Roles of Caveolin-1 in Angiotensin II–Induced Hypertrophy and Inward Remodeling of Cerebral Pial Arterioles

Shaikamjad Umesalma, Frederick Keith Houwen, Gary L. Baumbach, Siu-Lung Chan

Abstract—Angiotensin II (Ang II) is a major determinant of inward remodeling and hypertrophy in pial arterioles that may have an important role in stroke during chronic hypertension. Previously, we found that epidermal growth factor receptor is critical in Ang II–mediated hypertrophy that may involve caveolin-1 (Cav-1). In this study, we examined the effects of Cav-1 and matrix metalloproteinase-9 (MMP9) on Ang II–mediated structural changes in pial arterioles. Cav-1–deficient (Cav-1−/−), MMP9–deficient (MMP9−/−), and wild-type mice were infused with either Ang II (1000 ng/kg per minute) or saline via osmotic minipumps for 28 days (n=6–8 per group). Systolic arterial pressure was measured by a tail-cuff method. Pressure and diameter of pial arterioles were measured through an open cranial window in anesthetized mice. Cross-sectional area of the wall was determined histologically in pressurized fixed pial arterioles. Expression of Cav-1, MMP9, phosphorylated epidermal growth factor receptor, and Akt was determined by Western blotting and immunohistochemistry. Deficiency of Cav-1 or MMP9 did not affect Ang II–induced hypertension. Ang II increased the expression of Cav-1, phosphorylated epidermal growth factor receptor, and Akt in wild-type mice, which was attenuated in Cav-1−/− mice. Ang II–induced hypertrophy, inward remodeling, and increased MMP9 expression in pial arterioles were prevented in Cav-1−/− mice. Ang II–mediated increases in MMP9 expression and inward remodeling, but not hypertrophy, were prevented in MMP9−/− mice. In conclusion, Cav-1 is essential in Ang II–mediated inward remodeling and hypertrophy in pial arterioles. Cav-1–induced MMP9 is exclusively involved in inward remodeling, not hypertrophy. Further studies are needed to determine the role of Akt in Ang II–mediated hypertrophy. (Hypertension. 2016;67:00-00. DOI: 10.1161/HYPERTENSIONAHA.115.06565.) • Online Data Supplement

Key Words: angiotensin II • caveolin-1 • hypertrophy • matrix metalloproteinase-9 • mice

Chronic hypertension causes structural alterations of the vascular wall, including hypertrophy (increases in wall cross-sectional area) and inward remodeling (reduction of lumen external diameter [ED].) These changes in the cerebral circulation may become maladaptive, adversely affect local blood flow control, and therefore contribute to the increased risk of stroke. Despite decades of research efforts, the molecular mechanisms associated with structural changes of cerebral vasculature in chronic hypertension have not been fully elucidated.

One of the hallmark effects of angiotensin II (Ang II) on cerebral pial arterioles is hypertrophy. Recently, we found that Ang II induces hypertrophy in pial arterioles through signaling events that involve epidermal growth factor receptor (EGFR) activation and other signaling molecules, such as caveolin-1 (Cav-1) and c-Src. These results lead to the current study in which we examined the possible roles of Cav-1 and caveola-compartmentalized–specific signaling cascade in Ang II–mediated hypertrophy in pial arterioles. Caveolae are plasma membrane microdomains (lipid rafts) that serve as a signaling platform to facilitate the spatial localization of signal transduction events stimulated by Ang II. Cav-1 is the major structural, as well as signaling, protein component in vascular caveolae. In vascular smooth muscle cells (VSMCs), Ang II promotes the association of Ang II type 1 receptor (AT1R) with Cav-1, which in turn enables trafficking of AT1R into caveolin-enriched lipid rafts. This AT1R trafficking requires other signaling molecules, including reactive oxygen species and c-Src. AT1R transactivation of EGFR seems to take place in Cav-1–enriched caveolae, followed by activation of downstream EGFR-dependent signaling events for hypertrophy, including Akt/protein kinase B. These findings, however, have been performed in cell culture settings and have yet to be proven in cerebral vasculature in vivo. Therefore, the first goal of this study was to examine the role of Cav-1 in Ang II–mediated hypertrophy in pial arterioles using Cav-1 knockout mice that exhibit complete loss of Cav-1 within blood vessels. We also further investigated the possible involvement of the downstream signaling molecule Akt that has been shown to have a role in hypertrophy.

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From the Department of Pathology, University of Iowa College of Medicine, Iowa City (S.U., F.K.H., G.L.B.); and Department of Neurological Sciences, University of Vermont, Burlington (S.-L.C.).
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Correspondence to Gary L. Baumbach, Department of Pathology, University of Iowa Carver College of Medicine, 5231D RCP, 200 Hawkins Dr, Iowa City, IA 52242, E-mail g-baumbach@uiowa.edu or Siu-Lung Chan, Department of Neurological Sciences, University of Vermont, 149 Beaumont Ave, HSRF 416, Burlington, VT 05405, E-mail siu-lung.chan@uvm.edu
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Ang II also plays a critical role in inward remodeling of pial arterioles. Many studies have demonstrated that the molecular mechanism responsible for Ang II–induced inward remodeling differs in important aspects from that of hypertrophy. However, we previously found that redox-sensitive signaling is involved in both Ang II–mediated hypertrophy and inward remodeling in pial arterioles, suggesting a certain overlapping of mechanisms between the two. Because Cav-1 is also redox-sensitive and can be activated by reactive oxygen species derived from nicotinamide adenine dinucleotide phosphate oxidase, our second goal was to determine whether Cav-1 is involved in inward remodeling. In addition, matrix metalloproteinases (MMPs), a family of zinc-dependent extracellular proteinase involved in the degradation of basal lamina and extracellular matrix, have been implicated in vascular remodeling in hypertension. In particular, several studies suggest that activation of MMP9 in small vessels is also redox sensitive and Ang II dependent. Therefore, our third goal was to determine whether MMP9 is involved in Ang II–induced and Cav-1–mediated inward remodeling in pial arterioles.

Materials and Methods

Animals
Cav-1–deficient mice (Cav-1−/−, 12–14 weeks old; n=60) and background- and age-matched wild-type (WT, B6129SF2/J) mice (n=60) were purchased from Jackson Laboratory (Bar Harbor, ME). Male/ female ratio in each experiment and animal group was 50%. Cav-1−/− and WT mice were derived from heterozygous Cav-1+/− mice in Jackson Laboratories (Strain: STOCK Cav1tm1/Mls/J; Stock #004585). Male MMP9-deficient mice (MMP9−/−, Strain: B6.FVB[Cg]-Mmp9tm1Tvu/J; Stock #007084; 12–14 weeks old; n=40) and background- and age-matched WT (C57BL6/J) mice (n=40) were purchased from Jackson Laboratory. Animals were housed in pathogen-free facility at 24°C, exposed to 12 hours of light, and allowed free access to standard chow and water. All procedures were approved by Institutional Animal Care and Use Committee of the University of Iowa and in agreement with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Statistical Analysis
All data are presented as means±SEM. Differences were analyzed by 1-way ANOVA and post hoc test for ≥3 groups or t test for 2 groups using Graph Pad prism 6 (Graph Pad Software, Inc, San Diego, CA). Detailed description of experimental procedures are available in Materials and Methods in the online-only Data Supplement.

Results

Ang II Increases Cav-1 Expression in Cerebral Pial Vasculature

Western blotting of Cav-1 was performed to determine whether Ang II activates Cav-1 in pial arteries. Cav-1 protein expression was present in pial arteries from WT mice but nearly undetectable in Cav-1−/− mice (Figure 1A). In WT mice, Ang II significantly increased protein expression of Cav-1 in pial arteries compared with saline controls (Figure 1B). Furthermore, immunohistochemistry showed that Ang II increased Cav-1 expression in pial arteries from WT but not in Cav-1−/− mice (Figure 1C). These results suggest that Ang II activates Cav-1 in cerebral vasculature that was attenuated in Cav-1−/− mice.

It has been previously shown in VSMC culture studies that the association of AT1R with Cav-1 is critical for the transactivation of EGFR in caveolae. In this study, we showed that Ang II–induced increases in expression of phosphorylated EGFR were significantly reduced in pial arterioles in Cav-1−/− compared with WT mice (Figure S1 in the online-only Data Supplement). This result confirms our previous finding that Ang II increases caveolin-1 (Cav-1) expression in cerebral pial arteries in wild-type (WT) but not in Cav-1−/− mice. A, Representative Western blot illustrates the presence of Cav-1 in pial arteries from WT but not in Cav-1−/− mice. B, Representative images and densitometry of Cav-1 Western blotting in pial arteries from Ang II–treated WT mice. C, Representative photographs and staining density of Cav-1 immunohistochemistry in pial arterioles from Ang II–treated WT and Cav-1−/− mice. Results are means±SEM of 6 to 8 mice. *P<0.05 vs corresponding controls. Scale bar, 10 μm.
Ang II Increases Expression of MMP9
Ang II promotes VSMC migration through MMP9-dependent mechanisms.17 To investigate whether Ang II induces MMP9 expression in pial arteries, Western blotting of MMP9 was performed in saline or Ang II–treated WT mice. Baseline (saline treated) MMP9 expression was low in WT mice (Figure S2). Ang II increased MMP9 levels in pial arteries in WT mice (Figure S3A). Ang II mRNA expression was low in WT mice (Figure S3A). Ang II mRNA expression was low in WT mice (Figure S3A). Ang II significantly decreased ED (65±2 versus 75±4 μm) in WT but not in MMP9−/− mice (ED: 68±4 versus 68±2 μm in saline; Figure 4B). In addition, cross-sectional area of the pial arteriolar wall was significantly increased by Ang II in WT (482±35 versus 369±56 μm² in saline) and in MMP9−/− mice (478±45 versus 343±51 μm² in saline; Figure 4A). MMP9 deficiency did not significantly affect baseline and Ang II–induced increased blood pressure (Table 2). These findings suggest that MMP9 is involved in Ang II–induced inward remodeling, but not hypertrophy, in pial arterioles.

Deficiency of MMP9 Prevents Ang II–Mediated Inward Remodeling but Not Hypertrophy
MMP9−/− mice were used to determine whether MMP9 is involved in Ang II–mediated inward remodeling. Baseline (saline treated) MMP9 expression was low in WT mice (Figure S3A). Ang II–induced expression of MMP9 in pial arterioles from WT mice was significantly attenuated in MMP9−/− mice (Figure S3B). Ang II significantly decreased ED (65±2 versus 75±4 μm) in WT but not in MMP9−/− mice (ED: 68±4 versus 68±2 μm in saline; Figure 4B). In addition, cross-sectional area of the pial arteriolar wall was significantly increased by Ang II in WT (482±35 versus 369±56 μm² in saline) and in MMP9−/− mice (478±45 versus 343±51 μm² in saline; Figure 4A). MMP9 deficiency did not significantly affect baseline and Ang II–induced increased blood pressure (Table 2). These findings suggest that MMP9 is involved in Ang II–induced inward remodeling, but not hypertrophy, in pial arterioles.

Ang II Activation of Akt Is Cav-1–Dependent
Serine–threonine kinase Akt is known to be activated by EGFR and plays an important role in protein synthesis in the hypertrophic response.9,18 Thus, we examined the effects of Cav-1 deficiency on expression of Akt in cerebral vasculature. Ang II increased the protein expression of phosphorylated Akt in pial arteries in WT mice, which was significantly reduced in Cav-1−/− mice (Figure 5A). Moreover, immunochemical staining showed that phosphorylated Akt was present in pial arterioles in Ang II–treated WT mice and significantly reduced in Cav-1−/− mice (Figure 5B). These results suggest that Ang II activation of Akt is dependent on Cav-1.

Table 1. Physiological Data for WT and Cav-1–Deficient Mice Treated With Ang II (1000 ng/kg per min) or Saline for 4 Weeks

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT, Saline</th>
<th>WT, Ang II</th>
<th>Cav-1−/−, Saline</th>
<th>Cav-1−/−, Ang II</th>
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<tr>
<td>Systolic arterial pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Week 4</td>
<td>128±3</td>
<td>175±6*</td>
<td>127±2</td>
<td>178±4*</td>
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<td>Pial arteriolar pressure, mm Hg</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
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<td>47±3</td>
<td>39±2</td>
<td>40±3</td>
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<tr>
<td>Diastolic</td>
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<td>34±2</td>
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<tr>
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<td>11±1</td>
<td>8±1</td>
<td>10±1</td>
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<tr>
<td>Arterial blood gases</td>
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<tr>
<td>pH</td>
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<td>7.35±0.03</td>
<td>7.37±0.04</td>
<td>7.39±0.02</td>
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<tr>
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<td>29±3</td>
</tr>
<tr>
<td>PO₂</td>
<td>113±4</td>
<td>107±5</td>
<td>117±7</td>
<td>109±8</td>
</tr>
<tr>
<td>Age, wk</td>
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<td>13.4±0.7</td>
<td>18.0±0.5</td>
</tr>
<tr>
<td>Weight, g</td>
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<td>24.2±1.2</td>
<td>23.0±1.0</td>
<td>21.0±1.3</td>
</tr>
</tbody>
</table>

Systolic arterial pressure was measured by tail-cuff method in conscious mice at week 4 of Ang II treatment. Pial arteriolar pressure was measured under anesthetized conditions. Values are mean±SEM. Ang II indicates angiotensin II; Cav-1, caveolin-1; and WT, wild-type.

*P<0.05 vs corresponding saline controls.
Discussion

Chronic hypertension is one of the most important modifiable risk factors of stroke. Therefore, it is important to understand hypertension-induced changes in cerebral small vessels that are believed to be critical in local blood flow control. This study builds on our previous findings that EGFR-dependent signaling is critical in Ang II–mediated hypertrophy in pial arterioles and, in addition, demonstrates that Cav-1 and MMP9 play an important role in Ang II–induced inward remodeling. There are several novel findings; first, we demonstrated that Cav-1 is critical in Ang II–induced hypertrophy and inward remodeling in pial arterioles and, in addition, that both effects of Cav-1 seem to be pressure independent. Second, we showed that Ang II–induced expression of MMP9 is Cav-1 dependent. Importantly, MMP9 is a mediator of Ang II–induced inward remodeling, but not hypertrophy, in pial arterioles. Third, Akt activation may be a downstream signaling event of Ang II–induced Cav-1–mediated hypertrophy in pial arterioles. These findings, for the first time, suggest that Ang II mediates hypertrophy and inward remodeling in pial arterioles via two distinctively different Cav-1–dependent signaling pathways.

Chronic activation of renin–angiotensin system, in particular Ang II, plays an important role in regulating cerebral vascular structural alterations, including hypertrophy and inward remodeling. However, the molecular mechanisms of these changes are not completely understood. Previously, we showed that EGFR transactivation by Ang II is critical for hypertrophy in pial arterioles that may also involve Cav-1 signaling. This study aimed at further understanding the role of Cav-1 in this process. Surprisingly, Cav-1 deficiency inhibits not only Ang II–mediated hypertrophy but also inward remodeling, which is independent of EGFR. This finding suggests that Cav-1 mediates inward remodeling in pial arterioles by activating downstream signaling cascade that is independent of EGFR. Apart from that, Cav-1 is also involved in hypertrophy in pial arterioles mediated by EGFR, as supported by our data that Cav-1 deficiency reduced activation of EGFR. These interesting findings led us to further study the role of Cav-1 on inward remodeling and hypertrophy in pial arterioles separately.

Studies in hypertensive patients and animal models suggest that MMP9 activity plays a major role in arterial remodeling. MMP9 digests extracellular matrix and allows migration of VSMC, which is an early event of the remodeling process in vascular wall. Importantly, Ang II has been shown to induce MMP9 in a nicotinamide adenine dinucleotide phosphate oxidase–dependent manner. Therefore, we examined the role of MMP9 in Ang II–induced inward remodeling. Our results show that MMP9 is only involved in Cav-1–mediated inward remodeling and not hypertrophy. This agrees with the idea of MMP9-mediated VSMC migration rather than proliferation. These results further suggest that although Cav-1 has proproliferation properties, it stimulates hypertrophy in pial arterioles through a mechanism that
does not involve MMP9. In addition, the results strengthen the concept that the mechanism involved in inward remodeling differs from that of hypertrophy.

Recently, we have shown that EGFR transactivation by Ang II is critical for hypertrophy in pial arterioles. VSMC culture experiments show that transactivation of EGFR requires Cav-1 and takes place in Cav-1–enriched lipid rafts called caveolae.

The results of our present study support these previous studies that Cav-1 deficiency inhibits proatherogenic stimuli that normally act through caveolae and thereby prevents Ang II–induced cerebral arterial hypertrophy and remodeling.

The effect of Cav-1 and MMP9 deficiency on Ang II–induced hypertrophy and remodeling seemed to be pressure independent based on our tail-cuff results that the knockout animals developed similar level of hypertension by Ang II when compared with their WT counterparts. It is interesting to note that the WT control of Cav-1−/− mice had slightly higher baseline blood pressure than that of the WT control of MMP9−/− mice, possibly because of their different genetic background. The same reason may also be applied to explain that Cav-1−/− mice were more sensitive to the pressor effect of Ang II than that of MMP9−/− mice. Despite that, Cav-1−/− mice seemed to be more sensitive to the blood pressure–lowering effect of ketamine/xylazine because they had slightly lower cerebral arteriolar pressure than that of MMP9−/− mice measured in the anesthetized state.

A few limitations in this study are noteworthy. First, we did not develop a causal relationship between Cav-1 and MMP9 in remodeling of pial arterioles. This would be better determined in double Cav-1 and MMP9 knockout mice that were, unfortunately, not available to us. However, we did develop a compelling case for the notion that Cav-1 is essential in Ang II–mediated increase in MMP9 expression. Second, the use of Western blotting to determine protein expression of Cav-1, phosphorylated EGFR, Akt, and MMP9 was limited to larger pial arteries because pial arterioles contain insufficient amounts of protein. This has been partly resolved by immunohistochemistry. Third,
although we showed, in separate experiments, that Akt can be activated by Ang II and EGFR, further studies will be necessary to determine whether Akt is an important downstream signaling molecule that is involved in Ang II–Cav-1–EGFR–mediated hypertrophy in pial arterioles.

**Perspectives**

Ang II, the effector molecule of the renin–angiotensin system, is a major determinant of hypertrophy and remodeling in cerebral pial arterioles. However, the underlying molecular mechanisms are incompletely understood. In this study, we showed that Cav-1 is essential in Ang II–EGFR–mediated hypertrophy. Cav-1 is also an important signaling molecule in inward remodeling but seems to work through a different mechanism in MMP9. This study once again agrees with the concept that hypertrophy and inward remodeling of pial arterioles work through distinctly different mechanisms. Further in-depth studies are needed to understand how MMP9 affects inward remodeling and how potentially Akt signaling mediates hypertrophy in pial arterioles.

**Acknowledgments**

We thank Tom Gerhold for excellent technical assistance on open cranial window experiments and genotyping of genetic knockout mice.

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**Disclosures**

None.

**References**


**What Is New?**

- Caveolin-1 (Cav-1) is essential in both angiotensin II (Ang II)–induced hypertrophy and inward remodeling in cerebral pial arterioles. Matrix metalloproteinase-9 is critical in Cav-1–mediated inward remodeling but not hypertrophy. The epidermal growth factor receptor–Cav-1 signaling cascade is critical in Ang II–induced hypertrophy that may involve downstream signaling molecule Akt.

**What Is Relevant?**

- Ang II–induced pial arteriolar hypertrophy and inward remodeling are not completely understood and thought to be distinctive and independent. This study reveals that independent of epidermal growth factor receptor, Cav-1 critically contributes to Ang II–mediated matrix metalloproteinase-9 upregulation that only facilitates inward remodeling and not hypertrophy. Cav-1 also is involved in Ang II–induced hypertrophy in an epidermal growth factor receptor–dependent manner that may involve Akt.

**Novelty and Significance**

Although Ang II stimulated both hypertrophy and inward remodeling in cerebral pial arterioles via Cav-1–dependent manner, it seems that the signaling pathways for hypertrophy and remodeling downstream of Cav-1 differ in important ways. Cav-1–dependent matrix metalloproteinase-9, independent of epidermal growth factor receptor, is exclusively responsible for inward remodeling of pial arterioles, whereas Cav-1–dependent activation of epidermal growth factor receptor is responsible for hypertrophy that may also involve downstream signaling of Akt. This, once again, suggests that Ang II induces hypertrophy and inward remodeling through different mechanisms.
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ROLES OF CAVEOLIN-1 IN ANGIOTENSIN II-INDUCED HYPERTROPHY AND INWARD REMODELING OF CEREBRAL PIAL ARTERIOLES

Shaikamjad Umesalma¹, Ph.D.; Frederick Keith Houwen¹, M.D.; Gary Baumbach*¹, M.D.; Siu-Lung Chan*², Ph.D.
¹Department of Pathology, University of Iowa College of Medicine, Iowa City, IA 52242.
²Department of Neurological Sciences, University of Vermont, Burlington, VT 05405.

*¹Corresponding author:
Gary L. Baumbach, M.D.,
Department of Pathology, University of Iowa Carver College of Medicine,
5231D RCP, 200 Hawkins Drive, Iowa City, IA 52242, USA.
Tel: 319-384-9084
Fax: 319-384-8054
Email: g-baumbach@uiowa.edu

*²Corresponding author:
Siu-Lung Chan, Ph.D.,
Department of Neurological Sciences, University of Vermont,
149 Beaumont Ave., HSRF 416, Burlington, VT 05405, USA.
Tel: 802-656-4231
Fax: 802-656-8704
Email: siu-lung.chan@uvm.edu
Materials and Methods

Treatments

To study the effects of Cav-1 in Ang II-mediated remodeling in pial arterioles, mice were divided into two groups. In the first treatment group, WT (n=8) and Cav-1-/- mice (n=8) received Ang II (1000ng/kg/min, 28 days) via osmotic minipumps (Alzet, model 1004, Durect, Cupertino, CA, USA) and the second control group of WT and Cav-1-/-mice received saline (n=8/group). In a separate set of experiment, mice were infused with saline or Ang II (n=6/group) for western blot and immunohistochemical analysis. To investigate the role of MMP9 in Ang II-mediated inward remodeling, MMP9-/- and WT mice received the same dosing regimen of Ang II or saline via osmotic pumps (n=6/group). Minipumps were implanted subcutaneously in the midscapular region in mice during anesthesia with ketamine/xylazine (87.5/12.5 mg/mL, 10 mL/kg, i.p.).

Determination of systemic blood pressure

Systolic blood pressures were measured in conscious mice using an automated tail-cuff device (BP-2000, Visitech Systems, Apex, NC, USA). Mice were placed in mouse holders that allow measurements of systolic blood pressure under resting conditions. Prior to implantation of minipumps, mice were trained for 5 days, then blood pressure was measured at day 0 (baseline), 7, 14, 21, 28 days of Ang II treatment. Each day 30 measurements were made and averaged for each mouse.

Measurement of cerebral pial arteriolar pressure and diameter

After the treatment period, animals were anesthetized with ketamine/xylazine (87.5/12.5 mg/mL, 10 mL/kg. i.p.). Supplementary anesthetic (pentobarbital sodium, 50 mg/mL) was given during the experiment through a catheter connected to the femoral vein. We measured pressure and internal diameter in first-order arterioles on the cerebrum through an open skull preparation that we described previously in detail. Pial arteriolar systolic, diastolic, mean, and pulse pressure were measured continuously with a micropipette connected to a servo-null pressure-measuring device (model 5, Instrumentation for Physiology and Medicine, Inc.). Arterioles were monitored through a microscope connected to a closed-circuit video system with a final magnification of X356. Arteriolar diameter was measured from digitized images of arterioles using Image J (NIH, Bethesda, MD, USA).

Determination of pial arteriolar structure

About 30 minutes after completion of surgery, baseline pressure and diameter of pial arterioles were measured. Arterioles were then suffused with artificial cerebrospinal fluid containing ethylene diamine tetra acetic acid (EDTA, 67 mmol/L), which produces maximal dilatation of pial arterioles. Pressure-diameter relationships were obtained in deactivated pial arterioles between cerebral pressures of 10 and 50 mmHg. Arterioles were then fixed at physiological pressure in vivo by suffusion of vessels with glutaraldehyde fixative (2.25% glutaraldehyde in 0.10 mol/L cacodylate buffer) while maintaining pial arteriolar pressure at baseline levels. After mice were euthanized using an overdose sodium pentobarbital, the arteriolar segment was removed, processed and embedded in Spurr’s low viscosity resin while maintaining cross-sectional orientation. Cross-sectional area of the arteriolar wall was determined histologically, as described previously.
Western Blotting of Cav-1, pEGFR, pAkt and MMP9
Protein expression of Cav-1, EGFR, phosphorylated EGFR (pEGFR), Akt, pAkt and MMP9 was
determined in pial arteries using western blotting in saline- or Ang II-treated WT and Cav-1/-
mice. Pial artery segments were excised, snapped frozen in liquid nitrogen, and homogenized in
Laemmli buffer (25% Glycerin, 12.5% β-mercaptoethanol, 7.5% sodium dodecyl sulfate, 25% 1
mol/L TrisHCl pH 8.0, 0.25 mg/mL bromophenol blue) over liquid nitrogen. Multiple larger
branches of middle cerebral and basilar cerebral artery segments were pooled to obtain a
sufficient amount of protein for blotting. Protein concentrations were determined by Bradford
protein assay kit (Bio-Rad, Hercules, CA, USA). Equal quantities of protein were loaded onto a
15% (Cav-1) or 10% (all other proteins) polyacrylamide electrophoresis gel. After
electrophoresis (110 V, 90 min), the separated proteins were transferred to nitrocellulose
membrane (Bio-Rad). Membranes were blocked in 5% skim milk in Tris buffered saline tween
20 for 1 hour and then incubated overnight (4°C) with β-actin (1:2000; Cell Signaling, Danvers,
MA, USA), Cav-1, EGFR, pEGFR (1:1000; Santa Cruz, Dallas, TX, USA), Akt, pAkt (1:1000;
R & D systems, Minneapolis, MN, USA) and MMP9 (1:1000; abcam, Cambridge, MA, USA)
primary antibodies. Membranes were then incubated with the corresponding horseradish
peroxidase-conjugated with anti-rabbit and anti-mouse IgG secondary antibodies for 1h (1:2500
dilution, room temperature). Immunoreactive bands were detected and normalized to intensity of
β-actin bands and measured by densitometry using Image J.

Immunohistochemistry of Cav-1, pEGFR, pAkt and MMP9
Brains were fixed in 10 % paraformaldehyde for 24 hours and serially sectioned in 3 mm thick
coronal slices. Brain sections were then dehydrated in ethanol series and embedded in paraffin.
Sections for microscopy were cut at 5 μm. After deparaffinization and blocking of endogenous
peroxidase activity with 3 % hydrogen peroxide, the sections were incubated with rabbit
polyclonal antibody against Cav-1 (1:500), pEGFR (1:200), pAkt (1:200) and MMP9 (1:200) at
4°C overnight. Sections were visualized by Envision HRP System (Dako, Carpinteria, CA,
USA), using 3,3’-diaminobenzidine (Dako), followed by counterstaining with hematoxylin.
Negative controls were consecutive sections without primary antibodies. Immunoreactive cells
(brown staining) were counted in five different pial arteriolar regions across randomly selected
fields. The ratio of immunoreactive cells per total number of cells in the defined stained area was
used to calculate the number of immuno-positive cells and averaged from the five regions.
References
Figure S1. Deficiency of Cav-1 reduces phosphorylated EGFR (pEGFR) in cerebral vasculature. Representative images and densitometry of pEGFR western blotting in pial arteries from saline- or Ang II-treated WT and Cav-1/- mice (A); and representative images and staining density of pEGFR immunohistochemistry in pial arterioles from Ang II-treated WT and Cav-1/- mice (B). Results are mean ± SEM of 8 mice. *P<0.05 vs. WT control; #P<0.05 vs. WT with Ang II. Scale bar, 10 μm.
Figure S2. Representative images of MMP9 immunohistochemistry in pial arterioles from saline-treated WT and Cav-1/- mice. Scale bar, 10 µm.
Figure S3. Ang II increases MMP9 expression in cerebral arterioles. Representative images of MMP9 immunohistochemistry in pial arterioles from saline-treated WT and MMP9-/− mice (A). Representative images and straining density of MMP9 immunohistochemistry in pial arterioles from Ang II-treated WT and MMP9-/− mice (B). Results are mean ± SEM of 6 mice. *P<0.05 vs. WT. Scale bar, 10 µm.