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Retigabine protects the blood-brain barrier by regulating tight junctions between cerebral vascular endothelial cells in cerebral ischemia-reperfusion rats

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Abstract. – OBJECTIVE: To investigate the effect of retigabine on the blood-brain barrier permeability in rats with cerebral ischemia-reperfusion and its mechanism.

MATERIALS AND METHODS: A total of 90 Sprague-Dawley (SD) rats were selected to prepare a rat model of focal cerebral ischemia-reperfusion. The blood flow changes were detected using a laser Doppler flow meter, the percentage of the cerebral infarction volume was measured by means of the triphenyl tetrazolium chloride (TTC) staining, the effect of retigabine on the permeability of the blood-brain barrier after cerebral ischemia-reperfusion was examined via Evans blue (EB) staining, and the state of tight junctions between endothelial cells was determined via the transmission electron microscopy (TEM) technique. Immunohistochemistry was used to detect the effects of retigabine on the distribution and expressions of tight junction-associated proteins in the cerebral ischemia-reperfusion blood-brain barrier. Western blotting was adopted to examine the changes in the expressions of related proteins in cerebral ischemia-reperfusion tissues.

RESULTS: At 48 h and 96 h after cerebral ischemia-reperfusion, retigabine notably reduced the cerebral infarction volume of rats, and the tight junctions between microvascular endothelial cells in the ischemic area opened up, the permeability of the blood-brain barrier was remarkably increased, and the permeability of the blood-brain barrier was significantly reduced under the action of retigabine. The expressions of claudin-5, occludin, and ZO-1 in the blood-brain barrier of the ischemic brain tissue significantly declined, and retigabine notably increased the expressions of three proteins and their distributions along the microvessels. At 3 h, 24 h, 48 h, and 96 h after cerebral ischemia-reperfusion, the expressions of the MMP-2 protein and MMP-9 protein in the ischemic brain tissue were evidently increased, which were inhibited by retigabine. Moreover, the expressions of the PKCδ protein in the ischemic brain tissue were markedly increased, which were significantly inhibited by retigabine.

CONCLUSIONS: The regulatory roles of retigabine in the distribution and expressions of claudin-5, occludin, and ZO-1 may be associated with the inhibition of the expressions of the MMP-2, MMP-9, and PKCδ proteins.

Key Words
Retigabine, Blood-brain barrier, Cerebral ischemia-reperfusion, Tight junctions.

Introduction

Thrombolysis immediately after that the ischemic cerebrovascular disease occurs is the premise and basis for the successful treatment[1,2]. However, thrombolytic therapy is likely to cause cerebral edema and even cerebral hemorrhage primarily due to the increased permeability of the blood-brain barrier[3,4]. The blood-brain barrier comprises brain microvascular endothelial cells, astrocytes, pericytes, basement membranes, and other components. The tight connection between adjacent endothelial cells is a crucial functional unit for the maintenance of the integrity of the blood-brain barrier and a key factor determining the permeability[5]. Tight junction-associated proteins exert vital effects on the regulation of tight junction function, and their distribution and/or expression levels on vascular endothelial cells influence the opening or closing of tight junctions[6].

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Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes. According to studies[7,8], the ischemia-reperfusion injury is accompanied by activation of MMP-2 and MMP-9 that can degrade the cerebral vascular basement membrane and tight junction-associated proteins and increase the permeability of the blood-brain barrier. As a kind of Ca^{2+} and phospholipid-dependent phosphorylases, protein kinase C (PKC) can catalyze the phosphorylation of serine or threonine residues on a variety of protein substrates. Studies[9,10] have confirmed that PKC delta type (PKCδ) is a signaling molecule in cells that is rapidly activated in cerebral ischemia-reperfusion injury, and it regulates the inflammatory response, oxidative stress, and apoptosis triggered by reperfusion injury. PKCδ exhibits continuous high expression in the cerebral microvascular endothelial cells in the ischemic area, and the involvement of PKCδ in the regulation of the distribution or expressions of tight junction-associated proteins has been found in various endothelial cells[11,12].

At present, retigabine is regarded as an ancillary drug for partial epileptic seizures in adults, which is characterized by the stabilizing effect on the neuronal resting membrane potential and anti-excitability[13]. As a novel antiepileptic drug, retigabine also has potential clinical therapeutic value for diseases such as neuropathic pain, neurodegenerative diseases, and dystonia[14,15]. In the meantime, retigabine exerts antioxidant effects to reduce cell death when serum deprivation and oxygen deprivation are applied to culture hippocampal slices[16]. Scholars[17,18] have indicated that retigabine can exert a protective effect after cerebral ischemia, but its mechanism has not been completely clear. In this work, a rat model of cerebral ischemia-reperfusion was used to investigate whether retigabine can protect against cerebral ischemia-reperfusion in rats by improving the blood-brain barrier permeability after ischemia.

Materials and Methods

Laboratory Animals and Models

A total of 90 male Sprague-Dawley (SD) rats weighing 250-300 g were provided by the Qingdao University Laboratory Animal Center. This study was approved by the Animal Ethics Committee of Qingdao University Animal Center. An animal model of focal cerebral ischemia-reperfusion was established according to Longa middle cerebral artery occlusion (MCAO) method: rats were intraperitoneally injected with 10% chloral hydrate (0.3 mL/kg). After anesthesia, the rats were placed in the supine position and fixed in the operating table. A midline incision was made in the neck after routine disinfection to fully expose the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). The ECA was ligated at the bifurcation of ECA and CCA. The suture was made of heparin-coated nylon with a diameter of 0.26 mm. The nylon thread entered the ICA about 18-20 mm *via* CCA. If there is resistance, the MCA inlet was blocked. After the embolization was successful, the suture was fixed, and the nylon thread was pulled out after 2 h for reperfusion. The operation of the sham operation group was the same as that of the model group, but the nylon thread was not inserted in the former.

Detection of Cerebral Blood Flow Via a Laser Doppler flow meter

The anesthetized rats were placed in the prone position and fixed on the brain stereotactic device, and a small hole was drilled into the cerebral dura mater at the intersection of 1 mm behind the coronal suture and 5 mm to the right of the sagittal suture. The laser Doppler flow meter probe was inserted into the hole to measure changes in the blood flow in the middle cerebral artery of the rats. The baseline values of the cerebral blood flow under normal conditions and blood flow changes within 2 h of ischemia and at different time points after reperfusion were recorded. After MCAO, the brain flow was quickly reduced to less than 30% of the baseline value, and animals that did not meet the criteria were excluded.

Experimental Grouping

Rats were randomly divided into three groups, namely, Sham group, Saline group, and Retigabine group. Rats in Retigabine group were intraperitoneally injected with retigabine (10 mg/kg) at 1 h after operation. Rats in Saline group were intraperitoneally injected with the same dose of normal saline. According to different reperfusion time, rats in Saline group and Retigabine group were further divided into 3-h reperfusion group, 24-h reperfusion group, 48-h reperfusion group, and 96-h reperfusion group, with 10 rats in each group.

Measurement of the Volume of Cerebral Infarction

After excessive anesthesia in rats leading to death, the rats were quickly decapitated, and their

brains were taken and placed in a refrigerator at -20°C for 10 min. From the frontal pole, 6 pieces of brain tissues were cut from the front to the back every 2 mm, placed in 2% triphenyl tetrazolium chloride (TTC) solution and incubated in a water bath at 37°C for 30 min in the dark, followed by photographing with a digital camera. The percentage of the cerebral infarction volume was calculated *via* the image analysis software.

Determination of the Blood-Brain Barrier Permeability

2% Evans blue (EB) was injected into the femoral vein of the anesthetized rats at 2 mL/kg (body weight). After 2 h, the left ventricle was perfused with normal saline until the clear liquid was discharged. The rats were decapitated, and their brains were taken. The brain tissues were added with formamide solution (1 mL/100 mg) and extracted in a water bath at 60°C for 24 h. Then, the optical density value was measured at a wavelength of 620 nm using a microplate reader. A standard curve was made to obtain the EB content in brain tissues [μg/g (brain weight)].

Tight Junctions Observed Under a Transmission Electron Microscope

The anesthetized rats were perfused and fixed with 4% paraformaldehyde, and 1 mm^3 frontal parietal cortex of the ischemic area was taken, soaked in 2.5% glutaraldehyde and fixed in 2% osmium tetroxide, followed by dehydration with gradient alcohol and embedding in epoxy resin 618. Subsequently, ultrathin sections were double stained with uranyl acetate and lead citrate, and observed at 80 KV using the JEM-1200EX transmission electron microscope.

Immunohistochemistry

The anesthetized rats were perfused and fixed with 4% paraformaldehyde. Then they were decapitated to take the frontal parietal lobe, which was dehydrated with sucrose, embedded in OTC, cut into coronal serial sections (8 μm in thickness) using the frozen sectioner, diluted with the primary antibody at 1:150 and subjected to immunohistochemical staining [3,3'-Diaminobenzidine (DAB) method]. Images of immunohistochemistry results were collected using a Mode Images Advanced 3.2 image analysis system, and the results were quantitatively analyzed.

Western Blotting

After excessive anesthesia in rats leading to death, the rats were quickly decapitated, and the protein of the frontal parietal lobe in the ischemic area was extracted. The protein concentration was determined by homogenization centrifugation and bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). The protein was separated using *via* 7.5%, 10% and 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively and transferred onto the nitrocellulose membrane. Z0-1 (1:500), occludin (1:500), claudin-5 (1:500), MMP-2 (1:500), MMP-9 (1:500), PKCδ (1:500) and β-actin antibodies (1:5000) were added for incubation overnight at 4°C. Then, the corresponding secondary antibodies were added for incubation for 2 h at room temperature. Enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) was carried out, and Fluor Chem 2.0 image analyzer was applied to analyze the band integral optical density quantitatively.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA). All data were expressed as mean ± standard deviation. The *t*-test was used for the comparison between two groups. One-way analysis of variance and Bonferroni test were adopted for the comparison among multiple groups. $p<0.05$ represented that the difference was statistically significant.

Results

Influence of Blood Flow in the Blood Supply Area of the Rat Middle Cerebral Artery

Changes in the blood flow in the middle cerebral artery of rats were detected using the laser Doppler flow meter, and the cerebral blood flow decreased to less than 30% of the basal value represented that the cerebral ischemia model was successfully established. The results demonstrated that there was no significant change in the blood flow in the middle cerebral artery in Sham group. The blood flow in the middle cerebral artery in Saline group and Retigabine group was remarkable decreased, but there was no significant difference in the cerebral blood flow between the two groups at the same time point (Figure 1A).

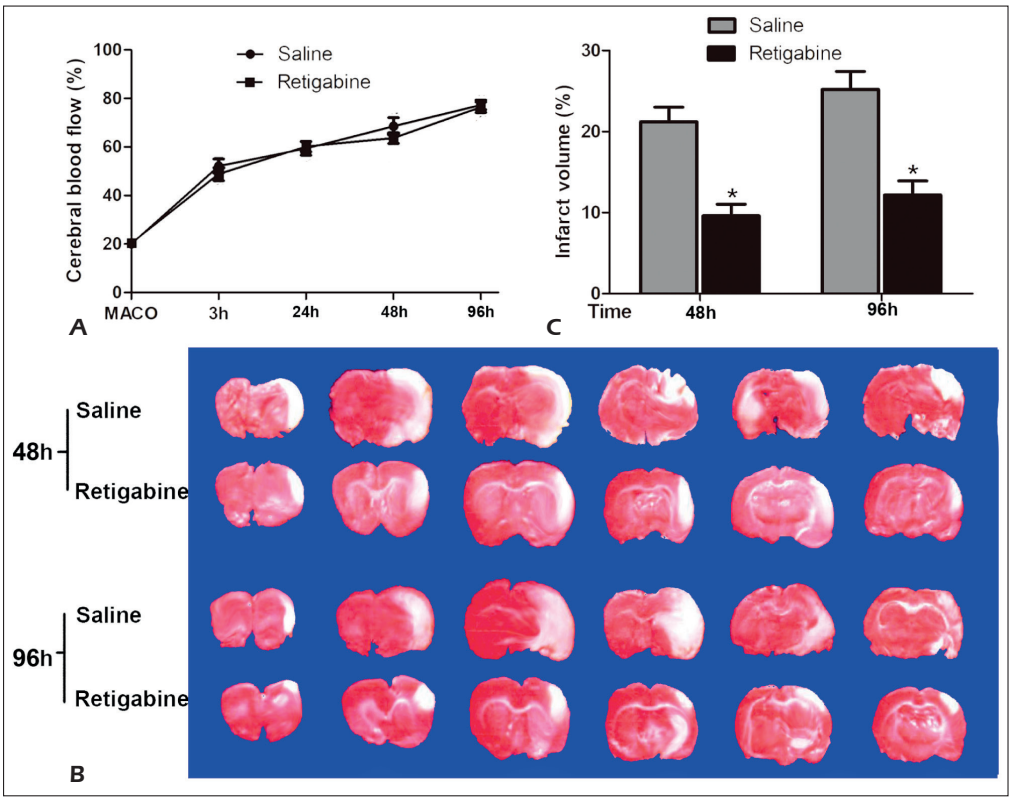

Figure 1. The beneficial effect of retigabine on rats with cerebral ischemia-reperfusion. **A**, Analysis of the blood flow in the middle cerebral artery of rats by the laser Doppler flow meter. **B**, TTC staining showed that the infarction lesion was located in the right frontal, apical and temporal cortexes and the lateral part of the new striatum. The normal tissue was red and the infarction lesion was white. **C**, Analysis of the infarction volume calculated by Shrimakura's method. *$p<0.05$ *vs.* Saline group.

Retigabine Evidently Reduced the Cerebral Infarction Volume After Cerebral Ischemia-Reperfusion

The brain tissue was stained by TTC. The experimental results manifested that after the middle cerebral artery experienced ischemia-reperfusion, the infarction lesion was located in the right frontal, apical and temporal cortexes and the lateral part of the new striatum. The normal tissue was red and the infarction lesion was white. The infarction volume was calculated by Shrimakura's method, which revealed that the cerebral infarction volume in Retigabine group was markedly smaller than that in normal Saline group at 48 h and 96 h after reperfusion (Figure 1B, 1C).

Retigabine Reduced the Blood-Brai n Barrier Permeability in Cerebral Ischemia-Reperfusion

EB permeability test was conducted to assess changes in the blood-brain barrier permeability. No EB was seen in the brain tissue in Sham group. In Saline group, EB could be found in the cerebral hemisphere ischemic area of rats, and the brain sections showed EB in the frontal cortex of the ischemic side and the lateral and central parts of the caudate putamen. The EB content in the ischemic brain tissue in Saline group was significantly higher than that in Sham group at each time point. The EB content in the ischemic brain tissue in 3 h and 48 h reperfusion groups was markedly higher than that in 24 h and 96 h groups. At 3 h and 24 h after reperfusion, the degree of EB staining in the ischemic brain tissue in Retigabine group was remarkably lower than that in Saline group (Figure2A, 2B).

Retigabine Changed the State of Tight Junctions Between Brain Microvascular Endothelial Cells

Observations under the transmission electron microscope identified that the narrowed vascular lumen and vascular destruction were seen in the ischemic brain tissue. The tight junctions showed clear and identifiable intercellular fis-

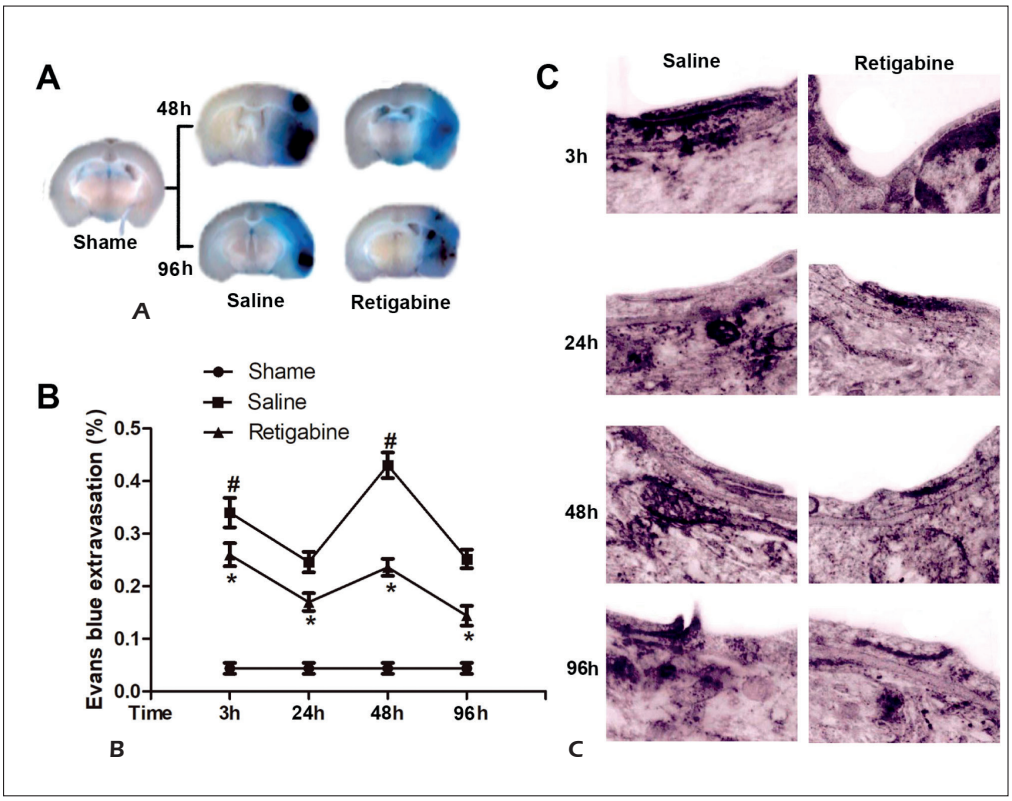

Figure 2. The effect of retigabine on blood brain barrier after cerebral infarction in rats. **A**, EB permeability test showed that retigabine decreased the permeability of BBBs of rats. **B**, Analysis of Evan blue extravasation between different groups. **C**, The results of TEM showed that the state of tight junctions between brain microvascular endothelial cells between different groups. **p*<0.05 *vs*. Saline group, #*p*<0.05 *vs*. 24 h and 96 h group.

sures. After the application of retigabine, the integrity of the tight junction structure could be restored. Then a tight junction dense band appeared, indicating that retigabine can change the open state of the tight junction of the blood-brain barrier (Figure 2C).

Retigabine Significantly Increased the Distribution and Expression of Tight Junction-Associated Proteins Claudin-5, Occludin, and Zonula Occludens-1 (ZO-1)

Immunohistochemistry was used to detect the distribution, changes in the expressions of tight junction-associated proteins, claudin-5, occludin, and ZO-1 at 3 h, 24 h, 48 h, and 96 h after cerebral ischemia-reperfusion and the effects of retigabine after application. The results denoted that the three proteins in the brain tissue of rats in Sham group were persistently expressed along the microvessels of the brain, showing a continuous distribution. In Saline group, the expressions of three proteins along the microvessels in the brain tissue of the ischemic area in rats were significantly lower than those in Sham group. The distribution and expressions of the three proteins along the capillaries after the application of retigabine were significantly increased compared with those in Saline group at the same time point (Figure 3).

Retigabine Notably Inhibited the Expressions of MMP-2 and MMP-9 Proteins

Western blotting was adopted to detect the changes in the expressions of MMP-2 and MMP-9 proteins at 3 h, 24 h, 48 h, and 96 h after cerebral ischemia-reperfusion and the effects of retigabine after application (Figure 4A). The expressions of the MMP-2 protein in rats in Saline group were markedly increased compared with those in Sham group at 3 h, 24 h, 48 h, and 96 h after reperfusion (Figure 4B). The expressions of MMP-9 protein in rats in retigabine group were significantly reduced compared with those in Saline group at 3 h, 24 h, 48 h, and 96 h after reperfusion (Figure 4C).

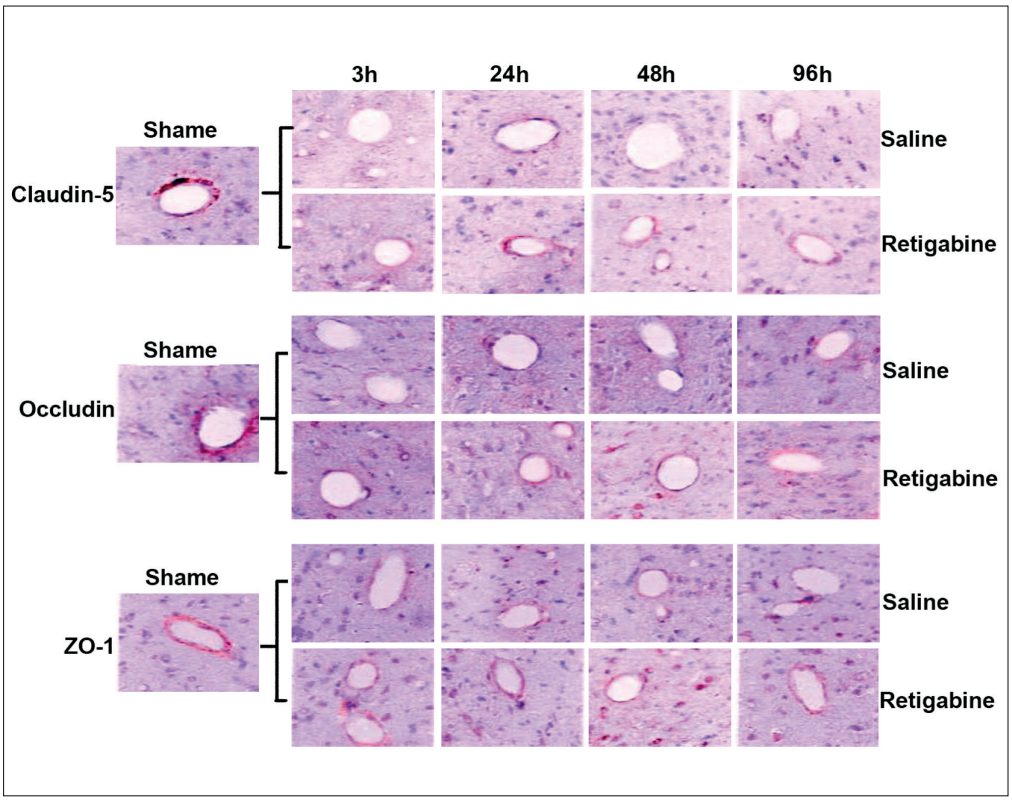

Figure 3. Immunohistochemistry showed that retigabine increased the distribution and expression of tight junction-associated proteins claudin-5, occludin, and ZO-1.

Retigabine Evidently Inhibited the Up-Regulation of the PKCδ Protein Expression

The changes in the expressions of the PKCδ protein at 3 h, 24 h, 48 h, and 96 h after cerebral ischemia-reperfusion and the effects of retigabine after application were detected using Western blotting (Figure 4D). It was found that the expressions of the PKCδ protein in rats in saline group were significantly higher than those in Sham group at 3 h, 24 h, 48 h, and 96 h after cerebral ischemia-reperfusion. After the retigabine intervention, the PKCδ protein expression was remarkably reduced compared with that in saline group (Figure 4E).

Discussion

In the beginning, cerebral ischemia-reperfusion causes a sharp increase in the cerebral blood flow. A large number of neutrophils adhere to the cerebral vascular endothelial cells to block microvessels, resulting in continuous hypoperfusion[19]. The decreased local blood supply in the ischemic area, metabolic disorders in the brain tissue and swelling vascular endothelial cells and astrocytes lead to an increase in the permeability of the blood-brain barrier[6]. As the reperfusion time goes by, numerous neutrophils ooze a lot, a large amount of microglia cells are activated, and various inflammatory cytokines are released, which directly damage endothelial cells, increase the permeability of the blood-brain barrier and cause secondary cerebral edema and even cerebral hemorrhage[20-22]. Studies[23-25] indicated that cerebral ischemia-reperfusion injury causes a bimodal change in the blood-brain barrier permeability. In this research, cerebral blood flow changes in rats with cerebral ischemia-reperfusion showed that the cerebral blood flow in the ischemic area was increased after reperfusion, and there was a significant difference compared with that after MCAO, but the cerebral blood flow in the ischemic area did not return to the baseline level within 96 h after reperfusion, suggesting that the reperfusion during this period is in a consistently low perfusion level.

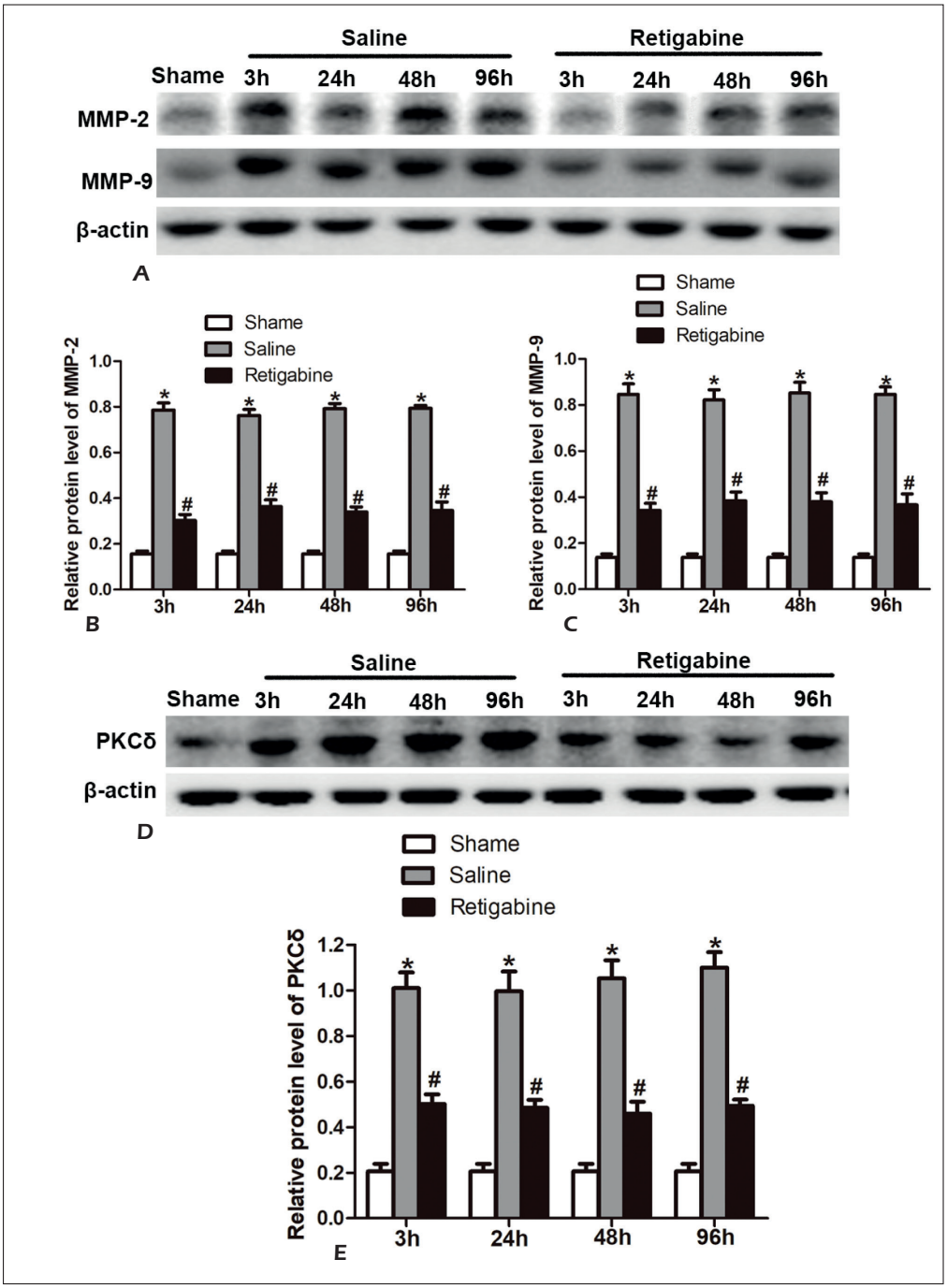

Figure 4. Retigabine notably inhibited the expressions of MMP-2, MMP-9 and PKCδ protein. **A**, Western blotting showed that retigabine inhibited the expressions of MMP-2, MMP-9. **B**, Semi quantitative analysis of protein level of MMP-2. **C**, Semi quantitative analysis of protein level of MMP-9. **D**, Western blotting showed that retigabine inhibited the expressions of PKCδ protein. **E**, Semi quantitative analysis of protein level of PKCδ. $p<0.05$. *vs.* Sham group, #$p<0.05$ *vs.* Saline group.w

The changes in the blood-brain barrier permeability at 3 h, 24 h, 48 h, and 96 h after reperfusion were observed. The results showed that the EB content in the ischemic brain tissue at 3 h and 48 h after reperfusion was significantly higher than that at 24 h and 96 h after reperfusion in Saline group, suggesting that the double-peak changes in the blood-brain barrier permeability of reperfu-

sion occur at about 3 h and 48 h after reperfusion. After retigabine was applied in rats with ischemia-reperfusion, the EB content in the ischemic brain tissue of rats was significantly lower than that in Saline group, indicating that retigabine significantly reduces the permeability of the blood-brain barrier in rats. The ultrastructure of brain microvascular endothelial cells was observed under the transmission electron microscope, and it was found that retigabine could change the open state of tight junctions between endothelial cells and restore the intact structure of tight junctions in the ischemic brain tissue, prompting that retigabine may influence tight junctions between endothelial cells and reduce the permeability of the blood-brain barrier of cerebral ischemia-reperfusion injury through the paracellular pathway.

To investigate the molecular mechanism by which retigabine regulates tight junctions, the changes in the distribution and expressions of tight junction-associated proteins, claudin-5, occludin, and ZO-1 were examined. As the first isolated tight junction transmembrane protein, occludin is tightly linked to claudin-5 and directly involved in the regulation of tight junction permeability[26]. ZO-1 is a member of the membrane-associated guanylate kinases (MAGUK) family (a family containing the guanylate kinase domain or the GUK domain) that interacts with occludin and claudin-5 in the cytoplasm, links the transmembrane protein to the cytoskeleton and is the basis of the supporting structure of tight junctions[27]. The results of this work revealed that the distribution and expressions of tight junction-associated proteins, claudin-5, occludin, and ZO-1 were markedly reduced at 3 h, 24 h, 48 h, and 96 h after cerebral ischemia-reperfusion, indicating that the increase in the blood-brain barrier permeability during this period has a correlation with the down-regulated expressions of the three proteins. Retigabine can up-regulate the expression levels of claudin-5, occludin, and ZO-1 as well as their protein expression levels, and restore the intact structure of tight junctions. Therefore, it was believed that retigabine may reduce the blood-brain barrier permeability through the paracellular pathway by affecting the distribution and expressions of claudin-5, occludin, and ZO-1.

When a cerebral ischemia-reperfusion injury occurs, the expressions of MMP-2 and MMP-9 proteins are increased, which may increase the permeability of the blood-brain barrier by degrading the vascular basement membrane and the tight junction protein[28,29]. Knockout by inhibitors of MMPs or MMP-9 in mice can reduce brain tissue damage and brain edema[30]. In this study, the changes in the expressions of MMP-2 and MMP-9 proteins were examined, so as to further analyze the mechanism of action of retigabine on the distribution and expressions of tight junction-associated proteins, claudin-5, occludin, and ZO-1. It was found that the expressions of the MMP-2 protein and MMP-9 protein were remarkably increased at 3 h, 24 h, 48 h, and 96 h after reperfusion, and retigabine significantly inhibited the expressions of MMP-2 and MMP-9. The above findings suggest that retigabine may reduce the hydrolysis of tight junction-associated proteins, claudin-5, occludin, and ZO-1, up-regulate the protein expression and reduce the blood-brain barrier permeability by inhibiting the expressions of MMP-2 and MMP-9 proteins.

PKC is an important signaling molecule in cells, whose main type is PKCδ that mediates the cerebral ischemia-reperfusion injury. Specific inhibition of PKCδ markedly reduces the number of damaged neurons caused by the cerebral ischemia-reperfusion injury[31]. The cerebral infarction volume of PKCδ knockout mice was significantly smaller than that of wild-type mice[32]. According to studies[33,34], the PKCδ phosphorylation of the activator protein 1 family is involved in the activation of MMP-2 and MMP-9. The activation of PKCδ in the blood cerebrospinal fluid barrier leads to an increase in MMP-9 activity, a decrease in the expression of tight junction-associated proteins, and an increase in the blood-cerebrospinal barrier permeability[33,34]. The application of PKCδ selective inhibitors can increase the associations of ZO-1 and occludin with the cytoskeleton, and reduce the damage to the tight junction structure[35]. The data of this paper demonstrated that the expressions of the PKCδ protein were evidently increased at 3 h, 24 h, 48 h, and 96 h after cerebral ischemia-reperfusion, indicating that cerebral ischemia-reperfusion injury may participate in the up-regulation of the expression or activity of MMP-2 and MMP-9 and changes in the tight junction protein phosphorylation/expression through the PKCδ pathway, thus increasing the blood-brain barrier permeability. Retigabine can significantly inhibit the up-regulation of the PKCδ protein expression, indicating that retigabine is involved in the regulation of the blood-brain barrier permeability of cerebral ischemia-reperfusion through the PKCδ pathway.

Conclusions

We observed that retigabine up-regulated the expressions of claudin-5, occludin, and ZO-1 and alters the distribution of these proteins, so as to protect the integrity of the tight junction structure of brain microvascular endothelial and reduced the permeability of the blood-brain barrier. The regulatory roles of retigabine in the distribution and expressions of claudin-5, occludin, and ZO-1 may be associated with the inhibition of the expressions of the MMP-2, MMP-9, and PKCδ proteins.

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Frerichs K, Baker J, Norbash A. Intra-arterial stroke thrombolysis and carotid stenting: methods for the treatment of ischemic cerebrovascular disease. Semin Roentgenol 2002; 37: 255-265.
2) Ali SF, Viswanathan A, Singhal AB, Rost NS, Forducey PG, Davis LW, Schindler J, Likosky W, Schlegel S, Solenski N, Schwamm LH. The TeleStroke mimic (TM)-score: a prediction rule for identifying stroke mimics evaluated in a Telestroke Network. J Am Heart Assoc 2014; 3: e838.
3) Wang YX, Li YQ, Chen Y, Zhang CH, Dong Z, Wang Z, Zhao SN, Li CH, Zhang PL. Analysis of related factors of orolingual angioedema after rt-PA intravenous thrombolytic therapy. Eur Rev Med Pharmacol Sci 2018; 22: 1478-1484.
4) Piccardi B, Arba F, Nesi M, Palumbo V, Nencini P, Giusti B, Sereni A, Gadda D, Moretti M, Fainardi E, Mangiafico S, Pracucci G, Nannoni S, Galmozzi F, Fanelli A, Pezzati P, Vanni S, Grifoni S, Sarti C, Lamassa M, Poggesi A, Pescini F, Pantoni L, Gori AM, Inzitari D. Reperfusion Injury after ischemic Stroke Study (RISKS): single-centre (Florence, Italy), prospective observational protocol study. BMJ Open 2018; 8: e21183.
5) Morita S, Furube E, Mannari T, Okuda H, Tatsumi K, Wanaka A, Miyata S. Heterogeneous vascular permeability and alternative diffusion barrier in sensory circumventricular organs of adult mouse brain. Cell Tissue Res 2016; 363: 497-511.
6) Wolburg H, Lippoldt A. Tight junctions of the blood-brain barrier: development, composition and regulation. Vascul Pharmacol 2002; 38: 323-337.
7) Liu Y, Huang Y, Xu Y, Qu P, Wang M. Memantine protects against ischemia/reperfusion-induced brain endothelial permeability. IUBMB Life 2018; 70: 336-343.
8) Zhang W, Song JK, Zhang X, Zhou QM, He GR, Xu XN, Rong Y, Zhou WX, DU GH. Salvianolic acid A attenuates ischemia reperfusion induced rat brain damage by protecting the blood brain barrier through MMP-9 inhibition and anti-inflammation. Chin J Nat Med 2018; 16: 184-193.
9) Wang ZK, Zhang LC, Zhao SH, Zhang JW, Shang LH, Zhang YL, Lin CR, Guo JK. [Effect of electroacupuncture on cerebral PKC isozyme expression levels in cerebral ischemia-reperfusion rats]. Zhen Ci Yan Jiu 2012; 37: 312-317.
10) Jiao H, Wang Z, Liu Y, Wang P, Xue Y. Specific role of tight junction proteins claudin-5, occludin, and ZO-1 of the blood-brain barrier in a focal cerebral ischemic insult. J Mol Neurosci 2011; 44: 130-139.
11) Bharath LP, Cho JM, Park SK, Ruan T, Li Y, Mueller R, Bean T, Reese V, Richardson RS, Cai J, Sargsyan A, Pires K, Anandh BP, Boudina S, Graham TE, Symons JD. Endothelial cell autophagy maintains shear stress-induced nitric oxide generation via glycolysis-dependent purinergic signaling to endothelial nitric oxide synthase. Arterioscler Thromb Vasc Biol 2017; 37: 1646-1656.
12) Lee SE, Jeong SI, Yang H, Park CS, Jin YH, Park YS. Fisetin induces Nrf2-mediated HO-1 expression through PKC-delta and p38 in human umbilical vein endothelial cells. J Cell Biochem 2011; 112: 2352-2360.
13) Ossemann M, de Fays K, Bihin B, Vandermeeren Y. Effect of a single dose of retigabine in cortical excitability parameters: a cross-over, double-blind placebo-controlled TMS study. Epilepsy Res 2016; 126: 78-82.
14) Qi Y, Wang J, Bomben VC, Li DP, Chen SR, Sun H, Xi Y, Reed JG, Cheng J, Pan HL, Noebels JL, Yeh ET. Hyper-SUMOylation of the Kv7 potassium channel diminishes the M-current leading to seizures and sudden death. Neuron 2014; 83: 1159-1171.
15) Kobayashi K, Nishizawa Y, Sawada K, Ogura H, Miyabe M. K(+)-channel openers suppress epileptiform activities induced by 4-aminopyridine in cultured rat hippocampal neurons. J Pharmacol Sci 2008; 108: 517-528.
16) Boscia F, Annunziato L, Taglialatela M. Retigabine and flupirtine exert neuroprotective actions in organotypic hippocampal cultures. Neuropharmacology 2006; 51: 283-294.
17) Rekling JC. Neuroprotective effects of anticonvulsants in rat hippocampal slice cultures exposed to oxygen/glucose deprivation. Neurosci Lett 2003; 335: 167-170.
18) Lee S, Yang Y, Tanner MA, Li M, Hill MA. Heterogeneity in Kv7 channel function in the cerebral and coronary circulation. Microcirculation 2015; 22: 109-121.
19) Li M, Qu YZ, Zhao ZW, Wu SX, Liu YY, Wei XY, Gao L, Gao GD. Astragaloside IV protects against focal cerebral ischemia/reperfusion injury correlating to suppression of neutrophils adhesion-related molecules. Neurochem Int 2012; 60: 458-465.
20) Teng X, Chen W, Liu Z, Feng T, Li H, Ding S, Chen Y, Zhang Y, Tang X, Geng D. NLRP3 inflammasome is involved in Q-VD-OPH induced necroptosis following cerebral ischemia-reperfusion injury. Neurochem Res 2018; 43: 1200-1209.
21) Liu J, Li C, Peng H, Yu K, Tao J, Lin R, Chen L. Electroacupuncture attenuates learning and memory impairment via activation of alpha7nAChR-mediated anti-inflammatory activity in focal cerebral ischemia/reperfusion injured rats. Exp Ther Med 2017; 14: 939-946.

22) Wang X, An F, Wang S, An Z, Wang S. Orientin attenuates cerebral ischemia/reperfusion injury in rat model through the AQP-4 and TLR4/NF-kappaB/TNF-alpha signaling pathway. J Stroke Cerebrovasc Dis 2017; 26: 2199-2214.
23) Janyou A, Wicha P, Jittiwat J, Suksamrarn A, Tocharus C, Tocharus J. Dihydrocapsaicin attenuates blood brain barrier and cerebral damage in focal cerebral ischemia/reperfusion via oxidative stress and inflammatory. Sci Rep 2017; 7: 10556.
24) Sarami FM, Sobhani ZS, Mohammadi MT, Aryafar M. Fullerenol nanoparticles decrease blood-brain barrier interruption and brain edema during cerebral ischemia-reperfusion injury probably by reduction of Interleukin-6 and matrix metalloproteinase-9 transcription. J Stroke Cerebrovasc Dis 2018:
25) Chen F, Liu Z, Peng W, Gao Z, Ouyang H, Yan T, Ding S, Cai Z, Zhao B, Mao L, Cao Z. Activation of EphA4 induced by EphrinA1 exacerbates disruption of the blood-brain barrier following cerebral ischemia-reperfusion via the Rho/ROCK signaling pathway. Exp Ther Med 2018; 16: 2651 2658.
26) Aggarwal A, Singh I, Sandhir R. Protective effect of S-nitrosoglutathione administration against hyperglycemia induced disruption of blood brain barrier is mediated by modulation of tight junction proteins and cell adhesion molecules. Neurochem Int 2018; 118: 205-216.
27) Wu L, Ye Z, Pan Y, Li X, Fu X, Zhang B, Li Y, Lin W, Li X, Gao Q. Vascular endothelial growth factor aggravates cerebral ischemia and reperfusion-induced blood-brain-barrier disruption through regulating LOC102640519/HOXC13/ZO-1 signaling. Exp Cell Res 2018; 369: 275-283.
28) Hannocks MJ, Zhang X, Gerwien H, Chashchina A, Burmeister M, Korpos E, Song J, Sorokin L. The gelatinases, MMP-2 and MMP-9, as fine tuners of neuroinflammatory processes. Matrix Biol 2017:
29) Rempe RG, Hartz A, Soldner E, Sokola BS, Alluri SR, Abner EL, Kryscio RJ, Pekcec A, Schlichtiger J, Bauer B. Matrix metalloproteinase-mediated blood-brain barrier dysfunction in epilepsy. J Neurosci 2018; 38: 4301-4315.
30) Wang X, Lee SR, Arai K, Lee SR, Tsuji K, Rebeck GW, Lo EH. Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator. Nat Med 2003; 9: 1313-1317.
31) Wang Z, Xue Y, Jiao H, Liu Y, Wang P. Doxycycline-mediated protective effect against focal cerebral ischemia-reperfusion injury through the modulation of tight junctions and PKCdelta signaling in rats. J Mol Neurosci 2012; 47: 89-100.
32) Wei H, Li Y, Han S, Liu S, Zhang N, Zhao L, Li S, Li J. cPKCgamma-modulated autophagy in neurons alleviates ischemic injury in brain of mice with ischemic stroke through Akt-mTOR pathway. Transl Stroke Res 2016; 7: 497-511.
33) Li C, Zhao Y, Yang D, Yu Y, Guo H, Zhao Z, Zhang B, Yin X. Inhibitory effects of kaempferol on the invasion of human breast carcinoma cells by downregulating the expression and activity of matrix metalloproteinase-9. Biochem Cell Biol 2015; 93: 16-27.
34) Lu Z, Lu N, Li C, Li F, Zhao K, Lin B, Guo Q. Oroxylin A inhibits matrix metalloproteinase-2/9 expression and activation by up-regulating tissue inhibitor of metalloproteinase-2 and suppressing the ERK1/2 signaling pathway. Toxicol Lett 2012; 209: 211-220.
35) Jiao H, Wang Z, Liu Y, Wang P, Xue Y. Specific role of tight junction proteins claudin-5, occludin, and ZO-1 of the blood-brain barrier in a focal cerebral ischemic insult. J Mol Neurosci 2011; 44: 130-139.