Impaired Assembly and Post-translational Regulation of 26S Proteasome in Human End-Stage Heart Failure

Sharlene M. Day, MD; Andras Divald, PhD; Ping Wang, MS; Frank Davis, BS; Sarah Bartolone, BS; Richard Jones, PhD; Saul R. Powell, PhD

Methods and Results—An enriched proteasome fraction was prepared from 7 human nonfailing and 10 failing heart explants. Native gel electrophoresis assessed docking of 19S to 20S proteasome revealing 3 proteasome populations (20S, 26S, and 30S proteasomes). In failing hearts, 30S proteasomes were significantly lower (P=0.048) by 37% suggesting diminished docking. Mass spectrometry–based phosphopeptide analysis demonstrated that the relative ratio of phosphorylated:non phosphorylated α7 subunit (serine_250) of the 20S proteasome was significantly less (P=0.011) by almost 80% in failing hearts. Rpt ATPase activity was determined in the enriched fraction and after immunoprecipitation with an Rpt6 antibody. ATPase activity (pmol PO4/μg protein per hour) of the total fraction was lowered from 291±97 to 194±27 and in the immunoprecipitated fraction from 42±12 to 3±2 (P=0.005) in failing hearts.

Conclusions—These studies suggest that diminished 26S activity in failing human hearts may be related to impaired docking of the 19S to the 20S as a result of decreased Rpt subunit ATPase activity and α7 subunit phosphorylation. (Circ Heart Fail. 2013;6:544-549.)

Key Words: heart failure ■ human ■ phosphorylation ■ ubiquitin proteasome system

Clinical Perspective on p 549

Recent studies have indicated that the UPS can become dysfunctional in a variety of cardiovascular disorders, including myocardial ischemia, diabetic cardiomyopathy, mutant surplus proteinopathies, and human end-stage heart failure. Although it is not clear whether the dysfunction causes the disease or is a consequence of the disease, it is clear that UPS dysfunction can be detrimental to the heart and other organs. On the basis of a proclivity for the dysfunction to preferentially affect the ATP-dependent function of the UPS, we have recently examined for oxidative damage to subunits of the 19S.
and have identified Rpt3/Rpt5 as 2 subunits vulnerable to oxidative
carbonylation in both human end-stage heart failure and
experimental myocardial ischemia.\textsuperscript{13,15} Oxidation of Rpt sub-
units is associated with loss of ATPase activity.\textsuperscript{16} In addition,
perturbations in protein kinase A–related and calcium/calcmod-
ulin-dependent protein kinase II–related signaling pathways in
heart failure have been described.\textsuperscript{17,18} Both of these kinases
have been shown to phosphorylate several subunits of the 20S
usually resulting in increased activity.\textsuperscript{19–21} On the basis of these
studies, we propose that the UPS dysfunction associated with
end-stage heart failure is because of diminished ATPase activity
of the Rpt subunits and altered phosphorylation of the 20S
subunits leading to diminished docking or association of 19S
regulatory particles with 20S proteasomes.

**Methods**

**Subjects**

**Human Heart Tissue Procurement**

Ventricular myocardial tissue from patients with advanced heart fail-
ure at the University of Michigan was collected at the time of cardiac
transplantation. Nonfailing ventricular myocardial tissue was collect-
ed from unmatched donors from the University of Michigan and other
regional hospitals through a protocol with the Gift of Life Michigan
Organ and Tissue Donation Program. This project was approved by the
University of Michigan Institutional Review Board (IRB) and subjects
gave informed consent. Each heart was perfused with ice-cold car-
dioplegia, samples were snap frozen in liquid N\textsubscript{2}, and stored at \(-80\textdegree\)C.
Patient demographics were recorded at the time of tissue collection.

**Laboratory**

**Preparation of Enriched Proteasome Fraction**

Enriched proteasome fraction was prepared as described by Gomes et al\textsuperscript{10} using differential ultracentrifugation. This procedure typically
results in the formation of a proteasome-containing fraction that is
>200-fold concentrated (additional detail in the online-only Data
Supplement).\textsuperscript{15}

**Native Gel Electrophoresis**

Enriched proteasome fraction was subjected to nondenaturing native
gel electrophoresis as described by Gomes et al\textsuperscript{19} using a 4%
polyacrylamide gel. After electrotransference, membranes were
probed with polyclonal anti-β\textsubscript{5} or anti-Rpt5 (Enzo Life Sciences,
Plymouth Meeting, PA) to assess the presence of 20S or 19S, re-
spectively. Immunoprecipitation (additional detail in the online-only
Data Supplement). 19S Rpt subunits were immunoprecipitated (IP)
with monoclonal anti-Rpt6 antibody (p45-110) (Enzo Life Sciences)
probed with polyclonal anti-

**ATPase Activity**

Enriched proteasome fraction and IP Rpt subunits were analyzed for
ATPase activity using the Sensolyte MG Phosphate Assay
Kit (Anaspec, Fremont, CA) according to the kit instructions.
Measurements were recorded during the 3- to 30-minute interval in the
enriched fraction and in the 2- to 20-minute interval in the IP
fractions to allow for completion of nonspecific formation of PO\textsubscript{4}.
All results are expressed as pmol PO\textsubscript{4}/µg protein per hour (additional
details in the online-only Data Supplement).

**Phosphopeptide Analysis of Enriched Proteasome Fraction**

Briefly, samples were quantitated using the Qubit fluorescence plat-
form (Invitrogen, Carlsbad, CA) with nano liquid chromatography/mass
spectrometry/mass spectrometry (Waters NanoAcquity HPLC
system, Milford, MA) interfaced to a Thermo Fisher LTQ Orbitrap
Velos (Waltham, MA). The 15 most abundant ions were selected for
mass spectrometry/mass spectrometry. Data were searched using a
local copy of Mascot. Mascot DAT files were parsed into the Scaffold
software for validation, filtering, and to create nonredundancy across
all samples. Data were filtered using a minimum protein value of 90%,
a minimum peptide value of 50% (Prophet scores), and requiring
at least 2 unique peptides per protein (in-depth protocol in the
online-only Data Supplement).

**Statistics**

Differences between nonfailing and failing hearts were determined
using Fisher exact test for dichotomous variables or a Mann–Whitney
rank-sum test for continuous variables (SigmaStat; Jandel Scientific,
San Rafael, CA), with \(P\leq0.05\) considered to be significant. All values
are expressed as the mean±SEM.

**Results**

**Characteristics of End-Stage Heart Failure Patients**

The demographics of patients from whom heart tissue sam-
iples were obtained are described in the Table. Age and race
were not significantly different between nonfailing and fail-
ing groups. A greater proportion of the donor hearts was from
women compared with failing hearts (\(P=0.05\)). The failing
samples were selected so as to minimize patients with left ven-
tricular assist devices as proteasome activity is enhanced with
mechanical unloading.\textsuperscript{15} As we are not aware of any studies
that show a sex bias in proteasome function in either failing or
nonfailing hearts, we think it unlikely that this sex discrepancy
would affect interpretation of the results. As expected, systolic
function (as measured by echocardiography) was markedly
lower in failing compared with nonfailing hearts.

**Docking of 19S to 20S is Decreased in End-Stage Human Heart Failure**

To evaluate docking of 19S to 20S, enriched proteasome fraction
was subjected to native gel electrophoresis. Membranes were
probed with antibodies to the β\textsubscript{5} or Rpt5 subunits to
detect presence of 20S or 19S, respectively. High molecular
weight bands were deemed to contain docked 26S or 30S if
recognized by both antibodies. In general, 3 proteasome pop-
ulations were detected: 20S (not docked), 26S (20S docked
with 1 19S), and 30S (20S docked with 2 19S; Figure 1A).
Overall docking, defined as the amount of 19S associated with
20S and included both 26S and 30S, was lower by almost 30%
(\(P=0.073\)) in failing hearts with docking of 30S significantly

**Table. Patient Demographics**

<table>
<thead>
<tr>
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<th>Control (n=7)</th>
<th>Failing (n=11)</th>
<th>(P) Values</th>
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<tr>
<td>Age, y</td>
<td>52±7</td>
<td>52±4</td>
<td>0.65</td>
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<tr>
<td>Sex, % women</td>
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<td>Race, % white</td>
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<tr>
<td>Ejection fraction, %</td>
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<td>15±1</td>
<td>&lt;0.001</td>
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<tr>
<td>Etiology of heart failure, % ischemic</td>
<td>...</td>
<td>45</td>
<td>...</td>
</tr>
<tr>
<td>LV assist device, %</td>
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<td></td>
<td></td>
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<tr>
<td>Beta blockers, %</td>
<td>14</td>
<td>45</td>
<td>0.33</td>
</tr>
<tr>
<td>Inotropic medications, %</td>
<td>0</td>
<td>91</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(LV\) indicates left ventricular.
lower by 37% (P=0.048; Figure 1B). Total 20S was lower by ≈20% but this was not significant. Decreased docking was not associated with significant differences between failing and nonfailing hearts in the protein abundance of any 20S or 19S subunits as detected by mass spectrometry (Table I in the online-only Data Supplement).

Rpt Subunit ATPase Activity is Lower in End-Stage Human Heart Failure
To determine function of the Rpt subunits in the base of the 19S, ATPase activity of the enriched proteasome fraction was determined and found to be lower by 34% (Figure 2A). Because the enriched fraction contains many other proteins, some of which may have ATPase activity, the Rpt subunits were IP with an antibody specific for Rpt6. Imunoprecipitation with an antibody specific for Rpt6 coimmunoprecipitates other Rpt subunits. Enriched proteasome fraction from both nonfailing and failing hearts was immunoprecipitated with anti-Rpt6 under non-denaturing conditions. The antigen:antibody complex was captured using the Mouse TrueBlot IgG beads. Equivalent volumes of the beads and the resultant supernatant were then subjected to polyacrylamide gel electrophoresis under denaturing conditions. The blot was then probed with an antibody to Rpt5. Heavy-chain contamination is ruled out by the blank (BL) which in this case was enriched fraction buffer (no protein) treated in a like manner with the Rpt6 antibody and the beads.

Phosphorylation of the α7 Subunit of the 20S is Lower in End-Stage Human Heart Failure
Phosphorylation of 20S subunits is likely necessary for docking of 19S to 20S proteasomes. Enriched proteasome fraction
was subjected to mass spectrometry phosphopeptide mapping to assess phosphorylation of 20S subunits. Phosphorylation of the α7 subunit at serine residues was detectable with a reliable signal in all samples. The relative percentage of phosphorylated protein (expressed as a total of phosphorylated/nonphosphorylated) was significantly lower (P=0.011) in failing compared with nonfailing hearts (Figure 3A). Chromatographic peak areas for phosphorylated and nonphosphorylated α7 for all hearts examined are presented in Table II in the online-only Data Supplement. Casein kinase II is the only kinase reported to phosphorylate α7 at serine. However, casein kinase II expression was actually higher by almost 70% (P=0.019) in the failing hearts (Figure 3B and C). Phosphorylated peptides were detected for Rpt6, Rpn6, and Rpn1 in some of the samples, but signals were too low for accurate quantification.

Discussion

Several studies have either directly or indirectly shown that the UPS is dysfunctional in human end-stage heart failure. This is unlike autophagy, which seems to be activated in both experimental models and in human end-stage heart failure perhaps to maintain protein quality control. On the basis of previous studies in experimental myocardial ischemia suggesting preferential effects on the ATP-dependent activities of the proteasome, we theorized that a persistent defect must be present in the 19S regulatory particle. We have shown that at least 1 of the Rpt subunits is vulnerable to oxidative damage in both experimental myocardial ischemia and in human end-stage heart failure. Proper function of the UPS requires docking of a 20S proteasome with 1 or 2 19S regulatory particles, which is dependent on ATPase activity of the Rpt subunits in the 19S, and likely phosphorylation of 20S subunits. In the present study, we show that UPS dysfunction in human end-stage heart failure is associated with diminished docking of the 19S to 20S, loss of ATPase activity of the Rpt subunits found in the base of the 19S, and decreased phosphorylation of the α7 subunit, the most heavily phosphorylated subunit of the 20S proteasome.

Diminished docking of the 19S to 20S has not been described previously in end-stage heart failure. The only study to examine docking in a cardiac pathology associated with UPS dysfunction does suggest that it can be diminished; however, this was in response to myocardial ischemia. Nonetheless, the results do indicate that there was less 19S associated with 20S. There are 2 main determinants of proper docking, the ATPase activity of the Rpt subunits within the base of the 19S and signaling events that alter phosphorylation of proteasome 19S and 20S subunits. The recent studies all suggest that proper function of the Rpt subunits is critical for assembly of complete 26S (or 30S) proteasomes. The current thinking is that on ATP binding, the C termini of the Rpt subunits interact with pockets between the 20S α subunits fostering docking of the 19S with a 20S and also opening the access gate and stimulating hydrolysis of peptides. The 2 most important Rpt subunits for this function seem to be Rpt2 and Rpt5, the latter being 1 of the subunits previously observed to be oxidized in human end-stage heart failure and experimental myocardial ischemia. In general, ATPasences have been observed to be sensitive to oxidative inactivation, thus, it is plausible that the diminished Rpt ATPase activity in failing heart is related to oxidative damage, consistent with a previous report in an in vitro cellular preparation exposed to an oxidizing environment.

The other determinant of docking is phosphorylation of 19S and 20S subunits. Several subunits have been observed to be phosphorylated, including α2, α3, α5, α7, β1, β2, β3, β5, β6, and β7 on the 20S and Rpt6 on the 19S. In general, phosphorylation of proteasome subunits tends to stabilize the proteasome and increase activity. Phosphorylation of Rpt6, in particular, seems to enhance docking and stabilize the 26S proteasome. One of our primary observations is that phosphorylation of the α7 subunit at serine is the major of the 2 known phosphorylation sites, is lower. Diminished phosphorylation of this subunit would be consistent with the observed decreased docking and reported diminished activity of the UPS in end-stage heart failure. Several kinases have been reported to phosphorylate various subunits of the 26S proteasome, including protein kinase A, calcium/calmodulin-dependent protein kinase II, and casein kinase II. In fact, it is likely that phosphorylation of multiple subunits by protein kinase A accounts for its ability to enhance assembly of 26S proteasomes.
proteasome in canine heart.27 However, only casein kinase II is known to phosphorylate the α7 subunit at serine, and thus was a reasonable prospect for analysis. Yet, we observe that like other kinases, casein kinase II is increased in human end-stage heart failure, and thus is contrary to the finding of decreased phosphorylation at this site. One possible explanation is increased phosphatase activity. Both PP1 and PP2A have been shown to dephosphorylate several proteasome subunits and in general decrease proteasome activity20; although, at this time it is not apparent which of these dephosphorylates serine250 of α7. In studies of human heart failure, upregulation of PP13,34 and increased phosphatase activity attributed to PP1 and PP2A have been consistently documented.4-7 Therefore, we hypothesize that increased phosphatase activity is responsible for relative dephosphorylation of serine250 of α7 observed in human end-stage heart failure compared with control hearts.

**Limitations**

The major limitation of any study using human samples is inherent variability in disease pathology, medical therapy, and availability and suitability of matched control heart tissue. By necessity, we are limited to a single time point, that is, explants from failing hearts at time of transplantation, thus the results of this study are applicable to end-stage heart failure only. In addition, the use of interventions to show cause and effect is limited and unfortunately, apical core samples obtained at time of left ventricular assist device do not provide enough tissue to prepare the enriched proteasome fractions. Several previous studies12,29-42 in experimental animal and cellular models have observed either increased UPS activity during development of hypertrophy and failure or have suggested that inhibition of the proteasome might be of clinical value. There is controversy as at least 1 study was unable to reproduce these results and observed the opposite.41 Because the current study was conducted on explanted end-stage failing hearts, it has little bearing on these previous studies, which examined relatively early events in the development of heart failure in short-term animal experimental models. All the previous studies in human heart, suggesting that the UPS is dysfunctional, were done on explanted end-stage failing hearts, thus the observations are relevant to this clinical phase only, which is difficult to model in experimental animals.13,23,24 A limitation of the proteomics studies is that only the most heavily phosphorylated subunit, α7, was detectable at a threshold sufficient to warrant quantitative comparison between groups. Therefore, we cannot exclude the possibility that there are biologically relevant differences in the phosphorylation of other proteasome subunits between nonfailing and failing hearts.

**Summary and Clinical Relevance**

This study examines the molecular basis for dysfunction of the UPS in end-stage heart failure. We observed decreased docking of the 19S to the 20S which could account for the previously reported decrease in proteasome activity in end-stage heart failure. We propose that the diminished docking is related to the observed loss of 19S Rpt subunit ATPase activity and decreased phosphorylation of the most heavily phosphorylated α subunit of the 20S. From a clinical perspective, a more thorough understanding of the mechanisms that underlie UPS dysfunction could lead to development of interventions that either prevent the oxidative damage that leads to loss of ATPase activity or augmentation of subunit phosphorylation by alteration of either kinase or phosphatase activities.

**Acknowledgments**

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**Sources of Funding**

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**Disclosures**

None.

**References**

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**CLINICAL PERSPECTIVE**

This study examined a potential mechanism for loss of proteasome function in human end-stage heart failure. The proteasome is a very large complex that has as 1 of its functions maintenance of protein quality control within the heart. In order for proteasome to accomplish this efficiently, this large complex must assemble completely and in proper order. We show that in human end-stage heart failure, a component that regulates the function of the proteasome does not combine efficiently with the main complex. We identify 2 potential mechanisms as to why this occurs with the thought that a more thorough understanding of these mechanisms could lead to interventions that could conceivably improve cardiomyocyte function in the failing heart.
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http://circheartfailure.ahajournals.org/content/suppl/2013/03/20/CIRCHEARTFAILURE.112.000119.DC1

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SUPPLEMENTAL MATERIAL

Supplemental Methods

**Preparation of enriched proteasome fraction.** The enriched 26S-proteasome fraction was prepared using the method described by Gomes et al.\(^1\) Briefly, approximately 1 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 100,000g for 1 h and the supernatant collected and centrifuged at 70,600g for 6 h. The resulting pellet was then resuspended in same buffer and applied to a 10 – 40% glycerol gradient and centrifuged at 100,000g for 22 h at 4°C. Fractions of 1 ml were collected and 26S proteasome containing fractions identified by activity analysis as described\(^2\) in the presence of ATP, 62.5 μmol/l. Fractions containing high activity were pooled and used for all future studies. Pooled fractions were then further concentrated 10-fold by centrifugation in Amicon\(^\text{®}\) Ultra-4 tubes (Millipore, Burlington MA) with a molecular weight cutoff of 10,000. In general, isolation in this manner enriched 26S-proteasome in excess of 200-fold.

**Immunoprecipitation.** Heart tissue was homogenized in buffer containing (mmole/L): Hepes 10, NaCl 137, KCl 4.6, KH\(_2\)PO\(_4\) 1.1, MgSO\(_4\) 0.6, EDTA 1, Digitonin 0.01%, pH 7.4, plus 1X protease inhibitors (Protease Cocktail Set 1, EMD-Calbiochem) and centrifuged at 10,000g, at 4°C, for 30 min. An aliquot containing 12 μg of enriched proteasome fraction was incubated with 3.6 μg of a monoclonal antibody specific for the Rpt6 subunit (clone P45-110; ENZO Life Sciences, Farmingdale, NY) in 200 μl buffer A, containing (mmole/L): KCl, 110, MgCl\(_2\) 1, Hepes 5, pH 7.4, at room temperature for 6 h. To minimize interference by the heavy and light chain IgGs, the subunit-immuno-complex was captured using the TrueBlot™ reagent kit (eBioscience, San Diego, CA) according to the literature protocol. Briefly, the antibody-subunit
complex was incubated with constant rotation with 24 μl of the pre-washed beads overnight at 4°C. The beads were then spun down and washed exhaustively with buffer B containing (mmoles/L): NaCl 150, Tris HCl 40, and EDTA 2, pH 7.5, prior to resuspension in 300 μl buffer A.

**ATPase activity.** The SensoLyte® MG Phosphate Assay Kit (Anaspec, Fremont, CA) is based on spectrophotometric quantification of the blue green complex formed between malachite green, molydate, and free phosphate versus known standards of phosphate. Both the enriched proteasome fraction and IP Rpt subunits were subjected to two cycles of centrifugal dialysis (Amicon® Ultra-0.5ml, 10,000 MWCO; Millipore, Carrigtwohill, Ireland) to remove phosphate prior to analysis.

**Phosphopeptide mapping of enriched proteasome fraction.** Samples were quantitated using the Qubit fluorescence platform (Invitrogen). 10 μg of each sample was solubilized in LDS buffer, heated at 85°C for 5 min and the full amount separated ~2 cm on a 10% Bis-Tris Novex mini-gel (Invitrogen) using the MES buffer system. The gel was stained with coomassie and each lane was excised into ten equally sized segments. Gel segments were processed robotically (ProGest,DigiLab) according to the following protocol:

1. washed with 25 mM ammonium bicarbonate followed by acetonitrile;
2. reduced with 10 mM dithiothreitol at 60°C followed by alkylation with 50mM iodoacetamide at room temperature;
3. digested with trypsin (Promega) at 37°C for 4 h;
4. quenched with formic acid and the supernatant was analyzed directly without further processing.
Samples were analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher LTQ Orbitrap Velos. Peptides were loaded on a trapping column and eluted over a 75 μm analytical column at 350 nL/min. Both columns were packed with Jupiter Proteo resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 60,000 FWHM resolution and MS/MS performed in the LTQ. The fifteen most abundant ions were selected for MS/MS. Data were searched using a local copy of Mascot with the following parameters:

1. enzyme: trypsin;
2. database: IPI Human v3.75 (concatenated forward and reverse plus common contaminants);
3. fixed modification: Carboxymethyl (C);
4. variable modifications: Oxidation (M), Acetyl (N-term), Pyro-Glu (N-term Q), Deamidation (N,Q), Phosphorylation (STY);
5. mass values: monoisotopic;
6. peptide mass tolerance: 10 ppm;
7. fragment mass tolerance: 0.9 Da;
8. max missed cleavages: 2.

Mascot DAT files were parsed into the Scaffold software for validation, filtering, and to create non-redundancy across all samples. Data were filtered using a minimum protein value of 90%, a minimum peptide value of 50% (Prophet scores) and requiring at least 2 unique peptides per protein. Phosphopeptide produce ion spectra were manually validated to be correct.
Supplemental Tables

Supplement table 1. Spectral counts for all proteasome subunits in failing and control hearts.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=4)</th>
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<td><strong>20S subunits</strong></td>
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<tr>
<td>α1</td>
<td>83 ± 5</td>
<td>74 ± 5</td>
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<td>α2</td>
<td>59 ± 4</td>
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<td>Rpt1</td>
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<td>52 ± 6</td>
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<td>80 ± 10</td>
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<tr>
<td>PA28α (11S)</td>
<td>8 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>PA28β (11S)</td>
<td>7 ± 3</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>
Spectral counting is the use of the number of product ion spectra obtained for each protein as a measure of protein abundance in a mixture. Spectral counts (SpC) correlate linearly with protein quantity. The table shows the mean and standard deviation of SpC observed to each proteasome subunit from four control and six failing hearts. The SpC value can only be used to compare a given protein to itself, and not to compare the levels between different proteins. The data indicate that the abundance of proteasome subunits is not significantly different between the two groups.
Supplement table 2. Chromatographic peak areas for phosphorylated (Ser\textsubscript{250}) and non-phosphorylated \(\alpha7\) in failing and control hearts.

<table>
<thead>
<tr>
<th></th>
<th>(PO_4\ \alpha7)</th>
<th>Non-(PO_4\ \alpha7)</th>
<th>(\frac{PO_4}{(PO_4 + \text{non-}PO_4\ \alpha7)}\times100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1.4 \times 10^8)</td>
<td>(6.6 \times 10^6)</td>
<td>95.4</td>
</tr>
<tr>
<td></td>
<td>(9.4 \times 10^7)</td>
<td>(1.2 \times 10^7)</td>
<td>88.9</td>
</tr>
<tr>
<td></td>
<td>(7.3 \times 10^7)</td>
<td>(1.4 \times 10^7)</td>
<td>83.5</td>
</tr>
<tr>
<td></td>
<td>(1.06 \times 10^8)</td>
<td>(3.1 \times 10^7)</td>
<td>77.3</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>(1.0 \pm 1.34 \times 10^8)</td>
<td>(1.6 \pm 0.53 \times 10^7)</td>
<td>86.3 ± 3.8</td>
</tr>
<tr>
<td><strong>Failing</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5.3 \times 10^7)</td>
<td>(2.2 \times 10^7)</td>
<td>70.7</td>
</tr>
<tr>
<td></td>
<td>(1.8 \times 10^8)</td>
<td>(6.9 \times 10^7)</td>
<td>71.7</td>
</tr>
<tr>
<td></td>
<td>(9.3 \times 10^7)</td>
<td>(2.3 \times 10^7)</td>
<td>79.9</td>
</tr>
<tr>
<td></td>
<td>(7.1 \times 10^7)</td>
<td>(3.9 \times 10^7)</td>
<td>64.5</td>
</tr>
<tr>
<td></td>
<td>(5.0 \times 10^7)</td>
<td>(4.1 \times 10^7)</td>
<td>54.9</td>
</tr>
<tr>
<td></td>
<td>(7.7 \times 10^7)</td>
<td>(6.5 \times 10^7)</td>
<td>54.2</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>(8.7 \pm 1.9 \times 10^7)</td>
<td>(4.3 \pm 0.8 \times 10^7)</td>
<td>66.0 ± 4.1*</td>
</tr>
</tbody>
</table>

Peak areas for both the ESLKEEDESDDDNM and ESLKEEDEpSDDDNM peptides (oxidized and non-oxidized methionine versions of each) were manually calculated in each sample, and across multiple gel bands per sample. *P<0.01 for failing vs control samples.
Reference List
