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

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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-Cav1\textsuperscript{tm1Mls/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 \textmu g/kg/min) via implanted osmotic mini-pump or sham-operated for the implant \textsuperscript{1}. 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 \textsuperscript{1,2}.

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 \textmu m 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\textsuperscript{2} 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
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 1 h 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 manufacturer’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 (10⁴ cells per cm²) 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 Tyr-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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   receptor and endoplasmic reticulum stress in vascular remodeling induced by

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**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; LVId: LV internal diameter in diastole; LVPWd: 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.