<0.01 vs the I/R group.

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Figure 2

Effects of BAR on neuronal apoptosis in ischemic cortex after 90 min of MCAO and 24 h of reperfusion. (A) Cell apoptosis in the penumbra cortex was detected by TUNEL staining. (B) The TUNEL-positive cells were counted in 10 randomly selected fields. (C) Western blot analysis of cleaved caspase-3, Bcl-2 and Bax protein expression levels. (D) Quantified results of Western blot analysis were normalized to β -actin expression (n = 8). **P<0.01 vs the sham group; #P<0.05 and ##P<0.01 vs the I/R group.

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Figure 3

Effects of BAR on lipid peroxide and antioxidant parameters in ischemic cortex after 90 min of MCAO and 24 h of reperfusion. MDA (A) and GSH (C) contents and SOD (B) and GSH-Px (D) activities in the penumbra cortex were determined. Data are expressed as means $\pm$ SD (n = 8). **P < 0.01 vs the sham group; ##P < 0.01 vs the I/R group.

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Figure 4

Effects of BAR on iNOS expression in ischemic cortex after 90 min of MCAO and 24 h of reperfusion. (A) Immunohistochemical analysis of iNOS protein expression in ischemic cortex, and (B) the ImageJ software was used to quantify the expression of iNOS in 10 randomly selected fields. (C) Western blot analysis was conducted to determine the protein level iNOS in ischemic cortex. (D) The density of the bands for iNOS was quantified by densitometric analysis. ** $P < 0.01$ vs the sham group; ## $P < 0.01$ vs the I/R group.

Figure 4

Effects of BAR on iNOS expression in ischemic cortex after 90 min of MCAO and 24 h of reperfusion. (A) Immunohistochemical analysis of iNOS protein expression in ischemic cortex, and (B) the ImageJ software was used to quantify the expression of iNOS in 10 randomly selected fields. (C) Western blot analysis was conducted to determine the protein level iNOS in ischemic cortex. (D) The density of the bands for iNOS was quantified by densitometric analysis. ** $P < 0.01$ vs the sham group; ## $P < 0.01$ vs the I/R group.

Figure 5

Effects of BAR on pro-inflammatory mediators in LPS-stimulated BV2 cells. (A) BV2 cells were incubated with various concentrations of BAR for 24 h, and the CCK-8 assay was performed to detect cytotoxicity. (B) BV2 cells were pretreated with BAR for 30 min prior to LPS (100 ng/mL) stimulation for another 6 h. ELISA was performed to measure the concentrations of TNF-α and IL-6 in the supernatant of cell culture medium. (C) BV2 cells were pretreated with BAR for 30 min and then exposed to LPS (100 ng/mL) for 24 h. Western blot analysis was conducted to detect iNOS and nuclear NF-κB p65 expression, and representative blots are shown. (D) Densitometric quantification data are expressed as the intensity ratio of target proteins to β-actin (n = 8). **P<0.01 vs the sham group; ##P<0.01 vs the I/R group.

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