# Estrogen Rescues Pre-existing Severe Pulmonary Hypertension in Rats

Soban Umar, Andrea Iorga, Humann Matori, Rangarajan D. Nadadur, Jingyuan Li, Federica Maltese, Arnoud van der Laarse and Mansoureh Eghbali

## Online data supplement

## Methods

**Animals and treatments.** Male and female Sprague-Dawley rats (350-400 g) were used. To induce PH, rats were treated with a single subcutaneous injection of MCT (60 mg/kg, Sigma). This model has been extensively used by many investigators and has been shown to be reproducible[1;2]. MCT was dissolved in 1N HCl, the pH was adjusted to 7.4, and diluted with PBS before injection. Continuous release E2 pellets (42.5 μg/kg/day, Innovative Research of America), Diarylpropionitrile (DPN,850 μg/kg/day, Tocris), 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol **(**PPT,850 μg/kg/day, Tocris), 4-[2-Phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP, 850 μg/kg/day, Tocris) for 10 days from day 21 to 30 were used. Placebo pellets (containing 5 compounds: cholesterol, lactose, cellulose, phosphates and cerates) were used for 10 days as vehicle for E2 from day 21 to 30. Placebo pellets had no effects as the disease progression in placebo-treated group was similar to RVF group. In some experiments animals were treated with E2 pellets together with subcutaneous administration of TNP-470 (30 mg/kg, Sigma) once every other day for 10 days[3]. A weight loss of more than 10% per day for 2 consecutive days and arterial oxygen tension of less than 80% were the criteria required to sacrifice the animals and counted as a 'loss' in survival measurements. Protocols received institutional review and committee approval.

**Cardiac and pulmonary hemodynamics.** B-mode, M-mode and pulmonary pulsed wave Doppler echocardiography was performed using a VisualSonics Vevo 770 equipped with a 30-MHz linear transducer. During the course of the experiment, serial echocardiography was performed to accurately monitor the stage of the disease by measuring cardiac and pulmonary hemodynamic parameters, as well as RV structure. RV

pressure was calculated from pulsed wave Doppler echocardiography of pulmonary artery flow using Mahan's regression equation: $MPAP = 79 - 0.45 \times PAAT^{4}$, MPAP is mean pulmonary artery pressure and PAAT is the pulmonary artery acceleration time. The RV pressure was also measured directly by inserting a catheter (1.4F Millar SPR-671) connected to a pressure transducer (Power Lab, ADInstruments) into the RV right before sacrifice. The values of RV pressure measured by both methods were very similar. The RV ejection fraction, RV free wall thickness and RV cavity dimensions were quantified using M-mode.

**Gross histologic evaluation.** The RV wall, the left ventricular wall and the interventricular septum were dissected and the ratio of the right ventricle to left ventricle plus septum weight [RV/(LV+IVS)] was calculated as an index of RV hypertrophy. Wet lung weight was determined by weighing the lung tissue.

**Real time PCR.** Total RNA from lungs and RV were isolated using Trizol (Invitrogen) and reverse transcribed with gene specific primers using Omniscript RT kit (Qiagen). Controls were: (1) the reaction without reverse transcriptase; and (2) $H_2O$ instead of cDNA.

**Western blot analysis.** RV and lungs were homogenized at 4ºC in (mM): 150 NaCl, 50 Tris-HCl, 1 EGTA. 1 EDTA, 1 NaF, 1 PMSF, 1 $Na_3VO_4$, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate (pH 7.4) supplemented with Protease and Phosphatase Inhibitor cocktails (Roche). The samples were centrifuged at 12,000 g for 10 min and the supernatants were collected. Protein concentration was measured and 100 μg of total protein was loaded on a 4-20% gradient Tris-HCl-SDS polyacrylamide gel, electrotransferred to nitrocellulose paper, blocked with 5% non-fat dry milk in 20 mM of

TBS with 0.1% Tween and incubated with primary antibodies. Blots were then indirectly labelled using infrared fluorophore-conjugated anti-rabbit and anti-mouse secondary antibodies for 1 h, and visualized with the Odyssey™ Imaging System (Li-Cor). Equal loading of protein onto each lane in the gel was confirmed by Vinculin or GAPDH.

**Immunocytochemistry and imaging.** Lungs were perfused *in situ* via the trachea cannula with buffered formaldehyde (4% paraformaldehyde in PBS, pH 7.4) at a pressure of 25 $cmH_2O$ for 5 min as shown in our previous study[2]. Whole hearts and lungs were fixed in 4% paraformaldehyde (PFA) in 0.1M $Na_2HPO_4$ and 23 mM $NaH_2PO_4$ (pH 7.4) for 4 h on ice. The tissue was then immersed in ice-cold 20% sucrose overnight to cryoprotect the tissue, was mounted using OCT, and transversal 6-7 μm sections were obtained with a cryostat. Tissue sections were stained with immunofluorescence, immunoperoxidase, standard hematoxylin-eosin and Masson trichrome staining. The images were acquired using a light microscope (Axiovert 135, Zeiss Germany) or with a laser scanning confocal microscope (Olympus).

- **Immunofluorescence staining.** Heart and lung cross-sections (4-6 μm) were fixed in acetone for 15 minutes at –20°C. The sections were then washed with PBS+0.1% Triton three times, incubated with 10% normal goat serum in PBS+0.1% Triton for 30 min to block the background. The sections were then incubated with primary antibodies in PBS+0.1% Triton+ 1% normal goat serum at $4^oC$ overnight. The sections were then washed with PBS+0.1% Triton three times, incubated with the appropriate secondary antibodies in PBS+0.1% Triton+ 1% normal goat serum at room temperature for 1 h. After washing the secondary antibodies with PBS+0.1% Triton three times, the sections were incubated with wheat germ agglutinin (WGA, 1:200 dilution) or TO PRO (1:1000

dilution, Invitrogen) in PBS+0.1% Triton+ 1% normal goat serum for 1 h at room temperature. The sections were then washed with PBS three times and mounted using Prolong gold (Molecular Probes) for imaging.

**-Immunoperoxidase staining.** Endogenous peroxidase activity was inhibited by incubating the lung sections with 0.3% $H_2O_2$ in PBS for 20 min at room temperature followed by washing with PBS thrice. The lung sections were then incubated with 10% normal goat serum in PBS containing 0.1% Triton for 30 min at room temperature to block the nonspecific binding. The sections were incubated with the appropriate primary antibodies in PBS+0.1% Triton+ 1% normal goat serum at $4^oC$ overnight, and washed 3 times with PBS+0.1% Triton. The sections were incubated with HRP-conjugated secondary antibody (1:200 dilution) in PBS+0.1% Triton+ 1% normal goat serum, for 1 h at room temperature, washed with PBS+0.1% Triton three times, and stained with 3,3'-diaminobenzidine (DAB) as a substrate (10X DAB solution + stable buffer) for 5-10 min. The DAB was rinsed with $dH_2O$ and stained with hematoxylin for 1 min, washed under running tap water for 10 min. Dehydration was then performed by incubating in 50%, 70%, 96%, 100% ethanol and xylene. Sections were mounted using Permount (Fisher Scientific) and examined under a light microscope.

**Histological Analyses**

Pulmonary arteriolar medial wall thickness was determined in lung sections using α-smooth muscle actin staining of pulmonary arterioles. The wall thickness of arterioles was quantified using ImageJ software by measuring the maximum thickness of arteriolar walls. The values are normalized to control and shown as % increase in wall thickness compared to control, where control is 100%.

Pulmonary angiogenesis was quantified by counting the number of small blood vessels (up to 50μm) stained with von Willebrand Factor in at least 10 high power fields (HPF) per section from 4 rats per group.

Percent tissue fibrosis in lung sections was determined using Masson trichrome stain for collagen with the use of a grid that divided the field of view into 100 squares, the number of collagenous tissue (blue stain) at the 100 intersection points in the grid was scored as 1 (present) or 0 (absent). Results are expressed as the percentage occupied by fibrosis to the total area examined.

**Reagents.** Primary antibodies used were: anti-smooth muscle actin (Thermo Scientific, 1:200), anti-PECAM (CD31, Millipore, 1:200), anti-von Willebrand Factor (Abcam, 1:200), anti-ED1 (CD68, Millipore, 1:200), and anti-vinculin (V9131, Sigma, 1:10,000) . Secondary antibodies used were goat anti-rabbit IgG-AlexaFluor-488 (1:1000) and goat anti-mouse IgG-AlexaFluor-568 (1:1000) for immunofluorescence, sheep anti-mouse IgG-HRP (1:200), and sheep anti-rabbit IgG-HRP (1:200) for immunoperoxidase stainings, goat anti-rabbit IgG-AlexaFluor-680 (1: 100,000, Invitrogen) and goat anti-mouse IgG-IR Dye 800 CW (1: 100,000, Odyssey, LI-COR) for western immunoblotting.

**Statistical Analysis.** One-way and two-way ANOVA tests were used to compare between groups and within the same group at different time points using SPSS13.0 for Windows. When significant differences were detected, individual mean values were compared by *post-hoc* tests, which allowed for multiple comparisons. P values less than 0.05 were considered statistically significant. Values are expressed as mean±SE.

## Results

### Estrogen reverses pulmonary inflammation and fibrosis in female rats with PH

It has been shown in previous studies that female rats develop less severe PH in response to chronic hypoxia or MCT and estrogen replacement prevents PH in female animals. We also found that females exhibited less severe PH 21 days after MCT and, as in males, a 10-day E2 therapy started at day 21 reversed RVSP and RVEF. .We examined whether reversal of pulmonary inflammation and fibrosis by E2 participated in E2-induced rescue of PH in female rats. Pulmonary ED1 positive macrophage/monocytes were significantly increased in PH lungs (47±1 cells per field in PH *vs.* 12±0.7 in CTRL, Fig. E2). E2 reversed the increase in inflammatory cells associated with PH in the lungs (13±0.7 in E2) (Fig. E3). Interestingly, the increase in ED1 cells in females with PH was still significantly lower than that in the males with PH. Next, we looked at the pulmonary fibrosis in female lungs. Pulmonary fibrosis was significantly increased in PH lungs of female rats (12±1 cells per field in PH *vs.* 2.4±0.2 in CTRL, Fig. E2) and to a lesser extent than in PH males..E2 reversed the increase in pulmonary fibrosis associated with PH in the lungs of females (2.5±0.05 in E2) (Fig. E3).

### Estrogen stimulates pulmonary angiogenesis in female rats with PH

We next examined whether stimulation of pulmonary neoangiogenesis by E2 participated in E2-induced rescue of PH in female rats. Pulmonary vessel density was significantly reduced by ~2.5-fold in PH (7±0.4 vessels per HPF in PH *vs.* 16±0.5 in CTRL, Fig. E3). E2 reversed the loss of blood vessels associated with PH in lung (14.8±1.13 in E2) .

**Supplemental Table E1.** Right ventricular hemodynamic parameters measured by M-mode echocardiography.

| | CTRL (day 30) | Adaptive RVH (day 14) | PH (day 21) | RVF (day 30) | E2 (day 30) |
|---|---|---|---|---|---|
| | 5 rats | 8 rats | 8 rats | 8 rats | 10 rats |
| **RV WT (mm)** | | | | | |
| Diastolic | 0.6±0.02$^{\#\dagger\dagger\wedge\wedge}$ | 1.09±0.04$^{*\P}$ | 1.30±0.13$^{**\P\P}$ | 1.22±0.04$^{**\P}$ | 0.81±0.05$^{\#\dagger\dagger\wedge}$ |
| Systolic | 0.83±0.01$^{\#\dagger\dagger\wedge\wedge}$ | 1.44±0.05$^{*\dagger\P}$ | 1.71±0.16$^{**\#\P\P}$ | 1.55±0.06$^{**\P}$ | 0.99±0.04$^{\#\dagger\dagger\wedge}$ |
| **RV diameter** | | | | | |
| **(mm)** | 1.63±0.04$^{\#\dagger\dagger\wedge\wedge}$ | 1.98±0.03$^{*\dagger\wedge\wedge\P}$ | 2.29±0.09$^{**\#\wedge\wedge\P\P}$ | 3.68±0.11$^{**\#\#\dagger\dagger\P\P}$ | 1.69±0.04$^{\#\dagger\dagger\wedge\wedge}$ |
| Diastolic | 1.10±0.05$^{\dagger\dagger\wedge\wedge}$ | 1.21±0.02$^{\dagger\dagger\wedge\wedge}$ | 1.88±0.07$^{**\#\#\wedge\wedge\P\P}$ | 3.21±0.18$^{**\#\#\dagger\dagger\P\P}$ | 1.17±0.02$^{\dagger\dagger\wedge\wedge}$ |
| Systolic | | | | | |
| **RVEF (%)** | 65.1±1.7$^{\#\dagger\dagger\wedge\wedge}$ | 71.1±1.6$^{*\dagger\dagger\wedge\wedge\P\P}$ | 39.0±0.6$^{**\#\#\wedge\wedge\P\P}$ | 30.4±1.7$^{**\#\#\dagger\dagger\P\P}$ | 61.5±0.8$^{\#\#\dagger\dagger\wedge\wedge}$ |
| **RVFS (%)** | 33.7±1.3$^{\#\#\dagger\dagger\wedge\wedge}$ | 38.6±1.1$^{**\dagger\dagger\wedge\wedge\P\P}$ | 17.7±0.3$^{**\#\#\wedge\wedge\P\P}$ | 12.9±0.3$^{**\#\#\dagger\dagger\P\P}$ | 30.6±0.6$^{\#\#\dagger\dagger\wedge\wedge}$ |

Values are means ± SEM. The criteria used to define adaptive RVH were increase in RV wall thickness (RV WT) and preserved RV ejection fraction (RVEF). CTRL, control; RVH, right ventricular hypertrophy; PH, pulmonary hypertension; RVF, right ventricular failure; E2, estrogen treatment; RV WT, right ventricular wall thickness; RVEF, right ventricular ejection fraction; RVFS, right ventricular fractional shortening. $^{*}P<0.05$ *vs.* CTRL, $^{**}P<0.001$ *vs.* CTRL; $^{\#}P<0.05$ *vs.* MCT Day 14, $^{\#\#}P<0.001$ *vs.* MCT Day 14; $^{\dagger}P<0.05$ *vs.* PH, $^{\dagger\dagger}P<0.001$ *vs.* PH; $^{\wedge}P<0.05$ *vs.* RVF, $^{\wedge\wedge}P<0.001$ *vs.* RVF; $^{\P}P<0.05$ *vs.* E2 and $^{\P\P}P<0.001$ *vs.* E2.

References

E1. Cowan, K. N., A. Heilbut, T. Humpl, C. Lam, S. Ito, and M. Rabinovitch. 2000. Complete reversal of fatal pulmonary hypertension in rats by a serine elastase inhibitor. *Nat.Med.* 6:698-702.

E2. Umar, S., Y. P. de Visser, P. Steendijk, C. I. Schutte, E. H. Laghmani, G. T. M. Wagenaar, W. H. Bax, E. Mantikou, D. A. Pijnappels, D. E. Atsma, M. J. Schalij, E. E. van der Wall, and A. van der Laarse. 2009. Allogenic stem cell therapy improves right ventricular function by improving lung pathology in rats with pulmonary hypertension. *Am J Physiol Heart Circ Physiol* 297:H1606-H1616.

E3. Sano, M., T. Minamino, H. Toko, H. Miyauchi, M. Orimo, Y. Qin, H. Akazawa, K. Tateno, Y. Kayama, M. Harada, I. Shimizu, T. Asahara, H. Hamada, S. Tomita, J. D. Molkentin, Y. Zou, and I. Komuro. 2007. p53-induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload. *Nature* 446:444-448.

E4. Chan, K. L., P. J. Currie, J. B. Seward, D. J. Hagler, D. D. Mair, and A. J. Tajik. 1987. Comparison of three Doppler ultrasound methods in the prediction of pulmonary artery pressure. *J.Am.Coll.Cardiol.* 9:549-554.

**Figure legends**

**Figure E1. E2 rescues severe PH by restoring RVP and RVEF and improving the weight gain. A.** RVP measured directly by inserting a catheter into the RV right before sacrifice and **B.** RVEF measured from M-mode echocardiography at end of each experiment (day 30 for CTRL, RVF and E2; day 21 for PH and day 42 for E2-W). **C.** Body weight gain from day 21 to day 30 in CTRL (white bar), RVF (black bar) and E2 (gray bar), $^{*}P<0.05$ *vs.* CTRL, $^{**}P<0.001$ *vs.* CTRL; $^{\dagger}P<0.05$ *vs.* PH, $^{\dagger\dagger}P<0.001$ *vs.* PH; $^{\wedge}P<0.05$ *vs.* RVF, $^{\wedge\wedge}P<0.001$ *vs.* RVF; $^{\#}P<0.05$ *vs.* E2.

**Figure E2. E2 therapy reverses lung inflammation and fibrosis in female rats with PH. A.** Magnified view (60X) of lung sections stained for ED1 in CTRL, PH and E2 in female rats showing ED1 postitive macrophage/monocytes (brown) in PH group. **B**. Quantification of ED1 positive cells (the alveolar macrophages as well as the perivascular macrophages) per field (20X) in CTRL, PH and E2 treated females. **C.** Masson trichrome staining of lung sections in CTRL, PH and E2 females, blue color indicates fibrosis. **D.** Quantification of lung fibrosis showing % lung fibrosis in CTRL, PH and E2 female groups. $^{**}P<0.001$ *vs.* CTRL; $^{\dagger\dagger}P<0.001$ *vs.* PH (n=3 animals per group).

**Figure E3. E2 therapy restores PH-induced loss of blood vessels in the lungs of female rats. A.** Single confocal images of lung sections of female rats immunostained for von Willebrand Factor (green, upper panel), overlay of von Willebrand Factor and Nuclei (stained red with TO PRO, middle panel) and at higher display magnification of the respective fields (lower panel). **B.** Quantification of vessels/high power field in CTRL (black bar), PH (red) and E2 (blue). $^{**}P<0.001$ *vs.* CTRL; $^{\dagger\dagger}P<0.001$ *vs.* PH (n=3 animals per group).

**Figure E4. Proposed mechanisms underlying E2-induced rescue of PH.** Injection of MCT induced PH in healthy rats after 21 days, which led to RVF if left untreated. E2 therapy starting at the PH stage not only prevented the transition to RVF, but also normalized the RV pressure and RVEF. Stimulation of cardiopulmonary angiogenesis together with suppression of lung inflammation and fibrosis by E2 are the key mechanisms in rescuing the lung and the heart.