Uncoupling Caveolae From Intracellular Signaling In Vivo

Jan R. Kraehling, Zhengrong Hao, Monica Y. Lee, David J. Vinyard, Heino Velazquez, Xinran Liu, Radu V. Stan, Gary W. Brudvig, William C. Sessa

Rationale: Caveolin-1 (Cav-1) negatively regulates endothelial nitric oxide (NO) synthase–derived NO production, and this has been mapped to several residues on Cav-1, including F92. Herein, we reasoned that endothelial expression of an F92ACav-1 transgene would let us decipher the mechanisms and relationships between caveolae structure and intracellular signaling.

Objective: This study was designed to separate caveolae formation from its downstream signaling effects.

Methods and Results: An endothelial-specific doxycycline-regulated mouse model for the expression of Cav-1-F92A was developed. Blood pressure by telemetry and nitric oxide bioavailability by electron paramagnetic resonance and phosphorylation of vasodilator–stimulated phosphoprotein were determined. Caveolae integrity in the presence of Cav-1-F92A was measured by stabilization of caveolin-2, sucrose gradient, and electron microscopy. Histological analysis of heart and lung, echocardiography, and signaling were performed.

Conclusions: This study shows that mutant Cav-1-F92A forms caveolae structures similar to WT but leads to increases in NO bioavailability in vivo, thereby demonstrating that caveolae formation and downstream signaling events occur through independent mechanisms. (Circ Res. 2016;118:48-55. DOI: 10.1161/CIRCRESAHA.115.307767.)

Key Words: caveolin-1 • cell • endothelial cell • eNOS • mice • nitric oxide • vascular function

Caveolae organelles are flask-shaped invaginations of the plasma membrane implicated in a variety of biological processes, including endocytosis, transcytosis, mechanosensing, and signaling.1–3 Caveolin-1 (Cav-1) is the main coat protein of caveolae in endothelial cells (EC) and is essential for the formation of caveolae.4 Despite the critical role of Cav-1 in caveolae assembly, Cav-1 knockout (KO) mice are viable and fertile, but show several cardiovascular and pulmonary phenotypes. Cav-1 KO mice exhibit impaired mechanosignaling and remodeling, myocardial hypertrophy, metabolic imbalances with elevated plasma lipids, pulmonary fibrosis, and hypertension and are protected from atherosclerosis.5–10 The precise mechanisms of how Cav-1 regulates these diverse phenotypes are unknown, and most data are rationalized based on the critical role of Cav-1 in caveolae formation and mechanosignaling or via Cav-1 serving as a scaffolding protein integrating extracellular signaling pathways to intracellular effectors, such as protein kinase A,11 G protein–coupled receptors,12,13 Rab5,13 and endothelial nitric oxide synthase (eNOS).14

eNOS-derived nitric oxide (NO) in the vascular system is a major regulator of vascular tone and, therefore, blood pressure. eNOS is post-translationally palmitoylated and trafficked to caveolae,15 where its activity is decreased because of the binding to Cav-1.14,16 Domain mapping studies revealed that the aa 82–101 of Cav-1 are critical for Cav-1 interacting with eNOS,14,16 whereas the aa T90, T91, and in particular F92 play a crucial role in the inhibitory action of this binding. After activation, eNOS is thought to be released from the inhibitory clamp of Cav-1 and phosphorylated by kinases, including protein kinase B (Akt).17 HSP90 (heat shock protein 90) and calmodulin binding leads to a fully activated eNOS,18 resulting in increased NO release. A hallmark of many vascular and pulmonary diseases is a decrease in the NO biogenesis or bioavailability via uncoupling. Therefore, understanding how to selectively activate eNOS could be beneficial.

Here, we report that inducible expression of a single point mutant of Cav-1 (Cav-1-F92A) in endothelium decreases systolic blood pressure. The reduction in blood pressure occurs contemporaneously with enhanced levels of NO bioactivity. Moreover, Cav-1-F92A does not interfere with the formation of caveolae or promote pulmonary fibrosis, myocardial dysfunction, or hypertrophy, as seen in global Cav-1 KO mice.
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Cav-1</td>
<td>caveolin-1</td>
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<tr>
<td>Cav-2</td>
<td>caveolin-2</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<tr>
<td>Hb</td>
<td>hemoglobin</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>ITA</td>
<td>tetracycline controlled transactivator</td>
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<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
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These data suggest that it is feasible to uncouple caveolae formation from its intracellular partners, and disrupting the inhibitory interaction between Cav-1 and eNOS could be a potential therapeutic approach.

Methods

Because of space limitations, a detailed description of the Materials and Methods is presented in the Online Data Supplement.

Results

Endothelial-Specific Expression of Cav-1-F92A

Previous studies have shown that Cav-1-F92A expression in ECs leads to an increased NO release.\(^\text{19,20}\) To further investigate this Cav-1 point mutation in EC in vivo, we generated an endothelial-specific, doxycycline (DOX)-controlled F92A Cav-1 transgenic mouse using a Cdh5-tetracycline controlled transactivator driver line\(^{21}\) bred to a tetracycline-responsive element–driven F92A Cav-1 construct tagged with the hemagglutinin (HA) epitope on its C terminus (Online Figure IA). The HA tag was necessary to distinguish endogenous Cav-1 from transgenic Cav-1 as the Cav-1 antibody does not discriminate between human and murine proteins. The expression of Cav-1-F92A was confirmed in whole lung lysates by immunoblotting (Figure 1A). The endothelial-specific expression was shown by isolating mouse lung endothelial cells using CD31 beads. The fractions bound to the beads (CD31\(^+\)) and the nonbound fraction (CD31\(^−\)) were separated and analyzed by immunoblotting (Figure 1B). Immunofluorescence of cross sections of the thoracic aorta with anti-α-SMA (alpha smooth muscle actin) and anti-HA and of en face images of the mesenteric artery stained with anti-PECAM1 (platelet endothelial cell adhesion molecule-1) and anti-HA show the EC-specific expression of Cav-1-F92A (Figure 1C). Based on en face images, we estimate that Cav-1-F92A is expressed in ≈30% of EC in a mosaic pattern. Moreover, breeding of the Cdh5-tetracycline controlled transactivator driver line\(^{21}\) to a TetO7-green fluorescent protein (GFP) reporter mouse\(^{22}\) (The Jackson Laboratory, No 018913) documents the mosaic activation of green fluorescent protein (GFP) in a population of EC (Figure 1B).

Blood Pressure Is Lower in Cav-1-F92A Transgenic Animals and Reversed by Doxycycline Treatment

Treatment of double transgenic animals with DOX (2 mg/mL) in the drinking water leads to time-dependent suppression of Cav-1-F92A expression at 3 and 7 days (Figure 2A), demonstrating DOX regulation of the transgene. Thus, single transgenic (control) and double transgenic (Cav-1-F92A) mice were implanted with telemetry devices for continuous monitoring of blood pressure. All mice were initially placed on water (−DOX) and then switched to DOX drinking water (+DOX). As seen in Figure 2B, the systolic blood pressure of Cav-1-F92A mice increases significantly after 7 days treatment with DOX by shutting off the transgene (Figure 2A). Blood pressure increases at a rate of 0.028 mm Hg/h on switching the drinking water to DOX (Online Table I). The absence or presence of DOX had no effect on systolic pressures in control mice (Figure 2B). Although diastolic pressure changes were not significantly different, the trends were toward an increase (Online Figure IIA). These differences may relate to the mosaic nature of the transgene expression or the dominant effects of Cav-1 on systolic pressure.\(^\text{23}\) Heart rates did not differ between the strains (Online Figure IIA). Treatment with L-N\(^\text{\textsuperscript{5}}\)-nitro-arginine-methyl-ester (L-NAME; 1 mg/mL) leads to significant increase of the systolic blood pressure of control and Cav-1-F92A mice (Figure 2C). The diastolic blood pressure changes in control and Cav-1-F92A mice significantly after 7 days treatment with L-NAME (Online Figure IIB). Heart rate decreases in control and Cav-1-F92A animals significantly after 7 days of L-NAME (Online Figure IIB).
Nitric Oxide Bioavailability Is Higher in Cav-1-F92A Transgenic Animals

The NO levels in whole blood were measured to assess whether the Cav-1-F92A transgene influenced blood pressure through eNOS-derived NO in vivo. Nitrosyl-hemoglobin (NO-Hb) was determined in venous blood by electron paramagnetic resonance. The 3-line hyperfine spectrum at $g = 2.01$ with a splitting constant of $\approx 17$ G is specific for the 5-coordinated complex.

**Figure 2.** Effects of suppression of Cav-1-F92A-HA expression and nitric oxide measurements. **A,** Left, Whole lung protein from double transgenic mice was analyzed for the expression of the transgene at day 0, day 3, and day 7 after treatment with doxycycline (DOX; 2 mg/mL) with 5% sucrose in the drinking water. Each time point was repeated in 3 animals. Right, Densitometry analysis of the HA intensity and the heat shock protein 90 (HSP90) loading control. **B,** Left, Averaged systolic blood pressure from 8 control and 8 Cav-1-F92A animals. The first 2 days baseline was recorded before drinking water was switched to doxycycline (2 mg/mL) with 5% sucrose. Dotted lines show the changes of the systolic blood pressure (slopes of the curves are listed in Online Table I). Right, Bar graph presentation of the same results. The bars present the average systolic blood pressure without and with doxycycline. **C,** Left, Averaged systolic blood pressure from 3 control and 3 Cav-1-F92A animals. The first 2 days baseline was recorded before drinking water was switched to L-N^2-nitro-arginine-methyl-ester (L-NAME) (1 mg/mL). Dotted lines show the changes of the systolic blood pressure (slopes of the curves are listed in the Online Table II). Right, Bar graph presentation of the same results. The bars present the average systolic blood pressure without and with L-NAME. **D,** EPR quantification of the peak to trough length of 4 mice each (representative traces are shown in the Online Figure IV). **E,** Quantification of the p-VASP/t-VASP ratio (immunoblot is shown in the Online Figure V). Cav-1 indicates Caveolin-1; HA, hemagglutinin; and VASP, vasodilator stimulated phosphoprotein.
of NO with hemoglobin as reported before\textsuperscript{23} and can be used as a readout for eNOS-derived NO because neither iNOS (inducible nitric oxide synthases) nor nNOS (neuronal nitric oxide synthases) contribute to the formation of NO-Hb under normal conditions.\textsuperscript{25} Mice expressing the Cav-1-F92A mutant show a significant increase (165 \%) of NO-Hb in their whole blood compared with WT (Figure 2D; representative traces in Online Figure III). As controls, venous blood from eNOS KO,\textsuperscript{26} eNOS S1176A (inactive), and eNOS S1176D (constitutively active) mice\textsuperscript{27} (Online Figure IV) was measured. Isolated blood from eNOS KO (66 \%) and the eNOS S1176A (41 \%) mutant shows significantly reduced NO-Hb levels, whereas eNOS S1176D have significantly higher (178 \%) NO-Hb levels compared with the WT. NO activates soluble guanylate

Figure 3. Stability of caveolae in the presence of Cav-1-F92A mutant. A, Left, Whole lung protein from single and double transgenic mice was analyzed for the expression of Cav-2 and Cavin-1. Cav-1 and HA immunoblotting as a control for the expression of the transgene. Heat shock protein 90 (HSP90) was used as loading control. Right, Quantification of the relative intensity of Cav-2 and Cavin-1 normalized to HSP90. B, Adenoviral reconstitution of immortalized Cav-1 KO mouse lung endothelial cells (MLECs) with either green fluorescent protein (GFP), Cav-1 wild-typeWT, or Cav-1-F92A mutant. Immortalized WT cells are loaded as control for the expected expression level of Cav-1 and Cav-2. Cav-2 immunoblotting as a readout for the stability of caveolae. C, Top, Immunoblot analysis of Cav-1 (WT, endogenous) and (HA, transgene) of the sucrose gradient fractions. HSP90 was used as marker for bulk fractions. Lower, Quantification of the immunoblot represented as \% band intensity per fraction. D, TEM (transmission electron microscopy) images for Cav-1 KO mouse embryonic fibroblasts (MEFs) adenoviral reconstituted with WT or Cav-1-F92A mutant. Scale bar is 500 nm. At least 35 individual images were analyzed per group for the quantification of caveolae/μm of plasma membrane. Cav-1 indicates Caveolin-1; and HA, hemagglutinin.
cyclase, the main NO receptor, and increases intracellular cGMP. cGMP activates protein kinase G and the subsequent phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser-239. Analysis of the p-VASP/t-VASP ratio in aorta lysates reveal that mice expressing Cav-1-F92A have a significantly higher (131%) phosphorylation status of Ser-239 of VASP compared with the WT controls (Figure 2E), thus indicating enhanced NO bioavailability.

Expression of Cav-1-F92A Stabilizes Endogenous Caveolin-2 and Does Not Affect Caveolae Properties
Previous studies have shown that the genetic loss of Cav-1 destabilizes caveolin-2 (Cav-2) and, to a lesser extent, destabilizes the caveolin adaptor, Cavin-1. Interestingly, Cav-1-F92A expression in EC enhances Cav-2 and Cavin-1 levels in whole lung lysates from transgenic mice (Figure 3A), demonstrating that Cav-1-F92A does not impair caveolae integrity, but likely stabilizes the protein complex. Additionally, Cav-2 stabilization as a readout of Cav-1 function was examined in immortalized Cav-1 KO mouse lung endothelial cell transduced with adenoviral WT Cav-1 (AdCav-1 myc) or Cav-1-F92A (AdCav-1-F92A HA). Expression of similar levels of WT and Cav-1-F92A increased Cav-2 protein levels compared with AdGFP transduced cells (Figure 3B). To further assess the integrity of the Cav-1–enriched domains, the flotation of Cav-1 and Cav-1-F92A on sucrose gradients was performed. Both WT and Cav-1-F92A fractionated into

Figure 4. Histology, echocardiography, and signaling. A, Histology of single (WT) and double transgenic (WT Tg) mice. First, Gross morphology of heart cross section through the ventricles. Second, Wall thickness of main coronary arteries. Third, Lung alveolar area. Fourth, Lung large bronchioles (B) and arteries (A). Scale bars: first panel, 1 mm, second to fourth panel, 200 μm. B, Echocardiography. Top, Representative M-mode images. Bottom, Quantification of the left ventricle diameter in diastole (LVD,d) and systole (LVD,s) and the thickness of the intraventricular septum wall (IVSW) and the posterior wall (PW). Bar graph presents the mean and the SEM for 5 WT and 5 WT+Tg animals. C, Signaling in heart and lung. Top, Representative immunoblots. Bottom, The quantification for protein kinase B (Akt) and mitogen-activated protein kinase 1 and 3 (ERK) activation by measuring the phospho/total ratio.
buoyant membrane domains similarly (Figure 3C). Finally, transmission electron microscopy of WT and Cav-1 KO mouse embryonic fibroblasts infected with Ad Cav-1 or AdCav-1-F92A was examined, and the morphology and numbers of caveolae/length of plasma membrane were indistinguishable (Figure 3D), demonstrating that Cav-1 F92A can generate caveolae de novo.

**Cav-1-F92A Does Not Recapitulate the Cav-1 KO Phenotypes**

In addition to enhanced NO levels and systolic hypertension, Cav-1 KO mice have mild pulmonary hypertension, fibrosis, and septal thickening and cardiac hypertrophy and dysfunction that worsens with age. To examine whether Cav-1-F92A mice exhibit any of these phenotypes, the lungs and hearts were histologically examined in aged mice (20–22 week; Figure 4A). Cav-1-F92A mice did not show histological evidence of pulmonary or cardiac abnormalities or cardiac function differences as assessed by echocardiography in aged mice (Figure 4B and Online Table V). Echocardiographic analysis showed no differences between the 2 groups in left ventricle dimensions in diastole or systole thickness of the intraventricular septum wall or posterior wall, parameters that were impaired in Cav-1 KO mice. Because of persistent eNOS activation, Cav-1 KO mice exhibit increased tyrosine nitration of proteins that contributes to their pulmonary hypertension; however, tyrosine nitration of proteins was not different between the 2 strains of mice in lungs and hearts (Online Figure VI). Finally, it is well described that Cav-1 KO mice show an increased phosphorylation of several downstream signaling pathways, such as Akt and ERK1/2. Densitometric analysis of p-Akt/t-Akt and p-ERK/t-ERK ratios shows no significant increase of either of these pathways (Figure 4C). Thus, the presence of Cav-1 F92A in ECs containing endogenous Cav-1 is sufficient to lower SBP and increase circulating NO levels; however, Cav-1 F92A mice are phenotypically distinct from mice lacking Cav-1 globally.

**Discussion**

This study was designed to answer 3 questions: (1) Is it possible to separate the formation of caveolae from downstream signaling in EC?; (2) Can a single point mutation in Cav-1 (F92A) disinhibit eNOS in vivo?, and (3) Would the persistent expression of Cav-1-F92A lead to Cav-1 KO phenotypes? The Cdh5-driven, DOX-regulated expression of Cav-1-F92A was advantageous to a global knock-in strategy because it allows titration of Cav-1-F92A in the endothelium. Here we demonstrate that Cav-1-F92A uncouples the intracellular function of Cav-1 as a negative regulator of eNOS function from Cav-1 regulation of caveolae assembly. Prior work in Cav-1 KO mice could not distinguish between phenotypes because of flattening of caveolae versus phenotypes associated with altered intracellular signaling. Our results lend credence to the idea that the intracellular role of Cav-1 is separable from its role in caveolae biogenesis.

In our mouse model, the expression of the F92A Cav-1 transgene is suppressed within 7 days, and during this period, the transgenic mice become normotensive again compared with the littermate single transgenic controls (ie, there are no significant differences between control and Cav-1-F92A mice). In comparison to blood pressure measurements in Cav-1 KO, the Cav-1-F92A animals do not display increased diastolic blood pressure.

Double transgenic mice show significantly higher levels of both circulating NO-Hb in vivo and phosphorylation of VASP-Ser-239 consistent with eNOS activation because of loss of endogenous Cav-1 suppression of eNOS (ie, removal of inhibition). Despite the higher concentrations of NO in these animals, neither lungs nor hearts displayed signs of fibrosis or hypertrophy akin to that seen in Cav-1 KO mice. Former studies have argued that excess NO can lead to S-nitrosylation of protein kinase G, thereby facilitating pulmonary fibrosis. However, our study indicates that increased NO levels are not sufficient for the development of fibrosis in the heart and lungs, but could contribute to this detrimental effect if other Cav-1 functions have been dysregulated in mice lacking Cav-1.

Previous work in Cav-1 KO mice found that these mice had lower systolic blood compared with controls, and the heart rate was significantly higher. These changes were associated with increased NO bioavailability (measured by electron paramagnetic resonance and VASP phosphorylation). Despite these findings, it is well known that Cav-1 KO animals exhibit both pulmonary and cardiac fibrosis and altered signaling. A robust readout of caveolae formation and function is measuring the Cav-2 protein level and, to a lower extent, Cavin-1 because both proteins are in a stable complex with Cav-1, and the loss of Cav-1 genetically destabilizes endogenous Cav-2 and Cavin-1. Whole lung or cardiac lysates show that EC expression of Cav-1-F92A indeed increased Cav-2 protein levels, suggesting that the point mutant F92A isoform did not disrupt the integrity of caveolae. Furthermore, re-expression of either Cav-1 WT or Cav-1-F92A into Cav-1 KO mouse lung endothelial cells led to a stabilization of Cav-2 protein similar to WT levels. Cav-1 or Cav-1-F92A proteins were distributed similarly on sucrose gradients and formed caveolae to the same extent, indicating that the F92A point mutant of Cav-1 integrates into the plasma membrane caveolae in manner similar to the WT protein. Recent biophysical data show that aa 102–134 are necessary to form the hairpin membrane domain of Cav-1, and phe- nylalanine at position 92 is in a juxtamembrane cytoplasmatic residue that will not affect the integration of aa 102–134 into the membrane.

In summary, our study shows that the expression of Cav-1-F92A leads to increases in NO release, without interfering with the formation of caveolae, indicating, that signaling and caveolae formation are independent functions of Cav-1 (Online Figure VII). These data complement data on the peptide cavoxin (a cell penetrating caveolin peptide which contains 3 point mutations at90A, T91A, and F92A) and highlight the significance of F92 for the inhibitory action of Cav-1 on eNOS function. Because neither the histological analysis nor the echocardiography showed any signs of cardiac hypertrophy or fibrosis in heart and lung, we surmise that prolonged therapeutic treatment with cavoxin or similar acting small molecule...
could lead to a new class of therapeutics to improve endothelial dysfunction.

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Disclosures

The authors declare that there are no conflicts of interest. The sponsors had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References


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**Novelty and Significance**

**What Is Known?**

- Caveolae are enriched in endothelial cells and serve as reservoirs for membrane expansion and mechanosignaling.
- Caveolin-1, a coat protein for caveolae, is essential for caveolae formation and suppression of nitric oxide release.

**What New Information Does This Article Contribute?**

- A mutant of caveolin-1 promotes hypotension.
- Hypotension is linked to increases in nitric oxide levels in blood and tissue.

Here we show that mutant caveolin-1 leads to increases in nitric oxide bioavailability in vivo supporting the idea that antagonizing the caveolin-1/endothelial nitric oxide synthase interaction can improve endothelial function.
Uncoupling Caveolae From Intracellular Signaling In Vivo
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SUPPLEMENTAL MATERIAL

Detailed Methods

Mice
Human Caveolin-1 (Cav-1) in pcDNA3.1 was mutated (F92A) by site directed mutagenesis, the C-terminus was PCR tagged with HA, before SalI/EcoRV cloned into pTet-Splice (TetO7 promoter). The linearized plasmid was injected into fertilized eggs of C57BL/6J mice (The Jackson Laboratory, #000664). A male founder was backcrossed 5 times before breed to Cdh5-tTA mice (tTA), a kind gift from Laura Benjamin. Single transgenic mice from the same litter were used as controls. For suppression of Cav-1-F92A expression 2 mg/ml doxycycline with 5 % sucrose was given in drinking water. The NOS inhibitor L-NAME was given at 1 mg/ml in the drinking water. The Institutional Animal Care Use Committee of Yale University approved all the experiments.

Isolation of Primary Mouse Lung Endothelial Cells (MLECs)
MLECs were isolated using a modified protocol published before. In brief, after lethal dose administration i.p. of ketamine/xylazine the whole lungs from 6-8 wk old mice were isolated, digested in collagenase I and sorted using CD31-dynabeads and a magnetic separator. The cell fraction bound to the CD31 beads (CD31+) and the non-bound fraction (CD31−) were lysed and analyzed by immunoblotting. A detailed step-by-step protocol (~6 pages) for the isolation of MLECs can be requested.

Immunofluorescence Staining of Vessels
Cross sections of thoracic aorta: Mice were euthanized using ketamine/xylazine. Mice were PBS and PFA (4 %) perfused by catheter placement in the left ventricle and punching the right atrium. The thoracic aorta was isolated, fixed O/N in PFA/PBS (4 %), then dried with sucrose (30 %) O/N before embedding in OTC media. Cryosections of 6 µm were stained with antibodies listed in table IV using a regular immunofluorescence protocol and imaged with a CLSM.
Whole-mount staining of the mesenteric artery: Mesenteric arteries (primary branch) were isolated from mice upon euthanization. Mesenteric arteries were isolated and fixed in 4 % PFA/PBS for 15 min at RT. Enface samples were blocked and incubated with primary antibodies in TNB blocking buffer. Respective secondary antibodies were applied prior to DAPI staining and subsequent imaging, as previously described.

Blood Pressure Measurement
Male mice, 10–12 wk old, were maintained under 1.75% (v/v) isoflurane anesthesia. The carotid artery was catheterized and a pressure transmitter (PA-C10, Data Sciences International) was implanted. After recovery the blood pressure was monitored continuously (one reading per minute) by oscillometric blood pressure measurement for 9 days. The first two days the baseline blood pressure was recorded, before the drinking water was switched to either doxycycline or L-NAME.
Electron Paramagnetic Resonance for Nitric Oxide Hemoglobin (NO-Hb)
Mice, 10–12 wk old, were euthanized by CO₂ (10 – 30% volume displacement per min). Whole blood was withdrawn from the inferior vena cava into 1-ml syringes and frozen immediately in liquid N₂. The frozen blood was transferred to a 77 K cold finger dewar. The three-line hyperfine spectrum of 5-coordinate nitrosyl hemoglobin was recorded at X-band with a Bruker ELEXYS E500 spectrometer equipped with a SHQ cavity in a similar setup published before⁴. ESR spectrometer settings were as follows: microwave frequency, 9.35 GHz; microwave power, 25 mW; modulation frequency, 100 kHz; modulation amplitude, 10 G; receiver gain, 70 dB; conversion time, 41 ms; time constant 20.5 ms; sweep time, 42 s. Quantification was performed by summing the peak-to-trough heights of the first two bands followed by normalization to the sample mass.

Adenoviral Reconstitution of Caveolin-1 WT and F92A mutant
Immortalized (retrovirus encoding the polyoma middle T-antigen) mouse lung endothelial cells (MLECs) isolated from WT and Cav-1 KO mice⁵ were maintained in EBM-2 medium supplemented with EGM-2 MV SingleQuots. The adenovirus for Cav-1 WT (myc-tagged) and Cav-1-F92A have been generated by sub cloning into the adenovirus shuttle plasmid. The sub cloning, production and titering of the virus was done at the Viral Vector Core of the University of Iowa.

Sucrose Gradient Fractionation
Mice were euthanized using ketamine/xylazine. Four lungs from double and single transgenic animals per experiment were isolated in MBS (25 mM MES, 150 mM NaCl, pH 7.5) with protease/phosphatase inhibitors (Roche cOmplete/Pefabloc). After mincing and homogenizing the tissue triton-x-100 was added to a final concentration of 1 % and incubated for 30 min under continuous rotation. The so prepared tissue lysate was then mixed 1 : 1 with 85 % sucrose (→ 42.5 %), aliquoted in ultracentrifugation tubes (14 x 89 mm: 12 ml) and overlaid with 35 % sucrose, followed by 15 % sucrose. The tubes were centrifuged for 18 h at 35,000 rpm in a SW40 rotor (Beckman Coulter) at 4°C.

Immunogold Transmission Electron Microscopy
Immortalized MLECs (as described before) were cultured in 10-cm dishes until confluency. The cells were fixed in 4 % PFA, followed by 0.1 % glutaraldehyde in PBS. Samples were pelleted and were cryoprotected by sucrose when frozen in liquid nitrogen. Thin sections (60 nm) were cut in liquid N₂ by a cryo-microtome. The imaging was performed on a FEI Tecnai Biotwin TEM.

Histology
Aged mice, 20 – 22 wk old, were euthanized using ketamine/xylazine and perfusion fixed as described before. Hearts and lungs were isolated and fixed O/N in PFA (4 %) before stored in 70 % EtOH. Specimens were paraffin embedded, sectioned and stained with hematoxylin/eosin.
Echocardiography
Aged mice, 20 – 22 wk old, were maintained under 1.75% (v/v) isoflurane anesthesia on a heat pad for maintaining the body temperature. Echocardiography was performed on a Vevo® 2100 (Visual Sonics). In brief, anatomical M-mode was used and all measurements were made during multiple consecutive cardiac cycles, and the average values were used for analysis. LV end-diastolic (LVD,d) and end-systolic (LVD,s) dimensions, as well as the thickness of the intraventricular septum wall (IVSW) and posterior wall (PW) were measured from the M-mode tracings. Diastolic measurements were taken at the point of maximum cavity, and systolic measurements were made at the point of minimum dimension.

Genotyping
Cdh5-tTA and Cav-1-F92A transgenes were genotyped by PCR with the primers and the PCR program (same for both transgenes) described in Table III.

Immunoblotting
For immunoblotting, tissues or cells were placed in lysis buffer (50 mM Tris-HCl, 1 % NP-40, 0.1 % SDS, 0.1 % Na-deoxycholate, 0.1 mM EDTA, 0.1 mM EGTA, 200 μM NaF, 20 μM Na-pyrophosphate, 2 mg/ml cOmplete protease inhibitor, 0.3 mg/ml Pefabloc phosphatase inhibitor, 40 mM β-glycerophospholate, 2 mM Na3VO4). Tissues were homogenized before sonication. Cell lysates were centrifuged for 15 min at top-speed at 4°C. Protein concentrations were determined (Bio-Rad Protein Assay). Equal amounts of protein were separated by 10 % SDS-PAGE or 4 -20 % gradient gels (Bio-Rad) and transferred to nitrocellulose membranes. After Ponceau S staining, the membranes were blocked with 1 % casein for 1 hr at RT under continuous rotation before incubation with the primary antibody O/N at 4°C. Table IV lists all primary antibodies used in this study. LI-COR compatible secondary antibodies (680 or 800 nm) were used based on species origin of the primary antibody.

Densitometry Analysis of Immunoblots
Densitometry Analysis of band intensities was performed using the Li-COR software Image Studio.

Reagents
Unless otherwise stated all reagents were obtained in the highest grade of purity from Sigma-Aldrich.

Statistics
Bar graphs represent the mean and their SEM. Statistical analysis was performed using Prism 6 (GraphPad Software). Means were compared with the Student’s t test. Differences with p < 0.05 were considered as statistically significant (indicated by a *).
Supplemental Figures with Figure Legends

Figure I A
Mouse model: Endothelial specific promoter Cdh5 of transgene 1 (Tg 1) drives the expression of the tetracycline-controlled transactivator (tTA) protein. In the absence of doxycycline (- DOX) tTA binds to the TetO7 promoter of Tg 2 and drives the expression of Cav-1-F92A HA (F92A) expression. In the presence of doxycycline tTA cannot bind the TetO7 promoter (Tet-off system).

Figure I B
Reporter Mouse for tTA. Cdh5-tTA was bred to a tTA reporter mouse (TetO7-GFP) as an independent readout, that the Cdh5-tTA is mainly responsible for the mosaic pattern and not the transgene (TetO7-Cav-1-F92A) itself. Whole-mount staining of mesenteric artery. En-face preparations were stained for the nucleus (blue), PECAM-1 (red) and GFP (green). Top panel, GFP alone. Bottom panel, merged.

Figure II
A, Left panels, Averaged diastolic blood pressure and heart rate from 8 control and 8 Cav-1-F92A animals. The first two days baseline was recorded, before drinking water was switched to doxycycline (2 mg/ml) with 5 % sucrose. Dotted lines show the changes of the systolic blood pressure (slopes of the curves are listed in Table I). Right panels, bar graph presentation of the same results. The bars present the average diastolic blood pressure and heart rate without and with doxycycline. B, Left panels, Averaged diastolic blood pressure from 3 control and 3 Cav-1-F92A animals. The first two days baseline was recorded, before drinking water was switched to L-NAME (1 mg/ml). Dotted lines show the changes of the diastolic blood pressure and heart rate (slopes of the curves are listed in Table II). Right panels, bar graph presentation of the same results. The bars present the average diastolic blood pressure and heart rate without and with L-NAME.

Figure III
Representative EPR traces for single (WT) and double transgenic (WT + Tg) animals.

Figure IV
As controls venous blood from eNOS KO, eNOS S1176A and eNOS S1176D mice was isolated and measured in the same way described before and compared to the WT. Plasma and blood cells (mostly erythrocytes) were isolated from WT mice. The cell fraction was incubated with an excess of NaNO2 and NaN2S2O4 to generate nitric oxide in situ to proof that the three hyperfine EPR bands are occurring from NO-Hb.

Figure V
Immunoblotting for p-VASP (S239) and t-VASP in the whole aorta lysate as a readout of bioavailability of nitric oxide. Cav-1 and HA immunoblotting as a control for the expression of the transgene. HSP90 was used as loading control. Each lane represents one animal (3 per group).

Figure VI
Immunoblotting for nitrotyrosine (see Table IV) on lung and heart lysate from 3 control and 3 Cav-1-F92A mice (one lane per animal). 50 µg were loaded.
Figure VII
Model of Cav-1-F92A membrane integration and signaling. A, domain structure of Cav-1. B, Cav-1 WT integrates in the membrane in a horseshoe loop structure (aa 102 – 134), whereas F92 is localized cytosolic and inhibits eNOS. C, Cav-1-F92A integrates exactly the same way into the plasma membrane as the WT, but the cytosolic A92 cannot inhibit eNOS, which leads to an increased NO release.
**Supplemental Tables**

**Table I**
Slopes of the changes of blood pressure and heart rate after doxycycline administration.

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<tr>
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<th>Control</th>
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<tr>
<td>systolic blood pressure</td>
<td>-0.009 mmHg/hr</td>
<td>+0.028 mmHg/hr</td>
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<tr>
<td>diastolic blood pressure</td>
<td>-0.008 mmHg/hr</td>
<td>+0.012 mmHg/hr</td>
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<tr>
<td>heart rate</td>
<td>+0.007 BPM/hr</td>
<td>-0.067 BPM/hr</td>
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**Table II**
Slopes of the changes of blood pressure and heart rate after L-NAME administration.

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<td>+0.096 mmHg/hr</td>
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<tr>
<td>diastolic blood pressure</td>
<td>+0.099 mmHg/hr</td>
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<tr>
<td>heart rate</td>
<td>-0.311 BPM/hr</td>
<td>-0.182 BPM/hr</td>
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**Table III**
Genotyping primers and PCR program

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<td>amplicon size</td>
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### Table IV
Antibodies used in the study

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Table V
Other readouts of the echocardiography. Mean and SEM is listed.

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<td>Stroke Volume (ml)</td>
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<td>Ejection Fraction (ml)</td>
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<td>Fractional Shortening (ml)</td>
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<td>Cardiac Output (ml)</td>
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<tr>
<td>LV Mass (mg)</td>
<td>137 ± 9</td>
<td>118 ± 10</td>
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Supplemental References


Figure IA

Tg 1

Cdh5

TetO7

Tg 2

tTA

F92A

~DOX

+ DOX

- - -
Figure IB

RepTetO

GFP

PECAM

GFP DAPI
Figure II

A

Diastolic blood pressure (mmHg) over 9 days with doxycycline administration.

B

Diastolic blood pressure (mmHg) over 9 days with L-NAME administration.

Legend:
- Control
- Cav-1-F92A

**gene on**
- **gene off**

Statistical significance:
- ns
- *
- **

[Graphs showing trends and comparisons between control and experimental groups]
Figure III

Control

Cav-1-F92A

Magnetic Field, G
Figure IV

- In situ
- Plasma
- eNOS S1176D
- eNOS S1176A
- eNOS KO

$a_N = 17.1 \text{ G}$
$g = 2.009$

Magnetic Field $G$

Bar chart showing
- WT
- eNOS KO
- eNOS S1176A
- eNOS S1176D

NO-Hb (%)
Figure V

- + - + - + Tg
p-VASP
t-VASP
HA
Cav-1
HSP90
Figure VI

**LUNG**

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**GAPDH**

**nitrotyrosine**

**HEART**

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**GAPDH**

**nitrotyrosine**
Figure VII

A

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B

Caveolin-1-WT

C

Caveolin-1-F92A

eNOS interaction domain (82-101)