Caveolin-1 Deletion Prevents Hypertensive Vascular Remodeling Induced by Angiotensin II

Steven J. Forrester,* Katherine J. Elliott,* Tatsuo Kawai, Takashi Obama, Michael J. Boyer, Kyle J. Preston, Zhen Yan, Satoru Eguchi, Victor Rizzo

Abstract—It has been proposed that membrane microdomains, caveolae, in vascular cells are critical for signal transduction and downstream functions induced by angiotensin II (AngII). We have tested our hypothesis that caveolin-1 (Cav1), a major structural protein of vascular caveolae, plays a critical role in the development of vascular remodeling by AngII via regulation of epidermal growth factor receptor and vascular endothelial adhesion molecule-1. Cav1+/− and control Cav1+/− mice were infused with AngII for 2 weeks to induce vascular remodeling and hypertension. On AngII infusion, histological assessments demonstrated medial hypertrophy and perivascular fibrosis of aorta and coronary and renal arteries in Cav1+/− mice compared with sham-operated Cav1+/− mice. AngII-infused Cav1+/− mice also showed a phenotype of cardiac hypertrophy with increased heart weight to body weight ratio compared with control Cav1+/− mice. In contrast, Cav1+/− mice infused with AngII showed attenuation of vascular remodeling but not cardiac hypertrophy. Similar levels of AngII-induced hypertension were found in both Cav1+/− and Cav1+/− mice as assessed by telemetry. In Cav1+/− mice, AngII enhanced tyrosine-phosphorylated epidermal growth factor receptor staining in the aorta, which was attenuated in Cav1+/− mice infused with AngII. Enhanced Cav1 and vascular endothelial adhesion molecule-1 expression was also observed in aorta from AngII-infused Cav1+/− mice but not in Cav1+/− aorta. Experiments with vascular cells further provided a potential mechanism for our in vivo findings. These data suggest that Cav1, and presumably caveolae, in vascular smooth muscle and the endothelium plays a critical role in vascular remodeling and inflammation independent of blood pressure or cardiac hypertrophy regulation.

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Online Data Supplement

Key Words: angiotensin II ▪ blood pressure ▪ endothelial cells ▪ hypertension ▪ signal transduction

Hypertension is a disease marked by chronic vascular dysfunction and inflammation facilitating cardiovascular remodeling and subsequent end-organ damage, which contribute to increased rates of morbidity and mortality.1,2 Past investigation in hypertensive patients and animal models has given evidence that mechanisms facilitating elevations in blood pressure and end-organ damage should be independent, at least partially.3 One of the key contributors to hypertension and the hypertensive response is the renin–angiotensin system and specifically the vasoactive peptide angiotensin II (AngII).4 In mouse models of AngII-induced hypertension, multiple distinct mechanisms involving endothelial cells (ECs),5 vascular smooth muscle cells (VSMCs),6,7 adventitial fibroblasts,8 or bone marrow–derived cells9 seem to mediate cardiovascular remodeling and end-organ damage but not hypertension. Our recent findings suggest that transactivation of epidermal growth factor receptor (EGFR) mediated by a caveolae-localized metalloproteinase, ADAM17, is required for cardiovascular remodeling independent of blood pressure regulation.7,10 However, this mechanism may or may not be limited to VSMCs.11 Caveolin-1 (Cav1) is a major structural component of caveolae, which are cholesterol-rich membrane microdomains that act as signaling platforms in facilitating specific signal transduction events including those activated by the AngII type 1 receptor.12,13 Cav1 is expressed in both vascular smooth muscle and the endothelium and is implicated in several cardiovascular diseases including atherosclerosis, dilated cardiomyopathy, pulmonary hypertension, and abdominal aortic aneurysm.13-15 Regarding hypertension, it has been well documented that Cav1 inhibits endothelial NO synthase activity and contributes to maintenance of myogenic tone in the vasculature.13 However, there are numerous conflicting reports using Cav1-deficient mice, which may not support a direct role for Cav1 in blood pressure regulation in hypertension.14 This could be because of vascular compensation associated with hypertrophic arterial remodeling, impaired endothelium-dependent hyperpolarization, and contribution of the 129SVJ
strain. However, endothelial inflammatory activation such as induction of vascular endothelial adhesion molecule-1 (VCAM-1) and atherosclerosis are attenuated in apolipoprotein-E Cav1 double deficient mice, which seems to involve endothelial Cav1. Regarding cardiac remodeling, Cav1-deficient mice have been reported to develop cardiac hypertrophy and fibrosis; however, there is conflicting data concerning the left ventricular wall thickness and cardiac function. Moreover, limited information is available on how the lack of Cav1 alters blood pressure and hypertensive cardiovascular remodeling in mouse models of hypertension such as those induced by AngII.

On the basis of the above information, we have tested our novel hypothesis that deletion of Cav1 will attenuate hypertensive vascular remodeling (vascular hypertrophy and perivascular fibrosis) independent of hypertension and cardiac hypertrophy in mice infused with AngII. Associated signaling mechanisms such as vascular EGFR transactivation and VCAM-1 induction have also been studied.

**Methods**

An expanded Methods section is available in the online-only Data Supplement.

**Animal Experiments**

All animal procedures were performed with previous approval of the Temple University Institutional Animal Care and Use Committee and in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eight- to 10-week-old male Cav1−/− and Cav1+/+ (C57Bl6) mice were infused with AngII (1 μg/kg/min) for 2 weeks via implanted osmotic minipump or sham-operated for the implant.

**Cell Experiments**

VSMCs were prepared from thoracic aorta of male Sprague-Dawley rats using the explant method as previously described. Sprague-Dawley rat aortic ECs were purchased from Cell Biologics. THP-1 human monocyte cells were obtained from the American Type Culture Collection.

**Statistical Analysis**

Data are presented as mean±SEM or SD where appropriate. Differences between the multiple groups were analyzed by 1-way or 2-way ANOVA, followed by the Tukey post hoc test. Statistical significance was set at P<0.05.

**Results**

Compared with sham-operated Cav1−/− mice, AngII-infused Cav1−/− mice showed a marked elevation in mean blood pressure assessed by telemetry. AngII-infused Cav1−/− mice exhibited increased mean arterial blood pressure similar to that observed in Cav1+/+ with AngII infusion, whereas diastolic blood pressure responded higher than in control mice (Figure 1A; Table S1 in the online-only Data Supplement). Heart weight to body weight ratio and echocardiogram were used to assess cardiac hypertrophy. Both Cav1+/+ and Cav1−/− mice infused with AngII showed a phenotype of cardiac hypertrophy including enhanced left ventricular volume (Figure 1B; Table S1). In addition, sham-operated Cav1−/− mice exhibited greater values in the heart weight ratio, interventricular septum thickness in diastole, interventricular septum thickness in systole, and left ventricular posterior wall thickness in systole compared with Cav1+/+ mice.

Although Cav1−/− mice developed cardiac hypertrophy in response to AngII, vascular alterations had differing results. AngII-infused Cav1+/+ mice exhibited increased medial thickness in the aorta, which was attenuated in Cav1−/− mice (Figure 2A). Cav1+/+ mice also developed marked medial hypertrophy and perivascular fibrosis in hearts and kidneys in response to AngII. However, Cav1−/− mice showed attenuation of these responses to AngII (Figure 2B).

To investigate a potential signaling mechanism that is critical for AngII-induced vascular remodeling, we performed immunohistochemistry with aortic sections. AngII-infused Cav1+/+ mice showed induction of phosphorylated EGFR at Tyr1068 (an autophosphorylation site), which was attenuated in Cav1−/− mice. AngII infusion also increased Cav1 staining in the endothelium and medial layers of the aortas in Cav1+/+ mice, which was attenuated in Cav1−/− mice. In addition, AngII-infused Cav1−/− mice showed increased VCAM-1 staining in the endothelium and adventitia, which was attenuated in Cav1−/− mice infused with AngII (Figure 3).

To support our in vivo findings, we have used cultured rat aortic VSMCs and ECs. AngII-induced fibrotic and hypertrophic responses are attenuated in VSMCs pretreated with Cav1-silencing adenovirus (Figure 4). Mitochondrial reactive oxygen species (ROS) seem critical for hypertension and cardiac hypertrophy induced by AngII. MitoTimer reporter, which encodes a mitochondria-targeted protein producing irreversible red fluorescence when oxidized, was used to...
determine whether Cav1 in VSMC is critical for mitochondrial ROS production. AngII-induced mitochondrial ROS production is attenuated by a mitochondria-specific ROS scavenger, mitoTempo, in VSMCs. On Cav1 silencing in VSMCs, both basal and AngII-induced mitochondrial ROS production are enhanced (Figure S1). Therefore, mitochondrial ROS production is independent of the mechanism by which Cav1 silencing inhibits AngII-induced vascular remodeling, while it may participate in hypertension and cardiac hypertrophy. In rat aortic ECs, tumor necrosis factor-α (TNFα) but not AngII is able to induce VCAM-1 expression in ECs. Cav1 silencing is able to partially reduce TNFα-induced VCAM-1 expression (Figure 5A). Moreover, Cav1 silencing prevented leukocyte adhesion to rat aortic ECs in response to TNFα (Figure 5B).

**Discussion**

Here, we report that Cav1 is a critical mediator of hypertensive vascular remodeling and inflammation in mice infused with AngII. Previous studies by others and our group have highlighted the critical roles Cav1 plays in mediating AngII-induced signal transduction in vitro, whereas its role in AngII-induced pathophysiological effects has been unclear. The present study builds a new concept that vascular Cav1 specifically mediates hypertensive vascular remodeling without altering hypertension. The vascular protective role of Cav1 silencing seems consistent with past reports using Cav1−/− mice in models of atherosclerosis, abdominal aortic aneurysm, and brain microvascular hypertrophy. In the present study, prevention of vascular remodeling in Cav1−/− mice

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**Figure 2.** Angiotensin II (AngII)-induced vascular remodeling is attenuated in Caveolin-1 (Cav1)-deficient mice. **A**, Representative staining of the thoracic aorta (×100) and quantification of the medial thickness are presented. **B**, Representative staining of the coronary and renal arteries (×200), quantification of medial area to internal arterial area of the arteries, and quantification of perivascular fibrosis area to vascular area of the arteries are presented. Values are represented as mean±SEM (n=4–6). *P<0.05 compared with control saline infusion. †P<0.05 compared with control Cav1+/+ mice.
infused with AngII correlated with reductions in EGFR activation in the vasculature. In addition, we found that Cav1 silencing has a protective effect against AngII-mediated increases in hypertrophy and collagen synthesis in vitro in VSMCs. We have previously studied the mechanism of VSMC signaling through Cav1 and demonstrated that Cav1 is a requisite component of ADAM17 activation and gene induction. We have also shown that ADAM17 activation is required for EGFR transactivation in VSMCs. Inhibition of ADAM17 or EGFR activity reduced hypertrophic and fibrotic responses to AngII in VSMCs. The mechanism of Cav1 acting via ADAM17-dependent EGFR activation is further supported by

Figure 3. Vascular epidermal growth factor receptor (EGFR) activation and vascular endothelial adhesion molecule-1 (VCAM-1) induction in response to angiotensin II (AngII) infusion was attenuated in Caveolin-1 (Cav1)-deficient mice. Aorta sections were immunostained with antibodies as indicated. The staining intensity was quantified in medial layers (EGFR-p and Cav1) or adventitia (VCAM-1). Values are represented as mean±SEM (n=4). *P<0.05 compared with control saline infusion. †P<0.05 compared with control Cav1+/+ mice.

Figure 4. Caveolin-1 (Cav1) silencing attenuates angiotensin II (AngII)–induced vascular smooth muscle cell (VSMC) remodeling in vitro. VSMCs were pretreated with adenovirus-encoding Cav1-silencing siRNA or control nonsilencing siRNA for 48 h and were stimulated with AngII (100 nmol/L) for 48 h. Extracellular collagen accumulation, total cell protein, and cell volume were evaluated. Values are represented as mean±SEM (n=4). *P<0.05 compared with basal. †P<0.05 compared with control AngII stimulation.
A recent study further suggests a critical link between Cav1 and EGFR. Hypoxia-inducible factor-1 was shown to upregulate Cav1 leading to EGFR transactivation at caveolae in cancer cells. AngII-induced vascular remodeling was attenuated in VSMC-specific hypoxia-inducible factor-1α-deficient mice. AngII can induce hypoxia-inducible factor-1α in VSMCs, which also increases ADAM17 gene induction. Cav1 was induced in the vasculature in response to AngII in the present and past studies. Therefore, it is intriguing to speculate that AngII promotes several feed-forward mechanisms including Cav1 induction to amplify the EGFR signaling pathway and subsequent vascular remodeling.

We are aware of the reported baseline phenotypes of Cav1−/− mice where enhanced arterial hypertrophy or fibrotic responses such as in lung and kidney have been reported. However, we did not observe any significant difference in baseline medial thickness in the aortas and only marginal enhancement of vascular medial area and perivascular fibrosis area in Cav1−/− mice. Although we do not have any mechanistic explanation for these discrepancies, it may be because of the genetic background of C57Bl/6 strain and the relatively young age at analysis. A protective role for Cav1 against fibrosis has been reasoned through its negative alterations on transforming growth factor-β activation and function. In contrast, a recent study using Cav1-deficient fibroblasts demonstrates that Cav1 promotes extracellular matrix remodeling and stiffness. Because transforming growth factor-β is critical for the AngII-induced cardiovascular fibrotic response, further clarification is desired for the potential cross talk between Cav1 and transforming growth factor-β in mediating perivascular fibrosis in hypertension.

In support of several previous reports, we did not notice any alterations in blood pressure in sham-operated mice of both Cav1+/+ and Cav1−/− genotypes. Increased systolic and mean blood pressure was found in both Cav1+/+ and Cav1−/− mice treated with AngII compared with control mice with no differences between the AngII groups. These data are consistent with past reports infusing 1-NAME in Cav1−/− mice. However, enhanced blood pressure responses have been reported with 1-NAME plus AngII infusion and high-fat diet in Cav1−/− mice, whereas reduced blood pressure response has been reported with AngII infusion in Cav1+/+ mice. In the present study, enhanced diastolic blood pressure was also observed in Cav1−/− mice in response to AngII infusion, which may involve the role of Cav1 in promoting AngII desensitization in arterial contraction.

A decrease in vascular remodeling should reduce the high blood pressure response to AngII. However, in our 2-week AngII infusion model with both genetic (Cav1 or ADAM17 deletion) and pharmacological (erlotinib or 4-phenylbutyrate) interventions, we have observed a suppression of vascular remodeling and no reduction in hypertension. One potential explanation is the relatively short duration of our studies. In mice with vascular hypoxia-inducible factor-1α silencing, AngII-induced vascular remodeling is attenuated at 4 weeks, whereas a reduction of blood pressure is far more noticeable at 4 weeks than at 2 weeks. Alternatively, the high concentration of AngII used in our protocol may maintain hypertension even with the reduction in vascular remodeling. Many published articles using a dose of AngII similar to ours indicate near-maximal hypertensive responses occurring within a few days. A study has looked at the time course of vascular remodeling in response to AngII in mice. The study shows that gradual arterial hypertrophy becomes noticeable at day 3 and keeps developing for 4 weeks. Suggesting that vascular remodeling is irrevocable for the establishment of hypertension with high dose of continued AngII infusion. By contrast, 4-phenylbutyrate reduced both cardiac fibrosis and hypertension induced by AngII with 60% less infusion rate than our studies. In addition, enhanced mitochondrial ROS production from VSMCs and presumably from ECs in response to AngII may also mediate hypertension in Cav1−/− mice infused with AngII.

At 22 months of age, Cav1−/− mice of different genetic backgrounds exhibited several pathophysiological cardiac phenotypes including cardiac hypertrophy, decreased...
contractility, and dilated cardiomyopathy. Our Cav1−/− mice at 10 week of age show moderate cardiac hypertrophy with preserved contractility, which is consistent with a past publication analyzing cardiac function of 6- to 8-week-old Cav1−/− mice. Several mechanisms for the basis of cardiac hypertrophy in Cav1−/− mice have been reported including an endothelium-dependent mechanism. Endothelial specific Cav1 re-expression in Cav1−/− mice rescues the cardiac phenotype. Nuclear factor-κB activation is critical for TNFα-induced VCAM-1 induction. The present study further suggests a role for endothelial Cav1 in mediating vascular inflammation in hypertension via VCAM-1 induction. Our findings are consistent with a reported attenuation of TNFα-induced lymphocyte adhesion to microvascular ECs by Cav1 silencing. Cav1 silencing also attenuated TNFα-induced VCAM-1 induction in human umbilical vein ECs. In addition, endothelial VCAM-1 induction in atherosclerosis was attenuated in Cav1−/− mice. In ECs, nuclear factor κB activation is critical for TNFα-induced VCAM-1 induction. Nuclear factor κB inhibition is likely involved in the suppression of VCAM-1 induction in ECs according to the literature.

Taken together, these data indicate that the mechanism of suppression of AngII-induced vascular remodeling with Cav1 silencing should involve inhibition of VSMC ADAM17/EGFR activation and suppression of endothelial inflammation via inhibition of VCAM-1 induction (Figure S2). VCAM-1 conditional knockout mice are available. Further inclusion of such mice and a rescue experiment with an EGFR agonist could be tested to support our conclusion. In addition, limitations of the present study include the lack of assessment of the developmental relationship between vascular remodeling and hypertension with lower AngII infusion and tibia length normalization of the heart weights in our assessments on cardiac hypertrophy.

Perspectives
Our findings highlight the complexity and critical role of Cav1 in mediating hypertensive vascular remodeling and inflammatory signaling. We propose that Cav1 is a needed component for EGFR transactivation contributing to hypertensive vascular remodeling. Our results also indicate a role for Cav1 in vascular inflammation as we noted a requirement for Cav1 in endothelial VCAM-1 induction. Vascular remodeling precedes end-organ damage in hypertension. In addition to atherosclerosis, Cav1 may serve as a novel therapeutic target in hypertension. Vascular specific targeting of Cav1 in hypertensive patients could provide a viable avenue in the treatment of this threatening disease. However, there is still more to uncover about Cav1. Specifically, a better understanding of how Cav1 contributes to normal physiological function compared with pathophysiological function in cells and tissues is a needed area of research before viable treatment options may be introduced.

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Disclosures
None.

References


Chow AK, Daniel EE, Schulz R. Cardiac function is not significantly diminished in hearts isolated from young caveolin-1 knockout mice. Am J Physiol Heart Circ Physiol. 2010;299:H1183–H1189. doi: 10.1152/ajpheart.00649.2014.


Bouzin C, Brouet A, De Vriese J, De Dewever J, Feron O. Effects of vascular endothelial growth factor on the lymphocyte-endothelium interactions:


**Novelty and Significance**

**What Is New?**
- Analyses of blood pressure and vascular pathology in the heart, kidney, and aorta with intervention established a role for caveolin-1 (Cav1) in angiotensin II–induced vascular remodeling independent of hypertension or cardiac hypertrophy in mice.
- The concept of vascular Cav1 in mediating the epidermal growth factor receptor pathway and vascular endothelial adhesion molecule-1 and subsequent vascular hypertrophy, fibrosis, and inflammation was presented.

**What Is Relevant?**
- Results indicating prevention of vascular remodeling but not hypertension by Cav1 silencing provide a foundation to seek a potential add-on therapy to current pressure-lowering treatments for hypertension.

**Summary**
In angiotensin II–infused Cav1-deficient mice, perivascular fibrosis and vascular hypertrophy were prevented compared with infused wild-type mice. Angiotensin II infusion showed vascular epidermal growth factor receptor activation and induction of Cav1 and vascular endothelial adhesion molecule-1, which were attenuated in Cav1-deficient mice. Cultured vascular cells were used to confirm the direct contribution of vascular Cav1 in hypertensive cellular pathophysiology.
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Short title: Caveolin-1 mediates organ damage by angiotensin II

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Online Methods

Animal Experiments
All animal procedures were performed with prior approval of the Temple University Institutional Animal Care and Use Committee and in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals. 8-10 week old male Cav1−/− mice in C57BL/6 background (B6.Cg-Cav1tm1Mls/J) and control C57BL/6J (Cav1+/+) mice were obtained from the Jackson Laboratory and housed under barrier conditions. Standard sterilized laboratory diet and water were available ad libitum. 8-10 week Cav1−/- and Cav1+/+ (C57Bl6) mice were infused with AngII (Bachem 1 µg/kg/min) via implanted osmotic mini-pump or sham-operated for the implant. Blood pressure and heart rate were evaluated in conscious mice at day 14 by telemetry (DSI equipped with ADInstrument 6 software) via carotid catheter (PA-C10). Evaluation of cardiac morphology and function was conducted with echocardiogram using the VisualSonics Vevo 2100. Extracted hearts, kidneys and aortas were fixed and used for histological assessment as described previously.

Evaluation of Vascular Remodeling
Vascular hypertrophy and perivascular fibrosis in heart and kidney samples were evaluated with Sirius Red (EMS, Hatfield OA) staining. Briefly, after de-paraffinization and re-hydration, sections (5 µm thick) were stained in equal parts Weigart’s Iron Hematoxylin A and B (EMS, Hatfield PA) for 10 min at room temperature. Sections were washed twice in distilled water for 3 min per wash. Sirius Red was added for 1 h at room temperature. Slides were washed twice in 0.01N HCL for 3 min per wash. Sections were then dehydrated and penetrated using ethanol and xylene, respectively. Thoracic aortas were stained with Masson’s trichrome to distinguish media area from adventitia. Briefly, after de-paraffinization and re-hydration, sections were incubated with Bouin’s fluid for 1 h at 56ºC. Sections were washed three time in distilled water for 3 min per wash was and incubated with working HE solution for 7.5 min followed by washing in distilled water for 30 sec. Sections were then incubated with Biebrich Scarlet-Acid Fuchsin solution for 5 min. After incubation with phosphotungstic-phosphomolybdic acid solution for 5 min, sections were stained with Aniline Blue stain solution for 5 min. Sections were washed in 1% acetic acid for 30 sec and distilled water for 30 sec. Sections were dehydrated and penetrated using ethanol and xylene, respectively. Images were visualized on an Olympus IX81 inverted microscope using an Olympus SC30 high resolution camera and were acquired with Olympus CellSens Entry 1.11 software. Analysis was conducted using ImageJ 1.50f software (http://rsb.info.nih.gov/ij).

To calculate vascular hypertrophy in the heart and kidney, the value of medial area was divided by the true area of the vessel. True area was calculated by vessel outer perimeter divided by $4\pi$. The value generated was the area of the vessel in true circular form. To calculate perivascular fibrosis, the value of fibrosis area was subtracted from vessel area and divided by the true area of the vessel. In total, 6-8 randomly selected samples per group were used for analysis. Three representative vascular images were analyzed per sample. Medial hypertrophy of thoracic aorta was quantified by measurements of medial thickness in 4 randomly-selected locations per
slide. Three representative vascular images were analyzed per sample. Adventitia of the aorta was not quantified as the area was occasionally damaged or removed during the dissection.

**Immunohistochemistry**

For immunohistochemistry, serial cross-sections were deparaffinized and blocked in 5% goat serum and 1% BSA for 1h at room temperature, incubated with primary antibody in PBS containing 1% BSA and 0.1% Tween 20 for 18 h at 4 °C, followed by biotinylated secondary antibody for 90 min at room temperature. Slides were incubated with avidin–biotin peroxidase complex for 30 min at room temperature and staining was visualized with the substrate diaminobenzidine (Vector) producing a brown color and counterstained with haematoxylin. An equal concentration of control IgG was used side-by-side with each antibody to ensure staining specificity. All images were visualized on an Olympus SC30 high resolution camera and were acquired with Olympus CellSens Entry 1.11 software using the same exposure time.

**Cell Experiments**

VSMCs were prepared from thoracic aorta of male Sprague-Dawley rats using the explant method as previously described. VSMCs were subcultured in DMEM with the addition of 10% fetal bovine serum, penicillin and streptomycin. Cells were made quiescent by incubation with serum-free medium for 2-3 days. To avoid any potential phenotypic alteration, VSMCs were renewed every 2-3 months and VSMCs from frozen stock were never used. The results were confirmed in at least 2 distinct cell preparations. For Cav1 silencing experiment, cells from passage 3 to 10 at 80~90% confluence were infected with adenovirus encoding Cav1 siRNA (100 moi) or control non-silencing siRNA as reported previously. To evaluate pro-fibrotic response, VSMCs were stimulated with 100 nmol/L AngII for 48 hours and extracellular cellular collagen content was quantified by Sirius Red collagen quantification kit (Chondrex) according to the manufacture’s protocol. VSMC total protein and cell volume measurements were used to assess a hypertrophic response induced by AngII as previously described.

Sprague-Dawley rat aortic endothelial cells (RAECs) were purchased from Cell Biologics and were cultured in DMEM with 10% fetal bovine serum, penicillin and streptomycin. For Cav1 silencing, RAECs from passage 4-8 were infected with adenoviruses encoding Cav1 siRNA in serum-free DMEM in the presence of 0.5 µg/mL poly-L-lysine for 48 hours prior to experimental stimulation. Since sub-cultured arterial endothelial cells lose expression of AngII type-1 receptors and AngII responses, RAECs were stimulated with 10 ng/mL tissue necrosis factor-α (TNFα) for 6 hours.

To observe leukocyte attachment to RAECs, THP-1 monocytes cultured in RPMI with 10% FBS, penicillin and streptomycin were suspended in serum-free DMEM with 0.2% BSA and 5 µg/mL Hoechst 33342 (ThermoFisher) for 30 min at 37°C. THP-1 cells re-suspended in DMEM with 0.2% BSA (10^4 cells per cm^2) were incubated with RAECs for 30 min at 37°C. Cells were then washed in PBS and fixed in 3.7% paraformaldehyde for 10 min at room temperature. Fixed cells were washed in PBS and subsequently imaged using a fluorescent inverted microscope. Three separate pictures were taken per condition using a 10x objective lens. Images were imported into imageJ where the
background was subtracted and an image threshold was generated. Stained THP-1 nuclei were counted to evaluate adhesion to RAECs.

**Immunoblotting**
Immunoblotting was performed as previously described. Upon cell stimulation for a specified duration, the reaction was terminated by the replacement of medium with 100 µL of 1xSDS lysis buffer. 40 µL of the cell lysates were subjected to SDS-PAGE gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane. The membranes were then exposed to primary antibodies overnight at 4 °C. After incubation with the peroxidase linked secondary antibody for 1 h at room temperature, immunoreactive proteins were visualized using a chemiluminescence reaction kit.

**Mitochondrial ROS measurement**
MitoTimer reporter assay was used to assess mitochondrial ROS production. MitoTimer gene encodes a mitochondria-targeted protein producing irreversible red fluorescence when oxidized. Adenovirus encoding MitoTimer gene was created from the plasmid DNA as reported. Serum-starved VSMCs grown on 8 well chamber slide were infected with mitoTimer adenovirus (25 moi) for 2 days together with adenovirus encoding Cav1 siRNA or control non-silencing RNA, and red fluorescent intensity was evaluated upon 4 hour stimulation of AngII (100 nmol/L).

**Antibodies**
Antibody against Tyr1068-phosphorylated EGFR for IHC (2234) was purchased from Cell Signaling. Antibody against VCAM-1 (ab134047) was purchased from Abcam. Antibody against Cav1 (610406) was purchased from BD biosciences. Antibody against GAPDH (MAB374) was purchased from Millipore.

**References**


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<td>DBP mmHg</td>
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<td>126±12*</td>
<td>101±9</td>
<td>156±30†</td>
</tr>
<tr>
<td>HR min</td>
<td>618±84</td>
<td>631±101</td>
<td>601±98</td>
<td>637±126</td>
</tr>
</tbody>
</table>

**Online Table S1.** Echocardiography and other characteristic of Cav1+/+ and -/- mice infused with AngII. Mean±SD (n=5-6), *p<0.05 compared with saline* or AngII† infusion. †p<0.05 compared with Cav1+/+ mice. IVSd: interventricular septum thickness in diastole; LVIDd: LV internal diameter in diastole; LVWd: LV posterior wall thickness in diastole; LVIDs: LV internal diameter in systole; LVv: LV volume; FS: fractional shortening. BW: body weight; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate.
Online Figure S1. Cav1 silencing does not block AngII-induced mitochondrial ROS production. VSMCs grown on 8-well chamber slides infected with adenovirus encoding mitoTimer (25 moi) were pretreated with mitoTempo (25 nM) or its vehicle (0.1% DMSO) for 30 min (A) or co-infected with adenovirus encoding Cav1 siRNA or control non-silencing RNA (100 moi) for 48 hours (B), and stimulated with Ang II (100 nmol/L) for 4 hours. Mito-timer images were obtained at 100x magnification under a confocal microscope using red channel. Images were loaded into the ImageJ program to analyze signal intensity. *p<0.05 compared with basal condition. †p<0.05 compared with control siRNA (n=6).
Online Figure S2. Schematic representation of the potential mechanism by which Cav1 contributes to vascular inflammation and remodeling.