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

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). Although some studies have found that E2 drives PAH progression, others have found a protective effect (3–6). The majority of these studies used right ventricular (RV) end systolic or mean pulmonary artery (PA) 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). 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

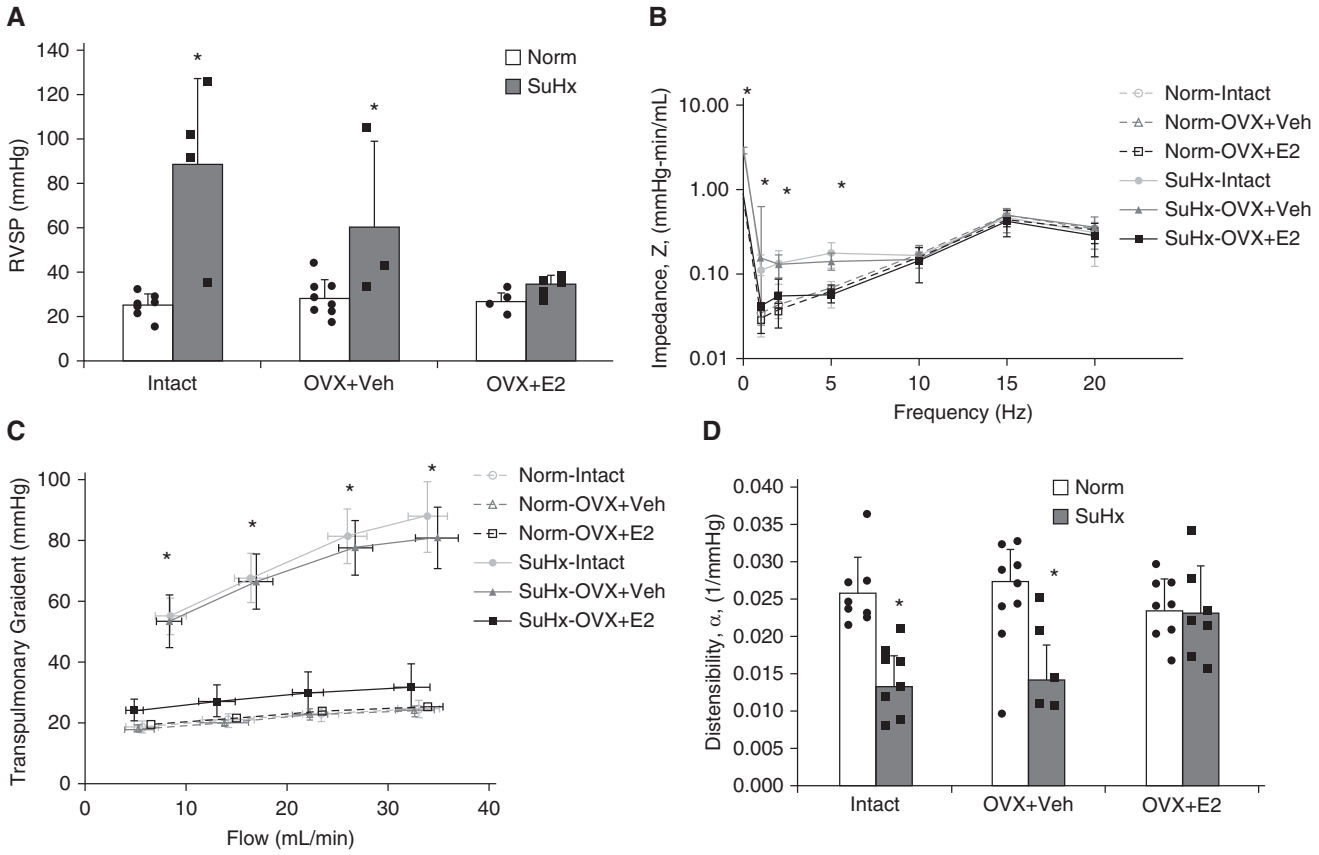

Figure 1. Exogenous estrogen treatment is protective for pulmonary arterial mechanics after Sugen/hypoxia (SuHx) exposure. (*A*) Right ventricular systolic pressure (RVSP) was measured via closed-chest right-heart catheterization in intact females (Intact) and ovariectomized females with continuous 17β-estradiol (E2) repletion (OVX+E2) or placebo (OVX+Veh) before either normoxia (Norm) or SuHx exposure; $n = 3$–8 per group. (*B*) The pulmonary vascular impedance magnitude (Z) was measured (logarithmic scale on the *y*-axis) for varying pulsatile flow frequencies in Intact, OVX+E2, and OVX+Veh isolated rat lungs after either Norm or SuHx exposure; $n = 4$–9 per group. (*C*) The transpulmonary gradient was measured for varying flow rates in Intact, OVX+E2, and OVX+Veh isolated rat lungs after either Norm or SuHx exposure; $n = 4$–9 per group. (*D*) Distal pulmonary artery distensibility in Intact, OVX+E2, and OVX+Veh isolated rat lungs after Norm or SuHx exposure; $n = 4$–9 per group. $^*P < 0.05$ versus Norm and SuHx-OVX+E2.

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frequencies, is necessary (8, 9). To date, no studies have quantified mechanical pulmonary vascular function in PAH using such an approach. A better understanding of the effects of E2 on mechanical pulmonary vascular function would help explain 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 (labeled Intact), ovariectomized rats replete with exogenous E2 in a continuous manner (75 $\mu g \cdot kg^{-1} \cdot day^{-1}$ via subcutaneous pellets [labeled OVX+E2]), or ovariectomized rats treated with 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 the effects of endogenous E2, we used only female rats. Seven weeks after SuHx exposure, RV systolic pressure (RVSP) was measured via closed-chest right-heart catheterization (3). This protocol was designed to generate a chronic, progressive, and severe model of PAH and has been well characterized. E2 repletion was initiated before 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 after treatment with the rho kinase inhibitor Y27632 (Y27, 10^{-5} M). Y27 was used to eliminate persistent vasoconstriction. The main PA and left atria were cannulated (10), and perfusion was carried out via a protocol adapted from one we previously developed for mice (11). Small variations in the steady flow rate were due to the flow pump's sensitivity to downstream impedance, which varied with individual animals but did not affect the distensibility calculations because the flow ranges achieved were equivalent.

To analyze PA remodeling, Verhoeff-Van Giesson immunohistochemical staining was performed on paraffin-embedded sections from lungs fixed with agarose-formalin (to a pressure of 23 cm H_2O) (12). Lungs fixed in formalin did not undergo *ex vivo* perfusion testing and were not treated with Y27. We determined the PA wall area by calculating the ratio between the area defined by the internal

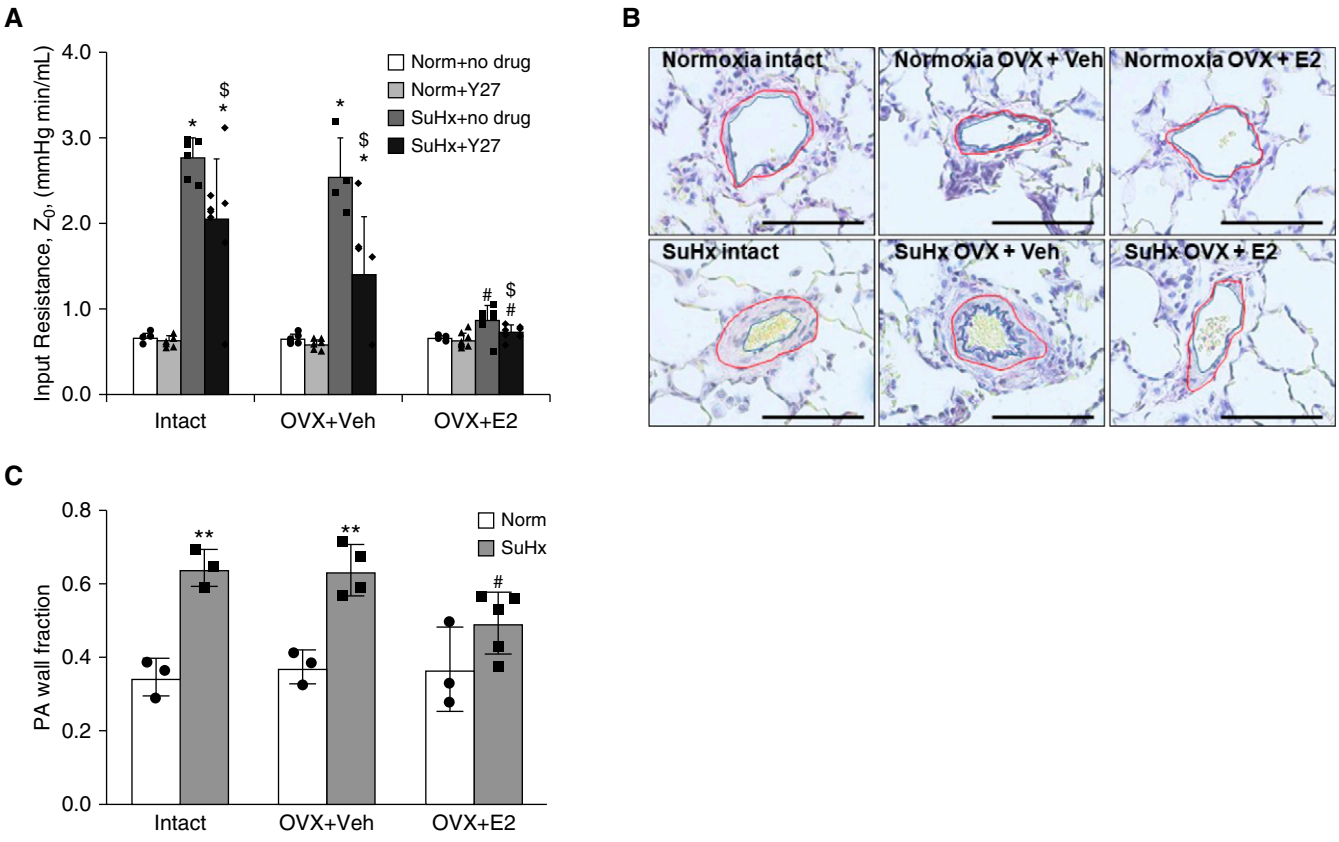

Figure 2. Exogenous estrogen prevents vasoconstriction and pulmonary artery (PA) remodeling after Sugen/hypoxia (SuHx) exposure. (*A*) Pulsatile perfusion of isolated rat lungs in intact females (Intact) and ovariectomized females with continuous 17β-estradiol (E2) repletion (OVX+E2) or placebo (OVX+Veh) before either normoxia (Norm) or SuHx exposure demonstrates increased impedance magnitude (Z) at 0 Hz (input resistance, Z_0) after SuHx exposure in the Intact and Veh groups, but not in the E2 groups. Treatment with the rho kinase inhibitor Y27 induced a stronger response in the SuHx-OVX+Veh group than in the Intact or OVX+E2 group; $n = 4$–9 per group, $^{*}P < 0.05$ versus Norm, $^{\#}P < 0.05$ versus SuHx-Intact and SuHx-OVX+Veh, and $^{\$}P < 0.05$ versus no Y27. (*B* and *C*) PA remodeling was assessed by Verhoeff-Van Giesson staining and subsequent determination of the PA wall fraction in Intact, OVX+E2, and OVX+Veh rat lungs after either Norm or SuHx exposure. The PA wall fraction was determined by dividing the PA wall area (area between the blue line and the red line) by the total vessel area (total area outlined by the red line). The red line denotes external elastic lamina, and the blue line denotes the internal lumen border. Note the decreased PA wall fraction in SuHx-OVX+E2 versus SuHx-Intact or SuHx-OVX+Veh rat lungs. Scale bars, 100 μm. (*B*) Representative images. (*C*) Quantification of the PA wall fraction; $n = 3$–5 rats per group; 20 vessels per rat were analyzed. $^{**}P < 0.01$ versus Norm and $^{\#}P < 0.05$ versus SuHx-Intact and SuHx-OVX+Veh.

lumen border and the external elastic layer (as identified by Verhoeff-Van Giesson 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 used nine animals per experimental group. One animal in the Intact SuHx group died before the terminal time point with symptoms of RV failure. Additional losses in data occurred owing to technical factors in the *in vivo* and *ex vivo* procedures. All data are presented as mean ± SD. Two-way ANOVA was used to compare differences between groups, and repeated-measures ANOVA was used to evaluate the effect of Y27 on pulmonary vascular mechanics. Although the sample sizes for individual groups were small for some endpoints, the combined sample sizes were sufficient for ANOVA to evaluate model assumptions. Residual plots and normal probability plots were examined, and there was no evidence of model assumption violations.

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

A steady-flow evaluation of pulmonary vascular mechanics *ex vivo* in isolated perfused lungs showed an increased transpulmonary gradient (TPG; determined as the main PA pressure minus the left atria pressure) after SuHx in Intact and OVX+Veh rats, whereas E2 repletion prevented this increase (Figure 1C). Consistent with these findings, distal PA distensibility determined from multipoint pressure–flow curves (13) decreased nearly 50% after SuHx exposure in both the Intact and OVX+Veh groups, whereas the OVX+E2 group exhibited preserved distensibility (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 after 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 (Figures 2B and 2C). We found that E2 repletion was associated with a 60% reduction in the PA wall area as compared with 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 rats with angioproliferative PAH. In particular, this is the first investigation of pulsatile pulmonary vascular mechanics in SuHx-PH rats. We demonstrated distal PA narrowing with relative preservation of proximal PA mechanics. Although 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 because RVSP can be confounded by other factors, and because impedance and distensibility are critical determinants of RV adaptation in PAH. Impedance measurements and the 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. Although 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 the two 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. In future studies, we will focus on the use of 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. ■

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Nonsteroidal Antiinflammatory Drugs Modify the Effect of Short-Term Air Pollution on Lung Function

To the Editor:

Air pollution, especially fine particulate matter (PM $<$2.5 μm [$PM_{2.5}$]), has been recognized as a major public health concern worldwide (1). Previous epidemiological studies demonstrated that short-term PM exposure can trigger acute respiratory events, including the decline of lung function (e.g., FEV_1 and FVC) (2). The activation of systemic inflammatory response is a widely accepted mechanism for the acute effect of PM exposure (1). Nonsteroidal antiinflammatory drugs (NSAIDs) are one of the most commonly prescribed medications for pain and inflammation. Previous studies revealed that daily use of aspirin, a common NSAID, was associated with a reduced risk of chronic obstructive pulmonary disease (3) and a significantly slower progression of emphysema over 10 years (4). Therefore, we hypothesized that the use of NSAIDs may be associated with a smaller decrease in lung function under short-term ambient PM exposure and tested this hypothesis in the longitudinal data of the Normative Aging Study (NAS).

Methods

Established in 1963, the NAS is a cohort of 2,280 male veterans from the Greater Boston area, where air pollution data have been collected since 1995 (5). Participants have been reevaluated every 3–5 years with use of on-site physical examinations and questionnaires, and they have provided detailed information about lifestyle and demographic factors at each visit. Spirometry was assessed with subjects in the standing position, wearing a noseclip, and using a 10-L, water-filled, survey-recording spirometer and an Eagle II minicomputer (Eagle Computer). Standard methods were used to obtain FVC (in L), FEV_1 (in L), percentage of vital capacity (FEV_1/FVC), and maximal midexpiratory flow rate (in L/s, calculated as the average flow rate between 25% and 75% of FVC) (6). All values were corrected to body temperature and pressure saturated with water vapor. Smoking status and pack-years were assessed at the time of pulmonary function testing. Usage of NSAIDs ("no"/"yes") at each visit was recorded and further

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