Published in final edited form as:

Behav Brain Res. 2019 January 01; 356: 8–17. doi:10.1016/j.bbr.2018.08.003.

Hippo/YAP signaling pathway mitigates blood-brain barrier disruption after cerebral ischemia/reperfusion injury

Pian Gong^{#a}, Zhan Zhang^{#b}, Changlin Zou^a, Qi Tian^a, Xuemei Chen^c, Michael Hong^d, Xi Liu^d, Qianxue Chen^a, Zhou Xu^a, Mingchang Li^{a,*}, and Jian Wang^{d,**}

^aDepartment of Neurosurgery, Renmin Hospital of Wuhan University, Wuhan, Hubei, 430060, PR China

^bDepartment of Respiratory Medicine, Renmin Hospital of Wuhan University, Wuhan, Hubei, 430060, PR China

^cDepartment of Anatomy, College of Basic Medical Sciences, Zhengzhou University, Zhengzhou 450000, Henan, PR China

^dDepartment of Anesthesiology and Critical Care Medicine, the Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

These authors contributed equally to this work.

Abstract

Ischemia/reperfusion (I/R) injuries commonly lead to breakdown of the blood-brain barrier (BBB). Restoration of the BBB can relieve neurologic damage caused by I/R injuries. The Hippo/YAP signaling pathway mediates cell proliferation, regulated cell death, and differentiation in various organisms and has been shown to participate in the restoration of the heart after I/R. In this study, we investigated whether the Hippo/YAP pathway plays a role in I/R injury in brain, especially in regard to I/R-induced BBB breakdown. The results of our study indicate that I/R injury led to an overall decrease in activity of the core proteins, YAP and TAZ, over a 24-h period. The most dramatic change was observed 1.5 h after reperfusion. In rats that underwent 1.5 h of reperfusion,

*Corresponding author. Mingcli@whu.edu.cn (M. Li). **Corresponding author at: Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University School of Medicine, 720 Rutland Ave, Ross Bldg 370B, Baltimore, MD 21205, USA. jwang79@jhmi.edu (J. Wang).

Authors' contributions

Mingchang Li, Pian Gong, and Zhan Zhang performed the study and wrote the paper. Pian Gong, Zhan Zhang, Changlin Zou, and Qi Tian participated in making the MCAO model and measuring neurologic deficit scores. Pian Gong, Zhan Zhang, Changlin Zou, Xuemei Chen, Xi Liu, and Michael Hong measured water content and cerebral infarct volume and performed Western blot, BBB permeability evaluation, and TUNELstaining. Pian Gong and Zhan Zhang analyzed the samples and data. Mingchang Li, Jian Wang, Qianxue Chen, Zhou Xu, Pian Gong, and Zhan Zhang conceived and designed the study. Mingchang Li, Qianxue Chen, Zhou Xu, and Jian Wang reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors have no competing interests to declare.

Availability of data and materials

They are available from the corresponding author on reasonable request during the current study.

Consent for publication

Not applicable.

Ethics approval

The protocols used were approved by the Institutional Animal Care and Use Committee of Wuhan University. All procedures adhered to the regulations specified by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All experiments were conducted to minimize the number of animals used and suffering caused.

intraperitoneal injection of YAP agonist dexamethasone activated YAP and TAZ and led to improved neurologic function, smaller brain infarct sizes, increased levels of tight junction proteins, decreased BBB permeability, decreased cerebral edema, and less apoptosis. Our results suggest that YAP exerts neuroprotective effects on the damaged brain that are likely related to restoration of the BBB.

Keywords

Signaling pathway; Ischemia/reperfusion injury; Blood-brain barrier

1. Introduction

Ischemic stroke is caused by the obstruction of blood flow to the brain and is a major cause of morbidity and mortality in adults worldwide. Thrombolytic therapy with tissue plasminogen activator (tPA) can be life-saving for patients with ischemic stroke. tPA helps to prevent the enlargement of blood clots, facilitating restoration of blood flow to the brain. However, tPA is associated with ischemic/reperfusion (I/R) injury [1,2], which aggravates ischemic dysfunction [3]. The application of tPA is associated with other complications as well, such as hemorrhagic transformation and rebleeding [4]. New therapy is needed not only to reverse stroke injury, but also to counteract the complications of I/R injury and tPA administration. Thus, it is important to identify the underlying mechanisms of I/R injury so that methods can be developed to counteract them.

Ischemic stroke involves several pathological processes, but one critical consequence is blood-brain barrier (BBB) dysfunction. Having a good knowledge of the BBB will be helpful to prevent BBB disruption after I/R injury. The BBB is a dynamic, complex structure that prevents brain damage and controls the exchange of ions and molecules between the brain and blood [5–7]. Brain microvascular endothelial cells closely interact with tight junction proteins, pericytes, astrocytic end-feet, neurons, and the extracellular matrix to form a neurovascular unit [8–10] that creates the barrier. An increase in permeability causes BBB dysfunction, which is a major mechanism of hemorrhagic transformation and I/R injury [3,11–14]. However, the concrete mechanism that underlies increased BBB permeability or BBB disruption in I/R injury is still under investigation.

The Hippo/YAP kinase cascade is a critical regulator of organ size, tissue regeneration, and tumor suppression [15–17]. The activation of the cascade induces the phosphorylation of YAP and TAZ, which are key downstream effectors and transcriptional co-activators. Phosphorylation is followed by the suppression of growth and tumorigenesis in respective organs [18,19]. YAP/TAZ activity is linked to many other signaling pathways involved in stem cell self-renewal, cell proliferation, and tumor promotion [20–23]. Studies indicate that YAP/TAZ may play an important role in maintaining the integrity and normal function of vascular endothelial cells, suggesting a potential mechanism for restoration of a damaged BBB [24,25]. The knockdown of YAP/TAZ or the administration of MnCl₂ has been shown to inhibit vascular endothelial cell proliferation via interactions with the JNK pathway. The suppression of JNK has been shown to damage the proliferative capacity of vascular

endothelial cells by decreasing the activity of YAP/TAZ [26]. The over expression of YAP may contribute to upregulation of cysteine-rich angiogenic inducer 61 (CYR61), connective tissue growth factor (CTGF), and ankyrin repeat domain 1 (ANKRD1), which would lead to inflammatory responses and the rapid multiplication of vascular endothelial cells [27]. Furthermore, YAP and TAZ are highly expressed in neural stem cells [28], and the knockdown of YAP/TAZ causes damage to astrocytes [29], suggesting that YAP/TAZ might influence neurons and offer protection against I/R injury-induced BBB disruption.

In our study, we show for the first time that stimulating YAP/TAZ can prevent the BBB damage caused by I/R injury. We also explored the mechanism by which the Hippo/YAP signaling pathway influences BBB disruption using YAP agonist dexamethasone (DXM) [30].

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats, weighing 250–280 g, were purchased from the Wuhan University Center for Animal Experiments and housed under controlled conditions (5 rats per cage, 22–25 °C, 50–60% relative humidity, 12-h light/dark cycle) with free access to water and food. The rats were allowed to adjust to the environment for 3–5 days before the operation, and their weights were properly controlled. The protocols used were approved by the Institutional Animal Care and Use Committee of Wuhan University. All procedures adhered to the regulations specified by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All efforts were made to minimize the number of rats used, as well as their suffering and mortality. The investigators were blinded to the treatment status of the animals during all surgical procedures.

2.2. I/R injury model

Transient cerebral ischemia was induced by endovascular occlusion of the left middle cerebral artery (MCA) as previously reported [11,31,32]. The rats were placed in the supine position and anesthetized with the proper concentration of isoflurane (approximately 3% in 100% O₂) via a face mask. An incision of approximately 15 mm was made in the middle of the neck, and the left carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were carefully isolated. The distal end of the ECA was ligated with 4-0 silk, and the ICA and left CCA were blocked by attaching two arterial clips. After the ICA clip was removed, a silicone-coated nylon monofilament (Sunbio Biotech, Beijing, China) was gently inserted via a bevel incision in the ECA to a point approximately 18.5 ± 0.5 mm from the bifurcation of the CCA, where a resistance was felt. After fixation of the monofilament, the other clip was removed. Finally, the skin was sutured. Consequently, ischemia of the MCA was induced in the relative area of the surgery. After 1.5 h, the monofilament was withdrawn, causing reperfusion. Sham-operated rats underwent surgery but without occlusion of the MCA. Rats that underwent I/R were housed at a constant 25 °C (temperature was maintained by an electric heater).

MCA occlusion (MCAO) was maintained in all rats of the I/R groups for 1.5 h. After surgery, the rats were returned to their cages (2 rats per cage). Subgroups of rats were sacrificed at 1.5 h after the reperfusion (I/R 3-h group), 4.5 h after reperfusion (I/R 6-h group), 10.5 h after reperfusion (I/R 12-h group), and 22.5 h after reperfusion (I/R 24-h group). Any rat with subarachnoid hemorrhage was excluded.

2.3. Animal groups

Two experiments were performed. In the first, we explored whether the Hippo/YAP signaling pathway is involved in BBB breakdown and its fluctuations. The rats were randomly [33] divided into the following five groups: sham group (sacrificed at 24 h after surgery), I/R 3-h group, I/R 6-h group, I/R 12-h group, and I/R 24-h group. In the second experiment, we examined whether the Hippo/YAP signaling pathway attenuates BBB disruption induced by I/R injury. The rats were divided into the following four groups: sham group (sacrificed at 3 h after surgery), I/R 3-h group, I/R 3-h + vehicle group (injected intraperitoneally with 50 ml/kg of saline simultaneously with the reperfusion), and I/R 3-h + DXM group (injected intraperitoneally with 50 mg/kg DXM (Selleck) dissolved in saline simultaneously with the reperfusion).

2.4. Neurologic deficit score

An investigator blind to the experimental groups assessed the rats using a previously published neurologic scoring system [34,35] as follows: 0, no deficits; 1, difficulty in fully extending the contralateral forelimb; 2, inability to extend the contralateral forelimb; 3, mild circling toward the contralateral side; 4, severe circling; and 5, falling onto the contralateral side. A higher score indicates a more critical I/R injury.

2.5. Brain water content

The brain water content after I/R injury was measured with the standard wet weight/dry weight method [36,37]. Each rat was anesthetized in a box filled with 5% isoflurane and 95% O₂, and the brain was extracted as quickly as possible. Each brain was divided into the ipsilateral and contralateral hemispheres. The hemispheres were weighed, and their respective wet weights were recorded (each brain thus had two wet weights). Then, the hemispheres were wrapped in pre-weighed aluminum foil and placed in an incubator at 110 °C for 24 h before the dry weights were measured. The brain water content was calculated as a percentage using the Billiot formula as follows: (wet weight — dry weight)/wet weight × 100% [38].

2.6. Cerebral infarct volume

The cerebral infarct area was determined by staining the brain with 2% 2,3,5-triphenyltetrazoliumchloride (TTC, Sigma, St. Louis, MO, USA; dissolved in phosphate-buffered saline [PBS] at 37 °C) [39,40]. The rats were anesthetized in a box filled with 5% isoflurane and 95% O₂. The brains were collected and stored at —20 °C for 10–20 min. A 2-mm section of the frontal pole was discarded, and the remaining brain tissue was sliced into eight coronal sections approximately 2 mm in thickness. Each section was placed into 2% TTC and stained at 37 °C for 30 min in the dark. Finally, the sections were fixed in 4% ice-

cold paraformaldehyde. Normal tissue stained red, while the infarct area remained pale gray. The stained tissues were imaged and analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA). The infarct volume percentage was calculated as the infarct area of the ipsilateral hemisphere/total area of ipsilateral hemisphere $\times$ 100% [41].

2.7. Western blotting

Using our established protocol [42], we extracted all proteins from the core ischemic and control areas in the cortex using RIPA lysis buffer containing phenylmethylsulfonyl fluoride (Thermo Fisher Scientific). Total protein concentration was analyzed by using a Cytoplasmic and Nuclear Protein Extraction Kit (Feremants, Shanghai, China). The proteins (20 μ g/lane) were separated by tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% (w/v) nonfat dry milk in TBST (Tris-buffered saline-0.1% Tween) at 25 °C for 3 h and then incubated with the following primary antibodies: (1) rabbit polyclonal anti-GAPDH antibody (1:2000, Santa Cruz Biotechnology, Dallas, TX, USA, 10494-1-AP); (2) rabbit polyclonal anti-p-actin antibody (1:2000, Abcam, Cambridge, MA, USA, ab8227); (3) rabbit polyclonal anti-YAP antibody (1:1000, Abcam, ab76252); (4) rabbit monoclonal anti-TAZ antibody (1:1000, Abcam, ab84927); (5) rabbit polyclonal anti-ZO-1 antibody (1:100, Santa Cruz Biotechnology, sc-10804); and (6) rabbit polyclonal anti-Claudin-3 antibody (1:100, Abcam, ab15102). The membranes were all incubated overnight at 4 °C, washed with 1 XTBST (3 washes, 15 min per wash), and then incubated with a secondary goat anti-rabbit anti-body (1:1000, Abcam, ab6721) for 2 h at room temperature. The membranes were washed three times after the secondary incubation. The protein electrophoresis bands were imaged on an Odyssey infrared scanner (LICOR Bioscience, Lincoln, NE, USA) and analyzed by using Image J software.

2.8. BBB permeability evaluation

Evans blue (EB; 2%, 3 ml/kg, Sigma) extravasation was used to reveal the BBB permeability [43]. The rats in the I/R 3-h group, I/R 3-h + vehicle group, and I/R 3-h + DXM group were administered 2% EB intravenously at a rate of 0.1 ml/min simultaneously with the reperfusion and then sacrificed 1.5 h later. The animals in the sham group also were administered 2% EB (0.1 ml/min) 1.5 h before being sacrificed. All rats were perfused with 300 ml of saline at 4 °C immediately before decapitation. The brains were collected, dissected into two hemispheres, weighed, and homogenized with 4 ml of 50% trichloroacetic acid solution. The tissue was centrifuged for 30 min at 14,000 rpm and then diluted with ethanol (1:3). The absorption of the BBB was measured at 632 nm on a spectrophotometer (Epoch&Take3, Biotek, Winooski, VT, USA) by an investigator blinded to group, and the EB extravasation index (EBI) was calculated. The EBI represents the ratio of the EB intensity of the I/R hemisphere to that of the other hemisphere based on a standardized curve.

2.9. TUNEL assay

We used terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) to detect apoptotic cells according to the manufacturer's instructions (in situ cell death detection kit POD, Boehringer, Germany). The stained tissue sections were analyzed under a

microscope by an investigator blinded to group. The number of positive cells was counted in 10 microscopic fields at $200\times$ magnification.

2.10. Statistical analysis

The data were analyzed with the SPSS for Windows 20.0 software package and GraphPad Prism Version 6.0 software (GraphPad Software Inc., La Jolla, CA, USA) and are expressed as means $\pm$ SD. The images were analyzed by Image J software. Differences among groups were determined by performing a one-way ANOVA, followed by Turkey *post-hoc* tests. Two-tailed *t* tests were used for comparisons between two groups. A two-tailed *p* value < 0.05 was considered statistically significant.

3. Results

3.1. Changes to the Hippo/YAP signaling pathway after I/R injury

The levels of both YAP and TAZ were lower in the rats that underwent MCAO than in sham-operated rats. Expression was lowest in rats from the I/R 3-h group ($n = 8$ rats per group, $p < 0.05$; Fig. 1A-D). The expression of YAP and TAZ in sham-operated rats was twice that of the I/R 3-h group. BBB breakdown was indicated by dramatic reductions in ZO-1 (Fig. 1A and E) and Claudin-3 (Fig. 1A and F). The expression level of ZO-1 and Claudin-3 in I/R groups was approximately 40% of that in the sham group. Because expression of the effectors in that pathway (i.e., YAP and TAZ) changed most in the I/R 3-h group, we went on to compare neurologic deficit score, water content, infarct volume, and apoptosis between the I/R 3-h group and the other I/R groups. The neurologic deficit scores and water content were higher in the I/R 3-h group than in the other three I/R groups; however, the difference was not statistically significant ($p > 0.05$; Fig. 2A and B). The sham group had a water content of 81.0%. However, water content was 82.5% in the I/R 3-h, 82.0% in the I/R 6-h group, 81.9% in the I/R 12-h group, and 81.3% in the I/R 24-h group. The infarct volume in the I/R 3-h group also appeared to be larger than that in the other I/R groups ($p > 0.05$; Fig. 2C and D). The infarct volumes in the I/R 3-h, I/R 6-h, I/R 12-h, and I/R 24-h groups were 25.8%, 22.3%, 23.0%, and 22.2%, respectively. The percentage of apoptotic cells in the I/R 3-h group (42%) was greater than that in the I/R 6-h (33%), I/R 12-h (34%), and I/R 24-h (35%) groups ($p < 0.05$; Fig. 3A and B). No neurologic deficiencies or ischemic areas were observed in the sham group.

Because differences in the Hippo/YAP signaling pathway were greatest at 3 h after the MCAO surgery, we selected the I/R 3-h group to further explore whether stimulation of the Hippo/YAP signaling pathway could lead to BBB restoration after I/R injury.

3.2. Activation of YAP or TAZ by DXM reduced MCAO-induced neurologic deficits

Our primary hypothesis was that a higher level of YAP or TAZ could improve neurologic function in rats that had undergone MCAO. Compared to rats in the I/R 3-h and I/R 3-h + vehicle groups, rats in the I/R 3-h + DXM group had markedly increased YAP and TAZ activation and significantly lower neurologic deficit scores ($n = 5$ rats per group, $p < 0.05$; Fig. 4A).

3.3. Activation of YAP or TAZ by DXM alleviated MCAO-induced brain edema

We measured hemispheric water content as an indicator of brain edema. Our results showed that brain water content was 79.2% in the sham group, 82.2% in the I/R 3-h group, 82.0% in the I/R 3-h + vehicle group, and 80.3% in the I/R 3-h + DXM group. The swelling associated with cerebral edema was lower in animals of the I/R 3-h + DXM group than in those of the other untreated or vehicle-treated I/R 3-h groups ($n = 6$ rats per group, $p < 0.05$; Fig. 4B). However, no difference was observed between the I/R 3-h group and the I/R 3-h + vehicle group ($p > 0.05$; Fig. 4B), indicating that restoration of BBB integrity occurs only after YAP activation. Thus, YAP or TAZ activation protected the brain in part by reducing cerebral edema after I/R injury.

3.4. Activation of YAP or TAZ by DXM reduced infarction volume after I/R injury

Our data indicated that DXM-mediated YAP regulation reduced infarct volume. Infarct volume percentage decreased from 25.5% and 25.8% in the I/R 3-h group and I/R 3-h + vehicle group, respectively, to 16.0% in the DXM-treated group ($n = 8$ rats per group, $p < 0.05$; Fig. 4C and D). No infarction was detected in the sham group.

3.5. Activation of YAP or TAZ preserved tight junction integrity after I/R injury

Damage to the BBB after I/R injury reduces elements such as endothelial cells, tight junction proteins, pericytes, astrocytic end-feet, and neurons, which are necessary for BBB integrity, as reflected by a decline in the expression of ZO-1 and Claudin-3 in the I/R groups. To examine whether the Hippo/YAP signaling pathway plays a role in regulating the molecules that maintain BBB integrity after I/R injury, we measured the levels of the tight junction proteins ZO-1 and Claudin-3. Western blot analysis showed that the expression levels of ZO-1 and Claudin-3 were upregulated in the DXM-treated I/R 3-h group compared to those in the untreated groups ($n = 8$ rats per group, $p < 0.05$; Fig. 5A-F). Thus, the Hippo/YAP signaling pathway was involved in normal BBB integrity, and this pathway could be a potential therapeutic target for accelerated recovery from I/R injury.

3.6. Stimulation of YAP or TAZ reduces I/R injury-induced BBB permeability

Extravasated EB in brain samples was significantly increased by I/R injury but mitigated by the administration of DXM ($n = 8$ rats per group, $p < 0.05$; Fig. 6A and B).

3.7. Stimulation of YAP or TAZ repressed apoptosis in the brain after I/R injury

The I/R 3-h group, I/R 3-h + vehicle group, and I/R 3-h + DXM group exhibited apoptotic cell percentages of 42.3%, 41.4%, and 28.5%, respectively. No significant difference in the percentage of TUNEL-positive cells was observed between the I/R 3-h group and the I/R 3-h + vehicle group ($n = 5$ rats per group, $p > 0.05$; Fig. 7A and B). Administration of DXM reduced the percentage of TUNEL-positive cells.

3.8. Mortality rates

The mortality rate was 2.85% (1/35) in the sham group (mostly due to individual differences among the rats), 11.42% (4/35) in the I/R 3-h + vehicle group, 8.57% (3/35) in the I/R 3-h

group, and 5.71% (2/35) in the I/R 3 h + DXM group. The mortality rate did not differ significantly among the four groups ($p > 0.05$).

4. Discussion

In our study, we hypothesized that the Hippo/YAP signaling pathway is linked to I/R injury. In support of this hypothesis, we found that the expression of YAP and TAZ is significantly reduced after MCAO-induced I/R injury but partially restored by treatment with DXM. YAP/TAZ activator DXM also reduced I/R-associated neurologic deficits, brain edema, and BBB breakdown.

To our knowledge, this study is the first to show that I/R injury alters YAP and TAZ and that activation of these proteins has a protective effect on the BBB in time-dependent MCAO models. The expression of the core pathway molecules YAP and TAZ decreased after experimental I/R injury, and the magnitude of the decrease was related to the length of the reperfusion process. The levels were lowest at 1.5 h after reperfusion, were slightly increased at 4.5 h, and plateaued at 6 h. Therefore, we used animals in the 1.5-h reperfusion group to elucidate the precise effect of the Hippo/YAP signaling pathway on BBB damage after I/R injury. BBB disruption, including vasogenic edema, has been shown to be closely related to the clinical symptoms present during the first 3 h after I/R [44]. We decided to determine whether the brain, specifically the BBB, could be protected by interrupting the signal pathway. Thus, DXM was applied to stimulate the expression of YAP or TAZ. As hypothesized, the application of DXM improved neurologic function, decreased brain infarct size, increased the levels of tight junction proteins, reduced BBB permeability and cerebral edema, and lessened apoptosis.

Recanalization therapy is the most effective method for treating ischemic stroke, which is inevitably followed by BBB disruption that can aggravate I/R injury. Injury to the BBB allows blood-borne cells, chemicals, and fluids to flow into the brain parenchyma [10], which may lead to complications, such as inflammatory responses and cerebral edema. For many years, researchers have attempted to discover approaches for restoring the BBB. One approach to maintain BBB integrity and protect it from multiple brain disorders is to increase the levels of mainly tight junction proteins and other related molecules or cascades [37]. However, whether this approach will repair the BBB is unclear. Therefore, new therapeutic targets for BBB recovery are needed.

The Hippo/YAP signaling pathway has been linked to the pathophysiologic processes of many ischemic diseases. A recent study revealed that Park2, which is a target gene of YAP, is essential for survival in human ischemic heart failure because of its roles in cardiomyocyte renewal and the recycling of damaged mitochondria [37,45]. Moreover, higher expression of the YAP target genes has been shown to contribute to the protection against I/R injury that is linked to heart failure in vivo [46]. YAP was also reported to be highly involved in the regeneration of neural stem cells, the proliferation of neural progenitor cells, and the differentiation, activation, and myelination of glial cells [47,48], indicating that YAP has underlying effects on diverse neurologic disorders. The Hippo/YAP signaling pathway closely interacts with the Wnt, Notch, Hedgehog, JAK/STAT, mitogen-activated protein

kinase, phosphoinositide-3-kinase-Akt, and nuclear factor κ B signaling pathways [49]. Of these, the Wnt, Notch, Hedgehog, and JAK/STAT pathways have been shown to exert active and dynamic influences on BBB; for example, the JAK/STAT pathway protects the BBB by inhibiting matrix metalloproteinase-3 released by astrocytes in I/R injury [50–54].

Altogether, these pathways influence cell proliferation and differentiation, tissue development, regeneration, and tumorigenesis and are involved in BBB disruption during I/R injuries.

In addition to the possible involvement of YAP/TAZ in BBB disruption after I/R injury, our study also showed that the Hippo/YAP signaling pathway may affect the injured brain mainly by protecting against BBB disruption. By activating YAP/TAZ, the modified tight junction proteins markedly decreased BBB permeability and relieved edema. The activation also repressed apoptosis in the damaged brain, indicating that cells of the BBB might be protected by YAP or TAZ activation. However, concrete target mechanisms are unknown and should be explored in the future. Additional studies also are needed to investigate the potential effects of YAP/TAZ on BBB breakdown in I/R injury and other neurologic disorders.

5. Conclusions

In conclusion, our study indicates for the first time that the Hippo/YAP signaling pathway reduces I/R injury in the brain, suggesting its potential to influence the prognosis of many types of neurologic disorders. Future studies are required to elucidate the specific roles of the pathway in underlying disorders. Our experiments revealed that DXM increased tight junction proteins and activated YAP, which provides protection against BBB disruption. However, the precise mechanism by which YAP interacts with the BBB in I/R injuries remains unclear. We believe that YAP/TAZ or perhaps the entire pathway has the ability to act as a therapeutic target for the treatment of I/R injury and other neurologic disorders.

Acknowledgements

We thank Claire Levine, MS, ELS, for assistance with manuscript preparation. We thank the Wang lab team members for insightful input.

Funding

This study was supported by grants from National Natural Science Foundation of China (81171112, 81371272 to M.C.L.). JW is supported by a Stimulating and Advancing ACCM Research (StAAR) award from the Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University.

Abbreviations:

BBB	blood-brain barrier
DXM	dexamethasone
EB	evansblue
EBI	evans blue extravasation index
I/R	ischemia/reperfusion

MCAO	middle cerebral artery occlusion
TTC	2,3,5-triphenyltetrazolium chloride
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling
ZO-1	cytoplasmic zonula occludens-1

References

- [1]. Francis JW, Ren J, Warren L, Brown RJ, Finklestein SP, Postischemic infusion of Cu/Zn superoxide dismutase or SOD:Tet451 reduces cerebral infarction following focal ischemia/reperfusion in rats, *Exp. Neurol* 146 (2) (1997) 435–443. [PubMed: 9270054]
- [2]. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, Logan A, Nadtochiy SM, Ord E, Smith AC, Eyassu F, Shirley R, Hu CH, Dare AJ, James AM, Rogatti S, Hartley RC, Eaton S, Costa A, Brookes PS, Davidson SM, Duchon MR, Saeb-Parsy K, Shattock MJ, Robinson AJ, Work LM, Frezza C, Krieg T, Murphy MP, Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS, *Nature* 515 (7527) (2014) 431–435. [PubMed: 25383517]
- [3]. Sifat AE Vaidya B, Abbruscato TJ, Blood-brain barrier protection as a therapeutic strategy for acute ischemic stroke, *AAPS J.* 19 (4) (2017) 957–972. [PubMed: 28484963]
- [4]. Takenaka K, Kato M, Yamauti K, Hayashi K, Simultaneous administration of re-combinant tissue plasminogen activator and edaravone in acute cerebral ischemic stroke patients, *J. Stroke Cerebrovasc. Dis* 23 (10) (2014) 2748–2752. [PubMed: 25307430]
- [5]. Zhang W, Zhang H, Mu H, Zhu W, Jiang X, Hu X, Shi Y, Leak RK, Dong Q, Chen J, Gao Y, Omega-3 polyunsaturated fatty acids mitigate blood-brain barrier disruption after hypoxic-ischemic brain injury, *Neurobiol. Dis* 91 (2016) 37–46. [PubMed: 26921472]
- [6]. Haddad-Tovoli R, Dragano N, Ramalho A, Velloso LA, Development and function of the blood-brain barrier in the context of metabolic control, *Front. Neurosci* 11 (2017) 224. [PubMed: 28484368]
- [7]. Theodorakis PE, Muller EA, Craster RV, Matar OK, Physical insights into the blood-brain barrier translocation mechanisms, *Phys. Biol* 14 (4) (2017) 041001. [PubMed: 28586313]
- [8]. Abbott NJ, Ronnback L, Hansson E, Astrocyte-endothelial interactions at the blood-brain barrier, *Nat. Rev. Neurosci* 7 (1) (2006) 41–53. [PubMed: 16371949]
- [9]. Zlokovic BV, The blood-brain barrier in health and chronic neurodegenerative disorders, *Neuron* 57 (2) (2008) 178–201. [PubMed: 18215617]
- [10]. Keaney J, Campbell M, The dynamic blood-brain barrier, *FEBS J.* 282 (21) (2015) 4067–4079. [PubMed: 26277326]
- [11]. Li M, Zhang Z, Sun W, Koehler RC, Huang J, 17beta-estradiol attenuates breakdown of blood-brain barrier and hemorrhagic transformation induced by tissue plasminogen activator in cerebral ischemia, *Neurobiol. Dis* 44 (3) (2011) 277–283. [PubMed: 21816222]
- [12]. Wang W, Li M, Chen Q, Wang J, Hemorrhagic transformation after tissue plasminogen activator reperfusion therapy for ischemic stroke: mechanisms, models, and biomarkers, *Mol. Neurobiol* 52 (3) (2015) 1572–1579. [PubMed: 25367883]
- [13]. Wang W, Li M, Wang Y, Li Q, Deng G, Wan J, Yang Q, Chen Q, Wang J, GSK-3beta inhibitor TWS119 attenuates rtPA-induced hemorrhagic transformation and activates the Wnt/beta-catenin signaling pathway after acute ischemic stroke in rats, *Mol. Neurobiol* 53 (10) (2016) 7028–7036. [PubMed: 26671619]
- [14]. Krueger M, Hartig W, Frydrychowicz C, Mueller WC, Reichenbach A, Bechmann I, Michalski D, Stroke-induced blood-brain barrier breakdown along the vascular tree-no preferential affection of arteries in different animal models and in humans, *J. Cereb. Blood Flow Metab* 37 (7) (2017) 2539–2554. [PubMed: 27683449]

- [15]. Zhao B, Tumaneng K, Guan KL, The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal, *Nat. Cell Biol* 13 (8) (2011) 877–883. [PubMed: 21808241]
- [16]. Johnson R, Haider G, The two faces of Hippo: targeting the Hippo pathway for regenerative medicine and cancer treatment, *Nat. Rev. Drug Discov* 13 (1) (2014) 63–79. [PubMed: 24336504]
- [17]. Yu FX, Zhao B, Guan KL, Hippo pathway in organ size control, tissue homeostasis, and cancer, *Cell* 163 (4) (2015) 811–828. [PubMed: 26544935]
- [18]. Zhu C, Li L, Zhao B, The regulation and function of YAP transcription co-activator, *Acta Biochim. Biophys. Sin. (Shanghai)* 47 (1) (2015) 16–28. [PubMed: 25487920]
- [19]. Ferraiuolo M, Verduci L, Blandino G, Strano S, Mutant p53 protein and the hippo transducers YAP and TAZ: a critical oncogenic node in human cancers, *Int. J. Mol. Sci* 18 (5) (2017).
- [20]. Chen L, Loh PG, Song H, Structural and functional insights into the TEAD-YAP complex in the hippo signaling pathway, *Protein Cell* 1 (12) (2010) 1073–1083. [PubMed: 21213102]
- [21]. Haider G, Dupont S, Piccolo S, Transduction of mechanical and cytoskeletal cues by YAP and TAZ, *Nat. Rev. Mol. Cell Biol* 13 (9) (2012) 591–600. [PubMed: 22895435]
- [22]. Fischer M, Rikeit P, Knaus P, Coirault C, YAP-mediated mechanotransduction in skeletal muscle, *Front. Physiol* 7 (2016) 41. [PubMed: 26909043]
- [23]. Wang J, Martin JF, Hippo pathway: an emerging regulator of craniofacial and dental development, *J. Dent. Res* 96 (11) (2017) 1229–1237. [PubMed: 28700256]
- [24]. Choi HJ, Kwon YG, Roles of YAP in mediating endothelial cell junctional stability and vascular remodeling, *BMB Rep.* 48 (8) (2015) 429–430. [PubMed: 26169195]
- [25]. Choi HJ, Zhang H, Park H, Choi KS, Lee HW, Agrawal V, Kim YM, Kwon YG, Yes-associated protein regulates endothelial cell contact-mediated expression of angiopoietin-2, *Nat. Commun* 6 (2015) 6943. [PubMed: 25962877]
- [26]. Wang L, Luo JY, Li B, Tian XY, Chen LJ, Huang Y, Liu J, Deng D, Lau CW, Wan S, Ai D, Mak KK, Tong KK, Kwan KM, Wang N, Chiu JJ, Zhu Y, Huang Y, Integrin-YAP/TAZ-JNK cascade mediates atheroprotective effect of unidirectional shear flow, *Nature* (2016).
- [27]. Wang KC, Yeh YT, Nguyen P, Limqueco E, Lopez J, Thorossian S, Guan KL, Li YJ, Chien S, Flow-dependent YAP/TAZ activities regulate endothelial phenol-types and atherosclerosis, *Proc. Natl Acad. Sci. U. S. A* 113 (41) (2016) 11525–11530. [PubMed: 27671657]
- [28]. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA, “Sternness”: transcriptional profiling of embryonic and adult stem cells, *Science* 298 (5593) (2002) 597–600. [PubMed: 12228720]
- [29]. Huang Z, Wang Y, Hu G, Zhou J, Mei L, Xiong WC, YAP Is a critical inducer of SOCS3, preventing reactive astrogliosis, *Cereb. Cortex* 26 (5) (2016) 2299–2310. [PubMed: 26679195]
- [30]. Sorrentino G, Ruggeri N, Zannini A, Ingallina E, Bertolio R, Marotta C, Neri C, Cappuzzello E, Forcato M, Rosato A, Mano M, Biccato S, Del SG, Glucocorticoid receptor signalling activates YAP in breast cancer, *Nat. Commun* 8 (2017) 14073. [PubMed: 28102225]
- [31]. Li M, Feng B, Wang L, Guo S, Zhang P, Gong J, Zhang Y, Zheng A, Li H, Tollip is a critical mediator of cerebral ischaemia-reperfusion injury, *J. Pathol* 237 (2) (2015) 249–262. [PubMed: 26011492]
- [32]. Wang M, Hong X, Chang CF, Li Q, Ma B, Zhang H, Xiang S, Heo HY, Zhang Y, Lee DH, Jiang S, Leigh R, Koehler RC, van Zijl PC, Wang J, Zhou J, Simultaneous detection and separation of hyperacute intracerebral hemorrhage and cerebral ischemia using amide proton transfer MRI, *Magn. Reson. Med* 74 (2015) 42–50. [PubMed: 25879165]
- [33]. Cheng T, Wang W, Li Q, Han X, Xing J, Qi C, Lan X, Wan J, Potts A, Guan F, Wang J, Cerebroprotection of flavanol (–)-epicatechin after traumatic brain injury via Nrf2-dependent and -independent pathways, *Free Radic. Biol. Med* 92 (2016) 15–28. [PubMed: 26724590]
- [34]. Pan LN, Zhu W, Li Y, Xu XL, Guo LJ, Lu Q, Wang J, Astrocytic Toll-like receptor 3 is associated with ischemic preconditioning-induced protection against brain ischemia in rodents, *PLoS One* 9 (6) (2014) e99526. [PubMed: 24914679]
- [35]. Wang J, Yu L, Jiang C, Chen M, Ou C, Wang J, Bone marrow mononuclear cells exert long-term neuroprotection in a rat model of ischemic stroke by promoting arteriogenesis and angiogenesis, *Brain Behav. Immun* 34 (2013) 56–66. [PubMed: 23891963]

- [36]. Jiang C, Zuo F, Wang Y, Wan J, Yang Z, Lu H, Chen W, Zang W, Yang Q, Wang J, Progesterone exerts neuroprotective effects and improves long-term neurologic outcome after intracerebral hemorrhage in middle-aged mice, *Neurobiol. Aging* 42 (2016) 13–24. [PubMed: 27143417]
- [37]. Jiang X, Andjelkovic AV, Zhu L, Yang T, Bennett M, Chen J, Keep RF, Shi Y, Blood-brain barrier dysfunction and recovery after ischemic stroke, *Prog. Neurobiol.* (2017).
- [38]. Zhang Z, Song Y, Zhang Z, Li D, Zhu H, Liang R, Gu Y, Pang Y, Qi J, Wu H, Wang J, Distinct role of heme oxygenase-1 in early- and late-stage intracerebral hemorrhage in 12-month-old mice, *J. Cereb. Blood Flow Metab* 37 (1) (2017) 25–38. [PubMed: 27317654]
- [39]. Li Q, Han X, Lan X, Hong X, Li Q, Gao Y, Luo T, Yang Q, Koehler RC, Zhai Y, Zhou J, Wang J, Inhibition of tPA-induced hemorrhagic transformation involves adenosine A2b receptor activation after cerebral ischemia, *Neurobiol. Dis* 108 (2017) 173–182. [PubMed: 28830843]
- [40]. Jiang C, Wang J, Yu L, Ou C, Liu X, Zhao X, Wang J, Comparison of the therapeutic effects of bone marrow mononuclear cells and microglia for permanent cerebral ischemia, *Behav. Brain Res* 250 (2013) 222–229. [PubMed: 23685323]
- [41]. Wang J, Liu X, Lu H, Jiang C, Cui X, Yu L, Fu X, Li Q, Wang J, CXCR4(+)CD45(–) BMMNC subpopulation is superior to unfractionated BMMNCs for protection after ischemic stroke in mice, *Brain Behav. Immun* 45 (2015) 98–108. [PubMed: 25526817]
- [42]. Lan X, Han X, Li Q, Wang J, (–)-Epicatechin, a natural flavonoid compound, protects astrocytes against hemoglobin toxicity via Nrf2 and AP-1 signaling pathways, *Mol. Neurobiol* 54 (10) (2017) 7898–7907. [PubMed: 27864733]
- [43]. Yang J, Li Q, Wang Z, Qi C, Han X, Lan X, Wan J, Wang W, Zhao X, Hou Z, Gao C, Carhuapoma JR, Mori S, Zhang J, Wang J, Multimodality MRI assessment of grey and white matter injury and blood-brain barrier disruption after intracerebral hemorrhage in mice, *Sci. Rep* 7 (2017) 40358. [PubMed: 28084426]
- [44]. Giraud M, Cho TH, Nighoghossian N, Maucourt-Boulch D, Deiana G, Ostergaard L, Baron JC, Fiehler J, Pedraza S, Derex L, Berthezene Y, Early blood brain barrier changes in acute ischemic stroke: a sequential MRI study, *J. Neuroimaging* 25 (6) (2015) 959–963. [PubMed: 25702824]
- [45]. Leach JP, Heallen T, Zhang M, Rahmani M, Morikawa Y, Hill MC, Segura A, Willerson JT, Martin JF, Hippo pathway deficiency reverses systolic heart failure after infarction, *Nature* 550 (7675) (2017) 260–264. [PubMed: 28976966]
- [46]. Matsuda T, Zhai P, Sciarretta S, Zhang Y, Jeong JI, Ikeda S, Park J, Hsu CP, Tian B, Pan D, Sadoshima J, Del RD, NF2 activates hippo signaling and promotes Ischemia/Reperfusion injury in the heart, *Circ. Res* 119 (5) (2016) 596–606. [PubMed: 27402866]
- [47]. Poon CL, Mitchell KA, Kondo S, Cheng LY, Harvey KF, The hippo pathway regulates neuroblasts and brain size in drosophila melanogaster, *Curr. Biol* 26 (8) (2016) 1034–1042. [PubMed: 26996505]
- [48]. Mindos T, Dun XP, North K, Doddrell RD, Schulz A, Edwards P, Russell J, Gray B, Roberts SL, Shivane A, Mortimer G, Pirie M, Zhang N, Pan D, Morrison H, Parkinson DB, Merlin controls the repair capacity of Schwann cells after injury by regulating Hippo/YAP activity, *J. Cell Biol* 216 (2) (2017) 495–510. [PubMed: 28137778]
- [49]. Luo K, Signaling cross talk between TGF-beta/smad and other signaling pathways, *Cold Spring Harb. Perspect. Biol* 9 (1) (2017).
- [50]. Phng LK, Potente M, Leslie JD, Babbage J, Nyqvist D, Lobov I, Ondr JK, Rao S, Lang RA, Thurston G, Gerhardt H, Nrp coordinates endothelial notch and Wnt signaling to control vessel density in angiogenesis, *Dev. Cell* 16 (1) (2009) 70–82. [PubMed: 19154719]
- [51]. Zhang J, Fukuhara S, Sako K, Takenouchi T, Kitani H, Kume T, Koh GY, Mochizuki N, Angiopoietin-1/Tie2 signal augments basal notch signal controlling vascular quiescence by inducing delta-like 4 expression through AKT-mediated activation of beta-catenin, *J. Biol. Chem* 286 (10) (2011) 8055–8066. [PubMed: 21212269]
- [52]. Wang W, Li M, Wang Y, Wang Z, Zhang W, Guan F, Chen Q, Wang J, GSK-3beta as a target for protection against transient cerebral ischemia, *Int. J. Med. Sci* 14 (4) (2017) 333–339. [PubMed: 28553165]

- [53]. Wang Y, Rattner A, Zhou Y, Williams J, Smallwood PM, Nathans J, Norrin/Frizzled4 signaling in retinal vascular development and blood brain barrier plasticity, *Cell* 151 (6) (2012) 1332–1344. [PubMed: 23217714]
- [54]. Yang B, Li W, Satani N, Nghiem DM, Xi X, Aronowski J, Savitz SI, Protective effects of autologous bone marrow mononuclear cells after administering t-PA in an embolic stroke model, *Transl Stroke Res.* 9 (2) (2018) 135–145. [PubMed: 28836238]

Fig. 1.

The key effectors of the Hippo/YAP signaling pathway, i.e., YAP and TAZ, decreased after I/R injury. (A, B) Western blots showed the expression of YAP, TAZ, ZO-1, and Claudin-3 at 3, 6, 12, and 24 h after the MCAO surgery. The levels of YAP and TAZ decreased after I/R injury. The decreases were greatest at 1.5 h of reperfusion. The expression levels of YAP and TAZ in the sham-operated rats were twice that of the I/R 3-h group. Expression levels of tight junction proteins ZO-1 and Claudin-3 also decreased after MCAO to approximately 40% of that in the sham group. (C-F) Quantified levels of YAP, TAZ, ZO-1, and Claudin-3 at

different times after I/R injury. β -act in and GAPDH were used as loading controls. ** p 0.01 vs. the sham group; # p 0.05 vs. the I/R 3-h group. The data were presented as means $\pm$ SD; $n = 8$ rats per group.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2.

Effect of I/R injury on neurologic deficits, brain water content, and infarct volume. (A) Neurologic deficit scores of rats were elevated in the I/R 3-h group. (B) Brain water content was slightly lower in the I/R 24-h group than in the I/R 3-h group. The brain water content was 81.0%, 82.5%, 82.0%, 81.9%, and 81.3% in the sham group, I/R 3-h group, I/R 6-h group, I/R 12-h group, and I/R 24-h group, respectively. (C) Representative rat brain slices stained with TTC. Unstained areas indicated injured tissue. (D) Quantification of infarct volume. The infarct volume in the I/R 3-h group was slightly larger than that at other time

points, but the difference was not statistically significant. Infarct volume percentages were 25.8%, 22.3%, 23.0%, and 22.2% in the I/R 3-h group, I/R 6-h group, I/R 12-h group, and I/R 24-h group, respectively. [#]*p* < 0.05 vs. the I/R 3-h group. The data are presented as means ± SD; n = 6 rats per group.

Fig. 3.

I/R injury induced apoptosis. (A) TUNEL staining in rat cortex at different times after I/R injury (scale bar = 100 μ m). (B) Quantitative analysis of apoptosis in these groups. Apoptotic cell percentages were 42%, 33%, 34%, and 35% in the I/R 3-h, I/R 6-h, I/R 12-h, and I/R 24-h groups, respectively. The degree of apoptosis was greater at 1.5 h of reperfusion (I/R 3-h group) than at later time points. $^{\#}p < 0.05$ vs. the I/R 3-h group. The data were presented as means $\pm$ SD; n = 5 rats per group.

Fig. 4.

Stimulation of YAP and TAZ by DXM reduced I/R-associated neurologic deficits, brain edema, and infarct volume. (A, B) Neurologic deficit scores (A) and brain water content (B) were lower in DXM-treated I/R 3-h rats than in vehicle-treated and untreated I/R 3-h rats. The brain water content was 79.2%, 82.2%, 82.0%, and 80.3% in the sham group, I/R 3-h group, I/R 3-h + vehicle group, and I/R 3-h + DXM group, respectively. (C) Representative images of TTC-stained brain slices from sham-operated and I/R-injured rats. (D) Quantification of the infarct volume showed that DXM reduced the infarct area.

Percentage infarct volume was 25.5% in the I/R 3-h group, 25.8% in the I/R 3-h + vehicle group, and 16% in the I/R 3-h + DXM group. * $p < 0.05$, ** $p < 0.01$ vs. the sham group; # $p < 0.05$, ## $p < 0.01$ vs. the I/R 3-h group; &p $p < 0.05$, && $p < 0.01$ vs. the I/R 3-h + vehicle group. The data were presented as means $\pm$ SD; n = 6 rats per group.

Fig. 5.

Activation of YAP and TAZ increased tight junction proteins at 1.5 h after I/R injury. (A, B) Western blot analysis of brain tissue showed protein levels of YAP, TAZ, ZO-1, and Claudin-3 in rats with I/R injury treated with or without DXM. (C-F) Quantification showed that DXM increased levels of YAP, TAZ, ZO-1, and Claudin-3. ** $p < 0.01$ vs. the sham group; # $p < 0.05$ vs. the I/R3-h group; & $p < 0.05$ vs. the I/R 3-h + vehicle group. The data were presented as means $\pm$ SD; $n = 8$ rats per group.

Fig. 6. Enhancement of YAP and TAZ mitigated the BBB permeability induced by I/R injury. (A) Representative images showed extravasation of Evans blue dye in rat brain slices. (B) Quantification showed that Evans blue dye extravasation was less in the DXM-treated I/R 3-h group than in the untreated and vehicle-treated I/R 3-h groups. * $p < 0.05$, ** $p < 0.01$ vs. the sham group; # $p < 0.05$ vs. the I/R 3-h group; & $p < 0.05$ vs. the I/R 3-h + vehicle group. The data were presented as means $\pm$ SD; $n = 8$ rats per group.

Fig. 7. DXM attenuated apoptosis induced by I/R injury. (A) TUNEL staining in the cortex of different groups (scale bar = 100 μ m). (B) Quantitative analysis of apoptosis in the groups. The percentage of apoptotic cells was 42.3% in the I/R 3-h group, 41.4% in the I/R 3-h + vehicle group, and 28.5% in the I/R 3-h + DXM group. $##p < 0.01$ vs. the I/R 3-h group; $&&p < 0.01$ vs. the I/R 3-h + vehicle group. The data were presented as means $\pm$ SD; n = 5 rats per group.