Reduced Levels of A20 Protein Prompted RIPK1-Dependent Apoptosis of Vascular Endothelial Cells and Blood–Brain Barrier Breakdown in CIRI

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Research Article

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Abstract

Blood–brain barrier (BBB) leakage is an important cause of the exacerbation of pathological features of ischemia and reperfusion. However, the specific mechanism of cell death of vascular endothelial cells is not clear. It was found that ischemic reperfusion resulted in RIPK1 activation in vascular endothelial cells and induced cells to undergo subsequent RIPK1-dependent apoptosis (RDA). Inhibition of RIPK1 significantly reduced BBB breakdown and brain damage.

The aim of this study is to investigate the mechanism of RIPK1 in the BBB leakage during the ischemia reperfusion procedure.

In this study, the role of RIPK1 in the development of cerebral ischemia reperfusion injury (CIRI) was investigated by immunohistochemical approaches on KO or mutant mice.

It was discovered by proteomics that autophagy activation resulting from ischemia and reperfusion significantly downregulated the level of A20 protein in vascular endothelial cells. A20 is an important protein that regulates RIPK1 and RDA. It was hypothesized that activation of autophagy in vascular endothelial cells caused by ischemic reperfusion led to a decrease in A20 protein, which, in turn, caused the activation of RIPK1 and the occurrence of RDA, leading to leakage of the blood–brain barrier.

The findings in this study revealed the role of RIPK1 in vascular endothelial cell death and BBB leakage upon cerebral ischemia reperfusion injury (CIRI), and these findings provide a novel perspective for the treatment of ischemic reperfusion.

Introduction

The blood–brain barrier (BBB) is an anatomical and biochemical barrier that protects the brain from potentially harmful substances (Wrobel and Toborek 2016). The BBB is a highly selective membrane barrier in the brain microvasculature that facilitates transport between the systemic circulation and the central nervous system (Wiranowska 1992). The BBB regulates homeostasis of the central nervous system (CNS) by forming a tightly regulated neurovascular unit (NVU) that includes endothelial cells (ECs), pericytes and astrocytic endfeet, which jointly maintain normal brain function (Charles et al. 1996; Go 1997; Lai and Kuo 2005). The presence of the BBB is capable of preventing some substances, mostly harmful, from entering the brain tissue from blood. The BBB acts as a physical and metabolic barrier between the CNS and the peripheral circulation, exerting regulatory and protective effects on the microenvironment of the brain (Rivest et al. 2000). Under normal circumstances, the primary function of the BBB is to establish and maintain homeostasis in the CNS. Once the BBB is destroyed, the brain is particularly vulnerable to infection and damage (Petito and Cash 1992; Eralinna et al. 1996; Woodman et al. 1999).

Reperfusion remains a leading cause of disability and death globally, and its therapeutic management is an extremely challenging problem in clinical practice. The recovery of blood supply, referred to as

reperfusion, has been considered a standard therapeutic option for ischemia (Cuevas et al. 1998). In addition to preventing the growth of infarction volume, reperfusion has been reported to aggravate ischemic damage, including early disruption of the blood–brain barrier (BBB) (Aoki et al. 2002; Pluta 2003; Pillai et al. 2009). One of the pathophysiological characteristics of cerebral ischemic reperfusion injury (CIRI) is the destruction of the BBB (Pluta 2003). During CIRI, BBB damage leads to the infiltration of inflammatory cells into the brain, which further aggravates cerebral inflammation and edema (Warach and Latour 2004; Dimitrijevic et al. 2006; McColl et al. 2008). Thus, increasing attention is turning towards the identification of potential targeted drugs to protect the BBB against ischemia–reperfusion injury.

Cerebral ischemia–reperfusion injury can result in brain microvasculature and blood–brain barrier (BBB) breakdown, leading to increased BBB permeability (Bederson et al. 1986; Abdullahi et al. 2018). Disruption of the BBB following CIRI results in brain edema, a primary event that affects both morbidity and mortality (Cheslow and Alvarez 2016). Subsequently, various mediators are released that enhance vasogenic and/or cytotoxic brain edema (Eltzschig and Eckle 2011). These include glutamate, lactate, H^+, K^+, Ca^{2+}, nitric oxide, arachidonic acid and its metabolites, free oxygen radicals, histamine, and kinins (Eltzschig and Eckle 2011). The permeability of the BBB to endogenous proteins, such as immunoglobulin G (IgG), is increased following experimental CIRI (Akiguchi et al. 1998; Michalski et al. 2010). An additional consequence of BBB disruption is the infiltration of leukocytes into brain tissue, accompanied by microglial activation and inflammation (Michalski et al. 2010).

Brain microvascular ECs are a key component in the pathophysiological mechanism of BBB dysfunction after ischemic reperfusion. However, the regulatory mechanism governing endothelial cell death is still unclear. Recently, it was implied that Receptor Interacting Protein Kinase 1 (RIPK1), which is a crucial necroptotic and apoptotic mediator in CIRI, might play an essential role in regulating endothelial cell death during the progression of CIRI (Chen J et al. 2019; Rehorova et al. 2019). RIPK1 is a serine/threonine protein kinase. The kinase activity of RIPK1 is stimulated by the tumor necrosis factor (TNF) death signal, and subsequent downstream necroptosis is activated (Chen AQ et al. 2019). Activated RIPK1 self-phosphorylates at serine 166, then recruits RIPK3 (Receptor Interacting serine/threonine Protein Kinase 3) and MLKL (Mixed Lineage Kinase domain Like protein) (Newton et al. 2016; Qu et al. 2017). The polymer RIPK1/RIPK3/MLKL leads to cell necroptosis during the progression of ischemia and reperfusion (Chen et al. 2018; Zhu and Sun 2018; Hribljan et al. 2019).

In this study, the physiological function of RIPK1 was investigated to elucidate the mechanism of blood–brain barrier destruction during the process of CIRI, which might contribute to the development or optimization of a clinical intervention approach.

Materials And Methods

Antibodies and Reagents

The following antibodies were employed: mouse anti-MLKL (Abcam), mouse anti-caspase 3 (Cell Signaling), mouse anti-cleaved caspase 3 (Cell Signaling), mouse anti-pRIPK1(S166) (Lifespan) and mouse anti-pMLKL(S345) (Novus). Secondary horseradish peroxidase HRP-conjugated antibodies were from Abcam.

Mice and Treatments

The pathogen-free male wild-type C57BL/6J mice (n = 24), *Ripk1*^{D138N} mice (n = 3) (on a C57BL/6J background) and *Ripk3*$^{-/-}$ mice (n = 3) (on a C57BL/6J background), 8 to 11 weeks of age, were provided by Model Organisms (Shanghai, China). Animal experiments were carried out in accordance with the guidelines of the China Animal Care and Use Committee of Tianjin Medical University.

The animals underwent the MCAO (middle cerebral artery occlusion) procedure to construct a CIRI model. Mice were injected with 3% pentobarbital sodium (80 mg/kg) intraperitoneally. The common carotid artery, internal carotid artery and external carotid artery were separated after the skin of the head was prepared and disinfected. Then, briefly, a monofilament (0.18 mm) was introduced into the common carotid artery under anesthesia, advanced to the origin of the MCA, and left there for 2 hours. After 2 hours of ischemia, the filament was pulled out to enable reperfusion, and the skin was sutured. The mice were placed on a heating pad and then transferred to an incubator after waking up. The brains of anesthetized mice were removed at 1 h, 6 h, 12 h, 24 h and 48 h after reperfusion for further experiments. Sham operations were performed following the same procedure, except that the surgery was stopped after the dura mater was opened.

shRNA design, cloning, and viral vector construction

siRNA sequences against A20 (NM_001270507) were designed using an online algorithm (http://sidirect2.rnai.jp) (5'-AGTTTCAACCGTCTTAATCAG-3', 5'-GATTAAGACGGTTGAAACTAG-3'). The indicated sequences were cloned downstream of a human Cdh5 promoter in a pAAV2-CMV-GFP vector provided by the Tianjin Institute of Pharmaceutical Research. A recombinant virus was produced by the Vector Builder (Tianjin Institute of Pharmaceutical Research) using a baculovirus system. In the A20 knockdown experiment, a total volume of 15 μl viral vector suspension was intravenously injected into C57BL/6J mice.

TTC staining

Wild-type, *Ripk1*$^{D138N/D138N}$ and *Ripk3*$^{-/-}$ mice were deeply anesthetized with sodium pentobarbital (80 mg/kg). The brains were removed quickly (within 10 min) and transferred into a -20°C refrigerator for 30 min, after which coronal brain sections (2 mm thickness) were stained with 2% red tetrazolium solution (Sigma-Aldrich, USA) for 30 min at 37°C in the dark. The container was slightly shaken every 5 min to ensure full staining. The brain slices were washed with PBS solution for 3–5 min and then fixed with 10% neutral formaldehyde for 6 hours, after which images were captured immediately.

Immunofluorescence (IF)

The activation of p-RIPK1 (S166) and cleaved caspase 3 (CC3) in mice treated with MCAO/R was detected by immunofluorescence using DAPI staining according to the manufacturer's instructions. CD31 was measured as an indicator of endothelial cells, and PI staining was employed to detect apoptosis. IgG was detected in this study to evaluate BBB permeability. The slides were observed using a fluorescence microscope (BX51; Olympus Corporation, Tokyo, Japan).

Immunoblotting

The brain tissues of treated mice were collected at 1, 6, 12, 24 and 48 hours after cerebral ischemia and reperfusion. The tissues were ground, and their protein levels were measured by Western blotting. Aliquots of protein extracts were subjected to 12% SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane (Amersham Biosciences) and then incubated with the desired primary antibodies (anti-A20 and anti-CC3) and secondary antibodies according to the manufacturer's instructions. Finally, the proteins were detected with Luminol ECL reagent (Thermo Scientific). Densitometry was performed using ImageJ software. All gels and densitometry shown are representative of at least three experiments unless otherwise indicated.

Statistical analyses

Data are expressed as the mean ± SEM. Significance was assessed with Student's t-test or one-way ANOVA followed by Bonferroni's post hoc test using Prism version 6.0 software (GraphPad). P values below 0.05 were considered significant.

Results

Initially, the activation of RIPK1 in vascular endothelial cells upon the occurrence of CIRI was investigated. The activation of RIPK1 was determined by phosphorylation of phosphorylation-serine 166. The data indicated that the activation of RIPK1 in vascular endothelial cells upon CIRI was significantly enhanced in wild-type mice compared to *Ripk1*$^{D138N/D138N}$ mutant mice, which are RIPK1 activation-deficient mutants (Fig. 1). Cluster of differentiation 31 (CD31) was detected in this assay as a marker indicating the expression of endothelial cells (Bujan et al. 1999). However, RIPK1 was also activated in the vascular endothelial cells of *Ripk3*$^{-/-}$ KO mice upon neuronal death signaling after CIRI.

In order to evaluate the permeability of the BBB during the process of ischemia and reperfusion, the penetration of immunoglobulin G (IgG), an endogenous protein, was monitored. An immunofluorescence assay indicated that IgG penetration was significantly increased 3 days after ischemia and reperfusion, suggesting the destruction of the BBB following the stimuli associated with CIRI (Fig. 2). Moreover, the permeability of the blood–brain barrier was remarkably weakened in *Ripk1*^{D138N} mutant mice, which inhibited RIPK1 activation after Middle cerebral artery occlusion (MCAO) (Fig. 2). These results demonstrated that RIPK1 activation may play an important role in BBB vascular endothelial cell death and BBB destruction.

RIPK1 was activated to a remarkable extent in vascular endothelial cells upon the occurrence of CIRI, and a series of MCAO animal experiments and immunofluorescence assays were performed to clarify whether RIPK1-induced BBB destruction was RIPK3 dependent. TTC staining data showed that the volume of cerebral infarction in *Ripk3* gene knockout mice remained similar to that of wild-type mice; however, the *Ripk1*^{D138N} mutant mice showed a significantly reduced volume of post-MCAO cerebral infarction (Fig. 3A).

Furthermore, the death of endothelial cells in MCAO experimental mice was assessed by PI staining. The results showed that *Ripk3* knockout did not inhibit endothelial cell death as effectively as *Ripk1*^{D138N} but achieved only partial inhibition (Fig. 3B), suggesting that RIPK3 may play a limited role in ischemia-reperfusion injury. Considering that RIPK3 was associated only with RIPK1-mediated necroptosis in CIRI, it was presumed that RIPK1-mediated programmed cell necrosis might not be the main cause of endothelial cell death in the BBB upon ischemia and reperfusion stimuli. Subsequently, it was speculated that RIPK1-dependent apoptosis (RDA), as opposed to RIPK1-mediated necroptosis, might play a dominant role in the death of vascular endothelial cells in the BBB.

The apoptosis marker cleaved caspase 3 (CC3) was employed in this study to evaluate the effect of RDA on BBB destruction during the progression of ischemia–reperfusion injury (Liu Z et al. 2017; Liu C et al. 2018). Immunoblotting data showed that an inactive mutant form of RIPK1 (*Ripk1*^{D138N}) inhibited the initiation of RDA in mice after MCAO treatment; however, the RDA features remained intact in the *Ripk3*$^{-/-}$ mice (Fig. 3C). The results suggested that the death of BBB endothelial cells in MCAO mice was caused mainly by apoptosis mediated by RIPK1 activation rather than programmed necrosis.

In order to clarify the molecular mechanism of RDA in vascular endothelial cell death after MCAO, protein mass spectrometry was performed to analyze the proteomic features of vascular endothelial cells isolated from mouse brains treated with MCAO. The analysis of up-regulated and downregulated proteins indicated that A20 protein was significantly downregulated (3-fold) in vascular endothelial cells after MCAO (Fig. 4A). The protein level of A20 in vascular endothelial cells was also measured by Western blot after MCAO treatment. Similarly, the mRNA level of A20 was quantified using PCR following MCAO treatment. The results showed that upon CIRI, the protein level of A20 was significantly reduced, but the mRNA level of A20 was not regulated (Fig. 4B and 4C), implying that the decrease in the A20 protein level may be the key cause of vascular endothelial cell death after ischemic reperfusion.

Subsequently, A20 knockdown mice were constructed on a C57BL/6J background to evaluate the physiological functions of A20 in vascular endothelial cell death after MCAO treatment. The results showed that the volume of cerebral infarction and the apoptosis of vascular cells were significantly increased in A20 knockdown mice (A20-EKD) during ischemic reperfusion (Fig. 5). This finding suggests that the degradation of A20 protein acts an essential feature in the initiation of RDA and might be deeply associated with vascular endothelial cell death and BBB destruction in the progression of ischemia–reperfusion injury.

Discussion

The blood–brain barrier (BBB) is central to the regulation of cerebral microcirculation due to its characteristic barrier properties and transport system. The BBB is principally composed of cerebral microvascular endothelial cells, which form tight junctions together and are interlaced with astrocytes, pericytes, and a basal lamina (Norsted et al. 2008). These cells possess specialized receptor-mediated transport mechanisms and barrier properties and contribute equally to the local control of cerebral microcirculation.

Cerebral ischemia–reperfusion injury can result in breakdown of the brain microvasculature and blood–brain barrier (BBB), leading to increased BBB permeability (Abdullahi et al. 2018). CIRI results in brain edema, a primary event that affects both morbidity and mortality following CIRI. Edema increases intracerebral pressure (ICP) and leads to secondary ischemic injuries by impairing cerebral perfusion and oxygenation (Hara et al. 1996). An additional consequence of BBB disruption is the infiltration of leukocytes into brain tissue, accompanied by microglial activation and inflammation (Ju et al. 2018). Blood–brain barrier (BBB) injury is recognized to play an important role in brain injury caused by ischemic reperfusion. Inhibition of BBB destruction and vascular endothelial cell death can greatly ameliorate brain injury (Tu et al. 2011). At present, the mechanism of endothelial cell death induced by ischemia and reperfusion is not completely clear.

It was found in this study that the activation of the kinase RIPK1 played an important role in ischemia–reperfusion injury, and ischemia–reperfusion injury was significantly ameliorated in kinase-deficient mutant *Ripk1*^{D138N} mice. In addition to the effect on neuronal cell death, the activation of RIPK1 was also associated with vascular endothelial cell death, suggesting that RIPK1 potentially features in the physiology of BBB destruction in CIRI. BBB permeability was investigated as an indicator of barrier breakdown in this study. The results indicated the crucial role of RIPK1 activation in BBB destruction upon MCAO treatment. The data showed that wild-type *Ripk1*$^{+/+}$ mice exhibited extensive post-MCAO BBB compared to *Ripk1*^{D138N} mutant mice. These observations imply that RIPK1 activation may play an important role in BBB vascular endothelial cell death and BBB destruction.

RIPK1 not only relates to necroptosis in which RIPK3 is recruited but also mediates RIPK1-dependent apoptosis in which caspase is activated. RIPK3 knockout mice (*Ripk3*$^{-/-}$) were employed in this study, and the TTC staining results indicated that Ripk3 gene knockout failed to ameliorate brain injury after MCAO treatment. Interestingly, RIPK1 was found to be activated in *Ripk3*$^{-/-}$ mice as detected by PI staining after MCAO treatment, suggesting that necroptosis induced by RIPK1 might not be the dominant contributor to endothelial cell death upon MCAO stimulation. Instead, it was speculated that RIPK1-dependent apoptosis might play a dominant role in endothelial cell death in CIRI. The results indicated that the level of cleaved caspase 3 (CC3) was maintained in endothelial cells of *Ripk3*$^{-/-}$ mice but significantly decreased in *Ripk1*^{D138N} mice. This finding suggested that, instead of necroptosis, the RDA (RIPK1-dependent apoptosis) of BBB vascular endothelial cells after ischemic reperfusion may be the main mechanism of BBB breakdown.

In order to investigate the molecular mechanism of RDA in vascular endothelial cells, a proteomic study was carried out. Protein A20 was significantly downregulated (3-fold) in vascular endothelial cells after MCAO. Zinc finger protein A20, encoded by the TNFAIP3 gene, possesses deubiquitinase activity (Jaattela et al. 1996). A20 is capable of activating NF-κB and initiating TNF-mediated apoptosis (Jaattela et al. 1996; Malewicz et al. 2003). Recently, it has been suggested that reducing A20 levels in mouse embryonic fibroblasts can promote RIPK1-dependent apoptosis and programmed necrosis. Accordingly, the regulatory effect of A20 on RIPK1 activity was investigated in this study. The data showed that the protein level of A20 was decreased by a remarkable degree in MCAO mice, but the mRNA level was maintained. It was speculated that the degradation of A20 protein might be a key cause of vascular endothelial cell death in MCAO mice. This hypothesis was tested in A20-EKD mice constructed with an AAV system (AAV-ShA20). The results confirmed that the degradation of A20 may play an essential role in the initiation of RIPK1-dependent apoptosis, resulting in endothelial cell death and BBB destruction.

In addition, the proteomic study also revealed that the protein p62, a substrate protein in autophagy, was significantly downregulated in MCAO mice, suggesting the activation of the autophagy system. It was reported that ischemia and reperfusion stimulated both autophagy and the ubiquitin proteasome system, two major intracellular degradation systems. The inhibition of the ubiquitin proteasome system can reduce the progression of ischemia–reperfusion injury, but the effect of its inhibition on autophagy has seldom been studied. It was presumed that inhibiting autophagy in CIRI might help ameliorate the destruction of the BBB.

Conclusion

The findings in this study demonstrated that the death of vascular endothelial cells induced by ischemic reperfusion was mediated mainly by the activation of RIPK1. The blood–brain barrier leakage and infarct volume of RIPK1 mutant mice (*Ripk1*^{D138N}) were significantly reduced compared to those of wild-type mice. RIPK1 is the main protein that mediates necroptosis; however, it was found that mice without the capacity for necroptosis (*Ripk3*$^{-/-}$) did not show the same high survival rate as RIPK1 kinase mutant mice (*Ripk1*^{D138N}). Additionally, the death of vascular endothelial cells in *Ripk3*$^{-/-}$ mice was not significantly improved, indicating that RIPK1-mediated programmed necrosis was not the main cause of blood–brain barrier injury.

Further investigations in this study revealed that RIPK1-dependent apoptosis might be responsible for the endothelial cell death that leads to BBB leakage after MCAO treatment. A20 was decreased during cerebral ischemia–reperfusion injury, and A20 acted as a key regulatory protein that inhibited RIPK1 and RIPK1-dependent apoptosis (RDA). The decrease in A20 may be related to degradation during autophagy, and autophagy is known to be activated by CIRI.

Owing to the crucial physiological functions of the BBB, there is an urgent demand for the development of therapeutic strategies to prevent blood–brain barrier dysfunction (i.e., provide vascular protection) in

ischemia and reperfusion. The autophagy system may undergo a distinct set of regulatory changes in response to CIRI.

Abbreviations

Middle-Cerebral-Artery Occlusion (MCAO), Cerebral Ischemia Reperfusion Injury (CIRI), Delayed Neuron Death (DND), blood brain barrier (BBB)

Declarations

Acknowledgements

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Ethics approval and consent to participate

All animal experiments were approved by the General Hospital of Tianjin Medical University and performed following the Guide for the Care and Use of Laboratory Animals and Stroke Treatment.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare.

Data availability

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

Contributions

All authors contributed to the study, conception, and design. Material preparation, data collection were performed by Ying Li, Chaonan Yang and Yuting Yang. The MCAO model mice were constructed by Huijie Li and Xiaohui Wu. Data analysis were performed by Ying Li and Min Gong. All authors read and approved the final manuscript.

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Figures

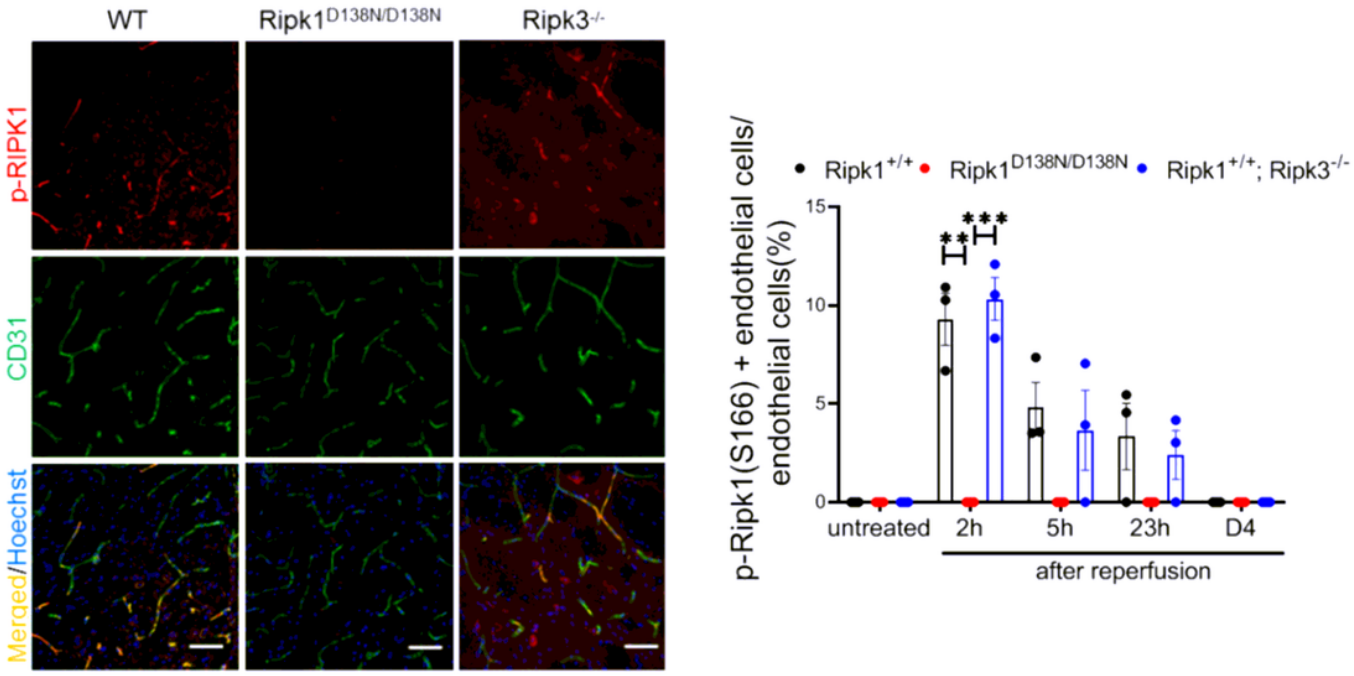

Figure 1

RIPK1 was activated by ischemic reperfusion in vascular endothelial cells of the blood–brain barrier. RIPK1 was significantly activated (p-Ser166) in vascular endothelial cells (p-S166). However, its activity was inhibited completely in the Ripk1D138N mice. Conditions: After MCAO/R, the brain tissues of wild-type mice, Ripk1D138N mutant mice and Ripk3-/- knockout mice were sectioned and stained. CD31 was detected as a specific vascular endothelial cell marker protein. Scale bar 50 μm.

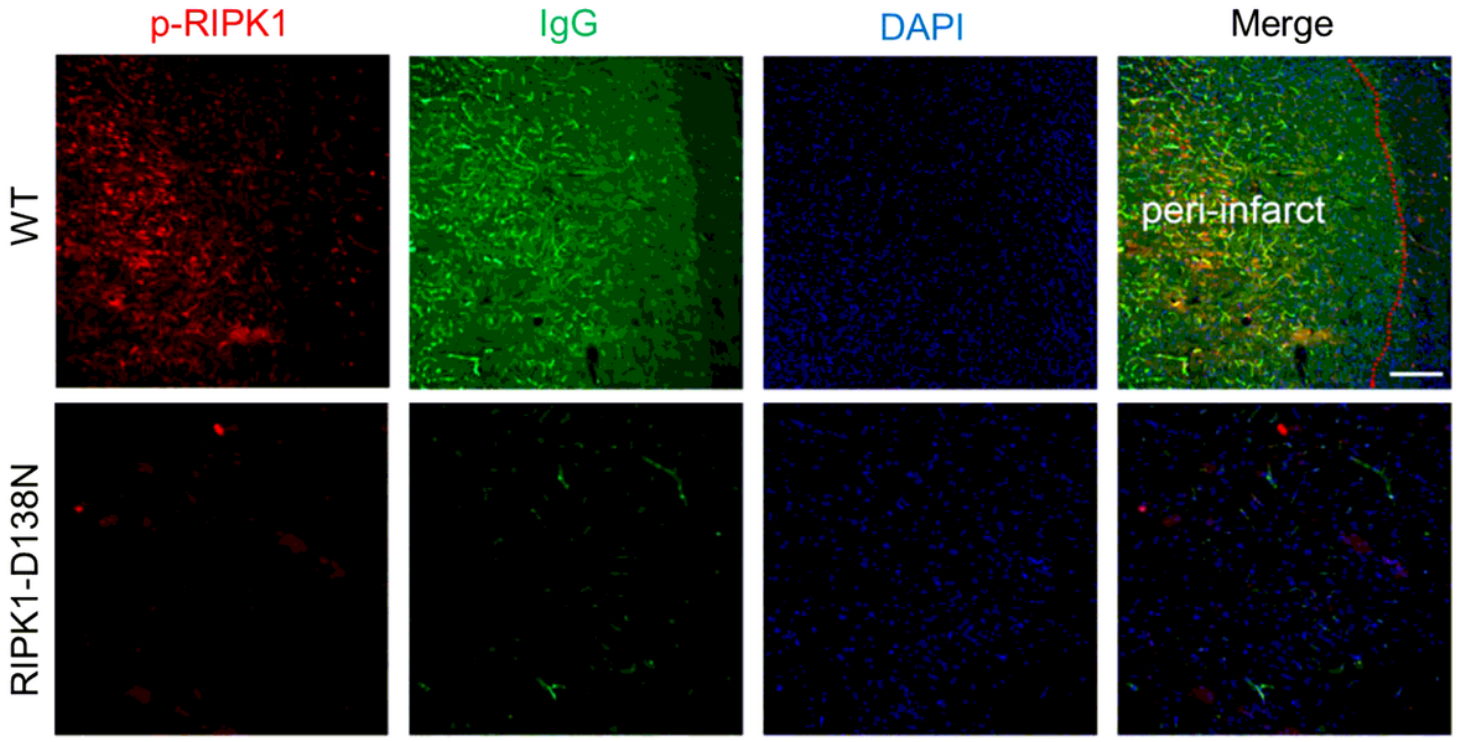

Figure 2

Inhibition of RIPK1 activity retarded blood–brain barrier leakage. The BBB was destroyed in wild-type mice after MCAO treatment (UP); however, it showed significantly increased tolerance to ischemia–reperfusion injury in mutant mice (Ripk1D138N). Conditions: After the MCAO/R experiment (3 days), the penetration of IgG in the brain was measured to evaluate BBB permeability.

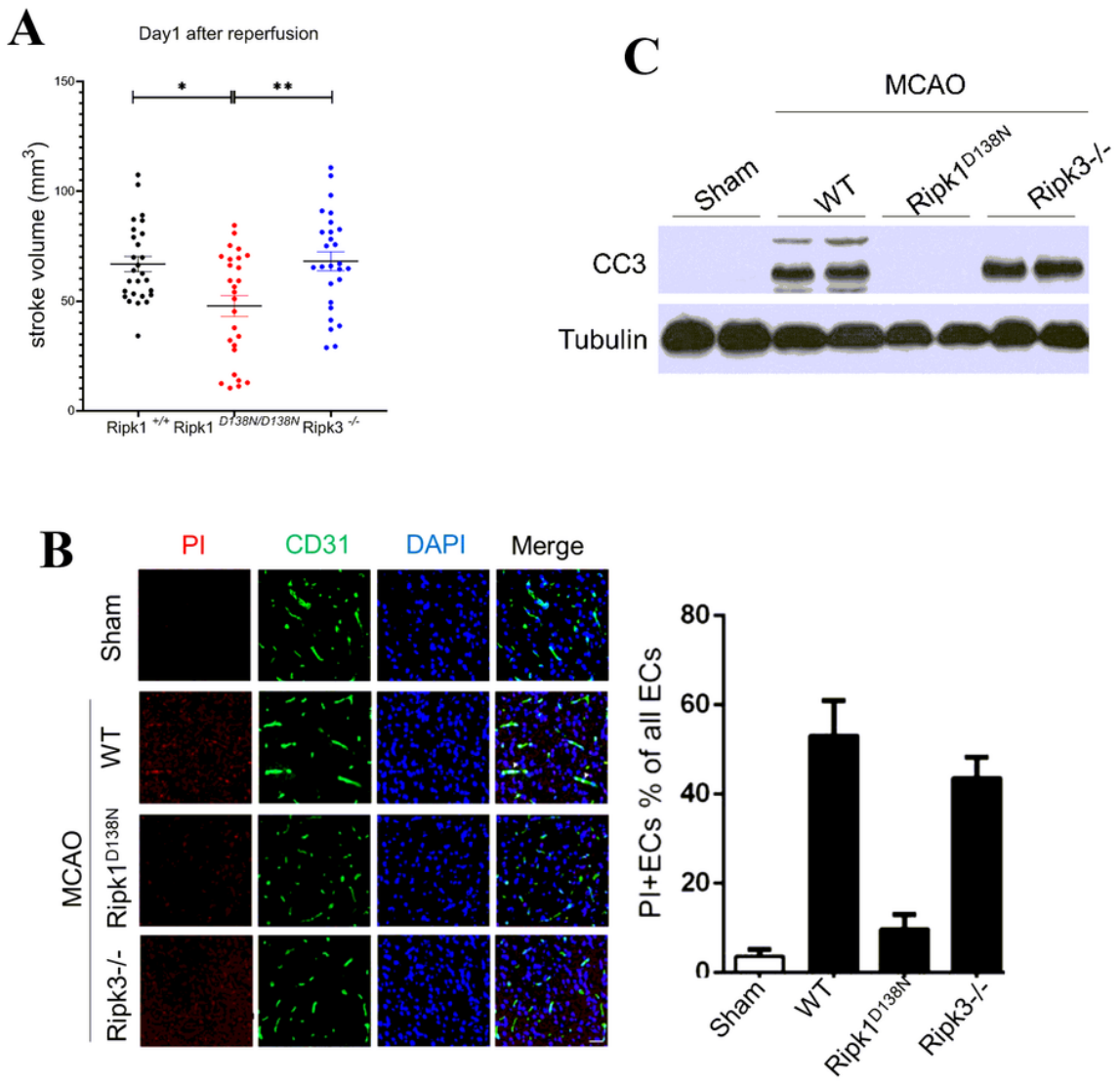

Figure 3

MCAO in RIPK3 knockout mice (Ripk3-/-). Panel A. Effect of RIPK3 knockout on cerebral infarction volume The results showed that Ripk3 gene knockout did not reduce the volume of cerebral infarction as Ripk1 mutation did (Ripk1D138N in Figure 1), suggesting that RIPK3 may play a limited role in ischemia–reperfusion injury. Conditions: After MCAO/R, the brains of mice were sectioned and stained with TTC. The infarct volume of mouse brains after TTC staining was quantified (n = 27). Panel B. BBB permeability in various mice upon MCAO treatment Images show the results of CD31 (green) and PI (red) staining in brain sections from MCAO-treated mice. The results indicated that Ripk3 knockout only partially inhibited endothelial cell death, in contrast to Ripk1D138N. Panel C. Effect of RIPK3 on the apoptosis of vascular endothelial cells The level of cleaved caspase 3 (CC3) in the vascular endothelial cells of Ripk1D138N mice was significantly decreased; however, the protein level of CC3 was maintained in Ripk3-/- mice. The results suggested that the apoptosis of BBB vascular endothelial cells in ischemic reperfusion is mainly caused by RIPK1-activated apoptosis rather than programmed necrosis. Conditions: Blood vessels from the brains of MCAO mice were isolated and ground, and the protein levels of CC3 were measured by Western blotting.

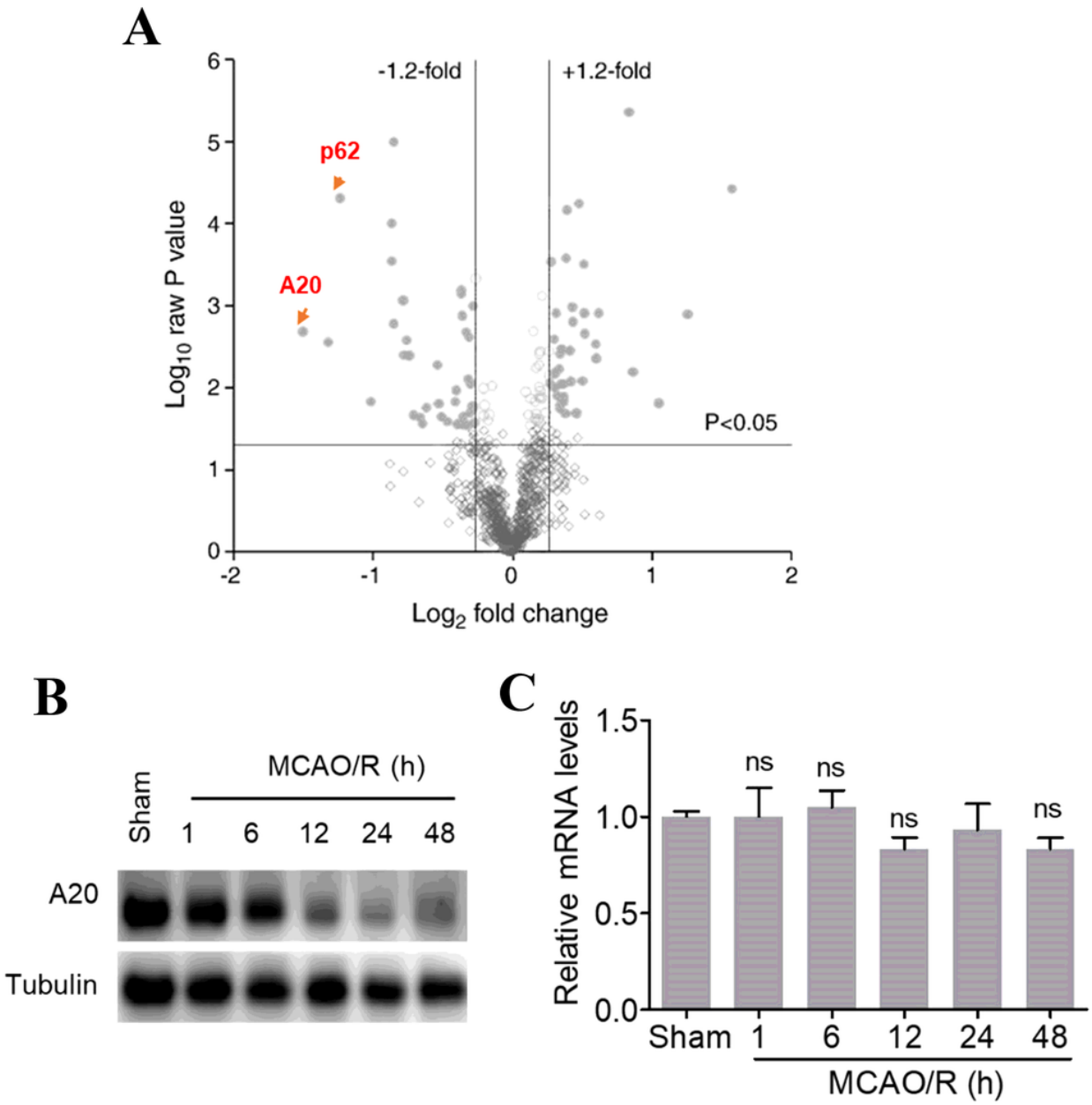

Figure 4

Investigation of the molecular mechanism of RDA in vascular endothelial cells. Panel A. Volcano plot of BBB vascular endothelial cells after MCAO treatment The volcano plot of protein mass spectrometry showed that protein A20 in vascular endothelial cells was significantly downregulated after MCAO (n = 3). Panel B. Protein level of A20 in vascular endothelial cells after MCAO treatment The results indicated that the protein level of A20 was remarkably downregulated after MCAO treatment. Conditions: After two hours of MCAO and different durations of reperfusion, the brain tissue was ground and assessed by

Western blotting. Panel C. mRNA level of A20 in vascular endothelial cells after MCAO treatment The results indicated that the mRNA level of A20 was retained during the process of MCAO. Conditions: After two hours of MCAO and different durations of reperfusion, brain tissues were collected and ground to measure the levels of A20 mRNA using qPCR. The results suggested that the decrease in A20 levels may be the key cause of RDA in vascular endothelial cells in ischemia–reperfusion injury.

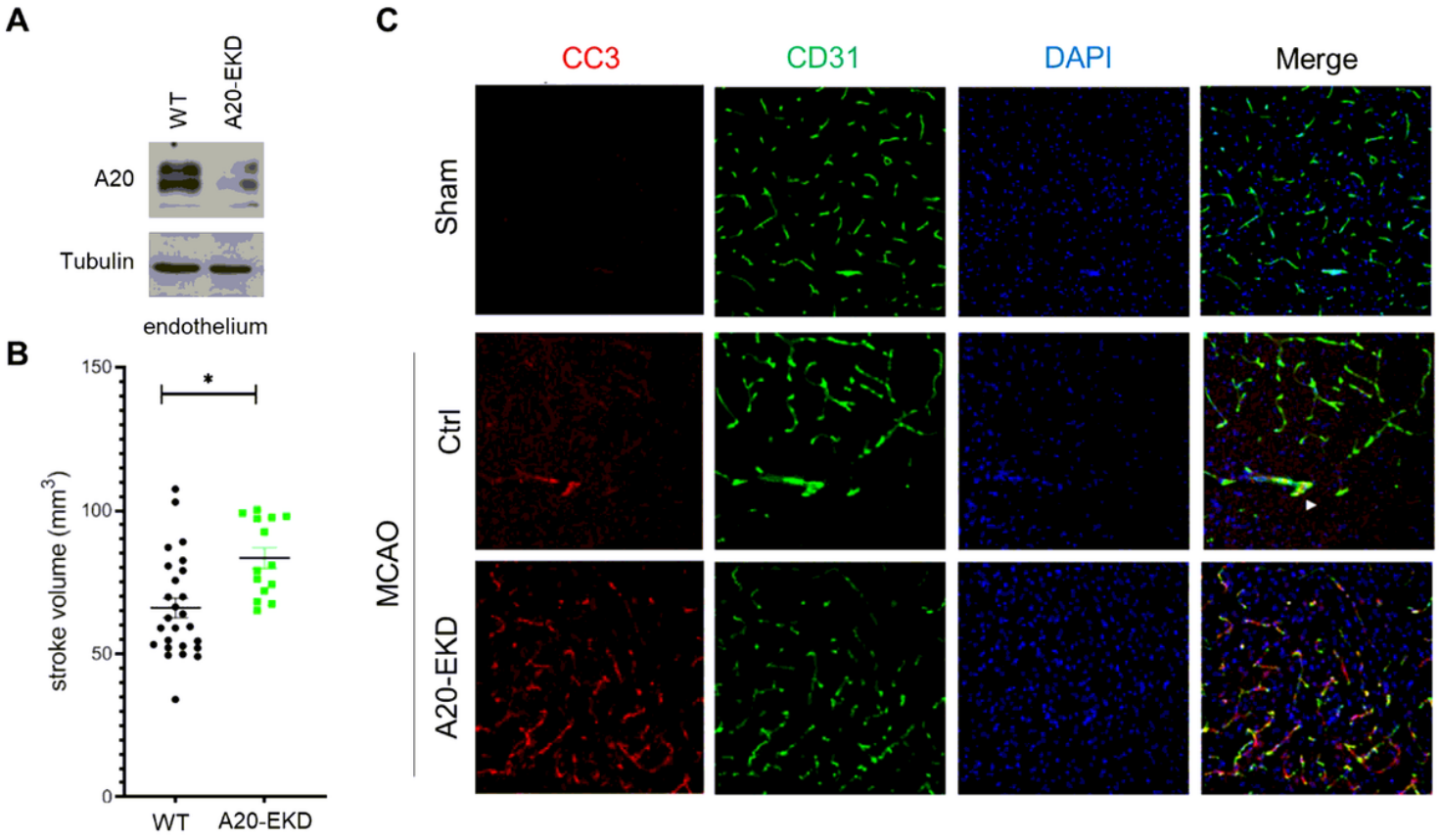

Figure 5

A20 knockdown enhanced RIPK1-dependent apoptosis in vascular endothelial cells after MCAO treatment. Panel A. Vascular endothelial cells (VECs) were isolated from brain tissues of wild-type and A20-EKD mice. The expression of A20 was measured by Western blotting. Panel B. After MCAO/R, the brains of wild-type and A20-EKD knockdown mice were sectioned and stained with TTC. T2-weighted imaging was used to measure the infarct volume (WT, n = 26; A20-EKD, n = 14). Panel C. Different RDA features in wild-type and A20 knockdown mice. CC3 immunofluorescence staining was performed on the brain tissue of mice after 24 hours of MCAO/R. A20 gene knockdown mice (A20-EKD) were constructed by adenovirus transfection (AAV-shA20) (Panel A). The volume of cerebral infarction during ischemia–reperfusion injury was increased in mutant mice (Panel B). In addition, the knockdown of A20 significantly enhanced apoptosis in vascular endothelial cells after MCAO treatment. The results suggested that the degradation of A20 protein may be the dominant mediator of RDA.

Supplementary Files

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