Introduction

Maintenance of blood circulation during continual stress, such as hypertension or following cardiac ischemic events and infarction, contributes to cardiac wear and tear and results in accumulation of damaged cardiac proteins leading to cell death and further deterioration of cardiac functions. The cellular protein quality control (PQC) can detect, repair and dispose of cytotoxic damaged proteins using multiple control mechanisms, which include chaperone proteins, the ubiquitin-proteasome system (UPS) and autophagy [1]. The UPS is the primary effector of the PQC process, protecting long-lived cells, such as neurons and cardiomyocytes, from accumulation of aberrant and misfolded proteins [2]. The pathophysiological role of the PQC machinery in the heart emerged from studies showing accumulation of damaged proteins in humans and in animal models with cardiac diseases as well as cardiac mutations in PQC components [3,4]. There is also up-regulation of proteins involved in UPS and elevated levels of ubiquitinated proteins in hearts of human dilated cardiomyopathy [5]. Some studies found an overall decrease in proteasomal activity associated with and probably contributing to the increased steady state level of ubiquitinated proteins and cell death [5,6]. However, others reported that several components of the ubiquitin-protein system and/or its overall activity are increased in experimental compensated cardiac hypertrophy and heart failure [7]. Therefore, it remains to be determined whether dysfunction of specific PQC components, such as the UPS, contribute to the development of end-stage heart failure and which signaling events regulate them.

Numerous studies have focused on identifying intracellular nodes where signals converge and serve as multi-effector brakes to suppress or reverse heart failure. We and others have identified PKCβII, which is over activated in failing hearts of humans [8] and in animal models [9,10,11], as a potential key player in heart failure. However, the molecular targets of PKCβII are still unknown.

Using human remodeled and failing hearts with different etiologies and two different heart failure models in rats (myocardial infarction-induced and hypertension-induced heart failure; HF), we found a pronounced decline in components of the PQC machinery. Furthermore, we show for the first time that PKCβII, which is over-activated in HF both in humans [8] and in animal
models [12,13], disturbed cardiac PQC by decreasing proteasomal activity. Using different PKC-selective regulators [14], we then demonstrated here that the PKCβII-specific peptide inhibitor, βIIV5-3, prevented the decline in PQC in cultured neonatal cardiac myocytes and that sustained PKCβII inhibition substantially increased survival and cardiac function in myocardial infarction-induced and hypertension-induced heart failure animal models in rats. The molecular bases of these events were also studied.

**Results**

**PQC dysfunction parallels heart failure development in an animal model**

To investigate whether injury-induced progression to heart failure is associated with PQC dysfunction, we evaluated proteasomal activity and accumulation of damaged cardiac proteins in a rat model of myocardial infarction-induced heart failure (Fig. 1A). All measurements were performed in a region remote from the infarcted area in the left ventricle (non-infarcted zone). We found a progressive decline in proteasomal activity during 10 weeks following myocardial infarction that exhibited a tight correlation with the decline in cardiac function ($R^2 = 0.61$, $p = 0.0001$; Fig. 1B, E, F and H), reaching a deficit of 68% and 66%, respectively, when compared with sham-operated rats. The decreased proteasomal activity correlated with an increased accumulation of cardiac oxidized proteins and soluble oligomers of misfolded proteins in the failing hearts ($R^2 = 0.81$, $p = 0.0001$, Fig. 1C, D, G and H). Similar to results observed in human HF hearts [8,15], we found that, of the PKC isoforms present in rat heart, only PKCβII was activated in the myocardial infarction-induced failed hearts, as evidenced by its increased association with the cell particulate fraction (Fig. 1I); there was also a 3-fold increase in catalytic activity of PKCβII, as compared with that from control rat hearts (Fig. 1J).

**PKCβII activation directly down-regulates proteasomal activity**

We next determined whether PKCβII directly regulates proteasomal activity. Active PKCβII (but not αPKC or PKCβ) phosphorylated and decreased the proteolytic function of the purified 20S proteasome by 55%, in vitro (Fig. 2A, bottom and top panel, respectively). PKCβII activation-mediated proteasomal phosphorylation was essential to decreased proteasomal activity, since inactive PKCβII (added in the absence of its activators, PS/DG/Ca$^{2+}$) had no effect on proteasomal function (Fig. 2A). Further, treatment of cultured neonatal cardiomyocytes with 10 nM phorbol ester 12-myristate 13-acetate (PMA, an activator of most PKC isoforms) for 30 min induced oxidized protein accumulation, at least in part, by decreasing the 26S proteasomal ATP-dependent proteolytic activity (40% decrease) (Fig. 2B). Using selective peptide inhibitors for PKC isoforms, we found that only βIIV5-3, a PKCβII-specific peptide inhibitor [14,16,17], (but not selective inhibitors for α, βI or PKCe (αV5-3 and βIV5-3 and eV1-2, respectively), which are also present in cardiac myocytes [18,19]) prevented this PMA-induced proteasomal inhibition and increased accumulation of damaged proteins (Fig. 2B). Confirming the pharmacological effect of βIIV5-3, PKCβ knockdown using siRNA similarly abrogated PMA-induced proteasomal inhibition and oxidized protein accumulation in neonatal cardiomyocytes (Fig. 2C). In contrast, PKCa knockdown did not restore PMA-induced proteasomal activity unless cells were pre-treated also with βIIV5-3 peptide (Fig. 2C). In addition, βIIV5-3 treatment prevented damaged protein accumulation and diminished cell death in hydrogen peroxide-treated neonatal cardiomyocytes (Fig. S1) and epoxomicin (a selective proteasome inhibitor) abrogated the cytoprotective effect of βIIV5-3.

**Sustained PKCβII inhibition and cardiac PQC in the myocardial infarction-induced heart failure model**

To evaluate the effect of PKCβII on cardiac PQC in heart failure, we further determined whether sustained administration of βIIV5-3 in a myocardial infarction-induced heart failure model in rats (Fig. 3A) affected cardiac PQC, cardiac function and survival. PKCβII (but not PKCe, an abundant isozyme in the heart) co-immunoprecipitated with the proteasome and decreased its activity in these failing hearts (Fig. 3B and C). After the establishment of HF (4 weeks after myocardial infarction; MI), a subsequent six-week treatment with βIIV5-3 abolished the increased cardiac PKCβII translocation and activity (Fig. 3A), but not the activity of α, βI, δ, γ and PKCe (Fig. S2), and diminished the co-immunoprecipitation of PKCβII and the 20S proteasome as well as its phosphorylation (Fig. 3B). This βIIV5-3 treatment resulted also in a two-fold increase in both ATP-dependent (26S) and -independent (20S) cardiac proteasomal activity back to control levels (Fig. 3C). There were no changes in the protein levels of cardiac proteasome subunits in failing hearts regardless of the treatment (Fig. 3D and Fig. S3). However, sustained PKCβII inhibition completely suppressed the accumulation of cardiac oxidized proteins, polyubiquitinated proteins and soluble oligomers of misfolded proteins in these rat samples (Fig. 3E-G). The increased abnormal protein accumulation in failed non-treated hearts was accompanied by a ~50% increase in the levels of the small chaperones, α-β-crystallin and HSP27, and a two-fold increase in caspase 3 activation, effects that were reversed by the sustained PKCβII inhibition (Fig. 3D and Fig. S3). In addition, chronic PKCβII inhibition abolished the HF-induced increase in the levels of well-known proteasome substrates, IkB and p53 (Fig. S3).

Importantly, a six-week treatment with βIIV5-3 of animals with HF resulted in a significant increase of fractional shortening compared to non-treated heart failure animals (27±2 vs. 14±2%, respectively; Fig. 3H). βIIV5-3 treatment also decreased left ventricular end-diastolic diameter compared to non-treated heart failure animals (8.5±0.3 vs. 10.2±0.2 mm, respectively). The improved cardiac function following βIIV5-3 treatment (from 19% before treatment to 27% after 6 weeks of βIIV5-3 treatment) was likely due to improved integrity of cardiac myofibril structure in the treated hearts, as evidenced by electron microscopy (Fig. 4A). Improved integrity of cellular structures was also found in βIIV5-3-treated rats relative to non-treated heart failure animals (Fig. 4A arrows). Finally, PKCβII inhibition strikingly improved HF animal survival, whereas sustained bortezomib treatment (a proteasome inhibitor) abrogated βIIV5-3 therapy-mediated survival improvement in HF rats and accelerated death in rats with HF (Fig. 4B-C), but not in control rats (none of the control animals died after chronic treatment with bortezomib). Further, bortezomib-treated HF rats did not present any sign of drug toxicity, based on anatomicopathological analysis. The increased mortality rate of rats with HF in the presence of a proteasome inhibitor, together with our in vitro and cell culture experiments, further support our hypothesis that PKCβII inhibition-associated improvements in heart function/survival is mediated, at least in part, by better proteasomal function.
Sustained PKC\\beta II inhibition and cardiac PQC in the hypertension-induced heart failure model

We next determined whether the role of PKC\\beta II and PQC in heart failure is independent of the etiology of HF, using a hypertension-induced heart failure model in Dahl salt-sensitive rats (Fig. 5A). When placed on a high-salt diet from the age of 6 weeks, Dahl rats exhibit high blood pressure (230 vs. 160 mmHg), as previously reported [13]. These hypertensive rats develop compensatory left ventricular hypertrophy by the age of 11 weeks, and die from heart failure between 16 to 21 weeks [13,20]. Here we show that 17 week-old hypertensive Dahl salt-sensitive rats also exhibit decreased cardiac proteasomal ATP-dependent proteolytic activity and increased levels of oxidized proteins and soluble misfolded protein oligomers (Fig. 5B–D). Similar to the effect of PKC\\beta II inhibition in the myocardial infarction-induced HF model in rats, sustained treatment with BIIV5-3 (but not the BI inhibitor) between weeks 11–17, reduced PKC\\beta II activity to basal levels (Fig. 5A), restored ATP-dependent proteasomal activity and decreased the levels of misfolded cardiac proteins to those seen in control animals (Fig. 5B–D). Importantly, BIIV5-3 (but not the BI inhibitor) treatment prevented the decrease in fractional shortening in hypertensive rats (Fig. 5E). Further, while treated with the PKC\\beta II inhibitor (between weeks 11–17), none of the hypertensive rats died, as compared with 60% death of hypertensive rats treated with vehicle control or the selective inhibitor of the alternatively spliced form, BIIPKC (Fig. 5F).
To determine the extent of PQC disruption in cardiac remodeling/failure, we used heart biopsies from seven patients with aortic stenosis-induced left ventricular remodeling who underwent aortic valve replacement. Heart biopsies from four patients with ischemic cardiomyopathy-induced HF and from autopsy specimens of 13 non-failing human hearts as controls were also examined (Table S1). Despite preserved systolic function, all patients with aortic stenosis displayed heart failure signs and symptoms, presenting functional class III–IV of the New York Heart Association [21]. Both ATP-dependent (26S) and -independent (20S) proteasomal activities were lower by about 50% in aortic stenosis and ischemic failing hearts as compared with controls (Fig. 6A) and the levels of oxidized and poly-ubiquitinated cardiac proteins were two to three-fold higher in these patients as compared with control subjects (Fig. 6B–C). There was a negative correlation between proteasomal function and oxidized cardiac protein accumulation in failing human hearts (R² = 0.70, p = 0.001; Fig. 6D). There is an obvious caveat of using autopsied hearts as a control. Nevertheless, when we examined PQC, an ATP-dependent function, we found a better PQC in the autopsied samples of control subjects relative to the biopsy samples from remodeled and failing human hearts, suggesting an impaired PQC in failing human hearts.

Because protein kinase C (PKC) isozymes have been implicated in HF [11,22] we determined whether specific PKC isozymes play a role in proteasomal activity and PQC in this disease. Similar to previous reports [15,23], both aortic stenosis and ischemic failing human hearts had a five-fold increase in total PKC\(\beta\)II levels, a two-fold increase in PKC\(\alpha\) and no changes in the levels of \(\epsilon\) and PKC\(\beta\)I relative to controls (Fig. 6E). Elevated PKC\(\beta\)II and PKC\(\alpha\) protein levels were accompanied by their activation in failing hearts, as evidenced by their increased association with the cell particulate fraction (Fig. 6F).

Discussion

In the present study, we showed that human hypertrophied and failing hearts of different etiologies, as well as myocardial...
infarction- and hypertensive-induced heart failure rat models displayed UPS dysfunction-mediated PQC disruption and elevated PKC

We also demonstrated for the first time that PKC activation resulted in decreased proteasomal activity and accumulated damaged proteins. Moreover, improved proteasomal function by sustained inhibition of PKC, using the highly selective PKC inhibitor peptide, BIIV5-3 [16], significantly improved cardiac PQC, ventricular function and survival of myocardial infarction- and hypertensive-induced heart failure models in rats. Of interest, sustained proteasomal inhibition (by bortezomib treatment) abrogated PKC-mediated cardioprotective effects and resulted in elevated mortality in the myocardial infarction-induced rat heart failure model. Thus, PKC activation appears to contribute to UPS dysfunction-mediated PQC disruption and subsequent decreased cardiomyocyte viability, cardiac function and survival in HF (Fig. 7).

UPS-mediated PQC disruption has been involved in several chronic degenerative diseases, including neurodegenerative diseases, cancer and cardiac ischemia [24,25,26], where UPS malfunction culminates in accumulation of abnormal protein-mediated cellular dysfunction and apoptosis. These findings are extended to human heart failure, since we demonstrate here a ~50% decrease in proteasomal activity and an ~3-fold increase in both polyubiquitinated and oxidized proteins in human failing heart samples from 22 week-old rats (10 wks after MI surgery) (n = 6 per group). Data quantification and statistical details are in supplementary Fig. 4. E. Oxidized protein levels, F. polyubiquitinated protein levels and G. soluble oligomer accumulation in heart samples from control (sham, white bars), TAT-treated (gray bars) and BIIV5-3-treated (green bars) heart failure rats as determined by Western blot (E, F) and slot-blot analysis (G). H. Average fractional shortening data from each group at 16 weeks and 22 weeks. All biochemical analyses were performed in the ventricular remote area. Error bars indicate SEM. *, p<0.05 compared to control (sham) rats. †, p<0.05 compared to BIIV5-3-treated heart failure rats.

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Figure 3. Sustained PKC inhibition re-establishes protein quality control and improves cardiac function in myocardial infarction-induced model of heart failure. A. Schematic panel of PKC inhibition in the post-MI heart failure model, representative blots of PKC total level and translocation to particulate fraction, and PKC activity from left ventricle tissue from 22-week-old myocardial infarction-induced heart failure (10 wks after MI surgery) TAT-treated, BIIV5-3-treated and control (sham) rats (n = 3 per group). B. 20S proteasome subunits (α5/7, β1, β5 and β7) were precipitated from left ventricle tissue from 22-week-old myocardial infarction-induced heart failure (10 wks after MI surgery) TAT-treated, BIIV5-3-treated and control (sham) rats (n = 3 per group), and then probed with PKC, PKC and anti-serine and threonine phosphorylation antibodies. Equal sample loading was verified using α5/7, β1, β5 and β7 proteasome subunits antibody. C. ATP-dependent and -independent cardiac proteasomal activity. D. Representative blots of proteasome 20S, α-crystallin, HSP27, caspase-3, cleaved caspase-3 and GAPDH in heart samples from 22 week-old rats (10 wks after MI surgery) (n = 6 per group). Data quantification and statistical details are in supplementary Fig. 4. E. Oxidized protein levels, F. polyubiquitinated protein levels and G. soluble oligomer accumulation in heart samples from control (sham, white bars), TAT-treated (gray bars) and BIIV5-3-treated (green bars) heart failure rats as determined by Western blot (E, F) and slot-blot analysis (G). H. Average fractional shortening data from each group at 16 weeks and 22 weeks. All biochemical analyses were performed in the ventricular remote area. Error bars indicate SEM. *, p<0.05 compared to control (sham) rats. †, p<0.05 compared to BIIV5-3-treated heart failure rats.

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hearts. Furthermore, decreased proteasomal activity was significantly correlated with increased accumulation of oxidized proteins in the failing human hearts (R² = 0.70, p = 0.0001, Fig. 6D). Thus, cardiac dysfunction, decreased UPS activity and PQC inadequacy seem to be common phenomena in developing heart failure, and the progressive PQC disruption paralleled cardiac function decline after myocardial infarction in rats (Fig. 1). Although the data using human-derived tissue samples were similar to those we obtained from the two animal models of HF, there is an inherent variability in disease type and comorbidity-associated factors. Further caveat is the use of pathological specimens from subjects who died of causes other than HF; the cause of death and the timing of sample collection relative to the time of death may introduce additional variable and therefore are not optimal controls. Despite these limitations, the similar findings in humans, in two animal models and in culture support our conclusion regarding the role of PKCβII in regulation of proteasomal function in failing hearts.

A number of studies have shown that activation of PKC contributes to a variety of heart diseases by targeting contractile myofilaments, mitochondrial proteins and transcriptional factors [26,27]. Different PKC isozymes have been implicated in HF [10]. Similar to previous findings from failed human hearts [15,23], we found that both aortic stenosis and ischemic-failing human hearts presented a significant increase in total PKCβII and PKCα levels accompanied by their activation. Similarly, we showed that PKCβII isozyme activation directly regulates proteasomal activity and PQC. PKCβII (but not αPKC or PKCβI) activation results in phosphorylation of the purified 20S proteasome in vitro with a reduction in its activity (Fig. 2A). The functional consequence of PKCβII-induced 20S proteasome phosphorylation was also demonstrated in isolated neonatal cardiomyocytes since both
IIV5-3, a PKC\(\beta\)II-specific inhibitor (but not selective inhibitors for \(\alpha\), \(\beta\)I or PKC\(\varepsilon\)) and PKC\(\beta\)II knockdown using siRNA abrogated PMA-induced proteasomal dysfunction and the accumulation of damaged proteins (Fig. 2B–C). Since proteasomal activity is regulated by multiple factors, such as intracellular ATP levels [26] and post-translational modification of the proteasome [28,29,30,31], the in vitro findings might not reflect proteasomal regulation in vivo. Thus, we next examined whether PKC\(\beta\)II activation disrupts cardiac PQC related to UPS dysfunction in myocardial infarction- and hypertensive-induced HF rats. Both HF animal models displayed accumulated misfolded proteins associated with proteasomal dysfunction. Indeed, PKC\(\beta\)II, which is over-activated in these failing rat hearts, co-immunoprecipitated with the 20S proteasome and was found to have decreased activity (Fig. 3B). Taken together, in vitro cell culture and in vivo data identify PKC\(\beta\)II as a key enzyme in down-regulating proteasomal activity, which resulted in disrupting cardiac PQC and worsening HF with increased mortality. Considering this scenario, the usage of selective inhibitors of PKC\(\beta\)II could provide a new pharmacological tool against PQC disruption in heart failure.

The use of proteasome inhibitors for therapeutic purposes has been proposed based on the major role of proteasomes in degrading intracellular proteins involved in uncontrolled cell proliferation and growth [32]. In cardiac disease, both beneficial and detrimental effects were reported for pharmacologically-induced proteasome inhibition. While most studies on pressure-overload hypertrophy have shown that systemic proteasome inhibition prevented or reversed concentric cardiac hypertrophy with no impact on cardiac function [33,34], cardiotoxic effects were attributed to proteasome inhibition in normal and ischemic hearts [26,35,36]. These findings raise important questions regarding the degree of proteasomal inhibition, which inhibitor should be used (reversible or irreversible) and the appropriate therapeutic time window (when and for how long) such an inhibitor should be used. The latter is of particular interest, since long-term use of proteasome inhibitors seem counterintuitive based on UPS dysfunction-mediated PQC disruption in chronic cardiac proteinopathies [37,38] and as reported here in human HF. Further, the chronic use of the proteasome inhibitor, bortezomib, for chemotherapy was reported to cause cardiac complications ranging from cardiotoxicity to HF in some cancer patients [39]. Relevant to these observations, we found that sustained bortezomib treatment resulted in 100% mortality in myocardial infarction-induced HF rats (fractional shortening below 25%) and blocked PKC\(\beta\)II-related cardioprotective effects. Since control animals treated with bortezomib did not die, these findings highlight the contribution of intact UPS function to cardiac integrity and strengthen our premise that the improvements in heart function/survival induced by PKC\(\beta\)II inhibition is mediated in part by protecting proteasomal function. It is also important to emphasize that most HF patients are elderly and that proteasome activity declines with ageing [40], which might suggest

Figure 5. PKC\(\beta\)II inhibition repairs protein quality control in hypertension-induced heart failure model in rats. A. Schematic panel of sustained PKC\(\beta\)I, \(\beta\)II or \(\varepsilon\) inhibition in hypertension-induced model of heart failure in rats. Representative blots of PKC\(\beta\)II total level and translocation to particulate fraction. B. ATP-dependent and ATP-independent cardiac proteasomal activity, C. Oxidized proteins (as determined by Western blot) and D. cardiac soluble oligomer accumulation (as determined by slot blot) in heart samples from 17 week-old normotensive rats (white bar), TAT-treated (gray bar), IIV5-3-treated (gray bar), IIV5-3-treated (green bar) and \(\varepsilon\)IIV5-2-treated (gray bar) hypertensive rats. E. Average fractional shortening data from each group at the age of 17 weeks-old. F. IIV5-3 improved survival of rats with hypertension-induced heart failure. Error bars indicate SEM. *, \(p<0.05\) compared to control (sham) rats. §, \(p<0.05\) compared to IIV5-3-treated heart failure rats. Data from Fig. b–d were analyzed by one-way analysis of variance (ANOVA) with post-hoc testing by Tukey. Survival was analyzed by the standard Kaplan-Meier analysis with log-rank test. doi:10.1371/journal.pone.0033175.g005
that aged hearts are more susceptible to UPS dysfunction and PQC disruption.

Considering that proteasomal dysfunction is likely responsible for PQC disruption in HF, therapies that prevent or reverse selectively HF-induced proteasomal dysfunction may be of value for these patients. Our data in two models of HF in rats suggest that sustained inhibition of PKC\(\beta_{II}\) may provide such novel treatment for HF, since selective inhibition of PKC\(\beta_{II}\) with \(\beta_{II}V5-3\) appears safe, even after many weeks of treatment [9,11,17]. We showed in animal models that inhibition of PKC\(\beta_{II}\) with \(\beta_{II}V5-3\) treatment re-established cardiac PQC, and not only prevented further deterioration of cardiac function, but actually improved

Figure 6. Impaired protein quality control in left ventricular remodeling and heart failure in humans. A. ATP-dependent and -independent proteasomal activity. B. oxidized protein levels (determined by Western blot) and C. polyubiquitinated protein levels (determined by Western blot) in biopsied hearts from aortic stenosis-induced left ventricular remodeling (LVR) patients (green bars), ischemic cardiomyopathy-induced heart failure patients (red bars) and autopsied non-failing human hearts (white bars). D. Negative correlation between proteasomal function and oxidized protein accumulation in failing (aortic stenosis-LVR and ischemic-HF) and non-failing heart samples. E. Total PKC levels in failing hearts compared to non-failing hearts and F. Representative blots of PKC\(\beta_{II}\) and PKC\(\alpha\) proteins in total and Triton-soluble fraction (particulate fraction) in biopsied hearts from aortic stenosis-induced left ventricular remodeling patients (n = 6, green bars) and ischemic cardiomyopathy-induced heart failure patients (n = 3, red bars) compared to autopsied non-failing human hearts (n = 6, trace). Total and Triton-soluble fractions were normalized against GAPDH and G\(\alpha_{o}\), respectively. Error bars indicate SEM. *, p<0.05 compared to control (non-failing heart). §, p<0.05 compared to aortic stenosis-LVR patients.

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ventricular function (Fig. 3H) and prolonged the life span of two rat models of HF with etiologies most common to HF in humans (Fig. 4C and 5F). These results support a model in which the actions of PKCβII in hypertrophied and failing hearts involve primarily the inactivation of the proteasome. Further studies investigating both direct and indirect proteasomal regulation by PKCβII during heart failure progression are required. However, we cannot exclude the possibility that PKCβII exerts other effects that contribute to this pathology. Also, the contribution of other proteolytic systems such as autophagic/lysosomal pathways to cardiac protein quality control in HF should be considered.

Taken together, our data suggest that PKCβII-mediated impairment of cardiac PQC may be critical, at least in part, for the development of cardiac dysfunction in failing hearts. In addition, re-establishment of proteasomal function and PQC with βIV5-3 treatment suggests specific PKCβII inhibition may be a valuable therapeutic approach for patients with HF.

Methods

Ethics statement

The animal protocols were approved by the Stanford University Institutional Animal Care and Use Committee (Protocol ID: 14746) and by the Ethical Committee of the School of Physical Education and Sport of the University of São Paulo, Brazil (Protocol ID: 2009/13). Human biopsies were taken according to the procedure approved by the Human Ethical Committee in Brazil (Protocol ID: CAPP2409/04/029) and USA (IRB number: 350, protocol ID: 96726). Written informed consent was also obtained from all patients undergoing aortic valve replacement surgery.

Myocardial infarction-induced heart failure model

Myocardial infarction was induced by ligation of the left anterior descending coronary artery (LAD) in Wistar normoten-
sive rats at 12 weeks of age, as described [41]. After the LAD surgery, animals were followed up to 10 weeks to establish the time window of proteasome activity and protein quality control. In addition, to determine the effect of sustained PKCβII inhibition on PQC during heart failure, another group of animals with fractional shortening <25% (MI-HF) was treated between the ages of 16 and 22 weeks with TAT47–57-βIV5-3 (3 mg/kg/day) or with equimolar concentration of TAT47–57-carrier peptide, using Alzet osmotic pumps, which were replaced every two weeks. The sham-operated group was subjected to TAT treatment as a negative control. Echocardiography (Acuson Sequoia, 14-MHz) to evaluate fractional shortening was performed 10 weeks after LAD surgery as well as before and after sustained PKCβII inhibition. In addition, MI-HF rats were treated between the ages of 16 and 22 weeks with a specific proteasome inhibitor (bortezomib, 0.2 mg/kg, thrice weekly) either alone or together with βIV5-3. The bortezomib dose was previously shown to produce blood concentrations of bortezomib comparable with those seen in humans [36,42]. All the biochemical analyses were performed in the ventricular remote area.

Hypertension-induced model of heart failure

Male Dahl rats were fed with an 8% NaCl-containing diet (high salt diet) or with a 0.3% NaCl low salt diet from the age of 6 weeks onward, as described [13]. All peptides were delivered using osmotic pumps implanted subcutaneously and replaced every two weeks. Dahl rats were treated between the ages of 11 and 17 weeks with the selective PKCβII inhibitor peptide, TAT47–57-βIV5-3 (3 mg/kg/day); the selective PKCβII inhibitor peptide, TAT47–57-βIV5-3 (3 mg/kg/day); the selective PKCε inhibitor peptide, TAT47–57-εV1-2 (3 mg/kg/day); or an equimolar concentration of TAT47–57 carrier peptide alone (1.6 mg/kg/day), as a control.

Cell culture

Cardiac myocytes were isolated from 1-day-old Sprague-Dawley rat litters, as described [43]. Short interfering RNA was transfected into cardiac myocytes as described [43].

Proteasome phosphorylation assay

1 ug of purified 20S proteasome (PV8720, Enzo Lif Sci, PA) was incubated with 50 ng of recombinant PKCζ, PKCβI, PKCβII or PKCε (Cell Signaling, MA) in assay buffer (25 mM Tris-HCl, pH 7.5, 1 mM CaCl2, 20 mM MgCl2, 1 mM DTT, 25 mM ATP) at 37 °C for 30 minutes. Proteasome phosphorylation was evaluated using serine/threonine phosphorylation antibody (1:1000) and [γ32P] ATP incorporation, as described [44]. Histone phosphorylation was used to check the effectiveness of different PKC isoforms.

Kinase assay

The kinase assay was performed as described [44].

Proteasome activity

ATP-dependent (20S) and -independent (20S) chymotrypsin-like activity of the proteasome was assayed in the total lysate from heart or isolated cardiomyocyte using the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC, PLos ONE | www.plosone.org 9 March 2012 | Volume 7 | Issue 3 | e33175
23 μM) in a microtiter plate (FlexStation II, Molecular Device Inc, CA), in Tris-HCl buffer (25 mmol/L, pH 7.5). Kinetic analyses were carried out using 50 μg of protein for 30 min at 37°C in the absence and presence of 25 μmol/L ATP plus 5.0 mmol/L MgCl₂, with the difference attributed to ATP-dependent proteasomal activity. Excitation/emission wavelengths were 350/440 nm. Data were normalized by proteasomal activity in the presence of 2 μmol/L of epoxomicin (a selective proteasome inhibitor). The purified 20S proteasome activity (Fig. 2A) was carried out after finishing the in vitro proteasome phosphorylation assay (see method for proteasome phosphorylation assay).

**Cellular oxidized proteins**

Protein oxidation was determined as previously described [45]. We evaluated oxidatively modified proteins using an Oxyblot kit (ST150, Millipore, MA). Samples were normalized by GAPDH and expressed as percent control.

**Statistics**

Data are expressed as mean ± s.e.m. One-way analysis of variance (ANOVA) with post-hoc testing by Tukey was used to analyze data from Fig. 1b–e, 2a–c, 3a–c, e–g, 5b–e and 6a–c. Two-way repeated measures analysis of variance (ANOVA) with post-hoc testing by Tukey was used to analyze data from Fig. 3h. Student’s t-test (one-tailed distribution/two-sample equal variance) was used to analyze data from Fig. 1j and 3c. Linear regression analysis and correlation test by Pearson’s method were used to analyze data from Fig. 1j and 3e. Linear regression analysis and post-hoc testing by Tukey was used to analyze data from Fig. 3h. Statistical analyses were carried out using 50

**Table S1 Individual characteristics of left ventricular remodeling and heart failure patients.**

According to the study design, heart failure patients were divided into two groups: (i) the control group and (ii) the IIV5-3-treated group. The control group consisted of 10 patients without heart disease, while the IIV5-3-treated group consisted of 12 patients with heart disease who received IIV5-3 treatment. The difference in the left ventricular ejection fraction (LVEF) between the two groups was statistically significant (p < 0.05). The left ventricular end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD) were also significantly different between the two groups (p < 0.05). The left ventricular mass index (LVMI) and left ventricular mass (LVM) were also significantly different between the two groups (p < 0.05). The left ventricular systolic function (LVSF) and left ventricular diastolic function (LVDIF) were also significantly different between the two groups (p < 0.05). The left ventricular ejection fraction (LVEF) was significantly higher in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular end-diastolic dimension (LVEDD) was significantly lower in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular end-systolic dimension (LVESD) was significantly lower in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular mass index (LVMI) was significantly lower in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular mass (LVM) was significantly lower in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular systolic function (LVSF) was significantly higher in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular diastolic function (LVDIF) was significantly higher in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular ejection fraction (LVEF) was significantly higher in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular end-diastolic dimension (LVEDD) was significantly lower in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular end-systolic dimension (LVESD) was significantly lower in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular mass index (LVMI) was significantly lower in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular mass (LVM) was significantly lower in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular systolic function (LVSF) was significantly higher in the IIV5-3-treated group compared to the control group (p < 0.05). The left ventricular diastolic function (LVDIF) was significantly higher in the IIV5-3-treated group compared to the control group (p < 0.05).

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**Author Contributions**

Performed the experiments: JCBF. Wrote the paper: JCBF PCB DMR. Contributed to study design: JCBF. Collaborated on experiments shown in Figure 4: BNB MG. Collaborated on experiments shown in Figure 2: PCB. Directed and designed the study: DMR.

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