

Supplemental Material

Supplemental Materials and Methods

Histology

Cardiac tissue was embedded in paraffin after overnight fixation in 4 % paraformaldehyde, sectioned in 10 μ m slices, and stained with hematoxylin/eosin or Masson's trichrome to analyze myocardial interstitial fibrosis. Serial mid-LV sections per heart was quantified at the level of papillary muscle using computer-assisted image analysis (Adobe Photoshop version 5.0). Cardiac tissue was also stained with wheat germ agglutinin (WGA, Alexa Fluor 488 conjugated) (1) as previously described to assess cell hypertrophy. The images were captured with fluorescence microscope and cardiomyocyte cross sectional area (CSA) was quantified with 400-600 cells per heart, using NIH Image J.

Confocal immunocytochemistry

Neonatal rat ventricular myocytes (NRVMs) were isolated, fixed, and stained for confocal microscopy as described previously (2). Cells were plated on imaging dishes and incubated in DMEM for adenovirus infection and for stimulation with ET1 as described in cell culture study. Cells were then fixed with ice-cold 50% v/v methanol and 50% v/v acetone, permeabilized with 0.1% saponin in PBS, and blocked in FBS. After incubation with primary antibodies (anti-FLAG; anti-PDE5A) at 4°C overnight, cells were subsequently incubated with secondary antibodies (Alexa Fluor 488-conjugated goat anti-mouse; Alexa Fluor 594-conjugated goat anti-rabbit). Imaging was performed on Zeiss confocal laser scanning microscope (UltraView; Perkin Elmer Life Science Inc.). Densitometry line scan analysis was performed by NIH Image J.

Quantitative real-time PCR

The isolation of total RNA from heart tissues or cardiac myocytes was carried out with TRIZOL Reagent (Invitrogen) followed by chloroform-isopropyl alcohol precipitation. Total RNA was reverse transcribed into cDNA with a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Life technologies), following the instruction. cDNA was subjected to PCR amplification using SYBR Green primers with the following specific sequences: mouse Nppb 5'-AAGTCCTAGCCAGTCTCCAGA-3'(forward) and 5'-GAGCTGTCTCTGGGCCATTTTC-3'(reverse); rat Nppb 5'-ACAATCCACGATGCAGAAGCT-3'(forward) and 5'-GGGCCTTGGTCCTTTGAGA-3'(reverse); rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'-GACATGCCGCCTGGAGAAAC-3'(forward) and 5'-AGCCCAGGATGCCCTTTAGT-3'(reverse). For TaqMan method, the probes for mouse Rcan1 (Mm01213407_m1), mouse GAPDH (Mm99999915_m1), rat Rcan1 (Rn00596606_m1) were used to evaluate the respective gene expression levels. The threshold cycle (Ct) value, which was determined using crossing point method, was normalized to GAPDH (Applied Biosystems, California, U.S.A) Ct value in each run.

Protein analysis

Lysates were prepared with lysis buffer (Cell Signaling Technology) from snap-frozen heart tissue or cardiac myocytes isolated from adult mice or neonatal rats. For non-reducing SDS PAGE, 100 mM N-ethyl maleimide (SIGMA) was added to the lysis buffer to prevent thiol oxidation during sample preparation. Protein concentration was measured by BCA method (Pierce) and SDS sample buffer was mixed with or without adding DTT to prepare samples under reducing or non-reducing condition, respectively (3). All protein extracts were run on Novex Tris-Glycine Gels (Life Technologies), then blotted onto

nitrocellulose membranes, and probed with each specific primary antibody such as PKG1 α (#13511, Cell Signaling Technology), monoclonal anti-FLAG M2 antibody (#14793, Cell Signaling Technology), phospho Ser92 PDE5A (GTX36930, GeneTex), or total-PDE5A (#2395, Cell Signaling Technology). Antibody binding was visualized with an infrared imaging system (Odyssey, Licor) and quantification of band intensity was performed using Odyssey Application Software 3.0. The percentage of dimeric PKG1 α was calculated by dividing the band intensity of disulfide dimer with the sum of dimer+monomer band intensities taken from the same gel lane.

PKG and PDE5 activity assay

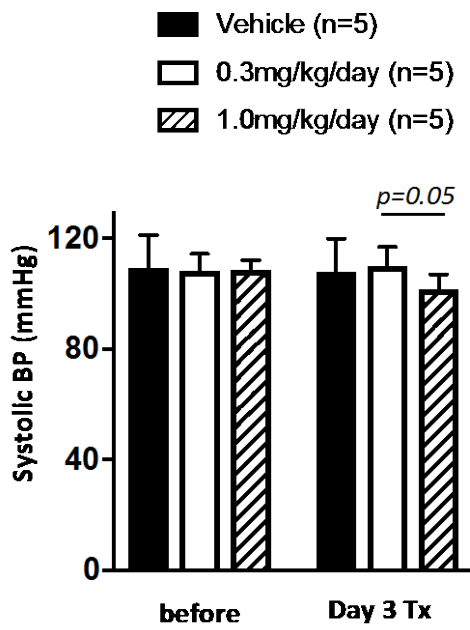
In vitro PKG activity was assayed in cell lysates either from heart tissues or neonatal rat ventricular myocytes which were pretreated with cGMP in the presence or absence of H₂O₂. The amount of phosphorylated PKG substrate was specifically measured by colorimetry (CycLex) as described (2). While this assay can detect the activity due to other kinases, we have shown it correlates with in-cell and tissue activity as indexed by PKG-phosphorylation proteins such as VASP (4), and is correspondingly altered (in either direction) when cGMP and thus PKG activity are specifically changed in cells and the intact heart in directions opposite to those of other kinases(5). PDE5 activity was assessed by fluorescence polarization assay under linear conditions with or without PDE5A inhibitor (sildenafil 1 μ M), following manufacturer's instructions (Molecular Devices). For myocyte cGMP assay, heart tissues were homogenized in trichloroacetic acid. Then, cyclic nucleotides were extracted with water-saturated ether. The aqueous phase was vacuum dried and the pellet was resuspended in sodium acetate buffer just before the measurement of intracellular cGMP level by cGMP enzyme immune assay system (Amersham Pharmacia Biotech).

PKG1 α -PDE5A Pull-down assay

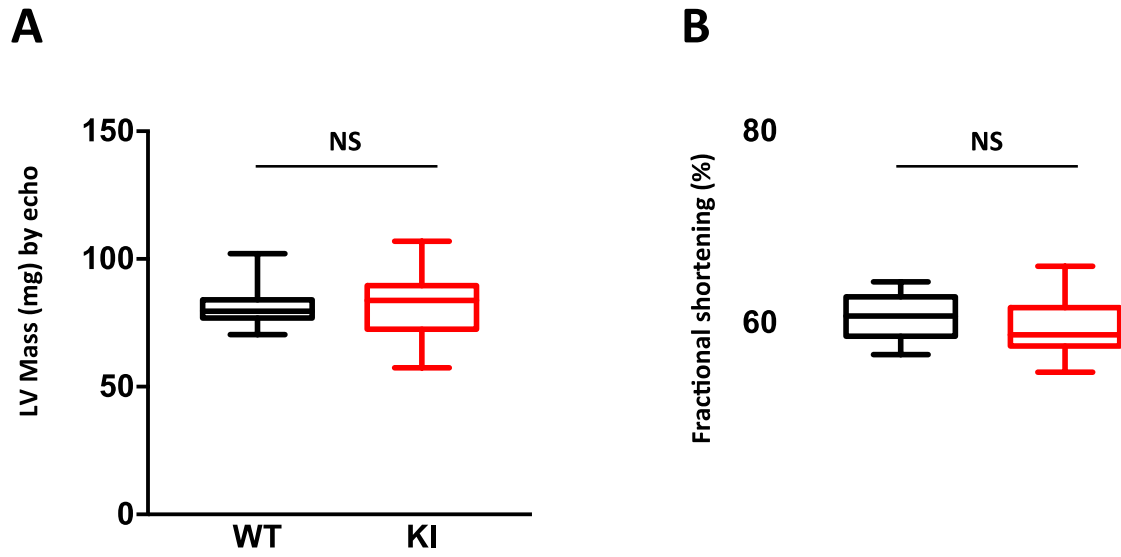
For assessing the effect of PKG oxidation on PKG-PDE5 interaction, NRVMs exogenously expressing either FLAG-tagged PKG1 α ^{WT} or PKG1 α ^{C42S} were treated with 8-Br-cGMP (100 μ M) for 20 min in the presence of H₂O₂ (10 μ M). A whole cell lysate prepared from infected NRVMs was incubated in mouse monoclonal anti-FLAG M2 antibody (F3165, Sigma-Aldrich) at 4°C with rotation. PKG antigen-FLAG antibody complexes were then incubated with pre-washed PureProteome Protein G magnetic beads (Millipore) following the manufacture's protocol. Prior to gel loading, the pre-formed PKG-containing complex was dissociated by suspension in a SDS sample buffer without adding DTT and magnetic beads were then removed. The eluted supernatant was used for the specific immunoprecipitation to detect endogenous PDE5 which combines with FLAG-tagged PKG1 α . The exogenous PKG1 α was then reverse blotted with rabbit monoclonal of anti-FLAG M2 antibody (#14793, Cell Signaling Technology) by using same western blot membranes.

1. Zhang M, Takimoto E, Lee DI et al. Pathological cardiac hypertrophy alters intracellular targeting of phosphodiesterase type 5 from nitric oxide synthase-3 to natriuretic peptide signaling. *Circulation*, 2012;942-51.
2. Nakamura T, Ranek MJ, Lee DI et al. Prevention of PKG1 α oxidation augments cardioprotection in the stressed heart. *J Clin Invest*, 2015:2468-72.
3. Burgoyne JR, Madhani M, Cuello F et al. Cysteine redox sensor in PKG1 α enables oxidant-induced activation. *Science*, 2007:1393-7.
4. Takimoto E, Belardi D, Tocchetti CG et al. Compartmentalization of cardiac beta-adrenergic inotropy modulation by phosphodiesterase type 5. *Circulation* 2007;115:2159-67.
5. Zhang M, Takimoto E, Hsu S et al. Myocardial remodeling is controlled by myocyte-targeted gene regulation of phosphodiesterase type 5. *J Am Coll Cardiol* 2010;56:2021-30.

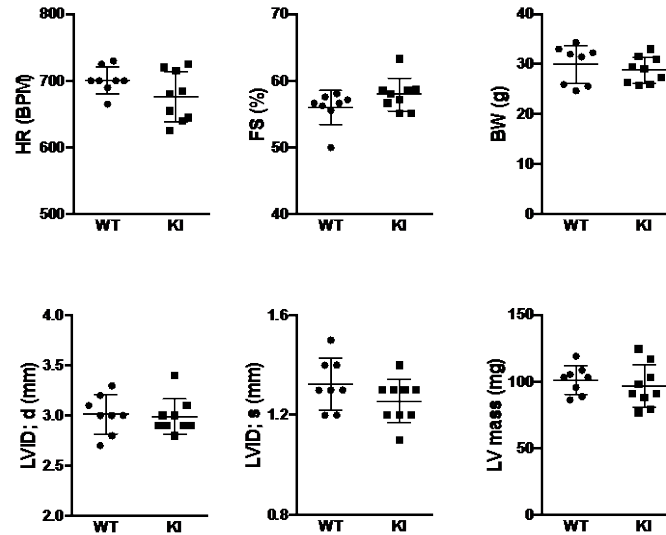
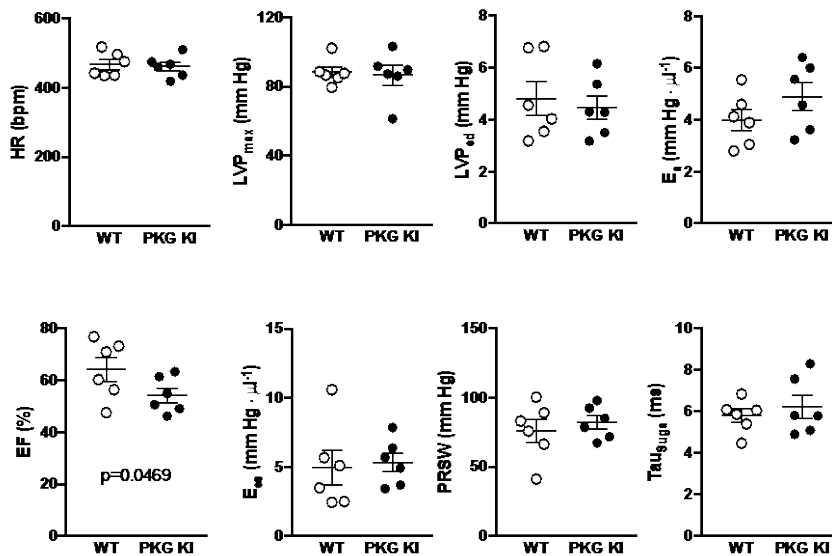
SUPPLEMENTAL FIGURES AND LEGENDS



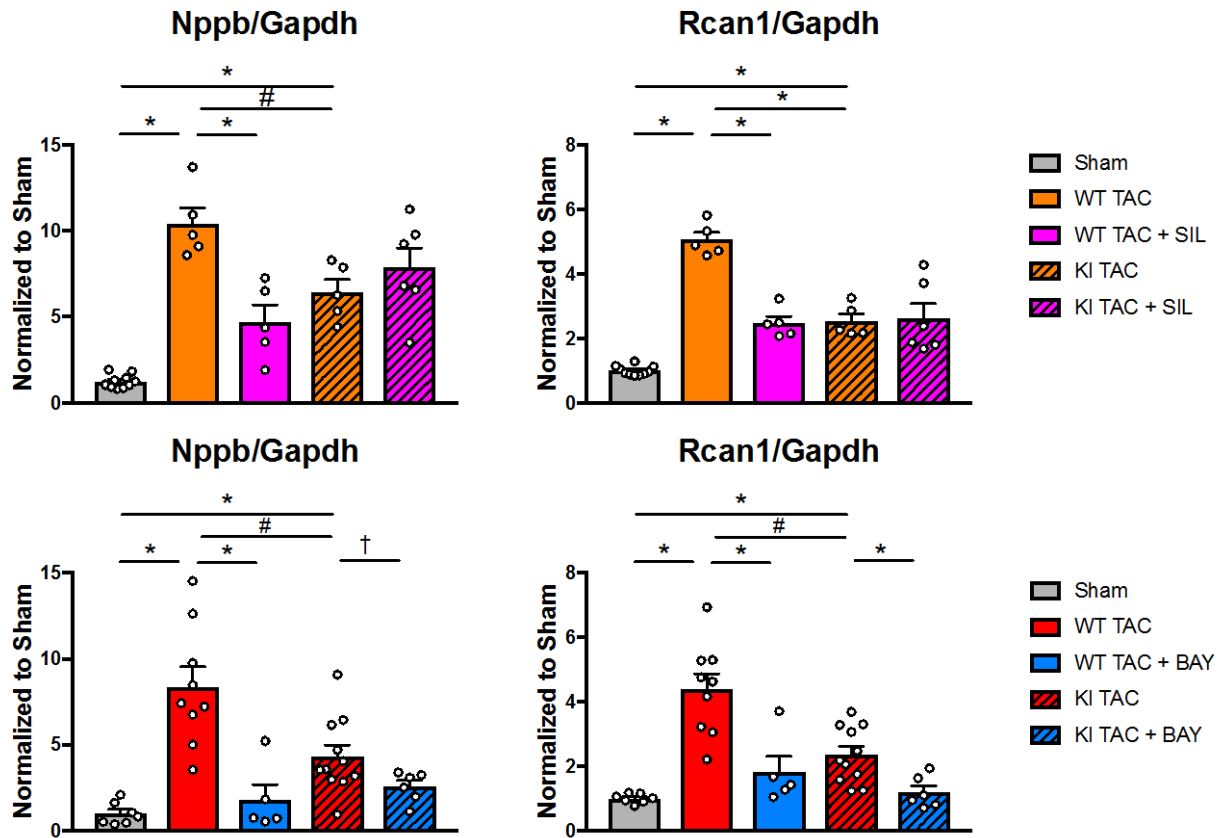
Supplemental Figure 1. Systolic blood pressure from tail-cuff monitor system in mice treated with vehicle, and two different doses of sGC activator BAY602770. The dose of 0.3 mg/kg/day was selected as the highest where no effect on blood pressure was observed.



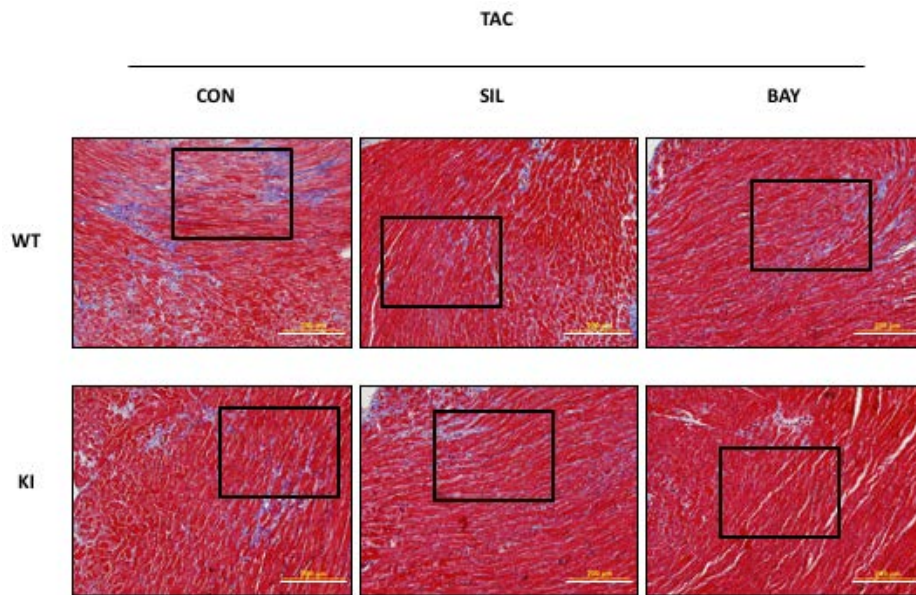
Supplemental Figure 2. Baseline left ventricular mass and fractional shortening in hearts from $\text{PKG1}\alpha^{\text{WT}}$ litter mate controls and $\text{PKG1}\alpha^{\text{C42S}}$ knock-in mice. These data are from mice at age 3-4 months.

A**B**

Supplemental Figure 3. Baseline cardiac function and morphology derived from echocardiography (A) and pressure-volume loop analysis (B) in PKG1 α ^{WT} controls and PKG1 α ^{C42S} knock-in mice measured at more than one year of age. None of the parameters were significantly different between the two groups. HR, heart rate; FS, fractional shortening; BW, body weight; LVD(d,s), diastolic and systolic LV diastolic dimension; LVP (max,ed), maximal and end-diastolic LV pressure; E_a, effective arterial elastance (total afterload); E_{es}, end-systolic elastance (contractility); PRSW, preload recruitable stroke work; and Tau (suga), time constant of relaxation.

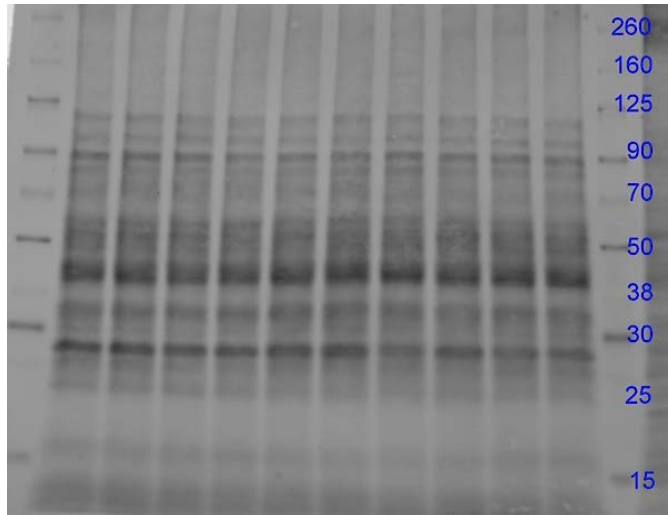


Supplemental Figure 4. Myocardial gene expression for natriuretic peptide type B (Nppb) and regulator of calcineurin-1 (Rcan1) each normalized to Gapdh for intact heart studies with hearts subjected to TAC +/- sildenafil (SIL) or sGC stimulation (BAY). Data for both mice expressing WT PKG1 α and the C42S KI mutation are shown. Both SIL and BAY were effective in suppressing these hypertrophic molecular markers in WT, but only BAY had an impact in mice with the C42S PKG1 α mutant. * $p < 0.001$; # $p < 0.01$; † $p = 0.07$ by Dunnett's post hoc test.

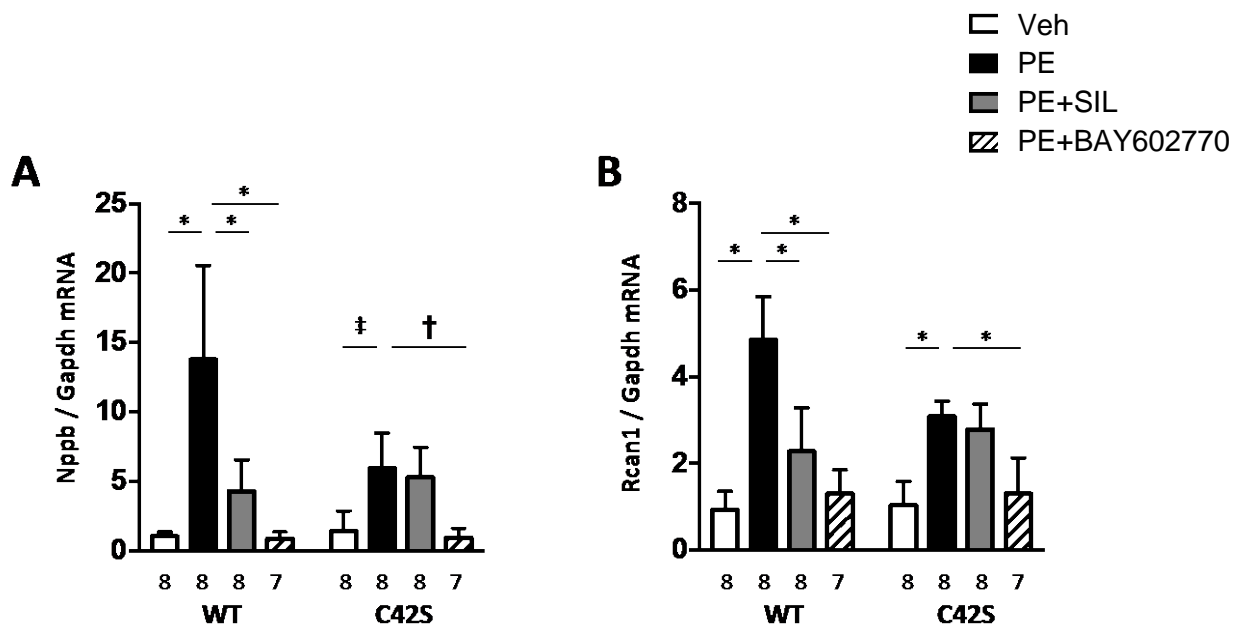


Supplemental Figure 5. Low magnification for Masson-Trichrome staining in Figure 1E.

Higher magnified panels in Figure 1E are identified by the square. Scale bar = 200 μ m

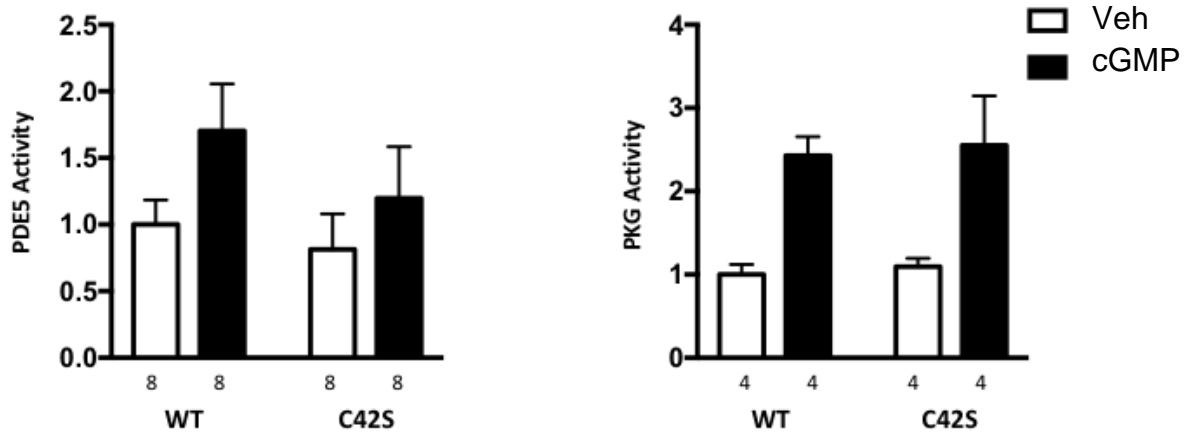


Supplemental Figure 6. Total protein staining in PDE5A expression gel displayed in Figure 2C. Gel demonstrates equal loading.



Supplemental Figure 7. Isolated myocytes expressing either PKG1 α ^{WT} or PKG1 α ^{C42S}, exposed to 48 hours of phenylephrine stimulation PE, PE + sildenafil (SIL), PE + BAY 60-2770, or vehicle (Veh). As shown in Figure 3A and 3B, hypertrophic gene expression increased by PE was diminished by both SIL and BAY in cells expressing the WT form of PKG1 α , but only BAY was effective in cells expressing the C42S redox dead mutation.

* p<0.001, † p<0.005; ‡p<0.05 (Tukey, post hoc test to 1-way ANOVA). Sample per group is shown.



Supplemental Figure 8. Activity levels of PDE5 and PKG in NRVMs exposed to cGMP in the absence of H₂O₂. A) cGMP treatment increased PDE5 activity in cells expressing either PKG^{WT} or PKG^{C42S}; p=0.16 for interaction of cGMP x genotype; <0.0001 for cGMP effect by two-way ANOVA. B) Treatment with cGMP also increased PKG activity similarly in two genotypes; p>0.5 for interaction of cGMP x genotype and genotype effect; <0.0001 for cGMP effect.

Supplemental Table

Results of multiple factor analysis of variance for data displayed in Figure 1A-D. For each variable, results for SIL or BAY treatments were obtained in two separate cohorts. To adjust for this, cohort is encoded by a categorical variable, and then experimental condition (Sham, TAC, TAC (BAY), or TAC (SIL)) as the second categorical variable. Table presents the pairwise probabilities for the condition, each ANOVA performed within genotype group (WT PKG1 α , or C42S PKG1 α).

WT PKG1 α

Left Ventricular Mass (echocardiography)

	SHAM	TAC	TAC (SIL)	TAC (BAY)
SHAM	1			
TAC	<0.001	1		
TAC (SIL)	<0.001	<0.001	1	
TAC (BAY)	0.052	<0.001	0.6	1

Fractional Shortening (echocardiography)

	SHAM	TAC	TAC (SIL)	TAC (BAY)
SHAM	1			
TAC	<0.001	1		
TAC (SIL)	0.14	<0.001	1	
TAC (BAY)	0.54	<0.001	0.97	1

End-Systolic Dimension (echocardiography)

	SHAM	TAC	TAC (SIL)	TAC (BAY)
SHAM	1			
TAC	<0.001	1		
TAC (SIL)	0.49	<0.002	1	
TAC (BAY)	0.98	0.001	0.90	1

Heart Weight/Body Weight (Post Mortem)

	SHAM	TAC	TAC (SIL)	TAC (BAY)
SHAM	1			
TAC	<0.001	1		
TAC (SIL)	<0.001	<0.001	1	
TAC (BAY)	<0.001	<0.001	0.99	1

C42S PKG1 α

Left Ventricular Mass (echocardiography)

	SHAM	TAC	TAC (SIL)	TAC (BAY)
SHAM	1			
TAC	<0.001	1		
TAC (SIL)	<0.001	0.33	1	
TAC (BAY)	0.43	<0.01	0.002	1

Fractional Shortening (echocardiography)

	SHAM	TAC	TAC (SIL)	TAC (BAY)
SHAM	1			
TAC	<0.001	1		
TAC (SIL)	<0.001	0.31	1	
TAC (BAY)	0.98	0.005	0.001	1

End-Systolic Dimension (echocardiography)

	SHAM	TAC	TAC (SIL)	TAC (BAY)
SHAM	1			
TAC	0.012	1		
TAC (SIL)	0.005	0.82	1	
TAC (BAY)	0.055	<0.001	0.05	1

Heart Weight/Body Weight (Post Mortem)

	SHAM	TAC	TAC (SIL)	TAC (BAY)
SHAM	1			
TAC	<0.001	1		
TAC (SIL)	<0.001	0.62	1	
TAC (BAY)	0.05	0.001	0.004	1