Caveolin-1 Deficiency Increases Cerebral Ischemic Injury

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Abstract—Caveolins (Cav), the principal structural proteins of the caveolar domains, have been implicated in the pathogenesis of ischemic injury. Indeed, changes in caveolin expression and localization have been reported in renal and myocardial ischemia. Genetic ablation of the Cav-1 gene in mice was further shown to increase the extent of ischemic injury in a model of hindlimb ischemia. However, the role of Cav-1 in the pathogenesis of cerebral ischemia remains unknown. Immunoblot and immunofluorescence analyses of rat brains subjected to middle cerebral artery occlusion revealed marked increases in endothelial Cav-1 and Cav-2 protein levels. To directly assess the functional role of caveolins in the pathogenesis of cerebral ischemic injury, we next investigated the effects of cerebral ischemia in caveolin knockout (KO) mice. Interestingly, Cav-1 KO mice showed a marked increase of cerebral volume of infarction, as compared with wild-type and Cav-2 KO mice. Immunofluorescence analyses showed an increased number of proliferating endothelial cells in wild-type ischemic brains, as compared with Cav-1 KO ischemic brains. Immunoblot analyses of wild-type ischemic brains showed an increase in endothelial nitric oxide synthase protein levels. Conversely, the protein levels of endothelial nitric oxide synthase remained unchanged in Cav-1 KO ischemic brains. TUNEL analysis also showed increased apoptotic cell death in Cav-1 KO ischemic brains, as compared with wild-type ischemic brains. Our findings indicate cerebral ischemia induces a marked increase in endothelial Cav-1 and Cav-2 protein levels. Importantly, genetic ablation of the Cav-1 gene in mice results in increased cerebral volume of infarction. Mechanistically, Cav-1 KO ischemic brains showed impaired angiogenesis and increased apoptotic cell death. (Circ Res. 2007;100:721-729.)

Key Words: caveolin ■ cerebral ischemia ■ angiogenesis ■ apoptosis
delivery was shown to accelerate endothelial cell differentiation and tubule formation, as well as to increase the number of capillary-like tubules, using human microvascular endothelial cells (HMEC-1) as a model system. Importantly, Cav-1 expression was considered essential for the development of collateral vessels in a mouse model of hindlimb ischemia. Indeed, Cav-1 KO mice subjected to femoral artery/vein ligation failed to recover a functional vasculature, and in some cases even lost their entire leg.19

Caveolar domains have also been implicated in the compartmentalization of signaling molecules involved in apoptosis. For instance, the caspase-3 proenzyme and its activated counterpart were both shown to localize within cardiac endothelial caveolae. Interestingly, like numerous signaling molecules, caveolar localization was suggested to maintain caspase-3 in an inactive state. Indeed, disruption of caveolar structure was reported to increase staurosporine-induced apoptotic cell death.21

Given their implications in angiogenesis and apoptosis, caveolin proteins may well act as key regulators of the patho-physiological processes of ischemic injury. Accordingly, changes in caveolin protein expression and localization have previously been reported in ischemic acute renal failure and myocardial ischemia/reperfusion.22,23 Importantly, the generation of Cav-1 KO mice strongly supports the functional role of caveolin proteins in the pathogenesis of ischemic injury. Indeed, genetic ablation of the Cav-1 gene was shown to increase the extent of ischemic injury in a model of hindlimb ischemia. However, the role of Cav-1 in the patho-physiology of cerebral ischemia remains unknown. In the present study, we first determined the natural behavior of caveolin protein expression in a rat model of middle cerebral artery occlusion (MCAO). Furthermore, to better determine the implications of caveolin proteins in the pathogenesis of cerebral ischemia, we subsequently investigated the outcome of a MCAO in caveolin KO mice.

**Materials and Methods**

**Animals**

This study was conducted according to the guidelines of the National Institute of Health and the Albert Einstein College of Medicine Institute for Animal Studies. Male Sprague-Dawley rats weighing 200 to 225 g were purchased from Taconic Farms (Hudson, NY). Male Cav-1 KO and Cav-2 KO mice were generated, as previously described. Male Sprague-Dawley rats weighing 200 to 225 g, as previously described.3 Briefly, the rats were initially anesthetized by inhalation of 5% halothane through a face-mask in oxygen-enriched air and later maintained at 2.5% of the same mixture. The left common carotid artery and left external carotid artery were exposed through a midline neck incision and the left external carotid artery was coagulated. A 4–0 monofilament suture (Ethicon, Somerville, NJ), whose tip had been rounded by heating near a flame, was inserted into the left external carotid artery and advanced into the left internal carotid artery past the MCA origin until the tip reached the proximal anterior cerebral artery, thus occluding the origin of the MCA. After three hours of MCAO, the filament was removed and blood flow was restored. Rats were killed by decapitation at either 48 hours, 1-week or 2-weeks postischemia (n=10 for each group). The Sham groups were subjected to the same procedure except for the occlusion of the MCA (n=10 for each group).

**Materials**

Mouse Cav-1 and Cav-2 monoclonal antibodies (mAbs) were the generous gifts of Dr Roberto Campos-Gonzalez (BD Pharmingen, San Diego, Calif). A rabbit polyclonal antibody (pAb) to Cav-1 and a mouse mAb to proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Rabbit pAbs to glial fibrillary-acidic protein (GFAP) and neurofilament heavy chain were respectively purchased from Dako Cytomation (Carpinteria, Calif) and Novus Biologicals (Littleton, Colo). A rabbit pAb to laminin was purchased from abcam (Cambridge, Mass). A mouse mAb to β-actin as well as the nuclear dye Hoechst were purchased from Sigma-Aldrich (St-Louis, Mo). A mouse mAb to eNOS, a rabbit pAb to inducible NOS (iNOS), a rabbit pAb to neuronal NOS (nNOS), a rabbit pAb to von Willebrand’s factor (vWF) as well as rabbit and mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were all purchased from BD-Pharmingen. A rabbit pAb to phospho(ser1177)-eNOS was purchased from Cell Signaling Technology (Danvers, Mass). Rabbit and mouse fluorescein (FITC)- and rhodamine (TRITC)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, Pa).

**Surgical Procedures**

Transient MCAO was induced in male Sprague-Dawley rats weighing 200 to 225 g, as previously described. Briefly, the rats were initially anesthetized by inhalation of 5% halothane through a face-mask in oxygen-enriched air and later maintained at 2.5% of the same mixture. The left common carotid artery and left external carotid artery were exposed through a midline neck incision and the left external carotid artery was coagulated. A 4–0 monofilament suture (Ethicon, Somerville, NJ), whose tip had been rounded by heating near a flame, was inserted into the left external carotid artery and advanced into the left internal carotid artery past the MCA origin until the tip reached the proximal anterior cerebral artery, thus occluding the origin of the MCA. After three hours of MCAO, the filament was removed and blood flow was restored. Rats were killed by decapitation at either 48 hours, 1-week or 2-weeks postischemia (n=10 for each group). The Sham groups were subjected to the same procedure except for the occlusion of the MCA (n=10 for each group).

**Figure 1.** Immunoblot analysis of Cav-1 and Cav-2 expression in the brains of Sham and MCAO rats at 48 hours (A), 1-week (B), and 2-weeks postischemia (C) (1 of 6 rats is shown for each group). Immunoblotting against β-actin is shown as an equal loading control. (R) represents the right hemisphere of the brain (contralateral) and (L) represents the left hemisphere of the brain (ischemic).
Permanent MCAO was induced in 8 to 10 week-old male wild-type (WT), Cav-1 KO and Cav-2 KO mice, as previously described (n=16 to 22 for each group). Briefly, mice were anesthetized with inhalation of 3% halothane initially and maintained at 1.5%. The left MCA was exposed by subtemporal craniotomy using an operating microscope. Two 11–0 silk sutures were passed under the artery and the artery was cut in between the 2 suture knots. Afterward, retracted soft tissue was replaced and wounds were sutured. All mice were killed by cervical dislocation at 72 hours postischemia.

Infarction Volume Measurement

Brains from WT, Cav-1 KO and Cav-2 KO mice were removed and cut into 2 mm-thick coronal section using a brain matrix (n=10 to 16 for each group). The brain sections were then immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich) for 30 minutes and then fixed with 4% phosphate buffered formalin. Each brain slice was scanned and the infarct area in each image was calculated using a video image analyzing system (NIH Scion Image, version 1.65) by an observer who was blinded to the study. Infarct volume corrected for edema was calculated by subtracting the noninfarcted area of the infarcted hemisphere from the normal contralateral hemisphere.

Immunoblot Analysis

Brains from Sham and MCAO rats as well as WT and Cav-1 KO mice were cut in half, to separate the left (ischemic) and the right (contralateral nonischemic) hemisphere of the brain (n=6 for each group). The brains were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. Proteins were then separated by SDS-PAGE (6% to 12% acrylamide) and transferred to nitrocellulose membranes. The membranes were then placed in blocking solution for 30 minutes and subsequently washed with 10 mmol/L Tris, 150 mmol/L NaCl and 0.05% Tween 20 (1X-TBS-Tween). The membranes were incubated with a given primary antibody for either 1 hour (Cav-1, Cav-2, β-actin) or 3 hours (eNOS, phospho(ser1177)-eNOS, iNOS, nNOS) at room temperature. Afterward, the mem-

Figure 2. Dual-label immunofluorescence analysis shows the colocalization of Cav-1 (A, red) and vWF (B, green) in rat ischemic hemispheres at 48 hours postischemia (C, yellow). However, Cav-1 (D and G, red) did not colocalize with either GFAP (E, green) or neurofilament heavy chain antibody (H, green) at 48 hours postischemia. Panels F and I represent the merged images (yellow) of Cav-1 with GFAP and neurofilament heavy chain antibody, respectively. All images were taken at the same magnification of ×40.

Figure 3. Representative TTC staining of coronal brain sections sliced rostral to caudal (from left to right) shows increased volume of infarction in Cav-1 KO mice, as compared with WT and Cav-2 KO mice (A). Quantitation of the volume of infarction is shown in panel B. *P<0.05 vs WT mice; †P<0.05 vs Cav-1 KO mice (n=10 to 16 for each group).
Immunofluorescence Analysis and Apoptosis Detection

Brains from Sham and MCAO rats, as well as WT and Cav-1 KO mice, were immersed in 4% paraformaldehyde for 24 hours and subsequently embedded in paraffin (n=4 to 6 for each group). Sections of 10 μm were cut and stained with Hematoxylin and Eosin (H&E). These sections were then rehydrated with graded alcohol to water and blocked overnight using HénBLKII (Aves Labs, Tigard, Ore). Paraffin from 10 μm-thick sections was removed by immersion in xylene. The sections were then rehydrated with graded alcohol to water and blocked overnight using HénBLKII (Aves Labs, Tigard, Ore). These sections were subsequently incubated with primary antibodies for 3 hours at room temperature. The primary antibodies were used at the following dilutions: Cav-1 mAb (1:100), Cav-2 mAb (1:100), vWF pAb (1:50), laminin pAb (1:50), PCNA mAb (1:50), GFAP pAb (1:100) and neurofilament heavy chain pAb (1:100). Hoechst dye was used at a concentration of 10 μg/mL. Afterward, the sections were washed with 1X-PBS and incubated for 1 hour at room temperature with secondary antibodies. Mouse and rabbit rhodamine (TRITC)- and fluorescein (FITC)-conjugated secondary antibodies were used at a dilution of 1:400. The sections were then mounted with ProLong Gold antifade reagent (Molecular Probes, Carlsbad, Calif). Detection of TUNEL-positive cells in WT and Cav-1 KO brains was performed using the TACS 2 TdT In Situ Apoptosis Detection Kit (Treivigen, Gaithersburg, Md), according to the manufacturer’s instructions. All sections were examined under a Nikon TE2000-S eclipse microscope (Morrell Instrument Company, Melville, NY). Immunofluorescence analyses of PCNA and laminin were performed in the border zone, whereas analysis of TUNEL-positive cells was performed in both the ischemic core and border zone.

Statistical Analysis

All data are expressed as mean ± S.E.M and the differences between groups were evaluated by either unpaired Student’s t-test or ANOVA followed by Tukey’s multiple-group comparisons test, where appropriate. Statistical significance was assumed at P<0.05.

Results

Increased Caveolin Protein Expression in Rats Subjected to MCAO

Immunoblot analyses showed marked increases in Cav-1 and Cav-2 protein levels in the ischemic hemisphere of MCAO rats at 48 hours, 1-week and 2-weeks postischemia (P<0.05; Figure 1). Dual-label immunofluorescence analysis at 48 hours post-ischemia demonstrated the colocalization of Cav-1 with the endothelial cell marker, vWF, in rat ischemic hemisphere (Figure 2). However, dual-label immunofluorescence analysis of rat ischemic hemisphere at 48 hours postischemia did not show colocalization of Cav-1 with either GFAP, an astrocyte marker, or neurofilament heavy chain antibody, a neuronal marker (Figure 2). Importantly, immunofluorescence analysis of rat brains at 1-week and 2-week postischemia gave identical results as the 48 hours postischemic group (data not shown). Furthermore, dual-label immunofluorescence analysis demonstrated identical localization of Cav-1 and Cav-2 in rat ischemic brains (supplemental Figure I in the online data supplement available at http://circres.ahajournals.org).

Caveolin-1 Deficiency Increases Cerebral Infarction Volume in Mice

To determine the cerebral volume of infarction, 2 mm-thick sections sliced rostral to caudal were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC). Interestingly, as shown in Figure 3A, the cerebral infarct observed in Cav-1 KO mice appears to be larger and to extend further caudally (from left to right). Accordingly, subtraction of the noninfarcted area of the infarcted hemisphere from the normal contralateral hemisphere reveal a marked increase of volume in infarction in Cav-1 KO ischemic brains (19.2±3.0 mm³) as compared with WT (10.2±2.6 mm³) and Cav-2 KO (7.0±1.3 mm³) ischemic brains (P<0.05; Figure 3B). Conversely, no significant differences of
volume of infarction were observed between the WT and Cav-2 KO ischemic brains (p=ns, Figure 3B).

**Caveolin-1 Deficiency Impairs Cerebral Angiogenesis in Mice**

Immunofluorescence analysis using antibodies against laminin showed an increased number of endothelial cells in WT ischemic hemispheres (16.3±1.6 cells/field), as compared with WT contralateral hemispheres (11.7±0.8 cells/field) (P<0.05; Figure 4). Conversely, Cav-1 KO contralateral and ischemic hemispheres showed similar numbers of endothelial cells (p=ns; Figure 4). Interestingly, immunofluorescence analysis further showed a decreased ratio of PCNA-positive nuclei/total nuclei in Cav-1 KO ischemic brains (13.8±1.2%), as compared with WT ischemic brains (27.0±2.9%) (P<0.05; Figure 5). Importantly, dual-label immunofluorescence analyses demonstrated that the percentage of PCNA-positive cells identified as endothelial cells was markedly decreased in Cav-1 KO ischemic brains (8.4±1.4%), as compared with WT ischemic brains (23.2±3.5%) (P<0.05; Figure 5). However, PCNA-positive cells did not colocalize with either GFAP or neurofilament heavy chain antibody in both Cav-1 KO and WT ischemic brains (supplemental Figure II in the online data supplement available at http://circres.ahajournals.org). No significant differences were observed in the immunofluorescence analysis of PCNA, GFAP and neurofilament heavy chain antibody between WT and Cav-1 KO contralateral hemispheres (data not shown).

Interestingly, immunoblot analysis demonstrated that WT ischemic brains display increases in both eNOS and phospho(ser1177)-eNOS proteins levels, as compared with Cav-1 KO ischemic brains (P<0.05; Figure 6). However, although the phospho(ser1177)-eNOS/total eNOS ratio appeared slightly increased in WT ischemic brains, no significant differences were observed among all groups (p=ns; Figure 6). Furthermore, nNOS and iNOS proteins levels remained unchanged in the ischemic brains of both WT and Cav-1 KO mice (p=ns; Figure 7).

**Caveolin-1 Deficiency Increases Cerebral Apoptotic Cell Death in Mice**

Cav-1 KO ischemic brains showed an increased number of TUNEL-positive cells (57.3±3.4 cells/field), as compared...
with WT ischemic brains (28.9±3.1 cells/field) (P<0.05; Figure 8).

Discussion

Our present results demonstrate marked increases in Cav-1 and Cav-2 protein levels as well as their specific colocalization with endothelial cells in the brains of rats subjected to transient MCAO. Our results further reveal, for the first time, that genetic ablation of the Cav-1 gene increases the cerebral volume of infarction in mice subjected to permanent MCAO. Mechanistically, impaired angiogenesis and increased apoptotic cell death appear to contribute to the increased cerebral ischemic injury observed in Cav-1 KO mice.

Caveolin proteins have previously been implicated in the pathophysiology of ischemic injury. Indeed, changes in caveolin protein expression and localization have been reported in several models of ischemic injury. For instance, Cav-1 expression was shown to be markedly increased in renal cortical/proximal tubules following ischemic acute renal failure.22 Furthermore, although their total protein expression remained unchanged, a dissociation of Cav-1 and Cav-3 from caveolae to the cytosol was reported in the hearts of rats subjected to myocardial ischemia-reperfusion.23 However, the natural behavior of caveolin protein expression in cerebral ischemia-reperfusion remained unclear. Our present results demonstrate marked increases in both Cav-1 and Cav-2 protein levels at 48 hours, 1-week, and 2-week postischemia. The present results differ from those of Shen et al (2006) who have recently reported decreased Cav-1 expression in the ischemic core of MCAO rat brains.27 Although unclear, these discrepancies might be due to variations in the experimental protocols, such as the age of the rats, the duration of ischemia (1 hour versus 3 hours), as well as the immunoblot analysis of homogenates of the entire ischemic hemisphere versus homogenates of the ischemic core only. Accordingly, Cav-1 expression appears to be differentially modulated in the ischemic core and penumbra area of rat ischemic brains.27 Our immunofluorescence analyses further reveal the selective colocalization of both Cav-1 and Cav-2 with the endothelial cell marker, vWF, in rat ischemic brains. For instance, we and others previously demonstrated the selective expression of Cav-1 and Cav-2 in endothelial cells of bovine and rat brains.28 These results suggest that increased caveolin protein levels might influence the angiogenic processes occurring in cerebral ischemia/reperfusion. We previously demonstrated that a marked increase in endogenous Cav-1 protein levels pre-
Mechanistically, our present results suggest that impaired angiogenesis might contribute to the increased cerebral ischemic injury observed in Cav-1 KO mice. Indeed, our immunofluorescence analyses demonstrate an increased number of endothelial cells in WT ischemic brains, as compared with Cav-1 KO ischemic brains. Most importantly, dual-label immunofluorescence analysis further demonstrates a marked increase in the number of proliferating endothelial cells (PCNA-positive) in WT ischemic brains, as compared with Cav-1 KO ischemic brains. These results are in accordance with those of Sonveaux et al (2004) who previously reported increased capillary density, as well as increased numbers of neo-vessels, in WT ischemic hindlimbs, as compared with Cav-1 KO ischemic hindlimbs.19 We have also previously reported a reduction of vessel density in Cav-1 KO mice, using a model of exogenous tumor cell injection.16 The contribution of impaired angiogenesis to the increased cerebral ischemic injury of Cav-1 KO mice is further supported by our findings of reduced volume of infarction in Cav-2 KO mice. Indeed, whereas Matrigel plugs implanted in Cav-1 KO mice showed dramatic reduction in both vessel infiltration and density, those implanted in Cav-2 KO mice conversely showed an enhanced angiogenic response.16

As previously suggested, the impaired angiogenesis observed in Cav-1 KO mice could be ascribed, at least in part, to the lack of caveolar domains.19 Accordingly, although Cav-1 is well recognized as a natural inhibitor of eNOS activity, Cav-1 KO ischemic hemispheres did not show increased eNOS activation. Conversely, WT ischemic hemispheres show significant increases in both eNOS and phospho(ser1177)-eNOS protein levels. However, although the phospho(ser1177)-eNOS/total eNOS ratio appears slightly increased in WT ischemic brains, no significant differences were observed among all groups. Nonetheless, cultured Cav-1 KO aortic endothelial cells were previously shown to display a marked inhibition of eNOS phosphorylation on vascular endothelial growth factor (VEGF) stimulation.19 Therefore, as previously suggested,19 the absence of caveolar domains might hinder the proper compartmentalization of the signaling molecules essential to NO synthesis and angiogenic processes. Accordingly, although its total expression remained unchanged, a dissociation of the VEGF receptor-2 (VEGFR2) from low-density to high-density membranes fractions was previously reported in cultured endothelial cells derived from Cav-1 KO mice.19 Furthermore, disruption of caveolar domains with MβCD was previously shown to inhibit VEGF-induced extracellular signal-regulated kinase (ERK) activation and cell migration in bovine aortic endothelial cells.31

The implication of caveolar domains in angiogenesis is further supported by our findings of reduced infarction volume in Cav-2 KO mice, as compared with Cav-1 KO mice. Indeed, unlike Cav-1 KO mice, Cav-2 KO mice still retain the ability to form caveolae through the homo-oligomerization of Cav-1.14

Importantly, although angiogenesis might play a crucial role in the expansion of an infarct, the implications of cerebral ischemia/reperfusion.29 Interestingly, intravenous delivery of the Cav-1 scaffolding domain peptide was previously shown to attenuate the protective effects of ischemic preconditioning in adult rat cardiomyocytes subjected to simulated ischemia/reperfusion.29 Interestingly, intravenous delivery of the Cav-1 scaffolding domain peptide was previously shown to exert cardio-protective effects in myocardial ischemia-reperfusion by increasing endothelium-derived NO release, as well as by reducing polymorphonuclear neutrophil adherence and infiltration.30 Collectively, these data, as well as our present results, suggest that decreased expression of Cav-1 might be further detrimental to the ischemic injury.

Figure 8. Representative images of TUNEL staining show increased apoptotic cell death in the Cav-1 KO ischemic brains (B), as compared with the WT ischemic brains (A). Quantitation of TUNEL-positive cells is shown in panel C. The quantitation represents the average number of TUNEL-positive cells/field of 20 fields per animal (n=6 for each group). *P<0.05 vs WT ischemic brains. Images were taken at the same magnification of ×40.
apoptosis in such a process cannot be overlooked. Hence, our present results further suggest that increased apoptotic cell death might also contribute to the increased cerebral ischemic injury observed in Cav-1 KO mice. As a matter of fact, Cav-1 KO ischemic brains show an increased number of TUNEL-positive cells, as compared with WT ischemic brains. Accordingly, although controversial, caveolin proteins have previously been reported to act as key regulators of apoptotic processes. Indeed, Cav-1 expression was previously shown to sensitize T24 bladder carcinoma cells to staurosporine-induced apoptosis.32 Conversely, Cav-1 expression was shown to suppress c-Myc-induced apoptosis in a human epithelial prostate cancer-derived cell line.33

Interestingly, caveolar domains have previously been maintained to prevent apoptotic signaling molecules in an inactive state, until reception of the appropriate stimulus.20 Disruption of caveolae with MJCD was shown to increase staurosporine-induced caspase-3 activity in cardiac endothelial cells.20 Importantly, we recently demonstrated that genetic ablation of the Cav-1 gene in a transgenic mouse model of prostate cancer resulted in increased apoptosis levels.21 Therefore, the lack of Cav-1 protein expression and caveolar domains might result in inappropriate compartmentalization and regulation of the numerous apoptotic signaling molecules and, consequently, result in the hyper-activation of apoptotic signaling pathways, leading to increased neuronal death in a model of cerebral ischemia.

In conclusion, this is the first report of increased expression of Cav-1 and Cav-2 as well as their colocalization with endothelial cells in the brains of rats subjected to cerebral ischemia/reperfusion. This increased expression of endothelial Cav-1 and Cav-2 suggests essential roles for the caveolin proteins in postsischemic angiogenesis. Interestingly, genetic ablation of the Cav-1 gene in mice subjected to MCAO results in increased volume of infarction. Mechanistically, impaired angiogenesis and increased apoptotic death appear to contribute to the increased ischemic injury observed in Cav-1 KO mice.

**Study Limitations**

Although we quantified the number of capillaries in both WT and Cav-1 KO contralateral and ischemic hemispheres, we did not determine the impact of collateralization on the cerebral volume of infarction. Therefore, in future studies, it will be interesting to determine whether genetic ablation of Cav-1 might affect the cerebral collateral circulation in mice.

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**Disclosures**

None.

**References**


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Online Figure I. Co-localization of Cav-1 (A, green), Cav-2 (B, red) and the nuclear dye marker Hoechst (C, blue) in rat ischemic hemispheres. Panel (D) represent the merged image of A, B, and C (yellow). Images were taken at the same magnification of 10X.

Online Figure II. Dual-label immunofluorescence analysis of PCNA (red) with either GFAP (A, green) or neurofilament heavy chain antibody (B, green) failed to show co-localization of PCNA with either astrocytes or neurons in Wild-Type ischemic brains. Images were taken at the same magnification of 40X.

Online Figure III. Immunoblot analysis of Cav-1 expression in the brains of Wild-Type mice subjected to permanent middle cerebral artery occlusion for 72 hrs. Immunoblotting against β-actin is shown as an equal loading control. (R) represents the right hemisphere of the brain (contralateral) and (L) represents the left hemisphere of the brain (ischemic).

Online Figure IV. Quantitation of the nuclear dye marker Hoechst shows increased nuclei counts in WT ischemic hemispheres as compared to WT contralateral hemispheres. Conversely, Cav-1 KO contralateral and ischemic hemispheres showed similar nuclei counts. *p<0.05 vs Wild-Type contralateral hemisphere (n=6 for each group).
Nuclei Counts

- Wild-Type
- Cav-1 KO

Contralateral
Ischemic

Number of Nuclei/Field

- 0
- 20
- 40
- 60
- 80
- 100
- 120

*