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Direct and indirect protection of right ventricular function by estrogen in an experimental model of pulmonary arterial hypertension

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[1]*Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, Wisconsin;* [2]*Department of Biostatistics and Medical Informatics, University of Wisconsin-Madison, Madison, Wisconsin;* [3]*Department of Medicine, University of Wisconsin-Madison, Madison, Wisconsin*

Liu A, Schreier D, Tian L, Eickhoff JC, Wang Z, Hacker TA, Chesler NC. Direct and indirect protection of right ventricular function by estrogen in an experimental model of pulmonary arterial hypertension. *Am J Physiol Heart Circ Physiol* 307: H273–H283, 2014. First published June 6, 2014; doi:10.1152/ajpheart.00758.2013.—Pulmonary arterial hypertension (PAH) results in right ventricular (RV) dysfunction and failure. Paradoxically, women are more frequently diagnosed with PAH but have better RV systolic function and survival rates than men. The mechanisms by which sex differences alter PAH outcomes remain unknown. Here, we sought to study the role of estrogen in RV functional remodeling in response to PAH. The SU5416-hypoxia (SuHx) mouse model of PAH was used. To study the role of estrogen, female mice were ovariectomized and then treated with estrogen or placebo. SuHx significantly increased RV afterload and resulted in RV hypertrophy. Estrogen treatment attenuated the increase in RV afterload compared with the untreated group (effective arterial elastance: 2.3 ± 0.1 mmHg/μl vs. 3.2 ± 0.3 mmHg/μl), and this was linked to preserved pulmonary arterial compliance (compliance: 0.013 ± 0.001 mm²/mmHg vs. 0.010 ± 0.001 mm²/mmHg; $P < 0.05$) and decreased distal muscularization. Despite lower RV afterload in the estrogen-treated SuHx group, RV contractility increased to a similar level as the placebo-treated SuHx group, suggesting an inotropic effect of estrogen on RV myocardium. Consequently, when compared with the placebo-treated SuHx group, estrogen improved RV ejection fraction and cardiac output (ejection fraction: 57 ± 2% vs. 44 ± 2% and cardiac output: 9.7 ± 0.4 ml/min vs. 7.6 ± 0.6 ml/min; $P < 0.05$). Our study demonstrates for the first time that estrogen protects RV function in the SuHx model of PAH in mice directly by stimulating RV contractility and indirectly by protecting against pulmonary vascular remodeling. These results underscore the therapeutic potential of estrogen in PAH.

cardiopulmonary hemodynamics; estrogen; sex difference; rv catheterization; pulmonary artery hypertension

PULMONARY ARTERIAL HYPERTENSION (PAH) is a female-dominated, devastating disease, characterized by extensive pulmonary arterial remodeling that increases right ventricular (RV) afterload (30). The RV functional response to increased afterload determines exercise capacity and survival in PAH patients (25), but the mechanisms and determinants of RV adaptation, dysfunction, and, finally, failure are unknown. Epidemiologically, despite the high incidence (a female-to-male ratio of 4:1) (1), female PAH patients have preserved RV function and improved survival rates compared with males (12, 14). Accumulating evidence suggests that estrogen underlies the sex differences in outcomes between male and female PAH patients (19, 35, 39, 40). However, how estrogen modulates the differences in RV response to changes in pulmonary artery (PA) structure and function remains elusive. Studying the mechanisms by which estrogen may protect RV function may contribute to therapy that targets RV function in men and women with PAH.

Recently, the protection estrogen provides to the RV in PAH has been recapitulated in preclinical animal models (19, 24). Lahm and colleagues (19) showed that estrogen prevented RV structural and functional deterioration in a rat model of hypoxic pulmonary hypertension (HPH). Nadadur and colleagues (24) showed that estrogen therapy reversed RV remodeling and prevented RV progression to failure in rats treated with monocrotaline. With elegant experimental designs, these studies have demonstrated that estrogen limits RV functional and structural remodeling, which is consistent with clinical observations (40). However, the mechanisms by which estrogen modulates RV structural remodeling are contradictory. For example, estrogen has been reported to prohibit or promote RV angiogenesis (19, 24). In addition, it remains unclear whether estrogen directly impacts the RV myocardium independent of its effects on RV afterload, because the RV function metrics used in these prior studies were load dependent (19, 24).

Our goals are to determine the role of estrogen in RV functional remodeling in response to PAH and to explore the underlying functional mechanisms of action of estrogen. We hypothesize that estrogen preserves RV function both by indirectly attenuating RV overload and by directly stimulating RV contractility. To test our hypothesis, we exposed ovariectomized female mice treated with estrogen to PAH created via a combination of 21 days of chronic hypoxia and weekly injection of the VEGF receptor inhibitor SU5416 (SuHx), developed by Ciuclan and colleagues (5). We recently demonstrated that SuHx-induced PAH leads to progressive RV dysfunction in male mice (43) by using a right heart catheterization technique to measure RV pressure and volume simultaneously (37). This approach enables us to assess the effects of estrogen

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on RV function independent of loading conditions. We found that estrogen protects RV function in an early stage of SuHx-induced PAH and confirmed our hypotheses that the protective mechanisms of estrogen are both stimulation of RV contractility and attenuation of RV overload.

METHODS

Animal preparations. Female C57BL/6 mice, 9 to 10 wk old, were ovariectomized (OVX) by a commercial vendor (Jackson Laboratory) to eliminate natural fluctuations in estrogen levels. After 3 to 4 wk, to ensure depletion of endogenous estrogen stores, mice were implanted subcutaneously with estradiol-17β pellets (0.1 mg per 21-day release; Innovation Research of America) to achieve a mean circulating estrogen level equivalent to the peak physiological estrogen level to obtain a maximum effect as done previously (28). Some OVX mice were implanted subcutaneously with placebo pellets as control for the effects of estrogen. Immediately after the pellet implantation, some mice ($n = 28$ mice treated with estrogen and $n = 28$ treated with placebo) were injected intraperitoneally with the VEGF receptor inhibitor SU5416 at 20 mg/kg weekly and exposed to normobaric hypoxia (10% O_2) for 21 days (SuHx). Other OVX mice ($n = 19$ treated with estrogen and $n = 19$ treated with placebo) were not injected with SU5416 and were kept in room air conditions for 21 days as control for the effects of SuHx. Of these animals, one group of mice (SuHx and control, estrogen and placebo treated, $n = 10$ each) were used for ultrasound and RV pressure and volume measurements; a second group of mice (SuHx only, estrogen and placebo treated; $n = 9$ each) were used for PA pressure measurement; and a third group of mice (all 4 conditions; $n = 9$ each) were used for PA stiffness measurements. All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Ultrasound measurements. In the first group of mice, transthoracic echocardiography was performed to examine left ventricle (LV) function and PA morphology in vivo. As described previously (9), mice were ventilated with 1% isofluorane + 99% O_2 and maintained on a heated platform for measurements of LV diameters from M-mode images obtained in a parasternal long-axis view using the leading edge-to-leading edge convention (Visual Sonics Vevo 770 ultrasonograph with a 30-MHz transducer). All parameters were measured over at least three consecutive cardiac cycles and averaged. LV fractional shortening was calculated from the LV diameter (LVD) at diastole (d) and systole (s) as [(LVDd − LVDs)/LVDd] × 100 and ejection fraction (EF) as [(LVVd − LVVs)/LVVd] × 100, where LVV is the LV volume estimated as $(LVD)^3 \times 7.0/(2.4 + LVD)$ at either systole or diastole. PA diameter was measured just distal to the pulmonary valve. Care was taken to measure PA diameter at its widest during the cardiac cycle. Mice were then allowed to recover before pressure and volume measurements were obtained.

RV pressure and volume measurement. RV pressure and volume (PV) were measured simultaneously following the procedures described by Tabima et al. (37). Briefly, mice were anesthetized with urethane (2.0 mg/g), intubated, and ventilated at room air with a tidal volume of ~250 µl and a respiratory rate of 200 breaths/min. The chest was opened, and a 1.2F admittance PV catheter was inserted into the RV to acquire PV loops. Signals were recorded at 1,000 Hz. After baseline recordings, a brief vena cava occlusion (VCO) was performed to alter venous return. Systemic pressure was monitored at the aortic arch via the right carotid artery. To examine the cardiac reserve, PV loops were also measured in mice ventilated with 10% O_2 acutely to create an additional increase in RV afterload.

Well-established RV function parameters were derived from baseline RV-PV loops, including RV end-systolic pressure (P_{es}), stroke volume, cardiac output (CO), EF, stroke work (SW), RV maximal and minimal derivative of pressure (dP/dt_{max} and dP/dt_{min}), relaxation factor (τ), and chamber compliance (3). To account for weight changes, cardiac index (CI) was calculated as CO normalized by body weight (BW). Load-independent indexes of systolic function such as preload recruitable SW (PRSW) and end-systolic elastance (E_{es}) were derived from PV loops with vena cava occlusion. To assess RV afterload and ventricular-vascular coupling (VVC) efficiency, effective arterial elastance (E_a), and E_{es}/E_a were calculated using standard methods (15, 31). Pressure-volume area (PVA; an estimation of myocardial oxygen consumption), external work, and ventricular mechanical efficiency were estimated based on the baseline RV pressure-volume relations as previously established (26). Total pulmonary vascular resistance (tPVR = P_{es}/CO) was used to estimate the resistance of pulmonary vasculature (the static component of RV afterload).

PA pressure measurement. In the second group of mice, PA pressure was measured following the procedures described by Tabima et al. (38). Briefly, after a mouse was anesthetized, intubated, and ventilated with room air, the chest was opened to expose the RV. A 1.0F pressure catheter (Millar Instruments, Houston, TX) was then inserted into the RV and advanced into the main PA just distal to the pulmonary valve. Maximal and mean PA pressure and pulse pressure (PP) derived from the pressure waveform were obtained.

Mechanical test on isolated proximal PA. In the third group of mice, proximal PA stiffness was measured ex vivo. Following the procedures detailed previously (42), immediately after euthanasia with pentobarbital (150 mg/kg ip), extralobar left PAs were excised from the pulmonary trunk to the first branches and cleared of surrounding tissue under a dissecting microscope. Each left PA was mounted between two aligned cannulas in a vessel chamber with no-flow setup. Pressure transducers were mounted in-line with the cannulas. Superfusate was continuously circulated and maintained at 37°C. Neither the perfusate nor the superfusate contained calcium or magnesium to eliminate the potentially confounding effects of smooth muscle cell (SMC) tone.

Before mechanical testing, PAs were stretched 140% axially to approximate in vivo stretch ratio and to prevent bulking at in vivo pressures. Vessels were allowed 30 min to equilibrate and then preconditioned from 5 to 50 mmHg at 1 Hz for at least 10 cycles. Cyclic, sinusoidal pressurization from 10 to 50 mmHg was then imposed on the left PA at a physiologically relevant frequency (10 Hz). Pressure and diameter data were recorded simultaneously using the in-line pressure transducers and a charge-coupled device camera connected to an inverted microscope at 4× magnification. Compliance was calculated as ΔA/ΔP, where ΔA is the change in cross-sectional area measured optically (via transillumination) due to the change in pressure ΔP from 10 to 50 mmHg.

Measurement of plasma estrogen concentration. After all hemodynamic measurements were completed in the first group of mice, ~1 ml blood was extracted to measure hematocrit (Hct) and, in a subset of these animals ($n = 5$ treated with estrogen; $n = 5$ treated with placebo), the level of plasma estrogen. The plasma estrogen concentration was determined in duplicate using a rodent estradiol ELISA kit (Calbiotech).

Histology. Immediately after hemodynamic measurements in the first group of mice, RV free wall and LV and septum (LV + S) were harvested, dissected, and weighed, separately. RV weight normalized by the BW and Fulton index (RV/LV + S) were used as indexes of RV hypertrophy. The uterus was also dissected and weighed to confirm the effects of exogenous estrogen.

Lung and PA tissues used for histology were fixed in 10% formalin and embedded in paraffin. Lung and PA sections of 5 µm were stained with hematoxylin and eosin for overall morphology, Picro Sirius Red for detection of collagen deposition, Verhoeff-Van Gieson stain for determination of medial thickening, and smooth muscle actin (Abcam, Cambridge, MA) for visualizing arterial muscularization. Degree of collagen deposition of proximal PA was characterized using two metrics: collagen area fraction defined as the ratio of the area positive for collagen divided by the total tissue area (17) and collagen thickness defined as the collagen area fraction multiplied by PA wall

thickness (17). Percentage of medial thickness, the medial layer normalized by the total wall thickness, was used as a measure for medial layer thickening of PAs ranging 20 μm to 300 μm. Arterial muscularization was measured as the number of α-SMA-positive vessels less than 80 μm in diameter per field of view at 20× power. At least five fields of view were examined from one histological section of each lung. Values from these five fields of view were averaged to obtain a single measurement for each lung (i.e., each mouse).

Hydroxyproline assay. The collagen content in RV was determined using a hydroxyproline (OHP) assay, as previously described (6, 16). Briefly, after RV tissue was homogenized and digested with papain (Worthington Biotech), tissue homogenate was hydrolysized with alkali. The free hydroxyproline in hydrolyzates were determined with a standard curve generated using known amounts of trans-4-hydroxy-L-proline. The degree of collagen deposition in RV was measured as the collagen content normalized by the total protein, determined with a bicinchoninic acid protein assay kit (Pierce Biotechnology).

Statistical analysis. All results were presented as means ± SE. The analysis of the hemodynamic data was conducted using a linear mixed effects model with repeated measures (on room air and acute hypoxia ventilation). The main effects and two-way interaction effects between exposure conditions (SuHx vs. control and estrogen vs. placebo) were included in the model. Two-way ANOVA was conducted to analyze the morphological (including body and heart weights and ratios and Hct), PA pressures, PA compliance, and histological data. Holm's step-down procedure was used to adjust for multiple comparisons. All P values were two-sided, and $P < 0.05$ was used to define statistical significance. Data analysis was conducted using SAS software (SAS Institute, Cary, NC) version 9.2.

RESULTS

Plasma estrogen level changes. The plasma level of estrogen measured with ELISA in the estrogen-treated group was 209 ± 21 pg/ml, slightly higher than the physiological levels of estrogen in mice as described previously (80–200 pg/ml) (28, 34). As expected, the plasma level of estrogen in the placebo-treated mice (8.8 ± 0.3 pg/ml) was at a similar level of male mice (11). The uterine weight in the estrogen-treated mice (102 ± 5 mg) was significantly higher than that in the placebo group (14 ± 2 mg) and confirmed the effects of plasma estrogen on tissues.

Morphologic changes. The BW was significantly decreased after SuHx exposure in both placebo- and estrogen-treated OVX female mice; the degree of weight loss was significantly less in the estrogen-treated group (Table 1). Accompanying the

Table 1. *Morphological parameters, hematocrit, and peak systolic arterial pressure in the systemic circulation*

	Control		SuHx	
	Placebo	Estrogen	Placebo	Estrogen
BW, g	24 ± 0.4	23.7 ± 0.4	19.9 ± 0.2*	21.4 ± 0.5*#
RV, mg	18.3 ± 0.6	17.9 ± 0.5	27.3 ± 1.8*	26.8 ± 1.1*
LV + S, mg	74.0 ± 1.6	69.7 ± 2.3	63.9 ± 2.0*	75.9 ± 3.2#
RV/BW, mg/g	0.76 ± 0.01	0.76 ± 0.01	1.37 ± 0.09*	1.25 ± 0.03*
LV + S/BW, mg/g	3.1 ± 0.05	3.0 ± 0.06	3.2 ± 0.10	3.5 ± 0.11*#
RV/LV + S, mg/mg	0.25 ± 0.01	0.26 ± 0.01	0.43 ± 0.02*	0.35 ± 0.01*#
Hct, %	45 ± 1	47 ± 1	65 ± 2*	62 ± 2*
Sys P_s, mmHg	98 ± 5	89 ± 2	95 ± 2	110 ± 4*#

Values are means ± SE. SuHx, SU5416-hypoxia; BW, body weight; RV, weight of right ventricle; LV + S, weight of left ventricle and septum; Hct, hematocrit; sys P_s, peak systolic arterial pressure measured in carotid artery. *$P < 0.05$ vs. control; #$P < 0.05$ vs. placebo.

weight loss after SuHx exposure, the weight of LV + S dropped in the placebo group. Estrogen treatment attenuated this decrease in LV + S weight. As a consequence, in the estrogen-treated group, LV + S normalized by BW unexpectedly increased after SuHx. The normalized weight of right ventricle (RV/BW) and the Fulton index (RV/LV + S) increased in both placebo- and estrogen-treated SuHx groups, indicating RV hypertrophy (Table 1). Note, because both BW and LV + S are significantly higher in the estrogen-treated SuHx group than in the placebo-treated SuHx group, we were unable to confirm that RV hypertrophy is lower in the estrogen-treated SuHx group compared with the placebo-treated SuHx group, as found previously in male rats (45).

Hematocrit changes. As expected, Hct increased in response to chronic hypoxia in both SuHx groups (Table 1). Previous studies in rats have shown that estrogen suppresses polycythemia in response to chronic hypoxia, leading to a smaller increase in Hct (4, 23). However, we did not observe this effect in our study.

RV hemodynamics measurement in mice ventilated with room air. RV P_{es} was significantly increased in both SuHx groups (Fig. 1), indicating pulmonary hypertension. The severity of SuHx-induced PAH in the placebo-treated OVX female mice was comparable with that created in male mice (of the same average BW) reported previously (43). Representative PV loops for the estrogen-treated control group, estrogen-treated SuHx group, and placebo-treated SuHx group are shown in Fig. 2. SuHx exposure significantly increased RV afterload measured by both E_a and tPVR in both the placebo- and estrogen-treated SuHx groups (Fig. 3). Correspondingly, RV contractility increased, as demonstrated by increases in the three preload independent contractility indexes (E_{es}, dP/dt_{max}-V_{ed}, and PRSW in Fig. 4 and Table 2). The increases in contractility effectively maintained VVC efficiency ($E_{es}/E_a >$ 0.5 in Fig. 4) and CO (Fig. 1). Interestingly, although the increase in RV afterload was significantly lower in the estrogen-treated SuHx group than that in the placebo-treated SuHx group, the increase in RV contractility was comparable between the two groups. Consequently, in the estrogen-treated SuHx group, CO and EF were significantly higher compared with the placebo-treated SuHx group (Figs. 1 and 4), suggesting that estrogen improved RV systolic function with SuHx-induced PAH.

To exclude an estrogenic effect on LV function that potentially contributes to the apparent increase in RV contractility and CO, we examined LV function in placebo and estrogen-treated SuHx groups using ultrasound before hemodynamic measurement. The ultrasound data did not show significant differences in LV EF or percent fractional shortening between the placebo- and estrogen-treated SuHx groups (data not shown), suggesting that the estrogen did not improve LV function. However, we cannot exclude potential estrogenic effects on the systemic vasculature that may have contributed to increased CO.

Indicators of RV diastolic function, dP/dt_{min} and τ, were significantly decreased by SuHx exposure (Table 2), consistent with a decrease of RV chamber compliance (Table 2). No significant differences in RV dP/dt_{min}, τ, or chamber compliance were found between placebo- and estrogen-treated groups.
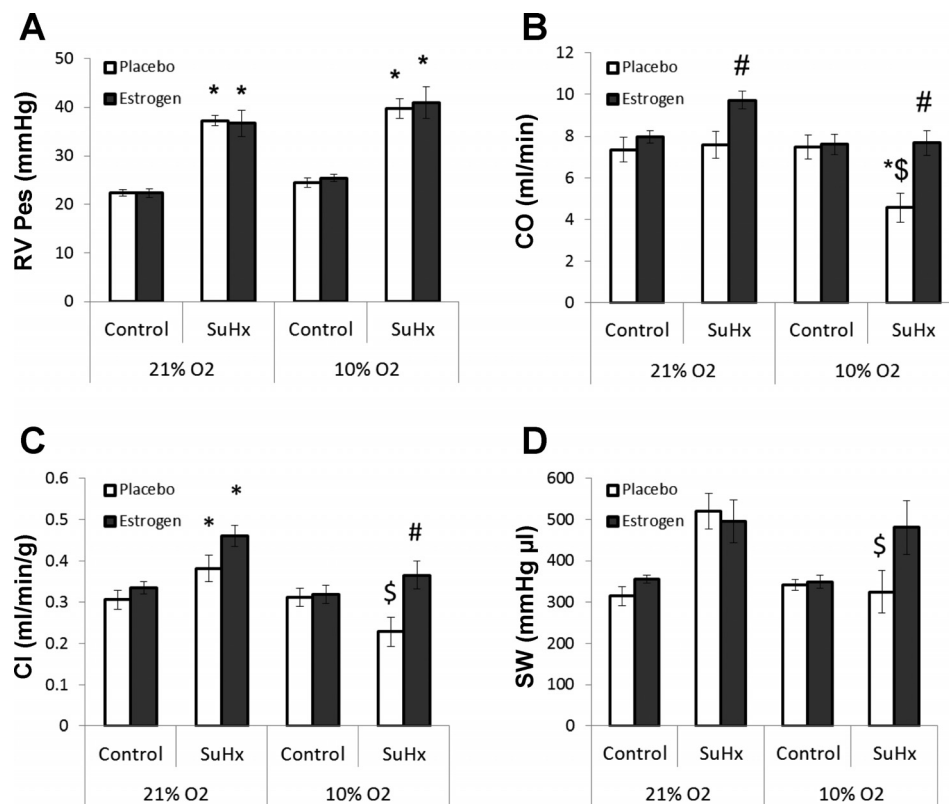
Fig. 1. Hemodynamic parameters in placebo- and estrogen-treated SU5416-hypoxia (SuHx) and control groups measured with 21% or 10% O_2 ventilation. *A*: right ventricular end-systolic pressure (RV P_{es}). *B*: cardiac output (CO). *C*: cardiac index (CI). *D*: stroke work (SW). *$P < 0.05$ vs. control; #$P < 0.05$ vs. placebo; $$P < 0.05$ vs. 21% O_2.

RV hemodynamic measurement in mice ventilated with 10% O_2. Hypoxic pulmonary vasoconstriction is an acute response of pulmonary arteries to low O_2, which results in a significant increase in vascular resistance and thus RV afterload. After hemodynamic measurements with room air ventilation, we exposed mice acutely to 10% O_2 to study the effects of estrogen on cardiac reserve. As expected, the acute exposure to low O_2 stimulated pulmonary hypoxic vasoconstriction (36) and increased RV afterload (E_a and tPVR) in all SuHx groups (Fig. 3). The relatively blunted increases in E_a and tPVR in the estrogen-treated SuHx group suggest that estrogen attenuates hypoxia-induced vasoreactivity. In response to increased afterload, all SuHx groups showed a corresponding increase in contractility (E_{es}, dP/dt_{max}-V_{ed}, and PRSW, see Fig. 4 and Table 3, although no statistical significance was reached in the estrogen-treated group and PRSW) such that VVC efficiency was preserved ($E_{es}/E_a > 0.5$; see Fig. 4).

RV P_{es} did not significantly increase in either control or SuHx groups in response to acute hypoxia (Fig. 1). Rather, CI significantly dropped in the placebo-treated SuHx groups. The decreases in CI and SW in response to the additional stress in the placebo-treated SuHx animals (Fig. 1) are a signature of cardiac dysfunction, suggesting that estrogen protects cardiac reserve in females with PAH. dP/dt_{min}, τ, and chamber compliance were not significantly different from those measured under room air (Table 3), suggesting that neither diastolic function nor chamber compliance were affected by acute hypoxia.

Ventricular and vascular elastance matching. To separate the direct (inotropic) effect of estrogen on the RV from the indirect, afterload-related (homeometric) effect of estrogen on the RV, we correlated E_a and E_{es} for all groups under both room and 10% O_2 ventilation (Fig. 5). We found a linear relationship between E_a and E_{es} among all control groups and the placebo-treated SuHx groups; the linear relationship suggests ventricular elastance is matched to vascular elastance via homeometric autoregulation. Interestingly, E_{es} for the estrogen-treated SuHx group, either with room air or 10% O_2 ventilation, was above the line, suggesting that the increase in E_{es} was higher than necessary to match the increase in afterload. This finding suggests a direct and stimulatory, i.e., inotropic, effect of estrogen on RV contractility.

To determine whether the inotropic effect of estrogen increases the energy demands on the RV, we calculated RV PVA, external work, and mechanical efficiency from pressure-volume loops. We found that when mice were ventilated in room air, SuHx-induced PAH increased the external work in both placebo- and estrogen-treated groups compared with the control groups; the external work tended to be higher in the estrogen-treated SuHx group compared with the placebo-treated SuHx group (Table 2). Correspondingly, PVA or oxygen consumption increased to a similar level in both placebo- and estrogen-treated SuHx groups. Although the mechanical efficiency was maintained in both SuHx groups, estrogen treatment significantly increased the mechanical efficiency compared with the placebo-treated SuHx group. When mice were ventilated with 10% O_2, both the external work and oxygen consumption significantly decreased such that mechanical efficiency was maintained in the placebo-treated SuHx group (Table 3). In the estrogen-treated SuHx group, the external work but not the oxygen consumption increased significantly compared with both the control and placebo-treated SuHx groups. Thus the mechanical efficiency tended to be higher in the estrogen-treated SuHx group compared with both
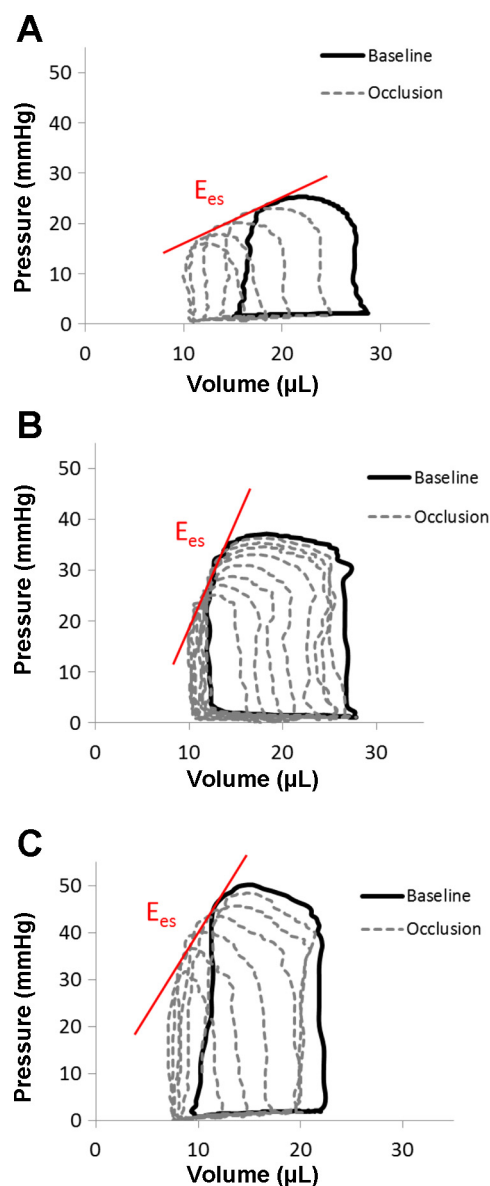
Fig. 2. Representative pressure-volume (PV) loops of the estrogen-treated control group (*A*), the estrogen-treated SuHx group (*B*), and the placebo-treated SuHx group (*C*). Note that PV loops of the placebo-treated control group are not shown because there are no significant differences between the estrogen- and placebo-treated control groups. E_{es}, end-systolic elastance.

RV and PA structural changes. SuHx exposure did not significantly increase collagen content in the RV compared with control groups (0.56 ± 0.05 μg/mg vs. 0.54 ± 0.03 μg/mg) by the hydroxyproline assay. In addition, no significant differences were found between estrogen- and placebo-treated SuHx groups (0.58 ± 0.05 μg/mg vs. 0.54 ± 0.07 μg/mg).

By semiquantitative analysis, collagen thickness but not collagen area fraction in the left PA was significantly lower in the estrogen-treated SuHx group compared with the placebo-treated SuHx group (Fig. 7), consistent with thinner left PA wall in the estrogen-treated SuHx group (27 ± 3 μm vs. 36 ± 2 μm; $P < 0.05$). Interestingly, via ultrasound main PA diameter was significantly larger in the estrogen-treated SuHx group compared with the placebo-treated SuHx group, likely due to the vasodilatory effect of estrogen (1.43 ± 0.11 mm vs. 1.33 ± 0.04 mm; $P < 0.05$).
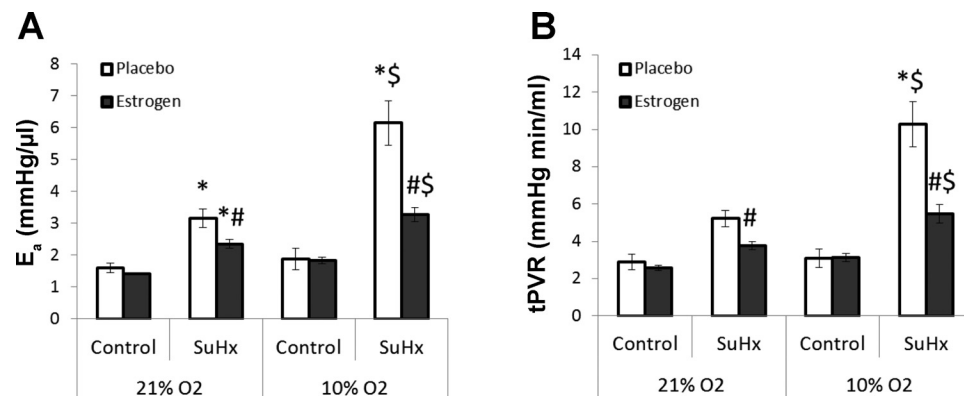
In the distal PAs ranging between 20 to 300 μm, no difference was found in the percentage of medial wall thickness between the estrogen-treated and placebo-treated SuHx groups (data not shown). In small distal PAs (<80 μm), SuHx exposure increased muscularization in the placebo-treated group compared with controls (Fig. 8), whereas estrogen treatment attenuated the SuHx-induced muscularization. No differences were found in small PAs between control groups.

DISCUSSION

We demonstrate for the first time that estrogen preserves RV function and attenuates PA remodeling in a mouse model of PAH. The protection of RV function was likely associated with both a direct effect of estrogen on RV contractility, which increased more than necessary to compensate for the increase in RV afterload, and an indirect effect, in which estrogen reduced RV afterload by limiting proximal PA stiffening and distal PA muscularization. Finally, estrogen treatment improved cardiac reserve, evident in an acutely low O_2 environment. Interestingly, the effects of estrogen only manifested in the SuHx group, suggesting that estrogen modulates the signaling pathways activated in response to pressure overload or VEGF receptor inhibition.

Estrogen is responsible for the sex differences in cardiac functional adaptation to PAH. Female PAH patients have better RV systolic function and thus better survival rates than male counterparts (12, 14). Evidence from postmenopausal women using hormone therapy suggests estrogen improves RV EF (40) and may underlie sex differences in RV response to PAH. Two recent studies using preclinical animal models of PAH also have shown that estrogen attenuated PAH and RV remodeling in male rats (19, 24, 39). Using OVX female mice, we showed that with SuHx exposure, the estrogen-treated group improved cardiac function (EF and CO). To dissect the contributions of estrogen and chromosomal differences to sex differences in RV functional adaption, we also compared RV functional and morphological parameters of the OVX female groups with prior data collected by us with the same techniques in a separate study on male control and SuHx-treated C57BL6 mice (43). From this comparison, we can observe that that male and placebo-treated OVX female groups had similar cardiac responses to SuHx exposure, including RV afterload (E_a), systolic function (EF and E_{es}), and hemodynamic alterations (P_{es} and CO). This finding further supports the conclusion that

the control and placebo-treated SuHx groups. These data suggest that increased inotropy by estrogen treatment is energetically efficient.

PA hemodynamic and functional measurements. Consistent with RV P_{es}, mean PA pressure was not significantly different between the placebo- and estrogen-treated SuHx groups (Fig. 6). The PP, which is associated with arterial compliance, was significantly higher in the placebo-treated SuHx group than the estrogen-treated SuHx group, suggesting that estrogen attenuated pulmonary vascular stiffening in PAH.

Our ex vivo mechanical tests on the left extralobar PA confirmed that PA compliance decreased in the placebo-treated SuHx group and estrogen treatment prevented the loss of compliance with PAH (Fig. 6).

Fig. 3. Right ventricular (RV) afterload in placebo- and estrogen-treated SuHx and control groups measured with 21% or 10% O_2 ventilation. *A*: effective arterial elastance (E_a). *B*: total pulmonary vascular resistance (tPVR). *$P < 0.05$ vs. control; #$P < 0.05$ vs. placebo; $$P < 0.05$ vs. 21% O_2.

estrogen is responsible for the sex differences in RV adaptation to PAH.

Estrogen directly enhanced RV contractility in response to PAH. Emerging evidence suggests that estrogen acts directly on RV myocardium independent of any effects on the pulmonary vasculature. Functional estrogen receptors have been identified in cardiac myocytes (7). In OVX female mice overexpressing the serotonin transporter (SERT+), estrogen treatment reduced RV hypertrophy despite higher RV systolic pressure and more pulmonary vascular remodeling than untreated mice (44). In one study on male rats treated with monocrotaline, estrogen protected rats from RV failure by enhancing RV angiogenesis, whereas in another study on male rats exposed to chronic hypoxia, estrogen attenuated hypoxia-induced RV angiogenesis (19, 24). However, in these two studies, the RV afterload in the untreated groups were different

(RVSP: 72 mmHg vs. 57 mmHg) and the hemodynamic context was not taken into account in the interpretation of results.

Similarly, if we ignore the effects of estrogen on vasculature, that is, focus exclusively on changes in E_{es}, we would erroneously conclude that estrogen has no effect on RV contractility. Instead, if we plot RV contractility index (E_{es}) versus RV afterload (E_a) (Fig. 5), we can observe that E_{es} is disproportionately higher than what is necessary to match E_a in the estrogen-treated SuHx groups. This suggests that estrogen has direct effects on RV contractility in addition to indirect, RV afterload-dependent effects. However, an increase in contractility is usually accompanied by an increase in myocardial oxygen consumption, which may worsen mortality in heart failure. From the PV loops, we calculated external work, PVA, and mechanical efficiency when the mice were ventilated to

Fig. 4. RV systolic function in placebo- and estrogen-treated SuHx and control groups measured with 21% or 10% O_2 ventilation. *A*: ejection fraction (EF). *B*: E_{es}. *C*: ventricular-vascular coupling efficiency (E_{es}/E_a). *$P < 0.05$ vs. control; #$P < 0.05$ vs. placebo; $$P < 0.05$ vs. 21% O_2.
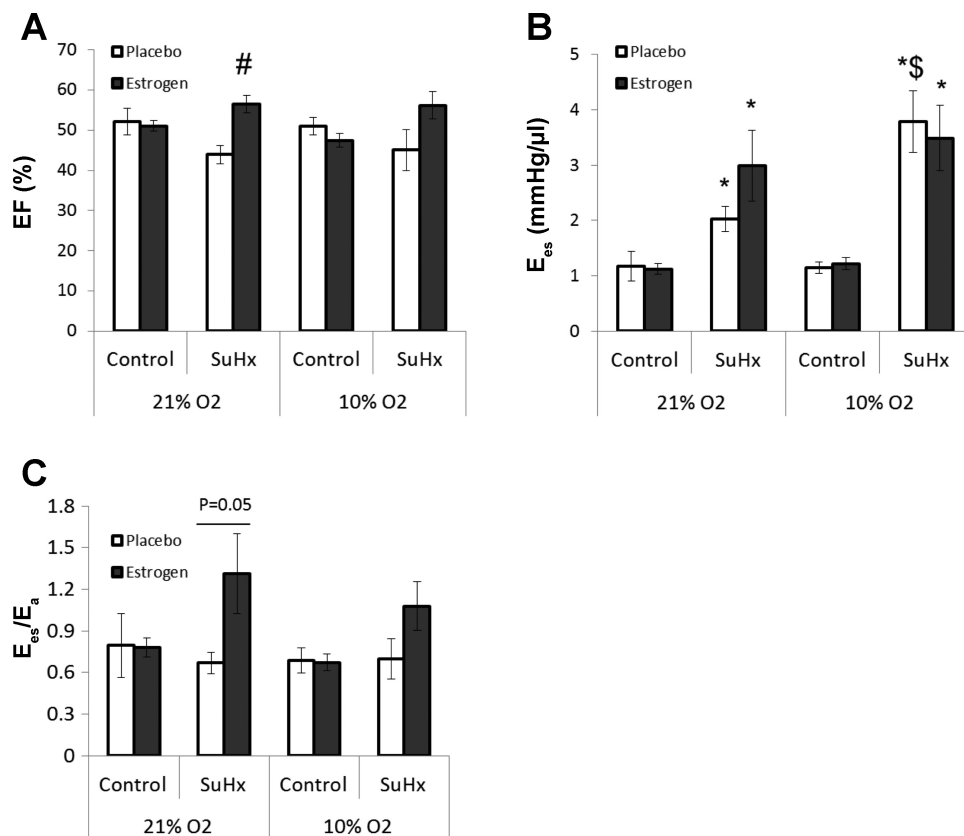
Table 2. *RV hemodynamic and function parameters measured in room air (21% O_2)*

	Control		SuHx	
	Placebo	Estrogen	Placebo	Estrogen
SV, μl	15.0 ± 1.2	16.1 ± 0.5	13.1 ± 1.1	16.1 ± 0.7
HR, beats/min	491 ± 9	499 ± 19	582 ± 8*	606 ± 10*
dP/dt_{max}-V$_{ed}$, mmHg^{-1}·s^{-1}·μl	62.9 ± 6.1	63.6 ± 5.4	114.6 ± 7.8*	123.0 ± 7.5*
PRSW, mmHg	14.2 ± 1.2	17.0 ± 1.1	29.2 ± 3.0*	28.3 ± 2.3*
EW, μl · mmHg	337 ± 29	362 ± 23	484 ± 41*	595 ± 59*
PVA, μl · mmHg	623 ± 58	611 ± 57	871 ± 72*	916 ± 141
Mechanical efficiency, %	55.8 ± 5.2	60.8 ± 2.3	56.4 ± 2.8	68.7 ± 4.6#
dP/dt_{min}, mmHg	−1,420 ± 67	−1,447 ± 58	−2,849 ± 134*	−2,901 ± 177*
τ, ms	8.4 ± 0.7	7.6 ± 0.6	5.5 ± 0.3*	5.2 ± 0.5*
CC, μl/mmHg	0.69 ± 0.06	0.71 ± 0.02	0.35 ± 0.03*	0.43 ± 0.03*

Values are means ± SE. SV, stroke volume; HR, heart rate; PRSW, preload-recruitable stroke work; EW, external work; PVA, pressure-volume area; dP/dt_{max} and dP/dt_{min}, RV maximal and minimal derivative of pressure, respectively; τ, relaxation factor; CC, chamber compliance. *$P < 0.05$ vs. control; #$P < 0.05$ vs. placebo.

room air and 10% O_2 (Tables 2 and 3). We found that when compared with the placebo-treated SuHx group, estrogen improved RV mechanical efficiency by increasing the external work without significantly affecting the oxygen consumption. These data suggest that estrogen treatment at this early stage of PAH is energetically beneficial for the RV.

Multiple mechanisms may underlie the inotropic effect of estrogen on RV myocardium. A change in the myosin heavy chain isoforms present in the RV could be responsible (13, 18), but we did not detect an increase in β-myosin heavy chain in any SuHx group (data not shown). We considered that the ability of estrogen to limit myocardial fibrosis could play a role (10), but we did not observe significant differences in collagen between estrogen- and placebo-treated groups. Because LV contraction contributes to about 20–40% of RV EF (32), we assessed LV function but found comparable fractional shortening and EF of LV in both estrogen- and placebo-treated groups (data not shown), thereby excluding the contribution of LV to improved RV contractility in the estrogen-treated group. Improved calcium handling (29) and altered phosphorylation of myofilaments (41), by which the RV adapts to PAH, may play a role in improving RV function in the estrogen-treated groups and warrant future exploration.

Estrogen reduced PA remodeling. PA remodeling, including both stiffening and narrowing, contributes to increased RV afterload in PAH. Our results showed that estrogen treatment significantly reduced the increase of RV afterload (E$_a$) compared with the placebo-treated SuHx group.

To understand how estrogen modulates pulmonary stiffness, we measured proximal PA compliance ex vivo and found that estrogen attenuated the loss of compliance with PAH. This finding is consistent with a previous study that showed that systemic arteries from OVX female mice were less compliant than those of intact female mice (8). We confirmed the impact of this compliance decrease in vivo by measuring pulmonary pressures in a subgroup of OVX mice treated with estrogen or placebo after 21 days of SuHx. We found that estrogen attenuated the increase in PP and systolic PA pressure, which indicate slower transmission rate of reflected pressure wave in PAH.

Multiple factors, including PA collagen content, diameter, and wall thickness, may be involved in the mechanisms by which estrogen modulates PA stiffness. Our previous study using transgenic mice with defects in collagen degradation (Col1a1 mice) suggested that collagen accumulation plays a critical role in hypoxic pulmonary hypertension-induced PA stiffening (27). It has been reported that estrogen is capable of reducing collagen secretion and turnover rate in SMC (2). Our current study supports the contribution of estrogen to collagen deposition since we found that estrogen reduced collagen deposition in left PA, compared with the placebo-treated SuHx group (Fig. 7). In addition, we found that estrogen attenuated PA wall thickening and increased PA diameter through mechanisms yet to be explored.

Our results showed that estrogen significantly attenuated the increase in tPVR in response to SuHx exposure (Fig. 3).

Table 3. *RV hemodynamic and function parameters measured in acute hypoxia (10% O_2)*

	Control		SuHx	
	Placebo	Estrogen	Placebo	Estrogen
SV, μl	14.8 ± 1.2	14.8 ± 0.8	7.6 ± 1.2*$	12.9 ± 0.9#
HR, beats/min	511 ± 18	514 ± 17	596 ± 8*	595 ± 20*
dP/dt_{max}-V$_{ed}$, mmHg^{-1}·s^{-1}·μl	61 ± 7	68 ± 4	216 ± 23*$	160 ± 16*
PRSW, mmHg	15.8 ± 1.2	16.9 ± 1.0	36.3 ± 3.9*	32.9 ± 3.8*
EW, μl · mmHg	362 ± 33	379 ± 29	307 ± 51$	538 ± 67*#
PVA, μl · mmHg	642 ± 51	672 ± 62	590 ± 104$	852 ± 137
Mechanical efficiency, %	57 ± 4	57 ± 2	54 ± 5	66 ± 4
dP/dt_{min}, mmHg	−1,539 ± 89	−1,569 ± 46	−2,729 ± 158*	−3,000 ± 167*
τ, ms	8.4 ± 0.6	8.7 ± 0.5	6.0 ± 0.5*	5.6 ± 0.5*
CC, μl/mmHg	0.65 ± 0.06	0.62 ± 0.03	0.19 ± 0.03*	0.32 ± 0.02*

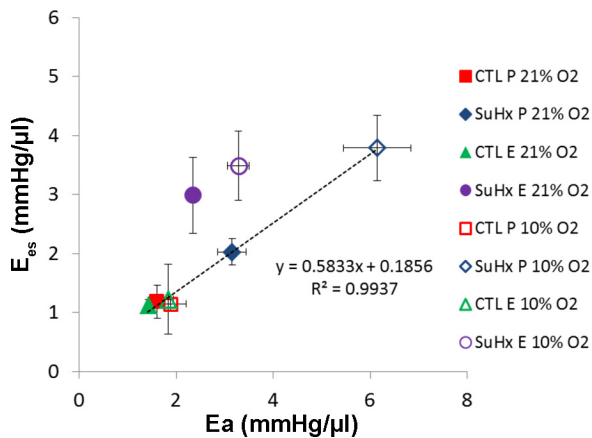Values are means ± SE. *$P < 0.05$ vs. control; #$P < 0.05$ vs. placebo; $$P < 0.05$ vs. 21% O_2.

Fig. 5. Correlation between E_{es} and E_a in placebo (P)- and estrogen (E)-treated SuHx and control (CTL) groups measured with 21% or 10% O_2 ventilation.

Resistance is largely determined by the luminal diameter of small, distal arteries as well as their level of SMC tone and reactivity. Muscularization of small PAs was reduced in the estrogen-treated SuHx group, which explains the attenuated increase in tPVR. In summary, estrogen reduced RV afterload by attenuating both proximal PA stiffening and distal PA muscularization in PAH.

Estrogen preserves cardiac reserve. Here we exposed mice acutely to 10% O_2 to study the effects of estrogen on cardiac reserve. We found that the placebo-treated SuHx group showed signs of RV dysfunction including reduced CO and inability to increase SW in response to acute stress. Increased muscularization in small pulmonary arteries likely escalated the effects of hypoxia-induced vasoconstriction in this group, with subse-
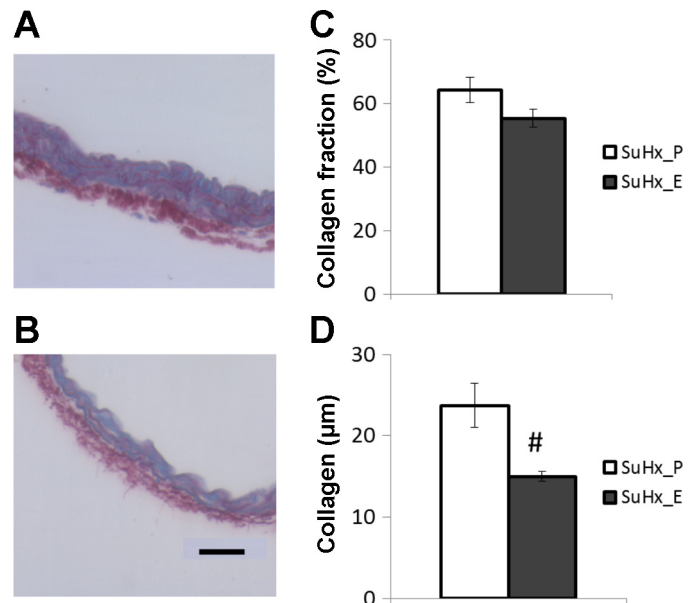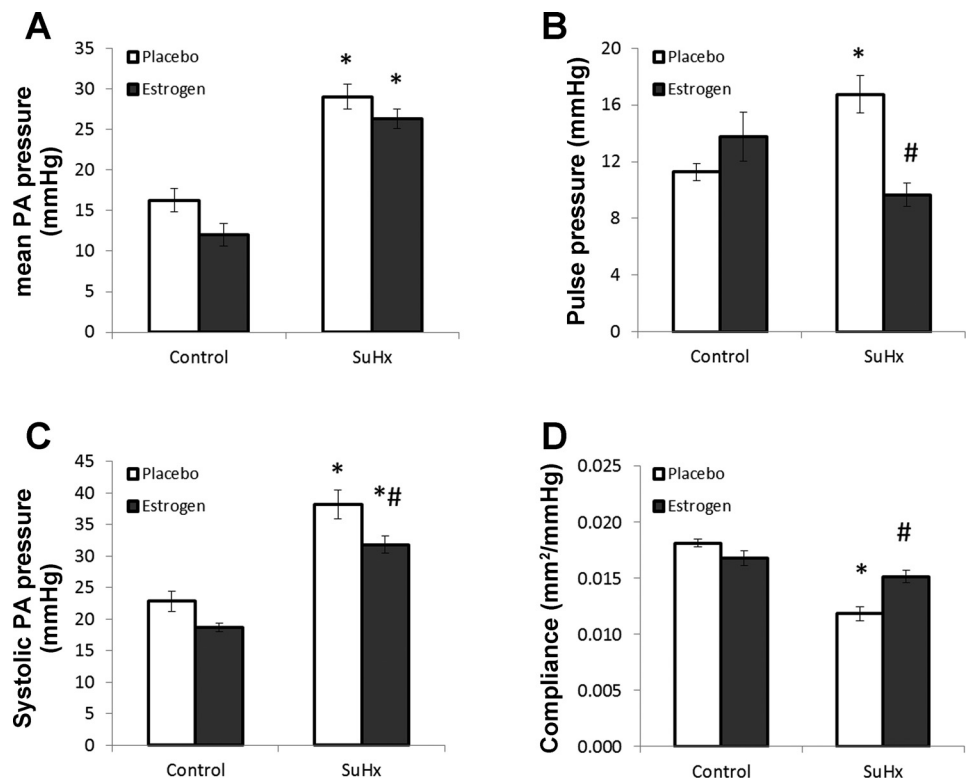

Fig. 7. Collagen concentration in the left pulmonary artery in the placebo (P)- and estrogen (E)-treated SuHx groups. Representative histological images of left pulmonary artery stained with picro-sirius red are shown: (A) placebo-treated SuHx group (*A*), estrogen-treated SuHx group (*B*), collagen area fraction (*C*), and collagen thickness (*D*) semiquantitatively analyzed from the histological images. Scale bar = 50 μm. #*P* < 0.05 vs. placebo.

quent RV dysfunction resulting from afterload excess without adequate RV hypertrophy to normalize wall stress.

In contrast, with acute stress, RV function remained intact in the estrogen-treated SuHx group. Our data suggest two mechanisms by which estrogen protected cardiac reserve. First,

Fig. 6. Pulmonary arterial pressures and compliance in the placebo- or estrogen-treated control and SuHx groups measured with 21% O_2 ventilation. *A*: mean pulmonary artery (PA) pressure. *B*: pulse pressure. *C*: systolic PA pressure measured at main PA. *D*: compliance of extra-lobar left PA. *P < 0.05 vs. control; #P < 0.05 vs. placebo.
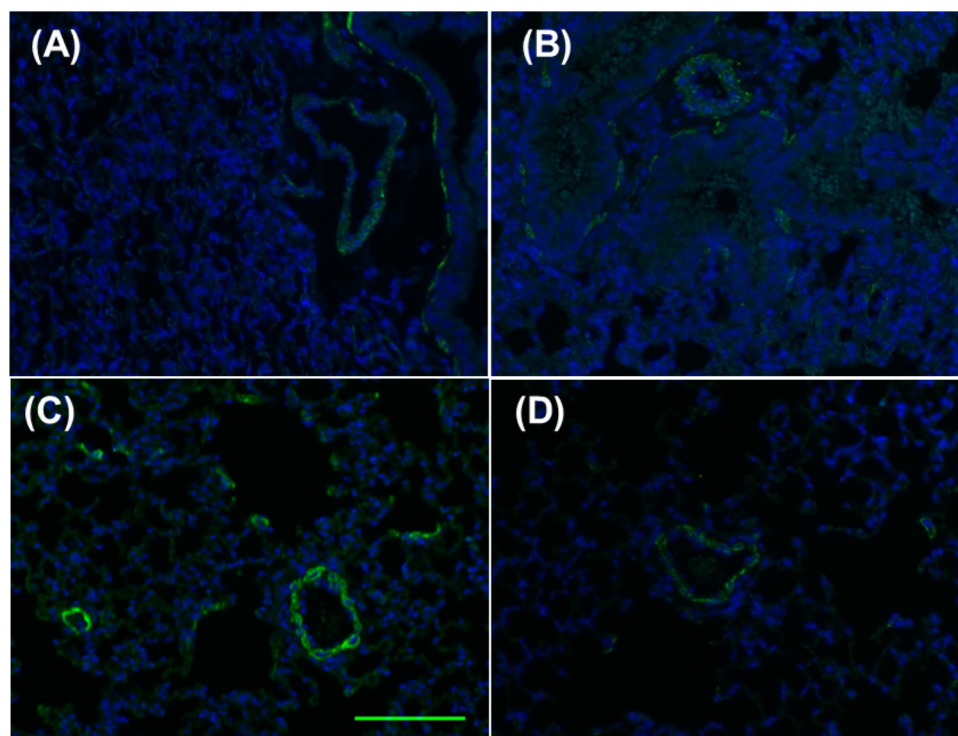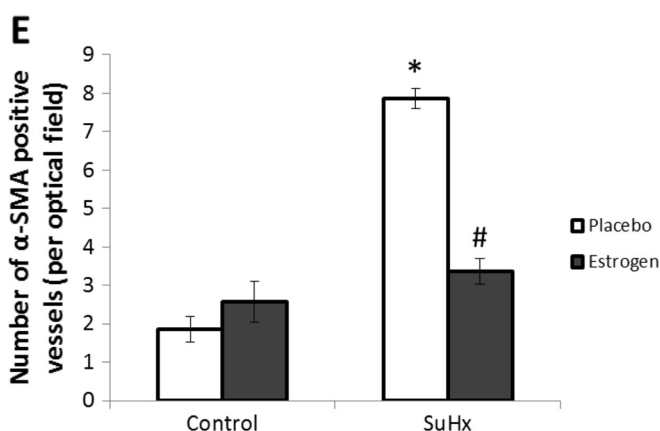
Fig. 8. Immunohistological images of lungs of placebo-treated control group (*A*), estrogen-treated control group (*B*), placebo-treated SuHx group (*C*), estrogen-treated SuHx group (*D*), and quantification of number of α- smooth muscle actin (SMA) positive vessels (*E*) from the immunohistological images. Green color indicates SMA-stain; blue indicates nuclei. Scale bar = 100 μm. $n = 2$ lungs for each control group; $n = 5$ lungs for each SuHx group. $*P < 0.05$ vs. control; $\#P < 0.05$ vs. placebo.

estrogen attenuated the acute increase in RV afterload. Estrogen lowered the degree of muscularization in small PAs, which likely contributed to attenuated pulmonary hypoxic vasoconstriction. In addition, a rapid vasodilatory effect of estrogen may have alleviated pulmonary hypoxic vasoconstriction. In PA rings ex vivo, only very high doses of exogenous estrogen (>500 μM) are capable of inducing rapid vasodilation (20). However, lower doses may be sufficient to exert a similar effect in vivo (21). Second, estrogen may have protected cardiac reserve via the suggested direct effects of estrogen on RV contractility (Fig. 5).

Unexpectedly, we did not observe increase in RV afterload (tPVR) in the control mice when exposed to acute hypoxia. In a previous study performed by our group using the same experimental techniques (33), exposure to acute hypoxia for 5 min significantly decreased the arterial oxygen saturation and partial pressure of arterial oxygen. Total PVR increased as a result of a drop in CO, and RV P_{es} did not increase. In the current study, there were no changes in CO and we observed

only a slight and not significant increase in RV P_{es}. The differences between the current and prior study are the sex of mice (OVX female vs. intact male) and O_2 level for acute hypoxia ventilation (10% vs. 8% O_2). We speculate that lower SMC reactivity and greater RV compensatory ability in female mice, coupled with the less severe hypoxia, led to the absence of a significant cardiopulmonary response to hypoxia in control mice in this study.

Limitations. In this study, we sought to establish a mean circulating estrogen level equivalent to the peak physiological estrogen level, which usually occurs at the proestrous phase of estrous cycle, to obtain a maximum effect as done previously (28). This provides a supraphysiological level of estrogen, which may have led to the higher systemic pressure and physiological LV hypertrophy found here in SuHx mice, as seen in pregnancy (22). Hemodynamic measurements in this study were performed during an open chest procedure, which potentially alters cardiopulmonary hemodynamics. However, because all groups underwent similar surgical procedures, the

comparisons between groups should remain valid. In addition, we used different groups of animals for hemodynamic measurements in RV and PAs, as well as for ex vivo mechanical tests on PA compliance. However, our group is experienced with these techniques and has obtained consistent results. We do not expect biological variations among groups of animals that would affect our interpretation of data.

Conclusions

Our study showed that estrogen protected the pulmonary vasculature from narrowing and stiffening and stimulated RV contractility, thereby improving RV function in the SuHx model of PAH in mice. The dual actions of estrogen on both the RV and pulmonary vasculature suggest that estrogen and associated signaling pathways may provide novel prevention and treatment targets for early-stage PAH patients. Our study also showed that estrogen treatment prevented RV dysfunction due to acute cardiopulmonary stress. This finding is clinically relevant to early-stage PAH patients, who have no overt RV dysfunction but may have reduced cardiac reserve. In addition, these results in C57BL6/J mice could serve as a baseline for future studies on molecular mechanisms by which estrogen acts on VVC using genetically modified mice.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.L. and N.C.C. conception and design of research; A.L., D.A.S., L.T., and T.A.H. performed experiments; A.L. and J.C.E. analyzed data; A.L., T.A.H., and N.C.C. interpreted results of experiments; A.L. prepared figures; A.L. drafted manuscript; A.L., J.C.E., Z.W., T.A.H., and N.C.C. edited and revised manuscript; A.L. and N.C.C. approved final version of manuscript.

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