Research Article

Pulmonary Circulation

Differential effects of integrin-linked kinase inhibitor Cpd22 on severe pulmonary hypertension in male and female rats

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Abstract

Pulmonary arterial hypertension (PAH) is a progressive fatal disease with no cure. Inhibition of integrin-linked kinase (ILK) reverses experimental pulmonary hypertension (PH) in male mice, but its effect on severe experimental PH in either male or female animals is unknown. We examined effects of ILK inhibitor Cpd22 on rats with SU5416/hypoxia-induced PH; treatment was performed at six to eight weeks after PH initiation. Five weeks after PH initiation, male and female rats developed similar levels of PH. Eight weeks after PH induction, vehicle-treated male rats had more severe PH than females. Cpd22-treated males, but not females, showed complete suppression of phospho-Akt in small pulmonary arteries (PAs), significantly lower PA medial thickness and percentage of fully occluded arteries, decreased systolic right ventricle (RV) pressure, PA pressure, RV hypertrophy, RV end-diastolic pressure, and improved RV contractility index compared to vehicle-treated group. Cpd22 suppressed proliferation of human male and female PAH pulmonary artery vascular smooth muscle cell (PAVSMC). 17β-estradiol had no effect as a single agent but significantly attenuated Cpd22-dependent inhibition of proliferation in female, but not male, PAH PAVSMC. Taken together, these data demonstrate that male rats develop more severe PH than females but respond better to Cpd22 treatment by reducing pulmonary vascular remodeling, PH, and RV hypertrophy and improving RV functional outcomes. 17β-estradiol diminishes anti-proliferative effect of Cpd22 in female, but not male, human PAH PAVSMC. These findings suggest potential attractiveness of ILK inhibition to reduce established PH in males and suggest that the combination with estrogen-lowering drugs could be considered to maximize anti-proliferative and anti-remodeling effects of ILK inhibitors in females.

Keywords

sex differences, pulmonary vascular disease, pre-clinical testing

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Introduction

Pulmonary arterial hypertension (PAH) is a progressive disease characterized by remodeling of small pulmonary arteries (PAs), increased PA pressure, right ventricle (RV) afterload, and death out of heart failure.[1,2] Currently approved therapies do not reverse pulmonary vascular remodeling or stop disease progression, and development of remodeling-focused anti-proliferative therapeutics is an area of unmet important needs.[3] PAH is predominantly female disease. Male patients, however, develop more severe disease and respond poorer to current therapies,[4–6]

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suggesting that responses to novel anti-proliferative therapeutics could also be gender-dependent.

Integrin-linked kinase (ILK) is pro-proliferative protein kinase that interacts with integrin beta 1 and beta 3 subunits and is involved in integrin-mediated signal transduction. ILK is activated by integrin engagement and by stimulation with growth factors and cytokines. Activated ILK, in turn, promotes cell proliferation via modulating numerous downstream targets, including activation of pro-proliferative/pro-survival protein kinase Akt and inhibition of growth suppressor HIPPO.[7–9] Activation of ILK in human cancers is associated with poor prognosis, and ILK inhibitors are under development as anti-proliferative therapeutic agents.[7,10,11] ILK1 is up-regulated in PA vascular smooth muscle cell (PAVSMC) in human PAH and experimental pulmonary hypertension (PH), which is required for increased cell proliferation, survival, pulmonary vascular remodeling, and overall PH,[9] and inhibition of ILK reverses experimental PH in male mice.[9] The benefits of ILK inhibition to target severe experimental PH, however, are not known, and sex-specific effects of pharmacological inhibition of ILK remain to be evaluated.

In this study, we used SU5416/hypoxia (SuHx) model of severe experimental PH and ILK inhibitor Cpd22. We report that there are sex-specific differences in PH development and in response to treatment. Our data show potential attractiveness of this therapeutic strategy to target established pulmonary vascular remodeling, RV dysfunction, and overall PH and suggest that sex-specific differences should be considered when targeting ILK in PAH.

Materials and methods

Animals

All animal procedures were performed under the protocols approved by the University of Pittsburgh Animal Care and Use Committee. Six- to eight-week-old male and female Sprague-Dawley rats (Charles River Laboratories, Taconic Biosciences, Inc., Hudson, NY) were randomly assigned to control and experimental groups (see Fig. 1a and 3a for experimental design). Rats from the experimental groups received subcutaneous injection of vascular endothelial growth factor receptor inhibitor SU5416 (Sigma-Aldrich, St. Louis, MO) (20 mg/kg in dimethyl sulfoxide (DMSO)) followed by three weeks of exposure to normobaric hypoxia (10% O_2) and two weeks of normoxia.[9,12,13] Five weeks post-SU5416 injection, rats were subjected to terminal hemodynamic analysis, or treated with vehicle (saline) or Cpd22 (20 mg/kg, intraperitoneal injection, five days/week)[9] for a period of three weeks (Fig. 3a), and terminal hemodynamic analysis was performed as described in several studies[9,12,13] Controls were same age and gender untreated rats maintained under normoxia. Upon the completion of data acquisition, lung and heart tissues were collected from dead animals for morphological analysis. Hearts were separated into RV and left ventricle (LV) + septum, and Fulton index was calculated as an RV/(LV + septum) ratio.

Terminal hemodynamic analysis was performed as described previously.[9,12,13] Briefly, animals were anesthetized with isoflurane (5% for induction, 2% during surgery, and 1% while performing pressure-volume (PV) loop measurements), and in vivo PV loop measurements of right ventricular (RV) function were performed by a PV catheter. A four-electrode PV catheter (Scisense, Inc., London, ON, Canada) attached to the data acquisition system (EMKA Instruments, Falls Church, VA, USA) was inserted into the apex of the RV. The data were collected using the EMKA data acquisition boxes and software (AD Instruments, Colorado Springs, CO, USA). Following ventricular assessment of PV loop relationships, a 20-mHz Doppler probe was placed over the PA and then over the aortic arch to assess CO and Doppler waveforms (DSPW; Indus Instruments, Houston, TX, USA).

Morphological and immunohistochemical analyses were performed as described in previous publications.[9,13–16] Briefly, lung tissues were fixed in 4% paraformaldehyde solution in phosphate-buffered saline (PBS) overnight, embedded in paraffin, and sectioned, and hematoxylin & eosin staining or immunostaining to co-detect phospho-S473 Akt, smooth muscle α-actin, and nuclei (4',6-diamidino-2-phenylindole, DAPI) was performed as described in literature.[9,13,14] Images were taken using an All-in-One Fluorescence Microscope (BZ-X810; Keyence Corporation, Itasca, IL, USA). Blinded analysis of medial wall thickness of small PAs (25–150 μm outer diameter; minimum of 5 rats/group, minimum of 12 PA/rat) was performed; percentage of fully, partially, and non-occluded small (20–50 μm outer diameter) PA was counted (a minimum of 5 rats/group, a minimum of 26 PA/rat).[9,13,16–18]

Cell cultures

Early-passage (3–8 passage) PAVSMCs isolated from small (≤1 mm outer diameter) PAs of male and female patients with PAH were provided by the University of Pittsburgh Vascular Medicine Institute Cell Processing Core and Pulmonary Hypertension Breakthrough Initiative. Tissue acquisition and cell isolation were performed in compliance with the University of Pittsburgh and University of Pennsylvania institutional review boards as described in previous studies.[9,15,19] Cells were maintained in complete LONZA growth media with SMGM-2 supplement, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza Group, Basel, Switzerland). Prior experiments, cells were incubated for 48 hours in basal media supplemented with 0.1% bovine serum albumin (BSA).

Cell proliferation analysis was performed as described in previous works.[9,20] Human PAH PAVSMCs were serum-deprived in Phenol Red-free medium (PromoCell, Heidelberg, Germany) supplemented with 0.1% BSA for

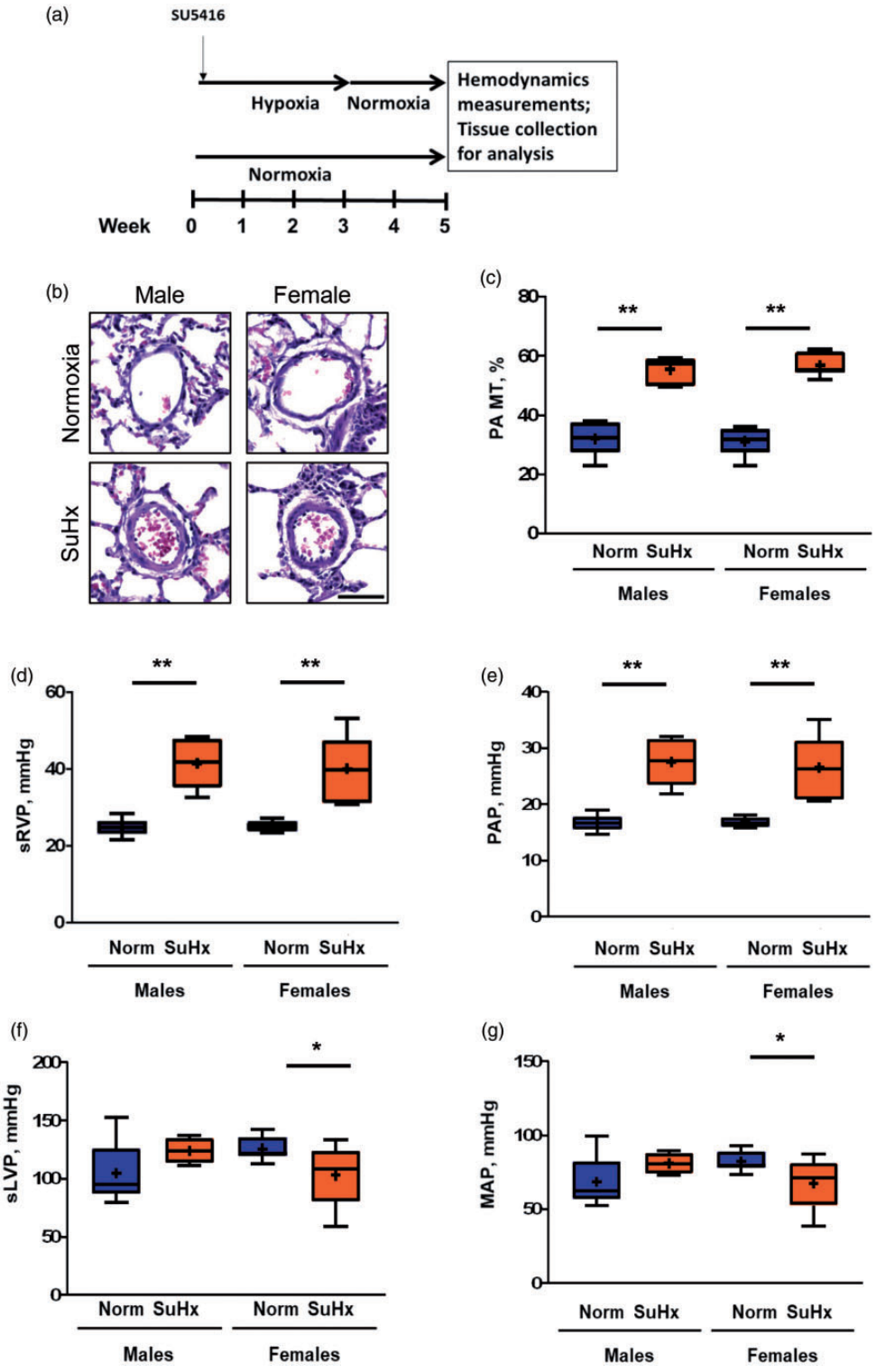

Fig. 1. Pulmonary vascular remodeling and pulmonary hypertension (PH) in SuHx-exposed rats five weeks after PH induction. (a) Schematic representation of experiment. Briefly, rats received single SU5416 injection and maintained for three weeks under hypoxia (10% O_2) and then for two weeks under normoxia (20% O_2). (b, c) Representative images of hematoxylin and eosin-stained small PAs (b) and PA MT analysis (c). Scale bar: 50 μm; $n = 5$–6 animals/group; minimum of 12 PA/animal. (d–g) Hemodynamics analysis was performed to measure sRVP (d), mean PAP (e), sLVP (f), and MAP (g) of rats with SuHx-induced PH (five weeks after induction) and age- and gender- matched controls. Data are means ± SE; $^{**}p < 0.01$ SuHx versus normoxia (Norm); $^{*}p < 0.05$ SuHx versus normoxia (Norm); "+" indicates mean value.

SuHx: SU5416/hypoxia; PAs: pulmonary arteries; PA MT: pulmonary artery medial thickness; sRVP: systolic right ventricular pressure; PAP: pulmonary arterial pressure; sLVP: systolic left ventricular pressure; MAP: mean arterial pressure.

48 hours. Treatment with Cpd22 (1 μM) and 17β-estradiol (E2) (1 nM), separately or in combination, or vehicle (DMSO) was performed for next 48 hours. Cells were then fixed in 4% paraformaldehyde in PBS, and cell proliferation was examined using Ki67 immunostaining (Cell Signaling Technology, Danvers, MA, USA). DAPI staining was performed to detect nuclei.[20] Images were taken using an All-in-One Fluorescence Microscope (BZ-X810; Keyence Corporation, Itasca, IL, USA).

Data analysis

Hemodynamic data were analyzed using Indus Instruments, IOX2, Emka, and MATLAB. Pulmonary arterial pressure (PAP) was calculated as systolic right ventricular pressure (sRVP) $\times 0.65 + 0.55$ mmHg.[21] Mean arterial pressure (MAP) was calculated as systolic left ventricular pressure (sLVP) * $0.65 + 0.55$ mmHg.[21] RV contractility index was calculated as [max dP/dT]/sRVP.[22] PA medial thickness (PA MT) was analyzed using Vascular Medicine

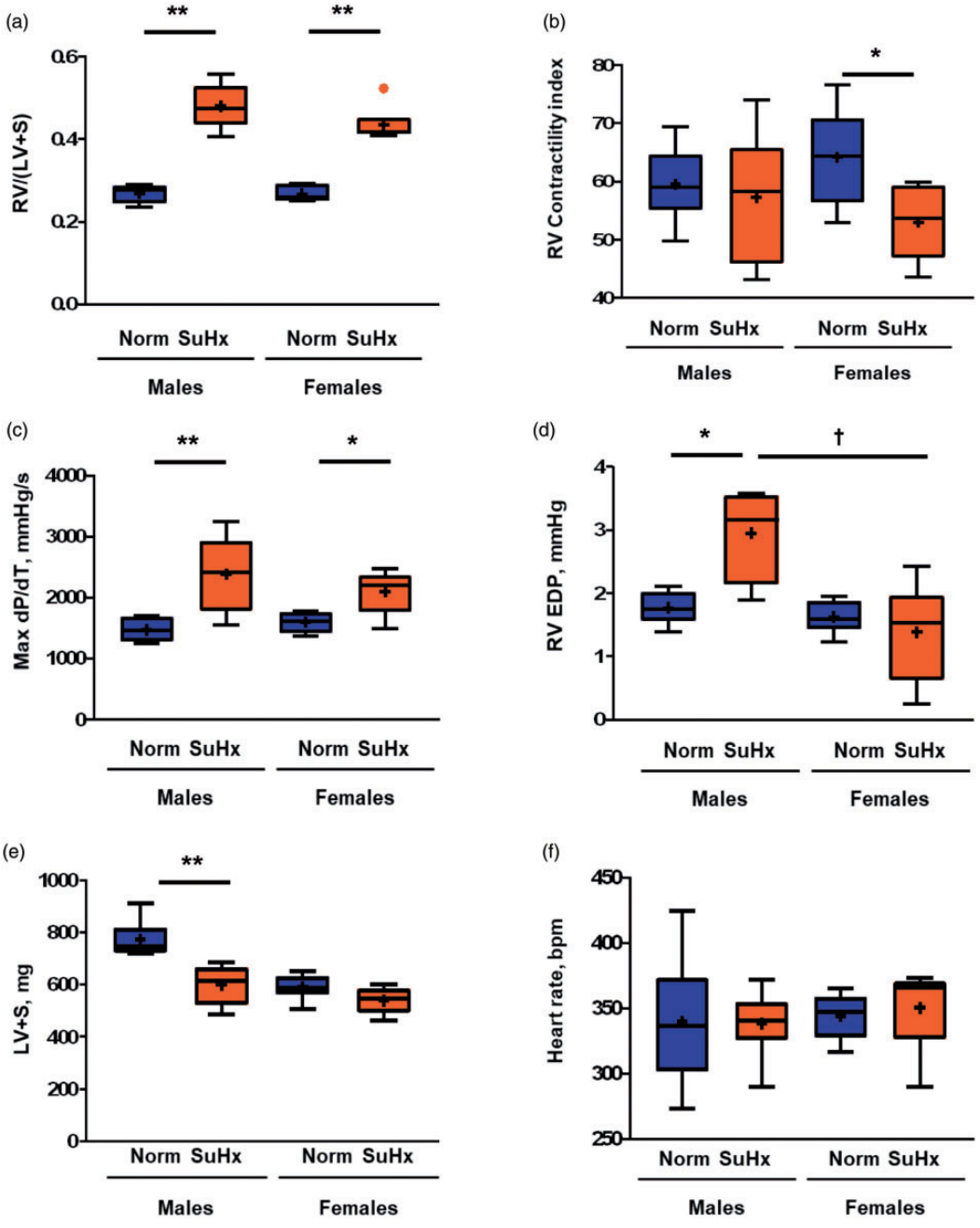

Fig. 2. RV hypertrophy and RV functional outcomes in SuHx-exposed rats five weeks after PH induction. (a) Fulton index, (b) RV contractility index, (c) max dP/dT, and (d) RV EDP. Data are means ± SE; $n = 5$–6 rats/group. *$p < 0.05$, **$p < 0.01$ for SuHx versus normoxia (Norm). †$p < 0.05$ females versus males. "+" indicates mean value.
SuHx: SU5416/hypoxia; RV: right ventricular; LV: left ventricle; EDP: end-diastolic pressure.

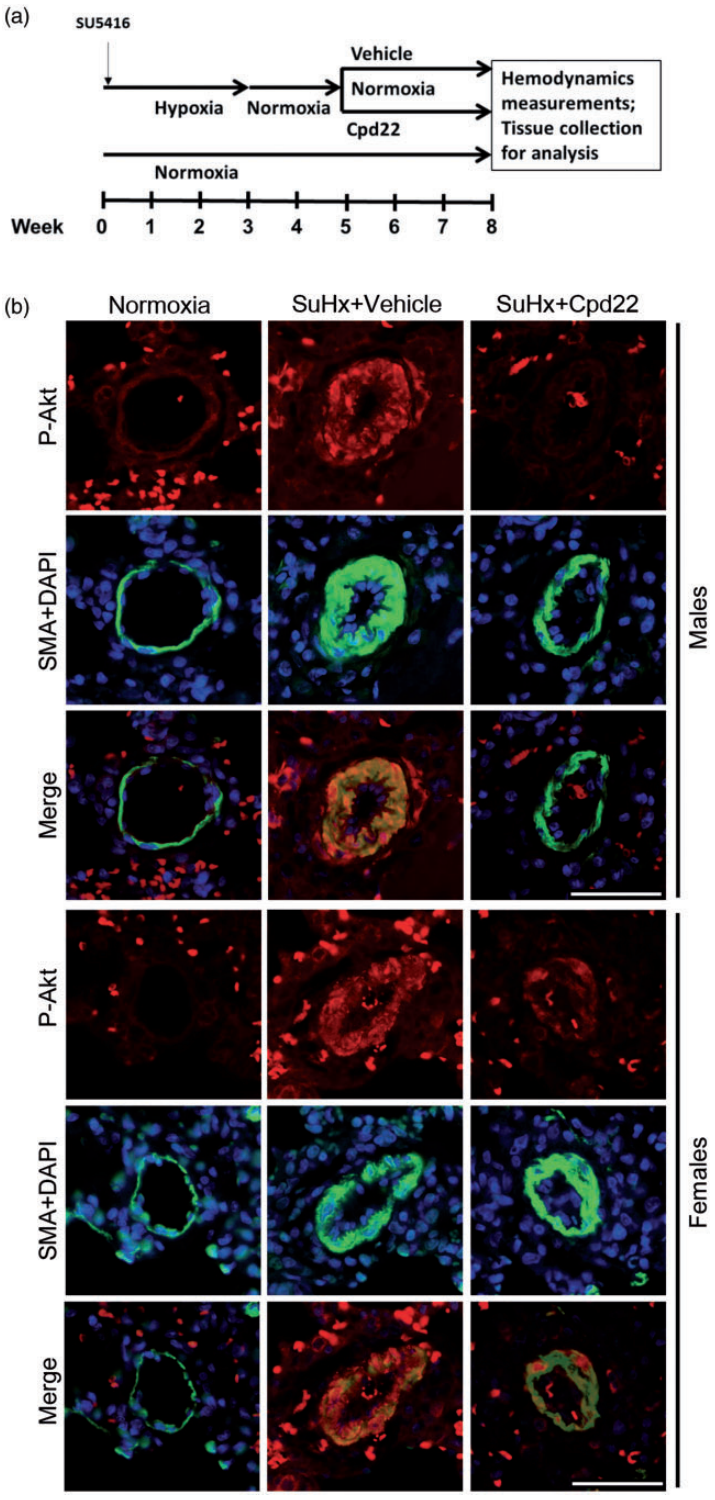

Fig. 3. (a) Schematic representation of experiment. Briefly, rats received single SU5416 injection and maintained for three weeks under hypoxia (10% O_2) and then for two weeks under normoxia (20% O_2). Then rats were treated with vehicle or Cpd22 for three weeks, five days/week. Controls were age- and gender-matched rats maintained under normoxia. (b, c) Effects of Cpd22 treatment on P-S473 Akt in small PAs from male and female rats with SuHx-induced PH. Immunohistochemical analysis of rat lungs at eight weeks after PH induction to detect P-S473 Akt (red) and smooth muscle α-actin (SMA) (green). DAPI (blue) staining was performed to detect nuclei. Images are representative from 3 rats/group, 10 PAs/animal. Scale bar: 50 μm. Merge image is produced by overlapping P-S473Akt, SMA, and DAPI images taken consequently from the same field. SuHx: SU5416/hypoxia; P-Akt: Phospho-Akt; SMA: smooth muscle alpha-actin.

Institute (VMI) calculator as described in Kelly et al.[16] Lesions were analyzed according to morphological grades as described in several works.[17,18,23] Morphological and histochemical data presented as box and whiskers graphs are generated by Prism (GraphPad Software, San Diego, CA, USA). Statistical comparisons among animal groups were performed by the Kruskal–Wallis test; statistical comparisons among cellular groups were performed by the analysis of variance (adjusted by Bonferroni) using Stata® software (StataCorp LLC, College Station, TX, USA). Statistical significance was defined as $p \leq 0.05$.

Results

First, we tested whether male and female rats develop significant PH after five weeks of PH initiation. Rats were subjected to SuHx exposure (SuHx groups) or maintained under normoxia (control groups). Five weeks after experiment initiation, hemodynamic and morphological analyses were performed (Fig. 1a). Both male and female animals from SuHx group showed comparable significant increases in PA MT (Fig. 1b and c), sRVP, and PAP (Fig. 1d and e), but not sLVP and MAP (Fig. 1f and g), showing that PH is already developed. Both, male and female rats with SuHx-induced PH also developed comparable levels of RV hypertrophy (Fulton index) (Fig. 2a) and had significant increases in RV contractility (max dP/dT) compared with same-age same-gender controls (Fig. 2c). Together, these data demonstrate that five weeks after PH induction, both male and female rats develop significant PH associated with pulmonary vascular remodeling, RV hypertrophy, and dysfunction. Interestingly, SuHx-exposed females, but not males, had significantly lower RV contractility index (Fig. 2b), while SuHx-exposed males, but not females, showed significant increase in RV end-diastolic pressure (EDP) (Fig. 2d) compared to respected control groups. Neither male nor female rats exposed to SuHx showed higher LV mass or differences in heart rates compared to normoxia-maintained controls (Fig. 2e and f).

Table 1. Morphological and hemodynamic parameters.

Group/time after experiment beginning	Control/ 5 weeks	Control/ 8 weeks	SuHx/ 5 weeks	SuHx + Vehicle/ 8 weeks	SuHx + Cpd22/ 8 weeks
Male PA MT, %	32.0 ± 2.30	29.0 ± 1.35	55.4 ± 1.85**	70.2 ± 3.12**	46.4 ± 3.78**,##
Female PA MT, %	31.1 ± 2.03	35.4 ± 2.50	56.9 ± 1.61**	57.7 ± 1.01**	53.7 ± 4.73*
Male sRVP, mmHg	24.8 ± 0.9	23.5 ± 0.9	41.4 ± 2.6**	64.9 ± 8.6**	39.2 ± 5.4**
Female sRVP, mmHg	25.0 ± 0.6	28.1 ± 1.1	40.0 ± 3.5**	45.2 ± 3.9*	46.4 ± 9.6
Male PAP, mmHg	16.7 ± 0.6	15.8 ± 0.6	27.5 ± 1.7**	42.7 ± 5.6**	26.1 ± 3.5**
Female PAP, mmHg	16.8 ± 0.4	18.8 ± 0.7	26.6 ± 2.3**	29.9 ± 2.5*	30.7 ± 6.2
Male sLVP, mmHg	104.8 ± 10.9	106.4 ± 7.2	124.0 ± 4.3	103.8 ± 11.5	106.9 ± 5.3
Female sLVP, mmHg	125.7 ± 4.3	128.5 ± 7.5	103.0 ± 11.0*	112.2 ± 6.6	116.4 ± 9.1
Male MAP, mmHg	68.7 ± 7.1	69.7 ± 4.7	81.2 ± 2.8	68.0 ± 7.5	70.0 ± 3.4
Female MAP, mmHg	82.2 ± 2.8	84.1 ± 4.9	67.5 ± 7.1*	73.5 ± 4.3	76.2 ± 5.9
Male Fulton index	0.269 ± 0.010	0.269 ± 0.008	0.481 ± 0.025**	0.635 ± 0.063**	0.417 ± 0.052*,#
Female Fulton index	0.267 ± 0.008	0.281 ± 0.010	0.435 ± 0.018**	0.438 ± 0.026**	0.463 ± 0.062**
Male RV contractility index	59.5 ± 2.7	52.5 ± 3.1	57.3 ± 4.6	44.0 ± 1.0*	52.6 ± 3.1#
Female RV contractility index	64.2 ± 3.5	60.8 ± 2.8	53.0 ± 2.7*	53.0 ± 3.1†	51.5 ± 2.9*
Male max dP/dT, mmHg/s	1476 ± 83.7	1246 ± 108	2389 ± 267**	2816 ± 336**	2022 ± 252**
Female max dP/dT, mmHg/s	1601 ± 74.6	1703 ± 82	2098 ± 150*	2402 ± 260	2233 ± 367
Male RV EDP, mmHg	1.77 ± 0.11	1.50 ± 0.19	2.95 ± 0.39*	2.78 ± 0.38**	1.49 ± 0.18##
Female RV EDP, mmHg	1.62 ± 0.11	1.36 ± 0.12	1.38 ± 0.32†	2.67 ± 0.38**	1.95 ± 0.40
Male heart rate, bpm	339.7 ± 20.7	315.3 ± 31.4	338.5 ± 10.9	314.9 ± 9.3	352.4 ± 18.9
Female heart rate, bpm	344.1 ± 7.5	346.0 ± 13.2	350.7 ± 13.1	341.4 ± 15.9	349.8 ± 11.5
Male body weight, g	403.0 ± 19.0	464.1 ± 12.2	284.7 ± 10.2*	460.3 ± 14.7	440.4 ± 9.8
Female body weight, g	273.1 ± 12.2	267.4 ± 3.0	243.6 ± 10.1†	250.9 ± 3.3†	251.1 ± 13.3†
Male LV + septum weight, mg	773.0 ± 29.4	902.8 ± 13.5	599.3 ± 31.3**	978.2 ± 42.3	883.1 ± 39.6
Female LV + septum weight, mg	590.8 ± 20.1	566.7 ± 16.0	539.8 ± 21.1	564.5 ± 10.6†	596.4 ± 37.1†

MAP: mean arterial pressure; PA MT: pulmonary artery medial thickness; PAP: pulmonary arterial pressure; RV: right ventricle; EDP: end-diastolic pressure; LV: left ventricle; sRVP: systolic right ventricular pressure; sLVP: systolic left ventricular pressure; bpm: beat per minute.
All values are expressed as mean ± SE from five to nine rats/group.
*$p < 0.05$, **$p < 0.01$ versus control (Contr); #$p < 0.05$, ##$p < 0.01$ versus SuHx; †$p < 0.05$ females versus males.

To evaluate potential benefits of ILK inhibitor Cpd22, we next induced PH by SuHx exposure and treated rats with vehicle or Cpd22 (ip. 20 mg/kg, five days a week) for three weeks starting at the beginning of sixth week, when PH is already developed (Fig. 3a). In agreement with previous studies,[13] vehicle-treated rats showed marked increase in phospho-S473 Akt, a molecular signature of ILK-dependent Akt activation,[9,11,24] compared to same-age controls (Fig. 3b). Vehicle-treated male rats had more severe PH compared to both, pre-treatment (five weeks of PH) and control groups, as evidenced by markedly higher PA MT, sRVP, PAP, RV hypertrophy, and max dP/dT and reduced contractility index (Table 1, Figs. 4–6), suggesting that PH continues to develop. Interestingly, vehicle-treated female rats from SuHx group developed less severe PH, RV hypertrophy, and RV contractility index than vehicle-treated males (Figs. 5a and b, 6a and b). Although both male and female vehicle-treated rats in SuHx groups showed significantly less non-occluded (Grade 0) small PAs compared to controls, male rats developed significantly more fully occluded (Grade 2) vessels compared to females (Fig. 4c), also supporting our observations that male rats in this model develop more severe PH and suggesting that sex-related similarities with human PAH are shared. Interestingly, male and female vehicle-treated rats had similar increases in RV EDT (Fig. 6d), suggesting that these RV functional abnormalities in female rats could be load-independent.

Treatment of male rats with Cpd22 suppressed S-473 Akt phosphorylation in small PAs, providing an evidence of successful ILK inhibition. Importantly, Cpd22 treatment markedly reduced pulmonary vascular remodeling in male rats as

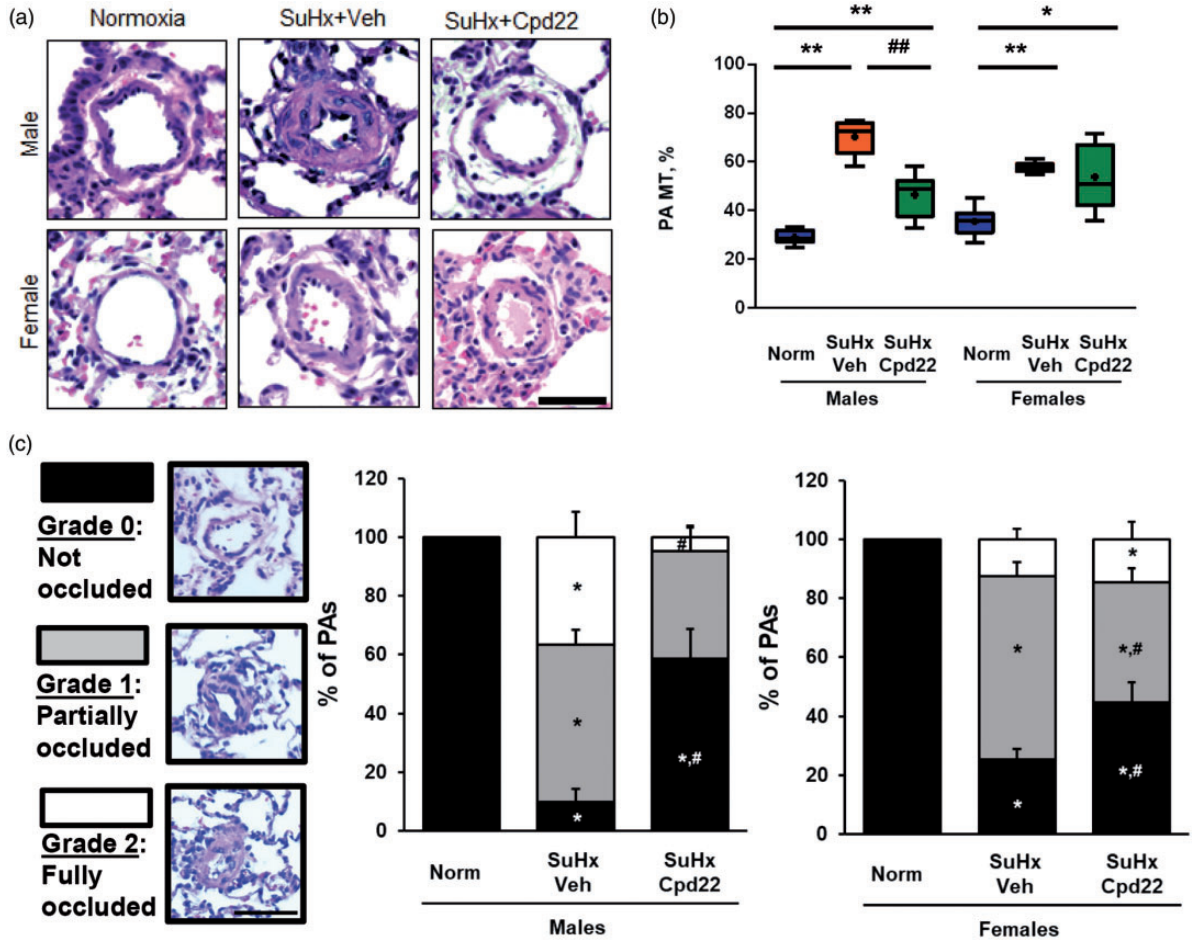

Fig. 4. Cpd22 attenuates pulmonary vascular remodeling in SuHx-exposed male, but not female, rats. (a, b) Representative images of hematoxylin and eosin-stained PA (a) and PA MT analysis (b). Scale bar: 50 μm; $n = 6$–9 animals/group; minimum of 12 PA/animal. Data are means ± SE; $^{**}p < 0.01$ versus normoxia (Norm), $^{\#\#}p < 0.01$ SuHx + Cpd22 versus SuHx + Veh. "+" indicates mean value. (c) % of not occluded (Grade 0, black), partially occluded (Grade 1, gray), and fully occluded PAs (Grade 2, white) in control (Norm), SuHx-exposed vehicle-treated (SuHx Veh), and SuHx-exposed Cpd22-treated (SuHx Cpd22) rats. Left panel: examples of Grade 0–3 PAs. Bar equals 50 μm. Middle and right panels: Data represent percentage of the arteries of each grade to total number of arteries taken as 100%. Data are from six to nine rats/group, ≥26 PA/rat. $^{*}p < 0.05$, $^{**}p < 0.01$ versus normoxia-maintained controls (Norm); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus SuHx + Veh.
SuHx: SU5416/hypoxia; PAs: pulmonary arteries; PA MT: pulmonary artery medial thickness.

evidenced by significantly lower PA MT (Fig. 4a and b), significantly increased percentages of non-occluded (Grade 0) small Pas, and significantly decreased percentage of fully occluded (Grade 2) vessels (Fig. 4c). This was accompanied by decreased sRVP, PAP, and max dP/dT (Figs. 5a, b, and 6c); significantly reduced RV hypertrophy (Fig. 6a) and RV EDP (Fig. 6d); and improved RV contractility index compared to the vehicle-treated group (Fig. 6b). In contrast, Cpd22 treatment of female rats just partially reduced phospho-S473 Akt in small PAs (Fig. 3b and c) and did not improve pulmonary vascular remodeling, PH, and/or RV hypertrophy and function compared to the vehicle-treated group (Figs. 4–6, Table 1). Together, these data demonstrate that Cpd22 attenuates pulmonary vascular remodeling, PH, and RV hypertrophy and improves RV functional parameters in male, but not female, rats with SuHx-induced PH, showing that there are sex-dependent benefits of ILK inhibition. We detected no significant differences in sLVP, MAP (Fig. 5c and d), heart rate, and LV + septum weight among control, vehicle-treated PH, and Cpd22-treated PH groups (Fig. 6e and f).

Because 17β-estradiol (E2) has pro-proliferative activity in PAVSMC and its metabolite 16OH-E2 is shown to increase proliferation of female, but not male, PAVSMC,[25,26] we reasoned that lack of anti-remodeling response in Cpd22-treated female rats could be due to the presence of estradiol. To test this hypothesis, we examined proliferation (Ki67) of human male and female PAH PAVSMC (see Table 2 for patients characteristics) treated with Cpd22 (1 μM) and E2 (1 nM) separately or in combination. As we expected, Cpd22 significantly inhibited proliferation of both male and female PAH PAVSMC (Fig. 7). Importantly, E2, while having no effect as a single agent, significantly attenuated Cpd22-induced inhibition of cell proliferation in female (Fig. 7a and c), but not male, PAH PAVSMC (Fig. 7a and b), showing that E2 impedes anti-proliferative effects of Cpd22 in female, but not male, PAVSMC.

In aggregate, our data demonstrate that male rats develop more severe PH than females but respond better to Cpd22 treatment. Our data also suggest that lack of anti-remodeling response to Cpd22 in females could be

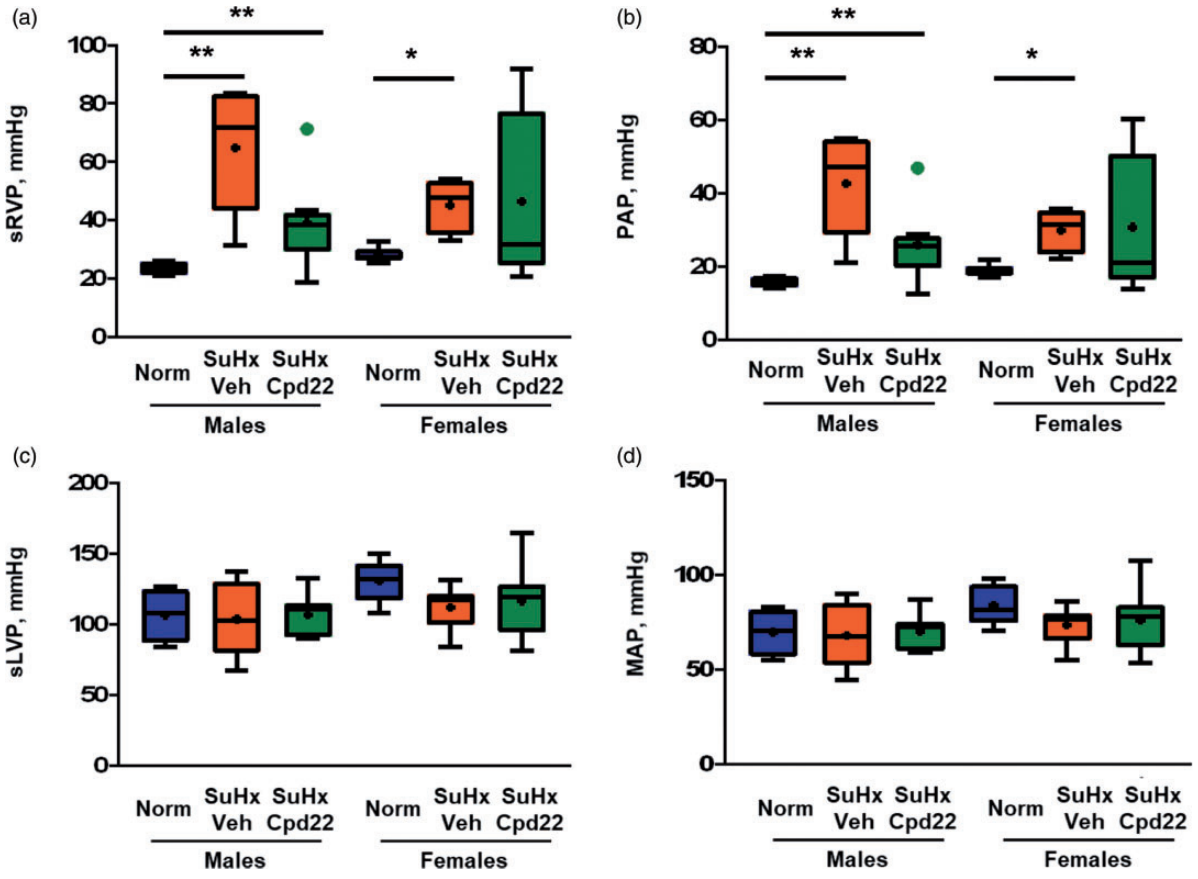

Fig. 5. Cpd22 attenuates SuHx-induced PH in male, but not female, rats. (a) sRVP, (b) PAP, (c) sLVP, and (d) MAP. Data are means ± SE from five to nine rats/group; "+" indicates mean value. *$p < 0.05$, **$p < 0.01$.
SuHx: SU5416/hypoxia; sRVP: systolic right ventricular pressure; PAP: pulmonary arterial pressure; sLVP: systolic left ventricular pressure; MAP: mean arterial pressure.

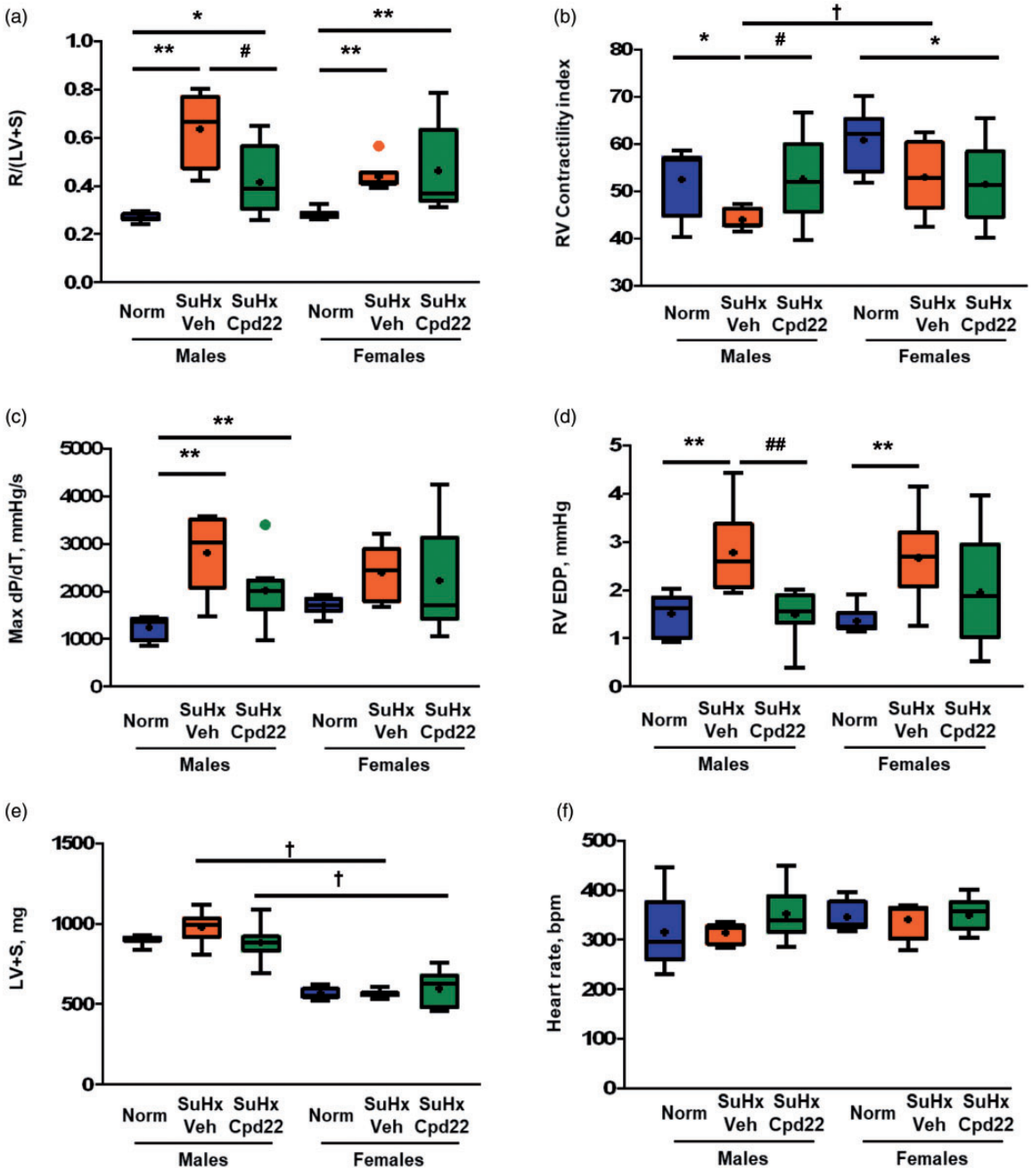

Fig. 6. Effects of Cpd22 treatment on RV hypertrophy and RV functional outcomes in SuHx-exposed rats. (a) Fulton index, (b) RV contractility index, (c) max dP/dT, (d) RV EDP, (e) LV + S, and (f) heart rate. Data are means ± SE from $n = 5$–9 rats/group; "+" indicates mean value; $^{*}p < 0.05$, $^{**}p < 0.01$ SuHx versus normoxia (Norm); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for SuHx + Cpd22 versus SuHx + Veh, $^{\dagger}p < 0.05$ females versus males. SuHx: SU5416/hypoxia; LV: left ventricle; RV: right ventricle; EDP: end-diastolic pressure; bpm: beat per minute.

explained, at least in part, by sex-dependent E2-induced protection of PAVSMC proliferation.

Discussion

The goal of this study was to test potential attractiveness of ILK inhibition to treat established PH in males and females using SuHx model of severe PH. Our major finding is that male rats develop more severe PH than females but show better therapeutic response to ILK inhibition.

There is a good consensus that female sex is a risk factor for PAH.[4,27,28] Male patients, however, have significantly higher disease severity, and male sex is an independent predictor of disease-associated mortality.[5] We found that male rats, while developing similar to females' PH at five weeks of disease initiation, have more severe pulmonary vascular

remodeling, PH, and RV hypertrophy at eight weeks after PH induction. Interestingly, while male rats developed elevated RV EDP at five weeks after PH initiation, at eight weeks post-PH initiation, RV EDP increases in males and females were similar. Although just a surrogate for diastolic function, delayed RV EDP response in females versus males may suggest delayed onset of overall PH, and longer follow up may determine whether females continue to develop PH and worsen systolic dysfunction as disease progresses. Overall, although different from the high-dose SU5416 (200 mg/kg) SuHx model developed by Tofovic et al.,[29] the differences between males and females which are observed by us are similar to those reported for other (monocrotaline and chronic hypoxia) models of experimental PH[30,31] and suggest that sex-related differences with human PAH may be shared.

Table 2. Human subjects characteristics.

Diagnosis	Gender	Age, years
Idiopathic PAH	Male	21
SS-PAH	Male	42
PAH	Male	55
Idiopathic PAH	Female	40
Idiopathic PAH	Female	49
PAH	Female	50

PAH: pulmonary arterial hypertension; SS-PAH: systemic sclerosis – pulmonary arterial hypertension.

Accumulating evidence demonstrates that response of PAH patients to therapies is also sex-dependent. Although developing more severe disease, male patients show less improvements of hemodynamics and right ventricular functions in response to current therapies, which is linked with worse overall long-term prognosis.[6] Our data, however, show that pharmacological inhibition of ILK with Cpd22 markedly attenuated pulmonary vascular remodeling, PH, and RV hypertrophy in males, which is in good agreement with our previous studies on male mice with SuHx-induced PH.[9] Female rats, treated with Cpd22, showed no significant decrease of pulmonary vascular remodeling, PH, RV

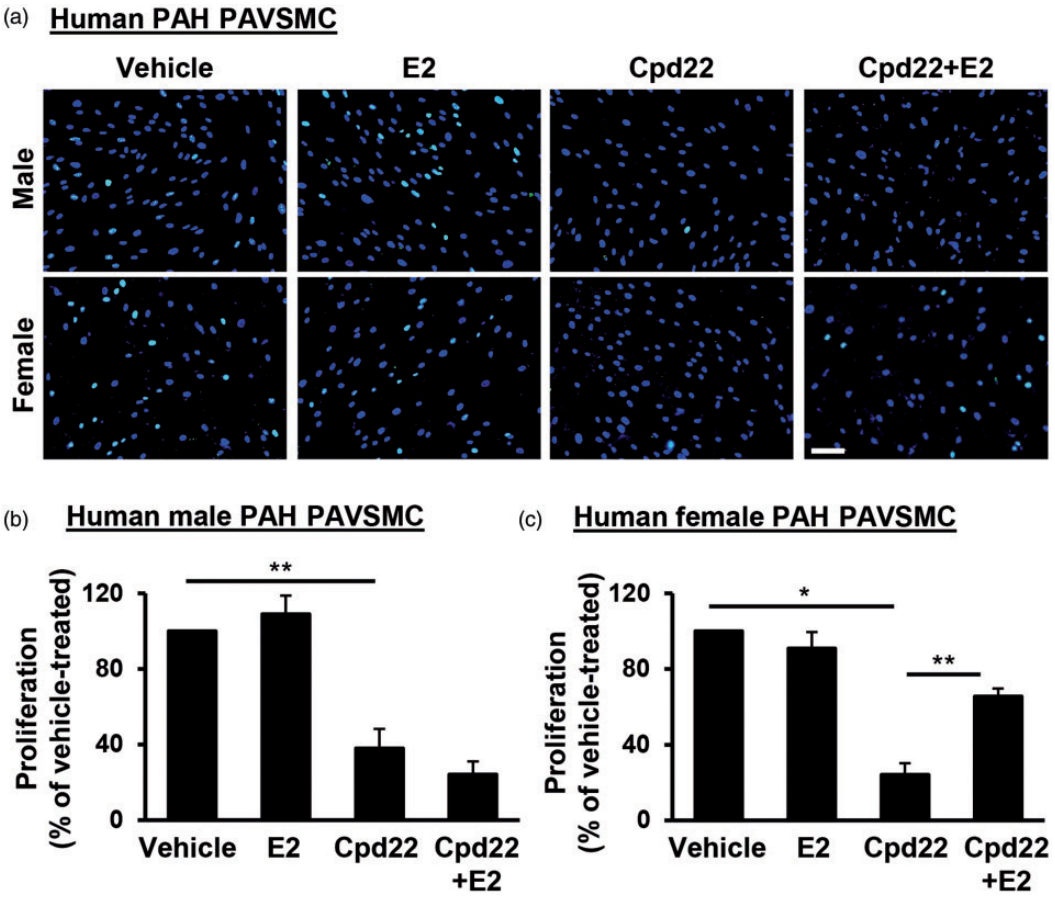

Fig. 7. 17β-estradiol (E2) attenuates inhibitory effect of Cpd22 on proliferation of PAVSMC from female, but not male, patients with PAH. (a) Images are representative from $n = 3$ subjects/condition. Bar equals 50 μm. green—Ki67; blue—DAPI; presented images produced by merging Ki67 and DAPI images captured on the same field. (b, c) Data are means ± SE from $n = 3$ subjects/group, a minimum of 100 cells/subject; data presesented as percentage to the vehicle-treated cells taken as 100%. *$p < 0.01$; **$p < 0.05$.
PAH: pulmonary arterial hypertension; PAVSMC: pulmonary artery vascular smooth muscle cell.

hypertrophy, and RV EDP and did not improve RV contractility. In aggregate, our study shows that male rats, while developing more severe disease, also show better anti-remodeling response to the ILK inhibition, suggesting that patients' sex should be considered in clinical trials with remodeling-focused anti-proliferative agents.

Such sex-related differences in response to Cpd22 treatment could be explained, at least in part, by the cross-talk between ILK and estrogen pathways in regulating cell proliferation. Indeed, recent studies show that 17β-estradiol (E2) acts as a pro-proliferative agent for human PAVSMC,[25] and E2 metabolite 16OH-E2 induces proliferation of female, but not male, rat PAVSMC.[26] Our data show that co-treatment with E2 attenuates Cpd22-induced inhibition of cell proliferation in female, but not male, PAVSMC from patients with PAH, supporting the potential role for estrogen in blunted response of female rats to the Cpd22 treatment. While the mechanisms by which E2 impedes anti-proliferative action of Cpd22 remain to be established, it should be noted that ILK activates pro-proliferative/pro-survival Akt and inhibits LATS1, a key component of growth-suppressor HIPPO cassette.[7–9,11,24,32–34] ILK inhibition by Cpd22, in turn, restores LATS1 and attenuates established PH in male mice via inhibiting Yap/Taz-Akt pathway.[9] Estrogen receptor α (ERα) is highly expressed in female PAVSMC in human PAH and experimental PH and supports estrogen-induced proliferation and overall PH via activation of ERK and Akt.[35,36] Although more studies are needed, it is possible that estrogen impedes anti-remodeling effects of Cpd22 in females by counter-balancing Akt inhibition, attenuating its anti-remodeling potential. In support of this hypothesis, our data show that Cpd22 suppresses SuHx-induced Akt phosphorylation in small PAs from male rats but fails to provide the same level of Akt inhibition in females. If this is the case, a combination of ILK inhibitors with estrogen-lowering or ERα-targeting drugs would be beneficial to attenuate pulmonary vascular remodeling and reduce established PH in females.

In conclusion, our study demonstrates that there are sex-specific differences in response to ILK inhibitor Cpd22 in SuHx rat model of PH. We show that, while developing more severe PH, male rats respond better to Cpd22 treatment. We also provide initial evidence that such lack of Cpd22 response in females may be caused by the estradiol-dependent prevention of Cpd22-induced inactivation of Akt in VSMC in small PAs, preserving pulmonary vascular remodeling and overall PH. Together with published studies, our data also suggest that combination of Cpd22 with anti-estrogen/ERα therapy could be beneficial to attenuate established PH in females.

Author contributions

Conception and design: EAG. Experimental work: YS, DAG, AR, EO, and HD. Analysis: YS, DAG, TVK, AR, TA, EO, RV, ALM, and EAG. Interpretation: YS, DAG, RV, ALM, HD, and EAG. Drafting the manuscript: YS and EAG.

Conflict of interest

The author(s) declare that there is no conflict of interest.

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