Ubiquitin Proteasome Dysfunction in Human Hypertrophic and Dilated Cardiomyopathies

Jaime M. Predmore, MS; Ping Wang, MS; Frank Davis; Sarah Bartolone; Margaret V. Westfall, PhD; David B. Dyke, MD; Francis Pagani, MD, PhD; Saul R. Powell, PhD; Sharlene M. Day, MD

Background—The ubiquitin proteasome system maintains a dynamic equilibrium of proteins and prevents accumulation of damaged and misfolded proteins, yet its role in human cardiac dysfunction is not well understood. The present study evaluated ubiquitin proteasome system function in human heart failure and hypertrophic cardiomyopathy (HCM).

Methods and Results—Proteasome function was studied in human nonfailing donor hearts, explanted failing hearts, and myectomy samples from patients with HCM. Proteasome proteolytic activities were markedly reduced in failing and HCM hearts compared with nonfailing hearts (P<0.01). This activity was partially restored after mechanical unloading in failing hearts (P<0.01) and was significantly lower in HCM hearts with pathogenic sarcomere mutations than in those lacking these mutations (P<0.05). There were no changes in the protein content of ubiquitin proteasome system subunits (ie, 11S, 20S, and 19S) or in active-site labeling of the 20S proteolytic subunit β-5 among groups to explain decreased ubiquitin proteasome system activity in HCM and failing hearts. Examination of protein oxidation revealed that total protein carbonyls, 4-hydroxynonenylated proteins, and oxidative modification to 19S ATPase subunit Rpt 5 were increased in failing compared with nonfailing hearts.

Conclusions—Proteasome activity in HCM and failing human hearts is impaired in the absence of changes in proteasome protein content or availability of proteolytic active sites. These data provide strong evidence that posttranslational modifications to the proteasome may account for defective protein degradation in human cardiomyopathies.

(Circulation. 2010;121:997-1004.)

Key Words: apoptosis ■ cardiomyopathy ■ heart failure ■ hypertrophy ■ myocardium ■ proteins

Proteolytic degradation is critical for maintaining a dynamic equilibrium of proteins and destroying damaged or misfolded proteins. As the major pathway for intracellular protein degradation, the ubiquitin proteasome system (UPS) requires precise control to sustain most biological processes. Regulation of proteasome function may occur by altered proteasome composition (ie, association of the 20S proteolytic core with different regulatory complexes such as the 19S or 11S) or by posttranslational modifications (ie, phosphorylation, oxidation) that affect proteasome assembly, stability, and activity. Proteasome regulation thus has the potential to provide highly dynamic responses to cellular signals and stresses.

Clinical Perspective on p 1004

Despite recognition that UPS function is dysregulated in many diseases, the importance of UPS function in cardiac diseases is only beginning to gain attention. Desmin-related cardiomyopathy mouse models provide compelling data for UPS dysfunction, in which cardiomyocyte accumulation of protein aggregates is postulated to inhibit proteasome function by restricting entry of ubiquitinated proteins into the proteasome. Another notable example is acute cardiac ischemia, in which proteasome inhibition is thought to occur as a result of accumulation of oxidized protein aggregates or oxidative injury to the proteasome. Both inhibition and activation of the UPS have been observed in models of cardiac hypertrophy.

There is growing evidence for UPS dysfunction in human heart failure. Accumulation of ubiquitinated proteins, soluble protein aggregates, and autophagic cell death in end-stage failing human hearts provides indirect evidence for proteasome dysfunction. However, only limited data are available on proteasome function in hypertrophied human hearts. ATP-dependent proteasome activity has not been assessed in human cardiac hypertrophy or failure.

The present study examines UPS function in cardiac tissue obtained from patients with end-stage heart failure...
or hypertrophic cardiomyopathy (HCM). Measurement of peptidase-specific fluorogenic substrate cleavage over a range of ATP concentrations revealed marked reductions in proteasome activity for both HCM and failing hearts and inhibition of activity in response to ATP compared with nonfailing controls. Proteasome subunit content, configuration, and access to the active sites within the proteolytic core were not different among groups. These findings suggest that posttranslational events are key contributors to proteasome dysfunction. Importantly, decreased proteasome activity in end-stage heart failure is observed at a point when total protein oxidation and oxidative modification of the 19S ATPase subunit Rpt 5 are increased. These results provide strong evidence that oxidative stress contributes to proteasome dysregulation in end-stage heart failure.

Methods
An expanded Methods section is available in the online-only Data Supplement.

Human Heart Tissue Procurement
Tissue from nonfailing, failing, and HCM hearts was collected as described in the Methods section in the online-only Data Supplement. Tissue collection from human hearts used in this study was approved by the University of Michigan institutional review board, and subjects gave informed consent.

Proteasome Activity Assay
An optimized method was used to determine heart tissue chymotrypsin-like and caspase-like activities23 (see the Methods section in the online-only Data Supplement).

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 al24,25 (see the Methods section in the online-only Data Supplement).

Measurement of Protein Oxidation Products
Protein carbonyls and 4-hydroxynonenylated proteins were analyzed as described14 (see the Methods section in the online-only Data Supplement).

Table. Demographics and Clinical Characteristics of Patients for Myocardial Tissue Analysis

<table>
<thead>
<tr>
<th></th>
<th>Nonfailing (n=6)</th>
<th>HCM (n=29)</th>
<th>Failing (n=33)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>59±4 (40–73)</td>
<td>49±3 (26–76)</td>
<td>54±2 (18–68)</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>4 (50)</td>
<td>14 (48)</td>
<td>26 (79)</td>
<td>0.03†</td>
</tr>
<tr>
<td>White, n (%)</td>
<td>7 (88)</td>
<td>28 (97)</td>
<td>27 (82)</td>
<td>0.2</td>
</tr>
<tr>
<td>Time since diagnosis, y</td>
<td>...</td>
<td>5.4±1.4 (0.2–40)</td>
<td>10.2±1.4 (0.4–30)</td>
<td>0.02</td>
</tr>
<tr>
<td>Nonischemic cause of heart failure, n (%)</td>
<td>...</td>
<td>...</td>
<td>17 (62)</td>
<td>...</td>
</tr>
<tr>
<td>LV assist device, n (%)</td>
<td>...</td>
<td>...</td>
<td>16 (48)</td>
<td>...</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>58±2</td>
<td>71±2</td>
<td>15±1</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>Maximum LV wall thickness, mm</td>
<td>11±0.8</td>
<td>23±1</td>
<td>10±0.4</td>
<td>&lt;0.001§</td>
</tr>
<tr>
<td>Known sarcomere gene mutation, n]</td>
<td>...</td>
<td>12/22</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Values represent mean±SEM (minimum to maximum). Each set of experiments used tissue from a subset of the patients listed above. Most tissue samples were used in >1 experiment.

*HCM versus nonfailing and failing.
†All pairwise comparisons.
‡Failing versus nonfailing and HCM.
§HCM versus nonfailing and failing.
||Seven HCM patients did not undergo clinical genetic testing.

Preparation of Enriched 26S Proteasome Fraction, Detection of Protein Carbonylation of Proteasome Subunits, and 2-Dimensional Gel Electrophoresis
An enriched 26S-proteasome fraction was prepared as described.26 Oxidation of proteasome subunits was detected with the protein carbonyl assay after separation of DNPH-tagged proteins on a 4% to 20% gel. Assignment of carbonyl reactive bands to specific subunits was made by 2-dimensional gel electrophoresis,26 followed by membrane stripping and recognition by antibodies specific for proteasome subunits (see the Methods section in the online-only Data Supplement).

Statistical Analysis
Data are expressed as mean±SEM unless otherwise indicated. Continuous variables were analyzed with 1-way ANOVA. The 26S proteasome activity over a range of ATP concentrations was analyzed with 2-way repeated-measures ANOVA with Bonferroni adjustment for multiple comparisons. Comparison of chymotrypsin-like activity between pre–left ventricular (LV) assist device (LVAD) and post-LVAD patients was assessed with paired t tests. Race and gender comparisons among patient groups (Table) were analyzed by χ² tests. When appropriate, data were log transformed before analysis. The statistical analyses were performed with Sigma Stat 3.0 (Aspire Software International, Ashburn, Va), with values of P<0.05 indicating significant differences.

Results
Distinct Characteristics of HCM and Dilated Cardiomyopathy
The patients with HCM were significantly younger compared with the nonfailing and failing groups, and there were more men in the failing group (Table). The duration of symptomatic disease was longer in the failing group compared with the patients with HCM (Table). As expected, systolic function and LV wall thickness (measured by echocardiography or magnetic resonance imaging) were markedly different among groups. Patients with end-stage heart failure had severely decreased systolic function and normal wall thickness, whereas patients with HCM displayed hyperdynamic function and marked hypertrophy.
Twenty-two patients with HCM had clinical genetic testing, of whom 12 carried a pathogenic sarcomere mutation, consistent with genotype results in large HCM cohorts. Mutations were identified in MYBPC (n=10; E542Q, R495Q, D1076fs, W1098X, IVS27+1 G>A, G1248_C1253 dup, IVS30+2 T>G [2 related probands], and E258K [2 unrelated probands]), TNNT2 (n=1: D86A), and TPM1 (n=1: I284V) within the HCM group. All of the patients with HCM exhibited severe LV outflow tract obstruction and New York Heart Association class III to IV symptoms, whereas patients undergoing LVAD implantation or transplantation exhibited New York Heart Association class IV symptoms. Ninety percent of patients with HCM and 55% of patients with heart failure were taking β-blockers at the time of tissue procurement. Forty-five percent of patients with heart failure were receiving intravenous inotropic therapy, and 52% were receiving antiarrhythmic therapy.

Ubiquitin Proteasome Peptidase Activity Is Markedly Impaired in HCM and Failing Hearts Compared With Controls

Proteasome peptidase activity in whole-heart protein extracts was measured over a range of ATP concentrations with synthetic fluorogenic peptides as substrates for chymotrypsin-like and caspase-like activities. Overall, ATP-dependent chymotrypsin-like activity was markedly decreased in failing and HCM hearts compared with nonfailing control hearts (P<0.001; Figure 1A). In nonfailing hearts, there was a small, nonstatistically significant rise in chymotrypsin-like activity from 0 to 14 μmol/L (P=0.37). In contrast, chymotrypsin-like activity decreased significantly in both HCM and failing hearts in response to ATP (P<0.001; range, 3.5 μmol/L to 1 mmol/L; data not shown for the entire concentration curve). Basal and ATP-dependent caspase-like activities were similarly reduced in failing and HCM hearts compared with nonfailing hearts (P<0.001; Figure 1B), with neither ATP-dependent activation nor inhibition in any of the 3 patient groups.

Paired heart tissue samples collected before and after LV unloading were analyzed from heart failure patients treated with LVADs as a bridge to cardiac transplantation. The mean time from LVAD implantation to transplantation was 30.78±11 weeks (range, 12.3 to 74 weeks), and the mean increase in LV ejection fraction was 11±4% (P<0.05). Proteasome activity increased significantly after mechanical unloading of the left ventricle (P<0.05 for [ATP] 0 to 28 μmol/L; Figure 1C), consistent with recently published data indicating upregulation of the
and failing hearts (Figure 1A of the online-only Data Supplement). Availability of the active site for the 20S subunit β-5, the peptidase with intrinsic chymotrypsin-like activity, was assessed with the novel cell-permeable fluorescent probe MV151, which irreversibly binds to the active sites on proteasome peptidases.24,25 No differences in β-5 active-site labeling were observed among groups (Figure 1B of the online-only Data Supplement). These data suggest that proteasome dysfunction in HCM and failing hearts cannot be explained by alterations in proteasome subunit content, configuration, or active-site accessibility within the proteolytic core.

**Oxidative Protein Damage and Oxidative Modification of 19S Proteasome Subunit Rpt 5 Are Increased in Failing Hearts**

Lack of a difference in the absolute or relative content of proteasome species in heart tissue from patients with HCM or heart failure suggests a posttranslational mechanism(s) to explain proteasome dysfunction. Oxidative injury to the proteasome is 1 such event that could interfere with protein substrate delivery to the 20S catalytic core. Accordingly, we assessed the degree of protein oxidation in human nonfailing, HCM, and failing hearts by measuring protein carbonyls and 4-hydroxynonenylated proteins. These oxidized derivatives were significantly ($P<0.05$) increased in failing hearts, but not in HCM hearts, compared with nonfailing hearts (Figure 3A and B). There was a nonsignificant reduction in total protein carbonyls after mechanical unloading with LVAD (Figure II of the online-only Data Supplement) that correlates with improved function as shown above. To determine whether the proteasomes themselves were oxidatively modified in failing hearts, we analyzed protein carbonylation of proteasome subunits in highly enriched 26S fractions prepared from nonfailing and failing heart samples. Sufficient tissue was not attainable from any single HCM sample for this analysis. A single 53-kDa band was more reactive with the DNPH antibody in the failing compared with the nonfailing samples (Figure 3C). The identity of this band was confirmed to be Rpt 5 by 2-dimensional gel electrophoresis and immunodetection (Figure 3D). These data support the hypothesis that oxidative modification to the proteasome itself could account in part for the proteasome dysfunction observed in failing human hearts.

**Discussion**

In this report, we show that 2 distinct human cardiac pathologies, HCM and heart failure, are associated with marked proteasome dysfunction compared with nonfailing controls. One earlier study described highly variable levels of proteasome peptidase activities in human hearts, with no significant difference between failing and nonfailing hearts.22 The use of freshly prepared protein homogenates from hearts preserved with cardioplegia before freezing and inclusion of ATP in our assays may explain the observed differences.23 An additional key finding in the present study is the identification of posttranslational proteasome oxidation, suggesting that oxidation may be an

---

**Akt and p53 Levels Are Increased in HCM and Failing Hearts**

In the next set of studies, potential pathways that could be affected by proteasome dysfunction were examined. Akt, a serine/threonine kinase, is the major mediator of mTOR activation and a key regulator of protein synthesis and cardiac hypertrophy. Akt is a direct target for degradation by the UPS, and Akt signaling is decreased in skeletal muscle atrophy.31,32 We found Akt protein expression to be significantly increased in failing and HCM hearts compared with controls (Figure 2A), consistent with the observed decrease in proteasome activity in these 2 groups.

Transcription factor p53 is a major mediator of apoptosis in response to various stimuli,33 including oxidative stress, and its stability is regulated by UPS degradation. p53 Protein expression of p53 was markedly increased in both HCM and failing hearts compared with controls, with more variable levels seen in the HCM group (Figure 2). However, the expression of 1 of the downstream targets of p53, proapoptotic factor Bax, was not significantly different among groups (data not shown).

**Proteasome Subunit Composition and Availability of Active Sites Are Not Altered in Human Cardiomyopathies**

Downregulation of certain proteasome populations and alteration of the proteasome particle configuration are potential mechanisms for proteasome dysfunction. Protein content of representative subunits from the 20S, 19S, and 11S proteasomes was therefore evaluated. No differences were observed in the content of α-7 (20S); Rpt 1, 3, and 4 (19S ATPases); Rpn 10 (19S ubiquitination recognition site); and PA28α (11S activator) among nonfailing, HCM, and failing hearts (Figure 1A of the online-only Data Supplement). Availability of the active site for the 20S subunit β-5, the peptidase with intrinsic chymotrypsin-like activity, was assessed with the novel cell-permeable fluorescent probe MV151, which irreversibly binds to the active sites on proteasome peptidases.24,25 No differences in β-5 active-site labeling were observed among groups (Figure 1B of the online-only Data Supplement). These data suggest that proteasome dysfunction in HCM and failing hearts cannot be explained by alterations in proteasome subunit content, configuration, or active-site accessibility within the proteolytic core.

---

**Figure 2.** Akt and p53 expression in human whole-heart homogenates. Representative immunoblots (left) and densitometric analysis (right) for Akt and p53. *$P<0.05$ for Akt, $P<0.001$ for p53 comparing both the HCM and failing groups with the nonfailing group.
important mechanism involved in proteasome dysfunction in human heart failure.

Our studies of proteasome peptidase activity yielded several unique and reproducible observations in human hearts. A small but statistically insignificant rise in proteasome activity is observed in nonfailing hearts in response to ATP, a magnitude lower than expected given the known dependence of the 26S proteasome on ATP for activity. The most striking observation with regard to proteasome activity was the marked inhibition of activity at low concentrations of ATP in HCM and failing human hearts compared with nonfailing hearts. A biphasic response of proteasome activity to ATP has previously been reported in rat hearts, but the reason for inhibition with increasing concentrations of ATP is unknown. Inhibition of activity by ATP in human HCM and failure is particularly notable because it distinguishes these disease states from the nonfailing control hearts. We speculate that the addition of ATP could promote the assembly of a damaged or dysfunctional 19S to the 20S core, resulting in restricted substrate entry. The observation of oxidative damage to the 19S subunit Rpt 5 supports this notion.

Mechanical circulatory support is a major advance in the treatment of refractory heart failure. LVAD support has many favorable effects on the myocardium, including increased myocyte contractile force, improved β-adrenergic responsiveness, normalization of Ca2+ cycling, and global changes in gene expression. We propose an additional benefit of mechanical unloading of the failing heart to be activation of the proteasome, analogous to findings with skeletal muscle unloading in which there is a shift in the balance from protein synthesis to degradation. Activation of the proteasome in heart failure by mechanical unloading may reduce the toxic accumulation of oxidized or damaged proteins and contribute to LV reverse remodeling.

Prior studies of protein degradation, aggregation, and autophagic cell death in human heart tissue have largely been limited to end-stage heart failure, with little direct evidence for proteasome dysfunction. Data on human cardiac hypertrophy are limited to 1 previous report demonstrating an increase in ubiquitin-related autophagy with aortic stenosis only when concomitant systolic dysfunction was present. Our study is the first to examine proteasome activity in human HCM. In contrast to the present work, proteasome activation occurred in response to hypertrophic stimuli in animal and cellular models. Differences in the nature of the inciting stimulus, rate of hypertrophy development, and disease duration may not adequately reflect the chronic and insidious nature of human hypertrophic heart disease. More than half the cases of familial HCM are linked to mutations in sarcomere genes, and this held true in the patients in this study. Proteasome activity was lower in patients with sarcomere gene mutations compared with those without mutations. Along with previous observations of the effects of myosin binding protein C truncation mutant expression on UPS function in neonatal cardiac myocytes, our results support the hypothesis that sarcomere mutant protein expression contributes to proteasome dysfunction in HCM. Decreased proteasome activity in human HCM also challenges the applicability of proteasome inhibition as a therapeutic strategy for suppressing cardiac hypertrophic growth.
Consistent with previous reports, polyubiquitinated proteins were increased in failing hearts compared with nonfailing controls. Somewhat surprisingly, this was not the case for the HCM hearts, despite a comparable magnitude of reduction in proteasome activity to the failing hearts. The reason for this finding is not clear, but possible explanations include differences in the duration of proteasome dysfunction or in the rate or magnitude of accumulation of damaged or misfolded proteins in HCM compared with failing hearts.

Increased expression of the key mediators of hypertrophy and apoptosis pathways, Akt and p53, respectively, is notable because both proteins are direct targets of the UPS and therefore proteasome dysfunction would be expected to increase their stability and steady-state expression. Elevated p53 expression has previously been observed in failing human hearts but has not been assessed in human hypertrophy. The importance of the finding of increased p53 expression in human HCM patients is highlighted by previous work showing that p53 is essential for the transition from hypertrophy to heart failure in an animal model of pressure overload by inhibition of hypoxia-inducible factor-1 and subsequent impairment of angiogenesis.

What is the mechanism for proteasome dysfunction in human heart failure and HCM? Studies on the cardiac proteasome reveal significant heterogeneity in the expression of different subunits and isoforms. Different proteasome subpopulations confer proteolytic substrate specificity and may fluctuate in response to environmental stresses. For example, upregulation of the 11S activated proteasome was recently described in an experimental rat model of diabetic cardiomyopathy. However, in our study, lack of a change in proteasome subunit expression or in the availability of proteolytic active sites invokes a posttranslational mechanism to explain proteasome dysfunction in the human diseased heart. One attractive hypothesis is that excess production of oxidized proteins could inhibit proteasome function by interfering with protein substrate delivery to the 20S catalytic core and/or oxidative modification of the proteasome itself. Increases in total protein oxidation and oxidative modification of the 19S subunit Rpt 5 with heart failure support this idea. These data are particularly intriguing given the recent observation that Rpt 5 plays a critical role in the assembly and activation of the 26S proteasome. The use of human heart tissue to identify potential disease mechanisms has obvious scientific value, although certain limitations should be acknowledged. In most cases, data are obtained at a single time point at an advanced stage of disease, which precludes tracking of disease pathways or determination of causality. In addition, statistical power is reduced by the limited availability of nonfailing donor heart tissue and the heterogeneity of clinical disease.

Conclusions
A marked and consistent decrease in proteasome activity in HCM and failing human hearts is observed in the absence of changes in proteasome protein content or availability of proteolytic active sites. A posttranslational defect in the regulation of protein substrate processing and/or oxidative damage to accessible proteasome subunits appears to be responsible for impaired protein degradation in these human disease states. Defective protein quality control has significant implications for disease pathogenesis in promoting the toxic accumulation of protein aggregates and increasing steady-state levels of prohypertrophic and proapoptotic factors. Future work in human tissue, complemented by animal models, is necessary to define the precise mechanisms for proteasome dysfunction and to establish a causal link to cardiomyopathy progression.

Acknowledgments
We thank Herman Overkleeft and Martijn Verdoe (Leiden University, Leiden, the Netherlands) for their kind gift of Bodipy TMR-Ah-L-VS (MV151), Kenneth Margulies (University of Pennsylvania) for providing samples of nonfailing human heart tissue, and John Younger (University of Michigan) and Ananda Sen (University of Michigan) for statistical consultation.

Disclosures
None.

Sources of Funding
This work was supported by NIH HL093338 (Dr Day), NIH HL67254 (Dr Westfall), and NIH HL68936 (Dr Powell).

References
1003
Predmore et al Proteasome Dysfunction in Human Cardiomyopathies


Cardiomyopathic diseases are the principal cause of heart failure and sudden cardiac death. Much focus toward understanding basic mechanisms of cardiomyopathies associated with heart failure has been on gene expression analysis. Although alterations in the transcriptome allow a cell to react to shifts in extracellular and intracellular signals, posttranslational events provide a much faster mechanism for activation or inhibition of signal transduction. For example, protein degradation is critical for maintaining a dynamic equilibrium of proteins within the heart and for eliminating damaged, misfolded, or oxidized proteins produced under various pathological conditions. Therefore, regulation of protein turnover is likely to be highly relevant to disease pathogenesis. In this study, we explored the function of the ubiquitin proteasome system, the major pathway for intracellular protein degradation, in human heart samples from patients with end-stage heart failure and from patients with hypertrophic cardiomyopathy. We observed that proteasome activity was markedly reduced in hypertrophic cardiomyopathy and failing human hearts compared with nonfailing donor hearts in the absence of changes in proteasome protein content or availability of proteolytic active sites. These findings suggest posttranslational dysregulation of the proteasome in these human disease states. In the failing hearts, there was an increase in total protein oxidation and in the degree of oxidative modification to one of the key 19S regulatory subunits. These results support the concept that defective protein quality control has a potentially important role in the pathogenesis of a broad spectrum of human cardiomyopathies.
Ubiquitin Proteasome Dysfunction in Human Hypertrophic and Dilated Cardiomyopathies
Jaime M. Predmore, Ping Wang, Frank Davis, Sarah Bartolone, Margaret V. Westfall, David B. Dyke, Francis Pagani, Saul R. Powell and Sharlene M. Day

Circulation. 2010;121:997-1004; originally published online February 16, 2010;
doi: 10.1161/CIRCULATIONAHA.109.904557

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/121/8/997

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2010/02/12/CIRCULATIONAHA.109.904557.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/
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 N₂ 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$_3$L$_3$VS (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\textsuperscript{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), 1 mmol/L DTT, 5 mmol/L MgCl\textsubscript{2}, 250 mmol/L sucrose, and 2 mmol/L ATP\textsuperscript{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\textsubscript{ex}532 and λ\textsubscript{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. 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 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 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. 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 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


