Molecular Cardiology

Protein Kinase G Positively Regulates Proteasome-Mediated Degradation of Misfolded Proteins

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Background—Proteasome functional insufficiency is implicated in a large subset of cardiovascular diseases and may play an important role in their pathogenesis. The regulation of proteasome function is poorly understood, hindering the development of effective strategies to improve proteasome function.

Methods and Results—Protein kinase G (PKG) was manipulated genetically and pharmacologically in cultured cardiomyocytes. Activation of PKG increased proteasome peptidase activities, facilitated proteasome-mediated degradation of surrogate (enhanced green fluorescence protein modified by carboxyl fusion of degron CL1) and bona fide (CryAB<sup>R120G</sup>) misfolded proteins, and attenuated CryAB<sup>R120G</sup> overexpression–induced accumulation of ubiquitinated proteins and cellular injury. PKG inhibition elicited the opposite responses. Differences in the abundance of the key 26S proteasome subunits Rpt6 and β5 between the PKG-manipulated and control groups were not statistically significant, but the isoelectric points were shifted by PKG activation. In transgenic mice expressing a surrogate substrate (GFPdgn), PKG activation by sildenafil increased myocardial proteasome activities and significantly decreased myocardial GFPdgn protein levels. Sildenafil treatment significantly increased myocardial PKG activity and significantly reduced myocardial accumulation of CryAB<sup>R120G</sup>, ubiquitin conjugates, and aberrant protein aggregates in mice with CryAB<sup>R120G</sup>-based desmin-related cardiomyopathy. No discernible effect on bona fide native substrates of the ubiquitin-proteasome system was observed from PKG manipulation in vitro or in vivo.

Conclusions—PKG positively regulates proteasome activities and proteasome-mediated degradation of misfolded proteins, likely through posttranslational modifications to proteasome subunits. This may be a new mechanism underlying the benefit of PKG stimulation in treating cardiac diseases. Stimulation of PKG by measures such as sildenafil administration is potentially a new therapeutic strategy to treat cardiac proteinopathies. (Circulation. 2013;128:365-376.)

Key Words: cardiomyopathies ■ cyclic GMP-dependent protein kinase ■ desmin ■ proteasome inhibitors ■ proteins

The ubiquitin-proteasome system (UPS) mediates the degradation of most intracellular proteins and regulates diverse cellular processes, including protein quality control. UPS-mediated proteolysis generally involves 2 steps: targeting of the substrate protein by ubiquitination and the subsequent degradation of the ubiquitinated protein by the 26S proteasome. The UPS is responsible for the removal of terminally misfolded proteins; however, the UPS, particularly the proteasome, can be overwhelmed by increased production of misfolded proteins, as observed in proteinopathy and ischemia/reperfusion injury, which causes proteasome functional insufficiency (PFI) and protein quality control inadequacy. Inadequate protein quality control causes cellular dysfunction and cell death. Adult cardiomyocytes are particularly vulnerable to the toxic effects of misfolded proteins because of their poor regenerative capability. The majority of failing human hearts with hypertrophic, dilated, or ischemic cardiomyopathy display increases in ubiquitinated proteins and abnormal protein aggregation in the form of, for example, preamyloid oligomer formation from unknown proteins and often decreased proteasome activities. These seminal findings implicate an important role for PFI in the progression of a large subset of cardiovascular diseases to congestive heart failure in humans. Indeed, experimental studies have revealed that PFI may play an important role in the development of at least a subset of heart diseases such as desmin-related cardiomyopathy (DRC) and myocardial ischemia/reperfusion injury. Hence, the improvement of proteasome function has potential as a new therapeutic strategy in the treatment of heart disease. However, adopting this strategy is hindered by the lack of an effective means to enhance proteasome function.

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Increased cGMP production and the resultant activation of cGMP-dependent protein kinase (PKG) has been demonstrated to reverse preexisting hypertrophy while inhibiting hypertrophic pathways.\textsuperscript{15,16} It remains untested whether PKG regulates UPS-mediated degradation of misfolded proteins. The present study tests the hypothesis that PKG activation in cardiomyocytes enhances proteasome function and facilitates the degradation of misfolded proteins. Our results support the hypothesis and suggest that stimulating PKG may become a therapeutic strategy to treat heart diseases with increased proteotoxic stress.

Methods

Animal Models
Protocols for animal care and use in this study were approved by the University of South Dakota Institutional Animal Care and Use Committee. The creation and validation of a transgenic (tg) mouse model expressing GFP\textsuperscript{dgn} were reported.\textsuperscript{17} GFP\textsuperscript{dgn} is a slightly shorter version of enhanced green fluorescence protein (GFP) modified by carboxyl fusing of degron CL.1 (GFPu).\textsuperscript{18} GFPu and GFP\textsuperscript{dgn} are surrogate for misfolded proteins and have proven to be UPS substrates.\textsuperscript{3,17} The line 708 tg mouse model expressing a missense mutant (R120G) of human PKG1 was created via deletion of the autoinhibitory domain of PKG1.\textsuperscript{21} The line 708 tg mouse model expressing a missense mutant (R120G) of human PKG1 was subcloned into a pShuttle-cytomegalovirus vector, which was used by ViraQest Inc and Biochrom Corporation (Baltimore, MD) following the manufacturer’s instructions and culturing as described.\textsuperscript{21} Adenoviruses harboring the expression cassette for β-crystallin (CryABR120G) was described.\textsuperscript{19} CryABR120G and GFP\textsuperscript{dgn} tg mice were maintained in the FVB/N inbred background. Genotypes were determined with polymerase chain reaction analysis.

Mouse Sildenafil Treatment
Age-matched male GFP\textsuperscript{dgn} mice received 2 consecutive intraperitoneal injections of sildenafil (10 mg/kg every 12 hours) or vehicle control. At 12 hours after the second injection, ventricular myocardium was sampled for total RNA isolation, protein extraction, and histological assessments. A cohort of age-matched (≈10 weeks old) line 708 male CryABR120G tg and non-tg (Ntg) littermate mice received chronic sildenafil infusion (10 mg·kg\textsuperscript{−1}·d\textsuperscript{−1}) via subcutaneous miniosmotic pumps (Alzet 2002 model) for 4 weeks before terminal experiments. The dose was determined from previous reports.\textsuperscript{15,20} Serial echocardiography was performed on this cohort as previously described.\textsuperscript{14}

Recombinant Adenoviruses Infection of Cultured Neonatal Rat Ventricular Myocytes
Neonatal rat ventricular myocytes (NRVMs) were isolated with the Cellutron Neomyocytes isolation system (Cellutron Life Technology, Farmingdale, NY). Neonatal rat ventricular myocytes (NRVMs) were isolated with the Cellutron Neomyocytes isolation system (Cellutron Life Technology, Farmingdale, NY). The cell culture medium was DMEM supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.

RNA Interference
The small interference RNA (siRNA) specific for rat PKG (PKG siRNA: 5’-AAGGTAACCGTACCCGAAGAA-3’) was purchased from Qiagen (Valencia, CA). The siRNA transfection using the Lipofectamine 2000 transfection reagent (Invitrogen) was generally started 24 to 48 hours after NRVMs were plated. The same amounts of luciferase siRNA (as control) and PKG siRNA (100 pmol) were applied to 2×10\textsuperscript{6} NRVMs.

Total Protein Extraction and Western Blot Analysis
Proteins were extracted from ventricular myocardium or cultured NRVMs. Protein concentration was determined with the use of bicinchoninic acid reagents (Pierce Biotechnology, Rockford, IL). SDS-PAGE, immunoblotting analysis, and densitometry were performed as described.\textsuperscript{21}

Reverse Transcription–Polymerase Chain Reaction
Total RNA isolated from ventricular myocardium was used for reverse transcription–polymerase chain reaction to assess GFP\textsuperscript{dgn} mRNA levels as described.\textsuperscript{14}

Cycloheximide Chase Assay
This assay was performed as described previously.\textsuperscript{13} Cycloheximide (100 μmol/L; Sigma-Aldrich) was used to block further protein synthesis.

PKG Activity Assay
A PKG activity assay kit from Cycled (catalog No. CY-1161; Nagano, Japan) was used to determine myocardial PKG activities.\textsuperscript{15}

Soluble/Insoluble Protein Extraction and Filter-Trap Assay
Extraction was done as described.\textsuperscript{14} Briefly, ventricular myocardium tissue was homogenized in PBS (pH 7.4) containing 2% Triton X-100, 5 mmol/L EDTA, 1 mmol/L phenylmethanesulfonfyl fluoride, and the protease inhibitor cocktail (Roche) and centrifuged at 10 000g in 4°C for 10 minutes. The soluble fraction (supernatant) was collected. The pellet was resuspended in 1× loading buffer (40 mmol/L Tris HCl [pH 8.8], 1% SDS, 8% glycerol), boiled for 5 minutes, and centrifuged at 3000g for 5 minutes; the supernatant was collected as the insoluble fraction. A total of 2.5 μg of the insoluble fraction proteins was filtered through nitrocellulose membrane (pore diameter, 0.22 μm, Millipore) with the use of a dot blot apparatus (BioRad) and immunoblotted for CryAB using mouse anti-6-CryAB antibodies (Enzo Life Sciences, Farmingdale, NY).

Fluorescence Confocal Microscopy
Microscopy was performed as described.\textsuperscript{21} Ventricular myocardium from GFP\textsuperscript{dgn} or CryABR120G tg mice was fixed with 3.8% paraformaldehyde and processed for obtaining 6-μm cryosections. The myocardial sections were stained with Alexa Fluor 568-conjugated phalloidin (Invitrogen) to reveal F-actin and to identify cardiomyocytes. CryAB-positive aggregates were stained with the rabbit anti-CryAB antibodies. GFP\textsuperscript{dgn} direct fluorescence (green) or CryAB immunofluorescence and the stained F-actin were visualized and imaged with a confocal microscope as described elsewhere.\textsuperscript{21}

Statistical Analysis
All continuous variables are presented as mean±SD. Unless otherwise indicated, differences between 2 groups were evaluated for statistical significance with a 2-tailed Student t test. When the difference among ≥3 groups was evaluated, 1-way ANOVA or, when appropriate, 2-way ANOVA followed by the Holm-Sidak test for pairwise comparisons was performed. A value of P<0.05 was considered statistically significant.

Results

PKG Manipulation Alters Proteasome-Mediated Proteolysis in Cardiomyocytes
We first tested the effect of PKG gain and loss of function on proteasome-mediated proteolysis in cultured NRVMs. Ser-239 of vasodilator-stimulated phosphoprotein (VASP)
is a well-established PKG target. As evidenced by the increased Ser-239–phosphorylated VASP (P-VASP; Figure I in the online-only Data Supplement), PKG gain of function was successfully achieved by either Ad-PKGcat infection or administration of the phosphodiesterase 5 inhibitor sildenafil, which raises cGMP levels and activates PKG. In Ad-GFPu– and Ad-RFP–coinfected cells, the ratio of GFPu to RFP was 50% to 60% lower in the PKG gain-of-function groups than in the respective controls (P<0.0001; Figure 1A and 1C), indicative of increased degradation of GFPu. Cycloheximide chase assays further showed that PKG activation shortened the half-life of GFPu by >50% (P<0.05; Figure 1B and 1D), demonstrating that PKG activation enhances UPS proteolytic function. Conversely, PKG loss of function in cultured NRVMs, which was achieved via siRNA-mediated PKG knockdown or treatment with KT5823 (Figure II in the online-only Data Supplement), increased the ratio of GFPu to RFP by a factor of 2.2 to 2.6 (P<0.0001) and elongated the half-life of GFPu proteins from <20 to >60 minutes (P<0.05; Figure III in the online-only Data Supplement), indicating that PKG activity is required for UPS-mediated degradation of a surrogate misfolded protein. Notably, PKG manipulation showed no discernible effect on the steady-state protein level of phosphatase and tensin homolog and β-catenin (Figure IV in the online-only Data Supplement), 2 bona fide native endogenous UPS substrates.

PKG Activation by Sildenafil Decreases GFPdgn Protein Levels in Mouse Hearts
To confirm in intact animals the findings from cultured NRVMs, we treated GFPdgn mice with sildenafil (10 mg/kg IP every 12 hours) for 24 hours and assessed cardiac GFPdgn expression. PKG activation was confirmed by increased P-VASP (P<0.0001; Figure 2A). Sildenafil treatment decreased myocardial GFPdgn protein levels by ≈50% (P<0.0001; Figure 2B) but showed no discernible effects on GFPdgn mRNA levels (P=0.545; Figure 2C). The decrease in GFPdgn in the cardiomyocyte compartment was confirmed by confocal microscopy (Figure 2D). These in vivo data confirm our in vitro findings that PKG activation by sildenafil stimulates UPS proteolytic function and facilitates the removal of a surrogate misfolded protein in cardiomyocytes.

PKG Stimulates Proteasome Peptidase Activities In Vivo and In Vitro
To probe whether PKG regulates the proteasome, we examined proteasome peptidase activities in sildenafil-treated GFPdgn mice and PKG-manipulated NRVMs. Sildenafil treatment increased myocardial ATP-dependent chymotrypsin-like activity by ≈50% (P<0.005) and ATP-independent and -dependent trypsin-like activities by ≈50% (P<0.01; Figure 3A). Compared with their respective controls, both
PKG\textsuperscript{cat} overexpression and sildenafil treatment caused significant increases in the ATP-dependent proteasomal chymotrypsin-like activity ($P<0.005$, $P<0.01$) and in the ATP-independent proteasomal trypsin-like activity in cultured NRVMs ($P<0.005$, $P<0.01$; Figure 3B and 3C). Conversely, PKG inhibition in cultured NRVMs by either PKG knockdown or KT5823 treatment decreased all 3 proteasome peptidase activities in both the absence and presence of ATP ($P<0.05$ or $P<0.01$; Figure V in the online-only Data Supplement).

Alterations of proteasome activities may result from altered proteasome abundance or posttranslational modifications. Thus, we assessed relative amounts of the Rpt6 of the 19S cap and of the $\beta_5$ subunit of the 20S core. No statistically significant changes in proteasome subunit abundance were detected from sildenafil-treated mouse hearts (Figure VI in the online-only Data Supplement) or PKG-manipulated cultured cardiomyocytes (Figure VII in the online-only Data Supplement), indicating that altered proteasome abundance is unlikely to be the underlying mechanism. Two-dimensional Western blot analyses from NRVMs infected with PKG\textsuperscript{cat} showed that the isoelectric point of a remarkable subpopulation of Rpt6 and $\beta_5$ displayed a shift toward the acidic side (Figure 3D), whereas PKG knockdown elicited a shift toward the basic end (Figure VIII in the online-only Data Supplement). These results indicate that posttranslational modifications, likely phosphorylation, of the proteasome may be a mechanism underlying PKG modulation of proteasomal function.

PKG Activation Enhances Proteasomal Degradation of a Human Disease-Linked Misfolded Protein

The results described above establish the ability of PKG to regulate proteasome-mediated proteolysis. Thus, we sought to further demonstrate the translational relevance of the newly discovered function of PKG by examining the impact of PKG manipulation on the removal of CryAB\textsuperscript{R120G}, a bona fide misfolded protein known to cause DRC in humans and mice.\textsuperscript{19,24} HA-CryAB\textsuperscript{R120G} was overexpressed in NRVMs via adenoviral gene delivery. Activation of PKG by PKG\textsuperscript{cat} overexpression or sildenafil significantly decreased the steady-state protein levels of CryAB\textsuperscript{R120G} (23 kDa) and its higher-molecular-weight modified species ($\approx$ 25–26 and $\approx$ 29–30 kDa; Figure 4A and 4B). The reduction of CryAB\textsuperscript{R120G} protein levels by PKG activation is proteasome dependent because the reduction was blocked in the presence of the proteasome inhibitor bortezomib (Figure 4C–4E). Conversely, PKG inhibition with PKG knockdown
or KT5823 increased the steady-state protein levels of CryAB\textsuperscript{R120G} and its higher-molecular-weight modified species (Figure IX in the online-only Data Supplement). Moreover, as revealed by the cycloheximide chase assay, PKG activation significantly shortened the CryAB\textsuperscript{R120G} protein half-life by >70% (P<0.01; Figure 4F and 4G). In contrast, PKG activation showed no discernible effects on the half-life of β-tubulin (Figure X in the online-only Data Supplement). These results further demonstrate that PKG activation enhances the proteasome-mediated degradation of misfolded but not native proteins.

Overexpression of misfolded proteins (eg, CryAB\textsuperscript{R120G}) causes PFI and accumulation of ubiquitinated proteins in cardiomyocytes. Here, we observed that CryAB\textsuperscript{R120G} overexpression–induced accumulation of ubiquitinated proteins in NRVMs was effectively attenuated by PKG activation via PKG\textsuperscript{cat} or sildenafil (P<0.005) and exacerbated by PKG inhibition with PKG knockdown or KT5823 (P<0.005; Figure 5). These findings support the conclusion that PKG positively regulates proteasome function and thereby facilitates the removal of misfolded proteins in cardiomyocytes.

**PKG Activation by Sildenafil Decreases Protein Aggregation and Slows Disease Progression in DRC Mice**

To test whether PKG activation by sildenafil can exert the same effect in mouse hearts as observed in cultured cardiomyocytes, we treated line 708 CryAB\textsuperscript{R120G} tg mice with sildenafil (10 mg kg\textsuperscript{-1} d\textsuperscript{-1}) for 4 weeks via miniosmotic pumps. PKG activation by sildenafil treatment was confirmed by the >100% higher myocardial PKG activity (P<0.005; Figure 6A) and ≈60% greater P-V ASP levels (P<0.01; Figure 6B and 6C) in the sildenafil-treated CryAB\textsuperscript{R120G} tg group than in the vehicle control CryAB\textsuperscript{R120G} tg group. Soluble and especially insoluble CryAB protein (Figure 6E–6H), but not mRNA levels (data not shown), in the CryAB\textsuperscript{R120G} tg hearts was significantly decreased by sildenafil treatment. Filter-trap assays showed that detergent-resistant aggregated CryAB proteins were increased by a factor of ≈16 in CryAB\textsuperscript{R120G} tg hearts compared with Ntg (P<0.005); these increases were attenuated by ≈65% by sildenafil treatment (P<0.005; Figure 7A and 7C). As expected, total high-molecular-weight ubiquitinated proteins (Figure 7B and 7D) and CryAB-positive protein

**Figure 3.** Protein kinase G (PKG) activation increases proteasome peptidase activities and decreases the isoelectric point (pl) of proteasome subunits. A, Changes in myocardial proteasome peptidase activities in GFPdgn mice treated with sildenafil as described in Figure 2. Crude protein extracts from ventricular myocardium were used for the indicated peptidase activity assays in presence or absence of ATP. B and C, Effects of PKG activation on proteasome peptidase activities in cultured neonatal rat ventricular myocytes (NRVMs). PKG activation by either forced expression of PKG\textsuperscript{cat} (B) or sildenafil treatment (C) in NRVMs was as described in Figure 1. Crude protein extracts from the cultured cells were used for proteasomal peptidase activity assays in the presence or absence of ATP. n=6 biological repeats. NS indicates not significant. *P<0.05; **P<0.01; ***P<0.005. D, Representative images of 2-dimensional Western blot analyses of Rpt6 and β5 proteasome subunits 72 hours after adenovirus (Ad)–PKG\textsuperscript{cat} or Ad–β-galactosidase (β-gal) infection. Isoelectric focusing gels (IFG) with a pH range from 7 to 10 were used for the first-dimension separation of proteins based on their pl. For the second-dimension separation based on molecular weights, the fully executed IFG was placed in a large center well that was flanked by regular small wells (arrowheads), which were used for 1-dimensional fractionation of the same source of protein samples used for the IFG. The 2-dimensional gel was transferred to polyvinylidene difluoride membrane and subjected to immunoblotting for the indicated proteins.
aggregates (Figure XI in the online-only Data Supplement) were significantly increased in CryABR120G tg hearts; importantly, these increases were remarkably attenuated by sildenafil treatment. These in vivo data provide compelling evidence that PKG activation by sildenafil facilitates CryABR120G removal and reduces aberrant protein aggregation, a key pathological process in disease with increased proteotoxic stress.

Serial echocardiography revealed that immediately before sildenafil treatment was initiated, there were no statistically significant differences in either end-diastolic left ventricle posterior wall thickness or fractional shortening among the 3 groups (Table I in the online-only Data Supplement). At the end of 4 weeks of treatment, compared with the vehicle-treated Ntg group, end-diastolic left ventricle posterior wall thickness was 18.2% greater (P<0.05) but fractional shortening was 15.3% smaller (P<0.05) in the vehicle-treated CryABR120G tg group; however, the differences in both end-diastolic left ventricle posterior wall thickness and fractional shortening between the sildenafil-treated tg and vehicle-treated Ntg groups were not statistically significant (P>0.05; Figure 7E and 7F). These results indicate that sildenafil treatment attenuates both cardiac hypertrophy and heart function decline in the DRC mice.

**PKG Activation Protects Cardiomyocytes From Proteotoxic Stress**

It has previously been shown that overexpression of CryABR120G in cardiomyocytes leads to cell injury, as manifested by increased cardiomyocyte death.25–27 As expected, we observed that overexpression of CryABR120G in cultured NRVMs led to increases in the level of cleaved (activated) caspase 3, significantly elevated leakage of lactate dehydrogenase to culture media (P<0.005), decreased survival of cardiomyocytes as revealed by MTT assays (P<0.005), and increased ratios of lactate dehydrogenase to MTT (P<0.005). Importantly, changes in all these parameters of cell injury were significantly attenuated by PKGcat overexpression or by treatment with sildenafil (P<0.05, P<0.005; Figure 8) and exacerbated by PKG inhibition achieved by...
PKG knockdown or KT5823 treatment (Figure XII in the online-only Data Supplement). These data show that PKG activation protects cardiomyocytes against proteotoxic stress triggered by overexpression of a bona fide misfolded protein.

Discussion

In the research field of UPS-mediated proteolysis, extensive attention was directed toward understanding the factors that control the ubiquitination of specific proteins. In contrast,
only a few reported studies have investigated whether and how proteasome function is regulated.2,28–30 However, increasing evidence suggests a role for PFI in the progression of at least a subset of heart diseases.5,11 Preserving proteasome function was shown to underlie cardiac protection of ischemic preconditioning.31,32 Previously, we experimentally demonstrated that improving proteasome-mediated degradation of misfolded proteins via a genetic approach alleviates cardiac pathological conditions characterized by an increased production of misfolded/damaged proteins.13,14 The present study demonstrates for the first time that PKG is an important regulator of proteasome-mediated proteolysis and that pharmacological activation of PKG by the phosphodiesterase 5 inhibitor sildenafil can stimulate proteasome activities, facilitate the clearance of a surrogate and a bona fide misfolded protein, and decrease aberrant protein aggregation in vitro and in vivo, thereby protecting cardiomyocytes against proteotoxic stress.

PKG Positively Regulates UPS-Mediated Degradation of Misfolded Proteins In Vitro and In Vivo

We have discovered that PKG activation by either genetic or pharmacological means was sufficient to destabilize a surrogate misfolded protein (GFPu) substrate of the UPS, whereas PKG inhibition stabilizes the GFPU proteins in cultured cardiomyocytes. Moreover, we have also shown that PKG activation by sildenafil treatment significantly decreases myocardial GFPdgn protein levels but displays no discernible effect on GFPdgn mRNA levels in GFPdgn tg mice. These findings suggest that PKG positively regulates the degradation of a misfolded protein by the UPS. We have further tested this postulate in both cultured cardiomyocytes and intact mice that overexpress CryABR120G, a bona fide misfolded protein known to cause human disease.24 We collected compelling evidence that PKG activation by either genetic or pharmacological methods facilitates whereas PKG inhibition by genetic and pharmacological means decreases the UPS-mediated degradation of CryABR120G in cultured NRVMs.

Sildenafil treatment showed no discernible effects on myocardial calpain activities in CryABR120G tg mice (Figure XIII in the online-only Data Supplement) or on the protein levels of the autophagosome marker LC3-II and a well-established autophagic substrate p62/SQSTM1 in GFPdgn mouse hearts (Figure XIV in the online-only Data Supplement). These data suggest that calpain and the autophagic-lysosomal pathway are unlikely to be involved in our observed effects.
UPS-mediated protein degradation takes 2 consecutive steps: ubiquitination and proteasomal cleavage. When the proteasome was inhibited, genetic PKG activation displayed no discernible effect on the levels of total ubiquitin conjugates in GFPu- or HA-CryABR120G–overexpressing NRVMs (Figure XV in the online-only Data Supplement), indicating that PKG activation does not appear to have a global effect on ubiquitination in cardiomyocytes. When GFPu or HA-CryABR120G proteins were probed in proteasome-inhibited NRVMs, we found that PKG activation showed no discernible effect on the levels of the higher-molecular-weight species of GFPu (P > 0.05) but significantly increased the level of multiple higher-molecular-weight species of CryABR120G (P < 0.005; Figure XVI in the online-only Data Supplement). These higher-molecular-weight CryABR120G species are likely ubiquitinated forms of CryABR120G; hence, these data suggest that PKG activation has differential effects on the ubiquitination of GFPu and CryABR120G species. Given that the UPS-mediated degradation of both GFPu and CryABR120G is facilitated by PKG activation, it is likely that facilitation of UPS-mediated misfolded protein degradation by PKG is due primarily to proteasome enhancement. This is consistent with the notion that the rate-limiting step for UPS-mediated degradation of misfolded proteins resides in the proteasome, not ubiquitination. This notion is supported by multiple lines of evidence. First, total ubiquitinated proteins are always increased in cells overexpressing misfolded proteins, as illustrated by the increases in ubiquitin conjugates in NRVMs overexpressing CryABR120G (Figure 5). Second, aberrant protein aggregation resulting from misfolded proteins impairs proteasome function, leading to PFI in vitro and in vivo. Finally, direct proteasomal enhancement via overexpression of a proteasome activator (PA28α) was sufficient to facilitate degradation of GFPdgn and CryABR120G and to protect against CryABR120G–based proteinopathy injury, demonstrating that PFI plays a major role in the genesis of CryABR120G–based cardiomyopathy. Here, we observed that while increasing proteasome function, PKG activation by PKGcat overexpression or sildenafil treatment significantly shortened the half-life of CryABR120G proteins, decreased the steady-state CryABR120G protein level, and attenuated the associated ubiquitin conjugate accumulation in cultured cardiomyocytes.

This is a highly significant discovery; currently, no pharmacological agent has been shown capable of increasing proteasome function and facilitating the removal of misfolded proteins in cardiomyocytes. The very favorable actions of PKG have the potential to be capitalized on to treat heart disease with increased proteotoxic stress. Indeed, we have further demonstrated in CryABR120G tg mice that long-term infusion of sildenafil activates PKG and decreases the soluble, the insoluble, and the detergent-resistant aggregated forms of CryAB, mimicking the previously reported effect of proteasomal enhancement by PA28α overexpression. Notably, PKG activation does not seem to alter the protein levels of bona fide native endogenous substrates of the UPS (eg, β-catenin and phosphatase and tensin homolog). These observations suggest that PKG stimulation activates selective pathways for misfolded protein degradation, making the said approach an attractive strategy to treat disease.

Potential Mechanisms by Which PKG Enhances Proteasome Function

The 26S proteasome is composed of a 20S proteolytic core (where the 3 peptidase activities reside) flanked at 1 or both

Figure 8. Protein kinase G (PKG) activation protects against proteotoxic stress in cardiomyocytes. PKG activation in neonatal rat ventricular myocytes (NRVMs) overexpressing CryABR120G was achieved by PKGcat overexpression (A and C) or sildenafil treatment (B and D), as described for Figure 5. The cultured cells were collected for Western blot analyses for caspase 3 and MTT assay, and the culture media were simultaneously collected for lactate dehydrogenase (LDH) assays. The LDH/MTT ratio is used to minimize the impact of potential variation resulting from the potential difference in the total cell number on a dish. β-Gal indicates β-galactosidase; and DMSO, dimethyl sulfoxide. *P < 0.05; **P < 0.01; ***P < 0.005.
ends by the 19S regulatory subcomplex. The 19S recognizes, binds to, and deubiquitinates polyubiquitinated proteins; unfolds them; and channels the unfolded polypeptide into the 20S. In the cell, both the 19S and 20S complexes can exist in free forms or associate with each other to form the 26S proteasome. The association of 19S with 20S requires ATP; hence, in vitro peptidase activity assays are often performed in the presence and absence of ATP to decipher the impact of the association of 19S proteasomes on individual peptidase activities. PKG inhibition by either PKG siRNA or KT5823 decreased all 3 proteasome peptidase activities in either the absence or presence of ATP, suggesting that a basal level of PKG activity is necessary for all proteasome peptidase activities. PKG gain of function did not show a discernible effect on caspase-like activities in vitro or in vivo. Interestingly, both overexpressing PKG and sildenafl treatment in cultured NRVMs could stimulate trypsin-like activity only in the absence of ATP. This indicates that raising PKG activities above the baseline can enhance the 20S proteasome trypsin-like activities, but not when 20S is associated with 19S in cultured cardiomyocytes. Notably, myocardial proteasomal trypsin-like activities, both ATP dependent and independent, were significantly increased in mice treated with sildenafl compared with the control treatment group, indicating that PKG activation by sildenafl may increase cardiac trypsin-like activity of both 20S and 26S proteasomes in vivo. The cause of the extra in vivo stimulating effect of sildenafl is unknown but might involve crosstalk between cGMP and cAMP signaling. Increased cGMP from sildenafl treatment could inhibit phosphodiesterase 3 and thereby raise cAMP levels and activate cAMP-dependent protein kinase (protein kinase A). Protein kinase A activation has been shown to increase 26S trypsin-like activity. In contrast, PKG gain of function both in vitro and in vivo could increase chymotrypsin-like activities only in 26S.

It is generally believed that the 26S proteasome is primarily responsible for the degradation of polyubiquitinated proteins. The chymotrypsin-like activity conferred by the β5 subunit of 20S is the most important among the 3 peptidase subunits in determining proteasome proteolytic function. Hence, our data from peptidase assays suggest that increased UPS function by PKG is attributable primarily to enhanced chymotrypsin-like activities of the 26S proteasome.

The regulation of PKG on cardiac proteasome function is unlikely by altering proteasome abundance because no discernible changes in the protein levels of representative subunits of 19S and 20S proteasomes were detected in PKG-manipulated cardiomyocytes and sildenafl-treated mouse hearts. This is also supported by our findings that PKG gain of function does not increase all 3 proteasome peptidase activities uniformly but rather differentially. This generates a hypothesis that posttranslational modification may be responsible. Indeed, we found that the isoelectric points of a representative 20S subunit (β5) and 19S proteasome subunit (Rpt6) were shifted to the acidic side (consistent with hyperphosphorylation) by PKG activation and conversely to the basic side (consistent with hypophosphorylation) by PKG inhibition. Notably, it has been reported that hyperphosphorylated proteasomes show higher activities. For example, protein kinase A was shown to increase nuclear proteasome function by phosphorylating Rpt6 at Ser120. Hence, a likely mechanism by which PKG activation enhances proteasome function is to directly or indirectly increase the phosphorylation of proteasome subunits such as β5 and Rpt6, thereby increasing the chymotrypsin-like activities of the 26S proteasome. It will be important to elucidate the mechanism underlying PKG-mediated positive regulation of proteasome function.

**PKG Activation Protects Cardiomyocytes Against Proteotoxic Stress**

Genetic enhancement of proteasomal degradation of misfolded proteins and the resulting inhibition of aberrant protein aggregation can protect the heart from proteotoxic stress. Hence, activation of PKG, which stimulates proteasomal degradation of misfolded proteins, should protect cardiomyocytes against the toxicity of misfolded proteins. This is exactly what we have observed in both DRC mice and cultured NRVMs. During the 4-week period of sildenafl trial, vehicle-treated, but not sildenafl-treated, CryABR120G tg mice displayed significantly greater LV posterior wall thickening and fractional shortening decline than the vehicle-treated Ntg control mice (Figure 7E and 7F), suggesting that sildenafl can slow DRC progression. By measuring caspase 3 activation, lactate dehydrogenase leakage, and cell survival, we found that PKG gain of function by PKG overexpression or sildenafl treatment significantly attenuated whereas PKG inhibition by PKG knockdown or KT5823 treatment significantly exacerbated cardiomyocyte injury induced by overexpression of CryABR120G.

Taken together, our results suggest that strategies to increase PKG activity may conceivably be used to treat disease with increased proteotoxic stress such as DRC and other proteinopathies. Currently, no treatment is available for these debilitating diseases. Food and Drug Administration–approved drugs (eg, sildenafl) are readily available to stimulate PKG. Moreover, this study provides a potentially new mechanism for phosphodiesterase 5 inhibition in treating heart diseases. Phosphodiesterase 5 inhibition by sildenafl elicits reverse remodeling and improves cardiac function in congestive heart failure animal models and human patients. Inadequate protein quality control, especially PFI, is suggested as a common pathogenic factor in the progression of a large subset of heart diseases. Accumulation of misfolded proteins, including preamyloid oligomers in cardiomyocytes, is sufficient to cause cardiomyopathy in mice, and conversely, improving proteasome function slows the progression of cardiac proteinopathy. Therefore, it is highly possible that improvements in protein quality control in cardiomyocytes of diseased hearts are a benefit of PKG stimulation.

**Acknowledgments**

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American Heart Association grants 0740025 N (to Dr Wang) and 11PRE5730009 (to Dr Ranek). The Imaging Core was supported by National Institutes of Health grant 5P20RR015567.

Disclosures
None.

References
23. Guazzi M, Vicenzi M, Arena R, Guazzi MD. PDE5 inhibition with sildenafil improves left ventricular diastolic function, cardiac geometry, and clinical status in patients with stable systolic heart failure: results of a
Circulation Perspectives

Proteasome functional insufficiency plays an important role in bona fide proteinopathy (e.g., desmin-related cardiomyopathy) and is implicated in the pathogenesis of many other heart diseases with increased production of misfolded proteins. Benign enhancement of proteasome function can be achieved by genetic approaches and protects against desmin-related cardiomyopathy and acute myocardial ischemia/reperfusion injury in mice; however, no pharmaceutics are currently available to do so. Here, we have shown that enhancement of proteasomal degradation of misfolded proteins can be achieved by protein kinase G gain of function. We have demonstrated that sildenafil activates protein kinase G, stimulates proteasome function, and reduces aberrant protein aggregation in intact mice. Food and Drug Administration–approved drugs are readily available to stimulate protein kinase G (e.g., sildenafil). Hence, our results suggest that strategies to increase protein kinase G activities may be used to treat desmin-related cardiomyopathy and other proteinopathy. Currently, no treatment is available for these debilitating diseases. Moreover, this study provides a potentially new mechanism for phosphodiesterase 5 inhibition in treating heart disease. Many recent studies have shown the beneficial effects of phosphodiesterase 5 inhibition in treating heart diseases. Proteasome functional insufficiency is suggested as a common pathogenic factor in the progression of most heart diseases. Accumulation of misfolded proteins, including preamyloid oligomers in cardiomyocytes as observed in failing human hearts, is sufficient to cause cardiomyopathy in mice, and conversely, improving proteasome function slows the progression of the cardiomyopathy. Therefore, it is highly possible that improving proteasomal removal of misfolded proteins in cardiomyocytes of diseased hearts is a benefit of protein kinase G stimulation.
Protein Kinase G Positively Regulates Proteasome-Mediated Degradation of Misfolded Proteins
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Online Supplementary Materials

Protein Kinase G Positively Regulates Proteasome-mediated Degradation of Misfolded Proteins

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Includes

Part I Supplementary Methods

Part II, Supplementary Table and Figures
Part I. Supplementary Methods

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from ventricular myocardium tissue using the TRIzol Reagent (Molecular Research Center, Inc., Cincinnati, OH) by following the manufacturer’s protocol. RNA concentration was determined using Agilent RNA 6000 Nano assay (Agilent technologies, Inc. Germany) following the manufacturer’s protocol. The SuperScript III First-Strand Synthesis kit (Invitrogen) was used to generate cDNA. GFPdgn transcript levels were assessed with the minimum number of cycles of PCR using primers: forward 5’-GGGCACAAGCTGGAGTACAACT-3’ and reverse 5’-ATGTTGTGGCGGATCTTGAG-3’. Primers corresponding to GAPDH were included as a control.

Total protein extraction and western blot analysis

Proteins were extracted from ventricular myocardium or cultured NRVMs. Protein concentration was determined using bicinchoninic acid (BCA) reagents (Pierce biotechnology, Rockford, IL). SDS-PAGE, immunoblotting analysis, and densitometry were performed as previously described. The following primary antibodies were used: total vasodilator-stimulated phosphoprotein (VASP), Ser239-phosphorylated-VASP (P-VASP), PKG (Cell Signaling), HA (Santa Cruz Biotechnology, Santa Cruz, CA), RPT6 (Biomol), β-tubulin (University of Iowa), α-actinin and ubiquitinated proteins (Sigma), and GFP, RFP, and proteasome subunit β5 (customized antibodies). The corresponding horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology) were used.

Two dimensional (2D) western blot analysis

Proteins were extracted from cultured cardiomyocytes with 2D lysis buffer (8 M Urea, 2 M Thiourea, 1% DTT, 2% CHAPS, 1% Carrier Ampholytes, and protease inhibitor cocktail). The supernatant was collected and protein concentrations determined. Equal amount of sample
was loaded into a gel strip (IPG gel strips, 11 cm, pH 7-10; Bio-Rad, Hercules, CA) for isoelectric focusing. For second dimension separation, the gel strips were electrophoresed through SDS-PAGE and then transferred to a PVDF membrane with a Trans-blot apparatus (Bio-Rad, Hercules, CA). The following primary antibodies were used: anti-proteasome subunit β5 (1:5,000; customized antibody) and anti-proteasome subunit Rpt6 (1:3,000; Biomol, Farmingdale, CA). The corresponding horseradish peroxidase conjugated secondary antibodies, goat anti-mouse or goat anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA), were applied for chemiluminescence-based analysis (GE Healthcare, Piscataway, NJ).

**Proteasomal peptidase activities**

Proteasome peptidase activity assays were performed according to the protocol reported by Saul R. Powell, with slight modifications. Briefly, snap-frozen tissues and cultured NRVMs were homogenized on ice in cytosolic extraction buffer (50 mM Tris-HCl pH 7.5, 250 mM Sucrose, 5 mM MgCl₂, 0.5 mM EDTA, and 1 mM DTT). Protein concentrations were determined with bicinchoninic acid (BCA) reagents (Pierce biotechnology, Rockford, IL) and equally concentrated in proteasome assay buffer (50 mM Tris-HCl pH 7.5, 40 mM KCl, 5 mM MgCl₂, and 1 mM DTT). Peptidase activities were determined in the presence and absence of 28 μM, 14 μM, and 14 μM ATP for Chymotrypsin-like, Caspase-like, and Trypsin-like activity, respectively. The following fluorogenic substrates were applied: Suc-LLVY-AMC (18 μM), Suc-LLE-AMC (45 μM), and AC-RLR-AMC (Bz) (40 μM), to Chymotrypsin-like, Caspase-like, and Trypsin-like activities, respectively. The plate was read in a Perkin Elmer plate reader, model 2030 (Waltham, MA) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

**Cycloheximide (CHX) chase assay**
CHX chase assays were performed as previously described. NRVMs subject to various experimental treatments were incubated in serum-free DMEM containing 100μM CHX (Sigma-Aldrich) to block further protein synthesis. The cells were collected at different consecutive time points after CHX administration and whole-cell lysates were analyzed by western blot analyses for the protein of interest.

PKG Activity Assay

A PKG activity assay kit purchased from Cyclex (Cat # CY-1161, Nagano, Japan) was used to measure myocardial PKG activity according to manufacturer’s guidelines in a 96-well plate as previously described. Briefly, 5 μg of whole myocardial lysate in the kinase buffer containing 125 μM ATP and 10 μM cGMP was added to each well of a 96-well microplate which had been pre-coated with recombinant PKG substrates, and incubated at 30°C for 30min. Wells were washed five times with wash buffer. HRP-conjugated anti-phosphorylated-G-kinase substrate antibodies (10H11) were then added to the well and incubated for one hour at room temperature. Wells were again washed five times to remove unbound antibodies. The HRP of the bound antibodies was then detected by its conversion of chromogenic substrate for 10 minutes at room temperature. Stop solution, 100 μl, was added to end the reaction. Samples were read in a plate reader for absorbance at 450 nm. Values were normalized to the NTG control.

Calpain activity assay

The calpain activity assay kit was purchased from Biovision (Cat # K240-100, Milpitas, CA). The assay was run following the manufacturer’s protocol. Briefly, whole cell lysate was collected from ventricular myocardium with the provided extraction buffer. In a 96-well plate 100 μg of sample, concentrated to 85μl, was incubated in the presence and absence of a calpain inhibitor (1μl, Z-LLL-FMK). The provided active calpain 1 (1μl) was utilized as a positive control.
Next, 10µl of 10x reaction buffer and 5µl substrate (Ac-LLY-AFC) were added to each well. The plate was incubated at 37°C for 60 minutes in the dark and read in a plate reader at excitation wavelength 400 nm and emission wavelength 505 nm. Values were calculated by subtracting the value of the calpain inhibited sample and then normalized to the NTG control.

**LDH and MTT assay**

Cultured NRVMs were plated in 6 cm dishes with 1 million cells per dish. Cardiomyocytes were cultured for 24-48 hours prior to PKG manipulation. Cells were cultured for an additional 72 hours (Ad-PKGcat and siPKG) or 48 hours (sildenafil and KT5823) with PKG manipulation. LDH leakage and MTT assay were assessed from the same culture dish to control for variance. For the LDH assay, the media was collected and analyzed per the manufacturer’s protocol (Roche, Indianapolis, IN) for LDH leakage as previously described. Briefly, the media and reaction mixture were combined in a 96 well microplate, incubated at room temperature, and read in an infinite M200 Spectrophotometer (Tecan, Switzerland) at an absorbance of 490 nm. The MTT assay was ran according to manufacturer’s protocol (Sigma-Aldrich, St. Louis, MO). Briefly, cells were washed in PBS, MTT working solution was added to the culture dish, incubated at 37°C, solubilized with acidic isopropanol, transferred to a 96 well plate, and read via the infinite M200 Spectrophotometer. The absorbance of the converted dye was read at a wavelength of 570 nm with background subtraction at 650 nm.

**Mini-osmotic pump treatment**

CryABR120G mice and cohorts of NTG controls were subcutaneously implanted with mini-osmotic pumps (model 2002, Alzet, Cupertino, CA) to deliver sildenafil (10 mg/kg/day) or vehicle control. Model 2002 pumps hold a volume of ~200 µl and deliver at a flow rate of 0.5 µl per hour over a two week period. To span the treatment period, the first pumps were removed after
two weeks and new pumps were implanted for another two weeks. Upon removal of the pumps, the residual pump volume was measured to assure efficient drug delivery. Echocardiography was assessed at baseline, 2 weeks of treatment, and 4 weeks of treatment.

References of online supplementary methods


**Part II. Supplementary Data** (1 Table, 16 Supplementary Figures)
Supplementary Table. Data from serial M-Mode Echocardiography of the Sildenafil-treated Mouse Cohort

<table>
<thead>
<tr>
<th>Baseline Measurements</th>
<th>NTG Vehicle (n=5)</th>
<th>CryABR120G TG Vehicle (n=7)</th>
<th>CryABR120G TG Sildenafil (n=8)</th>
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<tr>
<td></td>
<td>Mean</td>
<td>STD</td>
<td>Mean</td>
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<td>LVIDd (mm)</td>
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<td>0.97** †</td>
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LV=left ventricle, ID=internal diameter, d=at the end of diastole, s=at the end of systole, PW=posterior wall thickness, AW=anterior wall thickness, Vol=volume, EF=ejection fraction, FS=fractional shortening. LV mass was calculated from the echocardiography data. *p<0.05, **p<0.01 vs. the baseline of the same group, two-tailed paired Student’s t-test; †: p<0.05 vs. NTG Vehicle, one way ANOVA followed by Tukey’s test for pair-wise comparisons.
Supplementary Figure 1. PKGcat and sildenafil enhances PKG-mediated phosphorylation. NRVMs were cultured for 24 hours before PKG was stimulated; genetically through adenoviral infection (Ad-PKGcat, 10 MOI or control Ad-β-gal) (A-B) or pharmacologically (Sildenafil, 1 μM, or volume corrected vehicle DMSO) (C-D). The cells were harvested following an additional 72 hours of culture. Representative western blot analyses for PKG, Ser239-phosphorylated-VASP (P-VASP), and Total VASP (T-VASP) with β-tubulin probed as the loading control and a summary of the pooled densitometry. #:p<0.01 vs. respective control. N= 8 biological repeats per manipulation.
Supplementary Figure 2. PKG-mediated phosphorylation is reduced by siPKG and KT5823. NRVMs were isolated and cultured for 24 hours prior to PKG inhibition; genetically through siRNA transfection (siPKG, 100 pM or control siLuciferase, siLuc) (A-B) or pharmacologically (KT5823, 1 μM, or volume corrected vehicle DMSO) (C-D). The cells were harvested following an additional 72 hours culturing. Representative western blot analyses for PKG, Ser239-phosphorlated-VASP (P-VASP), and Total VASP (T-VASP) with β-tubulin probed as the loading control and a summary of the pooled densitometry. *:p<0.05, #:p<0.01 vs. respective control. N= 8 biological repeats per manipulation.
Supplementary Figure 3. PKG inhibition decreases the degradation of GFPu in cardiomyocytes. Cultured NRVMs were infected with Ad-GFPu and Ad-RFP as in main text Figure 1. Western blot analyses for the steady state protein levels of GFPu and RFP (A, C) were performed using total cell lysates from NRVMs collected at 72h after transfection of siRNA specific for PKG (siPKG) or for luciferase (siLuc, as control) (A) or after 48h of KT5823 (KT, 1M) or DMSO treatment (C). The CHX chase assays (B, D) were performed and the data are presented in the same manner as described in Figure 1. CHX treatment was started 24h after the transfection of siPKG/siLuc (B) or the treatment of KT/DMSO (D). *p<0.05 vs. the control group.
Supplementary Figure 4. PKG-mediated regulation of the UPS does not affect the degradation of normal endogenous substrates. Representative western blot analyses of the endogenous proteasome substrates indicated above with β-tubulin probed as the loading control. NRVMs were manipulated with Ad-PKG⁺cat (A), sildenafil (B), siPKG (C), or KT (D) and compared to their respective controls as described in Supplementary Figures I and II. No significance was detected between any experimental group and its respective control. N= 6 biological repeats.
Supplementary Figure 5. Proteasome peptidase activities are reduced by PKG inhibition. Chymotrypsin-like, caspase-like, and trypsin-like activities were assessed in the presence (+) and absence (-) of ATP from NRVM cell lysates. NRVMs were transfected with siLuc or siPKG (A), or treated with DMSO or KT (B) as described in Supplementary Figure II. NRVMs were harvested 48 hours (KT) or 72 hours (siPKG) after the onset of the respective treatment. *:p<0.05, #:p,0.01 vs. respective control. N=12 repeats per group.
Supplementary Figure 6. Myocardial proteasome subunit abundance is not altered by sildenafil treatment in GFPdgn mice. The key proteasome subunits Rpt6 and β5 were assessed by western blot analyses with β-tubulin as the loading control and a summary of the pooled densitometry. GFPdgn mice were subject to two consecutive intraperitoneal injections of sildenafil (10mg/kg x 2) or vehicle (DMSO) with an interval of 12 hours as described in Figure 3 of main text. Myocardial samples were collected at 12 hours after the second injection.
Supplementary Figure 7. Proteasome subunit abundance in cultured cardiomyocytes is not altered by PKG modulation. The key proteasome subunits RPT6 and Beta 5 were assessed by western blot analyses with β-tubulin as the loading control and a summary of the pooled densitometry. NRVMs were infected with Ad-gal or Ad-PKGcat (A), treated with sildenafil or DMSO (B), transfected with siPKG or siLuc (C), or treated with KT5823 or DMSO (D), as described in Supplementary Figures S1 and S2. NRVMs were collected 72 hours after PKGcat infection or siPKG transfection and 48 hours after treatment with sildenafil or KT. No significance was detected between any group. N= 8 biological repeats.
Supplementary Figure 8. The pl of key proteasome subunits is altered by PKG inhibition. Representative images of two dimensional western blots for β5 and Rpt6 subunits in cultured NRVMs. Cardiomyocytes were harvested after 72 hours of siPKG or control siLuc transfection, 100 pM. In gel for the 2nd dimension electrophoresis, the isofocusing gel strip (pH 7-10) was placed in the large well with the small wells on either side loaded with regular denatured proteins from the same sample (denoted by arrowheads). N= 6 per siRNA.
Supplementary Figure 9. PKG inhibition increases post-translationally modified HA-CryAB\textsuperscript{R120G}. NRVMs were cultured for 24h before PKG was inhibited through siPKG transfection (A) or treating with KT5823 (B). Following an additional 24h culturing, NRVMs were infected with either control Ad-\(\beta\)-gal or Ad-HA-CryAB\textsuperscript{R120G}. Representative images of western blot analyses of HA-CryAB\textsuperscript{R120G} and \(\beta\)-tubulin in NRVMs 72h after infection with Ad-HA-CryAB\textsuperscript{R120G} or Ad-\(\beta\)-gal. N= 6 per group.
Supplementary Figure 10. PKG activation does not alter the half-life of β-tubulin proteins in cultured NRVMs overexpressing HA-CryAB_{R120G}. Shown here are the pooled β-tubulin decay data from the Ad-PKG_{cat} (A) and sildenafil (B) treated NRVMs which were cultured and treated as described in Figure 5F and 5G of the main text with the representative images of CHX chase assays for β-tubulin are shown in main text Figure 5F and 5G, respectively.
Supplementary Figure 11. Representative confocal micrographs of immunofluorescence stained CryAB and F-actin. Line 708 CryAB<sub>R120G</sub> Ntg and tg mice were treated with sildenafil or vehicle control for 4 weeks as described in Figure 7 of main text. Cryosections of ventricular myocardium from the treated CryAB<sub>R120G</sub>tg mice were immunofluorescence stained for CryAB (green). Red fluorophore-conjugated Phalloidin was used to stain F-actin to identify cardiomyocytes. Scale bar=50μm.
Supplementary Figure 12. PKG inhibition exacerbates CryABR\textsuperscript{R\textsubscript{120G}} cytotoxicity in cultured NRVMs. PKG inhibition by either PKG siRNA or KT5823 treatment was achieved as described in Figure 2 of the main text. LDH and MTT assays were carried out as described in Figure 8 of the main text. *p<0.05, #p<0.01, ##, p<0.005 vs. the sLuc+\beta-gal group or the DMSO+\beta-gal group; $p<0.05$ vs. the sLuc+CryABR\textsuperscript{R\textsubscript{120G}} group or the DMSO+CryABR\textsuperscript{R\textsubscript{120G}} group; n=6 per group.
Supplementary Figure 13. Sildenafil does not change myocardial calpain activities in CryAB$^{R_{120G}}$ tg mice. The mouse cohort and sildenafil treatment are the same as described in Figure 7 of the main text. Difference among the three groups is not statistically significant (p=0.362). N=4 mice per group.
Supplementary Figure 14. PKG activation by sildenafil does not alter myocardial protein levels of LC3-II and p62/SQSTM1 in GFPdgn mice. GFPdgn tg mice were treated with either sildenafil or vehicle control for 24 hours as described in Figure 3 of the main text. Total protein extracts from ventricular myocardium were used for western blot analyses for LC3 (microtubule associate protein light chain 3) and p62. No statistically significant differences in LC3-II or p62 were observed between the two groups (p=0.452, 0.436). Each lane represents a mouse. N=4 mice/group.
Supplementary Figure 15. Effects of PKG activation on the levels of the total ubiquitin conjugates in GFPu or CryAB<sup>R120G</sup> overexpressing NRVMs when proteasome is inhibited. Cultured NRVMs were infected with Ad-CryAB<sup>R120G</sup> or Ad-GFPu (10 MOI) 24hrs after plating. Ad-β-gal was used as control for both groups. Infected groups were treated with sildenafil (data not shown) or infected with Ad-PKG<sup>cat</sup> (24hrs after infection with Ad-CryAB<sup>R120G</sup> or Ad-GFPu). Proteasome inhibition was by bortezomib (BZM) (10nM) for 24hrs after infection or administered simultaneously with sildenafil treatment. An uninfected and vehicle (Veh) treatment for BZM was used as control. Western bot analyses for total ubiquitinated proteins were performed using total cell lysate. Representative image of total ubiquitinated proteins in CryAB<sup>R120G</sup> overexpressing (A) or GFPu overexpressing (B) NRVMs are shown in the upper panel with pooled densitometry data shown below. *p<0.05, ***p<0.0001, vs. Veh. NS indicates no statistical significance; n = 4 biological repeats per group.
Supplementary Figure 16. Effects of PKG activation on the levels of the higher molecular species of GFPu or CryAB<sup>R120G</sup> in GFPu or CryAB<sup>R120G</sup> overexpressing NRVMs when proteasome is inhibited. NRVMs were cultured and treated as described in Supplementary Figure XIV. Western blot analyses for GFPu or HA-CryAB<sup>R120G</sup> proteins were performed using total cell lysate from GFPu or HA-CryAB<sup>R120G</sup> overexpressing NRVMs, respectively. Representative images of HA-CryAB<sup>R120G</sup> proteins (A) or GFPu proteins (B) NRVMs are shown in the upper panels with pooled densitometry data of the high molecular weight (HMW) species (>32kDa for HA-CryABR120G, >40kDa for GFPu) shown below. *p<0.05, **p<0.001, ***p<0.0001, vs. Veh; ## p<0.005; NS=not significant; n = 4 biological repeats per group.