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Astaxanthin Regulates PPAR-γ/NF-κB Pathway to Mitigate Nerve Injury after Cerebral Ischemia/Reperfusion in Rats

Miao Huo[1], Qian Zhang[2], Ziyu Zhao[1], Ruifen Xu[1], Xingxing Zheng[1], Guang Yang[1], Jiao Guo[1]*

[1]Department of Anesthesiology, Shaanxi Provincial People's Hospital, Xi'an, China
[2]Department of Burn and Plastic Surgery, Shaanxi Provincial People's Hospital, Xi'an, China
Email: *hxyylp@163.com

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Abstract

Purpose: This research evaluates the efficacy of astaxanthin (AX) on cerebral ischemia/reperfusion (I/R) injury in rats and elucidates the potential mechanism of its neuronal protective effect. **Methods:** Rats were subjected to a middle cerebral artery occlusion/reperfusion (MCAO/R) model. Fifty grown male Sprague-Dawley (SD) rats were divided into 5 groups, including sham operation group (Sham), MCAO/R group, MCAO/R+AX group, MCAO/R+AX+ Scramble group and MCAO/R+AX+ si-PPAR-γ group. The neurological score and cerebral infarction volume were evaluated after surgery. Rat microglia (RM) were stimulated by lipopolysaccharide (LPS) to form an inflammatory environment. LPS-induced RM cells were incubated with different concentrations of AX (1, 5 or 10 μg/mL), then cell viability, the expression of microglial activation markers, including cytokines (IL-1β, IL-6 and TNF-α), cluster of differentiation 68 (CD68), inducible nitric oxide synthase (iNOS) and CD206 and the expression of PPAR-γ and phosphorylated P65 (p-P65) proteins were determined. Cells were treated with pcDNA-PPAR-γ, as well as treatment with si-PPAR-γ or PPAR-γ antagonist GW9662 before AX treatment, and then cell activation mediators were tested. **Results:** AX inhibits LPS-induced RM cells activation and enhanced the expression level of PPAR-γ protein in way of dose-dependent, and pcDNA-PPAR-γ treatment had the same effect as AX. While si-PPAR-γ transfection or PPAR-γ suppressant GW9662 treatment reversed the effect of AX, and cut down the level of PPAR-γ protein and augmented the level of p-P65 protein. In addition, AX treatment alleviated the infarct volume, and sensorimotor and cognitive functions of MCAO/R model rats. **Conclusion:** AX alleviates LPS-induced microglial injury and has a protective effect on rat cerebral I/R injury by regulating the PPAR-γ/NF-κB pathway.

Keywords

Astaxanthin, Cerebral Ischemia/Reperfusion Injury, RM Cells, PPAR-γ, P65

1. Introduction

According to statistics, in 2019, there were 3.94 million new stroke cases and 2.19 million mortalities in China, posing seriously effect on the fitness of Chinese people [1]. Hypertension, smoking, diabetes, and hyperlipidemia are generally considered risk factors for stroke, and stroke is more common in older adults [2]. Reperfusion injury is a main reason for sickness and mortality in ischemic stroke. After cerebral I/R, a strong inflammatory response could be triggered, resulting in further neurological damage. Inflammation is also one of the key pathological mechanisms of reperfusion injury [3]. After reperfusion injury, microglia in the brain are activated and recruited to the peri-infarct area, and the activated microglia could express characteristic markers and release pro-inflammatory markers, such as inducible nitric oxide synthase (iNOS) and interleukin 6 (IL-6), which could aggravate brain injury [4].

Clinically, antihypertensive drugs are often used in combination with statins to treat stroke in people at cardiovascular risk [5]. Astaxanthin (AX) is a natural fat-soluble orange-red oxygenated carotenoid pigment belonging to a class of carotenoids called lutein [6], which has powerful anti-oxidative and anti-inflammatory capacities and plays a therapeutic role in a variety of diseases, such as atherosclerosis [7], osteoarthritis [8], diabetic nephropathy [9], etc. Furthermore, AX is reported to exert neuroprotective influences through anti-inflammatory and antioxidant activities in experimental animal models of cerebral ischemia [10]. A study showed that feeding AX to MCAO model rats could obviously reduce stroke volume, neurological deficits, and lipid peroxidation [11]. Meanwhile, AX could alleviate β-amyloid-induced neuronal apoptosis and oxidative stress by activating the ERK1/2 pathway and up-regulating the expression of heme oxygenase 1 [12].

As is reported that peroxisome proliferator-activated receptors (PPARs) are related to the ligand-activated transcription factors subclasses, which consists of three kinds of isoforms, PPAR-α, PPAR-β/δ and PPAR-γ. PPARs are closely associated with the expression of numerous genes regulating energy homeostasis, lipid metabolism, cell proliferation, inflammation, and vascular tissue functions [13]. PPAR-γ activation could prevent I/R injury in rat liver [14]. Contemporary, the report revealed that PPAR-γ overexpression could alleviate MCAO/R-induced neuronal apoptosis and inflammatory responses in rats by motivating phosphorylation of PI3K and AKT [15]. A recent study showed that AX could interact with PPARs, and that AX-mediated restructuring of PPARs has remedial influences on varieties of pathophysiological criteria [16]. However, the regulatory effect of AX on PPAR-γ in cerebral I/R injury is yet indistinct.

In this research, we observed that AX alleviated cerebral I/R injury induced rats neuroinflammation by targeting PPAR-γ/NF-κB pathway, providing a novel strategy for clinical therapy of cerebral I/R injury.

2. Materials and Methods

2.1. Cell Culture

Rat microglia (RM, R1900) line was provided by Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. RM cells were cultivated in Microglia Medium (MM, Cat. No. 1901, Zhong Qiao Xin Zhou) at 37˚C in a 5% CO_2 incubator. The blank control group consisted of cells that received medium only. While a dose of 1 μg/mL lipopolysaccharide (LPS, S11060, Shanghai yuanye Bio-Technology Co., Ltd, China) was utilized as an active inflammation control. Then, cells were incubated with 1 μg/mL, 5 μg/mL or 10 μg/mL AX (B25542, Shanghai yuanye Bio-Technology Co., Ltd), respectively.

2.2. Cell Transfection

The eukaryotic vectors pcDNA3.1-PPAR-γ and its isotype control were established by Sangon, Shanghai, China. RM cells were inoculated in 12-well plates under a specific mass of 5.0×10^4 cells/well after pre-transfection with 1 μg pcDNA3.1 plasmid for 24 h utilized Lipofectamine2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). Then, RM cells were digested 3 days after transfection.

PPAR-γ siRNA (si-PPAR-γ) and the scrambled siRNA (Scramble) were sourced from Shanghai GenaPharma Co., Ltd. Cells were transfected in term of the manufacturer's instructions and harvested 24 h later for subsequent experiments.

2.3. Detection of Cell Viability

Cell Counting Kit-8 (CCK-8) measurements (Sigma-Aldrich, St. Louis, MO, USA) were utilized to examine the RM cells viability. Briefly, cells were injected into 96-well microwells. After 24 h incubation, adding 10 μL of CCK-8 solubility product per well and incubating for 2 h with an additionally 5% CO_2 and 37˚C humidified. Precise measurement of 450 nm absorbance of each well by using a microplate reader.

2.4. Animal

The mature male Sprague-Dawley (SD) rats (weighted 260 ± 20 g) utilized in this research were acquired from the Animal Center of Xi'an Jiaotong University, and entire animal agreements were managed in terms of the "Guidelines for the Care and Use of Laboratory Animals", and the animal trials were confirmed by the Shaanxi Provincial People's Hospital Animal Research Committee. I/R injury was performed by middle cerebral artery occlusion/reperfusion (MCAO/R) as antecedently reported [17]. Briefly, rat cerebral ischemia was operated by 60-min

invertible MCAO upon isoflurane anesthesia. An automatic temperature control feedback system was utilized to sustain anal temperature at 36.5˚C ± 0.5˚C during surgery. Unilateral MCAO was executed by inserting a 6.0 mm monofilament (Doccol, Redlands, CA) through the external carotid stump into the right internal carotid artery 6 mm from the internal carotid/pterygopalatine bifurcation using a midline abdominal neck incision. 60 min occlusion followed by thread withdrawal for reperfusion. Fifty rats were casually separated into five groups: sham operation group (Sham), MCAO/R group, MCAO/R+AX group, MCAO/R+AX+ Scramble group and MCAO/R+AX+ si-PPAR-γ group, 10 per group. Sham group rats were subjected to the uniform surgical step, just the stitching was not got into the inner carotid artery. MCAO/R+AX group rats were subcutaneously injected with 10 mg/kg AX daily for 7 days before operation. MCAO/R+AX+ Scramble group or MCAO/R+AX+ si-PPAR-γ group rats were respectively injected with 10 μL of scrambled siRNA or PPAR-γ siRNA in the ventricle daily for 3 days before surgery. All of the rats were slaughtered at 7 d of reperfusion. The measurement of the MCAO/R-stimulated infarct was determined by triphenyl tetrazolium chloride (TTC) staining.

2.5. Infarct Volume Determination

After MCAO for 7 d, rat brain tissue was shifted and cut into 71-mm coronal slices, which were placed at 37˚C and dipped in a 2% 2,3,5-triphenyl-2H-tetrazolium chloride fluid for 15 min. At this point, normal brain tissues were dyed red, while the infarct tissues were dyed light gray. Subsequently, the sections were taken pictures and evaluated using Image-Pro Plus 6.0 (Media Cybernetics), and the infarct volume (percent) of the sections was computed after modification for dropsy.

2.6. Neurological Function Tests

All animals were tested for foot failure and a modified neurological severity score (mNSS) was procured at 1, 3, 5 and 7 days after MCAO/R operation. mNSS is a complex which utilized to appraise neurological characteristic grounded on motion, sensation, equilibrium, and reflex measuring on a range of 0 to 18 (normal score, 0; maximum deficit score, 18), and higher marks were intended to biggish nerve damage.

2.7. RT-qPCR

Trizol reactant (Invitrogen) was utilized to purify entire RNA from RM cells or brain tissues in terms of the manufacturer's explanations, NanoDrop ND-1000 (NanoDrop Technologies) was utilized to confirm the density of depurative RNA specimens. SuperScript IV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) was utilized for reverse transcription of RNA to cDNA. Additionally, real-time quantitative PCR experiments were performed with the Platinum™ Taq DNA Polymerase (Thermo Fisher Scientific) with SmartChip Real-Time PCR System (TaKaRa Bio, Dalian, China). Besides, the PCR response system involved 1.0 μL of RT primer, 1 μL of cDNA sample, 12.5 μL of Taq DNA

Polymerase, and double distilled H_2O was utilized to complement the remaining volume, and the primer sequences utilized in this research were as below: Cluster of differentiation 206 (CD206, sense: 5'-CTT CGG GCC TTT GGA ATA AT-3', antisense: 5'-TAG AAG AGC CCT TGG GTT GA-3'); Cluster of differentiation 68 (CD68, sense: 5'-GCT ACT GGC AGC CCC AGG G-3', antisense: 5'-GCT CTT GGT AGT CCT GTG G-3'); inducible nitric oxide synthase (iNOS, sense: 5'-CCC AGA GTT CCA GCT TCT GG-3', antisense: 5'-CCA AGC CCC TCA CCA TTA TCT-3'); GADPH (sense: 5'-GAG TCA ACG GAT TTG GTC GT-3', antisense: 5'-AGC ACT GTG TTG GCG TAC AG-3'). Genes expression levels were standardized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the $2^{-\Delta\Delta Ct}$ manner.

2.8. Western Blotting

RM cells and rats brain tissues were dissolved with RIPA lysis buffer (Elabscience Biotechnology, Wuhan, China). BCA protein assay kit (Elabscience Biotechnology) was utilized to determine the protein density. Afterwards, equivalent quantities of proteins (30 μg/lane) were isolated by 10% SDS-PAGE and electro-transferred onto 0.45 μm PVDF membranes (E-BC-R266, Elabscience Biotechnology). Subsequently, membranes were immerged in 5% non-fat milk for 2 h in an indoor environment, and then primary antibodies provided from Abcam comprising rabbit monoclonal anti-PPAR-γ (1:1000, ab272718), anti-phosphorylated P65 (p-P65, 1:1000, ab76302) and anti-P65 (1:2000, ab76311) and mouse monoclonal anti-GADPH (1:2000, ab9485) were respectively appended to petri dish co-cultivating with membranes overnight at 4˚C. Later, secondary antibody horseradish peroxidase (HRP)-tagged rabbit anti-mouse IgG (1:1000, #58802, Cell Signaling Technology, Boston, MA, USA) was affixed to membranes at indoor temperature for 2 h. GADPH was selected as the internal reference protein. In addition, proteins expression levels were determined by chemiluminescence, and ImageJ software (National Institutes of Health, Bethesda, MA, USA) was utilized to quantify proteins expression levels.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

The excretion of cytokines containing IL-1β, IL-6 and TNF-α in RM cells and rats brain tissues were tested by specific ELISA kits (TaKaRa Biotechnology) pursuant corresponding manufacturer's instructions.

2.10. Statistical Analysis

The experimental data were determined by SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and appeared as mean ± SEM. While $P < 0.05$ was recognized as statistical significance. The diversity between two unpaired instances was assessed by student's t test. The diversity among more than three groups was assessed by one-way ANOVA with Tukey's post hoc test or by two-way ANOVA with Bonferroni post hoc test.

3. Results

3.1. AX Impedes LPS-Induced RM Cells Activization

To study the effect of AX on microglia activization, RM cells were first co-cultivated with diverse concentration of AX. As shown in Figure 1(A), AX at doses

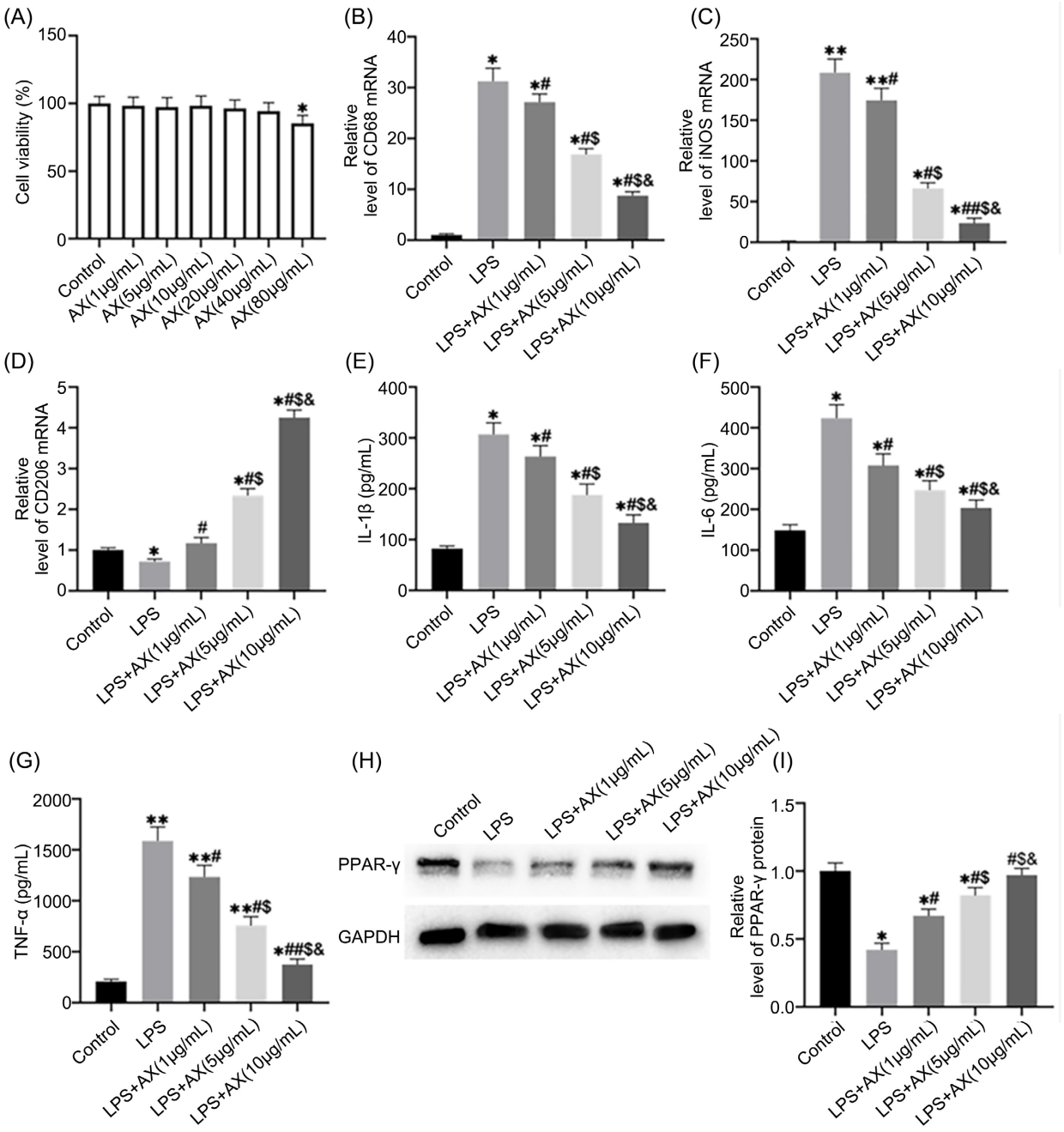


Figure 1. Effects of AX on LPS-stimulated microglia activation. (A) RM cells were incubated with different dose of AX (1, 5, 10, 20, 40 or 80 μg/mL), then cell viability was tested with CCK-8 kits. (B-D) RT-qPCR was utilized to determine the levels of CD68, iNOS and CD206 mRNA. (E-G) The secretion of cytokines IL-1β, IL-6 and TNF-α were tested by special ELISA kits. (H) Western blotting was utilized to determine PPAR-γ protein level. Values were represented as mean ± SEM. n = 6; *P < 0.05, **P < 0.01 vs. control group; #P < 0.05, ##P < 0.01 vs. LPS group; \$$P$ < 0.05 vs. AX (1 μg/mL) treatment group; &P < 0.05 vs. AX (5 μg/mL) treatment group.

ranging from 1 to 40 μg/mL were noneffective in cell viability. Compared with the control group, LPS induction increased the levels of CD68 (Figure 1(B)) and iNOS (Figure 1(C)) mRNA and the secretion of cytokines IL-1β, IL-6 and TNF-α (Figures 1(E)-(G)), and decreased the expression of CD206 mRNA (Figure 1(D)) and PPAR-γ protein (Figure 1(H)) levels. Compared with the LPS stimulation group, AX treatment decreased the levels of CD68 (Figure 1(B)) and iNOS (Figure 1(C)) mRNA and the secretion of cytokines IL-1β, IL-6 and TNF-α (Figures 1(E)-(G)), and increased the expression of CD206 mRNA (Figure 1(D)) and PPAR-γ protein (Figure 1(H)) levels. While the effect of high-dose AX (10 μg/mL) treatment group was superior than that of medium- (5 μg/mL AX) and low-dose (1 μg/mL AX) groups.

3.2. PPAR-γ Overexpression Suppresses LPS-Induced RM Cells Activation

To explore the influence of PPAR-γ protein on microglia activation, LPS-induced RM cells were co-cultivated with pcDNA-PPAR-γ. After that, the cell functions were determined. The results displayed that compared with the LPS+ Vector group, the expression of CD68 (Figure 2(B)) and iNOS (Figure 2(C)) mRNA levels and the secretion of cytokines IL-1β, IL-6 and TNF-α (Figures 2(E)-(G)) were enhanced, and the levels of PPAR-γ protein (Figure 2(A)) and CD206 mRNA (Figure 2(D)) were decreased in pcDNA-PPAR-γ treatment group.

3.3. AX Restrains LPS-Induced RM Cells Activation by Regulating the PPAR-γ/NF-κB Pathway

To further probe the regulatory mechanism of AX on microglial activation, LPS-induced RM cells were incubated with 10 μg/mL AX after transfection with si-PPAR-γ. Western blotting analysis displayed that compared with the control group, the level of PPAR-γ protein (Figure 3(A)) was decreased and the level of p-P65 protein (Figure 3(B)) was increased after LPS induction. Compared with the LPS induction group, PPAR-γ protein level was increased and p-P65 protein was decreased after AX treatment. While PPAR-γ interference downregulated the level of PPAR-γ protein and upregulated the level of p-P65 protein. As determined by RT-qPCR and ELISA assays, PPAR-γ interference increased the levels of CD68 (Figure 3(C)) and iNOS (Figure 3(D)) mRNA and the secretion of cytokines IL-1β, IL-6 and TNF-α (Figures 3(F)-(H)), and decreased the expression of CD206 mRNA (Figure 3(E)). All these findings suggested that the inhibitory effect of AX on LPS-induced microglial activation might be related to the PPAR-γ/NF-κB pathway.

3.4. PPAR-γ Antagonist GW9662 Abolishes the Inhibitory Effect of AX on RM Cells Activation

Similarly, compared with the LPS+AX group, PPAR-γ antagonist GW9662 treatment increased the expression of p-P65 protein level (Figure 4(A)), CD68 (Figure 4(B)) and iNOS (Figure 1(C)) mRNA levels and the secretion of cytokines

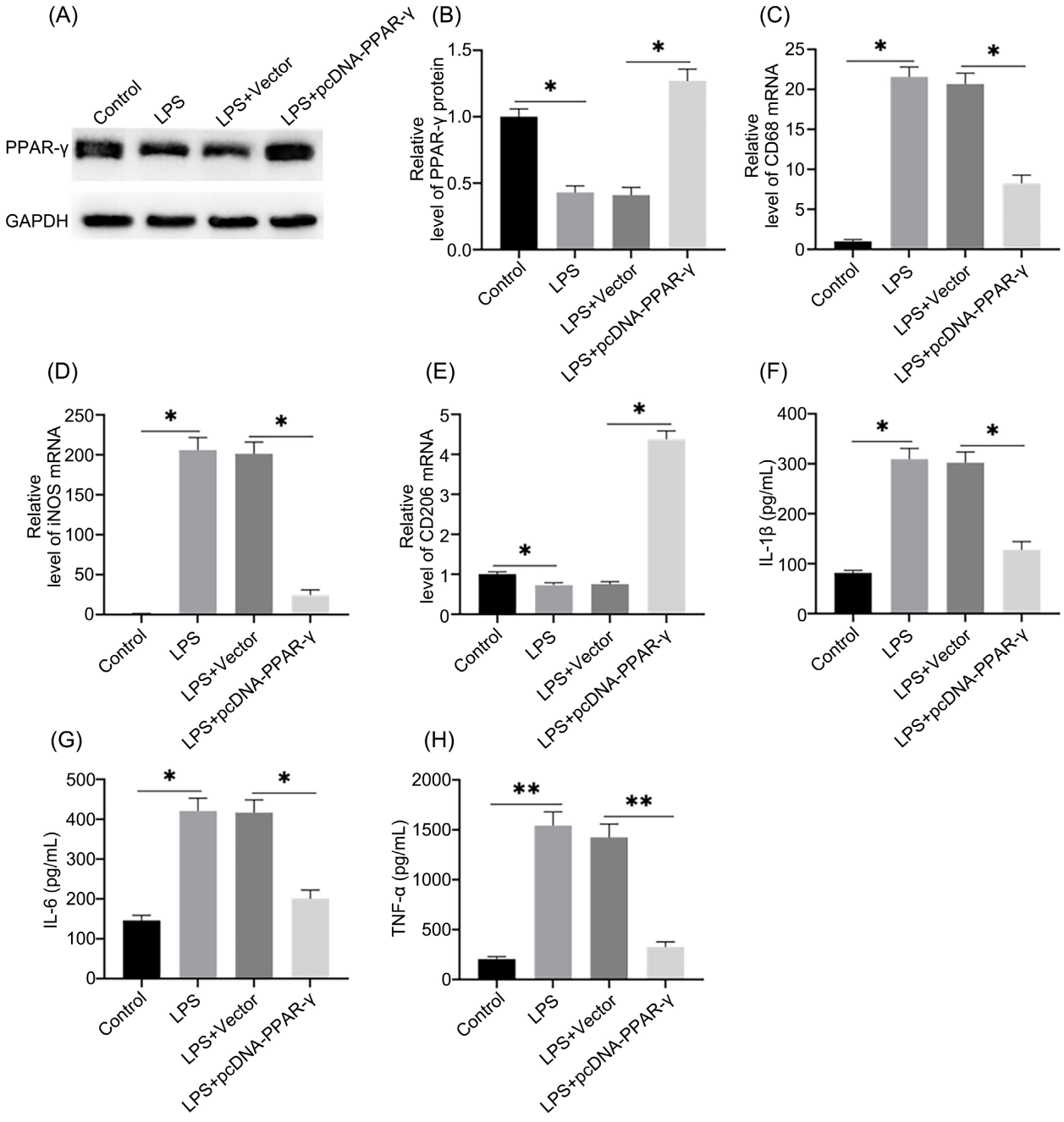


Figure 2. Effects of PPAR-γ overexpression on LPS-stimulated microglia activation. LPS-induced RM cells were co-incubated with pcDNA-PPAR-γ or empty vector. (A) Western blotting was utilized to determine PPAR-γ protein level. (B-D) RT-qPCR was utilized to determine the levels of CD68, iNOS and CD206 mRNA. (E-G) The secretion of inflammatory factors IL-1β, IL-6 and TNF-α were tested with ELISA kits. Values were represented as mean ± SEM. n = 6; *$P < 0.05$, **$P < 0.01$.

IL-1β, IL-6 and TNF-α (Figures 4(F)-(H)), and decreased the levels of PPAR-γ protein (Figure 4(A)) and CD206 mRNA (Figure 4(E)).

3.5. AX Exerts Neuroprotective Effect on Cerebral I/R Injury in Rats

In order to investigate the effect of AX on rat cerebral I/R injury, we firstly

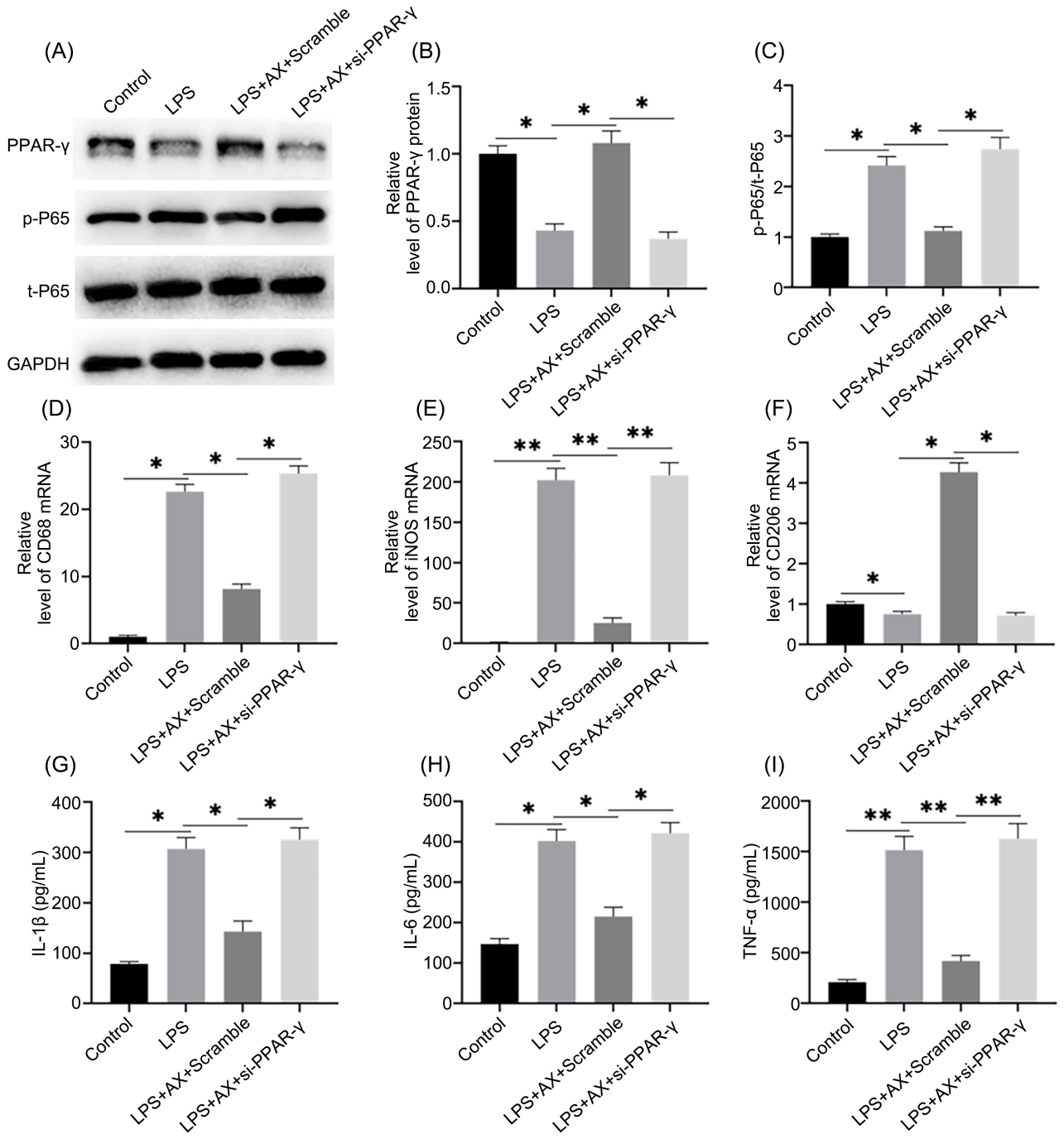


Figure 3. Mechanism of AX inhibiting LPS-induced microglial activation. LPS-induced RM cells were co-incubated with 10 μg/mL AX after pre-treatment with si-PPAR-*γ* or negative control scrambled siRNA. (A and B) Western blotting was utilized to determine the levels of PPAR-*γ* and p-P65 proteins. (C-E) RT-qPCR was utilized to detect the expression of CD68, iNOS and CD206 mRNA. (F-H) The secretion of inflammatory factors IL-1*β*, IL-6 and TNF-*α* were determined with ELISA kits. Values were represented as mean ± SEM. n = 6; **P* < 0.05, ***P* < 0.01.

established a rat MCAO/R model. The results revealed that compared with the sham operation group, the neurological damage of the rats in the MCAO/R model group was significantly severe (**Figure 5(A)**), and the levels of CD68 (**Figure 5(B)**) and iNOS (**Figure 5(C)**) mRNA and the secretion of cytokines

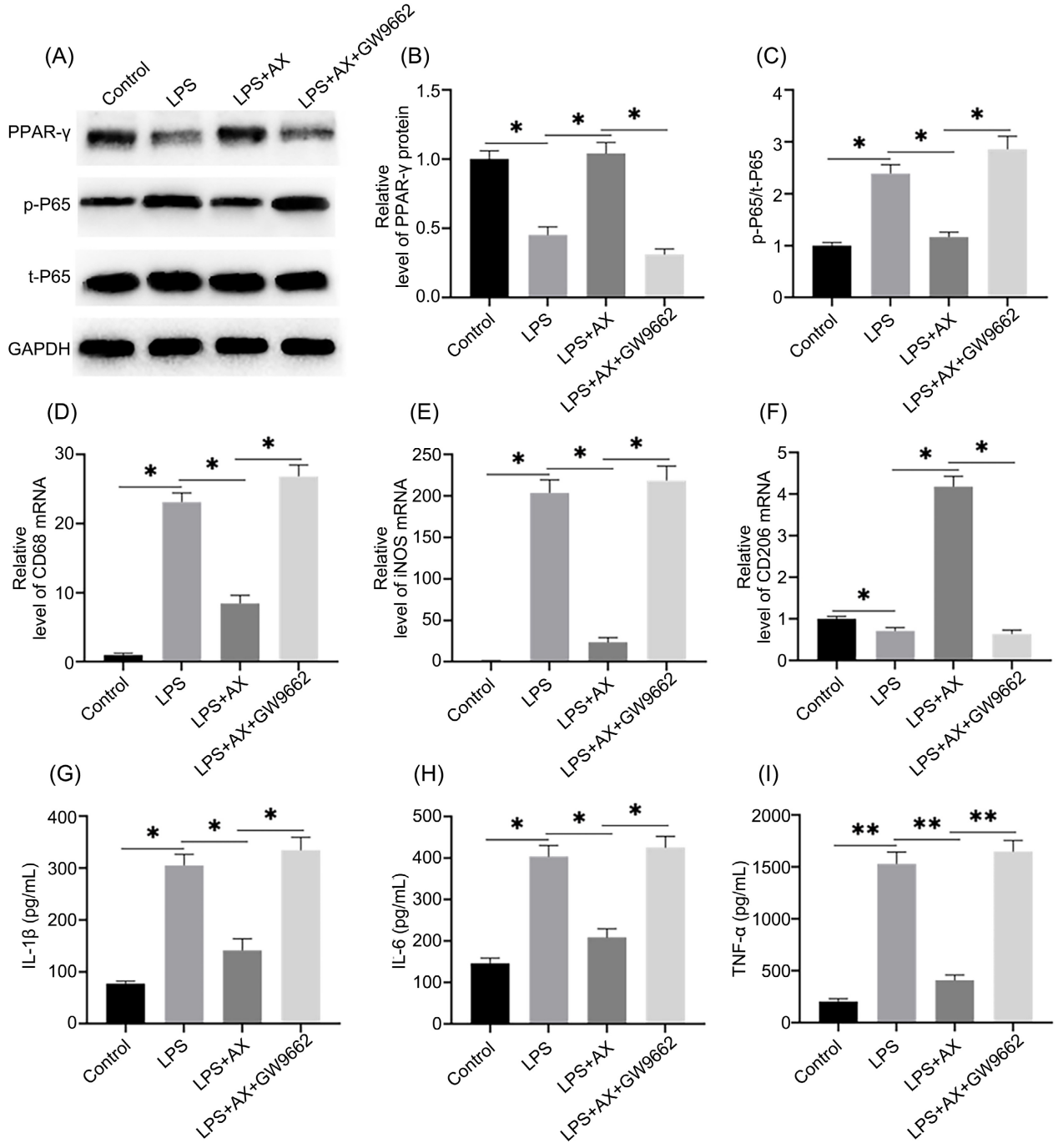


Figure 4. PPAR-γ antagonist GW9662 abrogates the inhibitory effect of AX on microglial activation. LPS-induced RM cells were co-incubated with 10 μg/mL AX or/and PPAR-γ antagonist GW9662. (A and B) Western blotting was utilized to determine the levels of PPAR-γ and p-P65 proteins. (C-E) RT-qPCR was utilized to detect the expression of CD68, iNOS and CD206 mRNA. (F-H) The secretion of inflammatory factors IL-1β, IL-6 and TNF-α were determined with special ELISA kits. Values were represented as mean ± SEM. n = 6; *$P < 0.05$, **$P < 0.01$.

IL-1β, IL-6 and TNF-α (Figures 5(E)-(G)) in rats brain tissues were distinctly enhanced. Compared with the MCAO/R model group, AX treatment prominently ameliorated the neurological damage of rats (Figure 5(A)), and dramatically decreased the expression of CD68 (Figure 5(B)) and iNOS (Figure 5(C))

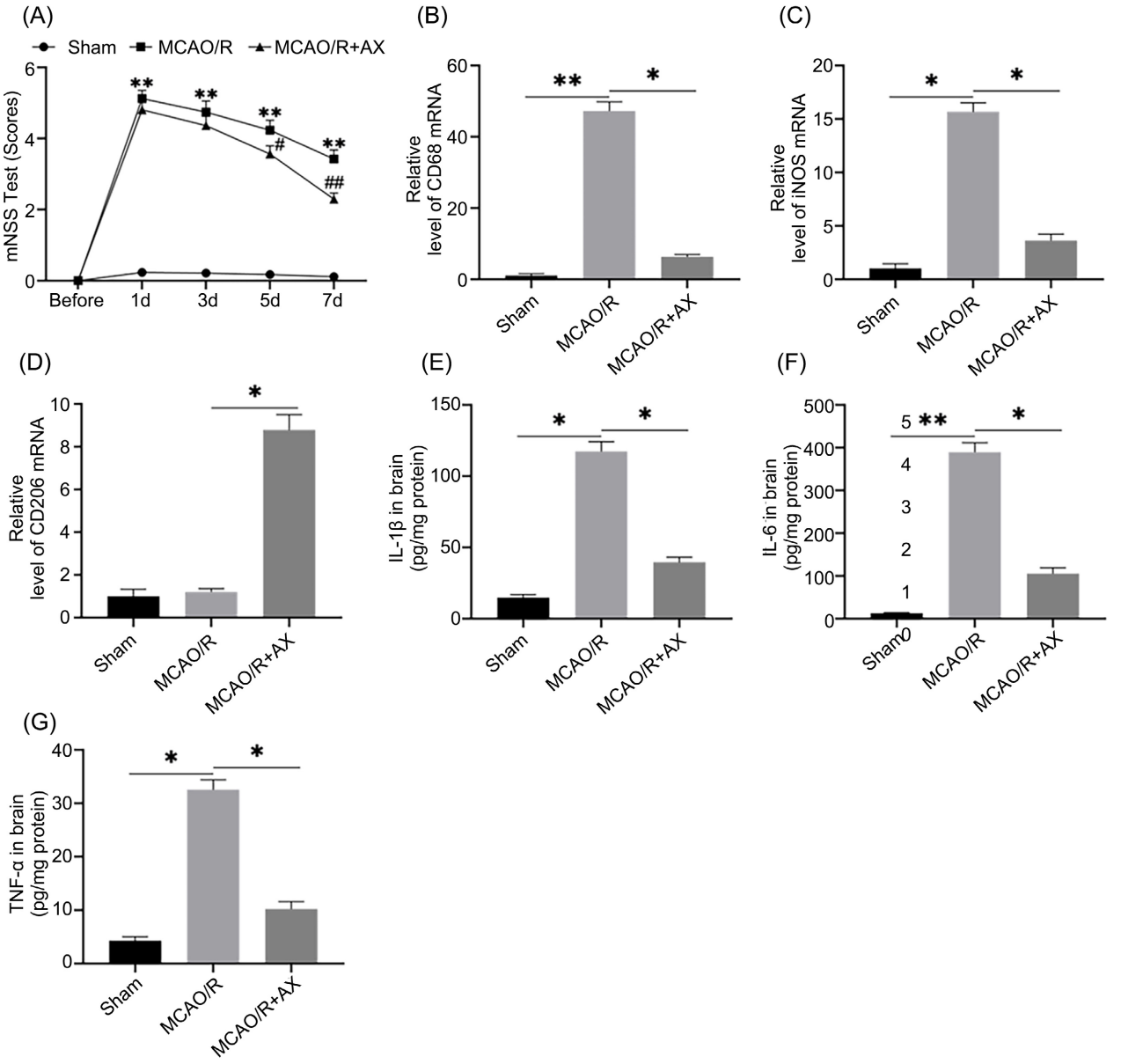


Figure 5. Efficacy of AX on cerebral I/R injury in rats. (A) Quantification of modified neurological severity scores(mNSS) at 1, 3, 5, and 7 days after MCAO/R. (B-D) RT-qPCR was utilized to test the mRNA levels of CD68, iNOS and CD206. (E-G) ELISA kits were utilized to determine the concentrations of IL-1β, IL-6 and TNF-α. Values were represented as mean ± SEM. n = 10; $^{*}P < 0.05$, $^{**}P < 0.01$.

mRNA and the secretion of cytokines IL-1β, IL-6 and TNF-α (Figures 5(E)-(G)) in rats brain tissues, and dramatically reduced the expression of CD206 mRNA (Figure 5(D)). These results suggested that AX protects rats from I/R-induced nerve injury.

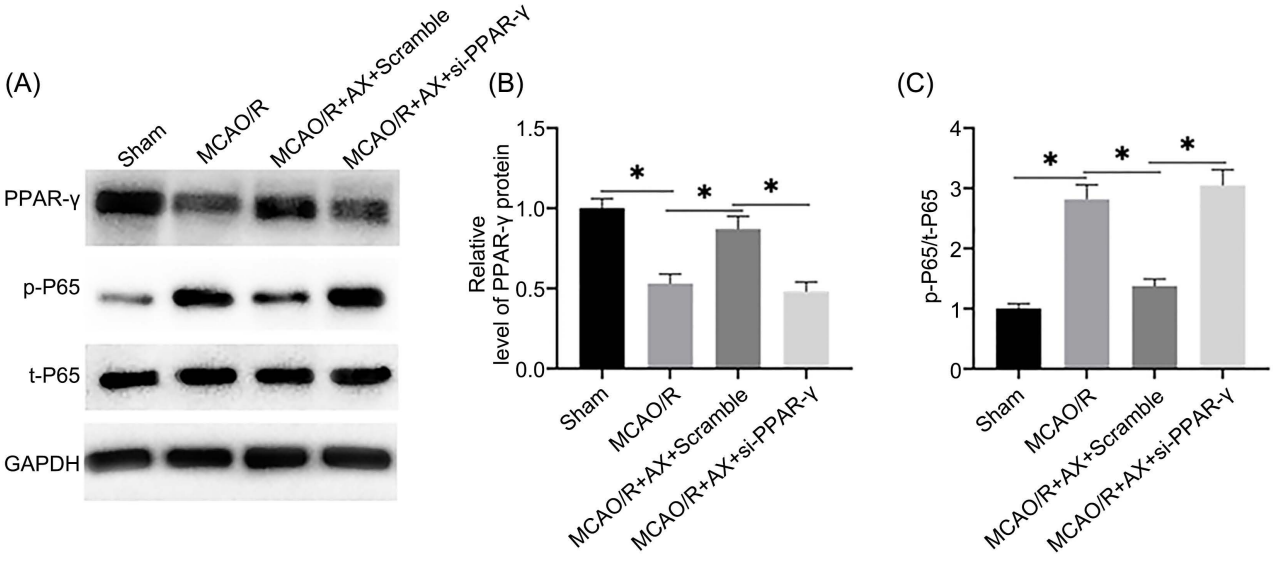
3.6. AX Ameliorates Rat Cerebral I/R Injury by Regulating the PPAR-γ/NF-κB Pathway

To investigate the pathogenesis of rat cerebral I/R injury, MCAO/R model rats were treated with AX with or without injection si-PPAR-γ. Western blotting re-

sults displayed that compared with Sham group, the level of PPAR-γ protein (**Figure 6(A)** & **Figure 6(B)**) & was prominently reduced and the level of p-P65 protein (**Figure 6(A)** & **Figure 6(C)**) was prominently increased in the MCAO/R model group rats. Compared with MCAO/R model group, AX treatment increased the level of PPAR-γ protein and decreased the level of p-P65 protein in rats brain tissues. While si-PPAR-γ injection reduced the level of PPAR-γ protein and enhanced the level of p-P65 protein. TTC staining results demonstrated that compared with Sham group, the rat brain infarct volume (**Figure 6(C)**) was prominently enhanced in MCAO/R model group, and AX treatment observably mitigated rat brain infarct volume, while si-PPAR-γ injection reversed the effect of AX. As determined by RT-qPCR and ELISA assays, compared with the AX treatment group, si-PPAR-γ injection markedly increased the expression of CD68 (**Figure 6(D)**) and iNOS (**Figure 6(E)**) mRNA and the excretion of cytokines IL-1β, IL-6 and TNF-α (**Figures 6(G)-(I)**) in rats brain tissues, and dramatically reduced the expression of CD206 mRNA (**Figure 6(F)**). The above results suggested that AX alleviates nerve injury after I/R in rats by regulating the PPAR-γ/NF-κB pathway.

4. Discussion

Generalized cerebral I/R injury is a type of common complications of clinical cardiopulmonary resuscitation and often causes delayed neuronal damage [18]. Microglia as one of the ample glial cell types in the brain, providing metabolic and nutritional sustainability to neurons and regulating synaptic activity upon regular physiological circumstances. After cerebral ischemia, activated microglia conduce to the inflammatory response and aggravate ischemic lesions [19]. A study claimed that AX inhibits neuroinflammation by inhibiting LPS-induced activation of mouse microglia [20]. Furthermore, previous studies displayed that AX has neuroprotective effects on focal cerebral I/R-induced brain injury in rats [11] [21]. In the current study, we first found that AX doses up to 80 μg/mL had no



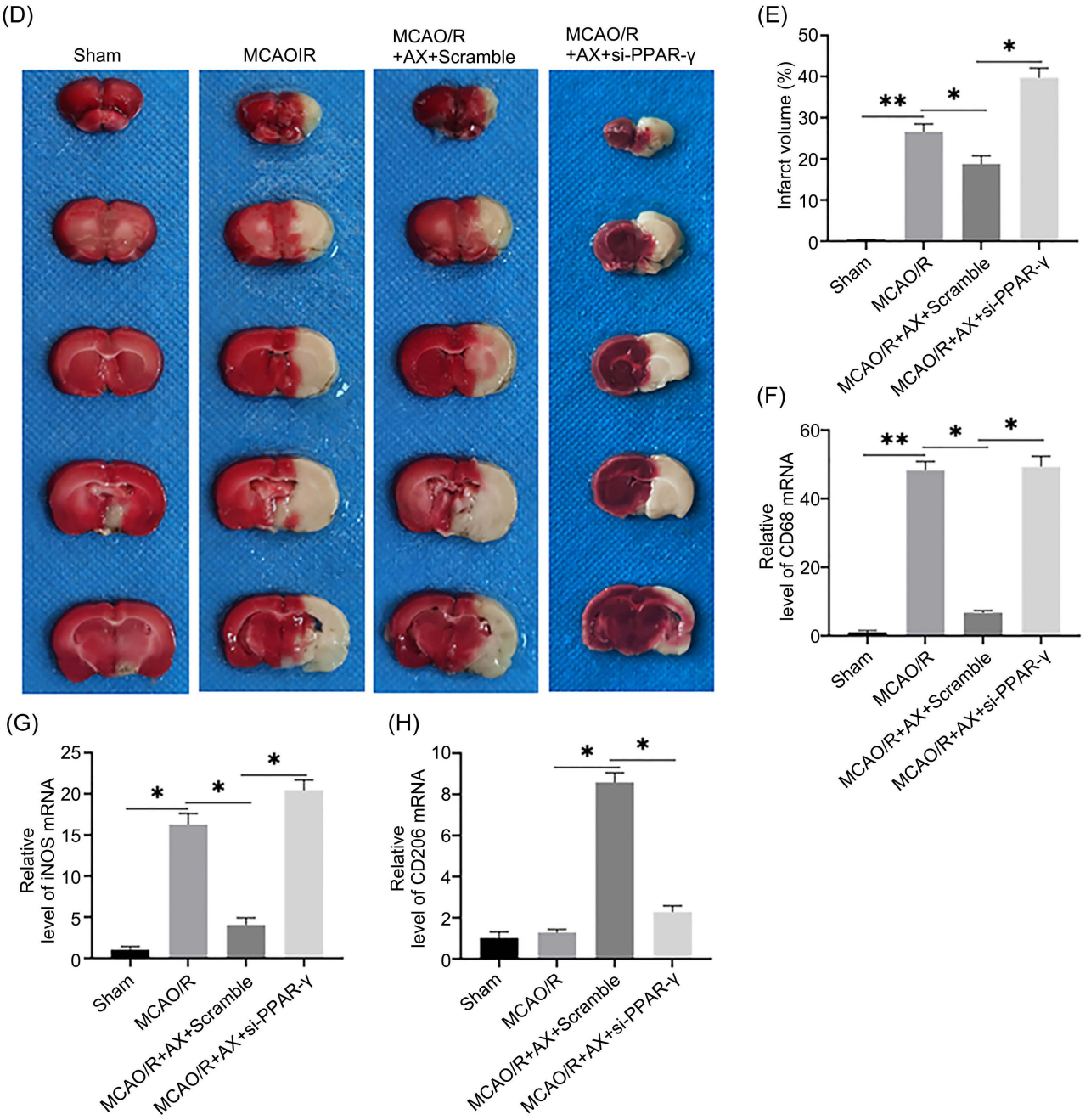


Figure 6. Mechanism of AX in alleviating cerebral I/R injury in rats. (A and B) Western blotting was utilized to determine the levels of PPAR-γ and p-P65 proteins in rats brain tissues. (C) TTC staining sections and quantification of the infarct volumes at 3 d after MCAO/R. (D-F) RT-qPCR was utilized to detect the mRNA levels of CD68, iNOS and CD206. (G-I) ELISA kits were utilized to test the concentrations of IL-1β, IL-6 and TNF-α. Values were represented as mean ± SEM. n = 10; $^{*}P < 0.05$, $^{**}P < 0.01$.

significant influence on rat microglia viability, which is consistent with a previous study that found that AX up to 150 μM was not toxic to the A172 human glioblastoma cell line, suggesting that AX is a relatively safe drug [22]. Simultaneously, we found that AX could dose-dependently reduce the levels of pro-inflammatory phenotype markers CD68 and iNOS mRNA and the excretion of cytokines IL-1β, IL-6 and TNF-α in LPS-stimulated RM cells.

An increasing number of studies showed that stroke and genetic defects are closely related, such as the dysregulation of histone deacetylase 4 in ischemic stroke could lead to impaired angiogenesis and neuronal death [23], and selective deletion of microglial transforming growth factor β-activated kinase 1 protects against long-term obesity resulting in ischemic stroke [24]. PPAR-γ is a ligand-inducible transcription factor remaining with the nuclear receptor superfamily, which can be expressed in microglia and is involved in regulating many biological processes related to cerebral ischemia [25]. PPAR-γ activation has powerful neuroprotective effects, PPAR-γ upregulation strengthens oxidative stress and inflammation response in an adult model of intracranial hemorrhage by downregulating pro-inflammatory cytokines, such as matrix metalloproteinase 9 and iNOS expression [26]. Moreover, it is reported that PPAR-γ activation converts microglia from M1 phenotype to M2 phenotype and repairs brain injury after cerebral I/R in rats [27]. Our research is consistent with these studies, we found that PPAR-γ overexpression alleviated LPS-induced inflammatory responses in RM cells. A study utilized CoA-BAP assay to analyze PPAR-γ binding and found that ASX binds to PPAR-γ in a dose-dependent manner and upregulates PPAR-γ expression, improving obesity and insulin resistance [28]. Besides, it is reported that in esophageal squamous cell carcinoma, AX has a protective effect on esophageal cancer by up-regulating PPAR-γ to activate the apoptotic pathway and inhibit oxidative stress [29]. However, the regulatory effect of AX on PPAR-γ in cerebral I/R injury is ill-defined. Our research found that the expression of PPAR-γ protein was decreased in LPS-induced RM cells, and AX promoted the level of PPAR-γ protein in RM cells in a dose-dependent manner.

Nuclear factor-κB (NF-κB) refers to a variety of cellular functions, including hematopoiesis, apoptosis, immune responses and inflammation [30]. It is showed that NF-κB could regulate the secretion of various pro-inflammatory factors in nerve cells, containing TNF-α, IL-6, and IL-1β [31]. The NF-κB pathway activation could lead to aggravation of cerebral I/R injury and apoptosis of hippocampal neurons in rats [32]. In addition, a study showed that upregulation of PPAR-γ expression ameliorated neuronal cell pyroptosis and protected rat cerebral I/R injury by impeding the phosphorylation of NF-κB pathway [33]. A previous research claimed that AX prevents against LPS-stimulated serious lung injury and sepsis via impeding the phosphorylation of NF-κB pathway [34]. Our current research discovered that AX treatment increased the expression of PPAR-γ protein and reduced the level of p-P65 protein. Meanwhile, PPAR-γ interference or PPAR-γ antagonist GW9662 treatment abolished the suppressing effect of AX on NF-κB pathway activation and microglial activation. *In vivo*, AX treatment alleviated the infarct volume of MCAO/R model rats, sensorimotor and cognitive functions, and neuroinflammation in the brain tissues of rats.

In conclusion, results in the current research illustrated that AX promotes the level of PPAR-γ protein and inhibits NF-κB pathway, alleviating nerve injury af-

ter cerebral I/R in rats. These outcomes gave a new direction for clinical therapy of cerebral I/R injury.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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