PDE5 inhibitor efficacy is estrogen dependent in female heart disease

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Inhibition of cGMP-specific phosphodiesterase 5 (PDE5) ameliorates pathological cardiac remodeling and has been gaining attention as a potential therapy for heart failure. Despite promising results in males, the efficacy of the PDE5 inhibitor sildenafil in female cardiac pathologies has not been determined and might be affected by estrogen levels, given the hormone's involvement in cGMP synthesis. Here, we determined that the heart-protective effect of sildenafil in female mice depends on the presence of estrogen via a mechanism that involves myocyte eNOS–dependent cGMP synthesis and the cGMP-dependent protein kinase Iα (PKGIα). Sildenafil treatment failed to exert antiremodeling properties in female pathological hearts from Gαq-overexpressing or pressure-overloaded mice after ovary removal; however, estrogen replacement restored the effectiveness of sildenafil in these animals. In females, sildenafil-elicited myocardial PKG activity required estrogen, which stimulated tonic cardiomyocyte cGMP synthesis via an eNOS/soluble guanylate cyclase pathway. In contrast, eNOS activation, cGMP synthesis, and sildenafil efficacy were not estrogen dependent in male hearts. Estrogen and sildenafil had no impact on pressure-overloaded hearts from animals expressing dysfunctional PKGIα, indicating that PKGIα mediates antiremodeling effects. These results support the importance of sex differences in the use of PDE5 inhibitors for treating heart disease and the critical role of estrogen status when these agents are used in females.

Introduction

Heart disease is the major cause of death in women as well as in men in developed countries. However, there are sex-specific clinical characteristics, some of which may be dependent on levels of the sex hormone estrogen. Heart failure in women occurs at an older age and responds better or worse to various treatments as compared with responses in men (1, 2). Considerable efforts have been made to establish guidelines for the prevention of cardiovascular disease in women (3, 4), but treatment strategies targeted at female heart disease remain to be established. cGMP-specific phosphodiesterase 5 (PDE5) inhibitors have provided beneficial cardioprotection against a broad range of heart diseases in experimental and clinical studies since our first report (5–11) and are expected to be new treatment options for heart failure. Their pharmacological actions arise via the blockade of cGMP hydrolysis and consequent cGMP-dependent kinase (PKG) activation. Importantly, lines of evidence from studies of the vasculature suggest that estrogen signaling and the NO/cGMP synthetic pathway are linked to this hormone's vasculoprotective effects (12–15). We hypothesized that the female response to PDE5 inhibitors in cardiac disease may be altered by the presence or absence of estrogen.

The present study demonstrates that the PDE5 inhibitor sildenafil ameliorates female cardiac pathologies caused by Gαq overexpression or pressure overload in an estrogen-dependent manner.

Female hearts maintain constitutive activation of eNOS by estrogen, providing a tonic synthesis of cGMP, which is targeted by PDE5, whereas male hearts show stress-responsive activation of eNOS. These results indicate that the levels of estrogen critically impact the efficacy of a PDE5 inhibitor on female heart disease, highlighting the potential need for sex-specific consideration in the use of PDE5 inhibitors in heart failure. Because there are large clinical trials testing the efficacy of a PDE5 inhibitor in patients with heart failure, our findings may have important clinical implications.

Results

Estrogen dependence of sildenafil efficacy in female failing hearts (Gαq overexpressors). Gq-coupled receptor activation is a key contributor to various cardiac pathologies and a critical target for the cGMP signaling that underlies the antihypertrophy and remodeling effects of PDE5 inhibition. To examine the impact of estrogen levels on cardiac disease modulation induced by the PDE5 inhibitor sildenafil, mice with cardiac Gαq overexpression (Gq/oe), with or without surgical ovariectomy (OVX), were treated with sildenafil for 2 weeks (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI70731DS1). The Gαq overexpressor develops heart failure with slight hypertrophy as early as 3 to 4 weeks after birth (16). We found that OVX reduced the efficacy of sildenafil with regard to improving cardiac function as compared with controls (Figure 1, A and B). In non-OVX females, sildenafil ameliorated cardiac dysfunction more than in males (Supplemental Figure 2). Exogenous supplementation of estrogen (estradiol,
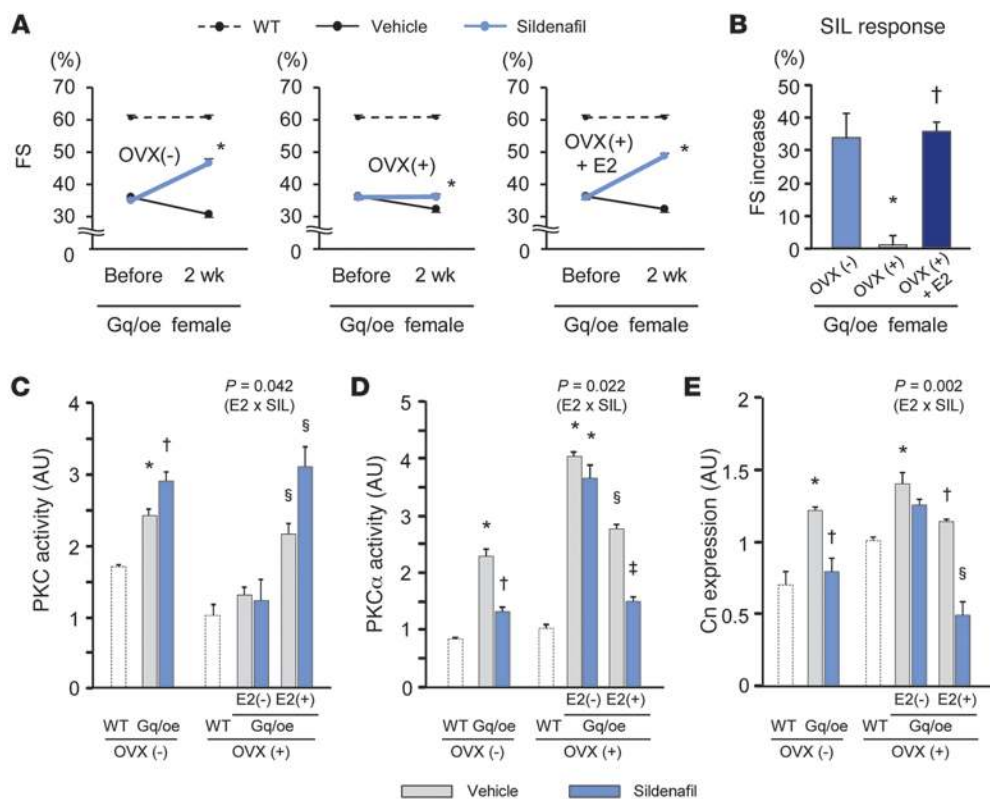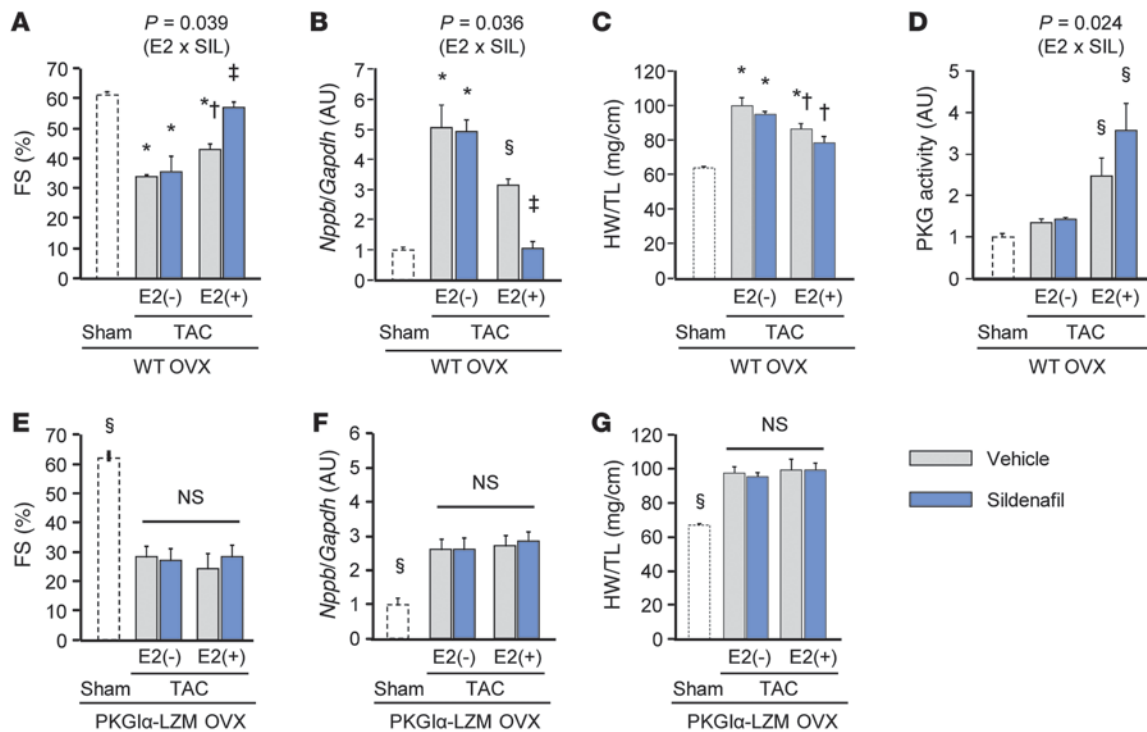
Figure 1

Estrogen dependence of sildenafil efficacy in female failing hearts (Gq overexpressors). (**A**) FS echocardiographic changes before and after 2 weeks of vehicle or sildenafil treatment in wild-type and Gq-overexpressing (Gq/oe) female mice subjected to sham operation [non-OVX, OVX(–)], OVX [OVX(+)], or OVX with estrogen replacement [OVX(+) + E2] (*n* = 7–8 per group). *P < 0.05 versus the 2-week vehicle treatment group. (**B**) Summary data for FS increased after 2 weeks of sildenafil (SIL) treatment (*n* = 7–8 per group). *P < 0.05 versus non-OVX group; †P < 0.05 versus OVX group. (**C**) Myocardial PKG activity. (**D**) PKCα activity. (**E**) Calcineurin (Cn) protein expression was normalized to GAPDH (*n* = 4–7 per group). *P < 0.05 versus the wild-type groups in non-OVX or OVX mice; †P < 0.05 versus the vehicle-without-E2 groups in non-OVX or OVX Gq/oe mice; ‡P < 0.05 versus the other groups among OVX Gq/oe mice; §P < 0.05 versus the other groups among OVX mice. *P* values shown are for interactions between E2 and SIL treatments (E2 × SIL); 2-way ANOVA.

E2) in OVX animals restored the efficacy of sildenafil (Figure 1, A and B, and Supplemental Figure 3A). The slight rise in cardiac hypertrophy in Gq/oe was itself normalized by E2 supplementation alone (Supplemental Figure 3B). A fetal gene marker of cardiac failure (BNP expression) as well as dysregulation of calcium-handling proteins (phospholamban phosphorylation and SERCA2a expression) (16, 17) were significantly improved by sildenafil in OVX mice with E2 rescue, but were unaffected in the absence of E2 (Supplemental Figure 3, C–E). Importantly, we found that OVX animals with E2 rescue as well as non-OVX animals showed very similar baseline characteristics and sildenafil responses, confirming that the appropriate dosage of exogenous E2 was used. These results suggest that the presence of estrogen critically impacts the response to PDE5 inhibition. We obtained similar results by more comprehensive analysis of cardiac function using pressure-volume (PV) analysis (Supplemental Figure 4). Sildenafil failed to increase myocardial PKG activity in OVX Gq/oe mice, but this activity was increased when E2 was exogenously provided (Figure 1C). This finding correlated with disparities in the repression of Gq signaling. We found that PKCα and calcineurin, both contributors to depressed cardiac performance (18) and maladaptive cardiac hypertrophy and remodeling (19), were markedly deactivated by sildena-

fil in OVX mice receiving E2, but were little impacted in OVX-only mice (Figure 1, D and E, and Supplemental Figure 3F).

Estrogen dependence of sildenafil efficacy in female pressure-overloaded hearts and the essential role of PKGIα. We next extended this paradigm to a more general disease model using 2 weeks of pressure overload induced by transverse aortic constriction (TAC) (Supplemental Figure 5). We found that sildenafil had antihypertrophic/remodeling effects in OVX mice receiving E2, but it had no effect in OVX mice without E2 supplementation (Figure 2, A and B). Hypertrophy following TAC was reduced by E2 itself and by E2 combined with sildenafil (Figure 2C). We found that myocardial PKG activation was again dependent on the presence of E2 in OVX TAC mice (Figure 2D), and PKCα and calcineurin were deactivated by sildenafil only if E2 was supplemented (Supplemental Figure 6, A and B). The similarity of these data to those in the Gq/oe model supports the idea that estrogen plays a general role in PDE5-cGMP regulation in the stressed heart.

To test whether this regulation was critically dependent on PKGIα, the predominant isoform in myocardium (20), TAC was used in mice with a knock-in mutation of PKGIα (*Prkg1*) harboring disrupted binding to leucine zipper–dependent interacting partners (PKGIα-leucine zipper mutant; PKGIα-LZM) (21, 22). Neither
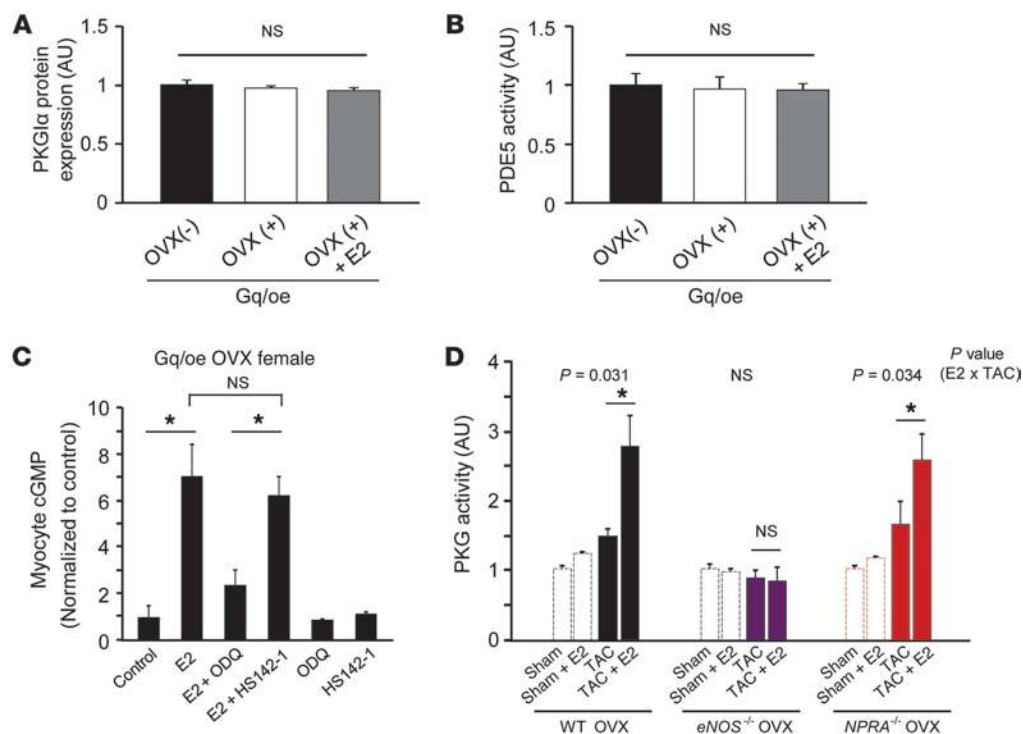
Figure 2
Estrogen dependence of sildenafil efficacy in female pressure-overloaded hearts and the essential role of PKGIα. Sildenafil response in OVX wild-type hearts exposed to 2 weeks of TAC with or without E2 replacement (**A–D**). (**A**) FS. (**B**) Myocardial BNP (*Nppb*) mRNA expression. (**C**) Heart weight (HW) normalized to tibia length (TL). (**D**) Myocardial PKG activity (*n* = 4–8 per group). Sildenafil response in OVX PKGIα-LZM hearts exposed to 2 weeks of TAC with or without E2 replacement (**E–G**). (**E**) FS. (**F**) *Nppb* mRNA expression. (**G**) HW/TL (*n* = 4–9 per group). *P < 0.05 versus the sham group; †P < 0.05 versus the vehicle-without-E2 group; ‡P < 0.05 versus the other groups in TAC; §P < 0.05 versus all other groups; *P* values shown are for interactions between E2 and SIL treatments; 2-way ANOVA.

sildenafil, E2, nor their combination ameliorated the phenotype of PKGIα-LZM OVX hearts exposed to 2 weeks of pressure overload (Figure 2, E–G, and Supplemental Figure 6, C and D), indicating that changes in protein binding–dependent PKGIα signaling are required to achieve the beneficial effects of E2 and sildenafil.

cGMP production by estrogen coupled to the eNOS/sGC pathway as an upstream regulatory mechanism. We next identified the mechanism by which estrogen altered PKG activity responses to sildenafil. Supplementation with E2 did not alter myocardial PKGIα protein expression (Figure 3A) or PDE5 activity (Figure 3B), suggesting that the regulatory system downstream of cGMP was likely unaltered. However, cGMP synthesis was markedly stimulated in response to E2 in the presence of PDE5 inhibition in cardiac myocytes from OVX Gq/oe hearts (Figure 3C). This increase was inhibited by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a soluble guanylate cyclase (sGC) inhibitor, but not by the natri-uretic peptide receptor A inhibitor HS142-1, suggesting func-tional coupling of E2 to NOS/sGC signaling. Consistent with this conclusion, we found that E2 supplementation in vivo failed to increase myocardial PKG activity in OVX hearts lacking eNOS (23) under pressure overload, while it increased PKG activity in OVX hearts lacking atrial natriuretic peptide (ANP) receptor (*Npra*^{-/-}) (24), similar to what was observed in wild-type hearts (Figure 3D). These results indicate that estrogen/eNOS signaling in cardiac myocytes from female hearts provides tonic cGMP synthesis whose hydrolysis is targeted by PDE5.

Gender differences in sildenafil responses and the mechanism involv-ing eNOS-cGMP synthesis and PKG activation under cardiac stress. Our previous work demonstrated that sildenafil ameliorated TAC-in-duced hypertrophic remodeling in male hearts (5–7), which have low levels of estrogen. Therefore, we tested whether male hearts were equipped with a PDE5-coupled cGMP regulatory system that is less estrogen dependent. In isolated male Gq/oe cardiac myocytes, E2 stimulated cGMP synthesis via the eNOS/sGC pathway (Figure 4A), as was observed in the female coun-terparts. Administration of E2 to male Gq/oe hearts in vivo pro-vided additive benefits to sildenafil treatment in terms of cardiac function (percentage of fractional shortening [FS]), repression of BNP, and deactivation of Gq-related signaling (*Rcan1* and PKCα), while sildenafil alone showed significant effects (Figure 4B and Supplemental Figure 7, A–D). Unlike Gq/oe OVX females, male Gq/oe myocardium had a resting increase in PKG activity com-pared with that seen in controls, and this was further increased by E2 administration as well as by sildenafil in an additive (no interaction) manner (Figure 4C). These changes correlated with molecular profiles (Supplemental Figure 7, B–D). The slight rise in cardiac hypertrophy in male Gq/oe hearts was again normal-ized by E2 administration alone (Supplemental Figure 7E). We observed similar additive beneficial effects of E2 and sildenafil treatments in male pressure-overload models (Supplemental Fig-ure 8). Thus, the primary difference in sildenafil efficacy between OVX females and males derives from the drug's dependence on
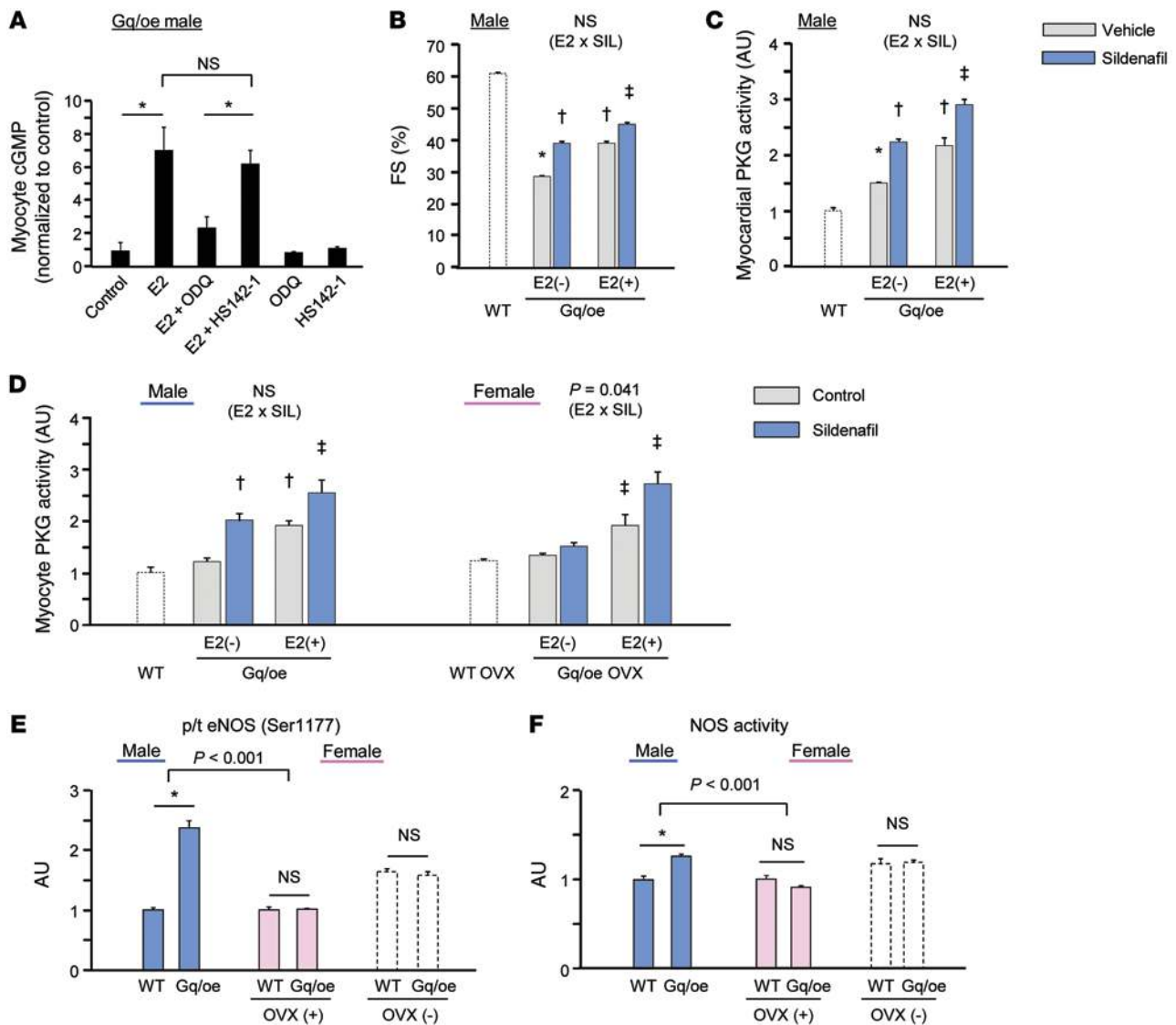
Figure 3

cGMP production by estrogen coupled to the eNOS/sGC pathway as an upstream regulatory mechanism. (**A**) PKGIα protein expression normalized to GAPDH. (**B**) PDE5 activity in Gq/oe female hearts subjected to sham operation (non-OVX), OVX, and OVX with E2 replacement ($n = 5–8$ per group). (**C**) Cardiac myocyte cGMP synthesis in OVX Gq/oe hearts. Isolated cardiac myocytes were treated with E2 (1 nM), E2 plus 10 μM ODQ sGC inhibitor, E2 plus 100 μg/ml HS142-1 (ANP receptor inhibitor), ODQ alone, or HS142-1 alone ($n = 3–4$ per group). (**D**) Myocardial PKG activity in OVX hearts lacking eNOS ($n = 5–6$ per group) or lacking NPRA ($n = 4–5$ per group) exposed to 2 weeks of TAC, with or without E2 replacement, as well as wild-type controls. *$P < 0.05$; P values shown are for interactions between E2 and TAC; 2-way ANOVA.

estrogen, as the estrogen levels themselves are comparable (25). We further examined this difference in isolated cardiomyocytes in acute settings. Male Gq/oe myocytes had no resting increase in PKG activity compared with wild-type control myocytes (likely reflecting the loss of neurohormonal stimulation with cell isolation) and compared with OVX female Gq/oe myocytes, and only male cells displayed a rise in this activity with sildenafil (Figure 4D). Estrogen augmented PKG activity in myocytes from both sexes, and in the presence of E2, both displayed further increases in this activity with sildenafil. These results support the rapid nongenomic action of estrogen in this regulation and also indicate that estrogen-independent cGMP synthesis induction exists in male Gq/oe cardiac myocytes, as PDE5 activity and PKGIα expression in male hearts were comparable to what we observed in female hearts (data not shown). In all, we examined the activation status of eNOS in Gq/oe hearts in male mice and in female mice with or without OVX. Importantly, in male hearts, Gq/oe itself induced a significant increase in eNOS phosphorylation at serine 1177, whereas the presence or absence of OVX, but not of Gq/oe, determined the phosphorylation in female hearts (Figure 4E). We found that myocardial NOS activity corresponded with the status of eNOS phosphorylation (Figure 4F). These results demonstrate the significant difference in PDE5-coupled cGMP regulation between the sexes: the eNOS-cGMP synthetic machinery is stress-responsive in male hearts and is tonic and estrogen dependent in female hearts.

Discussion

In the current study, we show that the anticardiac hypertrophy/remodeling efficacy of PDE5 inhibition in the female mouse heart depends on the presence of estrogen. Furthermore, we identified the mechanism underlying this regulation. Estrogen stimulates cGMP synthesis of cardiac myocytes via the eNOS/sGC pathway, serving as a predominant cGMP source that is coupled to PDE5 in female hearts, but not in male hearts. This is the first demonstration to our knowledge that estrogen levels critically determine drug efficacy in female cardiac pathology.

Estrogen's rapid non-nuclear signaling has been demonstrated in endothelial cells to be a key mechanism in this hormone's vasculoprotective effects (13–15). Chambliss et al. showed that estrogen-dendrimer conjugate (EDC), which activates estrogen receptors but remains non-nuclear, stimulates eNOS and vascular endothelial cell migration in vitro and protects against vascular injury in vivo (14). Our study shows that similar rapid signaling of estrogen critically impacted the intracellular cGMP pathway in female cardiac myocytes and thus the physiological response to a PDE5 inhibitor. Furthermore, our data from PKGIα-LZM mice provide in vivo evidence that PKG mediates estrogen's beneficial impact on the heart, which is consistent with previous findings in vitro (26). Interestingly, we found that estrogen's rapid action on cGMP signaling exists in male cardiac myocytes as well. This finding, however, is consistent with the report by Satoh et al. that estrogen reduces oxidative stress and ameliorates the Gq/oe male phenotype, as PKG

Figure 4
Sex differences in sildenafil responses and the mechanism involving eNOS-cGMP synthesis and PKG activation in Gq/oe hearts. (**A**) cGMP synthesis in cardiac myocytes isolated from male Gq/oe hearts, which were treated with 1 nM E2, E2 plus 10 μM ODQ, E2 plus 100 μg/ml HS142-1, ODQ alone, or HS142-1 alone (n = 3–4 per group). *P < 0.05. Sildenafil response as indicated by (**B**) FS and (**C**) myocardial PKG activity in male Gq/oe hearts with or without E2 treatment (n = 5–10 per group). *P < 0.05 versus the wild-type group; †P < 0.05 versus the vehicle-without-E2 group in Gq/oe mice; ‡P < 0.05 versus all other groups. (**D**) Myocyte PKG activity in response to acute sildenafil treatment for 10 minutes in cardiac myocytes isolated from Gq/oe males and OVX females with or without E2 treatment. Data were normalized to wild-type males. n = 5–9 per group. †P < 0.05 versus the vehicle-without-E2 group in Gq/oe mice; ‡P < 0.05 versus all other groups. P values shown are for interactions between E2 and SIL treatments; 2-way ANOVA. (**E**) Sex differences in phosphorylated eNOS at serine 1177/total expression for eNOS (p/t eNOS) and (**F**) NOS activity in response to cardiac Gq overexpression (n = 6–9 per group). *P < 0.05; P values shown are for interactions between sexes and genotypes; 2-way ANOVA.

reportedly reduces oxidative stress via the thioredoxin system (27, 28). In endothelial cells, membrane estrogen receptors are located within caveolae and lipid rafts and are complexed with caveolin-1, Gαi, and striatin, mediating the rapid non-nuclear effects of estrogen (29). The existence of and role for such membrane complexes in cardiac myocytes remain to be determined. We found that this estrogen-coupled cGMP synthesis is indispensable for sildenafil-elicited benefits in female, but not male, hearts. This sex difference could be due to differences in the eNOS activation mechanism: eNOS acti-

vation is induced by Gq signal stress alone in male hearts, but is not induced in the absence of estrogen in female hearts (see schematic diagram in Supplemental Figure 9). Although the precise mechanism by which Gq stress induces eNOS activation in male hearts remains uncertain, we speculate that androgens play a role in this regulation, as suggested by several recent vascular studies. Sieveking et al. reported that androgens play an important role in angiogenesis induction via the VEGF/Akt pathway under ischemic stress in male, but not in female, mice hind limbs (30). Another study by Yoshida

et al. revealed that the androgen receptor associates with the VEGF receptor KDR and PI3 kinase to activate the Akt/eNOS signaling pathway, and this association was augmented by androgen (31). These data support a role for androgen signaling in stress-responsive activation of eNOS, which is coupled to VEGF signaling. Since induction of VEGF by Gq stress, VEGF receptor expression, and androgen receptor expression are all reported in cardiac myocytes (32–36), androgen could mediate stress-responsive eNOS activation by enhancing VEGF signaling in cardiac myocytes as well. Future studies will be needed to elucidate the molecular mechanisms for stress activation of eNOS in males.

Growing data indicate that cGMP signal regulation by PDE5 in the heart has a significant impact on cardiac pathophysiology (5, 6, 37–39). PDE5 expression is induced in human failing hearts (40) and hypertrophied hearts (41), while overexpression of PDE5 in mice hearts indicates maladaptive cardiac remodeling (42, 43). PDE5 inhibitors have shown antiremodeling efficacy in various animal models (5–10) and are currently being tested for application in human heart disease. While a small clinical study showed long-term antiremodeling benefits of sildenafil in patients with systolic heart failure (11), the result was negative from a recent large, placebo-controlled, multicenter trial of sildenafil for the treatment of heart failure with a preserved ejection fraction (RELAX) (44). Our data suggest that the dependence of the sildenafil response on estrogen may have contributed to the negative results in the latter study, in which older individuals (median age 69 years) were examined, nearly half of whom were women. Importantly, our study reveals that exogenous estrogen restores a blunted sildenafil response. A similar synergy may exist and be of benefit in human heart disease as well, though hormone replacement therapy alone failed to provide this due to the complexity of estrogen regulation (45). A selective estrogen receptor modulator such as EDC, which selectively activates the non-nuclear effects of estrogen without increasing the risk of uterine or breast cancer, may prove to be an ideal candidate for testing this hypothesis.

In summary, our study indicates that female-specific treatment strategies may be required to achieve cardiac benefits from cGMP-enhancing therapies such as PDE5 inhibitors and further highlights the sex difference in cardiac pathophysiology.

Methods

Mice. Gq/oe mice were a gift from Gerald W. Dorn II (Washington University Center for Pharmacogenomics, St. Louis, Missouri, USA). PKGIα-LZM mice on a C57BL/6 background were previously reported (21, 22). Littermate wild-type mice were used as controls. eNOS-deficient mice on a C57BL/6 background and NPRA-deficient mice on a C57BL/6 background were obtained from The Jackson Laboratory.

OVX. Female animals underwent OVX at 6 to 10 weeks of age according to a standard protocol (46, 47). Briefly, anesthesia was induced by inhalation of isoflurane (1%–2%), which was supplemented by intraperitoneal injection of etomidate (5–10 mg/kg). Small dorsal incisions were made in the skin and peritoneum on each side, just above the ovaries. Each ovary was then taken from the cavity by pulling on the periovarian fat and was removed by cauterization through the distal uterine tube.

E2β-estradiol administration. 17β-estradiol (E2) was replaced by subcutaneous implantation of a 60-day release pellet containing 0.25 mg of E2 (Innovative Research of America) into female animals 1 week after OVX. The dose produces physiologically relevant concentrations of circulating E2 (46–48). E2 was administered to male animals in the same way.

Sildenafil administration. Sildenafil citrate (Viagra; Pfizer) was compressed into soft rodent chow (Transgenic Dough Diet; Bio-Serv) and was orally provided at a dose of 100 mg/kg/day for 2 weeks as described previously (5, 6).

TAC. Pressure overload was induced by TAC in female ovariectomized PKGIα-LZM mice, eNOS-deficient mice, NPRA-deficient mice, and their wild-type littermates and in male wild-type C57BL/6 mice, as reported previously (5, 6). Briefly, animals were anesthetized with isoflurane (2%–3%), intubated, and mechanically ventilated. The transverse aorta was constricted with a 26-gauge needle using 7-0 prolene suture, after which the chest was closed, and the animal was allowed to recover from anesthesia. The control mice were subjected to sham operations.

Echocardiography. In vivo cardiac morphology was assessed by transthoracic echocardiography (Acuson Sequoia C256, 13-MHz transducer; Siemens Medical Solutions) in nonanesthetized mice. M-mode left ventricular (LV) end-systolic and end-diastolic dimensions were averaged from 3 to 5 beats. LV percentage of FS and mass were calculated as previously described (5, 6). The studies and analysis were performed by investigators blinded to the genotype and heart condition.

In vivo hemodynamics. In vivo LV function was assessed by PV analysis in anesthetized mice as previously described (5, 6). Briefly, mice were anesthetized, intubated, and mechanically ventilated. The LV apex was exposed through an incision between the seventh and eighth ribs, and a 1.4-French PV catheter (SPR-839; Millar Instruments) was advanced through the apex. The absolute volume was calibrated, and PV data were assessed at steady state and during preload reduction. Data were digitized at 2 kHz and analyzed using custom software.

RNA and protein analysis. Total RNA was extracted from mice LV myocardium using an RNeasy Mini Kit (QIAGEN) and then reverse transcribed into cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Life Technologies). mRNA was analyzed by quantitative real-time PCR using either the TaqMan or SYBR green method. Real-time PCR reactions were performed, recorded, and analyzed using ABI PRISM 7900 (Applied Biosystems). *Rcan1*, *Atp2a2*, and *Gapdh* were assessed using a specific primer probe from Applied Biosystems. *Nppb* was assessed using SYBR green primers as previously described (7, 49).

Lysates were prepared from snap-frozen heart tissue with lysis buffer (Cell signaling Technology) and then subjected to gel electrophoresis and Western blot analysis as previously described (6). Primary antibodies included pan-calcineurin A, PKCα, serine 239 phospho-vasodilator-stimulated phosphoprotein (phospho-VASP), VASP, serine 1177 phospho-eNOS, eNOS, GAPDH (Cell Signaling Technology), PKGIα (N-16; Santa Cruz Biotechnology Inc.), caveolin-3 (BD Transduction Laboratories), serine 16 phospho-phospholamban (Badrilla), and phospholamban (Thermo Scientific).

Adult mouse cardiac myocyte isolation and cGMP measurement. Cardiac myocytes were isolated from Gq/oe hearts as previously described (49). Briefly, the heart was quickly excised and retroperfused through the aorta with the buffer (130 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 22 mM glucose) added to 1 mg/ml collagenase type 2 (Worthington Biochemical) and 0.05 mg/ml protease (Sigma-Aldrich). The myocytes were suspended in the buffer with increasing Ca^{2+} (final concentration of 1 mM) and incubated for 10 minutes with 500 μmol/l isobutylmethylxanthine (IBMX; Sigma-Aldrich) to avoid cGMP hydrolysis. Myocytes were then exposed to 1 nM of E2 (Sigma-Aldrich), E2 plus 10 μM of ODQ (Sigma-Aldrich), or E2 plus 100 μg/ml of HS142-1 (Kyowa Hakko) at room temperature. After 5 minutes, the cells were lysed for cGMP measurement using an enzyme immunoassay (EIA) kit (GE Healthcare) as previously described (5, 43).

PDE5A and PKG activity. PDE5 activity was assessed by fluorescence polarization assay (Molecular Devices) according to the manufacturer's instructions (5, 43). Myocardial PKG activity was assessed by VASP phosphorylation and/or EIA colorimetric assay (CycLex) as previously described

(5, 6, 43). Myocyte PKG activity was assayed in cardiac myocytes isolated from Gq/oe or wild-type hearts, which were incubated with E2 (1 nM), sildenafil (1 μM), or a combination of the two. After a 10-minute incubation, the cells were lysed, and PKG activity was determined.

PKCα activity. Membrane fractions of the LV myocardium were extracted using the ProteoExtract Subcellular Proteome Extraction Kit (EMD Biosciences), and PKCα activity was assessed by the translocation of PKCα to the membrane, expressed as PKCα/caveolin-3 protein expression as previously described (7).

Radiolabeled NOS activity assay. Ca^{2+}-dependent NOS activity was determined from myocardial homogenates by measuring [^{3}H]-L-arginine to [^{3}H]-L-citrulline conversion (Cayman Chemical) as previously described (23, 50, 51).

Statistics. All values were expressed as the mean ± SEM. Group data were compared using 1- or 2-way ANOVA, followed by a Holm-Sidak or Student-Newman-Keuls multiple comparisons test for between-group differences. A *P* value less than 0.05 was considered significant. Sample sizes and individual statistical results for the analyses are provided in the Figures and Supplemental Figures.

Study approval. All animal protocols were approved by the Animal Care and Use Committee of Johns Hopkins University.

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