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ORIGINAL RESEARCH

Neuroprotective Effects of Eugenol Acetate Against Ischemic Stroke

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Objective: To explore the neuroprotective effect of Eugenol Acetate (EA) on post-stroke neuroinflammation and investigate the underlying mechanisms.

Methods: For in vitro experiments, primary microglia were pre-incubated with EA for 2 hours, followed by lipopolysaccharide (LPS) stimulation for 24 hours or Oxygen-Glucose Deprivation (OGD) treatment for 4 hours. Real-time quantitative PCR, enzyme-linked immunosorbent assay (ELISA) and Western blot were performed to examine the expression levels of inflammatory cytokines in primary microglia. The activation of NF-κB signaling pathway was evaluated by immunofluorescence staining and Western blot. For in vivo experiments, middle cerebral artery occlusion (MCAO) was constructed to mimic ischemic brain injury on 8-week-old male C57BL/6J mice. The mice were continuously injected intraperitoneally with EA or vehicle after MCAO. Neurobehavioral tests and TTC staining were conducted to estimate the neurological deficits and infarct area. Moreover, the white matter integrity after MCAO was observed via immunofluorescence staining.

Results: EA significantly reduced the expression of pro-inflammatory cytokines in LPS or OGD treated primary microglia, and inhibited LPS-induced activation of the NF-κB signaling pathway. In addition, EA alleviated ischemic brain injury and improved neuromotor function of MCAO mice. Furthermore, long-term neurological deficits and white matter integrity were improved by EA treatment after MCAO.

Conclusion: EA alleviated ischemic injury and restored white matter integrity in MCAO mice, which might be associated with the inhibition of NF-κB signaling pathway in microglia. Therefore, EA might be a promising candidate for the treatment of ischemic stroke.

Keywords: Eugenol Acetate, Ischemic Stroke, Neuroinflammation, NF-κB Signaling Pathway

Introduction

Stroke is one of the most common causes of death and disability in the world, and ischemic stroke accounts for 60%-70% of the total number of strokes.[1] Currently, clinical methods for treating ischemic stroke include intravenous thrombolysis, endovascular thrombectomy, and other adjuvant treatments.[2] However, there exists inevitable limitations in these methods, including hemorrhagic transformation and a narrow treatment time window, which compromise the benefits.[3] Microglia-induced neuroinflammation plays a critical role in the pathogenesis of ischemic stroke,[4] which has been a promising target for the treatment of stroke.

Microglia are the resident macrophages in the central nervous system, maintaining the homeostasis of microenvironment and regulating the immune response.[5] Under pathological conditions, such as stroke, microglia are activated immediately to mediate the innate immune response, exerting neuroprotective or neurotoxic effects.[5,6] Activated microglia contribute to the destruction of blood-brain barrier (BBB) and induce neuronal cell death by releasing various pro-inflammatory cytokines and other neurotoxic

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mediators including reactive oxygen species (ROS), matrix metalloproteinases (MMPs), glutamate, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β). In addition, microglia excessively phagocytose damaged neurons leading to delayed neuronal death.[7] However, activated microglia can reduce neuronal apoptosis and enhance nerve regeneration by clearing tissue debris, further promoting the recovery of neurological function after stroke.[8,9] The crosstalk between microglia and neurons is a dynamic process regulated by microenvironmental factors and mediated by multiple pathways.[10] Our group has found that specific microglial subclusters exhibit different roles in the penumbra of ischemic stroke.[11] Disrupting peripheral myeloid cells communicated with microglia or inhibiting their downstream alleviates brain injury in ischemic stroke by limiting microglial inflammation.[12] Therefore, regulating the function of microglia might be a potential therapeutic strategy to treat ischemic stroke.

Traditional Chinese medicine has shown great potential for the treatment of stroke. We have shown that Imperatorin, extracted from the Traditional Chinese Medicine plants *Angelica sinensis* and *Angelica dahurica*, exerts anti-inflammatory effects by inhibiting the MAPK and NF-κB signaling pathways in ischemic stroke.[13] Meanwhile, Ginkgo biloba extract (EGb) improves neurological function recovery and reduces the expression of inflammatory cytokines in the MCAO model, which might be associated with suppressing the HIF-1α/HK2 signaling pathway and promoting the differentiation of Treg cells.[14] Syzygium aromaticum is a natural plant with important pharmacological effects, such as antibacterial, antioxidant, anti-inflammatory, analgesic, anticancer, and anesthetic effects.[15] Eugenol acetate (EA) is a polyphenol extracted from Syzygium aromaticum oil (Figure 1A), and accumulating data suggest that EA is a potential compound to treat inflammation-related diseases.[16,17] In this study, we demonstrated that EA could suppress post-stroke neuroinflammation mediated by activated microglia and mitigate ischemic brain injury, suggesting that EA might be a potential treatment strategy for ischemic stroke.

Materials and Methods

Reagents

Eugenol acetate (CAS: 93-28-7) was obtained from ALADDIN (Shanghai, China), and dissolved in 0.1% dimethyl sulfoxide (DMSO) for subsequent in vitro experiments. During the in vivo experiments, EA was diluted as follows: 45% Saline + 40% PEG300 + 10% DMSO + 5% Tween-80. LPS (Escherichia coli 055: B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Primary Microglia Culture

Primary microglia were prepared as previously described.[18] Briefly, primary microglia cells were isolated from newborn C57/BL6J mice. After being incubated with glia cultures, microglia cells were collected from the mixed glia cells 10–12 days later. Primary microglia cells were seeded in 6 or 12-well plates for following tests after shaking the bottles for 5 minutes. The purity of the primary microglia cells was determined by Iba-1 immunocytochemical staining. The concentration of LPS for subsequent experiment was 100 ng/mL.

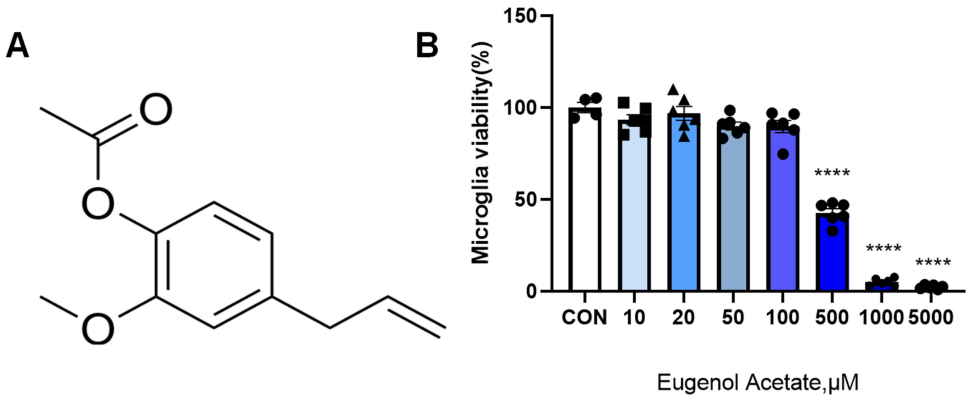

Figure 1 Effects of EA on the viability of primary microglia. (**A**) Chemical structure of EA. (**B**) Primary microglia were treated with different concentrations of EA (0, 10, 20, 50, 100, 500, 1000, 5000 μM). After 24 hours, CCK-8 assay was performed to detect the cell viability of the microglia. Control group n=4, other groups n=6. The values represent the means ± SEM.****p<0.0001 vs Control group.

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Oxygen-Glucose Deprivation (OGD)

Primary microglial cells were pre-treated with standard microglia medium (DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, 4.5 g/L glucose) with EA (50 and 100 μM) for 2 hours. Then the culture plates were placed in the anaerobic chamber flushed with a gas mixture of 95% N_2 and 5% CO_2 for 4 hours. After OGD treatment, the chamber was unsealed, and the cells were returned to normoxic conditions for 6 hours. For the control group, the culture medium was refreshed with standard microglial culture medium without exposure to hypoxic conditions.

Cell Viability Assessment

Microglial viability was estimated by cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Tokyo, Japan). Primary microglia cells were treated with different concentrations of EA (0, 10, 20, 50, 100, 500, 1000 and 5000 μM). After 24 hours incubation, CCK-8 was added for another 4 hours according instructions. Optical density (OD) was measured at 450 nm with a microplate reader (Bio-Rad, CA, USA). Cell survival rates=OD value of EA-treated cells/OD value of cells vehicle-treated cells.

Middle Cerebral Artery Occlusion (MCAO) Models

The animal study was approved by Animal Care and Use Committee of Nanjing Drum Tower Hospital (IACUC permit number: 2023AE01056), performed according to the Guidelines for the Ethical Review of Laboratory Animal Welfare (GB/T 35892–2018) and the General Requirements for Laboratory Animal Experiments (GB/T 35823–2018) of the People's Republic of China. Eight-week-old male C57BL/6J mice were obtained from the Animal Model Center of Nanjing University (Nanjing, Jiangsu, China). The MCAO model was performed as previously described.[19] Mice were anesthetized using 2.5% Avertin, and were randomly divided into 4 groups: sham operation group, MCAO model group, MCAO + 5 mg/kg EA treatment group and MCAO + 10 mg/kg EA treatment group. After the right common carotid artery and external carotid artery were surgically exposed, a 6/0 surgical suture was inserted into the origin of MCA, and the ipsilateral blood flow was monitored to below 30% of the baseline. The monofilament was withdrawn to allow blood reperfusion after 60 minutes occlusion.

Drug Treatment

Primary microglia were pretreated with EA (50 μM or 100 μM) for 2 hours, and then stimulated with LPS (100 ng/mL) for another 24 hours. For short-term in vivo experiments, EA (5 mg/kg or 10 mg/kg) was intraperitoneally injected at 15 minutes, 24 hours, and 48 hours after MCAO. In addition, EA (5 mg/kg or 10 mg/kg) was intraperitoneally injected for 28 days after MCAO during long-term in vivo experiments.

Quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA with the PrimeScript RT-PCR Kit (Vazyme, Nanjing, China). Real-time PCR was performed on a LightCycle® 96 Instrument Software system with SYBR Green Kit (Applied Biosystems, Foster City, CA, USA). The primer sequences were shown as previously reported.[20]

Western Blot

Total protein of the ischemic penumbra was extracted using RIPA lysis buffer (Bioworld, Minneapolis, MN, USA) and stored in −80°C. The proteins were separated by 8%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes. After being blocked with 5% skim milk, membranes were incubated with primary antibodies against IL-6 (1:1000, Cell Signaling Technology, Hertfordshire, UK), TNF-α (1:1000, Cell Signaling Technology, Hertfordshire, UK), iNOS (1:1000, Cell Signaling Technology, Hertfordshire, UK), COX-2 (1:1000, Bioworld Biotechnology, Minneapolis, MN, USA), IL-1β (1:1000, R&D Systems, USA), NF-κB p65 (1:1000, Cell Signaling Technology, Hertfordshire, UK), p-NF-κB p65 (1:1000, Cell Signaling Technology, Hertfordshire, UK), and GAPDH (1:5000, Bioworld Biotechnology, Minneapolis, MN, USA) at 4°C overnight. After incubation with the corresponding secondary antibodies for 2 hours at room temperature, the membranes were visualized

using the Gel-Pro System (Tanon Technologies, China). The quantification was analyzed by using ImageJ software (ImageJ-win64, NIH, United States).

Immunofluorescence Staining

Primary microglia and brain slices were used for immunofluorescence staining. Microglia cells were fixed with 4% paraformaldehyde for 20 minutes and blocked with 2% bovine serum protein for 2 hours. Brain was fixed with 4% paraformaldehyde and cut into 20 μM slices after gradient dehydration. Microglia or brain slices were incubated overnight at 4°C with primary antibodies against Iba1 (1:500, Abcam, Cambridge, UK), NF-κB (1:500, Cell Signaling Technology, Hertfordshire, UK) and Myelin Basic Protein (MBP, 1:1000, Abcam, Cambridge, UK), and incubated with corresponding secondary antibodies for 2 hours in dark at room temperature. DAPI was used to detect the nuclei. The images were obtained using an Olympus BX51 fluorescence microscope (Japan), and the relative fluorescence intensity of p65 and MBP were analyzed using ImageJ software.

Enzyme-Linked Immunosorbent Assays (ELISAs)

The ischemic penumbra region protein was extracted by RIPA, and the experiment was performed according to the manufacturer's instructions (Bioworld Biotechnology, Minneapolis, MN, USA). ELISAs were conducted to determine the protein levels of inflammatory factors (IL-1β, IL-6 and TNF-α). The OD value was measured at 450 nm (Bio-Rad, Hercules, CA, USA).

Measurement of Infarct Volume

2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining was performed to measure the infarct volume as previously described.[21] Three days after MCAO surgery, brains were cut into 6–7 slices and immersed in 2% TTC at 37°C, and photographed with a camera for analysis. The percentage of infarct was calculated as (volume of contralateral hemisphere-volume of ipsilateral normal hemisphere) / (volume of contralateral hemisphere) × 100% using ImageJ software (NIH).

Measurement of Neurological Deficits

Modified neurological severity score (mNSS) tests, comprising motor, sensory, and reflex tests, were conducted to measure neurological deficits of mice after MCAO. Motor balance and coordination were examined by the rotarod test. Forelimb grip strength was estimated by grip strength meter (GS3, Bioseb, France) at 3 days after MCAO.

Open Field Trial

The open-field trial was conducted in a box measuring 40 cm × 40 cm × 15 cm with a fixed camera. After being cleaned with 75% alcohol, mice were allowed to explore freely for 5 minutes while taking photos to count the total exercise distance.

New Object Recognition Trial

In training phase, mice were placed in an opaque box with two identical objects to recognize for 10 minutes. In testing phase, a new object with different shape and color was introduced, and the mice were allowed to explore freely for 5 minutes. The proportion of new objects compared to old ones was calculated.

Morris Water Maze Test

The Morris water maze (MWM) test was performed to examine spatial memory of mice 28 days after MCAO. Mice were tested in a circular tank to search the platform submerged beneath the surface of the water. The mice were trained to find the hidden platform in 60 seconds for 5 days in the acquisition test, and the escape latency was recorded. During the probe test, mice were allowed to swim for 60 seconds on the 6th day after the platform was removed. All trails were recorded by ANY-maze software (Stoelting, USA).

Statistical Analysis

All results were presented as mean ± SEM, and data were analyzed by using GraphPad Prism 8.0.2 software. Variations between experimental groups were established by performing Student's *t*-test, one-way analysis of variance (ANOVA), and two-way ANOVA followed by the post hoc test. A value of $P < 0.05$ was regarded as significant.

Results

Effects of EA on the Viability of Primary Microglia

To examine the safety and potential tolerability of EA (Figure 1A), the cytotoxicity was tested in primary microglia via CCK-8. Primary microglia were treated with EA (0 to 5000 μM) for 24 hours. As shown in Figure 1B, EA concentrations ranging from 10 to 100 μM exerted no impact on the microglial viability, whereas microglial viability was significantly decreased when exposed to higher concentrations (500 to 5000 μM). Therefore, concentrations of 50 and 100 μM were used in the following studies.

EA Attenuated Ischemic Injury and Neurological Dysfunctions in MCAO Mice

To investigate whether EA reduces the damage caused by ischemic stroke, EA was intraperitoneally injected at 15 minutes, 24 hours, and 48 hours after MCAO (Figure 2A). The results of TTC staining showed that EA significantly

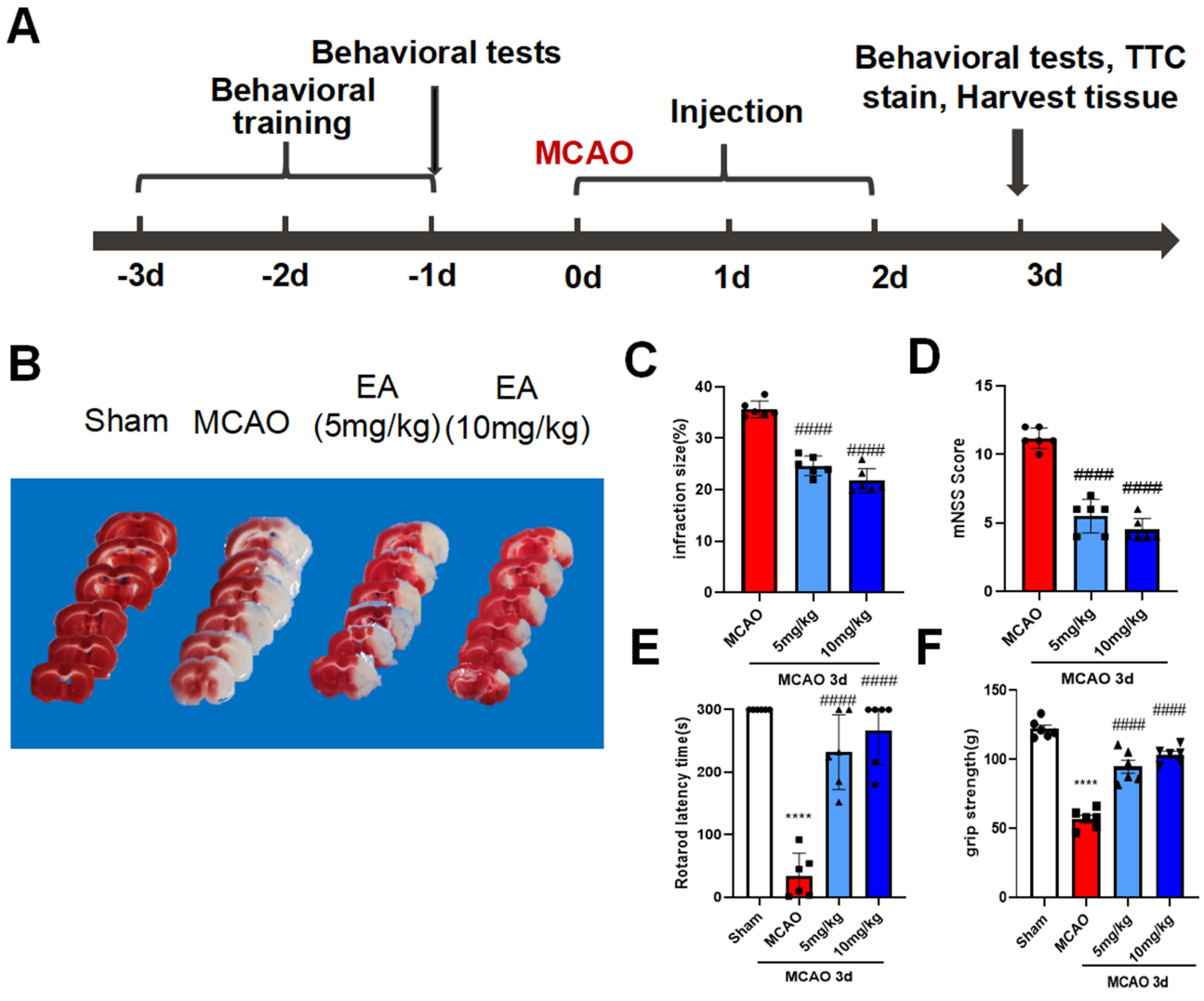

Figure 2 EA alleviated ischemic brain injury and ameliorated neurological deficits. (**A**) Timeline of short-term experiments. (**B**) Representative TTC staining and infarct volumes. (**C**) Infarct volume was determined in MCAO mice after EA treatment. (**D**) mNSS scores (**E**) The results of rotarod test (**F**) Grip strength test. The values were presented as the means ± SEM. n=6 per group. ****$p < 0.0001$ vs Sham group; #####$p < 0.0001$ vs MCAO group.

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decreased the infarct volume in the brains of MCAO mice (Figure 2B and C). Moreover, EA treatment ameliorated neurological deficits, including lower mNSS scores, better motor function and stronger grip strength (Figure 2D–F). These results suggested that EA could attenuate ischemic injury and neurological deficits in MCAO mice.

EA Attenuated Proinflammatory Cytokine Expression After MCAO

To estimate the effects of EA on neuroinflammation after brain ischemia, the mRNA levels of IL-1β, IL-6, and TNF-α were measured by RT-PCR. These pro-inflammatory cytokines were reduced in the ischemic penumbra tissue after EA treatment (Figure 3A). Additionally, the protein levels of IL-1β, IL-6, TNF-α, and COX-2 were downregulated after EA treatment in MCAO mice (Figure 3B–D). These results indicated that EA inhibited pro-inflammatory cytokine levels in ischemic penumbra tissue after MCAO.

EA Suppressed NF-κB Signaling Pathway After Ischemic Stroke

Since NF-κB pathways is a master mediator of the inflammation, we determined whether EA affected the NF-κB pathways in MCAO mice. The level of p-p65 was significantly increased after MCAO but was inhibited by EA treatment (Figure 4A and B). In addition, both p65 translocation and microglial activation were suppressed after EA treatment (Figure 4C and D). These data indicated that EA could inhibit the NF-κB pathway in the ischemic penumbra after ischemic stroke.

EA Improved Long-Term Neurological Function Recovery After Ischemic Stroke

To determine the effect of EA on long-term neurological function after ischemic stroke, EA was intraperitoneally injected for consecutive 28 days after MCAO, and behavioral tests were carried out to evaluate the sensorimotor and cognitive functions (Figure 5A). Shorter rotarod latency time, lower mNSS score, and stronger grip strength were observed in the MCAO mice treated with EA (Figure 5B–D). In the open field test, no significant difference in the duration of exercise was observed among different groups, indicating that EA treatment did not affect the exploratory behavior of mice (Figure 5E). In new object recognition tests, EA significantly upregulated the recognition index of MCAO mice (Figure 5F). In the MWM tests, platform crossing times and time spent in target quadrant were decreased in MCAO mice, while these metrics were significantly increased in the EA treatment group during the probe trial (Figure 5G–J). The integrity of white matter in MCAO mice was decreased,

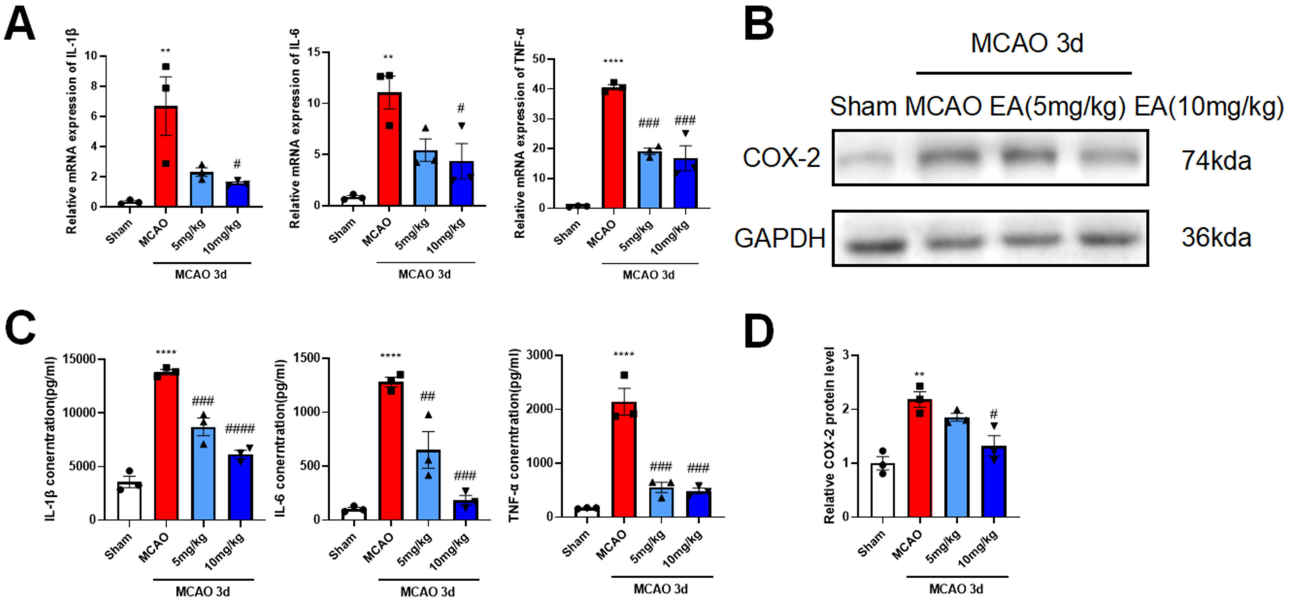

Figure 3 EA attenuated proinflammatory cytokine expression and after MCAO. (**A**) The mRNA levels of IL-1β, IL-6, and TNF-α in ischemic penumbra tissues were assessed by real-time PCR. (**B** and **D**) The protein levels of COX-2 in ischemic tissue were assessed by Western blot. (**C**) The concentrations of IL-1β, IL-6, and TNF-α in the protein extract of the ischemic penumbra tissue were detected by using the ELISAs. The values were presented as the means ± SEM. n = 3 per group. $^{**}p < 0.01$, $^{****}p < 0.0001$ vs control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ vs LPS-treated group.

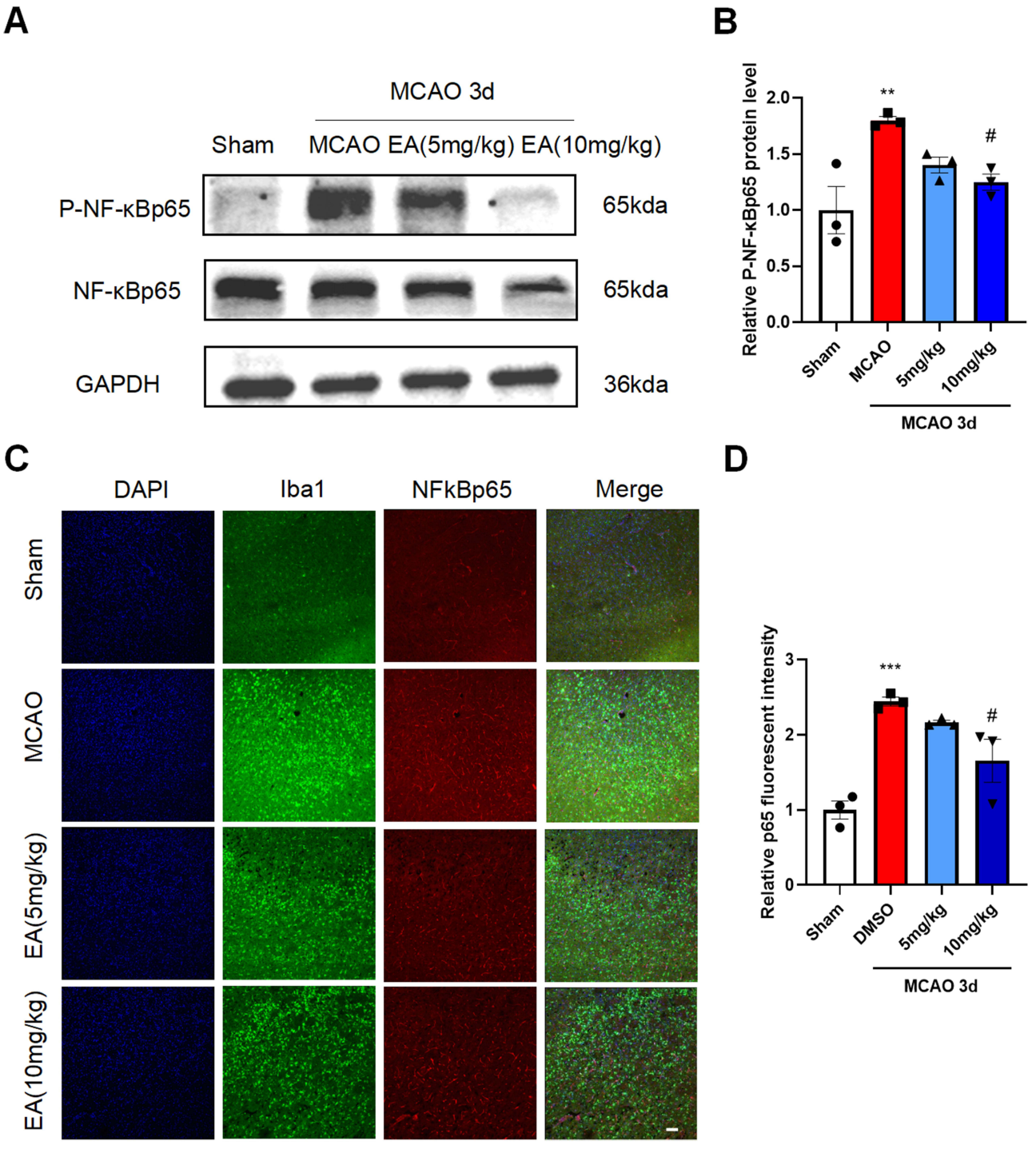

Figure 4 EA suppressed NF-κB signaling pathway in the ischemic penumbra after ischemic stroke. (**A** and **B**) The protein levels of p-NF-κBp65/NF-κBp65 in ischemic tissue were assessed by Western blot. n = 3 per group. (**C**) Representative images of tissue sections from the ischemic penumbra collected 3d after MCAO and stained with Iba1 and NF-κBp65. (**D**) Quantification analyzing of (**C**). Scale bar = 100 μM. The values were presented as the means ± SEM. ** $p < 0.01$, *** $p < 0.001$ vs Sham group; $^{\#}p < 0.05$ vs MCAO group.

as indicated by the MBP staining (Figure 5K), while EA treatment rescued the loss of white matter integrity. Collectively, our data shown that EA could improve long-term neurological function recovery and preserve white matter integrity following MCAO.

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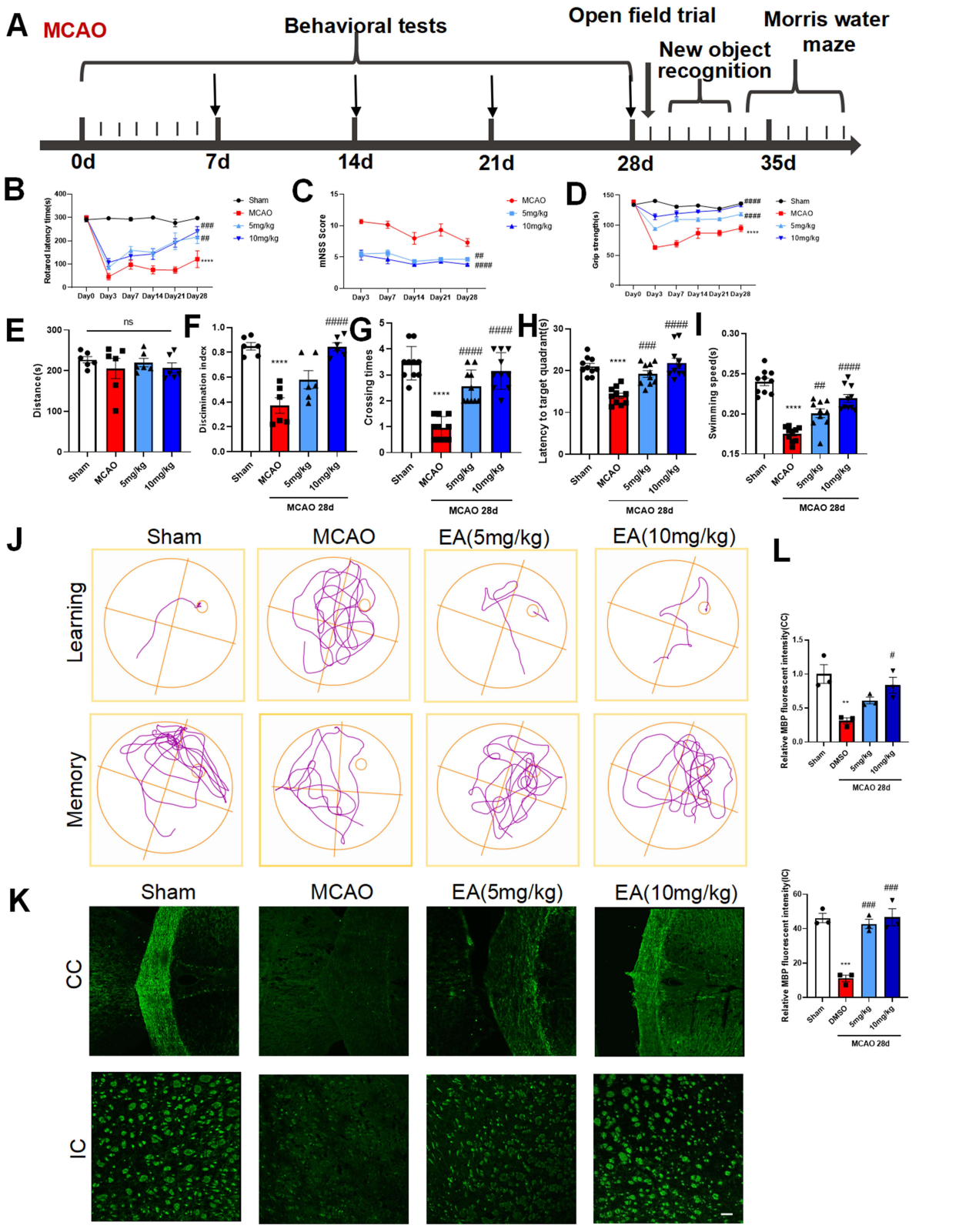

Figure 5 EA was obvious to improve long-term functional recovery after stroke in mice. (**A**) The timeline of long-term behavioral experiments. Sensorimotor functions were assessed before and up to 35 days after injury. (**B**) Rotarod latency time (**C**) mNSS score (**D**) Grip strength (**E**) the duration of exercise in open field tests were recorded. (**F**) In new object recognition tests, the percentage of time to explore new objects was recorded. (**G**–**I**) In probe tests, the number of target platform crossings (**G**), latency to the platform (**H**) and (**I**) were recorded in the probe trial. (**J**) Representative swim paths in the learning and memory phases of the MWM test. (**K**) Representative images and quantification analyzing of myelin basic protein in corpus callosum (CC) and internal capsule (IC). Scale bar: 100 μM. n = 6 per group. ****$p < 0.0001$ vs Sham group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ vs MCAO group; *ns* represented no significant statistical difference.

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EA Reduced the Production of Pro-Inflammatory Cytokine in LPS-Stimulated Microglia

To evaluate the effect of EA on microglia-mediated neuroinflammation in vitro, the levels of inflammatory cytokines were assessed in LPS-treated primary microglia. The mRNA levels of IL-1β, IL-6, TNF-α, iNOS, and COX-2 were significantly increased after LPS stimulation, while EA partially mitigated these effects (Figure 6A). Consistently, EA decreased the protein levels of IL-1β, IL-6, TNF-α, iNOS, and COX-2 (Figure 6B–D). These findings indicated that EA decreased the level of LPS-induced pro-inflammatory cytokine released in primary microglia.

EA Alleviated the Expression of Pro-Inflammatory Cytokine in Microglia Treated by OGD

OGD is an ideal model to stimulate the ischemic-reperfusion injury,[7] here, we have shown that the mRNA levels of IL-1β, IL-6, TNF-α, iNOS and COX-2 were significantly increased in OGD treated primary microglia. Consistently, EA treatment downregulated the expression of these pro-inflammatory factors (Figure 7A). Furthermore, EA reduced the protein levels of IL-1β, TNF-α, COX-2 and iNOS in OGD treated primary microglia (Figure 7B and C). These findings demonstrated that EA decreased the level of pro-inflammatory cytokine release in OGD-treated primary microglia.

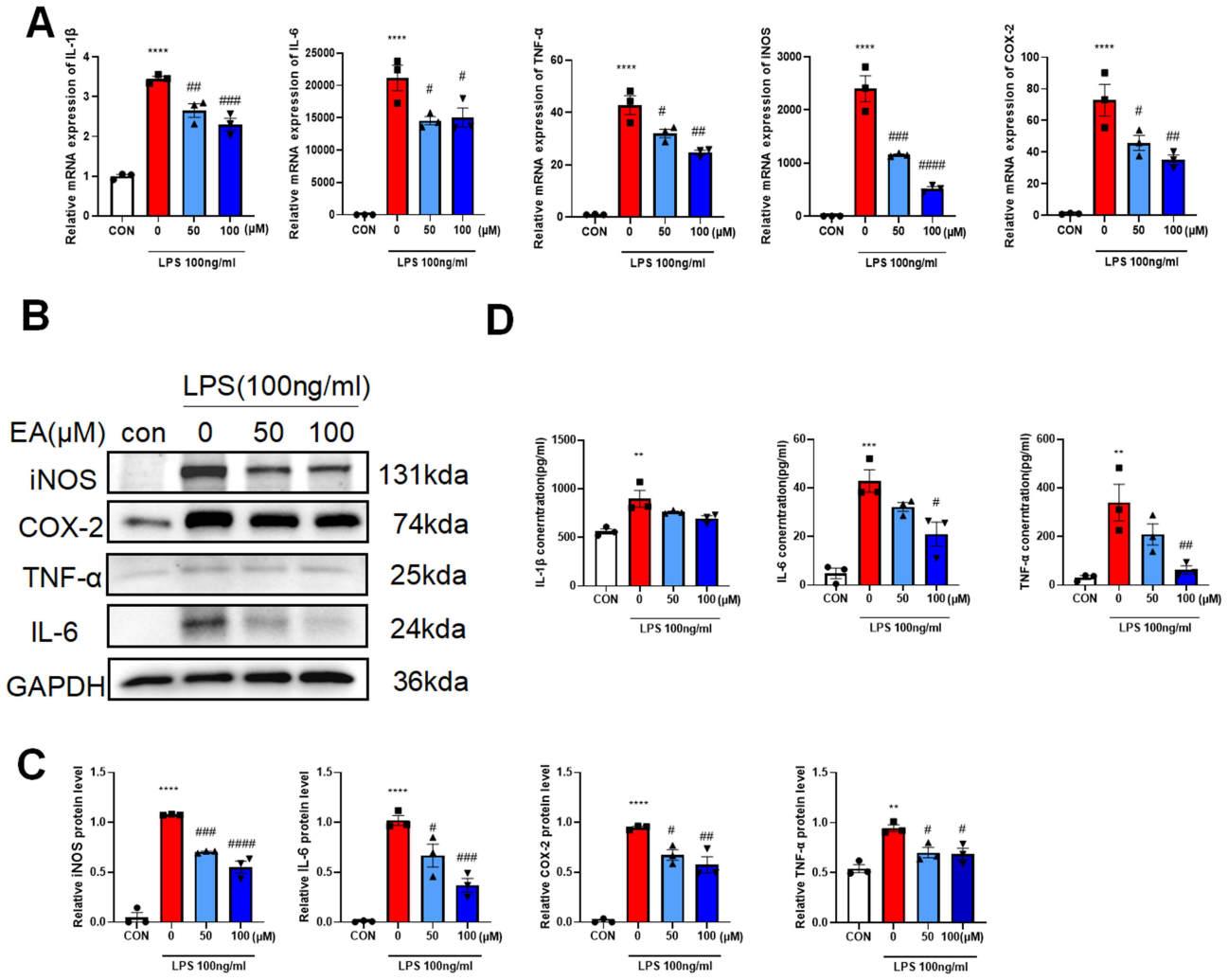

Figure 6 EA decreased the production of LPS-induced inflammatory cytokines expression in primary microglia. Microglia was pretreated with various concentrations of EA (50, 100 μM) for 2 hours followed by LPS treatment (100 ng/mL). (**A**) The mRNA levels of IL-1β, IL-6, TNF-α, iNOS, and COX-2 were measured by using real-time PCR. (**B** and **C**) The protein levels of IL-6, TNF-α, iNOS, and COX-2 were analyzed by Western blot. GAPDH as an internal reference. (**D**) The concentrations of IL-1β, IL-6 and TNF-α were detected by using the ELISAs. The values were presented as the means ± SEM. n=3, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ vs LPS-treated group.

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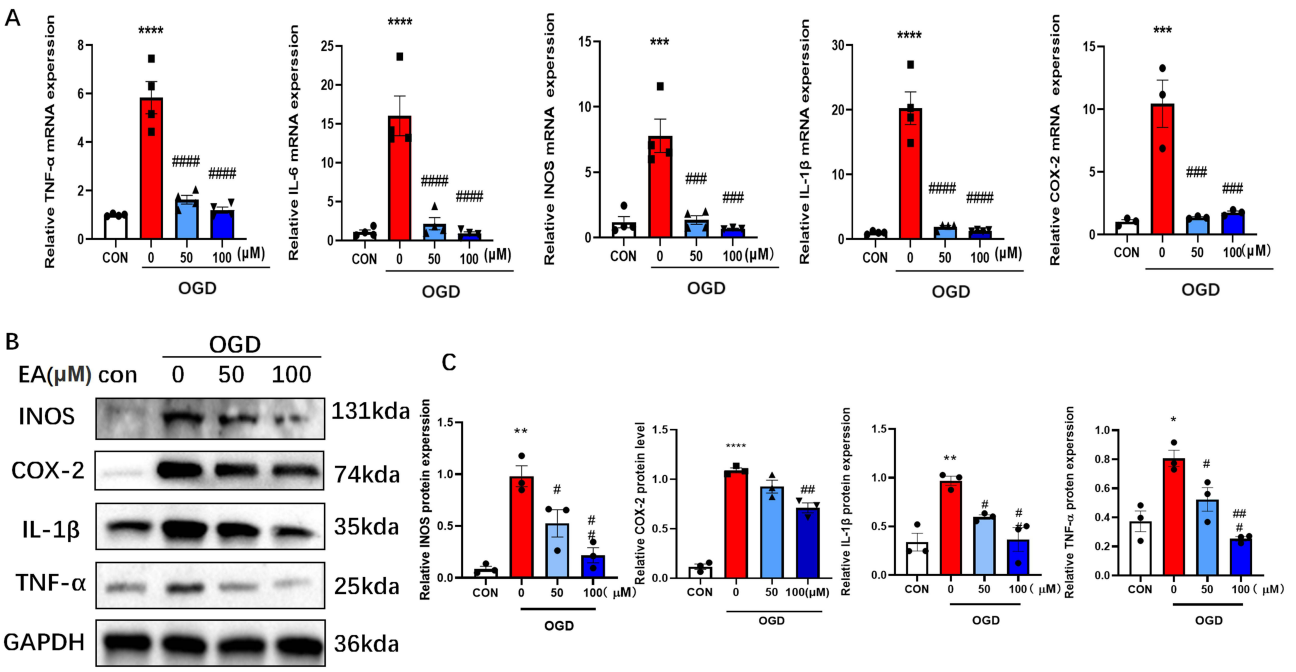

Figure 7 EA decreased the expression of inflammatory cytokines in OGD-induced primary microglia. Being pretreated with EA (50 and 100 μM) for 2 hours, the microglia was challenged with OGD for 4 hours. (**A**) The mRNA levels of IL-1β, IL-6, TNF-α, iNOS, and COX-2 were measured by using real-time PCR. (**B** and **C**) The protein levels of TNF-α, iNOS, IL-1β, and COX2 were analyzed by Western blot. GAPDH was used as an internal reference. The values were presented as the means ± SEM. n=3-4, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ vs OGD-treated group.

EA Inhibited NF-κB Signaling Pathway in LPS-Induced Microglia

Next, we examined the effects of EA on the NF-κB pathway in LPS-induced microglia. LPS treatment significantly increased the phosphorylation of p65 and IKBα, which was attenuated by EA pretreatment (Figure 8A and B). Additionally, EA alleviated LPS-induced morphological changes in primary microglia and disrupted p65 translocation from the cytoplasm to nuclei (Figure 8C and D). These results demonstrated that EA inhibited the NF-κB signaling pathway in LPS-stimulated microglia.

Discussion

The pathophysiological mechanisms of ischemic stroke are not fully defined, and limited therapeutic strategies are currently available in the clinic.[22] Although many neuroprotective strategies have been developed, clinical trials have not yielded optimistic results due to lack of efficacy or adverse side effects.[23] In this study, for the first time, we have shown that EA, a polyphenol extracted from *syzygium aromaticum* oil, rescues microglia-mediated neuroinflammation, restores the white matter integrity in MCAO mice, and inhibits the NF-κB signaling pathway in microglia, suggesting that EA might be a potential therapeutic agent for ischemic stroke treatment.

As an active component of the innate immune system, microglia participate in the progression of ischemic stroke.[24] Post-stroke neuroinflammation recruits immune cells, vascular, and molecular mediators.[25] This immune response aims to eliminate cellular damage,[26] remove necrotic cells and repair damaged brain tissue.[27] Upon activation, microglia release various chemokines and cytokine to trigger an immune response,[28] which leads to brain damage.[29] Therefore, anti-inflammatory therapy plays an increasing role in treating ischemic stroke. During inflammation, NF-κB pathway is activated, inducing the expression of IL-1β, IL-6 and TNF-α in microglia.[30] These inflammatory cytokines promote the production of iNOS, which stimulates the generation of nitric oxide (NO), COX-2, and ROS.[31] Chronic activation of microglia also produces neurotoxic mediators and pro-inflammatory cytokines, contributing to the progression of neurodegenerative lesions and diseases.[32–35] In our study, in response to LPS or OGD challenge, pro-inflammatory microglia were hyperactivated, and released inflammatory mediators and oxidative metabolites, including IL-1β, IL-6,

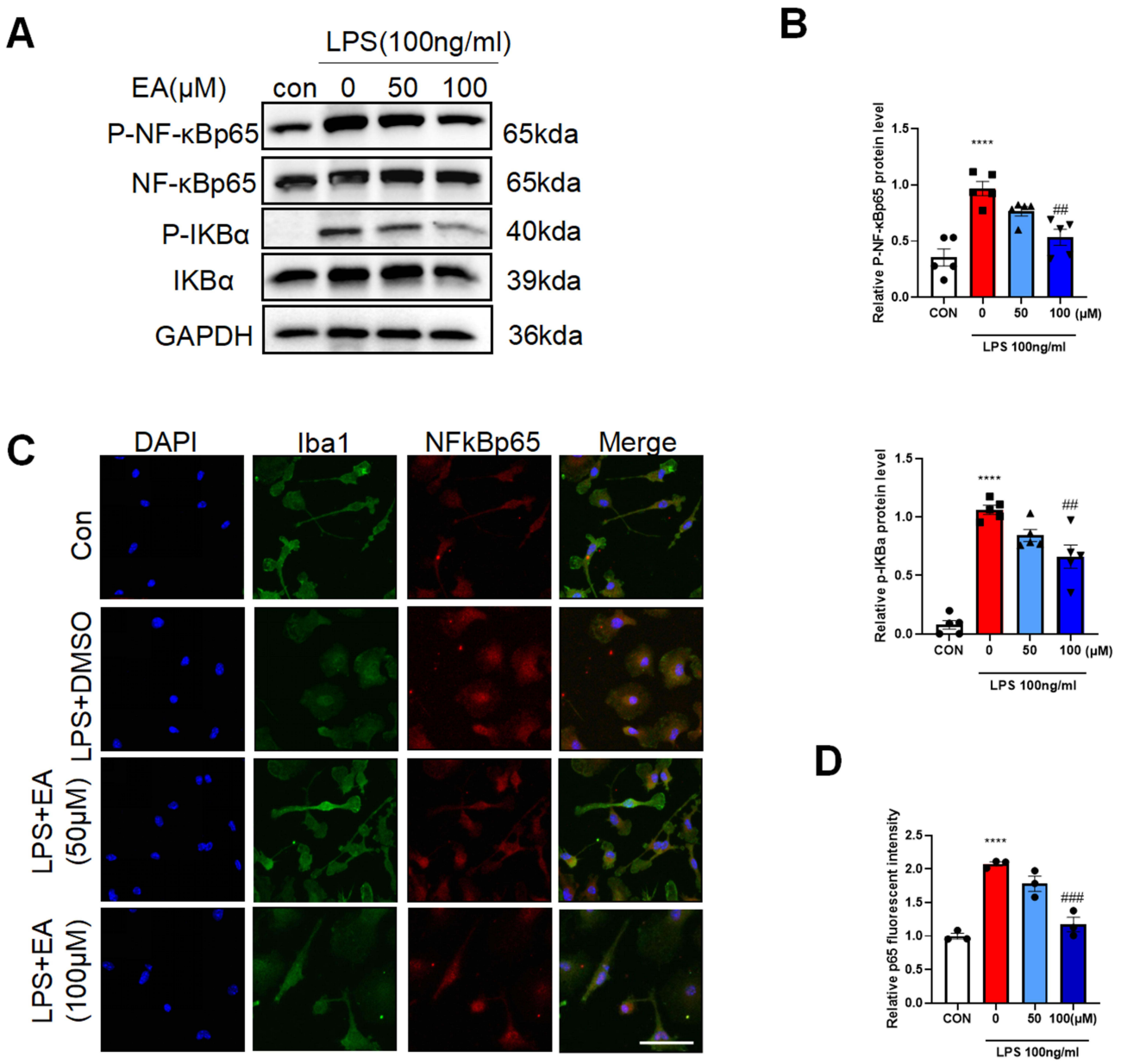

Figure 8 EA inhibited NF-κB signaling pathway in LPS-induced microglia (**A**) Western blot was used to detect the protein levels of p-NF-κBp65/NF-κBp65, p-IKBα/IKBα. n=3 per group. (**B**) Primary microglia were pretreated with EA (50, 100 μM) for 2 hours and then treated with LPS for 24 hours. (**C**) The morphological feature was examined by immunocytochemistry analysis by using Iba-1 antibody and NF-κBp65 antibody. (**D**) Scale bar = 100 μM. The values were presented as the means ±SEM. n=5 per group. $^{****}p < 0.0001$ vs control group; $^{\#\#}p < 0.01$ vs LPS-treated group.

TNF-α, and iNOS. EA significantly reduced the secretion of inflammatory cytokines in primary microglia induced by LPS or OGD. Thus, the neuroprotective effect of EA might result from its anti-inflammatory effect on microglia.

The NF-κB signaling pathway is the canonical signaling pathway for proinflammatory responses as well as microglial activation,[36,37] and plays an important role in both physiological and pathological conditions.[38] NF-κB is an important transcriptional regulator, consisting of subunits such as NF-κB1 (p50), NF-κB2 (p52), Rela (p65), RelB, and c-Rel.[39] Under physiological conditions, NF-κB is inhibited as a dimer in complex with its repressor protein IκB in the cytoplasm. IκB masks the nuclear localization signal of NF-κB, rendering it inactivate.[40] After ischemic damage, IκB is degraded by phosphorylation, resulting in its dissociation from the inactive complex and allowing NF-κB to translocate to the nucleus, where it induces the transcription of pro-inflammatory genes.[41] This process eventually aggravates brain injury.[42,43] Previous studies have shown that inhibition of the NF-κB signaling pathways downregulates the levels of inflammatory cytokines and alleviates cerebral

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infarction.[44] Quercetin reduces infarct volume and improves neurological function by regulating M1/M2 polarization through modulation of the PI3K/Akt/NF-κB signaling pathway in ischemic stroke.[45] Exogenous chemokine (C-X3-C motif) ligand 1 (CX3CL1) diminishes NLRP3 inflammasome and NF-κB signaling activation and promotes the functional recovery of stroke.[46] Our data showed that EA treatment significantly reduced the p-p65/p65 ratio in the in vitro and in vivo studies, suggesting that the therapeutic effect of EA on ischemic stroke might be related to the inhibitory effect on the NF-κB signaling pathway.

Chronic white matter structural injury is a risk factor for poor long-term outcomes after ischemic stroke.[47] Reversing white matter injury and restoring neurological function is critically important for the recovery after ischemic stroke.[48] M2 microglia-derived extracellular vesicles promote white matter repair, reducing brain atrophy volume and improving neurological function recovery in the MCAO model.[49] Moderate physical exercise alleviates functional impairment by promoting white matter recovery in ischemic stroke.[48] In this study, we have shown that EA promoted stroke recovery by improving neurological function and repairing white matter.

In conclusion, our study has demonstrated that EA treatment reduces ischemic injury and promotes the recovery of impaired neurological function. Furthermore, EA inhibits the NF-κB signaling pathway and attenuates microglia-mediated neuroinflammation. These findings indicate that EA might be developed as a potential therapeutic agent for ischemic stroke.

Conclusions

EA inhibits the NF-κB signaling pathway and alleviates microglia-mediated neuroinflammation, and promotes neurological recovery in MCAO mice, providing new insights for stroke drug development.

Data Sharing Statement

Data will be made available on request.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest that could influence the work reported in this paper.

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