Exogenous Estrogen Preserves Distal Pulmonary Arterial Mechanics and Prevents Pulmonary Hypertension in Rats

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To the Editor:

17β-estradiol (E2), the most abundant female sex steroid, has been implicated in the development and progression of pulmonary arterial hypertension (PAH) (1, 2). While some studies have found that E2 drives PAH progression, others found a protective effect (3-6). The majority of these studies used right ventricular (RV) end systolic or mean pulmonary artery pressure as the primary metric of pulmonary vascular function and RV afterload. However, these endpoints are affected by RV function and do not fully capture RV afterload (7, 8). In order to accurately determine the impact of E2 in PAH, a comprehensive assessment of pulmonary vascular function, including multipoint pressure-flow relationships and impedance to flow at physiological frequencies, is necessary (8, 9). To date, no studies have quantified mechanical pulmonary vascular function in these ways in PAH. A better understanding of the effects of E2 on mechanical pulmonary vascular function will help identify why prior studies yielded seemingly discrepant results and may help solve the “estrogen puzzle” of PAH. We investigated the impact of endogenous and exogenous E2 on pulmonary vascular mechanics in a rat model of PAH.

Healthy female Sprague-Dawley rats with cyclical endogenous E2 production (labelled “intact”), ovariectomized rats replete with exogenous E2 in a continuous manner (75 μg·kg⁻¹·day⁻¹ via subcutaneous pellets; labelled “OVX+E2”), or ovariectomized rats receiving vehicle (“OVX+Veh”) as previously described (3) were exposed to Sugen and hypoxia (SuHx) to generate experimental PH. Rats exposed to room air served as controls. Given the female predominance of PAH and to study effects of endogenous E2, we used only female rats. Seven weeks following SuHx exposure, RV systolic pressure (RVSP) was measured via closed-chest right heart catheterization (3). This protocol has been designed and well-characterized to
generate a chronic, progressive, and severe model of PAH. E2 repletion was initiated prior to SuHx exposure as a model of PAH prevention. Pulmonary vascular mechanics were subsequently assessed ex vivo via isolated lung perfusion with both pulsatile and steady flow at baseline and following treatment with rho kinase inhibitor, Y27632 (Y27, $10^{-5}$M). Y27 was used to eliminate persistent vasoconstriction. The main pulmonary artery (PA) and left atria (LA) were cannulated (10) and the perfusion protocol was adapted from our protocol developed for mice (11). Small variations in steady flow rate achieved were due to flow pump sensitivity to downstream impedance, which varied with individual animals, but did not affect distensibility calculations since flow ranges achieved were equivalent.

To analyze PA remodeling, Verhoeff-Van Giesson (VVG) immunohistochemical staining was performed on paraffin-embedded sections from lungs fixed with agarose-formalin (to a pressure of 23 cm of H$_2$O) (12). Lungs fixed in formalin did not undergo ex vivo perfusion testing nor were they treated with Y27. PA wall area was determined by calculating the ratio between the area defined by the internal lumen border and the external elastic layer (as identified by VVG staining) and then expressing this area as a percentage of the entire vessel area (determined by external elastic layer) in arteries $<200$ µm in diameter (identified by proximity to terminal bronchioles or alveolar ducts) (12). We employed 9 animals/experimental group. One animal in the intact SuHx group died prior to the terminal time point with symptoms of RV failure. Additional losses in data occurred due to technical factors with in vivo and ex vivo procedures. All data are presented as mean ± standard deviation. Two-way ANOVA was used to compare differences between groups; repeated measures ANOVA was used to evaluate the effect of Y27 on pulmonary vascular mechanics. While sample sizes for individual groups were small for some endpoints, combined sample sizes were sufficient for ANOVA to evaluate model
assumptions. Residual plots and normal probability plots were examined, and there was no
evidence for model assumption violations.

RVSP measured \textit{in vivo} increased following SuHx exposure in intact and OVX+Veh
groups. In contrast, \textit{in vivo} pressures were similar to normoxia (“Norm”) following SuHx in the
OVX+E2 group (\textbf{Figure 1A}). We next performed \textit{ex vivo} assessment of the opposition to
pulsatile flow across a range of oscillatory frequencies ranging from 0Hz (steady flow) well into
the physiological range of 15-20Hz (\textbf{Figure 1B}). We noted a significant increase in impedance at
0Hz ($Z_0$; measuring distal PA narrowing) and up to 10Hz (reflecting intermediate PA narrowing
and stiffening) following SuHx exposure in the intact and OVX+Veh groups (\textbf{Figure 1B}).
Interestingly, this was not seen in the SuHx - OVX+E2 group (\textbf{Figure 1B}). Increased impedance
following SuHx exposure was not seen at higher frequencies in any group. Consistent with this,
there was no difference in characteristic impedance ($Z_C$; reflecting proximal PA stiffness,
calculated as the average impedance from 5Hz to the highest frequency imposed [20Hz])
following SuHx exposure.

Steady flow evaluation of pulmonary vascular mechanics \textit{ex vivo} in isolated perfused
lungs showed increased transpulmonary gradient (TPG; determined as main PA pressure minus
LA pressure) following SuHx in intact and vehicle-treated OVX rats, while E2 repletion
prevented this increase (\textbf{Figure 1C}). Consistent with these findings, distal PA distensibility
determined from multipoint pressure-flow curves (13) decreased nearly 50% following SuHx
exposure in both the intact and OVX+Veh groups, whereas the OVX+E2 group exhibited
preserved distensibility (\textbf{Figure 1D}).

To determine the contribution of vasoconstriction to the increased resistance, increased
impedance, and decreased distensibility noted in the SuHx-intact and OVX+Veh groups, we
repeated the evaluation of pulmonary vascular mechanics after treatment with Y27. As expected, both groups demonstrated a decrease in $Z_0$ (Figure 2A), decrease in TPG (data not shown), and increase in distensibility (data not shown) in response to Y27. These findings demonstrate that vasoconstriction is a significant component of increased RV afterload following SuHx in the absence of continuous, exogenous E2 repletion.

To identify a structural correlate of the improvements in RVSP, $Z_0$, TPG and distensibility noted with E2 repletion in SuHx rats, we analyzed PA remodeling (Figure 2B&C). We found that E2 repletion was associated with a 60% reduction in PA wall area as compared to the intact and OVX+Veh groups.

This study is the first to comprehensively demonstrate that continuous, exogenous E2 treatment confers protection to pulmonary vascular mechanics in angioproliferative PAH in rats. In particular, this is the first investigation of pulsatile pulmonary vascular mechanics in SuHx-PH rats. We demonstrate distal PA narrowing with relative preservation of proximal PA mechanics. While exogenous E2 has previously been shown to reduce RVSP (14), we expand our prior observations beyond RVSP and now demonstrate the novel finding that E2 also attenuates PH-induced alterations in impedance and distensibility. This is important since RVSP can be confounded by other factors and since impedance and distensibility are critical determinants of RV adaptation in PAH. Impedance measurements and use of an *ex vivo* lung perfusion preparation allowed us to dissect for the first time how endogenous and exogenous E2 affects various pulmonary vascular compartments. Importantly, continuous exogenous E2 was superior to both physiologically cyclical, endogenous estrogen (intact females) and little to no estrogen (ovariectomized females) in limiting PH development. While several recent studies from our group and others demonstrated a protective effect of E2 on RV function (1, 3, 4), we
now identify a novel and direct protective effect of E2 on distal PA structure and function. In particular, exogenous, continuous E2 repletion attenuated the distal PA remodeling, increase in $Z_0$, increase in TPG, and decrease in distensibility induced by SuHx in intact and OVX female rats. A prior study performed in female mice found that E2 attenuated proximal, but not distal, PA remodeling (15), which highlights potentially important differences between species. The previously demonstrated beneficial effects of E2 on RV function and the currently demonstrated protective effects of E2 on distal PA structure and function suggest that exogenous E2 repletion could be a potent tool to combat PAH-induced changes in several compartments of the cardiopulmonary system. Future studies will focus on employing rescue protocols and male rats. Our findings highlight a key role for E2 in attenuating PA remodeling and dysfunction in PAH and identify the distal PA as the target of E2’s vasculoprotective effects in SuHx-PH rats. This provides a rationale and basis for further studies to understand the complex mechanisms by which E2 regulates pulmonary vascular mechanics.
References:
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Figure Legends:

**Figure 1:** Exogenous estrogen treatment is protective for pulmonary artery mechanics following Sugen/Hypoxia (SuHx) exposure.

A, Right ventricular systolic pressure (RVSP) was measured via closed-chest right heart catheterization in intact females (intact) and ovariectomized females with continuous E2 repletion (OVX+E2) or placebo (OVX+Veh) prior to either normoxia (Norm) or SuHx exposure, n=3-8 per group. B, Pulmonary vascular impedance magnitude (Z) was measured (logarithmic scale on y-axis) for varying pulsatile flow frequencies in intact, OVX+E2, and OVX+Veh in isolated rat lungs following either Norm or SuHx exposure, n=4-9 per group. C, Transpulmonary gradient (TPG) was measured for varying flow rates for intact, OVX+E2, OVX+Veh isolated rat lungs following either Norm or SuHx exposure, n=4-9 per group. D, Distal pulmonary artery distensibility for intact, OVX+E2, and OVX+Veh isolated rat lungs following Norm or SuHx exposure, n=4-9 per group. *p<0.05 vs Norm & SuHx-OVX+E2.

**Figure 2:** Exogenous estrogen prevents vasoconstriction and pulmonary arterial remodeling following Sugen/Hypoxia (SuHx) exposure.

A, Pulsatile perfusion of isolated rat lungs in intact females (intact) and ovariectomized females with continuous E2 repletion (OVX+E2) or placebo (OVX+Veh) prior to either normoxia (Norm) or Hypoxia-Sugen (SuHx) exposure demonstrates increased impedance magnitude (Z) at zero Hertz (input resistance, Z₀) following SuHx exposure in intact and Veh group but not E2. Treatment with rho kinase inhibitor, Y27, showed strongest response in SuHx OVX+Veh group compared to intact or OVX+E2, n=4-9 per group, *p<0.05 vs. Norm, #p<0.05 vs. SuHx Intact & SuHx OVX+Veh, Sp<0.05 vs. no Y27. B, C, Pulmonary artery (PA) remodeling was assessed by
Verhoeff-van Giesson staining and subsequent determination of PA wall fraction in intact, OVX+E2, and OVX+Veh rat lungs following either Norm or SuHx exposure. PA wall fraction was determined by dividing PA wall area (area between blue line and red line) by total vessel area (total area outlined by red line). Red line denotes external elastic lamina, blue line denotes internal lumen border. Note decreased PA wall fraction in SuHx OVX+E2 vs. SuHx intact or SuHx OVX+Veh rat lungs. Size bars = 100 μm. B demonstrates representative images, C demonstrates quantification of PA wall fraction, n=3-5 rats per group; 20 vessels per rat were analyzed. **p<0.01 vs. Norm, #p<0.05 vs. SuHx intact & SuHx OVX+Veh.
Exogenous estrogen treatment is protective for pulmonary artery mechanics following Sugen/Hypoxia (SuHx) exposure. A, Right ventricular systolic pressure (RVSP) was measured via closed-chest right heart catheterization in intact females (intact) and ovariectomized females with continuous E2 repletion (OVX+E2) or placebo (OVX+Veh) prior to either normoxia (Norm) or SuHx exposure, n=3-8 per group. B, Pulmonary vascular impedance magnitude (Z) was measured (logarithmic scale on y-axis) for varying pulsatile flow frequencies in intact, OVX+E2, and OVX+Veh in isolated rat lungs following either Norm or SuHx exposure, n=4-9 per group. C, Transpulmonary gradient (TPG) was measured for varying flow rates for intact, OVX+E2, and OVX+Veh isolated rat lungs following either Norm or SuHx exposure, n=4-9 per group. D, Distal pulmonary artery distensibility for intact, OVX+E2, and OVX+Veh isolated rat lungs following Norm or SuHx exposure, n=4-9 per group. *p<0.05 vs Norm & SuHx-OVX+E2.
FIGURE 2

A

B

C

Input Resistance, $Z_0$ (mmHg min/mL)

- Normoxia+no drug
- Normoxia+Y27
- SuHx+no drug
- SuHx+Y27

**Normoxia intact**

**Normoxia OVX + Veh**

**Normoxia OVX + E2**

**SuHx intact**

**SuHx OVX + Veh**

**SuHx OVX + E2**

PA wall fraction

- Norm
- SuHx