Supplemental Material
Data S1.

Supplemental Methods

SU5416 hyper-responsive phenotype characterization

Male and female Sprague Dawley (SD, Charles River laboratories, QC, Canada) rats weighing 150-200 g were used for this study. A total of 241 rats were utilized for the study (79 males, 38 females and 124 ovariectomized (OVX) females). PH was induced by a single subcutaneous injection of SU5416 (SU: 3-(3,5-dimethyl-1H-pyrrol-2-ylmethylene)-1,3-dihydropindol-2-one) (Tocris, Bristol, United Kingdom) in 0.5% carboxymethyl cellulose. Following SU treatment the rats were housed under normoxic condition for 7 weeks. PH phenotype was characterized by measuring RVSP, RV hypertrophy and lung vascular remodelling as described below (Fig S1 and S2). To study sex differences in penetrance of PH phenotype in male (n=34) and female (n=26) SDHR rats were injected with SU (Fig S1A) and 2 male rats died before the endpoint. To explore role of female sex hormones in penetrance of PH, OVX rats (n=14) and sham female SDHR rats (n=12) were treated with SU 2 weeks after OVX surgery (Fig S1B). One OVX rat died before endpoint.

Sex hormone treatment

Sex hormone replacement was performed using continuous slow-release pellets (17β-estradiol: 0.5mg pellet, s.c., 60-day release; progesterone: 150mg pellet, sc, 60-day release). At 2 days before, 2 days post or 4 weeks post-SU injection, rats were randomized to receive estradiol, progesterone or placebo pellets (Figures S1C, 1D, 2A and 2B). Rats were anaesthetized by isoflourane inhalation and slow-release pellets were implanted sub-cutaneously on the dorsal side at the base of the neck. Topical bupivacaine was applied immediately after wound closure and twice daily for one day post-surgery. Buprenorphine (s.c., 0.03mg/kg) was administered 1 hour prior to surgery and once daily for two days post-surgery. For estradiol pre-treatment (Fig S1C and 1D) experiments, 25 male SDHR rats
(Placebo: 13 rats; estradiol: 12 rats) and 26 OVX female SD\textsuperscript{HR} rats (Placebo: 13 rats; estradiol: 13 rats) were included and 4 male and 2 female placebo treated rats died before endpoint. For progesterone pre-treatment (Fig S1C and 1D) study, 20 male SD\textsuperscript{HR} rats (Placebo: 10 rats; progesterone: 10 rats) and 20 OVX female SD\textsuperscript{HR} rats (Placebo: 10 rats; progesterone: 10 rats) were included. Four male and 2 female placebo treated rats and 3 progesterone treated male rats died before endpoint. For early post-treatment (Fig S2A), 20 OVX female SD\textsuperscript{HR} rats (Placebo: 10 rats; estradiol: 10 rats) were included and 1 placebo treated rat died before endpoint. For 4-week treatment experiments, RVSP measurement was performed to confirm development of PH and rats with RVSP >35mmHg were included in the study and randomized to receive placebo or sex hormone containing pellets. Rats with RVSP <35mmHg at 4 weeks do not develop severe PH (based on extensive experience with this model) and; therefore, these rats were excluded from the experiments designed to investigate the effects of female sex hormones on established PH. Twenty five OVX female SD\textsuperscript{HR} rats (Placebo: 10 rats; estradiol: 15 rats) were included in the study of estradiol reversal treatment (Fig S1D), and 19 OVX female SD\textsuperscript{HR} rats (Placebo: 10 rats; progesterone: 9 rats) were included to study progesterone reversal treatment (Fig S1D).

**Measurement of RVSP and RV hypertrophy**

RVSP was measured using high-fidelity pressure catheters (Transonic-Scisense Inc., ON, Canada) at 4 weeks post-SU (Baseline) and at 7- or 8-weeks post-SU (end study). For RV catheterization, rats were anaesthetized by an intraperitoneal injection of xylazine (7 mg/kg) and ketamine (35 mg/kg). The pressure catheter was inserted into the right jugular vein and advanced through the superior vena cava and right atrium into the RV. Hemodynamic parameters were recorded and analyzed using the LabScribe3 software (iWorx, Dover, NH, USA). At 7 weeks, after data acquisition, animals were euthanized by exsanguination under anaesthesia. The heart was excised, and the ventricles were dissected from the atria, the aorta and the pulmonary trunk. The RV and left ventricle (LV) and septum (S) were separated, and RV hypertrophy was calculated by measuring the ratio of RV weight to LV plus septum weight (RV/LV+S, Fulton index). The operators acquiring the RVSP and RV
hypertrophy data were blinded to the treatment allocation. For 4-week RVSP measurement, rats were anaesthetized by isoflurane inhalation and topical bupivacaine was applied immediately after wound closure and twice daily for one day post-surgery. Buprenorphine (s.c., 0.03mg/kg) was administered 1 hour prior to surgery and once daily for two days post-surgery.

**Lung histological measurements**

The left lobe of the lung was inflated via the trachea with 50:50 OCT/saline solution (Tissue-Tek OCT; Qiagen, Mississauga, ON, Canada) and then removed. The left lobe was then cut into thick cross sections and fixed in 4% paraformaldehyde (PFA) for 24 h, rinsed and washed in PBS for 8 hr and stored in 70% ethanol until the day of paraffin embedding. Tissue blocks were sectioned (5μm thickness) with a microtome (Leica Microsystems, Concord, ON, Canada), placed onto poly-L-lysine-coated slides, dried at 37°C for 16 hours and then dewaxed and rehydrated through graded alcohols. For microscopy and quantitative morphometry of the lung, hematoxylin and eosin (H&E) staining was performed with standard protocols. Images were acquired by Panoramic DESK (3DHISTECH, Hungary) scanscope using Panoramic Scanner and analyzed using Panoramic Viewer (3DHISTECH, Hungary). Ten random high-power fields (100X magnification) for each rat were analyzed for media wall thickness, total vessel count and vascular occlusion. Media wall thickness as percent of external diameter was estimated as described previously.\textsuperscript{15} Percent Medial wall thickness \(= ((\text{distance between the internal and external lamina} \times 2)/\text{external diameter}) \times 100\). For total vessel count, all the vessels were counted from the 10 random fields. The numbers of normal and completely or partially occluded distal arterioles (<100 μm) were quantified from the random fields.

**Western blotting**

Right lung was collected at the end of study, flash-frozen in liquid nitrogen and stored at -80 °C until further processing. Lung lysates were prepared in CelLytic™ MT Cell Lysis Reagent (Sigma, ON, Canada) containing cOmplete™ protease inhibitor cocktail (Sigma, ON, Canada) and PhosSTOP™ (Sigma, ON, Canada) and using the TissueLyser (Qiagen, ON, Canada) two cycles of 25hz for 3 min.
The tissue lysate was then centrifuged at 12000xg for 10 min and the supernatant was collected. Protein concentration of the protein extract was determined colorimetrically by the DC Protein Assay kit (Bio-Rad, ON, Canada), using bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis of lung protein extract (50 µg) was performed with NuPAGE® Novex® 4-12% Bis-Tris Protein Gels (ThermoFisher Scientific, ON, Canada). Following transfer of the separated proteins to nitrocellulose membranes (NOVEX iBLOT Gel transfer Stacks, ThermoFisher Scientific, ON, Canada), blots were blocked with 2% BSA in PBS-T (PBS containing 0.1% Tween 20, pH 7.4). After blocking, blots were incubated with primary antibodies to cleaved caspase-3 (Cell Signalling Technologies, Cat# 9661S), progesterone receptor (Abcam, Cat# ab16661), BMPR2 (BD Biosciences, Cat# 612292), phospho-SMAD1/5/9 (Cell Signalling Technologies, Cat# 9511S) or β-actin (ThermoFisher Scientific, Cat# A5441) for overnight at 4 °C. Then the blots were washed for three times for 15 min with PBS-T and incubated with appropriate IRDye® anti-rabbit or anti-mouse secondary antibodies (LI-COR Biotechnology, NE, USA) in 2% BSA/PBS-T. Further, the blots were washed for three times for 15 min with PBS-T and imaged with Odyssey® imaging system (LI-COR Biotechnology, NE, USA). The blots were quantified using the Image Studio™ Software (LI-COR Biotechnology, NE, USA) and expressed as a percentage of control to reduce the variation between blots.

**Caspase 3/7 activity assay**

Caspase 3/7 activity in the lung lysates was assessed using Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega Cop, WI, USA) according to manufacturer’s protocol with slight modifications. Briefly, lung lysates were diluted to 1 µg/µL with CelLytic™ MT Cell Lysis Reagent. Then, 50 µL of diluted reagent (substrate and buffer combined) was added directly to 50 µL samples and incubated at 25 °C for 2.5 hr. Fluorescence was measured every 30 min using excitation wavelength of 480 nm and emission wavelength of 520 nm. Caspase activity was calculated using gain of fluorescence between 30 min intervals. Amount of metabolized substrate was determined from standard curve of Rhodamin 110.
Cleaved caspase 3 and von Willebrand Factor (vWF) immunohistochemistry

PFA fixed and paraffin embedded tissue were sectioned (5μm thickness) with a microtome (Leica Microsystems, Concord, ON, Canada), placed onto poly-L-lysine-coated slides, dried at 37°C for 16 hours and then dewaxed and rehydrated through graded alcohols. Antigen retrieval was performed using Citric Acid Based Antigen Unmasking Solution (Vector Labs, Cat# H3300) according to manufacturer’s protocol. Immunohistochemistry was performed using Rabbit specific HRP/DAB (ABC) Detection IHC Kit (Abcam, Cat# ab64261) according to manufacturer’s protocol. Sequential sections were used for cleaved caspase-3 (Cell Signalling Technologies, Cat# 9661S) and Von Willebrand Factor (vWF, Abcam, Cat# ab6994) immunohistochemistry. The primary antibodies (cleaved caspase-3 at 1:40 and vWF at 1:400) were diluted in 1% BSA in PBS and each section was incubated overnight at 4 °C with 80 μL diluted antibody. Images were acquired by Panoramic DESK (3DHISTECH, Hungary) scanscope using Panoramic Scanner and analyzed using Panoramic Viewer (3DHISTECH, Hungary). Ten random high-power fields (100X magnification) for each rat were analyzed for cleaved caspase-3 positive endothelial cells.

Cleaved caspase 3 and smooth muscle actin (SMA) immunofluorescence staining

PFA fixed and paraffin embedded tissue were sectioned (5μm thickness) with a microtome (Leica Microsystems, Concord, ON, Canada), placed onto poly-L-lysine-coated slides, dried at 37°C for 16 hours and then dewaxed and rehydrated through graded alcohols. Antigen retrieval was performed using Citric Acid Based Antigen Unmasking Solution (Vector Labs, Cat# H3300) according to manufacturer’s protocol. Slides were washed in PBS than permeabilized with 0.25% triton X-100 (Sigma Aldrich, Cat# T8787) in PBS for 15 min at room temperature. Slides were washed in PBS-T, prior to blocking with 5% goat serum (Rockland immunochemicals Inc. Cat# B304) – 2% bovine serum albumin (Wisent Inc. Cat# 800-095-EG) for 1h at room temperature. Slides were incubated overnight at 4°C with primary antibodies: rabbit anti-cleaved caspase 3 (Cell Signalling
Technologies, Cat# 9661S) 1:50 dilution, and mouse anti-actin (alpha smooth muscle; Sigma-Aldrich, Cat# A5228) 1:200 dilution. The slides were then washed 3x in PBS-T followed by incubation with secondary antibodies, goat anti mouse Alexa Fluor 488 (Thermofisher, Cat# A32723) and goat anti-rabbit Alexa Fluor 594 (Thermofisher, Cat# A-11037), at 1:400 dilution for 1h at room temperature. Samples were washed 3x in PBS-T, 1x in PBS than auto-fluorescence was further quenched using Vector TrueView Autofluorescence quenching kit (Vector Labs, Cat# VECTSP8400) for 4min immediately followed by 3x PBS wash. Slides were counterstained with DAPI (Sigma, Cat# D9542) for 10min at 5μg/ml. Cells were washed 3x in PBS-T and mounted using VECTASHIELD vibrance antifade mounting media (Vector Labs, Cat# VECTSP8400) and allowed to dry at room temperature for 1h. Slides were stored at 4C and imaged using the Zeiss Imager M2.

**Statistical analysis**

The study evaluated the effect of biological sex on the penetrance of the PAH phenotype by comparing response to SU alone between male and female SD rats. To study the role of female sex hormones in modifying penetrance of PAH, the response to SU alone in male or OVX female rats was compared between animals receiving placebo or female sex hormones (estradiol or progesterone). For statistical comparisons, Student’s t-test or One-Way ANOVA (>2 groups) were performed followed by Tukey multiple comparison test with significance level of p<0.05. For comparison of proportion change, odds ratio was calculated and Fisher’s exact test was performed to calculate statistical significance. Data are represented as mean ± standard error of mean unless otherwise stated. Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software Inc. CA, USA).
**Figure Legends:**

**Figure S1. Schematic diagrams demonstrating experimental procedures** for study of A) penetrance of pulmonary arterial hypertension (PAH) in male vs female SD<sup>HR</sup> rats in response to SU, B) effect of ovariectomized (OVX) on penetrance of PAH in response to SU; and effects of female sex hormones (17β-estradiol/ progesterone) pre-treatment on PAH penetrance in response to SU in C) male and D) OVX female SD<sup>HR</sup> rats.

**Figure S2. Schematic diagrams demonstrating experimental procedures** for study of the effects of female sex hormones (17β-estradiol/ progesterone) A) early post-treatment and B) delayed post-treatment on penetrance of pulmonary arterial hypertension (PAH) in response to SU5416 in ovariectomized (OVX) female SD<sup>HR</sup> rats.

**Figure S3. Mathematical derivation of cutoff values for separation of bimodal distribution of A) right ventricular systolic pressure (RVSP) and B) RV hypertrophy in the SD<sup>HR</sup> rats subjected to SU5416 in absence of hypoxia.**

**Figure S4. Representative low magnification images demonstrating vascular remodelling in the lungs of intact female non-responder (FNR) and responder (FR) SD<sup>HR</sup> rats in response to SU.**

**Figure S5. Representative low magnification images demonstrating vascular remodelling in the lungs of ovariectomized (OVX) female non-responder (FNR) and responder (FR) SD<sup>HR</sup> rats in response to SU5416.**
Figure S6. Bar graph showing plasma A) estradiol and B) progesterone concentration in placebo or estradiol/progesterone treated SD$^{HR}$ rats. Values represent mean±SD, *p<0.05 vs placebo.

Figure S7. Effect of continuous estradiol treatment, beginning 2 days before SU5416 injection, on body weights of SD$^{HR}$ rats. Bar graph demonstrating body weights of male or female SD$^{HR}$ rats treated with A) estradiol or B) progesterone at baseline and end study. Values represent mean±SD, n=12-14 per group, *p<0.05 vs placebo.

Figure S8. Representative low magnification images demonstrating cleaved caspase-3 positive vascular endothelial cells in the lungs of responder male SD$^{HR}$ rats at 7 weeks post SU5416 injection.

Figure S9. Representative low magnification images demonstrating cleaved caspase-3 positive vascular endothelial cells in the lungs of responder female SD$^{HR}$ rats at 7 weeks post SU5416 injection.

Figure S10. Representative immunofluorescence images demonstrating cleaved caspase-3 and smooth muscle acting staining in the lungs of vehicle or estradiol treated ovariectomized (OVX) female SD$^{HR}$ rats at 7 weeks post SU5416 injection. Arrows demonstrate cleaved caspase-3 positive cells in the intima.

Figure S11. Effect of continuous estradiol treatment, beginning 2-days before SU injection, on lung bone morphogenic protein receptor 2 (BMPR2) and phospho-SMAD1/5/9 expression. A) Images and bar graph demonstrating BMPR2 and phospho-SMAD1/5/9 expression in lung homogenates of male SD$^{HR}$ rats treated with placebo or estradiol. B) Images and bar graph
demonstrating BMPR2 and phospho-SMAD1/5/9 expression in lung homogenates of ovariectomized (OVX) female SD^{HR} rats treated with placebo or estradiol.

Figure S12. Effect of continuous progesterone treatment, beginning 2-days before SU injection, on lung bone morphogenic protein receptor 2 (BMPR2) and phospho-SMAD1/5/9 expression. A) Images and bar graph demonstrating BMPR2 and phospho-SMAD1/5/9 expression in lung homogenates of male SD^{HR} rats treated with placebo or progesterone. B) Images and bar graph demonstrating BMPR2 and phospho-SMAD1/5/9 expression in lung homogenates of ovariectomized (OVX) female SD^{HR} rats treated with placebo or progesterone.

Figure S13. Effect of continuous estradiol treatment, beginning 2 days after SU injection, on SU5416 induced pulmonary arterial hypertension (PAH). A) Right ventricular systolic pressure (RVSP) and B) RV hypertrophy of estradiol (E2) or placebo treated ovariectomized (OVX) female SD^{HR} rats at 7 weeks post SU5416 injection. C) Cleaved caspase-3 expression and D) caspase-3/7 activity in lung homogenates of placebo or estradiol (E2) treated OVX female SD^{HR} rats, n= 3-6 per group, *p<0.05 vs placebo.

Figure S14. Effect of continuous estradiol or progesterone treatment, beginning 4 weeks after SU injection, on lung progesterone receptor (PR) expression and body weights. A) Images and bar graph demonstrating increased PRa and PRb expression in lung homogenates of ovariectomized (OVX) female SD^{HR} rats treated with estradiol. Values represent mean±SD, n=4 per group, *p<0.05 vs placebo. B) Bar graph showing lower body weights of OVX female SD^{HR} rats treated with estradiol compared to placebo treated rats. Values represent mean±SD, n=9-14 per group, *p<0.05 vs placebo. C) Images and bar graph showing PRa and PRb expression in lung homogenates of OVX female SD^{HR} rats treated with progesterone. Values represent mean±SD, n=4 per group. D)
Bar graph showing no effect of progesterone treatment on body weights of OVX female SD$^{HR}$ rats.

Values represent mean±SD, n=9 per group.
**Figure S2.**

A

SD rats (Female)
OVX surgery

Week (-2)

Day-0

Day-2

SU5416 (20mg/kg, s.c.)

Pellet implantation
17β-Estradiol/Placebo

RVSP

Tissue collection

End point study

Week-7

B

SD rats (Female)
OVX surgery

Week (-2)

Day-0

Day-2

SU5416 (20mg/kg, s.c.)

RVSP and Pellet implantation
17β-Estradiol/
Progestosterone/Placebo

RVSP

Tissue collection

End point study

Week-8

Week-4
Figure S3

A

cutoff = 43.3, 28 (80%) RVSP+, 7 (20%) RVSP-

B

cutoff = 0.4286, 32 (78%) RVH+, 9 (22%) RVH-

RVSP

RV hypertrophy
Figure S6

A

Estradiol (pg/mL)

0 10 20 30

M-Placebo M-E2 OVX-f-Placebo OVX-F-E2

B

Progesterone (ng/mL)

0 20 40 60 80

M-Placebo M-P4 OVX-f-Placebo OVX-F-P4
Figure S7

A

![Graph A with bars showing body weight (g) for placebo and estradiol for male and female participants at baseline and end study.]

B

![Graph B with bars showing body weight (g) for placebo and progesterone for male and female participants at baseline and end study.]

- Graph A: * indicates a significant difference.

- Graph B: Placebo (black) and Estradiol (gray) for Male and Female participants.

- Placebo (black) and Progesterone (gray) for Male and Female participants.
Figure S9
Figure S11

A

BMPR2
pSMAD1/5/9
β-Actin

NR R E2

0.00 0.01 0.02 0.03

0.0 0.5 1.0 1.5 2.0

BMPR2/β-Actin
pSMAD/β-Actin

MNR MR M+E2

0.00 0.01 0.02 0.03

0.0 0.5 1.0 1.5 2.0

BMPR2
pSMAD1/5/9
β-Actin

B

FNR FR F+E2

0.00 0.01 0.02 0.03

0.0 0.5 1.0 1.5 2.0

BMPR2/β-Actin
pSMAD/β-Actin

FNR FR F+E2

0.00 0.01 0.02 0.03

0.0 0.5 1.0 1.5 2.0
Figure S12

A

BMPR2
pSMAD1/5/9
β-Actin

BMPR2/β-Actin

pSMAD/β-Actin

Placebo P4

0.0
0.1
0.2
0.3
0.4
0.5

0.0
0.1
0.2
0.3
0.4
0.5

Placebo P4

B

BMPR2
pSMAD1/5/9
β-Actin

BMPR2/β-Actin

pSMAD/β-Actin

Placebo P4

0.0
0.1
0.2
0.3
0.4
0.5

0.0
0.1
0.2
0.3
0.4
0.5

Placebo P4
Figure S13

A

RVSP (mmHg)

RV/LV+S

Placebo  E2

*  *

B

Placebo  E2

*  *

C

Caspase 3/7 activity (ng/mg/min)

Placebo  E2

*  *

D

Caspase 3/7 activity (ng/mg/min)

Placebo  E2

*  *
**Figure S14**

A. Western blot analysis showing PR-β-Actin in Placebo and E2 treatments.

B. Bar graph showing body weight in Placebo and E2 treatments.

C. Western blot analysis showing PR-β-Actin in Placebo and P4 treatments.

D. Bar graph showing body weight in Placebo and P4 treatments.