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Genetically induced moderate inhibition of 20S proteasomes in cardiomyocytes facilitates heart failure in mice during systolic overload

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ABSTRACT

The in vivo function status of the ubiquitin–proteasome system (UPS) in pressure overloaded hearts remains undefined. Cardiotoxicity was observed during proteasome inhibitor chemotherapy, especially in those with preexisting cardiovascular conditions; however, proteasome inhibition (PsmI) was also suggested by some experimental studies as a potential therapeutic strategy to curtail cardiac hypertrophy. Here we used genetic approaches to probe cardiac UPS performance and determine the impact of cardiomyocyte-restricted PsmI (CR-PsmI) on cardiac responses to systolic overload. Transgenic mice expressing an inverse reporter of the UPS (GFPdgn) were subject to transverse aortic constriction (TAC) to probe myocardial UPS performance during systolic overload. Mice with or without moderate CR-PsmI were subject to TAC and temporally characterized for cardiac responses to moderate and severe systolic overload. After moderate TAC (pressure gradient: ~40 mm Hg), cardiac UPS function was upregulated during the first two weeks but turned to functional insufficiency between 6 and 12 weeks as evidenced by the dynamic changes in GFPdgn protein levels, proteasome peptidase activities, and total ubiquitin conjugates. Severe TAC (pressure gradients >60 mm Hg) led to UPS functional insufficiency within a week. Moderate TAC elicited comparable hypertrophic responses between mice with and without genetic CR-PsmI but caused cardiac malfunction in CR-PsmI mice significantly earlier than those without CR-PsmI. In mice subject to severe TAC, CR-PsmI inhibited cardiac hypertrophy but led to rapidly progressed heart failure and premature death, associated with a pronounced increase in cardiomyocyte death. It is concluded that cardiac UPS function is dynamically altered, with the initial brief upregulation of proteasome function being adaptive; and CR-PsmI facilitates cardiac malfunction during systolic overload.

1. Introduction

The ubiquitin–proteasome system (UPS) is responsible for the degradation of most intracellular proteins for protein quantity and quality control [1]. Proteasome-mediated degradation occurs in the interior chamber of the 20S proteasome, which is composed of an axial stack of four heptameric rings: 2 outer α rings (α1–α7) and 2 inner β rings (β1–β7). The eukaryotic proteasome possesses three peptidase activities residing in 3 distinct subunits: chymotrypsin-like (β5), trypsin-like (β2), and caspase-like (β1). Clinically used proteasome inhibitors (bortezomib and carfilzomib) target the β5 subunit, thereby inhibiting the proteasome [2].

UPS dysfunction is implicated in a variety of cardiovascular diseases [1–4], including load-dependent cardiac disorders [5–7]. The pathogenic significance of impaired UPS-mediated protein degradation in cardiac hypertrophy and cardiomyopathy is underscored by the recent association of mutations in *TRIM63*, a ubiquitin ligase, with familial hypertrophic cardiomyopathy in humans [8]. Further characterization of the

Abbreviations: CHF, congestive heart failure; PsmI, proteasome inhibition; CR-PsmI, cardiomyocyte-restricted PsmI; LV, left ventricle; NTG, non-transgenic; PFI, proteasome functional insufficiency; PQC, protein quality control; TAC, transverse aortic constriction; TG, transgenic; UPS, ubiquitin–proteasome system.

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identified mutations suggests that they impair TRIM63 ubiquitin ligase activity [8]. Proteasome functional insufficiency (PFI) in cardiomyocytes has also been experimentally demonstrated to mediate the pathogenesis of cardiac proteinopathy and acute myocardial ischemia–reperfusion injury [9–11]. In pressure-overload, myocardial total ubiquitinated proteins are always increased whereas the proteasomal peptidase activities were reportedly both increased or decreased [6,12], indicating that the extent to which the UPS is affected during the progression of pressure overload cardiac hypertrophy remains undefined. The role of the proteasome in the development and progression of pathological hypertrophy also remains elusive. Pharmacological inhibition of the proteasome was found by some to facilitate maladaptive remodeling of stressed hearts [13,14]; however, there were also reports that systemic inhibition of the proteasome suppressed cardiac hypertrophy and attenuated maladaptive cardiac remodeling [6,15]. These controversies prompted us to use genetic approaches in the present study.

An inverse reporter mouse model of UPS performance is used here for the first time to determine dynamic changes in myocardial UPS performance at various stages of transverse aortic constriction (TAC)-induced left ventricle (LV) systolic overload. To determine the role of proteasome dysfunction, we also utilized a cardiomyocyte-restricted proteasome inhibition (CR-PsmI) mouse model [9]. Our results show that changes in myocardial UPS function and proteasome activities are stage-dependent during systolic overload and that CR-PsmI attenuates cardiac hypertrophy only in mice subject to severe TAC but not moderate TAC and, in both cases, facilitates cardiac failure.

2. Materials and methods

2.1. Transgenic mice

GFPdgn transgenic mice, an inverse reporter of UPS performance, were developed and initially characterized as reported [16]. The creation and initial characterization of the stable transgenic mouse lines of CR-PsmI were described [9]. Briefly, this is a stable mouse line expressing a protease-disabled Myc-tagged missense mutation (T60A) of the murine β5 subunit precursor of the 20S proteasome (T60A-β5) in cardiomyocytes, under the control of an attenuated murine *myh6* promoter.

2.2. Transverse aortic constriction (TAC)

TAC was performed as described [17]. The aortic arch was isolated and ligated against a 27-gauge needle for moderate TAC (mTAC, pressure gradient: ~40 mm Hg) or a 29-gauge needle for severe TAC (sTAC, pressure gradient: ~60 mm Hg). The needle was used as the constriction template and was withdrawn immediately after ligation is completed.

2.3. Left ventricular pressure-volume analysis

Left ventricular (LV) pressure–volume relationship was analyzed in mice as previously reported [9]. In brief, the mouse were anesthetized with 2% isoflorane in medical grade oxygen, intubated, and mechanically ventilated. A 1.2-F mouse pressure-volume catheter (Scisense, London, Ontario) was inserted into the LV via the right carotid artery. The animal was allowed to stabilize during steady state conditions for 10 min prior to data collection with a sampling rate of 1500 Hz with Ponemah software (Data Sciences International, Valley View, OH).

2.4. Protein extraction and western blot analysis

Proteins were extracted from LV myocardium. Bicinchoninic acid (BCA) reagents (Pierce biotechnology, Rockford, IL) were used to determine protein concentrations. SDS-PAGE, immunoblotting analysis, and densitometry were performed as previously described [18]. The following primary antibodies were used: green fluorescence protein (GFP, clone B2), GAPDH (Santa Cruz Biotechnology), RPT6 (Biomol), sarcomeric α-actinin, ubiquitin (Sigma), phosphatase and tensin homolog (PTEN), Ser473-phosphorylated-Akt, total Akt, caspase 3, cleaved caspase 3 (Cell Signaling), and PSMB5 (i.e., proteasome subunit β5, customized antibody). The corresponding horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Santa Cruz) were used respectively.

2.5. Proteasome peptidase activity assay

Proteasome peptidase activity assays were performed as reported [19]. Snap-frozen tissues were homogenized on ice in cytosolic extraction buffer (50 mmol/L Tris–HCl pH 7.5, 250 mmol/L Sucrose, 5 mmol/L MgCl2, 0.5 mmol/L EDTA, and 1 mmol/L DTT). Samples were then centrifuged at 8000 *g* for 10 min at 4 °C. The protein concentration of the supernatant was determined by a BCA assay. Proteasome assay buffer (50 mmol/L Tris–HCl pH 7.5, 40 mmol/L KCl, 5 mmol/L MgCl2, and 1 mmol/L DTT) was added to each well of a dark 96-well plate. ATP was added to certain wells to differentiate between peptidase activities in the presence and absence of ATP: chymotrypsin-like activity (28 μmol/L), caspase-like (14 μmol/L), and trypsin-like (14 μmol/L). Equal amounts of sample were loaded to each well, except for the blank wells. Proteasome inhibitors of the specific proteasome activities were applied to decipher the respective activities: chymotrypsin-like (MG132, 20 μmol/L), caspase-like (MG 132, 20 μmol/L), and trypsin-like (Epoxomicin, 5 μmol/L). Specific proteasome activity fluorogenic substrates were added for chymotrypsin-like (Suc-LLVY-AMC, 18 μmol/L), caspase-like (Suc-LLE-AMC, 45 μmol/L), and trypsin-like activities (AC-RLR-AMC (Bz), 40 μmol/L). The 96-well plates were incubated in a 37 °C incubator for 30, 60, 90, 120, 150, and 180 min. At each time point the plate was read in a plate reader (Perkin Elmer, model 2030, Waltham, MA) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Sample readings were subtracted from the blank reading. The proteasome inhibitor suppressible portion of peptidase activity is attributed to the proteasome.

2.6. Measurement of mRNA expression of the fetal gene program

This was done using either RNA dot blot analysis (for the mTAC experiments) or Real time reverse transcription PCR (for the sTAC experiments). Total RNA was isolated from LV myocardial samples using Tri-Reagent® (Molecular Research Center, Cincinnati, OH). RNA dot blot analysis was performed as previously reported [20]. Two micrograms of total RNA were loaded to each dot of a nitrocellulose membrane using a dot blot apparatus. Transcript-specific p32-labeled radioactive oligonucleotide probes were used. The mRNA of following murine genes were assessed: atrial natriuretic peptide (*NPPA*), B-type natriuretic peptide (*NPPB*), skeletal α-actin (*ACTA1*), α-myosin heavy chain (*MYH6*), β-myosin heavy chain (*MYH7*), sarcoplasmic reticulum calcium ATPase 2A (*ATP2A2*), and phospholamban (*PLN*), with the housekeeping gene glyceraldehyde phosphate dehydrogenase (*GAPDH*) probed for loading control. The radioactive probe-bound membrane was exposed to a phosphor screen and imaged and digitized using a Typhoon 860 imager and associated software (Molecular Dynamics).

Real time reverse transcription PCR (RT-PCR) was performed as previously described [21], for quantification of myocardial mRNA levels of the fetal gene program. The High Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA) was used for cDNA synthesis from 0.1 μg of total RNA by reverse transcription. The PCR reaction used the cDNA and thermal stable AmpliTaq Gold DNA polymerase. Predesigned TaqMan primers and probes specific for *NPPA*, *NPPB*, *MYH6*, *MYH7*, and *GAPDH* were obtained from Applied Biosystems.