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Article

Novel role of MCP-induced protein 1 in ischemic brain damage in animals of transient focal ischemia

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Abstract: Focal cerebral ischemia can lead to blood-brain barrier (BBB) breakdown, which is implicated in neuroinflammation and elevation of matrix metalloproteinases (MMPs). The role of the anti-inflammatory protein, monocyte chemotactic protein–induced protein 1 (MCPIP1) plays in the injury of BBB in stroke has not yet been reported. This study was conducted to identify and characterize the role MCPIP1 plays in BBB breakdown. Transient middle cerebral artery occlusion (MCAO) is induced in both wild-type and Mcpip1$^{-/-}$ mice for 2 hours of occlusion periods followed by reperfusion for 24 or 48 hours. BBB permeability was measured by FITC-dextran extravasation, MMP-9/3 expression was analyzed by western blot, and claudin-5 and zonula occludens-1 (ZO-1) were analyzed by immunohistochemistry and western blot. After MCAO in wild type mouse is induced, there is significantly increase in MCPIP1 mRNA and protein levels. Absence of MCPIP1 leaded to significant increase in FITC-dextran leakage in peri-infarct brain, significant upregulation of MMP-9, MMP-3 and reduced levels of tight junction components, claudin-5 and ZO-1 in the brain after MCAO. Our data demonstrate that absence of MCPIP1 exacerbates ischemia-induced blood-brain barrier disruption by enhancing the expression of matrix metalloproteinases and degradation of tight junction proteins. Overall data indicate that MCPIP1 is important protective role against BBB disruption in cerebral ischemia.

Keywords: Middle cerebral artery occlusion (MCAO); MCPIP1; blood-brain barrier; inflammatory response; ischemia; metalloproteinases.

1. Introduction

Stroke is a devastating neurological disease with high mortality and limited functional recovery. The inflammatory responses accompanying stroke contribute to secondary ischemic injury [1-5]. BBB breakdown is often a consequence of many neurological disorders such as multiple sclerosis, meningitis, encephalitis, amyloid angiopathy, traumatic brain injury and cerebral ischemia [6-7]. Blood-brain barrier (BBB) breakdown has been linked to post-stroke inflammatory responses and increased levels of various cytokines and chemokines [8]. These inflammatory factors induce the release of matrix metalloproteinases (MMPs), a family of extracellular enzymes which involve in degradation of tight junction proteins and increase in the permeability of the BBB[9].Upregulation and activation of MMP-9, MMP-3 and MMP-2 after cerebral ischemia leads to increased infarct size, BBB leakage and hemorrhagic activity [7, 10].

Monocyte chemotactic protein-induced protein 1 (MCPIP1) was originally found in human peripheral blood monocytes after treatment with monocyte chemotactic protein1 (MCP-1) [11]. Our

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previous results demonstrate that MCPIP1 can suppress macrophage activation and decrease the formation of proinflammatory cytokines, such as TNFα, IL-1β, IL-6 and MCP-1 through inhibitionof JNK and NF-κB proinflammatory signal pathway[7,15]. Our further studies indicated that upon LPS stimulation, MCPIP1 is upregulated in macrophages, endothelial cells and microglia [7,10] which is one of the mechanisms of LPS preconditioning/electroacupuncture pretreatment-induced ischemic brain tolerance [14,16]. The role of MCPIP1 in regulating ischemia/reperfusion-induced BBB breakdown after ischemic stroke has not been elucidated yet. Therefore, we examined the effect of ischemia in Mcpip1$^{-/-}$ mice. Our results demonstrate that the absence of MCPIP1 exacerbates ischemia-inducedblood-brain barrier disruption by enhancing the expression of matrix metalloproteinases and degrading of tight junction proteins after an ischemic stroke.

2. Materials and Methods

1.1 Animals

MCPIP1 deficient mice were producedas previously described [15]. Briefly, Mcpip1$^{-/-}$ mice were generated by homologous recombination in embryonic stem cells with C57/BL6 genetic background. Exons 3, 4, 5 and most part of 6 of mouse Mcpip1 were targeted with a LacZ-neomycin cassette in embryonic stem cells established from C57/BL6 mice and established Mcpip1$^{-/-}$ mice in pure C57/BL6 background. Results from immunoblotting suggested absence of MCPIP1 proteins in Mcpip1$^{-/-}$ mice. Eight to ten-week-old mice were selected. The experiment protocol was approved by the Institutional Animal Care and Use Committee of University of Central Florida. Mcpip$^{-/-}$ mice were backcrossed for at least 10 generations.All experiments were conducted by using littermate mice.

1.2 Induction of Cerebral Ischemia

Mice were randomly assigned to wild type group andMCPIP1 knockout group and experiments were performed in a blinded fashion. To induce focal brain ischemia, mouse transient middle cerebral artery occlusion (MCAO) was produced by filament blockageof the right MCA as previously described [14]. In brief, mice anesthesia was induced and maintained with 3% and 1.2% isoflurane, respectively, along withoxygen-enriched air through a facemask, and rectal temperature was kept between 36.5°C and 37.5°C during the course of the experiment by heating lamps. Unilateral MCAO was produced by inserting a 7-0 nylon monofilament into the internal carotid artery via an external carotid artery stump and filament is advanced up to 8-9mm into MCA from the CCA junction. MCA occlusion period lasts for 120 minutes followed by reperfusion.

1.3 Assessment of BBB Breakdown

To assess BBB breakdown we used a method modified from a described FITC-dextran assay [5]. After 2 h ischemia followed by 24 h reperfusion, fluoro-isothiocyanate dextran 70 kDa FITC-dextran (500mg/kg, Sigma, USA) was given by intravenous injection to each group mice and wait one minute before rapidly removing the brain and transfer it to 4% paraformaldehyde at 4 °C for 24h.The brains were sliced into 50 μm thick coronal sections and observed under a fluorescence microscope (Leica TCS SP5) for the BBB leakage study. Three fields were captured from each section and then analyzed. Each group has 6 mice.

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1.4 Quantitative Real-Time PCR

Quantitative Real Time-PCR was conducted according to previously established procedures [29].Briefly, genomic DNA is removed using DNase I (Ambion, USA) before 2.0 ug of total RNA from microglia of mouse brain tissue was reverse-transcribed to cDNA using a commercially available kit (Applied Biosystems, USA).Quantitative real-time PCR was performed with iCycler Thermal Cycler (Bio-Rad, USA) using 2 X SYBR Green master mixes (Bio-Rad, USA).Forty cycles proceeded under the following condition: 95 °C for 30 s, 60 °C for 30 s, proceeded by 10 min at 95 °C for polymerase activation. Quantification was performed by the delta cycle time method, with mouse β-actin used for normalization. The mouse specific primers (IDT, USA) are as follows, MCPIP1: F: 5′-CCCCCTGACGACCCTTTAG; R: 5′ GGCAGTGGTTTCTTACGAAGGA, β-actin: F: 5′- AAATCGTGCGTGACATCAAAGA; R: 5′- GGCCATCTCCTGCTCGAA.

1.5Western Blot

Western blot was carried out as previously reported[8]. Proteins from mouse brain tissue were extracted before concentrations were measured by the Bradford method (Bio-Rad, USA) with bovine serum albumincreating a standard curve. Proteins (50ug) were loaded to the wells of the SDS-PAGE for separation before being transferred from gel to nitrocellulose membranes in transfer buffer containing 0.1% SDS. The membrane were incubated with 5% nonfat dry milk in 0.05% Tween 20 in Tris-buffered saline (TTBS) for 2 h to prevent no-specific binding and then incubated in blocking buffer (1:1,000 diluted) containing primary antibodies against MMP-3, MMP-9 (Cell Signaling, USA), ZO-1, Claudin 5 and MCPIP1 (Santa Cruz, USA), in the 4ºC, gently rocking, overnight. After incubation, each membrane was washed three times for 10 min with TTBS and the membranes were incubated with secondary antibody (Santa Cruz, USA) for 1 h (ata 1:2,000 dilution in of in TTBS).Following three 10-min rinsed with TTBS, membranes were -incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) and the intensity of bands was quantified by AlphaImage 2200 (AlphaInnotech, USA) after exposure to x-ray film. Fold changes were calculated as a ratio of the expression in the wild type group and MCPIP1 knockout groupto the expression in the control group.β-actin was used for a loading control.

1.6 Immunohistochemistry

At 24 hours after I/R, mice were subjected to transcardial perfusion with cold phosphate-buffered saline, followed by 4% paraformaldehyde under anesthesia. Brains were removed and fixed by immersion in 4% paraformaldehyde at 4ºC. Coronal sections of 30 μm were made with a vibrating microtome (Leica Microsystems) in ice-cold phosphate-buffered saline. The sections were stored in anti-freeze solution at -20ºC for later use. The nonspecific binding sites on sections were blocked with 3% bovine serum albumin after the sections were rinsed, and primary antibody against GFAP, primary antibody against CD11b (BD Biosciences Pharmingen, San Diego, CA, USA), primary antibody against NSE (Invitrogen, USA), primary antibody against CD31, primary antibody against MCPIP1 (Santa Cruz Biotechnology, Dallas, Texas, USA), AlexaFluor®-488-conjugated secondary antibody (Invitrogen, USA) and AlexaFluor®-594-conjugated secondary antibody (Invitrogen, USA), monoclonal antibody conjugated to Alexa Fluor 594 against ZO-1(1:100, Invitrogen, USA), monoclonal antibody conjugated to Alexa Fluor 488 against Claudin-5 (1:100, Invitrogen, USA) were applied to the tissue section and incubated overnight. Sections were mounted in Vectashield

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mounting medium (Vector Laboratories, Burlingame, CA, USA) and scanned under a fluorescence microscope (Leica TCS SP5).Three fields were captured from each section and then analyzed. Each group has 6 mice.

3. Results

3.1. MCPIP1 induction in Mouse Brain after MCAO

To examine the potential effects of MCPIP1 in ischemic stroke we tested whether MCPIP1 was induced in the brain after ischemia/reperfusion (I/R) stress both at transcript and protein levels. After I/R stress, MCPIP1 mRNA and protein levels in the ipsilateral side of the brain cortex were significantly elevated compared to that of the sham group. Induction was detectable at 12h after MCAO and the transcript and protein levels reached 12.8 ±1.63 and 9.2±1.31 fold respectively, 24h after I/R stress in wild type mice (Fig. 1A, 1B), indicating that MCPIP1 may be an important factor after brain ischemic stress. There was no induction of MCPIP1 after I/R stress in Mcpip$^{-/-}$ mice (Fig. 1A, 1B).

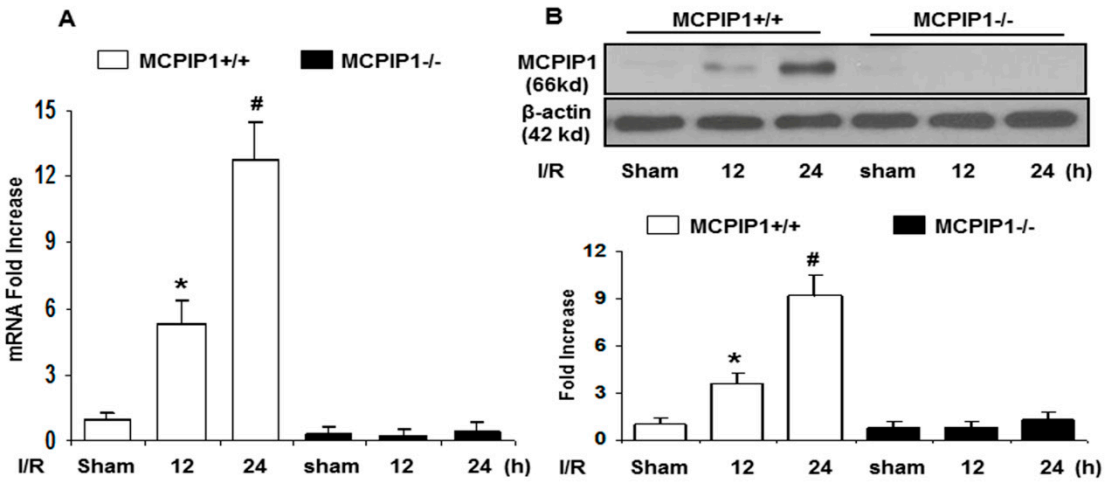

Figure 1. MCPIP1 mRNA and protein levels are elevated in the brain of mouse undergoing ischemic stroke.**(A)**, MCPIP1 mRNA expression in mouse brain with ischemic stress as measured by qRT-PCR. Values represent mean ± SD, *p<0.05, # p<0.01 versus sham control; n=6. **(B)**, MCPIP1 protein levels in mouse brain with ischemic stress as measured by Western blot. Results are representative of three independent experiments. Values represent mean ± SD, *p<0.05, # p<0.01 versus sham control; n=6.

3.2. Blood-Brain Barrier Disruption

BBB disruption has been strong implicated in the pathogenesis of acute ischemic stroke resulting in cerebral edema, brain hemorrhage, neuronal death and neurological deficits[9]. To determine whether MCPIP1 plays a critical role in regulating ischemic brain damage we examined BBB permeability by FITC-dextran extravasation in brain. As shown in Figure 3, FITC-dextran leakage was clearly seen in the peri-infarct cortex of the brain after a 2-hour MCAO followed by 24-hour reperfusion (Fig. 2b, 3d). Measurement of fluorescence intensity in peri-infarct cortical regions of the ischemic hemisphere showed that after 2 hour ischemia followed by 24 hours reperfusion Mcpip1$^{-/-}$ mice had a significantly higher BBB leakage than that in the wild-type (Fig. 2B).

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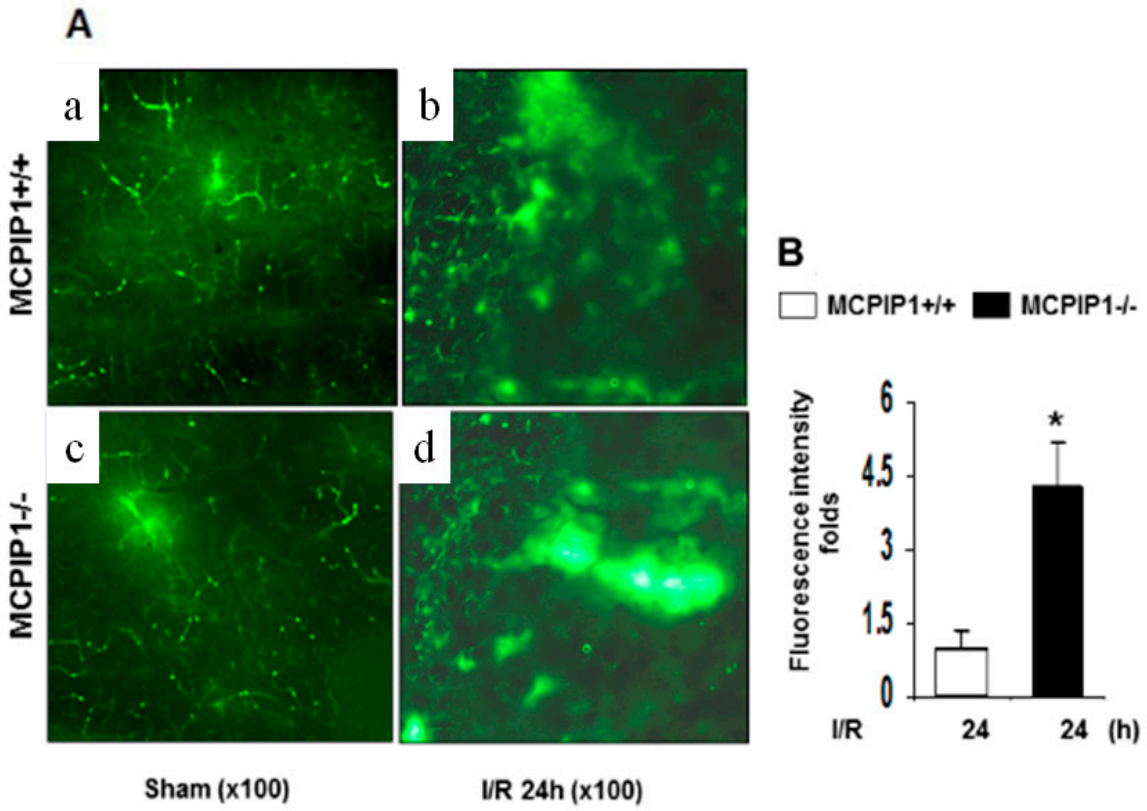

Figure 2. MCPIP1 deficiency exacerbates ischemia-induced cortical blood-brain barrier (BBB) disruption. (A), Representative FITC-dextran perfusion in peri-infarct cortical capillary networks from wild-type and Mcpip1$^{-/-}$ mice undergoing 2h brain ischemia followed by 24h reperfusion. (B), Measurement of fluorescence intensity in peri-infarct cortical regions of the ischemic hemisphere. Mcpip1$^{-/-}$ mice had a significant increase in BBB leakage after 2 hour ischemia followed by 24 hours reperfusion. Values represent mean ± SD,* $p<0.05$ versus wild type group. n=6 per group.

3.3 Matrix Metalloproteinase Elevation

Cerebral ischemic injury-induced inflammatory cytokines such as TNFα, IL-1β are known to stimulate the expression of matrix metalloproteinases (MMPs) [17]. To investigate whether the absence of MCPIP1 enhanced the expression of MMPs, at 12 and 24h after MCAO, the expression of matrix metalloproteinase wasmeasuredin the brain of wild-type and Mcpip1$^{-/-}$ mice. The results showed that the expression of MMP-3/9 after brain ischemia in mice lacking MCPIP1 was significantly higher versus the wild-type mice after ischemia (Fig. 3)

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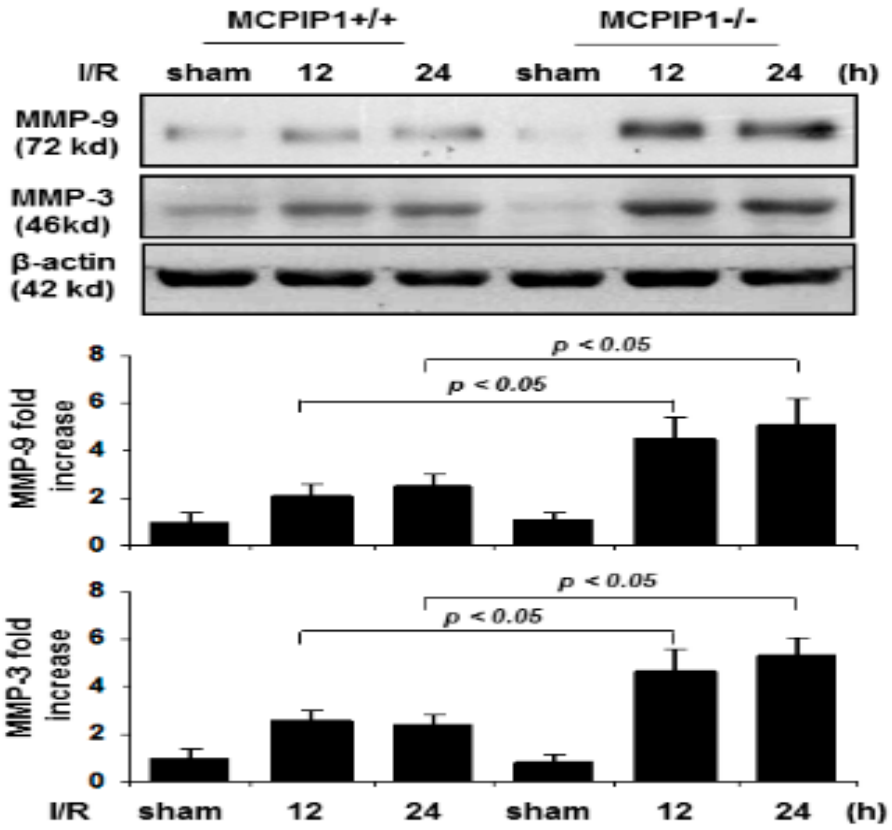

Figure 3. Representative western blots of MMP-9 and MMP-3 in peri-infarct cortical regions of the ischemic hemisphere at 12h and 24h after MCAO. Densitometric analysis shows that expression of MMP-9 and MMP-3 was significantly higher in MCPIP1 deficient mice as compared to that of wild type after MCAO. Values represent mean ± SD, * p<0.05 versus wild type group. n= 6 per group.

3.4 Tight junction degradation

The BBB is mainly formed by specialized brain endothelial cells that are held together by well-developed tight junctions and creates a dynamic interface between the blood and the brain (9). To determine whether the absence of MCPIP1 caused a loss of tight junction components, immunostaining was performed to examine ischemia/reperfusion-induced changes in levels of the tight junction proteins (ie: claudin-5 and ZO-1) that are known to be critical for maintaining BBB integrity. In WT mice, claudin-5 and ZO-1 levels in peri-infarct ischemic cortical regions were found to be significantly lower at 24 hours after ischemic stroke (Fig. 4A, 4B). The levels of tight junction proteins claudin-5 and ZO-1 were significantly lower in Mcpip1$^{-/-}$ mice versuswild-type mice at24h after MCAO (Fig. 4C). Confirming the immunostaining results, the immunoblot analysis showed that claudin-5 and ZO-1 levels were significantly lower in Mcpip1$^{-/-}$ mice as compared with that of the wild-type 24 h after MCAO (Fig. 4D). Immunohistochemical analysis of peri-infarct microvessels with antibodies against tight junction claudin-5 and endothelial marker CD31 showed that claudin-5 level was significantly lower in the endothelial cells in Mcpip1$^{-/-}$ mice as compared with their levels in the wild-type 24h after MCAO (Fig. 4A, 4B). These results indicated that absence of MCPIP1 exacerbated the BBB disruption of mice undergoing ischemic stroke.

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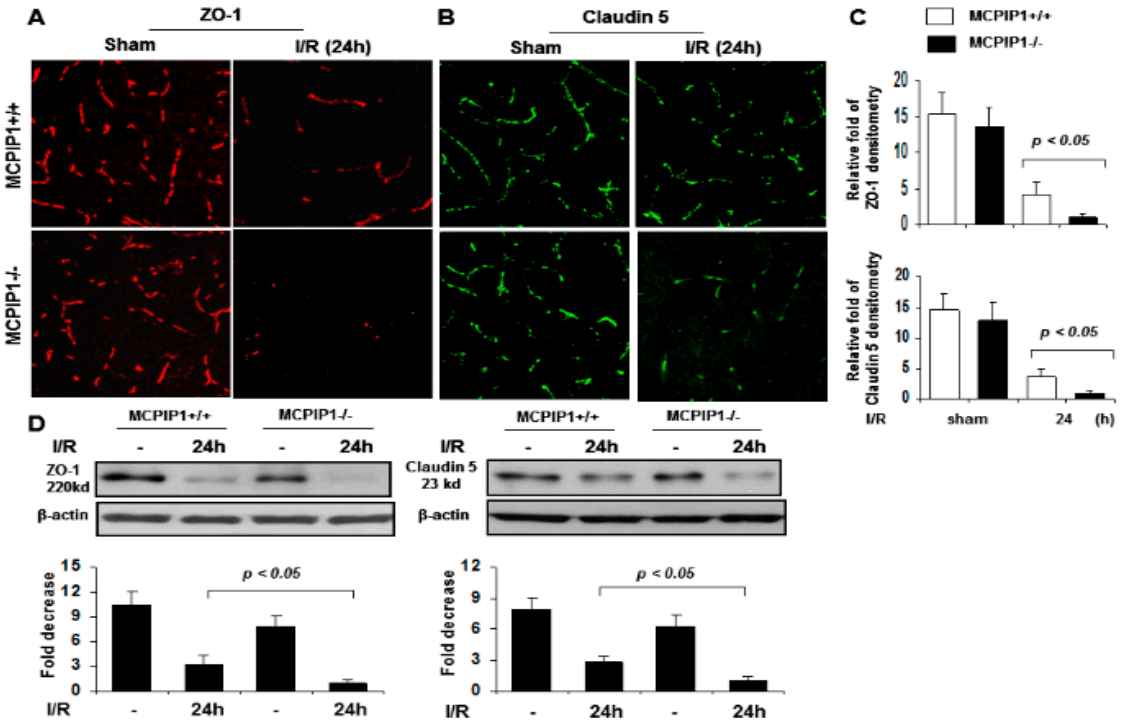

Figure 4.ZO-1 and Claudin-5 Degradation after Brain Ischemia. (A) and (B), Representative immunostaining images of ZO-1 and claudin-5 in prei-infarct cortical regions of the ischemic hemisphere. Cerebral ischemia induces rapid ZO-1 and claudin-5 degradation at 24h after MCAO in vivo. (C), Densitometric analysis shows that levels of ZO-1 and claudin-5 were significantly lower in MCPIP1 deficient mice as compared to that of wild type after MCAO. Values represent mean ± SD. $p<0.05$ as significant difference between groups indicated. (D), ZO-1 and claudin-5 protein levels in prei-infarct cortical regions of the ischemic hemisphere as measured by Western blot. The levels of ZO-1 and claudin-5 were significantly lower in Mcpip1-/- mice as compared to that of wild type after 2 hour ischemia followed by 24 hours reperfusion. Results are representative of three independent experiments. Values represent mean ± SD. $p<0.05$ as significant difference between groups indicated. n=6 per group.

4. Discussion

The BBB comprises a functional neurovascular unit and dynamic interface between the blood capillaries and the neuronal network. This is the first study to provide direct demonstration that MCPIP1 probably plays a pivotal part in protecting the brain from cerebral ischemia-induced BBB disruption and brain damage. Inflammation is a key tache in the pathogenesis of ischemic stroke. Ischemic stroke-induced cerebral inflammatory responses can produce substantial brain injury, elevation of MMP levels and BBB dysfunction. The post-ischemic neuroinflammatory response involves microglial and astrocytic activation and upregulation of inflammatory mediators [18-20]. Proinflammatory cytokines and chemokines is an important factor that cause stroke-related brain injury [3,21].During ischemia, a variety of cell types, including endothelial cells, microglia, astrocytes and neurons are activated and produce cytokines such as TNF-α, IL-1β, IL-6, and chemokines such as CINC and MCP-1[3]. In particular, cytokines that are upregulated in inflammation like TNF-α and certain other interleukins can trigger the early events that cause BBB

breakdown and subsequent development of cerebral edema [22]. It is shown in the clinical data that the main cause of stroke death in patients that suffered a malignant middle cerebral artery infarction was focal brain edema [23-24]. Increase in brain water content was observed in animal model of cerebral infarction within hours and brain water content reached the peak at 24 hour after stroke[25].Progressive post-ischemic edema would lead to increased intracranial pressure with compression of the brain stem, occlusive hydrocephalus and secondary ischemic damage, further clinical deterioration, coma, and death within 2 to 5 days after stroke [8,26-28]. In previous studies we identified that MCPIP1, an important inducible anti-inflammatory regulator [12,15], is significantly induced in the brain by LPS and electroacupuncture and arouse LPS and electroacupuncture pretreatment-induced ischemic brain tolerance [14,16]. In the present study our resultsindicate that deficiency of MCPIP1 significantly promoted brain BBB leakage and from our previous results demonstrated that absence of MCPIP1 significantly increases the infarct volume and brain edema compared to wild type after ischemia/reperfusion injury [14]. Collectively，those results indicate MCPIP1 plays a very important role in attenuating brain damage caused by stroke.

The causes of pathologic BBB opening are not fully characterized, but studies have suggested that proteolytic enzymes degrading the vascular basement membrane results in disruption of the BBB [8,28].MMP-2 activation is a key factor in the initial reversible opening phase of the BBB and activated MMP-2 can degrade tight junction proteins [17]. The second phase of BBB opening occurs after 24 to 48 hours reperfusion depending on the ischemia duration; the longer the duration, the earlier and more disruptive the BBB opening [29].It has been manifested that MMP-3 and MMP-9 which is induced by cytokines produced during inflammation mediate the second phase of BBB opening. We have previously found that absence of MCPIP1 caused an increase in production of inflammatory cytokines in the brain after stroke [14]. The results obtained from the present study showed that the absence of MCPIP1 caused a significant increase in the expression of MMP-3 and MMP-9 in the brain after ischemic stroke indicating that MCPIP1 probably is a crucial neuroprotective role against ischemic stroke by inhibiting the induction of MMP-3 and MMP-9 via inhibition of inflammatory cytokine production.

There exists a complex network of tight junction proteins consist of claudins and ZO-1 functions to hold adjacent endothelial cell together by linking transmembrane proteins to the actin cytoskeleton. Expression of these tight junction proteins is modified in several neurological diseases including stroke [30], and they are vulnerable to attack by MMPs. In this study, we proved that focal cerebral ischemia/reperfusion-induced BBB structural integrity damage and BBB dysfunction is exacerbated by absence of MCPIP1.Despite substantial research, the molecular mechanisms regulatingthe ischemia/reperfusion-induced biphasic BBB opening are not completely understood. Among tight junctionproteins, claudin-5, ZO-1 are two most widely studied components of the BBB function and both are crucial to maintain the BBB structural integrity and permeability. The degradation of claudin-5 as well as ZO-1 has highcorrelationwith the dynamic process of BBB break down followingcerebral ischemia [31].The results from the present study showed that the absence of MCPIP1 increased the degradation of the tight junction proteins to enhance the BBB disruption, suggesting that MCPIP1 is an important factor inprotecting BBB in acute stroke.

5. Conclusions

Our data demonstrate that absence of MCPIP1 exacerbates ischemia-induced blood-brain barrier disruption by enhancing the expression of matrix metalloproteinases.

Author Contributions: Resources, P.E.K.; data curation, J.L.; writing—original draft preparation, J.L., Z.J. and J.Q.L. writing—review and editing, Z.J., J.L. and J.Q.L.; supervision, P.E.K.; project administration, P.E.K.; funding acquisition, Z.J..

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Conflicts of Interest: The authors declare no conflict of interest.

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