Reverse right ventricular structural and extracellular matrix remodeling by estrogen in severe pulmonary hypertension

Rangarajan D. Nadadur, Soban Umar, Gabriel Wong, Mansour Eghbali, Andrea Iorga, Hummann Matori, Rod Partow-Navid, and Mansoureh Eghbali

Department of Anesthesiology, Division of Molecular Medicine, University of California at Los Angeles, Los Angeles, California

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Nadadur RD, Umar S, Wong G, Eghbali M, Iorga A, Matori H, Partow-Navid R, Eghbali M. Reverse right ventricular structural and extracellular matrix remodeling by estrogen in severe pulmonary hypertension. J Appl Physiol 113: 149–158, 2012. First published May 24, 2012; doi:10.1152/japplphysiol.01349.2011.—Chronic pulmonary hypertension (PH) leads to right-ventricular failure (RVF) characterized by RV remodeling. Ventricular remodeling is emerging as an important process during heart failure and recovery. Remodeling in RVF induced by PH is not fully understood. Recently we discovered that estrogen (E2) therapy can rescue severe preexisting PH. Here, we focus on whether E2 (42.5 μg·kg⁻¹·day⁻¹, 10 days) can reverse adverse RV structural and extracellular matrix (ECM) remodeling induced by PH using monocrotaline (MCT, 60 mg/kg). RV fibrosis was evident in RVF males. Intact females developed less severe RV remodeling compared with males and ovariectomized (OVX) females. Novel ECM-degrading disintegrin-metalloproteinases ADAM15 and ADAM17 transcripts were elevated 2-fold in all RVF animals. E2 therapy reversed RV remodeling in all groups. In vitro, E2 directly inhibited ANG II-induced expression of fibrosis markers as well as the metalloproteinases in cultured cardiac fibroblasts. Estrogen receptor-β agonist diarylpropionitrile (DPN) but not estrogen receptor-α agonist 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)tris(phenol (PPT) was as effective as E2 in inhibiting expression of these genes. Expression of ECM-interacting cardiac fetal-gene osteopontin (OPN) also increased 9-fold in RVF males. Intact females were partially protected from OPN upregulation (≈2-fold) but OVX females were not. E2 reversed OPN upregulation in all groups. Upregulation of OPN was also reversed in vitro by E2. Plasma OPN was elevated in RVF (∼1.5-fold) and decreased to control levels in the E2 group. RVF resulted in elevated Akt phosphorylation, but not ERK, in the RV, and E2 therapy restored Akt phosphorylation. In conclusion, E2 therapy reverses adverse RV remodeling associated with PH by reversing fibrosis and upregulation of novel ECM enzymes ADAM15, ADAM17, and OPN. These effects are likely mediated through estrogen receptor-β.

pulmonary hypertension; right ventricular failure; ventricular remodeling; estrogen; extracellular matrix

RIGHT VENTRICULAR FAILURE (RVF) is a common cause of death in patients suffering from long-standing pulmonary arterial pressure overload caused by pulmonary hypertension (PH) (5, 47, 48). Chronic PH first leads to compensated hypertrophy of the right ventricle (RV) that ultimately progresses to severe decompensated RV failure (RVF) (5, 47, 48). This RVF is accompanied by extensive RV structural and extracellular matrix (ECM) remodeling (5, 47, 48). End-stage heart failure has long been regarded as a terminal state of cardiac pathological remodeling that is almost impossible to reverse by any available therapy (17). However, some therapies have been shown recently to be somewhat effective in reversing the adverse remodeling of the left ventricle (LV) induced by pressure and volume overload (44). Unfortunately there is still no ideal therapy that halts or reverses the progression of RV remodeling secondary to PH (5, 47, 48).

RV remodeling is generally characterized by extensive fibrosis and additional changes in the expression of cardiac ECM-associated proteins (5, 47, 48). These expression changes are a key event in promoting the adverse remodeling of the RV (5, 47, 48). Together, these structural and ECM changes affect contractility, signaling, and electrical conduction (25).

The cardiac ECM provides structural support and facilitates mechanical, electrical, and chemical signals during homeostasis and in response to stress or injury (7). Matrix metalloproteinases (MMPs) and the related “a disintegrin and metalloproteinase” (ADAM) family degrade ECM anchoring proteins but are strictly regulated in normal physiological conditions (7). Altering the homeostasis of the ECM plays an important role in various cardiac pathologies, including dilated cardiomyopathy, myocardial infarction, and hypertensive cardiac hypertrophy (46).

Many changes in ECM protein expression in RV hypertrophy and failure have been reported, including altered collagen and metalloproteinase expression (31, 45). Although not all ADAMs show altered regulation during cardiac stress, ADAM15 and ADAM17 are emerging as two metalloproteinases in the heart responsible for pathological remodeling in LV stress and failure (13, 23, 52). However, their expression during RVF induced by PH and recovery has not been characterized. Because these two ADAMs share function and regulation with metalloproteinases well-established as responsible for adverse RV remodeling, they are likely to be involved during RVF as well (8, 22, 37). Another ECM protein well characterized during LV dysfunction is osteopontin (OPN), a matricellular cardiac fetal gene that is reactivated during cardiac stress and plays a role in heart failure. Again, its role in PH-induced RVF has not been characterized (20, 30, 40, 55, 56).

During cardiac stress induced by PH, the cardiac fibroblasts undergo a transition to activated myofibroblasts that are responsible for fibrosis and the secretion of metalloproteinases and other ECM enzymes that contribute to adverse ventricular remodeling (32). Recently we discovered that E2 therapy can rescue severe preexisting PH in male rats primarily by reversing pulmonary vascular remodeling (46). Pedram et al. (32) recently showed that E2 therapy can directly inhibit the transition to myofibroblasts in vitro, preventing the secretion of many of adverse remodeling proteins. Additionally, E2 treatment has been shown to directly mitigate adverse ECM remodeling in left ventricular hypertrophy and failure by attenuating altered collagen, and metalloproteinase
expression in vivo (45, 51). Here we explore whether the rapid restoration of RV function by E2 therapy during PH-induced RVF is at least in part due to a direct effect of E2 on the adverse ECM remodeling of the RV.

Using the well-established model of PH-induced RVF using monocrotaline (MCT) in rats (9, 48), we hypothesize that markers of adverse RV remodeling including fibrosis, ADAM15, and OPN are increased during RVF and are directly reversed with E2 therapy. We also explore the effects of E2 on RV remodeling in intact and ovariectomized (OVX) females.

METHODS

Animals

Male and female (intact and OVX) rats (Sprague-Dawley, 350–400 g) were used. All 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 (NIH Pub. No. 85–23, Revised 1996).

Experimental Protocols

**In vivo.** RVF was induced secondary to chronic PH in rats either with a single subcutaneous injection of MCT (60 mg/kg, Sigma) to induce PH or saline (CTRL) at day 0. MCT was dissolved in 1 N HCl; the pH was adjusted to 7.4 and diluted with PBS before injection. Severe PH was evident in MCT-injected rats at day 21. At this time point, the rats were either left untreated to develop right ventricular failure (RVF group) by day 30 or received estrogen therapy using continuous release 17β-estradiol (E2) pellets (42.5 μg·kg−1·day−1, Innovative Research of America) for 10 days. The E2-treated rats were kept for an additional 12 days after E2 withdrawal at day 30 until the point of full recovery previously observed (46) (E2 group, Fig. 1A).

**In vitro.** Neonatal rat ventricular myocytes (NRVMs) and fibroblasts were isolated as previously described and cocultured (42). Cells were serum starved for 24 h and then were serum replenished and either left untreated (CTRL) or treated with ANG II (100 nM, Sigma A9525) in the presence or absence of E2 (10 nM), selective estrogen receptor-β agonist diarylpropionitrile (DPN, 10 nM), or selective estrogen receptor-α agonist 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, 10 nM) for 48 h (ANG II, ANG II + E2, DPN, PPT groups) (Fig. 1B). Gene expression was measured by RT-PCR. All in vitro studies were performed in triplicate three independent times.

Cardiac and Pulmonary Hemodynamics

Hemodynamics were measured as previously described (46). Briefly, B-Mode and M-Mode echocardiography was performed under isoflurane anesthesia using a VisualSonics Vevo 770 equipped with a 30-MHz linear transducer. The Vevo770 software calculated RV ejection fraction (RVEF) and RV fractional shortening (RVFS) using RV M-mode echocardiogram. The RV pressure was measured directly by inserting a catheter (1.4F Millar SP-671) connected to a pressure transducer (Power Lab, ADInstruments) into the RV immediately before the rat was euthanized. The rats were sedated using a mixture of ketamine/xylazine (80 mg/kg ketamine, 8 mg/kg xylazine) administered intraperitoneally. After cardiac catheterization, rats were euthanized by removing the heart while they were under anesthesia.

Real-Time PCR

Total RNA from right ventricle or from cells was isolated using Trizol (Invitrogen). Total RNA (2 μg) was reverse transcribed with gene specific primers using Omniscript RT kit (Qiagen). GAPDH was used as an internal reference gene. The sequences of the primers are as follows: OPN forward primer 5′-TACGCCGGCCAGTGA-3′, OPN reverse primer 5′-ATGGCTTGTCCTCCCTGTTGCT-3′ (accession no. NM_017061.2); GAPDH forward primer 5′-CCCTATGTCTCTTCTTGTGGTGA-3′, ADAM15 forward primer 5′-CTCATGTCCTCTTTGTGGTGA-3′, ADAM15 reverse primer 5′-GGACACCTGACGACATGCCA-3′ (accession no. NM_020308); ADAM15 forward primer 5′-GTGTGCAACCCGCAACTGCTG-3′, ADAM15 reverse primer 5′-CAGCAGTCAATGAAACATCAG-3′ (accession no. NM_020306); transforming growth factor (TGF)-β reverse primer 5′-GATACCCTGAGCTGCTGTGCT-3′, TGF-β reverse primer 5′-CTCTCTGCTGAGCTGAACTCAG-3′ (accession no. NM_020306); collagen type 1 forward primer 5′-GACCGATGATGGTCAGTTCGCA-3′, collagen type 1 reverse primer 5′-AAGGTACCTGCTGATACCCGACAT-3′ (accession no. NM_053304.1); lysil oxidase forward primer 5′-CAGCCAAGCAGACAGAAGGCTG-3′, lysil oxidase reverse primer 5′-CGCAGATAGACAGAAGGCTG-3′ (accession no. NM_017068). Single peak was detected from the first derivative of fluorescence (dF/dT) vs. temperature plots (melting curve) indicating amplification of a single product. Agarose gel electrophoresis at the end of the reaction also confirmed the amplification of a single product of the expected size. Controls were 1) reaction cocktail without reverse transcriptase tested in a regular 40 cycle PCR; and 2) H2O instead of cDNA tested in parallel to real-time PCR.

Immunofluorescence Staining

Heart cross sections (6 μm) were fixed in acetone for 15 min at −20°C. The sections were then washed with PBS + 0.1% Triton three times, incubated with 10% normal goat serum in PBS + 0.1% Triton...
for 30 min to block the background. The sections were incubated with primary antibodies in PBS + 0.1% Triton + 1% normal goat serum at 4°C overnight, washed with PBS + 0.1% Triton three times, and incubated with the appropriate secondary antibodies in PBS + 0.1% Triton + 1% normal goat serum at room temperature for 1 h. After washing the secondary antibodies, the sections were mounted using Prolong gold (Molecular Probes) for imaging.

**Trichrome Staining and Histologic Analysis**

Standard Masson trichrome staining was performed on RV tissue sections (Sigma-Aldrich). Percent tissue fibrosis in RV sections was determined using the stain for collagen with the use of a grid that divided the field of view into 100 squares; the number of collagenous tissue (blue stain) at the 100 intersection points in the grid was scored as 1 (present) or 0 (absent). Results are expressed as the percentage occupied by fibrosis to the total area examined.

**Plasma Osteopontin ELISA**

Concentration of OPN in plasma samples was determined by a quantitative sandwich enzyme immunoassay technique by rat OPN ELISA (27360, IBL-America) with a measurement range of 0.07–4.75 ng/ml. Total plasma was diluted to 10 μg/μl of protein and 100 μl of each sample was loaded onto the precoated plate. Absorbance at a wavelength of 450 nm was read with a spectrophotometer and plasma concentrations were calculated using a relative standard curve.

**Western Blotting**

Right ventricles were isolated and homogenized at 4°C in (in mM) 150 NaCl, 50 Tris·HCl, 1 EGTA, 1 EDTA, 1 NaF, 1 1% Na3VO4, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate (pH 7.4) supplemented with protease and phosphatase inhibitor cocktails (Roche). The samples were centrifuged at 12,000 g for 10 min and the supernatants were collected. Protein concentration was measured and 100 μg of total protein was loaded on a 4–20% gradient Tris·HCl/SDS polyacrylamide gel, electrotransferred to nitrocellulose paper, and incubated with primary antibodies. Blots were then indirectly blocked with 5% nonfat dry milk in 20 mM TBS with 0.1% Tween, SDS polyacrylamide gel, electrotransferred to nitrocellulose paper, 100 μl of each sample was loaded onto the precoated plate. Absorbance at a wavelength of 450 nm was read with a spectrophotometer and plasma concentrations were calculated using a relative standard curve.

**Reagents**

The primary antibodies used were anti-ADAM17 (1:200, Calbiochem), anti-Akt (1:500, Cell Signaling rabbit polyclonal), anti-phospho-Akt (1:200 Cell Signaling, rabbit polyclonal); anti-ERK1/2 (1:500, Cell Signaling, rabbit polyclonal), anti-phospho ERK1/2 (1:200, Cell Signaling, mouse monoclonal), anti-OPN (Santa Cruz Biotechnology, 1:200 or Abcam, 1:200), and anti-αC1 (1:200, Alomone Labs). The secondary antibodies used were goat anti-rabbit Alexa 488 (1:1,000, Invitrogen) and goat anti-mouse Alexa 568 (1:1,000, Invitrogen) for immunocytochemistry, or goat anti-rabbit IgG-Alexa Fluor680 (1:100,000, Invitrogen) and goat anti-mouse IgG-IR Dye800CW (1:100,000, Odyssey, LI-COR) for Western blot.

**Statistics**

One-way ANOVA tests were used to compare between groups using SPSS13.0 for Windows. When significant differences were detected, individual mean values were compared by post hoc tests that allowed for multiple comparisons. P values <0.05 were considered statistically significant. Values are expressed as means ± SE.

**RESULTS**

**Adverse RV Remodeling and RV Fibrosis Induced by PH is Reversed by E2 Therapy**

A single injection of MCT induced RV failure in ~30 days (see Figs. 1A, 2A, and 7A). As we recently described, E2 therapy rescued advanced pulmonary hypertension and restored RV function in male rats by improving RV pressure from 72.0 ± 1.4 to 31.1 ± 0.9 mmHg (Fig. 2A), RV ejection fraction from 30.4 ± 1.7% to 61.5 ± 0.8%, and RV fractional shortening from 13.35 ± 0.47% to 31.81 ± 1.02% (46). In fact, estrogen therapy resulted in 100% survival whereas all untreated rats died by day 32 (46). Here we explored the direct effects of E2 therapy on RV remodeling. E2 treatment was able to completely reverse PH-induced RV fibrosis in males in vivo (RV fibrosis 0.47% to 31.1% in CTRL, RVF, and E2. **P < 0.05 vs. CTRL; ##P < 0.05 vs. RVF.

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Fig. 2. E2 restores RV function and reverses RV fibrosis associated with pulmonary hypertension (PH). A: RV pressure in CTRL, RVF, and E2. B: trichrome staining of RV cross sections. Red indicates cardiomyocytes and blue shows collagen deposition (fibrosis). C: quantification of %RV fibrosis in CTRL, RVF, and E2. D and E: RV lysyl oxidase (D) and collagen I relative transcript expression (E) in CTRL, RVF, and E2. **P < 0.05 vs. CTRL; ##P < 0.05 vs. RVF.
associated with a ~2 fold upregulation of ADAM15 and ADAM17 transcripts in male rats (2.13 ± 0.19 for ADAM15 and 1.85 ± 0.04 for ADAM17, normalized to their corresponding CTTLs, all \( P < 0.05 \) vs. CTRL, Fig. 3A). E2 therapy fully restored ADAM15 and ADAM17 expression to normal levels in vivo (0.47 ± 0.07 for ADAM15, 1.3 ± 0.14 for ADAM17, all \( P < 0.05 \) vs. RVF, Fig. 3A). Western blotting also revealed that ADAM17 was upregulated in the RV of RVF animals and reversed with E2 therapy (1.00 ± 0.01 in CTRL, 1.59 ± 0.15 in RVF, 1.18 ± 0.16 in E2, all \( P < 0.05 \) Fig. 3, B and C).

**Estrogen Therapy Reverses the Expression of Remodeling Enzymes In Vitro Through Estrogen Receptor \( \beta \)**

E2 therapy was also able to reverse ANG II-induced upregulation of TGF-\( \beta \) and collagen type I in vitro in cocultured cardiac NRVMs and fibroblasts (1.75 ± 0.09 in ANG II, 0.97 ± 0.12 in ANG II + E2 for TGF-\( \beta \); 1.82 ± 0.22 in ANG II, 1.04 ± 0.20 ANG II + E2 for collagen I, normalized to CTRL, all \( P < 0.05 \), Fig. 4, A and B). Estrogen receptor-\( \alpha \) agonist DPN was as effective as E2 in reversing ANG II-induced expression of TGF-\( \beta \) and collagen I (0.88 ± 0.09 for TGF-\( \beta \), 0.87 ± 0.09 for collagen 1, all \( P < 0.05 \) vs. ANG II, Fig. 4, A and B). ANG II treatment of cocultured cardiac NRVMs and fibroblasts also resulted in increased expression of ADAM15 and ADAM17 transcripts (1.76 ± 0.14 and 1.81 ± 0.16-fold, respectively; \( P < 0.05 \) vs. CTRL, Fig. 4, C and D). E2 restored upregulation of ADAM15 and ADAM17 in vitro (1.05 ± 0.15 and 0.92 ± 0.19 respectively; \( P < 0.05 \) vs. ANG II, Fig. 4, C and D). Angiotensin II-induced upregulation of ADAM15 and ADAM17 was reversed by DPN (0.95 ± 0.14 for ADAM15, 0.92 ± 0.17 for ADAM17, all \( P < 0.05 \) vs. RVF, Fig. 4, C and D) but not with PPT (2.45 ± 0.36 for ADAM15, 2.17 ± 0.20 for ADAM17, all \( P = \text{NS} \) vs. CTRL, Fig. 4, C and D).
Upregulation of PH-Induced RV OPN Expression in RVF is Reversed by E2 Therapy

The transcript levels of OPN, a matricellular fetal gene, were upregulated more than ninefold in RVF male rats (1.00 ± 0.10 CTRL to 9.33 ± 2.07 in RVF, *P < 0.05, Fig. 5A). Similar to ADAMs, OPN expression returns to control levels following E2 therapy (0.45 ± 0.16, *P < 0.05, Fig. 5A). Western blotting revealed four isoforms of OPN, 32 kDa, 44 kDa, 50 kDa, and 66 kDa, which were all upregulated in the RV in RVF group (Fig. 5, B and C). Estrogen was able to reverse the protein expression of the 32-kDa, 44-kDa, and 50-kDa isoforms (Fig. 5, B and C). The full-length OPN is partially downregulated but shows no statistical significance in response to E2 therapy (Fig. 5, B and C). Immunocytochemistry staining of RV cross sections shows localization of OPN exclusively to the extracellular space (Fig. 5D). The total OPN expression observed is elevated in RVF and reversed with E2 therapy (Fig. 5D).

OPN plasma levels have also shown to be increased in PH, supporting the view that OPN has some potential as a biomarker (26). Here we explored whether E2 therapy could reverse increased plasma OPN levels observed in PH. Plasma OPN levels increased significantly ~1.5 fold in RVF males (1.47 ± 0.19 normalized to CTRL, *P < 0.05, Fig. 5E). Circulating OPN levels were restored to control levels (0.62 ± 0.14, *P < 0.05 vs. RVF, Fig. 5E) following E2 therapy. In vitro, OPN expression is elevated in ANG II-treated cocultured cardiac NRVMs and fibroblasts, and reversed in the presence of E2 (2.74 ± 0.19-fold in ANG II + E2; all *P < 0.05, Fig. 5F).

Akt Signaling is Involved in the Progression of PH-Induced Adverse RV Remodeling and Both Akt and ERK in Estrogen-Mediated Reverse Remodeling

AKT and ERK signaling are known to be regulated by E2 (60). We explored whether these signaling cascades are involved in RV remodeling secondary to PH and are regulated by E2. The progression to RVF was associated with upregulation of Akt phosphorylation (pAkt/Akt = 1.00 ± 0.10 in CTRL, 1.35 ± 0.03 in RVF, *P < 0.05, normalized to CTRL, Fig. 6, A and B) but not ERK phosphorylation (pERK1/ERK1 = 1.00 ± 0.07 in CTRL, 1.17 ± 0.13 in RVF; pERK2/ERK2 = 1.00 ± 0.02 in CTRL, 0.97 ± 0.02 in RVF; all *P = NS, Fig. 6, C and D). With E2 therapy, Akt phosphorylation is significantly decreased (pAkt/Akt = 1.07 ± 0.06, *P < 0.05 vs. RVF, Fig. 6, A and B) as is ERK phosphorylation (pERK1/ERK1 = 0.75 ± 0.07, *P < 0.05 vs. RVF and CTRL, pERK2/ERK2 = 0.94 ± 0.13, *P = NS, Fig. 6, C and D).

Less Severe PH-Induced RV Remodeling in Intact Female Rats is Abolished by Ovariectomy

Intact female rats developed less severe PH compared with male and OVX rats as the RV pressures was significantly lower (62.53 ± 3.34 mmHg in females vs. 77.61 ± 3.49 mmHg in OVX and 72 ± 1.4 mmHg in male; *P < 0.05 for all RVF groups vs. corresponding CTRL; *P < 0.05 for RVF females vs. RVF males and RVF OVX-females; *P = NS for RVF male vs. RVF OVX, Fig. 7A). E2 therapy was able to reverse elevated RV pressure in

**Fig. 5. Osteopontin (OPN) expression is elevated in the RV of RVF group and is reversed with E2-therapy. A: OPN relative transcript expression measured in CTRL, RVF, and E2. **P < 0.05 vs. CTRL; ###P < 0.05 vs. RVF. B: Western blot showing 4 different length isoforms of OPN (bottom 4 bands, 32 kDa, 44 kDa, 50 kDa, and 66 kDa) and vinculin (top band) representative samples. C: quantification of OPN protein expression normalized to appropriate CTRL and vinculin protein from Western blot in CTRL, RVF, and E2 groups. **P < 0.05 vs. corresponding CTRL; ###P < 0.05 vs. corresponding RVF. D: staining of RV cross sections double labeled with L-type calcium channel α1c (green) and OPN (red). E: OPN plasma levels measured by sandwich-ELISA, normalized to CTRL in CTRL, RVF, and E2. **P < 0.05 vs. CTRL; ###P < 0.05 vs. RVF. F: OPN transcript expression in NRVM/fibroblast culture in CTRL, ANG II treated, and ANG II + E2 treated. **P < 0.05 vs. CTRL; ###P < 0.05 vs. ANG II.**
increased in RVSP and adverse RV remodeling in both intact and OVX females. In vitro, E2 reversed ANG II-induced expression of TGF-β, collagen I, OPN, ADAM15, and ADAM17. Estrogen receptor-β agonist DPN, but not estrogen receptor-α agonist PPT, also reversed ANG II-induced expression of these markers. These in vitro data suggest a possible direct action of E2 on the RV through estrogen receptor-β.

**RV Failure Secondary to Chronic PH**

PH is a chronic lung disorder, the progression of which causes RV hypertrophy and failure leading to sudden cardiac death (47). Current treatments aim to delay the progression of the disease, but there is still no definitive ideal therapy for preexisting PH (3). Although RV failure secondary to chronic PH is a cause of significant mortality, the specifics of PH-induced RV failure are still being elucidated (10, 19, 47). As a result, improving the lung condition has been the target of current therapies, but there is a lack of treatments targeting the failing RV. As the mechanisms of PH-induced RVF are illuminated, there is potential for developing RV-specific therapies for PH. These therapies have the potential to improve the survival of patients suffering from chronic PH (10).

As PH-induced RV failure progresses and adverse cardiac remodeling begins, the cardiac ECM composition is drastically altered (25, 45). These changes in the ECM can disrupt cardiac function in several ways. For example, deposition of collagen and other ECM proteins can inhibit contractility and electrical signal conduction. The degradation of integrins can have an effect on chemical signaling (35). However, RV failure also leads to the activation of a fetal gene program, a reexpression of several genes normally only present in the developing heart (4). It is thought that the purpose of these reexpressed genes is partially to support cardiac structure and function during failure (11).

**E2 Therapy and Cardiac Remodeling**

We have recently shown that E2 therapy can rescue severe preexisting pulmonary vascular disease associated with PH (46). E2 has been shown to directly inhibit cardiac fibrosis. Pedram et al. (32) recently showed that E2 can directly prevent cardiac fibrosis by inhibiting the transition of fibroblasts to myofibroblasts through an estrogen receptor-β-mediated mechanism (32). Females have also been shown to be protected from PH-induced RV failure (34). The role of E2 in preventing or reversing the changes in RV structure associated with PH is an area of current interest.

The fact that intact females develop less severe adverse RV remodeling (including RV pressures, fibrosis, and expression of ECM remodeling enzymes) compared with OVX females (Fig. 7) further supports a protective role of endogenous estrogen (Fig. 7). E2 therapy resulted in reversal of RV remodeling associated with RV dysfunction in both males and females (OVX and intact). These data suggest that endogenous estrogen plays a protective role in PH-induced RVF, and that exogenous estrogen therapy could have a positive effect as postmenopausal hormone replacement therapy to mitigate PH and the resulting RV remodeling. Our in vitro studies show that estrogen receptor-β agonist DPN, but not estrogen receptor-α agonist PPT, is almost as effective as E2 in reversing ANG-II induced overexpression of TGF-β and collagen I. Our data support the view that the direct inhibitory effect of E2 on...
cardiac fibrosis is mainly mediated through estrogen receptor-β and are in agreement with Pedram et al. (32).

**E2 Therapy Reverses PH-Induced ADAM15 and ADAM17 Upregulation in the RV**

Among the ECM-associated proteins, the MMP family has been well documented for its direct role in causing adverse remodeling associated with PH-induced RV remodeling (45). The role of ADAMs in this remodeling is a much more recently emerging field. The ADAM family, like the related MMP family, is responsible for breaking down ECM anchoring proteins, but in addition to the metalloproteinase function, ADAMs also have a disintegrin domain (7). Integrins play a role in ECM interactions between cardiac myocytes and fibroblasts, and also provide overall structural integrity and mediate cell-cell communication in the heart (7). Among their many functions, integrins have been found to be responsible for cardioprotective signaling in the failing myocardium, as well as in the prevention of fibrosis leading to heart failure, and integrins are known to be downregulated during PH-induced RV remodeling (18, 39, 41). Although ADAM15 and ADAM17 have been less studied, they are emerging as two of the most important ADAMs present in the heart during LV dysfunction, as not all ADAMs show altered regulation during cardiac stress (13, 23, 52). Here we found that both ADAM15 and ADAM17 are upregulated in RVF males but are restored to control levels with E2 treatment. Both ADAMs are also upregulated in both OVX and intact females. Interestingly E2 therapy is not as effective in reversing expression in intact females. We also show that ADAM15 and ADAM17 expression is reversed by E2 therapy through estrogen receptor-β in our in vitro model of cardiac stress.

E2 has been shown to inhibit metalloproteinase transcription, specifically MMP-2 via the MAP kinase pathway (28). E2 may have a similar inhibitory effect on other metalloproteinases, including ADAMs. It is possible that E2 therapy reverses the progression to RV failure by directly inhibiting adverse ECM remodeling metalloproteinases in the cardiac ECM (Fig. 8).

**Elevated RV OPN Expression in PH is Reversed by E2 Therapy**

The ECM interacting matricellular protein OPN, originally described for its role in bone tissue, is expressed in the developing heart but is virtually nonexistent in a healthy adult heart (43). OPN has been shown to reexpress in the heart in response to stress (6, 55, 56). OPN also plays a role in the
inflammatory response in the heart, although its exact role in inflammation is still not fully understood (36). We observed elevated OPN expression in the RV of RVF both in males and females, although to a much lesser degree in intact females (~2-fold in intact females vs. ~8-fold in male and OVX females). E2 therapy was able to reverse increased OPN expression in all three groups to normal levels in vivo. OPN expression was also elevated in an in vitro model of cardiac stress and reversed with E2 therapy.

OPN is known to have several isoforms (58). Western blotting of RV tissue from males revealed at least four major isoforms of OPN; we observed OPN at 66 kDa, 50 kDa, 44 kDa, and 32 kDa. Full-length OPN is known to be cleaved into 40- and 32-kDa isoforms with thrombin or MMP treatments (15, 61). OPN has been shown to be involved with cell migration, fusion, and motility, and all these isoforms of OPN appear to play a role in cell adhesion and migration, suggesting their role in ECM integrity (1, 14). Others have also observed a 50-kDa OPN that appears to be predominantly secreted from cells (50). Numerous other isoforms are suggested to be formed by cleavage and posttranslation modifications such as glycosylation and phosphorylation (59).

Here we found four main isoforms in the RV, which were all upregulated (~2- to 5-fold) in RVF male rats. The 32-, 44-, and 50-kDa isoforms were significantly downregulated by E2 therapy. Although full-length OPN appeared partially downregulated by E2 therapy, there was no statistical significance. It is possible that E2 is involved in regulating the cleavage of OPN into its active isoforms but has a less pronounced effect on total OPN expression.

We speculate that OPN plays a role in mitigating adverse ECM remodeling in the failing RV. OPN knockout mice have been shown to demonstrate altered collagen deposition (24). Furthermore, OPN has directly been shown to inhibit metalloproteinases, specifically MMP-2 and MMP-9, through IL1-beta and PKC-zeta (57). MMP-2 and MMP-9 are known to be important proteins in advancing the adverse remodeling of the RV during failure and causing many of the negative structural changes that accompany RV failure (45). It is possible that OPN is reexpressed to support the ECM that is being degraded in a failing heart. Since E2 and OPN both have been observed to inhibit metalloproteinases, it is possible that there is a more direct link between E2 therapy and fetal gene reexpression. Further study on the connection between E2, ADAMs, and fetal genes like OPN is warranted.

**OPN as a Potential Plasma Marker for PH**

Several cardiac fetal genes, especially the natriuretic peptides brain natriuretic peptide (BNP), NT-proBNP, and atrial natriuretic factor (ANF), are emerging as clinical plasma markers of heart failure (49). The plasma ratio of MMP-1 to its inhibitor TIMP-1 is already a well-established marker of PH-induced RVF (16). Schumann et al. recently showed in human samples that plasma levels of TIMP-4, tenascin-C, MMP-2, and NT-proBNP, all ECM interacting proteins, are elevated in PH and are correlated with disease severity (38). OPN seems to be another promising biomarker for PH-induced RVF (26). Here we observed that OPN plasma levels significantly increase in the RVF group. E2 therapy was associated with complete reversal of increased plasma OPN levels. It is important to note that the plasma OPN observed may also be partially due to elevated OPN expression in the lung during pulmonary hypertension as recently reported in addition to the OPN in the RV we report here (53). Regardless of the origin of the plasma OPN, our data further support the role of OPN as a potential biomarker for PH-induced RVF and recovery.

**Akt and ERK Signaling During PH-Induced RV Failure and Estrogen-Mediated Recovery**

Akt and ERK signaling are critical mediators of cardiac hypertrophy, survival, and stress. Akt signaling is known to be involved in both cardioprotection as well as in several models of cardiac stress, including ischemia, transaortic constriction, and in human patients with left ventricular assist devices (2, 21, 33). Recently, Drake et al. (12) showed that Akt signaling is increased during right heart failure PH. We also find increased Akt phosphorylation in the RV of RVF males (Fig. 6). With E2 therapy, we observe a significant decline in Akt phosphorylation (Fig. 6). Similarly, ERK is known to play a role in left
ventricular pathological heart hypertrophy (29). Interestingly, we saw no increase in ERK1/2 signaling during RVF. However, with E2 therapy there is a decline in ERK1 phosphorylation. These results provide some insight into the molecular signature of PH-induced RVF and E2-mediated recovery.

Limitations

This study is not a global analysis of ECM remodeling enzymes during RVF and their response to E2 therapy. The focus of this study was to instead examine the role of E2 therapy in reversing adverse RV remodeling and to identify some novel enzymes involved in the remodeling process. It would be interesting to pursue a more global analysis of ECM and fetal genes involved in the remodeling process of the RV. Here we observed that OPN plasma levels significantly increase in the RVF group. It would be interesting to explore whether circulating OPN is predominately of cardiac or pulmonary origin. Some additional studies to measure OPN concentration in blood from the aortic root and from the coronary sinus would provide some insight into the source of circulating OPN. Regardless of the origin of OPN in the plasma, it still serves a valuable role as a circulating biomarker in this model and has also been shown in patients with PH (27, 54).

Conclusions

We show significant RV structural and ECM remodeling in PH-induced RVF in males and to a lesser extent in female rats. Protection from PH-induced RV remodeling in intact female rats is abolished by ovariectomy. Matricellular protein and fetal gene OPN, as well as disintegrin metalloproteinases ADAM15 and ADAM17, show elevated expression in RVF group. E2 therapy reversed adverse RV remodeling in both male and female rats. In vitro, E2 therapy directly inhibits expression of remodeling enzymes most likely through estrogen receptor-β. These in vitro studies support the direct action of E2 on the myocardium in addition to its effects on the pulmonary vasculature. OPN also shows promise as a plasma biomarker of PH that correlates with recovery.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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