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Priming the proteasome by protein kinase G: a novel cardioprotective mechanism of sildenafil

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Abstract

The proteasome mediates the degradation of most cellular proteins including misfolded proteins, pivotal to intracellular protein hemostasis. Proteasome functional insufficiency is implicated in a large subset of human failing hearts. Experimental studies have established proteasome functional insufficiency as a major pathogenic factor, rationalizing proteasome enhancement as a potentially new therapeutic strategy for congestive heart failure. Protein kinase G activation known to be cardioprotective was recently found to facilitate proteasomal degradation of misfolded proteins in cardiomyocytes; sildenafil was shown to activate myocardial protein kinase G, improve cardiac protein quality control and slow down the progression of cardiac proteinopathy in mice. This identifies the first clinically used drug that is capable of benign proteasome enhancement and unveils a potentially novel cardioprotective mechanism for sildenafil.

Keywords

bortezomib; cyclic GMP-dependent protein kinase; desmin-related cardiomyopathy; methocramine; phosphodiesterase inhibitor; pilocarpine; protein quality control; sildenafil; ubiquitin proteasome system

Proteasome-mediated proteolysis plays a pivotal role in intracellular protein catabolism by removal of individual misfolded proteins for the purpose of protein quality control (PQC) and highly regulated degradation of native proteins, including signaling proteins, that are no longer needed by the cell at a given time and place. Controlled degradation of signaling proteins enables the ubiquitin-proteasome system (UPS) to participate actively in the regulation of virtually all cellular processes. PQC acts to minimize the amount and toxicity of misfolded proteins in the cell whereas the degradation of normal cellular proteins regulates a variety of cellular processes by protein quantity control [1]. For a protein, especially a native protein (i.e., properly folded protein) to be degraded by the proteasome, it

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must be covalently attached by a chain of ubiquitin via an ATP-dependent process known as ubiquitination. The ubiquitinated protein is then shuttled to, and degraded by, the 26S proteasome which consists of two types of subcomplexes: the barrel-shaped catalytic core particle (also known as the 20S proteasome) and the 19S regulatory subcomplex which caps one or both ends of the 20S core to control substrate entry into the proteolytic chamber of the 20S [2]. The binding of the 19S to the 20S and degradation of ubiquitinated proteins by the 26S proteasome require and consume ATP. Normally, polyubiquitinated proteins are very efficiently degraded by the 26S proteasome; hence, the level of steady state polyubiquitinated proteins is quite low in the cell. Notably, the 20S proteasome may be able to degrade unstructured proteins, such as oxidized proteins, in an ubiquitin- and ATP-independent fashion [3].

As recently reviewed comprehensively by Drews and Taegtmeyer [4], targeting the UPS is increasingly implicated as new therapeutic considerations and opportunities for cardiology despite many controversies remaining to be clarified at bench. Some experimental reports suggest that systemic submaximal proteasome inhibition might be beneficial to certain cardiac pathological conditions, especially those with elevated inflammatory responses [4]. It should be pointed out that the beneficial effects were observed mostly in early stage disease models with no or little evidence of heart failure [4]. Importantly, multiple lines of evidence support that proteasome functional insufficiency (PFI) occurs in at least a subset of human heart diseases during their progression to congestive heart failure (CHF) and experiments using animal models have demonstrated that PFI plays a major pathogenic role in cardiac proteinopathy and acute ischemia–reperfusion (I/R) injury [5–9], suggesting that improvement of cardiac proteasome function may represent potentially a new therapeutic strategy to treat at least a subset of heart disease. Excitingly, studies are emerging to demonstrate that proteasome function can be enhanced by not only genetic means but pharmacological interventions as well [6,7,10,11]. This review article will highlight these recent advances seemingly to pave a new avenue to future cardiology.

PFI is likely a major pathogenic factor for congestive heart failure

Even under normal conditions, a polypeptide in heart muscle cells is under enormous challenge to attain and maintain its native conformation. Nonstop mechanical stress and resultant thermal stress and metabolic demand, for example, are obviously among the many factors challenging protein folding experienced by cardiomyocytes in a beating heart more than many other cell types in the body. Hence, protein misfolding and unfolding (collectively known as protein malfolding) are inevitable but cardiomyocytes have evolved a set of sophisticated PQC mechanisms to keep the level of malfolded proteins low in the cell and to minimize their toxicity. It is conceivable that the production of malfolded proteins can be markedly increased in cardiomyocytes under many pathological conditions, including both inherited and acquired diseases, which demands augmented PQC capacity and efficiency to achieve proteostasis. Hence, as the main pathway for PQC to timely remove terminally misfolded proteins in a highly targeted fashion, the UPS must be tuned up to meet the increased demand in a diseased heart. Although the molecular mechanisms underlying the ubiquitination process of misfolded proteins are poorly understood, the ubiquitination step does not seem to be the rate-limiting step in UPS-mediated degradation of misfolded

proteins in diseased hearts. This is because steady state ubiquitinated proteins are often, if not always, found to be increased in human hearts with end-stage heart failure as well as in the heart of animal models of human heart disease [12]. This is consistent with the notion that myocardial proteasome function may become insufficient during the progression from a large subset of heart disease to CHF and this insufficiency may cause PQC inadequacy and thereby contributes to CHF pathogenesis. This perhaps has something to do with the relatively low proteasome abundance in cardiomyocytes at baseline. It was described that murine heart tissue has significantly lower proteasome abundance than the liver [13,14].

PFI is implicated in diseased human hearts

At present, there is neither serum biomarker nor any other noninvasive or minimally invasive method available for estimating myocardial proteasome function in living humans although specimen obtained via cardiac biopsy can potentially be used for *in vitro* detection of myocardial proteasomal activity. Nonetheless, scientists have attempted to measure the expression of proteasome subunits as well as proteasomal peptidase activities in the myocardial specimen from explanted human hearts or from the heart being installed left ventricular assist device (LVAD). The findings of these studies rather consistently suggest proteasome impairment in multiple forms of human heart disease [15–17]. For instance, Predmore *et al.* recently investigated myocardial proteasome function in human heart failure and hypertrophic cardiomyopathy and found a significant reduction of proteasome activity, characterized by approximately 40% inhibition of both chymotrypsin and caspase-like 26S proteasome activities in failing and hypertrophic cardiomyopathy hearts compared with nonfailing hearts [16]. They failed to detect any alteration in proteasome abundance or active-site availability of the proteolytic subunits [16]. The LVAD are used clinically as an effective bridge to heart transplantation and has been demonstrated to exert beneficial effects on myocardial function recovery due to mechanical unloading. Paired samples collected at LVAD installation and 31 weeks after LVAD from the same patients with heart failure showed approximately 50% increase in myocardial chymotrypsin-like 26S proteasome activity resulting from LVAD installation [16]. In addition, another study took ventricular myocardium samples from patients with idiopathic dilated cardiomyopathy after LVAD and demonstrated a marked increase in myocardial 20S proteasome activity [15]. Similar results were observed in patients with CHF. Heart tissue from CHF patients displayed a significant reduction of nuclear and sarcoplasmic 20S proteasome expression, whereas the situation was reversed after ventricular unloading with a significant increase in sarcoplasmic 20S proteasome expression [17]. A more recent report showed evidence for impairment of 26S proteasome assembly in human hearts with end-stage heart failure [5]. Results of these emerging studies indicate proteasome impairment or PFI in at least a subset of diseased human hearts, suggesting that PFI might contribute to cardiovascular disease progression. This novel hypothesis has not been directly tested in humans yet because there is not a proven proteasome activator available for *in vivo* use although proteasome inhibitors have been approved for use in clinic to treat certain types of malignancy for nearly a decade [18]. Notably, cardiac toxicity including heart failure has been observed in a significant proportion of patients who had received proteasome inhibitor bortezomib for chemotherapy [18,19], supporting the notion that PFI is detrimental to the heart.

It is important to point out that accompanied with PFI in diseased hearts is increased production of misfolded proteins. Various pathological stresses may pose direct challenges to protein folding and refolding; for example, oxidative modification of cellular proteins resulting from increased oxidative stress seen in myocardial I/R can damage the protein thereby causing misfolding. Moreover, the most common and powerful cardiac response to increased stress is cardiac hypertrophy which requires inevitably increased protein synthesis. It has been reported that approximately 30% of the newly translated polypeptides are never able to make to mature proteins but eliminated by co-translational degradation mediated by the proteasome [20]. A recent study from Ping's group has elegantly demonstrated that myocardial global protein turnover rate is remarkably increased in mice during isoproterenol-induced cardiac hypertrophy and remodeling [21]. Hence, it can be inferred that cardiac hypertrophy intrinsically generates misfolded proteins and increases the burden to the proteasome, let alone that increased protein content in a hypertrophic cell demands greater PQC capacity to maintain proteostasis. Therefore, it is not a big surprise that inadequate PQC and PFI are observed in a vast majority of failing human hearts [22].

PFI plays a major pathogenic role in animal models of heart diseases

The myocardial PFI implicated in human cardiomyopathy and CHF has been recapitulated in animal models of several forms of heart disease. Mimicking increased production of misfolded proteins in cardiomyocytes, a transgenic mouse model was created to achieve with cardiomyocyte-restricted overexpression of a missense mutation (R120G) of a small heat shock protein α B-crystallin (CryAB^{R120G}) [23], a *bona fide* misfolded protein linked to human desmin-related myopathy [24]. The CryAB^{R120G} transgenic mice develop cardiomyopathy, heart failure and premature death, which recapitulates most aspects of human desmin-related cardiomyopathy (DRC), thereby serving as a *bonafide* mouse model of cardiac proteinopathy [23]. A stable transgenic line of CryAB^{R120G} mice display no discernible abnormal cardiac phenotype at 1 month of age, develop cardiac hypertrophy and dysfunction at 3 months, and die of CHF between 5 and 7 months [23]. Aberrant protein aggregation resulting from expression of misfolded proteins was previously shown to impair proteasome function in cultured cells [25]. Since aberrant protein aggregation is a prominent feature in the cardiomyocytes of the CryAB^{R120G} mice, Chen *et al.* used them along with a UPS function reporter mouse model to demonstrate for the first time that aberrant protein aggregation resulting from increased production of misfolded proteins is sufficient to cause PFI *in vivo* [26]. The PFI is attributable to defective delivery of substrates to the 20S proteasome [26]. It should be noted that driven by the very robust transgenic *myh6* promoter, the cardiac expression of the misfolded protein CryAB^{R120G} in this widely used transgenic mouse model is likely higher than that in the heart of human patient harboring this mutation or that of an animal model with the mutation specifically knocked in the genomic locus of the *cryab* gene. It will be interesting and important to determine if PFI occurs in the heart of a heterozygous knock-in model once such model becomes available. Nevertheless, for proof-of-principle studies, the CryAB^{R120G} transgenic mice remain a useful model to mimic cardiac conditions with accumulation of misfolded proteins in cardiomyocytes. Indeed, cardiac PFI has also been demonstrated subsequently in mouse models of acute myocardial I/R injury and in knock-in mice that mimic human familial hypertrophic cardiomyopathy [8,27,28]. Several studies further showed that proteasome

inhibition is sufficient to cause cardiac dysfunction in normal animals or facilitate adverse cardiomyocyte remodeling in stressed hearts [8,29,30].

The development of a transgenic mouse model with benign enhancement of cardiac proteasome function through cardiomyocyte-restricted overexpression of proteasome activator 28alpha (PA28 α) has made it possible for the first time to test the necessity of PFI in pathogenesis. The study by Li *et al.* using cross-breeding between PA28 α overexpression mice and CryAB^{R120G} mice has revealed that enhancing proteasome function can significantly reduce aberrant protein aggregation, slow down the progression of proteinopathy and delay premature death in the CryAB^{R120G} mice [7], representing the first comprehensive demonstration in any organ or disease that PFI is pathogenic. In the same report, Li *et al.* also provide compelling evidence that enhancement of proteasome function can protect against myocardial I/R injury [7]. Conversely, Tian *et al.* have provided the first genetic evidence that cardiomyocyte-restricted modest inhibition of the 20S proteasome (CR-PSMI) by overexpression of a peptidase-disabled mouse proteasome $\beta 5$ subunit (T60A- $\beta 5$) exacerbates infarct size, worsens cardiac dysfunction and increases cardiomyocyte apoptosis in an acute myocardial I/R setting. Taken together, there are multiple lines of strong evidence that PFI plays a major role in cardiac dysfunction, promising that pharmacological enhancement of cardiac proteasome function might be a potential therapeutic approach to treat heart disease.

PKG activation protects the heart

The cGMP and cGMP-dependent protein kinase (PKG) signaling pathway was initially appreciated for its induction of vascular smooth muscle relaxation and platelet disaggregation. The pathway starts by nitric oxide (NO) or natriuretic peptides activation of soluble guanylate cyclase or particulate guanylate cyclase, respectively. The guanylate cyclase subsequently catalyzes the synthesis of cGMP from GTP. Phosphodiesterases (PDEs), such as PDE5 and PDE1, hydrolyze cGMP and thereby keep the balance of intracellular cGMP level. cGMP activates multiple cellular targets including PKG (Figure 1), cGMP-gated ion channels and certain PDEs. The PKG families are encoded by two separate genes, namely *PRKG1* and *PRKG2*. Only *PRKG1* is expressed in cardiovascular system. Activated PKG phosphorylates multiple downstream cellular targets [31,32]. Emerging evidence revealed function of this signaling pathway in inhibiting cardiac hypertrophy [33]. Intrigued by this inspiring finding, researchers developed multiple compounds to target this pathway for treating cardiovascular disease. These compounds share similar characteristics of activating PKG although their upstream target components are different [32].

Sildenafil, a specific PDE5 inhibitor, is a proven safe drug widely used for erectile dysfunction. However, its application is not limited to human penis [34]. A growing number of studies revealed its powerful cardioprotection against myocardial I/R injury [35], doxorubicin cardio-toxicity [36,37], cardiac hypertrophy [33] and heart failure [38]. Many theories have been proposed to elucidate the underlying mechanism, pointing out that such protection likely involves complex pathways related to nitric oxide synthase [39], activation of PKG [40], ERK activation [41], Bcl-2 and inhibition of RhoA/Rho-kinase pathway [42].

Specifically, Madhani *et al.* demonstrated that sildenafil induced phosphorylation of phospholemman contributes to the cardioprotective effect in reperfusion injury via stimulation of the Na⁺/K⁺-ATPase in a PKG dependent manner [43]. Takimoto *et al.* revealed that sildenafil induced PKG1 activation suppresses multiple hypertrophy signaling pathways including the calcineurin/NFAT, PI3K/AKT and ERK1/2 signaling pathways [33]. A more recent study by Sasaki *et al.* showed sildenafil induced PKG activation is estrogen dependent in female hearts, which stressed the importance of sex difference in treating cardiac diseases using sildenafil [44]. Hence, PDE5 inhibition and PKG activation may take multiple pathways to exert cardiac protection.

Recently published work in our laboratory reveals that PKG activation by genetic or pharmacological means can enhance proteasome-mediated degradation of misfolded proteins and thereby protects the heart via improving PQC in cardiomyocytes. Given the emerging role of PFI in cardiac pathogenesis, these discoveries implicate a novel molecular mechanism for cardioprotection elicited by sildenafil and other PDE5 inhibitors. In the subsequent sections, we will highlight these discoveries, elaborate on their clinical implication and attempt to synthesize them into a potentially new mechanism by which sildenafil and alike protect the heart.

PKG facilitates proteasomal degradation of misfolded proteins

PKG promotes UPS-mediated degradation of misfolded proteins in cardiomyocytes

The 26S proteasome is responsible for implementing peptide cleavage in UPS-mediated protein degradation. *In vitro* proteasome peptidase activity assays are commonly used to estimate proteasome activities. However, these conventional *in vitro* assays using synthetic small peptide substrates, albeit simple and useful to some extent, are often incapable of accurately evaluating *in vivo* UPS proteolytic function status under many pathophysiological conditions. This shortcoming is overcome by the creation and employment of various full-length reporter proteins that are specifically degraded by the UPS [25,45–46]. One such surrogate substrate protein is GFPu (or GFPdgn) which is an enhanced green fluorescence protein (GFP) modified by carboxyl fusion of degron CL1, an ubiquitination signal sequence analogous to a signature conformation of misfolded proteins [25,45,47–48]. Taking advantages of this surrogate substrate or inverse reporter, Ranek *et al.* first evaluated the effect of PKG activation on UPS proteolytic function in cultured neonatal rat ventricular myocytes (NRVMs) [11]. PKG activation was achieved either by sildenafil treatment or a genetic approach, namely, overexpression of a constitutively active PKG (PKG^{cat}). The PKG^{cat} was generated from human PKG1 α lacking the autoinhibitory domain. PKG activation was verified by the increased Ser-239 phosphorylation of vasodilator-stimulated phosphoprotein, a well-proven PKG target [49]. They tested the effect of PKG activation on cultured NRVMs co-infected with recombinant adenoviruses harboring the expression cassettes of GFPu and a red fluorescence protein (RFP) by monitoring the changes in GFPu protein levels and GFPu/RFP protein ratios. In this experimental system, GFPu is a proven UPS substrate but RFP is not, but the expression of both GFPu and RFP is controlled by exactly the same regulatory elements; therefore, GFPu/RFP protein ratio can serve as a better parameter than GFPu alone to reflect reversely UPS proteolytic function. They found

that PKG activation induced remarkably decreases in GFPu/RFP ratio. Using cycloheximide chase assays which measure the degradation rate of a protein when protein synthesis is shut down, they further demonstrated that PKG activation destabilized GFPu protein, as indicated by a shorter half-life of GFPu in PKG activated cells. Conversely, PKG loss of function achieved by either siRNA-mediated PKG knockdown or administration of PKG inhibitor KT5823, elevated the protein levels of GFPu and GFPu/RFP ratios and elongated the half-life of GFPu proteins. The faster GFPu degradation induced by PKG activation does not appear to be caused by a decrease in UPS load because PKG activation did not decrease the levels of total ubiquitin conjugates in GFPu expressing NRVMs when the proteasome was inhibited [11].

They next tested the effect of PKG activation on a *bona fide* misfolded protein, CryAB^{R120G}, which is known to cause cardiac proteinopathies in human and mice [23,24]. Similar results were obtained in cultured NRVMs expressing CryAB^{R120G}. PKG activation remarkably facilitated proteasome-dependent degradation of CryAB^{R120G} and significantly attenuated the accumulation of ubiquitin conjugates resulted from CryAB^{R120G} overexpression in cultured NRVMs; and conversely, PKG inhibition exacerbated the accumulation of ubiquitinated proteins. These results indicate that PKG activation not only is sufficient to facilitate UPS-mediated degradation of misfolded proteins but also is required for efficient removal of misfolded proteins by the UPS in cardiomyocytes.

PKG activation allays proteotoxic stress induced cellular injuries in cultured cardiomyocytes

Previous studies showed that CryAB^{R120G} overexpression triggers cellular injuries and cardiomyocyte death, as evidenced by elevated protein levels of cleaved caspase 3, lactate dehydrogenase leakage and deceased cell survival assessed by the MTT assay [50,51]. Ranek *et al.* observed that PKG activation by PKG^{cat} overexpression or sildenafil treatment reversed the changes of the three parameters in cultured NRVMs overexpressing CryAB^{R120G}. Conversely, PKG inhibition by PKG knockdown or KT5823 treatment deteriorated the cytotoxic effect of CryAB^{R120G}. These data suggest that PKG activation enables cardiomyocytes to eliminate proteotoxicity resulted from overexpression of a *bona fide* misfolded protein.

PKG activation by sildenafil reduces the protein level of a stably expressed surrogate misfolded protein in mouse hearts

To further verify the findings from cultured cardiomyocytes, Ranek *et al.* treated GFPdgn mice with intraperitoneal injections of sildenafil [11]. The activation of PKG in the mouse heart by sildenafil was verified by both myocardial PKG activity assays and detection of Ser237-phosphorylated vasodilator-stimulated phosphoprotein. Protein levels, but not transcript levels, of GFPdgn were markedly reduced in mouse hearts by sildenafil. Furthermore, confocal microscopy verified that the reduction of GFPdgn protein was primarily in the cardiomyocyte compartment. These *in vivo* data are in agreement with the *in vitro* results, indicating that sildenafil-induced PKG activation enhances UPS-mediated degradation of surrogate misfolded proteins in cardiomyocytes.

Additionally, Ranek *et al.* also performed assays to test whether macroautophagy, another major protein degradation mechanism, plays a role in the reduction of myocardial GFPdgn protein in sildenafil treated mice. Western blot analyses failed to detect significant changes in myocardial protein levels of autophagic marker LC3-II (lipidated form of microtubule binding protein 1 light chain 3) and autophagy substrate p62/SQSTM1 in mice receiving sildenafil treatment, compared with the vehicle control treatment group. These data indicate that the autophagy-lysosome pathway is unlikely involved in the observed effects of sildenafil.

Potential mechanism by which PKG enhances UPS degradation of misfolded proteins

PKG activation can suppress general protein synthesis; hence, it is plausible that a decrease in total substrate load to the UPS could spare the UPS for a faster degradation of overexpressed proteins. However, this possibility is ruled out by the findings that PKG activation neither significantly decreased the level of total ubiquitin conjugates nor discernibly increased the ubiquitinated forms of GFPu or CryAB^{R120G} in cultured NRVMs overexpressing GFPu or CryAB^{R120G} when proteasomal degradation was blocked [11]. The degradation of GFPu/GFPdgn by the 26S proteasome requires them to be ubiquitinated first; hence, both enhanced ubiquitination and primed proteasomes could be the potential underlying cause for PKG activation to shorten GFPu half-life. However, the ubiquitination step is unlikely the target because PKG activation failed to increase total ubiquitinated proteins or the high molecular weight species of GFPu in GFPu expressing NRVMs when the proteasome is blocked by a proteasome inhibitor [11]. This infers that PKG facilitating the degradation of misfolded proteins may be through enhancing proteasomal degradation of ubiquitinated misfolded proteins. Therefore, Ranek *et al.* performed proteasomal peptidase activity assays to examine whether manipulating PKG alters the activity of the proteasome.

The proteasome possesses three types of peptidase activities: chymotrypsin-like, trypsin-like and caspase-like activities, residing respectively in $\beta 5$, $\beta 2$ and $\beta 1$ subunits of the 20S proteasome. These peptidase activities in crude tissue extracts or cell lysates are measurable using peptidase-specific synthetic fluorogenic peptides as substrates. Although the degradation of a full length protein in general requires the 26S proteasome consisting of both the 20S and the 19S particles, these synthetic peptide substrates are small enough to enter the 20S catalytic core without the assistance from the 19S; nevertheless, the addition of ATP in the assay system, which allows the assembly of the 26S proteasome, enhances the cleavage of these synthetic fluorogenic substrates [52,53]. Based on this principle, Ranek *et al.* tested the effect of PKG activation on proteasome peptidase activity in mice and cultured NRVMs. Sildenafil treated GFPdgn mice showed increased ATP-dependent chymotrypsin-like activity and ATP-independent and -dependent trypsin-like activities. PKG manipulation, either by PKG^{cat} overexpression or sildenafil treatment, induced significant increases in the ATP-dependent chymotrypsin-like activity and in the ATP-independent trypsin-like activity in cultured NRVMs. Conversely, PKG inhibition by either PKG knockdown or KT5823 treatment suppressed all three peptidase activities of the proteasome. According to previous studies, the chymotrypsin-like activity is the most important one among the three peptidases in determining proteasome proteolytic function [8,54]. Therefore, these results suggest that

both *in vitro* and *in vivo* PKG activation enhance proteasome function by improving specific peptidase subunit activities.

This raises an important question: how PKG activation increases proteasome peptidase activities? Obviously, increases in proteasome abundance can increase proteasome peptidase activity. Additionally, a growing body of evidence supports a critical role of post-translational modifications, such as ubiquitination, phosphorylation and oxidization, in the regulation of proteasome activities the cell [55,56]. For instance, ubiquitination is well-known for its role in targeting substrate proteins for proteasomal degradation but ubiquitination of proteasome subunits has also been shown to regulate the function of proteasome *per se*. Myocardial I/R injury causes increased production of reactive free radicals, which results in oxidative modification of multiple cellular components including critical subunits of the proteasome, impairing myocardial proteasome function [55]. More importantly, phosphorylation of critical subunits of proteasome was reported to regulate proteasome assembly and activities. Both protein kinase A (PKA) and calcium/calmodulin-dependent kinase II were reported to directly phosphorylate proteasome subunits (e.g., Rpt6), thereby enhancing proteasome activity [57,58]. The enhanced proteasome activity by PKG activation via sildenafil or PKG manipulation is unlikely attributable to altered proteasome abundance because no discernible changes in expression levels of key subunits of the proteasome were observed. The underlying mechanism likely lies in the post-translational modifications of proteasome by PKG because two-dimensional electrophoresis showed an apparent shift of the pI of key proteasome subunits to the acidic side both in sildenafil and pilocarpine induced PKG activation. The pI shift is consistent with increased phosphorylation of the proteasome, which has previously been shown to increase proteasome activity. It will be important to determine whether PKG directly or indirectly phosphorylates specific proteasome subunits.

Muscarinic 2 receptors stimulate cardiac proteasomes in a PKG-dependent manner

The parasympathetic nervous system (PNS) innervates the heart via the vagus nerve which releases acetylcholine to activate cardiac muscarinic (M) receptors. The M2 receptor, one of the five subtypes of M receptors, is mainly responsible for PNS control of cardiac function [59,60]. Previous studies have demonstrated that parasympathetic stimulation exerts effective cardioprotection in several animal models of heart failure [61]; conversely, mice with M2 receptor deficiency exhibited impaired ventricular contractility under chronic adrenergic stress, indicating that the PNS/M2 pathway confers a beneficial protection on ventricular function [62]. Prompted by the fact that PKG is a downstream kinase in the M2 receptor intracellular signaling cascade in cardiomyocytes [60], Ranek *et al.* investigated the impact of the PNS/M2 receptor activity on cardiac proteasome function [10]. They treated GFPdgn transgenic mice with pilocarpine (an M receptor agonist), methoctramine (an M2 receptor antagonist) or saline (vehicle control). The pilocarpine treatment markedly reduced, whereas the methoctramine treatment significantly increased, the steady state protein levels of myocardial GFPdgn in the ventricles. Neither of these two groups had a significant effect on the mRNA levels of myocardial GFPdgn. The M receptor manipulation induced GFPdgn protein level changes were accompanied by reciprocal alterations in myocardial proteasome peptidase activities. These data indicate that the PNS plays an important role in regulating

cardiac UPS function and M stimulation may promote proteasomal degradation of misfolded proteins in the heart.

To further investigate the effect and downstream mediator of M2 stimulation on cardiac proteasomes, Ranek *et al.* performed additional studies using cultured NRVMs [10]. Pilocarpine induced a significant reduction of the GFPu/RFP ratio compared with vehicle alone treatment. Cycloheximide chase assay further validated a shorter half-life of GFPu in pilocarpine-treated NRVMs. Proteasome peptidase assays also revealed markedly increases in the 26S proteasome chymotrypsin-like activity and the 20S proteasome trypsin-like activity in NRVMs treated with pilocarpine whereas no significant alteration of key proteasome subunits was detected. These *in vitro* data further solidify the notion that activation of the M receptor on ventricular cardiomyocytes primes the proteasome for degradation of misfolded proteins. This priming action is mediated by PKG activation because further experiments demonstrated pilocarpine treatment activated PKG in the cell and the promoting effects of pilocarpine on both proteasome peptidase activities and GFPu degradation were abolished by PKG inhibition. Taken together, both PDE5 inhibitor sildenafil and M receptor agonist pilocarpine can exert similar proteasome priming effects, providing compelling rationale to perform a preclinical test for impact of a PKG-activating drug on a disease in which PFI is a known major pathogenic factor.

Sildenafil treatment slows down the progression of DRC in transgenic mice

Caused primarily by mutations in the desmin gene or desmin-related genes (e.g., CryAB^{R120G}), desmin-related myopathy is featured pathologically by the presence of desmin-positive protein aggregates in muscle cells. The desmin-related myopathy often affects the heart and the resultant cardiomyopathy is referred to as DRC, arguably the best studied cardiac proteinopathy [63]. It has been well-established in animal models that PFI plays a major pathogenic role in DRC [7,26,64]. Genetic enhancement of proteasome function was shown to allay effectively PQC inadequacy, attenuate cardiac pathology and functional deficiency, and delay overall disease progression in CryAB^{R120G}-based DRC mice [7]. Since PKG activation by sildenafil can enhance proteasome function *in vitro* and *in vivo* and protect against cytotoxicity exerted by CryAB^{R120G} in cultured cardiomyocytes, it was highly anticipated to determine whether sildenafil treatment yields therapeutic benefit on DRC in animals. Hence, as the first step into a preclinical trial, CryAB^{R120G} transgenic mice were treated with sildenafil for 4 weeks via subcutaneous implantation of mini-osmotic pumps. Serial echocardiography showed sildenafil treatment effectively blocked left ventricle fractional shortening reduction and left ventricle posterior wall thickening, the two major pathological parameters in the DRC mice, providing strong evidence that sildenafil treatment can slow down the progression of DRC in this transgenic mouse model. More detailed biochemical and pathological examinations of mouse hearts revealed that sildenafil's therapeutic effects are associated with attenuated PQC impairment and enhanced degradation of CryAB^{R120G}, as indicated by significant reduction of soluble and insoluble CryAB proteins in absence of changes of transgenic mRNA, marked reduction of detergent-resistant aggregates and significant attenuation of the increases in total high-molecular-weight ubiquitinated proteins and CryAB-positive protein aggregates in CryAB^{R120G}

transgenic hearts. These findings unequivocally show that sildenafil treatment can effectively protect against proteotoxic stress in mouse hearts.

Priming the proteasome does not increase degradation of native cellular proteins

Since the UPS is also responsible for the degradation of most normal cellular proteins when they are no longer needed, an automatic concern is whether enhancement of proteasome proteolytic function would promote degradation of native proteins in the cell and thereby perturb normal cellular processes as it facilitates removal of misfolded proteins. However, we submit that this concern is very likely invalid. This postulate is based on the following observations: proteasome enhancement by PA28 α overexpression in either cultured cells or intact mice did not appear to affect the steady state protein levels of native proteins (e.g., β -catenin, GATA4) that are known substrates of the UPS [6,7]; cardiac proteasome enhancement by PA28 α overexpression in mice is benign as no discernible changes in baseline myocardial gene expression and cardiac morphology and function were detected in PA28 α overexpression mice within one year of age, the longest time monitored [7] and genetic and pharmacological PKG activation, which has now been shown to enhance proteasome activity, did not significantly change the half-life of native proteins in cultured cardiomyocytes [11].

This postulate is actually consistent with the current prevalent model of how the UPS targets native protein for degradation. Based on current model, a native protein must be ubiquitinated before it can be recognized and degraded by the 26S proteasome. Priming the proteasome by either PA28 α overexpression or PKG activation does not alter the ubiquitination process of native proteins; hence their degradation is unlikely promoted by increased proteasome activity. That priming the proteasome facilitates the degradation of misfolded proteins indicates that the rate-limiting step of UPS-mediated degradation of misfolded proteins resides in the proteasome step, which is in stark contrast to degradation of native proteins. Consistent with this notion, increased levels of steady state ubiquitinated proteins are always found in the cell or tissues with overproduction of misfolded proteins while overexpression of a wild type protein rarely leads to a discernible increase in total ubiquitinated proteins [65].

Conclusion

In summary, multiple lines of emerging evidence suggest that increased proteotoxic stress and inadequate PQC resulting from factors such as PFI are highly prevalent in failing human hearts. Studies using animal models of heart disease have established an essential role for PFI in cardiac pathogenesis. Proteasome enhancement via either PA28 α overexpression or PKG activation protects against heart disease with increased proteotoxic stress. The exciting discovery that PKG activation enhances proteasome function suggests that the cardioprotective effects of PKG activating agents, such as PDE5 inhibitor sildenafil and M receptor agonist pilocarpine, may be mediated in part by improving proteasomal removal of damaged proteins in cardiomyocytes (Figure 1) but this postulate remains to be fully

established. It also remains to be fully understood how the proteasome is primed by PKG activation.

Future perspective

At present, no treatment is available for targeting elevated proteotoxic stress; however, proteotoxic stress is not only the primary cause of *bona fide* proteinopathies (e.g., DRC), which may be rare genetic disorders, but also involved in major pathological processes (e.g., reperfusion injury) seen in the natural course or during intervention of common forms of heart disease such as ischemic heart disease. It should be emphasized that preamyloid oligomers, which are both a consequence and a further cause of PFI, have been detected a vast majority of explanted failing human hearts but not normal hearts [22,66], indicating the high prevalence of increased proteotoxic stress and PFI in the development of CHF. These recent advances highlighted in this review show great promise, and encourage further effort, to establish priming the proteasome via PKG activation as a new therapeutic strategy and translate it to the clinic to treat heart disease with increased proteotoxic stress. Given that a number of PKG-activating drugs such as PDE5 inhibitors are already in clinical use, it is rather tempting to predict that in the foreseeable future the clinical indication of these PKG activating drugs could be broadened to include proteinopathies and other more common types of heart disease with increased proteotoxic stress.

Besides PDE5, other PDEs can also hydrolyze cGMP in cardiomyocytes. For example, PDE1 was found to play a significant role in degrading cGMP in human cardiomyocytes and is upregulated in diseased hearts [67,68]. A PDE1-selective inhibitor was shown to inhibit cardiomyocyte hypertrophic responses *in vitro* and *in vivo* and attenuate myocardial fibrosis in pathological cardiac remodeling [68,69]. It will be important and interesting to test whether inhibition of other cGMP-hydrolyzing PDEs (e.g., PDE1) enhances proteasome function.

By stimulating guanylate cyclase and increasing the synthesis of cGMP, NO can also activate PKG (Figure 1); hence NO increasing agents might also exert a proteasome priming effect. However, NO has been shown to cause O-GlcNAcylation and peroxynitrite modifications to proteasome subunits and thereby modulates 26S proteasome activities in at least noncardiac cells [70–74]. Zhang *et al.* has demonstrated that O-GlcNAcylation of proteasome subunits inhibits 26S proteasome activity *in vitro* and in cultured NRK cells [75]. Liu *et al.* showed more recently that by increasing O-GlcNAcylation of Rpt2 (an AAA-ATPase subunit of the 19S proteasome), NO serves as an endogenous inhibitor of the 26S proteasome in vascular endothelial cells [70]. Formation of peroxynitrite (ONOO-) requires simultaneous increase of both NO and superoxide [76]. Zou and colleagues have elegantly demonstrated in various disease conditions that increased 26S proteasome activity in vascular endothelial cells is associated with peroxynitrite modification of the 19S proteasome [71,73]. Hence, the effect of NO on proteasome function in at least vascular endothelial cells is likely context-dependent. It will be interesting to determine the net effect of NO on proteasomal function in cardiomyocytes.

From existing evidence, we speculate that priming the proteasome by sildenafil via PKG activation could be potentially upstream or, in some cases, downstream of some of the previously reported actions associated with its cardioprotection. For example, suppressing the calcineurin-nuclear factor of activated T-cells (NFAT) pathway was associated with inhibition of pressure overloaded cardiac hypertrophy by sildenafil [33]. Inadequate PQC has been implicated in pressure overloaded cardiomyopathy and PQC inadequacy derived from either CryAB deficiency or PFI has been shown to activate the calcineurin-NFAT pathway [29,77–78]. Therefore, by enhancement of proteasomal degradation of misfolded proteins via PKG activation, sildenafil can improve PQC in stressed myocardium, which could take away at least one factor that activates the calcineurin-NFAT pathway, thereby contributing to the hypertrophy inhibition effect. Hence, it will be interesting and perhaps worthwhile to further determine whether this newly identified mechanism for sildenafil-triggered cardioprotection could overlap or be complementary to its other actions unveiled previously.

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EXECUTIVE SUMMARY

Proteasome functional insufficiency is a major pathogenic factor for heart failure

- Both increased burden to proteasomes and proteasome impairment occur in human hearts with cardiomyopathies and heart failure.
- Cardiac proteasome functional insufficiency (PFI) is observed in animal models of cardiac proteinopathy and myocardial ischemia/reperfusion (I/R) injury.
- Intermittent systemic proteasome inhibition is associated with cardiac malfunction in animals and humans.
- Genetic enhancement of cardiac proteasomes via PA28 α overexpression allays cardiac proteotoxic stress and protects against cardiac proteinopathy and I/R injury in mice.

Protein kinase G activation protection protects the heart

- Cardiac protein kinase G (PKG) can be activated via PDE5 inhibition or muscarinic (M) receptor stimulation.
- Both PDE5 inhibitor sildenafil and M receptor agonist have been shown to protect the heart against pathological hypertrophy and I/R injury.

PKG facilitates proteasomal degradation of misfolded proteins

- Both genetic and pharmacological PKG activation increases proteasome peptidase activities.
- PKG activation facilitates proteasomal degradation of misfolded proteins and attenuates cytotoxicity elicited increased misfolded proteins in cultured cardiomyocytes.
- PKG activation by sildenafil or pilocarpine in mice markedly reduced the myocardial protein levels of a proven surrogate substrate of the ubiquitin-proteasome system.
- PKG activation leads to post-translational modifications to key proteasome subunits, consistent with increased phosphorylation known to prime the proteasome.
- M2 receptor stimulation increases proteasomal function in a PKG-dependent manner.

Sildenafil treatment slows down the progression of desmin-related cardiomyopathy in transgenic mice

- Chronic administration of sildenafil via mini osmotic pumps increased myocardial PKG activity.
- Sildenafil effectively reduced the myocardial protein levels of a stably overexpressed misfolded protein (CryAB^{R120G}) in mice.
- Sildenafil attenuated aberrant protein aggregation triggered by CryAB^{R120G}.

- The sildenafil treatment blocked reduction of cardiac contractile function and cardiac hypertrophy in a *bona fide* mouse model of cardiac proteinopathy.

Priming the proteasome does not increase degradation of native cellular proteins

- Proteasome enhancement by either PA28 α overexpression or PKG activation does not alter the steady state protein levels of native cellular proteins *in vitro* and *in vivo*.
- The safety profile of PKG activation by drugs like sildenafil is well demonstrated in humans.
- Transgenic mice with cardiac proteasome enhancement via PA28 α overexpression show no abnormality.

Conclusion

- Proteasomal degradation of misfolded proteins can be facilitated by PKG activation but the precise mechanism remains to be determined.
- Improving proteasomal degradation of misfolded proteins in cardiomyocytes may be an unappreciated mechanism underlying cardioprotection by PKG activating agents.
- It represents a great opportunity to launch clinical trials on PKG activation agents (e.g., sildenafil) for their efficacy in treating cardiac proteinopathies such as desmin-related cardiomyopathy.
- There is a strong rationale to explore more effective strategies (e.g., PDE1 inhibition) than PDE5 inhibition to activate PKG in the heart.

Figure 1. A schematic illustration of protein kinase G activation priming the proteasome to facilitate degradation of misfolded proteins and allay proteotoxic stress in cardiomyocytes

Increases in cGMP by the M2 receptor agonists or PDE5-selective inhibitor sildenafil activate PKG, which in turn primes the proteasome and facilitates proteasomal degradation of misfolded proteins and thereby allays proteotoxic stress in cardiomyocytes and prevents it from causing cardiac injury and dysfunction. Increased cardiac proteotoxic stress can result either from genetic mutations encoding misfolded proteins (e.g., mutations in the desmin and CryAB genes linked to desmin-related cardiomyopathies) or from acquired disease such as ischemic heart disease in which increased oxidative stress from I/R damages cellular proteins and causes protein misfolding as well as proteasomal impairment. Cardiac hypertrophy, the most common and dominant cardiac response to increases in various stresses, requires elevated protein synthesis which inevitably increases production of misfolded proteins, increasing proteotoxic stress.

I/R: Ischemia-reperfusion; M2: Muscarinic 2; PKG: Protein kinase G; PTM: Post-translational modification.