

Phosphodiesterase 5 Associates With β 2 Adrenergic Receptor to Modulate Cardiac Function in Type 2 Diabetic Hearts

Toni M. West, PhD; Qingtong Wang, MD, PhD; Bingqing Deng, MD; Yu Zhang, PhD; Federica Barbagallo, PhD; Gopireddy R. Reddy, PhD; Dana Chen, BS; Kyle S. Phan, BS; Bing Xu, BS; Andres Isidori, MD, PhD; Yang K. Xiang, PhD

Background—In murine heart failure models and in humans with diabetic-related heart hypertrophy, inhibition of phosphodiesterase 5 (PDE5) by sildenafil improves cardiac outcomes. However, the mechanism by which sildenafil improves cardiac function is unclear. We have observed a relationship between PDE5 and β 2 adrenergic receptor (β 2AR), which is characterized here as a novel mechanistic axis by which sildenafil improves symptoms of diabetic cardiomyopathy.

Methods and Results—Wild-type and β 2AR knockout mice fed a high fat diet (HFD) were treated with sildenafil, and echocardiogram analysis was performed. Cardiomyocytes were isolated for excitation-contraction (E-C) coupling, fluorescence resonant energy transfer, and proximity ligation assays; while heart tissues were implemented for biochemical and histological analyses. PDE5 selectively associates with β 2AR, but not β 1 adrenergic receptor, and inhibition of PDE5 with sildenafil restores the impaired response to adrenergic stimulation in HFD mice and isolated ventriculomyocytes. Sildenafil enhances β adrenergic receptor (β AR)-stimulated cGMP and cAMP signals in HFD myocytes. Consequently, inhibition of PDE5 leads to protein kinase G-, and to a lesser extent, calcium/calmodulin-dependent kinase II-dependent improvements in adrenergically stimulated E-C coupling. Deletion of β 2AR abolishes sildenafil's effect. Although the PDE5- β 2AR association is not altered in HFD, phosphodiesterase 3 displays an increased association with the β 2AR-PDE5 complex in HFD myocytes.

Conclusions—This study elucidates mechanisms by which the β 2AR-PDE5 axis can be targeted for treating diabetic cardiomyopathy. Inhibition of PDE5 enhances β 2AR stimulation of cGMP and cAMP signals, as well as protein kinase G-dependent E-C coupling in HFD myocytes. (*J Am Heart Assoc.* 2019;8:e012273. DOI: 10.1161/JAHA.119.012273.)

Key Words: cardiac myocyte • cell signaling • diabetic cardiomyopathy • EC coupling • echocardiography • pathophysiology • pharmacology

Patients with diabetes mellitus in the United States are 2 to 4 times more likely to develop heart diseases than patients without diabetes mellitus, with diabetes mellitus placing a patient in a high-risk category for the development of heart failure (HF).¹ Studies suggest that differences in the cause of diabetic-related HF^{2,3} and many of the medications

patients with diabetes mellitus take to manage their condition^{2,4-6} can both contribute to worse HF outcomes. For instance, several publications have shown that patients with diabetes mellitus and HF with preserved ejection fraction (HFpEF) are 70% to 80% more likely to be hospitalized than HFpEF patients without diabetes mellitus.⁷⁻⁹ This highlights the need for development of more effective treatments for diabetic-related HF, with an emphasis on individualized care.

Studies have shown that the phosphodiesterase 5 (PDE5) inhibitor, sildenafil, is effective at reversing symptoms in murine HF models¹⁰⁻¹² and improving cardiac contractility in men with diabetic-related cardiac hypertrophy.^{13,14} However, the RELAX (Phosphodiesterase-5 Inhibition to Improve Clinical Status and Exercise Capacity in Diastolic Heart Failure) trial showed that sildenafil has little effect in the general HF population,¹⁴⁻¹⁶ further begging a critical question: how does PDE5 recover cardiac contractile function in the heart? Earlier studies indicate that inhibition of PDE5 leads to pleiotropic beneficial downstream effects in different animal models of cardiac diseases, including reduced cardiac hypertrophy and altered protein expression,^{11,17} improved cardiac contractile

From the Department of Pharmacology, University of California, Davis School of Medicine, Davis, CA (T.M.W., Q.W., B.D., Y.Z., F.B., G.R.R., D.C., K.S.P., B.X., Y.K.X.); Sun-Yet Sen Memorial hospital, Sun-Yet Sen University, Guangzhou, China (B.D.); College of Pharmacy, Guangzhou Medical University, Guangzhou, China (Y.Z., B.X.); Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy (F.B., A.I.); VA Northern California Health Care System, Mather, CA (Y.K.X.).

Correspondence to: Yang K. Xiang, PhD, Department of Pharmacology, University of California at Davis, 2426 Tupper Hall, One Shields Avenue, Davis, CA 95616. Email: ykxiang@ucdavis.edu

Received February 6, 2019; accepted June 18, 2019.

© 2019 The Authors. Published on behalf of the American Heart Association, Inc., by Wiley. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

Clinical perspective

What Is New?

- Phosphodiesterase 5 is in a protein complex with β_2 adrenergic receptor in cardiomyocytes. Inhibition of phosphodiesterase 5 promotes cGMP in a β_2 adrenergic receptor-dependent fashion, leading to improvements in contractility in mice fed a high fat diet by enhancing cGMP-protein kinase G signaling, as well as phosphodiesterase 3-dependent cAMP signaling.

What Are the Clinical Implications?

- The β_2 adrenergic receptor-phosphodiesterase 5 axis represents a promising therapeutic target for treating diabetic-related heart hypertrophy.

function via increased myofilament-mediated relaxation,¹² improved mitochondrial function and reduced oxidative stress,^{18–21} and improved anti-inflammatory and antiapoptotic responses during cardiac ischemia.¹⁹ In models using streptozotocin or a high fat diet (HFD) to induce diabetes mellitus or the related cardiomyopathy, the PDE5 inhibitor vardenafil improves heart function through nitric oxide synthase-cGMP-PKG (protein kinase G) activation.^{22,23} Meanwhile, PDE5 has been linked to the soluble guanylyl cyclase pathway, whereas PDE9 is linked to the particulate guanylyl cyclase pathway for differential regulation of local cGMP signaling induced by different neurohormonal stimuli.²⁴ Still, it is unclear how PDE5-dependent regulation of cGMP can be influenced by elevated neurohormonal stimulation in diabetic hearts, which may offer new strategies to effectively target a subpopulation of patients.

Previous work has shown that PDE5 inhibition leads to changes in adrenergic modulation in the heart,²⁵ in which β_3 adrenergic receptor (β_3 AR) has been linked to the effects of inhibition of PDE5, leading to a negative inotropic response through PKG phosphorylation of troponin I in mouse hearts.²⁶ Recent studies indicate that PDE5 is also linked to cardiac β_2 adrenergic signaling, which negatively modulates chronotropy in neonatal and atrial nodal cells.²⁷ Meanwhile, we have recently shown that β_2 adrenergic receptor (β_2 AR) plays an important role in E-C coupling during diabetic-related cardiomyopathy, with β_2 AR knockout (β_2 KO) being cardioprotective.^{23,28} This is due to β_2 AR complexing with insulin receptor,²⁹ which leads to transactivation of β_2 AR.^{28,30} Concurrently, diabetic cardiomyocytes have an increased expression in phosphodiesterase, which is correlated with a decrease in PKA (protein kinase A) activity.²⁸ We speculate a potential connection between cardiac adrenergic signaling and PDE5 that may be relevant to diabetic cardiomyopathy, including a reduced cardiac inotropy. Moreover, the interaction between different phosphodiesterase isoforms is essential in

regulation of cardiac adrenergic signaling to control both cAMP and cGMP concentrations in cardiomyocytes.³¹ For instance, the binding of cGMP to phosphodiesterase 3 (PDE3) can act as a competitive inhibitor for binding and breakdown of cAMP, whereas the binding of cGMP to PDE2 can act as a stimulator to increase cAMP hydrolysis. In this way, treatments such as sildenafil that increase cGMP concentrations may also indirectly modulate cAMP concentrations via cross-talk with PDE2 and PDE3.³¹ Given the clinical research showing that PDE5 inhibition is effective in enhancing cardiac function in male diabetic cardiomyopathy, we hypothesize that there is a functional association between PDE5 and β ARs, contributing to reduced cardiac contractility during development of diabetic cardiomyopathy; and inhibition of PDE5 with sildenafil can improve adrenergic stimulation of both cAMP and cGMP signals and enhance E-C coupling of diabetic myocytes.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Reagents and Materials

All reagents and materials were obtained from Millipore-Sigma unless otherwise specified.

Experimental Animals

UC Davis's Institutional Animal Care and Use Committee approved animal protocols in accordance with institutional and National Institutes of Health (NIH) guidelines and were approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Male wild-type (WT) C57BL/6J mice were purchased from Jackson Laboratories, and β_2 adrenergic receptor knockout (B2KO) C57BL/6J mice were bred in-house. Starting at 6 to 7 weeks old, mice received either a 10% fat diet (Research diets Inc., D12450J), deemed normal chow (NC), or a HFD containing 60% fat by calories (D12492J), deemed HFD. $n \geq 10$ mice per group. Mice that were gavaged received either vehicle (1% carboxymethyl cellulose) or 100 mg/kg per day of crushed sildenafil citrate tablets in vehicle at 20 μ L/10 g body weight starting 3.5 months after the special diet was started. All gavaged NC mice were treated with vehicle only. Adult mice were euthanized at 8 weeks old (no special diet employed) or after 4.5 to 5 months of special diet.

Intraperitoneal Glucose Tolerance Test

Mice underwent fasting for 6 hours before intraperitoneal glucose tolerance testing. Blood glucose readings were taken

from a tail nick with a Bayer Breeze2 standard glucometer at 0, 15, 30, 60, and 120 minutes after injection with 1 mg/g IP glucose solution. The area under the curve was calculated using GraphPad Prism 7.

Echocardiography

Short-axis M-mode and ventricular tissue Doppler were performed under isoflurane anesthesia (2–2.5% for induction and 1–1.5% for maintenance) in pure oxygen flow using a Vevo 2100 imaging system (VisualSonics) with a 22- to 55-MHz MS550D transducer. Body temperature, ECG, and respiration rate were monitored during the test to maintain the health of the animal and reduce variation, as previously described.^{32,33}

Parasternal short-axis M-mode was also used to assess sympathetic reserve of the mice. Once each mouse's heart rate had equilibrated within the target heart rate between 400 and 500 beats per minute, the mouse was injected with (-)-isoproterenol hydrochloride (0.2 mg/kg IP), followed by sildenafil citrate (1.6 mg/kg IP, Millipore-Sigma). Readings were taken once a minute for 20 minutes beginning immediately before injection with isoproterenol. All echocardiogram analysis was performed with group identifiers removed from the imaging.

Histology

Hearts were perfused with 10% formalin, dehydrated on an Autotechnicon Mono Tissue Processor (Technicon Corporation), embedded in paraffin with a Leica HistoEmbedder, and sectioned using a Leica RM2255 Microtome. Sections were stained with hematoxylin and eosin and Masson's trichrome according to the manufacturer's protocols. Apoptosis detection was performed using the Promega DeadEnd Fluorometric terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) system according to manufacturer's instructions. Hematoxylin and eosin and Masson's trichrome images were collected on a Nikon eclipse with a Nikon DS-Qi1Mc camera. TUNEL images were collected on a Zeiss LSM 700 confocal. Images were analyzed using Image J (NIH) by a group-blinded researcher.

Adult Rabbit and Mouse Ventriculomyocytes

Thirteen- to 14-week-old male New Zealand white rabbits (no special diet implemented) were purchased from Harlan Laboratories (Indianapolis, IN) and euthanized under general anesthesia (induction with 2 mg/kg propofol and 2–5% isoflurane in 100% oxygen). After thoracotomy, the heart was excised and rinsed in Minimum Essential Medium without calcium. Proceeding steps were performed as previously described,³⁴ with the heart being digested with 150 mL of

recirculated perfusion buffer containing 150 mg collagenase II and 8 mg protease XIV until digestion was complete. The ventricles were cut into a dish containing perfusion buffer with BSA, cut into pieces, titrated for 30 seconds with a 3-mL transfer pipette, filtered through a 250- μ m mesh, and centrifuged at 500 rpm for 1 minute. The cell pellet was resuspended and recovered in a gradient of calcium buffers to a concentration of 1 mmol/L.

The method implemented for mouse cardiomyocyte isolation has been previously described.³⁵ Adult mice were injected with 5000 units/kg heparin (Fresenius Kabi) and anesthetized in 2% to 5% isoflurane. The heart was pre-digested with 15 mL of perfusion buffer containing 2.5 mg of collagenase II (Worthington), 0.5 mg protease XIV, and 0.1% BSA. The heart was then digested with 20 mL of perfusion buffer containing 10 mg collagenase II, 2 mg protease XIV, 0.1% BSA, and 50 μ mol/L CaCl_2 , which was recirculated through the system until the heart was digested. The heart was cut below the atria into a dish containing 5 mL of digestion buffer and dissociated using forceps. The supernatant was placed in 5 mL of stop buffer (12.5 μ mol/L CaCl_2 and 10% fetal bovine serum [FBS] in perfusion buffer) and was centrifuged at 500 rpm for 1 minute. Remaining particles of tissue were further digested with 5 mL of digestion buffer for 5 minutes at 37°C twice more to ensure maximal yield of cells. Cell pellets were resuspended and recovered in a gradient of calcium buffers to a concentration of 1 mmol/L.

Neonatal Mouse Cardiomyocytes

Neonatal pups that were less than 24 hours old were collected from WT, β_1 adrenergic receptor (β_1 AR)-knockout β_1 KO, β_2 KO, and β_3 AR knockout (B3KO) FVB mice bred in-house. Hearts were minced and rotated in calcium- and bicarbonate-free Hank's buffer with HEPES (136.9 mmol/L NaCl, 5.36 mmol/L KCl, 0.81 mmol/L MgSO_4 , 5.55 mmol/L glucose, 0.44 mmol/L KH_2PO_4 , 0.34 Na_2HPO_4 , 20 mmol/L HEPES) containing 1 mg/mL of collagenase type II (Worthington) for 10 minutes at 37°C. Tissue pieces settled for 1 minute before the supernatant was expunged to remove epithelial cells. Fresh collagenase type II-containing calcium- and bicarbonate-free Hank's buffer with HEPES was then used in conjunction with a 10-mL pipette to triturate the tissue and was followed by another 10-minute incubation at 37°C. The tissue was then further triturated; the supernatant was collected and placed in 2 mL of horse serum to stop digestion and centrifuged at 800 rpm for 3 minutes to pellet cells. To ensure maximal yield of cells, remaining tissue pieces were further dissociated with another round of digestion, using the same procedure as outlined above. All cell pellets were combined through resuspension in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and plated on

a 10-cm Petri dish for 1 hour at 37°C. Cardiomyocytes were then gently washed from the Petri dish and collected, while fibroblasts that remained attached to the Petri dish were discarded. The collected cardiomyocytes were plated on laminin-coated glass coverslips in DMEM with 10% FBS for 24 hours at 37°C before adenovirus infection with the cGi500³⁶ fluorescence resonant energy transfer (FRET) biosensor in serum-free DMEM for 48 hours.

Coimmunoprecipitation

Isolated rabbit adult ventriculomyocytes (AVMs) were incubated in DMEM + 10% FBS for 2 hours. The media was changed to serum-free DMEM containing adenovirus coding for either Flag-tagged mouse β 2AR (Flag-m β 2AR) or cyan fluorescent protein- and Flag-tagged mouse β 1AR (Flag-m β 1AR) for 40 hours. Viruses were produced using the AdEasy system³⁷ (Qbiogene). Cells were lysed with IP lysis buffer (25 mmol/L HEPES, 150 mmol/L NaCl, 5 mmol/L EDTA, 10% glycerol, 1% Triton-X) containing inhibitors (100 mmol/L Na₃F, 1 mmol/L Na₂VO₄, 1 mmol/L glycerol, 2.5 mmol/L NaP₂O₇, 10 μ g/mL leupeptin, 1 mmol/L phenylmethane sulfonyl fluoride, 10 μ g/mL aprotinin). Coimmunoprecipitation was performed for 15 hours at 4°C using Flag-M2 beads. Western blot was used to detect PDE5 and Flag by a group-blinded researcher.

Western Blotting

Left ventricular tissues were lysed as previously described²⁹ using FastPrep-24 lysing matrix D beads (MP Biomedicals) in lysis buffer (25 mmol/L Tris HCl pH=7.6, 150 mmol/L NaCl, 1% IGEPAL, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA) containing protease and phosphatase inhibitors (100 mmol/L NaF, 1 mmol/L Na₂VO₄, 1% glycerol, 2.5 mmol/L NaP₂O₇, 1 mmol/L phenylmethane sulfonyl fluoride, 10 μ g/mL aprotinin, 10 μ g/mL pepstatin A, and 20 μ g/mL E-64, 5 μ g/mL bestatin). Protein concentrations were equilibrated after BCA assay. For most blots, 50 μ g of each lysate was resolved by SDS-PAGE using a 4% to 20% gradient gel and transferred to 0.45 μ m polyvinylidene fluoride. For phospholamban blots, lysates were further treated with 10% β -mercaptoethanol, sonicated, and heated at 37°C for 1 hour before resolution of 15 μ g of protein per sample by SDS-PAGE and transfer onto 0.2 μ m of nitrocellulose membrane (Bio-Rad). Membranes were incubated with primary antibody, followed by IRDye 680CW or 800CW secondary antibodies and scanned on an Odyssey scanner (Li-Cor Biosciences). Primary antibodies implemented were phosphodiesterase 5A (PDE5A; sc-398747, Santa Cruz), Flag (F7425, Millipore-Sigma), phosphodiesterase 3A (NIH, PDE3A-CT aa1098-1115),³⁸ phospho-serine 282 and total cardiac-

specific myosin-binding protein c (p282 and cMyBP-C and total cMyBP-C, generous gift from Dr Sakthivel Sadayappan,³⁹ Loyola University), phospho-serine 23/24 and total troponin I (p23/24 and total TnI; 4004 and 4002, Cell Signaling), sarcoplasmic-endoplasmic reticulum calcium ATPase 2A (SERCA2A; 2A7-A1, Thermo), phospho-serine 16 and total phospholamban (p16 and total PLB; A010-12 and A010-14, Badrilla), and glyceraldehyde 3-phosphate (GAPDH; 97166, Cell Signaling). Band intensities were quantified using ImageJ (NIH), divided by GAPDH intensities and normalized to the average of the first group.

Proximity Ligation Assay

Cells were fixed with buffered 4% paraformaldehyde for 10 minutes and stored in ddPBS at 4°C. Cells were permeabilized (0.3% Triton-X, 2% goat serum in ddPBS) and incubated at 4°C overnight in primary antibodies. Primary antibodies instituted included PDE5A (2395, Cell Signaling) with β 2AR (SC-271322, Santa Cruz) for PDE5A: β 2AR PLA, phosphodiesterase 3A (NIH, PDE3A-CT aa1098-1115)³⁸ with β 2AR (SC-570, Santa Cruz) for PDE3A: β 2AR PLA, and PDE5A (2395, Cell Signaling) with phosphodiesterase 3A (NIH, PDE3A-CT aa1098-1115)³⁸ for PDE5:PDE3 PLA. The manufacturer's protocol was followed for proceeding steps. Images were collected on a Zeiss LSM 700 confocal and analyzed on ImageJ by a group-blinded researcher.

Adult Cardiomyocyte Contractility and Calcium Transient

Mouse group identity was masked from the researcher performing the assay. Mouse AVMs were suspended in isolation buffer containing 1 mmol/L CaCl₂ and placed on ice, and 10 mg/L Fluo-4-AM (Thermo-Fisher) was added. Cells were incubated at room temperature for 12 minutes, and then the supernatant was replaced with fresh buffer. Cells were incubated at room temperature for another 15 minutes and then placed on ice for 15 minutes or more. As previously described,³⁰ the cells were then transferred into beating buffer and were paced using platinum electrodes with 50 V at 1 Hz with a stimulator. Cells that were not sharply beating in pace and flashing green in pace were excluded from analysis.

A Zeiss Axio Observer A1 inverted fluorescence microscope with Hamamatsu Orca-Flash 4.0 camera acquired phase contrast and fluorescein isothiocyanate images to assess contractility and calcium transient, respectively. The cells were treated with 100 nmol/L isoproterenol and/or 1 μ mol/L sildenafil. 10 μ mol/L Forskolin + 100 μ mol/L IBMX were similarly added to act as positive control. Cells were pretreated with 100 nmol/L ICI-118551, 100 nmol/L CGP-20712, 1 μ mol/L KN-93, 1 μ mol/L PKI 14-22^{myr}, or

100 nmol/L DT2. The percentage of myocyte contractility was defined as (maximal cell length—minimal cell length)/maximal cell length \times 100, assessed by MetaMorph software. Analysis of calcium transient dynamics was performed by a group-blinded researcher using an in-house routine written in interactive data language as previously described.⁴⁰

Fluorescence Resonant Energy Transfer

Mouse group identity was masked from the researcher performing the assay. Mouse adult cardiomyocytes that were intended for FRET assays were plated, as previously described,⁴¹ on laminin-coated glass coverslips with mouse FRET media (10.7 g/L M1018, 6.25 μ mol/L blebbistatin, 10 mmol/L HEPES, 0.2% BSA, 4 mmol/L NaHCO₃, 1 \times penicillin-streptomycin-glutamate [PSG]) containing 10% FBS and incubated at 37°C for 5 hours. The media was then changed to serum-free mouse FRET media that contained adenovirus coding for an appropriate FRET biosensor, and cells were incubated at 37°C for 30 to 50 hours, based on biosensor expression. FRET biosensors implemented in this study were the A-kinase activity reporter-3 (AKAR3), Epac-based sensor #H187 (Epac-S^{H187}), and cGMP indicator-500 nmol/L (cGi500), which have all been previously described.^{36,42–45} Viruses were produced using the AdEasy system³⁷ (Qbiogene).

For both neonatal and adult cardiomyocytes, the media was changed to serum-free media without virus. Coverslips were transferred to glass bottom culture dishes (MatTek) containing ddPBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 1.8 mmol/L KH₂PO₄, pH=7.4). For adult cells, ddPBS was supplemented with 1 μ mol/L blebbistatin. A Zeiss Axio Observer A1 inverted fluorescence microscope with Hamamatsu Orca-Flash 4.0 camera acquired FRET images. Images were recorded by excitation at 430 to 455 nm and emission on 2 filters (475DF40 for cyan and 535DF25 for yellow). Cells were pretreated with 100 nmol/L ICI-118551 or 100 nmol/L CGP-20712 as indicated. The change in FRET ratio was measured after 100 nmol/L isoproterenol and/or 1 μ mol/L sildenafil citrate were added. 10 μ mol/L Forskolin+100 μ mol/L IBMX were similarly added to act as positive control for AKAR3 or Epac-S^{H187}, while 50 μ mol/L sodium nitroprusside acted as positive control for cGi500. If no response was seen to initial drug addition, the appropriate positive control drug was added. Only cells that depicted a response were analyzed. Images were subjected to background subtraction and were acquired every 30 seconds with an exposure time of 200 ms. The donor/acceptor FRET ratio was normalized to the baseline.

Statistical Analysis

All values are presented as mean \pm SEM. Statistical tests implemented were calculated using GraphPad Prism 7.

$P\leq 0.05$ was considered significant. Unpaired *t* tests were performed for comparisons of 2 groups. For comparisons between ≥ 3 groups with a group $n=3$, Kruskal–Wallis tests with Dunn's post-tests were performed. For comparisons between ≥ 3 groups with $n>3$ per group, 1-way ANOVAs were executed with Tukey post-tests. For comparisons between groups over time, a repeated measures ANOVA with Sidak post-test was implemented. Post hoc tests were only performed on ANOVAs, which had a significant *F* value. Area under the curve was calculated by determining the total peak area above $y=0$ for each mouse. For left ventricular wall thickness, fibrosis, and TUNEL measurements, average values from multiple readings for each mouse were determined before plotting data on the overall graph.

Results

Sildenafil Treatment Improves Cardiac Reserve in Response to Sympathetic Stimulation in Diabetic Hearts

High fat diet (HFD) mice displayed a reduction in glucose tolerance, as well as a reduced cardiac ejection fraction and fractional shortening associated with cardiac remodeling in the form of increased hypertrophy, fibrosis, and myocyte apoptosis (Figure 1A through 1K). Meanwhile, chronic phosphodiesterase 5 (PDE5) inhibition with sildenafil in diabetic mice improved basal heart function, which correlated with reduced hypertrophy, fibrosis, and myocyte apoptosis (Figure 1C through 1K), similarly to previous reports in diabetic humans and rats.^{22,23} Moreover, while HFD mice displayed a reduced cardiac reserve in response to adrenergic stimulation with isoproterenol, acute treatment with sildenafil restored cardiac reserve in response to adrenergic stimulation (Figure 1L and 1M). Chronic PDE5 inhibition with sildenafil also restored cardiac reserve in response to adrenergic stimulation (Figure 1L and 1M). These results suggest that besides improved basal diabetic heart function, sildenafil can also improve cardiac reserve in response to sympathetic stimulation in diabetic cardiomyopathy.

Western blots were then performed to determine the changes in E-C coupling proteins. First, NC and HFD heart tissue samples were compared. As we have previously observed,²⁸ HFD mouse hearts have a reduction in expression of SERCA2A, which leads to an increase in the ratio of phospholamban-to-SERCA2A. A reduction in phosphorylation of p23/24 TnI and p16 PLB could also be similarly observed. Meanwhile, PDE5A and PDE3A showed no significant change between NC and HFD mouse hearts. A PKG substrate of p282 of cMyBP-C promotes myocyte contractility upon phosphorylation.⁴⁶ The expression of total cMyBP-C, as well as the levels of p282 cMyBP-C, did not show significant changes between NC and HFD mouse hearts (Figure 2A and 2B). When

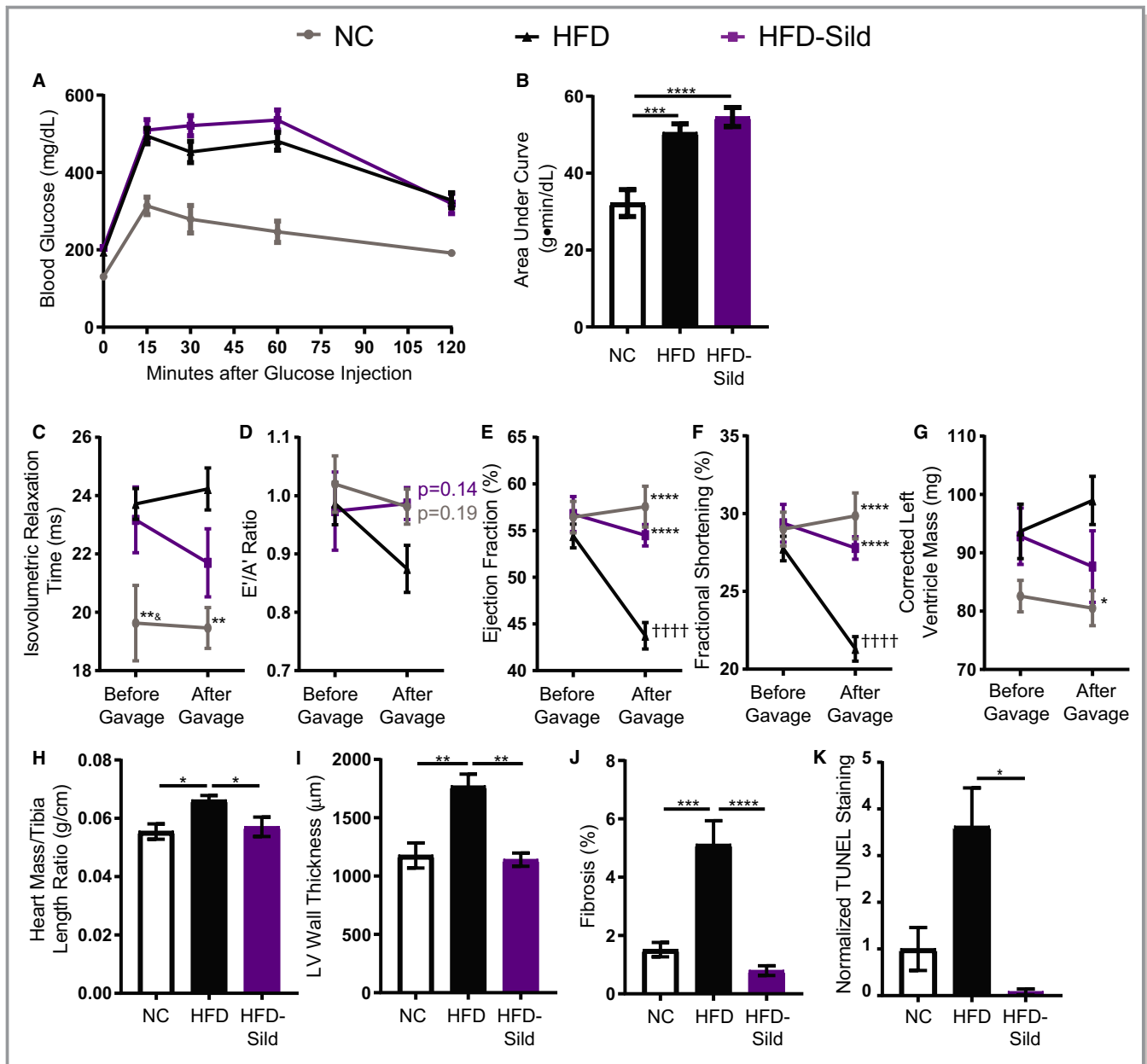


Figure 1. Sustained in vivo sildenafil treatment in mice fed a high fat diet (HFD) improves basal and adrenergic-induced heart function and morphology. **A**, Intraperitoneal glucose tolerance testing (IPGTT) and **(B)** area under the curve of IPGTT from wild-type (WT) mice on a special diet for 4.5 months and gavaged for 1 month with either vehicle or 100 mg/kg per day of sildenafil citrate. $***P \leq 0.001$, $****P \leq 0.0001$ by 1-way ANOVA and Tukey post-test. $n \geq 9$ mice per group. **C**, Isovolumetric relaxation time, **(D)** E'/A' ratio, **(E)** ejection fraction, **(F)** fractional shortening, and **(G)** corrected left ventricular (LV) mass of WT mice after 3.5 and 4.5 months of normal chow or HFD, as measured by M-mode and tissue Doppler echocardiogram. Between the 2 readings, all mice were gavaged daily with either vehicle only or 100 mg/kg sildenafil. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ compared with HFD at the same time point; $^{\&}P \leq 0.05$ compared with HFD-sildenafil at the same time point; $^{\dagger\dagger\dagger}P \leq 0.0001$ compared with the same group before gavage by a repeated measures ANOVA and Sidak post-test. $n \geq 9$ mice per group. **H**, Heart mass to tibia length ratio of gavaged mice. $n = 5$ mice per group. $*P \leq 0.05$ by 1-way ANOVA and Tukey post-test. **(I)** Quantification of LV wall thickness of gavaged mice measured from hematoxylin and eosin slices. $n = 5$ mice per group. $**P \leq 0.01$ by 1-way ANOVA and Tukey post-test. **J**, Quantification of fibrosis percentage in gavaged mice, measured from 6 areas of each mouse left ventricle stained with Masson's trichrome. $n = 5$ mice per group. $***P \leq 0.001$, $****P \leq 0.0001$ by 1-way ANOVA and Tukey post-test. **K**, Quantification of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells in heart slices from gavaged mice. $*P \leq 0.05$ by Kruskal-Wallis test with Dunn's post-test. $n = 3$ mice per group. **L**, Ejection fraction and **(M)** fractional shortening of HFD mice gavaged with sildenafil (HFD-sildenafil) or vehicle only (HFD) at baseline and after isoproterenol followed by sildenafil injections. $n \geq 9$ mice per group. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, and $****P \leq 0.0001$ by 1-way ANOVA and Tukey post-test.

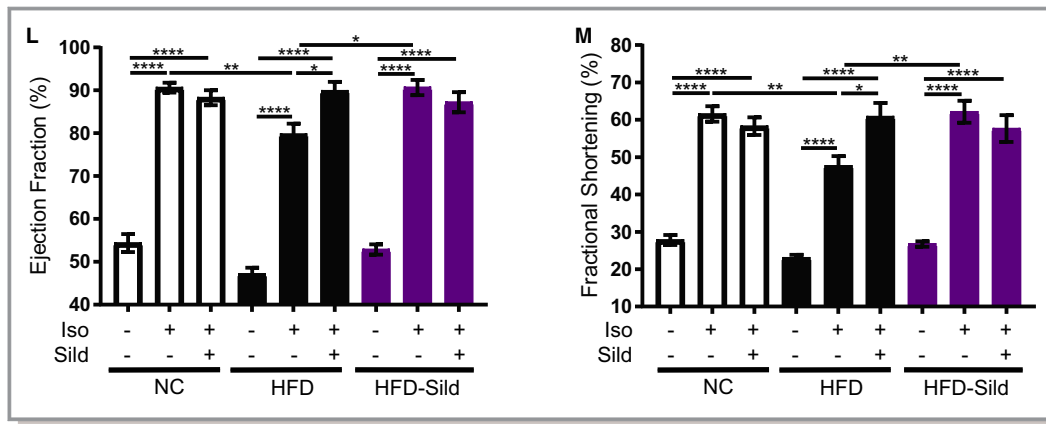


Figure 1. Continued

comparing sildenafil-treated HFD animals with their HFD counterparts, the expression of PDE5A and PDE3A in hearts did not significantly change. Interestingly, chronic sildenafil treatment did increase the expression of total cMyBP-C, as well as the levels of p282 cMyBP-C (Figure 2C and 2D). Sildenafil also increased expression of TnI while decreasing PLB expression, but did not change the expression of SERCA2A in HFD hearts (Figure 2C and 2D). In addition, sildenafil led to an increase in p16 and PLB/total PLB (Figure 2C and 2D). These data suggest that sustained sildenafil treatment *in vivo* alters the expression and activities of proteins in a manner that favors improved E-C coupling and heart function. These include increases in myofilament protein expression and myofilament calcium sensitivity, as well as increases in SERCA calcium reuptake activity.

To determine whether the increases in whole-heart sympathetic responses produced by sildenafil in WT HFD mice were caused by improvements in E-C coupling, AVMs were treated *ex vivo* with isoproterenol and/or sildenafil and their contractility, calcium transient amplitude, and calcium half-time recovery (τ) were recorded. HFD myocytes displayed an impaired excitation-contraction response to isoproterenol stimulation while acute treatment with sildenafil fully rescued isoproterenol-induced contractility and calcium transient dynamics in HFD AVMs (Figure 3A through 3C). Meanwhile, chronic therapy with sildenafil also restored isoproterenol-induced contractility and calcium transient dynamics in HFD AVMs. Additional treatment with sildenafil on myocytes isolated from sildenafil-treated mice did not further enhance isoproterenol-induced contractility or calcium transient. These data suggest that either acute *in vitro* or sustained *in vivo* sildenafil treatment is sufficient to rescue adrenergic stimulation of contractility in diabetic cardiomyocytes with improvements of calcium amplitude and reuptake. This opposes previous reports in which sildenafil attenuates isoproterenol-induced contractility response in myocytes from 6- to 8-week-

old mice.^{25,26} As control, E-C coupling was measured in myocytes from 8-week-old mice. The addition of sildenafil reduced contractility and calcium transient dynamics induced by isoproterenol in young AVMs (Figure 3D through 3F). These data suggest that a remodeling of PDE5 in modulation of adrenergic stimulation for E-C coupling during development of diabetic cardiomyopathy occurs.

Sildenafil Bolsters Adrenergically Stimulated Cyclic Nucleotide Concentration in HFD AVMs

FRET-based analysis of cGMP with the cGi500 biosensor in AVMs was then applied to understand the mechanism underlying sildenafil's effect on E-C coupling (Figure 4A). Sildenafil alone did not affect the baseline levels of cGMP in AVMs isolated from any group. Isoproterenol induced robust increases in cGMP in NC AVMs, which were significantly impaired in HFD AVMs (Figure 4A). Acute addition of sildenafil enhanced isoproterenol-induced cGMP in HFD AVMs (Figure 4A). Chronic treatment with sildenafil *in vivo* also recovered isoproterenol-induced increases in cAMP in HFD-sildenafil AVMs, while acute addition of sildenafil to AVMs *in vitro* did not further enhance HFD-sildenafil AVM responses induced by isoproterenol (Figure 4A). Eight-week-old mouse ventriculomyocytes responded in a similar fashion to the 6-month-old NC cells (Figure 4B). These data together suggest an increased coupling between PDE5 and adrenergic regulation in diabetic cardiomyopathy.

Adrenergically induced cAMP signal and PKA activity in NC, HFD, and HFD-sildenafil AVMs were also assessed with FRET-based biosensors EpacS^{H187} and AKAR3, respectively. Sildenafil alone did not affect cAMP signal nor PKA activity (Figure 4C through 4E). Stimulation with isoproterenol alone induced robust increases in cAMP signal and PKA activity in NC AVMs. Similar increases in cAMP as seen in 6-month-old NC AVMs were also seen upon stimulation of 8-week-old

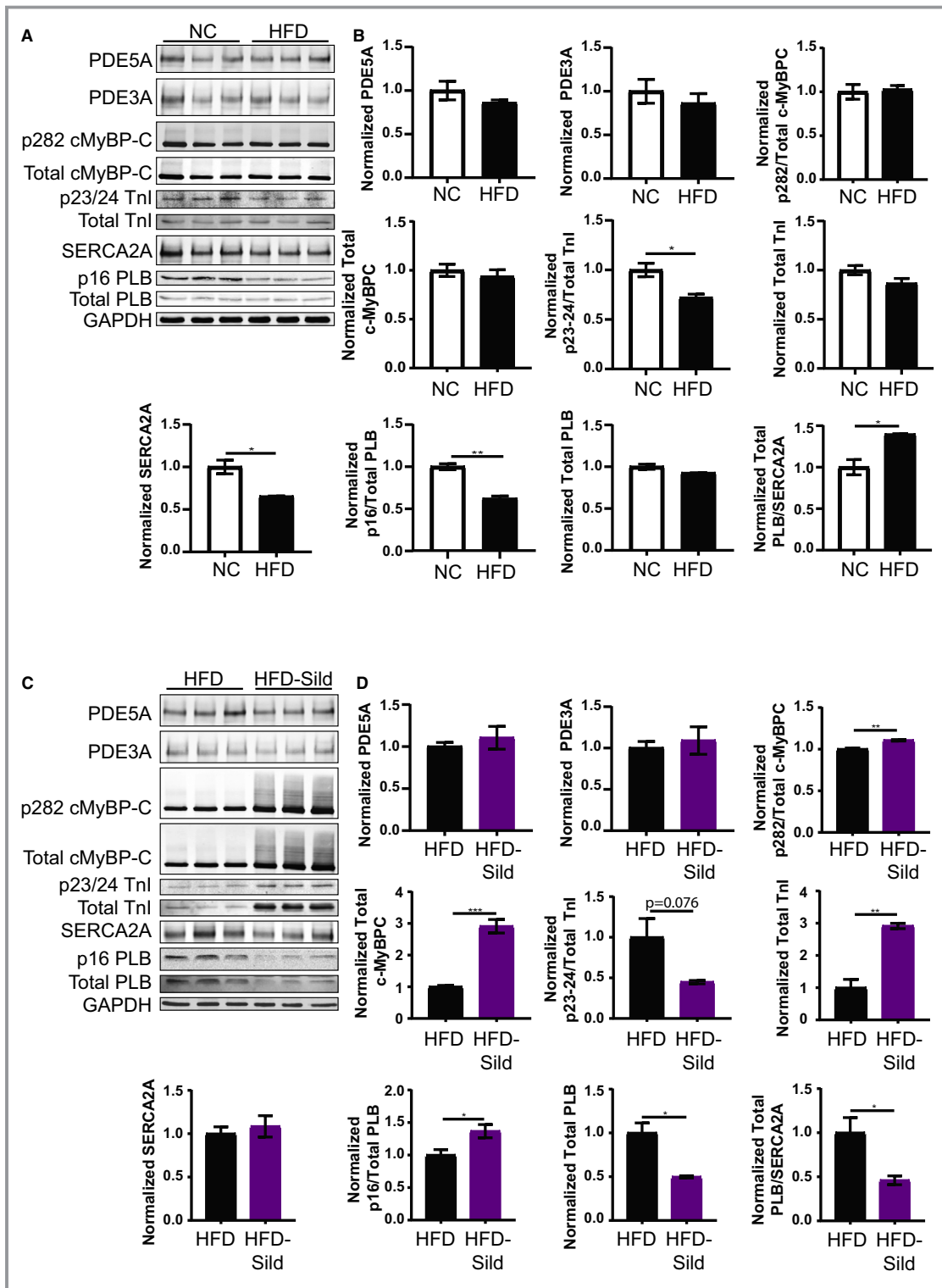


Figure 2. Sildenafil treatment improves E-C coupling protein activation and expression in mice fed a high fat diet (HFD). Images and quantification of Western blots for phosphodiesterase 5A (PDE5A), phosphodiesterase 3A (PDE3A), phospho-serine 282 (p282) and total cardiac myosin binding protein C (cMyBP-C), phospho-serine 23/24 (p23/24) and total troponin I (TnI), sarcoplasmic-endoplasmic reticulum calcium ATPase 2A (SERCA2A), and phospho-serine 16 (p16), and total phospholamban (PLB) for normal chow (NC) and HFD mice (**A** and **B**) followed by that for HFD and HFD-sildenafil mice (**C** and **D**). Total protein normalized to glyceraldehyde 3-phosphate (GAPDH). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ by unpaired t test. $n=3$ mice per group.

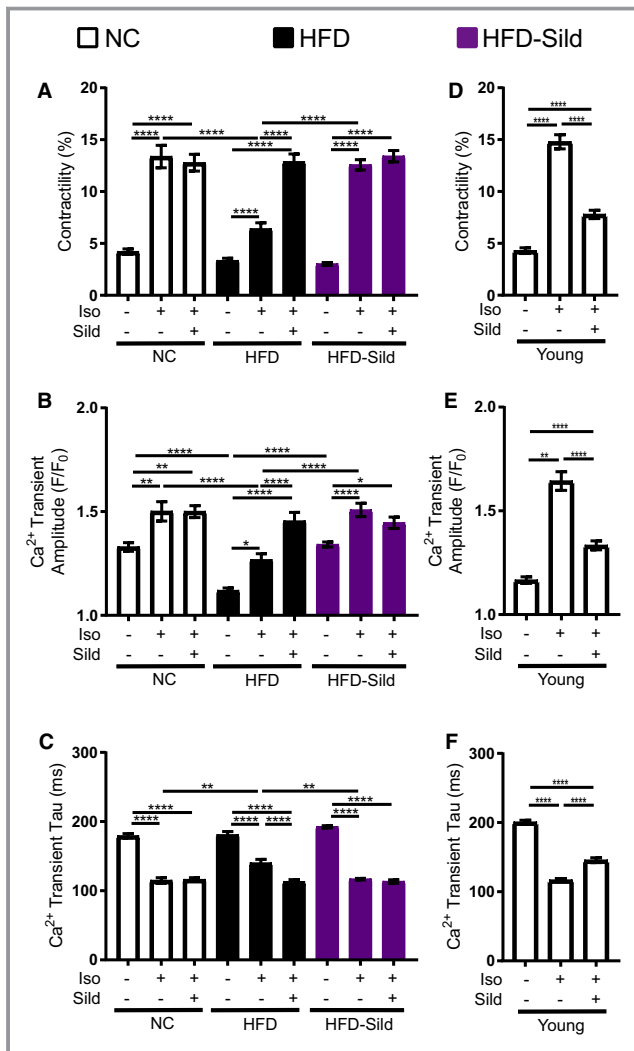


Figure 3. Sustained *in vivo* or acute *in vitro* sildenafil treatment is sufficient to rescue adrenergic response in diabetic adult ventricular myocytes (AVMs). **(A)** Contractility, **(B)** calcium transient amplitude, and **(C)** calcium transient half-time recovery (tau) of AVMs from wild-type mice fed normal chow (NC) or high fat diet (HFD) for 4.5 months and gavaged with either sildenafil or vehicle only for 1 month. $n \geq 20$ cells per group. **(D)** Contractility, **(E)** calcium transient amplitude, and **(F)** calcium transient half-time recovery in isolated adult cardiomyocytes from young 8-week-old mice. $n \geq 15$ cells per group. For all graphs, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, and $****P \leq 0.0001$ by 1-way ANOVA and Tukey post-test.

mouse AVMs (Figure 4D). When compared with the increases seen in healthy controls, the HFD AVM response was significantly impaired (Figure 4C through 4E). Acute addition of sildenafil rescued isoproterenol-induced cAMP signal and partially enhanced PKA activity in HFD AVMs (Figure 4C through 4E). Chronic treatment with sildenafil *in vivo* also rescued isoproterenol-induced increases in cAMP signal and partially recovered PKA activity in HFD-sildenafil AVMs; additional sildenafil *in vitro* did not further enhance the

response induced by isoproterenol in AVMs from sildenafil-treated animals (Figure 4C through 4E). These data suggest that both acute and sustained inhibition of PDE5 not only change cGMP signal but also cAMP signal and PKA activity in diabetic AVMs in response to adrenergic stimulation in HFD myocytes. In comparison, in both age-matched NC and young mouse control AVMs, sildenafil did not further enhance cGMP nor cAMP concentrations induced by isoproterenol (Figure 4A through 4E).

Sildenafil Improves E-C Coupling in Diabetic Myocytes in a PKG-Dependent Manner

Based on the results from FRET assays, the function of downstream kinases of cGMP and cAMP that are important for E-C coupling were evaluated. WT HFD AVMs were pretreated with the PKG inhibitor, DT2, the PKA inhibitor, PKI, or the calcium/calmodulin kinase II (CaMKII) inhibitor, KN93, and then their contractility and calcium transient dynamics were measured in response to isoproterenol and/or sildenafil. Inhibition of PKG caused a dramatic reduction in contractility that was accompanied by a slowing of tau but not a significant change in calcium transient amplitude (Figure 4F through 4H). To a lesser extent, inhibition of CaMKII also significantly attenuated contractility response to isoproterenol+sildenafil. CaMKII inhibition did not affect calcium transient dynamics to isoproterenol+sildenafil (Figure 4F through 4H). In contrast, inhibition of PKA did not affect contractility nor calcium cycling response to isoproterenol+sildenafil (Figure 4F through 4H). Together, these data indicate that *in vitro* sildenafil improves E-C coupling primarily through PKG in HFD AVMs.

PDE5 Interacts With β_2 Adrenergic Receptor (B2AR) and Its Signaling Cascade

To determine whether sildenafil would affect adrenergically stimulated cGMP concentration in a β AR-subtype-specific manner, FRET analysis of cGMP concentration was performed in WT, β_1 KO, β_2 AR knockout, and β_3 KO neonatal cardiomyocytes after treatment with sildenafil and/or isoproterenol. In WT cardiomyocytes, sildenafil significantly enhanced cGMP concentrations induced by isoproterenol. Deletion of β_2 AR abolished the effect of sildenafil, whereas deletion of either β_1 AR or β_3 AR did not do so (Figure 5A through 5D). These data suggest that sildenafil enhances cGMP induced by β_2 AR in neonatal cardiomyocytes. To determine whether this interaction also occurs in diabetic cardiomyopathy, AVMs from HFD mice were treated with the β_1 AR inhibitor, CGP-20712, or the β_2 AR inhibitor, ICI-118551, and adrenergically stimulated cGMP levels were measured by FRET (Figure 5E).

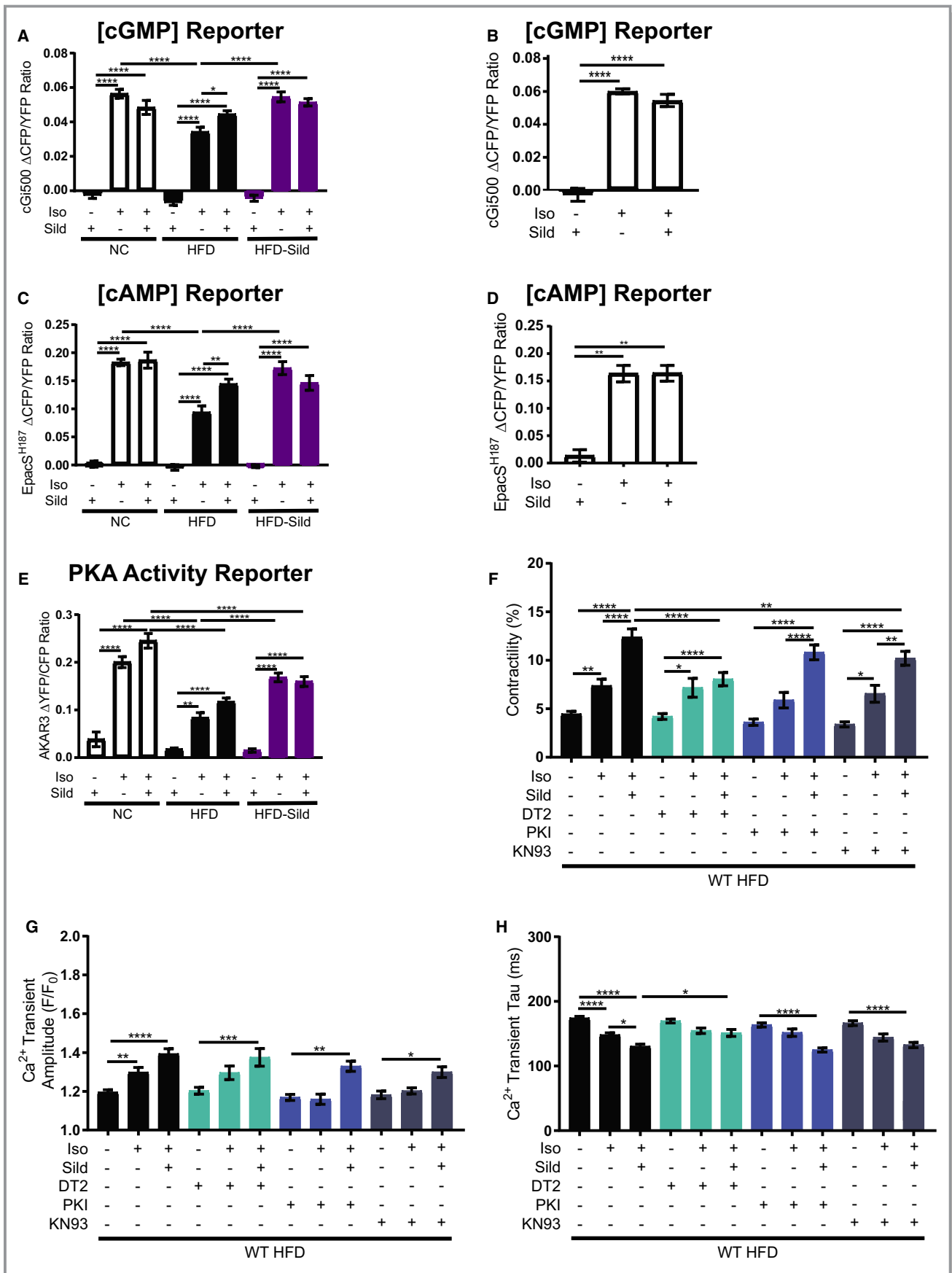


Figure 4. Sildenafil rescues cyclic nucleotide concentration, increases PKA (protein kinase A) activity, and improves adrenergic E-C coupling response in a PKG (protein kinase G)- and calcium/calmodulin kinase II-dependent manner. Average maximal fluorescence resonant energy transfer (FRET) response of the cGMP probe, cGi500 in adult ventriculomyocytes (AVMs) from gavaged mice (A) and from young 8-week-old mice (B). Average maximal FRET response of the cAMP probe, EpacS^{H187} in AVMs from gavaged mice (C) and from young 8-week-old mice (D). Average maximal FRET response of the PKA activity probe, A-kinase activity reporter-3 in AVMs from gavaged mice (E). In all FRET response graphs, $n \geq 15$ cells per group; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$ by 1-way ANOVA with Tukey post-test. Contractility (F), calcium transient amplitude (G), and calcium transient tau (H) of AVMs from wild-type mice fed an HFD for 4.5 months. Cells were pretreated with and without DT2 peptide, myrisoylated PKI peptide (PKI), or KN93 before stimulation with isoproterenol and sildenafil. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ by 1-way ANOVA and Tukey post-test. $n \geq 25$ cells per group.

While inhibition of β 1AR led to a significant reduction in response to isoproterenol, the addition of sildenafil could still cause a significant rescue of signal. Meanwhile, HFD cells treated with the β 2AR inhibitor had no additional response to the addition of sildenafil. In contrast, the addition of sildenafil to isoproterenol in NC AVMs inhibited with CGP-20712 or ICI-118551 showed no significant difference to the response to isoproterenol alone (Figure 5E). This suggests that PDE5 has an important functional interaction with β 2AR in HFD AVMs.

In vivo echocardiogram studies were then performed to test whether β 2AR is necessary for sildenafil-mediated modulation of adrenergic stimulation. As previously observed,²⁸ β 2KO mice fed a HFD are protected from decreases in basal heart function and worsening cardiac fibrosis and hypertrophy that is seen in WT HFD mice (Figure 5F through 5I). In contrast, both WT and β 2KO mice fed HFD had significant reductions in response to isoproterenol when compared with age-matched WT NC controls (Figure 5J and 5K). Additional administration of sildenafil enhanced the increase in ejection fraction and fractional shortening induced by isoproterenol in WT HFD mice, but not in WT NC controls. In contrast, sildenafil did not boost isoproterenol-induced ejection fraction and fractional shortening in β 2KO HFD mice, which suggests that sildenafil improves diabetic heart stress response in a β 2AR fashion (Figure 5J and 5K).

Consistent with the in vivo observations, HFD feeding led to impaired E-C coupling after isoproterenol stimulation, which is rescued by sildenafil treatment (Figure 6A through 6C). Deletion of β 2AR normalized the isoproterenol-induced E-C coupling in HFD AVMs. Treatment with sildenafil did not cause an additional response in the E-C coupling parameters, again supporting that sildenafil improves E-C coupling in diabetic myocytes in a β 2AR-dependent manner. To ensure that the results collected from β 2KO HFD myocytes were not compensatory from genetic deletion, AVMs from HFD WT mice were pretreated with the β 1AR inhibitor, CGP-20712, or the β 2AR inhibitor, ICI-118551. While CGP-20712 abolished isoproterenol response completely, ICI-118551 did not significantly affect isoproterenol response (Figure 6D through 6F), consistent with the primary regulatory role of β 1AR in

sympathetic E-C coupling response.⁴⁷ However, when sildenafil is added to isoproterenol in ICI-118551-treated AVMs, there is not a significant enhancement in the isoproterenol response like that in WT HFD cells without pretreatment. Addition of sildenafil to isoproterenol in ICI-118551-pretreated AVMs is associated with a slowing of calcium tau, but not a significant decrease in calcium amplitude (Figure 6D through 6F). These data reiterate that sildenafil improves sympathetically induced E-C coupling dynamics in a β 2AR-dependent fashion. Moreover, these results underscore the importance of β 1AR in sympathetically induced contractility, where modulation of the β 2AR signal only further affects downstream signaling.

To ascertain whether PDE5 physically interacts with β 2AR, coimmunoprecipitation was performed on rabbit AVMs expressing either Flag-m β 2AR or Flag-m β 1AR, where Flag was pulled down. PDE5A was pulled down with Flag- β 2AR but not with Flag-m β 1AR (Figure 7A and 7B), depicting that PDE5A specifically interacts in a protein complex with β 2AR. Proximity ligation assay was then performed between PDE5A and β 2AR in AVMs from NC and HFD mice to determine how this interaction may change. There is no significant change in colocalization of PDE5A and β 2AR between these groups (Figure 7C), but there is a significant increase in the interaction of both PDE5A and β 2AR with PDE3A in HFD cardiomyocytes (Figure 7D and 7E). This suggests that during disease development there is a change in the interaction of the β 2AR:PDE5A complex with the dual cAMP/cGMP phosphodiesterase PDE3.

Discussion

Phosphodiesterase 5 (PDE5) has been linked to cardiac adrenergic signaling regulation in both physiological and pathological conditions. The important finding in this study is that PDE5A exists in a protein complex with β 2 adrenergic receptor (β 2AR) and mediates the impacts of sildenafil on adrenergic response in diabetic cardiomyopathy in a β 2AR-specific manner. The inhibition of PDE5 by sildenafil not only rescues adrenergically induced cGMP but also cAMP in diabetic cardiomyocytes through an increased interaction

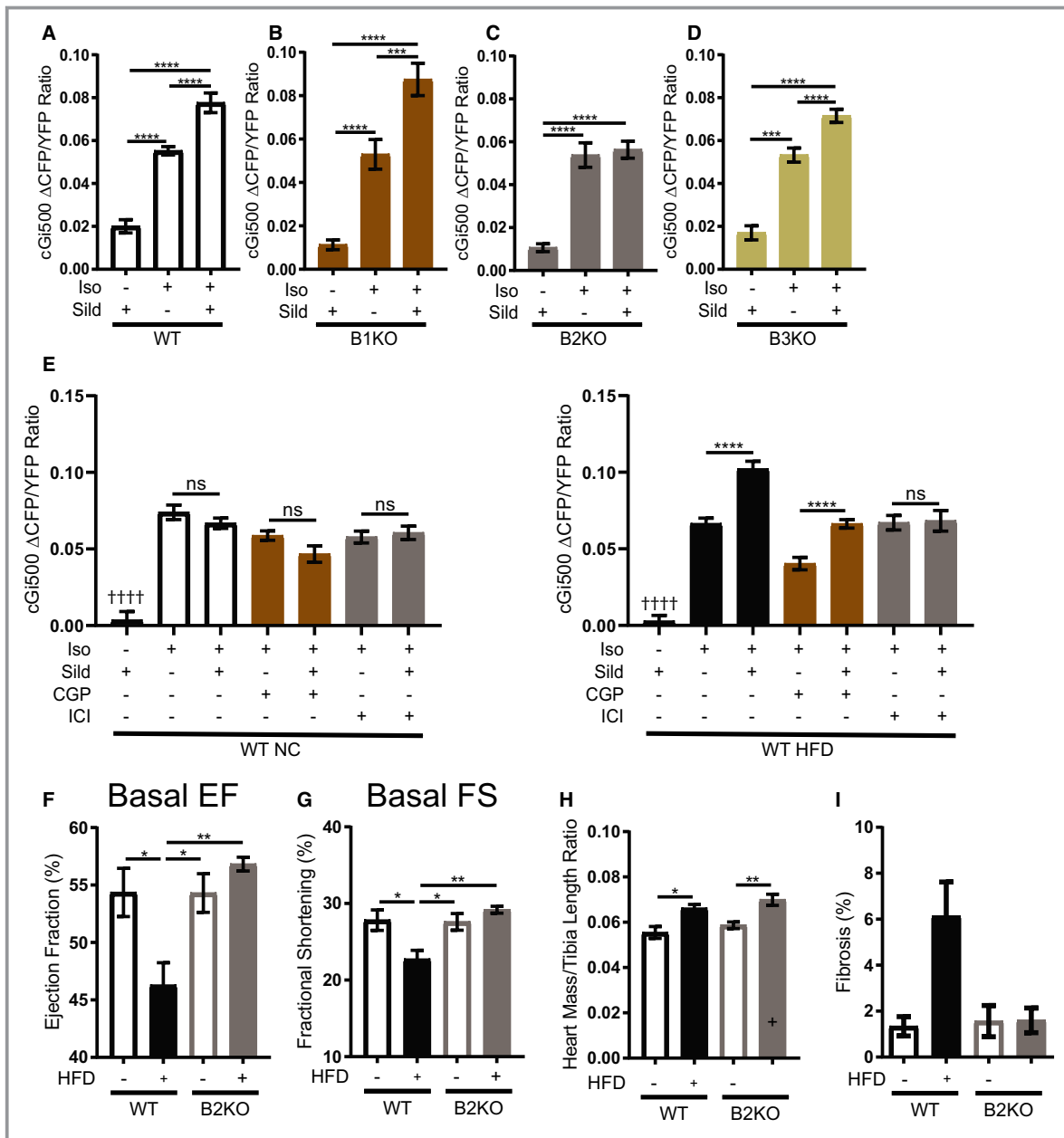


Figure 5. Phosphodiesterase 5 preferentially interacts with β_2 adrenergic receptor (β_2 AR) and its signaling cascade. **A** through **D**, Average maximal fluorescence resonant energy transfer (FRET) response of the cGMP probe to isoproterenol, cGi500 in isolated neonatal cardiomyocytes from wild-type (WT), β_1 adrenergic receptor (β_1 AR) knockout (β_1 KO), β_2 AR knockout (β_2 KO), and β_3 adrenergic receptor knockout (β_3 KO) mice. $n \geq 30$ cells per group. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ by 1-way ANOVA with Tukey post-test. **E**, Average maximal FRET response of the cGMP probe to isoproterenol, cGi500 in isolated NC and HFD ACMs with pretreatment of the β_1 AR inhibitor, CGP-20712 (CGP), or the β_2 AR inhibitor, ICI-118551 (ICI). $n \geq 20$ cells per group. $\dagger\dagger\dagger\dagger P \leq 0.0001$ compared with all other groups, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ by 1-way ANOVA with Tukey post-test. **F**, Ejection fraction and **(G)** fractional shortening echocardiogram measurements of WT and β_2 KO mice fed normal chow (NC) or high fat diet (HFD) after 4.5 months of special diet. **H**, Heart mass/tibia length ratio of WT and β_2 KO mice fed NC or HFD for 4.5 months. $n \geq 7$ mice per group. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ by 1-way ANOVA with Tukey post-test. **I**, Interstitial cardiac fibrosis percentage in WT and β_2 KO mice fed NC or HFD for 4.5 months. $n = 3$ mice per group. Kruskal–Wallis test with Dunn’s post-test performed. **J**, Ejection fraction and **(K)** fractional shortening echocardiogram measurements of WT and β_2 KO mice fed NC and HFD for 4.5 months after isoproterenol injection followed by sildenafil injection. $n \geq 8$ mice per group. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ by 1-way ANOVA with Tukey post-test.

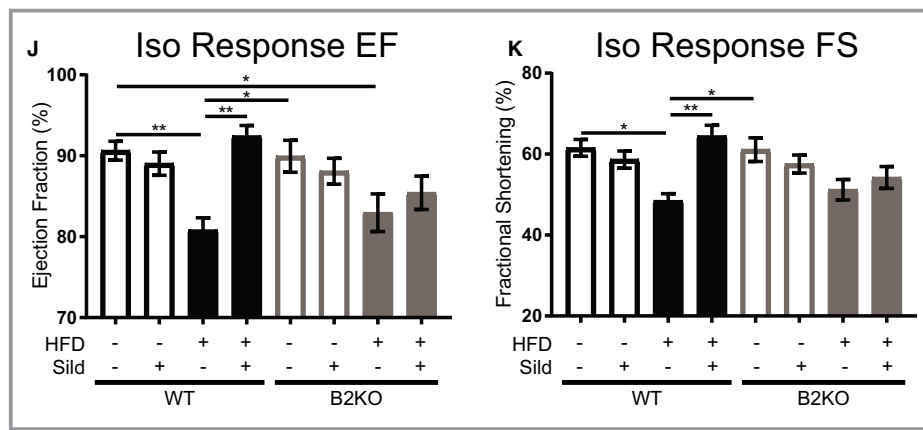


Figure 5. Continued.

with PDE3. Sildenafil boosts E-C coupling dynamics of diabetic AVMs through activation of PKG and, to a lesser extent, CaMKII (Figure 8A). Furthermore, chronic therapy with sildenafil *in vivo* improves cardiac function at baseline and after adrenergic stimulation. Deletion of β_2 AR abolishes the effects of sildenafil on adrenergic modulation. These data reveal a novel molecular mechanism of a functional PDE5- β_2 AR complex underlying the effects of sildenafil in healthy and diseased hearts.

Biochemical, proximity ligation, and FRET-based live cell imaging assays show that PDE5 selectively associates with cardiac β_2 AR but not β_1 AR and specifically enhances β_2 AR-induced cGMP signaling in myocytes to improve E-C coupling in a model of diabetic cardiomyopathy. These data, for the first time, place PDE5 in a receptor complex to facilitate the hydrolysis of cGMP, which exerts local control over a pool of cGMP after adrenergic stimulation in both neonatal and HFD myocytes. A previous report also linked a pool of cGMP promoted by PDE5 inhibition to β_3 AR in young adult cardiomyocytes, which interestingly leads to PKG-dependent reduction of isoproterenol-induced contractility via phosphorylation of Tnl.²⁶ However, a specific interaction between PDE5 and β_3 AR remains to be determined. Our observation also parallels the well-documented association of cAMP-specific PDE4 with both β_1 AR and β_2 AR in AVMs,⁴⁸ suggesting that both cyclic nucleotides are subjected to tight regulation in space and time. Future use of PDE5 inhibitors in the study of developmental- and disease-related roles that PDEs have in the heart will be a valuable tool to disentangle this important signaling network.

Our data show that inhibition of PDE5, a cGMP-specific PDE, also enhances adrenergic stimulation of cAMP levels in diabetic cardiomyocytes. This could have been caused by cross-talk between cAMP and cGMP through the dual substrate-specific PDEs, PDE3, and/or PDE2. We have previously shown that inhibition of PDE5 leads to a decrease in cAMP in neonatal cardiomyocytes through an activation of

PDE2.²⁷ In contrast, PDE3, which is well expressed in cardiomyocytes,⁴⁹ breaks down both cAMP and cGMP through competitive inhibition.⁵⁰ While proteins important for cAMP signal management are scaffolded by A-kinase anchoring proteins, proteins used in cGMP signal management attach to the leucine-zipper motif in PKG.⁵¹ Moreover, in compartments that contain PKA regulatory subunit I, cGMP increases cAMP concentration, which suggests that PDE3 is the predominant phosphodiesterase. In compartments that contain PKA regulatory subunit II, cGMP decreases cAMP, which suggests that PDE2 is the predominant phosphodiesterase in this compartment.⁵² Therefore, the delicate association of different PDEs allows for a fine-tuned interplay between cAMP and cGMP.^{53,54}

Our data indicate an increase in association between PDE3 and PDE5- β_2 AR complex in diabetic cardiomyocytes, supporting that the increase in cGMP caused by the inhibition of PDE5 by sildenafil can inhibit the breakdown of cAMP by PDE3 and leads to an enhancement of isoproterenol-induced cAMP concentration by sildenafil. However, in cardiomyocytes from mice subjected to transaortic constriction, there are switches of PDE2/3 between β_1 AR and β_2 AR. The cAMP from β_2 AR is sensitive to PDE2 in transaortic constriction myocytes when compared with the sham controls.⁵³ This suggests that β_2 AR interacts more with PDE2 in a model of cardiac hypertrophy after increased afterload, which is contrary to the results in the current model of diet-induced hypertrophy. These data indicate again that, dependent on cellular context, PDE5 can switch coupling to different signaling cascades to modulate levels of specific pools of cyclic nucleotides in a given cardiomyocyte/heart. This difference may underlie the reasons for why sildenafil is effective at reducing symptoms of HF in men with diabetes mellitus^{13,14} but not in the wider population of patients with general HF.¹⁴⁻¹⁶ Future work to determine how the localization of β ARs and PDEs in diabetic cardiomyocytes affects

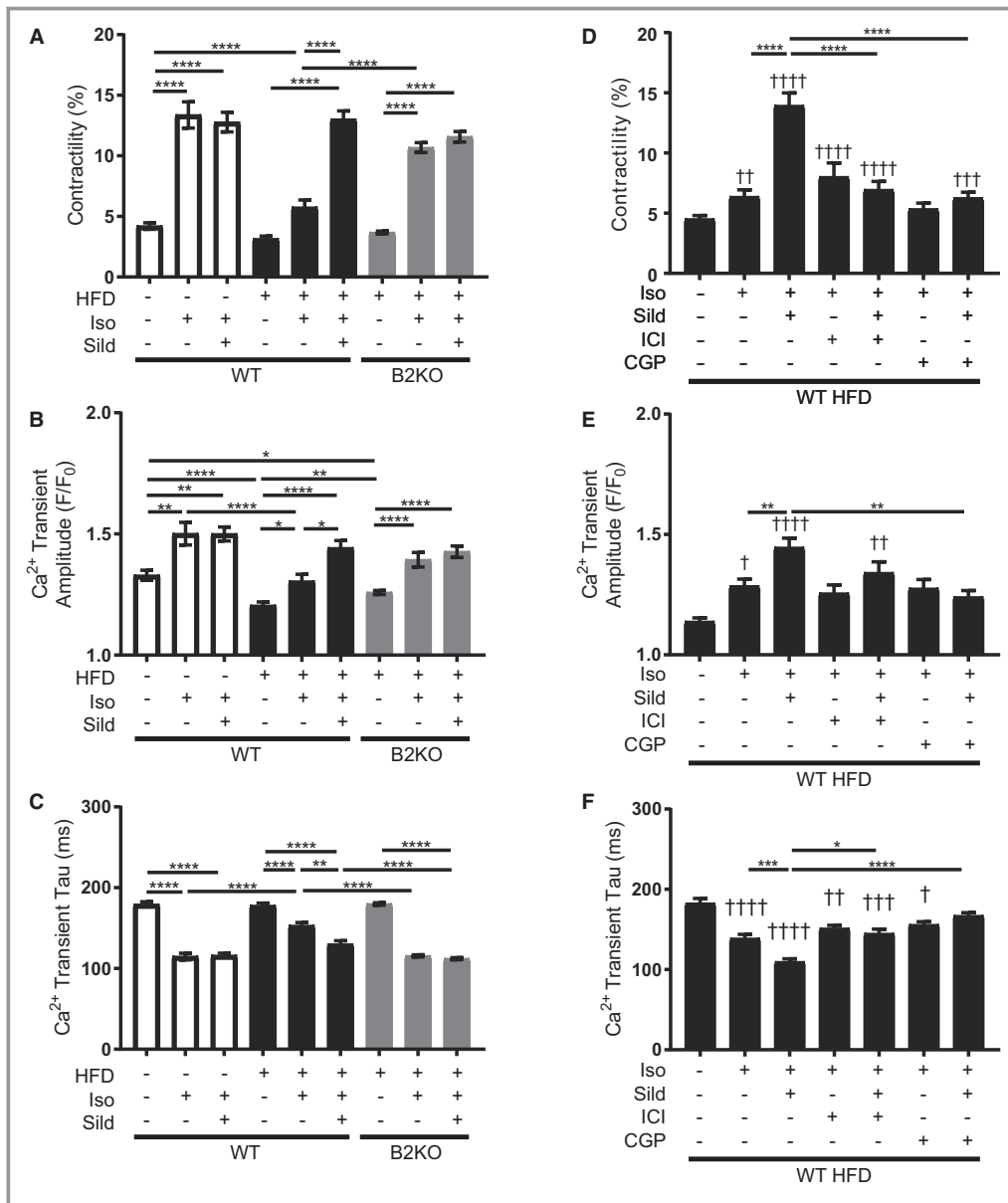


Figure 6. Sildenafil acts through β_2 adrenergic receptor (β_2 AR) to improve adrenergic excitation-contraction coupling response in high fat diet (HFD) adult ventriculomyocytes (AVMs). **A**, Contractility, **B** calcium transient amplitude, and **C** calcium transient tau of AVMs from wildtype (WT) and β_2 AR knockout (β_2 KO) mice fed NC or HFD for 4.5 months and treated ex vivo with drugs, as indicated. $n \geq 20$ cells per group. **D**, Contractility, **E** calcium transient amplitude, and **F** calcium transient tau of AVMs from WT mice fed HFD for 4.5 months. Cells were pretreated with and without ICI-118551 (ICI) or CGP-20712 (CGP) before stimulation with isoproterenol and/or sildenafil. $n \geq 25$ cells per group. For all graphs, $^\dagger P \leq 0.05$, $^\ddagger P \leq 0.01$, $^\ddagger\ddagger P \leq 0.001$, $^\ddagger\ddagger\ddagger P \leq 0.0001$ compared with basal; and $* P \leq 0.05$, $** P \leq 0.01$, $*** P \leq 0.001$, $**** P \leq 0.0001$ by 1-way ANOVA and Tukey post-test.

subcellular cyclic nucleotide signaling will be highly informative to understanding diabetic-related heart disease. In healthy individuals, β_2 AR is found mostly in the T-tubule structures of the cardiomyocytes.⁵⁵ During HF and to a lesser extent during aging, T-tubules shrink in size,^{56,57} with the structural proteins important for T-tubule maintenance, junctophilin and caveolin, being reduced in expression.^{57,58} As this

structural change occurs, β_2 ARs move from the shrinking T-tubules to the crest of the cell, which can interact with different proteins, leading to changes in signal propagation.⁵⁵ Meanwhile, β_1 AR but not β_2 AR expression is reduced during HF; the ratio of β_2 AR: β_1 AR increases. While β_2 AR signaling does not trigger changes in E-C coupling in healthy cardiomyocytes, it plays a significant role in modulating E-C coupling

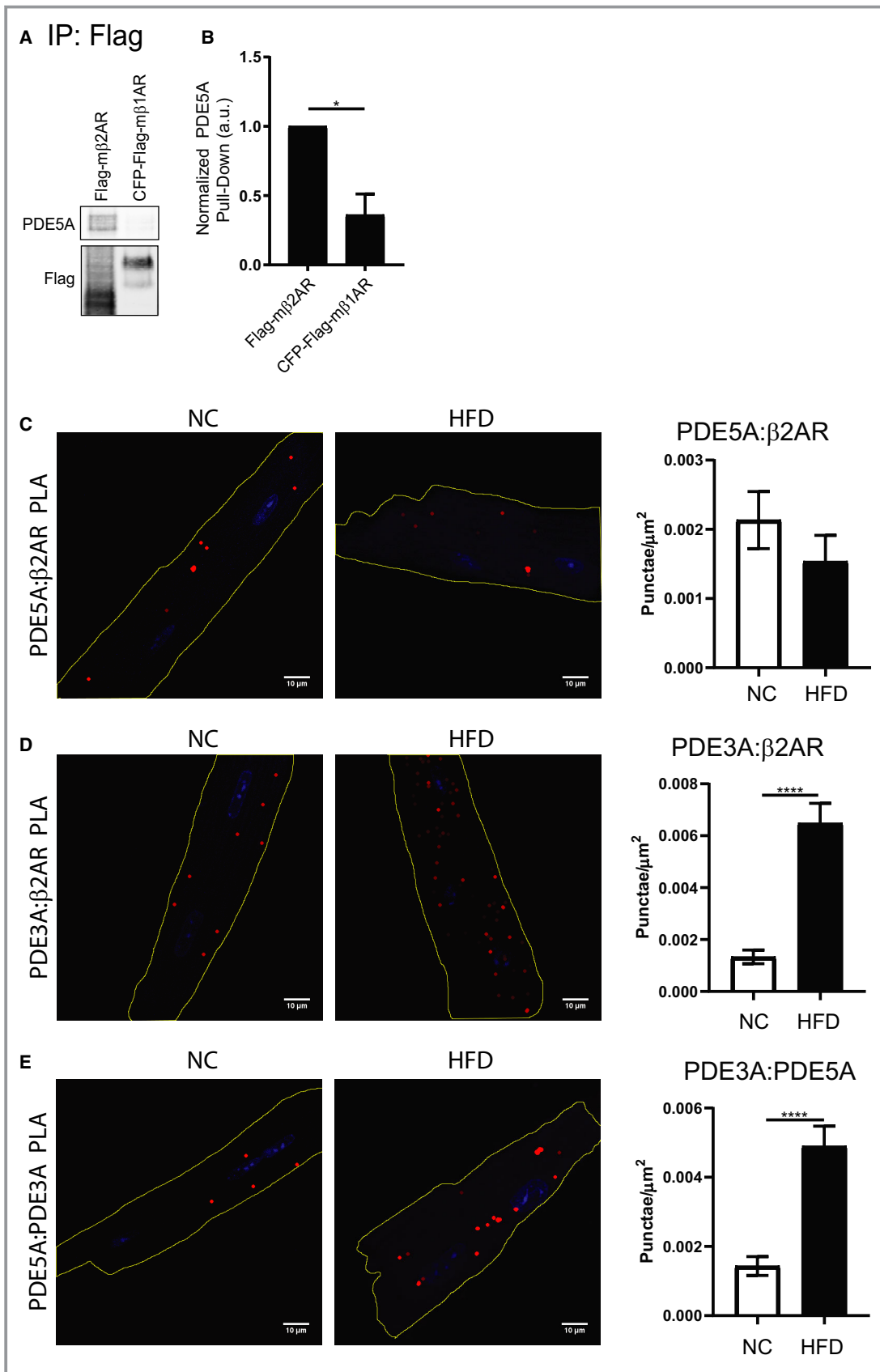


Figure 7. Phosphodiesterase 3 (PDE3) displays an increased association with the phosphodiesterase 5 (PDE5)– β_2 adrenergic receptor (β_2 AR) protein complex in high fat diet (HFD) cardiomyocytes. **A**, Representative images and **(B)** quantification of coimmunoprecipitation of Flag-tagged β ARs from rabbit adult ventriculomyocytes (AVMs) expressing Flag-m β_2 AR or CFP (cyan fluorescent protein) – and Flag-tagged mouse β_1 adrenergic receptor (β_1 AR), with detection of PDE5A. $n=3$ rabbits. * $P\leq 0.05$ by unpaired t test. Proximity ligation assay (PLA) representative images and quantification in normal chow (NC) and HFD AVMs for the interaction between PDE5A and β_2 AR (**C**), between PDE3A and β_2 AR (**D**), and between PDE3A and PDE5A (**E**). Scale bar=10 μ m. Red=PLA signal. Blue=DAPI. $n=18$ cells per group. **** $P\leq 0.0001$ by unpaired t test.

dynamics and arrhythmogenesis in HF.⁵⁹ Future studies exploring the development- and age-related changes in β AR/phosphodiesterase coupling will be informative in developing more effective treatment strategies, especially for pediatric patients with HF, where there is growing evidence that PDE3 inhibition has varying outcomes based on age.^{60,61}

In the current study, sildenafil significantly enhanced cGMP signal induced by isoproterenol stimulation in both neonatal and HFD cardiomyocytes. However, sildenafil did not affect cGMP signal induced by isoproterenol stimulation in young

adult 8-week-old and 6-month-old NC cardiomyocytes despite the presence of the β_2 AR/PDE5 complex. This is juxtaposed to data depicting that addition of sildenafil to isoproterenol causes a decrease in adrenergically stimulated E-C coupling in 8-week-old myocytes while it causes no change in 6-month-old NC myocytes. The discrepancy between signaling and contractile function at different ages is currently not understood. Nevertheless, these data underlie the complex and dynamic changes of β AR complex during aging and disease development and indicate that PDE5 may switch association to different PDEs such as PDE3 to modulate specific pools of

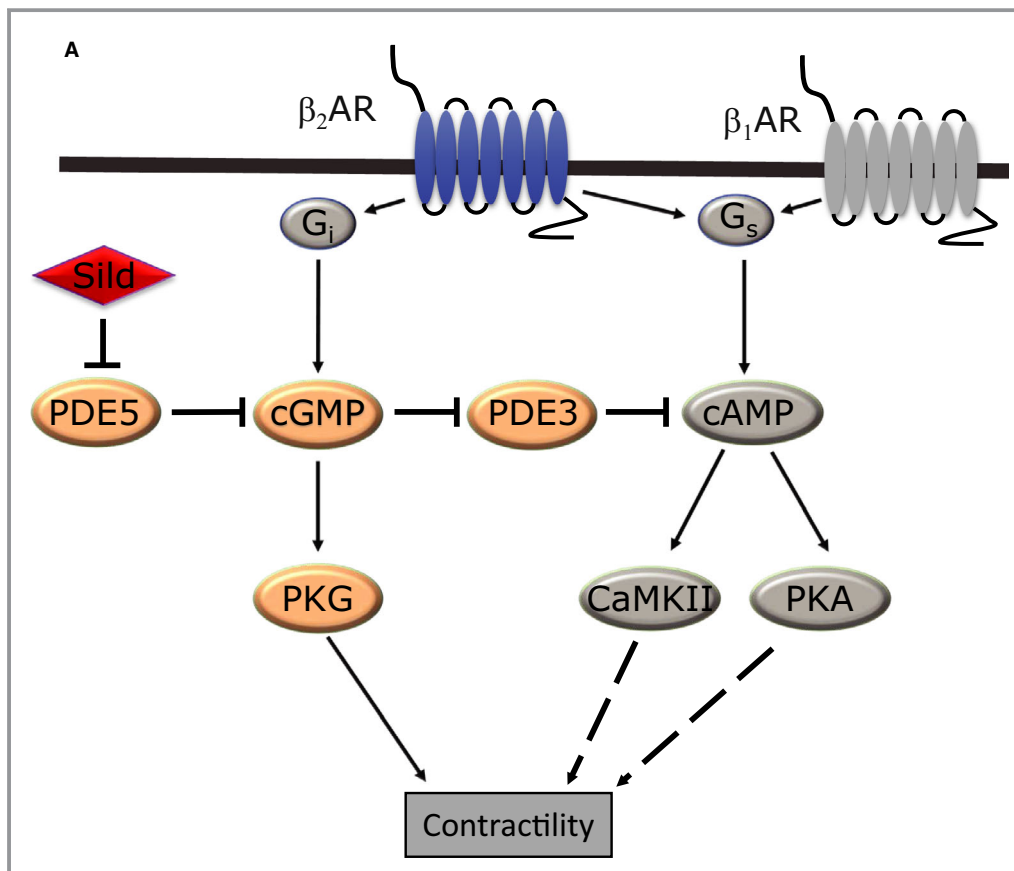


Figure 8. Inhibition of phosphodiesterase 5 (PDE5) by sildenafil improves contractility in a β_2 adrenergic receptor (β_2 AR)–dependent mechanism **(A)** depiction of the pathway sildenafil uses in mice fed a high fat diet to improve function. CaMKII indicates calcium/calmodulin kinase II; G_i , inhibitory G protein; G_s , stimulatory G protein; PKA, protein kinase A; PKG, protein kinase G; PDE3, phosphodiesterase 3; β_1 AR, β_1 adrenergic receptor.

cyclic nucleotides to affect E-C coupling in cardiomyocytes. Future study into where these pools are located, what PDEs control them, and how they change with age will be able to identify possible new targets for pharmacological intervention in pediatric HF care.

Of note, despite the increases in cAMP signal in diabetic cardiomyocytes, both CaMKII and PKA are only marginally involved in sildenafil's effect on isoproterenol-induced cardiomyocyte contractility. Future study into the roles that PKA and CaMKII activity play in diabetic cardiomyopathy may provide insight into changes in E-C coupling protein expression profile.⁶² In contrast, PKG is the major downstream kinase involved in sildenafil-induced enhancement of E-C coupling in HFD AVMs after isoproterenol stimulation. Our data indicate that the increased PKG activity for phosphorylation of myofilament proteins may contribute to the improvement of E-C coupling in HFD myocytes, consistent with previous studies in various HF models.⁶³

Conclusions

Our data are consistent with previous studies showing that the benefits of the PDE5 inhibitor are dependent on activation of NO-cGcGMP-PKG signaling cascades in diabetic-related cardiac diseases.^{22,23} In those studies, inhibition of PDE5 leads to potentiation of NO-cGMP signaling cascades and subsequent pleiotropic effects in the myocardium ranging from reduced hypertrophy, altered cytoskeletal gene expression, enhanced myofilament relaxation, improved mitochondrial function, and reduced oxidative stresses, as well as anti-inflammatory and apoptotic responses during ischemic stress.^{18–21} Here, we reveal an additional benefit of PDE5 inhibition in increasing cardiac E-C coupling. Moreover, we have revealed a novel network linking PDE5 to PDE3, as well as cardiac β_2 AR, indicating that some of the previously reported beneficial effects of PDE5 inhibition may be the result of modulation by adrenergic stimulation and also dependent on increases in cAMP signal in diabetic hearts. Future study into the link among β_2 AR, PDE5, and PDE3 may shed light on how cyclic nucleotide levels affect development of diabetic cardiomyopathy. Together, our data provide a novel PDE5- β_2 AR axis as a promising treatment target for improving heart function in diabetes mellitus.

Acknowledgments

We would like to thank Mark Jaradeh in Dr Donald M. Bers's laboratory at the University of California, Davis, for isolating rabbit AVMs for our use. We thank Dr Sakthivel Sadayappan from the University of Cincinnati for the generous gift of cMyBP-C antibodies and Dr Vincent Manganiello (deceased) from NIH for the generous gift of PDE3A antibody.

Author Contributions

West, Barbagallo, Isidori, and Xiang conceived the experimental design. West, Deng, and Zhang isolated mouse AVMs, bred the needed mice, and cared for mice within the study. West, Wang and, Reddy performed myocyte contraction, calcium cycling, and FRET assays. Xiang, Xu, and West performed coimmunoprecipitations. West performed Western blots, stained histology samples, and performed echocardiogram assays. Chen analyzed contractility and calcium cycling data. Phan performed histological analysis. Xiang provided overall supervision of the study. West and Xiang procured funding for the study and wrote the original article. West and Xiang had full access to all of the data in the study and take responsibility for its integrity and the data analysis.

Sources of Funding

This study was supported by NIH grants R01-HL127764 and R01-HL112413 (Xiang), and the US Veteran's Administration Merit grant 01BX002900 (Xiang). West is a recipient of the American Heart Association predoctoral fellowship. Xiang is an established American Heart Association investigator.

Disclosures

None.

References

1. Benjamin EJ, Virani SS, Callaway CW, Chang AR, Cheng S, Chiuve SE, Cushman M, Delling FN, Deo R, de Ferranti SD, Ferguson JF, Fornage M, Gillespie C, Isasi CR, Jimenez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Lutsey PL, Matchar DB, Matsushita K, Mussolino ME, Nasir K, O'Flaherty M, Palaniappan LP, Pandey DK, Reeves MJ, Ritchey MD, Rodriguez CJ, Roth GA, Rosamond WD, Sampson UKA, Satou GM, Shah SH, Spartano NL, Tirschwell DL, Tsao CW, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS, Muntner P. Heart Disease and Stroke Statistics-2018 Update: A Report From the American Heart Association. *Circulation*. 2018;137:e67–e492.
2. Nassif M, Kosiborod M. Effect of glucose-lowering therapies on heart failure. *Nat Rev Cardiol*. 2018;15:282.
3. Lindman BR, Dávila-Román VG, Mann DL, McNulty S, Semigran MJ, Lewis GD, de las Fuentes L, Joseph SM, Vader J, Hernandez AF, Redfield MM. Cardiovascular phenotype in HFpEF patients with or without diabetes: a RELAX trial ancillary study. 2014;64:541–549.
4. Scirica BM, Bhatt DL, Braunwald E, Steg PG, Davidson J, Hirshberg B, Ohman P, Frederich R, Wiviott SD, Hoffman EB, Cavender MA, Udell JA, Desai NR, Mosenzon O, McGuire DK, Ray KK, Leiter LA, Raz I. Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. *N Engl J Med*. 2013;369:1317–1326.
5. Monami M, Dicembrini I, Mannucci E. Dipeptidyl peptidase-4 inhibitors and heart failure: a meta-analysis of randomized clinical trials. *Nutr Metab Cardiovasc Dis*. 2014;24:689–697.
6. Simpson SH, Majumdar SR, Tsuyuki RT, Eurich DT, Johnson JA. Dose-response relation between sulfonylurea drugs and mortality in type 2 diabetes mellitus: a population-based cohort study. *Can Med Assoc J*. 2006;174:169–174.
7. Aguilar D, Deswal A, Ramasubbu K, Mann DL, Bozkurt B. Comparison of patients with heart failure and preserved left ventricular ejection fraction among those with versus without diabetes mellitus. *Am J Cardiol*. 2010;105:373–377.
8. MacDonald MR, Petrie MC, Varyani F, Östergren J, Michelson EL, Young JB, Solomon SD, Granger CB, Swedberg K, Yusuf S, Pfeffer MA, McMurray JJV; and

- for the CI. Impact of diabetes on outcomes in patients with low and preserved ejection fraction heart failure: an analysis of the Candesartan in Heart failure: assessment of Reduction in Mortality and morbidity (CHARM) programme. *Eur Heart J*. 2008;29:1377–1385.
9. Owan TE, Hodge DO, Herges RM, Jacobsen SJ, Roger VL, Redfield MM. Trends in prevalence and outcome of heart failure with preserved ejection fraction. *N Engl J Med*. 2006;355:251–259.
 10. Eskesen K, Olsen NT, Dimaano VL, Fritz-Hansen T, Sogaard P, Chakir K, Steenbergen C, Kass D, Abraham TP. Sildenafil treatment attenuates ventricular remodeling in an experimental model of aortic regurgitation. *SpringerPlus*. 2015;4:592.
 11. Nagayama T, Hsu S, Zhang M, Koitabashi N, Bedja D, Gabrielson KL, Takimoto E, Kass DA. Sildenafil stops progressive chamber, cellular, and molecular remodeling and improves calcium handling and function in hearts with pre-existing advanced hypertrophy caused by pressure overload. *J Am Coll Cardiol*. 2009;53:207–215.
 12. Prisyazhna O, Burgoyne J, Scotcher J, Grover S, Kass D, Eaton P. Phosphodiesterase 5 inhibition limits doxorubicin-induced heart failure by attenuating protein kinase G α oxidation. *J Biol Chem*. 2016;291:17427–17436.
 13. Giannetta E, Isidori AM, Galea N, Carbone I, Mandosi E, Vizza CD, Naro F, Morano S, Fedele F, Lenzi A. Chronic inhibition of cGMP phosphodiesterase 5A improves diabetic cardiomyopathy: a randomized, controlled clinical trial using magnetic resonance imaging with myocardial tagging. *Circulation*. 2012;125:2323–2333.
 14. Santi D, Giannetta E, Isidori AM, Vitale C, Aversa A, Simoni M. Therapy of endocrine disease. Effects of chronic use of phosphodiesterase inhibitors on endothelial markers in type 2 diabetes mellitus: a meta-analysis. *Eur J Endocrinol*. 2015;172:R103–R114.
 15. Wang H, Anstrom K, Ilkayeva O, Muehlbauer MJ, Bain JR, McNulty S, Newgard CB, Kraus WE, Hernandez A, Felker GM, Redfield M, Shah SH. Sildenafil treatment in heart failure with preserved ejection fraction: targeted metabolomic profiling in the RELAX trial. *JAMA Cardiol*. 2017;2:896–901.
 16. Redfield MM, Chen HH, Borlaug BA, Semigran MJ, Lee KL, Lewis G, LeWinter MM, Rouleau JL, Bull DA, Mann DL, Deswal A, Stevenson LW, Givertz MM, Ofili EO, O'Connor CM, Felker GM, Goldsmith SR, Bart BA, McNulty SE, Ibarra JC, Lin G, Oh JK, Patel MR, Kim RJ, Tracy RP, Velazquez EJ, Anstrom KJ, Hernandez AF, Mascette AM, Braunwald E. Effect of phosphodiesterase-5 inhibition on exercise capacity and clinical status in heart failure with preserved ejection fraction: a randomized clinical trial. *JAMA*. 2013;309:1268–1277.
 17. Koitabashi N, Aiba T, Hesketh GG, Rowell J, Zhang M, Takimoto E, Tomaselli GF, Kass DA. Cyclic GMP/PKG-dependent inhibition of TRPC6 channel activity and expression negatively regulates cardiomyocyte NFAT activation: novel mechanism of cardiac stress modulation by PDE5 inhibition. *J Mol Cell Cardiol*. 2010;48:713–724.
 18. Koka S, Aluri HS, Xi L, Lesnfsky EJ, Kukreja RC. Chronic inhibition of phosphodiesterase 5 with tadalafil attenuates mitochondrial dysfunction in type 2 diabetic hearts: potential role of NO/SIRT1/PGC-1 α signaling. *Am J Physiol Heart Circ Physiol*. 2014;306:H1558–H1568.
 19. Koka S, Das A, Salloum FN, Kukreja RC. Phosphodiesterase-5 inhibitor tadalafil attenuates oxidative stress and protects against myocardial ischemia/reperfusion injury in type 2 diabetic mice. *Free Radic Biol Med*. 2013;60:80–88.
 20. Koka S, Xi L, Kukreja RC. Chronic treatment with long acting phosphodiesterase-5 inhibitor tadalafil alters proteomic changes associated with cytoskeletal rearrangement and redox regulation in Type 2 diabetic hearts. *Basic Res Cardiol*. 2012;107:249.
 21. Varma A, Das A, Hoke NN, Durrant DE, Salloum FN, Kukreja RC. Anti-inflammatory and cardioprotective effects of tadalafil in diabetic mice. *PLoS One*. 2012;7:e45243.
 22. Mátyás C, Németh BT, Oláh A, Török M, Ruppert M, Kellermayer D, Barta BA, Szabó G, Kökény G, Horváth EM, Bódi B, Papp Z, Merkely B, Radovits T. Prevention of the development of heart failure with preserved ejection fraction by the phosphodiesterase-5A inhibitor vardenafil in rats with type 2 diabetes. *Eur J Heart Fail*. 2017;19:326–336.
 23. Radovits T, Bömicke T, Kökény G, Arif R, Loganathan S, Kécsán K, Korkmaz S, Barnucz E, Sandner P, Karck M, Szabó G. The phosphodiesterase-5 inhibitor vardenafil improves cardiovascular dysfunction in experimental diabetes mellitus. *Br J Pharmacol*. 2009;156:909–919.
 24. Lee DI, Zhu G, Sasaki T, Cho GS, Hamdani N, Holeywinski R, Jo SH, Danner T, Zhang M, Rainer PP, Bedja D, Kirk JA, Ranek MJ, Dostmann WR, Kwon C, Margulies KB, Van Eyk JE, Paulus WJ, Takimoto E, Kass DA. Phosphodiesterase 9A controls nitric-oxide-independent cGMP and hypertrophic heart disease. *Nature*. 2015;519:472.
 25. Takimoto E, Belardi D, Tocchetti CG, Vahebi S, Cormaci G, Ketner EA, Moens AL, Champion HC, Kass DA. Compartmentalization of cardiac β -adrenergic inotropy modulation by phosphodiesterase type 5. *Circulation*. 2007;115:2159–2167.
 26. Lee DI, Vahebi S, Tocchetti CG, Barouch LA, Solaro RJ, Takimoto E, Kass DA. PDE5A suppression of acute β -adrenergic activation requires modulation of myocyte beta-3 signaling coupled to PKG-mediated troponin I phosphorylation. *Basic Res Cardiol*. 2010;105:337–347.
 27. Isidori AM, Cornacchione M, Barbagallo F, Di Grazia A, Barrios F, Fassina L, Monaco L, Giannetta E, Gianfrilli D, Garofalo S, Zhang X, Chen X, Xiang YK, Lenzi A, Pellegrini M, Naro F. Inhibition of type 5 phosphodiesterase counteracts β_2 -adrenergic signalling in beating cardiomyocytes. *Cardiovasc Res*. 2015;106:408–420.
 28. Wang Q, Liu Y, Fu Q, Xu B, Zhang Y, Kim S, Tan R, Barbagallo F, West T, Anderson E, Wei W, Abel ED, Xiang YK. Inhibiting insulin-mediated beta2-adrenergic receptor activation prevents diabetes-associated cardiac dysfunction. *Circulation*. 2017;135:73–88.
 29. Fu Q, Xu B, Liu Y, Parikh D, Li J, Li Y, Zhang Y, Riehle C, Zhu Y, Rawlings T, Shi Q, Clark RB, Chen X, Abel ED, Xiang YK. Insulin inhibits cardiac contractility by inducing a G(i)-biased β_2 -adrenergic signaling in hearts. *Diabetes*. 2014;63:2676–2689.
 30. Fu Q, Xu B, Parikh D, Cervantes D, Xiang YK. Insulin induces IRS2-dependent and GRK2-mediated β_2 AR internalization to attenuate β AR signaling in cardiomyocytes. *Cell Signal*. 2015;27:707–715.
 31. Zhao CY, Greenstein JL, Winslow RL. Interaction between phosphodiesterases in the regulation of the cardiac β -adrenergic pathway. *J Mol Cell Cardiol*. 2015;88:29–38.
 32. Qi Y, Xu Z, Zhu Q, Thomas C, Kumar R, Feng H, Dostal DE, White MF, Baker KM, Guo S. Myocardial loss of IRS1 and IRS2 causes heart failure and is controlled by p38 α MAPK during insulin resistance. *Diabetes*. 2013;62:3887–3900.
 33. Zhang X, Szeto C, Gao E, Tang M, Jin J, Fu Q, Makarewich C, Ai X, Li Y, Tang A, Wang J, Gao H, Wang F, Ge XJ, Kunapuli SP, Zhou L, Zeng C, Xiang KY, Chen X. Cardiotoxic and cardioprotective features of chronic β -adrenergic signaling. *Circ Res*. 2013;112:498–509.
 34. Pogwizd SM. Nonreentrant mechanisms underlying spontaneous ventricular arrhythmias in a model of nonischemic heart failure in rabbits. *Circulation*. 1995;92:1034–1048.
 35. Zhou YY, Wang SQ, Zhu WZ, Chruscinski A, Kobilka BK, Ziman B, Wang S, Lakatta EG, Cheng H, Xiao RP. Culture and adenoviral infection of adult mouse cardiac myocytes: methods for cellular genetic physiology. *Am J Physiol Heart Circ Physiol*. 2000;279:H429–H436.
 36. Russwurm M, Mullershausen F, Friebe A, Jäger R, Russwurm C, Koesling D. Design of fluorescence resonance energy transfer (FRET)-based cGMP indicators: a systematic approach. *Biochem J*. 2007;407:69–77.
 37. Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, Sharff KA, Luu HH, Haydon RC, Kinzler KW, Vogelstein B, He TC. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. *Nat Protoc*. 2007;2:1236.
 38. Beca S, Ahmad F, Shen W, Liu J, Makary S, Polidovitch N, Sun J, Hockman S, Chung YW, Movsesian M, Murphy E, Manganiello V, Backx PH. Phosphodiesterase type 3A regulates basal myocardial contractility through interacting with sarcoplasmic reticulum calcium ATPase type 2a signaling complexes in mouse heart. *Circ Res*. 2013;112:289–297.
 39. Sadayappan S, Gulick J, Osinska H, Barefield D, Cuello F, Avkiran M, Lasko VM, Lorenz JN, Maillet M, Martin JL, Brown JH, Bers DM, Molkenin JD, James J, Robbins J. A critical function for Ser-282 in cardiac myosin binding protein-C phosphorylation and cardiac function novelty and significance. *Circ Res*. 2011;109:141–150.
 40. Pereira L, Cheng H, Lao DH, Na L, van Oort RJ, Brown JH, Wehrens XH, Chen J, Bers DM. Epac2 mediates cardiac β_1 -adrenergic dependent SR Ca(2+) leak and arrhythmia. *Circulation*. 2013;127:913–922.
 41. Reddy GR, West TM, Jian Z, Jaradeh M, Shi Q, Wang Y, Chen-Izu Y, Xiang YK. Illuminating cell signaling with genetically encoded FRET biosensors in adult mouse cardiomyocytes. *J Gen Physiol*. 2018;150:1567–1582.
 42. Allen MD, Zhang J. Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochem Biophys Res Commun*. 2006;348:716–721.
 43. Klarenbeek J, Goedhart J, van Batenburg A, Groenewald D, Jalink K. Fourth-generation Epac-based FRET sensors for cAMP feature exceptional brightness, photostability and dynamic range: characterization of dedicated sensors for FLIM, for ratiometry and with high affinity. *PLoS One*. 2015;10:e0122513.
 44. Takao K, Okamoto K-I, Nakagawa T, Neve RL, Nagai T, Miyawaki A, Hashikawa T, Kobayashi S, Hayashi Y. Visualization of synaptic Ca₂₊/calmodulin-dependent protein kinase II activity in living neurons. *J Neurosci*. 2005;25:3107–3112.

45. DiPilato LM, Zhang J. The role of membrane microdomains in shaping [small beta]2-adrenergic receptor-mediated cAMP dynamics. *Mol Biosyst.* 2009;5:832–837.
46. Sadayappan S, Osinska H, Klevitsky R, Lorenz JN, Sargent M, Molkenin JD, Seidman CE, Seidman JG, Robbins J. Cardiac myosin binding protein c phosphorylation is cardioprotective. *Proc Natl Acad Sci USA.* 2006;103:16918–16923.
47. Fu Q, Shi Q, West TM, Xiang YK. Cross-talk between insulin signaling and G protein-coupled receptors. *J Cardiovasc Pharmacol.* 2017;70:74–86.
48. Richter W, Day P, Agrawal R, Bruss MD, Granier S, Wang YL, Rasmussen SGF, Horner K, Wang P, Lei T, Patterson AJ, Kobilka B, Conti M. Signaling from β_1 - and β_2 -adrenergic receptors is defined by differential interactions with PDE4. *EMBO J.* 2008;27:384–393.
49. Ding B, Abe JI, Wei H, Huang Q, Walsh RA, Molina CA, Zhao A, Sadoshima J, Blaxall BC, Berk BC, Yan C. Functional role of phosphodiesterase 3 in cardiomyocyte apoptosis: implication in heart failure. *Circulation.* 2005;111:2469–2476.
50. Degerman E, Belfrage P, Manganiello VC. Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). *J Biol Chem.* 1997;272:6823–6826.
51. Kokkonen K, Kass DA. Nanodomain regulation of cardiac cyclic nucleotide signaling by phosphodiesterases. *Annu Rev Pharmacol Toxicol.* 2017;57:455–479.
52. Stangherlin A, Gesellchen F, Zoccarato A, Terrin A, Fields LA, Berrera M, Surdo NC, Craig MA, Smith G, Hamilton G, Zaccolo M. cGMP signals modulate cAMP levels in a compartment-specific manner to regulate catecholamine-dependent signaling in cardiac myocytes. *Circ Res.* 2011;108:929–939.
53. Perera RK, Sprenger JU, Steinbrecher JH, Hubscher D, Lehnart SE, Abesser M, Schuh K, El-Armouche A, Nikolaev VO. Microdomain switch of cGMP-regulated phosphodiesterases leads to ANP-induced augmentation of beta-adrenoceptor-stimulated contractility in early cardiac hypertrophy. *Circ Res.* 2015;116:1304–1311.
54. Barbagallo F, Xu B, Reddy GR, West T, Wang Q, Fu Q, Li M, Shi Q, Ginsburg KS, Ferrier W, Isidori AM, Naro F, Patel HH, Bossuyt J, Bers D, Xiang YK. Genetically encoded biosensors reveal PKA hyperphosphorylation on the myofilaments in rabbit heart failure. *Circ Res.* 2016;119:931–943.
55. Nikolaev VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, Lohse MJ, Korchev YE, Harding SE, Gorelik J. β_2 -adrenergic receptor redistribution in heart failure changes cAMP compartmentation. *Science.* 2010;327:1653–1657.
56. Guo A, Zhang C, Wei S, Chen B, Song LS. Emerging mechanisms of T-tubule remodelling in heart failure. *Cardiovasc Res.* 2013;98:204–215.
57. Kong CHT, Bryant SM, Watson JJ, Gadeberg HC, Roth DM, Patel HH, Cannell MB, Orchard CH, James AF. The effects of aging on the regulation of T-tubular ICa by caveolin in mouse ventricular myocytes. *J Gerontol A Biol Sci Med Sci.* 2018;73:711–719.
58. Minamisawa S, Oshikawa J, Takeshima H, Hoshijima M, Wang Y, Chien KR, Ishikawa Y, Matsuoka R. Junctophilin type 2 is associated with caveolin-3 and is down-regulated in the hypertrophic and dilated cardiomyopathies. *Biochem Biophys Res Commun.* 2004;325:852–856.
59. DeSantiago J, Ai X, Islam M, Acuna G, Ziolo MT, Bers DM, Pogwizd SM. Arrhythmogenic effects of $\beta(2)$ -adrenergic stimulation in the failing heart are due to enhanced SR Ca load. *Circ Res.* 2008;102:1389–1397.
60. Price JF, Towbin JA, Dreyer WJ, Moffett BS, Kertesz NJ, Clunie SK, Denfield SW. Outpatient continuous parenteral inotropic therapy as bridge to transplantation in children with advanced heart failure. *J Card Fail.* 2006;12:139–143.
61. Berg AM, Snell L, Mahle WT. Home inotropic therapy in children. *J Heart Lung Transplant.* 2007;26:453–457.
62. Drews O, Tsukamoto O, Liem D, Streicher J, Wang Y, Ping P. Differential regulation of proteasome function in isoproterenol-induced cardiac hypertrophy. *Circ Res.* 2010;107:1094–1101.
63. Rainer PP, Kass DA. Old dog, new tricks: novel cardiac targets and stress regulation by protein kinase G. *Cardiovasc Res.* 2016;111:154–162.