Regulation of Caveolin-1 Expression Determines Early Brain Edema After Experimental Focal Cerebral Ischemia

Kang-Ho Choi, MD, PhD; Hyung-Seok Kim, MD, PhD*; Man-Seok Park, MD, PhD*; Joon-Tae Kim, MD, PhD; Ja-Hae Kim, MD; Kyung-Ah Cho, PhD; Min-Cheol Lee, MD, PhD; Hong-Joon Lee, PhD; Ki-Hyun Cho, MD, PhD

Background and Purpose—Most patients with cerebral infarction die of brain edema because of the breakdown of the blood–brain barrier (BBB) in ischemic tissue. Caveolins (a group of proteins) are key modulators of vascular permeability; however, a direct role of caveolin-1 (Cav-1) in the regulation of BBB permeability during ischemic injury has yet to be identified.

Methods—Cav-1 expression was measured by immunoblotting after photothermotic ischemia. A direct functional role of Cav-1 in cerebral edema and BBB permeability during cerebral ischemia was investigated by genetic manipulation (gene disruption and re-expression) of Cav-1 protein expression in mice.

Results—There was a significant correlation between the extent of BBB disruption and the Cav-1 expression. In Cav-1–deficient (Cav-1−/−) mice, the extent of BBB disruption after cerebral ischemia was increased compared with wild-type (Cav-1+/+) mice, whereas the increase in cerebral edema volume was ameliorated by lentiviral-mediated re-expression of Cav-1. Furthermore, Cav-1−/− mice had significantly higher degradation of tight junction proteins and proteolytic activity of matrix metalloproteinase than Cav-1+/+ mice. Conversely, re-expression of Cav-1 in Cav-1−/− mice restored tight junction protein expression and reduced matrix metalloproteinase proteolytic activity.

Conclusions—These results indicate that Cav-1 is a critical determinant of BBB permeability. Strategies for regulating Cav-1 represent a novel therapeutic approach to controlling BBB disruption and subsequent neurological deterioration during cerebral ischemia. (Stroke. 2016;47:1336-1343. DOI: 10.1161/STROKEAHA.116.013205.)

Key Words: blood-brain barrier ■ brain edema ■ brain ischemia ■ caveolin-1 ■ tight junction proteins

Stroke is one of the leading causes of death and disability worldwide. Although research aimed at cellular mechanisms of ischemic injury has been a mainstay in the field, cell death is not a direct cause of patient death in the acute phase. Rather, most patients who have a cerebral infarction die of brain edema and hemorrhagic transformation in ischemic brain tissues.1,2 Brain edema ultimately causes enlargement of the infarction after ischemic stroke and clinical deterioration via a mass effect.3 Hemorrhagic transformation is a well-recognized complication that limits the use of or negates the effects of thrombolytic treatment and occasionally results in death.4

Both vasogenic cerebral edema and hemorrhagic transformation are consequences of damage to the blood–brain barrier (BBB).5 Thus, vascular integrity represents an attractive target for the effective treatment of cerebral ischemia. Nonetheless, therapeutic options for manipulating BBB integrity in the clinical setting are limited. Understanding the molecular mechanism(s) underlying BBB breakdown in cerebral ischemia could significantly affect the clinical management and prognosis of stroke. An important direction in the field of cerebral ischemia research is identifying strategies for protecting the BBB against damage and avoiding edema.

Recent studies have shown that caveolins (a group of proteins) are key modulators of vascular permeability.6–11 This family of integral membrane proteins comprises the principal components of caveolar membranes; they function as scaffolding proteins within the caveolar membrane that compartmentalize and concentrate signaling molecules.12–14 Caveolin proteins serve as both positive and negative regulators of intracellular signaling.15–17 Their role in cerebral ischemic injury and BBB dysfunction is controversial and remains largely unknown. To date, a direct
role of Cav-1 in cerebral edema after ischemic insult in mice lacking and re-expressing the Cav-1 gene remains to be demonstrated. The aim of this study was to investigate brain edema and BBB permeability during cerebral ischemia in Cav-1–deficient (Cav-1−/−) mice with and without Cav-1 re-expression.

Materials and Methods

Animals

All animal protocols were carried out in accordance with the Chonnam National University guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Chonnam National University (permit number: 2012-27). All experiments were carried out in accordance with the guidelines laid down by the National Institutes of Health (Bethesda, MD) about the care and use of animals for experimental procedures. Animals were maintained on a 12-hour light/dark cycle and allowed free access to food and water. Cav-1−/− mice (a kind gift from Dr K.A. Cho, Department of Biochemistry and Molecular Biology, Chonnam National College of Medicine, Gwangju, Korea) and control wild-type (Cav-1+/+) littermates were generated through heterozygous mating and housed in a local facility. All procedures were approved by the local authorities in accordance with National Animal Care regulations.

Lentiviral Vector and Transfection

The recombinant lentivirus was produced by Macrogen Lentivector Institute (Seoul, Korea). To construct red fluorescence protein–tagged Cav-1, full-length Cav-1 cDNA was amplified from a human cDNA library and fused at its C-terminus with sequences encoding monomeric red fluorescence protein. Briefly, the amplified Cav-1 fragment was digested with BamHI and inserted into the lentivector, Lenti-hCMV-IRESpuro, which contains the mouse cytomegalovirus (CMV) promoter, using the internal ribosome entry site (IRES) system. The resultant Cav-1–red fluorescence protein gene fusion was validated by nucleotide sequencing. Culture supernant containing viral particles was harvested 48 hours after transfection, clarified through a 0.45-mm membrane filter (Nalgene, Rochester, NY), and stored at −70°C until use. Transfection efficiency, which was assessed by fluorescence imaging and immunoblotting, was ≈80%, respective of the amount of plasmid used in the transfection. A nonspecific plasmid encoding β-galactosidase was used to maintain identical amounts of DNA in each transfection.

To evaluate the effect of Cav-1 re-expression in vivo, endothelial cells (ECs) obtained from the cerebral artery of normal BALB/c mice were transduced with the lentiviral Cav-1 expression vector (Lenti-hCMV-IRESpuro) and then transplanted 5×10^5 cells intravenously into the femoral vein of Cav-1 knockout mice. This cell dose was chosen because we confirmed with optical bioluminescence imaging that systemic injection with 5×10^5 cells is sufficient to target the hypoxic infarcted brain. All mice used in these studies were of the C57BL/6J genetic background.

Surgical Procedures

Focal cortical ischemia was induced by photothrombosis of the cortical microvessels using Rose Bengal (Sigma Chemical Co, St. Louis, MO) with cold light (Zeiss KL1500 LCD, Germany). Each animal was anesthetized with 5% isoflurane and maintained with 2% isoflurane in an oxygen/air mixture using a gas anesthesia mask in a stereotaxic frame (Stoelting, Wood Dale, IL). Body temperature was maintained during surgery at 37±0.5°C using a heating pad controlled by a rectal probe. For illumination, a 4.5-mm fiber-optic bundle from a cold light source was positioned onto the exposed skull 0.5 mm anterior to bregma and 3.7 mm lateral to the midline over the left sensorimotor cortex, as previously described. The brain was illuminated for 10 minutes after infusion of 50 mg/kg Rose Bengal in normal saline into the right femoral vein via a microinjection pump within 1 minute. The scalp was sutured, and the mice were allowed to wake before being returned to their home cages. For the sham surgery, 12 animals received illumination after infusion of normal saline instead of Rose Bengal.

Measurement of BBB Integrity

Vascular permeability was assessed by measuring extravasation of Evan blue (EB) by quantitative fluorescence. BBB integrity was assessed 6, 12, 24, and 72 hours after ischemia (n=3 per time point) using EB dye as described previously. Fluorescence intensity of EB in the supernatants was determined using a fluorescent microplate reader. Extravasated EB dye is expressed as micrograms per gram (μg/g) of brain tissue.

In Vivo Optical Imaging

To examine the feasibility of intravenous cell transplantation for the infarcted brain, we explored in vivo optical bioluminescence imaging using cells engineered to express a firefly luciferase gene. To assess the correlation between the cell number and the bioluminescent signal, increasing numbers of cells (5×10^3, 5×10^4, 5×10^5, 5×10^6, and 5×10^7 cells) were transplanted immediately after ischemia through femoral vein in Cav-1−/− mice (n=6 for each group).

Next, we determined the optimal time point of cell transplantation before ischemia in mice. It was defined as the time point of cell injection that led to the strongest light signal in the infarcted brain 12 hours after ischemic insult. On the basis of our preliminary experiments, 5×10^5 cells were injected intravenously 1, 3, 4, or 7 days before ischemia. Further details of the method of optical imaging are provided the online-only Data Supplement.

Gelatin Zymography

Gelatin zymography was performed as described previously to determine the effect of regulation of Cav-1 expression on matrix metalloproteinase (MMP) activity. To quantify the relative expression levels of MMP-9 and MMP-2 as detected by gelatin zymography, the gels were digitized, and the area of lysis for each band detected was quantified in square millimeter using ImageJ software (National Institutes of Health).

Statistical Analysis

Details of the method of statistical analysis are provided the online-only Data Supplement.

Results

BBB Disruption After Ischemic Injury

Temporal changes in BBB integrity in the injured cortex of photothrombotic mice were assessed by EB extravasation after focal ischemic insult. EB was undetectable in the nonischemic mouse brain. After an ischemic event, EB extravasation increased within 6 hours, peaked at 12 hours after insult in the ischemic hemisphere, and then gradually decreased (Figure 1A and 1B).

Increased Cav-1 Protein Expression After Photothrombosis

Immunoblot analysis revealed marked increases in Cav-1 protein levels in the ischemic hemisphere of photothrombotic mice.
6, 12, 24, and 72 hours after ischemia (P<0.01; Figure 1C and 1D). Cav-1 expression rapidly increased after ischemic insult, with peak levels observed 12 hours after insult, as assessed by densitometry, followed by a gradual decrease (Figure 1D). Thus, Cav-1 expression paralleled BBB breakdown in mice after ischemic injury. In fact, there was a significant correlation between BBB disruption and Cav-1 expression (correlation coefficient=0.943; P=0.005, Spearman rank correlation analysis).

**Localization of Intravenously Administered Cells in the Infarcted Brain**

For selection of optimal cell dose, different numbers of cells were transplanted in mice. The percentage of mortality after cell injection was significantly different in the mice transplanted over 5×10^6 cells: 5×10^3 (0%), 5×10^4 (0%), 5×10^5 (0%), 5×10^6 (50%), and 5×10^7 (100%). We found a linear correlation between the number of cells reaching 5×10^5 and the bioluminescent signal. Thus, 5×10^5 cells were chosen as the optimal cell dose. The strongest light signal was obtained 3 days after transplantation across all groups (Figure 2A). Optical bioluminescence imaging obtained at 12 hours after ischemic insult revealed that the optimal time for cell transplantation was 3 days before ischemia (Figure 2B).

**Cav-1 Expression in the Ischemic Brain After Intravenous Transplantation of Cav-1−/− ECs**

To determine whether we could engineer Cav-1−/− mice to express ectopic Cav-1, ECs transfected with a Cav-1 lentiviral expression vector were transplanted into Cav-1−/− mice. As seen in Figure 3, Cav expression was restored in Cav-1−/− mice after transplantation. Importantly, the ischemic brains of transplanted mice showed increased levels of Cav-1 compared with Cav-1−/− mice (P<0.01; Figure 3B). Densitometric analysis confirmed that the transfection efficiency was sufficient for ectopic Cav-1 expression in the ischemic brain. There was no significant difference in Cav-1 expression levels between the brains of Cav-1+/+ and ReCav-1 mice (P=nonsignificant [ns]).

To determine whether restored Cav expression resulted from the effect of transfected ECs itself, ECs without Cav-1 transfection were transplanted into Cav-1−/− mice. As seen in Figure 3C, Cav expression was not restored in Cav-1−/− mice after transplantation. There was no significant difference in Cav-1 expression levels in the brains of Cav-1−/− and EC-transplanted mice (P=ns; Figure 3D).

**Regulation of Cav-1 Expression Determines Vasogenic Edema in Mice**

To determine the extent of BBB disruption, BBB permeability was quantified by EB extravasation. As shown in Figure 4A, brain edema in Cav-1−/− mice after ischemia seemed to be larger and cover a greater area than that in Cav-1+/+ mice. EB extravasation after ischemia was also significantly increased in Cav-1−/− mice (12.6±1.3 μg/g tissue) compared with the Cav-1+/+ group (7.9±0.7 μg/g tissue; P<0.01; Figure 4B). Notably, EB extravasation in ReCav-1 mice was significantly lower (9.3±0.7 μg/g tissue) 12 hours after ischemia than that in the Cav-1−/− group (P<0.01). There was an inverse relationship between Cav-1 expression and the extent of BBB disruption (correlation coefficient, 0.999; P=0.025, Spearman rank correlation analysis; Figures 3 and 4). There were no significant differences in BBB permeability between the brains of Cav-1+/+ and ReCav-1 mice at 6, 12, 24, and 72 hours after ischemia.

![Figure 1](http://stroke.ahajournals.org/)

**Figure 1.** Photograph (A) and quantification (B) of Evan blue extravasation in a mouse model of photothrombosis. Blood–brain barrier disruption peaked 12 hours after ischemic insult. C, Representative blot showing caveolin-1 (Cav-1) and β-actin expression in the cortex of sham-operated mice and at the lesion site 6, 12, 24, and 72 hours after ischemia. D, Quantification of Cav-1 expression. Cav-1 expression increased significantly 6, 12, 24, and 72 hours after ischemia. Peak Cav-1 expression occurred 12 hours after ischemic insult. *P<0.05 and †P<0.01 vs sham, Student unpaired t test. Data represent mean±SEM (n=3 mice per group).
significant differences in the extent of cerebral edema between Cav-1+/+ and ReCav-1 mice ($P=\text{ns}$). As a control, there were no differences in BBB disruption in the contralateral hemisphere among Cav-1+/+, Cav-1−/−, and ReCav-1 mice based on EB extravasation analysis ($P=\text{ns}$; Figure 3B).

Next, we performed in vivo near-infrared optical imaging to evaluate BBB disruption. The near-infrared dye Cy5.5 does not cross the intact BBB. Cy5.5 was used as optical tracers to localize BBB disruption based on the tracer tissue extravasation. The average fluorescence intensity signal was significantly higher in Cav-1−/− mice compared with Cav-1+/+ mice ($P<0.01$; Figure 4). Comparatively, the average fluorescence intensity signals of ReCav-1 and Cav-1+/+ mice were similar for the acute phase of ischemic stroke ($P=\text{ns}$; Figure 4C).

**Regulation of Cav-1 Expression Determines Tight Junction Protein Degradation and MMP-9 Activity**

We next examined the mechanisms underlying the BBB disruption after regulation of Cav-1 expression. Consistent with previous reports in other ischemic models, protein expression levels of claudin-5, occludin, zona occludens-1, and JAM-A were decreased after photothrombotic ischemia (Figure I in...
We proceeded to evaluate whether Cav-1 regulation could induce alterations in tight junction (TJ) protein expression and MMP activity. Figure 5 indicates that transplantation of transfected ECs expressing Cav-1 to Cav-1−/− mice trends in the direction of reversing the Cav-1 deficiency–induced loss in TJ proteins, including claudin-5, occludin, zona occludens-1, and JAM-A. Although the reversal effect on JAM-A was the smallest, it was statistically significant (P<0.05). That neither Cav-1−/− nor ReCav-1 affected the level of β-actin, a housekeeping gene, demonstrates that there was no general effect on protein expression.

Next, we investigated whether Cav-1–induced alteration of TJ protein expression was related to the proteolytic activities of MMP-9 and MMP-2. MMPs have been shown to proteolytically disrupt TJ proteins after ischemic stroke. Therefore, we compared MMP activity using gelatin zymograms for sham, Cav-1+/+, Cav-1−/−, ReCav-1, and EC-transplanted mice 12 hours after ischemic insult. MMP activity was significantly increased after photothrombotic ischemia (P<0.01; Figure 6). Cav-1+/+ mice had significantly higher MMP-9 and MMP-2 activities than Cav-1−/− mice, and this was attenuated by transfection of ECs with a Cav-1 expression plasmid (P<0.05). However, transplantation of ECs without Cav-1 transfection to Cav-1−/− mice did not significantly affect MMP-9 activity compared with that in Cav-1−/− mice (P=ns; Figure 6B). Although there was no significant difference of MMP-2 activity between ReCav-1 and EC-transplanted mice, ReCav-1 tended to have lower MMP-2 activity than EC-transplanted mice (P=ns; Figure 6C). The results indicate that regulation of Cav-1 expression influences TJ protein expression and MMP activity in cerebral ischemia.

**Discussion**

Here, we report, for the first time, a direct relationship between Cav-1 expression and the cerebral edema in the focal ischemic brain. Deletion of the Cav-1 gene increased BBB disruption,
Choi et al  Cav-1 as a Critical Determinant of Brain Edema

1341

degradation of TJ proteins, and proteolytic activity of MMP-9 and MMP-2 in infarcted mice subjected to photothermogony. Furthermore, we provide the first evidence that intravenously transplanted cells selectively localized in the infarcted brain through the disrupted BBB, and lentiviral-mediated re-expression of Cav-1 in Cav-1−/− mice ameliorated vasogenic cerebral edema, TJ protein degradation, and MMP activity during cerebral ischemia. These results indicate that Cav-1 is a critical determinant of BBB permeability by regulating of TJ protein expression and MMP activity.

The role of Cav-1 in brain injury and BBB dysfunction remains controversial. Genetic studies strongly support a functional role for this protein in cerebral ischemia. Deletion of the Cav-1 gene increases the extent of ischemic injury in models of hindlimb ischemia and middle cerebral artery occlusion.28,29 Conversely, Cav-1 gene deletion reduces early brain injury after intracerebral hemorrhage and increases neural stem cell proliferation in the subventricular zone.30,31 On BBB permeability, Cav-1 silencing leads to downregulation of TJ protein expression and increased MMP activity and BBB permeability in cerebrovascular ECs and Cav-1 knockout mice.3,22 In contrast, Cav-1 upregulation was found to mediate TJ protein degradation and BBB breakdown in cerebrovascular ECs, a cortical cold injury rat model, and thiamine-deficient mice.3,32 Knockdown of Cav-1 with siRNA inhibited tissue-type plasminogen activator–induced MMP-9 upregulation and prevented the redistribution of claudin-5 induced by oxygen-glucose deprivation in cerebrovascular ECs.33,34

We found a strong correlation between Cav-1 levels and BBB breakdown, which indicates that Cav-1 is a critical determinant of BBB permeability. Our results provided direct evidence of a protective effect of Cav-1 against cerebral edema in mice subjected to ischemic insult. Re-expression of Cav-1 alone in Cav-1−/− mice, with no other accompanying changes, resulted in less BBB disruption and restored Cav-1 and TJ protein expression, supporting a direct relationship between Cav-1 and cerebral edema. These results suggest that Cav-1 plays a pivotal role in regulating BBB permeability during cerebral ischemic injury.

There is some evidence of a protective role for Cav-1 against BBB breakdown during focal cerebral ischemia. As integral membrane proteins, caveolins and the more recently discovered cavins (also known as PTRF) are structural components of caveolae that are essential for caveolae formation.35 Caveolins physically interact with a large number of other proteins via the caveolin scaffold domain.36 Proteins with a caveolin-binding site include nitric oxide synthase, MMPs, aquaporin, and endothelial growth factor receptor. Caveolins

Figure 5. Expression of tight junction (TJ) proteins in a mouse model of photothermal injury. Representative immunobots showing claudin-5 (A), occludin (B), zona occludens-1 (ZO-1) (C), and JAM-A (D) expression in the cortex of Cav-1+/+, Cav-1−/−, and Cav-1–re-expressing (ReCav-1) mice at the lesion site 12 hours after photothermal ischemia. Transplantation of transfected ECs expressing Cav-1 restored TJ protein expression in the ischemic brain compared with Cav-1−/− mice. *P<0.05 and †P<0.01 vs Cav-1+/+ mice. Data represent mean±SEM (n=3 mice per group repeated 3×).
help regulate membrane-initiated intracellular signaling and caveolin-mediated bidirectional signal transduction.15–17 Cav-1 also acts as an important mediator of anti-inflammatory effects by inhibiting expression of the proinflammatory cytokine nuclear factor-κB and activating protein-1.38

Current study indicates that Cav-1 protects the integrity of the BBB mainly by preventing degradation of TJs and inhibiting MMP activity. Along the same lines, Cav-1 has been shown to protect TJ proteins from degradation via the downregulation of MMPs.8 Furthermore, an isolated caveolin scaffold domain fragment can inhibit MMP-2 activity in a dose-dependent manner, resulting in degradation of the neurovascular matrix, leading to edema and tissue injury.10 Moreover, Cav-1 knockout mice exhibit significantly higher MMP-2 activity than wild-type mice.10 In addition, overexpression of Cav-1 in Cav-1−/− mice, and this was attenuated by transfection of endothelial cells (ECs) with a caveolin-1 (Cav-1) expression plasmid. Bar graph showing band intensities for MMP-9 (B) and MMP-2 (C). *P<0.05 and †P<0.01 vs Cav-1−/− mice. Data represent means±SEM (n=3 mice per group). ReCav-1 indicates Cav-1 re-expressing.

Collectively, these data suggest that decreased expression of Cav-1 during cerebral ischemia may protect against BBB damage and reduce cerebral edema. The results of this study highlight Cav-1 as a critical determinant of BBB permeability and possibly a novel therapeutic target not only for treating cerebral edema but also for potentially increasing BBB integrity and protecting TJ proteins in disease states that are characterized by BBB damage, such as stroke, inflammatory diseases, tumors, and toxic or metabolic encephalopathies.

There are several limitations of this study. The results should be interpreted with the caution because of the limited sample size. It is important to note that animal models have many shortcomings and may reflect only certain clinical features of ischemic injury. Thus, the current findings need to be confirmed in other ischemic models. Further studies addressing the significance of Cav-1 upregulation in a more physiological ischemic model, such as middle cerebral artery occlusion, are needed, as are investigations of the molecular mechanism of Cav-1 involvement in BBB breakdown. Future studies are needed in the field of cerebral ischemic injury, and some investigations are being performed in our laboratory.

Sources of Funding
This study was supported by a grant of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI12C1631; K.-H. Choi).

Disclosures
None.

References


Regulation of Caveolin-1 Expression Determines Early Brain Edema After Experimental Focal Cerebral Ischemia
Kang-Ho Choi, Hyung-Seok Kim, Man-Seok Park, Joon-Tae Kim, Ja-Hae Kim, Kyung-Ah Cho, Min-Cheol Lee, Hong-Joon Lee and Ki-Hyun Cho

Stroke. 2016;47:1336-1343; originally published online March 24, 2016; doi: 10.1161/STROKEAHA.116.013205

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/47/5/1336

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2016/03/24/STROKEAHA.116.013205.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/
Supplemental Methods

Immunoblot analysis

Brain tissue was homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. Proteins were separated by SDS-PAGE (6% to 12% acrylamide) and then transferred to a nitrocellulose membrane. The membrane was placed in blocking solution for 30 min and then washed with a solution containing 10 mmol/L Tris, 150 mmol/L NaCl and 0.05% Tween 20 (1X-TBS-Tween). The membrane was incubated overnight at 4°C with primary antibodies against Cav-1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), claudin-5, occludin, JAM-A (1:1000, Santa Cruz Biotechnology), or zona occludens (ZO)-1 (1:1000; Invitrogen, Carlsbad, CA, USA). Afterward, the membrane was washed and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000, Santa Cruz Biotechnology). To control sample loading, the membranes were stripped and reprobed with β-actin antibody (1:1000, Santa Cruz Biotechnology). Immunoreactive proteins were detected using Super-Signal chemiluminescence substrate. Expression levels were quantified using ImageJ software (NIH) using the mean gray value for each band.

In vivo optical imaging

To obtain bioluminescence images, anesthetized animals were placed in the light-tight chamber of the IVIS100 imaging system (Caliper Life Sciences, Hopkinson, MA, USA) equipped with a cooled charge-coupled device (CCD) camera.

To evaluate the utility of in vivo near-infrared optical imaging for assessing BBB disruption,
we used the ART Optix MX2 optical imaging system (Advanced Research Technologies Inc., Montreal, QC, Canada). Prior to photothrombosis, mice were imaged in the ART Optix to obtain a background image. Animals subjected to photothrombosis were injected with 100 nM Cy5.5 near-infrared fluorescent probe (GE Healthcare, Milwaukee, WI, USA) via the tail vein. A 670-nm pulsed laser diode with a repetition frequency of 80 MHz and a 12-ps light pulse was used to excite the Cy5.5 probe. The fluorescence signal was collected at 700 nm by a highly sensitive time-correlated single-photon counting system and detected through a fast photomultiplier tube.

For in vivo imaging, the mice were anesthetized for induction with 1.5% isoflurane and maintained in 1.0% isoflurane in a nitrous oxide/oxygen/isoflurane mixture (69/30/1%) administered through an inhalation mask, and the near-infrared fluorescence (NIRF) images were observed by the OptiView analysis system (ART Inc.) without the skull. Average fluorescence intensity (FI) and lifetime values of the same selected region of interest (ROI) in the head were obtained noninvasively.

**Statistical analysis**

All data represent the mean ± standard error of the mean (SEM). Differences between groups were evaluated using the one-way analysis of variance (ANOVA) followed by a Tukey post hoc test or Student’s t-test. A two-sided probability value ($P < 0.05$) was considered statistically significant. All measurements were taken by observers blinded to the individual treatments. All statistical analyses were performed using PASW 18.0 for Windows (SPSS, Inc., Chicago, IL, USA).
Supplemental Figure and Figure legend

Supplemental Figure I

TJ protein expression after ischemia in a mouse model of photothrombosis. Representative immunoblots and relative quantification of claudin-5 (A), occludin (B), ZO-1 (C), and JAM-A (D) expression in the cortex of sham and Cav-1\(^{+/+}\) mice at the lesion site 12 h after photothrombotic ischemia. \(*P < 0.05\) vs. sham. Data represent the mean ± SEM (n = 6 mice/group).