Estrogen Rescues Preexisting Severe Pulmonary Hypertension in Rats

Soban Umar[1], Andrea Iorga[1], Humann Matori[1], Rangarajan D. Nadadur[1], Jingyuan Li[1], Federica Maltese[1], Arnoud van der Laarse[2], and Mansoureh Eghbali[1]

[1]Department of Anesthesiology, Division of Molecular Medicine, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, California; and [2]Department of Cardiology, Leiden University Medical Center, Leiden, The Netherlands

Rationale: Pulmonary hypertension (PH) is characterized by progressive increase in pulmonary artery pressure leading to right ventricular (RV) hypertrophy, RV failure, and death. Current treatments only temporarily reduce severity of the disease, and an ideal therapy is still lacking.

Objectives: Estrogen pretreatment has been shown to attenuate development of PH. Because PH is not often diagnosed early, we examined if estrogen can rescue preexisting advanced PH.

Methods: PH was induced in male rats with monocrotaline (60 mg/kg). At Day 21, rats were either treated with 17-β estradiol or estrogen (E2, 42.5 μg/kg/d), estrogen receptor-β agonist (diarylpropionitrile, 850 μg/kg/d), or estrogen receptor α-agonist (4,4′,4″-[4-Propyl-(1H)-pyrazole-1,3,5-triyl] trisphenol, 850 μg/kg/d) for 10 days or left untreated to develop RV failure. Serial echocardiography, cardiac catheterization, immunohistochemistry, Western blot, and real-time polymerase chain reaction were performed.

Measurements and Main Results: Estrogen therapy prevented progression of PH to RV failure and restored lung and RV structure and function. This restoration was maintained even after removal of estrogen at Day 30, resulting in 100% survival at Day 42. Estradiol treatment restored the loss of blood vessels in the lungs and RV. In the presence of angiogenesis inhibitor TNP-470 (30 mg/kg) or estrogen receptor-β antagonist (PHTPP, 850 μg/kg/d), estrogen failed to rescue PH. Estrogen receptor-β selective agonist was as effective as estrogen in rescuing PH.

Conclusions: Estrogen rescues preexisting severe PH in rats by restoring lung and RV structure and function that are maintained even after removal of estrogen. Estrogen-induced rescue of PH is associated with stimulation of cardiopulmonary neoangiogenesis, suppression of inflammation, fibrosis, and RV hypertrophy. Furthermore, estrogen rescue is likely mediated through estrogen receptor-β.

Keywords: pulmonary hypertension; estrogen; neoangiogenesis; estrogen receptors; inflammation

Pulmonary hypertension (PH) is a chronic lung disease characterized by pulmonary vascular remodeling and progressive

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Despite significant advances in cardiopulmonary research, pulmonary hypertension (PH) still remains a difficult disease to treat, as therapeutic strategies to simultaneously reduce pulmonary vascular damage and prevent right ventricular (RV) dysfunction are lacking. This debilitating disease occurs more frequently in women, yet both animal studies in classical models of PH and limited clinical data suggest protective effects of estrogens: the estrogen paradox in pulmonary hypertension.

What This Study Adds to the Field

Estrogen not only exerts beneficial effects on lung pathology but also protects the RV from PH-related complications in RV structure and function in rats treated with monocrotaline. This study demonstrates that estrogen rescues severe preexisting PH in rats, suggesting a possible role of estrogen for treatment of severe PH in patients. Because the rescue action of estrogen is likely mediated by estrogen receptor-β, selective estrogen receptor-β ligands may have value for rigorous preclinical evaluations and could provide a novel treatment modality for patients with pulmonary hypertension.

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increase in pulmonary artery pressure leading to right ventricular (RV) hypertrophy and RV failure (RVF). End-stage RVF has long been regarded as a terminal state of pathological cardiopulmonary remodeling, including fibrosis and chamber dilation, being unresponsive to available therapies. Advanced PH is most often treated with aggressive nonpharmacological therapies, such as lung transplantation, but this approach is only feasible for a fraction of patients. In the past decade, cell and gene therapies have shown great potential for treatment of PH in animal models (1, 2) and humans (3). However, effective pharmacological therapy for treatment of patients with advanced PH would be much more practical and much more cost effective. Several agents have been identified to attenuate the development of PH when the therapy is started before the initiating stimuli (4–6). Unfortunately, up to now, there has been no ideal pharmacological therapy to reverse advanced PH.

Although the incidence of PH remains higher in female patients (7), in various animal models females have been shown to be protected against PH (8–10), a phenomenon known as the "estrogen paradox" of PH. This sex difference in susceptibility to experimental PH has been suggested to be in part due to the action of estrogen (E2), as ovariectomy exacerbates PH and pretreatment with E2 and its metabolites attenuates the progression of different animal models of PH (4, 9, 11).

Because PH is not always diagnosed early, we tested the more clinically relevant hypothesis that E2 may also reverse preexisting

severe PH and explored the molecular mechanisms involved in the rescue of preexisting PH by E2 therapy. We used the well-established model of monocrotaline (MCT)-induced PH in rats, which has been commonly used by many investigators (1, 12). We found that a 10-day E2 treatment significantly reversed MCT-induced preexisting PH by restoring lung and heart structure and function, resulting in 100% survival even after withdrawal of E2. Restoration of angiogenesis by E2 both in RV and lung is one of the key mechanisms in E2-induced rescue of PH. Furthermore, this rescue action of E2 is likely mediated through an estrogen receptor (ER)-β−dependent mechanism. Some of the results of these studies have been previously reported in the form of abstracts (13, 14).

METHODS

Animals and Treatments

Male and female Sprague-Dawley rats (350–400 g) were used. Details of the treatments and criteria for survival measurements are given in the online data supplement. Protocols received institutional review and committee approval. The investigation conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (15).

Cardiac and Pulmonary Hemodynamics

B-mode, M-mode, and pulmonary pulse-wave Doppler echocardiography were performed using a VisualSonics Vevo 770 equipped with a 30-MHz linear transducer to accurately monitor the stage of the disease (see online data supplement for details).

Gross Histologic Evaluation

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

Real-Time Polymerase Chain Reaction

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

Western Blot Analysis

Standard Western blot analysis was performed using lung and RV lysates. The details of the Western blot analyses and the antibodies used are presented in the online data supplement.

Immunohistochemistry and Imaging

Whole hearts and lungs were fixed in 4% paraformaldehyde in 0.1 M Na_2HPO_4 and 23 mM NaH_2PO_4 (pH 7.4) for 4 hours on ice. The tissue was then immersed in ice-cold 20% sucrose overnight to cryoprotect, was mounted using OCT, and transversal 6- to 7-μm sections were obtained with a cryostat. Tissue sections were stained with immunofluorescence, immunoperoxidase, standard hematoxylin-eosin, and Masson trichrome staining. The images were acquired using a light microscope (Axiovert 135; Zeiss, Germany) or with a laser-scanning confocal microscope (Olympus).

Statistical Analysis

One-way analysis of variance was used to compare between groups and within the same group at different time points using SPSS13.0 for Windows. When significant differences were detected, individual mean values were compared by *post hoc* tests (Bonferroni correction) that allowed for multiple comparisons. *P* values less than 0.05 were considered statistically significant. Values are expressed as mean ± SE.

RESULTS

Estrogen Reverses PH by Improving Cardiac and Pulmonary Structure and Function and Leads to 100% Survival

Male Sprague-Dawley rats were injected with a single dose of monocrotaline (MCT, 60 mg/kg, Figure 1A) to induce PH as has been extensively used by many investigators (1, 12). Two weeks after MCT injection, signs of PH started to appear together with RV hypertrophy, but the RV ejection fraction (RVEF) was still preserved (adaptive RV hypertrophy; see Table E1 in the online supplement). By day 21, severe PH had already been well established (PH group), as peak RV systolic
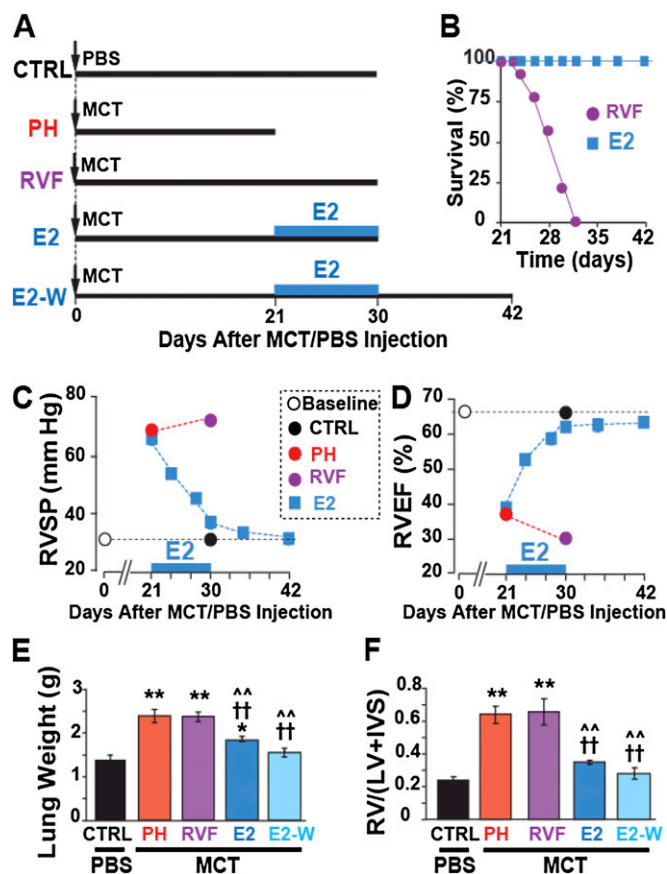

Figure 1. Estrogen (E2) rescues severe pulmonary hypertension (PH). (*A*) Experimental protocol: male rats were injected with monocrotaline (MCT) or phosphate-buffered saline (PBS) at Day 0. The *thick horizontal lines* represent the length of each experimental group. At Day 21, animals were either killed (PH group), or left untreated to develop right ventricular (RV) failure (RVF group). Both E2 and E2-W groups received E2 only from Day 21 to Day 30. All of the rats in the E2 group were killed at Day 30, whereas the rats in the E2-W group were kept for another 12 days until Day 42 after E2 withdrawal at Day 30. (*B*) Survival plot. (*C*) RV systolic pressure (RVSP) and (*D*) RV ejection fraction (RVEF) at baseline (*open circle*) and after a single MCT/PBS injection in control mice (CTRL; *black circle*), PH (*red circle*), RVF (*purple circle*), and E2-treated (*blue squares*). For simplicity, RVSP and RVEF are only shown at the end of the experiment, except for the E2-treated group, for which these parameters are shown as a function of time. The black dotted lines represent the baseline levels. (*E*) Lung weight and (*F*) RV hypertrophy index in CTRL (*black bar*), PH (*red bar*), RVF (*purple bar*), E2 (*dark blue bar*) and E2-W (*light blue bar*). *P < 0.05 versus CTRL, **P < 0.001 versus CTRL; ††P < 0.001 versus PH; ^^P < 0.001 versus RVF (n = 6–8 rats per group). IVS = interventricular septum; LV = left ventricular wall.
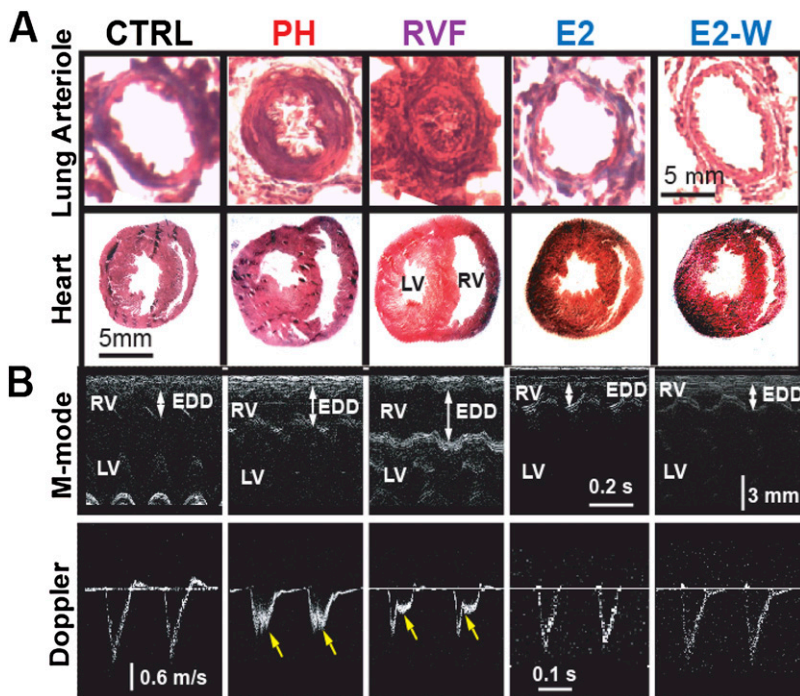
Figure 2. Reversal of cardiopulmonary structure and function by estrogen (E2). (*A*) hematoxylin-eosin staining for lung arterioles and heart cross-sections in male rats. (*B*) Echocardiographic images of M-mode (*upper panels*) showing right ventricular (RV) end-diastolic diameter (EDD), left ventricular (LV) and interventricular septum (IVS), and pulse-wave Doppler (*lower panels*) in male rats. *Yellow arrows* show mid-systolic notch present in pulmonary hypertension (PH) and RV failure (RVF) only.

pressure (RVSP) had increased from 31.1 ± 0.9 in control rats (CTRL) to 67.7 ± 1.1 mm Hg in the PH group (Figure 1C), and RVEF was reduced from $65.1 \pm 1.7\%$ in CTRL rats to $39.0 \pm 0.6\%$ in the PH group (Figure 1D). The severity of PH at Day 21 was further confirmed by (*1*) the increase in lung weight (from 1.37 ± 0.1 g in CTRL to 2.37 ± 0.09 g in PH, Figure 1E), (*2*) RV hypertrophy (RV/[LV+IVS] increased from 0.23 ± 0.02 in CTRL to 0.64 ± 0.05 in PH, Figure 1F), (*3*) pulmonary arteriolar medial hypertrophy (Figure 2A), and (*4*) appearance of a midsystolic notch on Doppler echocardiography of pulmonary artery flow (Figure 2B). These data clearly demonstrate the development of malignant PH by Day 21 (16) and agree with previous studies using this time point as an initiating point for therapy of advanced PH (12). Therefore, we started E2 therapy at Day 21 in one group of rats by subcutaneously implanting continuous-release E2 pellets (42.5 μg/kg/d) for 10 days from Days 21 to 30 (E2 group), and another group was left untreated to develop RV failure (RVF group) by Day 30. E2 not only prevented the progression to RVF but also it gradually improved RVSP and RVEF, as these parameters had returned to almost CTRL levels within 10 days of E2 treatment. In untreated animals, however, the RVSP significantly increased even further to 72.0 ± 1.4 mm Hg and RVEF significantly decreased to $30.4 \pm 1.7\%$ (Figures 1C and 1D). However, the lung weight (Figure 1E) and RV hypertrophy index (Figure 1F) did not increase after 21 days any further. E2 therapy was also associated with reversal of lung weight (1.83 ± 0.05 g vs. 2.37 ± 0.14 g at Day 21, Figure 1E) and regression of RV hypertrophy (RV/[LV+IVS] = 0.34 ± 0.01 g vs. 0.64 ± 0.05 at Day 21, Figure 1F). Moreover, pulmonary arteriolar medial hypertrophy was completely reversed (Figure 2A).

The improvements in lung and RV structure and function mediated by E2 led to 100% survival, as all E2-treated animals were alive at Day 30, whereas in untreated rats, the mortality started at Day 24 and sharply increased to 45% by Day 28 and to 75% by Day 30 (Figure 1B). Although RVF was associated with a significant reduction in weight gain during the last 10 days from Days 21 to 30 (9 ± 5 g vs. 39 ± 4 g in CTRL), E2 treatment significantly improved weight gain in this time interval (26 ± 5 g, Figure E1C).

Estrogen's Rescue of PH Persists Even after Estrogen Withdrawal

To investigate whether the improved structural and functional changes achieved by E2 therapy were maintained once E2 was no longer available, some E2-treated rats were kept for another 12 days until Day 42 after E2 withdrawal at Day 30 (E2-W group). Surprisingly, not only were the RVSP and RVEF maintained but also they improved even further and were fully restored to normal levels as in CTRL rats by Day 42 (Figures 1C and 1D; Figures E1A and E1B). The E2-induced reduction in lung weight and RV hypertrophy index decreased even further after E2 withdrawal: at Day 42, these parameters were not significantly different from the CTRL (lung weight = 1.54 ± 0.11 g in E2-W vs. 1.37 ± 0.1 g in CTRL; RV/[LV+IVS] = 0.28 ± 0.03 in E2-W vs. 0.23 ± 0.02 in CTRL; Figures 1E and 1F). All rats with PH treated with E2 for 10 days survived to at least 42 days after MCT injection, whereas none survived after Day 32 in the untreated PH group (Figure 1B). Hence, these data confirmed that E2 rescue of PH persisted even after discontinuation of E2 therapy, suggesting that short-term E2 therapy was sufficient to cure the disease.

Estrogen Reverses Pulmonary Inflammation and Inflammatory Signaling

To gain insight into the cellular and molecular mechanisms of E2-induced rescue of PH, we first investigated whether suppression of inflammation by E2 could be involved in E2-induced rescue of PH. Lung sections were stained for ED1 (CD68), a marker for macrophages/monocytes. The number of ED1-positive inflammatory cells (both the alveolar macrophages and the perivascular macrophages) in rats with PH was increased fivefold (62.5 ± 3.16 in PH vs. 12.6 ± 1.42 cells/field in CTRL), and E2 fully reversed this increase (15.4 ± 1.4 cells/field, Figures 3A and 3B). Next, we examined the possible regulation of the proinflammatory cytokine IL-6 by E2 in the lungs (17). E2 partially reversed PH-induced approximately 22-fold up-regulation of IL-6 transcripts. IL-6 protein up-regulation in PH was also
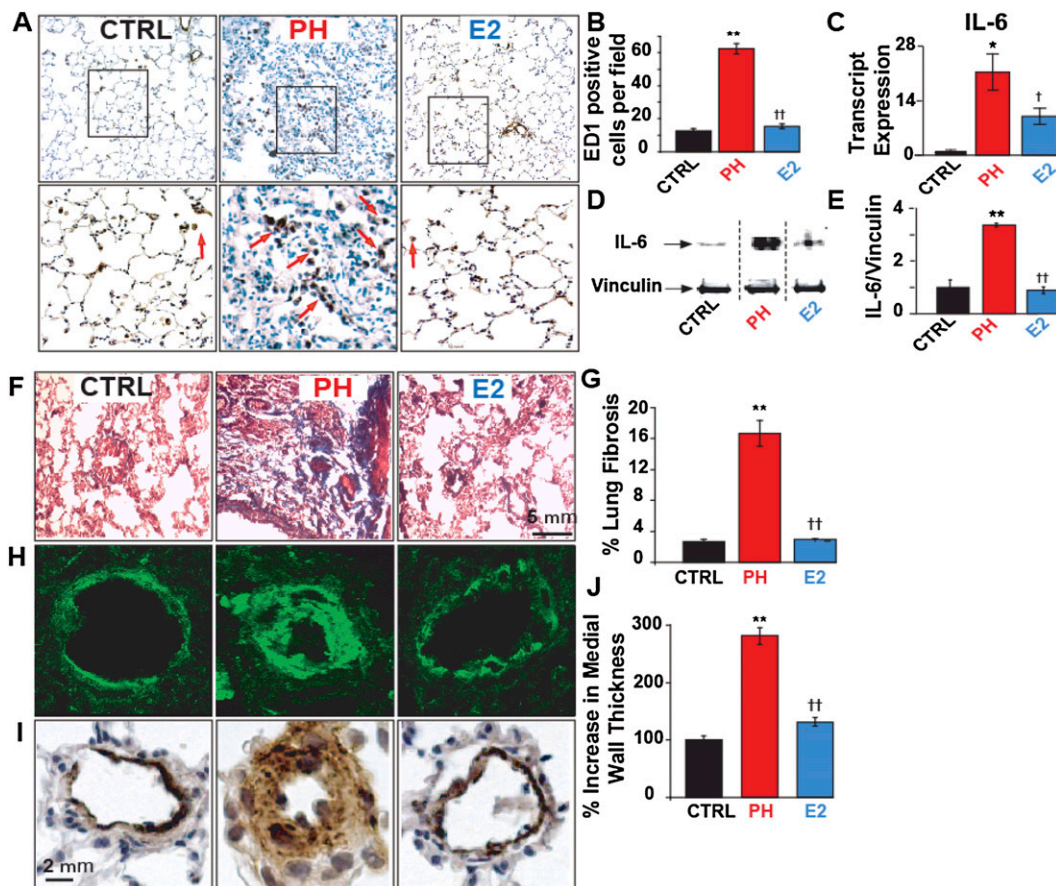
Figure 3. Reversal of lung inflammation and remodeling by estrogen (E2). (*A*) Lung sections of male rats stained for ED1 in control (CTRL), pulmonary hypertension (PH), and estrogen (E2) groups (*upper panel*) and at higher magnification of the respective fields (*lower panel*). *Red arrows* indicate the ED1-positive cells. (*B*) ED1-positive cells quantification per field in CTRL (*black bar*), PH (*red bar*), and E2 (*blue bar*). (*C*) Relative transcript expression of lung IL-6 in CTRL (*black bar*), PH (*red bar*), and E2 (*blue bar*), normalized to CTRL. (*D*) Representative immunoblots of lung lysates from CTRL, PH, and E2 labeled with anti–IL-6 and anti-vinculin antibodies. In this immunoblot and the subsequent immunoblots, all samples from CTRL, PH, and E2 groups were run on the same gel. Because we are only showing representative lanes from a total of three to five samples per group, some of the intervening lanes were not shown and are separated by a *dotted line*. (*E*) Western blot analysis of IL-6 protein in lung lysates normalized to vinculin in CTRL (*black bar*), PH (*red bar*), and E2 (*blue bar*). (*F*) Masson trichrome staining of lung sections in CTRL, PH, and E2; *blue color* indicates fibrosis. (*G*) Quantification of lung fibrosis showing percent lung fibrosis in CTRL, PH, and E2 groups. (*H*) Immunofluorescence labeling of pulmonary arterioles stained for smooth muscle actin (*green*) and (*I*) immunoperoxidase labeling of pulmonary arterioles stained with anti–smooth muscle actin antibody (*brown*) together with hematoxylin-stained nuclei (*blue*). (*J*) Bar graph for quantification of pulmonary arteriolar medial wall thickness in CTRL, PH, and E2 groups. $*P < 0.05$ versus CTRL, $**P < 0.001$ versus CTRL; $^{\dagger}P < 0.05$ versus PH, $^{\dagger\dagger}P < 0.001$ versus PH (n = 4 animals per group).

fully restored by E2 (Figures 3C–3E). In the PH group, lung fibrosis had increased considerably (16.6 ± 1.65% in PH vs. 2.6 ± 0.35% in CTRL), and E2 mediated a complete reversal of this fibrosis (2.93 ± 0.15%) (Figures 3F and 3G). Pulmonary arteriolar medial hypertrophy in the lungs of the PH group was significantly increased (281 ± 14% in PH vs. 100 ± 7% in CTRL) and was reversed by E2 (131 ± 7%) (Figures 3H–3J). RV fibrosis has been observed in biopsies from RV endomyocardial tissue of patients with PH (18). We also observed the presence of mild to moderate fibrosis in the RV sections of PH, but this was completely absent in E2-treated animals (data not shown). E2 was also able to reverse pulmonary fibrosis and inflammation in the lungs of females with PH (Figure E2).

Estrogen Stimulates Pulmonary and Cardiac Angiogenesis

Stimulation of pulmonary neoangiogenesis has been suggested as a potential therapeutic strategy for treatment of PH (19). We examined whether stimulation of pulmonary neoangiogenesis by E2 participated in E2-induced rescue of PH. Pulmonary vessel density of small vessels up to 50 μm was significantly reduced by approximately threefold in PH (5.5 ± 0.5 vessels per high-power field in PH vs. 16.5 ± 0.5 in CTRL, Figures 4A and 4B). E2 reversed the loss of blood vessels associated with PH in lung (14.3 ± 1.4) (Figure 4B). Similar to male rats, E2 was also

able to reverse the loss of vessels in the lungs of female rats with PH (Figure E3). Next, we examined whether E2 could also restore the loss of RV microvessels as reported in patients with PH as well (20). RV capillary density was significantly reduced by approximately 40% in PH (0.65 ± 0.06 microvessels per cardiomyocyte in PH vs. 0.95 ± 0.07 in CTRL, Figures 5A–5D). E2 not only completely reversed the loss of capillaries in RV but also significantly enhanced capillary density by approximately 50% compared with healthy CTRL (1.54 ± 0.12 in E2 vs. 0.95 ± 0.07 in CTRL) (Figure 5D). To further confirm the direct role of angiogenesis in the rescue action of E2, the animals were treated with E2 alone or E2 together with the angiogenesis inhibitor TNP-470 (30 mg/kg, Figure 6A). In the presence of TNP, E2 failed to rescue PH as RVSP (59.4 ± 1.8 mm Hg), RVEF (37.1 ± 1.8%), and RV hypertrophy index (0.68 ± 0.09) had not significantly improved after 10 days of E2 therapy (Figures 6B–6F). In fact, RVEF and RV hypertrophy index were not significantly different from PH. (Figures 6E and 6F). These data strongly support the vital role of angiogenesis in the rescue action of E2 in PH.

Involvement of Estrogen Receptor β in the Rescue of PH by E2

To investigate whether ERs are involved in the rescue action of E2, rats were treated with a selective agonist of ERα, 4,4′,4″-(4-
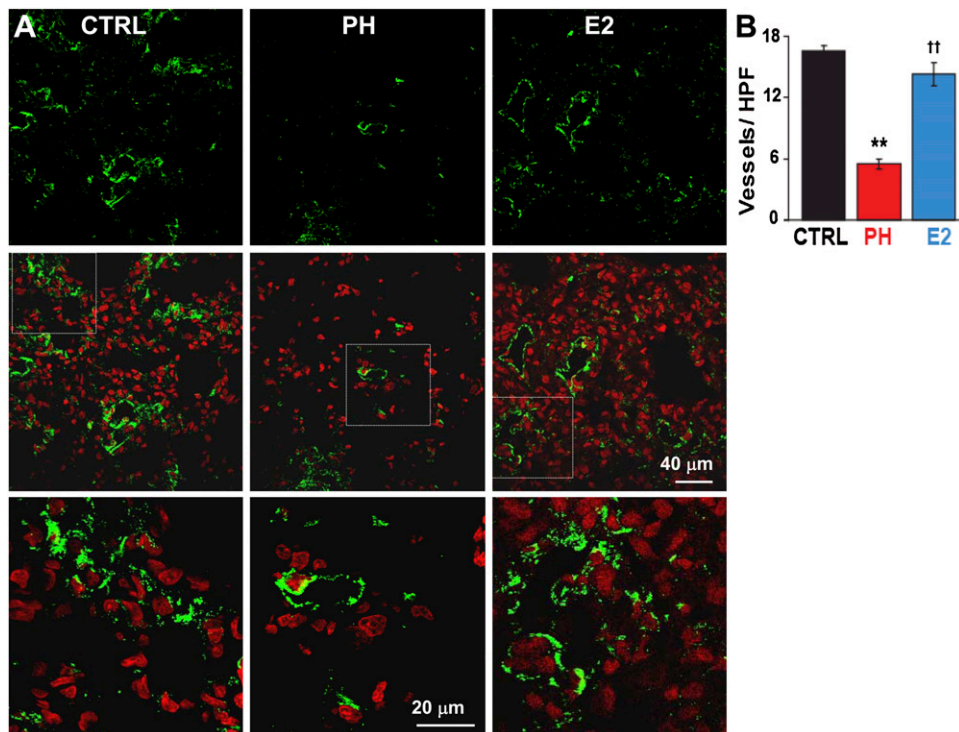
Figure 4. Stimulation of pulmonary neo-angiogenesis by estrogen (E2). (*A*) Single confocal images of lung sections of male rats immunostained for von Willebrand Factor (*green, upper panels*), overlay of von Willebrand factor and nuclei (stained *red* with TO PRO, *middle panels*), and at higher magnification of the respective fields (*lower panels*). (*B*) Quantification of vessels per high power field (HPF) in control (CTRL; *black bar*), pulmonary hypertension (PH; *red bar*), and E2 (*blue bar*). **$P < 0.001$ versus CTRL; $^{††}P < 0.001$ versus PH (n = 4 animals per group).

Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) (0.85 mg/kg/d), or a selective agonist of ERβ, diarylpropionitrile (0.85 mg/kg/d) at Day 21 after MCT injection for 10 days (Figure 7A). Diarylpropionitrile was as effective as E2, if not even better, in rescuing PH (RVSP = 34 ± 1 mm Hg, RV/[LV+IVS] = 0.30 ± 0.01, RVEF = 64 ± 4%, and lung weight = 1.5 ± 0.4 g), whereas PPT was not as effective as E2 (RVSP = 56 ± 1mm Hg, RV/[LV+IVS] = 0.60 ± 0.01, RVEF = 40 ± 4%, and lung weight = 2.0 ± 0.1 g). PPT was only able to reduce RVSP significantly compared with PH, but RVEF, lung weight, and RV hypertrophy index were not significantly different from PH (Figures 7B–7E). To further confirm the role of ERβ in the rescue action of E2, we treated rats with PH with E2 in the presence of the ERβ-specific antagonist PHTPP for 10 days. Interestingly, E2 could not rescue PH in the presence of PHTPP, as the beneficial effects of E2 on RV pressure, RV ejection fraction, lung weight, and RV hypertrophy were all abolished (RVSP = 60 ± 4 mm Hg, RV/[LV+IVS] = 0.58 ± 0.05, RVEF = 42 ± 1.7%, and lung weight = 1.93 ± 0.01 g) (Figures 7B–7E). These results suggest that the rescue of PH by E2 is most likely mediated via the ERβ.

DISCUSSION

The present study shows that E2 is a rescue agent for advanced preexisting PH. E2 reversed PH by improving cardiac and pulmonary structure and function leading to 100% survival even after withdrawal of E2. Our approach of starting E2 treatment after the establishment of fatal PH has major advantages over previous studies (4, 11) that used E2 therapy before the initiation of the disease, as our approach (*1*) is more practical for patients who already have severe PH, as this disease is not often diagnosed early; and (*2*) requires short duration and low dose of E2, thereby minimizing the chance of side effects, especially in women. Regarding the possible mechanisms involved in E2-induced rescue of PH, our data strongly support that the rescue action of E2 is mainly mediated through ERβ because (*1*) selective estrogen receptor-β agonist is able to rescue PH as

efficiently as E2, and (*2*) E2 fails to rescue PH in the presence of ERβ selective antagonist (Figure 7). Stimulation of lung and RV angiogenesis by E2 is the other key mechanism involved in E2-induced rescue of PH (Figures 4–6). The beneficial effects of E2 in PH also seem to result from interplay of several factors, including suppression of lung inflammation and fibrosis as well as reversal of RV hypertrophy (Figure E4). Direct actions of E2 on the vessel wall of pulmonary arteries and arterioles have been described (21), but whether the improved RV function is simply a result of improved lung function or a direct action of E2 on the heart is not clear. As E2 was shown to attenuate LV hypertrophy of ventricular myocytes (22), we speculate that in addition to the action of E2 on the lung, E2 also directly acts on the RV to reverse RV hypertrophy (Figure 1F) and restores RV contractility and function (23) (Figure 1D).

Reverse Remodeling of Pulmonary Inflammation and Fibrosis by E2 Therapy

Inflammation plays an important role in the progression of PH in animal models as well as in patients with PH of various origins (24), and E2 has been shown to have antiinflammatory activity in many tissues such as the lung (25). The number of ED1-positive inflammatory cells was increased considerably in PH, and E2 treatment led to suppression of these inflammatory cells in the lungs both in males and females. E2 treatment also suppressed the up-regulation of lung IL-6 transcript and protein levels induced by PH (Figure 3). Therefore, suppression of inflammation and inflammatory mediators by E2 could be involved in E2-induced rescue of PH.

Sex differences have recently been proposed to exist in the development of lung fibrosis, as female mice are better protected against pulmonary fibrosis than male mice (26). In the present study, the increase in pulmonary fibrosis in our male PH group was almost completely reversed by E2. Reversal of cardiac fibrosis by E2 has been shown recently to be mediated by ERβ through suppression of transforming growth factor-β signaling. We believe that E2 may act through a similar ERβ-mediated pathway to reverse lung fibrosis as suggested by the recent reports (27, 28).
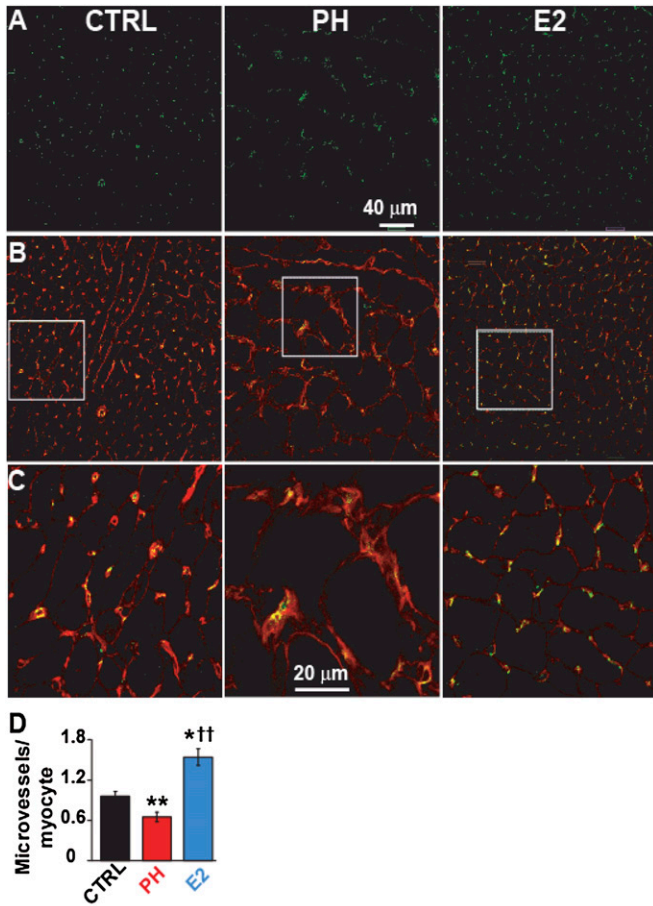
Figure 5. Stimulation of cardiac neoangiogenesis by estrogen (E2). (A–C) Single confocal images of right ventricular sections of male rats immunostained for CD31 (green, A), overlay of CD31 and WGA (red, B) and at higher display magnification (C). (D) Quantification of microvessels/cardiomyocyte in control (CTRL; black bar), pulmonary hypertension (PH; red), and E2 (blue). *P < 0.05 versus CTRL, **P < 0.001 versus CTRL; ††P < 0.001 versus PH (n = 4 animals per group).

Figure 6. Estrogen (E2) fails to rescue pulmonary hypertension (PH) in the presence of angiogenesis inhibitor. (A) Experimental protocol. (B) Right ventricular systolic pressure (RVSP) and (C) RV ejection fraction (RVEF) at the end point of each experiment, except for E2-treated groups, for which the data are presented as a function of time. The solid lines represent the baseline levels. (D–F) RVSP, RVEF, and RV hypertrophy index RV/(left ventricular[LV]+ interventricular septum [IVS]) for control (CTRL; open bar), PH (shaded gray bar), E2 (gray bar), and E2+TNP (solid bar) groups at Day 30 except for PH group, which was at Day 21. *P < 0.05 versus CTRL, **P < 0.001 versus CTRL; †P < 0.05 versus PH, ††P < 0.001 versus PH; #P < 0.05 versus E2, ##P < 0.001 versus E2 (n = 3–8 animals per group).

Stimulation of Pulmonary and Cardiac Angiogenesis by Estrogen

Loss of small blood vessels and impaired pulmonary angiogenesis contribute to the elevation of pulmonary pressures and progression of the PH. In the RV, loss of myocardial microvessels together with increased oxygen demand in patients with PH with normal coronary arteries has been shown to result in RV ischemia (20). The decrease in capillary density in the RV has been proposed to be due to insufficient up-regulation of angiogenic factors (29). Here we found that PH was associated with loss of small blood vessels both in lungs and in RV. E2 therapy reversed the loss of vessels associated with PH in the lungs in both male and female rats. E2 not only reversed the loss of blood vessels observed in the RV of the PH group, it also stimulated the growth of new capillaries beyond their levels in healthy CTRL rats. Stimulation of coronary angiogenesis in the LV has been shown to be a key event in maintaining heart function during the development of LV hypertrophy (30). These findings are not surprising as E2 is known to promote angiogenesis in several tissues (31–33). Therefore, increased myocardial blood vessels together with increased angiogenesis in the pulmonary circulation by E2 therapy may underlie the decrease in PH severity leading to improvements in cardiopulmonary
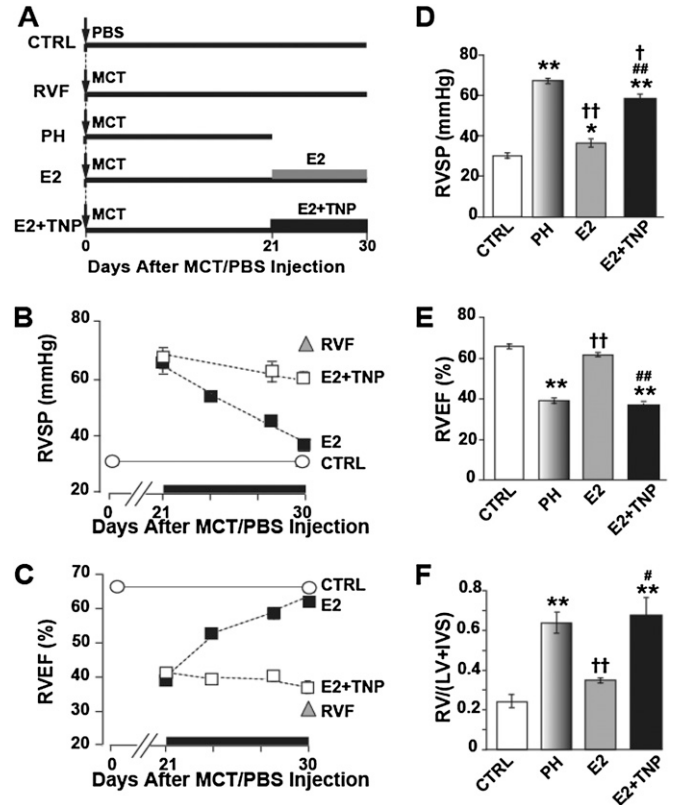
structure and function. The fact that in the presence of TNP, a potent angiogenesis inhibitor, E2 failed to rescue PH strongly supports that stimulation of angiogenesis in both lung and RV by E2 is one of the key mechanisms in E2-induced rescue of PH.

The Rescue Action of Estrogen Is Likely Mediated through ERβ

E2 exerts its biological effects mainly via ERα and ERβ. Both receptor subtypes are present in lungs and heart (17, 34). ERβ has been shown to protect the lungs in a trauma-hemorrhage model (17) and the heart against ischemia/reperfusion injury and against pressure overload-induced LV failure (26, 35, 36). ERβ-knockout mice demonstrated abnormal vascular function and hypertension, increased mortality, and aggravation of heart failure (37, 38). Our data strongly support that the rescue action of E2 is mainly mediated through ERβ because (1) selective estrogen receptor β-agonist is able to rescue PH as efficiently as E2, and (2) E2 fails to rescue PH in the presence of an ERβ-selective antagonist. The ER–mediated action of estrogen has also been recently reported in another experimental model of PH induced by hypoxia (39). These researchers show that E2 attenuates hypoxia-induced pulmonary hypertension through
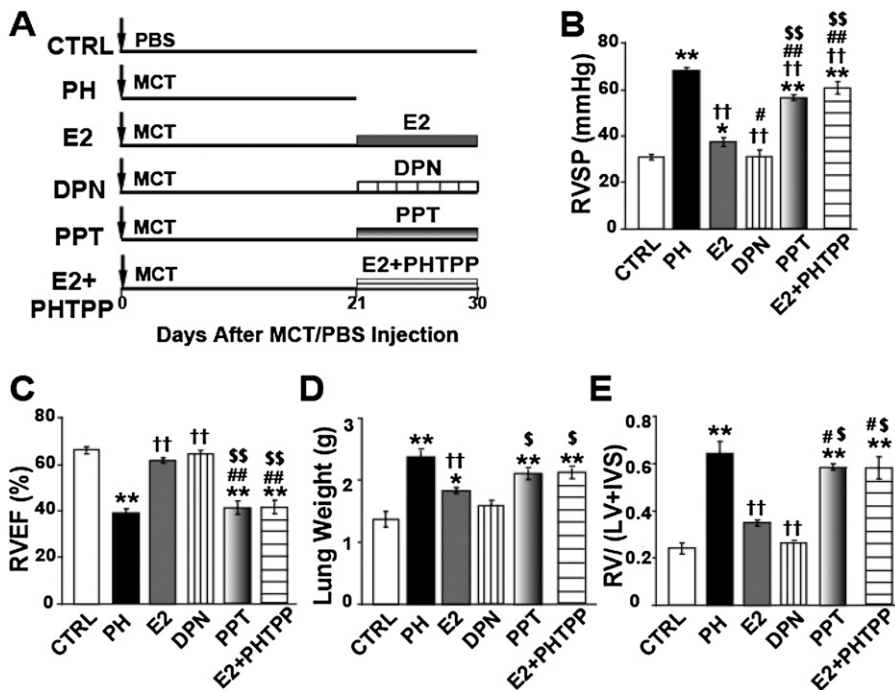
Figure 7. Selective estrogen receptor (ER)β agonist diarylpropionitrile (DPN) is as effective as estrogen (E2) in rescuing severe pulmonary hypertension (PH), and E2 fails to rescue PH in the presence of ERβ antagonist, PHTPP. (*A*) Experimental design. (*B–E*) Right ventricular systolic pressure (RVSP) (*B*), RV ejection fraction (RVEF) (*C*), lung weight (*D*), and RV hypertrophy index (*E*), at Day 30 except for PH, which was at Day 21 after monocrotaline (MCT) injection, for control (CTRL; *open bar*), PH (*solid bar*), E2 (*gray bar*), DPN (ERβ-agonist, *vertical striped bar*), PPT (ERα agonist, *shaded gray bar*), and E2+PHTPP (ERβ antagonist, *horizontal striped bar*) groups in male rats. *$P < 0.05$ versus CTRL, **$P < 0.001$ versus CTRL; ††$P < 0.001$ versus PH; #$P < 0.05$ versus E2, ##$P < 0.001$ versus E2; $$P < 0.05$ versus DPN, $$$P < 0.001$ versus DPN (n = 3–8 animals per group).

an ER-dependent mechanism, as the preventive effect of E2 is abolished in the presence of E2 receptor antagonist ICI. The importance of receptor-specific actions of E2 in the protection against PH and RV dysfunction is in agreement with many studies demonstrating that the estrogen-mediated protection against vascular injury, hypertension, cardiac remodeling, and apoptosis are mediated through ERβ (26, 37, 40). However, Tofovic and colleagues speculated that the preventive action of E2 against PH is independent of its receptors because E2 metabolites have weak affinity for ERs (9). This speculation has been challenged recently as in the presence of COMT inhibitor OR-486, which inhibits the conversion of E2 to catechol and methoxyestrogen metabolites that exert their effects in an ER-independent manner, E2 was still able to attenuate the progression of PH and its beneficial effects were even potentiated (39).

The Estrogen Paradox in PH

The fact that E2 is rescuing PH may seem surprising due to the higher prevalence of idiopathic PH in young women (41). Contrary to humans, female rats with PH induced by either MCT or chronic hypoxia exhibit less severe PH than their male counterparts (1, 8, 12). This sex difference in susceptibility to PH has been suggested to be partly due to the protective action of E2, as E2 replacement attenuates the progression of PH in ovariectomized animals (42). Likewise, E2 treatment in male rats also slows down the development of PH (4). In addition to E2, estradiol metabolites, such as 2-methoxyestradiol (9), natural soybean-derived phytoestrogen, genistein (43), and a selective estrogen receptor modulator, raloxifene (44), all slow down the progression of MCT-induced or chronic hypoxia–induced PH in rodents.

How to explain the higher prevalence of idiopathic PH in young women? We favor the speculation by Lahm and colleagues (21), which states that a defect in the ERs, ER signaling, estrogen metabolism, or genetic susceptibility in certain women could make them more prone to this disease (45). Future studies are needed to investigate the possible correlation between plasma estrogen levels, pulmonary levels of ERβ and ERα, and the severity of PH in these women. The fact that (*1*)

postmenopausal women have an increased risk for the development of pulmonary hypertension (46), and that (*2*) hormone replacement therapy may prevent the development of PH in these patients further supports the beneficial role of E2 in patients with PH (47). Other investigators have also suggested that severe PH has now become overwhelmingly a disease of postmenopausal women (48). In fact, despite higher incidence of the disease in female patients, as shown in the REVEAL registry of patients with PH (7), there is a clear shift in the mean age of diagnosis toward older age, particularly in the female patients from the United States (7).

Other Sex Steroids and PH

Similar to estrogen, progesterone also prevents the progression of experimental PH (49). Although testosterone has been shown to be a pulmonary vasodilator in isolated pulmonary arteries (49–51), male rats, which have higher levels of testosterone, do much worse than female rats in different experimental models of PH (8–10). Hansmann and colleagues showed male apoE$^{-/-}$ mice on high-fat diets developed more severe PH associated with insulin resistance and lower plasma adiponectin levels compared with female apoE$^{-/-}$ mice (52). Because testosterone inhibits the secretion of adiponectin in adipocytes, they hypothesized that elevation of this vasoprotective adipocytokine accounted for the less severe vascular phenotype in female apoE$^{-/-}$ mice. They treated male apoE$^{-/-}$ mice with rosiglitazone and documented eightfold higher plasma adiponectin levels, improved insulin sensitivity, and complete regression of PH (52). Taken together, testosterone does not seem to be beneficial in experimental PH. Low levels of testosterone in women are also unlikely to be the cause of female predominance of PH in patients (53).

Dehydroepiandrosterone (DHEA) can be metabolized to estrogens and androgens. In fact, Homma and colleagues detected higher plasma levels of estradiol and testosterone in DHEA-treated than in DHEA-untreated animals (54). Because testosterone does not seem to be beneficial in experimental PH, we speculate that the beneficial action of DHEA in experimental PH must have been due to conversion of DHEA to estrogen.

ERβ-Selective Ligands as Novel Therapeutic Agents for Patients with PH

Our findings raise the intriguing concept that E2 therapy may be of significant benefit for patients with severe PH. The rescue action of E2 is most likely mediated by ERβ, which has become a favorable therapeutic target due to its antiproliferative activity as opposed to ERα that promotes epithelial proliferation and has proestrogenic effects in the breasts and uterus (55, 56). Therefore, selective ERβ ligands could provide a novel treatment modality for patients with PH by minimizing estrogenic side effects, a concept that most definitely warrants further investigation.

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