Temporal alterations in pericytes at the acute phase of ischemia/reperfusion in the mouse brain

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Abstract

Pericytes, as the mural cells surrounding the microvasculature, play a critical role in the regulation of microcirculation; however, how these cells respond to ischemic stroke remains unclear. To determine the temporal alterations in pericytes after ischemia/reperfusion, we used the 1-hour middle cerebral artery occlusion model, which was examined at 2, 12, and 24 hours after reperfusion. Our results showed that in the reperfused regions, the cerebral blood flow decreased and the infarct volume increased with time. Furthermore, the pericytes in the infarct regions contracted and acted on the vascular endothelial cells within 24 hours after reperfusion. These effects may result in incomplete microcirculation reperfusion and a gradual worsening trend with time in the acute phase. These findings provide strong evidence for explaining the "no-reflow" phenomenon that occurs after recanalization in clinical practice.

Key Words: acute ischemic stroke; alpha-smooth muscle; cerebral blood flow; microcirculation; no-reflow phenomenon; pericytes; platelet-derived growth factor receptor beta; vascular endothelial cells

Introduction

Ischemic stroke is a serious disease of the central nervous system (CNS) causing high morbidity and mortality (Benjamin et al., 2017). Although our understanding of the pathological alterations in ischemic stroke, such as neuronal degeneration and glial activation, has progressed, the pathological impact of ischemic stroke remains poorly understood. Recent stroke research has shifted the focus from the neuron centered view to the microvascular system. Increasing evidence suggests that successful neuroprotection depends on microvascular protection. In addition to the recognized role of astrocytes and microglia in the pathogenesis of stroke, pericytes play an important role in the progression and recovery after stroke (Gautam and Yao, 2018; Wen et al., 2021). It is increasingly recognized that neuroprotection cannot be achieved without the protection of microvessels.

In the CNS, pericytes account for the highest proportion of cells (ElAli et al., 2014; Courtney and Sutherland, 2020). However, a recent in-depth exploration of pericytes provided further insights into the study of microcirculation regulation (Dalkara et al., 2019). "No-reflow", a typical phenomenon that occurs with ischemic stroke, which has been observed depends on microvascular protection. Several studies (Peppiatt et al., 2006; Yemisci et al., 2009) made outstanding contributions to the study of the capillary contracting effect of pericytes on the phenomenon of no-reflow. Their contractility is mediated by a unique cytoskeletal organization formed by filaments of actin that allows pericyte deformability with the consequent mechanical force transferred to the extracellular matrix to change the diameter of the vessel (Yemisci et al., 2009; Hall et al., 2014; Alarcon-Martinez et al., 2019; Kureli et al., 2020).

Pericytes are pialeral cells that are mainly located on the microvessels, which are distributed on the precapillary arterioles, capillaries, and postcapillary venules (van Dijk et al., 2015). As observed in the CNS phenotype (Armulik et al., 2005, 2011), pericytes extend around the capillaries, mostly in the circumferential direction on the arteriole side and branch points of the capillary bed, and more longitudinally in the middle of the capillary bed, and they have a stellate shape on the venule side (Hartmann et al., 2015).

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Identification of pericyte biochemical markers is of great significance to the field of cerebrovascular biology. It is difficult to identify pericytes with molecular markers because of the lack of specific pericyte markers. In general, the vascular smooth muscle cells and pericytes both rely on the expression of at least two markers (Yang et al., 2017). One of the classic markers used for pericyte identification is alpha-smooth muscle actin (α-SMA), whose expression is related to the pericyte regulatory function of capillary blood flow (van Bruggen et al., 2008). Another marker, pericyte receptor beta (PDGFR-β), is another common marker of pericytes. It is widely expressed on pericyte surfaces and is necessary for pericyte proliferation and survival (Armulik et al., 2011). After ligand binding and receptor dimerization, phosphorylated tyrosine in the intracellular domain of PDGFR-β recruits scaffold proteins to induce multiple signaling pathways (Dubrac et al., 2018).

In view of these findings, 2,3,5-triphenyltetrazolium chloride (TTC) staining and laser speckle measurement of cerebral blood flow (CBF) were conducted in this study to evaluate the changes in cerebral infarction volume and CBF, respectively, after cerebral ischemia/reperfusion (Li et al., 2018; Trojan-Marcus et al., 2020; Zhang et al., 2020). Fluorescence histochemistry and immunohistochemistry were used to analyze the expression changes in pericytes and endothelial cells after cerebral I/R. Here, PDGFR-β, α-SMA, and CD31 were selected to label pericytes and endothelial cells in the microvasculature (Sweeney et al., 2016; Grant et al., 2019). Through this study, we hope to characterize the expression changes in the relationship between pericyte contraction of microvessels and reperfusion time after early stroke from the perspective of histochemistry, which may contribute to a better understanding of the role of pericytes in blocking ischemic tissue reperfusion. Furthermore, we attempted to classify the diversity of mural cell phenotypes. By staining α-SMA and PDGFR-β, distinct pericyte morphologies were observed and classified in the mouse brain.

Materials and Methods

Ethics statement

This study was approved by the Animal Care Committee, Beijing University of Traditional Chinese Medicine, China (approval No. BUCM-4 20191015074007) on October 15, 2019. All experiments related to the operation were performed in accordance with the National Institutes of Health Laboratory Animal Care Methods and Use Guidelines.

Animals

Generally, male mice have better physical health indicators than female mice (Gibson et al., 2006), thus 48 male C57BL/6 mice (6–8 weeks old, weighing 22–25 g, specific pathogen free level) were purchased from Sibeifu (Beijing) Laboratory Animal Breeding Co., Ltd. (Beijing, China). All experimental operations were performed under the same conditions between 9:00 am and 6:00 pm. All mice were kept under a 12-hour light/dark cycle with controlled temperature and humidity and had free access to food and water. Except for the occlusion of the middle cerebral artery. After the operation, the brain was carefully dissected after perfusion, fixed with 4% paraformaldehyde and clamped with microvascular arterial clamps. A sensory monofilament was placed on the skull to measure CBF. At 2 (hyperacute phase), 12, and 24 hours (acute phase) after I/R, one speckle contrast image was calculated for every 100 ms using a laser speckle imager. After the operation, the ischemic hemisphere was homogenized in radioimmunoprecipitation assay (RIPA) buffer (Shanghai Sangon, China) and then incubated with 2% TTC solution at 37°C for 20–30 minutes. Subsequently, the brain slices of each group were imaged and the photos were analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Bethesda, MD, USA). The infarct volume is expressed as a percentage of total infarct volume/total brain volume.

Fluorescence immunohistochemistry

Fluorescence immunohistochemistry was used to examine colocalization of PDGFR-β and α-SMA, PDGFR-β and CD31 (a marker for endothelial cells in the microvasculature), and PDGFR-β and CD31 in brain capillaries and pericytes. The sections were washed 3 times with 0.1 M phosphate buffer (pH 7.4), and then incubated with the corresponding secondary antibodies and 4,6-diamidino-2-phenylindole (1:50,000; Molecular Probes, Eugene, OR, USA) solution for 1.5 hours at room temperature. The secondary antibodies were: donkey anti-rabbit Alexa Fluor 594 (1:1500; Thermo Fisher Scientific, Waltham, MA, USA), goat anti-α-SMA (1:1500; Abcam, Cat# ab7817, RRID: AB_262054), and goat anti-CD31 (1:1000; RD, St. Louis, MO, USA, Cat# AF6326, RRID: AB_2161028).

Western blot assay

The ischemic hemisphere was homogenized in radioimmunoprecipitation assay (RIPA) buffer (Shanghai Sangon, China) and clarified by centrifugation (12,000 × g, 15 minutes, 4°C). The protein concentration was determined using a bichinchoninic acid protein quantification kit (Cat# CW0014, CWbio, Beijing, China). Equal amounts of protein were then resolved in 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, and then transferred to polyvinylidene fluoride membranes (Millipore Corporation, Billerica, MA, USA). The membranes were blocked for 1 hour with 5% nonfat dry milk at room temperature and then incubated overnight with the primary antibodies rabbit anti-PDGFR-β (1:1000; Abcam, Cat# ab32570, RRID: AB_777165), mouse anti-α-SMA (1:1500; Abcam, Cat# ab7817, RRID: AB_262054) at 4°C. After three washes, the membranes were incubated for 1 hour in the secondary antibody (1:5000; Proteintech, Cat# SA00001-1, RRID: AB_2722565) and goat anti-rabbit IgG (H+L) (1:5000; Proteintech, Cat# SA00001-2, RRID: AB_2722564), respectively. The signal was visualized with high-sensitivity enhanced chemiluminescence luminous liquid and the images were captured using an Azure Bioimaging system (Aureo C500, Azure Biosystems, Dublin, Ireland). The protein bands' intensities were analyzed using the Image-Pro Plus 6.0 software and normalized to the value of the corresponding tubulin (mouse, 1:5000; Abcam, Cat# ab8226, RRID: AB_306371) as an internal control.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). One-way analysis of variance followed by least significant difference test was used to decide the significance of differences among multiple independent samples. Differences were considered statistically significant at P < 0.05.
Results

Temporal alterations in CBF and infarct volume after cerebral I/R

The changes in the CBF before and after the operation were determined in all mice by laser speckle contrast imaging, which showed that the MCAO induced a significant CBF decline (28.63 ± 2.07% of the baseline, Figure 1A–F). The CBF after 2 hours of I/R (I/R 2 h) was significantly increased compared with that during the MCAO (65.09 ± 2.38% of the baseline, Figure 1F), however, it gradually decreased after 12 and 24 hours, returning to the level during the MCAO (Figure 1A–H). Furthermore, the brain infarct volume was significantly increased in the I/R groups relative to the sham group (I/R 2 h and 12 h: P < 0.01; I/R 24 h: P < 0.001), and the severity of ischemia positively correlated with the reperfusion time, with the highest severity observed after 24 hours of reperfusion (r = 0.9852, P < 0.0001; Figure 1I, J and J1).

![Figure 1](image1.png)

Figure 1 | The level of perfusion deficits increases with time after ischemia/reperfusion.

(A–E) Representative CBF pseudocolor images in the ischemic cortex before and during the MCAO, and 2, 12, and 24 hours after ischemia/reperfusion. The color bar represents the CBF capacity (in perfusion units). As the reperfusion time increased, the CBF decreased in the relative CBF in the region of interest (ROI); % of the baseline of each animal group. (G, H) CBVs in contralateral and ipsilateral ROI before and during MCAO, and 2, 12, and 24 hours after ischemia/reperfusion. (J) Representative images of 2,3,5-triphenyltetrazolium chloride (TTC)-stained brain slices. The volume of cerebral infarction (white) increased with the prolongation of reperfusion time. (J) Quantitative analysis of cerebral infarct volume. Data are reported as mean ± SEM (n = 6 per group). **P < 0.01, vs. pre-MCAO; ##P < 0.05, ###P < 0.01, vs. during MCAO; ****P < 0.001 (one-way analysis of variance followed by least significant difference test). CBF: Cerebral blood flow; MCAO: Middle cerebral artery occlusion; ROI: region of interest.

Selection of the observation sites and successful marking of pericytes

Initially, we planned to focus on the cortex-penetrating arterioles and the branches from the epithelial layer to the middle cortex because in our previous study, the in vivo two-photon imaging study of CBF focused on this area of the brain (Zhang et al., 2020). In view of the locations of cerebral infarction we observed, we decided to examine the cortex and hippocampus.
Three-dimensional reconstruction shows the spatial relationship between the expression of the pericyte marker α-SMA at different time points after cerebral ischemia/reperfusion. In previous studies of neurovascular units, pericytes have been shown to play a crucial role in maintaining blood-brain barrier integrity and modulation of angiogenesis. These functions depend on appropriate interactions and signaling between pericytes and other cells at the blood-brain barrier, especially endothelial cells (Huang, 2016; Caporarello et al., 2019). Pericytes and endothelial cells form numerous direct contacts, including peg-socket contact, adhesion plaques, N-cadherin junctions, and gap junctions (Gautam and Yao, 2018). CNS pericytes exert a significant influence on terminal arterioles, capillaries, and postcapillary venules (Attwell et al., 2016; Sweeney et al., 2016; Zheng et al., 2021), where they make specific focal contacts with the endothelium and localize membrane of blood microvessels.

Pericytes are closely related to cerebral capillaries. Microvessels of the CNS have the highest pericyte coverage of any organ. Pericytes are vascular mural cells embedded within the vascular basement membrane of blood microvessels (Sweeney et al., 2016; Zheng et al., 2021), where they make specific focal contacts with the endothelium and localize to terminal arterioles, capillaries, and postcapillary venules (Attwell et al., 2016; Caporarello et al., 2019). Pericytes and endothelial cells form numerous direct contacts, including peg-socket contact, adhesion plaques, N-cadherin junctions, and gap junctions (Gautam and Yao, 2018). CNS pericytes exert a large variety of functions, including CBF regulation, maintenance of the blood-brain barrier integrity, and modulation of angiogenesis. These functions depend on appropriate interactions and signaling between pericytes and other cells at the blood-brain barrier, especially endothelial cells (Huang, 2020). We used pericycle markers and a microvascular marker and found that a large number of pericytes were expressed in the periphery of the vascular endothelium. In previous studies of neurovascular units, pericytes have been shown to be closely related to cerebral capillaries.

Figure 4 | The expression of the pericycle marker α-SMA at different time points after cerebral ischemia/reperfusion.

Figure 5 | Three-dimensional reconstruction shows the spatial relationship between PDGFR-β and α-SMA expression and the capillaries.

Figure 6 | Changes in the expression of the pericycle marker PDGFR-β at different time points after cerebral ischemia/reperfusion.

Discussion

In the present study, we demonstrated the pathological changes in mouse brain CBF and cerebral infarction at different time points after cerebral I/R. Taking advantage of this experimental model, we showed the morphology and the changes in different pericycle markers in normal brain tissue and after cerebral I/R (Figure 7). The contraction of pericytes plays an important role in the restoration of CBF. Thus, pericytes have become a promising target for the treatment of ischemic stroke.

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neglected for a long time. Furthermore, in previous studies of stroke, our observations of microvessels have increasingly focused on pericytes (Yang et al., 2017; Dalkara et al., 2019). Therefore, it is of great significance to study the relationship between pericytes and the CNS under physiological and pathological conditions.

Pericyte labeling and its limitations

While pericytes have many important functions in the CNS vasculature, their involvement in the regulation of CBF remains controversial. The morphological and genetic markers of pericytes compared with smooth muscle cells are not exclusive. Several markers have been used to identify pericytes, including α-SMA, desmin, NG-2, PDGFR-β, and CD13 (Smyth et al., 2018; Uemura et al., 2020). Currently, there is no single molecular marker that can be used to unequivocally and exclusively label pericytes (Costa et al., 2017). Pericytes are heterogeneous regarding their distribution, phenotype, marker expression, origin, and function (Senà et al., 2018), and their expression is dynamic. For example, α-SMA is not appreciably expressed by CNS pericytes under normal circumstances (Ozerdem and Stallcup, 2003). Furthermore, the claim that normally there is no detectable α-SMA in pericytes contradicts both early histology studies (Herman and D’Amore, 1985) and the observations that pericytes can constrict capillaries (Hall et al., 2014; Rungta et al., 2018). This may be related to the labeling method (Alarcon-Martinez et al., 2018). Presently, the state-of-the-art identification of pericytes in tissue preparations relies on at least two pericyte molecular markers; therefore, we must keep in mind that unambiguous positive identification of pericytes is still a problem.

We used immunofluorescence to stain brain tissue sections to label and observe pericytes. However, pericyte contraction is a dynamic process, hence dynamic observation in vivo is a better way to observe pericyte contraction of capillaries. For example, a two-photon microscope was used to compare pericyte transgenic mice before and after modeling (Hall et al., 2014). This is a great inspiration for our future research on the relationship between pericytes and microcirculation.

The no-reflow phenomenon

The no-reflow phenomenon refers to the observation that when an organ is made ischemic by occlusion of a large artery supplying it, restoration of patency in that artery does not restore perfusion to the microvasculature supplying the parenchyma of that organ (Kloner et al., 2018; Haylkym et al., 2021). We showed that following 2 or more hours of ischemia, parts of the brain did not reperfuse after the proximal flow was restored. The size of perfusion deficits increased with longer periods of ischemia. These nonperfused brain regions were often interspersed within areas of perfusion and sometimes resulted in a mottled appearance. However, the contralateral flow was also decreased. When modeling cerebral ischemia on one side of a mouse brain, it will affect the overall CBF. Additionally, C57BL/6 mice have poor development of the posterior communicating arteries that connect the carotid artery and the vertebrobasilar arterial system in the brain, thus we speculated that this is the cause of the decrease in the contralateral CBF. In our previous research (Ma et al., 2019), the cerebral I/R injury that resulted in blood-brain barrier leakage caused an increase in brain water content and brain edema in the ipsilateral hemisphere. Hence, we think that the edema of the brain tissue on the ischemic side had a squeezing and compressive effect on the contralateral side, causing different degrees of CBF decline in the contralateral brain tissue. Brain capillary pericytes have been suggested to play a role in the regulation of CBF under physiological and pathophysiological conditions (Peppiatt et al., 2006). Furthermore, pericytes have been shown to cause capillary constriction under ischemic conditions, and thus have been suggested to be involved in the “no-reflow” phenomenon (Yemisci et al., 2009; Hall et al., 2014; Zagrean et al., 2018; Uemura et al., 2020). Other studies suggest that swelling of glial cells, increased blood viscosity, and erythrocyte aggregation are the main culprits in causing no-reflow in the brain (Chiang et al., 1968). Although pericytes are involved in the no-reflow phenomenon, the molecular and cellular mechanisms involved in this process remain to be examined.

The significance of the expression of the two pericyte markers PDGFR-β and α-SMA

We subcategorized the pericytes into two groups based on the appearance of two markers: mesh and thin-stranded pericytes, with the latter being the canonical pericyte type, as all cells examined in microvessels had protruding ovoid cell bodies, a feature historically used to define pericytes in the brain and other organs (Kisler et al., 2017). Mesh pericytes are α-SMA-positive, and thin-stranded pericytes were PDGFR-β-positive. In contrast to the normal, the pericytes in the ischemic area have a contraction effect on the capillaries, which is an important cause of the no-reflow phenomenon. PDGFR-β: Platelet-derived growth factor receptor beta; α-SMA: alpha-smooth muscle actin.
The western blot results showed that, slightly different from α-SMA, the protein expression of PDGFR-β in the model group was higher than that in the sham operation group, but there was no positive correlation with the reperfusion time. Therefore, we hypothesized that the shrinkage of pericytes positively correlates with the expression of α-SMA. It is uncertain whether over a long period of time, cerebral pericyte death in rigor occurs and contributes to a long-lasting reduction in blood flow (Hall et al., 2014), and whether preventing pericyte death would produce a greater restoration of flow (O’Farrell et al., 2017). To reduce no-reflow therapeutically by preventing pericyte constriction of capillaries, it is necessary to develop agents that act selectively on pericytes.

Conclusion
Our current results demonstrated that after stroke, ischemia causes pericytes to contract capillaries, and as the reperfusion time increases, the pericytes’ contractile force increases. Thus, this study provides more neurological information for evaluating the clinical no-reflow phenomenon after vascular recanalization, i.e., pericytes regulate CBF by changing the diameter of microvessels. Furthermore, we demonstrated that different pericyte biomarkers have different expression patterns.

Author contributions: Study conception and design: FFC, WZB; model establishment: ZJ, XL, HF3; histochemical staining and sample observation: ZJ, IW, YS, CYM, MW; data analysis: YZ, QGW, XQW; manuscript draft: ZJ, FFC, WZB. All authors read and approved the final manuscript.

Conflicts of interest: YZ is an employee of Shineway Pharmaceutical Group Ltd. No other actual or potential conflict of interest is declared.

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