Genetically Induced Moderate Inhibition of the Proteasome in Cardiomyocytes Exacerbates Myocardial Ischemia-Reperfusion Injury in Mice

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ABSTRACT

**Rationale:** Both cardiomyocyte-restricted proteasome functional enhancement and pharmacological proteasome inhibition (PSMI) were shown to attenuate myocardial ischemia/reperfusion (I/R) injury. The role of cardiac proteasome dysfunction during I/R and the perspective to diminish I/R injury by manipulating proteasome function remain unclear.

**Objectives:** We sought to determine proteasome adequacy in I/R hearts, create a mouse model of cardiomyocyte-restricted PSMI (CR-PSMI), and test CR-PSMI impact on I/R injury.

**Methods and Results:** Myocardial I/R were modeled by ligation (30min) and subsequent release of the left anterior descending artery in mice overexpressing GFPdgn, a validated surrogate proteasome substrate. At 24h of reperfusion, myocardial proteasome activities were significantly lower while total ubiquitin conjugates and GFPdgn protein levels were markedly higher in all regions of the I/R hearts than the sham controls, indicative of proteasome functional insufficiency. CR-PSMI in intact mice was achieved by transgenic (tg) overexpression of a peptidase-disabled mouse β5 subunit (T60A-β5) driven by an attenuated mouse mhc6 promoter. Overexpressed T60A-β5 can replace endogenous β5 and inhibits proteasome chymotrypsin-like activities in the heart. Mice with moderate CR-PSMI showed no abnormalities at the baseline but displayed markedly more pronounced structural and functional damage during I/R, compared with non-tg littermates. The exacerbation of I/R injury by moderate CR-PSMI was associated with significant increases in the protein level of PTEN and protein kinase Cδ (PKCδ), decreased Akt activation, and reduced PKCε.

**Conclusions:** Myocardial I/R causes proteasome functional insufficiency in cardiomyocytes and moderate CR-PSMI augments PTEN and PKCδ, suppresses Akt and PKCε, increases cardiomyocyte apoptosis, and aggravates I/R injury in mice.

**Keywords:** proteasome inhibition; myocardial reperfusion injury; transgenic mouse; Akt; PKCδ,

**Non-standard Abbreviations:**

- AAR: area at risk
- BZ: border zone: the zone between ischemic and non-ischemic area
- CR-PSMI: cardiomyocyte-restricted proteasome inhibition
- GFPdgn: enhanced green fluorescence protein with carboxyl fusion of degron CL1
- I/R: ischemia-reperfusion
- Ntg: non-transgenic
- PFI: proteasome functional insufficiency
- PKC: protein kinase C
- PQC: protein quality control
- PSMI: proteasome inhibition
- PTEN: phosphatase and tensin homolog
- RA: the remote area, i.e., the blood flow of this area is not directly reduced
- T60A-β5: a mouse mutant β5 subunit with its Threonine60 mutated to Alanine
- TG, Tg, tg: transgenic
- UPS: ubiquitin-proteasome system

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INTRODUCTION

The ubiquitin-proteasome system (UPS) mediates the degradation of most proteins in the cell. The UPS is pivotal to protein quality control (PQC) because it is responsible for degrading all abnormal proteins, including terminally misfolded proteins. Through targeted degradation of normal proteins that are no longer needed (e.g., activated kinases and their regulators), the UPS also regulates virtually all cellular processes.\(^1,2\) PQC and the regulatory protein degradation are essential to maintaining normal cell function and cell survival; hence, cardiac UPS dysfunction can cause not only cardiac malfunction but also increased cardiomyocyte death in the heart.\(^3,5\) In general, UPS-mediated proteolysis involves ubiquitination of target proteins and the degradation of the ubiquitinated proteins by the proteasome. The most studied proteasome is the 26S proteasome which consists of a 20S proteasome flanked by the 19S proteasome at one or both ends. Proteasomal proteolysis takes place in the interior chamber of the 20S. The 20S is composed of an axial stack of four rings: two anti-parallel inner \(\beta\) rings flanked by two outer \(\alpha\) rings. Each \(\alpha\) or \(\beta\) ring is formed by 7 subunits, known as \(\alpha_1\) through \(\alpha_7\) and \(\beta_1\) through \(\beta_7\). Three peptidase activities: chymotrypsin-like, trypsin-like, caspase-like have been identified in eukaryotic proteasomes, residing respectively in \(\beta_5\), \(\beta_2\), and \(\beta_1\) subunits. Most proteasome inhibitors, including the clinically used bortezomib, target the \(\beta_5\) subunit, inhibit its catalytic activity, and thereby inhibit the 20S proteasome.\(^2\)

Based largely on the in vitro proteasome peptidase activity assays, alterations of proteasome function were associated with a variety of cardiac pathological conditions,\(^6,7\) e.g., load-dependent cardiac disorders,\(^8-10\) hypertrophic or dilated cardiomyopathy,\(^10-14\) and ischemic heart disease.\(^14-20\) Apparently, the altered proteasome activities in a pathological condition can be a pathogenic factor contributing to progression of the disease, a compensatory response, or simply an epiphenomenon; hence, it is important to test these possibilities in a given disease, for a better understanding of the role of UPS dysfunction in pathogenesis. To do so, we must answer at least two important questions: (1) is the altered proteasome function sufficient to maintain proteostasis in the cell, and (2) what is its impact on the progression of the disease?

To help examine questions like the first one, we previously developed a stable transgenic (tg) mouse model expressing a modified green fluorescence protein (GFPdgn) that is a verified surrogate substrate of the UPS.\(^21\) We used the GFPdgn tg mice to unveil proteasome functional insufficiency (PFI), despite a marked increase of proteasome peptidase activities, in the heart of mouse models of cardiac proteinopathy.\(^22,23\) However, proteasome functional sufficiency has not been determined in many other cardiac pathological conditions, e.g., myocardial ischemia/reperfusion (I/R) injury, although most reports show decreased proteasome peptidase activities in I/R hearts.\(^16,20,24\) Thus, in the first part of the present study, we inquired PFI occurrence in mouse hearts with acute regional myocardial I/R in vivo.

To answer the second question, we must manipulate proteasome function specifically in cardiomyocytes without affecting other cell types of the heart and other organs/systems. However, an animal model of cardiomyocyte-restricted proteasomal inhibition (CR-PSMI) has not been described although we have recently reported a mouse model of cardiomyocyte-restricted proteasome functional enhancement.\(^25,26\) We used the latter model to demonstrate that proteasome functional enhancement in cardiomyocytes protects against acute myocardial I/R injury,\(^26\) contradicting to several reports which demonstrated via pharmacologic means a protective effect of ubiquitous proteasome inhibition (PSMI) against myocardial I/R injury.\(^18,27-30\) To address this apparent discrepancy, we have established here the first tg mouse model of CR-PSMI and used it to test the impact of CR-PSMI on acute I/R injury in intact mice.

In the present study, we demonstrated that regional myocardial I/R induces PFI in cardiomyocytes of the heart. Second, we discovered that overexpression of a protease-disabled missense (T60A) mutant
precursor of β5 subunit in cardiomyocytes can effectively replace endogenous β5, and thereby inhibit proteasome chymotrypsin-like activity; thus, we established a stable tg mouse model of CR-PSMI. Finally, we demonstrated that moderate CR-PSMI is well tolerated by mice at the baseline condition but it augments the pro-apoptotic kinase protein kinase Cδ (PKCδ) and the phosphatase and tensin homolog (PTEN) signaling, suppresses PKCε and the activation of pro-survival kinase Akt, aggravates cardiomyocyte apoptosis, and exacerbates myocardial I/R injury.

METHODS

A detailed Methods section can be found in the online-only Data Supplement.

Transgenic mice.

All mice used in this study are in the FVB/N inbred background. The creation and characterization of the tg mice with expression of a modified green fluorescence protein (GFPdgn) have been previously described. GFPdgn was engineered via carboxyl fusion of degron CL1 to an enhanced green fluorescence protein (GFP) and is a proven substrate for the UPS. To establish a genetic model of CR-PSMI, we created stable tg mouse lines that overexpress a Myc-tagged missense mutation (T60A) of the murine precursor of β5 subunit of the 20S proteasome (hereafter known as T60A-β5) under the control of an attenuated murine mhc6 promoter. The latter consists of a full-length mhc6 promoter in which 3 GATA sites and 2 TREs (thyroid response elements) were ablated but other cis-acting regions important for cardiac-specific expression were left intact.

LV pressure–volume relationship analysis.

To assess left ventricle (LV) pressure–volume (P-V) relationship, mice were anesthetized with isoflurane (2%) in medical grade oxygen, then intubated and mechanically ventilated. The LV was catheterized via the right carotid artery with a 1.2-F mouse P-V catheter (Scisense, London, Ontario). The instrumented animal was stabilized for 10min and then data were recorded with a sampling rate of 1,500 Hz with Ponemah software (Data Sciences International, Valley View, OH) during steady-state conditions. For subsequent analysis of P-V loops Ponemah software was used. The raw conductance volumes were corrected for parallel conductance by the hypertonic saline bolus.

In vivo myocardial I/R and assessment of infarct size.

Regional myocardial I/R was modeled in mice by surgical ligation of the left anterior descending coronary artery (LAD) for 30min followed by releasing of the ligation for 24h. Determined by pilot studies, this protocol yields an average area at risk (AAR) of 40% and an average infarct size of 55% in young wild type FVB/N mice (Online Figure I). Infarct size was determined as previously reported.

Terminal deoxynucleotidyl transferase end-labeling (TUNEL) assay.

The heart was excised 24h after reperfusion. Two tissue samples corresponding respectively to the AAR and the remote area (RA) of the LV free wall were collected, fixed in 4% paraformaldehyde in PBS for 24h at 4°C, and further processed for TUNEL assays as previously described.

Statistical Analysis.

All continuous variables are presented as mean±SD. Differences between two groups were evaluated for statistical significance using two-tailed Student’s t-test. When difference among 3 or more groups was evaluated, one-way analysis of variance (ANOVA) or when appropriate, 2-way ANOVA, followed by the Holm-Sidak test for pair-wise comparisons were performed. The P value <0.05 were considered statistically significant.
RESULTS

PFI in the heart of mice with acute focal myocardial I/R

To determine the impact of acute myocardial I/R on cardiac proteasome function, GFPdgn tg mice were subject to surgical LAD ligation for 30 min followed by release of the ligation to allow reperfusion. At 24h after reperfusion, myocardial samples were collected respectively from AAR (the area undergone ischemia during coronary ligation), the remote area (RA), and the border zone (BZ, a 1-mm-wide zone between AAR and RA) of the LV free wall for assessing proteasome peptidase activities and the steady state ubiquitinated proteins. For the sham surgery control group, the tissue of the entire LV free wall was collected. Crude protein extracts were used for measuring 20S proteasome peptidase activities. All three peptidase activities were significantly decreased in all the three zones of I/R hearts, compared with the sham controls (Figure 1A). The total ubiquitinated proteins, especially the high molecular weight species, were significantly increased in all zones of I/R hearts, compared with the sham controls (Figure 1B, 1C).

To test if the decreased proteasome peptidase activities affect on UPS proteolytic function, we measured cardiac expression of GFPdgn, a surrogate protein substrate for the UPS. A significant increase in GFPdgn protein levels in all three zones of I/R hearts was observed quantitatively using western blot analyses (Figure 2A, 2B) and qualitatively via confocal microscopy (Figure 2C). RT-PCR analyses demonstrated that the steady state GFPdgn mRNA levels were not increased (Figure 2D, 2E). In the absence of increases in synthesis, the increased GFPdgn protein levels indicate that the removal of abnormal proteins by the UPS is impaired. This impairment is presumably caused by PFI because 20S proteasome peptidase activities were significantly decreased in the I/R hearts.

Establishment of a mouse model of moderate CR-PSMI

To date, no animal model of CR-PSMI has been reported but such a model would benefit remarkably investigation into the pathophysiological significance of PFI in cardiomyocytes of intact animals. Hence, we sought to create one.

As mentioned earlier, proteasome peptidase activities reside in β1, β2, and β5 subunits of the 20S. The N-terminal threonine of the three matured β-subunits performs the nucleophilic attack for peptide bond hydrolysis. The catalytically active N-terminal threonine residue resides in the interior face of the 20S chamber. The C termini of β subunits are on the outer surface of proteasomes so that carboxyl fusion of an “epitope tags” to a β subunit would not affect the configuration and presumably activity of the proteasome. In both yeast and cultured mammalian cells, the activity of β5 subunit appears to be the most important component for proteasome activities. For proteasome assembly and maturation the hierarchy of importance among the three proteolytic subunits is β5>β2>β1. Thus, we decided to create a catalytically inactive mutant β5 subunit through mutating its N-terminal catalytic threonine to alanine. All 3 peptidase subunits are synthesized in the cell as precursors. The N-terminus is post-translationally processed by autocatalytic activities during proteasome assembly and maturation, which exposes the N-terminal threonine. Various mutation studies further confirmed the importance of Thr76 in β5 precursor of yeast (also known as pre2). Replacing Thr76 with Ala (T76A) in pre2 decreased its chymotrypsin-like activity markedly but had little effect on trypsin-like and caspase-like activities. This indicates that T76A-pre2 can incorporate into the 20S proteasome, functioning as a dominant negative mutant.
Alignment of β5 sequences across species shows that T60 of mouse β5 (Psmb5) corresponds to T76 of yeast pre2. Thus, we chose to create a T60A-β5 from mouse wild type (WT-) β5 precursor cDNA. We differentiated T60A-β5 from endogenous β5, with addition of a myc tag to the carboxyl terminus of T60A-β5. Our pilot studies confirmed that overexpression of T60A-β5 in mammalian cell lines and neonatal rat ventricular myocytes led to expression-level-dependent decreases in the chymotrypsin-like activity and inhibition of proteasomal proteolytic function (data not shown). Hence, we generated stable tg mouse lines that overexpress myc-tagged T60A-β5 precursor under the control of an attenuated mouse Mhc6 promoter (Figure 3A). Basic characterization of these mice revealed that overexpressed T60A-β5 precursor was successfully converted to mature T60A-β5, decreased endogenous β5 (Figure 3B, C), incorporated into the 20S proteasome as revealed by co-immunoprecipitation with the endogenous β5 and α4 subunits of the 20S (Figure 3D), and decreased the chymotrypsin-like activity in the heart by approximately 60% and 65% in tg line 1 and line 2, respectively (Figure 3E). Immunofluorescence confocal microscopy revealed that myc-T60A-β5 proteins are enriched in the nucleus and at the Z-line levels in the cytoplasm (Online Figure II), demonstrating the distribution pattern of endogenous 20S proteasomes observed in the cardiomyocytes.39 No abnormality in cardiac function, morphology, or growth was detected during the first 6 months of life of thesetg mouse lines (data not shown). Consistent with the moderate nature of CR-PSMI in these tg lines, myocardial total ubiquitinated proteins in T60A-β5 tg mice at the baseline do not differ from Ntg littermates (Online Figure III). Notably, as described above, both tg lines exhibit the same phenotype. The experiments described hereafter used tg line 1.

Moderate CR-PSMI exacerbates cardiac dysfunction in I/R mice

To determine the effect of CR-PSMI on I/R injury, T60A-β5 tg and non-tg (Ntg) littermate mice were subject to the ischemia (30min) and reperfusion (24hrs) protocol as described above. To verify T60A-β5 expression and its impact on proteasome function in the AAR, BZ and RA of I/R hearts, we assessed the expression of endogenous β5 and tg T60A-β5 proteins and the changes of the activities of the β5-dependent and independent peptidases (Figure 4). Compared with Ntg sham, endogenous β5 protein levels were significantly decreased in the AAR and BZ but not RA of the Ntg I/R hearts. Compared with the tg sham, both endogenous β5 and tg T60A-β5 proteins were expressed at a relatively lower level in all three zones of I/R hearts (Figure 4A, 4B). As a result, β5-dependent chymotrypsin-like activities were markedly lower in all three zones of I/R hearts in the T60A-β5 tg mice than the Ntg mice (Figure 4C). Proteasome caspase-like activities, which are β5-independent, showed no significant difference between the tg and the Ntg groups (Figure 4D).

In a separate cohort, LV function was measured at the terminal experiment using P-V relationship analysis. As summarized in Table 1 and illustrated by Online Figure IV, no statistically significant difference in any of the parameters examined was observed between the Ntg sham and the Tg sham groups. Compared with the Ntg sham, the Ntg I/R group demonstrated marked impairment of systolic and diastolic function. The systolic function impairment was reflected by significant decreases in LV end-systolic pressure (Pes), the maximal rate of pressure increasing (dP/dtmax), ejection fraction (EF), strokes work (SW), and the preload recruited stroke work (PRS W) and by significant increases in end-systolic volume (Ves). The impairment of diastolic function was indicated by significant increases in end-diastolic pressure (Ped) and Tau and by decreases in the maximal rate of pressure decreasing (dP/dtmin). As a result, both stoke volume (SV) and cardiac output per minute (CO) were significantly smaller in the Ntg I/R group, compared with the Ntg sham group. Importantly, the I/R induced changes in all parameters except Tau were significantly exacerbated in the T60A-β5 Tg I/R group. These findings demonstrate that CR-PSMI aggravates cardiac malfunction induced by regional myocardial I/R.
Ischemia and infarct size determination showed that the LAD ligation induced ischemic area or AAR was not significantly different between the Ntg I/R and the Tg I/R groups but at 24 hours after reperfusion, the infarct size of the Tg I/R group was significantly greater than that of the Ntg I/R group (Figure 5). These results indicate further that moderate CR-PSMI exacerbates I/R injury.

To explore the potential mechanism underlying the detrimental effect of moderate CR-PSMI on I/R hearts, we first examined the prevalence of cardiomyocyte apoptosis via the TUNEL assay and the detection of activated/cleaved caspase 3 in various areas of the LV. Essentially no TUNEL positive cardiomyocytes were detected in Ntg or Tg sham control hearts but I/R induced an increase in TUNEL positive cardiomyocytes in the AAR and the remote areas of both Ntg and Tg hearts. The increases were more pronounced in the T60A-β5 Tg I/R hearts than Ntg I/R hearts (Figure 6A, 6B). Western blot analysis revealed that the level of cleaved caspase3 was significantly increased in the AAR, BZ, and RA of the I/R hearts of Ntg and Tg groups. The increase was more pronounced in the T60A-β5 Tg hearts than Ntg hearts (Figure 6C, 6D).

**DISCUSSION**

Myocardial I/R injury is an important pathological process occurring both in the natural course and during the intervention (e.g., revascularization) of ischemic heart disease, in addition to heart transplantation. Although significant progress has been made toward understanding I/R injury the molecular mechanisms underlying I/R are incompletely understood and finding effective clinical intervention to reduce the injury remains an important challenge. Several previous studies have suggested a role of altered cardiac proteasome function in I/R injury; however, these reports often contradict one another, regarding the adequacy of the altered proteasome function in maintaining cardiac proteostasis during I/R and the feasibility to inhibit proteasome function for ameliorating I/R injury. Taking advantage of a previously validated UPS reporter mouse, we demonstrated here for the first time that PFI occurs in mouse hearts undergoing regional myocardial I/R. Moreover, we established the first animal model of CR-PSMI and used this model to illustrate that a moderate inhibition
of proteasome function in cardiomyocytes can be tolerated in mice at the baseline condition but it remarkably exacerbates I/R injury potentially via increasing PTEN and PKCδ protein levels, suppressing PKCε and Akt signaling, and increasing cardiomyocyte death.

PFI in cardiomyocytes of I/R hearts

In previous reports, proteasome functional status in I/R hearts was assessed primarily by measuring proteasome peptidase activities, the expression of some proteasome subunits, and the abundance of ubiquitin conjugates. Virtually all reports showed increases in ubiquitin conjugates. Most reports showed decreased 20S proteasome activities in I/R hearts. We confirmed these findings in the GFPdgn tg mouse LV with regional I/R. The decreases of proteasome peptidase activities observed in I/R myocardium are likely attributable to both decreased proteasome abundance, as evidenced by the decreases of β5 in both the BZ and AAR of Ntg I/R hearts, and posttranslational modifications such as oxidation of proteasome subunits as previously reported.17, 20 Notably, the degree (10%~40%) of decreases in the 20S proteasome peptidase activities observed in the RA, BZ, and AAR of I/R hearts alone should not be sufficient to impair the degradation of native proteins if the terminally damaged/misfolded proteins, which need proteasomes for their removal, are not increased. This is because previous reports have shown that it takes much more severe inhibition of the 20S proteasome (~75%) to accumulate surrogate proteasome substrates.21, 43 During I/R the production of terminally misfolded/damaged proteins (e.g., oxidized proteins) is inevitably increased; hence, the global increase of ubiquitinilated proteins in I/R hearts is consistent with PFI but does not necessarily demonstrate PFI. This is because global increases of ubiquitinilated proteins in the cell can also be caused by many other factors, such as increased production of ubiquitinilated proteins (i.e., increased ubiquitination), decreased deubiquitination, increased atypical ubiquitination that yields ubiquitinilated proteins not degradable by the proteasome, and impaired macroautophagy.2 Therefore, we probed the sufficiency of UPS function using GFPdgn, a validated surrogate substrate for the UPS. To our surprise, besides in cardiomyocytes of the AAR and the BZ, GFPdgn was also accumulated in cardiomyocytes of the RA where blood perfusion was not manipulated. These findings indicate that PFI occurs in the cardiomyocytes of I/R hearts.

Moderate CR-PSMI is well tolerated at the baseline but is detrimental during I/R

Multiple pharmacological agents are capable of inhibiting proteasome function and a pharmacological approach can have a few advantages. It is relatively cost- and time-efficient, and easier to achieve different degrees of inhibition. However, it is extremely difficult, if not impossible, to achieve homogeneous CR-PSMI in intact animals without affecting proteasomal function in other tissues and organs using pharmacological inhibitors, regardless to the specificity issues of proteasome inhibitors. Therefore, a genetic approach to specifically inhibit the 20S proteasome fulfills the purpose of this study better.

In the present study, we created the first tg mouse model of CR-PSMI via cardiac overexpression of a dominant negative mutant β5 subunit. Our baseline characterization on cardiac growth/development, the fetal gene program, and heart function of these mice (data not shown) has revealed that moderate CR-PSMI does not cause discernible abnormal phenotype in mice during the first 6 months of life (the longest time observed). However, CR-PSMI exacerbates myocardial I/R induced cardiac functional and structural impairment. At the function side, our P-V relationship analysis showed that at 24hr reperfusion following 30min ischemia, as expected, Ntg mice displayed significant decreases in both LV contractility and relaxation. The I/R induced LV functional impairment, especially the systolic malfunction, was more pronounced in T60A-β5 tg mice. Similarly, the same I/R procedure caused a greater infarct size in T60A-β5 tg mice than Ntg littermates. Consistent with these findings, chemotherapy using proteasome inhibitor bortezomib is generally well tolerated by multiple myeloma patients without preexisting cardiac conditions, whereas the therapy was associated with the development of heart failure and other cardiac conditions.
dysfunction in elderly patients or patients with preexisting cardiac conditions. The present findings are also in agreement with our recent report that cardiomyocyte-restricted proteasome functional enhancement via PA28α overexpression protects against I/R injury in mice. Taken together, through both gain-of-function (our previous study) and loss-of-function (the present study) approaches, we have now demonstrated that PFI in cardiomyocytes contributes to myocardial I/R injury. This conclusion is indirectly supported by several recent studies which suggest preserving cardiac proteasome function protects against I/R injury.

Notably, the above conclusion appears to tangentially dispute several previous reports which show a protective effect of proteasome inhibitors on I/R hearts in vivo or ex vivo. This is because PSMI in the present study is cardiomyocyte-restricted while the previously reported in vivo PSMI was achieved by systemic administration of pharmacological inhibitors and was ubiquitous to the body and/or to all tissue/cell types of the heart. Inflammation and the innate immune responses play a major role in I/R injury. Indeed, there is evidence that the protective effect of pharmacological PSMI observed in previous studies is attributable to its anti-inflammatory effects, such as inhibition of the NFκB pathway and leukocyte infiltration. Apparently, non-cardiomyocyte compartments of the heart (e.g., vasculature) and other systems (e.g., leukocytes and the immune system) play an important role in I/R associated inflammatory responses. In aggregate, it is suggested that the benefit of ubiquitous PSMI during myocardial I/R is derived primarily from the inhibition of proteasomes in non-cardiomyocytes. Hence, it is reasonable to speculate that measures to selectively inhibit the proteasome in non-cardiomyocyte compartments would be more effective in protecting against myocardial I/R injury than ubiquitous PSMI.

Potential mechanisms underlying the detrimental effect of CR-PSMI

Oxidative stress is markedly increased during reperfusion, resulting in a burst of production of oxidized proteins, including oxidative modifications of proteasome subunits and thereby inhibiting proteasome function. The removal of oxidized proteins is 20S proteasome dependent. Therefore, it is not surprising that PFI occurs in the cardiomyocytes of I/R hearts. Hence, as supported by the findings that I/R triggered increases of total ubiquitinated proteins were remarkably aggravated by CR-PSMI (Online Figure V), additional CR-PSMI further impairs the degradation of oxidized/misfolded proteins in cardiomyocytes, exacerbating cardiomyocyte dysfunction and even causing cell death. Indeed, both TUNEL assays and detection of activated form of caspase 3 revealed that CR-PSMI significantly increased the prevalence of apoptotic cardiomyocytes in I/R hearts.

Beyond PQC, UPS-mediated protein degradation also regulates cell signaling pathways. PTEN is degraded by the UPS and it negatively regulates Akt. The Akt-mediated pro-survival signaling is activated and plays a protective role during myocardial I/R. The effect of PSMI on PTEN and Akt activation in the cardiomyocytes of I/R hearts has rarely been reported. Here we found that myocardial PTEN was significantly increased, and consistently, Akt activation was suppressed by moderate CR-PSMI in mice under the basal condition (Online Figure VI). Moreover, Akt activation during I/R, as indicated by the increase of Ser473-phosphorylated Akt, was abolished by moderate CR-PSMI. This is perhaps mediated by increased expression of PTEN and PKCδ as both PTEN and PKCδ can inhibit Akt. These results suggest that suppressing Akt activation may have contributed to CR-PSMI induced increases in cardiomyocyte apoptosis in I/R hearts. Ischemic preconditioning (IPC) can powerfully protect against subsequent I/R injury. Two recent reports suggest that preservation of proteasome function contributes to the IPC protection. By activating on the mitochondrial pathway of cell death, PKCδ is a well-known mediator of I/R injury, whereas PKCε protects against I/R injury. It was shown that IPC enhanced PKCδ degradation by the UPS and pharmacologically induced PSMI resulted in mitochondrial accumulation of PKCδ and a loss of IPC protective effects. In the present study, we...
observed that genetically induced CR-PSMI increased the abundance of PKCδ and decreased PKCε in I/R hearts, suggesting that CR-PSMI induced exacerbation of I/R injury might also be related to upregulation of PKCδ and downregulation of PKCε in the heart.

In summary, the present study demonstrates that PFI occurs in cardiomyocytes during myocardial I/R, that moderate CR-PSMI exacerbates cardiac dysfunction and cardiomyocyte death during regional myocardial I/R, and that the detrimental effects of CR-PSMI during myocardial I/R are associated with suppressing the activation of pro-survival kinase Akt and PKCε while enhancing pro-apoptotic pathways mediated by PKCδ.

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DISCLOSURE
None.
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TABLE 1.

In vivo LV function based on P-V loop measurements in Ntg and T60A-β5 Tg mice treated with and without I/R (mean±SD)

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<th>Parameters</th>
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<th>Tg</th>
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<td>Sham (n=6)</td>
<td>I/R (n=7)</td>
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<tr>
<td>HR, beats/min</td>
<td>532 ± 35</td>
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<td>Pes, mmHg</td>
<td>120 ± 15.2</td>
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<td>PRSW, mmHg</td>
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HR, heart rate; Pes, end-systolic pressure; Ves, end-systolic volume; Ped, end-diastolic pressure; Ved, end-diastolic volume; SV, stroke volume; SW, stroke work; CO, cardiac output; %EF, ejection fraction; dP/dtmax, the maximal rate of pressure increasing; dP/dtmin, the maximal rate of pressure decreasing; Tau, relaxation time constant calculated by Glantz method; PRSW, preload recruited stroke work (slope of stroke work- Ved relationship). *P< 0.05, **P< 0.01 vs. Ntg sham; †P 0.05, ††P<0.01 vs. Tg sham; ‡P<0.05, ‡‡P<0.01 vs. Ntg I/R.
Novelty and Significance

What Is Known?

- Myocardial proteasome peptidase activities were altered by acute ischemia/reperfusion (I/R).
- Both genetically achieved cardiomyocyte-restricted proteasome functional enhancement and pharmacologically induced ubiquitous proteasome inhibition (PSMI) paradoxically protect against I/R injury.

What New Information Does This Article Contribute?

- The establishment of the first animal model of cardiomyocyte-restricted PSMI (CR-PSMI), revealing that moderate CR-PSMI is well tolerated by mice.
- CR-PSMI aggravates acute I/R injury.

Elevated oxidative stress during I/R inevitably increases oxidized proteins. Oxidized proteins are degraded by the 20S proteasome in a ubiquitin-dependent or independent manner. Unfortunately, the 20S can be modified and functionally impaired by oxidative stress. Hence, PFI likely occurs in the cardiomyocytes during I/R but this has not been directly demonstrated. Although enhancing proteasome function can protect against I/R injury, proteasome inhibitors have also been shown to reduce myocardial I/R injury. To address this apparent paradox, it is important to assess the impact of CR-PSMI on myocardial I/R injury. Here we report that a peptidase-inactivated mutant β5 subunit of the 20S, when expressed in cardiomyocytes, can replace endogenous β5 and effectively inhibits proteasome function. These studies establish the first animal model of CR-PSMI and show that moderate CR-PSMI suppresses the activation of a key survival kinase Akt potentially via increasing PTEN. This augments the pro-apoptotic kinase PKCδ, promotes cardiomyocyte apoptosis, and thereby aggravates myocardial I/R injury in mice. These findings indicate that in cardiomyocytes PFI plays an important pathogenic role in acute I/R injury, suggesting that the previously observed protective effects of proteasome inhibitors could be related to their activity in the non-cardiomyocyte compartment. Thus, proteasome inhibitors may be more effective in reducing I/R injury if their effects on cardiomyocytes can be minimized.
FIGURES and LEGENDS

Figure 1. Myocardial ischemia/reperfusion (I/R) impairs proteasome peptidase activities in mouse hearts. GFPdgn tg mice were subject to myocardial I/R created by ligation (30min) and subsequent release of the ligation (24h) of the anterior descending artery. Crude protein extracts from myocardial tissues of the left ventricle (LV) of the sham surgery group or of the LV area at risk (AAR), border zone (BZ), and remote area (RA) of the I/R group were used for proteasomal peptidase activity assays (A) and western blot analysis for ubiquitinated proteins (B, C). n=6 mice/group; *p<0.05, **p<0.01 vs. sham.
Figure 2. Increase of GFPdgn protein levels in GFPdgn tg mouse hearts after in vivo focal I/R. A and B, Western blot analyses show increases in the myocardial GFPdgn protein level in the indicated zones (RA, BZ, and AAR) of the LV free wall after I/R. Representative images (A) and densitometry data (B) are presented. N=4 mice per group; *p<0.05, **p<0.01 vs. sham. C, Representative images of myocardial GFPdgn direct green fluorescence in the indicated zones of the LV. Nuclei were stained blue with DAPI. Scale bar=100µm. D and E, RT-PCR assays show that I/R did not increase GFPdgn mRNA levels in the area at risk and the remote area. N=6 mice/group.
Figure 3. Baseline characterization of a tg mouse model of moderate cardiomyocyte-restricted proteasome inhibition (CR-PSMI). Tg mouse lines with a moderate expression of Myc-tagged T60A-β5 precursor under the control of an attenuated mhc6 promoter were created. A, Schematic illustration of the transgenic (TG) construct used for fertilized egg microinjections to create the T60A-β5 Tg mouse founders. B, Sample images of western blot analyses for Myc (upper panel) and the β5 subunit (lower panel) of the 20S proteasome in ventricular myocardium of mice from 2 independent tg lines (Line 1, TG-1; Line 2, TG-2) at 8 weeks. C, A summary of myocardial endogenous β5 (Endo-β5) protein expression in T60A-β5 TG and NTG littermate mice at 8 weeks. D, Representative images of immunoblot analyses (IB) for the indicated proteins in immunoprecipitated (IP) 20S proteasomes from the ventricular myocardium of Myc-T60A-β5 TG and NTG littermate mice at the baseline condition. Antibodies against α3 subunit of the 20S were used for IP 20S proteasomes from crude protein extracts from ventricular myocardium. E, Expression of T60A-β5 suppressed the chymotrypsin-like activity of 20S proteasomes in the heart. **p<0.01 vs. Ntg; n=4 mice/group.
Figure 4. Effects of T60A-β5 tg expression in cardiomyocytes on endogenous β5 expression and proteasomal peptidase activities of I/R and sham hearts. T60A-β5 tg and Ntg littermate mice were subject to myocardial I/R as described in Figure 1. Crude protein extracts from the indicated zones of the LV were used for western blot analyses of endogenous β5 and tg T60A-β5 (A, B) or for proteasomal chymotrypsin-like (C) and caspase-like (D) activity assays. A and B, Representative images (A) and a summary of densitometric data (B) of the western blot analyses for the endogenous β5 expressions. n=4. C and D, Changes in the indicated proteasomal peptidase activities. n=6. *p< 0.05, **p<0.01, ***p<0.005 vs. Ntg sham; #p< 0.05, ##p<0.01.
Figure 5. Moderate CR-PSMI significantly increases the infarct size in I/R mice. T60A-β5 Tg and Ntg littermates at 10~12 weeks of age were subject to LAD ligation and release as described in Figure 1. Infarct size was determined at 24h after reperfusion. A, Representative comparison of infarct size between a Tg mouse heart and a littermate Ntg mouse heart. B and C, Morphometric analysis of area at risk (B) and infarct size (C) in T60A-β5 Tg and Ntg mouse hearts. n=6, *p<0.05 vs. Ntg.
Figure 6. Evaluation of cardiomyocyte apoptosis in Ntg and T60A-β5 Tg mice undergone myocardial I/R. A, Representative images of TUNEL staining (green) in the sham and the indicated zones of the LV of I/R hearts. Nuclei were stained blue with DAPI. Cardiomyocytes are identified by the green fluorescence resulting from Alexa-568-phalloidin staining. Scale bar=50µm. B, The number of TUNEL-positive nuclei is expressed as a percentage of total nuclei detected by DAPI staining. n=6 mice/group, *P<0.05 vs. Ntg. C and D, Western blot analysis myocardial levels of the cleaved (i.e., activated) caspase-3 in all zones of the T60A-β5 Ntg and Tg mouse hearts. N=4; *p< 0.05, **p<0.01, ***p<0.005 vs. Ntg sham; #p< 0.05, ##p<0.01.
Figure 7. Effects of CR-PSMI on Akt activation and PKCδ protein expression in sham and I/R hearts.  

A ~ C, Representative images (A) and a summary of densitometry data of western blot analyses for total Akt and S473-phosphorylated Akt (p-S473-Akt, B) and PTEN (C) in sham hearts and in the indicated zones of I/R hearts. GAPDH was probed for loading control. 

D ~ F, Representative images (D) and a summary of densitometry data of western blot analyses for PKCδ (E) and PKCε (F) in sham hearts and in the indicated zones of I/R hearts. β-Tubulin was probed for loading control. n=4 mice/group; *p< 0.05, **p<0.01, ***p<0.005 vs. Ntg sham; #p< 0.05, ##p<0.01, ###p<0.005.
Genetically Induced Moderate Inhibition of the Proteasome in Cardiomyocytes Exacerbates Myocardial Ischemia-Reperfusion Injury in Mice
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Detailed Methods

Transgenic mice
All mice used in this study are in the FVB/N inbred background. The creation and characterization of the tg mice with expression of a modified green fluorescence protein (GFPdgn) have been previously described.1 GFPdgn was engineered via carboxyl fusion of degron CL1 to an enhanced green fluorescence protein (GFP) and is a proven substrate for the UPS.1, 2

To establish a genetic model of CR-PSMI, we created stable tg mouse lines that overexpress a missense mutation (T60A) of the murine precursor of β5 subunit of the 20S proteasome (hereafter known as T60A-β5) under the control of an attenuated murine mhc6 promoter that was generously donated by Dr. Jeffrey Robbins of University of Cincinnati (Cincinnati, OH). The promoter consists of a full-length murine mhc6 promoter in which 3 GATA sites and 2 TREs (thyroid response elements) were ablated but other cis-acting regions important for cardiac-specific expression were left intact.3

LV pressure–volume relationship analysis
For the invasive assessment of pressure–volume (P-V) relationship, mice were anesthetized with isoflurane (2%) in medical grade oxygen, then intubated and ventilated using a pressure controlled respirator at a tidal volume of 200 µl and a frequency of 130 strokes/minute. Body temperature was monitored with a rectal thermometer and maintained at 37°C. The LV was catheterized in a retrograde fashion via the right carotid artery with a 1.2-F mouse P-V catheter (Scisense, London, Ontario). The instrumented animal was stabilized for 10min and the data were recorded with a sampling rate of 1,500 Hz with Ponemah software (Data Sciences International, Valley View, OH) during steady-state conditions. For subsequent analysis of P-V loops Ponemah software was used. The raw conductance volumes were corrected for parallel conductance by the hypertonic saline bolus.

Proteasome peptidase activity assays
Myocardial samples were homogenized at 4°C in 10 volumes HEPES buffer (50 mM, pH 7.5) containing: KCl 20 mM, MgCl2 5mM, DTT 1mM. Cell debris was removed by centrifugation for 15 minutes at 12,000 g, and the supernatants were immediately used for protein concentration assay and then determination of peptidase activities. The following synthetic fluorogenic substrates: Suc-LLVY-AMC (18µM), Z-LLE-AMC (45µM) (CALBIOCHEM, San Diego, CA), and Ac-RLR-AMC (40µM, BIOMOL Plymouth Meeting, PA) were used respectively for measuring chymotrypsin-like, caspase-like, and trypsin-like peptidase activities in the absence or presence of a proteasome inhibitor, MG-132 (20µM, A.G. Scientific, Inc. San Diego, CA) for chymotrypsin-like and caspase-like activities, or epoxomycin (5µM, CALBIOCHEM, San Diego, CA) for trypsin-like activity. Measurements of each specimen are performed in triplicates. A 5µg of crude protein extract is added to 200µl of the HEPES buffer containing the fluorogenic substrate to each well in 96-well plates, and incubated at 37°C. The fluorescence intensity was measured at 60min of incubation using a Perkin Elmer 2030 Multilabel Microplate Reader with the excitation wave length of 380nm and emission wave length at 460nm. The portion of peptidase activity inhibited by the proteasome inhibitor is attributed to the proteasome.

Western blot analysis
This was performed as previously described.4 Crude proteins were extracted from myocardial tissues. The protein concentration was determined using bichinchoninic acid (BCA) reagents (Pierce biotechnology, Rockford, IL). Equal amounts of samples were subjected to sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF
membrane using a Trans-blot apparatus (Bio-Rad, Hercules, CA). The membranes were blocked with 5% non-fat-dry milk in PBS containing 0.1% Tween-20 (PBS-T) for 1 hour at room temperature and then probed with appropriate primary antibodies overnight at 4°C. Primary antibodies include: ubiquitin (U5379, Sigma-Aldrich, Saint Louis, MO), GAPDH (G8795, Sigma-Aldrich), GFP(B2) (sc-9996, Santa Cruz Biotechnology), β-tubulin (sc-55529, Santa Cruz), murine proteasome β5 subunit (customized), c-Myc (MB600-336, Novus Biological, Littleton, CO), cleaved caspase 3, total Akt (9272S, Cell Signaling), Ser473-phosphorylated Akt (9271S, Cell Signaling), and PKCδ (sc-213, Santa Cruz) and GAPDH (G8795, Sigma). The corresponding horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology) were used respectively for chemiluminescence-based western blot analyses. The bound secondary antibodies were detected using either enhanced chemiluminescence (ECL-Plus) detection reagents (GE Healthcare, Piscataway, NJ) or, for weak signals, ECL Advance Western Blotting Kit (GE Healthcare) and visualized with a VersaDoc3000 imaging system (Bio-Rad). The signal was quantified with the Quantity One software (Bio-Rad).

**Immunostaining and fluorescence confocal microscopy**

These were performed as previously described. 4 Mouse tissues were fixed with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) by perfusion fixation, saturated with 40% sucrose solution and embedded in Tissue-Tek O.C.T. (Sakura Finetek. USA, Inc, Torrance, CA), and then underwent tissue sectioning at 5 micron thickness. The tissue cryosections were permeabilized with 1% of Triton-X100 in PBS for 1 hour, quenched with 0.1M glycine in PBS for 1 hour, and blocked with 0.5% BSA for 1 hour. The specimens were then incubated with primary antibodies overnight at 4°C. The primary antibodies used for immunostaining include: rabbit anti-c-Myc (MB600-336, Novus Biological) and mouse monoclonal anti-sarcomeric α-actinin (A5044, Sigma). The secondary antibodies used are the Alexa-Fluor 488 donkey anti-rabbit Ig, and the Alexa-Fluor 568 donkey anti-mouse Ig (Molecular Probes). The immunostaining was visualized using a fluorescence confocal microscope (Olympus Fluoview 500) and the images were captured and digitalized using the associated software.

**Semi-quantitative reverse transcription-(RT-) polymerase chain reaction (PCR) analyses**

These were performed as we previously described. 5 Total RNA was isolated from ventricular myocardium tissue using the TRI-Reagent (Molecular Research Center, Inc., Cincinnati, CA) following the manufacturer’s protocol. The concentration of RNA was determined using Agilent RNA 6000 Nano assay (Agilent technologies, Inc. Germany) following the manufacturer’s protocol.

Semi-quantitative RT-PCR is a PCR amplification technique that employs both reverse transcriptase and thermostable Taq DNA polymerase. The first step is reverse transcription which synthesizes complementary DNA (cDNA) from the total mRNA template. The subsequent step is traditional PCR amplifying the synthesized cDNA to detectable levels. For reverse transcription reaction, 1μg of RNA was used as a template to generate cDNA using the SuperScript III First-Strand Synthesis kit (Invitrogen) and was carried out according to the manufacturer’s instructions. For PCR amplification reaction, 2μl of solution resulting from the reverse transcription reaction and specific primers towards the gene of interest were used. The transcript levels of GFPΔg were semi-quantitated by PCR at the minimum of cycles that can detect PCR products. The sequences of the specific primers were as follows, GFPΔg: forward 5'-TCT ATA TCA TGG CCG ACA AGC AGA-3' and reverse 5'-ACT GGG TGC TCA GGT AGT GGT TGT-3'; GAPDH: forward 5'-GCC GTA TTG GGC GCC TGG TCA-3' and reverse 5'-AAC
ATA CTC AGC ACC GGC CTT ACCC-3’. Relative mRNA levels were normalized with GAPDH mRNA levels.

In vivo myocardial I/R and assessment of Infarct Size:
These were carried out as we previously described.6 Mice were anesthetized with isoflurane and ventilated through intubation. A thoracotomy was performed at the fourth intercostal space to expose the heart. I/R was produced in mice by surgical ligation of the left anterior descending coronary artery (LAD) for 30min followed by releasing of the ligation for 24h. At the end of reperfusion, the LAD was re-ligated at the original position and the heart was perfused with phthalocyanine blue and cut transversely into 6 slices. The slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) at 37°C for 15 minutes. Each section was weighed and digitally photographed. The phthalocyanine blue–stained area is defined as the remote area (RA), the area unstained by phthalo blue is known as the area at risk (AAR), the TTC unstained area (white) is the infarct area. The RA, the AAR, the infarct area, and the total ventricular areas from both sides of each section were measured using Image-Pro plus software. The AAR was expressed as the percentage of the total ventricular weight and the infarct size was expressed as a percentage of the AAR. For the sham surgery group, the same surgical procedures were performed except for the LAD ligation part.

Immunoprecipitation of 20S proteasomes
Ventricular myocardial samples from T60A-β5 Ntg and Tg littermate mice at the baseline condition were individually homogenized in RIPA buffer and centrifuged at 10,000xg in 4°C for 10 minutes. The supernatant (the crude protein extracts) was collected and protein concentration is determined using the BCA reagents. The antibody specifically against α3-subunit of the 20S proteasome, generously donated by Dr. Peipei Ping of UCLA,7 was used to immunoprecipitate the 20S proteasome.7 The precipitates were then subject to SDS-PAGE and immunoblot using a primary antibody specifically against murine β5 (binds to both endogenous and tg T60A-β5; customized) subunits of the 20S proteasome, c-Myc (binds to tg myc-tagged T60A-β5; MB600-336, Novus Biological, Littleton, CO), or α4 subunit of the 20S (Enzo Life Sciences, Inc. Farmingdale, NY). The reason to probe α4 is to make sure a comparable amount of immunoprecipitated 20S proteasomes are compared between the Tg and Ntg groups.

Terminal deoxynucleotidyl transferase end-labeling (TUNEL) assay
This was performed as previously described.4 The heart was excised 24 hours after reperfusion. Two tissue samples corresponding respectively to the AAR and the remote area (RA) from the LV free wall of each animal were collected (n=6mice/group), fixed in 4% paraformaldehyde in PBS for 24h at 4°C, embedded in Tissue-Tek O.C.T. Compound (Sakura finetek. USA, Inc, Torrance, CA), and frozen and stored at -80°C before sectioning.

TUNEL staining was performed on 5µm cryosections using the In Situ Cell Death Detection kit (Roche Diagnostics) as previously described.4 Images were captured with a confocal fluorescence microscope (Olympus Fluoview 500) in 3 fields for each zone of each animal using a 40x objective. The number of TUNEL positive cells (green) was analyzed using Image-pro Plus 4.5 software, expressed as a percentage of the total nuclei population. Cardiomyocytes (red) were stained with phalloidin labeling, and nuclei (blue) were stained by DAPI.

Statistical Analysis
All quantitative data were presented as mean ± S.D. Differences between groups were evaluated for significance using Student’s t-test for unpaired two group comparison or one-way
or two-way ANOVA followed by the Scheffé's test when appropriate. The p-value <0.05 is considered statistically significant.

References for Supplemental Methods

Supplementary Figures I ~ VI

Online Figure I. Average area at risk (AAR) and infarct size in FVB/N mice induced by the ischemia/reperfusion (I/R) protocol used in the present study. Wild type FVB/N mice at ~10 weeks of age were subject to left anterior descending artery (LAD) ligation (ischemia) for 30min and subsequent release of the ligation (reperfusion) for 24 hours. The mice were sacrificed at 24 hours of reperfusion and the AAR and infarct size determination as described in the Methods section. **A**, TTC staining images of two representative hearts. **B** and **C**, A summary of the indicated parameters from a pilot study (n = 4 mice).
Online Figure II. Immunofluorescence confocal micrographs of the distribution of transgenic T60A-β5 proteins in ventricular myocardium. From a transgenic (tg) mouse line with overexpression of Myc-tagged T60A-β5 precursor under the control of an attenuated mhc6 promoter and a non-tg (Ntg) littermate, perfusion-fixed ventricular myocardium was collected and subject to cryo-sectioning and immunofluorescence confocal microscopy. Representative confocal micrographs of ventricular myocardium immune-stained for Myc (green) and α-actinin (actnn, red) are shown. Scale bar=10μm.
Online Figure III. Western blot analysis for total ubiquitinated proteins in ventricular myocardium of T60A-β5 tg and Ntg mice. Total protein extracts from ventricular myocardium of T60A-β5 Tg and Ntg littermate mice were subject to western blot analyses. β-tubulin was probed as loading controls. The level of total ubiquitinated proteins in ventricular myocardium was not statistically significant between T60A-β5 Tg and Ntg mice (P>0.25, t-test, n=6 mice/group).
Online Figure IV. Representative pressure-volume loops of T60A-β5 Ntg (A, B) and Tg (C, D) mice subject to I/R (B, D) or sham surgery (A, C). Data from detailed analyses of P-V loops are presented in Table 1 of the main text.
Online Figure V. Western blot analyses for total ubiquitinated proteins in the ventricular myocardium of T60A-β5 Tg and Ntg mice that were subject to myocardial ischemia/reperfusion (I/R). Acute focal I/R were induced as described in the main text. Representative images (A) and a bar graph summarizing the pooled densitometry data (B) are shown. *p<0.01 vs. Ntg, n=6 mice per group, ANOVA.
Online Figure VI. Western blot analyses for the indicated proteins in the ventricular myocardium of T60A-β5 Tg and Ntg mice at the baseline condition. Myocardial tissues were collected from the ventricle of T60A-β5 Tg and Ntg littermate mice at approximately 8 weeks of age. Total protein extracts were used for western blot analyses for the indicated proteins. GAPDH was probed for loading control. Representative images (A) and a bar graph showing pooled densitometry data (B) are presented. PTEN, phosphatase and tensin homolog; pS473-Akt, Ser473 phosphorylated Akt; PKCδ and PKCe, protein kinase C isoform δ and ε, respectively. *P<0.05 vs. Ntg, n=3 mice for each group, t-test.