Differential Regulation of Proteasome Function in Isoproterenol-Induced Cardiac Hypertrophy

Oliver Drews,* Osamu Tsukamoto,* David Liem, John Streicher, Yibin Wang, Peipei Ping

**Rationale:** Proteasomal degradation is altered in many disease phenotypes including cardiac hypertrophy, a prevalent condition leading to heart failure. Our recent investigations identified heterogeneous subpopulations of proteasome complexes in the heart and implicated multiple mechanisms for their regulation.

**Objective:** The study aimed at identification of molecular mechanisms changing proteasome function in the hypertrophic heart.

**Method and Results:** Proteasome function, expression, and assembly were analyzed during the development of cardiac hypertrophy induced by β-adrenergic stimulation. The analysis revealed, for the first time, divergent regulation of proteasome function in cardiac hypertrophy. Proteasome complexes have 3 different proteolytic activities, which are ATP-dependent for 26S complexes (19S assembled with 20S) and ATP-independent for 20S core particles. The 26S activities were enhanced in hypertrophic hearts, partially because of increased expression and assembly of 19S subunits with 20S core complexes. In contrast, caspase- and trypsin-like 20S activities were significantly decreased. Activation of endogenous cAMP-dependent protein kinase (PKA) rescued the depressed 20S functions, supporting the notion that PKA signaling is a positive regulator of protein degradation in the heart. Chymotrypsin-like 20S activity was stably maintained during cardiac remodeling, indicating a switch in proteasome subpopulations, which was supported by altered expression and incorporation of inducible β subunits.

**Conclusions:** Three novel mechanisms for the regulation of proteasome activities were discovered in the development of cardiac hypertrophy: (1) increased incorporation of inducible subunits in 20S proteasomes; (2) enhanced 20S sensitivity to PKA activation; and (3) increased 26S assembly. PKA modulation of proteasome complexes may provide a novel therapeutic avenue for restoration of cardiac function in the diseased myocardium. (Circ Res. 2010;107:1094-1101.)

**Key Words:** heart disease ■ protein degradation ■ cellular homeostasis

Recent investigations have identified heterogeneous subpopulations of proteasome complexes in mammalian tissues,1–4 demonstrating the potential for fine tuning of the protein degradation machinery in various cell types, including cardiac myocytes.5–7 However, our knowledge regarding regulatory pathways signaling to cardiac proteasomes is limited, and there is lack of a system-wide characterization linking proteasome function to cardiac phenotypes. It remains unclear whether dysfunction of proteasome complexes contributes to the pathogenesis of cardiac diseases and, if so, which molecular mechanisms governing proteasome function accomplish these pathological transitions.

The sympathetic system is activated during the development of left ventricular hypertrophy (LVH) and heart failure.8–10 Chronic stimulation of β-adrenergic receptors leads to their desensitization, which attenuates downstream signal transduction,11 including reduction of intracellular cAMP required for cAMP-dependent protein kinase (PKA) activation.12–14 PKA-specific phosphorylation, in turn, is a critical mediator for heart failure progression.15 Mounting evidence in the literature supports a role for proteasome complexes in modulating the β-adrenergic system, because its key elements are degraded by the proteasome-dependent pathway.16,17 In contrast, little is known regarding how the β-adrenergic system regulates the proteolytic capacity of proteasome complexes and whether enhanced β-adrenergic signaling augments proteasome function in the heart.

The cardiac proteasome system modulates myocardial tissue mass potentially through its function as a major checkpoint for protein quality control, because it receives a much higher workload during the development of LVH, which largely includes protein synthesis.18 Inhibition of the proteasome pathway in a β-adrenergic–stimulated murine model resulted in prevention or regression of LVH.19 In a canine model of chronic cardiac hypertrophy induced by...
aortic banding, upregulation of trypsin- and chymotrypsin-like 26S proteasome activities were reported.20

The term proteasome refers to several complexes with multiple functions and specificities. The 26S proteasomes are composed of the 19S regulatory particles and the 20S proteolytic cores. The regulatory particles contain ATPases and recognition sites for polyubiquitinated proteins, which are subject to degradation.21–23 They are further subdivided into the 19S base and lid. It is the general opinion that 26S activities are coupled to 20S activities in their regular cellular functions, except that they are ATP-dependent. Consequently, conclusions on proteasome function are widely drawn after partial analyses of 26S or 20S activities. However, degradation of oxidized proteins without prior ubiquitination has been reported to be a specific function of 20S proteasomes.24,25 Both 20S and 26S proteasomes have caspase-, trypsin-, and chymotrypsin-like proteolytic activities, which are linked to the subunits β1, β2, and β5, respectively.26–29 In heart, we have demonstrated that heterogeneity exists at the level of 20S proteasomes, because of differential incorporation of the β subunits β1i, β2i, and β5i.1 When incorporated, those subunits replace the corresponding β1, β2, and β5 subunits and consequently alter the proteolytic activities of proteasome complexes.1,30,31

The complexity of cardiac proteasomes provides dynamic regulatory venues for proteasome function. Previous investigations demonstrated effective reduction of LVH via inhibition of proteasome function.19,20,32 In this study, we aimed at gaining comprehensive molecular insights of proteasome function in the diseased heart. Therefore, both 26S and 20S activities were analyzed in a catalytic subunit–specific fashion in a β-adrenergic–stimulated murine model of LVH,2,19,33 and linked to the expression and incorporation of 19S and 20S proteasome subunits. We hypothesized that altered assembly and activities of proteasomes are involved in LVH and are potentially linked to the desensitization of the β-adrenergic pathway.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Animal Model and Experimental Groups
Cardiac hypertrophy was induced by isoproterenol (Iso) treatment of male ICR mice using microosmotic pumps. Three groups were studied along the development of hypertrophy: acute (30 minutes), short-term (24 hours), and established hypertrophy (7 days).

Proteasome Activities
Proteins were extracted from heart homogenates, and activities were assayed according to modified published protocols.1,2 Catalytic subunit–specific measurements were performed using the following fluorescently tagged substrates: Z-LLE-AMC (β1), Boc-LSTR-AMC (β2/β2i), and Suc-LLVY-AMC (β5/β1i/β5i). The 26S activities were distinguished from 20S activities through their ATP dependence. Proteasome-specific activities were distinguished from nonproteasomal degradation of the substrates by subtracting proteolytic activities in presence of the specific proteasomes inhibitors epoxomicin and Z-Pro-Nle-Asp-H. Dependence of proteasome activities on endogenous PKA was determined by cAMP activation of PKA and specific PKA inhibition using H-89. Activities were measured for at least 5 animals and results shown as mean±SEM.

Quantitative Analysis of Proteasome Subunits, Proteasome Complexes, and Ubiquitinated Proteins
The pools of total proteasome subunits and ubiquitin–protein conjugates were analyzed after separation of equal protein amounts on SDS-PAGE by Western blotting. Total protein staining served for normalization. Proteasome assembly was derived from immunoprecipitated proteasomes using a custom polyclonal antibody raised in rabbit against the 20S subunit α3. Quantitative analyses were performed after ECL detection and laser densitometry. At least 5 samples per condition were immunoblotted and averaged to determine differences between sham and Iso-treated animals.

Results
Development of LVH in Response to Chronic β-Adrenergic Receptor Stimulation
In the present study, alterations in proteasome degradation and assembly were investigated during the development of LVH induced by Iso treatment for up to 7 days in mice, which is an established model for cardiac hypertrophy.12,19,33 As shown in Figure 1, acute catecholamine treatment successfully increased left ventricular systolic function. This increase persisted even after 7 days Iso treatment, indicating no signs of heart failure. The mitral valve Doppler E/A ratio (ratio of early to late diastolic filling), which is an early indicator for heart failure, also remained unchanged (Online Figure I). The heart weight/body weight ratio was increased by ~50% after 7 days treatment, demonstrating a state of compensated hypertrophy in response to chronic β-adrenergic stimulation.

β-Adrenergic Stimulation Acutely Reduced the Pool of Polyubiquitinated Proteins in LVH
Polyubiquitinated proteins were quantified during the development of LVH, because previous studies suggested a role for

| Non-standard Abbreviations and Acronyms |
|-----------------|-----------------|
| Iso             | isoproterenol   |
| LVH             | left ventricular hypertrophy |
| PKA             | cAMP-dependent protein kinase |

Figure 1. Development of cardiac hypertrophy caused by chronic β-adrenergic stimulation. Ejection fraction (EF) (top) and heart weight/body weight (HW/BW) ratio (bottom) were measured after 30 minutes, 24 hours, and 7 days of Iso treatment in mice. The ejection fraction was increased significantly after 30 minutes treatment and remained elevated even after 7 days of treatment. The ratio increased by ~50% after 7 days Iso treatment.
Proteasomal degradation in LVH. After 30 minutes and 24 hours of β-adrenergic stimulation, no significant difference in the concentration of polyubiquitinated proteins was detected (Figure 2). In contrast, polyubiquitinated proteins were reduced by ~44% in the hypertrophic myocardium after chronic catecholamine treatment for 7 days (Figure 2).

Enhanced 26S and Partially Decreased 20S Proteasome Function in LVH

Proteasome activities were studied in a subunit- and complex-specific fashion. Caspase-, trypsin-, and chymotrypsin-like activities were measured, representing subunit-specific degradation by β1, β2, and β5 respectively. Furthermore, ATP-dependent 26S activities were distinguished from ATP-independent 20S activities. In the myocardium of acutely β-adrenergic–stimulated animals, none of the proteasome activities were altered (Figure 3), as would be expected from the results showing an unchanged pool of ubiquitinated proteins (Figure 2). After chronic β-adrenergic stimulation, a multilayered regulation of proteasome activities was detected (Figure 3). Proteasome activities were differentially regulated, with those of 26S proteasomes being increased by up to 89%, whereas the 20S activities were decreased by up to 41% compared with the activities in hearts of sham-treated mice. The only exception was the chymotrypsin-like 20S activity, which remained unchanged in the hypertrophic heart. All proteasome activities were distinguished from background proteolytic activities by utilization of specific proteasome inhibitors as detailed in Methods. Thus, the altered degradation is to be attributed to proteasome complexes. The uniform increase in 26S activities corresponded to the observed decrease in the pool of ubiquitinated proteins in the chronic β-adrenergic–stimulated myocardium (Figure 2).

Alterations in the Pools of Proteasome Subunits During the Development of LVH

Changes in proteasome activities in response to cellular stimuli are generally preceded by corresponding alterations in proteasome subunit expression. After 30 minutes of catecholamine treatment, the pools of all 20S subunits bearing proteolytic sites (β1, β2, and β5) were significantly increased (Figure 4, left; Table 1). The abundance of the inducible 20S subunit β1i was increased by ~65% as well, although proteasome activities were unaffected during the acute phase of β-adrenergic stimulation (Figure 3). However, proteasome subunits are subjected to a cascade of intermediate steps before proteasome assembly is completed. Therefore, their abundance is not directly reflecting proteasome activities. After 24 hours of Iso treatment, the pools of β1 and β1i remained elevated, but the elevation in β5 abundance was less pronounced and β2 was back to the level in sham-treated animals (Table 1). Independent changes in the pools of catalytic 20S subunits indicated distinct regulation of those subunits. In parallel, the 19S subunit Rpt1 was elevated by ~35% at 24 hours of Iso (Table 1), which preceded the increase of 26S activities after 7 days of Iso treatment as well (Figure 3). After chronic β-adrenergic stimulation and the development of LVH, β1i abundance was ~78% increased and thus at comparable levels to the previous time points during the treatment. The amount of the inducible subunit β5i was similarly increased by ~59%. In contrast, the amounts of all constitutive subunits bearing proteolytic sites were back to levels indistinguishable...
from sham-treated mice (Table 1). Of the 19S subunits, Rpt4 abundance was elevated in addition to Rpt1 and Rpt6 by almost 2.5-fold (Table 1), indicating increased assembly of 26S proteasomes and potentially reflecting the overall elevated ATP-dependent 26S activities in LVH as shown in Figure 3. A more detailed analysis including the 19S lid subunits Rpn6 and Rpn11 in hypertrophic hearts demonstrated that cytosolic concentrations of 19S subunits were increased overall (Table 1).

**Shift in the Assembly of 20S Core Particles and Increased Association With 19S Regulatory Particles in LVH**

Whether changes in the pools of proteasome subunits translated into altered proteasome assembly and consequently activities was analyzed by coimmunoprecipitation using subunit α3 as bait. It is a structural 20S subunit at the interface to the 19S particles and remained unchanged during the development of LVH (Table 1). Cardiac proteasome assembly was unaffected after 30-minute and 24-hour-adrenergic stimulation (Figure 4, right; Table 2), although the pools of several proteasome subunits were significantly increased (Figure 4, left; Table 1). Thus, proteasome assembly did not parallel changes in total proteasome subunits, but reflected unchanged proteasome activities during the early phases (Figure 3). In the hypertrophic heart, a significantly increased association of 20S subunits with the regulatory 19S base subunits Rpt1, Rpt4, and Rpt6 was detected (Table 2), indicating increased assembly of 26S proteasomes and matching the uniform elevation of 26S activities (Figure 3), as well as the reduction of ubiquitinated proteins, at this stage (Figure 2). Again, a more detailed analysis including the 19S lid subunits Rpn11 and Rpn12 specifically in hypertrophic hearts further indicated increased 26S assembly (Table 2). Association of the structural 20S subunit α7 with α3, as well as its total abundance, remained unchanged in LVH (Tables 1 and 2), indicating that the total pool of 20S proteasomes remained largely unaffected by the

Table 1. Alteration of Proteasome Subunit Pools in the Myocardium After β-Adrenergic Stimulation

<table>
<thead>
<tr>
<th>Subunit</th>
<th>30 Minutes</th>
<th>24 Hours</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Iso</td>
<td>Sham</td>
</tr>
<tr>
<td><strong>20S Proteasomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α3</td>
<td>1.00±0.12</td>
<td>1.08±0.12</td>
<td>1.00±0.08</td>
</tr>
<tr>
<td>α7</td>
<td>1.00±0.02</td>
<td>0.90±0.02</td>
<td>1.00±0.04</td>
</tr>
<tr>
<td>β1</td>
<td>1.00±0.11</td>
<td>1.60±0.11*</td>
<td>1.00±0.13</td>
</tr>
<tr>
<td>β2</td>
<td>1.00±0.06</td>
<td>1.32±0.08*</td>
<td>1.00±0.03</td>
</tr>
<tr>
<td>β5</td>
<td>1.00±0.23</td>
<td>2.59±0.22*</td>
<td>1.00±0.16</td>
</tr>
<tr>
<td>β1i</td>
<td>1.00±0.11</td>
<td>1.65±0.12*</td>
<td>1.00±0.10</td>
</tr>
<tr>
<td>β5i</td>
<td>1.00±0.12</td>
<td>1.03±0.08</td>
<td>1.00±0.12</td>
</tr>
<tr>
<td><strong>19S Proteasomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rpt1</td>
<td>1.00±0.03</td>
<td>1.10±0.03</td>
<td>1.00±0.08</td>
</tr>
<tr>
<td>Rpt4</td>
<td>1.00±0.01</td>
<td>1.09±0.02</td>
<td>1.00±0.01</td>
</tr>
<tr>
<td>Rpt6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rpn6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rpn12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Initially, catalytic 20S subunits were generally induced (Figure 4, left). In contrast, 19S base, lid and the inducible catalytic 20S subunits were elevated in the hypertrophic heart. Mean±SEM. *P<0.01 and †P<0.05 (n=5). Significant changes in subunit concentrations >10% are in bold. ND indicates not determined.
chronic catecholamine treatment. Remarkably, the detailed analysis of the composition of proteasome revealed increased incorporation of the inducible proteolytic 20S subunits β1i and β5i increased significantly (Figure 4, right). In addition, higher levels of the 19S subunits Rpt1, Rpt4, Rpt6, and Rpn12 were associated with 20S subunits, indicating increased 26S activity was specifically upregulated by PKA by cAMP (Figure 5). In contrast, the caspase-like 20S activities in LVH, but it also distinguished between 20S and 26S proteasome complexes. Neither PKA activation nor inhibition affected 26S activities in LVH (Figure 5, right).

Discussion

Several studies suggest that proteasomes have the capacity to adjust their activities through multiple molecular mechanisms. Previously, we have demonstrated that proteasome heterogeneity exists in the heart in form of subpopulations1,2 and that PKA is capable of modulating proteasome activities in vitro.5,35 Here, we present first evidence that proteasome heterogeneity is dynamically regulated, 20S activities are uncoupled from 26S activities, and 20S is dependent on endogenous PKA during the development of a cardiac disease (Figure 6). The proteasome system controls the abundance of several key elements of the β-adrenergic pathway through their degradation.16,17 Our study suggests novel mechanisms regulating protein degradation by proteasomes through a feedback-loop originating from the β-adrenergic system.

The initial cellular response to β-adrenergic stimulation in the heart increased the pools of 20S subunits with catalytic activity (Table 1). This increase was concomitant with higher cardiac contractility (Figure 1) and elevated levels of oxidized proteins (Online Figure II). Thus, the increased levels of catalytic 20S subunits were possibly induced by the oxidative stress of the catecholamine treatment.36 Oxidized proteins are potential substrates of 20S proteasomes.24,25 The levels of oxidized proteins normalized after 24 hour Iso treatment, without 20S activities and assembly becoming significantly elevated (Online Figure II; Figures 3 and 4). Thus, it seems that the myocardium adapted to the oxidative stress through a proteasome-independent pathway and that proteasome regulation had no observable effect on the total level of oxidized proteins.

During the development of hypertrophy, a shift in the composition of proteasome subpopulations, uniformly increased 26S activities as well as unequal regulation of 20S activities were observed (Figure 6). A disease induced reorganization of 20S subpopulations was also found during the

### Table 2. Rearrangement in 20S Proteasome Complexes and Increased 26S Assembly in the Hypertrophic Myocardium

<table>
<thead>
<tr>
<th></th>
<th>30 Minutes</th>
<th>24 Hours</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>20S Proteasomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α7</td>
<td>1.00±0.04</td>
<td>1.14±0.09</td>
<td>1.00±0.01</td>
</tr>
<tr>
<td>β1</td>
<td>1.00±0.15</td>
<td>1.22±0.21</td>
<td>1.00±0.05</td>
</tr>
<tr>
<td>β2</td>
<td>1.00±0.03</td>
<td>1.00±0.03</td>
<td>1.00±0.18</td>
</tr>
<tr>
<td>β1i</td>
<td>1.00±0.12</td>
<td>0.91±0.18</td>
<td>1.00±0.04</td>
</tr>
<tr>
<td>β5i</td>
<td>1.00±0.04</td>
<td>1.07±0.12</td>
<td>1.00±0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>19S Proteasomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rpt1</td>
<td>1.00±0.10</td>
<td>0.92±0.09</td>
<td>1.00±0.15</td>
</tr>
<tr>
<td>Rpt4</td>
<td>1.00±0.05</td>
<td>1.03±0.03</td>
<td>1.00±0.10</td>
</tr>
<tr>
<td>Rpt6</td>
<td>ND</td>
<td>ND</td>
<td>1.00±0.11</td>
</tr>
<tr>
<td>Rpn11</td>
<td>ND</td>
<td>ND</td>
<td>1.00±0.17</td>
</tr>
<tr>
<td>Rpn12</td>
<td>ND</td>
<td>ND</td>
<td>1.00±0.02</td>
</tr>
</tbody>
</table>

Changes in proteasome assembly were detectable after 7 days of Iso treatment. Incorporation of the inducible proteolytic 20S subunits β1i and β5i increased significantly (Figure 4, right). In addition, higher levels of the 19S subunits Rpt1, Rpt4, Rpt6, and Rpn12 were associated with 20S subunits, indicating increased 26S assembly. Mean±SEM. *P<0.01 and †P<0.05 (n=5). Significant changes in subunit incorporation greater than 10% are in bold. ND indicates not determined.
The study indicated that subtype-specific degradation is as important as the rate of degradation. In the immune response, inducible subtype-specific degradation by proteasomes is potentially essential for antigen processing. Furthermore, repression of inducible proteasome subunits or their mutation seems to be part of tumor survival strategies. Tumor sensitivity to proteasome inhibitors was linked to the expression of inducible subunits. For these reasons and because proteasome inhibitors exhibited tissue-specific toxicity in clinical studies, specific targeting of proteasome subpopulations has been initiated. Our study demonstrates that a therapeutic strategy targeting specific proteasome subpopulations in the heart is preferential because it would aim precisely at distinct proteasome functions.

The majority of proteasome inhibitors interfere with the chymotrypsin-like activity of both 20S and 26S proteasomes, but the susceptibility of cardiac proteasome subpopulations to these inhibitors is distinct. Therefore, detailed knowledge about proteasome dynamics in cardiac disease phenotypes is essential for the development of therapeutic strategies. In recent publications, proteasome inhibitors were used to prevent or even induce a regression of LVH in animal models. Although the studies suggested a role for proteasome function in the development of cardiac hypertrophy, details about the regulatory mechanisms for proteasome degradation in LVH remained obscure. Our results indicate that inhibition of the chymotrypsin-like activity of proteasomes in the development of LVH is potentially beneficial, because 26S activities were significantly increased and uncoupled from 20S activities (Figure 6). Additionally, the chymotrypsin-like 20S activity remained at normal levels in LVH, whereas the other 20S activities were reduced. Thus, a selective inhibitor of chymotrypsin-like proteasome activity would not further decrease those 20S activities, which were already reduced by the disease and potentially minimize the divergent regulation of proteasomes.
proteasome activities. Altogether, the results suggest that elevated 26S activities, which were also reported in a pressure overload model,\textsuperscript{10,20,32} are a maladaptation attributable to increased 26S assembly during the development of the disease. This assumption is also supported by the abnormally reduced pool of polyubiquitinated proteins in LVH (Figure 2) to which key elements of the β-adrenergic system belong.\textsuperscript{16,17}

A novel link between proteasome regulation and the β-adrenergic system was found in cAMP dependent PKA signaling. Specifically in hypertrophic hearts, 20S proteasomes were highly responsive to activation of endogenous PKA by cAMP (Figure 5). Our previous studies suggested that cardiac proteasome complexes are potential targets of PKA by indicating enhanced 20S activities and phosphorylation after treating highly purified 20S proteasomes with PKA in vitro.\textsuperscript{3,5,33} Furthermore, there is evidence that PKA phosphorylates proteasome subunit Rpt6 in nuclear extracts of cultured normal rat kidney cells.\textsuperscript{46} In heart, PKA potentially confers ischemic protection through maintaining proteasome activities,\textsuperscript{47} which are generally reduced by ischemic injury.\textsuperscript{7,47} In animal models of chronic β-adrenergic stimulation of the heart, reduced adenylyl cyclase activity, reduction in the cAMP pool, and reduced PKA activity have been published previously.\textsuperscript{12-14} In the present study, reduced caspase- and trypsin-like 20S activities in hypertrophic hearts were specifically rescued by endogenous PKA activation through cAMP after 7 days of chronic β-adrenergic stimulation. In contrast, 26S and chymotrypsin-like 20S activities were not responsive to PKA activation in sham-treated and in β-adrenergic–stimulated hearts. The insensitivity of 26S proteasomes to PKA in LVH could have multiple reasons. Analogous to PKA phosphorylation of the ryanodine receptor RyR2 in heart failure, activated PKA could be locally associated with 26S proteasomes or the phosphorylation site stabilized by the 26S complex.\textsuperscript{15} Alternatively, PKA sensitivity and activity loss of 26S proteasomes could occur at a later stage of cardiac disease, because their assembly requires additional steps.\textsuperscript{21,22} With 20S activities failing and potentially preceding 26S dysfunction in cardiac hypertrophy, our findings propose a previously missing link regarding investigations in animal models of cardiac hypertrophy\textsuperscript{16,20,32} and those of heart failure studies in humans.\textsuperscript{45,49} Taken together, increased 26S activities during the development of cardiac hypertrophy in the present study appear to act as compensatory mechanisms for 20S dysfunction.

In summary, our study revealed 3 uncharacterized mechanisms in proteasome regulation during the development of Iso-induced cardiac hypertrophy. First, inducible subunits were increasingly incorporated in 20S proteasomes, which was concomitant with heterogenous shifts in 20S activities. Second, 20S proteasomes became PKA sensitive, evident by the rescue of decreased caspase- and trypsin-like 20S activities. Third, 19S increasingly associated with 20S proteasomes, correlating with increased 26S activities and reduction of ubiquitinated proteins. The discoveries demonstrate that dynamic changes in proteasomal degradation are evident processes during the development of myocardial hypertrophy and provide novel therapeutic targets to promote cardiac function in this disease.

Sources of Funding

This study was supported in part by American Heart Association Grant 0625062Y (to O.D.), NIH Program Project P01-080111 (to P.P. and Y.W.), and multi-PI NIH grant R01 HL098954 (to P.P. and Y.W.).

Disclosures

None.

References


pathogenesis of cardiac disease, including hypertrophy. To gain insights in the role of proteasomes during the pathogenesis of cardiac hypertrophy, we comprehensively characterized proteasome function and regulation. In cardiac hypertrophy induced by chronic β-adrenergic stimulation, a uniform increase of 26S proteasome function responsible for targeted protein degradation was measured. Increased 26S function was associated with elevated 26S assembly. In contrast, 20S function of proteolytic core complexes was nonuniformly decreased and associated with alternative 20S assembly. The observed abnormalities in proteasomal function (hyper and hypo) in cardiac hypertrophy potentially describe a novel compensatory mechanism. Recovery of proteasome dysfunction is considered to be cardioprotective. To this end, up to 56% of lost 20S function during the pathogenesis of hypertrophy.

Proteasomes are macromolecular machines that selectively degrade targeted proteins, including elements of the β-adrenergic pathway. They are a heterogenic group of multi-protein complexes with multiple mechanisms regulating their function. Proteasome dysfunction potentially contributes to the pathogenesis of cardiac disease, including hypertrophy. To gain insights in the role of proteasomes during the pathogenesis of cardiac hypertrophy, we comprehensively characterized proteasome function and regulation. In cardiac hypertrophy induced by chronic β-adrenergic stimulation, a uniform increase of 26S proteasome function responsible for targeted protein degradation was measured. Increased 26S function was associated with elevated 26S assembly. In contrast, 20S function of proteolytic core complexes was nonuniformly decreased and associated with alternative 20S assembly. The observed abnormalities in proteasomal function (hyper and hypo) in cardiac hypertrophy potentially describe a novel compensatory mechanism. Recovery of proteasome dysfunction is considered to be cardioprotective. To this end, up to 56% of lost 20S function in hypertrophic hearts could be rescued through a PKA-dependent pathway. Because PKA is regulated through β-adrenergic signaling, we hereby report a novel feedback loop between the proteasome and the β-adrenergic pathway. In conclusion, the study identifies novel mechanisms and targets to promote cardiac function during the pathogenesis of hypertrophy.
Differential Regulation of Proteasome Function in Isoproterenol-Induced Cardiac Hypertrophy

Oliver Drews, Osamu Tsukamoto, David Liem, John Streicher, Yibin Wang and Peipei Ping

*Circ Res.* 2010;107:1094-1101; originally published online September 2, 2010; doi: 10.1161/CIRCRESAHA.110.222364

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/107/9/1094

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/09/02/CIRCRESAHA.110.222364.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* 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 Research* is online at:
http://circres.ahajournals.org/subscriptions/
Detailed Methods
Animal model and experimental groups.
Male 8-10 week old ICR mice were randomly divided into two groups: sham and Iso. Three stages of Iso treatment were analyzed: acute (30min), short-term (24h) and established hypertrophy (7 days). Mice of the acute group were injected 15 mg/kg Iso i.p., whereas micro-osmotic pumps (alzet, model 1002), which release Iso at a rate of 30 mg/kg/day, were implanted subcutaneously in the other two groups.\(^1\) Sham treated animals were treated in parallel and received PBS i.p. or via micro-osmotic pumps. Body temperature was maintained at 37°C using temperature controlled heat pads. Cardiac function was measured by transthoracic echocardiography under anesthesia (isofluorane 2.5% in 95% O\(_2\)/5% CO\(_2\)) using the VisualSonics Vevo 770 equipped with a 45-MHz linear transducer. Electrocardiograms (ECGs) were obtained using built-in ECG electrode-contact pads (VisualSonics). Standard Lead II ECGs were acquired using the BioBench at 0.1 kHz. Averaged ECGs were obtained from representative 10 to 20 seconds recording periods. Hearts were removed after euthanasia, perfused with PBS, weighted and quickly frozen in liquid nitrogen before storage at -80°C.

Proteasome activities. Proteins were extracted from heart homogenates and activities assayed according to modified published protocols.\(^2\),\(^3\) Briefly, murine hearts were homogenized with hand-held (Potter-Elvehjem) homogenizers in 1.5ml buffer containing 20mmol/L HEPES (pH 7.5), 150mmol/L NaCl, 1mmol/L MgCl\(_2\), 0.5mmol/L EDTA, 1mmol/L DTT. Proteasomes were collected in the supernatant after 1h centrifugation at 100,000xg. The caspase-, trypsin- and chymotrypsin-like activities of proteasomes were assayed using 25µg extract and fluorescently tagged substrates (Z-LLE-AMC, Boc-LSTR-AMC and Suc-LLVY-AMC). All assays were carried out in a total volume of 100µl. The ATP-dependent 26S assays were performed in the homogenization buffer after the addition of 50µmol/L ATP. The ATP-independent 20S assay buffer for the chymotrypsin-like proteasome activity was 25mmol/L HEPES (pH 7.5), 0.5mmol/L EDTA, and 0.03% SDS. For the caspase- and trypsin-like 20S activities, the buffer composition was 25mmol/L HEPES (pH 7.5), 0.5mmol/L EDTA, and 0.05% NP-40, and 0.001% SDS. Released AMC was measured using a Fluoroskan Ascent fluorometer (Thermo Electron) at an excitation wavelength of 390nm and an emission wavelength of 460nm for up to 90min. Each assay was conducted in the absence and presence of a specific proteasome inhibitor to subtract non-proteasomal proteolysis (10µmol/L epoxomicin for chymotrypsin-like, 20µmol/L epoxomicin for trypsin-like, and 30µmol/L Z-Pro-Nle-Asp-H for caspase-like activity). To assess the impact of endogenous PKA on proteasome activities, endogenous PKA was activated for 30min before measuring proteasome activities by the addition of cAMP to a final concentration of 20µmol/L, which is in the upper physiological range in cardiomyocytes.\(^4\) PKA specificity was shown by inhibiting PKA activation using H-89 (Calbiochem). The inhibitor was added 15min prior to cAMP addition and the final concentration in the assays was 50µmol/L. Volumes and the concentration of solutes besides cAMP and H-89 were identical in all samples. All samples were assayed in triplicate per animal for at least five animals. Results were calculated as means ± SEM for the biological replicates.

Quantitative analysis of proteasome subunits, proteasome complexes and ubiquitinated proteins. Proteins were extracted as described for the proteasome assays to link activities to expression. The pools of total proteasome subunits and ubiquitin-protein conjugates were analyzed after the separation of equal protein amounts on SDS-PAGE by Western Blotting. To control equal loading and normalize the signal, total protein was stained by Ponceau S. For co-immunoprecipitation, the buffer contained 25mmol/L Tris (pH 7.5), 5mmol/L MgCl\(_2\), 1mmol/L DTT, 2 mmol/L ATP, 0.1% NP-40, 15% glycerol (w/v), 1% phosphatase inhibitor 1 & 2 (Sigma), and protease inhibitor cocktail (Roche). Those homogenates were also subjected to 1h at 100,000xg at 4°C. Supernatants were pre-cleared before co-immunoprecipitation of proteasome
complexes over night at 4°C using a custom polyclonal antibody raised in rabbit against the 20S subunit α3 and protein A/G agarose beads (Santa Cruz). Immunoprecipitates were washed four times, including twice after adding 150 mmol/L NaCl to the buffer. Quantitative analysis by immunoblotting was performed after SDS-PAGE. Primary antibodies were obtained from BIOMOL International and Abcam Inc.. Secondary HRP-conjugated antibodies were obtained from Sigma (goat α-mouse), BD Biosciences (goat α-rabbit) and Santa Cruz Biotechnology (rabbit α-goat). ECL detection (GE Healthcare) was performed prior to laser densitometry on a Typhoon 9300 (GE Healthcare) and quantification using the Scion Image software (National Institutes of Health). At least 5 samples per condition were immunoblotted and averaged to determine differences between sham and Iso treated animals.

**Quantification of protein oxidation.** The detection of oxidized proteins was performed indirectly by chemical derivatization, because i) oxidized residues are generally too small to detect them reproducibly as epitopes and ii) the derivatization captures the oxidative state immediately during or after homogenization of the tissue. Carbonyl groups were detected by the OxyBlot Protein Oxidation Detection Kit (Chemicon International, Inc.) according to the manufacture’s protocol using 15μg sample. Quantification was performed by software analysis as described above after SDS-PAGE, immunoblotting using an anti-DNP antibody, and ECL detection. All signals were normalized against total protein expression.

**Supplemental References**

Online Figure I: E/A ratio showed no signs of heart failure. E/A ratios (ratio of early to late diastolic filling) were measured by echocardiography before and after 7 days Iso treatment, demonstrating that there was no decrease in cardiac function of the hypertrophic heart.
Online Figure II: Significant increase of protein oxidation in the acute β-adrenergic stimulated myocardium. Oxidized proteins were quantified in cardiac homogenates of mice subjected to β-adrenergic stimulation (S – sham, Iso – isoproterenol). At 30min, oxidized proteins were increased by 57%. After 24h and 7days, protein oxidation was not significantly different.