17β-Estradiol Attenuates Conduit Pulmonary Artery Mechanical Property Changes With Pulmonary Arterial Hypertension

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Abstract—Pulmonary arterial hypertension (PAH), a rapidly fatal vascular disease, strikes women more often than men. Paradoxically, female PAH patients have better prognosis and survival rates than males. The female sex hormone 17β-estradiol has been linked to the better outcome of PAH in females; however, the mechanisms by which 17β-estradiol alters PAH progression and outcomes remain unclear. Because proximal pulmonary arterial (PA) stiffness, one hallmark of PAH, is a powerful predictor of mortality and morbidity, we hypothesized that 17β-estradiol attenuates PAH-induced changes in mechanical properties in conduit proximal PAs, which imparts hemodynamic and energetic benefits to right ventricular function. To test this hypothesis, female mice were ovariectomized and treated with 17β-estradiol or placebo. PAH was induced in mice using SU5416 and chronic hypoxia. Extra-lobar left PAs were isolated and mechanically tested ex vivo to study both static and frequency-dependent mechanical behaviors in the presence or absence of smooth muscle cell activation. Our static mechanical test showed significant stiffening of large PAs with PAH ($P<0.05$). 17β-Estradiol restored PA compliance to control levels. The dynamic mechanical test demonstrated that 17β-estradiol protected the arterial wall from the PAH-induced frequency-dependent decline in dynamic stiffness and loss of viscosity with PAH ($P<0.05$). As demonstrated by the in vivo measurement of PA hemodynamics via right ventricular catheterization, modulation by 17β-estradiol of mechanical proximal PAs reduced pulsatile loading, which contributed to improved ventricular–vascular coupling. This study provides a mechanical mechanism for delayed disease progression and better outcome in female PAH patients and underscores the therapeutic potential of 17β-estradiol in PAH. (*Hypertension*. 2015;66:1082-1088. DOI: 10.1161/HYPERTENSIONAHA.115.05843.) • Online Data Supplement

Key Words: arterial stiffness ■ 17β-estradiol ■ pulmonary arterial hypertension ■ sex difference ■ viscoelasticity

Pulmonary arterial hypertension (PAH) is a fast progressing vascular disease characterized by proximal pulmonary arterial (PA) stiffening and distal PA occlusion, which increases right ventricular (RV) afterload and ultimately leads to RV failure. PAH disproportionally affects women, with a female to male ratio of ≈4:1[1]; however, female PAH patients have better RV function[2] and a higher survival rate than male patients.[3] Both animal and clinical studies suggest that 17β-estradiol plays an important role in the sex differences in PAH.[4,5] However, the mechanistic role of 17β-estradiol in modifying PAH development and progression remains incompletely known.

17β-Estradiol modifies age- and hypertension-related arterial stiffening in systemic arteries.[6,7] 17β-Estradiol deficiency (because of ovariectomy or menopause) increases arterial stiffness,[8,9] and 17β-estradiol therapy restores stiffness (and its inverse, compliance) toward healthy values.[6,7] Stiffening of large, proximal PAs, a common feature of PAH, occurs early in disease progression[10] and is a powerful predictor of mortality in PAH.[11,12] PA compliance primarily affects the oscillatory component of RV afterload; loss of PA compliance (PA stiffening) increases RV afterload and thus affects RV adaption to PAH. In addition, the stiffening of large proximal PAs reduces their ability to dampen the pulsatility of pressure and flow to distal PAs, which may exacerbate distal PA remodeling and thus progression of PAH.[13,14] To understand how and to what extent 17β-estradiol affects proximal PA stiffening and PAH progression, the first step is to comprehensively investigate the effects of 17β-estradiol on PAH-induced biomechanical changes in proximal PAs.

Most prior studies on the effects of 17β-estradiol on PA biomechanical properties have focused on its acute effects on PA vasoconstriction in healthy animals.[15–17] The chronic effects of 17β-estradiol on biomechanical properties of remodeled PAs remain underexplored. Therefore, we tested the hypothesis that 17β-estradiol attenuates PAH-induced changes in proximal PA mechanical properties, which imparts hemodynamic and energetic benefits to RV adaption. We

found that 17β-estradiol protected PAs from PAH-induced loss of arterial compliance and viscoelasticity by modulating vessel morphology and constituents. This finding provides a mechanical mechanism for delayed disease progression and better outcomes in female PAH patients.

Methods

Animals

Female C57/BL6 mice (9–10 weeks old) were ovariectomized and treated with 17β-estradiol or placebo. PAH was induced using SU5416 and chronic hypoxia (SuHx). PA hemodynamics was measured in vivo via RV catheterization. Subsequently, left extra-lobar PAs were isolated and mechanically tested ex vivo to study both static and frequency-dependent behaviors in the presence or absence of smooth muscle cell (SMC) activation. All protocols and procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee. Detailed methods are provided in the online-only Data Supplement.

Results

In Vivo Hemodynamics

SuHx caused hypertension as expected (Table). 17β-Estradiol did not affect PAH severity, which is important because quantitative comparison of PA stiffness and elastic modulus is often confounded by differences in mean transmural pressure.[18] Because cardiac output was preserved in both SuHx groups, total pulmonary vascular resistancesignificantly increased ($P<0.05$) in both SuHx groups, and 17β-estradiol did not affect this increase (Table). In contrast, 17β-estradiol limited the increase in pulse pressure (PP) and decrease in global compliance index (stroke volume/PP) induced by SuHx (Table), which suggests that 17β-estradiol modulates conduit PA stiffness.

Ex Vivo Mechanics

Vasoreactivity

The receptor-independent vasoconstrictor KCl caused a dose-dependent reduction in PA diameter measured ex vivo

(Figure 1A). In control groups, 17β-estradiol treatment tended to reduce the magnitude change, suggesting that 17β-estradiol attenuated vasoreactivity in healthy arteries. In contrast, in the SuHx groups, 17β-estradiol treatment increased the magnitude change ($P=0.05$ versus placebo-treated SuHx group at 50 mmol/L KCl), suggesting that 17β-estradiol sensitizes diseased PAs to vasoconstriction by KCl.

Functional contraction ratio, a measure of arterial contractility, exhibited a downward parabolic relationship with transmural pressures ranging from 10 to 50 mm Hg (Figure 1B). 17β-Estradiol enhanced the maximal functional contraction ratio in the SuHx groups but had no effect in the control groups, further indicating that 17β-estradiol sensitizes diseased conduit PAs to vasoconstriction.

Compliance

SuHx reduced conduit PA compliance, and 17β-estradiol treatment restored it to control levels (Figure 2A). SMC activation increased total PA compliance in all groups (Figure 2B). A sigmoid relationship between PA compliance and pressure was observed. Without SMC tone, PA compliance peaked at a pressure of ≈25 mm Hg (Figure 2C). 17β-Estradiol treatment attenuated the loss of maximal compliance seen in the placebo-treated SuHx group, but did not restore maximal compliance to control levels. SMC activation increased the maximal compliance and shifted it to higher pressures in all groups (Figure 2D). 17β-Estradiol further enhanced the effect of SMC activation on compliance and restored the maximal compliance to control levels.

Static Mechanical Properties

SuHx shifted the stress–strain relationships upwards and to the left (Figure 3A). 17β-Estradiol treatment attenuated the shift of the stress–strain curves, although the 17β-estradiol-induced change was small compared with the SuHx-induced change. Compared with the stress–strain curve without SMC tone, isobaric SMC contraction resulted in a more linear stress–strain relationship in the low strain regime (Figure 3B). SMC activation had no effect in the high strain regime, indicating that

Table.   Effects of 17β-Estradiol on Pulmonary Hemodynamics in PAH

Parameters	CTL_P	CTL_E	SuHx_P	SuHx_E
mPAP, mm Hg	16.7±0.8	16.7±1.2	27.2±0.8*	24.1±1.5*
sPAP, mm Hg	21.5±1.4	22.4±1.3	37.5±1.2*	30.8±1.8*
dPAP, mm Hg	11.2±0.7	11.7±1.0	19.3±0.7*	19.2±1.4*
PP, mm Hg	12.0±0.5	12.0±0.5	18.2±1.0*	11.6±1.1†
SV, μL	19.7±1.6	21.7±1.7	17.3±1.3	19.1±0.2
CO, mL/min	10.6±0.8	10.0±0.8	9.8±0.7	9.5±0.9
CI, mL/min/g	0.48±0.04	0.47±0.03	0.48±0.04	0.43±0.04
SV/PP, μL/mm Hg	1.76±0.13	1.89±0.12	0.98±0.12*	1.68±0.23†
tPVR, mm Hg min/mL	1.6±0.1	1.5±0.1	2.9±0.3*	2.8±0.5*

Mean±SE values (n=6–10 per group). CI indicates cardiac index; CO, cardiac output; CTL, control; dPAP, diastolic pulmonary arterial pressure; E, estradiol-treated; mPAP, mean pulmonary arterial pressure; P, placebo-treated; PAH, pulmonary arterial hypertension; PP, pulse pressure; sPAP, systolic pulmonary arterial pressure; SuHx, SU5416 and chronic hypoxia; SV, stroke volume; and tPVR (=mPAP/CO), total pulmonary vascular resistance.

*$P<0.05$ vs control.
†$P<0.05$ vs placebo.

collagen remains the primary load-bearing wall component at high pressures and strains.

SuHx decreased the transition strain, a threshold above which collagen engages and bears load (Figure S2A in the online-only Data Supplement). 17β-Estradiol treatment attenuated this decrease. A similar trend was observed with SMC activation (Figure S2B).

Dynamic Mechanical Properties

SuHx increased dynamic elastic modulus at all frequencies (Figure 4A), and 17β-estradiol tended to attenuate this increase in elastic modulus. SuHx caused the dynamic modulus to decrease progressively with frequency in a range of 1 to 10 Hz, such that the dynamic modulus at 10 Hz was significantly lower than that at 0.1 Hz. 17β-Estradiol tended to attenuate the rate of frequency-dependent decrease.

Similarly, SuHx significantly increased dynamic stiffness, and 17β-estradiol significantly reduced the increase ≤5 Hz (Figure 4B). Because the frequency-dependent reduction in dynamic stiffness only occurred in the placebo-treated SuHx group, no difference was detected between the 17β-estradiol- and placebo-treated SuHx groups at 10 Hz. Damping capacity increased with frequency in all groups (Figure 4C). SuHx significantly reduced the damping capacity, and 17β-estradiol restored damping capacity to control levels, indicating that 17β-estradiol protects the PAH-induced reduction in wall viscosity. We did not find any significant differences in dynamic elasticity, dynamic stiffness, or damping capacity at 10 Hz between with and without SMC activation (data not shown).

Morphological and Histological Data

SuHx increased RV weight normalized by body weight (Table S1), which is evidence of RV hypertrophy. 17β-Estradiol had a limited effect on the normalized RV weight. PA outer diameter measured optically at 15 mm Hg was significantly larger in the 17β-estradiol-treated SuHx group compared with both the control and placebo-treated SuHx groups (Table S1). The wall thickness measured by histology increased significantly in SuHx groups, and this increase was attenuated by 17β-estradiol (Table S1). The same trend was observed in the medial wall thickness.

SuHx increased the area fraction of collagen measured histologically, and 17β-estradiol attenuated this increase (Figure S1M and S1N). Elastin did not significantly change with PAH or with 17β-estradiol treatment (data not shown). SuHx increased proteoglycans in both 17β-estradiol- and placebo-treated groups (Figure S1O and S1P). Changes in nitrotyrosine, a biomarker of oxidative stress, were not detected (8%–10% of the vessel wall). Smoothelin, a biomarker for the fully differentiated, contractile phenotype of SMCs,[19] increased significantly in the medial layer of the 17β-estradiol-treated SuHx group compared with the control groups (Figure S1Q and S1R); no significant difference was detected between the 17β-estradiol- and placebo-treated SuHx groups.

Discussion

Here we investigated the effects of 17β-estradiol on changes in conduit PA mechanical properties in mice with PAH via ex vivo mechanical testing. The novel findings include the following: (1) 17β-estradiol enhanced vasoreactivity with PAH; (2) 17β-estradiol attenuated stiffening with PAH; and (3) 17β-estradiol preserved wall viscosity. Our in vivo pulmonary hemodynamics data indicate that 17β-estradiol reduced RV pulsatile load and improved ventricular–vascular coupling efficiency. Because conduit PA mechanical properties play an important role in distal PA remodeling and RV adaption to PAH, the protective effects of 17β-estradiol likely contribute to improved outcomes for female PAH patients compared with male patients.

17β-Estradiol Enhances Conduit PA Vasoreactivity in PAH

Vasoconstriction is an important contributor to PAH.[20] Both endogenously and exogenously administered estrogen attenuated vasoconstriction of healthy PA induced by vasoactive agents or hypoxia.[16,17] We hypothesized that these effects of 17β-estradiol are altered by PAH. Our results show that in the PAH-remodeled PA, 17β-estradiol heightened drug-induced vasocontractility. Without 17β-estradiol, the ability of conduit PAs to respond to vasoconstrictors was impaired, as previously observed in both hypoxic- and monocrotaline-treated rats.[21] This suppressed vasoreactivity can result from dedifferentiation of pulmonary SMCs to a more proliferative/synthetic and less contractile phenotype in PH.[21]
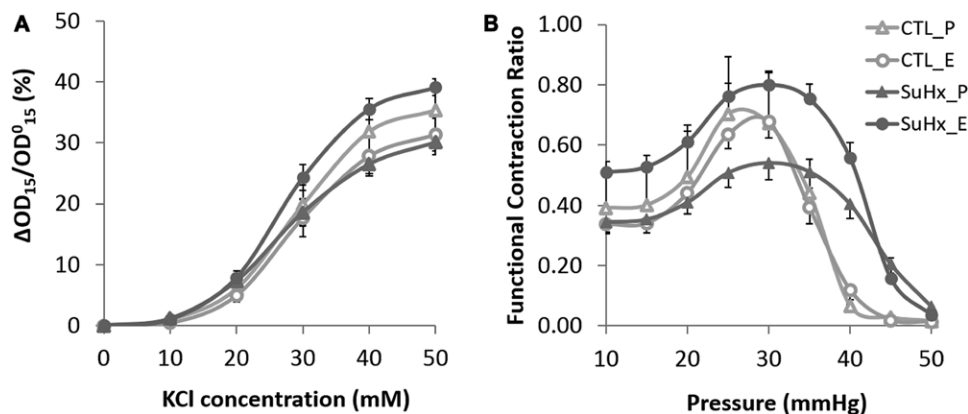

Figure 1. 17β-Estradiol enhances conduit pulmonary artery (PA) vasoreactivity in pulmonary arterial hypertension (PAH). **A**, Dose-dependent response to receptor-independent vasoconstrictor KCl; **B**, Pressure dependence of functional contraction ratio at 50 mmol/L KCl. CTL indicates control; E, estradiol-treated; P, placebo-treated; and SuHx, SU5416 and chronic hypoxia.
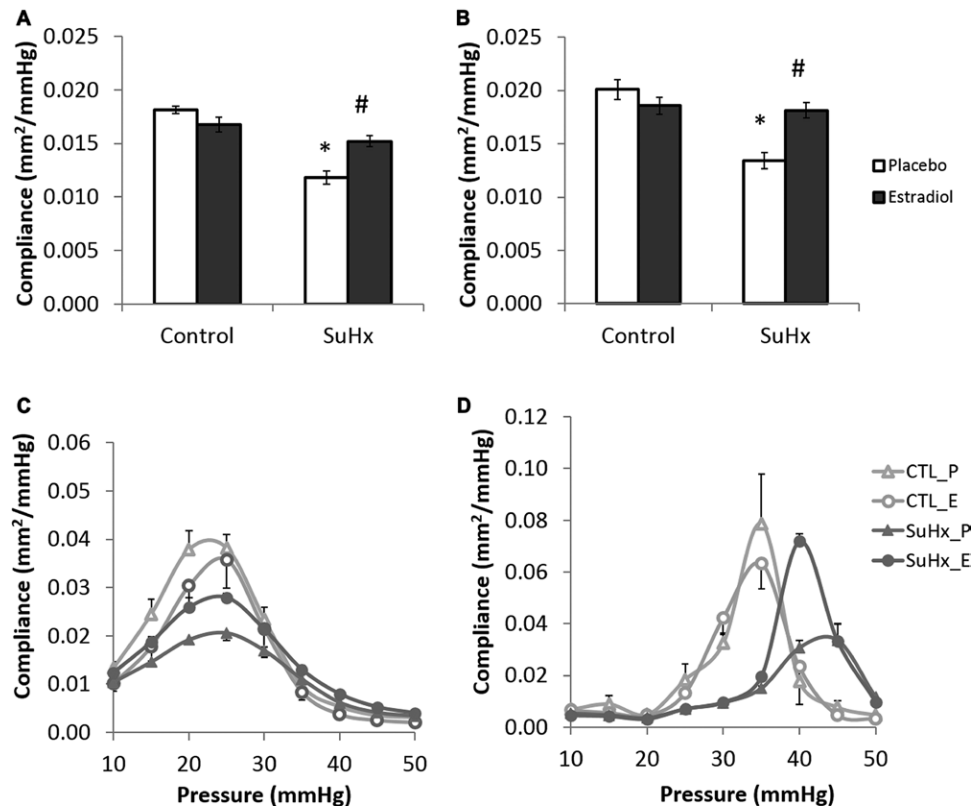
Figure 2. 17β-Estradiol preserves conduit pulmonary artery (PA) compliance in pulmonary arterial hypertension (PAH; mean±SE). Total PA compliance in the pressure range of 5 to 50 mm Hg in the absence of smooth muscle cell tone (**A**) and with smooth muscle cell activation at 50 mmol/L KCl (**B**); pressure–compliance curves in the absence of smooth muscle cell tone (**C**) and with smooth muscle cell activation at 50 mmol/L KCl (**D**). *$P<0.05$ vs control; #$P<0.05$ vs placebo. CTL indicates control; E, estradiol-treated; P, placebo-treated; and SuHx, SU5416 and chronic hypoxia.

This view is supported by the evidence of altered growth responses of pulmonary SMCs from PAH patients to antiproliferative bone morphogenetic proteins and transforming growth factor-β.[21] Here we observed that 17β-estradiol upregulates smoothelin in the PA medial layer of diseased animals (Figure S1R), suggesting that estradiol prevents SMCs dedifferentiation in PAH.

17β-Estradiol Attenuates PA Stiffening in PAH

Premenopausal women have greater arterial distensibility than age-matched men and postmenopausal women.[22] 17β-Estradiol protects systemic arteries from age- and hypertensive-related stiffening.[6–8] Here we show that 17β-estradiol attenuates loss of PA compliance in PAH, consistent with our previous study.[4] Furthermore, we demonstrate that SMC activation significantly altered the pressure–compliance relationship, and estrogen enhanced the effects of SMC activation on PA compliance. The increase in PA compliance with SMC activation is consistent with previous studies[23] and can be attributed to disengagement of collagen at a given pressure. The rightward shift of the pressure–compliance curves enables the PAs to maintain compliance at
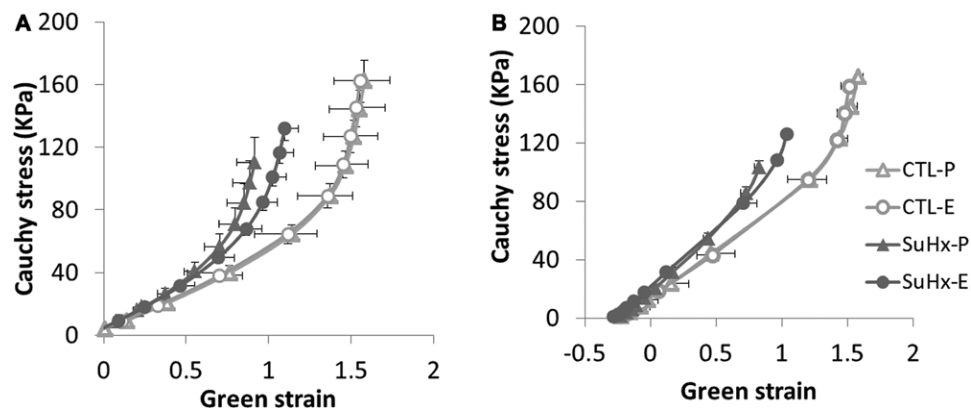

Figure 3. 17β-Estradiol attenuates changes in conduit pulmonary artery (PA) static mechanical properties in pulmonary arterial hypertension (PAH). Stress–strain curves in the absence of smooth muscle cell tone (**A**) and with smooth muscle cell activation at 50 mmol/L KCl (**B**). CTL indicates control; E, estradiol-treated; P, placebo-treated; and SuHx, SU5416 and chronic hypoxia.
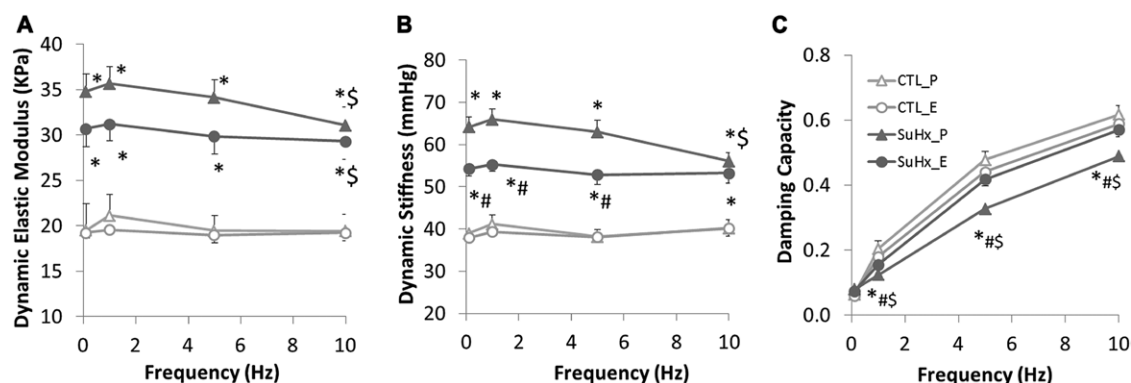
Figure 4. 17β-Estradiol attenuates changes in conduit pulmonary artery (PA) dynamic mechanical properties in pulmonary arterial hypertension (PAH). Dynamic elastic modulus (**A**), dynamic stiffness (**B**), and damping capacity (**C**). *$P<0.05$ vs control; #$P<0.05$ vs placebo; $$P<0.05$ vs 0.1 Hz. CTL indicates control; E, estradiol-treated; P, placebo-treated; and SuHx, SU5416 and chronic hypoxia.

higher pressures. These findings suggest that increased conduit PA SMC reactivity is a protective adaptation to hypertension and that 17β-estradiol enhances the benefits of SMC activation. However, as increased SMC reactivity in distal pulmonary vasculature is detrimental in PAH,[20] whether 17β-estradiol also sensitizes distal PA SMC reactivity warrants further exploration.

Arterial compliance is primarily influenced by material properties and morphology.[24] Our results show that rather than significantly reducing wall elasticity, chronic administration of 17β-estradiol increased diameter and attenuated wall thickening, which compensated for the increase in elastic modulus in PAH. 17β-Estradiol also significantly reduced the PAH-induced collagen accumulation and postponed early engagement of collagen, both contributing to attenuating arterial stiffening with PAH. In addition, as suggested by the pressure–compliance curves, differences in compliance can result from the pressure ranges in which the conduit PA operates in vivo, which would engage different wall components in normal and hypertensive groups. For the 17β-estradiol-treated hypertensive group, the conduit PA operates around the maximal region of compliance, which further reduces the difference in compliance between the control and the 17β-estradiol-treated PAH groups in vivo.

17β-Estradiol Protects PA Viscoelasticity in PAH

Viscoelastic properties of the arterial wall determine the relationship between pulsatile pressure and flow in cardiovascular system. Arterial viscoelasticity deteriorates with aging and hypertension.[25,26] A recent study showed that carotid artery viscoelastic deterioration is a significant risk factor for coronary artery disease.[27] Women after menopause had increased arterial viscoelastic deterioration compared with age-matched men, suggesting that 17β-estradiol protects against viscoelastic deterioration. Our group recently reported that PAH modified frequency-dependent viscoelastic behaviors in conduit PAs of male mice.[28] Here, we are the first to address the interaction between viscoelasticity and 17β-estradiol in PAs.

We found that 17β-estradiol tended to attenuate the PAH-induced increase in dynamic elasticity and stiffness for all frequencies studied.17β-Estradiol also prevented the frequency-dependent decline in the dynamic modulus and

dynamic stiffness in the hypertensive groups. The biomechanical mechanisms for frequency-dependent changes in elasticity and stiffness remain unknown. Because 17β-estradiol attenuated collagen deposition and limited the increase in SMC content in PAs with PAH, we speculate that interactions between collagen, vascular cell types, and intracellular SMC proteins are important contributors to frequency-dependent arterial mechanics.

Although previous studies have shown that damping capacity, a measure of arterial wall viscosity, decreases in PAH,[28] we show that 17β-estradiol preserved damping capacity. Changes in extracellular matrix components, such as collagen, proteoglycans, and SMC content have been linked with changes in arterial viscosity.[28–30] Here, we found a positive correlation between collagen content and damping capacity at 0.1 Hz (Figure S3A). The relationship became negative at higher frequencies (Figure S3B), suggesting that the contribution of collagen content to arterial viscosity is frequency-dependent. Furthermore, we found a negative correlation between the medial wall thickness and wall viscosity in conduit PAs at a physiological frequency (Figure S4). Although this finding is not consistent with Bia's study on healthy carotid and aortic arterial segments,[30] we speculate that this inconsistency is because of inherent differences between systemic and pulmonary arteries or differences between healthy and diseased states. It is difficult to attribute the change in wall viscosity to changes in any single PA constituent because arterial viscoelasticity is likely affected by interactions between cells and matrix proteins,[31] organization of matrix,[29] and hemodynamic loading.[32]

17β-Estradiol Is an Important Contributor to Sex Disparities in PAH Outcomes

The pulsatile pulmonary pressure– flow relationship provides insight into the impact of changes in conduit PA mechanical properties on RV afterload, RV–PA coupling efficiency, and distal arterial remodeling, which play major roles in the progression of PAH. Our study shows that 17β-estradiol restored PP without significantly affecting the elevation of mean pressure and total pulmonary vascular resistance, indicating that 17β-estradiol had limited effects on distal occlusive remodeling in early PAH. This is expected because considerable changes in arterial stiffness occur in early PAH with a small change in

pulmonary resistance.[10] The ability of 17β-estradiol to preserve PA compliance likely contributed to attenuated RV pulsatile load in PAH (Figure S5), which may explain the improved RV function and ventricular–vascular coupling in the 17β-estradiol-treated SuHx group observed in our previous study.[4]

Loss of large PA compliance with PAH also has consequences for distal PAs. In particular, loss of compliance can result in significantly higher PP (by 2- to 3-fold), which has been linked to endothelial cell dysfunction and SMC hypertrophy.[13,14] Here, we report that 17β-estradiol restores large artery compliance and thus the PP (Figure S5), which should lessen remodeling of the distal PAs, a key feature in PAH disease progression.

The conduit PA mechanical property changes we found in the placebo-treated SuHx group were also found in the male SuHx group in our previous study.[28] We also found similar cardiac response to SuHx exposure in the current placebo-treated female and prior male groups.[4] Because the similar mean PA pressure excluded the impact of pressure on mechanical and structural remodeling of conduit PA between the 17β-estradiol- and placebo-treated SuHx groups, we conclude that the estrogenic modulation of arterial wall mechanical properties is, at least partially, responsible for sex disparities in PAH outcomes.

Study Limitations

First, this study focuses on the effects of 17β-estradiol on the mechanical properties of conduit PAs. The conclusions derived from this study cannot directly apply to other forms of estrogens, such as conjugated equine estrogens and synthetic estrogens, without further confirmation. In addition, whether the protection of estradiol on PAs is mediated through its metabolites, such as 2-methoxyestradiol[33] or estrogen receptors,[34] is not clear and warrants further study. Second, for better comparison, we tested material properties of isolated conduit PAs under the same condition (same pressure and frequency range) rather than under their respective conditions in vivo. However, changes in material properties may be an adaptation to the differences in pressures and flow. For example, the reduction in arterial wall viscosity in the placebo-treated SuHx group may be a compensatory mechanism for the increased PP.[32] Finally, the effects of 17β-estradiol were studied using relatively young adult mice with mild PAH, and so the results may not be applicable to aged animals or more severe disease. Investigations into the interaction of 17β-estradiol, age, and PAH severity are warranted.

Perspective

Our study demonstrated that 17β-estradiol attenuated stiffening and limited viscoelastic deterioration of conduit PAs in PAH. The modulation in arterial mechanical properties by 17β-estradiol improved pulmonary hemodynamics and RV–PA coupling efficiency. As large PA stiffening is a powerful predictor of morbidity and mortality,[11,12] a key implication is that exogenous 17β-estradiol may act as a novel therapy to prevent PA stiffening and thus delay the progression of PAH. This finding is profound because commonly used antihypertensive medications in PAH are based largely on their vasodilating effects on smaller resistance vessels and have little

effect on large arterial stiffness.[35] In addition, this study suggests that the effects of 17β-estradiol on PA stiffness and viscoelasticity are important contributors to sex differences in PAH progression and outcomes.

Disclosures

None.

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Novelty and Significance

What Is New?

- Pulmonary arterial wall stiffening, a hallmark of pulmonary arterial hypertension (PAH), occurs early in PAH and has been associated with increased mortality and mobility in advanced PAH. Our study demonstrated that estrogen attenuates PAH-induced arterial stiffening.
- Frequency-dependent behaviors of the pulmonary arterial wall, including dynamic elasticity, stiffness, and viscosity, are altered in PAH. 17β-Estradiol protects conduit pulmonary arteries (Pas) from PAH-induced deterioration in viscoelasticity.

What Is Relevant?

- The modulation by 17β-estradiol of conduit PA stiffness and viscoelasticity provides a mechanical mechanism by which 17β-estradiol contributes to sex differences in PAH progression and outcomes.

- Our data suggest that 17β-estradiol may be an effective therapy for preventing or reducing PA stiffening in PAH.

Summary

Our study demonstrates that 17β-estradiol attenuates arterial stiffening and limits viscoelastic deterioration of conduit PAs in PAH. The attenuation of arterial mechanical property changes by 17β-estradiol also preserves pulmonary hemodynamics and right ventricular–PA coupling efficiency.

Online Supplement

17β-estradiol attenuates conduit pulmonary artery mechanical property changes with pulmonary arterial hypertension

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Short title: Estradiol attenuates PA mechanical property changes with PAH

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Methods

Animal preparation

Female C57/BL6 mice (9-10 weeks) were ovariectomized at the Jackson Laboratory (Bar Harbor, ME) to eliminate natural fluctuations in 17β-estradiol levels. After one week, to ensure depletion of stored 17β-estradiol, mice were implanted subcutaneously with 17β-estradiol pellets (0.1 mg/21 days release, Innovative Research of America). The dosage of exogenously delivered 17β-estradiol was chosen to mimic peak physiological 17β-estradiol levels as done in our previous study[1]. Some mice were implanted with placebo pellets as control for 17β-estradiol effects. Immediately after pellet implantation, PAH was induced in half of the mice (n = 10 treated with 17β-estradiol and n = 10 treated with placebo) via a weekly injection of the VEGFR inhibitor SUGEN 5416 (Sigma) at a dosage of 20 mg/kg combined with normobaric hypoxia at 10% O_2 for 21 days. The other half (n = 10 treated with 17β-estradiol and n =10 treated with placebo) were kept in room air without SuHx exposure. All animals were housed at room temperature with a 12-hour light and dark cycle and with free access to water and standard chow.

All mice were used for mechanical testing of the extralobar left pulmonary artery (LPA). A subset of mice (SuHx and control, 17β-estradiol and placebo-treated, n = 6-7 per group) was also used for pulmonary hemodynamic measurements before vessel isolation. All protocols and procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Hemodynamic measurements

In a subset of mice, pulmonary pressure and flow were measured simultaneously in situ following procedures described by Tabima et al. [2]. Briefly, after a mouse was anesthetized (with urethane at 2 g/kg to maintain heart rate), intubated and ventilated with room air, the chest was opened to expose the RV. A 1.2F pressure catheter (Millar Instruments, Houston, TX) was then inserted into the RV and advanced into the main PA just distal to the pulmonary valve. Simultaneously, blood flow velocity was measured at the same location with transthoracic echocardiography (Visual Sonics Vevo 770 ultrasonograph with a 30-MHz transducer) in Doppler mode. Pressure and flow waveforms were collected at 1000 Hz and synchronized using a customized hemodynamic workstation (Cardiovascular Engineering Inc., Norwood, MA).

Mean PA pressure (mPAP), pulse pressure (PP = systolic P – diastolic P), stroke volume (SV), cardiac output (SV × HR), total pulmonary vascular resistance (tPVR = mPAP/CO), and global pulmonary arterial compliance (SV/PP) were obtained.

Ex vivo mechanical testing

If hemodynamic measurements were not obtained, mice were euthanized with an overdose of 50 mg/kg pentobarbital by intraperitoneal injection after 21 days of SuHx or room air exposure. If hemodynamic measurements were obtained, mice were euthanized by exsanguination under anesthesia. For all mice, median sterontomy was then performed to harvest lungs and heart en bloc. In a petri dish with Ca^{2+} and Mg^{2+}

free PBS solution, the LPA was cleared of surrounding tissues and isolated from the main PA to the first intralobar branch under a dissecting microscope.

The LPA was then mounted between two aligned cannulas in a vessel chamber with no-flow setup. Two pressure transducers were mounted in-line with the cannula to obtain the pressures at both ends of the vessel. The superfusate (PSS or Ca^{2+} and Mg^{2+} free PBS) was continuously circulated and maintained at 37 °C. The perfusate (PSS) was aerated with an air-CO_2 mixture to main the pH at 7.4 and supplied symmetrically to both ends of the vessel with a static pump (LSI; Burlington, VT) and a pulsatile pump (EnduraTec TestBench; Bose Corporation; Eden Prairie, MN) to achieve static or cyclic pressurization.

After mounting but prior mechanical testing, the LPA was stretched 140% axially at the approximate in vivo stretch ratio to prevent buckling at high pressure. The vessel was allowed 30 minutes to equilibrate, and then preconditioned at 1Hz for 10 cycles. The outer diameter (OD) and the wall thickness were measured at 10X magnification at 5 and 15 mmHg using a video dimension analyzer. During vasoreactivity and mechanical tests, pressure and outer diameter were recorded simultaneously using the in-line pressure transducers (ATS300, Harvard Apparatus) and a CCD camera connected to an inverted microscope at 4X magnification (±2.5 μm).

Protocol 1: Vasoreactivity to KCl

Following equilibrium and preconditioning, the baseline LPA diameter was recorded at a constant transmural pressure of 15 mmHg. Increasing concentration of KCl (10, 20, 30, 40, 50 mM) was added to the superfusate (PSS) every 30 min or when the rate of change of vessel diameter was less than 2 μm/min. The OD of the LPA was continuously recorded to generate a cumulative dose response curve to KCl.

Protocol 2: mechanical tests with activated SMC

The mechanical test was performed on the LPA with SMC activated at a KCl concentration of 50 mM. In the static mechanical test, the transmural pressure was increased in a stair-wise manner between 5 and 50 mmHg with a step size of 5 mmHg. In dynamic mechanical tests, the vessel was pressurized with a cyclic sinusoidal waveform from 10 to 50 mmHg at 10 Hz.

Protocol 3: mechanical tests with passive SMC

The superfusate was replaced with fresh Ca^{2+}-and Mg^{2+}-free PBS. The LPA was allowed to equilibriate at 15 mmHg for 20 min and then preconditioned between 5 and 50 mmHg at 1 Hz for 10 cycles. Both static and dynamic tests were then performed. In the dynamic test, the cyclic sinusoidal pressure ranged from 10 to 50 mmHg at frequencies of 0.1, 1, 5, and 10 Hz.

The dose response curve is defined as the percent change in OD at various concentrations of KCl relative to the OD at 0 mM KCL. We calculated:

$$\frac{\Delta OD_{15}}{OD_{15}^0} = \frac{OD_{15} - OD_{15}^0}{OD_{15}^0}$$

where the subscript of OD indicates the transmural pressure and the superscript indicates the concentration of KCl. That is, OD_{15} is the external diameter at various concentrations of KCl and with a transmural pressure of 15 mmHg, and OD_{15}^0 is the external diameter at zero mM KCl and 15 mmHg.

Arterial contractility in response to 50 mM KCl was evaluated with the functional contraction ratio as defined previously [4]:

$$\frac{\delta OD}{OD} = \frac{OD - OD'}{OD}$$

where OD is outer diameter at any given pressure in the SMC passive state and OD' is the outer diameter at the same pressure in the SMC activated state.

Cauchy stress (σ) and Green's circumferential strain (ε) were calculated as described previously [5].

$$\varepsilon = \frac{1}{2}(\lambda^2 - 1)$$

$$\sigma = \frac{P \times ID}{h}$$

Where λ is the stretch, defined as the ratio of OD at the given pressure to the OD at 5 mmHg in the passive state ($\lambda = \frac{OD}{OD_5}$); h is the wall thickness caculated as $h = \frac{1}{2}\left(OD - \sqrt{(OD)^2 - (OD_{15})^2 + (OD_{15} - 2h_{15})^2}\right)$ as previously described [5]; and ID is the inner diameter calculated as ID = OD - 2h. Transition strain was approximated as the intersection point of two lines that best fit the low and high strain regions of the stress-strain curve, respectively [6]. PA compliance was defined as the area (A = $\pi OD^2/4$) change in response to pressure change (C = $\Delta A/\Delta P$). Here we calculated PA compliance using the areas and pressures at 5 and 50 mmHg. Since arterial compliance is a function of pressure, we also calculated PA compliance at each incremental change of pressure at 5 mmHg from 5 to 50 mmHg.

Damping capacity (D) is defined as the ratio of energy loss to total energy in the artery wall over a pressurized cycle, as described previously [5]:

$$D = \frac{W_D}{(W_D + W_S)}$$

where W_D and W_s are the dissipated energy and stored energy in the arterial wall, respectively, and cycle ($W_D + W_s$) is the total energy. Damping capacity was quantified using the area of the pressure-diameter relationship hysteresis. Dynamic elastic

modulus and structural stiffness were derived as the slope of the line best-fit to the loading cycle of the stress-strain and pressure-stretch hysteresis loops, respectively.

Histological analysis of PA structure

After mechanical testing, the LPA was fixed in 10% formalin, embedded in agar gel, and sectioned to 5 µm thickness. The slides were stained with hematoxylin and eosin (H&E) to examine vessel morphology (total and medial wall thickness), verhoff Van Geisen (VVG) to identify elastin, alcian blue to detect proteoglycans, and picro-sirius red to measure collagen. To measure changes in SMC phenotype and endothelial cell function with PAH and/or 17β-estradiol treatment, we performed immunohistochemistry using smoothelin (cat. no. 28562, Santa Cruz Biotechnology, CA), a biomarker for contractile phenotype of SMC [7]. To test the hypothesis that oxidative stress increases with PAH and is attenuated with 17β-estradiol treatment, we assessed immunostaining of nitrotyrosine (ab42789, Abcam), a stable biomarker of oxidative stress [8]. All slides for immunohistology were counterstained with hematoxylin on a DAB substrate.

Images were captured on an inverted microscope (TE-2000; Nikon, NY) at 10X using a Spot camera and analyzed using imaging analysis software Metavue (Optical Analysis Systems, NH). To avoid the artifacts induced by fixation and sectioning, only intact regions of artery wall were chosen for analysis. Wall thickness was measured as the intimal-to-adventitial distance and averaged over 5-6 positions, as described previously [3]. To quantify the area fraction of a protein of interest, the area positive for staining was identified using thresholding and normalized to the total area of PA wall at the region of interest. The content of the protein of interest was calculated as the product of area faction and the total wall thickness of the PAs. Values from 3-4 regions of interest were averaged to obtain a single measurement for each PA. PAs from 4-6 mice per group were assessed.

Statistical analysis

All results are presented as mean ±SE. Two-way analysis of variance was used to compare hemodynamic parameters, powers, morphological and histological data between the 17β-estradiol -treated and placebo-treated PAH and control groups. Tukey multiple comparisons were used for post hoc analysis. A linear mixed effects model with animal specific random effects was used to compare vasoreactivity response, static test compliance and stretch, and dynamic test parameters between experimental conditions. Tukey's Honestly Significant Difference (HSD) method was used to control the false positive rate when conducting multiple comparisons. Dunnett's method was used as multiple testing adjustment when comparing dynamic test parameters between experimental and control conditions. Model assumptions for all outcome parameters were evaluated using normal probability plots. All p-values are two-sided and $P<0.05$ was used to determine statistical significance. Statistical analyses were conducted using SAS software (SAS Institute, Cary NC) version 9.3.

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Supplemental Table

Table S1. Effects of 17β-estradiol on morphological changes of the right ventricle and conduit pulmonary artery in PAH

Parameters	CTL_P	CTL_E	SuHx_P	SuHx_E
BW (g)	22.5±0.3	22.1±0.5	20.4±0. 3*	21.8±0.5†
RV/BW (mg/g)	0.89±0.03	0.85±0.03	1.68±0.1*	1.53±0.04*
PA diameter (μm)	752±10	722±11	730±10	784±13*†
Total wall thickness (μm)	36±3	36±1	53±2*	41±4†
Medial wall thickness (μm)	14.5±0.8	13.8±0.5	23.9±1.1*	18.6±2.3

Mean ± SE values (n = 6-7 per group). RV here indicates right ventricular weight; BW, body weight. *, $P<0.05$ vs Control; †, $P<0.05$ vs Placebo.

Supplemental Figures
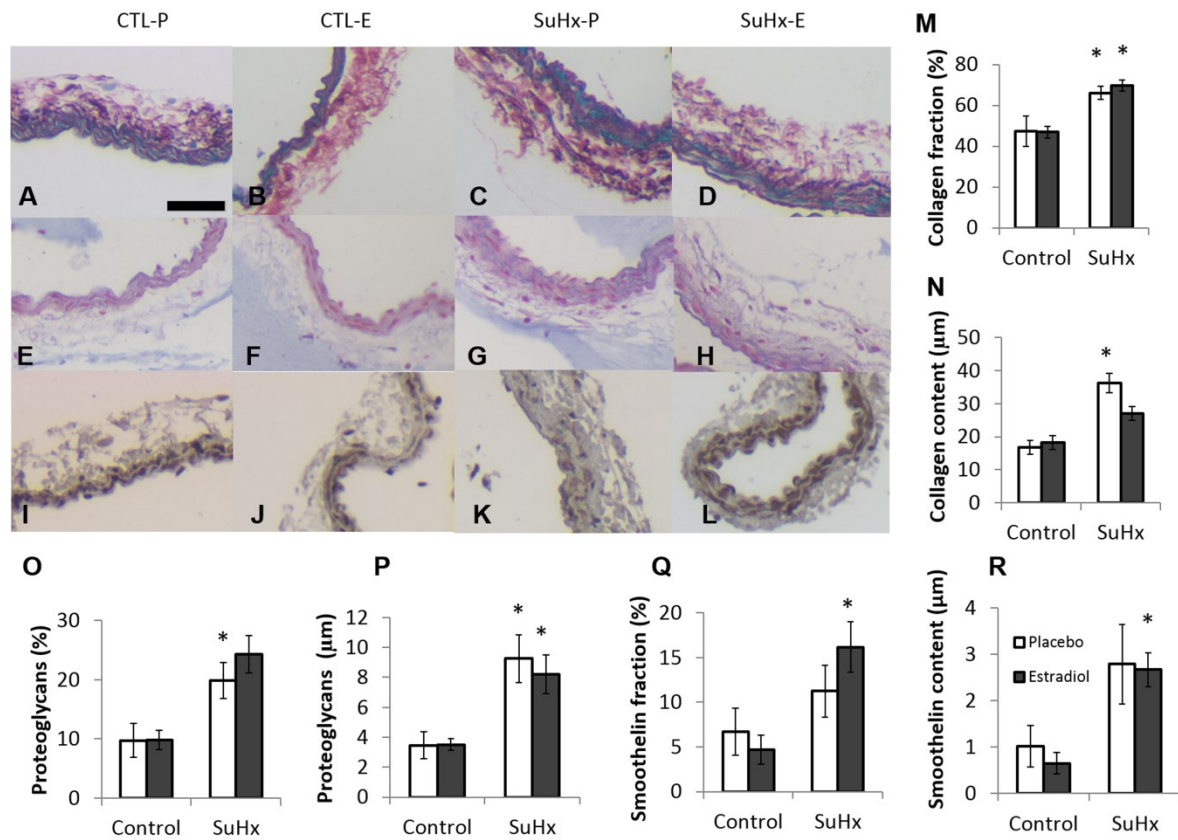
Figure S1. Representative conduit PA histology and semi-quantitative analysis in the placebo- and 17β-estradiol-treated control and SuHx groups. (A-D) Sirius red staining for collagen (red), (E-H) Alcian blue staining for proteoglycans (red), and (I-L) immunohistochemistry for smoothelin (brown); (M) collagen area fraction, and (N) content; (O) proteoglycan area fraction, and (P) content; (Q) smoothelin area fraction, and (R) content. *, P<0.05 vs. Control; #, P<0.05 vs. Placebo. Scale bar = 50 µm.
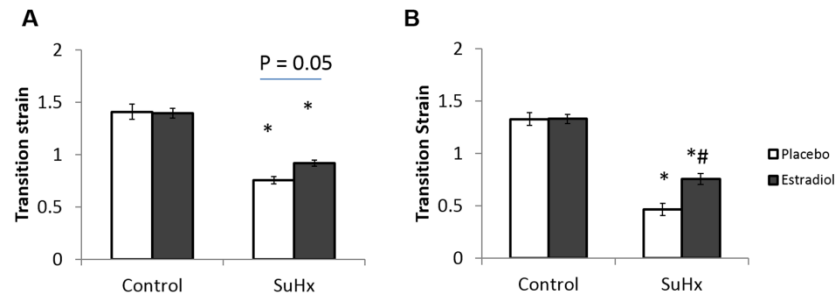
Figure S2. 17β-estradiol attenuates the decrease of transition strain in conduit PAs in PAH (A) in the absence of SMC tone and (B) with SMC activation at 50 mM KCl. *, P<0.05 vs. Control; #, P<0.05 vs. Placebo.
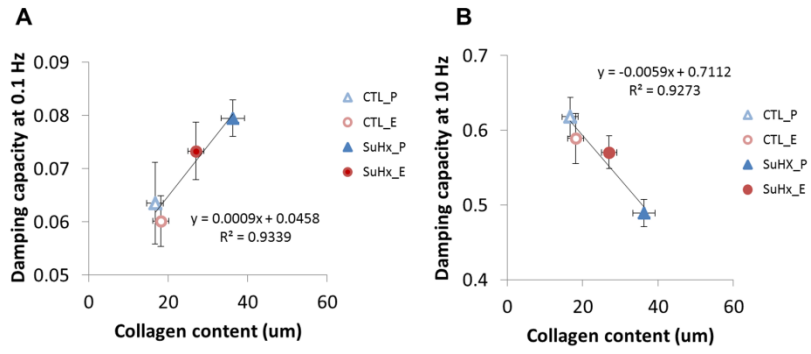
Figure S3. Frequency-dependence of relationship between conduit PA collagen content and conduit PA damping capacity. Correlation of collagen content and damping capacity (A) 0.1 Hz and (B) 10 Hz.

$y = -0.0113x + 0.7667$
$R^2 = 0.8982$
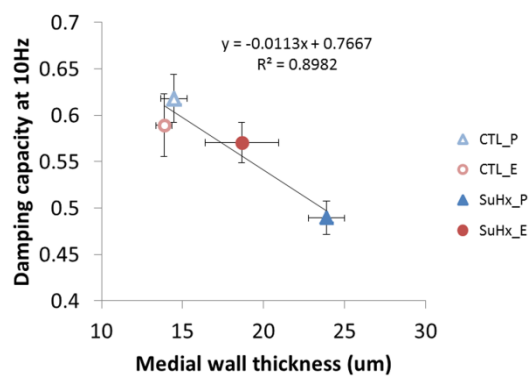
Figure S4. Negative correlation of conduit PA medial wall thickness and damping capacity at 10 Hz.
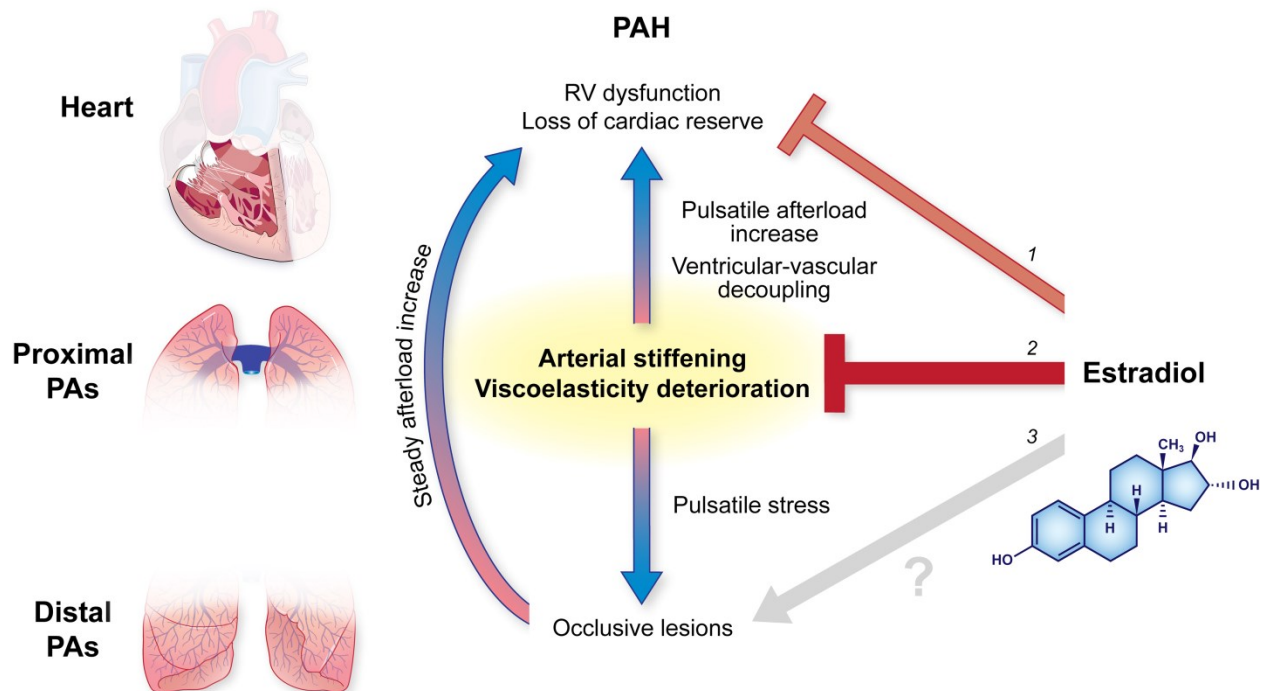
Figure S5. A simplified diagram summarizing the mechanical interactions between the right ventricle (RV), conduit PAs and pulmonary microvasculature in PAH and how 17β-estradiol modifies these interactions. In PAH, increase of RV steady afterload due to distal PA occlusion and increase of RV pulsatile afterload due to conduit PA stiffening lead to ventricular-vascular decoupling, RV dysfunction, and loss of cardiac reserve. In addition, loss of compliance and viscoelasticity of conduit PAs increases pulsatile stress, which promotes distal PA occlusion and contributes to PAH progression. The potential effects of 17β-estradiol on the RV, conduit PAs and pulmonary microvasculature in PAH are summarized as: 1. 17β-estradiol protects RV function and cardiac reserve [1,9]; 2. 17β-estradiol attenuates conduit PA stiffening and viscoelasticity deterioration, which is the focus of this study; and 3. the effects of 17β-estradiol on distal PAs remain controversial -- while some studies indicate 17β-estradiol protects the pulmonary vasculature [10,11], other studies suggest that 17β-estradiol promotes occlusive lesions in distal PAs [12–14].

17β-Estradiol Attenuates Conduit Pulmonary Artery Mechanical Property Changes With Pulmonary Arterial Hypertension

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