4-Methoxybenzylalcohol protects brain microvascular endothelial cells against oxygen-glucose deprivation/reperfusion-induced injury via activation of the PI3K/AKT signaling pathway

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Abstract. Damage to the blood-brain barrier (BBB) during the process of cerebral ischemic injury is a key factor that affects the treatment of this condition. The present study aimed to assess the potential effects of 4-methoxybenzyl alcohol (4-MA) on brain microvascular endothelial cells (bEnd.3) against oxygen-glucose deprivation/reperfusion (OGD/Rep) using an *in vitro* model that mimics *in vivo* ischemia/reperfusion injury. In addition, the present study aimed to explore whether this underlying mechanism was associated with the inhibition of pro-inflammatory factors and the activation status of the PI3K/Akt signaling pathway. bEnd.3 cells were subjected to OGD/Rep-induced injury before being treated with 4-MA, following which cell viability, lactate dehydrogenase (LDH) release and levels of nitric oxidase (NO) were detected by colorimetry, pro-inflammatory factors including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6, were detected by ELISA. The expression levels of occluding and claudin-5were evaluated by immunofluorescence staining. The expression levels of AKT, phosphorylated (p)-Akt, endothelial nitric oxide synthase (eNOS) and p-eNOS were also measured by western blot analysis. After bEnd.3 cells were subjected to OGD/Rep-induced injury, cell viability and NO levels were significantly decreased, whilst LDH leakage and inflammatory factor (TNF-α, IL-1β and IL-6) levels were significantly increased. Treatment with 4-MA significantly ameliorated cell viability, LDH release and the levels of NO and pro-inflammatory factors TNF-α, IL-1β and IL-6 as a result of OGD/Rep. Furthermore, treatment with 4-MA upregulated the expression of occludin, claudin-5, Akt and eNOS, in addition to increasing eNOS and AKT phosphorylation in bEnd.3 cells. These results suggest that 4-MA can alleviate OGD/Rep-induced injury in bEnd.3 cells by inhibiting inflammation and by activating the PI3K/AKT signaling pathway as a possible mechanism. Therefore, 4-MA can serve as a potential candidate for treating OGD/Rep-induced injury.

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Introduction

The blood-brain barrier (BBB) consists of a highly selective semipermeable border of endothelial cells that prevents solutes in the circulating blood from crossing into the extracellular fluid of the central nervous system where neurons reside (1). BBB is comprised of cerebral endothelial cells, astrocytes and pericytes. The tight junctions (TJ) between adjacent endothelial cells are responsible for the low paracellular permeability and high electrical resistance of the BBB. They regulate the movement of polar solutes and macromolecules across the barrier (2). Significant advances in BBB research over the past decade has led to the discovery of an increasing number of structural and regulatory proteins in TJ (3). TJ is comprised of a wide range of different protein complexes, including occludin, claudin-5 and ZO-1. Under normal circumstances, TJ can effectively prevent harmful substances from entering the brain (4). However, during ischemia-reperfusion injury, TJ become disrupted, causing the loss of BBB function and subsequent aggravation of brain damage (5).

Ischemic stroke accounts for ~85% of all cases of stroke and is one of the most common diseases worldwide with a high disability and mortality rates (6). During cerebral ischemia, pro-inflammatory factors, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β IL-1β and IL-6, in addition to the rate-limiting enzymes of nitric oxide (NO), become activated (7). During the early stages of cerebral ischemia, the PI3K-AKT signaling pathway is immediately activated, where endothelial nitric oxide synthase (eNOS) becomes phosphorylated downstream (8). Co-existence of inflammatory factors and NO activates the matrix metalloproteinase (MMP) family (9). MMP2/9 is the most important matrix degrading enzyme in the body (10), which can degrade almost all components of the extracellular matrix and basement membrane (11).

MMP2/9 activation promotes the destruction of endothelial cells during blood reperfusion, specifically the degradation of TJ proteins (12), which leads to cerebral hemorrhage and cerebral edema. Eventually, the BBB is destroyed and the barrier effect disappears. Therefore, targeting BBB integrity appears to be a promising therapeutic approach for ischemic stroke.

To the best of our knowledge, 4-methoxybenzyl alcohol (4-MA) is one of the main active compounds that can be isolated from Gastrodia elata Blume (GEB). Previous studies revealed a significant protective effect of GEB on cerebral ischemia/reperfusion injury (13,14). In addition, 4-MA has been demonstrated previously to cross the BBB freely in both healthy rats and rats with cerebral ischemia reperfusion injury (MCAO/R), where it remained in the brain tissue and cerebrospinal fluid for 30 min after MCAO/R induction (15). 4-MA has also been demonstrated to protect the brain against ischemic injury by decreasing the permeability of the BBB after MCAO/R, where the underlying mechanism may be associated with the inhibition of NOS activity, upregulation of TJ protein expression and downregulation of aquaporin-4 protein expression (16). However, the potential effects of 4-MA on brain microvascular endothelial cells, especially their barrier function during oxygen-glucose deprivation/reoxygenation (OGD/R) insult and the underlying mechanisms remain poorly elucidated.

In the present study, an *in vitro* oxygen-glucose deprivation/reperfusion (OGD/Rep) model was established to investigate the effects of 4-MA on BBB integrity and activation of PI3K/AKT pathway following ischemic-reperfusion injury. In addition, the expression levels of key components of the PI3K/AKT pathway and TJs were determined, whilst cell viability, lactate dehydrogenase (LDH) release, NO levels, pro-inflammatory factors TNF-α, IL-1β and IL-6 were also measured to explore the potential molecular mechanisms of 4-MA against ischemic-reperfusion injury.

Materials and methods

Materials. Mouse brain microvascular endothelial cells (bEnd.3) were obtained from the American Type Culture Collection. DMEM, FBS (cat. no. 10437028) and penicillin/streptomycin (cat. no. 15140-122) were purchased from Gibco, Thermo Fisher Scientific, Inc. Occludin (cat. no. sc-5562) and claudin-5 (cat. no. sc-28670) primary antibodies were purchased from Santa Cruz Biotechnology, Inc. FITC-conjugated secondary antibodies (cat. no. bs-0296R-FITC), DAPI (cat. no. C02-04002) and β-actin primary antibody (cat. no. bs-0061R) were purchased from BIOSS. LY294002 (cat. no. S1737) was purchased from Beyotime Institute of Biotechnology. Primary antibodies against eNOS (cat. no. 32027S), phosphorylated (p)-eNOS (cat. no. 9571S), AKT (cat. no. 9272S) and p-AKT (cat. no. 4060S) were purchased from Cell Signaling Technology Inc. Horseradish peroxidase (HRP)-conjugated Affinipure Goat Anti-Rabbit IgG (cat. no. SA00001-2) secondary antibody was obtained from Proteintech Group, Inc. Additionally, 96-well and six-well plates were purchased from Corning Inc. Bicinchoninic acid (BCA) protein assay kit (cat. no. A045-4-2), lactate dehydrogenase assay kit (LDH; cat. no. A020-2-2), nitric oxide (NO) assay kit (cat. no. A013-2-1), MTT cell proliferation and cytotoxicity assay kit (cat. no. G020-1-1), tumor necrosis factor-α (TNF-α; cat. no. H052), Interleukin (IL)-1β (cat. no. H002) and IL-6 ELISA assay kits (cat. no. H007) were purchased from Nanjing Jiancheng Bioengineering Institute. 4-MA (cat. no. M107568) was obtained from Aladdin.

Cell culture and treatment. bEnd.3 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and incubated at 37˚C under 5% CO_2. A fresh medium was added every 2 days. Cell growth curves of $1x10^3$, $5x10^3$, $1x10^4$ cells/ml were produced. The OD value at 570 nm of each density was measured daily for 6 days using MTT assay. To select for the optimal conditions for OGD/Rep cell models, cell viability after Oxygen-glucose (OGD) for 1 h/reperfusion (Rep) for 1 h, OGD for 2 h/Rep for 2 h, OGD for 3 h/Rep for 3 h, OGD for 4 h/Rep for 4 h and OGD for 6 h/Rep for 4 h were also determined by MTT. Oxygen-glucose deprivation/reperfusion (OGD/Rep) *in vitro* model was established for the treatment of bEnd.3 cells as described previously (17). Briefly, bEnd.3 cells were first incubated in glucose-free DMEM (Thermo Fisher Scientific, Inc.) without FBS and subsequently transferred into a Tri-Gas incubator (HF100; Heal Force Bio-meditech Holdings, Ltd.) with 1% O_2, 94% N_2, and 5% CO_2 for 6 h at 37˚C. After OGD, glucose-free DMEM was replaced with high-glucose DMEM (Thermo Fisher Scientific, Inc.) with 10% FBS and the cells were incubated under 95% O_2, 5% CO_2 and maintained 4 h at 37˚C. For cell experiments, the solvent used for drugs are 0.05% DMSO. The control and OGD/Rep groups were treated with 0.05% DMSO. To assess the safety of 4-MA, bEnd.3 cells were treated with 800, 400, 200, 100, 50 and 25 μM 4-MA for 36 h at 37˚C before cell viability was determined using MTT assay. To deduce the optimal dose of 4-MA, bEnd.3 cells were treated with 800, 400, 200, 100, 50 and 25 μM 4-MA for 36 h at 37˚C before OGD/Rep induction before cell viability was determined using MTT assay.

Cell viability. In total, 1x104 bEnd.3 cells/ml were seeded into 96-well plates before cell viability was determined using MTT assay. Firstly, 20 μl MTT solution was then added into each well so that the final concentration of MTT per well is 0.5 mg/ml. The plate was then incubated at 37˚C for 3 h. After incubation, 150 μl DMSO was add into each well and the plate was wrapped in foil, which was shaken on an orbital shaker for 10 min at 37˚C. Absorbance at 570 nm was read in each well using a microplate reader.

LDH release, NO levels and pro-inflammatory factors. In total, $1x10^4$ bEnd.3 cells/ml were seeded into 96-well plates. The OGD/Rep-induced damage to cells were then treated with 200, 100 and 50 μM 4-MA for 36 h at 37˚C. In the media supernatant, LDH release and levels of NO were detected by colorimetry whilst the levels of pro-inflammatory factors TNF-α, IL-1β and IL-6 were measured using respective ELISA kits according to the protocols from the respective manufacturers.

Immunofluorescence assay. bEnd.3 cells were seeded into six-well plates at a density of $1x10^4$ cells/ml. bEnd.3 cells that underwent OGD/Rep were treated with 200 μM 4-MA for 36 h. The culture medium was then discarded and the cells

were washed three times with PBS for 5 min each. The bEnd.3 cells were subsequently fixed in 4% paraformaldehyde at room temperature for 1 h and washed with PBS three times for 5 min each. Permeabilization was then performed using 0.5% Triton X-100 for 30 min at room temperature and the cells were washed with PBS again three time for 5 min each. A total of 10% goat serum (Beijing Solarbio Science & Technology Co., Ltd.) was used for blocking at room temperature for 1 h. Subsequently, 200 μl occludin (dilution, 1:100) and claudin-5 (dilution, 1:25) antibodies was added and the cells were incubated at room temperature for 1 h at 4˚C. After washing three times with PBS for 5 min each, 200 μl FITC-conjugated secondary antibody (dilution, 1:200) was added and incubated at room temperature for 2 h. The cells were counterstained with 0.5 μg/ml DAPI (cat. no. C02-04002) at room temperature for 5 min. In total, threes fields of view were randomly selected per well before images were taken using a fluorescence microscope (magnification, x100; Olympus Corporation). ImageJ image analysis software (version 15.1P; National Institutes of Health) was used to measure the integrated optical density (IOD) and picture area (Area) of each field of view and the mean optical density (MOD) was calculated using the following formula: MOD = IOD/Area. The average value of MOD in four fields of view is the result of immunofluorescence determination of the sample, where the MOD represents the expression level of occludin and claudin-5.

Western blot analysis. bEnd.3 cells were seeded into six-well plates at a density of $1x10^4$ cells/ml. bEnd.3 cells that underwent OGD/Rep were treated with 200 μM 4-MA or 200 μM 4-MA + 50 μM LY294002 for 36 h. The cells were then washed with ice-cold PBS before ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology) was added and the cell lysates were collected into a pre-cooled microcentrifuge tube, which was maintained under constant agitation for 30 min at 4˚C. Following centrifugation in a microcentrifuge at 4˚C for 20 min at 14,006 x g, the supernatant was collected and the pellet was discarded. After that, 60 μg protein loaded per lane was separated by 10% SDS-PAGE and were transferred onto PVDF membranes, membranes were blocked with 5% skimmed milk powder at room temperature for 1 h. The membranes were then incubated with eNOS (dilution, 1:1,000), p-eNOS (dilution, 1:1,000), Akt (dilution, 1:1,000) or p-Akt (dilution, 1:2,000) antibodies for 2 h at room temperature. The membranes were washed with TBS-0.1% Tween-20 three times for 8 min each and incubated with HRP-conjugated Affinipure Goat Anti-Rabbit IgG secondary antibody (dilution, 1:1,000) for 1 h at room temperature. The membrane was developed using an ECL Detection Reagent (cat. no. B500023; Proteintech Group, Inc.) and exposed using an Imaging System (cat. no. EC3; UVP Inc.). Western blotting data was quantified by ImageJ software (version no. 1.48; National Institutes of Health).

Statistical analysis. Results were presented as the mean ± standard deviation, representative of six experimental repeats. Differences between groups were assessed using one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism 6.0 software (GraphPad Software Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of 4-MA on cell viability, LDH release and the levels of NO and pro-inflammatory factors. Cell growth curves after using the cell densities of $1x10^3$, $5x10^3$ and $1x10^4$ cells/ml were plotted. The cell growth rates of $1x10^4$ cells/ml density was found to be optimal (Fig. 1A). Compared with 100±13.87% in the control group, treatment of cells with OGD/Rep for OGD 1 h/Rep 1 h, OGD 2 h/Rep 2 h, OGD 3 h/Rep 3 h, OGD 4 h/Rep 4 h, and OGD 6 h/Rep 4 h reduced cell viability to 93.91±7.50, 85.06±4.57 ($P<0.05$), 77.02±4.28 ($P<0.01$), 69.21±4.22 ($P<0.01$) and 56.95±1.99% ($P<0.01$), respectively (Fig. 1B). Since the survival rate in the OGD 6 h/Rep 4 h model was found to be 56.95±1.99%, the OGD 6 h/Rep 4 h model was used for subsequent experiments.

Treatment with 4-MA at concentrations of 800, 400, 200, 100, 50 and 25 μM exhibited little to no effects on bEnd.3 cells (Fig. 1C). Subsequently, it was revealed that 4-MA with dose of 200 μM had the highest efficacy on bEnd.3 cells after OGD/R (Fig. 1D). Compared with that in the OGD/Rep group (57.97±7.20), 36 h treatment with 4-MA at doses of 200, 100 and 50 μM significantly increased cell viability to 87.77±15.26 ($P<0.01$), 83.90±10.04 ($P<0.01$) and 79.74±11.69% ($P<0.05$), respectively (Fig. 1D).

LDH release from the cells into the medium has been frequently used as an indicator of cell injury (18). Compared with that in the control group (131.44±23.56), LDH release was found to be significantly increased in the OGD/Rep group to 266.87±37.75 U/l ($P<0.01$). Compared with that in the OGD/Rep group (266.87±37.35), 36 h treatment with 200, 100 and 50 μM 4-MA significantly reduced LDH release in cells that underwent OGD/Rep to 201.09±11.58 ($P<0.01$), 215.45±19.42 ($P<0.05$) and 217.00±15.14 U/l ($P<0.05$), respectively (Fig. 1E).

Activation of eNOS has been previously reported to mediate protection from stroke by preserving cerebral blood flow and inhibiting inflammation, platelet aggregation, thrombosis and apoptosis (19). Compared with that in the control group (1.44±0.27), NO level was significantly reduced in OGD/Rep group (0.92±0.13 μM/l; $P<0.01$; Fig. 1F). Compared with that in the OGD/Rep group, 36 h treatment with 200 and 100 μM 4-MA significantly increased NO levels, to 1.16±0.18 μM/l ($P<0.01$) and 1.11±0.11 μM/l ($P<0.05$), respectively (Fig. 1F).

Compared with those in the control group (37.33±3.65, 5.52±1.05, 18.91±1.86 for TNF-α, IL-1β and IL-6, respectively), the levels of TNF-α, IL-1β and IL-6 were significantly elevated in OGD/Rep group to 45.09±3.71 ($P<0.01$), 7.37±0.60 ($P<0.01$) and 23.44±1.73 ng/l ($P<0.01$), respectively (Fig. 1G-I). Compared with those in the OGD/Rep group, 36 h treatment with 200 μM 4-MA significantly reduced the levels of TNF-α, IL-1β and IL-6 to 37.89±2.93 ($P<0.05$), 3.57±1.11 ($P<0.01$) and 18.20±2.73 ng/l ($P<0.05$), respectively (Fig. 1G-I).

Data from the cell viability and LDH release assays, in addition to measurements of NO and pro-inflammatory factor levels suggest that 36 h treatment with 200 μM 4-MA exhibited the strongest effect on protecting bEnd.3 cells against OGD/Rep-induced injury.

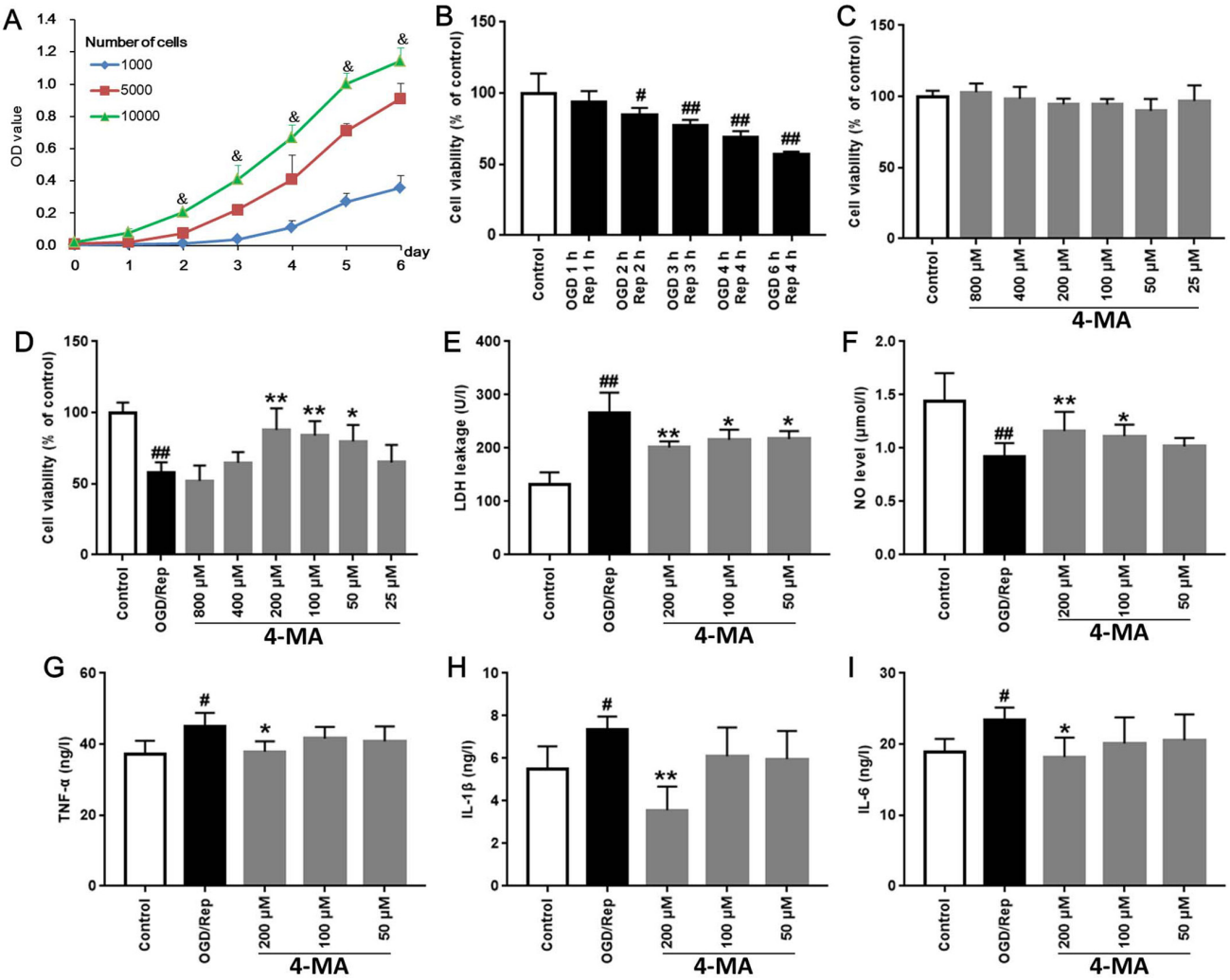

Figure 1. Effects of 4-MA on cell viability, LDH release, NO levels and pro-inflammatory factors after OGD/Rep in bEnd.3 cells. (A) Cell growth curves at seeding densities of $1x10^3$, $5x10^3$, $1x10^4$ cells/ml were measured daily for 6 days using MTT. (B) To select the conditions for OGD/Rep cell models, cell viability after OGD 1 h/Rep 1 h, OGD 2 h/Rep 2 h, OGD 3 h/Rep 3 h, OGD 4 h/Rep 4 h and OGD 6 h/Rep 4 h were determined by MTT. (C) To assess the safety of 4-MA, bEnd.3 cells were treated with 800, 400, 200, 100, 50 and 25 μM 4-MA for 36 h and cell viability was determined using MTT assay. (D) To deduce the optimal dose of 4-MA, bEnd.3 cells were treated with 800, 400, 200, 100, 50 and 25 μM 4-MA for 36 h before OGD/Rep induction, and cell viability was determined using MTT assay. (E) LDH release and (F) NO levels were detected by colorimetry. The levels of pro-inflammatory factors (G) TNF-α, (H) IL-1β and (I) IL-6 were measured using respective ELISA kits. $^{\&}$P<0.05 vs. $1x10^3$ and $5x10^3$ cells/ml; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. Control; *P<0.05 and **P<0.01 vs. OGD/Rep. OD, optical density; TNF-α, tumor necrosis factor-α; IL, interleukin; NO, nitric oxide; LDH, lactate dehydrogenase; OGD/Rep, oxygen-glucose deprivation/reperfusion; 4-MA, 4-methoxybenzyl-alcohol.

4-MA enhances occludin and claudin-5 expression. Compared with those in control group, OGD/Rep significantly reduced the average optical densities of claudin-5 and occluding in bEnd.3 cells (P<0.01; Fig. 2). However, treatment with 200 μM 4-MA significantly increased the average optical densities of claudin-5 and occludin in bEnd.3 cells compared with those in the OGD/Rep group (P<0.01; Fig. 2).

4-MA activates the PI3K/AKT signaling pathway. Compared with that in the control group, AKT and eNOS phosphorylation were significantly downregulated in the OGD/Rep group (P<0.01). After treatment, 200 μM 4-MA significantly upregulated AKT and eNOS phosphorylation (P<0.01), which was abolished by LY294002 (P<0.01; Fig. 3A, B and C). These results suggest that 4-MA can activate the PI3K/AKT signaling pathway in bEnd.3 cells after OGD/Rep-induced injury and that PI3K/Akt signaling pathway could be inhibited by LY294002.

Discussion

After ischemic stroke, the integrity of the blood-brain barrier is compromised. The BBB serves to restrict the passage of pathogens, restrict the diffusion of solutes and large or hydrophilic molecules, whilst allowing the diffusion of hydrophobic molecules, including O_2, CO_2, hormones and small polar molecules, from the blood circulation into the cerebrospinal fluid (20). Brain microvascular endothelial cells differ from peripheral endothelial cells with regards to the expression of specific ion transporters and receptors, where they contain fewer gap and pinocytotic vesicles (21). There are several factors that can cause BBB destruction, including MMPs, inflammatory factors, trauma and tumor-infiltrating immune cells (22). Damage can be caused by reductions in TJ protein expression and function (23), degradation of the basement membrane, increased permeability and the entry of harmful substances such as free radicals into

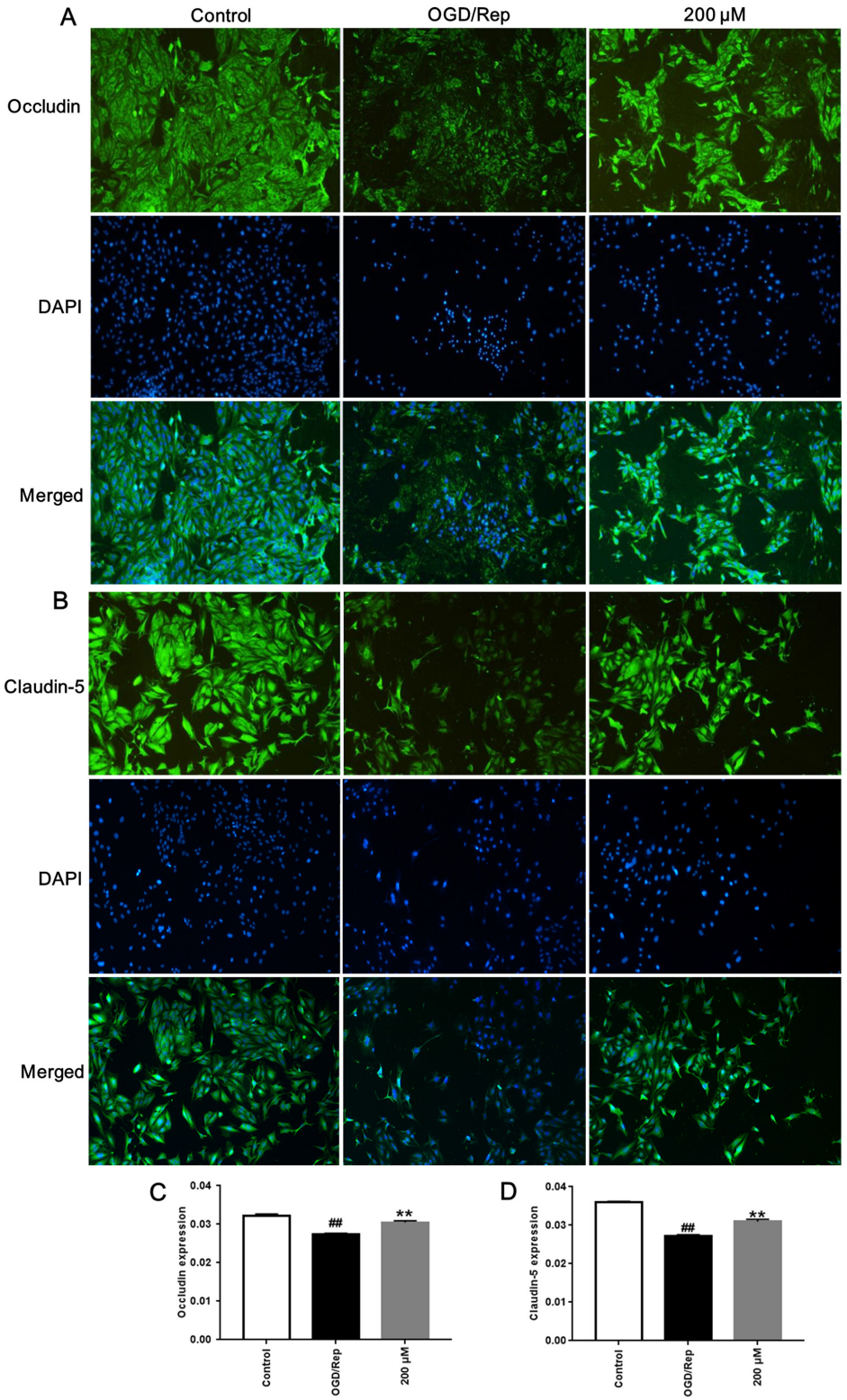

Figure 2. Effects of 4-MA treatment on expressions of occludin and claudin-5 expression in bEnd.3 cells after OGD/Rep injury. Expression of (A) occludin and (B) claudin-5 was determined using immunofluorescence staining. Magnification, x100. Expression of (C) occludin and (D) claudin-5 was subsequently quantified. $^{\#\#}$P<0.01 vs. Control; **P<0.01 vs. OGD/Rep. 4-MA, 4-methoxybenzyl-alcohol; OGD/Rep, oxygen-glucose deprivation/reperfusion.

the brain (24). Therefore, protection of BBB integrity serves a vital role in the prevention and treatment of ischemic stroke.

In the present study, an OGD/Rep *in vitro* model was established to mimic ischemic stroke (25), a cell model with a survival rate of 50-60% is the most suitable. If the survival rate of the model group is too high, it will cause no difference compared with the control group. If the survival rate of the model group is too low, it is difficult for the drug to have a prevention and treatment effects. LDH is rapidly released into the cell culture supernatant when the plasma membrane is damaged, which is a key feature of cells undergoing apoptosis, necrosis or other forms of cellular damage (26). Results

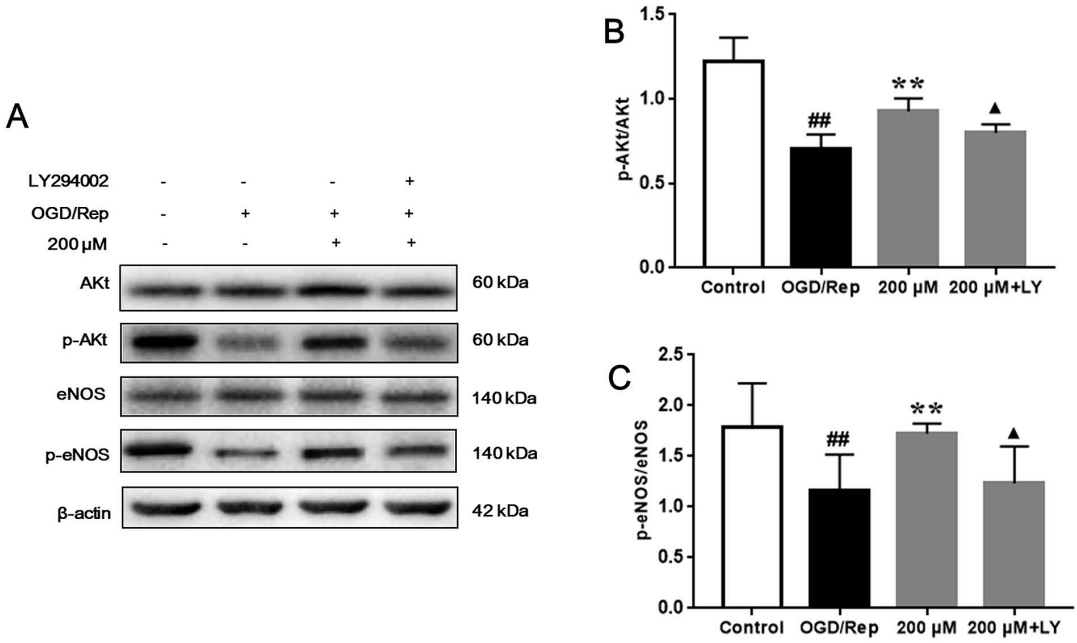

Figure 3. Effects of 4-MA on the PI3K/AKT signaling pathway in bEnd.3 cells after OGD/Rep induction. (A) Representative western blotting images showing the expression of AKT and eNOS in addition to the phosphorylation levels of AKT and eNOS, in the various treatment groups. Quantitative analysis of the density of (B) p-AKT/AKT and (C) p-eNOS/eNOS. (D) eNOS expression and (E) eNOS phosphorylation are quantified. ##P<0.01 vs. Control; **P<0.01 vs. OGD/Rep;▲P<0.05 vs. 200 μM. 4-MA, 4-methoxybenzyl-alcohol; OGD/Rep, oxygen-glucose deprivation/reperfusion; p-, phosphorylated; eNOS, endothelial nitric oxide synthase; LY, LY294002.

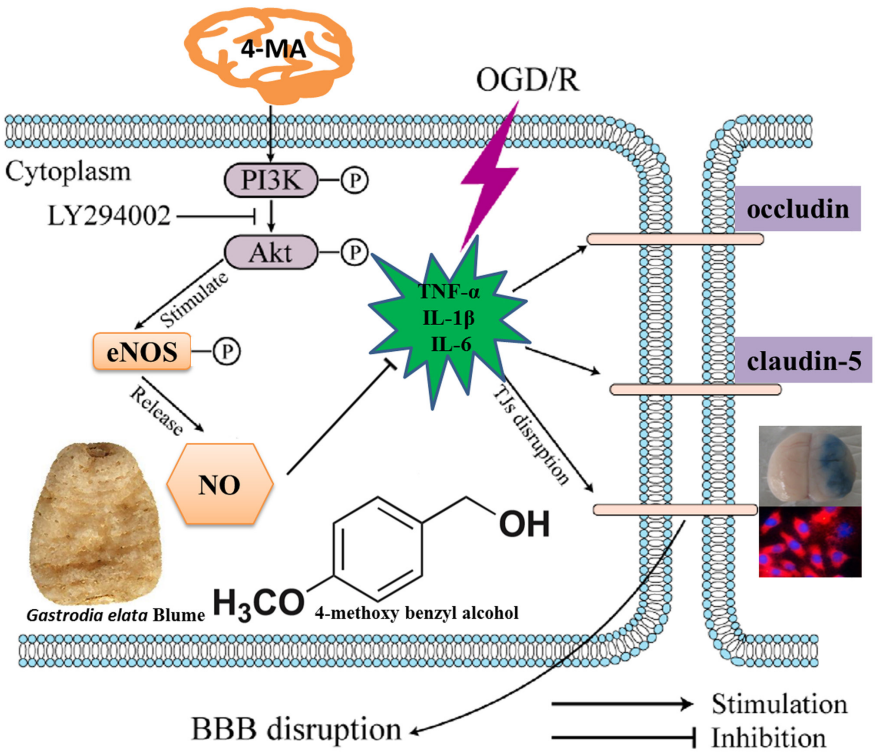

Figure 4. Proposed protective effects of 4-MA against OGD/Rep-induced injury and associated mechanism. 4-MA inhibits the production of inflammatory factors (TNF-α, IL-1β, IL-6) and increased the expression of TJ proteins occluding and claudin-5, ameliorates OGD/Rep-induced brain microvascular endothelial barrier dysfunction through activating the PI3K/AKT signaling pathway. After the PI3K/AKT signaling pathway is activated, downstream eNOS is phosphorylated to produce a large amount of NO. NO can expand blood vessels, increase blood supply in ischemic areas and have a protective effect on the brain. BBB, blood-brain barrier; TNF-α, tumor necrosis factor-α; IL, interleukin; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; LDH, lactate dehydrogenase; OGD/R, oxygen-glucose deprivation/reperfusion; 4-MA, 4-methoxybenzyl-alcohol; TJ, tight junction; p-, phosphorylated.

from the present study showed that 4-MA can reduce LDH levels after OGD/Rep induction. A number of pathogenic factors, including infection, tissue injury or cardiac infarction, can induce inflammation to mediate tissue damage (27).

The etiologies of inflammation can be either infectious or non-infectious (28). Immune cells produce and release a large number of cytokines, including IL-1β, IL-6 and TNF-α and chemotactic factors. After ischemic stroke, pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α can induce changes in the integrity of the BBB, which increases permeability (29). This pro-inflammatory environment can lead to a more robust release of other inflammatory mediators, such as the chemokine MCP-1 (30). Due to the excessive stimulation of immune cells, large quantities of leukocyte adhesion to endothelial cells occurs, resulting in the excessive activation of the anti-infection response (31). Data from the present study suggest that 4-MA can inhibit the release of pro-inflammatory factors following OGD/Rep injury.

TJ membrane proteins were first identified in retinal ECs, which include occludin, claudin-1, claudin-2, claudin-5 and junctional adhesion molecule-A (32). Occludin is not essential for the formation of TJ, since occludin-deficient embryonic stem cells can differentiate into epithelial cells and can form well-developed tight junction structures (33). Claudin-5 is specifically expressed in the brain tissue, where it regulates the integrity and permeability of the BBB (34). The results from the present study demonstrated that 4-MA can enhance TJ protein expression following OGD/Rep injury.

The PI3K/AKT signaling pathway is involved in multiple biological processes, including cell proliferation, survival and apoptosis (35,36). PI3K is a heterodimer composed of a p85 regulatory subunit and a p110 catalytic subunit that regulates a variety of cellular responses (37). Activation of class I PI3Ks generates phosphatidylinositol-3,4,5-trisphosphate, as a second messenger that serve as a docking platform. At the plasma membrane, AKT is phosphorylated at Thr-308 by 3-phosphoinositide-dependent kinase 1 whereas 3-phosphoinositide-dependent kinase 2 has also been suggested to phosphorylate AKT (38). BBB damage is the critical pathological process of ischemic stroke. PI3K/Akt pathway was involved in altering BBB permeability in various cerebral pathological conditions, where the activation of PI3K/Akt pathway can result in neuroprotection in cerebral ischemia (39,40). The results of the present study suggest that 4-MA can activate the PI3K/AKT signaling pathway in bEnd.3 cells after OGD/Rep-induced injury.

In summary, the present study demonstrates that 4-MA can protect against OGD/Rep-induced cerebral microvascular endothelial cell injury and the disruption of BBB integrity *in vitro*. 4-MA can attenuate the production of inflammatory mediators and activate the PI3K/AKT signaling pathway. These findings suggest that 4-MA may have the potential for treating cerebral ischemic events (Fig. 4).

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Availability of data and materials

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

Authors' contributions

QL and LY designed the study. WW performed all the experiments. QL and XD interpreted the data and drafted the manuscript. All authors read and approved the final version of the manuscript. XD can authenticate the raw data in this study.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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