Regulation of the proteasome by ATP: implications for ischemic myocardial injury and donor heart preservation

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Submitted 12 March 2012; accepted in final form 20 May 2013

Majetschak M. Regulation of the proteasome by ATP: implications for ischemic myocardial injury and donor heart preservation. Am J Physiol Heart Circ Physiol 305: H267–H278, 2013. First published May 24, 2013; doi:10.1152/ajpheart.00206.2012.—Several lines of evidence suggest that proteasomes are involved in multiple aspects of myocardial physiology and pathology, including myocardial ischemia-reperfusion injury. It is well established that the 26S proteasome is an ATP-dependent enzyme and that ischemic heart disease is associated with changes in the ATP content of the cardiomyocyte. A functional link between the 26S proteasome, myocardial ATP concentrations, and ischemic cardiac injury, however, has been suggested only recently. This review discusses the currently available data on the pathophysiological role of the cardiac proteasome during ischemia and reperfusion in the context of the cellular ATP content. Depletion of the myocardial ATP content during ischemia appears to activate the 26S proteasome via direct regulatory effects of ATP on 26S proteasome stability and activity. This implies pathological degradation of target proteins by the proteasome and could provide a pathophysiological basis for beneficial effects of proteasome inhibitors in various models of myocardial ischemia. In contrast to that in the ischemic heart, reduced and impaired proteasome activity is detectable in the postischemic heart. The paradoxical findings that proteasome inhibitors showed beneficial effects when administered during reperfusion in some studies could be explained by their anti-inflammatory and immune suppressive actions, leading to reduction of leukocyte-mediated myocardial reperfusion injury. The direct regulatory effects of ATP on the 26S proteasome have implications for the understanding of the contribution of the 26S proteasome to the pathophysiology of the ischemic heart and its possible role as a therapeutic target.

26S proteasome; 20S proteasome; myocardial infarction; heart transplantation; protein degradation

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Introduction

The ubiquitin-proteasome pathway of protein degradation (UPP) is the major nonlysosomal proteolytic system in eukaryotes. The UPP plays important roles in all aspects of biology, including the regulation of protein turnover, cell cycle control, antigen presentation and inflammation. In this pathway, ubiquitin-protein ligase systems, which traditionally consist of a ubiquitin-activating enzyme (E1), a ubiquitin-carrier or conjugating enzyme (E2), and a ubiquitin-protein ligase (E3), catalyze the covalent ligation of ubiquitin to intracellular proteins (=ubiquitination or ubiquitylation). After binding of ubiquitin to the substrate protein, a polyubiquitin chain can be formed in which the COOH-terminus of each ubiquitin molecule is linked to a specific lysine residue of the previous ubiquitin (61, 125). While reversible ubiquitylation regulates several intracellular processes, a polyubiquitin tag serves as a signal for degradation of the target protein by the ATP-dependent 26S proteasome (14, 61, 63, 125, 142).

The UPP has been shown to contribute to the pathophysiology of various disease processes and recently evolved as a drug target. Selective proteasome inhibitors have been developed, and the proteasome inhibitors bortezomib and carfilzomib have been approved by the Food and Drug Administration for the treatment of multiple myeloma and mantle cell lymphoma (18, 139). Furthermore, there is substantial interest in the development of new therapeutics that target other enzyme components of the UPP, such as E1, E3s, or deubiquitylating enzymes (DUBs) (27, 89, 97, 148).

Several lines of evidence suggest that the UPP is also involved in various myocardial pathologies, such as cardiac proteinopathies, hypertrophy, heart failure, and ischemic heart disease (87, 111, 112, 124, 145, 154). Moreover, bortezomib has already been used off-label for the treatment of cardiac allograft rejection in patients and in phase 1 clinical trials for the treatment of cardiac amyloidosis (23, 35, 37, 98).
It is well established that the 26S proteasome is an ATP-dependent enzyme and obvious that ischemic heart disease is associated with changes in the ATP content of the cardiomyocyte. Thus it is surprising that a possible link between the proteasome, myocardial ATP levels and ischemic cardiac injury has been suggested only recently (46).

The purpose of this review article is to discuss the possible regulation of the proteasome by ATP during myocardial ischemia and subsequent reperfusion. For information on other important aspects of the contribution of the UPP to myocardial ischemia and reperfusion injury, such as the involvement of the ubiquitin-protein ligase system or DUBs, the reader is referred to previous review articles on this topic (20, 30, 82, 111, 115, 140, 151, 152, 154, 155).

The imbalance between myocardial oxygen demand and supply during ischemia results in a progressive depletion of the cellular ATP content. The normal ATP concentration in the heart is ~4–6 mM, and time course measurements in the ischemic myocardium after coronary artery ligation documented that the myocardial ATP content decreases to 35, 16, 9, and 7% of normal after 15, 30, 40, and 60 min of ischemia, respectively (13, 16, 70, 81). Similarly, time-course measurements of ATP levels in isolated perfused hearts demonstrated a decline down to ~10% of normal within 20–30 min of no-flow ischemia and to less than 50% during 1 h of low-flow ischemia, followed by a poor recovery of ATP levels during subsequent reperfusion for up to 60 min (22, 51, 123). While myocardial ATP rapidly depletes during normothermic ischemic cardiac arrest, ATP levels decline with a half-life of ~8 h after cardioplegic arrest and cold ischemic storage of the heart in University of Wisconsin solution (46, 58). Thus the understanding of the influence of fluctuations of cellular ATP levels on 26S proteasome assembly, stability, and function appears essential for an assessment of the pathophysiological role of the cardiac proteasome during ischemia and reperfusion.

Regulation of the Proteasome by ATP

The UPP has been discovered during the study of energy-dependent proteolysis in early 1980. The ATP-requiring mechanisms in the ubiquitylation reaction have been solved during the discovery of the ubiquitin protein ligase system (26, 62). In the ubiquitylation reaction ATP is required to activate the COOH-terminal glycine of ubiquitin through the generation of an adenylate intermediate by an E1 enzyme, which is followed by the formation of an E1-ubiquitin thiolester and the release of AMP and inorganic pyrophosphate (62). Interestingly, the $K_m$ value of E1 for ATP lies in the low micromolar range (54, 93), which is far below the normal ATP concentration in the cell. In contrast, the precise role of ATP in the regulation of 26S proteasome assembly, stability, and function is still not fully understood and remains of major biological interest.

26S proteasome assembly and stability. Studies in the late 1980s uncovered that ATP is also required for the assembly and stability of the 26S proteasome complex (6, 40). The 26S proteasome is formed from a ~700 kDa cylinder-shaped multimeric proteasome core particle (20S proteasome), which is singly or doubly capped at its ends by a 19S regulator complex that confers Mg$^{2+}$/ATP dependency and ubiquitylated substrate specificity (12, 43, 61, 104, 118). The 20S core particle is composed of four stacked rings. Each ring consists of seven different $\alpha$- and $\beta$-type subunits ($\alpha_{1-7}, \beta_{1-7}, \beta_{1-7}, \alpha_{1-7}$). In eukaryotic 20S core particles, the proteolytic active sites are located in the subunits $\beta_1$, $\beta_2$, and $\beta_3$ of the inner rings. Based on the ability to hydrolyze short fluorogenic peptides, the activities of the $\beta_1$, $\beta_2$, and $\beta_3$-subunits are referred to as caspase-like, trypsin-like, and chymotrypsin-like, respectively. These subunits can be replaced by inducible subunits $\beta_{1i}, \beta_{2i}$, and $\beta_{3i}$. As two of these subunits are encoded within the MHC class II region, they are referred to as immuno-(i)-subunits (43, 48, 50, 52, 119, 127).

The 700-kDa 19S regulator complex consists of at least 17 subunits. It is arranged as a base that is formed out of six AAA (ATPases associated with different cellular activities family) ATPase subunits (Rpt1–6) and three non-ATPase subunits (Rpn1, 2, and 10) that interacts with the 20S core particle and a lid consisting of eight non-ATPase subunits (43, 47, 102).

The regulation of eukaryotic 26S proteasome assembly is a highly regulated and complex process; many aspects of its assembly pathway remain to be determined (43). Nevertheless, it is known that ATP binding and ATP hydrolysis play multiple and distinct roles in the regulation of 26S proteasome assembly and stability (29, 77, 90).

Studies with cell extracts and purified proteasomes from various sources have shown that the 19S regulator and 20S core particle can be released from 26S proteasomes by ATP depletion and that readdition of ATP to 19S regulators and 20S core particles leads to the reformation of 26S proteasomes (8, 31, 40, 60, 90, 95, 110). As nonhydrolyzable ATP analogs were able to substitute for ATP during 26S proteasome assembly from purified 20S core particles and 19S regulators, ATP binding appears to be sufficient in this process (90).

Furthermore, studies on the dissociation kinetics of immobilized human 26S proteasomes suggested that a high-affinity ATP binding site and a low-affinity ATP binding site, which is virtually not saturable under physiological conditions, are required to fully stabilize the 26S complex. As omission of Mg$^{2+}$ as well as replacement of ATP with a nonhydrolyzable analog resulted in the dissociation of ~60–70% of 26S complexes and also in the loss of low affinity ATP binding, only the low affinity binding site appears to require ATP hydrolysis (95).

The dissociation of immobilized human 26S proteasomes shows a pseudolinear kinetic when the ATP concentration is reduced from 5 mM, the physiological ATP concentration in the heart (13, 81), down to ~0.5 mM. The disassembly of the remaining 26S proteasomes follows a simple hyperbolic kinetic with a $K_d$ in the low micromolar range (95). Because a similar relationship could also be established between ATP concentrations and 26S proteasome content in solution assays, these data imply that in the physiological millimolar range of ATP concentrations, fluctuations of cellular ATP levels result in corresponding changes of the cellular content of 26S proteasomes. Approximately 30% of 26S proteasome complexes remain stable even if ATP concentrations decrease to critically low levels (46). Although the molecular basis for these two-phase dissociation kinetics of the 26S proteasome is currently unknown, it appears to be directly related to interactions with ATP because these effects were detectable in purified enzyme preparations in the absence of other molecular chaperones that may contribute to the formation of the 26S proteasome complex (96).
Nevertheless, a $K_d$ of E1 for ATP in the low micromolar range in combination with the stability of a significant proportion of 26S proteasomes at low ATP concentrations indicate that the UPP is still functional under conditions that are associated with a severe depletion of the cellular energy supply.

26S proteasome activity. Besides the requirement of ATP for 26S proteasome formation and stabilization, ATP binding and ATP hydrolysis also regulate hydrolysis of peptides and degradation of ubiquitylated proteins by the 26S proteasome. ATP is required for 19S regulator-induced opening of the substrate access gates of the 20S proteasome and for unfolding of structured substrate proteins to permit access to the catalytic sites in the 20S core particle (43, 86, 90, 128).

The most commonly used method to evaluate the activity of the proteasome in purified enzyme preparations or cell extracts is to measure the activities of the various peptidase sites using fluorogenic peptide substrates (74, 88). Because the distinct catalytic sites of the proteasome function as a single, coordinated multicatalytic enzyme and studies in yeast suggested that the chymotrypsin-like site is the most important catalytic site, assaying of the chymotrypsin-like peptidase activity has been considered to be sufficient to assess the proteolytic activity of the proteasome (5, 59, 74, 75). It should be noted, however, that the contribution of the different proteolytic sites to the overall ability to degrade proteins varies with the protein substrate in mammalian proteasomes (73, 74).

While the ATP concentration does not affect the peptidase activity of free 20S core particles (46), dose response curves for ATP from solution assays with 26S proteasomes are more difficult to interpret because withdrawal of ATP leads to their disassembly. Nevertheless, such studies reported saturation kinetics with maximal 26S proteasome peptidase activities at concentrations between 25 and 100 μM of ATP when tested between 0 and 0.5–1 mM ATP (29, 90). When purified 26S proteasomes were tested at higher ATP concentrations, however, chymotrypsin-like, trypsin-like, and caspase-like peptidase activities showed bell-shaped dose responses with maximal activities in the lower micromolar range and a continuous decrease when ATP was raised up to physiological ATP concentrations in the millimolar range (24, 46, 66).

This relationship between ATP concentrations and proteasome peptidase activities has also been detected when proteasome peptidase activities were measured in cell free cardiac extracts (46, 113). As the increase in 26S proteasome peptidase activities with reduction of the ATP concentration from 5 mM down to the micromolar range coincides with the disassembly of the 26S proteasome under these conditions, it could be possible that this phenomenon is due to higher peptidase activities of free 20S proteasomes when released from the 26S proteasome, compared with their activity when incorporated into the 26S proteasome complex.

Several observations, however, argue against this possibility. First, it is known that ATP-dependent 19S regulator binding to the 20S core complex induces conformational changes that result in the opening of the substrate access pores of the 20S core particle, thus facilitating hydrolysis of peptide substrates (43, 49, 80, 86). Second, variation of the ATP or Mg$^{2+}$ concentration does not affect the stability and the peptidase activity of the 20S proteasome (46). Third, 26S proteasome disassembly and 26S proteasome peptidase activities show distinct kinetics in response to variations of ATP/Mg$^{2+}$ concentrations (46). Fourth, measurements of 26S proteasome peptidase activities at very low ATP concentrations (< 10 μM) or in the absence of ATP result in low peptidase activities, comparable with their activities at ATP concentrations above 2 mM (46, 66). Thus the maximal 26S proteasome peptidase activity that is detectable at concentrations of 20–100 μM of ATP is likely attributable to 26S proteasomes which remain intact at very low ATP concentrations. The observation that variation of the Mg$^{2+}$ concentration results in the same activation of 26S proteasome peptidase activities that has been detected with variation of the ATP concentration further supports this assumption and suggests that ATP hydrolysis is required to maximally activate 26S proteasome peptidase activities (46). While the exact molecular mechanisms underlying the regulation of 26S proteasome stability and activity by ATP remain to be determined, recent findings suggest that distinct conformational states of the 19S ATPase subunits with high, low, and no affinity for ATP and the stoichiometry of their nucleotide binding could be responsible (129).

Whereas ADP, AMP, adenosine, and inorganic pyrophosphate cannot substitute for the effects of ATP on 26S proteasome peptidase activities or proteasomal degradation of intact proteins, the 26S proteasome has a broad specificity for nucleotide triphosphates with a relative nucleotide preference for ATP > CTP >> GTP > UTP (6, 24, 34, 64, 65). Although all NTPs can substitute for ATP during degradation of intact proteins by the 26S proteasome, GTP and UTP are unable to promote assembly of the 26S proteasome complex (6, 24). Furthermore, all NTP pools show similar depletion kinetics during anoxia in cardiomyocytes and during myocardial ischemia in vivo (45, 132, 133). Thus the large excess of ATP over nonadenine nucleotides (< 5% of ATP) in cardiomyocytes suggests that ATP is the primary regulator and that possible effects of nonadenine nucleotides on assembly, stability, and function of the 26S proteasome are negligible (45).

Figure 1 shows a simplified model of the suggested regulation of the 26S proteasome by ATP. This model is based on observations from solid phase affinity immobilization assays and chymotrypsin-like, trypsin-like, and caspase-like proteasome peptidase activity measurements with purified enzyme preparations and cell and tissue extracts (24, 46, 66, 95, 113). The observed relationships between 26S proteasome stability, activity, and ATP concentrations have implications for the interpretation of proteasome peptidase activity measurements in biological samples and the understanding of the role of the 26S proteasome under conditions that are associated with alterations of the cellular ATP content. The direct ATP dependency of the stability and activity of the 26S proteasome suggests that activity measurements should be performed at the actual tissue/cell ATP concentration to be able to interpret its possible regulation and role in the pathophysiology of interest. Furthermore, these relationships suggest that 26S proteasome activities at a normal cellular ATP concentration correspond to a suppressed functional state. This further implies that pathological conditions which are associated with depletion of the cellular ATP content, such as ischemia or anoxia, likely result in a reduced cellular ratio between 26S proteasomes and free.
Regulation of Myocardial Proteasome Activity During Cardiac Ischemia and Reperfusion

Besides the biochemical evidence that changes in ATP levels influence proteasome assembly, stability, and function, several previous studies have measured proteasome peptidase activities in myocardial extracts and in purified myocardial 20S proteasome preparations to assess the regulation and pathophysiological role of the cardiac proteasome during ischemia and reperfusion. These studies used left anterior descending coronary artery (LAD) occlusion, the isolated perfused heart, and heterotopic heart transplantation as model systems (Table 1). Whereas chymotrypsin-like peptidase activity was measured by all investigators, only three previous studies determined caspase-like proteasome peptidase activity measurements in the absence of Mg2+/ATP to assess the function of the free 20S proteasome (19), other studies also determined the ATP (28 μM-5 mM)-dependent proportion of the peptidase activities to calculate 26S proteasome activity (25, 32, 33, 116) or assessed total proteasome activity as the ATP-dependent and proteasome inhibitor-sensitive proportion of the total peptidase activity (94).

Irrespective of the applied method, the reported proteasome peptidase activities suggest impairment of the 20S/26S proteasome during reperfusion of postischemic hearts. As this phenomenon was also detectable when proteasome activities were normalized to the myocardial proteasome content, these data suggest that the proteasome peptidase activities are under direct control of the cellular ATP concentration. As ATP levels decrease from physiological levels (∼5 mM) to ∼0.5 mM, 26S proteasomes disassemble into free 20S core particles and 19S regulators in direct correlation to the ATP concentration. This reduces the physiological ratio between 26S proteasomes and free 20S core particles. Approximately 30% of 26S proteasomes remain stable down to the low micromolar range of ATP concentrations. While 20S proteasome stability and activity are not affected by changes of ATP levels, peptidase activities of the 26S proteasome increase severalfold when the ATP concentration is reduced from physiological levels, with peak activities at ATP concentrations between 10 and 100 μM. Further reduction of the ATP concentration results in the disassembly of the remaining 26S proteasomes, leading to a reduced peptidase activity in solution assays. Whether 19S regulators that are released form 26S proteasome complexes (shown as transparent icons) remain stable and contribute to the reformation of 26S proteasome complexes when ATP levels recover is unknown.

Table 1. Proteasome peptidase activities during myocardial ischemia and reperfusion

<table>
<thead>
<tr>
<th>Model</th>
<th>Specimen</th>
<th>Activity During Ischemia</th>
<th>Activity During Reperfusion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAD, I/R 30 min/50 min</td>
<td>Extract</td>
<td>N/D</td>
<td>CT-L ↓, T-L ↓, Casp-L ↓</td>
<td>(19)</td>
</tr>
<tr>
<td>LAD, I/R 30 min/50 min</td>
<td>20S</td>
<td>N/D</td>
<td>CT-L ↓</td>
<td>(19)</td>
</tr>
<tr>
<td>LAD, I/R 30 min/180 min</td>
<td>Extract</td>
<td>CT-L ↓</td>
<td>CT-L ↓</td>
<td>(32)</td>
</tr>
<tr>
<td>LAD, I/R 50 min/300 min</td>
<td>Extract</td>
<td>CT-L ↑</td>
<td>CT-L ↓</td>
<td>(7)</td>
</tr>
<tr>
<td>IPH, I/R 15–30 min/60 min</td>
<td>Extract</td>
<td>CT-L ~↑/↓</td>
<td>CT-L ~↑</td>
<td>(116)</td>
</tr>
<tr>
<td>IPH, I/R 30 min/60 min</td>
<td>Extract</td>
<td>CT-L ↓</td>
<td>N/D</td>
<td>(25)</td>
</tr>
<tr>
<td>IPH, I/R 30 min/60 min</td>
<td>Extract</td>
<td>N/D</td>
<td>CT-L ↓</td>
<td>(32)</td>
</tr>
<tr>
<td>IPH, I/R 30 min/60 min</td>
<td>Extract</td>
<td>N/D</td>
<td>CT-L ~↑/↑, Casp-L ~</td>
<td>(113)</td>
</tr>
<tr>
<td>HTx, c/I 180 min/4–24 h</td>
<td>Extract</td>
<td>CT-L ~↑, T-L ~</td>
<td>CT-L ~↑, T-L ~</td>
<td>(94)</td>
</tr>
</tbody>
</table>

The proteasome peptidase activities that have been measured in each study are listed. CT-L, chymotrypsin-like proteasome peptidase activity; T-L, trypsin-like proteasome peptidase activity; Casp-L, caspase-like proteasome peptidase activity; ↓, ↑, ~, reduced, increased, or unchanged proteasome activities, respectively, as compared with normal hearts; N/D, not determined; LAD, I/R, left anterior descending coronary artery occlusion/reperfusion; I/R, ischemia (I) and reperfusion (R) time; IPH, isolated perfused heart; HTx, heterotopic heart transplantation; c/I, cold (5°C) ischemia; 20S, purified 20S proteasome.
likely reflect changes in the enzymatic properties of the proteasome (94).

Inactivation of the trypsin-like 20S proteasome peptidase activity could be confirmed in purified 20S proteasome preparations from postischemic hearts and has been attributed to its inactivation by the lipid peroxidation product 4-hydroxy-2-nonenal (19, 41). As purified 20S proteasomes derived from postischemic hearts did not show impairment of chymotrypsin-like and caspase-like peptidase activities, the reduced chymotrypsin-like and caspase-like activities that were detectable in heart extracts have been attributed to endogenous and as yet unidentified proteasome inhibitors (19, 41). Although the activities of purified 26S proteasomes from ischemic or postischemic hearts have not been determined, oxidative damage of the 19S regulator subunit Rpt5 during reperfusion of postischemic hearts has been detected and associated with reduced ATP-dependent 26S proteasome peptidase activity (32, 68).

Aforementioned studies, however, compared proteasome peptidase activities in extracts from normal, ischemic, and postischemic hearts that were measured at the same ATP concentration in the activity assay. In the consideration of the rapid alterations in myocardial ATP content during ischemia and the ATP dependency of the 26S proteasome, it becomes apparent that measurements of proteasome activities that have been performed at a standard ATP concentration should be interpreted with caution when absolute proteasome activities are placed in a pathophysiological context.

To date, information on the regulation of the myocardial proteasome by ATP during ischemia and reperfusion has been provided only in a heart transplantation model (Table 1). Inhibition of the myocardial proteasome in extracts from postischemic hearts, which showed incomplete recovery of myocardial ATP levels, could be confirmed when chymotrypsin-like peptidase activities were measured at the actual myocardial ATP concentration (9). Total chymotrypsin-like proteasome peptidase activities that were measured at the actual tissue ATP concentration, however, increased by 200–500% as the myocardial ATP content decreased during ischemia (46). This implies that the regulation of the 26S proteasome by ATP, which is detectable in extracts from normal and postischemic hearts and in purified enzyme preparations (46, 66, 113), occurs in the ischemic heart, leading to a severalfold activation of the 26S proteasome. This activation of the 26S proteasome remains concealed when proteasome activity measurements are performed without adjustment of the assay ATP concentration.

Although measurements of proteasome peptidase activities may not necessarily reflect the ability of the proteasome to degrade natural proteins, studies in cell systems suggested that this regulation of the 26S proteasome by ATP also occurs in intact cells and is reflected by corresponding changes of the endogenous pool of ubiquitin-protein conjugates and of artificial and natural proteasome substrate proteins (66, 73). In agreement with the activation of the cardiac proteasome as ATP levels decline during ischemia and its impairment during reperfusion are studies that reported decreased amounts of myocardial ubiquitin-protein conjugates during ischemia and increased ubiquitin-protein conjugates during reperfusion, as assessed by Western blot experiments with cardiac extracts (19, 46, 94, 116). Increased myocardial ubiquitin-protein conjugates have also been detected in cardiac extracts from patients with ischemic heart disease by Western blot analysis (141). Thus these observations may provide evidence for functional relevance of the changes in cardiac proteasome activity during ischemia and reperfusion at the protein level. The biological relevance of globally increased ubiquitin-protein conjugates, however, remains to be determined. Furthermore, the regulation of the endogenous ubiquitin-protein conjugate pool size is complex and determined by the equilibrium between the activities of the ubiquitin-protein ligase system, DUBs, and the 26S proteasome. While increased activities of the sum of ubiquitin-protein ligase systems has been detected in the rat heart during ischemia (94), information on the regulation of the individual enzyme activities of E1, E2, and E3 enzymes and of deubiquitylating enzymes in the heart during ischemia and reperfusion is currently lacking. Nevertheless, previous studies on the regulation of the endogenous ubiquitin-protein conjugate pool in cell systems and organs suggested that the ubiquitin-protein conjugate pool size is predominantly controlled by ubiquitylation and deubiquitylation rates, whereas potential modulation of the degradation rates would have little effects (53, 107). Thus a comprehensive assessment of the relationship between the ubiquitin-protein conjugate pool and the activities of the enzyme components of the UPP is required to determine the relative contribution of the 26S proteasome to the observed changes of endogenous ubiquitin-protein conjugates during cardiac ischemia and reperfusion.

Nevertheless, the currently available data suggest that ischemia and reperfusion lead to opposite changes in myocardial proteasome activity. While ATP depletion during ischemia likely upregulates 26S proteasome activity, partial or complete recovery of the myocardial ATP content, oxidative damage of the 19S regulator and 20S core particle, as well as other regulatory mechanisms during reperfusion appear to reduce and impair 26S/20S proteasome function in postischemic hearts.

Effects of Proteasome Inhibitors in the Ischemic and Postischemic Heart

Further information on the pathophysiological role of the proteasome and its potential as a drug target during cardiac ischemia and reperfusion has been provided by studies that tested the effects of various proteasome inhibitors (Table 2). The proteasome inhibitors that were used in these studies belong to the major classes of peptide aldehydes (MG-132), boronates (bortezomib, Pyz-Phe-boroLeu), epoxyketones (epoxomycin), and β-lactones (lactacystin, PS-519). These inhibitors reversibly (peptide aldehydes and boronates) or irreversibly (peptide epoxyketones, β-lactones) interact with the catalytically active sites. They predominantly inhibit the chymotrypsin-like site but also have coinductive effects on the trypsin-like and/or caspase-like sites. In addition, allostERIC proteasome inhibitors (PR-11, PR-39) were used, which affect proteasome activity through their interaction with the 20S proteasome α7-subunits (42). The biochemical and pharmacological properties of these proteasome inhibitors have been previously reviewed in detail elsewhere (75, 76).

It should be noted, however, that the majority of these studies did not assess myocardial ATP content or cardiac proteasome activities (10, 21, 44, 67, 91, 99, 116, 117, 131, 150); proteasome inhibitors were employed based on their anti-inflammatory (10, 21, 44, 99, 117, 131) and immune-
Table 2. Effects of proteasome inhibitors during myocardial ischemia and reperfusion

<table>
<thead>
<tr>
<th>Model</th>
<th>Species</th>
<th>Inhibitor (Dose)</th>
<th>Time Point and Route of Administration</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPH I/R 20/45 min</td>
<td>Rat</td>
<td>PS-519 (0.01–1 mg/kg)</td>
<td>Preischemic perfusion</td>
<td>Preserved contractility, preserved coronary perfusion*</td>
<td>(21)</td>
</tr>
<tr>
<td>IPH I/R 30/60 min</td>
<td>Rat</td>
<td>MG-132 (6–25 μM)</td>
<td>Preischemic perfusion</td>
<td>Reduced heart rate × pressure product</td>
<td>(116)</td>
</tr>
<tr>
<td>LAD I/R 60/180 min</td>
<td>Pig</td>
<td>PS-519 (1 mg/kg)</td>
<td>Preischemic ic</td>
<td>No effect on heart rate × pressure product</td>
<td>(33)</td>
</tr>
<tr>
<td>LAD I/R 30 min/24 h</td>
<td>Mouse</td>
<td>PS-519 (1 mg/kg)</td>
<td>Series 1: 30 min pre-LAD ip</td>
<td>Preserved contractility</td>
<td>(117)</td>
</tr>
<tr>
<td>LAD I/R 30 min/72 h</td>
<td>Mouse</td>
<td>PS-519 (1 mg/kg)</td>
<td>Series 2: with reperfusion ip</td>
<td>Series 1: Reduced infarct size and preserved contractility</td>
<td>(131)</td>
</tr>
<tr>
<td>LAD I/R 90 min/6 h</td>
<td>Dog</td>
<td>Bortezomib (0.0875 mg/kg)</td>
<td>1 h pre-/5 h post-LAD iv</td>
<td>Series 2: No effect on infarct size</td>
<td>(150)</td>
</tr>
<tr>
<td>LAD I/R 30 min/24 h</td>
<td>Rat</td>
<td>PR-39/PR-11 (10 nmol/kg)</td>
<td>With reperfusion im</td>
<td>Reduced arrhythmia</td>
<td>(150)</td>
</tr>
<tr>
<td>LAD I/R 90 min/6 h</td>
<td>Dog</td>
<td>Epoxomicin (2.5 μg/kg)</td>
<td>Preischemia ic</td>
<td>Reduced infarct size</td>
<td>(67)</td>
</tr>
<tr>
<td>LAD I/R 45/60 min</td>
<td>Mouse</td>
<td>Epoxomicin (0.5 mg/kg)</td>
<td>Preischemia ip</td>
<td>Reduced infarct size</td>
<td>(44)</td>
</tr>
<tr>
<td>HTx cl 0–48 h</td>
<td>Rat</td>
<td>Pyz-Phe-boroLeu* (0.5–1 mg·kg⁻¹·day⁻¹)</td>
<td>Series 1: days 1–16 post-Tx ip</td>
<td>No effect on infarct size</td>
<td>(7)</td>
</tr>
<tr>
<td>HTx cl/R 12–24 h/4 h</td>
<td>Rat</td>
<td>Epoxomicin (50 μM)</td>
<td>Addition to preservation solution</td>
<td>Prolonged allograft survival</td>
<td>(134)</td>
</tr>
<tr>
<td>LAD 1 24 h</td>
<td>Dog</td>
<td>Bortezomib (0.0875 mg/kg)</td>
<td>1 h post-LAD iv</td>
<td>Reduced edema, preserved ultrastructure</td>
<td>(91)</td>
</tr>
<tr>
<td>LAD 1 24 h</td>
<td>Dog</td>
<td>Bortezomib (0.0875 mg/kg)</td>
<td>1 h post-LAD iv</td>
<td>Reduced edema, preserved ultrastructure</td>
<td>(96)</td>
</tr>
<tr>
<td>LAD 1 7 days</td>
<td>Mouse</td>
<td>PR-39 (1 μg·kg⁻¹·day⁻¹)</td>
<td>Post-LAD for 7 days ip</td>
<td>Reduced arrhythmia</td>
<td>(10)</td>
</tr>
<tr>
<td>LAD 1/R 30 min/24 h</td>
<td>Rat</td>
<td>PR-39/PR-11 (10 nmol/kg)</td>
<td>With reperfusion im</td>
<td>Reduced infarct size</td>
<td>(150)</td>
</tr>
<tr>
<td>LAD I/R 90 min/6 h</td>
<td>Dog</td>
<td>Epoxomicin (2.5 μg/kg)</td>
<td>Preischemia ip</td>
<td>Reduced infarct size</td>
<td>(150)</td>
</tr>
<tr>
<td>LAD I/R 90 min/6 h</td>
<td>Dog</td>
<td>Epoxomicin (0.5 mg/kg)</td>
<td>Preischemia ip</td>
<td>Reduced infarct size</td>
<td>(150)</td>
</tr>
<tr>
<td>LAD I/R 30 min/24 h</td>
<td>Rat</td>
<td>PR-39/PR-11 (10 nmol/kg)</td>
<td>With reperfusion im</td>
<td>Reduced infarct size</td>
<td>(150)</td>
</tr>
<tr>
<td>LAD I/R 90 min/6 h</td>
<td>Dog</td>
<td>Epoxomicin (2.5 μg/kg)</td>
<td>Preischemia ip</td>
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<td>(150)</td>
</tr>
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<td>Rat</td>
<td>PR-39/PR-11 (10 nmol/kg)</td>
<td>With reperfusion im</td>
<td>Reduced infarct size</td>
<td>(150)</td>
</tr>
<tr>
<td>HTx cl 0–48 h</td>
<td>Rat</td>
<td>Epoxomicin (50 μM)</td>
<td>Addition to preservation solution</td>
<td>Reduced edema, preserved ultrastructure, prolonged viability</td>
<td>(9)</td>
</tr>
</tbody>
</table>

MVT, malignant ventricular tachycardia; SCD, sudden cardiac death; iv, intravenously; ic, intracoronally; ip, intraperitoneally; im, intramyocardially. *Effects dependent of the presence of leukocytes. #Pyz-Phe-boroLeu is the active ingredient in bortezomib.

Benefits of the proteasome inhibitors PR-39, PR-11, and PS-519 after ischemia and reperfusion have been associated with reduced NK-κB activation in postischemic hearts through inhibition of 26S proteasome-mediated degradation of phosphorylated and ubiquitinated IkBα (10, 44, 99, 105, 117, 131, 137, 138). However, Pye et al. (117) demonstrated that activation of NK-κB occurs in postischemic hearts but not in the ischemic heart before reperfusion. PS-519 not only attenuated decreased myocardial function and reduced infarct size by 90% in the 3 h reperfusion heart but also prevented a 50% decrease in regional myocardial function after 1 h of ischemia and thus maintained myocardial function of the ischemic heart at a level comparable to normal hearts (117). This suggests that inhibition of the cardiac proteasome by PS-519 during a time frame when myocardial ATP levels rapidly decline, and subsequently result in the activation of the cardiac 26S proteasome, confers additional cardioprotection independent of NK-κB.

In agreement with these protective actions of PS-519 in the ischemic heart are findings from a myocardial infarction model in which administration of the reversible proteasome inhibitor bortezomib prevented ventricular tachyarrhythmias and a decline in myocardial GRK2 levels in the epicardial border and infarct zones. This suggests that enhanced proteasomal degradation of GRK2 in the ischemic myocardium contributes to the increased susceptibility for sudden cardiac death after myocardial infarction (67, 109, 150). Consistent with the observed effects of proteasome inhibitors after LAD occlusion with or without reperfusion are suppressive properties (91, 146) or to assess whether they affect G protein receptor kinase 2 (GRK2) expression during ischemia (67, 150). Nevertheless, in vivo inhibition of the proteasome by PS-519 and bortezomib was confirmed using whole blood or leukocyte proteasome peptidase activity measurements as a surrogate for their pharmacological efficacy in some studies (67, 117, 150). Furthermore, the basic arginine/proline-rich peptide PR-39 has been reported to prevent proteasome-mediated degradation of IkBα without effects on proteasomal degradation of total cellular protein (10, 44).

Initial evidence for beneficial effects of proteasome inhibitors during myocardial ischemia and reperfusion has been provided in an isolated perfused rat heart model (21). Intravenous administration of PS-519 before excision and instrumentation of the heart, followed by ischemia and reperfusion of the isolated perfused heart in the presence of autologous polymorphonuclear leukocytes (PMN), dose-dependently reduced cardiac PMN infiltration and preserved coronary perfusion and myocardial contractility. As reperfusion of the hearts in the absence of PMN did not result in myocardial reperfusion injury in this model, the observed effects can be attributed to inhibition of PMN-mediated myocardial injury by PS-519.

Subsequently, the effects of proteasome inhibitors have been studied in vivo models of coronary artery ligation in various species. When administered before or after coronary artery ligation or with initiation of reperfusion, various proteasome inhibitors were found to reduce infarct size, preserve contractility, and reduce the occurrence of malignant cardiac arrhythmias (10, 44, 67, 99, 117, 131, 150).
cardiomyocytes, it reduced viability of cultured neonatal ven-
cally relevant concentrations during culture of primary adult
Although bortezomib did not show cytotoxic effects at clini-
Dose-dependent cytotoxicity has been also described for vari-
in vivo and induce apoptosis of tumor cells in vitro (3).
known that proteasome inhibitors possess antineoplastic effects
some subunits, and cardiac proteasome subtypes with differ-
chondrial serine protease HtrA2/Omi (4, 75). Furthermore,
cathepsins, chymase, dipeptidyl peptidase II, and the mito-
reperfusion, could be explained with indirect cardioprotection
reperfusion, could be explained with indirect cardioprotection
Effects of Enhancement of Proteasome Activity in the
Several small molecule activators of the 20S proteasome are
known; however, their low aqueous solubility limits the use of
these compounds (143). Thus, when compared with the mul-
tiple reports on proteasome inhibition in the ischemic and
postischemic heart, information on the effects of cardiac pro-
tase activation is sparse. Reduced postischemic myocardial
function, ultrastructural abnormalities of cardiomyocytes, and
decreased ATP synthesis (103).
Several observations may account for these contradictory
findings. Most available proteasome inhibitors are not protea-
some specific (75). While MG-132 is known to inhibit the
proteasome, calpain and cathepsins, bortezomib also inhibits
cathepsins, chymase, dipeptidyl peptidase II, and the mito-
chondrial serine protease HtrA2/Omi (4, 75). Furthermore,
proteasome inhibitors show differential selectivity for protea-
some subunits, and cardiac proteasome subtypes with differ-
etial susceptibilities for MG-132 and bortezomib have been
isolated (28, 79). This suggests that adverse effects of MG132
and bortezomib could be related to their intrinsic target selec-
tivity and inhibitory efficacy in the heart.
The UPP regulates normal growth and apoptosis, and it is
known that proteasome inhibitors possess antineoplastic effects
in vivo and induce apoptosis of tumor cells in vitro (3).
Dose-dependent cytotoxicity has been also described for vari-
ous proteasome inhibitors in H9c2 cardiomyoblasts (103).
Although bortezomib did not show cytotoxic effects at clini-
cally relevant concentrations during culture of primary adult
cardiomyocytes, it reduced viability of cultured neonatal ven-
tricular cardiomyocytes at high concentrations (103). While
high concentrations of MG-132 have been associated with
cardiac apoptosis in the isolated perfused heart, PS-519 did not
affect cardiac apoptosis after LAD ligation and reperfusion
(114, 117). Thus it appears possible that the drug-specific
cytotoxicity also contributes to the cardiotoxic effects of MG-
132 and bortezomib, which have been observed in some
studies.

Besides the possibility that differences in proteasome inhib-
tor specific off-target effects, their intrinsic toxicity profiles,
and selectivity for certain proteasome subunits account for
adverse effects in the heart, it is conceivable that the patho-
physiological consequences of proteasome inhibition also de-
pend on the functional status of the cardiac proteasome.
Among numerous other functions, proteasomes have been
shown to be involved in the regulation of normal connexin
and cardiac myofibrillar protein turnover (36, 122, 135). This may
explain that proteasome inhibition under normal conditions or
during insignificant ischemic periods impairs the dynamic
equilibrium between protein degradation and synthesis and
thus can result in disturbed cardiac contractility and excitabil-
ity.
The findings that proteasome inhibition under conditions
that are associated with severe or prolonged periods of ATP
depletion protects cardiomyocytes in vitro (92, 130) and in
vivo (Table 2) correlates with the pathological increase in 26S
proteasome activity during ischemia. This further suggests that
increased proteasomal degradation of endogenous protein sub-
strates, such as GRK2 (67, 150), type-2 ryanodine receptors
(108), connexin 43 (57), and possibly of myofibrillar proteins
(33, 36), contributes to ischemic myocardial injury.

The paradoxical findings from some studies that proteasome
inhibition in the postischemic heart was beneficial, although
myocardial proteasome activity is already impaired during
reperfusion, could be explained with indirect cardioprotection
through immune suppressive drug actions, e.g., by inhibition of
NK-κB activation, which result in attenuated leukocyte-med-
ated myocardial reperfusion injury.

Effects of Enhancement of Proteasome Activity in the
Postischemic Heart
Several small molecule activators of the 20S proteasome are
known; however, their low aqueous solubility limits the use of
these compounds (143). Thus, when compared with the mul-
tiple reports on proteasome inhibition in the ischemic and
postischemic heart, information on the effects of cardiac pro-
tase activation is sparse. Reduced postischemic myocardial
dysfunction and infarct size have recently been described after
LAD occlusion and reperfusion in transgenic mice with en-
hanced cardiac proteasomal function (85). Furthermore,
several lines of evidence suggest that ischemic preconditioning
also results in an activation of the myocardial proteasome
activity by ~50% and preserves 26S proteasome function in
the postischemic heart through multiple mechanism, such as
diminished oxidative damage of the 19S regulator or interac-
tions with protein kinases A and C (7, 25, 116). The
reported proteasome activity measurements, however, have
been performed at a standard ATP concentration in the enzyme
assay and thus do not reflect the effects of ATP on myocardial
proteasome function during ischemia and reperfusion. As isch-
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The various effects of ATP on 26S proteasome assembly, stability, and function make the cardiac proteasome a moving target during ischemia and reperfusion. Studies on the myocardial ATP content during ischemia, in combination with the effects of ATP on 26S proteasome stability/activity in cell free systems and corresponding observations in intact cells, suggest that depletion of the myocardial ATP content during prolonged ischemia likely results in the activation of the 26S proteasome (9, 46, 66). The effects of proteasome inhibition during prolonged cardiac ischemia in the absence of leukocytes support the concept that the subset of 26S proteasomes, which remains stable at critically low ATP levels, functions as a destructive protease. This implies that a sufficient energy supply prevents the heart from proteasomal autodestruction. In contrast, during reperfusion of the ischemic heart, findings from enzyme activity assays with cell-free extracts suggest impairment and inhibition of the proteasome.

Besides the roles of the UPP in the regulation of global protein turnover and quality control and its contribution to cardiac autophagy and proteotoxicity, it is also involved in the regulation of important signaling molecules in the ischemic and postischemic heart, such as NK-κB, GRK2, type-2 ryanodine receptors, or 5'-AMP-activated protein kinase (11, 14, 39, 67, 83, 108, 120, 150, 155). Furthermore, recent studies on the roles of a number of E3s (muscle ring finger 1, murine ubiquitin-related protein-1, carboxy terminus of Hsp70-interacting protein) in overexpression systems suggested that modulating ubiquitylation and subsequent proteasomal degradation of the substrate proteins could lead to beneficial as well as to detrimental effects on myocardial ischemia-reperfusion injury (84, 101, 136, 144, 147, 153). This demonstrates that alterations of the UPP will affect a multitude of cell functions and explains why the predominant mechanisms through which changes in proteasome activity influence cardiac function and myocardial injury during ischemia and reperfusion are currently unknown.

Irrespective of the underlying molecular mechanisms, the majority of studies that evaluated the effects of proteasome inhibitors during cardiac ischemia and reperfusion reported cardioprotective effects (Table 2). Thus increased myocardial 26S proteasome activity during ischemia and prevention of pathologically enhanced protein degradation could provide a pathophysiological rationale for cardioprotective effects of proteasome inhibitors when administered before or during ischemia. On the contrary, cardioprotective effects of proteasome inhibitors during the reperfusion period appear paradoxical. It is unknown, however, whether impaired proteasome function in the postischemic heart also impairs substrate specificity of the 26S/20S proteasome, which could result in the degradation of proteins that are not physiological proteasome substrates and might be preventable by proteasome inhibition. Furthermore, proteasome inhibitors have anti-inflammatory and immune-suppressive actions, which attenuate leukocyte-mediated myocardial reperfusion injury. Therefore, proteasome inhibitors could be beneficial during reperfusion when the pathophysiological consequences of leukocyte-mediated reperfusion injury outweigh those of an additional reduction of the already impaired cardiac proteasome activity. Thus it might be speculated that proteasome inhibitors could be useful to limit infarct size and prevent malignant arrhythmias in patients with myocardial ischemia/infarction, in particular when revascularization is not possible in a timely manner.

Currently available proteasome inhibitors, however, have well-documented toxicities and side effects, which correspond to the degree of proteasome inhibition, as assessed by proteasome peptidase activity measurements in whole blood or extracts from peripheral blood leukocytes. Based on preclinical toxicity studies, the maximum safe level of proteasome inhibition by bortezomib is ~90%, because proteasome inhibition beyond this threshold resulted in severe gastrointestinal toxicities and lethal hemodynamic consequences (1, 2, 56). In a dose-escalating phase-1 study in patients with solid tumors and lymphomas, the dose-limiting toxicity of bortezomib was peripheral neuropathy (56). Accordingly, a dose of bortezomib that resulted in 70% proteasome inhibition and did not cause dose-limiting adverse effects was recommended (56). Analyses of the safety of bortezomib from phase-3 clinical trials in multiple myeloma patients revealed hematologic toxicity as the most common adverse event (17). Adverse cardiac effects have been reported in 8–11% of multiple myeloma patients treated with bortezomib (17, 38, 55). Nonetheless, initial reports of advantageous effects of bortezomib in patients with cardiac allograft rejection and amyloidosis justify further exploration of proteasome inhibitor treatment during cardiac ischemia (35, 121). As new proteasome inhibitors with improved pharmacological characteristics are under development and the recently Food and Drug Administration approved proteasome inhibitor carfilzomib appears to possess a favorable toxicity profile (78, 126, 149), it is possible that drugs with appropriate pharmacokinetics, i.e., short duration of action, could show a reasonable efficacy-to-toxicity ratio.
In cardiac transplantation, the problem of viability is a consistent limitation (69, 106). Cold ischemic storage of donor hearts is currently limited to 4–6 h because longer ischemic periods reduce organ survival and function. Interestingly, activation of the cardiac proteasome with depletion of the tissue ATP content during prolonged cold ischemic storage resulted in proteasome activities at 5°C that were comparable with the normal myocardial proteasome activities under normothermic conditions (46). This suggests a profound imbalance between proteasomal protein degradation and protein resynthesis in the hypothermic ischemic heart. This phenomenon, in combination with the advantageous effects of epoxomicin during cold ischemic storage and subsequent transplantation of hearts, implies proteasome inhibition as a promising approach to improve preservation and prolong ischemic tolerance of donor hearts.

The direct regulatory effects of ATP on the 26S proteasome have implications for the understanding of its contribution to the pathophysiolo-gy of the ischemic heart and its possible role as a therapeutic target. Further characterization of the molecular mechanisms through which ATP regulates 26S proteasome activity during ischemia and reperfusion, as well as the development of agents that may permit cardioselective targeting of the proteasome, are likely to provide novel therapeutic opportunities for ischemic heart disease, heart preservation, and transplantation.

GRANTS

This work was supported, in part, by a grant from the American Heart Association.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.M. prepared figures, drafted manuscript, edited and revised manuscript, and approved final version of manuscript.

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