Chronic hypoxia induces right heart failure in Caveolin-1 -/- mice*

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*Running Head: RV failure in hypoxic Cav-1 -/- mice

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AUTHORS CONTRIBUTIONS

JAC and EMB led the project and designed and performed most of the experiments. AIR measured superoxide production in tissue samples. AG performed real-time PCR experiments. NSZ assisted with hemodynamic measurements. YW measured eNOS activity in tissue samples. SS and HCC provided intellectual input and critical reading of the manuscript. P.M.B. supervised the project. JAC, EMB and PMB wrote the manuscript.
ABSTRACT

Caveolin-1 (Cav-1) -/- mice develop mild pulmonary hypertension as they age. In this study we sought to determine the effect of chronic hypoxia, an established model of pulmonary hypertension, on young Cav-1 -/- mice with no measurable signs of pulmonary hypertension. Exposure of Caveolin-1 -/- mice to chronic hypoxia resulted in an initial rise in right ventricular (RV) systolic pressure (RVSP) similar to wild-type (WT) mice. By three weeks RVSP decreased in the caveolin-1 -/- mice whereas it was maintained in WT mice. The drop in RVSP in Cav-1 -/- mice was accompanied by decreased cardiac output, increased RV hypertrophy, RV interstitial fibrosis, decreased RV SERCA2a mRNA and decreased RV function compared to WT mice. Importantly, minimal differences were noted in pulmonary vascular remodeling between WT and Cav-1 -/- mice and left ventricular function was normal in hypoxic Cav-1 -/- mice. Mechanistically, increased endothelial nitric oxide synthase uncoupling, and increased tyrosine nitration of protein kinase G were detected in the right ventricle of Caveolin-1 -/- mice. These hemodynamic, histologic, and molecular changes were prevented in Caveolin-1 -/- mice expressing an endothelial specific caveolin-1 transgene or by nitric oxide synthase inhibition. These data suggest that in Cav-1 -/- mice increased oxidative/nitrosative stress due to eNOS uncoupling modifies the response of the RV to pressure overload, accelerating the deterioration of RV function.

Key Words: pulmonary hypertension, endothelial nitric oxide synthase, nitrosative stress, cGMP-dependent protein kinase
INTRODUCTION

Right ventricular (RV) heart failure is the most common cause of death among patients with pulmonary arterial hypertension (PAH).\(^\text{7, 20}\) Unfortunately, while newer therapies improve clinical outcomes such as hemodynamics and 6 minute walking distance, mortality rates remain high, suggesting that there are still significant barriers to the treatment of RV failure in PAH. A better understanding of the underlying mechanisms causing the shift from compensated RV hypertrophy to maladaptive remodeling and dilatation could lead to the development of RV-specific therapies for PAH, potentially improving survival.

An extraordinary number of recent reports have revealed important roles for caveolin-1 (cav-1) in the cardiovascular system.\(^\text{5}\) Targeted ablation of cav-1 decreases angiogenic capacity,\(^\text{21}\) increases neointimal hyperplasia,\(^\text{12}\) impairs arterial vasomotor tone,\(^\text{8, 18}\) and results in cardiac hypertrophy.\(^\text{6}\) More recent studies reveal that genetic ablation of cav-1 results in the spontaneous development of PAH\(^\text{30}\) and cav-1 expression is decreased in human PAH.\(^\text{30}\) Interestingly, many of these complex observations, including the spontaneous development of PAH, are reported to be secondary to hyperphosphorylation of endothelial nitric oxide synthase (eNOS), eNOS uncoupling, and endothelial dysfunction.\(^\text{27, 28, 31}\)

In this study, we examined the role of endothelial cav-1 in the development of chronic hypoxia-induced pulmonary hypertension (PH). This is opposed to our previous study in which we examined the effect of endothelial cav-1 on the spontaneous development of PH in Cav-1 \(-/-\) mice (17). We observed that Cav-1 \(-/-\) mice develop right ventricular failure in response to 3-weeks of chronic hypoxia, which was rescued by re-expression of caveolin-1 in the endothelium of Cav-1 \(-/-\) mice (Cav-1 RC mice). Interestingly, we observed no difference in RV pressures between the Cav-1 \(-/-\), WT or Cav-1 RC mice during the first two weeks of hypoxia. These findings led us to explore potential mechanisms underlying the differential response of the Cav-1 \(-/-\) vs. WT mice or Cav-1 RC mice to chronic hypoxia.
METHODS

Materials. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), Deferoxamine, peg-SOD, Sodium Diethyldithiocarbamate and L-NAME were from Sigma (St. Louis, MO). Lucigenin was from Alexis (San Diego, CA).

Mice. Cav-1 -/- and endothelial reconstituted Cav-1 -/- mice (Cav-1 RC) were generated as described. (8, 29) Male hybrid littermate mice (8 weeks old) from WT, Cav-1 -/- or Cav-1 RC were used in this study. WT, Cav-1 -/-, and Cav-1 RC mice were exposed to 1-3 weeks of chronic hypoxia (FIO2=0.10) or room air. A group of WT and Cav-1 -/- mice were additionally treated with oral L-NAME (1 mg/ml; Fisher) dissolved in drinking water during the 3-weeks of chronic hypoxia. All of the experiments performed were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Hemodynamic measurements. Mice were anesthetized with Pentobarbital sodium (50 mg/kg i.p.) and ventilated via a tracheotomy with room air (175 breaths/min, 175 μl tidal volume). The heart was exposed via a thoracotomy and right and left ventricular systolic pressure (RVSP and LVSP) were determined by placing a 1F solid state pressure transducing catheter (Millar Instruments Inc., Houston, TX) directly into the RV or LV respectively. RV and LV contractility was assessed by Contractility Index (CI; dP/dtmax normalized for RVSP or LVSP). (13, 23, 24, 26) CI and the relaxation time constant (tau) were computed using the blood pressure module of the LabChart software (AdInstruments). Cardiac Output was determined using a 20-MHz Doppler ultrasound probe (Indus Instruments, Houston, TX) placed directly at the aortic trunk and analyzed by the Doppler Signal Processing Workstation (Indus Instruments).

Determination of RV and LV hypertrophy. RV hypertrophy (RVH) was determined by the Fulton Index (ratio of the weight of the right ventricle to the weight of the left ventricle plus septum) as previously described. (32)

Superoxide detection. Superoxide was measured in RV homogenates by monitoring lucigenin (5 μM) chemiluminescence. (19) The specificity of the assay for O2- was confirmed by adding pegylated-SOD (100 units/ml). Residual activity after treatment with PEG-SOD was considered non-specific and subtracted from the raw values.
**NOS activity assay.** The activity of eNOS was determined in detergent solubilized homogenates of RVs by measuring the conversion of $[^{14}\text{C}]$arginine to $[^{14}\text{C}]$citrulline as previously described.\(^{(2)}\)

**Real time PCR.** RNA was isolated after homogenization of snap frozen RVs using the RNeasy Mini Kit (Qiagen). RNA was reverse transcribed using random hexamers and reverse transcriptase enzyme (Applied Biosystems). Taqman primer/probe mix for SERCA2a, and β2 microglobulin as endogenous control was from Applied Biosystems. Target gene expression was normalized to β2-microglobulin.

**Histology.** After sacrifice, mice were perfused intracardially with phosphate buffered saline to clear the vasculature of blood. Hearts were then removed and utilized for morphometric analysis or biochemical assays or placed into 4% paraformaldehyde for 3-4 h. Lungs were inflated via the trachea with 4% paraformaldehyde and the trachea were tied closed to maintain inflation. The lungs were then placed in 4% paraformaldehyde for 3-4 h. Hearts and lungs were dehydrated in 30% sucrose for 24 h, paraffin embedded, and sectioned (5μm). Lung sections were immunostained with HRP-conjugated undiluted smooth muscle alpha actin (SMA) monoclonal antibody (DAKO). HRP-conjugated mouse IgG was used as a negative control. Sections were then stained with hematoxylin. Masson Trichrome staining was performed by the University of Pittsburgh Research Histology Laboratory.

**Pulmonary vascular remodeling.** Pulmonary vascular remodeling was assessed in lung sections stained for smooth muscle alpha actin by counting the number of partially and fully muscularized peripheral arterioles (35-100 mm) per high power field (200x total magnification). For each mouse, at least 20 high power fields were analyzed in multiple lung sections.

**Determination of Fibrotic Area.** The degree of fibrosis was examined by staining paraffin embedded heart sections for collagen using Masson Trichrome method. RV fibrotic area was quantified by Masson Trichrome staining of a section from the mid-ventricle and computer-assisted color thresholding and measurement of stained and total areas (Image J, NIH).

**Immunoprecipitations.** PKG was immunoprecipitated from RV lysates using PKG mAb conjugated to protein G dynabeads (9).
Western blot. Immunoprecipitated samples or tissue homogenates were separated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were blocked in TBST (Tris buffered saline, 0.1% Tween 20), 5% non-fat dry milk for 30 min, followed by incubation in primary antibody. Membranes were washed in TBST before incubation for 1h with horseradish peroxidase-conjugated secondary antibodies. Membranes were washed and developed using enhanced chemiluminescence substrate (Pierce).

Statistical Analysis. Statistical analyses were performed using Graphpad Prism software. Data were analyzed by Student’s T-test when comparing two groups or by one-way ANOVA and Bonferroni post-hoc tests when comparing 3 or more groups. P<0.05 was considered significant.

RESULTS

Cav-1 -/- mice develop RV failure in response to chronic hypoxia. 8-week-old wild-type (WT) mice, Cav-1 -/- or Cav-1 RC mice were exposed to chronic hypoxia for 1-, 2-, or 3-weeks. Age matched mice maintained in room air served as normoxic. RVSP was increased significantly in WT, Cav-1 -/- or Cav-1 RC mice exposed to 1 or 2 weeks of chronic hypoxia. However, RVSP was significantly less in the Cav-1 -/- mice exposed to 3 weeks hypoxia as compared to hypoxic Cav-1 -/- mice exposed to 2 weeks hypoxia or WT mice or Cav-1 RC mice exposed to three weeks hypoxia (Fig.1A). Despite the lower RVSP in the Cav-1 -/- mice, exposure of Cav-1 -/- mice to 3-weeks hypoxia resulted in significantly more RV hypertrophy compared to WT or Cav-1 RC mice, as assessed by the weight of the RV free wall (table 1) and the ratio of the weight of the RV free wall to left ventricle + septum (Fulton index) (Fig.1B). Chronic hypoxia had no effect on LVSP in WT, Cav-1 RC or Cav-1 -/- mice (table 1).

In PH increased pulmonary arterial pressures are due in part to muscularization of small pulmonary arterioles. Assessment of the number of fully or partially muscularized distal arterioles in mice exposed to three weeks hypoxia shows that there was no significant difference in the number of partially or fully muscularized arterioles in WT vs. Cav-1 -/- or Cav-1 RC mice (Fig 1C-F), although the number of fully muscularized arterioles trended higher in the Cav-1 -/- mice. These data suggest that there were only
minor differences in the extent of pulmonary arterial remodeling in Cav-1-/- mice compared to WT or Cav-1 RC mice.

The drop in RVSP after 3-weeks hypoxia coupled with increased RV hypertrophy in the Cav-1-/- mice led us to explore whether they were suffering from RV failure. There was no difference in cardiac output (CO) in normoxic Cav-1-/- vs. WT or Cav-1 RC mice. Cav-1-/- mice exposed to 3-weeks of hypoxia, however, suffered from a 50% drop in CO (12.16 +/- 1.71 ml/min normoxic vs. 5.59 +/- 0.60 ml/min hypoxic) (Fig 2A). In contrast, chronic hypoxia had no effect on CO in WT or Cav-1 RC mice. The heart rate was similar in all groups, demonstrating that the drop in CO in Cav-1-/- mice was due to decreased stroke volume and not to changes in heart rate (table 1). Consistent with the development of heart failure, Cav-1-/- hypoxic mice suffered from significant weight loss (cachexia), losing approximately 10% of their body weight after 3-weeks hypoxia, where as hypoxic WT and Cav-1 RC mice maintained their body weight (table 1).

We next analyzed pressure recordings to determine the contractility index (CI; dP/dtmax normalized for RVSP or LVSP) (13, 23, 24, 26) and relaxation time constant (tau), two measures of ventricular function. Cav-1-/- mice exposed to 3-weeks hypoxia demonstrated a decrease in CI (Fig.2B) and tau (Fig.2C) in the RV indicative of decreased RV function. We observed no decrease in RV function in WT, Cav-1-/- or Cav-1 RC mice exposed to 1 or 2 weeks chronic hypoxia (table 1). Likewise, there was no difference in CI or tau in the LV of normoxic or 3-week hypoxic WT, Cav-1-/-, or Cav-1 RC mice.

As further evidence of RV failure we next examined the RVs for signs of fibrosis. Figure 3A-C shows representative photomicrographs of Masson Trichrome stained RVs from hypoxic WT, Cav-1-/- and Cav-1 RC hearts. Quantification of the fibrotic area shows that Cav-1-/- RVs have an increase in the area of interstitial fibrosis compared to WT or Cav-1 RC mice (Fig.3D), providing further evidence of RV failure in these mice.

Finally, SERCA2a mRNA expression, which is known to decrease in heart failure (1), was lower in the RV free wall of Cav-1-/- mice after 3-weeks of hypoxia compared to their WT or Cav-1 RC counterparts (Fig.3E). There was no difference in SERCA2a mRNA levels in the LV of WT vs. Cav-1-/-
or Cav-1 RC mice after 3 weeks of hypoxia (Fig.3F). Taken together, these data reveal that Cav-1-/- mice develop RV failure in response to chronic hypoxia.

**Increased oxidative and nitrosative stress in the hearts of Cav-1-/- mice.** We and others have demonstrated that eNOS is hyperactive in Cav-1-/- mice.(17, 27, 28, 31) Therefore, we wished to determine whether eNOS phosphorylation and activity is increased in the RV free wall of hypoxic Cav-1-/- mice compared to hypoxic WT and Cav-1 RC mice. eNOS phosphorylation was increased approximately 2-fold in both normoxic and hypoxic Cav-1-/- mice when compared to WT mice or Cav-1 RC mice (Fig.4A,4B). Surprisingly, despite this increase in eNOS phosphorylation, eNOS activity was not different between normoxic Cav-1-/- and normoxic WT or Cav-1 RC mice, and was significantly decreased in the RV of hypoxic Cav-1-/- mice (Fig.4C) compared to the other groups. There was no difference in eNOS activity in the LV of WT, Cav-1-/- or Cav-1 RC mice in normoxia or hypoxia (Fig4D).

The discrepancy between eNOS phosphorylation and eNOS activity in the RV of Cav-1-/- mice led us to test the hypothesis that loss of endothelial cav-1 leads to eNOS uncoupling in the RV of Cav-1-/- mice. Total superoxide production was increased in the RV of normoxic Cav-1-/- mice by 1.5-fold compared to WT or Cav-1 RC mice. In hypoxia, total superoxide production was 1.4-fold, 1.5-fold, and 3.2-fold higher in WT, Cav-1 RC and Cav-1-/- mice, respectively, and L-NAME inhibitable superoxide accounted for approximately 50% of the increase in superoxide production the Cav-1-/- RV (Fig.5A).

When examining L-NAME inhibitable superoxide production alone, we observed a 1.9-fold in the RVs of normoxic Cav-1-/- mice compared to WT or Cav-1 RC mice. In response to chronic hypoxia, L-NAME inhibitable superoxide production increased approximately 2.5-fold in the RVs of WT or Cav-1 RC mice. In contrast, L-NAME inhibitable superoxide increased by 7-fold in the RVs of hypoxic Cav-1-/- mice (Fig.5B). These data strongly suggest that eNOS is uncoupled in the RV of Cav-1-/- mice, leading to superoxide production at the expense of nitric oxide production, and that eNOS uncoupling is enhanced upon hypoxic exposure.
Increased nitrosative and oxidative stress leads to tyrosine nitration of the cGMP-dependent protein kinase (PKG) and inhibition of its enzymatic activity (31). PKG is also known to play a role in cardioprotection (15, 25). Thus, we wished to determine whether Cav1 -/- mice exhibit increased tyrosine nitration of PKG in the RV free wall. PKG was immunoprecipitated from RV lysates from normoxic or hypoxic WT, Cav-1 -/-, and Cav-1 RC mice. Western blot analysis of immunoprecipitates revealed that PKG from hypoxic Cav-1 -/- mice exhibit a greater than two-fold increase in nitrotyrosine compared to either WT or Cav-1 RC mice (Fig.5C) whereas there was no difference in PKG nitration between the normoxic groups (Fig.5D).

**NOS inhibition prevents hypoxia-induced RV failure in Cav-1 -/- mice.** The previous results implicate eNOS as a possible player in the development of chronic hypoxia-induced RV failure in the Cav-1 -/- mice. In order to substantiate a role for eNOS, WT or Cav-1 -/- mice were exposed to normoxia or 3-weeks of hypoxia with or without treatment with the NOS inhibitor L-NAME (1mg/ml in drinking water, ad libitum) at a dose that was previously shown to prevent spontaneous PH in Cav-1 -/- mice (30). NOS inhibition resulted in normalization of the response to chronic hypoxia in Cav-1 -/- mice, preventing the drop in RVSP (Fig.6A), and decreasing RVH to that of WT hypoxic levels (Fig.6B). L-NAME had no notable effect on the development of PH in WT mice as was previously reported (11). Importantly, L-NAME treatment prevented the drop in CO (Fig.6C) as well as changes in CI (Fig.6D) and tau (Fig.6E) in hypoxic Cav-1 -/- mice. Data further reveal that treatment of hypoxic Cav-1 -/- mice with L-NAME significantly attenuated superoxide production (Fig.7A) and PKG nitration in the RV free wall of hypoxic Cav-1 -/- mice (Fig.7B). These data suggest that oxidative/nitrosative stress from uncoupled eNOS contributes to chronic hypoxia-induced RV failure in Cav-1 -/- mice.

**DISCUSSION**

Cor pulmonale (right heart failure) is a long-term consequence of PH, and is closely associated with PH-mediated mortality. The mechanism(s) by which PH leads to RV failure are not well understood.
This lack of understanding is compounded by the fact that some conditions are associated with greater cardiac dysfunction in the setting of the same or lower pulmonary arterial pressures. In this study, genetic deletion of cav-1 led to the development of RV failure in the setting of chronic hypoxia-induced PH. This is supported by data demonstrating decreased cardiac output, decreased RV function, decreased RV SERCA2a mRNA expression, and RV fibrosis in the Cav-1 -/- mice while maintaining normal LV function.

Interestingly, our data suggest that RV failure in the Cav-1 -/- mice was not due to a difference in pressure overload between WT or Cav-1 RC mice and Cav-1 -/- mice. Indeed, the RV afterload in the Cav-1 -/- mice never exceeded that of the RV afterload in the WT or Cav-1 RC mice and only minor differences in pulmonary arterial remodeling were noted. We interpret this to mean that the RV failure was due to a difference in how the Cav-1 -/- RV responded to increased afterload compared to how the WT or Cav-1 RC RV responded to the same increased afterload. In other words, in the face of the same pressure overload, the Cav-1 RV fails whereas the WT or Cav-1 RC RV does not. Furthermore, chronic hypoxia in mice is rarely associated with RV failure, and the RV pressures in the hypoxic Cav-1 -/- mice, when compared to examples in the literature, do not appear to warrant RV failure. That being said, we can not rule out the possibility that between weeks 2 and 3 there was an increase in RVSP that went undetected and that this contributed to the RV failure.

In the pressure-overloaded left ventricle, it is established that stresses such as neurohormonal activation, oxidative and nitrosative stress, and cardiomyocyte apoptosis, when coupled with pressure overload, accelerate functional deterioration of the heart. It remains largely unknown how right heart failure is modified by such additional stresses. Our data suggest that one such stress, namely increased oxidative/nitrosative, accelerates this progression in Cav-1 -/- mice. Previous studies demonstrate eNOS uncoupling in normoxic Cav-1 -/- mice and that cav-1 preferentially inhibits uncoupled eNOS. We also observed increased eNOS uncoupling in the RV of normoxic Cav-1 -/- mice compared to normoxic WT or mice. We further demonstrate that chronic hypoxia significantly enhances eNOS uncoupling in the RVs of hypoxic Cav-1 -/- compared to WT mice. Reconstitution of the endothelium of Cav-1 -/- mice
with cav-1 (Cav-1 RC mice) or treatment of hypoxic Cav-1 -/- mice with the NOS-inhibitor L-NAME attenuated this increase in superoxide production and PKG nitration in the RV wall. The decrease of oxidative/nitrosative stress in these mice was associated with normalization of CO, CI, and tau. These data suggest that increased oxidative/nitrosative stress in the RV modifies the response of the RV to pressure overload, accelerating the deterioration of RV function. Our data further implicate the RV endothelium as the source of NOS-derived superoxide in the RV free wall since reconstitution of the endothelium with cav-1 prevented increased RV ROS production. It can not be ruled out however, that the endothelium from other vascular beds or the endocardium contributes in some way to this effect.

The mechanism of eNOS uncoupling was not explored in this study. A previous study, however, demonstrates that in a model of cor pulmonale preservation of eNOS coupling can be achieved using PDE5A inhibition with chronic sildenafil.(13) Also, BH4 has been shown to prevent the cardiopulmonary phenotype of Cav-1 -/- mice. (27) Indeed, as mentioned above, a recent study demonstrates that cav-1 binds more avidly to pterin free eNOS thus suppressing its oxidase activity.(14) This study is consistent with such a mechanism in that re-expression of cav-1 in the endothelium rescued eNOS activity and superoxide production to WT levels.

One consequence of uncoupled eNOS was increased PKG nitration in the RV of hypoxic Cav-1 -/- mice when compared to hypoxic WT or Cav-1 RC mice, or to hypoxic Cav-1 -/- mice treated with L-NAME. A recent study demonstrates that nitration of PKG leads to decreased PKG activity.(31) The study also shows that PKG nitration is increased in the lungs of Cav-1 -/- mice and that inhibition of NOS-derived superoxide or scavenging of ROS could prevent PKG nitration and thus protect PKG activity. Furthermore, it has been demonstrated that PKG plays a cardioprotective role in the heart. Inhibition of cGMP-hydrolyzing PDE-5 by sildenafil ameliorated cardiac hypertrophy, fibrosis, and systolic dysfunction in a murine model of left ventricular pressure overload.(25) In another study, activation of PKG directly modified passive stiffness of the LV wall, thus improving diastolic function.(15) These data suggest that the PKG nitration observed in the current study could be one mechanism by which eNOS uncoupling in the Cav-1 -/- mice could contribute to RV failure and supports
the concept that preservation of PKG signaling results in improved RV function in the setting of pulmonary hypertension.

In summary, our study suggests that eNOS uncoupling in the RV, leading to oxidative/nitrosative stress, modifies the response of the RV to pressure overload in Cav-1 -/- mice. eNOS uncoupling in the RV may thus represent a novel therapeutic target for the treatment of RV failure in PH.

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GRANTS

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DISCLOSURES

None

AUTHORS CONTRIBUTIONS

JAC and EMB led the project and designed and performed most of the experiments. AIR measured superoxide production in tissue samples. AG performed real-time PCR experiments. NSZ and PMB assisted with hemodynamic measurements. YW measured eNOS activity in tissue samples. SS and HCC provided intellectual input and critical reading of the manuscript. P.M.B. supervised the project. JAC, EMB and PMB wrote the manuscript.

REFERENCES


**FIGURE LEGENDS**

**Figure 1. Effects of chronic hypoxia on young Cav-1 -/- mice.** (A) Effect of hypoxia on RVSP in WT vs. Cav-1 -/- and Cav-1 RC mice over time (n=6-7 for 7 and 14 day mice, n= 8 for 21 day mice and normoxic controls, *P<0.05 vs. 21 day WT Hypoxic). (B-C) Chronic hypoxia induces a greater degree of RV hypertrophy (n=8 mice per group *P<0.05) in Cav-1 -/- mice compared to Cav-1 RC or WT mice. (D) LVSP in normoxic and hypoxic WT Cav-1 -/-, and Cav-1 RC mice. (E-F) Photomicrographs show representative partially and fully muscularized arterioles (scale bar = 50 μm). (G-H) Quantitation of the number of (G) partially and (H) fully muscularized arterioles per high power field (n=8 mice per group, n.s = not significant). Data represent the mean +/- SD.

**Figure 2. Cav-1 -/- mice develop RV failure in response to chronic hypoxia.** (A) Exposure to 3 weeks of chronic hypoxia leads to a decrease in CO in Cav-1 -/- mice (n=6-7 mice per group, *P<0.05, n.s.=not significant). (B) Heart rate of normoxic and hypoxic WT, Cav-1 -/-, or Cav-1 RC mice during CO measurement. (C-D) Contractility index and (E-F) relaxation time constant (tau) in the LV and RV of normoxic and hypoxic WT, Cav-1 -/-, or Cav-1 RC mice (n=8 mice per group, *P<0.05 vs. all other groups). Data shown are mean +/- SD.

**Figure 3. Molecular changes associated with heart failure in the RV of hypoxic Cav-1 -/- mice.** Representative photomicrographs of (A) WT, (B) Cav-1 -/-, and (C) Cav-1RC hypoxic RV stained with Masson’s Trichrome (scale bar represents 200μm). (D) Quantitation of fibrotic area in the RV wall of WT vs. Cav-1 -/- or Cav-1 RC mice after three weeks hypoxia (n=4 mice per group, *P<0.05 vs. WT hypoxic mice). (E) Decreased expression of SERCA2a in the RV of hypoxic Cav-1 -/- mice (n=4 mice per group, *P<0.05 vs. WT and Cav-1 RC hypoxic mice or normoxic Cav-1 -/- mice). Data shown are mean +/- SD.
Figure 4. Effect of hypoxia on eNOS phosphorylation and activity. Western blot analysis of the RV free wall from (A) normoxic or (B) hypoxic WT, Cav-1 -/- and Cav-1 RC mice each (WB are representative of a total of 6 mice). Densitometric analysis reveals that cav-1 -/- mice exhibit similarly increased eNOS phosphorylation compared to WT or Cav-1 RC mice under normoxic and hypoxic conditions (n=6 mice per group; *P<0.05; n.s.=not significant). (C) Decreased eNOS activity in hypoxic Cav-1 -/- RV free wall compared to WT or Cav-1 RC mice. (n=6 mice per group; *P<0.05 compared to hypoxic WT or Cav-1 RC mice; †P<0.05 vs. normoxic Cav-1 -/- mice). Data shown are mean +/- SD.

Figure 5. eNOS uncoupling and PKG nitration in the RV of hypoxic Cav-1 -/- mice. (A) Superoxide production in the RV of normoxic and hypoxic WT, Cav-1 -/- or Cav-1 RC mice divided into L-NAME inhibitable superoxide and superoxide from sources other than NOS (N=normoxia, H=hypoxia). (B) NOS-derived superoxide quantified as the amount of L-NAME-inhibitable superoxide in the RV of normoxic and hypoxic WT, Cav-1 -/-, and Cav-1 RC mice. PKG nitration in the RV of (C) normoxic or (D) hypoxic WT, Cav-1 -/-, and Cav-1 RC mice. Blot shows the results from 2-3 separate animals in each group and is quantified in the histogram. *P<0.05 compared to hypoxic WT or Cav-1 RC mice. Data shown are mean +/- SD.

Figure 6. Chronic L-NAME treatment prevents RV failure in Cav-1 -/- mice. (A) RVSP, (B) RV hypertrophy and (C) cardiac output in WT and Cav-1 -/- mice exposed to normoxia, hypoxia, or hypoxia + L-NAME (N=normoxia, H=hypoxia, H+L= hypoxia + L-NAME, n=5-8 mice per group, *P<0.05; n.s.= not significant). (D) contractility index and (E) the relaxation time constant (tau) in WT and Cav-1 -/- mice exposed to normoxia, hypoxia, or hypoxia + L-NAME (n=8 mice per group for normoxic and hypoxic Cav-1 -/- mice; n=4 for hypoxic Cav-1 -/- mice treated with L-NAME). Data represent the mean +/- S.D. *P<0.05 vs. Normoxic Cav-1 -/- mice. †P<0.05 vs. hypoxic Cav-1 -/- mice.
Figure 7. Chronic L-NAME treatment attenuates oxidative/nitrosative stress in hypoxic Cav-1 -/- mice. (A) Fold change in superoxide production in the RV free wall of WT and Cav-1 -/- mice exposed to normoxia, hypoxia, or hypoxia + L-NAME (n=5-6 per group; *P<0.05 vs. hypoxic Cav-1 -/- mice). (B) PKG nitration in the RV free wall of WT and Cav-1 -/- mice exposed to normoxia, hypoxia, or hypoxia + L-NAME. Blot shown is representative of 4 animals in each group and is quantified in the histogram (*P<0.05 vs. hypoxic Cav-1 -/- mice). (A-E) Data shown are the mean +/- SD.
Figure 1

A

B

C

D

E

F

G

H
Figure 3

A) Hypoxic WT

B) Hypoxic Cav-1 -/-

C) Hypoxic Cav-1 RC

D) % Fibrotic Area

E) SERCA2a mRNA (Fold WT Nom Control)
Figure 4

A. Normoxia

B. Hypoxia

C.
Figure 7

**A**

Superoxide (Fold Change)

WT

N  H  H+L  N  H  H+L

Cav-1 -/-

**B**

Nitrotyrosine PKG (normalized to control)

WT

N  H  H+L  N  H  H+L

Cav-1 -/-

IP: PKG

IB: NT

IB: PKG
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<th>Table 1. Compiled Morphometric and Hemodynamic Measures</th>
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**BW=** Body Weight; **HR=** Heart Rate; **RVSP =** Right Ventricular Systolic Pressure; **CO =** Cardiac Output; **RV/LV+S =** Fulton Index

CI = Contractility Index; **Tau =** Ventricular Relaxation Time Constant

Data represent the mean +/- SD; *P<0.05 vs. WT Hypoxia (21d), Cav-1 RC Hypoxia (21d), or Cav-1 -/- Hypoxia (21d) + L-NAME