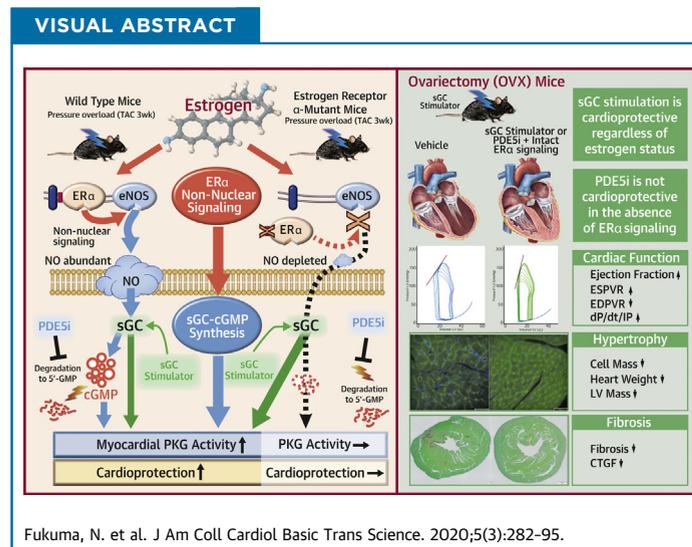


PRECLINICAL RESEARCH

Estrogen Receptor- α Non-Nuclear Signaling Confers Cardioprotection and Is Essential to cGMP-PDE5 Inhibition Efficacy



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HIGHLIGHTS

- The role of ER α non-nuclear signaling in female heart failure was investigated, using a novel mouse line in which ER α non-nuclear signaling was selectively disrupted by inhibiting the interaction between ER α and striatin, a scaffold protein residing at caveolae.
- ER α non-nuclear signaling was linked to myocardial PKG activity in female hearts and ameliorated cardiac maladaptive remodeling induced by pressure overload.
- ER α non-nuclear signaling was indispensable to the therapeutic efficacy of cGMP-PDE5 inhibition in heart failure but not to that of sGC stimulation.
- sGC stimulation potentially ameliorated cardiac remodeling, regardless of estrogen conditions, in sharp contrast to PDE5 inhibition.
- The study provided the first *in vivo* evidence for the role of ER α non-nuclear signaling in heart failure, linking it to cGMP-PKG pathways. The data also supported the advantage of sGC stimulation over PDE5 inhibition as a potential therapeutic strategy in treating heart failure in post-menopausal women, highlighting the need for female-specific therapeutic strategies.

SUMMARY

Using genetically engineered mice lacking estrogen receptor- α non-nuclear signaling, this study demonstrated that estrogen receptor- α non-nuclear signaling activated myocardial cyclic guanosine monophosphate-dependent protein kinase G and conferred protection against cardiac remodeling induced by pressure overload. This pathway was indispensable to the therapeutic efficacy of cyclic guanosine monophosphate-phosphodiesterase 5 inhibition but not to that of soluble guanylate cyclase stimulation. These results might partially explain the equivocal results of phosphodiesterase 5 inhibitor efficacy and also provide the molecular basis for the advantage of using a soluble guanylate cyclase simulator as a new therapeutic option in post-menopausal women. This study also highlighted the need for female-specific therapeutic strategies for heart failure. (J Am Coll Cardiol Basic Trans Science 2020;5:282-95) © 2020 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Heart failure is one of the leading causes of death in many developed countries, including the United States, and the number of patients continues to increase worldwide. Despite decades of advances in the standard of care for treating heart failure, its mortality remains high (1). The cyclic guanosine monophosphate (cGMP) signaling pathway plays a central role in the maintenance of cardiovascular homeostasis; its aberrant regulation has been implicated in the pathophysiology of heart failure. Restoring or augmenting cGMP signaling has emerged as new therapeutic strategy, including enhancing its synthesis and inhibiting its degradation. The former is achieved by soluble guanylate cyclase (sGC) stimulation or neprilysin inhibition, and the latter occurs by cGMP-phosphodiesterase (PDE) inhibition.

Among the small molecules that activate cGMP signaling pathways, PDE5 inhibitors (PDE5i) have long been clinically used to treat pulmonary hypertension. Although the PDE5i sildenafil provides cardiac benefits in various experimental models (2-6), clinical studies testing the efficacy of PDE5i in patients with heart failure have shown equivocal results (7-9). Negative data come from mostly female patients and positive data come from mostly male

patients. This may be attributable to attenuated nitric oxide (NO) production due to reduced levels of estrogen in older adult patients. We previously demonstrated that reduced levels of estrogen critically hampered the anti-remodeling efficacy of PDE5i in female rodent heart failure, which suggested that estrogen-coupled cGMP signaling is essential to the efficacy of PDE5 inhibition (10). In contrast, sGC stimulators have been gaining attention as a potential therapeutic approach for heart failure by enhancing the cGMP signaling pathway, and a new sGC stimulator Vericiguat (Bayer and Merck Sharp & Dohme, Branchburg, New Jersey) is being tested for heart failure in a Phase III clinical study (11). It is of significant clinical importance to determine whether its efficacy is affected by estrogen conditions.

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Estrogen plays a significant role in maintaining homeostasis of the cardiovascular system and vascular protection in women (12-19). Estrogen and its receptors classically bind and translocate to the nucleus, where they directly modulate transcription. Estrogen receptors (ERs) also have extra-nuclear actions that

ABBREVIATIONS AND ACRONYMS

cGMP = cyclic guanosine monophosphate

E2 = estradiol

ECs = endothelial cells

EDC = estrogen dendrimer conjugate

eNOS = endothelial nitric oxide synthase

ER = estrogen receptor

LV = left ventricular

NO = nitric oxide

PaPE = pathway-preferential estrogen

PDE5i = phosphodiesterase 5 inhibitor

PKG = cGMP-dependent protein kinase G

sGC = soluble guanylate cyclase

TAC = transverse aortic constriction

VO₂ = oxygen consumption rate

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involve activation of kinase signaling, which leads to rapid cellular responses, known as non-nuclear signaling (20). ER α is one of the predominant ER subtypes, which has both nuclear and non-nuclear actions. The roles for ER α nuclear actions have been clarified using unique mouse models of ER α , including a model lacking AF-1 function (ER α AF-1⁰ mice) and a model lacking AF-2 function (ER α AF-2⁰ mice), as well as a complete gene deletion model (21,22). In particular, the dissection of ER α AF-2⁰ that lacks nuclear function has provided evidence that the nuclear actions of ER α mediate protective effects against atherosclerosis, diabetes, and bone demineralization, which are conferred by estrogen (22-25).

In contrast, non-nuclear actions of ER α have been shown to mediate endothelial NO production, vasodilatation, and endothelial healing (re-endothelialization) from the study of a mouse that had a point mutation at its palmitoylation site (C451A-ER α), and therefore, a membrane-specific loss of function (20,26). Other studies used pharmacological tools to study non-nuclear actions, including estrogen dendrimer conjugates (EDCs) or pathway-preferential estrogens (PaPEs) (20), and demonstrated the role for vasculo-protection and endothelial NO production without growing uterine or breast cancer cells (21,27-30). However, in light of chronic arterial protection, Guivarc'h et al. (31) performed a study that compared nuclear actions versus non-nuclear actions of ER α in models of chronic hypertension and atherosclerosis using EDCs, PaPEs, C451A-ER α , and ER α AF-2⁰, and demonstrated the prominent protective role of the former. However, non-nuclear actions of ER α might play a pivotal role in the heart. Menazza et al. (26) used an ex vivo model of myocardial ischemia-reperfusion and found that EDCs ameliorated the injury via mechanisms that potentially involved NO and protein S-nitrosylation (26). We hypothesized that non-nuclear actions of ER α might play a role in pathological cardiac remodeling because of the coupling of the non-nuclear actions to endothelial nitric oxide synthase (eNOS) and thus potentially to cGMP-dependent protein kinase G (cGMP-PKG) signaling in the heart.

The present study used a novel mouse line, which we recently reported, in which ER α non-nuclear signaling was disrupted by inhibiting the interaction between ER α and striatin while maintaining intact genomic signaling (32). We investigated the role of ER α non-nuclear signaling in heart failure remodeling and in the cGMP-PKG enhancing therapeutic interventions. We found that ER α non-nuclear signaling protected against cardiac remodeling and was essential to the therapeutic efficacy

of cGMP-PDE5 inhibition but not to that of sGC stimulation.

METHODS

ANIMALS. All animal procedures were approved by Institutional Animal Care and Use Committee at the University of Tokyo. The animal care and experiments were in accordance with the guidelines of the National Institutes of Health. Animals were maintained with food and water ad libitum and kept in cages with a 12-h light-dark cycle in a temperature-controlled laboratory. Sildenafil citrate (100 mg/kg/day) was fed mixed with soft rodent chow (Transgenic Dough Diet, Bio-Serv, Flemington, New Jersey) for 3 weeks as described previously (6,33). The sGC stimulator (Riociguat: BAY 632521 [Irvine, California]; 3 mg/kg/day) was prepared in specific solution (Transcutol 10%, Cremophor 20%, water 70%) and given by oral gavage every day for 3 weeks as described previously (34).

The KRR^{KI/KI} mice (KRR knock-in mutant ER α) were generated by GenOway (Lyon, France) as previously described (35). The mutations were introduced in the exon 3 of ESR1, replacing amino acids 235K, 237R, and 238R: GCC, GCT, and GCA of mouse ER α (corresponding 231K, 233R, and 234R of human ER α) with alanine. These mutations were located in region C, the DNA-binding domain of ER α . Heterozygous male and female animals were mated, and all of the offspring were genotyped by polymerase chain reaction as previously described (35). Homozygous mutants (KRR^{KI/KI}) and their littermate controls (KRR^{WT/WT}, wild type littermate) were used in this study (10).

OVARECTOMY AND ESTROGEN REPLACEMENT.

Ovariectomy. Ovariectomy was performed in female mice at the age of 6 to 8 weeks by a standard bilateral back approach procedure (36,37). Briefly, animals were anesthetized with an intraperitoneal injection of 100 mg/kg of etomidate and placed in a prone position. Two small skin incisions (1 cm in length) were made on both lateral back sides, caudal to the last rib and 1 cm lateral from the vertebra. To reach the peritoneal space, the body wall and peritoneal muscle layer were incised. The ovary was identified with oviducts, uterus, and fat tissues, and then exteriorized with forceps. After a ligature was applied between the ovary and the edge of uterine horn for hemostasis, the ovary was carefully excised.

Estrogen replacement. One week after ovariectomy, 60-day time-release estradiol (E2) pellets (0.25 mg, Innovative Research of America, Sarasota, Florida) or placebo-containing pellets were implanted subcutaneously as previously described (10). The size

of the uterus was checked to confirm the successful excision of ovaries with or without E2 supplementation, as previously described (31).

TRANSVERSE AORTIC CONSTRICTION. Animals underwent transverse aortic constriction (TAC) or sham surgery (sham) at 8 to 10 weeks as previously described (6,33). Briefly, animals were anesthetized with isoflurane (1% to 1.5%) and 100 mg/kg of etomidate, intubated, and mechanically ventilated. The transverse aorta was constricted with a 27-gauge needle using 7-0 prolene suture, and animals' chests were closed. Animals were killed 3 weeks after TAC. Total heart weight was measured, and the left ventricular (LV) tissue was harvested. Snap frozen heart samples are stored at -80°C until analyses.

ECHOCARDIOGRAPHIC STUDY. Transthoracic echocardiography was performed (VEVO2100, 9 to 18 MHz transducer, Visualsonics Inc., Toronto, Ontario, Canada) with conscious mice. M-mode LV end-systolic and end-diastolic dimensions were measured, and the percentage of LV fractional shortening was calculated as previously described (10). Studies were performed by investigators blinded to genotypes and heart conditions. Serial studies were performed on the day of and at 1 and 3 weeks after TAC surgery.

HEMODYNAMIC STUDY USING PRESSURE VOLUME ANALYSIS. In vivo LV function was assessed by pressure–volume analysis in anesthetized mice as previously described (6,33). The LV apex was exposed through an incision between the seventh and eighth ribs. A 1.4-F pressure–volume catheter (SPR-839, Millar Instruments; Houston, Texas) was inserted from the LV apex and advanced into the LV lumen to lie along the longitudinal axis. The absolute volume was calibrated and pressure–volume data were assessed at the steady state and during the pre-load reduction phase. Data were analyzed with the LabChart application (AD Instruments, Dunedin, New Zealand).

CARDIOMYOCYTE ISOLATION AND NOS ACTIVITY MEASUREMENT. Cardiomyocytes were isolated from $\text{KRR}^{\text{WT}/\text{WT}}$ and $\text{KRR}^{\text{KI}/\text{KI}}$ mouse hearts as previously described (38). Briefly, the heart was quickly excised and retroperfused through the ascending aorta with modified Tylode's solution (130 mM sodium chloride, 5.4 mM potassium chloride, 0.33 mM sodium dihydrogen phosphate (NaH_2PO_4), 0.5 mM magnesium chloride (MgCl_2), 22 mM glucose) with 1 mg/ml of collagenase type 2 (Worthington Biochemical, Lakewood, New Jersey) and 0.05 mg/ml of protease (Sigma-Aldrich, St. Louis, Missouri). Cardiomyocytes were incubated in modified Tylode's solution with 1

nM of E2 (Sigma-Aldrich) or without E2 for 40 min at 37°C , then cells were lysed and NOS activity was measured using an enzyme immunoassay kit (Ultra-sensitive NOS Assay Kit; Oxford Biomedical Research, Rochester Hills, Michigan) according to the manufacturer's protocol.

MEASUREMENT OF E2 CONCENTRATION IN SERUM AND HEART BY ENZYME IMMUNOASSAY. Serum and heart E2 levels were measured using an E2 enzyme immunoassay kit (Arbor Assays Estradiol Serum EIA kit [Arbor Assays, Inc., Ann Arbor, Michigan]) according to the manufacturer protocols. For serum E2 measurement, blood samples were centrifuged at 4°C , and supernatants were collected.

For measuring E2 concentration in heart tissue, we followed the published protocol by Iorga et al. (39). LV samples were trimmed in 80 to 100 mg of tissues. Samples were then snap frozen, pressed into powders, and were diluted in E2 enzyme immunoassay buffer at a concentration of 100 mg powder/ml buffer. Amounts per 100 mg heart tissue were calculated.

RNA AND PROTEIN ANALYSIS. Total RNA was extracted from mouse LV heart samples by using TRIreagent (Molecular Research Center Inc., Cincinnati, Ohio). The mRNA was reverse transcribed into cDNA using a high-capacity RNA-to-cDNA Kit (Applied Biosystems, Life Technologies, Rockville, Maryland).

The primer sequences were as follows:

CTGF: forward AGCCTCAAACCTCAAACACC, reverse CAACAGGGATTGACCAC

Nppb: forward AAGTCCTAGCCAGTCTCCAGA, reverse GAGCTGTCTCTGGGCCATTTC

GAPDH: forward CATGGCCTTCCGTGTTCTTA, reverse CCTGCTTACCACCTTCTTGAT

GREB1: forward GACCGTCTACTACCTCGTCCA, reverse GCCAGGAGCGTAGGAAGAT

AGT: forward CGGAGGCAAATCTGAACAAC, reverse TCCTCCTCTCTGCTTTGAG

C3: forward CGGCATAGAGAAGAGGCAAG, reverse AAGGCAGCATAGGCAGAGC

cDNAs were amplified with Thunderbird qPCR Mix (Toyobo Inc., Osaka, Japan), and relative expression levels of target genes were measured using Light Cycler480 (Roche Inc., Basel, Switzerland) as previously described (38,40). Each sample was run in duplicate, and the results were normalized to glyceraldehyde 3-phosphate dehydrogenase.

PKG ACTIVITY ANALYSIS. An enzyme immunoassay colorimetric assay (CycLex, MBL International Corporation, Woburn, Massachusetts) was performed to

evaluate PKG activity as previously described. Proteins were extracted from whole heart samples according to the previous description (2).

HISTOLOGY. Heart samples were fixed with 10% formalin and embedded in paraffin. Samples were sliced into 4- to 5-mm slices. Sections were stained with Picrosirius red for interstitial fibrosis and fast-green for counterstaining. The LV short axis at the levels of papillary muscle was analyzed. Cardiomyocyte cell size was measured using wheat germ agglutinin (Biotin Conjugated Triticum vulgare Lectin, EY Laboratories, Inc., San Mateo, California) stained sections. Forty to 50 cells per slide were measured, and the average value in each sample was calculated.

ANALYSES OF METABOLIC STATUS. To assess the metabolic status of $KRR^{KI/KI}$ mice after pressure overload and cGMP pathway stimulation, the oxygen consumption rate (VO_2) and locomotor activities were measured using an oxygen/carbon dioxide metabolic measurement system (MK-5000, Muromachi, Chuoku, Tokyo, Japan), as previously described (41). The VO_2 was normalized to body weight.

STATISTICAL ANALYSIS. All data are presented as mean \pm SEM. D'Agostino and Pearson's normality test was applied for determining whether sample distributions were normally distributed or skewed. If the values were normally distributed, a 1- or 2-way analysis of variance was applied, with Tukey's post hoc method used for pairwise comparisons. The Kruskal-Wallis test with Dunn's post hoc method for pairwise comparisons was done if data were not normally distributed. A p value <0.05 was considered statistically significant. Statistical analyses were performed with GraphPad Prism 7 (GraphPad Software, La Jolla, California).

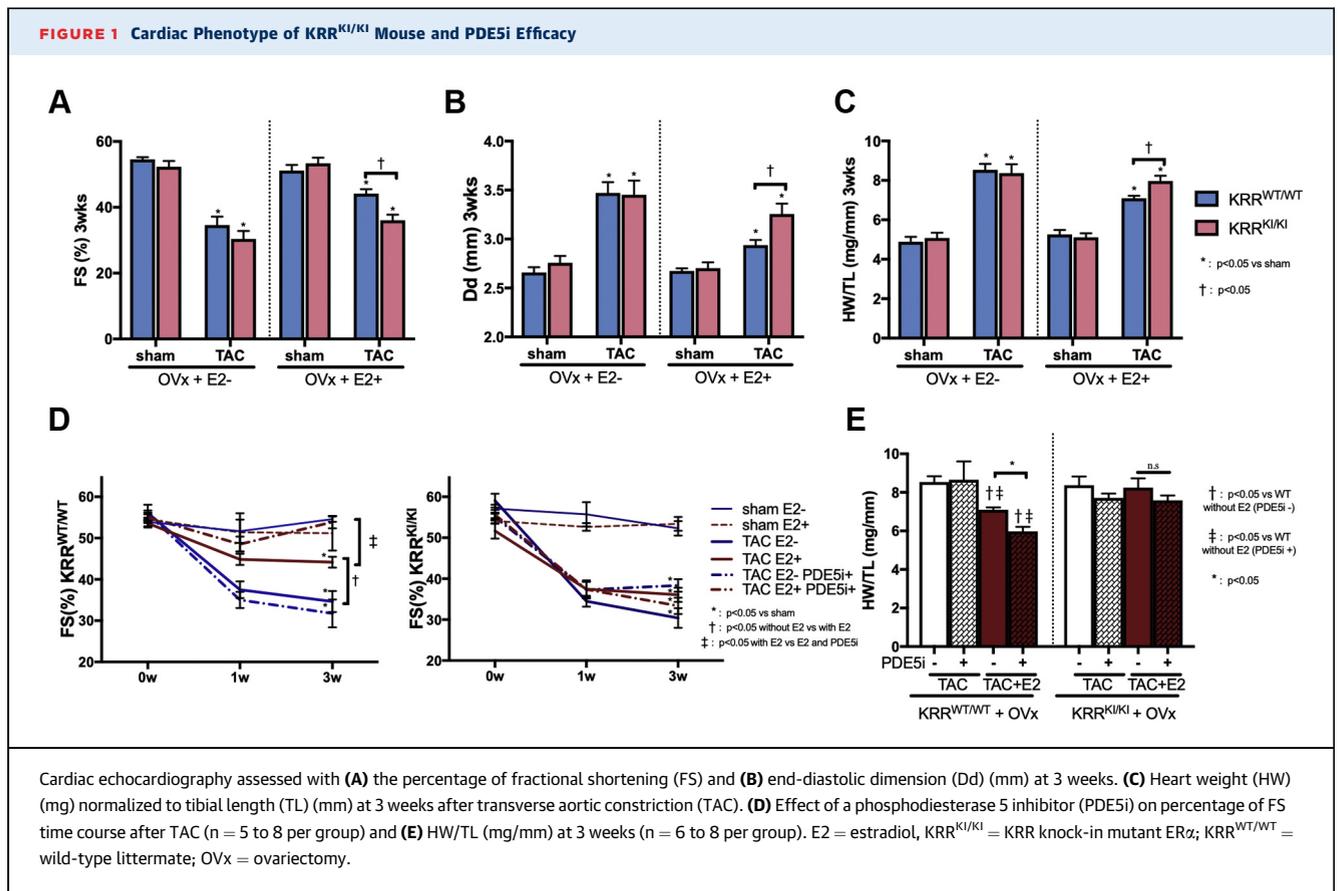
RESULTS

LOSS OF E2-STIMULATED NOS ACTIVATION IN CARDIAC MYOCYTES WITH PRESERVED NUCLEAR ACTIONS IN $KRR^{KI/KI}$ ANIMALS. To confirm loss of non-nuclear actions in $KRR^{KI/KI}$ hearts, we assessed E2-stimulated NOS activity in cardiac myocytes isolated from $KRR^{KI/KI}$ animals. As expected, short-term E2 stimulation failed to activate NOS in $KRR^{KI/KI}$ myocytes, whereas increased NOS activity was found in wild-type myocytes (Supplemental Figure 1), which indicated that the non-nuclear signaling was ablated in cardiac myocytes by the introduction of KRR mutations as reported in endothelial cells (ECs) (30). To exclude the possibility of potential alteration of nuclear actions by introducing the point mutations in the C domain, we examined myocardial expression

levels of estrogen responsive element-containing genes including *Greb1*, *C3*, and *Agt* in the presence or absence of E2 (E2 vs. ovariectomy) (35,42). These genes were significantly up-regulated in response to E2 in $KRR^{KI/KI}$ hearts, similar to $KRR^{WT/WT}$ hearts (Supplemental Figure 2), which suggested that the nuclear actions were preserved in $KRR^{KI/KI}$ hearts. Uterus phenotype was consistently comparable between the genotypes (Supplemental Figures 3A and 3B). Ovariectomy led to uterine atrophy in both genotypes, and E2 replacement induced hypertrophy in $KRR^{KI/KI}$ mice, similar to $KRR^{WT/WT}$ mice.

ER α NON-NUCLEAR SIGNALING PLAYS A PIVOTAL ROLE IN ESTROGEN-CONFERRED CARDIO-PROTECTION AND IS ESSENTIAL TO cGMP-PDE5 BENEFITS. To determine the role of ER α non-nuclear signaling in cardiac remodeling and in the therapeutic efficacy of cGMP-PDE5 inhibition, animals lacking ER α non-nuclear signaling ($KRR^{KI/KI}$) were ovariectomized, supplemented with or without E2, and exposed to 3 weeks of LV pressure overload by TAC surgery.

The effects of concomitant PDE5i treatment were then determined. $KRR^{KI/KI}$ mice showed a baseline cardiac phenotype that was indistinguishable from littermates $KRR^{WT/WT}$ at this age, regardless of estrogen conditions, as assessed with echocardiography and terminal heart weight measurement (Figures 1A to 1C). However, in the presence of E2, $KRR^{KI/KI}$ hearts that underwent TAC revealed lower cardiac function (percent of fractional shortening), a larger LV cavity, end-diastolic dimension (Dd), and more of an increase in heart weight normalized to tibial length (HW/TL) compared with $KRR^{WT/WT}$ mice, whereas both genotypes developed severe and dilative LV remodeling without E2 supplementation (Figures 1A to 1C). This suggested that ER α non-nuclear signaling was necessary for E2-conferred cardioprotection against LV remodeling after pressure overload. To rule out the possibility that these physiological impacts might result from potential differential estrogen regulations between the genotypes, we measured estrogen levels in serum and in the myocardium. The serum estrogen levels were approximately 120 pg/ml with E2 pellet implantation in both genotypes, whereas the removal of ovaries resulted in serum estrogen levels of approximately 10 pg/ml (Supplemental Figures 4A and 4B). The levels achieved with the pellet were in the super-physiological range because 20 to 60 pg/ml is the range of the mouse estrous cycle. However, these levels were consistent with previous studies that used the same dose of estrogen pellets (0.25 mg, 60-day release) to study the effects of estrogen on the



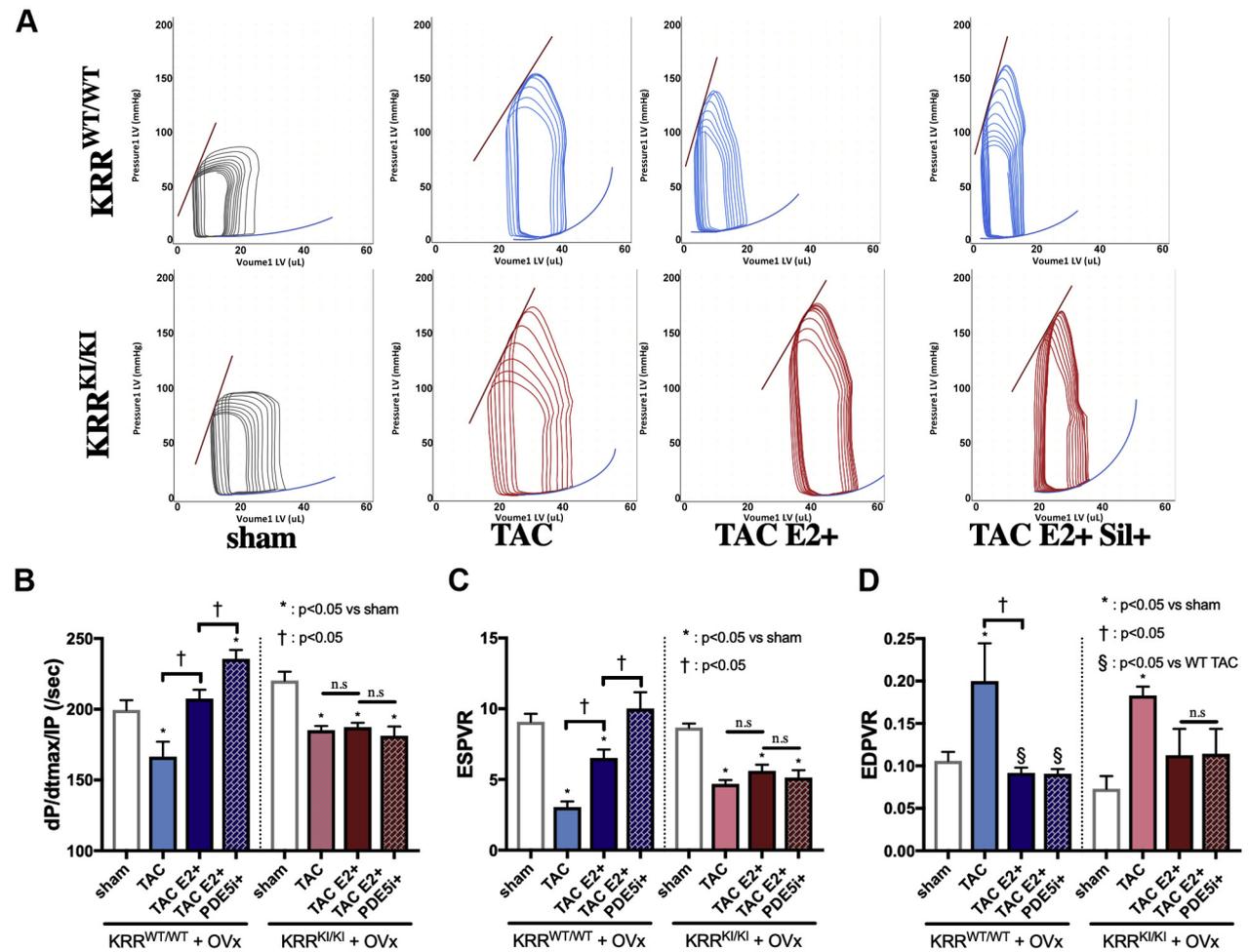
cardiovascular system (35-37,43). TAC did not significantly alter serum estrogen levels. We further determined myocardial estrogen levels, which were approximately 15 pg/100 mg heart in either genotype after ovariectomy, and increased to approximately 40 pg/100 mg heart with estrogen supplementation without any significant change by TAC (Supplemental Figures 4C and 4D).

Serial measurement of the percentage of fractional shortening by echocardiography and terminal heart weight showed that concomitant PDE5i treatment significantly improved global LV systolic function and inhibited a heart weight increase in KRR^{WT/WT} TAC mice with E2 supplementation. However, PDE5i failed to provide these effects without E2, which was consistent with our previous report (10). Importantly, PDE5i no longer had such anti-remodeling effects on KRR^{KI/KI} TAC hearts despite E2 supplementation (Figures 1D and 1E), which suggested the pivotal role of ER α non-nuclear signaling in the therapeutic efficacy of PDE5 inhibition in failing hearts.

In contrast, the cardioprotective effects of PDE5i in KRR^{KI/KI} male mice were not abrogated as assessed with the percentage of fractional shortening and

heart weight (mg/mm) (Supplemental Figures 5A to 5C), which indicated that ER α non-nuclear signaling in males was not essential for cardioprotection provided by cGMP-PDE5 signaling.

CARDIAC FUNCTIONAL ASSESSMENT BY PRESSURE-VOLUME LOOP ANALYSIS. We performed invasive pressure-volume loop analysis to assess LV cardiac functions (Figure 2A). E2 alone ameliorated cardiac systolic function assessed with dP/dt max/IP (dP/dt max normalized to instantaneous pressure), end-systolic pressure volume relationship (ESPVR), and ejection fraction (Figures 2B and 2C, Supplemental Figure 6A), as well as diastolic function assessed with end-diastolic pressure volume relationship (EDPVR) (Figure 2D) in KRR^{WT/WT}. Concomitant PDE5i treatment provided further improvement in systolic function (Figures 2B and 2C). In contrast, in KRR^{KI/KI} mice, neither E2 nor PDE5i improved cardiac performance. Among all groups, heart rate was comparable (approximately 600 beats/min) (Supplemental Figure 6B), and TAC groups showed increased LV afterload (Ea: effective arterial elastance, 8 to 10 mm Hg/ μ l in TAC groups compared with 5 mm Hg/ μ l in sham groups) (Supplemental Figure 6C). In line with echocardiographic data, these

FIGURE 2 Cardiac Functional Assessment by PV Loop Analysis

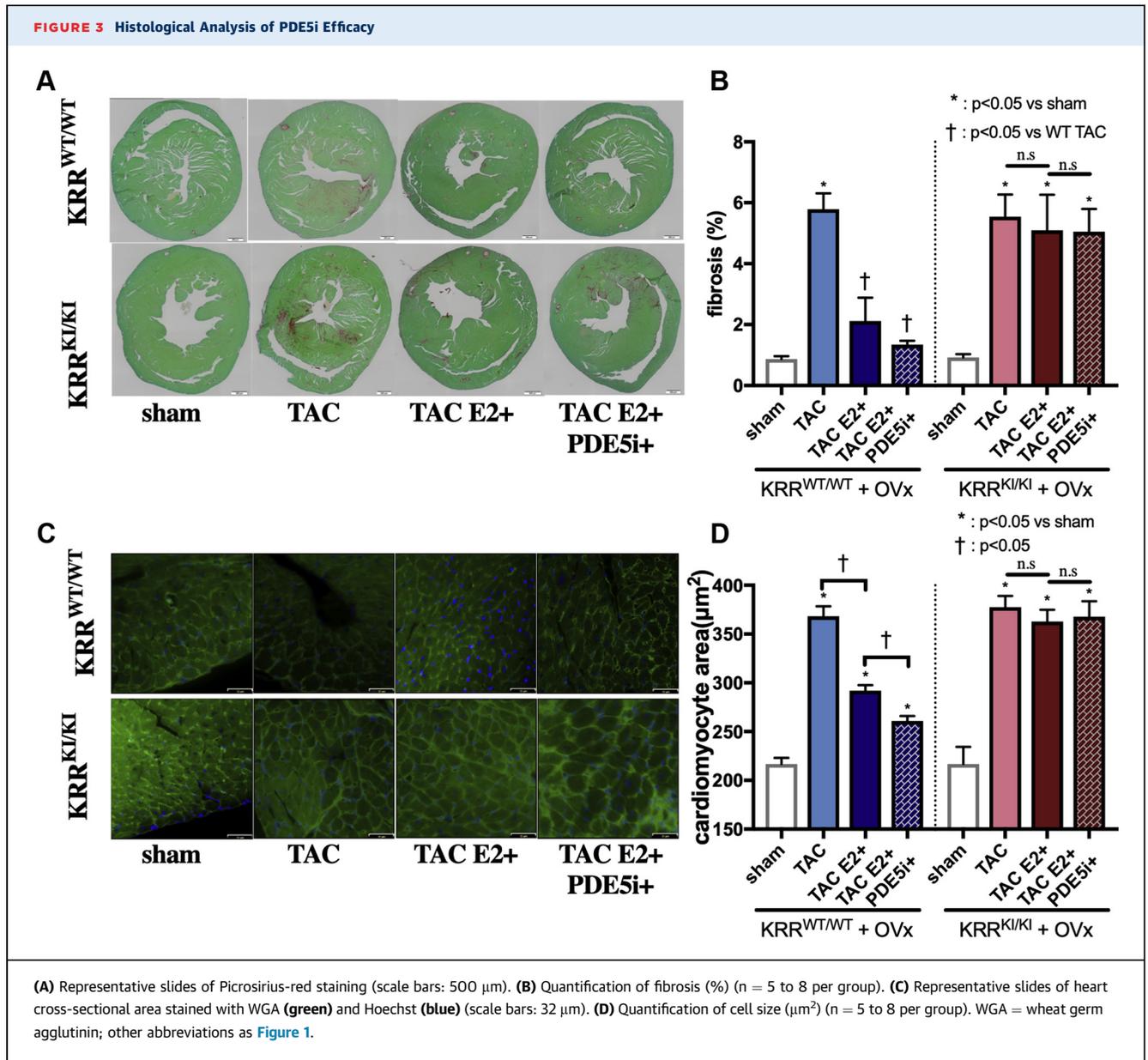
(A) Representative pressure–volume (PV) loops during pre-load reduction. (B) dP/dt max/IP (per second). (C) ESPVR. (D) EDPVR ($n = 6$ to 8 per group). dP/dt max/IP = dP/dt max normalized to instantaneous pressure; EDPVR = end-diastolic pressure volume relationship; ESPVR = end-systolic pressure volume relationship; WT = wild-type. Other abbreviations as Figure 1.

physiological results supported the necessity of ER α non-nuclear signaling in heart failure remodeling and in the efficacy of PDE5i.

HISTOLOGICAL ASSESSMENT REVEALED THE ROLE OF THE ER α NON-NUCLEAR PATHWAY IN CARDIAC REMODELING. We performed histological analyses to assess fibrosis and hypertrophy after TAC surgery. Picosirius red staining revealed that TAC-induced fibrosis was significantly reduced by E2 alone or by PDE5i in the presence of E2 in KRR^{WT/WT} hearts but was unaltered by either treatment in KRR^{KI/KI} hearts (Figures 3A and 3B). Cell size assessment using wheat germ agglutinin (WGA) staining demonstrated E2 alone attenuated cell size increase by TAC, which was

further reduced by PDE5i. Again, neither E2 nor PDE5i with E2 had an impact on cardiomyocyte size in KRR^{KI/KI} TAC hearts (Figures 3C and 3D).

E2 AND PDE5i FAILED TO ACTIVATE MYOCARDIAL PKG WITHOUT THE MEMBRANE-INITIATED ER α NON-NUCLEAR PATHWAY. We examined molecular marker genes for hypertrophy (*Nppb*) and fibrosis (*CTGF*) in these hearts. Although TAC-induced *Nppb* (brain natriuretic peptide (BNP)) upregulation was attenuated by E2 alone and was further inhibited by PDE5i + E2 in KRR^{WT/WT} hearts, *Nppb* induction was not inhibited by either in KRR^{KI/KI} hearts (Figure 4A). The *CTGF* induction by TAC was also inhibited by E2 or PDE5i + E2 in KRR^{WT/WT} hearts. Interestingly, in

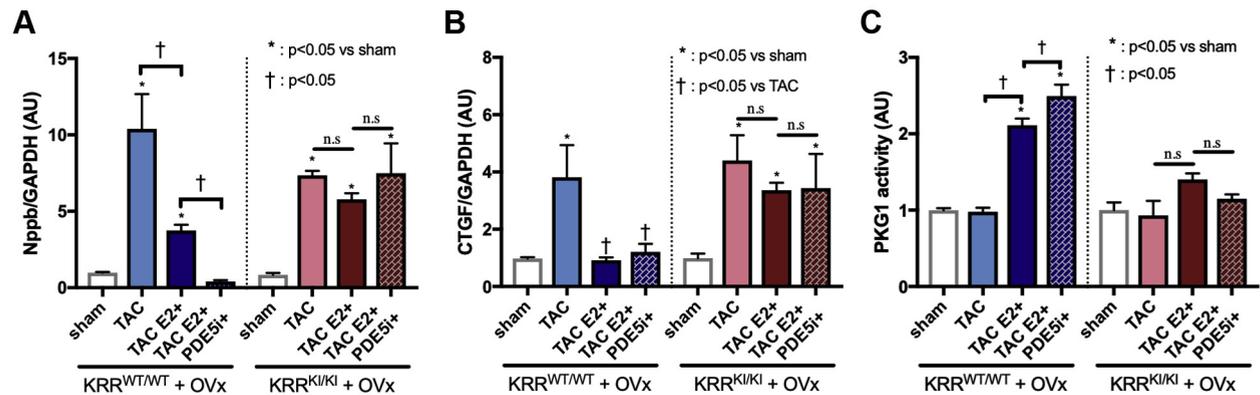


KRR^{KI/KI} hearts, CTGF induction by TAC was significantly attenuated by E2 alone, with no additional effects from PDE5i (Figure 4B), which suggested that estrogen's genomic effects might contribute to the regulation of CTGF.

To assess the impact of the non-nuclear signaling of estrogen signaling on the cGMP signaling pathway, we next determined myocardial PKG activity. Importantly, myocardial PKG activity remained at baseline levels with E2 or with E2 + PDE5i in KRR^{KI/KI} hearts, whereas PKG was activated by E2 alone and further augmented by co-treatment with PDE5i in KRR^{WT/WT} mice (Figure 4C). These results indicated

that estrogen's non-nuclear pathway via ER α critically affected myocardial PKG levels and was essential to the PKG activation elicited by PDE5i in female hearts after pressure overload.

SGC STIMULATION AMELIORATES CARDIAC FUNCTION, REGARDLESS OF E2 STATUS. Although PDE5i blocks degradation of cGMP that is coupled to the NO-sGC pathway to activate the cGMP signaling pathway, cGMP production is enhanced independently of NO by sGC stimulators. One of them, vericiguat, is currently being tested in a Phase III clinical study. We tested if the efficacy of sGC stimulation was affected

FIGURE 4 Molecular Marker Genes and PKG1 Activity

Myocardial fetal gene expressions of (A) *Nppb* and (B) *CTGF* (n = 5 to 8 per group). (C) PKG1 activity (n = 5 to 8 per group). PKG = cyclic guanosine monophosphate (cGMP)-dependent protein kinase G. Abbreviations as Figure 1.

by E2 conditions using the same protocol but without E2 supplementation. The sGC stimulator potently ameliorated cardiac remodeling induced by 3-week TAC in mice that underwent ovariectomy in either genotype, as assessed by serial echocardiographic studies assessed with the percentage of fractional shortening FS (%) and Dd (mm) (Figure 5A, Supplemental Figure 7). Terminal heart weight assessment revealed a TAC-induced increase in heart weight was potently inhibited by sGC stimulation without E2 in either genotype (Figure 5B) and improved the expression profiles of *Nppb* and *CTGF* (Supplemental Figures 8A and 8B), as well as histological findings (Supplemental Figures 9A to 9D). Cardiac systolic and diastolic performance (Figures 5C to 5E, Supplemental Figure 10A) was also potently improved by sGC stimulation without altering heart rate or LV afterload (Ea) (Supplemental Figures 10B and 10C). Importantly, the anti-remodeling benefits from sGC stimulator treatment were associated with a marked increase in myocardial PKG activity (Figure 5F). Serum or myocardial levels of estrogen were unaltered by co-treatment of an sGC stimulator (serum E2 concentration mean \pm SEM [pg/ml]: ovariectomized KRR^{WT/WT} TAC and ovariectomized KRR^{WT/WT} TAC + sGC treatment 12.4 ± 4.3 vs. 16.5 ± 7.6 ; p = 0.989, ovariectomized KRR^{KI/KI} TAC vs. ovariectomized KRR^{KI/KI} TAC + sGC treatment 10.0 ± 3.2 vs. 15.1 ± 5.2 ; p = 0.974).

METABOLIC STATUS WAS UNAFFECTED WITH cGMP SIGNALING PATHWAY STIMULATION IN KRR^{WT/WT} AND KRR^{KI/KI} MICE. Because of the reported metabolic phenotype of estrogen's non-nuclear signaling,

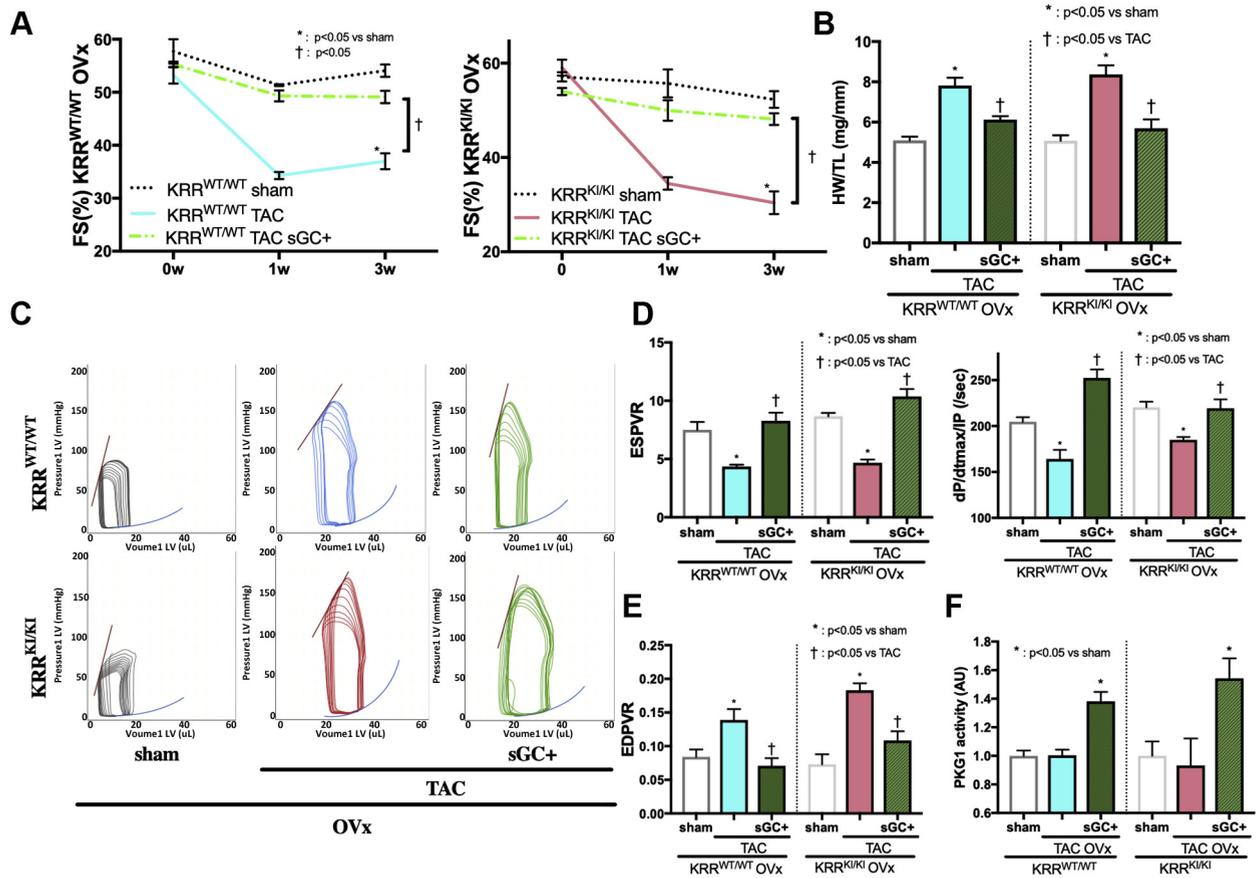
we assessed body weight after TAC (Supplemental Table 1), daytime and night VO₂ (ml/h/kg), and locomotor activities (counts/min) to check whether cGMP pathway stimulation by sGC stimulator affected metabolic status.

KRR^{KI/KI} and KRR^{WT/WT} animals started to show body weight differences at around 8 weeks of age, when reaching sexual maturity, and the difference became more evident with age (35). In the present study, we used 8- to 10-week old animals to induce pressure overload when we observed an approximate 10% nonsignificant borderline increase in body weight in KRR^{KI/KI} mice compared with KRR^{WT/WT} mice (Supplemental Table 1). KRR^{KI/KI} mice showed lower locomotor activities at night and lower VO₂ at daytime than KRR^{WT/WT} mice at baseline. In both genotypes, TAC groups showed significantly lower VO₂ and locomotor activities, but sGC stimulation did not change either parameter despite the improvement of heart failure phenotype (Supplemental Figures 11 and 12). These results suggested that enhancing cGMP pathways with a sGC stimulator improved cardiac remodeling during pressure overload without affecting locomotor activities or VO₂.

DISCUSSION

In the present study, we used a novel genetically engineered mouse model and demonstrated that ER α non-nuclear signaling is a pivotal contributor to the protection against cardiac remodeling conferred by estrogen and is essential to the therapeutic efficacy of cGMP-PDE5i in heart failure but not to that of sGC stimulation (Supplemental Figure 13).

FIGURE 5 Efficacy of sGC Stimulation in Estrogen-Deprived Models



Effect of soluble guanylate cyclase (sGC) stimulation on (A) percentage of FS time course and (B) HW/TL (mg/mm) at 3 weeks (n = 5 to 8 per group). (C) Representative PV loops during pre-load reduction. (D) ESPVR, dP/dt max/IP (per second) and (E) EDPVR (n = 6 to 8 per group). (F) PKG1 activity after sGC stimulator treatment (n = 6 to 8 per group). Abbreviations as in Figures 1 to 3.

Extensive research on estrogen identified estrogen's non-nuclear signaling as a significant contributor to the beneficial effects of estrogen on the cardiovascular system (44). ER α is 1 of the major ERs that mediates estrogen's protective effects against cardiac ischemic injury or vascular injury (45,46); its endothelial non-nuclear action is coupled to the NO-cGMP signaling pathway and/or S-nitrosylation of proteins (27,32,47,48). Previous studies used non-nuclear selective modulators to target this signaling, including EDCs and structurally novel estrogens named PaPEs. Chambliss et al. (29) reported that EDCs activate eNOS via ER α and stimulate EC proliferation and migration, providing vascular benefit (29). Recently, Madak-Erdogan et al. (49) developed PaPEs, which interacted with ERs to activate the non-nuclear pathway preferentially over the nuclear pathway and demonstrated the repair of vascular

endothelium following carotid artery injury by PaPEs. Such effects on the vasculature by the non-nuclear pathway was also demonstrated by a study that used a genetically engineered mouse model that had a point mutation of the palmitoylation site of the ER α (C451A-ER α), and therefore, lacked non-nuclear signaling (26). Importantly, a recent work by Guivarc'h et al. (31) used PaPEs and C451A-ER α mice and demonstrated that chronic vasculo-protection of estrogen against hypertension or atherosclerosis was attributable to nuclear actions of estrogen rather than non-nuclear actions. These results suggested the exquisite balance of complexity for the nuclear and non-nuclear actions of estrogen, which provides cardiovascular benefits.

This was the first study to demonstrate the protective role of ER α non-nuclear signaling in cardiac function and remodeling to pathological stressors by

using pathway-specific deletion animals. It was reasonable to postulate that myocardial NO-cGMP-PKG might primarily contribute to the benefits from this signaling; however, it remains to be determined which cell type is the key to the estrogen-elicited NO-cGMP-PKG in the heart. Our previous work demonstrated that short-term E2 application to isolated cardiomyocytes induced a cGMP increase in cardiac myocytes (10). In the present study, we found that estrogen-stimulated NOS activity was absent in cardiomyocytes of $KRR^{KI/KI}$ mutants. These findings supported the potential role of cardiomyocyte non-nuclear ER α signaling in this regulation. In contrast, a work by Menazza et al. (47) supported the role of endothelial ER α non-nuclear function. Using tissue-specific ER α deletion models, they observed that EDCs ameliorated ischemia-reperfusion injury in cardiac-specific, but not in endothelial-specific, deletion models, which could involve protein S-nitrosylation. It was also possible that ER α non-nuclear signaling of other diverse cell types might be involved in the observed cardiac benefits other than cardiac myocytes and ECs. Our previous work showed that $KRR^{KI/KI}$ mutants gained more body weight and were metabolically deranged when growing old, via a brain-mediated mechanism (35). Lu et al. (32) showed that ER α non-nuclear signaling regulated various genes involved in cell migration, proliferation, and regulation of inflammatory cells (32). All these cell types could have significant impact on cardiac remodeling.

We previously demonstrated that the therapeutic efficacy of PDE5i is estrogen-dependent in female hearts (10). The present study further unraveled the specific role of ER α non-nuclear signaling in this regulation. PDE5i-elicited myocardial PKG activity in females required intact ER α non-nuclear signaling. Importantly, our data revealed myocardial PKG activity was increased by estrogen alone in an ER α non-nuclear pathway-dependent fashion that was associated with anti-remodeling benefits. These results suggested that cGMP-PKG coupled to ER α non-nuclear signaling might play a role in the cardiac benefits from estrogen in female hearts. In contrast, $KRR^{KI/KI}$ male hearts showed similar LV remodeling to $KRR^{WT/WT}$ male hearts. More importantly, the cardioprotective effects of PDE5i were not abrogated in $KRR^{KI/KI}$ male hearts. These results suggested that ER α non-nuclear signaling hearts might not play a significant role in LV remodeling in males and was dispensable for the anti-remodeling efficacy of PDE5i, in sharp contrast to female hearts. The mechanism for this difference between males and females remains an open question and warrants future studies.

However, we speculate that androgens might function in male hearts, as do estrogens in female hearts. For example, a previous study by Sieveking et al. (50) revealed that androgens played an important role in angiogenesis under ischemic stress of hind limbs in male mice but not in female mice. Yoshida et al. (51) reported that androgen signaling activated the Akt/eNOS signaling pathway.

As a translational insight, our data could partially explain the negative results of clinical studies of PDE5i, in which significant numbers of older adult women were enrolled. It was likely that impaired cGMP production in low-estrogen conditions in older adult women might have critically limited the therapeutic efficacy of PDE5i. Because estrogen's non-nuclear signaling pathway does not reportedly induce endometrial carcinoma cell growth or proliferation of breast cancer cells (29), a pathway-selective future hormone therapy could make PDE5i an attractive treatment option for heart failure in women. Other PDE families could also work for PKG activation with non-nuclear signaling. Inhibition of PDE9 was also demonstrated as one of the key regulators for cGMP signaling, independent of NOS activity (52). Activating estrogen non-nuclear signaling and PDE9 inhibition might work simultaneously with additive PKG activation in female hearts.

Thus far, the effects of the sGC stimulator were mainly examined in male animal models. In the present study, we tested the efficacy of sGC stimulation for the first time in female animals and found that sGC stimulation potentially ameliorated female ovariectomized heart failure without estrogen supplementation, in sharp contrast to PDE5i. The difference was attributable to their NO dependency for cGMP elevating function, wherein sGC stimulation did not require NO to activate cGMP-PKG. In a SOCRATES-REDUCED (Phase IIb Safety and Efficacy Study of Four Dose Regimens of BAY1021189 in Patients With Heart Failure With Reduced Ejection Fraction Suffering From Worsening Chronic Heart Failure) Phase II study, a sGC stimulator (vericiguat) was administered to 456 patients with heart failure reduced ejection fraction (<45%) for 12 weeks, and the promising results were obtained with high-dose groups, including better composite endpoints and improved N-terminal pro-B-type natriuretic peptide (53). Currently, the international Phase III VICTORIA (Vericiguat Global Study in Subjects With Heart Failure With Reduced Ejection Fraction) trial is ongoing to test the efficacy of high doses (11). Our findings had important clinical implications, indicating that the sGC stimulator provided potent cardioprotective benefits in post-menopausal patients.

STUDY LIMITATIONS. First, direct enzyme immunoassay did not provide accurate measurement, particularly at the lower concentration range; therefore, it might not be reliable for measuring estrogen levels in mice, considering the lower levels of estrogen in this species versus humans (54). The limited specificity of enzyme immunoassays also might be due to the nonspecific binding of C-reactive protein factors and another “ill-defined matrix” (55). However, enzyme immunoassays have been used in many rodent studies, including the recent one by Iorga et al. (39), due to its simple straight-forwardness. However, an ideal method is mass spectrometry, which requires a larger amount of specimens and a group of experts devoted to measuring estrogen levels. The numbers we obtained were in line with previous publications that used enzyme immunoassays. Most importantly, our conclusions were not affected by these measurement results by design because all the animals underwent ovariectomy and were supplemented with or without exogenous E2.

Second, we did not examine whether all the nuclear signaling pathways were intact in $KRR^{KI/KI}$ mice. Because of uterus morphology and its response to estrogen, as well as several estrogen response element (ERE)-containing gene inductions, were preserved, genomic actions were reasonably preserved. However, we could not completely exclude the possibilities of some interference on other genomic actions.

Third, cell type-specific analyses remain to be assessed because we studied a whole body knocked-in mouse model. Because ER α non-nuclear signaling might be different in ECs, cardiomyocytes, and other cell types, future studies using cell type-specific mouse models should clarify the key cell types to this regulation. Because the estrogen signal network is complicated, disruption of ER α non-nuclear signals might affect other ER signaling or genomic pathways. In this context, ER α non-nuclear signaling might cooperate with ER β because of the protective role of

ER β in cardiomyocytes under pathological stress (56). Thus far, estrogen-signaling interactions between subtypes or genomic and non-nuclear signals remain to be clarified.

CONCLUSIONS

The ER α non-nuclear pathway plays a pivotal role in the cardioprotective mechanism by estrogen and is indispensable to the therapeutic efficacy of cGMP-PDE5 inhibition. In contrast, sGC stimulation potentially ameliorates heart failure remodeling, regardless of estrogen status.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE:

Estrogen exerts physiological effects via genomic and non-genomic pathways. The non-nuclear pathway of ER α plays a pivotal role in heart failure remodeling. An intact non-nuclear pathway is required for the therapeutic efficacy of cGMP-PDE5 inhibition in females, whereas estrogen status does not affect the efficacy of cGMP-sGC stimulation.

TRANSLATIONAL OUTLOOK: The study might provide a potential explanation for the negative results of PDE5 inhibition in heart failure and might also provide a molecular basis for the advantage of using a sGC simulator as a new therapeutic option in postmenopausal women. This study also highlighted the need for a female-specific therapeutic strategy.

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APPENDIX For supplemental figures, please see the online version of this paper.