Early View

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Endothelial PHD2 Deficiency Induces Nitrative Stress via Suppression of Caveolin-1 in Pulmonary Hypertension

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**Running Title:** PHD2 deficiency induces nitrative stress in PH

**Take home message:** Endothelial PHD2 deficiency decreases Caveolin-1 expression leading to augmented nitrative stress, which contributes to obliterative pulmonary vascular remodeling and severe PH.
Abstract

Nitrative stress is a characteristic feature of the pathology of human pulmonary arterial hypertension (PAH). However, the role of nitrative stress in the pathogenesis of obliterative vascular remolding and severe PAH remains largely unclear. Our recent studies identified a novel mouse model \( Egln1^{Tie2\text{Cre}} \), \( Egln1 \) encoding prolyl hydroxylase 2 (PHD2)] with obliterative vascular remodeling and right heart failure, which provides us an excellent model to study the role of nitrative stress in obliterative vascular remodeling. Here we show that nitrative stress was markedly elevated whereas endothelial Caveolin-1 expression was suppressed in the lungs of \( Egln1^{Tie2\text{Cre}} \) mice. Treatment with a superoxide dismutase mimetic, manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTmPyP) or endothelial Nos3 knockdown using endothelial cell-targeted nanoparticle delivery of CRISPR-Cas9/gRNA plasmid DNA inhibited obliterative pulmonary vascular remodeling and attenuated severe PH in \( Egln1^{Tie2\text{Cre}} \) mice. Genetic restoration of Cav1 expression in \( Egln1^{Tie2\text{Cre}} \) mice normalized nitrative stress, reduced PH and improved right heart function. These data suggest that suppression of Caveolin-1 expression secondary to PHD2 deficiency augments nitrative stress through eNOS activation, which contributes to obliterative vascular remodeling and severe PH. Thus, reactive oxygen/nitrogen species scavenger might have therapeutic potential for the inhibition of obliterative vascular remodeling and severe PAH.

Key words: Caveolin-1, Endothelial cells, eNOS, HIF, nitrative stress, oxidative stress, reactive oxygen/nitrogen species, pulmonary vascular remodeling.
Introduction

Pulmonary arterial hypertension (PAH) is a devastating disease characterized by persistent increase of pulmonary vascular resistance and obstructive pulmonary vascular remodeling, which lead to right-sided heart failure and premature death (1–3). Histopathological features of PAH from different etiologies include increased vessel wall thickness, vascular fibrosis, augmented oxidative/nitrative stress, microvascular occlusion and complex plexiform lesions (4, 5). Given the molecular mechanisms of obliterate pulmonary vascular remodeling are not fully understood, the current PAH therapies targeting mainly vasoconstriction with little effect on vascular remodeling are not effective in curing the disease and promoting survival (6, 7).

There are accumulating evidence of oxidative/nitrative stress in the lungs of idiopathic PAH (IPAH) patients. Xanthine oxidase activity involved in the generation of superoxide anions (reactive oxygen species, ROS) is markedly increased in IPAH lungs (8), and they contributes to chronic hypoxia-induced PH (9). NADPH oxidases including Nox1, 2, and 4, which also produce high levels of ROS, have been demonstrated to be involved in the development of PH (10). 8-Hydroxyguanosine (a biomarker of oxidative damage caused by reaction of superoxide with guanine) staining is prominent in endothelial cells (ECs) within the plexiform lesions of IPAH patients (11). In contrary, antioxidants such as manganese superoxide dismutase (MnSOD, a key mitochondrial antioxidant enzyme coded by the SOD2 gene) activity was lower in IPAH lungs (11, 12). Excessive production of nitric oxide (NO) and superoxide leads to formation of the damaging reactive nitrogen species (RNS)
peroxynitrite (13, 14). Elevated levels of peroxynitrite (i.e., nitrative stress) are detrimental, which induce cell damage and death of pulmonary vascular cells, including ECs and smooth muscle cells (SMCs) (10, 15). In addition, peroxynitrite modifies Tyrosine residue resulting in tyrosine nitration of proteins (formation of 3-nitrotyrosine) and thus modifies their functions (13, 14, 16). Peroxynitrite levels are elevated in the lungs of IPAH patients (11, 12), which are in part due to tissue hypoxia and inflammation (15). Immunohistochemical staining show ubiquitous and marked increase of 3-nitrotyrosine (11). Our previous studies showed that Caveolin-1 (Cav1) deficiency results in eNOS activation and a marked increase of nitric oxide levels, as well as augmented production of superoxide, which form peroxynitrite leading to prominent nitrative stress in pulmonary vasculature (16, 17). The resultant augmentation of nitrative stress causes protein kinase G (PKG) tyrosine nitration which impairs its kinase activity, leading to enhanced vasoconstriction and vascular remodeling and thus PH as seen in Prkg1−/− (Prkg encodes PKG) mice (16, 18, 19). PKG tyrosine nitration was markedly increased in the lungs of IPAH patients (16).

Recently, we have reported the unprecedented mouse model of severe PH [Tie2Cre-mediated disruption of Egln1, encoding hypoxia inducible factor (HIF) prolyl hydroxylase 2 (PHD2), designated as Egln1Tie2Cre, CKO] with progressive obliteratorive vascular remodeling including vascular occlusion and plexiform-like lesion and right heart failure, which recapitulates many features of clinical PAH including IPAH(20, 21). However, it is unknown whether oxidative/nitrative stress is also augmented and involved in the pathogenesis of PH in this severe PH model. In the present study, we for
the first time demonstrate endothelial PHD2 deficiency induces extensive oxidative/nitrative stress via downregulation of endothelial Cav1 expression in a HIF-2α dependent manner. ROS scavenger treatment, genetic disruption of EC-specific Nos3 or restoration of Cav1 expression markedly decreased nitrative stress and attenuated PH in CKO mice.
Methods

Animal Models

All the mice used in this study are C57BL/6J background. $\text{Egln1}^{\text{Tie2Cre}}$ (CKO) and $\text{Hif2a/Egln1}^{\text{Tie2Cre}}$ (EH2) mice were generated as described previously (20). $\text{Cav1}^{\text{Tg}}$ mice were obtained from Dr. William Sessa’s lab from Yale University (22, 23). CKO mice were bred with $\text{Cav1}^{\text{Tg}}$ mice to generate $\text{Egln1}^{\text{Tie2Cre}/\text{Cav1}^{\text{Tg}}}$ (CKO/Tg) and $\text{Egln1}^{\text{f/f}/\text{Cav1}^{\text{Tg}}}$ (Tg) mice. Both male and female $\text{Egln1}^{\text{f/f}}$ (designed as WT), CKO, CKO/Tg, and Tg littermates at the age of 5 weeks to 3.5 months were used in these studies. For ROS scavenger treatment, both male and female CKO mice at the age of 5 weeks were treated with manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTmPyP, Mn) (5 mg/kg) (16) intraperitoneally daily for 9 weeks. PBS was used as a control. The same PBS group was used in our previous publication (24). The animal care and study protocols were reviewed and approved by the Institutional Animal Care and Use Committees of Northwestern University Feinberg School of Medicine, and the University of Arizona.

Echocardiography

Echocardiography was performed in the Core facilities at Northwestern University Feinberg School of Medicine and the University of Arizona as described previously (20, 25, 26). Transthoracic echocardiography was performed on a VisualSonics Vevo 2100 or 3100 ultrasound machine (FujiFilm VisualSonics Inc) using an MS550D (40 MHz) transducer. The right ventricle wall thickness during diastole (RVWTD) was obtained from the parasternal short axis view at the papillary muscle level using M-mode. The
RV cross-sectional area was obtained from the parasternal short axis view at the papillary muscle level using B-mode. Pulmonary artery (PA) acceleration time and PA ejection time were obtained from the parasternal short axis view at the aortic valve level using pulsed Doppler mode. The left ventricle fractional shortening (LV FS) and the cardiac output (CO) were obtained from the parasternal short axis view using M-mode.

**Hemodynamics measurement**

To measure the right ventricular systolic pressure (RVSP), mice were anesthetized with Ketamine/Xylazine cocktail. A 1.4F pressure transducer catheter (Millar Instruments) was inserted through the right jugular vein into the right ventricle. RVSP was recorded and analyzed by AcqKnowledge software (Biopac Systems Inc.) as described previously (20, 25, 26).

**ECs isolation**

Mouse lung tissues were well-perfused with PBS to remove blood. The tissues were then minced and incubated with collagenase A (1.0 mg/ml in HBSS, Roche) in a shaking water bath at 37°C for 45 mins, and then were dispersed by gentleMACS Dissociator (Miltenyi Biotec), followed by filtering through a 40 μm cell strainer. Cells were then incubated with anti-mouse CD31 antibody (BD, cat#550274, 1.5 μg/ml) on ice for 1 hour and followed by incubation with sheep anti-rat IgG Dynabeads M-450 (Invitrogen, Cat#11035, 50 μl) for 30 mins. Beads bound ECs (CD31+ cells) were pulled down by magnet and lysed for protein isolation (25, 26).

**Histological assessment**
Mouse lung tissues were perfused with PBS and fixed with 10% formalin via tracheal instillation at a constant pressure (15 cm H\(_2\)O) and embedded in paraffin wax. Lung sections were stained with a Russel-Movat pentachrome staining kit (American MasterTech) according to the manufacturer’s protocols. For assessment of PA wall thickness, PAs from 40 images at 20X magnification were quantified by Image J. Wall thickness was calculated by the distance between internal wall and external wall divided by the distance between external wall and the center of lumen (20, 25, 26).

**Immunofluorescent staining**

Mouse lung tissues were perfused with PBS, inflated with 50% OCT in PBS, and embedded in OCT for cryosectioning. For immunofluorescent staining of Cav1 and \(\alpha\)-nitrotyrosine (NT) (16), lung sections (5 \(\mu\)m) were fixed with 4% paraformaldehyde and blocked with 0.1% Triton X-100 and 5% normal goat serum at room temperature for 1 hour. After 3 washes with PBS, the slides were incubated with anti-Cav1 (Santa Cruz Biotechnology, Cat#sc-894, 1:100) or anti-NT (Millipore, Cat#05-233, 1:300), anti-CD31 antibody (BD Bioscience, Cat#550274, 1:25), anti-\(\alpha\)-SMA (Abcam, Cat#Ab5694, 1:300), anti-CD45 (BioLegend, Cat#103101, 1:100), anti-Periostin (Abcam, Cat#14041, 1:100) at 4°C overnight then incubated with Alexa 594-conjugated anti-mouse IgG, Alexa 488 or 647-conjugated anti-rat or anti-rabbit IgG (Life Technology) at room temperature for 1 h. Nuclei were counterstained with DAPI contained in Prolong Gold mounting media (Life Technology).

Lung sections from formalin-fixed mouse samples were dewaxed and dehydrated, followed by boiling in 10 mmol/L sodium citrate (pH 6.0) for 10 minutes for antigen
retrieval. Slides were incubated with anti-α-SMA (Abcam, Cat #ab5694, 1:300) at 4°C overnight followed by Alexa 594 or 488 conjugated anti-rabbit IgG at room temperature for 1 h. Nuclei were counterstained with DAPI. Images were taken using the Zeiss Confocal microscope LSM880 and tiled scanning at 20X magnification. α-SMA⁺ vessels were quantified in 40 fields per lung (20, 25, 26).

**RNA isolation and quantitative RT-PCR analysis**

One lobe of lung tissue was homogenized by a TissueLyser (Qiagen) in Trizol solution (Life Technology). RNA was isolated by phenol:chloroform, followed by cleanup with Qiagen RNeasy mini kit (Qiagen). One microgram of RNA was transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative RT-PCR analysis was performed on an ABI ViiA 7 Real-time PCR system (Applied Biosystems) with the FastStart SYBR Green Master kit (Roche Applied Science). Target mRNA expression was determined using the comparative cycle threshold method of relative quantitation. Cyclophilin A was used as an internal control. Information for mouse Cav1 and Cyclophilin A primers was described previously(20), mouse Nos3 primers are

5’-AGGCAATCTTCGTTCCAGCCA-3’ and 5’-TAGCCCGCATAGCGTATCAG-3’.

**Immunoprecipitation and Western Blotting**

Lung tissues were collected from 3.5 months old mice for homogenization. 500 µg of lysates/mouse were then immunoprecipitated with anti-PKG antibody(16) and protein conjugated-Sepharose beads and blotted with anti-NT antibody (Millipore,
Cat#05-233, 1:2000). The same membrane was also blotted with anti-PKG antibody (Cell Signaling Technology, Cat# 13511S, 1:1000) as a loading control. Other Western blotting assays were performed using anti-Cav1 (Santa Cruz Biotechnology, Cat#sc-894 or sc-53564, 1:2000), anti-NT (Millipore, Cat#05-233, 1:2000), anti-eNOS (BD Biosciences, Cat# 610297, 1:1000). Anti-β-actin (Sigma, Cat #A2228, 1:10,000) was used as a loading control.

**Measurement of reactive oxygen/nitrogen species (ROS/RNS) levels**

Lung tissues were perfused free of blood with PBS, followed by collection of the left lobe. Fifty mg of lung tissue was homogenized in PBS on ice. Tissue homogenates were spun at 10,000g for 5 min in 4°C. Fifty µl of homogenates were then used for determination of tissue ROS/RNS levels (OxiSelect™ In Vitro ROS/RNS Assay Kit, Cat#STA-347-T) according to the manufacture’s guide. 2’,7’-dichlorodihydrofluorescein (DCF) in the samples was measured fluorometrically against DCF standards on a fluorescence plate reader (TECAN Infinite 200 Pro). The free radical content in homogenates is determined based on DCF standard curve. The relative DCF values were normalized by the mean value of WT group.

**Nanoparticle delivery of CRISPR plasmid DNA for EC-targeted knockdown of Nos3 in adult mice.**

To induce genome editing and knockdown of Nos3 in ECs, plasmid DNA expressing CRISPR/Cas9 under the control of CDH5 promoter and 2 mouse Nos3-specific guide RNAs (gRNAs) driven by U6 promoter was delivered to Egln1Tie2Cre mice by PLGA-based nanoparticles (Mountview Therapeutics LLC) as
described previously (27). Briefly, 2 gRNA sequences downstream of the start codon ATG of Nos3 gene encoding the 1/3 N-terminus of eNOS were designed manually (Genscript program failed to identify potent gRNAs at this region) and the single complementary DNA oligonucleotides corresponding to the gRNA sequences were commercially synthesized (Integrated DNA technologies). The oligo gases were then phosphorylated, annealed, and cloned sequentially plasmid pSpCas9-2U6-GFP containing 2 U6 promoters. Positive clones were confirmed by DNA sequencing. For EC-specific genomic editing, the CAG promoter was replaced with the human CDH5 promoter (CRISPR\textsuperscript{CDH5}). The DNA sequences corresponding to the gRNAs of mouse Nos3 were: 5’-TCAGCCTATTGGCTGACGAG-3’ (reverse oligo 5’-CTCGTCAGCCAATAGGCTGA-3’), and 5’-GGATCTGATTGCTTCAGTCA-3’ (reverse oligo 5’-TGACTGAAGCAATCAGATCC-3’).

To deliver the CRISPR\textsuperscript{CDH5} plasmid DNA into adult mice following the manufacture’s instruction, PLGA-based nanoparticles were mixed with plasmid DNA and incubated at RT for 10 min. The mixture was then administered retro-orbitally to CKO mice at 7 weeks of age (25 μg plasmid DNA/mouse) and again at 8 and 9 weeks of age (total 3 injections as these gRNAs are not potent). Mixture of nanoparticles:control plasmid DNA expressing a random RNA oligo was administered as a control to a separate cohort of mice. Mice were then housed until the age of 3.5 months for hemodynamic measurement, and tissue collection.

**Determination of genome editing efficiency in mouse lung ECs**
Freshly isolated ECs from mouse lungs were digested with proteinase K overnight. The supernatants were collected after centrifugation at 20,000g for 10 min and extracted with phenol:chloroform mixture followed by ethanol precipitation to isolate genomic DNA. SYBR Green (Roche Applied Science)-based quantitative PCR analysis of genomic DNA was carried out with the Quant Studio 6-Flex Real-Time PCR system (Applied Biosystem) for determination of genome indel efficiency as described previously(27). The sequences of the forward and reverse primers are 5’-CACGTACTCAGAGGCCTCAA-3’ & 5’-AATCCTCAGGCTATTGGCTGAC-3’. The primer sequences of the control Nme1 DNA are 5’-CCCCTTCTTTACTGGCCTGG-3’ and 5’-GCTATCCCACCAGCCTGTIT-3’ for normalization.

**Statistical analysis**

Statistical significance was determined by one-way ANOVA with a Tukey post hoc analysis that calculates P values corrected for multiple comparisons. Two-group comparisons were analyzed by the unpaired 2-tailed Student t-test. P <0.05 denoted the presence of a statistically significant difference. All bar graphs represent mean ± SD.
Results

Prominent nitrative stress in \textit{Egln1}^{Tie2Cre} mouse lungs and pulmonary vascular ECs

Given that nitrative stress is a characteristic feature of the pathology of clinical PAH, we tested whether nitrative stress is also prominent in the lung of \textit{Egln1}^{Tie2Cre} (CKO) mice. Quantification of total free radical levels with the OxiSelect™ \textit{In Vitro} ROS/RNS Assay Kit of lung homogenates showed that CKO lungs exhibited markedly elevated ROS/RNS levels (Figure 1A). Western blotting demonstrated markedly increased levels of nitrotyrosine (NT), indicative of nitrative stress, in CKO mouse lungs (Figure 1B and 1C). We then performed the NT immunostaining together with markers for ECs (CD31), inflammatory cells (CD45), fibroblasts (periostin), SMCs (\(\alpha\)-SMA) to determine what cell types were the NT positive cells in the CKO lungs. Our data showed that NT was highly expressed in the pulmonary vascular ECs, CD45\(^+\) inflammatory cells and relatively low expression in pulmonary vascular SMCs and periostin\(^+\) fibroblasts (Figure 1D-1E and supplementary Figure S1A and 1B). Our previous studies demonstrated that nitration–dependent impairment of PKG activity is involved in PH development(16). To further determine whether PHD2 deficiency also affects PKG nitration, we performed immunoprecipitation assay using anti-PKG antibody and blotted with anti-NT antibody. The data showed that PKG nitration was markedly increased in the lungs of CKO mice (Supplementary Figure S2). Together, these data demonstrate that nitrative stress is markedly augmented in the pulmonary vascular lesions in CKO mice.
ROS scavenger treatment inhibited obliterative vascular remodeling and attenuated PH in Egln1<sup>Tie2Cre</sup> mice

We next determined whether depletion of ROS/RNS in CKO mice affected PH development. CKO mice at age of 5-weeks (with established PH phenotype) were treated with the ROS/peroxynitrite scavenger MnTMPyP (Mn), i.p., which disrupts superoxide and thus peroxynitrite formation (16, 28), for 9 weeks. Mn treatment markedly reduced the levels of NT in the pulmonary vasculatures and lungs of CKO mice (Figure 2A). Hemodynamics measurement showed that RVSP was reduced to ~50 mmHg in Mn-treated CKO mice compared to ~77 mmHg in PBS-treated CKO mice (Figure 2B). RV hypertrophy, determined by weight ratio of RV versus left ventricular plus septum (LV+S), was also attenuated in Mn-treated CKO mice (Figure 2C). Lung pathology examination also revealed inhibition of occlusive vascular remodeling evident by diminished neointima formation (Figure 2D), and decreased pulmonary arterial wall thickness and muscularization of distal pulmonary vessels in Mn-treated CKO mice compared to PBS treated mice (Figure 2E-G). Together, these data suggest the involvement of oxidative/nitrative stress in the development of severe PH in CKO mice.

Nos3 knockdown in ECs reduced nitrative stress, vascular remodeling and severe PH in Egln1<sup>Tie2Cre</sup> mice

Peroxynitrite is formed by nitric oxide (NO) and superoxide reaction. To determine whether NO mediates the nitrative stress and PH development in CKO mice, we treated CKO mice with a pan-inhibitor (L-NAME) of nitric oxide synthases in the drinking
water (1 mg/ml), however, all the 7 CKO mice died within 1 weeks (data not shown), suggesting that the NO bioavailability is critical for the maintenance of vascular homeostasis in this severe PH model. Nos3 (encoding eNOS) but neither Nos1 nor Nos2 was upregulated in the CKO lungs (Figure 3A and Supplementary Figure S3) and normalized in lungs of Egln1 and Hif2a double knockout mice (Egln1Tie2Cre/Hif2aTie2Cre, EH2), suggesting eNOS mediates nitrative stress in CKO mice. To answer this question, we leveraged nanoparticle delivery of CRISPR-Cas9 plasmid DNA(27) expressing Cas9 under the control of CDH5 promoter and Nos3-specific gRNAs driven by U6 promoter to specifically knockdown Nos3 in ECs of CKO mice (Figure 3B). We observed ~30% genome editing efficiency in lung ECs of Nos3 gRNA plasmid DNA-treated CKO mice (Supplementary Figure S4), and diminished eNOS protein expression (Figure 3C). Immunostaining also showed diminished eNOS expression in lung ECs of these mice compared with control mice (Figure 3D), demonstrating successful knockdown of Nos3 in CKO lung ECs. Accordingly, NT levels were also markedly decreased in lung ECs of these Nos3 gRNA plasmid DNA-treated CKO mice (Figure 3C and 3E). Hemodynamics measurement showed that Nos3 knockdown in ECs significantly reduced RVSP and RV/(LV+S) ratio in these CKO mice (Figure 3F and 3G). Pulmonary vascular remodeling via assessing pulmonary arterial wall thickness (Figure 3H and 3I) and muscularization of distal pulmonary vessels (Figure 3J and 3K) was also markedly attenuated in these mice. Additionally, all the 5 Nos3 gRNA plasmid DNA-treated mice survived compared to 50% mortality of control CKO mice during the same period.
These data demonstrate that endothelial eNOS is the primary source of nitrative stress, which contributes to the development of occlusive remodeling and severe PH seen in the CKO mice.

**Decreased expression of endothelial Cav1 secondary to PHD2 deficiency in a HIF-2α-dependent manner**

Cav1 deficiency activates eNOS and increases ROS and RNS production, leading to nitrative stress, which post-translationally modifies PKG through tyrosine nitration and impairs its kinase activity leading to PH(16, 19). To find out whether dysregulation of Cav1 signaling is involved in the generation of nitrative stress and PH development in CKO mice, we first checked the expression level of Cav1 in the lungs of CKO mice. As shown in **Figure 4A**, Cav1 mRNA levels were markedly decreased in the lungs of CKO mice compared to that from similar age WT mice. HIF-2α deletion in CKO (EH2) mice restored Cav1 mRNA expression (**Figure 4A**). Western blotting also demonstrated reduced Cav1 protein levels in CKO lungs which were reversed in EH2 mice and in fact increased in EH2 mouse lungs compared to WT lungs (**Figure 4B** and **4C**). By performing immunofluorescent staining, we found that Cav1 is co-stained with CD31+ cells (i.e. ECs) in the pulmonary vasculature of WT mice whereas diminished in pulmonary vascular lesions in CKO mice and restored in pulmonary vascular ECs of EH2 mice (**Figure 4D**), suggesting that endothelial Cav1 is reduced by PHD2 deficiency dependent on HIF-2α activation in Egln1\textsuperscript{Tie2Cre} mice.

**Restored Cav1 expression inhibited nitrative stress in Egln1\textsuperscript{Tie2Cre/Cav1Tg} mice**
To determine whether Cav1 deficiency is responsible for the augmentation of nitrative stress via eNOS activation and the pathogenesis of PH in CKO mice, we generated a mouse model with Cav1 transgenic expression under control of the preproET-1 promoter in the genetic background of CKO mice (Egln1Tie2Cre/Cav1Tg, CKO/Tg)(23) (Figure 5A). Western blotting and immunostaining demonstrated that reduced Cav1 expression seen in CKO mouse lungs was restored in CKO/Tg mice (Figure 5B and 5C). Restored Cav1 expression in CKO mice drastically reduced the levels of ROS/RNS in the lungs of CKO/Tg mice compared to CKO mice (Figure 6A). Further examination of NT levels via Western Blotting and immunostaining showed that NT levels were inhibited in the lung and pulmonary vascular ECs of CKO/Tg mice compared to CKO mice (Figure 6B and 6C), suggesting that Cav1 deficiency is responsible for the increased oxidative/nitrative stress in CKO mouse lungs.

**Cav1 transgenic expression attenuates PH seen in Egln1Tie2Cre mice**

We next determined if Cav1 transgenic expression in CKO mice would rescue the hypertensive pulmonary phenotype. Hemodynamic measurement demonstrated a partial decrease of RVSP in CKO/Tg at age of 3.5 months compared to CKO mice (Figure 7A). RV hypertrophy was also attenuated in CKO/Tg mice (Figure 7B). Pathological examination showed that CKO/Tg mice had reduced pulmonary vascular remodeling, including decreases of neointima and occlusive lesions (Figure 7C), pulmonary arterial wall thickness (Figure 7D), and muscularization of distal pulmonary vessels in comparison of CKO mice (Figure 7E and 7F).
Cav1 transgenic expression normalizes RV and pulmonary artery functions in $Eglnt^{Tie2Cre}/Cav1^{Tg}$ mice

We next evaluate whether Cav1 transgenic expression will inhibit right heart dysfunction seen in CKO mice. As shown in Figure 8A-8C, echocardiography showed that $CKO/T_g$ mice had marked decreases of RV wall thickness compared to CKO mice. $CKO/T_g$ mice also exhibited marked improvements of RV contractility assessed by RV fraction area change (RV FAC) (Figure 8D and Supplementary Video 1-2). However, we did not observe significant change of heart rate, cardiac output and left ventricular fraction shortening (LV FS) in $CKO/T_g$ mice compared with CKO mice (Supplementary Figure S5A-5C). Pulmonary artery (PA) function assessed by PA acceleration time/ejection time (AT/ET) showed that $CKO/T_g$ mice exhibited improved PA function compared to CKO mice (Figure 8E). These data demonstrated that Cav1 transgenic expression partially but significantly rescues the defective PA and RV functions seen in CKO mice.
Discussion

The present study demonstrated that nitrative stress was markedly augmented in the lungs, especially in vascular lesions of CKO mice, recapitulating the pathological feature of clinical PAH. Augmentation of nitrative stress was due to endothelial Cav1 deficiency and eNOS activation. Cav1 transgenic expression in CKO/Tg mice reduced nitrative stress levels and attenuated PH evident by reduction of RVSP, pulmonary vascular remodeling, and RV hypertrophy, as well as improvements of RV and PA function. Both pharmacological scavenging of ROS/RNS and genetic knockdown of endothelial Nos3 via EC-targeted nanoparticle delivery of CRISPR-Cas9 technology in adult mice diminished nitrative stress and markedly reduced the severity of pulmonary vascular remodeling and PH in CKO mice. These findings suggest that restoration of Cav1 expression and ROS/RNS scavenging represent potential therapeutic strategies for the treatment of severe PAH, including IPAH (Figure 8F). The clinical relevance is validated by our previous observation that PHD2 expression was diminished in ECs of occlusive pulmonary vessels in IPAH patients (20).

Prominent oxidative/nitrative stress is a pathological feature of clinical PAH (10, 15, 16). Our recent study has for the first time shown that CKO mice develop spontaneously severe PH with obliterative pulmonary vascular remodeling including occlusive vascular lesions and formation of plexiform-like lesions and marked elevation of RVSP ranging from 70-100 mmHg as well as development of right heart failure (20). Here we show a marked increase of ROS/RNS in CKO mouse lungs and prominent anti-NT staining indicative of extensive nitrative stress in pulmonary
vascular lesions, especially in ECs and inflammatory cells. This study provides further evidence of the CKO mice as an excellent mouse model of human PAH. Furthermore, treatment of the mice with the ROS scavenger MnTMPyP inhibited occlusive pulmonary vascular remodeling and attenuated PH, demonstrating the pathogenic role of oxidative/nitrative stress in contributing to the severity of PH.

Nitrative stress is formed by excessive superoxide and NO which chemically react to form peroxynitrite. We found that expression of Nos3 but neither Nos1 nor Nos2 was markedly increased in CKO lungs. To specifically inhibit peroxynitrite formation, we employed the EC-targeted nanoparticle delivery of CRISPR plasmid DNA technology to selectively disrupt Nos3 expression in ECs in adult CKO mice, which resulted in diminished nitrative stress in CKO lungs and especially in pulmonary vascular ECs. These data demonstrate that endothelial cells are the primary sources of nitrative stress via eNOS expression and activation secondary to PHD2 deficiency in CKO lungs. Given that peroxynitrite is more stable and diffusible than NO, EC-derived nitrative stress can easily spread into other cell types in the lesions such as in pulmonary vascular SMCs, fibroblasts, and infiltrated proinflammatory cells. Thus, EC-specific Nos3 knockdown results in decreased nitrative stress not only in ECs but also in whole lungs. Additionally, EC-derived peroxynitrite diffuses to pulmonary vascular SMCs and induces tyrosine nitration of PKG expressed in SMCs, which impairs its kinase activity leading to pulmonary vascular remodeling and PH development. Consistently, we observed reduced RVSP, pulmonary vascular remodeling and RV hypotrophy in endothelial Nos3-disrupted CKO mice. Together, these data provide unequivocal
evidence of nitrative stress derived from endothelial dysfunction in contributing to obliterative pulmonary vascular remodeling and severe PH development.

Previous studies have shown decreases of \textit{CAV1} mRNA and protein levels in lung ECs of patients with PAH(16). Cav1 deficiency is also evident in the arterial lesions of PH rats including both Sugen5416/hypoxia-induced PH and monocrotaline-induced PH(29, 30). Our previous study provides the first genetic evidence that Cav1 deficiency induces PH in mice(17). A recent study shows that Cav1 deficiency in ECs promote more severe PH in response to hypoxia (31). Consistently, \textit{CAV1} mutation is associated with heritable PAH in patients (32). Together, these studies provide clear evidence of CAV1 deficiency in the pathogenesis of PH. Our other study has shown that genetic deletions of both \textit{Cav1} and \textit{Nos3} or treatment of \textit{Cav1}−/− mice with either NO Synthase inhibitor L-NAME or ROS/RNS scavenger MnTMPyP inhibited PH seen in \textit{Cav1}−/− mice, suggesting chronic activation of eNOS secondary to Cav1 deficiency is the mechanism of PH induced by CAV1 deficiency (16). Others also show that L-NAME treatment inhibits PH in \textit{Cav1}−/− mice (33). It has been shown that eNOS activation secondary to Cav1 deficiency leads to peroxynitrite formation which causes PKG nitration in \textit{Cav1}−/− mice. PKG nitration impairs its kinase activity and thereby induces pulmonary vascular remodeling and PH(16). eNOS activation and PKG nitration is prominent in lung tissues of IPAH patients (16, 34). These studies provide unequivocal evidence that Cav1 deficiency induces nitrative stress leading to PKG nitration and thus PH.
In CKO mice, we observed elevated levels of ROS/RNS, PKG nitration and decrease of endothelial Cav1 expression. We demonstrated that Cav1 deficiency in CKO mice is involved in the regulation of nitrative stress as Cav1 transgenic expression normalizes the levels of ROS/RNS and inhibits nitrative stress evident by diminished NT levels in pulmonary vascular ECs and thus inhibits oblitative pulmonary vascular remodeling and reduces PH as well as improves RV and PA functions in CKO mice. Especially, we observed complete inhibition of occlusive vascular remodeling without neointima formation in CKO/Tg mice, endothelial Nos3 knockdown CKO mice, and MnTMPyP-treated CKO mice. However, the medial thickness was less affected in these mice, which is consistent with the markedly reduced but still elevated RVSP. Together, our data demonstrate that endothelial Cav1 deficiency secondary to PHD2 deficiency mediates the augmentation of nitrative stress in CKO mouse lungs via activation of endothelial Nos3 which leads to oblitative pulmonary vascular lesions and severe PH.

The current studies demonstrated that ROS/RNS scavenger MnTMPyP treatment attenuated both RVSP and vascular remodeling in CKO mice, which is consistent with previous observation that antioxidant N-acetylcysteine (NAC) treatment reduced PH induced by chronic hypoxia in rats(35). However, TEMPOL, another anti-oxidant has a beneficial effect on reducing RVSP but not arterial remodeling in the hypoxia and SU5416/hypoxia induced PH rats (36). Thus, whether antioxidant therapies are effective in preclinical models of PH is still controversial, which might be due to the selected agents, doses and designing of experiments(37). For example, depending on
the concentration and lung cell types, TEMPOL can be pro-oxidant or anti-oxidant (38). Other studies also show that high concentrations of, or extended exposure to TEMPOL increases ROS levels (39, 40). Further studies are warranted to address this important question of whether antioxidants should be given to PAH patients for treatment of the disease.

In summary, we have demonstrated prominent nitrative stress in vascular lesions of CKO mice, which is mediated by endothelial Cav1 deficiency ascribed to HIF-2α activation and resultant eNOS activation. ROS/RNS scavenger treatment, CRISPR-mediated endothelial Nos3 knockdown or transgenic expression of Cav1 inhibits nitrative stress and oblitative pulmonary vascular remodeling and attenuates PH in CKO mice. Thus, Cav1 deficiency-induced oxidative/nitrative stress secondary to PHD2 deficiency is a part of the mechanisms of severe PH in CKO mice as seen in PAH patients.
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Author contributions

Z.D. and Y.Y.Z. conceived the experiments. Z.D., B.L., D. Y., J.D., N.M., D.D., K.R., Y. P. and M.M.Z designed, carried out experiments, and analyzed the data. Z.D., R.V. and Y.Y.Z. analyzed and interpreted the data. Z.D. wrote the manuscript. Z.D. and Y.Y.Z. supervised the project and revised the manuscript. All authors approved the manuscript.

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Reference


Figure 1. Augmented nitrative stress in pulmonary vascular lesions in *Egln1<sup>Tie2<sup>Cre</sup></sup> mice. (A) DCF measurement demonstrating marked increase of ROS/RNS in the lungs of *Egln1<sup>Tie2<sup>Cre</sup></sup> (CKO) mice. Lung tissues were collected from 3.5 months old WT and
CKO mice for 2', 7'-dichlorodihydrofluorescein (DCF) assay. (B, C) Western blotting demonstrating increase of nitrotyrosine modification of proteins in the lung of CKO mice. NT, anti-NT antibody. Anti-β-actin was used as a control. (D, E) Representative micrographs of immunostaining showing prominent nitrative stress in pulmonary vascular lesions in CKO mice. Lung sections were immunostained with anti-NT antibody (red) for detection of nitrative stress. Anti-CD31 antibodies were used to label vascular ECs (green, D). Anti-CD45 antibodies were used to label inflammatory cells (green, E). Nuclei were counterstained with DAPI (blue). V, vessel. Scale bar, 50 µm. *, P < 0.05. **, P < 0.01. Student t-test. (A, C).
Figure 2. MnTMPyP treatment reduced PH in Egln1Tie2Cre mice. (A) Immunostaining against NT showed that MnTMPyP (Mn) treatment reduced nitrotyrosine modification in CKO lungs. CKO mice at age of 5 weeks were treated with either Mn or PBS for 9 weeks. (B, C) RVSP was measured, and RV hypertrophy was determined in CKO mice at age of 3.5 months (3.5M) after 9 weeks treatment with
Mn or PBS. *Egln1*^+/−* (WT) mice were used as controls. RVSP and RV hypertrophy were also determined in 5 weeks (5WK) old *CKO* mice without treatment. (D, E) MnTMPyP (Mn) treatment inhibited neointima formation and decreased PA wall thickness. Lung sections were processed for Russel-Movat pentachrome staining. V, vessel. (F, G) Quantification of α-SMA immunostaining demonstrating decreased muscularization of distal pulmonary vessels in Mn-treated CKO mice. Lung cryosections were immunostained with anti-α-SMA (red) and counterstained with DAPI (blue). Arrows point to muscularized distal pulmonary vessels. *, $P < 0.05$, **, $P < 0.01$, ***, $P <0.001$, ****, $P < 0.0001$. One-way ANOVA with Tukey post-hoc analysis (B, C, E, G). Scale bar: 50μm.
Figure 3. Endothelial Nos3 knockdown attenuated PH in Egln1Tie2Cre mice. (A) Quantitative RT-PCR analysis demonstrating Nos3 mRNA expression is upregulated in Egln1Tie2Cre (CKO) mouse lungs compared to WT lungs and restored in Egln1Tie2Cre/Hif2αTie2Cre (EH2) lungs. Lung tissues were collected from 3.5 months old
mice. (B) A diagram showing the strategy of knockdown of endothelial Nos3 in CKO mice. Mixture of nanoparticles:CRISPR plasmid DNA was administered to CKO mice at age of 7, 8, and 9 weeks to disrupt Nos3 expression in ECs. (C) eNOS protein and NT levels were markedly reduced in Nos3 gRNA plasmid DNA-treated CKO mice compared with control vector DNA-treated mice. (D) Immunostaining against eNOS demonstrated that eNOS was reduced in the lung ECs of Nos3 gRNA plasmid DNA-treated CKO mice. (E) Endothelial Nos3 knockdown attenuated NT levels in the lung ECs of CKO mice. (F) Hemodynamic measurement showing that Endothelial Nos3 knockdown attenuated RVSP in the CKO mice. (G) RV hypertrophy was reduced in endothelial Nos3 knockdown CKO mice. (H and I) Endothelial Nos3 knockdown in CKO mice reduced neointima formation and decreased PA wall thickness. Lung sections were processed for Russel-Movat pentachrome staining. V, vessel. (G, H) Quantification of α-SMA immunostaining demonstrating decreased muscularization of distal pulmonary vessels in endothelial Nos3 knockdown CKO mice. Lung cryosections were immunostained with anti-α-SMA (red) and counterstained with DAPI (blue). Arrows point to muscularized distal pulmonary vessels. *, $P < 0.05$, **, $P < 0.01$, ***, $P <0.001$, ****, $P < 0.0001$. One-way ANOVA with Tukey post-hoc analysis (C, D, F, H). Scale bar: 50µm.
Figure 4. Endothelial Cav1 expression was suppressed in Egln1^{Tie2Cre} mouse lungs in a HIF-2α dependent manner. (A) Quantitative RT-PCR analysis demonstrating decreased Cav1 mRNA expression in CKO mouse lungs compared to WT lungs, which is restored in *Egln1^{Tie2Cre}/Hif2α^{Tie2Cre}* (EH2) lungs. Lung tissues were collected from 3.5 months old mice. (B, C) Decreased Cav1 protein expression in CKO mouse lungs compared to WT lungs. Cav1 protein expression in EH2 mouse lungs was markedly increased compared to WT and CKO mouse lungs. (D) Immunostaining demonstrating diminished Cav1 expression in pulmonary vascular ECs of CKO mice and restoration
in EH2 mice. Lung sections were immunostained with anti-Cav1 (red) and CD31 (green). Nuclei were counterstained with DAPI (blue). *, P < 0.05, **, P < 0.01, ***, P < 0.001. One-way ANOVA with Tukey post-hoc analysis (A and C). Scale bar: 50μm.

**Figure 5. Cav1 transgenic expression in Egln1^{Tie2Cre} mice.** (A) A diagram showing the strategy of generating Egln1^{Tie2Cre}/Cav1^{Tg} (CKO/Tg) mice by breeding Cav1 transgenic mice (Cav1^{Tg}) into the genetic background of CKO mice. (B) Western
Immunostaining demonstrated that Cav1 expression was downregulated in lung ECs of CKO mice compared to WT and restored in CKO/Tg mice. Lung sections were immunostained with anti-Cav1 (red) and CD31 (green). Nuclei were counterstained with DAPI (blue). *, P < 0.05, **, P < 0.01, ****, P < 0.0001. One-way ANOVA with Tukey post-hoc analysis (B).
Figure 6. Cav1 transgenic expression inhibited excessive ROS/RNS in Egln1^{Tie2Cre} mouse lungs. (A) DCF measurements demonstrating the increased production of ROS/RNS seen in CKO mouse lungs was inhibited in CKO/Tg mouse lungs. There is no marked difference in lung generation of ROS/RNS in Tg mice compared to WT mice. WT and CKO data shared with Figure 1A. (B) Western Blotting demonstrated the reduction of NT levels in CKO/Tg mice compared to CKO mice. (C) Representative immunostaining showed that CKO/Tg mice exhibited reduced NT levels compared to CKO mice. Lung sections were immunostained with anti-NT (red) and CD31 (green). Nuclei were counterstained with DAPI (blue). *, P < 0.05, **, P < 0.01. One-way ANOVA with Tukey post-hoc analysis (A).
Figure 7. Transgenic expression of Cav1 inhibited severe PH seen in Egln1Tie2Cre mice. (A, B) CKO/Tg mice exhibited marked decreases of RVSP (A) and RV hypertrophy (B) compared to CKO mice at age of 3.5-months. (C, D) Representative micrographs of Russel-Movat pentachrome staining showing inhibited obliterative vascular remodeling including diminished neointima formation and reduced wall...
thickness in the lung of CKO/Tg mice. (E, F) Immunostaining of α-smooth muscle actin (α-SMA) demonstrating inhibition of muscularization of distal pulmonary vessels in the lungs of CKO/Tg mice. Scale bar: 50μm. ***, P < 0.001. One-way ANOVA with Tukey post-hoc analysis (A, B, D and F).
Figure 8. Cav1 transgenic expression improved right heart and pulmonary artery functions in Egln1Tie2Cre/Cav1Tg mice. (A) Representative echocardiography B-mode showing reduced RV chamber size in CKO/Tg mice compared to CKO mice. (B)
Representative echocardiography M-mode showing reduced RV wall thickness at diastolic stage in CKO/Tg mice compared to CKO mice. (C) RV wall thickening seen in CKO mice was reduced in CKO/Tg mice. (D) RV contractility was improved by restored endothelial Cav1 expression in CKO/Tg mice compared to CKO mice. (E) Pulmonary arterial function assessed by PA AT/ET ratio was rescued in CKO/Tg mice. (F) A diagram showing that endothelial PHD2 deficiency induces decreases of endothelial Cav1 expression and eNOS activation resulting in augmented nitrative stress which contributes to obliterative pulmonary vascular remodeling and severe PH. Restoration of endothelial Cav1 expression or ROS/RNS scavenging may be effective therapeutic approaches for the treatment of PAH. KD, knockdown. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. One-way ANOVA with Tukey post-hoc analysis (C-E).
Supplementary Figure S1. Nitrative stress in SMCs and fibroblasts in the pulmonary vascular lesions of *Egln1*\(^{Tie2}^{Cre}\) mice. (A, B) Representative micrographs of immunostaining showing relatively low levels of NT expression in SMCs and fibroblasts in pulmonary vascular lesions of CKO mice (3.5 months old). Lung sections were immunostained with anti-NT antibody (red) for detection of nitrative stress. Anti-α-SMA antibody was used to label SMCs (green, A). Anti-Periostin antibody was used to label fibroblasts (white, B). Nuclei were counterstained with DAPI (blue). The strong positive NT signal in (A) was from pulmonary vascular ECs not SMCs.
Supplementary Figure S2. Immunoprecipitation assay demonstrating that PKG nitrotyrosine modification was markedly increased in Egln1<sup>Tie2Cre</sup> mice. Lung tissues were collected from 3.5 months old mice for homogenization. 450 µg of lysates/mouse were then immunoprecipitated with anti-PKG antibody and blotted with anti-NT antibody. The same membrane was also blotted with anti-PKG antibody as a loading control. IgG was also used as a control for precipitation.

Supplementary Figure S3. Nos3 was upregulated in Egln1<sup>Tie2Cre</sup> mice. RNA-sequencing analysis demonstrated the upregulation of Nos3 in CKO mice and restoration in Egln1<sup>Tie2Cre/Hif2a<sup>Tie2Cre</sup></sup> (EH2) lungs. RNA-sequencing analysis was performed with lung tissues of 3.5 months old WT, CKO and EH2 mice.

Supplementary Figure S4. Genomic editing efficiency of Nos 3 in lung ECs after nanoparticle delivery of CRISPR-Cas9 plasmid DNA. Mixture of
nanoparticles: Nos3 CRISPR plasmid DNA was administered to CKO mice at age of 7, 8, and 9 weeks (total 3 injections) and lung tissues were collected at 14 weeks of age for EC isolation. Genomic DNA isolated from ECs was used for quantitative PCR analysis with a primer specific for the predicted indel site. The genomic editing efficiency in the lung ECs was approximately 30%. ****, P < 0.0001, Student’s t test.

Supplementary Figure S5. Echocardiography analysis showing similar left heart function and cardiac output in CKO/Tg mice and CKO mice. At age of 3.5 months, the mice were subject to echocardiography to assess heart rate (A), left ventricular fraction shortening (LV FS), indicative of contractility (B), and cardiac output (C). bpm= beats per minute. One-way ANOVA with Tukey post-hoc analysis (A-C).

Supplemental Video 1, Echocardiography M-Mode of CKO mice.
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Supplemental Video 2, Echocardiography M-Mode of CKO/Tg mice.
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