Curcumin ameliorates ischemic stroke injury in rats by protecting the integrity of the blood-brain barrier

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Abstract. The blood-brain barrier (BBB) is critical for proper cerebral homeostasis and its dysfunction during ischemic stroke can result in significant neurological injury. The major goal of the present study was to identify whether curcumin pretreatment possessed protective effects on BBB integrity during the 24 h of acute ischemic brain injury. To investigate the protective effects of curcumin, male Sprague-Dawley rats were divided into multiple groups, including sham, middle cerebral artery occlusion/reperfusion (MCAO/R) vehicle and curcumin pretreated MCAO/R groups. The effects of curcumin were measured by analyzing neurological deficits, infarct size, BBB permeability and expression levels of permeability-related proteins in the brain. It was found that curcumin pretreatment significantly improved neurological scores, decreased infarct size, and protected synaptic remodeling of hippocampal neurons and upregulated the protein expression level of tight junction proteins, ZO-1, occludin and claudin-5 in ischemic rat brains. Furthermore, curcumin pretreatment before stroke was shown to downregulate the phosphorylation of NF-κB and MMP-9, which are central mediators of inflammation. The results from the present study indicated that curcumin pretreatment ameliorated ischemic stroke injury by protecting BBB integrity and synaptic remodeling, as well as inhibiting inflammatory responses.

Introduction

Stroke, a common neurological disorder associated with a high risk of disability and mortality (1), disrupts the blood-brain barrier (BBB), where it can induce neuronal injury through brain edema and inflammation (2). There is increasing evidence that inflammation contributed to the progression of ischemia-induced secondary brain damage, leading to aggravated dysregulation of the BBB, as well as cerebral edema (3,4). Thus, identification of novel drugs to protect against ischemic stroke and to understand the underlying mechanisms involved is important to combat this pathology (5).

The BBB consists of a variety of cell types and acts as a border between the brain and the blood circulating through the body (6). It is well-known that BBB impairment is a consequence of ischemic stroke (7,8). Controlling the BBB during stroke has both neuronal and vascular protective effects (9). Previous studies have suggested that NF-κB and MMP-9, which are central mediators of inflammation, play pivotal roles in inflammatory damage following experimental ischemic stroke (10,11).

There are numerous types of MMPs. The MMP that damages the BBB following stroke is gelatinase MMP-9 (12). Under normal, steady state conditions, MMP-9 protein expression levels in endothelial cells are low and are present as inactive zymogens. In animals experiencing cerebral ischemia and reperfusion, inflammatory mediators are upregulated and activate leukocytes, which can secrete MMP-9 (13). When MMP-9 protein levels in the endothelial cells are elevated, extracellular matrix components and substrates in the BBB are damaged, primarily through degradation of type IV collagen. Structural damage to the BBB will increase permeability and result in vascular-derived brain edema, and toxic substances are introduced into the BBB and harm brain tissue (14). Notably, NF-κB directly regulates the transcription of MMP-9, and inhibition of NF-κB activity reduces the protein expression level of MMP-9, as well as inflammation (15,16). Reduced MMP-9 protein expression in endothelial cells, as well as
decreased inflammation, reduces BBB disruption and cerebral injury caused by ischemic stroke (17).

Curcumin, a pleiotropic agent extracted from the rhizome of Curcuma longa (18), exerts pharmacological effects against stroke via its anti-inflammatory and anti-inflammatory action (19). Previous research has revealed that curcumin could pass through the BBB and significantly decrease water content, infarct size and BBB leakage in a middle cerebral artery occlusion/reperfusion (MCAO/R) rat model (20-23). The post-ischemic neuroprotective effects of curcumin have been investigated; however, the potential mechanisms behind these effects are not fully known.

The aim of the present study was to assess how curcumin affected brain injury, the BBB status and the protein expression level of phosphorylated (p)NF-κBp65 and MMP-9 in a rat MCAO/R stroke model. We hypothesized that pretreatment with curcumin would protect BBB integrity after MCAO/R in the brain via its anti-inflammatory effects, including the attenuation of pNF-κBp65 and MMP-9 in endothelial cells and increase the expression level of tight junction proteins.

Materials and methods

Animal and establishment of the stroke model. A total of 90 3-month-old male Sprague-Dawley (SD) rats, weighing 250-280 g were provided by Xuzhou Medical University (Jiangsu, China). The rats were housed in a temperature-controlled (22±2˚C) setting, including 55±10% humidity and a 12 h light/dark cycle with free access to food and water. All rats were sufficiently adapted to their environments before subjected to surgery. The MCAO/R stroke model used in the present study was established as previously described (24). Briefly, rats were intraperitoneally anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg). The ventral midline neck was cut to expose the right common (CCA), right internal (ICA) and right external (ECA) carotid arteries. The ECA branches were then ligated. Next, a 4-0 nylon monofilament containing a round tip (Guangzhou Jialing Biotechnology Co., Ltd.) was inserted along the CCA into the ICA. This was performed until resistance was detected. Approximately 90 min following MCAO surgery, the monofilament was removed, followed by reperfusion for 24 h, and 3 and 7 days post-MCAO. The exact same surgery was performed in the sham group rats; however, filament insertion was not performed.

The SD rats were randomized into three individual groups, including the sham (n=30), the MCAO/R with vehicle (n=30) and the MCAO/R plus curcumin groups (n=30). Curcumin (300 mg/kg; Sigma-Aldrich; Merck KGaA) was dissolved in 2% dimethyl sulfoxide (vehicle) and intraperitoneally administered 30 min prior to MCAO/R surgery (25). All the experiments were performed following guidelines written by the Institutional Animal Care and Use Committee of China. The studies were also approved by the Ethics Committee for the Use of Experimental Animals at Xuzhou Medical University (assurance nos. 2015-46 and 2015-47).

Assessing neurological deficits. The modified Neurological Severity Scale (mNSS) has a total score of 18 points and is divided into 4 parts as follows: Motion, sensation, balance, and reflex. The score of normal rats was 0. The higher the score, the more severe the symptoms of neural power deficiency. Neurological functions were evaluated 24 h, and 3 and 7 days following MCAO/R using a modified Neurological Severity Scale (mNSS), as previously described (26). This scale includes measurement of balance, reflex, sensory and motor skills. The mNSS is similar to the contralateral neglect tests performed for humans. Neurological functions were measured on a scale from 0 to 18, where normal was scored as 0 and maximum deficit was scored as 18. A mNSS score of 0 was indicative of the sham group.

Measuring infarct size. The rats were anesthetized 24 h post-MCAO/R and 2 mm coronal slices were prepared. The issues were stained at 37˚C for 30 min using 2% solution 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich; Merck KGaA) diluted in PBS. Following which, the tissues were fixed using 4% paraformaldehyde in a 4˚C refrigerator overnight. Red staining was associated with undamaged regions after MCAO/R and white stained areas revealed regions experiencing an infarct. The relative infarction volume percentage (RIVP) was calculated using the following formula: RIVP=total infarct area/total area x100%. Infarct size was quantified using ImageJ software 1.8.0 (National Institutes of Health).

Rapid golgi staining. The brains were removed from the rats and processed using the rapid Golgi staining kit (FD Neurotechnologies, Inc.), based on the instructions provided by the manufacturer. Briefly, serial sections (100-µm) from the hippocampus were prepared on a freezing microtome and dehydrated in an ascending absolute ethanol series (50, 70 and 90%), washed in xylene and mounted in neutral balsam. Next, 5 pyramidal neurons extracted from each rat (3 rats/group; 20 brain sections from each group) were measured from area CA1 of the hippocampus. A camera lucida drawing tube, attached to an Olympus BX51 microscope (x400; Olympus Corporation) was used to select neurons for analyses. To analyze the neurons, the soma center was considered as the reference dot. Total dendritic length and number were measured every 50 µm.

Evaluation of BBB permeability. To measure BBB permeability, Evans blue (EB) dye was used as a tracer, as previously described (27). Briefly, the rats were injected with 2% EB solution diluted in normal saline through the tail vein (2 ml/kg of body weight; Sigma-Aldrich; Merck KGaA) 24 h post-surgery. The EB dye was allowed to circulate for 2 h. Next, the rats were anesthetized and transcardially perfused using 0.9% sodium chloride. The brains were removed, divided into left and right hemispheres, then the right hemisphere was immersed in formamide (10 ml/kg; Sigma-Aldrich; Merck KGaA) at 60˚C for 24 h. Cortical proteins were formamide extracted and centrifuged (2,500 x g) for 10 min at 4˚C. A total of 1 ml supernatant was measured in a spectrophotometer at 620 nm to compare EB content in the brain tissue with standard EB solution.

Immunofluorescence. Following brain perfusion, the tissues were further post-fixed overnight in 4% paraformaldehyde and 4˚C refrigerator, then dehydrated in 30% sucrose. Cerebral
peri-ischemic cortices were sliced into 20-µm slices and washed in PBS. The slices were then incubated in blocking solution 0.1% Triton X-100 in 0.1M PBS, 10% bovine serum albumin (cat. no. Pro-422, pH 7.6; Prospec-Tany Technogene Ltd.) in a 4°C refrigerator for 2 h, followed by overnight incubation at 4°C with anti-ZO-1 (1:100; cat. no. ab96587; Abcam) and anti-claudin-5 (1:100; cat. no. 352588; Thermo Fisher Scientific, Inc.) antibodies. After washing in PBS, the tissues were incubated with goat biotin-conjugated anti-cat IgG antibody (1:10,000, cat. no. LS-C68537; LifeSpan BioSciences, Inc.) and counterstained with 4',6-diamidino-2-phenylindole (Beyotime Institute of Biotechnology). Lastly, the sections were analyzed using a spectral confocal microscope DMI6000 (Leica Microsystems GmbH).

Western blot analysis. Peri-ischemic cortical proteins were extracted from the rat brains by homogenizing the tissues in RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology), supplemented with protease and phosphatase inhibitors. The samples were centrifuged at 7,000 × g (EP5417R; Eppendorf) for 20 min at 4°C. A BCA assay kit (Thermo Fisher Scientific, Inc.) was used to measure the concentration of each sample. Nuclear and cytoplasmic extraction kits (cat. no. 78833; Thermo Fisher Scientific, Inc.) for nuclear protein extraction. An equal amount (0.06 mg) of protein was separated using 6 and 10% SDS-PAGE before being transferred to a PVDF membrane (EMD Millipore). Membranes were blocked with 5% skimmed milk for 90 min. Following blocking, the membranes were incubated overnight at 4°C with anti-pNF-κBp65 (1:1,000; cat. no ab222494; Abcam), anti-NF-κBp65 (1:1,000; cat. no. ab228497; Abcam), anti-MMP-9 (1:1,000; cat. no. ab38898; Abcam), anti-β-actin (1:5,000; cat. no. ab1033; ABclonal Biotech Co., Ltd.), anti-occludin (1:1,000; cat. no. ab216327; Abcam), anti-claudin-5 (1:500; cat. no. 35288; Thermo Fisher Scientific, Inc.) and anti-ZO-1 (1:1,000; cat. no. ab96587; Abcam) antibodies. After several washes with TBS/0.1% Tween-20 and incubation with the goat anti-cat IgG antibody (1:10,000, LS-C68537, LSBio) for 2 h at room temperature, the immuno-reactive bands were visualized using an ECL kit (Beyotime Institute of Biotechnology). The protein bands were then analyzed using ImageJ software version 1.8.0 (National Institutes of Health).

Statistical analysis. All the data are presented as the mean ± SEM. Shapiro-Wilk normality test was used to determine if the data was normally distributed. Statistically significant differences were analyzed using one-way ANOVA for multiple groups or an unpaired Student’s t-test for comparisons of 2 groups were applied. All statistical analyses were performed using GraphPad prism v5 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin ameliorates early brain injury and improves neurological performance in MCAO/R rats. A schematic diagram representing experimental procedures is shown in Fig. 1A (reperfusion was performed 24 h, and 3 and 7 days following MCAO/R). As depicted in Fig. 1B, vehicle-treated MCAO/R rats exhibited poor and reduced neurological function as compared with that in the sham group. Based on the mNSS scale, the MCAO/R rats treated with curcumin showed a significant improvement in neurological performance at all the measured time points (24 h, and 3 and 7 days following reperfusion), where the most optimal effect was noted 24 h post-surgery (P<0.05; Fig. 1B). As the lowest mNSS score
was observed 24 h following reperfusion, this time point was selected for subsequent experiments.

TTC staining was used to measure infarct volume 24 h post-reperfusion. The results showed an absence of infarct in the sham group, but significant ischemic injury in the MCAO/R group compared with that in the sham group (P<0.001; Fig. 1C). However, curcumin pre-treatment significantly reduced infarct size compared with that in the vehicle treated MCAO/R group (P<0.05; Fig. 1D). Therefore, it was concluded that pretreatment with curcumin protected rats against cerebral injury and improved neurological deficits in MCAO/R rats.

Curcumin protects synaptic remodeling of hippocampal neurons against MCAO/R. As shown in Fig. 2A-E, neurons were selected according to the following criteria: The body and dendrites of the neuron were completely impregnated, the neuron was relatively separated from the surrounding neurons and the neuron was located in the hippocampal CA1 area. Golgi staining and quantitative analysis indicated that rats in the vehicle-treated MCAO/R group showed a significant decrease in the number of hippocampal dendrites, as well as total dendritic length as compared with that in the sham group (P<0.01). The rats that were treated with curcumin before MCAO/R had significantly reduced MCAO/R-induced atrophy in the hippocampal neurons, as there was an increase in both dendrite number and length (P<0.05). These data revealed that curcumin could protect hippocampal neuron remodeling when subjected to MCAO/R.

Curcumin pretreatment protects against BBB permeability induced by MCAO/R. The brain sections were viewed using a EB tracer, as shown in Fig. 3A. Leakage of EB dye was significantly attenuated in the curcumin group compared with that in the MCAO/R vehicle group. Levels of formamide extracted from EB dye were further analyzed. Fig. 3B demonstrated that MCAO/R induced significant EB extravasation as compared with that in the sham group at 24 h (P<0.001). Curcumin pretreatment significantly reduced EB extravasation levels (P<0.05) compared with that in the vehicle-treated MCAO/R group. This indicated that curcumin could protect MCAO/R rats from disrupted BBB permeability resulting from ischemic damage.

Curcumin suppresses the MCAO/R-induced inflammatory response. The inflammatory response is activated as a result of BBB injury (28); therefore, it was determined whether curcumin pretreatment could affect the protein expression levels of known inflammatory mediators. Following reperfusion for 24 h, the protein expression levels of both pNF-κBp65 and MMP-9 were increased. However, when exposed to curcumin pretreatment, there was a significant reduction in the expression levels of both pNF-κBp65 (P<0.01; Fig. 4A) and MMP-9 (P<0.05; Fig. 4B) compared with the vehicle groups. Taken together, pretreatment with curcumin suppressed the
inflammatory response, which was revealed by the reduction of key inflammatory mediators.

Curcumin preserves in-situ protein expression level of claudin-5 and ZO-1 in MCAO/R rats. Subsequently, the effects of curcumin pretreatment on BBB permeability were further investigated to confirm the earlier result. Immunohistochemistry, using claudin-5 and ZO-1 antibodies, was performed on the tissues extracted from the rats in the sham and MCAO/R groups, 24 h following reperfusion. As seen in Fig. 5A and B, claudin-5 and ZO-1 protein expression levels were markedly decreased in the cortex of the rats, 24 h following MCAO/R injury as compared with that in the sham group. However, in the group of curcumin pretreated rats, both claudin-5 and ZO-1 expression levels were increased as compared with that in the MCAO/R rats treated with vehicle. These data suggested that curcumin could reverse the attenuation of claudin-5 and ZO-1, which typically occurs when the BBB is disrupted following ischemic brain injury.

Curcumin treatment prevents the degradation of BBB tight junction proteins. Lastly it was investigated how curcumin pretreatment affected BBB integrity and permeability in rats, which had experienced a stroke. The protein expression levels of tight junction proteins, ZO-1, occludin and claudin-5 were analyzed, which play an important role in BBB integrity (29). Western blot analysis followed by densitometry analysis revealed that MCAO/R induced significant reduction of ZO-1, occludin and claudin-5 protein expression levels, 24 h following reperfusion as compared with that in the sham rats. Notably, the protein expression levels of these tight junction proteins were significantly increased following curcumin pretreatment (P<0.05; Fig. 6A-C). Therefore, curcumin may protect the integrity of the BBB by inhibiting the degradation of tight junction proteins induced by MCAO/R.

**Discussion**

In the present study, the effects of curcumin on reperfusion-induced BBB disruption in a rat model of MCAO/R was investigated. The results revealed that pretreatment with curcumin protected rats from MCAO/R and the resulting neurological deficits, reduced infarct size, as well as nerve injury. To obtain a mechanistic insight behind these effects, it was identified that curcumin repressed the inflammatory response and restored levels of tight junction proteins early enough to protect against BBB deterioration.

Curcumin belongs to a class of yellow polyphenols derived from turmeric medicinal plants (25). A previous study has shown that curcumin exerted an array of biological functions, including anti-tumor, anti-immunity, anti-oxidation, anti-fibrosis and anti-inflammatory effects (30). Recently, a study has indicated that curcumin harbored protective effects against neuronal damage following cerebral ischemia (31). In addition, previous studies have shown that following curcumin treatment, neurological scores were improved, early brain injury was ameliorated, and NF-κB and MMP-9 protein expression levels in brain tissue of injured rats were decreased (25,32,33). Furthermore, both the number of hippocampal neuronal dendrites and total dendritic length were increased and EB content in the brain tissue was significantly decreased (34-36). The results from the present study provides additional results by revealing the beneficial effects of curcumin pretreatment against MCAO/R injury. Abnormal hippocampal neurotransmitters may affect neural plasticity, which may be associated with the pathophysiology of ischemic cerebral infarction (37). In the present study, it was found that in the MCAO/R rat model, hippocampal neuronal remodeling could be significantly disrupted following ischemia and reperfusion. It was also confirmed that the administration of curcumin protected the BBB structure, inhibited the inflammatory response of the hippocampus, and remodeled hippocampal neurons against...
MCAO/R (37-39). The protective effect of curcumin on hippocampal neurons provides evidence of its advantage in the treatment of ischemic stroke.

In most unstimulated cells, the inactive p65 component of NF-κB remains in the cytoplasm as a dimer, which can bind to IkB. The protein expression levels of tumor necrosis factor (TNF-α) and IL-1β were increased at the early stages of the inflammatory response following cerebral ischemia and reperfusion (30,40). As the initiating factors of the inflammatory response, both TNF-α and IL-1β can stimulate leukocytes and activate the c signaling pathway (33,41). The IkB protein is phosphorylated and targeted for degradation via the proteasome pathway, where the p65 component of NF-κB is translocated to the nucleus to form pNF-κBp65 and increases the secretion of adhesion molecules via microvascular endothelial cells and leukocytes (42). Leukocytes can adhere to vascular endothelial cells and produce neutrophil-derived oxidant and MMP-9 (13), resulting in BBB damage from MMP-9 (25,32). Previous studies have also showed that MMP-9 protein was expressed in endothelial cells and the main cellular source of brain MMP-9 are brain microvascular endothelial cells in the initial phase following focal cerebral ischemia (43,44). Based on this, it could be concluded that inhibition of NF-κB activation could reduce ischemia-reperfusion injury in rats (45). The protein expression levels of pNF-κBp65 were analyzed in the nucleus. The results showed that curcumin pretreatment effectively reduced the protein expression levels of pNF-κBp65, inhibited the transcriptional activity of NF-κB in cerebral ischemia/reperfusion (I/R) and may have a strong anti-inflammatory effect.

Activation of MMP-9 leads to the degradation of key proteins located in the cerebral blood vessels, mainly through the degradation of type IV collagen. The extracellular matrix components and substrates in the BBB are destroyed, resulting in the extravasation of water in the capillaries and plasma...
proteins in the peripheral blood, resulting in an increase of water in the cell space and formation of vascular-derived brain edema. There is an introduction of toxic substances into the BBB and damage to brain tissue, which destroys the integrity of the vascular structure (43,46,47). Previously, the association between MMP-9 and ischemic brain injury has gained interest (46,47). When an inflammatory reaction occurs in the body, activated white blood cells secrete substances that can be toxic, including IL-8, TNF, MMPs, nitric oxide and reactive oxygen species. Among these, MMP-9 is a key protein, which causes damage to the BBB. Inhibition of MMP-9 has been reported to prevent damage to the brain by maintaining BBB integrity in elderly humans (48). In the present study, curcumin pretreatment was found to significantly reduce MMP-9 protein expression levels induced following MCAO/R. This is consistent with previous reports showing that MMP-9 inhibition mediated BBB protection in a mouse model of ischemic stroke (16). Therefore, it is reasonable to conclude that curcumin pretreatment could reduce BBB damage at least via the reduction of MMP-9 protein expression level to protect against damage from cerebral ischemia.

The BBB is composed of highly selective tight junctions between endothelial cells, made primarily of the membrane-associated accessory proteins, including occludin, ZO-1 and claudin-5 (4,49,50). The results from the present study revealed that pretreatment with curcumin blocked the decrease of tight junction protein expression, which is instrumental in maintaining BBB integrity and function. In summary, the present study revealed that pretreatment with curcumin prior to stroke could inhibit the central pro-inflammatory mediator NF-κB, reduce the protein expression level of MMP-9 and attenuate BBB damage, indicating its neuroprotective effects. These data provide novel targets to investigate its protective effects underlying cerebral ischemic injury and provide a new direction to determine therapeutics for brain insults by restoring the BBB.

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Availability of data and materials

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

Author’s contributions

XZ and SH conceived the design of the study. SW and TG performed the experiments and analyzed the data. TG, WQ, YL, JG and CL performed the experiments and analyzed the data. YS and BY collected the data. YS, BY, SH and XZ performed data analysis and/or wrote part of the paper. SW, XZ and SH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All the experiments were performed following guidelines written by the Institutional Animal Care and Use Committee of China. The studies were also approved by the Ethics Committee for the Use of Experimental Animals at Xuzhou Medical University (assurance nos. 2015-46 and 2015-47).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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