Nitrosation-dependent caveolin 1 phosphorylation, ubiquitination, and degradation and its association with idiopathic pulmonary arterial hypertension

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Abstract: In the present study, we tested the hypothesis that chronic inflammation and oxidative/nitrosative stress induce caveolin 1 (Cav-1) degradation, providing an underlying mechanism of endothelial cell activation/dysfunction and pulmonary vascular remodeling in patients with idiopathic pulmonary arterial hypertension (IPAH). We observed reduced Cav-1 protein despite increased Cav-1 messenger RNA expression and also endothelial nitric oxide synthase (eNOS) hyperphosphorylation in human pulmonary artery endothelial cells (PAECs) from patients with IPAH. In control human lung endothelial cell cultures, tumor necrosis factor α -induced nitric oxide (NO) production and S-nitrosation (SNO) of Cav-1 Cys-156 were associated with Src displacement and activation, Cav-1 Tyr-14 phosphorylation, and destabilization of Cav-1 oligomers within 5 minutes that could be blocked by eNOS or Src inhibition. Prolonged stimulation (72 hours) with NO donor DETANONOate reduced oligomerized and total Cav-1 levels by 40%–80%, similar to that observed in IPAH patient-derived PAECs. NO donor stimulation of endothelial cells for >72 hours, which was associated with sustained Src activation and Cav-1 phosphorylation, ubiquitination, and degradation, was blocked by NOS inhibitor L-NAME, Src inhibitor PP2, and proteasomal inhibitor MG132. Thus, chronic inflammation, sustained eNOS and Src signaling, and Cav-1 degradation may be important causal factors in the development of IPAH by promoting PAEC dysfunction/activation via sustained oxidative/nitrosative stress.

Keywords: Src, endothelial nitric oxide synthase (eNOS), endothelial dysfunction, oxidative stress, pulmonary arterial hypertension (PAH).

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INTRODUCTION

Caveolin 1 (Cav-1) is a key regulator of endothelial cell function and vascular homeostasis.¹⁻³ Through its association with membrane cholesterol, Cav-1 is essential for the formation of caveolae,⁴ the nonclathrin endocytic vesicles that mediate transcellular transport (transcytosis) of macromolecules.⁵ Cav-1 has also been shown to regulate numerous signaling pathways (e.g., endothelial nitric oxide

synthase [eNOS], Ca²⁺, Rho)⁶ that control the integrity of junctional adhesion complexes,⁷ neoangiogenesis,⁸ and cellular proliferation.⁹ Evidence pointing to a loss of Cav-1 protein expression in the underlying mechanism of endothelial cell activation/dysfunction and the resultant cardiovascular disease phenotype has been steadily accumulating.^{10,11} For instance, we showed that Cav-1 knockout

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(Cav-1^{-/-}) mice have poorly perfused and disorganized microvessels in the lungs and that this contributes to a moderate increase in pulmonary vascular resistance in aged mice.¹² In addition, Cav-1 expression was shown to be significantly reduced in plexiform lesions in lung sections from patients with idiopathic pulmonary hypertension,¹³ although it remains unclear at present whether the loss of Cav-1 in endothelial cells induces hypertensive arteriopathy and formation of focal plexigenic vascular lesions.¹³ Interestingly, Austin et al.¹⁴ recently identified a genetic Cav-1 mutation in skin-punch biopsy fibroblast cultures from patients with pulmonary arterial hypertension (PAH) that was associated with reduced Cav-1 expression. However, the mechanisms responsible for changes in total Cav-1 protein levels in familial or idiopathic disease—that is, whether changes in expression are due to altered transcription, translation, trafficking, or protein turnover—have not been investigated.

In the plasma membrane of endothelial cells, Cav-1 regulates and is regulated by eNOS and Src signaling.¹⁵⁻¹⁷ Interactions between Cav-1, eNOS, and Src not only permit the endothelium to transiently produce NO (via eNOS activation) in response to vasodilator agonists but also prevent unfettered activation of eNOS and resultant oxidative stress via peroxynitrite production that can damage the vasculature.¹⁸ Maintenance of this delicate and somewhat fragile balance between NO output and Src activity by phospho-Cav-1-dependent feedback inhibition of eNOS and Src¹⁵⁻¹⁷ appears to be a critical mechanism involved in endothelial cell homeostasis. NO-mediated Src activation and phosphorylation of Cav-1 increases the affinity of Cav-1 for eNOS¹⁷ as well as the Src-inactivating kinase Csk,¹⁵ thereby promoting the sequestration and inactivation of eNOS and Src at the plasma membrane. This feedback regulatory mechanism, which appears to be critical for maintaining endothelial vasodilatory and homeostatic functions, led us to hypothesize that under conditions of oxidative stress as during chronic inflammation, chemical modification and targeted degradation of Cav-1 may underlie persistent vascular dysfunction and remodeling as observed in patients with idiopathic PAH (IPAH) and scleroderma.^{10,11,19-22}

The findings of the present study indicate that prolonged exposure of endothelial cells to the inflammatory cytokine tumor necrosis factor α (TNF- α) or NO donor induces Cav-1 S-nitrosation (SNO) at Cys-156 within the C-terminus. Following nitrosation, Src, which normally binds to this residue,²³ becomes displaced and activated, resulting in Src-dependent phosphorylation of Cav-1 N-terminal Tyr-14 and leading to destabilization of membrane-

associated Cav-1 oligomers. We propose a model in which sustained Cav-1 nitrosation and phosphorylation increases its ubiquitination and degradation by the proteasome. Since both eNOS and Src inhibitors blocked oxidative stress-induced Cav-1 degradation, these findings suggest that aberrant Cav-1-regulated eNOS and Src signaling may play a significant role in initiating and sustaining endothelial cell activation/dysfunction associated with inflammatory vascular disease, in particular IPAH.

MATERIAL AND METHODS

Cell culture and reagents

Human lung microvascular endothelial cells (HLMVECs) and mouse lung microvascular endothelial cells (MLMVECs). HLMVECs were purchased from Lonza (Walkersville, MD), cultured on 0.2% gelatin-coated dishes, grown using the Vasculife VEGF-Mv Medium Complete kit (Vasculife basal medium [475 mL], Vasculife VEGF-Mv LifeFactors kit; LifeLine, Frederick, MD), and incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air. MLMVECs from wild-type (WT; B6/129Sj2) and Cav-1^{-/-} mice (Jackson Laboratory, Bar Harbor, ME) were isolated as described elsewhere.²⁴

Reagents. Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise, including carbobenzoxy-L-leucyl-L-leucinal (MG132), 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2), and N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME). The ubiquitin antibody used (P4D1) and the green fluorescent protein (GFP) antibody used (B-2) were from Santa Cruz Biotechnology (Dallas, TX). β -Actin, Cav-1, and p-Cav-1 Tyr-14 antibodies were purchased from BD Biosciences (San Jose, CA); p-Src Tyr418 and p-Src Tyr530 antibodies were purchased from Cell Signaling Technology (Danvers, MA); and antibody for Src (monoclonal) was purchased from Santa Cruz Biotechnology. DETANONOate ((Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate) was purchased from A.G. Scientific (San Diego, CA). *n*-Octyl- β -D-glucopyranoside (ODG) buffer was purchased from RPI (Mt. Prospect, IL); S-nitroso-N-acetyl-D,L-penicillamine (SNAP) was purchased from Cayman Chemicals (Ann Arbor, MI).

Human embryonic kidney (HEK) 293 cells. HEK cells were purchased from the American Type Culture Collection (Rockville, MD). They were cultured in Dulbecco's modified eagle medium from Invitrogen (Grand Island, NY) supplemented with 10% fetal bovine serum from Serum

Source International (Charlotte, NC) and 1% penicillin/streptomycin from Invitrogen.

Human pulmonary arterial endothelial cells (HPAECs; Cleveland Clinic). HPAECs were obtained from human lung tissues from either unused explanted donor lungs or explanted lungs from confirmed subjects with PAH undergoing lung transplantation at the Cleveland Clinic, as described elsewhere.²⁵ The study was approved by the Cleveland Clinic Institutional Review Board (IRB).

Human pulmonary microvascular endothelial cells (HPMVECs; Amsterdam). HPMVECs were isolated from PAH patients (autopsies and lung transplantations) and controls (material from lung tumor surgeries) by mechanical homogenization and treatment with 0.3% collagenase type 2 (Sigma-Aldrich), as approved by the IRB and with consent. The tissue suspension was filtered through 70- μ m nylon mesh and purified by CD31-labeled magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Pure HPMVEC cultures were seeded on 1% gelatin-coated culture flasks and maintained in EGM-2 growth medium plus Bulletkit (Lonza). Passage numbers between four and six were used for experiments. HPMVECs were cultured in growth medium until confluence was reached and kept confluent for 72 hours to allow maturation of cell adhesions. Then the cells were put on ice, washed with ice-cold phosphate-buffered saline (PBS), and lysed with lysis buffer (Roche, San Francisco, CA) containing Triton X-100 and a protease/phosphatase-inhibitor cocktail. The lysates were centrifuged at 17,075 g, and supernatants were stored at -80°C .

Western blotting and immunoprecipitation

HPAECs, HLMVECs, and HEK cells were lysed on ice in lysis buffer containing 20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 60 mM *n*-octylglucoside, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and protease inhibitor cocktail. Insoluble materials were removed by centrifugation for 5 minutes at 16,100 g at 4°C , and lysates were boiled in lysis buffer with 6 $\times$ Laemmli sample buffer (Boston BioProducts, Ashland, MA) and 30 mM dithiothreitol. Protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation was performed, and proteins were blotted onto nitrocellulose membranes and blocked with 5% blotting-grade nonfat dry milk (Bio-Rad, Hercules, CA) in Tris-buffered saline (TBS) with 0.05% Tween 20 for

1 hour. Membranes were probed with primary antibodies overnight at 4°C , washed three times in TBS with Tween, and incubated with secondary species-specific horseradish peroxidase-conjugated antibodies for 1 hour at room temperature in blocking buffer. Membranes were washed three times, and proteins were visualized with enhanced chemiluminescence substrate (Pierce). For immunoprecipitation studies, cells were lysed in ODG buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 20 mmol/L NaF, 2% octyl-D-glucoside, 5% glycerol, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium orthovanadate, and protease inhibitor. Resulting lysates were added to magnetic sheep anti-mouse immunoglobulin (Ig) G-coated Dynabeads (Invitrogen) that were preincubated for 30 min at 4°C with nonspecific mouse IgG or anti-Cav-1 monoclonal antibodies. Samples were rotated for 1 hour at 4°C , placed on a magnetic particle concentrator (Invitrogen), and washed with cold PBS without calcium/magnesium supplemented with protease-inhibitor cocktail and sodium orthovanadate. Samples were washed three times, 6 $\times$ Laemmli sample buffer was added, and the samples were then either boiled for 5 minutes or loaded without boiling onto SDS-PAGE gels for Western blot analysis.

Immunostaining and confocal microscopy

Formalin-fixed, paraffin-embedded (FFPE) lung tissue sections were processed for fluorescence microscopy as follows. Lung tissues sectioned at 20- μ m thickness were rehydrated and subjected to antigen retrieval in citrate buffer. After cooling, sections were postfixed in methanol, endogenous peroxidase activity was blocked, and antibody staining was performed by first blocking nonspecific binding with 5% donkey serum for 2 hours and then sections were incubated with primary antibodies diluted in blocking solution overnight at 4°C . After being washed in PBS with 0.05% Tween 20 PBS, sections were incubated with fluorophore-conjugated secondary antibodies raised in donkey (Jackson ImmunoResearch Laboratories, West Grove, PA). Primary antibodies were diluted as follows: mouse monoclonal vascular endothelial (VE)-cadherin, 1:1,000 (BD Transduction Laboratories, San Diego, CA); platelet endothelial cell adhesion molecule 1 (PECAM-1) monoclonal antibody hec7, 1:1,000 (gift of W. A. Muller, Northwestern University, Chicago, IL). Negative controls and appropriate isotype-matched control antibodies were included in all immunostaining experiments. Confluent HLMVECs grown on collagen-coated glass coverslips or MLMVECs purified from WT or Cav-1^{-/-} mice grown on Matrigel-coated coverslips at 37°C . Cells were washed, fixed with 4% paraformaldehyde, permeabilized with 0.1%

Triton X-100, and stained with the nuclear marker 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 1 µg/mL). Nonconfocal DAPI images were acquired using Hg lamp excitation and a UV filter set. Confocal microscopy was performed using a 63× 1.2 N.A. objective on a Zeiss LSM 510 META microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) with a 488-excitation laser line and pinhole set to achieve 1 Airy unit.

Generation of Cav-1 cysteine mutants and transfections

Cells were transfected using Lipofectamine 2000 on the basis of manufacturer's protocols.

Methods for pEGFP-N1-CAV1-WT plasmid subcloning. Full-length *Homo sapiens* Cav-1 complementary DNA (cDNA) was subcloned into pEGFP-N1 (C-terminal-tagged GFP) vector (Clontech, Mountain View, CA) via restriction sites 5'-*NheI* and 3'-*KpnI*. Polymerase chain reaction (PCR) was performed with Phusion (Finnzymes, Espoo, Finland) to generate the full-length Cav-1 PCR fragment without the STOP codon using the primer pairs Cav1-*NheI*-F (5'-ACTAGCTAGCGACCGCCATGTCTGGGGGCAAATAC-3') and Cav1-*KpnI*-R (5'-ACTGGGTACCGTTATTTCTTTCTGCAAGTTGATGCG-3'). The bold and italicized base pairs shown in the primers indicate the restriction sites. The resulting PCR fragment was digested with *NheI* and *KpnI* (Invitrogen or New England Biolabs, Ipswich, MA) and ligated to pEGFP-N1, also digested with the same restriction enzymes, using T4 DNA Ligase (New England Biolabs). The ligated reaction was transformed into DH5α chemically competent cells (Invitrogen), and the transformed cells were plated onto Luria-Bertani (LB) plates supplemented with 25 mg/mL kanamycin. After overnight incubation at 37°C, single colonies were cultured in LB with kanamycin overnight. Plasmid DNA was obtained using the PureLink Quick Plasmid Mini Prep kit (Invitrogen) and analyzed by restriction digest to confirm the presence of Cav-1 cDNA. Further amplification of plasmid DNA was done using the NucleoBond Xtra Midi kit (Clontech). The resulting plasmid was verified for DNA purity and accuracy by DNA quantification, gel analysis, and sequencing analysis.

Double or multiple amino acid mutation Cav-1 plasmid subcloning. Several steps of PCR were used to create the multiple mutations using the single amino acid-mutated plasmid as the starting cDNA template. Several fragments of PCR were generated using primer sets that targeted other amino acid mutations. Subsequent steps of diges-

tion and ligation, transformation, colony screening, and plasmid DNA isolation and amplification were performed as described. The resulting plasmid was verified for DNA purity and accuracy by DNA quantitation, gel analysis, and sequencing analysis. The PCR thermocycling conditions were as follows: initial denaturation at 98°C for 30 seconds; 30 cycles of denaturation, annealing, and extension at 98°C for 10 seconds, 67°C or melting temperature plus 3° of the lowest primer in a primer pair for 30 seconds, and 72°C for 15 seconds; and final extension at 72°C for 10 minutes followed by holding at 4°C.

H. sapiens Cav-1 primer sequences. Bold and italicized base pairs indicate the sequence of restriction enzymes, while the underlined base pairs indicate the amino acid being mutated. Primers and sequences were as follows: for CAV1-WT, Cav1-*NheI*-F (5'-ACTAGCTAGCGACCGCCATGTCTGGGGCAAATAC-3') and Cav1-*KpnI*-R (5'-ACTGGGTACCGTTATTTCTTTCTGCAAGTTGATGCG-3'); for CAV1-C133S, Cav1-C133S-F (5'-GTTGTACCATCCATTAAGAGC-3') and Cav1-C133S-R (5'-GCTCTTAATGGATGGTACAAC-3'); for CAV1-C143S, Cav1-C143S-F (5'-GAGATTGAGTCCACCA-GCCGTGTC-3') and Cav1-C143S-R (5'-GACACGGCTGG-TGGACTGAATCTC-3'); for CAV1-C156S, Cav1-C156S-F (5'-TACGTCCACACCGTCTCTGACCCACTC-3') and Cav1-C156S-R (5'-TTCAAAGAGTGGGTGAGAGACGGTGTG-3'); and for CAV1-C133/143/156S, pEGFP-N1-CAV1-C143S was used as template, and C133S then C156S primers were used in PCR.

Reverse-transcription (RT) PCR

Total RNA from endothelial cells were isolated using TRIzol reagent. RT was performed using oligo(dT) primers and superscript RT (Invitrogen) following the manufacturer's instructions. Human p38 isoforms and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified using the following primer sets: p38 (sense, 5'-GATCAGTTGAAGCTCATTTTAA-3'; antisense, 5'-CACTTGAATAATTTTGGAGAGT-3'); p38 (sense, 5'-AGCCATATCTGGCAAGAAGCTGGA-3'; antisense, 5'-AAGTGTCCGAGTCCAAGTCCACAT-3'); p38 (sense, 5'-TTGAATTGGATGCGCTACACGCAG-3'; antisense, 5'-AGGGCTTGCAATTGGTCAGGATAGA-3'); p38 (sense, 5'-TGTGCAGAAGCTGAACGACAAAGC-3'; antisense, 5'-TGCCATGCAAGATGAGTCCCTACA-3'); and GAPDH (sense, 5'-TATCGTGAAGGACTCATGACC-3'; antisense, 5'-TACATGGCAACTGTGAGGGG-3'). RT product (2 L) was amplified in a 20-L volume containing 100 pmol of primers and 2.5 U of TaqDNA polymerase. To quantify fold change in Cav-1 expression TaqMan probes, GAPDH

(Hs02758991_g1) and Cav-1 (Hs00971716_m1) from Invitrogen were used, and the analysis was run on Applied Biosystems 7500 FAST (Invitrogen) according to the manufacturer's instructions. We looked for fold change in hypertension/control. Cav-1 expression levels were normalized to GAPDH expression in each sample. The graph represents fold change in expression in hypertension compared with control samples.

Biotin switch assay

S-nitrosylated protein was detected by the biotin switch method as described elsewhere²⁶ using the Cayman Chemical S-Nitrosylated Protein Detection kit. Briefly, endothelial cells were treated with DETANONOate for the indicated time. Samples were immunoprecipitated using streptavidin-conjugated antibodies (Dynabeads M-280 streptavidin from Invitrogen). Biotinylated Cav-1 was resolved by nonreducing SDS-PAGE, transferred, and detected by immunoblotting with mouse anti-Cav-1 (1 : 1,000). Analysis of SNO-Cav-1 by biotin switch assay was performed as described elsewhere.²⁶ Samples were immunoprecipitated using streptavidin-conjugated antibodies (Dynabeads M-280 streptavidin from Invitrogen). Biotinylated Cav-1 was resolved by nonreducing SDS-PAGE, transferred, and detected by immunoblotting with mouse anti-Cav-1 (1 : 1,000).

Mass spectrometry

Mass spectrometry analysis was performed by the Chicago Biomedical Consortium/University of Illinois at Chicago Research Resources Center Proteomics and Informatics Services Facility. The in-gel tryptic digestion was carried out by following the protocol described by Kinter and Sherman.²⁷ Briefly, Coomassie-stained gel bands were cut into 1-mm³ pieces, rinsed, and dehydrated with acetonitrile, and the protein was reduced with dithiothreitol (DTT) and alkylated with iodoacetamide in the dark prior to overnight digestion with Promega Gold trypsin at 37°C in 100 mM ammonium bicarbonate. The peptides were extracted with ammonium bicarbonate and acetonitrile, concentrated, and analyzed using a Thermo LTQ-FT Ultra mass spectrometer equipped with a Dionex 3000 nanoflow high-performance liquid chromatography system controlling a reverse-phase column (Agilent Zorbax 300SB-C18, 3.5 μ m, 75 μ m $\times$ 150 mm) at a flow rate of 250 nL/min. The peptides were separated and eluted with a linear gradient of 10%–60% solution B (95% acetonitrile, 0.1% formic acid) for 60 minutes.

Transmission electron microscopy

For ultrastructural analysis, cells grown on Matrigel-coated plastic dishes were fixed with Karnovsky fixative, stained

with OsO₄, dehydrated, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a transmission electron microscope.

Densitometry and statistics

Densitometry of protein bands was performed with ImageJ software (<http://rsbweb.nih.gov/ij/>). Comparison of two groups was conducted using Student's *t* test, and three or more groups were compared with analysis of variance with Bonferroni post hoc testing to correct for multiple testing and to keep the family-wise error rate below 0.05. Experimental data are presented as mean $\pm$ standard error of the mean (SEM). All tests were performed two-sided and non-blinded and were executed with GraphPad Prism software for Mac (ver. 6; GraphPad Software, La Jolla, CA). A *P* value of <0.05 was considered statistically significant.

RESULTS

Reduced Cav-1 protein but not mRNA expression in IPAH endothelial cells

Consistent with previous studies and various animal models of PAH,^{14,28,29} PAECs isolated from excised human lung tissue from patients with IPAH (*n* = 4)^{25,30} exhibited reduced oligomerized and total Cav-1 protein levels (Fig. 1A, 1B) but increased Cav-1 mRNA expression (Fig. 1C), suggesting that Cav-1 degradation is faster than its synthesis in the PAH endothelia. Consistent with the known role of Cav-1 in the mechanism of eNOS inactivation,³¹ reduction in expression and oligomerization of Cav-1 paralleled an increase in eNOS phosphorylation at active site Ser 1177 despite a slight reduction in the total level of eNOS protein (Fig. 1A). That Cav-1 mRNA expression was upregulated twofold in IPAH PAECs suggests that there is increased turnover of Cav-1 protein in IPAH patient-derived PAECs. Furthermore, we observed reduced Cav-1 expression in tissue sections (deidentified FFPE sections of cadaver lung tissue diagnosed with PAH; *n* = 6) compared with that in healthy donor lung tissue (*n* = 6). Immunostaining and confocal microscopy revealed reduced Cav-1 expression in PECAM-1-positive endothelial cells in pulmonary arterioles from patients with IPAH (Fig. 1D). While the observed decrease in total Cav-1 protein expression in IPAH PAECs was not found in isolated and cultured microvascular endothelial cells from excised lungs diagnosed with PAH, there was a trend toward reduced oligomerization (Fig. S1).

Inflammatory mediator TNF- α induces eNOS-dependent Cav-1 SNO

In view of findings shown in Figure 1, we hypothesized that inflammation and oxidative stress that accompanies

Figure 1. Reduced caveolin 1 (Cav-1) oligomer and monomer protein level in pulmonary artery endothelial cells (PAECs) from patients with idiopathic pulmonary arterial hypertension (IPAH). **A**, Representative Western blot of PAEC lysates obtained from a healthy donor (left lane) and a patient with IPAH (right lane). The lower panel reveals an increase in phospho-Ser-1177 endothelial nitric oxide synthase (p-eNOS) relative to total eNOS expression in IPAH endothelial cells, indicative of eNOS hyperactivation/dysfunction. **B**, Summarized data. Asterisk indicates $P < 0.05$ versus control; $n = 5$. **C**, Quantitative polymerase chain reaction indicates increased Cav-1 messenger RNA (mRNA) in IPAH PAECs compared with control. Asterisk indicates $P < 0.05$; $n = 3$. **D**, Immunohistochemical staining and confocal microscopy of Cav-1 (green) and platelet endothelial cell adhesion molecule 1 (PECAM-1; red) in formalin-fixed, paraffin-embedded control and PAH lung sections. Note the significant colocalization of Cav-1 and PECAM-1 in control lung pulmonary arteriole and reduced Cav-1 staining in the endothelial cells of the remodeled pulmonary arteriole of the PAH lung section. Staining is representative of 6 PAH and control lung samples. M_r : relative molecular mass; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride.

PAH¹⁹ may promote chemical modification and degradation of Cav-1, thus altering its ability to negatively regulate eNOS signaling in the endothelium. Consistent with this assumption, we observed that brief challenge with TNF- α , a powerful proinflammatory cytokine known to activate eNOS,³² significantly increased SNO of Cav-1 (Fig. 2A). The dependence of Cav-1 SNO on NOS activity was shown by its abrogation in cells pretreated with the NOS inhibitor L-NAME (Fig. 2A). To pinpoint the SNO to a particular thiol residue, each of the critical thiol residues implicated in the regulation of Cav-1 targeting to the plasma membrane (C-133, C-143, and C-156) was mutated to serine (Fig. 2B). The mutants were then transfected into HEK cells overexpressing eNOS (HEK-eNOS cells).¹⁴ In addition, we prepared a triple mutant-encoding construct. Analysis of each of the transfected lines revealed that the C156S Cav-1 mutation was sufficient to abolish Cav-1 SNO, whereas the C133S and C143S mutants

showed only a modest reduction in SNO once normalized to total Cav-1 expression level (Fig. 2C).

SNO of Cav-1 promotes Src dissociation and activation

We next interrogated whether the nitrosation of Cav-1 on Cys-156 regulates the interaction/binding and activity Src, which is known to phosphorylate and affect Cav-1-regulated signaling.¹⁵⁻¹⁷ As shown in Figure 3A, treatment of HEK cells expressing WT-Cav-1-GFP with SNAP significantly reduced Src/Cav-1 coimmunoprecipitation. Furthermore, TNF- α -induced Src activation was shown to be NOS dependent (Fig. 3B). The ratio of Src active-site phospho-Tyr416 to inactive-site phospho-Tyr527, which predicts Src activity in human lung endothelial cells,^{12,33} increased in cells challenged with TNF- α alone and was blocked in cells pretreated with the NOS inhibitor L-NAME (Fig. 3B). Indeed, we found, concurrently with the

Figure 2. Tumor necrosis factor α (TNF- α) induces caveolin 1 (Cav-1) S-nitrosation on Cys-156. **A**, Biotin switch assay indicates TNF- α -dependent, L-NAME-inhibitable nitrosation of Cav-1. TNF- α (2–20 ng/mL) was added to human lung microvascular endothelial cells (HLMVECs) 5 minutes before lysis. L-NAME was added 30 minutes prior to TNF- α . **B**, Biotin switch assay performed on human embryonic kidney cells overexpressing endothelial nitric oxide synthase that were expressing wild type (WT)–Cav-1, C133S–Cav-1, C143S–Cav-1, and C156S–Cav-1 mutants in pEGFP vector. C133S-, C143S-, C156S, and triple (C133S/C143S/C156S)–Cav-1 mutants in pEGFP vectors were made. **C**, Nitrosation was promoted by adding S-nitroso-N-acetyl-D,L-penicillamine (SNAP; 20 μ M) at room temperature for 5 minutes. After lysis and the biotin switch, samples were immunoprecipitated with Cav-1 and blotted for streptavidin. Right panels show quantified densitometry of Western blots using ImageJ software. Bars show mean $\pm$ standard deviation. Asterisks indicate $P < 0.05$ versus control (**A**) or WT (**C**); $n = 3$. GFP: green fluorescent protein.

demonstration that Cys-156 is required for Cav-1/Src interaction, that mutation of Cys-156 to serine (C156S) was sufficient to abolish Cav-1/Src interaction (Fig. 3C). A diminishment in the association of Cav-1 and Src produced by nitrosation—or as shown in Figure 3D by expression of the Cav-1 C156S mutant—led to increased Src activity (Src-pTyr416) and Cav-1 phosphorylation at Tyr-14.

Cav-1 SNO is accompanied by destabilization of Cav-1 oligomers

To form caveolae and regulate signaling via specific scaffolding interactions with a multitude of target proteins known to interact with Cav-1,¹ Cav-1 organization as a multimeric homotypic structure is thought to be essential.³⁴ As shown in Figure 4, TNF- α , possibly via Cav-1

SNO, destabilized and disrupted Cav-1 oligomers in an L-NAME-sensitive manner, indicating that eNOS activation and subsequent Cav-1 SNO may promote Cav-1 oligomer destabilization and accumulation of Cav-1 monomers. Furthermore, in addition to NO signaling, Src activity also appears to participate in the destabilization of Cav-1 oligomers since PP2, a specific inhibitor of Src, blocked the effects of TNF- α to an extent comparable to that observed in the presence of L-NAME (Fig. 4A). Again, modification of the Cys-156 residue appears to mediate this effect since the C156S mutation (but not C133S or C143S) mimicked the effect of SNO (i.e., decreased oligomerization) as shown in Figure 4B. Taken together, these observations indicate that Cav-1 SNO followed by Src-dependent phosphorylation of Cav-1 Tyr-14 induces the destabilization of Cav-1 oligomers, resulting in the accumulation of Cav-1 in its monomeric form. It also implies that modifications to

Figure 3. Nitric oxide (NO) donor and caveolin 1 (Cav-1)-C156S mutant decrease Cav-1/Src interaction and increase Src activity. **A**, Human embryonic kidney (HEK) cells were transfected with wild type (WT)-Cav-1-green fluorescent protein (GFP) and stimulated with either NO donor or S-nitroso-N-acetyl-D,L-penicillamine (SNAP; 20 μ M, 5 minutes) or left untreated. Cells were lysed, Src was immunoprecipitated, and bound Cav-1 was quantified by Western blot. Asterisks indicate $P < 0.05$ versus control; $n = 3$. **B**, Analysis of active/inactive Src as indicated. pTyr⁴¹⁹/pTyr⁵³⁰ Western blots from tumor necrosis factor α (TNF- α)-stimulated human lung microvascular endothelial cells with or without NO synthase (NOS) inhibitor pretreatment indicates that L-NAME blocks TNF- α -induced Src activation. Asterisks indicate $P < 0.05$ versus TNF- α alone; $n = 3$. **C**, HEK cells overexpressing endothelial NOS (eNOS; HEK-eNOS cells) were transfected with empty vector (EV), WT-Cav-1, and C156S-Cav-1 mutant in pEGFP vector. Samples were blotted for total Cav-1 (upper row) or immunoprecipitated with anti-Src monoclonal antibody and blotted for Cav-1 and Src (lower rows). **D**, HEK-eNOS cells were transfected with WT-Cav-1 and C156S-Cav-1 mutant and blotted for Src-pTyr⁴¹⁸, total Src, Cav-1-pTyr¹⁴, and total Cav-1. Asterisks indicate $P < 0.05$ versus WT-Cav-1; $n = 3$.

Cys-156 may disrupt the oligomeric structure and function of Cav-1 polymers in the plasma membrane.

Differential effects of short- and long-term exposure to NO on Cav-1 oligomer stability

As shown in Figure 5A, brief (minutes) and prolonged (days) exposure to the NO donor SNAP dramatically reduced the structural integrity of Cav-1 oligomers and the overall stability of the protein, respectively. Short-term NO exposure rapidly (within 5–10 minutes) reduced Cav-1 oligomerization, which was followed, after 72 hours of continuous NO challenge, by an overall reduction in to-

tal Cav-1 protein level (monomers and oligomers). This observation suggests that Cav-1 and its ability to interact with Src and eNOS may be regulated by NO. It also indicates that prolonged exposure to NO may alter Cav-1-regulated signaling by provoking its degradation, consistent with reports of derailed Cav-1/eNOS interactions in conditions characterized by chronic inflammatory signaling and oxidative stress.^{35–37}

NO-dependent loss of Cav-1 protein was mitigated by MG132 (Fig. 5B), a proteasome inhibitor,³⁸ suggesting that the decrease in Cav-1 expression as a consequence of persistent NO challenge occurs via proteolytic degrada-

Figure 4. Caveolin 1 (Cav-1) oligomerization is reduced by tumor necrosis factor α (TNF- α) in a nitric oxide (NO)- and Src-dependent manner. **A**, Human lung microvascular endothelial cells were pretreated with either nitric oxide synthase inhibitor L-NAME or Src inhibitor PP2 30 minutes prior to being challenged with TNF- α for 5 minutes. Cav-1 oligomer/monomer distribution was analyzed by Western blot of nonboiled samples. Quantification of densitometry using ImageJ (mean $\pm$ SD; asterisk indicates $P < 0.05$ versus untreated; $n = 3$). **B**, Western blot analysis of Cav-1 expressed in human embryonic kidney cells (EV = empty green fluorescent protein [GFP] vector, wild type [WT]-, C133S-, C143S-, C156S-, and triple [C133S/C143S/C156S]-Cav-1-GFP mutants) showed that Cys-156 is required to maintain oligomer stability. Bar graphs show quantified densitometry of Cav-1 oligomer/monomer. Asterisks indicate $P < 0.05$ versus WT-Cav-1; pound signs indicate $P < 0.05$ versus C133S- and C143S-Cav-1; $n = 3$.

tion. Importantly, PP2, a specific Src inhibitor, also inhibited NO-stimulated Cav-1 proteolytic degradation (Fig. 5B, middle panel), thus implicating sustained Cav-1 Tyr-14 phosphorylation by active Src tyrosine kinase in the mechanism of Cav-1 degradation. This evidence suggests that Src displacement from Cav-1 on nitrosation of Cys-156 may be upstream of Cav-1 phosphorylation by Src and that, in tandem, SNO and phosphorylation decrease Cav-1 oligomerization (Fig. 5C) and promote Cav-1 degradation (Fig. 5D). In agreement with the possibility that Cav-1 SNO followed by Src-dependent phosphorylation targets Cav-1 for proteasomal degradation, we found that NO, particularly after 48- and 72-hour exposures, markedly increased Cav-1 ubiquitination (Fig. 5E), the characteristic signature of proteasomal targeting. PP2 strongly inhibited Cav-1 ubiquitination, providing evidence for the involvement of Src family tyrosine kinase activity in NO-mediated degradation of Cav-1 (Fig. 5E). Further evidence for the ubiquitination of Cav-1 is provided in Figure S2. Using mass spectrometry-assisted peptide mapping, we identified the residue that is ubiquitinated following NO ex-

posure as Lys-86. Taken together, experimental evidence demonstrates that short- and long-term exposure to NO may have markedly distinct effects on Cav-1 stability and regulated signaling. Sustained Cav-1 SNO was associated with Src-dependent phosphorylation of Tyr-14, ubiquitination of Lys-86, and proteasomal degradation.

Functional implications of Cav-1 degradation

Confocal imaging experiments showed that WT-Cav-1-GFP transfection into Cav-1^{-/-} mouse lung endothelial cells was associated with formation of punctate vesicle-like structures that decorated the membrane and cytoplasmic compartments (Fig. 6A, left panel). However, expressed C156S-Cav-1-GFP accumulated as large aggregates in the perinuclear region (Fig. 6A, right panel) with reduced localization in the plasma membrane where Cav-1 is known to assemble into functional oligomers and promote the formation of caveolae. In parallel, electron microscopy analysis of Cav-1^{-/-} endothelial cells confirmed the requirement of Cav-1 for formation of caveolae (Fig. 6B, 6C). Restoration of Cav-1 expression by transfection of WT-

Figure 5. Nitric oxide (NO) donors induce Src-dependent caveolin 1 (Cav-1) oligomer destabilization and Cav-1 degradation. **A**, Human lung microvascular endothelial cells (HLMVECs) treated with S-nitroso-N-acetyl-D,L-penicillamine (SNAP; 10 μ M) showed reduced oligomerization at 5 minutes (0.08 hours) and a decrease in total Cav-1 expression at 72 hours. **B**, HLMVECs either untreated or pretreated with the Src inhibitor PP2 (10 μ M) or the proteasome inhibitor MG132 (20 μ M) were challenged every 24 hours with NO donor DETANONOate (250 μ M) for 24, 48, or 72 hours. Nonboiled samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted for Cav-1 and actin. **C**, **D**, Quantified densitometry of Western blots reveal a significant decrease in the Cav-1 oligomer/monomer ratio (**C**) and a significant decrease in total Cav-1 (monomer + oligomer; **D**) after 72 hours of DETANONOate, which is blocked by PP2 and MG132. Asterisks indicate $P < 0.05$ versus untreated by analysis of variance; $n = 3$. **E**, Ubiquitination of immunoprecipitated Cav-1 from HLMVEC lysates after challenge with NO donor DETANONOate (250 μ M) in the presence or absence of PP2 (10 μ M; left panels) and reprobe of membrane with anti-Cav-1 antibody (right panels). Immunoblots show that ubiquitination (left panel) and degradation (right panel) of Cav-1 (oligomers and monomers) following treatment with DETANONOate is blocked by PP2 (representative of 3 experiments).

Cav-1 cDNA rescued caveolae formation, as demonstrated in Figure 6B (left panel). In clear contrast, transfection of C156S-Cav-1 mutant cDNA was unable to rescue caveolae formation in Cav-1^{-/-} endothelial cells (Fig. 6B, 6C). Thus, Cav-1 Cys-156 is a requirement for stability as an oligomer and caveolae formation. Consistent with these findings, WT-Cav-1-GFP expressed in HEK cells was detectable primarily in its high-molecular-weight oligomeric

form in nonboiled SDS-PAGE, whereas C156S Cav-1-GFP was detected as a monomeric protein (Fig. 6D).

DISCUSSION

Cav-1 expression is essential for caveolae formation^{5,39} and the endocytosis and transcytosis of macromolecular cargo (e.g., albumin,⁴⁰⁻⁴³ insulin,⁴⁴ myeloperoxidase,⁴⁰ and cholesterol⁴⁵) into and through the vascular endothelium.⁴⁶

Figure 6. Confocal and transmission electron microscopy reveals that the C156S caveolin 1 (Cav-1) mutant fails to rescue caveolae formation in Cav-1^{-/-} mouse lung endothelial cells (MLECs). **A**, Cav-1^{-/-} MLECs transfected with wild type (WT)-Cav-1-green fluorescent protein (GFP) complementary DNA (cDNA) restored membrane-associated Cav-1 localization, whereas the C156S-Cav-1-GFP mutant accumulated in perinuclear intracellular compartments. **B**, Representative transmission electron microscopy micrographs show rescue of caveolae in Cav-1^{-/-} endothelial cells following transduction of WT-Cav-1, whereas no caveolae were observed in Cav-1^{-/-} MLECs transduced with C156S-Cav-1 cDNA. **C**, Quantitative analysis of electron micrographs indicate near-complete rescue of caveolae formation by WT-Cav-1 compared with WT control and Cav-1^{-/-} mouse lung endothelial cells and lack of caveolae formation in cells transduced with C156S-Cav-1. **D**, Western blot analysis of WT-Cav-1-GFP, C156S-Cav-1-GFP, and empty GFP vector (EV) expression in human embryonic kidney cells. Note the absence of stable high-molecular-weight oligomers in C156S-Cav-1-transfected cells.

In addition, studies during the last 15 years have unveiled critical functions of Cav-1 as a membrane-associated scaffolding protein involved in the regulation of receptors, ion channels, and signaling enzymes.^{15-17,47-55} In the current investigation, we describe a novel posttranslational modification, SNO of Cav-1 Cys-156. When sustained by chronic inflammatory cytokine signaling (e.g., TNF- α) or direct NO stimulation, Cav-1 SNO leads to Src-dependent Tyr-14 phosphorylation, Lys-86 ubiquitination, and Cav-1 degradation via the proteosomal pathway. We propose, on the basis of these findings, that chronic vascular inflammation induces Cav-1 degradation via ubiquitination and proteolysis through the proteosomal pathway. These findings are consistent with reduced Cav-1 expression and endothe-

lial cell activation/dysfunction in group 1 idiopathic pulmonary artery hypertension (IPAH) patients characterized by hypertensive arteriopathy and plexiform lesions.^{13,56} The mechanism by which Cav-1 degradation may promote the plexigenic endothelial cell phenotype is currently under investigation.

First, as noted in PAECs harvested from the excised lungs of IPAH patients undergoing transplant surgery,⁵⁷ Cav-1 levels were significantly reduced and there was a concurrent increase in eNOS phosphorylation at active site Ser-1177, indicative of its activation. We noted a twofold increase in Cav-1 mRNA, suggesting that the decrease in Cav-1 expression in IPAH PAECs occurs at the protein rather than the transcriptional level. Interestingly, we did

not observe a statistically significant reduction in Cav-1 protein level or oligomerization in human PAH MVECs. However, lung sections from patients with PAH showed a significant reduction in Cav-1 immunostaining of PECAM-1-positive cells associated with vessels <100 μ m in diameter, suggesting that at least endothelial cells of the smaller muscularized microvessels may be similarly affected by oxidative stress. Further study of patient-derived endothelial cells from different vascular beds in association with tissue immunohistochemistry is required to better understand whether inflammation and/or graded oxidative stress differentially affects macro- versus microvascular endothelial cell Cav-1 expression.

In normal human lung endothelial cell cultures, we demonstrated that the proinflammatory cytokine TNF- α induces Cav-1 nitrosation within minutes, an event that we surmised links eNOS hyperactivation to the sustained proteolytic loss of Cav-1 in chronic inflammatory states. We observed in experiments that employed the NOS inhibitor L-NAME that TNF- α -induced nitrosation of Cav-1 was eNOS dependent. Additionally, by generating single-residue mutations to C-terminal Cys residues, experiments pinpointed Cys-156 as the primary residue in Cav-1 modified by NO. The significance of this finding with regard to Cav-1 stability and signaling was then investigated. We showed that Cav-1 nitrosation or mutation of Cys-156 to Ser (C156S) decreased Cav-1 association with Src tyrosine kinase, indicating that Cav-1 nitrosation is sufficient to activate Src by releasing it from its inhibitory association with the Cav-1 C-terminus. Src displacement from Cav-1 was also shown to promote activation (as demonstrated by Western blot studies shown in Fig. 3B) and resultant phosphorylation of Cav-1 Tyr-14 (Fig. 3D).

Interestingly, we observed that Cav-1 nitrosation and phosphorylation was associated with its accumulation in monomeric form (i.e., 22 kDa) in nonreduced SDS gels. As a structural protein, Cav-1 is thought to function as a polymeric chain of oligomers.⁵⁸ In conjunction with the cavin family of proteins, Cav-1 is stabilized in cholesterol-enriched membrane microdomains and therein is thought to account for the flask-shaped curvature of plasmalemmal vesicles, or caveolae.^{59,60} Destabilization, disassembly, and/or dissociation of Cav-1 polymers was suggested to regulate membrane curvature and invagination⁶¹ and—if sustained, as suggested by the current study—disruption/dissolution of caveolae due to Cav-1 degradation. The mechanism of Cav-1 nitrosation and phosphorylation-dependent destabilization of oligomers is unclear. Phosphorylation of Cav-1 has been suggested to introduce significant charge repulsion within the polymer due to

negative charges in closely apposed N-terminal phospho-Tyr-14 residues within the oligomeric clusters of Cav-1.⁶¹ As shown in the current study (Figs. 4, 5), both L-NAME (NOS inhibitor) and PP2 (Src inhibitor) blocked TNF- α -induced Cav-1 monomerization and degradation. Therefore, NO and downstream Src signaling-induced destabilization and degradation of Cav-1 may be an important determinant of the structural and functional integrity of caveolae as well as Cav-1 regulation of endothelial cell signaling.

Our studies demonstrate that NO donors are sufficient to promote Cav-1 monomerization. Inhibition of Src activity downstream of NO prevented Cav-1 oligomer destabilization and pretreatment of cells with the proteasome inhibitor MG132 blocked NO-dependent Cav-1 degradation. Taken together, these observations establish a sequence of events that connect nitrosative stress to Cav-1 degradation. According to our findings, Cav-1 Cys-156 nitrosation is associated with Src displacement and activation. Src phosphorylation of Cav-1, if sustained, is associated with degradation of Cav-1. Consistently, we detected Cav-1 ubiquitination in samples exposed to the NO donor DETANONOate and observed that PP2 could block Cav-1 ubiquitination and degradation. Importantly, mass spectrometry indicated Lys-86 within the caveolin scaffolding domain (which is known to be important for eNOS binding)⁶² as a site of Cav-1 ubiquitination; other Lys residues in Cav-1 have also been shown to be ubiquitinated.^{63,64} In the absence of Cav-1-mediated feedback regulation of eNOS activity, eNOS has been shown to be hyperphosphorylated on Ser-1179 (see Fig. 1B). Hyperactivation of eNOS leads to its uncoupling,²¹ thereby promoting peroxynitrite production in lieu of NO,¹⁸ which we hypothesize promotes persistent activation and dysfunction of the vascular endothelium.

Cav-1-null endothelial cells harvested from the lungs of Cav-1^{-/-} mice lack caveolae.^{24,65} Rescue experiments in which the Cav-1 C156S mutant was reintroduced failed to restore caveolae formation, whereas WT-Cav-1 cDNA was sufficient to induce formation of caveolae (Fig. 6B). This result is not surprising in view of our finding that Cav-1 C156S is unable to form stable oligomeric structural units (Figs. 3B, 6D).

In summary, our studies demonstrate that Cav-1 degradation occurs following sustained NO production, as with chronic inflammatory stimuli. Decreased Cav-1 expression results in uncontrolled activation of at least two key endothelial cell-signaling proteins: eNOS and Src, whose persistent activation may perpetuate Cav-1 proteolytic degradation by promoting Cav-1 oligomer destabilization and ubiquitination. As observed in IPAH lung sec-

tions and PAECs, Cav-1 expression is reduced and, in parallel, eNOS is hyperactivated. Further study of the mechanisms regulating Cav-1 expression level and the consequences of Cav-1 degradation in endothelial cells due to chronic vascular inflammation on altered endothelial cell function and vascular arteriopathy/remodeling, as noted in patients with IPAH, type 2 diabetes, COPD, and so on, is warranted.

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