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RESEARCH ARTICLE

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Endothelial IK and SK channel activation decreases pulmonary arterial pressure and vascular remodeling in pulmonary hypertension

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Abstract

Endothelial cells (ECs) from small pulmonary arteries (PAs) release nitric oxide (NO) and prostacyclin, which lower pulmonary arterial pressure (PAP). In pulmonary hypertension (PH), the levels of endothelium-derived NO and prostacyclin are reduced, contributing to elevated PAP. Small-and intermediate-conductance Ca^{2+}-activated K^+ channels (IK and SK)—additional crucial endothelial mediators of vasodilation—are also present in small PAs, but their function has not been investigated in PH. We hypothesized that endothelial IK and SK channels can be targeted to lower PAP in PH. Whole-cell patch-clamp experiments showed functional IK and SK channels in ECs, but not smooth muscle cells, from small PAs. Using a SU5416 plus chronic hypoxia (Su + CH) mouse model of PH, we found that currents through EC IK and SK channels were unchanged compared with those from normal mice. Moreover, IK/SK channel-mediated dilation of small PAs was preserved in Su + CH mice. Consistent with previous reports, endothelial NO levels and NO-mediated dilation were reduced in small PAs from Su + CH mice. Notably, acute treatment with IK/SK channel activators decreased PAP in Su + CH mice but not in normal mice. Further, chronic activation of IK/SK channels decreased PA remodeling and right ventricular hypertrophy, which are pathological hallmarks of PH, in Su + CH mice. Collectively, our data provide the first evidence that, unlike endothelial NO release, IK/SK channel activity is not altered in PH. Our results also demonstrate proof of principle that IK/SK channel activation can be used as a strategy for lowering PAP in PH.

KEYWORDS
calcium signaling, endothelium, potassium channels, pulmonary artery, pulmonary hypertension

INTRODUCTION

Pulmonary hypertension (PH) is a progressive and debilitating disease characterized by elevated pulmonary arterial pressure (PAP) and pulmonary vascular resistance. Findings from the Registry to Evaluate Early and Long-term Pulmonary Arterial Hypertension Disease (REVEAL) report that, on average, patients with PAH have a survival rate of 85% in the first year and 49% within 7 years.[1] A lack of proper diagnosis and treatment of PH results in progressive right heart failure with a median survival rate of 2.8 years.[2,3] Loss of endothelial function in small, resistance-sized pulmonary arteries (PAs) is a crucial contributor to the pathogenesis of PH.[4] Under healthy conditions, endothelial cells (ECs) release vasodilatory mediators, including nitric oxide (NO) and prostacyclin, which relax the surrounding smooth muscle cells (SMCs) and maintain a low PAP. The levels of endothelium-derived vasodilatory mediators are drastically reduced in PH.[5–8] Current treatment options circumvent endothelial dysfunction in PH by directly increasing the levels of NO or prostacyclin in the extracellular milieu. However, these treatment options have limited efficacy and are associated with off-target effects on other cell types. An alternative strategy involving activation of an endothelial mechanism to lower PAP might be associated with fewer side effects. Therefore, identifying an endothelial mechanism that is not impaired in PH and could be easily activated could prove vital for designing a treatment strategy that lowers PAP in an endothelium-dependent manner.

In this regard, endothelial intermediate (IK)- and small (SK)-conductance, Ca^{2+}-activated K^+ channels remain largely unexplored as therapeutic targets in PH. Studies in systemic arteries indicate a crucial role for IK and SK channels in endothelium-dependent vasodilation[9] and blood pressure regulation.[10] Increases in endothelial Ca^{2+} activate IK and SK channels, hyperpolarizing EC and nearby SMC membranes and causing vasodilation. Moreover, the activity of endothelial IK/SK channels is reduced in cardiovascular disorders, including hypertension, obesity, and diabetes.[11–16] We previously reported that functional IK and SK channels are present in ECs from small PAs and that IK/SK channel activation dilates PAs.[10] However, direct recordings of endothelial IK/SK channel activity in PH are not available in the literature. Assessing the activity of endothelial IK/SK channels in PH is a crucial first step in determining whether these channels can be targeted to lower PAP in PH.

NO is the predominant vasodilator molecule in small PAs.[17,18] Under normal conditions, an increase in endothelial Ca^{2+} activates endothelial NO synthase (eNOS). Endothelium-derived NO then diffuses to SMCs, increasing cyclic guanosine monophosphate (cGMP) levels and cGMP-dependent kinase (PKG) activity to cause SMC relaxation.[19] Endothelial Ca^{2+} elevation, eNOS activity, and NO-cGMP signaling are impaired in PAs from mouse models of PH and PH patients.[6,18] Moreover, the levels of prostacyclin, another endothelium-derived vasodilator, are also reduced in the lungs of PH patients.[7] Although IK/SK channels are crucial for endothelium-dependent dilation of systemic arteries, whether they can be targeted to lower PAP in PH is not known.

Here, we tested the hypothesis that endothelial IK/SK channel activation lowers PAP in PH utilizing a SU5416 plus chronic hypoxia (Su + CH) mouse model of PH. Su + CH mice showed elevated PAP and pulmonary arterial lesions. Acute treatment with the IK/SK channel agonist SKA-31 lowered PAP and right ventricular (RV) systolic pressure (RVSP) in Su + CH mice but not in control mice. Moreover, chronic treatment with SKA-31 reduced pulmonary artery lesions and RV hypertrophy in Su + CH mice. Notably, the activity of IK and SK channels was not impaired in PH, and IK/SK channel-induced vasodilation of small PAs was also unaffected. Collectively, our data demonstrate that the endothelial IK/SK channel pathway for vasodilation is not altered in PH and provide proof-of-principle that IK/SK channel activation could be a therapeutic strategy for lowering PAP and PA remodeling in PH.

METHODS

Drugs

NS309, TRAM-34 and apamin were purchased from Tocris Bioscience. U46619 was purchased from Cayman Chemicals (Ann Arbor). SU5416, SKA-31, and sodium nitroprusside (SNP) were purchased from Sigma Aldrich.

Animal protocols

All animal studies were approved by the University of Virginia Animal Care and Use Committee. Male C57BL6/J mice (10–14 weeks old; The Jackson Laboratory) were used for this study. Mice were housed in an enriched environment and maintained under a 12:12 h light/dark photocycle at ~23°C with fresh tap water and a standard chow diet available *ad libitum*. Mice were killed with pentobarbital (90 mg kg^{-1}; i.p.; UVA Hospital Pharmacy) followed by cranial dislocation for lung tissue harvesting.

Mouse model of PH

PH was induced by exposing C57BL6/J mice to chronic hypoxia (CH; 10% O_2: 4 weeks) with concurrent treatment with the receptor tyrosine kinase inhibitor, SU5416 (20 mg kg^{-1}), administered subcutaneously (s.c.) once a week.[20] SU5416 was dissolved in DMSO/PEG 400 (50/50). Mice were exposed to CH conditions in a vinyl hypoxic chamber (Coy Laboratory Products; Inc.) connected to an auto purge airlock inlet. Oxygen concentration in the glove box was regulated by an oxygen controller and oxygen sensor (Coy Laboratory Products, Inc.). Control mice were maintained in room air for 4 weeks.

For PA remodeling studies, mice in the experimental group were treated with the IK/SK channel activator, SKA-31 (30 mg kg^{-1}; i.p.), twice a day for the final 7 days of Su + CH exposure. Control mice were exposed to Su + CH for 4 weeks but were injected with vehicle instead of SKA-31. At the end of week 4 of Su + CH exposure, mice were euthanized with pentobarbital (90 mg kg^{-1}; i.p.; UVA Hospital Pharmacy) and left lungs were perfused with the NO donor and fast-acting vasodilator, SNP (50 μmol/L; Sigma Aldrich), followed by incubation overnight at room temperature with 4% paraformaldehyde (PFA) for histology and immunostaining.

Measurement of RVSP and fulton index

Mice were anesthetized with pentobarbital (50 mg kg^{-1}; i.p.), and bupivacaine HCl (100 μl of 0.25% solution; s.c.) was used to numb the dissection site on the mouse. RVSP was measured as an indirect indicator of PAP.[18,21] Mice were cannulated with a Mikro-Tip pressure catheter (SPR-671; Millar Instruments), connected to a bridge amp (FE221) and a PowerLab 4/35 4-channel recorder (ADInstruments), through the external jugular vein into the right ventricle. RV pressure was acquired and analyzed using LabChart8 software (ADInstruments). In the subgroup of mice treated with SKA-31 (30 mg kg^{-1}; i.p.), RVSP was measured before SKA-31 injection and 20 min after. A stable 3-min recording was acquired for all animals, and a 1-min continuous segment was used for data analysis. Where necessary, traces were digitally filtered using a low-pass filter at a cut-off frequency of 50 Hz. At the end of experiments, mice were euthanized, and their hearts were isolated for RV hypertrophy analysis. RV hypertrophy was determined by calculating the Fulton index, a ratio of the RV heart weight over the left ventricular (LV) plus septum (S) weight (RV/LV + S).

Measurement of PAP

PAP was evaluated using an IPL-1 ex vivo murine lung perfusion system (Harvard Apparatus) as previously described.[22,23] Briefly, mice were anesthetized with isoflurane, after which a tracheostomy was performed and animals were ventilated with room air at 150 strokes/min and a stroke volume of 200 ml. Animals were exsanguinated by transecting the inferior vena cava. The main pulmonary artery was cannulated through the right ventricle, and the left ventricle was tube-vented through a small incision at the apex of the heart. The lungs were then perfused at a constant flow rate of 0.5 ml min^{-1} with Krebs-Henseleit buffer (11 mmol/L glucose; 1.2 mmol/L $MgSO_4$; 1.2 mmol/L KH_2PO_4; 4.7 mmol/L KCl; 118 mmol/L NaCl; 1.25 mmol/L $CaCl_2$, and 25 mmol/L $NaCO_3$). The perfusate buffer and isolated lungs were maintained at 37°C using a circulating water bath. Once properly perfused and ventilated, the isolated lungs were maintained on the system for a 10-min equilibration period, after which hemodynamic and pulmonary parameters were recorded using the PULMODYN data acquisition system (Hugo Sachs Elektronik).

Pressure myography

Mouse fourth-order PAs (~50 μm) were cannulated on glass micropipettes in a pressure myography chamber (The Instrumentation and Model Facility; University of Vermont) and pressurized to a physiological pressure of 15 mmHg.[24] Briefly, freshly dissected lungs were placed in ice cold HEPES buffer in a dissection plate. The left lung was stretched and pinned down, with ventral side facing up. First, pulmonary veins, and then the airway were cut open to gain access to the PAs underneath. Once PAs were visible, they were gently separated from surrounding tissue.[25] Arteries were superfused with bicarbonate-physiological salt solution (PSS; 119 mmol/L NaCl; 4.7 mmol/L KCl; 1.2 mmol/L KH_2PO_4; 1.2 mmol/L $MgCl_2$ hexahydrate; 2.5 mmol/L $CaCl_2$ dihydrate; 7 mmol/L dextrose; and 24 mmol/L $NaHCO_3$) at 37°C and bubbled with 20% O_2/5% CO_2 to maintain the pH at 7.4. Approximately 20% PAs from normal mice develop myogenic tone, whereas ~65% PA from Su + CH mice show myogenic tone at 15 mmHg. Due to the relatively small percentage of PAs from normal mice showing myogenic tone, we preconstricted all PAs with 30 nmol/L U46619 (a thromboxane A2 receptor agonist). More than 95% arteries showed a constriction in response to U46619 in both normal and PH groups. U46619 was added to the chamber immediately, before the arteries could develop myogenic tone. Before

measuring vascular reactivity, arteries were treated with NS309 (1 μmol/L), a direct activator of endothelial IK/SK channels, to assess endothelial health. Arteries that failed to dilate completely after treatment with NS309 were discarded. Changes in arterial diameter were recorded at a 60-ms frame rate using a charge-coupled device camera and edge-detection software (IonOptix LLC).[11,26] At the end of each experiment, Ca^{2+}-free PSS (119 mmol/L NaCl; 4.7 mmol/L KCl; 1.2 mmol/L KH_2PO_4; 1.2 mmol/L $MgCl_2$ hexahydrate; 7 mmol/L dextrose; 24 mmol/L $NaHCO_3$; and 5 mmol/L EGTA) was applied to assess the maximum passive diameter. Percent constriction was calculated by:

$$[(\text{Diameter}_{\text{before}} - \text{Diameter}_{\text{after}})/\text{Diameter}_{\text{before}}] \times 100 \quad (1)$$

where $\text{Diameter}_{\text{before}}$ is the diameter of the artery before treatment and $\text{Diameter}_{\text{after}}$ is the diameter after treatment. Percent dilation was calculated by:

$$[(\text{Diameter}_{\text{dilated}} - \text{Diameter}_{\text{basal}}) / (\text{Diameter}_{\text{Ca-free}} - \text{Diameter}_{\text{basal}})] \times 100 \quad (2)$$

where $\text{Diameter}_{\text{basal}}$ is the stable diameter before drug treatment, $\text{Diameter}_{\text{dilated}}$ is the diameter after drug treatment, and $\text{Diameter}_{\text{Ca-free}}$ is the maximum passive diameter.

Immunostaining

Isolated left lungs were perfused with phosphate-buffered saline (PBS) containing 50 μmol/L SNP and fixed in 4% PFA overnight at room temperature before paraffin embedding and sectioning. After sectioning paraffinized lungs, sections (5-μm thick) were deparaffinized and incubated in endogenous peroxidase (30%) for 30 min at room temperature, followed by a 5-min wash with double-distilled H_2O. For antigen retrieval, sections were microwaved in a citrate-based antigen unmasking solution (Vector Laboratories) for 20 min with brief pauses to avoid boiling. After allowing slides to cool in the antigen unmasking solution for 1 h at room temperature, sections were permeabilized by incubating with 0.2% Triton-X for 30 min on a shaking rocker at room temperature, then blocked by incubating with 0.2% Triton-X, 5% normal donkey serum (ab7475; Abcam) and fish skin gelatin (FSG; Sigma Aldrich) for 1 h. Thereafter, sections were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-α-actin primary antibody (1:500; F3777; Sigma Aldrich) and washed first in PBS containing FSG for 5 min and then in PBS alone for 5 min (three times). After washing in PBS, nuclei were stained by incubating with 0.3 μmol/L 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) for 10 min at room temperature. Images were acquired along the z-axis (optical slice thickness; 0.1 μm) using an Andor Dragonfly 505 confocal spinning-disk system (Oxford Instruments) and a Leica DMi8 microscope with a X40 objective (NA 1.1; Leica Microsystems). Images were extracted with IMARIS version 9.3 and analyzed with Image J. Data were normalized by dividing the collagen fiber area by the arterial diameter.

Isolated ECs and SMCs were fixed by incubation with 4% PFA at room temperature for 15 min and then permeabilized by treatment with PBS containing 0.2% Triton-X for 1 h. The cells were then treated with 5% normal donkey serum for 1 h. ECs were subsequently incubated with monoclonal CD31 antibody (1:100; #RM5201; Invitrogen[18]) for 1 h at room temperature. SMCs were incubated with FITC-conjugated anti-α-actin antibody (1:500; F3777; Sigma Aldrich[18,27] for 1 h at room temperature. After washing the cells three times with PBS, nuclei were stained by incubating with 0.3 μmol/L DAPI (Invitrogen) for 10 min at room temperature in the dark.

Patch-clamp analysis of freshly isolated ECs and SMCs from small PAs

ECs were freshly isolated from PAs following a previously established protocol.[21,24] Briefly, PAs were incubated in dissociation solution (55 mmol/L NaCl; 80 mmol/L Na glutamate; 6 mmol/L KCl; 2 mmol/L $MgCl_2$; 0.1 mmol/L $CaCl_2$; 10 mmol/L glucose; 10 mmol/L HEPES; pH 7.3) containing Worthington neutral protease (0.5 mg/ml; Worthington Biochemical Corporation) for 30 min at 37°C. With our protocol for EC isolation, more than 95% of cells are CD31-positive.

For SMC isolation,[27] arterial segments were transferred to a 12 × 75 mm borosilicate glass culture tube containing 1 ml dissociation solution (145 mmol/L NaCl; 4 mmol/L KCl; 1 mmol/L $MgCl_2$; 10 mmol/L HEPES; 0.05 mmol/L $CaCl_2$,10 mmol/L glucose; pH 7.3) and 0.5 mg/ml bovine serum albumin (BSA) and incubated for 10 min at room temperature (~24°C). The solution was then replaced with 1 ml dissociation solution containing 1 mg/ml papain (Sigma Aldrich) and 0.5 mg/ml dithiothreitol (Sigma Aldrich), and incubation was continued at 37°C for 20 min. Thereafter, 0.5 ml of the papain solution was carefully removed without displacing the arterial segments and replaced with 0.5 ml dissociation solution containing 2 mg/ml collagenase type IV (Worthington

Biochemical Corporation), 0.5 mg/ml elastase (Sigma Aldrich) and 1 mg/ml soybean trypsin inhibitor (Sigma Aldrich). After incubating at 37°C for 5 min, the enzyme solution was removed and replaced with cold dissociation solution containing BSA. The tube containing digested arteries was placed on ice, and the solution was gently triturated every 15 min for 1 h to yield a single-cell suspension. SMCs were identified by their elongated shape and α-actin immunostaining.

Whole-cell currents were measured at room temperature using conventional whole-cell patch-clamp electrophysiology. The bath solution consisted of 10 mmol/L HEPES, 134 mmol/L NaCl, 6 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$ hexahydrate, and 7 mmol/L dextrose (adjusted to pH 7.4 with NaOH). The intracellular solution consisted of 10 mmol/L HEPES, 30 mmol/L KCl, 10 mmol/L NaCl, 110 mmol/L K-aspartate and 1 mmol/L $MgCl_2$ (adjusted to pH 7.2 with NaOH). The pipette solution for conventional patch clamp consisted of 10 mmol/L HEPES, 123.2 mmol/L KCl, 10 mmol/L NaCl, 5.5 mmol/L $MgCl_2$, 0.2 mmol/L $CaCl_2$ and 5 mmol/L HEDTA (adjusted to pH 7.2 with 16.8 mm KOH) and contained 3 μm free-Ca^{2+} and 1 mmol/L free-Mg^{2+}, as calculated using the Max-Chelator program (Chris Patton; Stanford University). The voltage-clamp protocol involved 250-ms voltage ramps from −100 to +50 mV from a holding potential of −50 mV. Patch electrodes were pulled from borosilicate glass (O.D.: 1.5 mm; I.D.: 1.17 mm; Sutter Instruments) using a Narishige PC-100 puller (Narishige International USA; Inc.) and polished using a MicroForge MF-830 polisher (Narishige International USA). The pipette resistance was 3–5 mΩ. IK and SK channel currents were elicited by adding 1 μmol/L NS309 (IK/SK channel activator) to the bath solution. IK/SK channel currents were inhibited by adding 1 μmol/L TRAM-34 (IK channel inhibitor) and 300 nmol/L Apamin (Apa; SK channel inhibitor) to the bath solution. The effect of each drug was studied 5 min after its addition. Data were acquired using a Multiclamp 700 B amplifier connected to a Digidata 1550 B system and analyzed using Clampfit 11.1 software (Molecular Devices).

Histology

Isolated left lungs were perfused with PBS containing 50 μmol/L SNP and fixed by incubating overnight at room temperature in 4% PFA. Tissue samples were then paraffin embedded, sectioning to a thickness of 5 μm, and stained with Masson's Trichrome (Polysciences, Inc.). Collagen fiber thickness was calculated based on the area of collagen fiber staining (blue) using NIH Image J software. Data were normalized by dividing collagen fiber area by arterial diameter. Histology images were captured using a Leica DMIL LED microscope with a Leica DMC6200 camera and LAS X Software (Leica Microsystems Inc.).

NO imaging with DAF-FM

NO was imaged by fluorescence microscopy using DAF-FM (4-amino-5 methylamino-2′,7′-difluorofluorescein diacetate), which forms a fluorescent triazole compound after binding NO. DAF-FM was dissolved in HEPES-PSS containing 0.02% pluronic acid[17] to obtain a solution with a final concentration of 5 μmol/L. Fourth-order PAs were pinned down *en face* on a Sylgard block and loaded with 5 μmol/L DAF-FM for 20 min at 30°C in the dark. DAF-FM was excited at 488 nm, and emission was collected with a 525/36-nm band-pass filter. Images were captured across the z-axis (slice thickness; 0.1 μm) from the surface of the endothelium to the bottom where the EC layer encounters the SMC layer. Baseline NO release was studied in PAs from control (normoxic) and Su + CH mice. Custom-designed software written by Dr. Adrian Bonev (SparkAn) was used to analyze DAF-FM images[26] (https://github.com/vesselman/SparkAn). Arbitrary fluorescence intensity per cell was measured by manually drawing an outline around each EC, establishing the entire cell as a region of interest. Background fluorescence (intensity without laser) was subtracted from the arbitrary fluorescence value obtained for each cell, and the fluorescence values of each cell were averaged to obtain a single fluorescence number for the specific field.

Statistics

Results are presented as means ± standard error of the mean. Data were obtained from at least three mice in experiments performed on at least two independent groups. All data are presented graphically using CorelDraw x9 (Corel Corp.) and were analyzed statistically using OriginPro (version 7.5; OriginLab Corp.), Prism (version 8; GraphPad Software Inc.) and MATLAB R2019b (MathWorks). The normality of data was determined by performing a Shapiro–Wilk test. Data were analyzed using two-tailed, paired or independent *t*-tests for comparison of data collected from two different treatments, or one-way or two-way analysis of variance for analysis of statistical differences among more than two different treatments.

Statistical significance was determined as a p value less than 0.05; individual p-values (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) are indicated in figure legends.

RESULTS

Endothelial IK and SK channel activity in small PAs is not altered in a mouse model of PH

eNOS activation and prostacyclin release are impaired in PH.[5–8,18,28–30] However, direct studies of endothelial IK/SK channel activity in PH have not been performed. We tested the possibility that ionic currents through IK and SK channels are impaired in PH. To this end, we used mice exposed to chronic hypoxia (CH; 4 weeks; 10% O_2) together with the vascular endothelial growth factor receptor antagonist SU5416 (20 mg kg^{-1}; s.c.; once a week), which is known to cause a more profound PH phenotype than CH alone.[18,20] Mice exposed to SU5416 plus CH (Su + CH) showed elevated RVSP, an indirect in vivo indicator of PAP, compared with normoxic (N) mice (Figure 1a). The Fulton index, a ratio of RV weight to left ventricle and septal (LV + S) weight, was also higher in mice exposed to Su + CH, confirming the development of right ventricle hypertrophy in these mice (Figure 1b). Previous studies have established that endothelial Ca^{2+} influx, eNOS activity, and eNOS-mediated vasodilation are impaired in this model of PH.[18]

Whole-cell patch-clamp experiments were performed in freshly isolated ECs from small, 4th order PAs (~50 μm; Figures 1c,d). IK/SK channel currents induced by the IK/SK channel agonist NS309 (1 μmol/L) were recorded following application of a 250-ms voltage ramp from −100 to +50 mV. Outward currents at 0 mV were compared following sequential addition of 1 μmol/L NS309, 1 μmol/L TRAM-34 (IK channel inhibitor), and 300 nmol/L apamin (Apa; SK channel inhibitor; Figure 1e). Outward currents in the presence of NS309, NS309 + TRAM-34, or NS309 + TRAM-34 + Apa were not different between ECs from N and Su + CH groups (Figure 1f), supporting the idea that endothelial IK and SK channel activity is not altered in this model of PH. Additionally, IK and SK channel currents in the presence of 3 μmol/L free cytosolic Ca^{2+} were not different between ECs from N and Su + CH groups, indicating that the Ca^{2+}-sensitivity of IK and SK channels is not altered in this model of PH (Figures 1g,h).

Endothelial IK/SK channel-mediated dilation is unaltered in PAs from PH mice

The unaltered activity of endothelial IK and SK channels in freshly isolated ECs from small PAs of Su + CH mice suggested that IK/SK channel-mediated dilation would also be unaltered in PH. To confirm this, we recorded endothelium-dependent dilation of small PAs in response to the IK/SK channel agonist NS309 (0.3–1 μmol/L) using pressure myography [17,18] and found that NS309-induced dilation of small PAs was not different between N and Su + CH mice (Figures 2a and 2b). Similarly, in PAs that developed myogenic tone, NS309 (1 μmol/L)-induced dilation was not different between N and Su + CH groups (Figure 2c). Given that endothelial IK/SK channel activation is known to cause dilation via electrical communication from ECs to SMC through myoendothelial gap junctions,[31,32] the absence of a change in NS309-induced vasodilation also provides evidence supporting unaltered electrical communication between ECs and SMC in PH.

We then compared the dilator effect of basal (absence of agonist) IK/SK channel and eNOS activity in small PAs from N and Su + CH mice. Exposure to TRAM-34 (1 μmol/L) and Apa (300 nmol/L) constricted small PAs by ~10%, a constriction that was not different between N and Su + CH mice (Figure 2d), indicating that the dilatory effect of basal IK/SK channel activity is not altered in PH. NOS inhibition with L-NNA (100 μmol/L) constricted PAs from N mice by ~20%, but was unable constrict PAs from Su + CH mice, demonstrating the dilatory effect of basal eNOS activity in PAs from N mice and its absence in PAs from Su + CH mice (Figure 2e). Endothelial NO levels in PAs, measured using the fluorescent NO indicator 4-amino-5 methylamino-2′,7′ difluorofluorescenin diacetate (DAF-FM; 5 μmol/L), were lower in Su + CH mice compared with N mice (Figure 2f). Endothelial NO levels in response to purinergic receptor and transient receptor potential vanilloid 4 (TRPV4) channel agonists, which increase NO levels in ECs, were also lower in PAs from SU + CH mice.[18] Together, these data establish that eNOS-mediated dilation is impaired in PH, but endothelial IK/SK channel-mediated dilation remains intact.

Acute treatment with IK/SK channel activator decreases PAP in mice exposed to su + CH

Unaltered endothelial IK/SK channel-mediated dilation of small PAs in PH led us to hypothesize that IK/SK

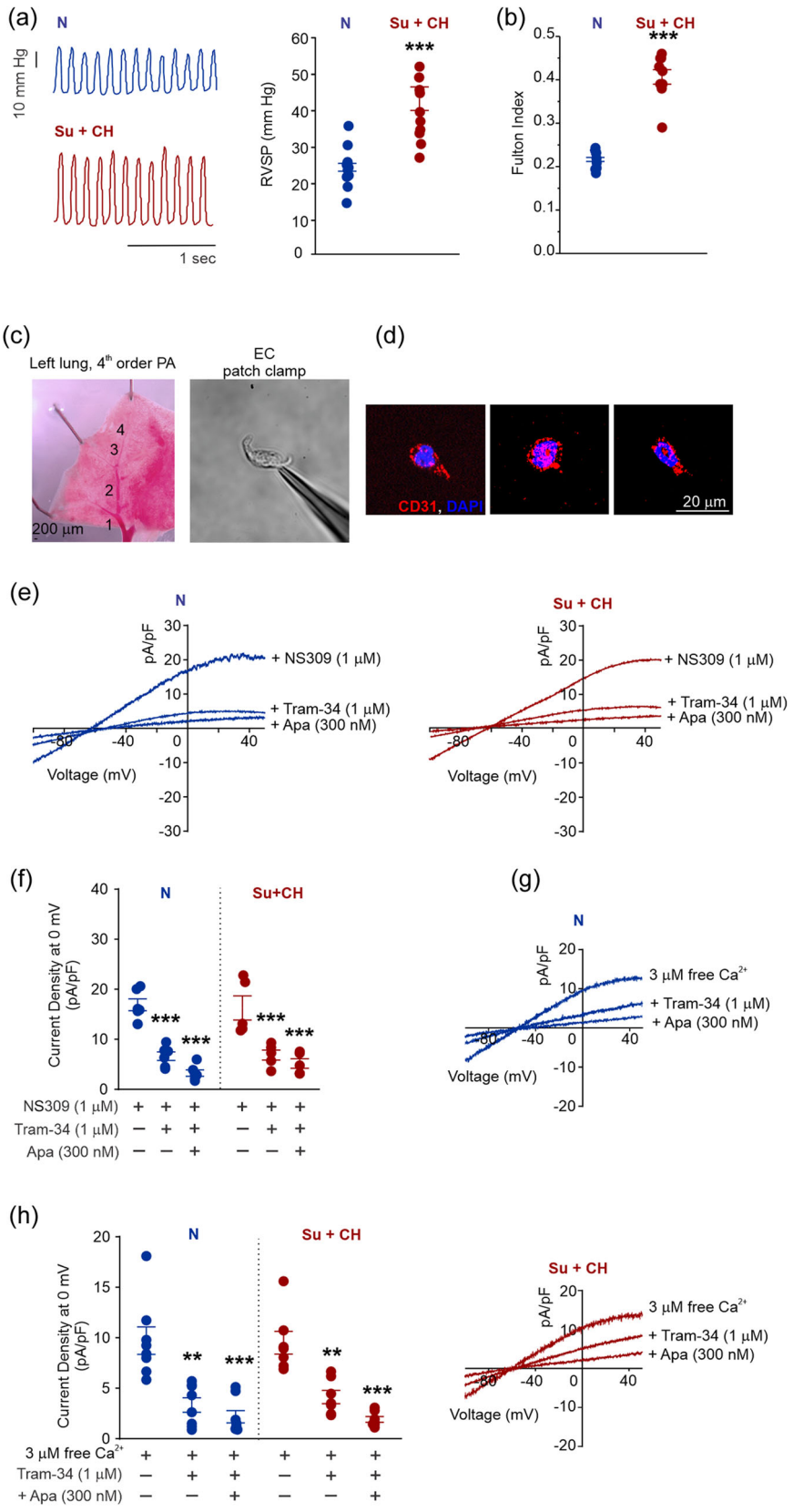

FIGURE 1 Endothelial IK/SK channel activity is not impaired in PAs from a mouse model of PH. (a) Representative right ventricular systolic pressure (RVSP; mm Hg) traces (left) and averaged RVSP values (right) in normoxic (N) mice and mice exposed to chronic hypoxia (CH; 4 weeks; 10% O_2) and SU5416 (Su + CH; $n = 12$; ***$p < 0.001$; *t*-test). (b) Average Fulton Index values in N and Su + CH mice ($n = 12$; ***$p < 0.001$; *t*-test). (c) Images showing 4th-order PAs (left) and ECs isolated from PAs in a patch-clamp experiment (d) Left, Representative images showing immunostaining for the EC marker CD31 and nuclear staining with DAPI in freshly isolated ECs from small PAs. (e) Left, Representative traces showing IK/SK currents in freshly isolated ECs from N (left) and Su + CH (right) mice in the presence of IK/SK channel activator NS309 (1 µmol/L), followed by sequential addition of the IK channel inhibitor TRAM-34 (1 µmol/L) and SK channel inhibitor apamin (Apa; 300 nmol/L). Currents were recorded in the whole-cell configuration of the patch-clamp technique. (f) Current density (pA/pF) plot of IK/SK currents in freshly isolated ECs from PAs of N and Su + CH mice in the presence of NS309 (1 µmol/L), followed by sequential addition of TRAM-34 (1 µmol/L) and Apa (300 nmol/L; $n = 5$–7; ***$p < 0.001$ vs. N [+NS309]; ***$p < 0.001$ vs. Su + CH [+NS309]; two-way ANOVA). (g) Left, Representative traces showing IK/SK currents in freshly isolated ECs from N (top) and Su + CH (bottom) mice in the presence of 3 µmol/L free cytosolic Ca^{2+}, followed by sequential addition of the IK channel inhibitor TRAM-34 (1 µmol/L) and SK channel inhibitor apamin (Apa; 300 nmol/L). Currents were recorded in the whole-cell configuration of the patch-clamp technique. (h) Current density (pA/pF) plot of IK/SK currents in freshly isolated ECs from PAs of N and Su + CH mice in the 3 µmol/L free cytosolic Ca^{2+}, followed by sequential addition of TRAM-34 (1 µmol/L) and Apa (300 nmol/L; $n = 5$–7; **$p < 0.01$ vs. Basal; ***$p < 0.001$ vs. Basal; two-way ANOVA). ANOVA, analysis of variance.

channel agonists would decrease PAP in PH. For in vivo studies, SKA-31 was used to activate IK/SK channels instead of NS309 (used for activating IK/SK channels in *ex vivo* studies) because of its favorable metabolism.[33] SKA-31 administration (30 mg kg^{-1}; i.p.) significantly reduced RVSP in Su + CH mice but not in N mice (Figure 3a). PAP, measured in an isolated perfused lung preparation as described previously,[18] was decreased by activation of IK/SK channels with NS309 (1 μmol/L) in Su + CH mice, but not in N mice (Figure 3b). These results provide the first proof-of-principle evidence that activation of endothelial IK/SK channels can be used as a strategy to reduce PAP in PH. Moreover, these findings identify an endothelial mechanism that is not altered in PH and can be activated to lower PAP in PH.

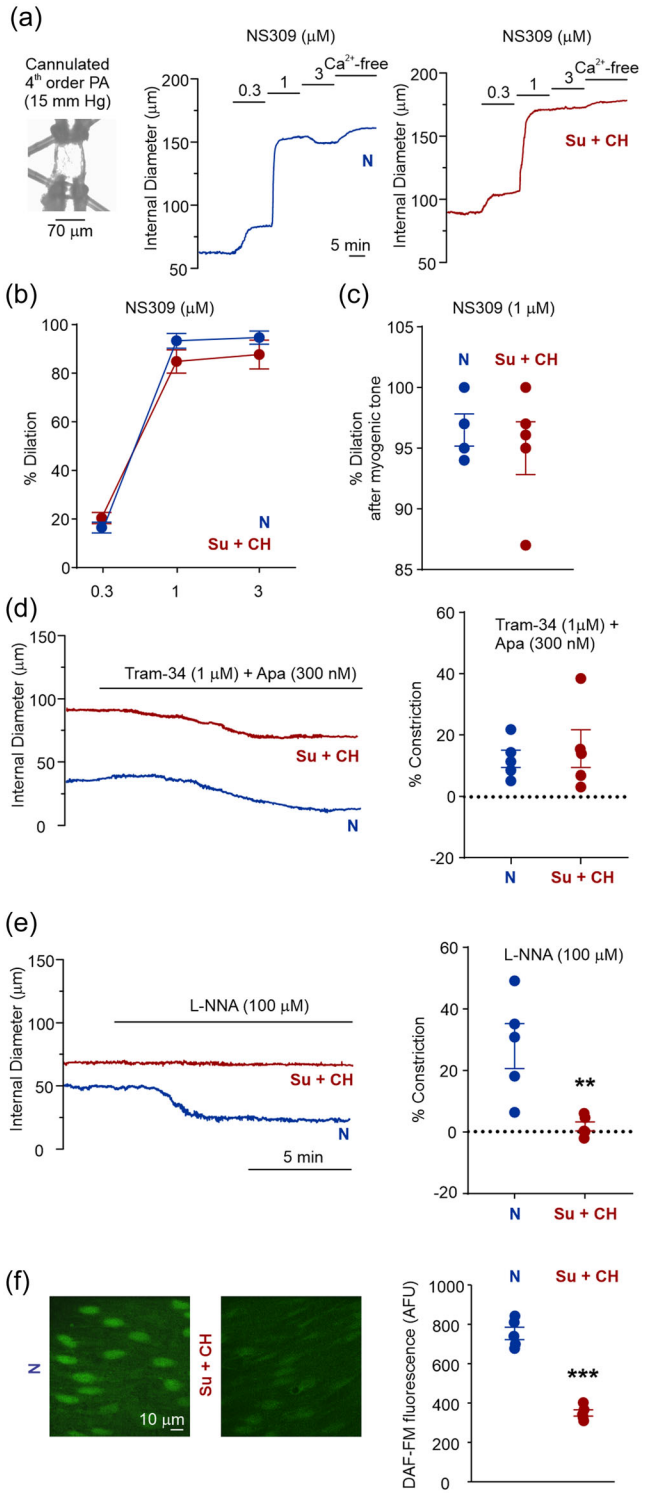

FIGURE 2 IK/SK channel-mediated dilation of small PAs is intact in a mouse model of PH. (a) Fourth-order PAs (left) were pressurized to 15 mmHg and preconstricted with the thromboxane A2 receptor agonist U46619 (30 nmol/L). Pressure myography traces (center and right) of PAs in response to NS309 (0.3–3 μmol/L). (b) Averaged percent dilation of PAs in response to NS309 (0.3–3 μmol/L; $n = 5$–6). (c) Percent dilation of PAs that developed myogenic tone to NS309 (1 μmol/L; $n = 4$). (d) Pressure myography traces (left) and averaged percent constriction (right) to TRAM-34 (1 μmol/L) + Apa (300 nmol/L; $n = 5$) in small PAs from N and Su + CH mice. (e) Pressure myography traces (left) and averaged percent constriction (right) to the NOS inhibitor L-NNA (100 μmol/L; $n = 5$; $^{***}p < 0.01$ vs. N; *t*-test). (f) DAF-FM fluorescence analysis of endothelial NO levels in *en face* preparations of PAs from N and Su + CH mice ($n = 5$; $^{***}p < 0.001$ vs. N; *t*-test).

SMCs from PAs do not express functional IK/SK channels under normal conditions or in PH

To rule out a possible role for SMC IK/SK channels in SKA-31/NS309-induced lowering of PAP, we tested whether SMCs from small PAs exhibit IK/SK channel currents. NS309-induced ionic currents were measured in freshly isolated SMCs (Figure 4a) from PAs in whole-cell patch-clamp experiments (Figures 4b,c). TRAM-34 (1 μmol/L) and Apa (300 nmol/L) did not reduce NS309 (1 μmol/L)-induced outward currents in SMCs from PAs of control or Su + CH mice. Moreover, outward currents at 0 mV in the presence of NS309 or NS309 + TRAM-34 + Apa were not different between SMCs from PAs of N and Su + CH mice. These data confirm the absence of functional IK/SK channels in SMCs from PAs under normal conditions and in PH, and support the idea that SMCs do not contribute to the dilatory or PAP-lowering effects of IK/SK channel activators in PH.

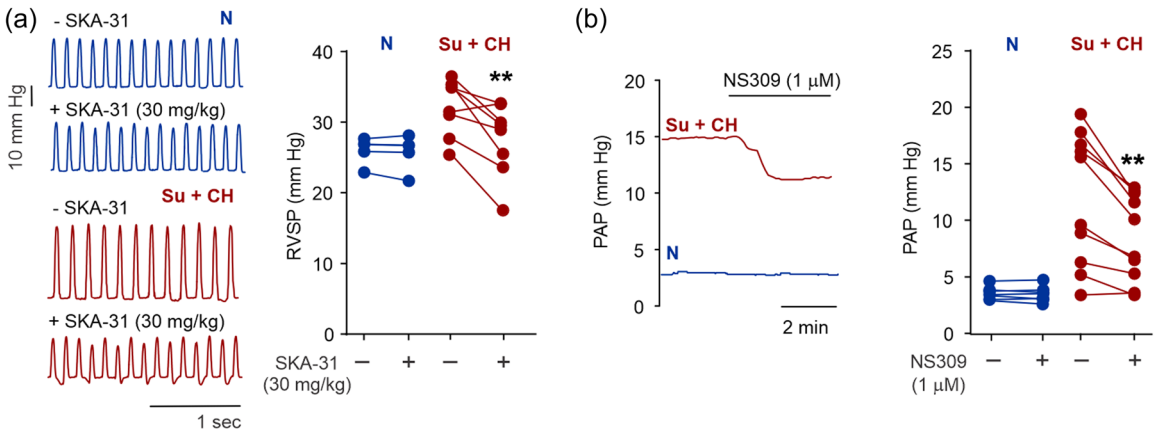

FIGURE 3 Acute administration of an IK/SK channel activator reduces PAP in PH. (a) Left, Representative RVSP (mm Hg) traces in N and Su + CH mice before and after acute treatment with the IK/SK channel activator SKA-31 (30 mg kg^{-1}; intraperitoneally; i.p.). Right, Average RVSP values in N and Su + CH mice before and after acute treatment with SKA-31 (30 mg kg^{-1}; i.p.; n = 8; $^{**}p < 0.01$ vs. no SKA-31 [Su + CH]; paired *t*-test). (b) Left, PAP (mm Hg) traces in isolated perfused lungs from N and Su + CH mice before and after acute treatment with NS309 (1 μmol/L). Right, Average PAP (mm Hg) in isolated perfused lungs from N and Su + CH mice before and after acute treatment with NS309 (1 μmol/L; $n = 10$; $^{**}p < 0.01$ vs. no SKA-31 [Su + CH]; paired t-test).

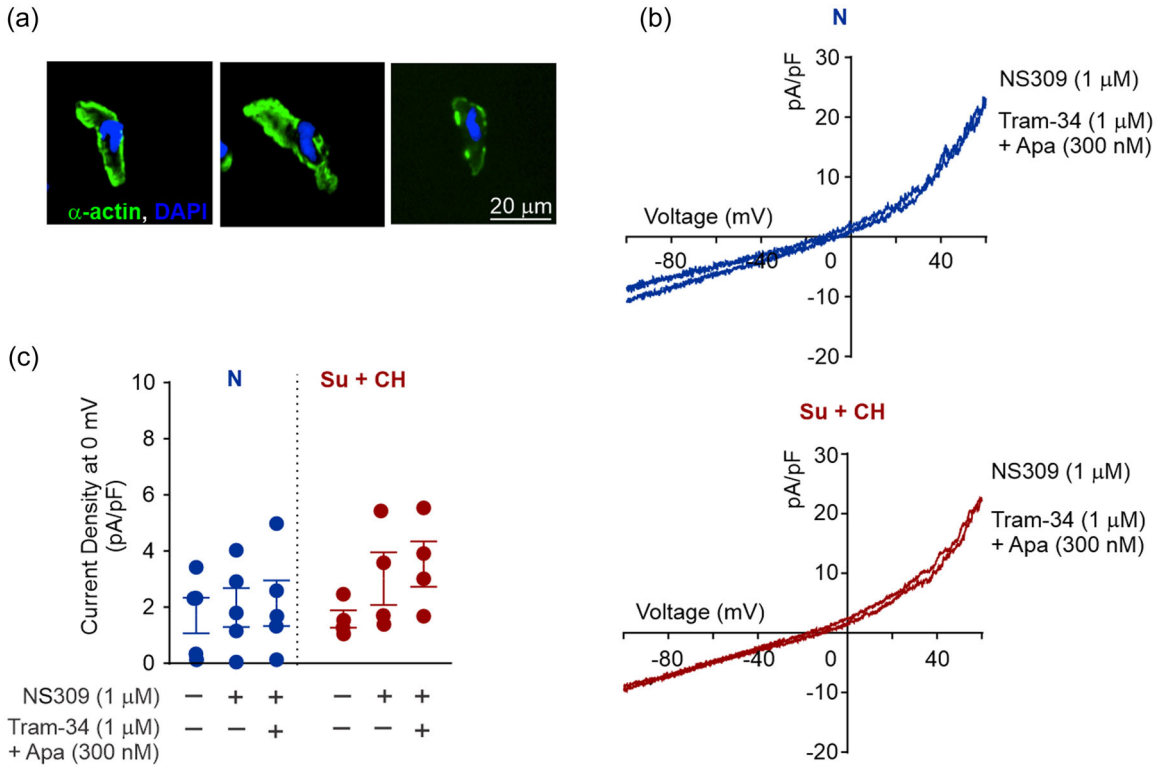

FIGURE 4 SMCs in small PAs from N and Su + CH mice do not express functional IK/SK channels. (a) Representative images showing immunostaining for the SMC marker α-actin and nuclear staining with DAPI in freshly isolated SMCs from small PAs. (b) Representative traces showing ionic currents in freshly isolated SMCs from N (top) and Su + CH (bottom) mice in the presence of NS309 (1 μmol/L), followed by addition of TRAM-34 (1 μmol/L) and Apa (300 nmol/L). Currents were recorded in the whole-cell configuration of the patch-clamp technique. (c) Current density (pA/pF) at 0 mV in freshly isolated SMCs from N and Su + CH mice at basal level and in the presence of NS309 (1 μmol/L), followed by TRAM-34 (1 μmol/L) and Apa (300 nmol/L; $n = 4$).

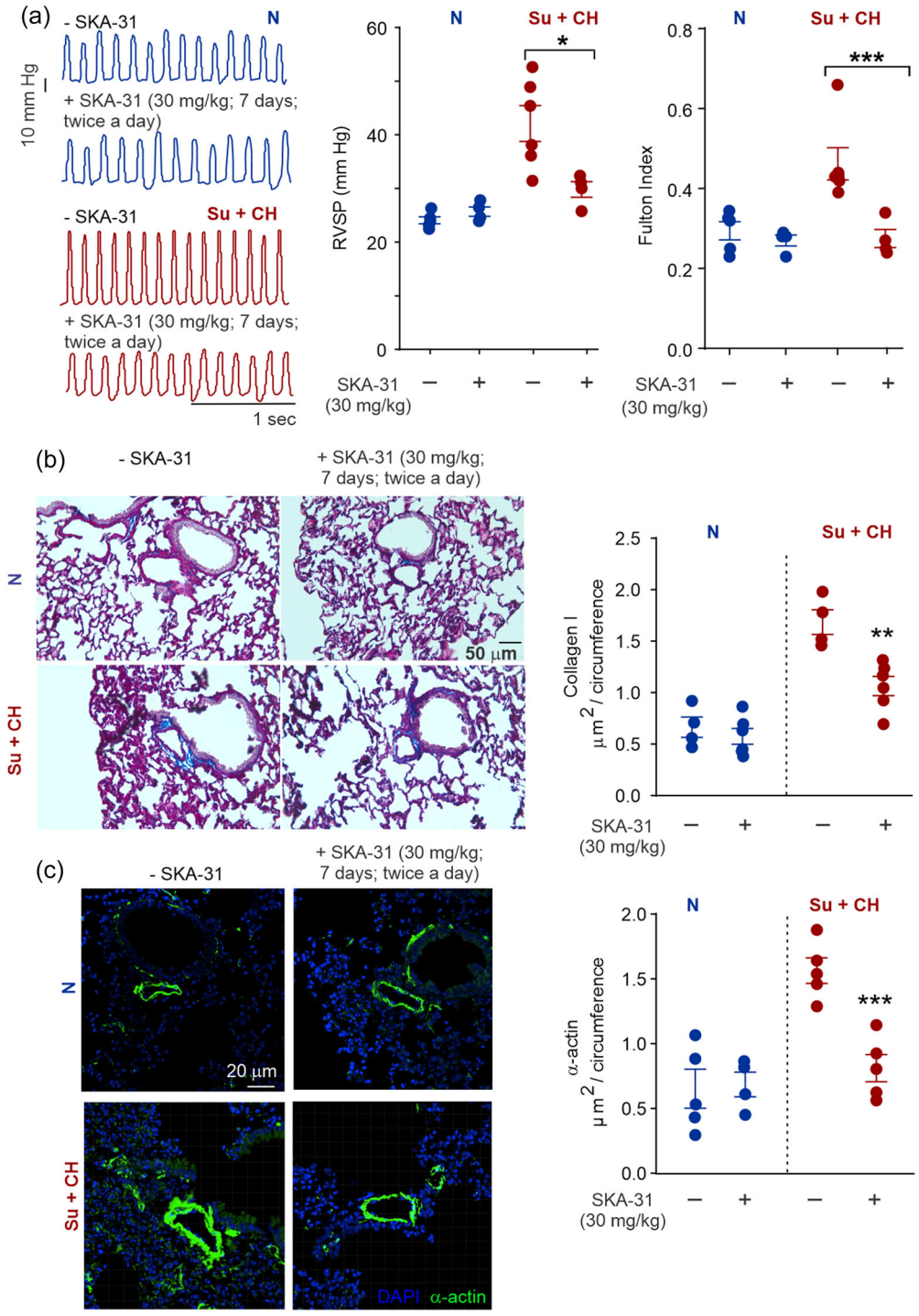

FIGURE 5 Chronic treatment with an IK/SK channel activator decreases RVSP and vascular remodeling in PH. (a) Representative RVSP traces (left; mm Hg) and averaged RVSP values (center) in N and Su + CH mice after chronic (7 days; twice a day) treatment with SKA-31 (30 mg kg^{-1}; i.p.; n = 4; *$p < 0.05$ vs. no SKA-31 [Su + CH]; t-test) or vehicle (-SKA-31). Right, Average Fulton Index values in N and Su + CH mice in the absence or presence of chronic treatment with SKA-31 (30 mg kg^{-1}; i.p.; $n = 4$; ***$p < 0.001$ vs. no SKA-31 [Su + CH]; *t*-test). (b) Left, Masson Trichrome staining for collagen I in 5-µm-thick lower lobe lung sections from N and Su + CH mice before and after treatment with SKA-31 (30 mg kg^{-1}; i.p.; 7 days; twice a day). Right, Averaged collagen I area in PAs from N and Su + CH mice before and after treatment with SKA-31. Data were normalized by dividing by arterial circumference ($n = 4–6$; **$p < 0.01$ vs. no SKA-31 [Su + CH]; two-way ANOVA). (c) Left, Representative images showing immunostaining for the SMC marker α-actin and nuclear staining with DAPI in PAs from N and Su + CH mice before and after treatment with SKA-31 (30 mg kg^{-1}; i.p.; 7 days; twice a day). Right, Averaged α-actin area in PAs from N and Su + CH mice before and after treatment with SKA-31. Data were normalized by dividing by arterial circumference (PAs; $n = 5$; ***$p < 0.001$ vs. no SKA-31 [Su + CH]; two-way ANOVA). ANOVA, analysis of variance.

Chronic IK/SK channel activation decreases PA remodeling in a mouse model of PH

Pulmonary arterial remodeling is a hallmark of PH. Based on our finding that acute treatment with IK/SK channel activators reduces PAP in a mouse model of PH, we hypothesized that chronic treatment with an IK/SK channel activator would reduce PA remodeling in PH. Su + CH mice were treated with SKA-31 (30 mg kg^{-1}; i.p.) twice a day for 7 days during the fourth week of exposure to Su + CH. Chronic SKA-31 treatment significantly lowered RVSP and the Fulton index in Su + CH mice (Figure 5a), demonstrating beneficial effects on PAP and RV hypertrophy. A morphological analysis using Masson's Trichrome staining (Figure 5b) for collagen I and labeling of muscle fibers by immunostaining for α-actin (Figure 5c) revealed enhanced remodeling of small PAs in Su + CH mice, a characteristic PH phenotype. Chronic treatment with SKA-31 reduced collagen content and α-actin immunostaining in Su + CH mice. These data provide the first evidence that chronic IK/SK channel activation has beneficial effects on PA remodeling in PH.

DISCUSSION

Under physiological conditions, endothelium-dependent dilation of PAs occurs through spatially localized release of diffusible mediators from ECs (NO or prostacyclin) that act on nearby SMCs.[18,34–36] The release of NO or prostacyclin from ECs is severely impaired in PH.[5–8,18,28–30] Therefore, currently used vasodilators lower PAP by directly elevating the levels of NO, prostacyclin, or their downstream mediators. However, elevated extracellular NO or prostacyclin can act on other cell types and have undesirable side effects. One potential strategy for limiting the side-effects of vasodilator therapy is to activate an endothelial vasodilator pathway that is not altered in PH. Our studies used direct measurements of IK/SK channel currents in ECs from small PAs to provide evidence that endothelial IK/SK channel activity is not altered in the Su + CH mouse model of PH. The preserved functionality of IK/SK channels is also reflected in unaltered IK/SK channel-dependent dilation of PAs in PH. Moreover, pharmacological activation of IK/SK channels reduced RVSP and PAP in this model, and a week-long treatment with an IK/SK channel activator reduced pulmonary arterial remodeling and RV hypertrophy—hallmarks of PH. Taken together, our findings indicate that pulmonary endothelial IK/SK channels represent potential therapeutic targets that, because they are unaltered in PH, can be activated to reduce PAP and decrease PA remodeling.

Endothelial IK/SK channel currents have not been studied in PH. We previously provided evidence for functional IK/SK channel currents in freshly isolated ECs from small PAs and showed that activation of endothelial IK/SK channels dilates small PAs.[24] Although the loss of endothelium-dependent vasodilation is well-established in PH, this effect has mostly been attributed to impaired eNOS and prostacyclin signaling. Here, we provide the first direct evidence that, unlike eNOS, endothelial IK/SK channel activity is not impaired in PH. Importantly, pharmacological activation of IK/SK channels reduces RVSP and PAP in Su + CH mice but not in normal mice, indicating that IK/SK channel activation does not reduce PAP to below physiological levels. Lack of an effect of SKA-31 on RVSP in normal mice could be explained by[1] "high-flow"-induced PA dilation, which helps maintain a low resistance in normal mice, and addition of a dilator is unable to cause further dilation; and/or[2] lower potency of SKA-31 compared to NS309 in activating IK/SK channels.[37,38] In direct contrast to its effects on resting PAP, IK/SK channel activation with SKA-31 lowered resting systemic blood pressure,[33] possibly pointing to differences in basal resistance between pulmonary (low-resistance and high flow) and systemic (high resistance and low flow) vasculatures. In a clinical setting, PH is often accompanied by increased systemic blood pressure. Although the effect of IK/SK channel activation on PAP in PH was not previously known, IK/SK channel agonists have been shown to lower systemic blood pressure in systemic hypertension.[37] Together with these previous findings, our data imply that IK/SK channel activation in PH may have additional beneficial effects on systemic blood pressure for PH patients with systemic hypertension.

Two different IK/SK channel activators were used for in vivo RVSP versus ex vivo PAP measurements. While NS309 is a highly potent activator (EC_{50} for IK and SK channels is ~20 nmol/L and ~600 nmol/L, respectively), it also has a short half-life in vivo.[38] SKA-31, although a less potent activator of IK/SK channels than NS309, has a longer half-life. Therefore, SKA-31 is preferred over NS309 for in vivo studies.[37] Previous studies in the literature have also used NS309 for ex vivo studies [9,24] and SKA-31 for in vivo studies.[39,40] Our PAP measurements with NS309 and RVSP measurements with SKA-31 confirmed that two different IK/SK channel activators have desirable effects on PAP and RVSP in the Su + CH mouse model of PH.

The endogenous mechanisms responsible for activation of endothelial IK/SK channels in PAs have not been investigated. eNOS and IK/SK channels appear to be the predominant mediators of endothelium-dependent dilation in systemic and PAs. Both eNOS and IK/SK channels can be activated by increases in intracellular Ca^{2+}. Multiple Ca^{2+} signals that activate IK/SK channels in systemic arteries have been identified.[24,26,41–47] In PAs, Ca^{2+} influx through endothelial TRPV4 channels[48] activates eNOS but not IK/SK channels.[24] This selective activation of eNOS by TRPV4 channels in PAs is attributable to the spatial proximity of TRPV4 channels with eNOS but not IK/SK channels. Although the activity of IK/SK channels is similar between systemic and PAs, the Ca^{2+} signals that activate endothelial IK/SK channels in PAs are unknown. Our new findings demonstrate that IK/SK channels have a dilatory effect on PAs under basal conditions, and that this dilatory effect is not altered in PH. Therefore, the discovery of endogenous mechanisms that activate endothelial IK/SK channels in PAs could unveil additional therapeutic targets for lowering PAP in PH through endothelium-dependent dilation.

Previous studies on the functional effects of endothelial IK/SK channels in PH have focused on large PAs. Simonsen and colleagues showed that exposure to chronic hypoxia decreases the dilatory response of large PAs to the IK/SK activator, NS4591, in rats.[49] In another study, the dilation of large PAs (~560 μm internal diameter) in response to the IK/SK channel agonist, NS309, was shown to increase by ~3-fold in mice exposed to chronic hypoxia.[50] The differences between our findings on endothelial IK/SK channel-dependent dilation of PAs in PH and previously published findings could be explained by the size of the PAs (small vs. large) used in the respective studies. Indeed, we recently showed that the mechanisms underlying endothelium-dependent dilation are different in large (>200 μm) and small (<100 μm) PAs.[10,18,21] It should be noted that the regulation of PAP occurs at the level of small PAs (~50–100 μm). Therefore, studies of IK/SK channel-dependent dilation in small PAs provide more relevant information on the effect of endothelial IK/SK channel activity on PAP. Additionally, our patch-clamp studies provide the first direct recordings of endothelial IK/SK channel activity in PH and demonstrate that PH does not affect endothelial IK/SK channel activity in small PAs.

Remodeling of small PAs is a crucial contributor to elevated PAP in PH. Our histological studies provide evidence that remodeling of PAs in Su + CH mice is reduced by chronic treatment with the IK/SK channel agonist, SKA-31. The precise mechanism responsible for the decrease in PA remodeling by IK/SK channel activation is not clear. Previously published data suggest that K^+ channels in SMCs can regulate SMC proliferation in the systemic vasculature.[51] However, our findings confirm that functional IK/SK channels are not present in SMCs from PAs. Therefore, we postulate that the effect of SKA-31 on SMC proliferation and PA remodeling is a consequence of the decrease in PAP following the administration of SKA-31. IK channels have been shown to inhibit proliferation of human airway SMCs,[52] T-lymphocytes,[53] and fibroblasts.[54] In contrast, other studies have suggested that IK channel overexpression can lead to the proliferation of systemic vascular SMCs.[55] Similarly, global SK channel knock-in mice show increased proliferation and angiogenesis,[41,56] and IK/SK channel inhibitors alleviate vascular remodeling in the systemic vasculature.[57,58] Thus, the effects of IK/SK channel activity on SMC proliferation and arterial remodeling appear to be divergent in systemic and pulmonary vasculatures. These findings are consistent with the well-established structural and functional differences between systemic and pulmonary circulations.

In conclusion, this study provides compelling evidence that IK/SK channel activity in small PAs is not altered in a mouse model of PH. Activation of IK/SK channels dilates PAs and reduces PAP in PH. IK/SK channel activation also has beneficial effects on PA and cardiac remodeling, which are hallmarks of PH. These findings identify an endogenous signaling mechanism that is not altered in PH and can be targeted to decrease PAP and vascular remodeling in PH, establishing that IK/SK channel activators with desirable pharmacokinetic and pharmacodynamic properties could be used therapeutically to lower PAP and reduce vascular remodeling in PH. Importantly, identifying endogenous activators of endothelial IK/SK channels in PAs could present additional therapeutic targets for the treatment of PH.

AUTHOR CONTRIBUTIONS

Swapnil K. Sonkusare conceptualized the study. Swapnil K. Sonkusare and Zdravka Daneva designed experiments. Zdravka Daneva performed right ventricle pressure measurements, pressure myography, histology and immunostaining experiments and analysis. Yen-Lin Chen performed patch clamp and immunostaining experiments and analysis. Swapnil K. Sonkusare and Abhishek Bazaz performed NO imaging and analysis. Vamsi Manchikalapudi assisted with histology. Huy Q. Ta and Victor E. Laubach performed ex vivo pulmonary arterial pressure measurements and analysis. Swapnil K. Sonkusare and Zdravka Daneva drafted the manuscript and revised it. All authors approved the final version of the manuscript submitted for publication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

All animal studies were approved by the University of Virginia Animal Care and Use Committee.

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