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SUPPLEMENTAL MATERIAL

Supplemental Methods

Human heart tissue procurement: Ventricular myocardial tissue from patients with advanced heart failure at the University of Michigan was collected at the time of cardiac transplantation and/or left ventricular assist device implantation. Tissue from patients with HCM (University of Michigan) was collected from the proximal intraventricular septum at the time of myectomy. Nonfailing ventricular myocardial tissue was collected from unmatched donor hearts from the University of Michigan (n=3), the National Disease Research Interchange (NDRI) (n=2), and as a kind gift from Kenneth Margulies (University of Pennsylvania) (n=3). Co-morbidities in the donors that may have precluded use of their hearts for cardiac transplantation included age, hypertension, diabetes, minor coronary artery disease, alcohol or tobacco use, and an incidental finding of a malignant but localized renal tumor. Prior to tissue retrieval, all hearts were perfused with ice-cold cardioplegia. Samples from each heart were snap frozen in liquid N_2 at the time of arrival and stored at -80°C. Hearts from NDRI were shipped on ice in cardioplegia solution and received in our laboratory within 11-14 hours after cross clamp. One heart retrieved at the University of Michigan was on ice for 3 hours before freezing (due to the clinical situation). All other hearts were harvested in the operating room immediately after organ explantation. Patient demographic data were recorded at the time of tissue collection.

Clinical genetic testing: Genetic mutation analysis (performed at the Laboratory for Molecular Medicine, Harvard Partners Health Care, Boston, MA) was available on a subset of HCM patients (n=13). Genetic testing consisted of complete sequencing of the coding regions and splice sites of 8 sarcomere genes (MYH7, MYBPC3, TNNI3, TNNT2, TPM1, MYL2, MYL3, and ACTC) for all patients.

Chemicals and reagents: Fluorogenic substrates Suc-LLVY-AMC and Z-LLE-AMC, proteasome inhibitor lactacystin, and all antibodies used to detect proteasome subunits and polyubiquinated proteins were from Biomol International (Plymouth Meeting, PA). Antibodies for rabbit and mouse GAPDH were from Abcam and Chemicon respectively. The activity-based fluorescent proteasome probe, Bodipy TMR-Ah_3L_3VS (MV151) was a kind gift from Herman Overkleeft and Martijn Verdoes (Leiden University, The Netherlands).

Proteasome activity assay: Freshly prepared cytosolic protein (60 μg) was assayed over a range of ATP concentrations in a 96 well plate with a final volume of 250 μL/well. The plate was scanned once per minute for 45 min at an excitation wavelength of 380 nm and emission wavelength of 440 nm in a Spectramax M5 plate reader (Molecular Diagnostics). Data were reported as end-point readings at 45 min, as cleavage of the substrate was linear over this time.

Immunoblotting and densitometry analysis: Protein concentration was determined by the Bradford method. Protein extracts were denatured by boiling and sonication,

resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blocked in 5% milk. Blots were probed with primary antibodies for 1-18 hours, followed by fluorescent-tagged secondary antibodies for 1 hour. Imaging and densitometry analyses were performed using the LI-COR Odyssey laser scanner or ImageQuant TL software and band intensity was normalized to GAPDH as a protein loading control unless otherwise specified.

Active site labeling and in-gel detection of the 20S proteasome: Active site labeling of proteasome catalytic sites was performed as described by Verdoes et al[1, 2]. Whole heart protein lysates (10 μg) were incubated for 1 hr at 37° C with MV151 (300 nmol/L) in a buffer containing 50 mmol/L Tris (pH 7.5), 1mmol/L DTT, 5 mmol/L $MgCl_2$, 250 mmol/L sucrose, and 2mmol/L ATP[1, 2]. For assessment of background labeling, 10 μg of protein lysate was boiled for 3 min with 1% SDS before incubation with MV151. Reaction mixtures were boiled and sonicated and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescently-labeled β-5 subunit was accomplished using the Typhoon Variable Mode Imager (Amersham Biosciences) at λ_{ex}532 and λ_{em}560. Densitometric analysis was performed using Quantity One 1-D Analysis software.

Measurement of protein oxidation products: Protein carbonyls were detected using a commercially available kit (Oxyblot, ONCOR, Gaithersburg, MD). An aliquot of cytosolic protein was derivatized with dinitrophenylhydrazine (DNPH) under acid denaturing conditions. Denatured proteins were separated on a 4-20% SDS-PAGE gel and transferred to PVDF membrane. Membranes were probed with a primary antibody for

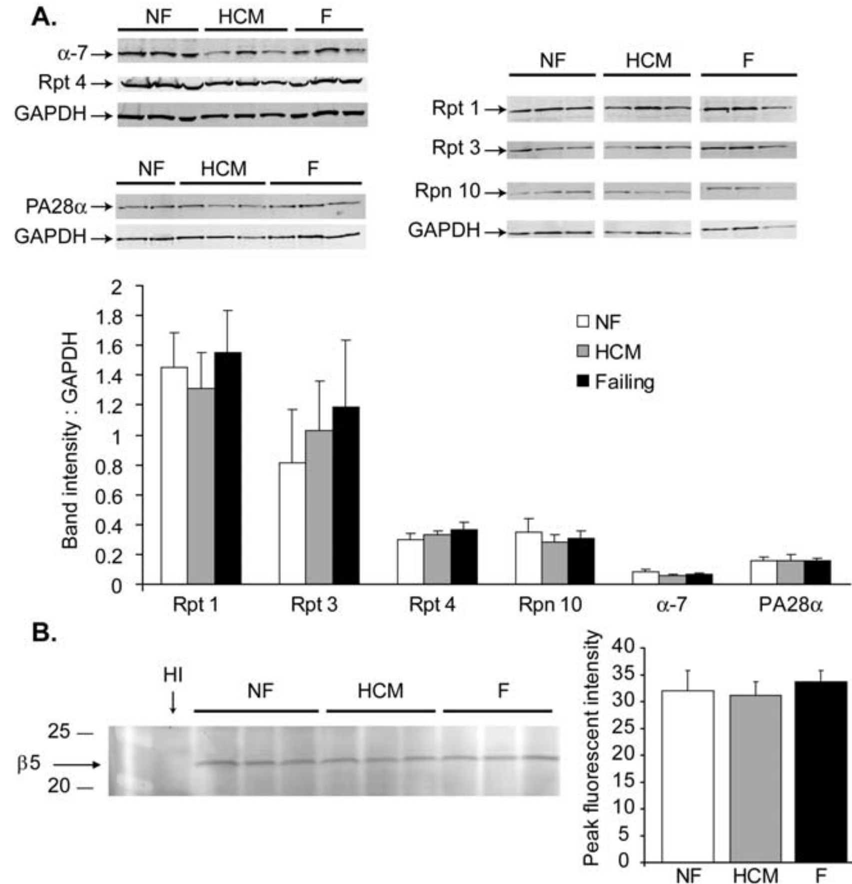
DNPH supplied in the kit, and a secondary horseradish-peroxidase conjugated antibody, and then developed using chemiluminescence. 4-Hydroxynonylated proteins (4HNE) were analyzed by probing membranes with a monoclonal antibody (Oxis International Inc. Foster City, CA).

Preparation of enriched proteasome fraction. The enriched 26S-proteasome fraction was prepared using the method described by Gomes *et al*[3]. Briefly, 1.5 g of frozen heart tissue was pulverized and homogenized in (mmol/l): Tris-HCl, 50, pH 7.5; ATP, 2; $MgCl_2$, 5; DTT, 1. The homogenate was centrifuged at 100,000*g* for 1 h and the supernatant collected and centrifuged at 70,600*g* for 6 h. The resulting pellet was then resuspended in same buffer and applied to a 10 – 40% glycerol gradient and centrifuged at 100,000*g* for 22 h at 4°C. Fractions of 1 ml were collected and 26S proteasome containing fractions identified by activity analysis as described[4] in the presence of ATP, 62.5 μmol/l. Thus the activity detected was considered to be representative of total chymotryptic activity. Activity was concentrated in fractions 13 thru 20 which were pooled and used for all future studies. Pooled fractions were then further concentrated 10-fold by centrifugation in Amicon® Ultra-4 tubes (Millipore, Burlington MA) with a molecular weight cutoff of 10,000. Isolation in this manner enriched 26S-proteasome in excess of 300-fold.

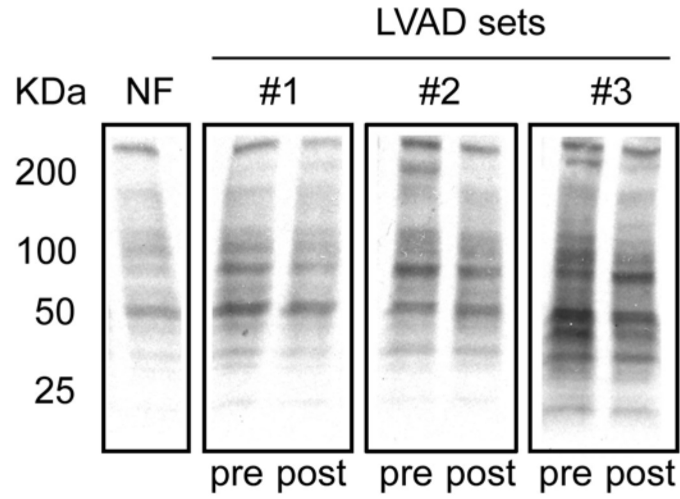
2D gel electrophoresis. The enriched proteasome fraction was reacted with 2,4-dinitrophenylhydrazine (DNPH) as previously described[5] with the exception that neutralization was omitted. Following tagging of carbonyls the enriched proteasome

fraction was separated using 2D gel electrophoresis as described by Gomes *et al*[3]. The enriched 26S proteasome fraction was desalted by 20% TCA/80% acetone precipitation. Following drying the pellet was resuspended in IPG rehydration buffer containing: urea, 8 mol/l; CHAPS, 4%; DTT, 50 mmol/l; Bio-Lytes (BioRad), 0.2%; bromophenol blue, 0.001%. Five μg of the resuspended protein was loaded onto a 7cm IPG strip, pH 3 to 10 (BioLytes, BioRad) overnight. The rehydrated strip was then subjected to isoelectric focusing on the 1st dimension using the Protean Isoelectric Focusing Cell (Bio-Rad) at 250V for 5 hours, then 3000V for 1 hour, then 8000V for 7 hours (total of 49375 V hours). Following this, proteins were first reduced in Equilibration buffer I containing: urea, 6 mol/l; SDS, 2%; Tris-HCl, 0.375 mol/l, pH 8.8; glycerol, 20%; DTT, 130 mmol/l, for 10 min followed by alkylation in Equilibration Buffer II containing: urea, 6 mol/l; SDS, 2%; Tris-HCl, 0.375 mol/l, pH 8.8; glycerol, 20%; iodoacetamide, 135 mmol/l, for 10 min. The proteins were then separated in the 2nd dimension using gel electrophoresis on a 12% Protean II (BioRad) precast gel.

Supplemental Figures and Figure Legends



Supplemental Figure 1. *Proteasome subunit protein quantification in human whole heart homogenates.* **A.** Representative immunoblots probed for proteasome subunits from the 20S core (α-7), 19S regulatory cap (Rpt 1, 3 and 4 and Rpn 10), and 11S activator (PA28α) (top). Densitometric analysis of subunit protein content relative to GAPDH as a protein loading control (bottom). There were no significant differences in protein expression of any of the subunits among groups. N=5-12 per group. **B.** Active-site labeling of the 20S proteasome subunit β-5 (chymotrypsin-like activity) and in-gel detection using the fluorescent and cell-permeable proteasome inhibitor, MV151. Heat-inactivated protein (HI) was used as a negative control. There were no significant differences in the availability of the β-5 active site among groups; n=5 per group.



Supplemental Figure 2. Total protein carbonyls in paired failing heart samples at the time of LVAD and after mechanical unloading at the time of transplantation, showing a non-statistical decrease after LVAD.

Supplemental References

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