MMP-9 Upregulation is Attenuated by the Monoclonal TLR2 Antagonist T2.5 After Oxygen−Glucose Deprivation and Reoxygenation in Rat Brain Microvascular Endothelial Cells

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Background: Blood-brain barrier (BBB) disruption plays a key role in the pathophysiology of acute ischemic stroke. Matrix metalloproteinases-2/9 (MMP-2/9) have been shown to participate in the disruption of the BBB and hemorrhagic transformation after cerebral ischemia. Toll-like receptor 2 (TLR2) may also be correlated with endothelial cell injury during ischemia-reperfusion events. However, the correlation between MMP-2/9 and TLR2 on endothelial cells after ischemia has not yet been evaluated. The aim of the study was to evaluate the impact of TLR2 and MMP-2/9 on tight junction proteins (TJs) after oxygen−glucose deprivation and reoxygenation (OGDR). *Materials and methods:* Rat primary brain microvascular endothelial cells (BMECs) were cultured. Quantitative real-time PCR and western blotting were used to measure the mRNA and proteins expression of TLR2 and MMP-2/-9. The protein expression of TJs was detected by western blotting and immunofluorescence. *Results:* MMP-9 significantly increased after OGDR. Protein and mRNA expression of TLR2 was also upregulated. However, claudin-5, occludin, collagen-IV, and ZO-1 were decreased after OGDR. When monoclonal anti-TLR2 antibody (T2.5) was added to BMECs after OGDR, MMP-9 was significantly downregulated, whereas occludin and collagen-IV had a tendency to increase. *Conclusion:* TLR2 antagonist T2.5 is able to downregulate the expression of MMP-9, and may constitute a therapeutic option for restoration of the BBB after OGDR.

Key Words: Brain microvascular endothelial cells—toll like receptor-2—matrix metalloproteinases-2/9—blood-brain barrier—tight junction proteins—anti-TLR2 antibody (T2.5)

Introduction

Acute ischemic stroke is a major cerebrovascular disease in which a thrombus or embolism suddenly obstructs blood vessels supplying the brain, leading to ischemia and glucose deprivation of cerebral tissue, and subsequent neurologic symptoms.[1] Cerebral ischemia triggers blood-brain barrier (BBB) damage, cellular and vasogenic edema, neuronal death, and astroglial activation. Disrupting the BBB may exacerbate changes in homeostasis within the central nervous system (CNS) and promote hemorrhage transformation (HT) after reperfusion.[2] However, the molecular mechanisms underlying these

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Received June 5, 2018; revision received August 29, 2018; accepted September 8, 2018.

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1052-3057/$ - see front matter

https://doi.org/10.1016/j.jstrokecerebrovasdis.2018.09.014

changes are not well understood. BBB integrity is maintained by brain microvascular endothelial cells (BMECs), astrocyte endfeet, pericytes, and the extracellular matrix.[2] BMECs are connected by tight junctions (TJs) and adherens junctions (AJs). Occludin and claudin proteins in the TJ and cadherin proteins in the AJ are anchored to the actin cytoskeleton via multiple accessory proteins, such as the zonula occludens family (ZO-1, ZO-2, and ZO-3).

Matrix metalloproteinases (MMPs) are matrix-degrading enzymes that play an important role in the CNS. Experimental studies suggest that MMPs can degrade the neurovascular matrix and disrupt BBB TJs. Therefore, MMPs (chiefly MMP-9) exacerbate BBB injury, brain edema, and HT in acute ischemic stroke.[3,4]

Toll-like receptors (TLRs) are receptors against common exogenous pathogens, such as bacteria and viruses. TLRs are ubiquitous and expressed in both mammalian immune-related cells and nonimmune cells. Some TLRs are also present in brain cells, including astrocytes, microglia, oligodendrocytes, neurons and endothelial cells.[5] Recently, it has been demonstrated that TLR2 and TLR4 play a role in noninfectious immune-mediated injury, such as ischemic brain injury.[6,7]

Cerebral endothelial cells express a complex system of signaling molecules that regulate endothelial functions precisely[8]. This study aimed to investigate the expressions of MMP-2/9, TLR2, and TJ proteins in BMECs after oxygen–glucose (OGD) deprivation and reoxygenation (OGDR) treatment in vitro. Additionally, the relationship between TLR2 and MMP-2/9 was studied when monoclonal anti-TLR2 antibody (T2.5) was added to BMECs after OGDR.

Materials and Methods

Culture of Primary BMECs

The study protocol was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, Bethesda, Maryland), and approved by the Animal Care Committee of Kunming University of Science and Technology, China. Eight Sprague-Dawley neonatal rats aged 3-5 days were used (Animal Center of Kunming Medical University, Kunming, Yunnan, China). Cerebral cortices were cut into small pieces and digested in two steps with 0.2% collagenase (Vetec Sigma-Aldrich (Shanghai), China), 0.2% dispase (Sigma, Ronkonkoma, NY) and 25% bovine serum albumin (Amresco, LLC, Solon, OH) followed by centrifugation.[9] Endothelial cells were selected with 4 μg/ml puromycin (Amresco) for 24 hours. BMECs were identified with immunofluorescence staining using the specific von Willebrand factor marker (vWF, 1:50, Proteintech, Rosemont, IL) (Fig 1, A,B). The cells in Figure 1A were the first generation primary BMECs, but the cells in Figure 1B were the third or fourth generation.

OGD and OGDR Treatment

Primary cell cultures were treated with OGD to mimic acute ischemia in vitro.[10] Briefly, glucose-free Dulbecco's modified Eagle medium (DMEM; without pyruvic acid) was used to replace the normal DMEM/high glucose medium, and cells were placed in an oxygen deprived (94% N_2, 5% CO_2, 1%O_2) incubator at 37 °C (CO_2 incubator 3131, Thermo Fisher Scientific, Waltham, MA). Cells were split into groups and subjected to OGD for 1 hour, 2 hours, 3 hours, 6 hours, and 24 hours. Control groups were incubated with a normal DMEM/high glucose medium without fetal bovine serum (FBS) at 37 °C in 95% air and 5% CO_2. Immediately after OGD treatment, the cells were collected separately for subsequent analyses. To create the OGDR conditions, cells were first subject to OGD for 2 hours, and then returned to normoxic conditions with normal medium and oxygen concentration for 1 hour, 2 hours, 3 hours, 6 hours, and 24 hours.

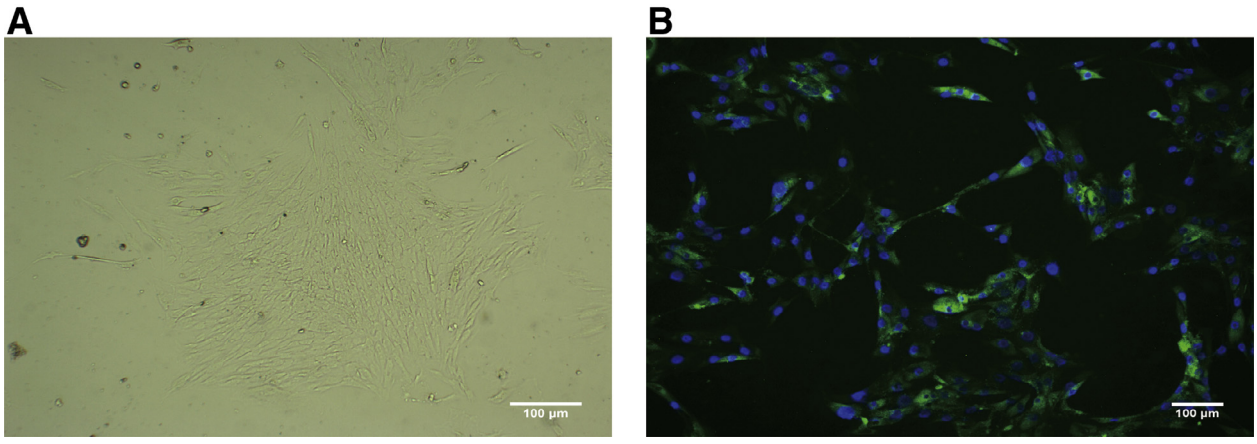

Figure 1. *Primary brain microvascular endothelial cells. (A) BMECs morphology under an inverted microscope; (B) positive immunofluorescence staining for von Willebrand factor.*

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from treated and control cells using an Eastep Total RNA Extraction Kit (Promega, Shanghai, China). Total RNA was transcribed to cDNA (GoScript Reverse Transcriptase System, Promega, Madison Promega, Shanghai, China, WI), and the subsequent products were amplified using SYBR Premix Ex TaqTM II (Tli RNaseH Plus; Takara, Dalian) on a Roche LightCycler 480 real-time PCR system under the following conditions: 95 °C for 30 seconds, 40 cycles at 95 °C for 5 seconds, 55 ° C for 30 seconds and 72 °C for 30 seconds; 95 °C for 5 seconds, 60 °C for 60 seconds and 95 °C. Primers for TLR-2, MMP-2, MMP-9 and β-actin were designed with the following sequences:

TLR-2 (NM_198769.2) forward: 5′-GGCCACAG-GACTCAAGAGCA-3′, reverse:5′-AGAGGCCTAT-CACAGCCATCAAG-3′;

MMP-2(NM_031054.2) forward: 5′-ACCTTGACCA-GAACACCATCGAG-3′, reverse: 5′- CAGGGTC-CAGGTCAGGTGTGTA -3′;

MMP-9(NM_031055.1) forward: 5′- CATGCGCT-GGGCTTAGATCA-3′, reverse: 5′- GAGGCCTTG-GGTCAGGTTTAGAG-3′;
β-actin (NM_031144.3) forward: 5′-GGAGAT-TACTGCCCTGGCTCCTA-3′, reverse: 5′- GACT-CATCGTACTCCTGCTTGCTG -3′.

Each sample was measured in triplicate. The comparative ΔΔCt method was applied to calculate the relative value of mRNA expression. Briefly, mean Ct values were normalized to β-actin and the difference was determined as ΔCt. The difference between the mean ΔCt values of treated and control groups was calculated and defined as ΔΔCt. The comparative expression level of mRNA was expressed as $2^{-\Delta\Delta Ct}$.

Immunofluorescence

Primary rat BMECs were cultured on coverslips. After treated with OGD, cells were fixed using ice-cold acetone for 10 minutes, and then washed 3 times for 5 minutes each time with phosphate-buffered saline. Subsequently, cells were blocked with 5% bovine serum albumin for 30 minutes, and coverslips were incubated with primary antibody (anti-vWF [1:50, Proteintech, Rosemont, IL], ZO-1[1:200, invitrogen, Carlsbad, CA], claudin-5[1:100, Abcam Biotechnology, San Francisco, CA], occludin [1:100, Abcam Biotechnology], collagen-IV [1:200, Abcam Biotechnology]). Goat anti-rabbit IgG H&L (FITC) secondary antibody (1:1000) was purchased from Abcam. DAPI was used to stain cell nuclei (Beijing Solarbio Science & Technology Co., Ltd, Beijing).

Western Blot Analysis

Before BMECs were collected, medium was discarded and cells were washed 3 times using pre-cooled phosphate-buffered saline for 5 minutes each time. BMECs were treated with RIPA lysis buffer (Beyotime Biotechnology, Nantong, Jiangsu) containing a protease inhibitor cocktail (Calbiochem, San Diego, CA) and PhosSTOP (Roche, Rockford, IL) on ice for 30 minutes, and then centrifuged at 12,000 g for 10 minutes at 4 °C. The supernatant was placed in a labeled 1.5 ml centrifuge tube at −20 °C. Subsequently, protein concentration was determined with a bicinchoninic acid assay (Beyotime Biotechnology, Nantong, Jiangsu). Proteins were separated using western blot and transferred to PVDF membranes (Merk Millipore). After blocking with tris buffered saline (TBS) (Sangon Biotech, Shanghai,China) and 0.01% tween 20 with 5% w/v nonfat milk, the membranes were incubated with primary rabbit poly-antibody at 4 °C overnight. Anti-TLR2 (1:1000), anti-MMP-9 (1:1000), anti-MMP-2 (1:1000) and anti-occludin (1:1000) antibodies were purchased from Abcam. Anti-claudin-5 (1:1000) and anti-collagen-IV (1:2000) antibodies were purchased from Millipore and Abcam Biotechnology, respectively. Anti-ZO-1 (1:4000) was purchased from Invitrogen. β-actin was obtained from Proteintech, Rosemont, IL. The membranes were washed 3 times for 10 minutes each time with TBS-T, and were incubated with goat anti-rabbit secondary antibody (1:5000; Proteintech, Rosemont, IL) at room temperature for 2 hours. Immunoreactions were observed with an ECL Plus developer (Proteintech, Rosemont, IL), and photographed in a membrane imaging system (Bio-Rad, Hercules, CA). Bands were semi-quantitatively calculated using ImageJ software. The values were normalized to β-actin.

Statistical Analysis

Data are presented as mean ± standard deviation. Differences in means among multiple groups were analyzed using 1-way ANOVA, followed by the least significant difference test. *P*-values of less than 0.05 were considered statistically significant.

Results

TJs in BMECs were Downregulated After OGD

Endothelial cells are one of the most important cells of BBB. They are irregular and polygonal in shape and are in close contact with each other (Fig 1, A). In order to observe the impact of OGD on BMECs, we evaluated TJs

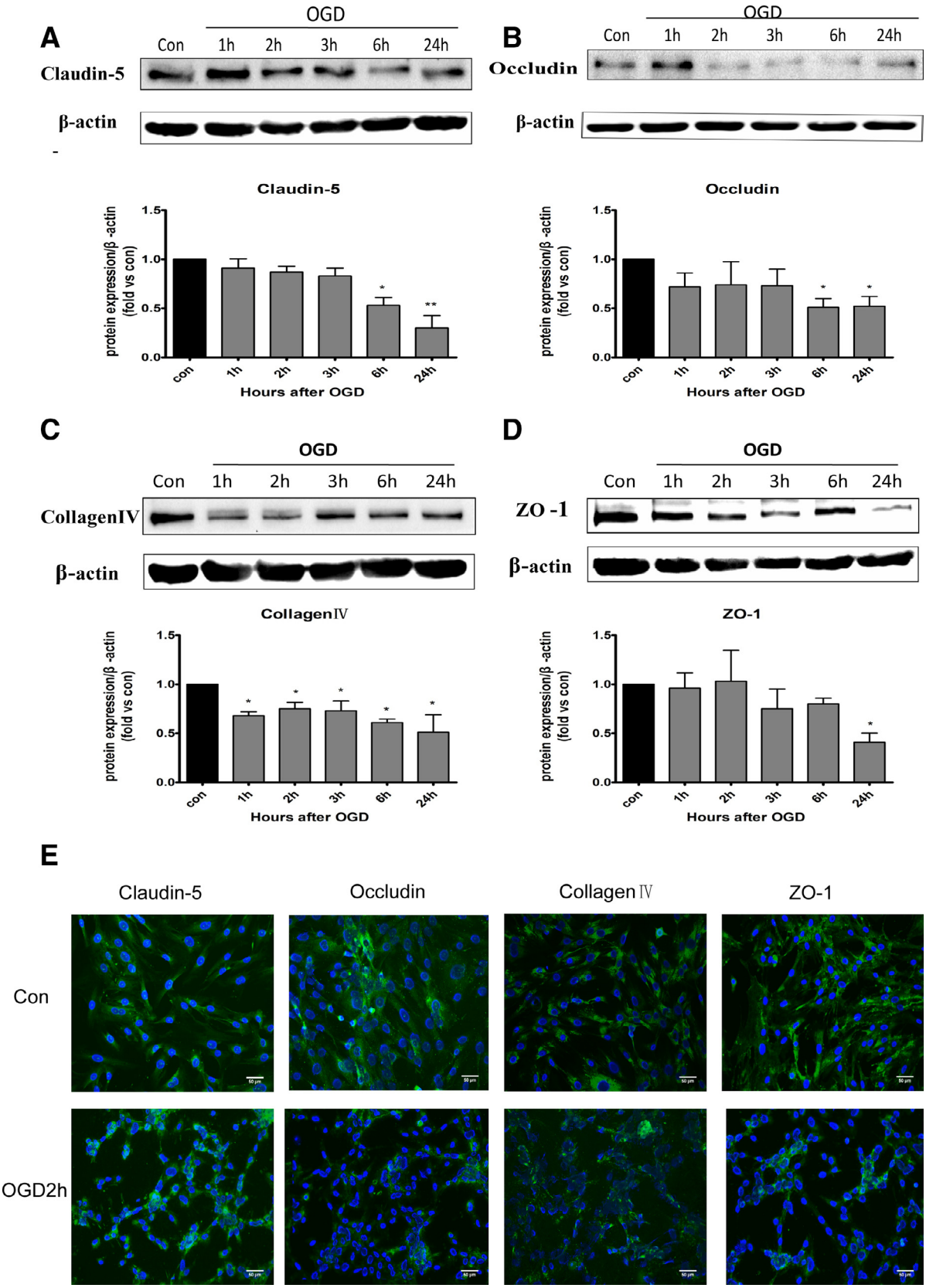

Figure 2. *Expression of TJ proteins claudin-5, occludin, collagen-*Ⅳ*, and ZO-1 in rat primary BMECs after 1-24 hours of OGD. Abbreviations: Con, control group; OGD, oxygen–glucose deprivation; ZO-1: zona occludens-1.*

using western blot and immunofluorescence. Compared with control groups, claudin-5 and occludin were significantly downregulated in BMECs at 6 hours of OGD (Fig 2, A,B). The expression of collagen-IV in BMECs was decreased after 1 hour of OGD (Fig 2, C), while ZO-1 was only downregulated after 24 hours of OGD (Fig 2, D). Immunofluorescence staining (E) was used to detect the expression of TJs in BMECs after 2 hours of OGD. The immunofluorescence intensity of claudin-5, occludin, ZO-1, and collagen-IV was attenuated, and the cell structure was integrated to a certain extent. Compared with the control group, the immunofluorescence intensity of claudin-5, occludin, ZO-1, and collagen-IV in 2 hours OGD group was attenuated. The cells in the 2-hour OGD group maintained their structure (Fig 2, E), whereas those in the 3-hour OGD group lost their structure (figures were not shown).

Expression of MMP-9 was Increased in BMECs After OGDR

As shown above, TJs were degraded and the structure of BMECs was damaged to a certain extent after 2 hours of OGD. Therefore, we evaluated the effects of reoxygenation after 2 hours of OGD to study the impact of OGDR on MMP-2/9, TLR2, and TJ proteins expression. BMECs were disposed by 2 hours of OGD and then placed in normoxic conditions with normal medium and oxygen for 1 hour, 2 hours, 3 hours, 6 hours, and 24 hours. qRT-PCR showed that MMP-9 mRNA significantly increased after 2 hours of OGDR, and western blot indicated that MMP-9 protein increased after 3 hours of OGDR (Fig 3, B,D). However, the expression of MMP-2 mRNA and protein did not change after OGDR (Fig 3, A,C).

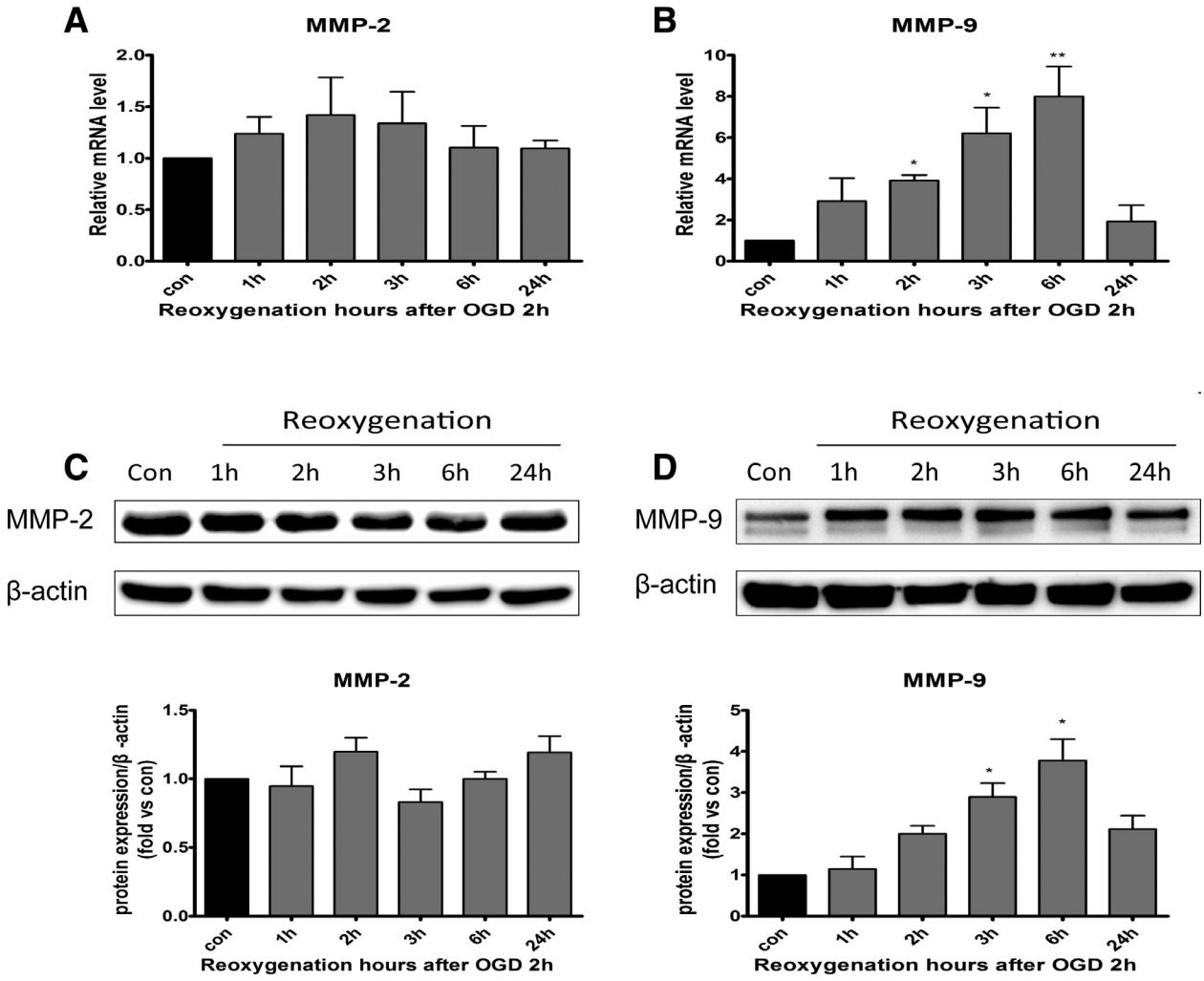

Figure 3. *Expression of MMP-2/9 in rat primary BMECs after OGDR. Abbreviations: MMP-2, matrix metalloproteinases-2; MMP-9, matrix metalloproteinases-9; OGDR, oxygen−glucose deprivation reoxygenation.*

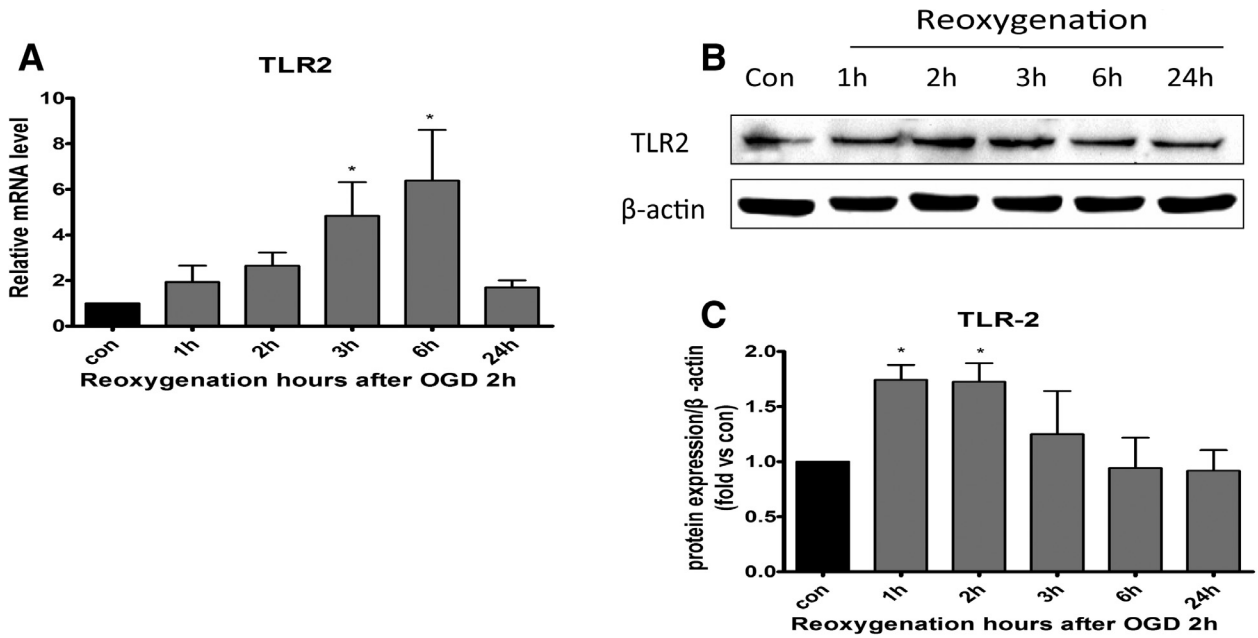

Figure 4. *Expression of TLR2 in rat primary BMECs after OGDR. Abbreviations: OGDR, oxygen−glucose deprivation reoxygenation; TLR2, TOLL like receptor 2.*

Expression of TLR2 was Upregulated in BMECs After OGDR

TLR2 mRNA and protein levels in BMECs were also measured after OGDR by qRT-PCR and western blot, respectively. TLR2 mRNA and protein significantly increased after 3 hours and 1 hour of OGDR, respectively (Fig 4, A-C). Subsequently, TLR2 mRNA returned to control levels at 24 hours of OGDR, while TLR2 protein returned to control levels at 3 hours of OGDR.

TJ Proteins were Downregulated in BMECs After OGDR

Compared with control cells, protein expression of claudin-5 and occludin was decreased at 3 hours and 6 hours of reoxygenation, respectively (Fig 5, A,B). At 1 hour post-reoxygenation, protein expression of collagen-IV and ZO-1 was downregulated (Fig 5, C,D).

TLR2 Antagonist T2.5 Downregulated MMP-9 Expression

To investigate whether the monoclonal anti-TLR2 antibody (T2.5) (Invivogen, San Diego, CA) influenced expression of MMP-2/9 and restored BMECs TJs, 50 μg/ml T2.5 was added to the cells when OGDR began. As shown in Figure 6, MMP-9 was downregulated when T2.5 was added at 6 hours and 24 hours. The expressions of occludin and collagen-IV tended to increase following addition of T2.5; however, these trends did not reach statistical significances (Fig 6, F-K).

Discussions

Acute ischemic cerebral injury involves a number of different mechanisms. Although there are developments in the pathophysiology of cerebral ischemia, treatments for acute ischemic stroke remained limited.[11] The only approved therapy for acute ischemic stroke is intravenous recombinant tissue plasminogen activator (tPA).[12] However, the therapeutic application of tPA is time-dependent and is associated with hemorrhagic complications.[12,13] Moreover, tPA may activate MMPs and contribute to BBB damage.[14] New therapies that focus on protecting the BBB after acute ischemic stroke are urgent required.

TJs consist of claudin, occludin and ZO proteins. Claudin-5 is vital to the integrity of TJs in BMECs. Studies have demonstrated that damage to claudin-5 alone is enough to result in a change in the function of the BBB.[15] Occludin is a tetraspan membrane protein localized with TJ strands that can increase electrical resistance across the membrane and decrease paracellular permeability.[3] We adopted an OGD model in vitro to study TJ proteins expression in BMECs under hypoxia. The present study showed that OGD results in the degradation of TJs in primary BMECs. Western blot revealed that TJ proteins claudin-5, occludin, ZO-1, and collagen-IV were degraded at different durations of OGD. Collagen-IV may be more sensitive to hypoxia than claudin-5, occludin, and ZO-1 in BMECs, given the significant decrease observed at 1 hour of OGD. ZO-1 was downregulated only after 24 hours of OGD, suggesting some tolerance to ischemia. The fluorescence intensity of four proteins in BMECs was attenuated after 2 hours of OGD. Notably, the structure of BMECs

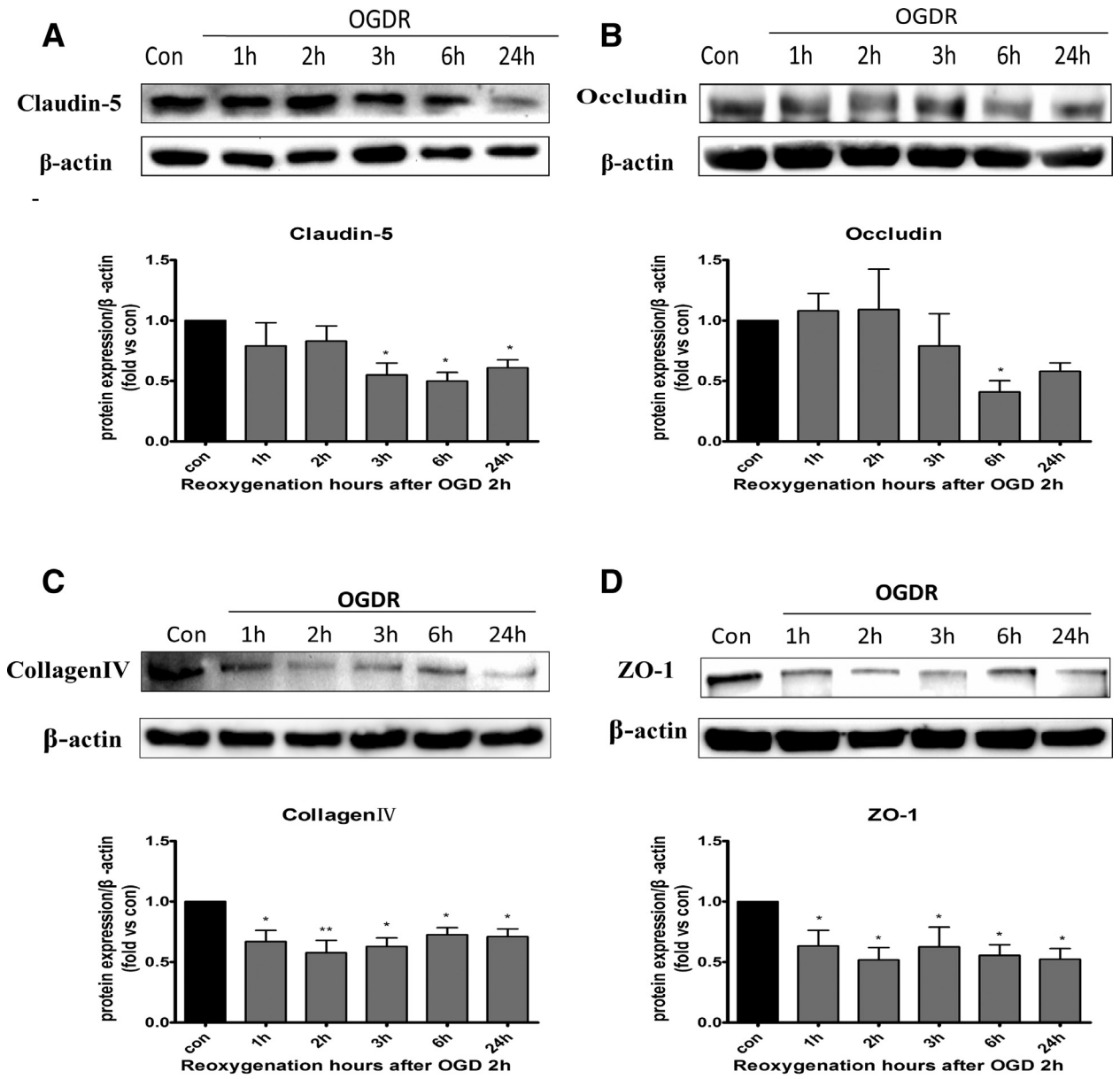

Figure 5. *Expression of TJ proteins in rat primary BMECs in response to OGDR: Abbreviations: BMECs, brain microvascular endothelia cells; OGDR, oxygen−glucose deprivation reoxygenation; ZO-1, zona occludens-1.*

was visibly destroyed after 3 hours of OGD, though it was observed by invert microscopy (not shown). Therefore, we investigated the effects of reoxygenation following 2 hours of OGD. The results showed that claudin-5, occludin, collagen-IV and ZO-1 do not recover within the study timeframe. Together, these results suggest that molecular mechanisms may play a role in the damage noted to TJs after OGDR.

MMPs are a family of zinc-dependent interstitial enzymes that can degrade constituents of the CNS. Researchers have shown that MMPs play a dual role in ischemic stroke. In the acute phase after cerebral ischemia, MMPs destroy the BBB causing BBB leakage, brain edema, and HT by downregulating basal lamina proteins (such as laminin, collagen-IV, and so on) and TJ proteins (such as occludin and claudin-5).[16,17]Subsequently, however, MMPs may remodel the neurovascular unit after the acute phase.[17] Some studies have demonstrated that MMP-2 exerts a critical role in the early degradation of the BBB after cerebral ischemia.[3] Endothelial cells are known to consecutively express and secrete MMP-2.[18] This study also showed that MMP-2 was expressed in BMECs but there was no change after OGDR. It has reported that MMP-2 was normally expressed in astrocytic end feet and increased after MCAO followed by reperfusion.[19] Stimulated astrocytes by lipopolysacchaide (LPS, a ligand of TLR2) produced activated MMP-2.[19] Some reports suggested that MMP-9 plays a key role in the subsequent

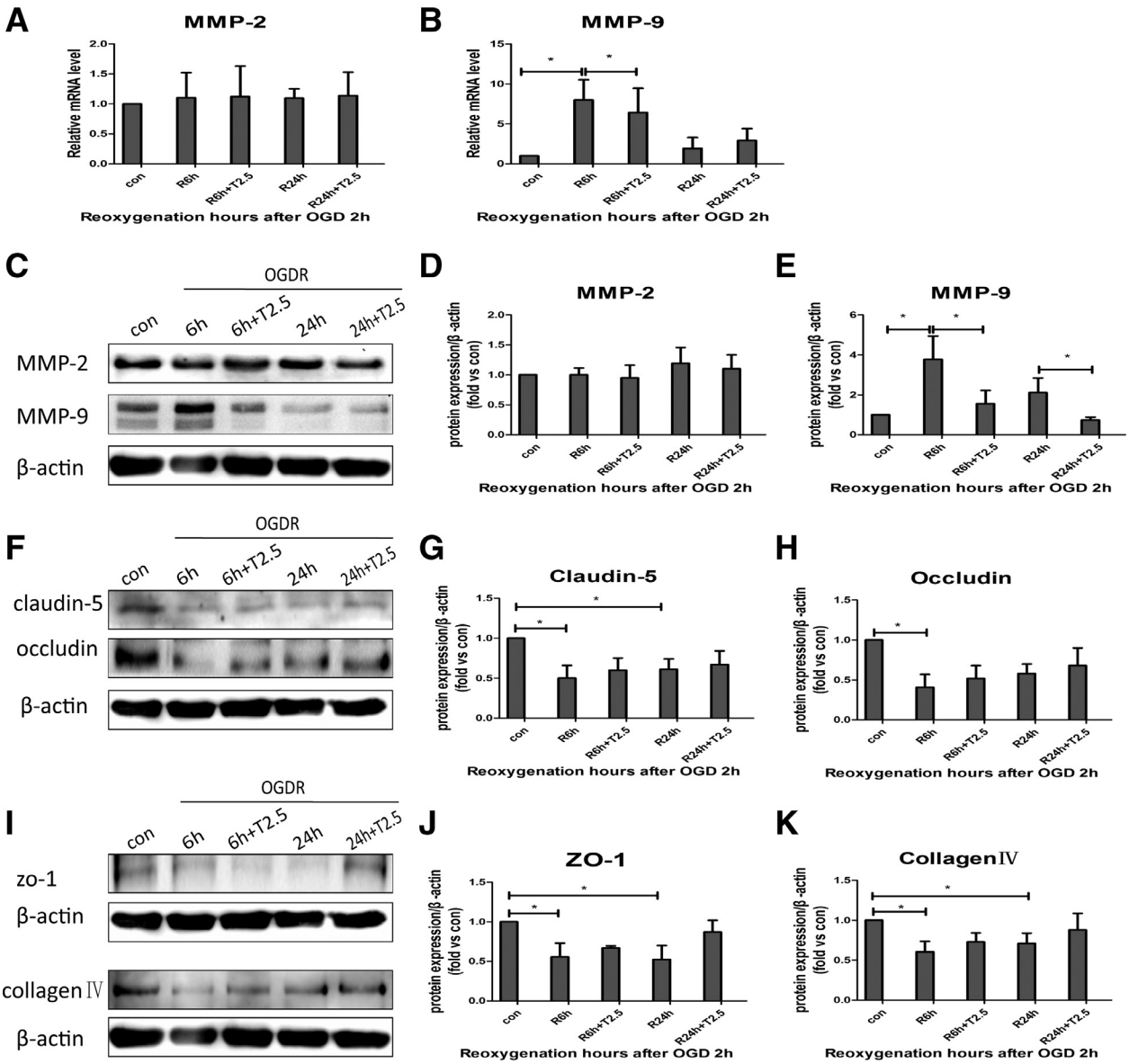

Figure 6. *Expression of TJ proteins in rat primary BMECs following OGDR and T2.5. Abbreviations: BMECs, brain microvascular endothelia cells; MMP-2, matrix metalloproteinases-2; MMP-9, matrix metalloproteinases-9; OGD, oxygen−glucose deprivation; OGDR, oxygen−glucose deprivation reoxygenation; ZO-1, zona occludens-1.*

degradation of BBB after ischemic stroke.[3] However, our data show that MMP-9 is significantly upregulated early after OGDR; these findings are consent with the notion that BMECs are the main cellular source of brain MMP-9 in the initial phase (within 24 hours) after focal cerebral ischemia.[3] It has repeatedly demonstrated that MMP-9 expression is elevated in ischemia or reperfusion in vivo.[20–23] BMEC injury may produce free radicals and proinflammatory cytokines, leading to an upregulation or activation of MMPs.[1] Recent literature has suggested that TJ proteins are substrates for MMPs.[24] Both MMP-2 and MMP-9 can degrade collagen-IV and laminin of the BBB basal lamina.[25] Activated MMP-9 can also degrade TJ proteins (claudin-5, occludin, ZO-1).[26] Therefore, we speculate that the upregulation of MMP-9 leads to damage of TJs in BMEC TJs after OGDR.

TLRs belong to transmembrane pattern-recognition receptors, which play a pivotal role in innate immunity against microbial pathogens.[5] Recent studies have reported that TLR-2 is expressed in various cells, including neurons, astroctyes, micoglia, and endothelial cells in CNS. There is differing regarding the role of TLR2 in acute cerebral ischemia. Recent reports have shown that some TLRs are involved in events following cerebral ischemia. For example, a number of studies have demonstrated that the upregulation of TLR2 in cerebral ischemia is detrimental to outcome.[6,27,28] Nevertheless, about the role of TLR2 in ischemia remains controversial.[29–31] Reductions in

neuronal death, accumulation of inflammatory cells, and decrease of the infarct volumes and brain edema have been shown with the monoclonal anti-TLR2 antibody (T2.5) after middle cerebral artery occlusion (MCAO) in mice.[27] Our study demonstrated that TLR2 was expressed in primary rat BMECs and increased after OGDR. Both MMP-9 and TLR2 were upregulated on BMECs treated with OGDR. Tissues injury in stroke released the numerous endogenous molecules, which were recognized by TLRs. It has reported that TLRs take part in the noninfectious immune-mediated diseases of CNS, such as stroke, by binding their endogenous ligands.[32] The activation of TLRs activate downstream MAPK or NFκB signaling pathway and then produced multiple cytokines, chemokines and proinflammatory cytokines,[32] which may regulate the expression or activation of MMP-2/9. T2.5 masked the ligand-binding domain in TLR2 and antagonize TLR2 function through inhibition of ligand-TLR2-complex formation.[33] So, we speculate that TLR2 may influence the structure and function of the BBB through MMP-9 regulation in BMECs. The results showed that an anti-TLR2 antibody (clone T2.5) was able to downregulate MMP-9 expression in BMECs after OGDR. Therefore, T2.5 may help to recover the integrity of TJs after OGDR. However, there were still existed in defects. Firstly, it was better provided that cells were prepared from TLR2-KO mice or to use siRNA technique in these kinds of studies. Secondly, the study lacks in vivo data, despite using primary BMECs. Subsequent research should focus on evaluating the results described in this study using an in vivo model.

Conclusions

In conclusion, OGD and OGDR result in the degradation of TJs in BMECs. TLR2 and MMP-9 were upregulated in BMECs after OGDR. Blocking TLR2 with T2.5 can downregulate MMP-9, and possibly protect TJs.

Acknowledgments

The study was supported by grants from the National Natural Science Foundation of China (Grant No. 81460188); the Applied Basic Research Project in Yunnan Province of China (No. 2011FB148); the Applied Basic Combined Special Project of the Department of Science and Technology of Yunnan Province and Kunming Medical University (2013FB201); the Young and Middle-Aged Academic and Technological Leaders and Reserved Talents of Yunnan Province of China (No. 2012HB028); the Health System Leaders of Yunnan Province of China (No. D-201235); and the Academician Wang Long-de Workstation.

References

1. Lakhan SE, Kirchgessner A, Tepper D, et al. Matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke. Front Neurol 2013;4:32.
2. Neuwelt E A, Bauer B, Fahlke C, et al. Engaging neuroscience to advance translational research in brain barrier biology. Nat Rev Neurosci 2011;12:169-182.
3. Jin R, Yang G, Li G. Molecular insights and therapeutic targets for blood-brain barrier disruption in ischemic stroke: critical role of matrix metalloproteinases and tissue-type plasminogen activator. Neurobiol Dis 2010;38:376-385.
4. Sandoval KE, Witt KA. Blood-brain barrier tight junction permeability and ischemic stroke. Neurobiol Dis 2008;32:200-219.
5. Hamanaka J, Hara H. Involvement of Toll-like receptors in ischemia-induced neuronal damage. Cent Nerv Syst Agents Med Chem 2011;11:107-113.
6. Tang SC, Arumugam TV, Xu X, et al. Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits. Proc Natl Acad Sci 2007;104:13798-13803.
7. Shichita T, Ago T, Kamouchi M, et al. Novel therapeutic strategies targeting innate immune responses and early inflammation after stroke. J Neurochem 2012;123:29-38.
8. Krizbai IA, Deli MA. Signalling pathways regulating the tight junction permeability in the blood-brain barrier. Cell Mol Biol (Noisy-le-grand) 2003;49:23-31.
9. Burek M, Salvador E, Forster CY. Generation of an immortalized murine brain microvascular endothelial cell line as an in vitro blood brain barrier model. J Vis Exp 2012;66:e4022.
10. Lv M, Liu Y, Zhang J, et al. Roles of inflammation response in microglia cell through Toll-like receptors 2/interleukin-23/interleukin-17 pathway in cerebral ischemia/reperfusion injury. Neuroscience 2011;176:162-172.
11. Donnan GA, Fisher M, Macleod M, et al. Stroke. Lancet 2008;371:1612-1623.
12. Group N I O N. Intracerebral hemorrhage after intravenous t-PA therapy for ischemic stroke. N Engl J Med 1995;333:1581-1587.
13. Clark WM, Wissman S, Albers GW, et al. Recombinant tissue-type plasminogen activator (Alteplase) for ischemic stroke 3 to 5 hours after symptom onset. The ATLANTIS Study: a randomized controlled trial. Alteplase Thrombolysis for Acute Noninterventional Therapy in Ischemic Stroke. JAMA 1999;282:2019-2026.
14. Wang X, Lee S R, Arai K, et al. Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator. Nat Med 2003;9:1313-1317.
15. Cui L, Zhang X, Yang R, et al. Neuroprotection of early and short-time applying atorvastatin in the acute phase of cerebral ischemia: down-regulated 12/15-LOX, p38MAPK and cPLA2 expression, ameliorated BBB permeability. Brain Res 2010;1325:164-173.
16. Adibhatla RM, Hatcher JF. Tissue plasminogen activator (tPA) and matrix metalloproteinases in the pathogenesis of stroke: therapeutic strategies. CNS Neurol Disord Drug Targets 2008;7:243-253.
17. Cunningham SA, Rodriguez JM, Arrate MP, et al. JAM2 interacts with alpha4beta1. Facilitation by JAM3. J Biol Chem 2002;277:27589-27592.
18. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 2003;92:827-839.
19. Rosenberg G A, Cunningham L A, Wallace J, et al. Immunohistochemistry of matrix metalloproteinases in reperfusion injury to rat brain: activation of MMP-9 linked to stromelysin-1 and microglia in cell cultures. Brain Res 2001; 893:104-112.

20. Rosenberg GA, Estrada EY, Dencoff JE. Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain. Stroke 1998;29:2189-2195.
21. Asahi M, Asahi K, Jung J C, et al. Role for matrix metalloproteinase 9 after focal cerebral ischemia: effects of gene knockout and enzyme inhibition with BB-94. J Cereb Blood Flow Metab 2000;20:1681-1689.
22. Park KP, Rosell A, Foerch C, et al. Plasma and brain matrix metalloproteinase-9 after acute focal cerebral ischemia in rats. Stroke 2009;40:2836-2842.
23. Harris AK, Ergul A, Kozak A, et al. Effect of neutrophil depletion on gelatinase expression, edema formation and hemorrhagic transformation after focal ischemic stroke. BMC Neurosci 2005;6:49.
24. Stamatovic SM, Johnson AM, Keep RF, et al. Junctional proteins of the blood-brain barrier: new insights into function and dysfunction. Tissue Barriers 2016;4:e1154641.
25. Suofu Y, Clark JF, Broderick JP, et al. Matrix metalloproteinase-2 or -9 deletions protect against hemorrhagic transformation during early stage of cerebral ischemia and reperfusion. Neuroscience 2012;212:180-189.
26. Lakhan SE, Kirchgessner A, Tepper D, et al. Matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke. Front Neurol 2013;4:32.
27. Ziegler G, Freyer D, Harhausen D, et al. Blocking TLR2 in vivo protects against accumulation of inflammatory cells and neuronal injury in experimental stroke. J Cereb Blood Flow Metab 2011;31:757-766.
28. Ziegler G, Harhausen D, Schepers C, et al. TLR2 has a detrimental role in mouse transient focal cerebral ischemia. Biochem Biophys Res Commun 2007;359:574-579.
29. Lu C, Liu L, Chen Y, et al. TLR2 ligand induces protection against cerebral ischemia/reperfusion injury via activation of phosphoinositide 3-Kinase/Akt signaling. J Immunol 2011;187:1458-1466.
30. Hua F, Ma J, Ha T, et al. Differential roles of TLR2 and TLR4 in acute focal cerebral ischemia/reperfusion injury in mice. Brain Res 2009;1262:100-108.
31. Bohacek I, Cordeau P, Lalancette-Hebert M, et al. Toll-like receptor 2 deficiency leads to delayed exacerbation of ischemic injury. J Neuroinflammation 2012;9:191.
32. Fadakar K, Dadkhahfar S, Esmaeili A, et al. The role of Toll-like receptors (TLRs) in stroke. Rev Neurosci 2014;25:699-712.
33. Meng G, Rutz M, Schiemann M, et al. Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. J Clin Invest 2004;113:1473-1481.