

## Rescue of Pressure Overload-Induced Heart Failure by Estrogen Therapy

Andrea Iorga, PhD; Jingyuan Li, MD, PhD; Salil Sharma, PhD; Soban Umar, MD, PhD; Jean C. Bopassa, PhD;\* Rangarajan D. Nadadur, BS; Alexander Centala, BS; Shuxun Ren, MD; Tomoaki Saito, PhD;† Ligia Toro, PhD; Yibin Wang, PhD; Enrico Stefani, MD, PhD; Mansoureh Eghbali, PhD

**Background**—Estrogen pretreatment has been shown to attenuate the development of heart hypertrophy, but it is not known whether estrogen could also *rescue* heart failure (HF). Furthermore, the heart has all the machinery to locally biosynthesize estrogen via aromatase, but the role of local cardiac estrogen synthesis in HF has not yet been studied. Here we hypothesized that cardiac estrogen is reduced in HF and examined whether exogenous estrogen therapy can rescue HF.

**Methods and Results**—HF was induced by transaortic constriction in mice, and once mice reached an ejection fraction (EF) of  $\approx 35\%$ , they were treated with estrogen for 10 days. Cardiac structure and function, angiogenesis, and fibrosis were assessed, and estrogen was measured in plasma and in heart. Cardiac estrogen concentrations ( $6.18 \pm 1.12$  pg/160 mg heart in HF versus  $17.79 \pm 1.28$  pg/mL in control) and aromatase transcripts ( $0.19 \pm 0.04$ , normalized to control,  $P < 0.05$ ) were significantly reduced in HF. Estrogen therapy increased cardiac estrogen 3-fold and restored aromatase transcripts. Estrogen also rescued HF by restoring ejection fraction to  $53.1 \pm 1.3\%$  ( $P < 0.001$ ) and improving cardiac hemodynamics both in male and female mice. Estrogen therapy stimulated angiogenesis as capillary density increased from  $0.66 \pm 0.07$  in HF to  $2.83 \pm 0.14$  ( $P < 0.001$ , normalized to control) and reversed the fibrotic scarring observed in HF ( $45.5 \pm 2.8\%$  in HF versus  $5.3 \pm 1.0\%$ ,  $P < 0.001$ ). Stimulation of angiogenesis by estrogen seems to be one of the key mechanisms, since in the presence of an angiogenesis inhibitor estrogen failed to rescue HF (ejection fraction =  $29.3 \pm 2.1\%$ ,  $P < 0.001$  versus E2).

**Conclusions**—Estrogen rescues pre-existing HF by restoring cardiac estrogen and aromatase, stimulating angiogenesis, and suppressing fibrosis. (*J Am Heart Assoc.* 2016;5:e002482 doi: 10.1161/JAHA.115.002482)

**Key Words:** angiogenesis • aromatase • estrogen • fibrosis • heart failure

Heart failure (HF) is often preceded by heart hypertrophy, which is usually triggered by external stressors. While heart hypertrophy is presumed to be compensatory at the beginning, it often progresses to chronic HF when insults persist.<sup>1</sup> Advanced chronic HF is most often treated with

aggressive nonpharmacological therapies such as heart transplantation or implantable left ventricular assist devices. Effective pharmacological therapies for treatment of HF patients would possess major advantages because of their noninvasive and more cost-effective nature. Several agents such as a phosphodiesterase 5A inhibitor as well as the  $G\beta\gamma$  small molecule inhibitor gallein have been shown to halt the progression of cardiac remodeling and failure.<sup>2–4</sup> However, effective pharmacological intervention that can rescue HF at an advanced stage is still necessary.

The heart has all the machinery for local biosynthesis of estrogen via the help of aromatase. However, it is not known whether local heart E2 biosynthesis is disrupted in HF. In addition, estrogen pretreatment has been shown to attenuate the development of heart hypertrophy,<sup>5</sup> but it is not known whether E2 could *rescue* advanced HF. Here we found that HF induced by pressure overload is associated with lower local heart (but not plasma E2) concentrations, and that cardiac aromatase transcripts were significantly downregulated in HF. We hypothesized that local heart E2 concentration is reduced in HF possibly because of the downregulation of cardiac

From the Division of Molecular Medicine, Departments of Anesthesiology (A.I., J.L., S.S., S.U., J.C.B., R.D.N., A.C., S.R., T.S., L.T., Y.W., E.S., M.E.), Molecular & Medical Pharmacology (L.T.), and Physiology (Y.W., E.S.), David Geffen School of Medicine at University of California Los Angeles, Los Angeles, CA.

\*Dr Jean C. Bopassa is currently located at the Department of Physiology, University of Texas Health Science Center, San Antonio, TX 78229.

†Dr Tomoaki Saito is currently located at the Medical Science Division, Japan Patent Office, Ministry of Economy, Trade and Industry, Japan.

**Correspondence to:** Mansoureh Eghbali, PhD, Department of Anesthesiology, UCLA School of Medicine, BH-160CHS, Los Angeles, CA 90095. E-mail: meghbali@ucla.edu

Received July 28, 2015; accepted November 22, 2015.

© 2016 The Authors. Published on behalf of the American Heart Association, Inc., by Wiley Blackwell. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

aromatase, and therefore exogenous E2 therapy after the onset of advanced HF may rescue pre-existing HF. We now show that short-term E2 therapy starting after the onset of advanced HF restores cardiac hemodynamics and the ejection fraction from  $\approx 35\%$  in HF to  $\approx 55\%$ . We demonstrate that the beneficial effects of E2 seem to result from the interplay of various factors, including stimulation of angiogenesis and suppression of fibrosis.

## Methods

### Animals

Wild-type male and female CD-1 and male C57Bl/6 mice 3 to 4 months old were used. All protocols received institutional review and committee approval from the Division of Laboratory Animal Medicine of UCLA.

### Experimental Protocol

Healthy mice with an ejection fraction of  $\approx 60\%$  were randomly subjected to sham or transaortic constriction (TAC) surgery as described previously.<sup>6</sup> Serial echocardiography was performed to monitor the heart structure and function throughout the course of the experiment. HF was achieved 6 to 8 weeks after the TAC surgery with an ejection fraction of  $\approx 35\%$ . Once TAC mice reached HF, mice were randomly euthanized (HF group), or were assigned to one of the following treatment groups for 10 days: E2 via a subcutaneous 10-day continuous-release pellet of 0.03 mg E2/kg per day (Innovative Research of America, E2-RES group), the angiogenesis inhibitor TNP-470 (30 mg/kg, Sigma, once every other day) alone, TNP-470 together with E2 pellets (E2+TNP), or placebo pellets (containing 5 compounds: cholesterol, lactose, cellulose, phosphates, and cerates, which were used as vehicle for E2).

### Plasma and Heart Estrogen Measurements

Plasma and heart E2 measurements were performed using the estradiol enzyme immunoassay (Cayman Chemical). Plasma measurements were conducted according to manufacturer's protocol. For determining estrogen concentrations from the heart, hearts were first frozen and powdered with a mortar and pestle. Eighty milligrams powdered heart tissue was weighed from each heart, lysed in estradiol enzyme immunoassay buffer, and sonicated in a total volume of 500  $\mu\text{L}$  estradiol enzyme immunoassay buffer. This lysate was then subjected to standard estradiol enzyme immunoassay procedure according to manufacturer's protocol and the concentration values were obtained were based on a standard curve in units of pg/mL. As each 500  $\mu\text{L}$  lysate sample

contained 80 mg of heart tissue, 1 mL of lysates would contain 160 mg of heart tissue. The concentration values are thus expressed as pg/160 mg heart.

### Cardiac Hemodynamics

Serial B-Mode and M-Mode echocardiography was performed using a VisualSonics Vevo 2100 equipped with a 30-MHz linear transducer to accurately monitor the stage of the disease by measuring cardiac hemodynamic parameters and assessing heart structure.<sup>7</sup> The left ventricular (LV) ejection fraction, LV wall thickness, and LV cavity dimensions were quantified using M-mode. The left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and heart rate (HR) were recorded directly by inserting a catheter (1.4F Millar SPR-671) connected to a pressure transducer (Power Lab, ADInstruments) into the LV right before euthanizing. The left ventricular developed pressure (LVDP) and rate pressure product (RPP) were calculated as  $\text{LVDP} = \text{LVSP} - \text{LVEDP}$ ,  $\text{RPP} = \text{HR} \times \text{LVDP}$ . The maximum rate of the LV pressure rise ( $dP/dt_{\text{max}}$ ) and decline ( $-dP/dt_{\text{min}}$ ) were directly calculated from the recordings.

### Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

For RT-qPCR quantification, hearts were excised and rinsed in ice-cold phosphate-buffered saline. The heart weight was measured, the atrias were then removed, and the ventricles were snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . For RNA extraction, hearts were powdered with a mortar and pestle on dry ice, suspended in 2 mL Trizol (Invitrogen), and homogenized with a Polytron (Kinematica). RNA quality was assessed via gel electrophoresis. For cDNA synthesis, RNA concentration was assessed using a spectrophotometer (Beckman) and 2  $\mu\text{g}$  of RNA with purity (A260/A280) of 1.7 to 2.0 were reverse transcribed using the Omniscript RT kit (Qiagen) according to manufacturer's protocol in a final reaction volume of 20  $\mu\text{L}$ . The real-time qPCR was performed using IQ SYBR Green supermix (BioRad) according to manufacturer's protocol in a final volume of 25  $\mu\text{L}$  using a BioRad CFX RT-qPCR machine. For all assays, there were at least 3 samples/group assayed in duplicate. Threshold cycle (Cq value) was determined using CFX Manager, and the Cq value of the gene of interest was normalized to the Cq value of its own internal control gene (GAPDH). Controls consisted of: the reaction cocktail without reverse transcriptase and  $\text{H}_2\text{O}$  instead of cDNA tested by RT-qPCR.

### RT-qPCR Primer Validation

Prior to the RT-qPCR experiments, each primer set was validated to ensure that it yields a single sharp peak in the

**Table 1.** Real-Time Polymerase Chain Reaction Primer Sequences

Gene	Primer Sequences
<i>GAPDH</i>	Forward: 5'-CCTGCACCACCAACTGCTTAG -3'
	Reverse: 5'-ATGACCTTGCCACAGCCTTG -3'
$\beta$ -MHC	Forward: 5'-CTCAACTGGGAAGAGCATCCA -3'
	Reverse: 5'-CCTTCAGCAAACCTCTGGAGGC-3'
<i>Vegfa</i>	Forward: 5'-ACACGGTGGTGAAGAAGAG -3'
	Reverse: 5'-CAAGTCTCCTGGGGACAGAA -3'
<i>Hif1a</i>	Forward: 5'-TCATGTCACAGAGCCTCCT -3'
	Reverse: 5'-GCGGAGAAAGAGACAAGTCC -3'
<i>TGF-<math>\beta</math>1</i>	Forward: 5'-CCTGCAAGACCATCGACATGG-3'
	Reverse: 5'-TGGTTTTCTCATAGATGGCGTT-3'
<i>Fibrosin 1</i>	Forward: 5'-AACACGAACCCTGAGCTGCCA-3'
	Reverse: 5'-TCATGTAAGCCACACGAACGTG-3'
<i>Collagen I</i>	Forward: 5'-GACCGATGGATTCCCGTTTCA-3'
	Reverse: 5'-AAGGTCAGCTGGATAGCGACAT -3'
<i>Collagen III</i>	Forward: 5'-AATTCTGCCACCCCGAACTCAA-3'
	Reverse: 5'-TCCATCTTGACGCTTGGTTAG -3'
<i>LOX</i>	Forward: 5'-CACGAGCAGCAGAAATGGGC -3'
	Reverse: 5'-CGCAGTACCAGCCTCAGCGA-3'
<i>ANF</i>	Forward: 5'-CTGATGGATTCAAGAACCTGCT- 3'
	Reverse: 5'-CTCTGGGCTCCAATCCTGTC-3'
<i>SERCA</i>	Forward: 5'-CCTTCTACCAGCTGAGTCATTT-3'
	Reverse: 5'-CAGATGGAGCCACGAGCCA-3'

RT-qPCR melting curve. Primer sequences are listed in Table 1. The controls for each primer set consisted of the reaction cocktail without reverse transcriptase and H<sub>2</sub>O instead of cDNA tested simultaneously by RT-qPCR. All the RT-qPCR products, including the negative controls, were then subjected to gel electrophoresis to ensure amplification of a single product of the expected molecular size without any product amplified in the negative control reactions.

### Immunohistochemistry and Imaging

Whole hearts were fixed in 4% paraformaldehyde and transversal 6 to 7  $\mu$ m sections were obtained with a cryostat. Tissue sections were used for standard eosin/hematoxylin and Masson's Trichrome (Sigma) staining according to manufacturer's protocol and images were acquired with a light microscope (Nikon). For immunofluorescent confocal microscopy, sections were labeled with anti-CD-31 (04-1074; Millipore, 1:200 dilution) and wheat germ agglutinin (Invitrogen, 1:500 dilution) and images were acquired with a confocal scanning microscope (Nikon).

### Western Blot

Whole heart cell lysates were prepared by homogenizing the hearts in 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, 1 mmol/L EGTA, and 1 mmol/L EDTA supplemented with Phosphatase and Protease Inhibitor cocktails (Roche). The samples were then centrifuged at 12 000g for 10 minutes and the supernatants were collected. The protein concentrations were measured and 50  $\mu$ g of protein were treated with sodium dodecyl sulfate/dithiothreitol loading buffer prior to gel electrophoresis. The blots were probed with anti-ER $\alpha$  (sc-542, Santa Cruz Biotechnology), -ER $\beta$  (sc-53494, Santa Cruz Biotechnology), -Stat3 (#9139, Cell Signaling), -pStat3 (Y705, #9145, Cell Signaling), -Akt (sc-8132, Santa Cruz Biotechnology), -pAkt (Ser473, #4051, Cell Signaling), -GAPDH (NB300-327, Novus Biologicals), and -Vinculin (V9131, Sigma-Aldrich). Quantification of protein levels was achieved using Adobe Photoshop.

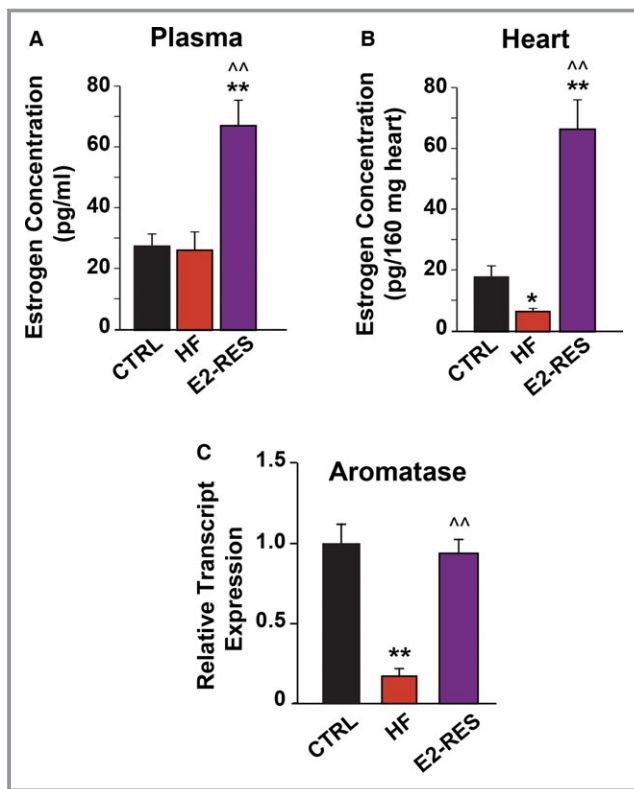
### Statistical Analysis

Means of normally distributed measures were compared on the appropriate scale across groups using the fixed-effects ANOVA model assuming equal variances or allowing nonequal variances as appropriate. For the nonequal variance models, we conservatively estimate error degrees of freedom using the Satterthwaite correction. Normality was evaluated by examining residual error quantile-quantile plots and the corresponding Shapiro-Wilk test under the above models. Constant variance was evaluated by examining plots of the residual errors versus the means under the above models. Variables that did not follow the normal distribution were compared across groups nonparametrically using the Kruskal-Wallis test. Post hoc comparisons were adjusted for multiple testing using the Holm criterion. The Holm criterion states that the kth ordered *P*-value must be  $<0.05 \times (k/3)$  if the overall type I error is to be  $<0.05$  for a given outcome.

### Results

#### Local Heart Estrogen and Cardiac Aromatase Expression Levels Are Significantly Lower in HF Mice

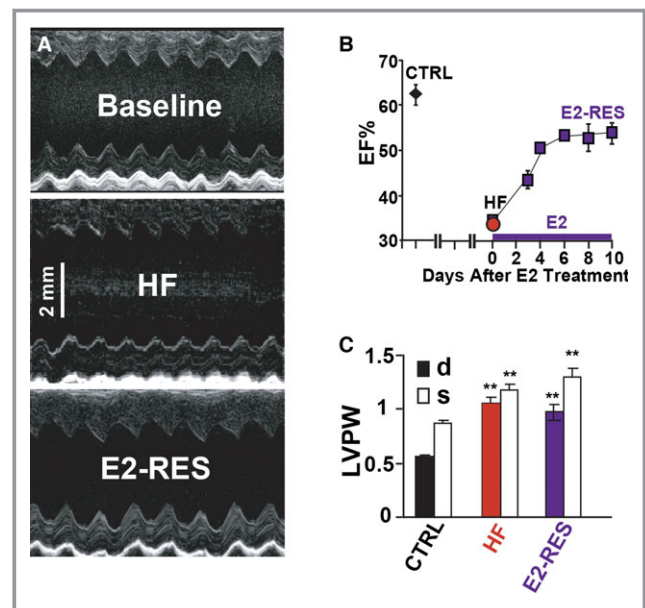
Figure 1A demonstrates that plasma estrogen concentrations remained unchanged in HF ( $26.29 \pm 6.10$  pg/mL in HF and  $27.43 \pm 4.27$  pg/mL in control [CTRL]). Interestingly, however, the local estrogen concentrations in the heart tissue were significantly lower in HF compared to CTRL ( $6.18 \pm 1.12$  pg/160 mg of heart tissue versus  $17.79 \pm 1.28$  pg/160 mg of heart in CTRL, Figure 1B). Additionally, cardiac aromatase transcript levels were also  $\approx 5$ -fold lower in HF compared to



**Figure 1.** Local heart E2 concentrations and cardiac aromatase transcripts are reduced in HF. A, Estrogen concentrations in plasma and (B) in heart ( $n=6-13$  mice/group) in control (CTRL) and after treatment with E2 (E2-RES). C, Relative transcript expression of cardiac aromatase normalized to CTRL. GAPDH was used as an internal control ( $n=4-5$  hearts/group). \* $P<0.05$  vs CTRL; \*\* $P<0.001$  vs CTRL; <sup>^^</sup> $P<0.001$  vs HF. CTRL indicates control; E2-RES, E2-induced rescue; HF, heart failure.

CTRL ( $0.19\pm0.04$  in HF, normalized to CTRL, Figure 1C). Estrogen therapy increased cardiac and plasma E2 concentrations to similar levels ( $61.46\pm3.75$  pg/160 mg of heart and  $66.48\pm9.65$  pg/mL, Figure 1A and 1B), and restored aromatase transcripts to CTRL ( $0.95\pm0.08$ , normalized to CTRL, Figure 1C).

We then explored whether exogenous treatment of estrogen could *rescue* pre-existing HF. Figure 2A illustrates a typical example of an M-mode echocardiogram from the same male mouse at the baseline before the TAC operation (Baseline), and in HF before and after E2 treatment. E2 therapy restored the ejection fraction of HF mice in a surprisingly efficient manner from  $33.2\pm1.1\%$  in HF to  $53.1\pm1.3\%$  within 10 days of E2 treatment (Figure 2B). As expected, HF was associated with a significant increase in LV posterior wall thickness when compared to CTRL, and E2 therapy did not affect this parameter as it remains unchanged versus HF (Figure 2C). The cardiac function of the HF mice treated with placebo progressively declined. Some placebo-treated mice either died or were euthanized because of poor health before the end of the 10-day



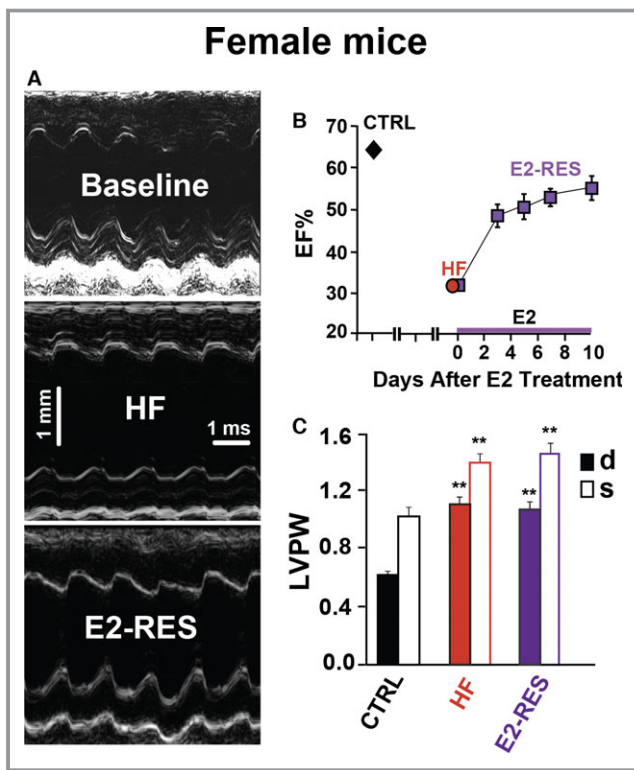
**Figure 2.** Estrogen therapy rescues HF in male mice by restoring heart function. A, Examples of M-mode images of the parasternal short axis view by echocardiography from the same mouse before TAC (Baseline), after TAC in HF, and after treatment with E2 (E2-RES). B, Averaged EF in CTRL (black diamond), HF (red circle) and after E2 treatment as a function of time (purple squares). C, Left ventricular posterior wall thickness (LVPW) at the end of diastole (filled bars) and systole (empty bars). Values and the number of animals are given in Table 2. \*\* $P<0.001$  vs CTRL. CTRL indicates control; EF, ejection fraction; HF, heart failure; TAC, transaortic constriction.

treatment period according to the criteria for early experiment termination and euthanasia at UCLA, while others survived with placebo for 10 days with worsening cardiac function.

E2 was also able to rescue cardiac function in C57/BL6 mice, the most common strain used for TAC studies, to similar levels as CD-1 mice (data not shown). Furthermore, E2 therapy was also able to restore cardiac function in female mice. Intact age-matched female mice were subjected to TAC and once the ejection fraction reached  $\approx 35\%$ , estrogen therapy was commenced at the same dose as in male mice for 10 days. Figure 3 demonstrates that E2 was able to rescue the myocardial contractility of female mice with HF just as in male mice ( $55.35\pm2.78\%$  with E2,  $31.66\pm0.15\%$  in HF versus  $65.53\pm1.24\%$  in CTRL).

E2 partially reversed LV structural morphology changes occurring in HF as reflected in the whole heart (Figure 4A) and eosin/hematoxylin images (Figure 4B), as well as in the heart weight to body weight ratio (mg/g:  $5.39\pm0.14$  CTRL;  $10.15\pm0.3$  HF, and  $7.88\pm0.44$  in E2-RES, Figure 4C). E2 therapy also decreased the cardiomyocyte cross-sectional diameter by  $\approx 10\%$  ( $1\pm0.01$  in CTRL versus  $1.73\pm0.04$  in HF to  $1.53\pm0.02$  in E2, normalized to CTRL, Figure 4D). In pathological heart hypertrophy, fetal genes such as  $\beta$  myosin



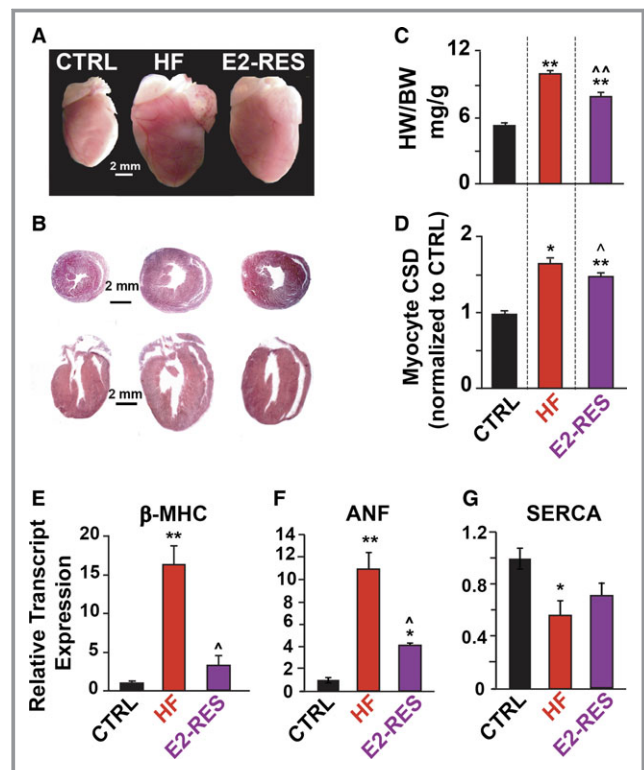


**Figure 3.** Estrogen therapy rescues HF in female mice. A, Examples of M-mode images of the parasternal short axis view by echocardiography from the same mouse before TAC (Baseline), after TAC in HF, and after treatment with E2. B, Averaged EF in CTRL (black diamond), HF (red circle) and after E2 treatment (E2-RES) as a function of time (purple squares). C, Left ventricular posterior wall thickness (LVPW) at the end of diastole (filled bars) and systole (empty bars).  $^{**}P < 0.001$  vs CTRL. CTRL indicates control; EF, ejection fraction; E2-RES, E2-induced rescue; HF, heart failure; TAC, transaortic constriction.

heavy chain ( $\beta$ -MHC), atrial natriuretic factor (ANF), and sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) are known to be regulated.<sup>8,9</sup> In HF, both  $\beta$ -MHC and atrial natriuretic factor were greatly upregulated.  $\beta$ -MHC was upregulated  $\approx 16$ -fold from  $1.00 \pm 0.24$  in CTRL to  $16.26 \pm 2.44$  in HF (normalized to CTRL), while atrial natriuretic factor was upregulated  $\approx 10$ -fold to  $10.90 \pm 1.15$  versus  $1.00 \pm 0.23$  in CTRL. E2 therapy restored  $\beta$ -MHC levels similar to CTRL and significantly downregulated atrial natriuretic factor to  $4.09 \pm 0.24$  (Figure 4E and 4F). SERCA was significantly downregulated in HF (from  $1.00 \pm 0.08$  in CTRL to  $0.56 \pm 0.12$  in HF), and partially restored to  $0.72 \pm 0.09$  with E2 (Figure 4G).

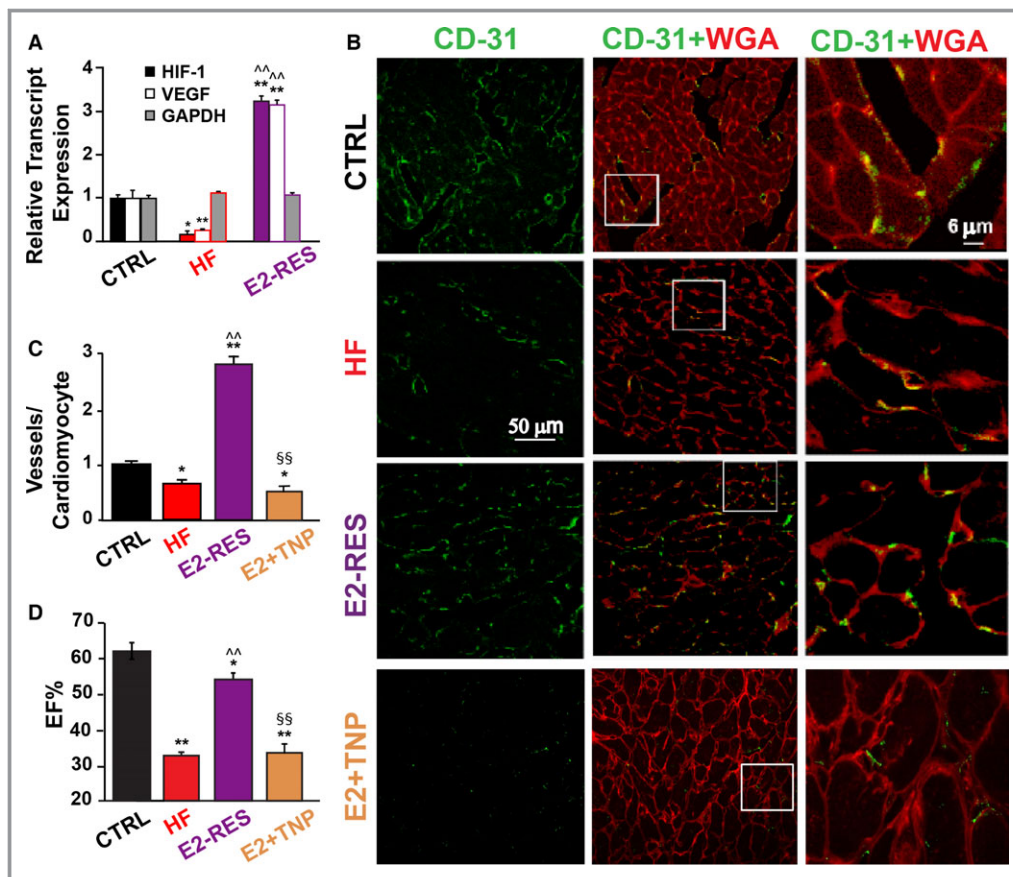
### Stimulation of Cardiac Angiogenesis by E2 Therapy Is a Key Mechanism in E2-Induced Rescue of HF

Reduced cardiac angiogenesis in HF has been previously reported.<sup>10,11</sup> Thus, we examined whether stimulation of cardiac



**Figure 4.** E2-induced rescue of HF is associated with a decreased heart weight to body weight ratio, decreased cardiomyocyte diameter, and reversed expression of fetal gene transcripts. A, Images of the whole heart as well as (B) hematoxylin and eosin staining of cross-sections and longitudinal sections of hearts in CTRL, HF, and E2-RES. C, The heart weight to body weight ratio (HW/BW,  $n=6-9$  animals per group). D, Cardiomyocyte cross-sectional diameter (CSD, normalized to CTRL). Relative transcript expression of (E)  $\beta$ -MHC, (F) atrial natriuretic factor (ANF), and (G) SERCA normalized to CTRL. GAPDH was used as an internal control and its transcripts were similar in all conditions.  $^{*}P < 0.01$  vs CTRL  $^{**}P < 0.001$  vs CTRL;  $^{\wedge}P < 0.001$  vs HF;  $^{\wedge}P < 0.05$  vs HF ( $n=3-5$  animals/group). CTRL indicates control; E2-RES, E2-induced rescue; HF, heart failure;  $\beta$ -MHC,  $\beta$ -myosin heavy chain; SERCA, sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase.

angiogenesis by E2 could be one of the mechanisms participating in the E2-induced rescue of HF. The transcript levels of 2 angiogenic markers, vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1a (HIF1a), were both significantly downregulated ( $\approx 5$ -fold) in HF mice. E2 treatment was not only able to reverse the downregulation of VEGF and HIF1a transcript levels, but also upregulated both transcripts  $\approx 3$ -fold higher than in healthy controls ( $3.24 \pm 0.1$  in E2 versus  $0.26 \pm 0.06$  in HF, Figure 5A). Quantification of capillary density also revealed that E2 therapy significantly enhanced capillary density compared to the HF group ( $2.83 \pm 0.14$  in E2 versus  $0.66 \pm 0.07$  in HF, normalized to CTRL, Figure 5B and 5C). To further confirm the role of angiogenesis in the E2-induced rescue of HF, HF mice were treated with the angiogenesis inhibitor TNP-470 alone or together with E2 (E2+TNP, Figure 5D). All the HF animals



**Figure 5.** Stimulation of cardiac angiogenesis by E2. A, Relative transcript expression of HIF1a and VEGF normalized to CTRL. GAPDH was used as an internal control and its transcripts were similar in all conditions. B, Single confocal images of LV sections immunostained for CD-31 (green, left panels), the overlay of CD31 and wheat germ agglutinin WGA (red, middle panels) and at higher display magnification of the white squares (right panels). C, Quantification of capillary density as microvessels per cardiomyocyte. D, EF% at the end of the experiment. \* $P < 0.01$  \*\* $P < 0.001$  vs CTRL; ^^ $P < 0.001$  vs HF; §§ $P < 0.001$  vs E2 (n=3–4 hearts/group). CTRL indicates control; EF, ejection fraction; E2-RES, estrogen rescue; HF, heart failure; LV, left ventricular; VEGF, vascular endothelial growth factor.

treated with TNP-470 alone died shortly after commencing treatment. E2 failed to rescue HF in the presence of TNP-470 as ejection fraction ( $29.3 \pm 2.1\%$ ) was not significantly improved by 10 days of E2 therapy (Figure 5D). TNP-470 also prevented E2 from stimulating the capillary density ( $0.53 \pm 0.07$  in E2+TNP versus  $2.83 \pm 0.14$  in E2, Figure 5C). Next, we assessed whether exogenous estrogen could promote cardiac angiogenesis in healthy mice, in the absence of TAC. Figure 6 demonstrates that in the absence of TAC stimulus, E2 was also able to significantly induce cardiac angiogenesis by about 30% in healthy mice.

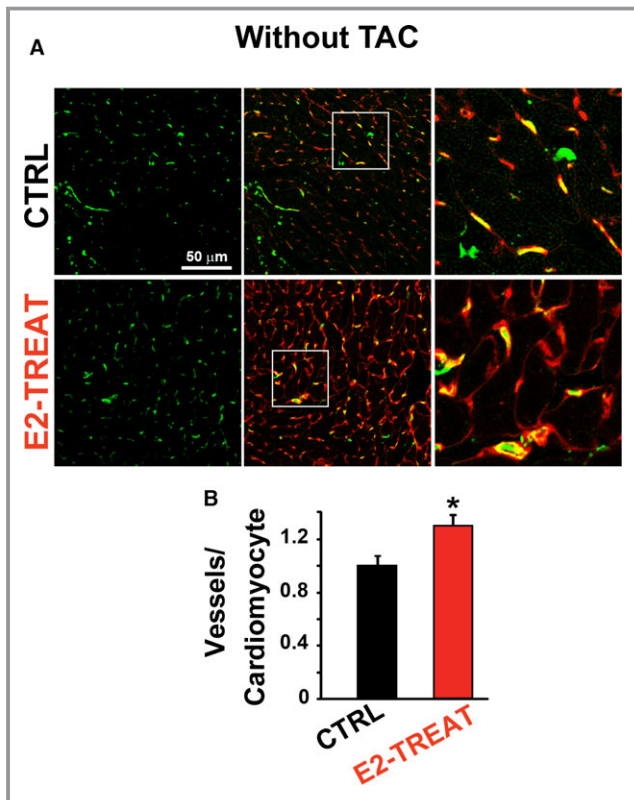
### Estrogen Reverses HF-Induced Myocardial Fibrosis

Extensive myocardial fibrosis has been identified in both severe HF patients as well as in different experimental models of HF.<sup>12</sup> Masson's Trichrome staining revealed that the interstitial and perivascular fibrosis observed in HF was absent in E2-treated

mice (Figure 7A). Mice in HF had  $45.5 \pm 2.8\%$  fibrous tissue in cardiac sections versus  $1.2 \pm 0.4\%$  in CTRL, while E2 therapy significantly decreased fibrotic scarring to  $5.3 \pm 1.0\%$  in E2 (Figure 7B). RT-PCR revealed that the transcript levels of several profibrotic markers were significantly upregulated in HF (from  $1.0 \pm 0.2$  to  $1.8 \pm 0.1$  for collagen I;  $1.0 \pm 0.2$  to  $1.5 \pm 0.1$  for collagen III;  $1.0 \pm 0.9$  to  $4.3 \pm 0.6$  for transforming growth factor- $\beta$ 1;  $1.0 \pm 0.5$  to  $3.6 \pm 0.2$  for fibronin I and  $1.0 \pm 0.3$  to  $2.9 \pm 0.3$  for lysyl oxidase), while E2 treatment restored these transcripts to levels comparable to the CTRL group (Figure 7C).

### Estrogen-Induced Cardioprotection Is Associated With Activation of Protein Kinase B Pathway and Estrogen Receptor- $\beta$

It has previously been shown that E2 protects the heart via regulation of the protein kinase B (Akt) and Stat3 pathways.<sup>13,14</sup> While Stat3 is not regulated in our model in



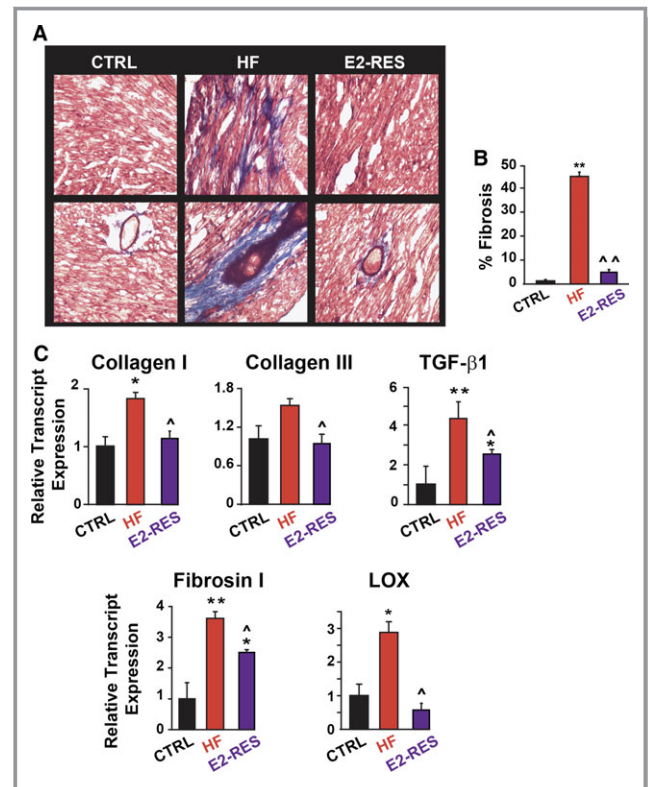
**Figure 6.** Stimulation of cardiac neoangiogenesis by E2 in the absence of TAC in healthy mice. A, Single confocal images of LV sections immunostained for CD31 (green, left panels), the overlay of CD31 and WGA (red, middle panels) and at higher display magnification of the white squares (right panels). B, Quantification of capillary density as microvessels per cardiomyocyte. \* $P < 0.05$  vs CTRL ( $n = 4$  hearts/group). CTRL indicates control; LV, left ventricular; TAC, transaortic constriction; E2-TREAT, estrogen treatment group; WGA, wheat germ agglutinin.

advanced HF and after E2 rescue (Figure 8A and 8B), Akt phosphorylation is reduced approximately by half in HF (from  $1.00 \pm 0.15$  in CTRL to  $0.55 \pm 0.08$  in HF, normalized to CTRL), while E2 therapy normalized phospho-Akt expression to CTRL levels ( $1.14 \pm 0.15$  with E2 rescue, Figure 8C and 8D).

As E2 mainly acts through its classical receptors  $ER\alpha$  and/or  $ER\beta$ , which are both present in the heart,<sup>15</sup> we investigated the  $ER\alpha$  and  $ER\beta$  expression in HF and with E2 rescue. Both  $ER\alpha$  and  $ER\beta$  transcript and protein levels remain unchanged in HF, while E2 treatment interestingly resulted only in increased  $ER\beta$  protein expression from  $1.00 \pm 0.08$  in CTRL and  $1.03 \pm 0.07$  in HF to  $1.29 \pm 0.07$  with E2 (normalized to CTRL, Figure 9).

### E2 Therapy Restores Cardiac Hemodynamic Parameters of HF Mice

Direct catheterization revealed that LVDP was reduced in HF from  $97.8 \pm 2.6$  mm Hg in CTRL to  $86.2 \pm 2.7$  mm Hg in HF. Consistent with the improvement of mechanical performance



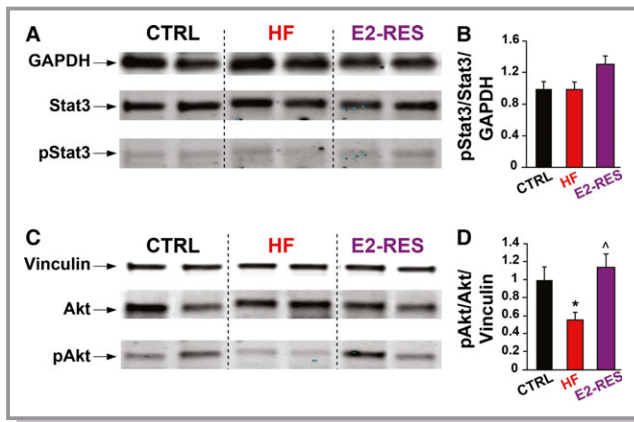
**Figure 7.** E2-induced rescue of HF is associated with suppression of fibrosis. A, Representative Masson's Trichrome staining of LV sections (interstitial, top panels; perivascular, lower panels). B, Quantification of overall fibrosis expressed as percentage of each high-power field. C, Relative transcript expression of profibrotic genes normalized to CTRL. GAPDH was used as an internal control and its transcripts were similar in all conditions. \* $P < 0.05$  vs CTRL; \*\* $P < 0.001$  vs CTRL; ^ $P < 0.05$  vs HF; ^^ $P < 0.001$  vs HF ( $n = 3-5$  hearts/group). CTRL indicates control; E2-RES, estrogen rescue; HF, heart failure; LOX, lysyl oxidase; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

of the heart after E2 therapy, LVDP also increased to  $131 \pm 4.6$  mm Hg (Table 2). E2 also improved the RPP from  $36\ 251.9 \pm 3531.7$  mm Hg  $\times$  beats/min in HF to  $65\ 455 \pm 5533$  mm Hg  $\times$  beats/min after E2 treatment, Figure 10A). The contractile and relaxation defects induced by HF were also corrected by E2 therapy, as the maximum rate of LV pressure rise ( $dP/dt_{max}$ ) and decline ( $dP/dt_{min}$ ) were restored with E2 treatment to values similar to healthy hearts in spite of the sustained presence of the TAC stress stimulus (Figure 10B, Table 2). As expected, RPP and  $dP/dt$  could not be restored by E2 in the presence of the angiogenesis inhibitor TNP-470 (Figure 10).

### Discussion

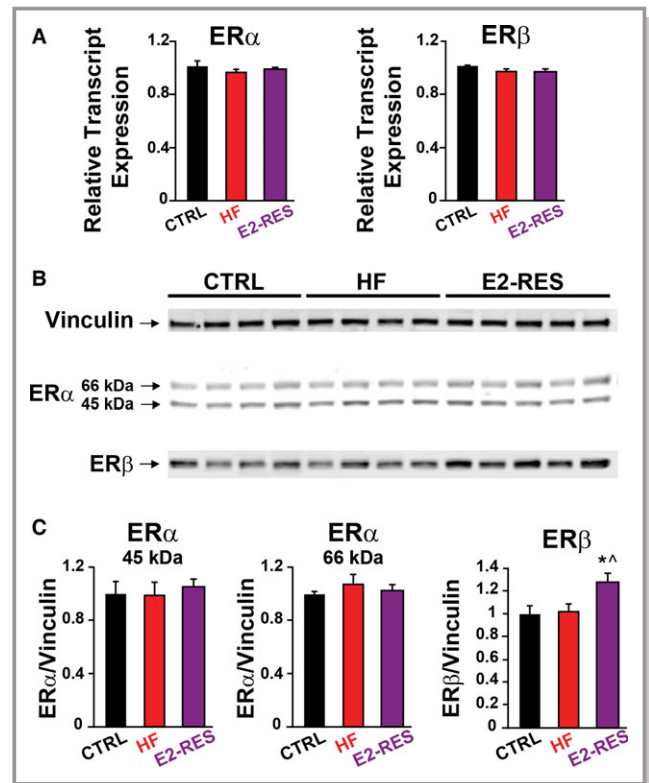
HF is a multifactorial and multiphase disease with a major impact on human health. Despite the advances in





**Figure 8.** E2 therapy regulates Akt, but not Stat3 phosphorylation. A, Representative Western blot for whole heart lysates for (A) Stat3 and phospho-Stat3 and (C) Akt and phospho-Akt. All the samples in each experiment were blotted on the same Western blot; some intervening lanes are not shown as the figure depicts 2 representative lanes/group. B and D, Bar graphs representing the quantification of fluorescent signal of phosphorylated protein expression as normalized to the corresponding total protein expression and then to the corresponding loading control (GAPDH for phospho-Stat3 and Vinculin for phospho-Akt) and to CTRL levels. GAPDH and Vinculin were used as loading control. \* $P<0.05$  vs CTRL and ^ $P<0.05$  vs HF (n=3–5 hearts/group). CTRL indicates control; E2-RES, estrogen rescue; HF, heart failure.

nonpharmacological and pharmacological interventions, as well as novel cell and gene therapies, chronic HF still continues to be a major cause of morbidity and mortality. Here we show for the first time that local heart E2 is significantly reduced in HF. Furthermore, cardiac aromatase transcripts were also downregulated in HF, while remaining unchanged in plasma (Figure 1). Despite the presence of sustained pressure overload, pharmacological intervention using E2 therapy *after* the onset of HF restored ejection fraction of TAC-induced HF from  $\approx 35\%$  to  $\approx 55\%$  both in male and female mice (Figures 2 and 3). E2 therapy also restored heart E2 and aromatase transcript levels. Starting E2 treatment *after* the establishment of HF has major clinical advantages over previous studies that used E2 therapy *prior* to the initiation of the disease.<sup>5</sup> As HF is not often diagnosed early, our approach is more practical for patients who already suffer from HF. E2-induced rescue of HF is strongly correlated with stimulation of LV angiogenesis, as the angiogenesis inhibitor TNP-470 prevented the beneficial action of E2 (Figure 5). In addition, the beneficial effects of E2 in HF were also associated with the reversal of myocardial fibrosis (Figure 6). E2-induced rescue of HF was also associated with upregulation of phospho-Akt as well as activation of estrogen receptor- $\beta$  (Figures 8 and 9).



**Figure 9.** E2 therapy upregulates ER $\beta$  protein levels. A, Relative mRNA transcript levels normalized to CTRL. GAPDH was used as an internal control. Representative Western blot of whole heart lysates for (B) the 45- and 66-kDa isoforms ER $\alpha$ , and ER $\beta$ . C, Bar graphs representing the quantification of fluorescent signal of ER $\alpha$  or ER $\beta$  normalized to the corresponding Vinculin and to CTRL. Vinculin was used as a loading control. \* $P<0.05$  vs CTRL and ^ $P<0.05$  vs HF (n=4–5 hearts/group). CTRL indicates control; E2-RES, estrogen rescue; ER- $\alpha/\beta$ , estrogen receptor- $\alpha/\beta$ ; HF, heart failure.

### Regulation of Cardiac E2 and Aromatase in HF

Here we show that cardiac E2 levels are reduced in HF while plasma E2 remains the same (Figure 1). Interestingly, aromatase transcripts are also reduced in HF; therefore, reduced cardiac E2 could be caused by a reduction in aromatase levels, a lower uptake of plasma E2 by cardiac tissue, or because of impaired myocardial perfusion as a result of decreased capillary density. Most importantly, E2 therapy restored cardiac E2 levels as well as cardiac aromatase transcripts. The normalization of cardiac aromatase transcripts by E2 therapy could be result of improved heart function or attributable to increased E2 availability in the heart. E2 therapy could directly normalize aromatase transcripts by binding to a half ERE site, or indirectly through transcription factors as our analysis of the 5-kb promoter region of aromatase gene shows several half ERE as well as Sp1 and Ap1 elements.

The role of aromatase in the heart is not well understood. In postmenopausal women, aromatase plays an important



**Table 2.** E2 Therapy Restores Cardiac Function

	CTRL	HF	E2-RESC
	15 mice	25 mice	16 mice
IVS, mm			
Diastolic	0.58±0.01	0.98±0.04**	0.94±0.07**
Systolic	0.90±0.03	1.08±0.06*	1.22±0.09*
LV PW, mm			
Diastolic	0.56±0.01	1.05±0.05**	0.97±0.08**
Systolic	0.87±0.02	1.18±0.06**	1.29±0.08**
LV diameter, mm			
Diastolic	4.57±0.08	4.44±0.09	4.66±0.12
Systolic	3.06±0.06	3.74±0.08**	3.39±0.11* <sup>^</sup>
LV volume, mm <sup>3</sup>			
Diastolic	93.5±5.49	91.0±4.47	101.9±6.10
Systolic	36±2.53	60.9±3.35*	48.3±3.54*
LV %EF	61.5±0.74	33.2±1.13**	53.1±1.3** <sup>^^</sup>
LV %FS	33±0.53	15.7±0.58**	27.3±0.84** <sup>^^</sup>
	10 mice	7 mice	7 mice
LVSP	104.7±3.9	95.6±5.5	135±4.6** <sup>^^</sup>
LVEDP	6.9±3.8	9.5±4.9	4±2
LVDP	97.8±2.66	86.2±2.7*	131±4.6** <sup>^^</sup>
RPP	60 868.6±2412.8	36 251.9±3531.7**	65 455±5533.2 <sup>^^</sup>
(dP/dt) <sub>Max</sub>	11 036.6±495.2	4871.4±511.9**	8541.2±638.5* <sup>^^</sup>
(dP/dt) <sub>Min</sub>	8390.9±469.3	4429.2±280.1**	7862.6±754.9 <sup>^^</sup>
HR	622.8±19	421.7±40.4**	499.2±35.1

Values are mean±SEM. Intraventricular septum (IVS), LV posterior wall (LV PW), LV chamber dimensions, and LV mass were obtained from M-mode images of parasternal short axis view similar to those shown in Figure 2A. The number of animals is given in each section. CTRL indicates control; (dP/dt)<sub>Max</sub>, maximum rate of LV pressure rise; (dP/dt)<sub>Min</sub>, maximum rate of LV pressure decline; E2-RESC, estrogen rescue; HF, heart failure; HR, heart rate; LV %EF, left ventricular ejection fraction; LV %FS, left ventricular fractional shortening; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; RPP, rate pressure product.

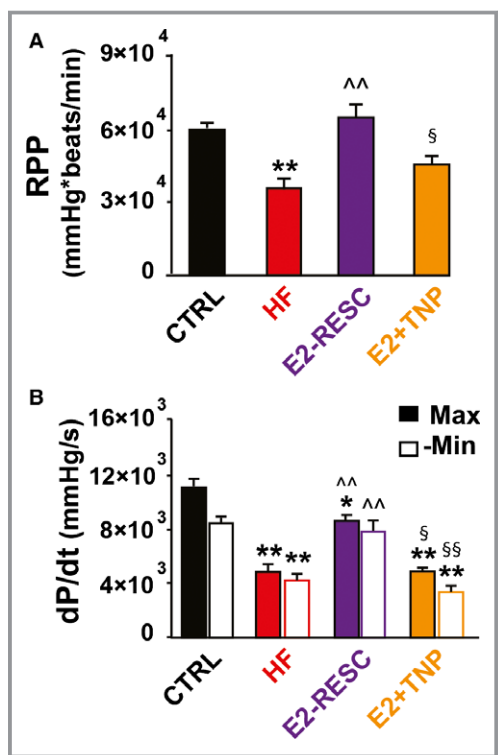
\**P*<0.05 vs CTRL; \*\**P*<0.001 vs CTRL; <sup>^</sup>*P*<0.05 vs HF and <sup>^^</sup>*P*<0.001 vs HF.

role, as its peripheral activity becomes the primary source of E2 synthesis.<sup>16</sup> The ablation of aromatase has also been demonstrated to be associated with increased adiposity<sup>17</sup> and progressive insulin resistance,<sup>18,19</sup> which play significant roles in the development of cardiovascular diseases such as atherosclerosis and diabetic cardiomyopathy. Therefore, restoring aromatase in the heart by E2 therapy could be an important process in preserving the heart function.

### Stimulation of Cardiac Angiogenesis by E2

Increased cardiac angiogenesis has been shown to be a key event in maintaining LV function during adaptive hypertrophy induced by pressure overload; however, the imbalance between cardiac growth and neoangiogenesis eventually leads to the transition from compensated heart hypertrophy to HF.<sup>11</sup> VEGF is an important angiogenic factor and a critical

determinant of capillary growth and density.<sup>20</sup> We have shown that E2 strongly stimulated VEGF expression and growth of blood vessels in the LV of HF mice (Figure 5). The fact that E2 failed to rescue HF in the presence of the angiogenesis inhibitor TNP (Figure 5D) strongly supports the critical role of angiogenesis in the rescue action of E2. We also show that E2 can stimulate angiogenesis in healthy mice, in the absence of TAC (Figure 6). This is not surprising as E2 has been shown to be pro-angiogenic in various tissues and organs such as the uterus, breast, brain, and limbs.<sup>21–24</sup> However, the 30% increase in angiogenesis in healthy mice that did not undergo TAC is not as dramatic as the 300% observed in HF mice treated with E2. Since the existing angiogenesis meets the demands of the heart under basal conditions (in the absence of TAC), there is only a marginal increase in angiogenesis by E2. In HF, despite the larger left ventricle size and obvious need for more blood vessels to support the heart muscle,



**Figure 10.** E2 therapy restores systolic function and corrects contraction and relaxation defects induced by HF. A, RPP and (B)  $dP/dt_{Max}$  (filled bars) and  $dP/dt_{Min}$  (open bars). The values and the number of animals are given in Table 2. \* $P < 0.05$  vs CTRL; \*\* $P < 0.001$  vs CTRL; ^^ $P < 0.001$  vs HF; § $P < 0.05$  vs E2; §§ $P < 0.001$  vs E2. CTRL indicates control; E2-RESC, estrogen rescue; HF, heart failure; RPP, rate pressure product.

angiogenesis is drastically decreased. Therefore, the 300% increase in neoangiogenesis by E2 therapy is a vital process in restoring the heart function.

Furthermore, angiogenesis has been shown to play an important role in maintaining cardiac health, since insufficient blood flow could result in aggravating ischemic heart disease. Many studies have shown that induction of new vessel growth via many angiogenic factors including vascular endothelial factor A (VEGFA), fibroblast growth factor-2, angiopoietin-2, or stem cell therapy can improve cardiac function in various animal models of heart disease.<sup>25,26</sup> In this study, we have highlighted the pivotal role played by E2 in rescuing HF. We have demonstrated that E2 therapy results in a vast increase in angiogenesis, thus reinforcing the concept that estrogen-driven angiogenesis may play a critical role in improving cardiac function. We have also shown that blocking angiogenesis by using the angiogenesis inhibitor TNP together with E2 treatment does not improve cardiac function, further demonstrating a potential role of angiogenesis in the development of HF.

## Reverse Remodeling of Cardiac Fibrosis by E2 Therapy

End-stage HF has long been regarded as a terminal state of cardiac pathological remodeling, consisting of fibrosis and extracellular matrix remodeling that are almost impossible to reverse by any currently available therapy. E2 treatment has been demonstrated to mitigate adverse extracellular matrix remodeling in LV hypertrophy and failure by decreasing collagen deposition and metalloproteinase expression.<sup>27</sup> Here we show that E2-induced rescue of HF is associated with reversal of LV fibrosis by reversing increased expression of the profibrotic genes collagen I, III, transforming growth factor- $\beta$ 1, lysyl oxidase, and fibronectin I (Figure 7). Although attenuation of fibrotic deposition in the heart with tetrahydrobiopterin (a NOS coupler), tempol (a broad antioxidant), as well as FTY-720 (an immunomodulator for treating multiple sclerosis<sup>28</sup>) in the TAC model has been demonstrated, our study reports the reversal of extensive fibrosis with E2 therapy.

## The Involvement of Akt and Estrogen Receptor- $\beta$ in the Rescue Action of E2

Previous findings by Weinberg et al<sup>9</sup> showed that SERCA mRNA levels were depressed  $\approx 40\%$  in male, but not in female rats with ascending aortic stenosis-induced LV hypertrophy as compared with control rats. Here we show that SERCA transcript levels were also significantly downregulated in HF and partially restored by E2 (Figure 4), thus indicating the protective role of E2 via SERCA activation in this model of pressure overload as well. Previous work by Patten et al<sup>29</sup> further showed that estrogen pretreatment decreased Stat3 activation up to 48 hours post-TAC. In our model, however, we do not see any significant differences in Stat3 activation in HF or following E2 treatment (Figure 8). Because the mechanism by which E2 influences the activation of the Stat transcription factor family has not yet been elucidated in cardiomyocytes,<sup>29</sup> it is possible that its regulation occurs only early on post cardiac insult and its activation stabilizes after more prolonged periods of time such as in our model.

Lastly, we investigated whether E2 can exert its cardioprotective effect via Akt activation. We show that Akt phosphorylation is reduced approximately by half in HF, while E2 therapy normalized phospho-Akt expression to control levels (Figure 8). These data are in agreement with previous studies that reported a pressure overload-induced decrease in Akt phosphorylation,<sup>13,14</sup> which was significantly restored by estrogen.<sup>14</sup>

Estrogen mainly acts through its classical receptors ER $\alpha$  and/or ER $\beta$ , and both receptors are present in the heart.<sup>15</sup> The beneficial role of ER $\beta$  in cardioprotection has been

highlighted in recent years,<sup>30</sup> as it has been shown to be responsible for the antihypertrophic and antifibrotic effects of E2.<sup>5,30</sup> Here we show that E2 therapy upregulated ER $\beta$  expression by  $\approx$ 20%, which could also be one of the mechanisms through which E2 rescues HF.

### The Beneficial Action of E2 on the Heart

Our data show that E2 therapy restores systolic function and corrects contraction and relaxation defects associated with HF in spite of the sustained presence of the TAC banding (Figure 10A and 10B). These hemodynamic data, together with stimulation of cardiac angiogenesis by E2 (Figure 6) and reversal of LV fibrosis (Figure 7), support the view that the improvement of LV function is mediated via the action of E2 on the heart. However, the direct action of E2 on the vessel wall could not be excluded, as E2 promotes vasodilation by acting on the endothelial and smooth muscle cells.<sup>31</sup> E2 directly stimulates endothelial nitric oxide synthase activity and thus has a beneficial effect on vasculature. Many reports have shown that E2 can cause arterial dilation of elastic and muscular arteries in vivo via estrogen receptor-mediated activation of ERK and PI3K, and stimulation of nitric oxide production.<sup>32</sup> Furthermore, E2 has also been shown to promote dilation of canine coronary arteries in an endothelium-independent manner not mediated by the classic intracellular ERs;<sup>33</sup> thus, unloading of the heart may be an important explanatory factor for the rescue effect of E2 therapy. Therefore, it is possible that vasodilation caused by E2 on the vessel wall has an additional contribution to the observed E2-induced rescue of HF and results in beneficial effects on cardiovascular function and improved cardiac hemodynamics.

We have also found that treatment with E2 increases the LV diameter and the LV volume compared to CTRL, suggesting a dilatation of the LV as compared to CTRL. An elevation of LVEDP is usually associated with an increase in LV diameter. In our findings, however, we did not observe an increased LVEDP after E2 treatment. On the contrary, we obtained a reduction in the filling pressure compared to CTRL. Since the HR is not statistically significant between the E2-treated and CTRL groups, the elevated LVSP observed in the E2 group can be undoubtedly associated with a Starling effect caused by a much higher relaxation of the myocardium. Together, our hemodynamic results indicate that after decompensated HF, E2 treatment rescues the heart function by increasing the myocardial relaxation, which favors the myocardial contractility.

Here we show that E2 rescues HF induced by pressure overload both in male and female mice. In ovariectomized rats, E2 replacement 2 weeks post-aortic banding has been shown to significantly inhibit pressure overload-induced

increases in heart hypertrophy and to ameliorate impairment of LV function.<sup>14</sup> Therefore, we speculate that E2 therapy should improve cardiac function of OVX mice with HF as well. The beneficial action of estrogen is not limited to only pressure overload induced by TAC. Estrogen has been shown to be protective in other models of HF such as ischemia/reperfusion injury,<sup>34</sup> volume overload,<sup>35</sup> as well as right ventricular failure secondary to pulmonary hypertension.<sup>36</sup>

We also show that E2-induced rescue of HF is associated with the reversal of adverse LV remodeling. The beneficial effects of E2 treatment in HF seem to result from an interplay of different factors, among which restoration of cardiac aromatase levels, stimulation of cardiac neoangiogenesis, and reversal of fibrosis are key mechanisms. However, the long-term benefits of E2-induced cardioprotection in HF remain to be evaluated, as it is yet unknown whether the beneficial effects of short-term E2 therapy persist after E2 withdrawal. Furthermore, the mechanism of E2 action (genomic versus nongenomic) also remain to be determined. Here we speculate that E2-induced cardioprotection is via a genomic pathway, as E2 exerts its beneficial effects on the heart in 10 days.

Even though E2 may have unwanted long-term side effects, our findings with relatively short-term E2 treatment raise the exciting possibility to expand the application of E2 for the treatment of chronic HF within a relatively safe time frame, a concept that most definitely warrants further investigation. Because E2 reverses LV dysfunction associated with a number of cardiovascular diseases, our findings have broad clinical implications.

### Acknowledgments

We would like to thank Dr Jeffrey A. Gornbein and Daniela Markovic, Biostatisticians at the UCLA Department of Biomathematics Statistical/Biomathematical Consulting Clinic, for their assistance with the statistical analysis and statistical methods section.

### Sources of Funding

This work has been supported by NIH and NRSA training grants T32-GM065823 and HD00722 (Iorga), NIH grants HL089876, HL089876S1, and HL119886 (Eghbali), HL107418 (Toro, Stefani), and HL088640 (Stefani).

### Disclosures

None.

### References

- Chien KR, AAG. In: Barunwald E, ed. *Heart Disease: A Textbook of Cardiovascular Medicine*. Philadelphia, PA: Saunders Company; 1996:1626–1648.
- Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER, Bedja D, Gabrielson KL, Wang Y, Kass DA. Chronic inhibition of cyclic GMP phospho-



- diesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med*. 2005;11:214–222.
3. Sasaki H, Nagayama T, Blanton RM, Seo K, Zhang M, Zhu G, Lee DI, Bedja D, Hsu S, Tsukamoto O, Takashima S, Kitakaze M, Mendelsohn ME, Karas RH, Kass DA, Takimoto E. PDE5 inhibitor efficacy is estrogen dependent in female heart disease. *J Clin Invest*. 2014;124:2464–2471.
  4. Kamal FA, Mickelsen DM, Wegman KM, Travers JG, Moalem J, Hammes SR, Smrcka AV, Blaxall BC. Simultaneous adrenal and cardiac g-protein-coupled receptor-G $\beta\gamma$  inhibition halts heart failure progression. *J Am Coll Cardiol*. 2014;63:2549–2557.
  5. van Eickels M, Grohe C, Cleutjens JP, Janssen BJ, Wellens HJ, Doevendans PA. 17 $\beta$ -Estradiol attenuates the development of pressure-overload hypertrophy. *Circulation*. 2001;104:1419–1423.
  6. Rockman HA, Ross RS, Harris AN, Knowlton KU, Steinhilber ME, Field LJ, Ross J Jr, Chien KR. Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. *Proc Natl Acad Sci USA*. 1991;88:8277–8281.
  7. Zhou YQ, Foster FS, Parkes R, Adamson SL. Developmental changes in left and right ventricular diastolic filling patterns in mice. *Am J Physiol Heart Circ Physiol*. 2003;285:H1563–H1575.
  8. Dorn GW, Robbins J, Sugden PH. Phenotyping hypertrophy: eschew obfuscation. *Circ Res*. 2003;92:1171–1175.
  9. Weinberg EO, Thienelt CD, Katz SE, Bartunek J, Tajima M, Rohrbach S, Douglas PS, Lorell BH. Gender differences in molecular remodeling in pressure overload hypertrophy. *J Am Coll Cardiol*. 1999;34:264–273.
  10. Sano M, Minamino T, Toko H, Miyauchi H, Orimo M, Qin Y, Akazawa H, Tateno K, Kayama Y, Harada M, Shimizu I, Asahara T, Hamada H, Tomita S, Molkenin JD, Zou Y, Komuro I. p53-Induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload. *Nature*. 2007;446:444–448.
  11. Shiojima I, Sato K, Izumiya Y, Schiekofer S, Ito M, Liao R, Colucci WS, Walsh K. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *J Clin Invest*. 2005;115:2108–2118.
  12. Shirani J, Pick R, Roberts WC, Maron BJ. Morphology and significance of the left ventricular collagen network in young patients with hypertrophic cardiomyopathy and sudden cardiac death. *J Am Coll Cardiol*. 2000;35:36–44.
  13. Bhuiyan MS, Shioda N, Fukunaga K. Ovariectomy augments pressure overload-induced hypertrophy associated with changes in Akt and nitric oxide synthase signaling pathways in female rats. *Am J Physiol Endocrinol Metab*. 2007;293:E1606–E1614.
  14. Tagashira H, Bhuiyan S, Shioda N, Fukunaga K. Distinct cardioprotective effects of 17 $\beta$ -estradiol and dehydroepiandrosterone on pressure overload-induced hypertrophy in ovariectomized female rats. *Menopause*. 2011;18:1317–1326.
  15. Grohe C, Kahlert S, Lobbert K, Vetter H. Expression of oestrogen receptor alpha and beta in rat heart: role of local oestrogen synthesis 6. *J Endocrinol*. 1998;156:R1–R7.
  16. Purohit A, Reed MJ. Regulation of estrogen synthesis in postmenopausal women. *Steroids*. 2002;67:979–983.
  17. Jones ME, Thorburn AW, Britt KL, Hewitt KN, Wreford NG, Proietto J, Oz OK, Leury BJ, Robertson KM, Yao S, Simpson ER. Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity. *Proc Natl Acad Sci USA*. 2000;97:12735–12740.
  18. Takeda K. Sarcolemmal slow conductance increase of frog sartorius fibers during hyperpolarization. *Jpn J Physiol*. 1975;25:515–524.
  19. Ling S, Dai A, Dilley RJ, Jones M, Simpson E, Komesaroff PA, Sudhir K. Endogenous estrogen deficiency reduces proliferation and enhances apoptosis-related death in vascular smooth muscle cells: insights from the aromatase-knockout mouse. *Circulation*. 2004;109:537–543.
  20. Flamme I, Breier G, Risau W. Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (flk-1) are expressed during vasculogenesis and vascular differentiation in the quail embryo. *Dev Biol*. 1995;169:699–712.
  21. Ardelt AA, McCullough LD, Korach KS, Wang MM, Munzenmaier DH, Hum PD. Estradiol regulates angiotensin-1 mRNA expression through estrogen receptor-alpha in a rodent experimental stroke model. *Stroke*. 2005;36:337–341.
  22. Rodger FE, Young FM, Fraser HM, Illingworth PJ. Endothelial cell proliferation follows the mid-cycle luteinizing hormone surge, but not human chorionic gonadotrophin rescue, in the human corpus luteum. *Hum Reprod*. 1997;12:1723–1729.
  23. Elkin M, Orgel A, Kleinman HK. An angiogenic switch in breast cancer involves estrogen and soluble vascular endothelial growth factor receptor 1. *J Natl Cancer Inst*. 2004;96:875–878.
  24. Kyriakides ZS, Petinakis P, Kaklamanis L, Sbarouni E, Karayannakos P, Iliopoulos D, Dontas I, Kremastinos DT. Intramuscular administration of estrogen may promote angiogenesis and perfusion in a rabbit model of chronic limb ischemia. *Cardiovasc Res*. 2001;49:626–633.
  25. Ng YS, D'Amore PA. Therapeutic angiogenesis for cardiovascular disease. *Curr Control Trials Cardiovasc Med*. 2001;2:278–285.
  26. Taimeh Z, Loughran J, Birks EJ, Bolli R. Vascular endothelial growth factor in heart failure. *Nat Rev Cardiol*. 2013;10:519–530.
  27. Mahmoodzadeh S, Dworatzek E, Fritschka S, Pham TH, Regitz-Zagrosek V. 17 $\beta$ -Estradiol inhibits matrix metalloproteinase-2 transcription via MAP kinase in fibroblasts. *Cardiovasc Res*. 2010;85:719–728.
  28. Liu W, Zi M, Tsui H, Chowdhury SK, Zeef L, Meng QJ, Travis M, Prehar S, Berry A, Hanley NA, Neyses L, Xiao RP, Oceandy D, Ke Y, Solaro RJ, Cartwright EJ, Lei M, Wang X. A novel immunomodulator, FTY-720 reverses existing cardiac hypertrophy and fibrosis from pressure overload by targeting NFAT (nuclear factor of activated T-cells) signaling and periostin. *Circ Heart Fail*. 2013;6:833–844.
  29. Patten RD, Pourati I, Aronovitz MJ, Alsheikh-Ali A, Eder S, Force T, Mendelsohn ME, Karas RH. 17 Beta-estradiol differentially affects left ventricular and cardiomyocyte hypertrophy following myocardial infarction and pressure overload. *J Card Fail*. 2008;14:245–253.
  30. Pedram A, Razandi M, O'Mahony F, Lubahn D, Levin ER. Estrogen receptor-beta prevents cardiac fibrosis. *Mol Endocrinol*. 2010;24:2152–2165.
  31. Tostes RC, Nigro D, Fortes ZB, Carvalho MH. Effects of estrogen on the vascular system. *Braz J Med Biol Res*. 2003;36:1143–1158.
  32. Han G, Ma H, Chintala R, Miyake K, Fulton DJ, Barman SA, White RE. Nongenomic, endothelium-independent effects of estrogen on human coronary smooth muscle are mediated by type I (neuronal) NOS and PI3-kinase-Akt signaling. *Am J Physiol Heart Circ Physiol*. 2007;293:H314–H321.
  33. Sudhir K, Chou TM, Mullen WL, Hausmann D, Collins P, Yock PG, Chatterjee K. Mechanisms of estrogen-induced vasodilation: in vivo studies in canine coronary conductance and resistance arteries. *J Am Coll Cardiol*. 1995;26:807–814.
  34. Lagranha CJ, Deschamps A, Aponte A, Steenbergen C, Murphy E. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. *Circ Res*. 2010;106:1681–1691.
  35. Gardner JD, Murray DB, Voloshenyuk TG, Brower GL, Bradley JM, Janicki JS. Estrogen attenuates chronic volume overload induced structural and functional remodeling in male rat hearts. *Am J Physiol Heart Circ Physiol*. 2010;298:H497–H504.
  36. Umar S, Iorga A, Matori H, Nadadur RD, Li J, Maltese F, van DER LA, Eghbali M. Estrogen rescues preexisting severe pulmonary hypertension in rats. *Am J Respir Crit Care Med*. 2011;184:715–723.