FORUM REVIEW ARTICLE

Targeting the Ubiquitin-Proteasome System in Heart Disease: The Basis for New Therapeutic Strategies

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Abstract

Significance: Novel therapeutic strategies to treat heart failure are greatly needed. The ubiquitin-proteasome system (UPS) affects the structure and function of cardiac cells through targeted degradation of signaling and structural proteins. This review discusses both beneficial and detrimental consequences of modulating the UPS in the heart. **Recent Advances:** Proteasome inhibitors were first used to test the role of the UPS in cardiac disease phenotypes, indicating therapeutic potential. In early cardiac remodeling and pathological hypertrophy with increased proteasome activities, proteasome inhibition prevented or restricted disease progression and contractile dysfunction. Conversely, enhancing proteasome activities by genetic manipulation, pharmacological intervention, or ischemic preconditioning also improved the outcome of cardiomyopathies and infarcted hearts with impaired cardiac and UPS function, which is, at least in part, caused by oxidative damage. **Critical Issues:** An understanding of the UPS status and the underlying mechanisms for its potential deregulation in cardiac disease is critical for targeted interventions. Several studies indicate that type and stage of cardiac disease influence the dynamics of UPS regulation in a nonlinear and multifactorial manner. Proteasome inhibitors targeting all proteasome complexes are associated with cardiotoxicity in humans. Furthermore, the type and dosage of proteasome inhibitor impact the pathogenesis in nonuniform ways. **Future Directions:** Systematic analysis and targeting of individual UPS components with established and innovative tools will unravel and discriminate regulatory mechanisms that contribute to and protect against the progression of cardiac disease. Integrating this knowledge in drug design may reduce adverse effects on the heart as observed in patients treated with proteasome inhibitors against noncardiac diseases, especially cancer. *Antioxid. Redox Signal.* 21, 2322–2343.

Introduction

ALL COMPONENTS OF A living organism are subject to continuous synthesis and degradation, including the human heart. This revolutionizing hypothesis dates back to Rudolf Schoenheimer's studies on the biochemistry of lipids and proteins, summarized in a monograph titled "The Dynamic State of Body Constituents" (186). In the concluding paragraph, Schoenheimer states: "The new results imply that not only the fuel but the structural materials are in a steady state of flux." Many decades later, the ubiquitin-proteasome system (UPS) assumes a prominent role in this now-established hypothesis, as it is identified as a highly specialized protein deg-

radation machinery that marks proteins before their efficient removal (94, 95, 210). Through this targeted selection of protein substrates, the UPS participates in the regulation of apparently all essential cellular signaling events, including oxygen sensing, antioxidant response, and apoptosis (75, 209, 212). Since its discovery, it has become increasingly apparent that cardiac UPS function is (i) highly dynamic, (ii) critical for a healthy myocardium, and (iii) its modulation can alter the outcome of cardiac disease (10, 49, 132). In this review, we address the current state of research, suggesting that the UPS may serve as a novel therapeutic target in improving strategies to cope with cardiac disease, which remains the leading cause of death and disability worldwide.

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Molecular Basis and Targets for Proteasome Inhibition and Stimulation in the Heart

20S proteasome inhibition

The UPS is a multilayered machinery that is compartmentalized by hundreds of structural and functional proteins (Fig. 1) (30). The primary effort to target the UPS *via* pharmaceutical agents is directed against the activities of 20S proteasome complexes, which are responsible for degradation of substrate proteins when assembled into 26S proteasomes (17). Notably, 20S proteasomes may degrade oxidized proteins without previous targeting by ubiquitination (Fig. 2) (80, 189). The plural in the term “20S proteasomes” must be emphasized, because two populations are known since early on: a constitutive and an inducible form (71). Since its discovery, the inducible 20S proteasome is assigned a role in immune response and antigen processing, but more recent research associates it with broader function, including stress response and the degradation of proteins damaged by oxidation (3, 11, 119). In the mammalian heart, both 20S proteasome populations and subpopulations with mixed assembly exist under unstimulated conditions (Fig. 3) (50). The subpopulations differ in their incorporation of proteolytic subunits. In cardiac tissue, six proteolytic subunits are co-expressed by six genes. Analyses of purified cardiac 20S proteasomes by two-dimensional electrophoresis show that the proteolytic subunits $\beta 1$, $\beta 2$, and $\beta 5$ are incorporated at much higher levels than $\beta 1i$, $\beta 2i$, and $\beta 5i$ (47, 232).

Each 20S proteasome consists of two outer α - and two inner β -rings with each ring containing seven distinct α - or β -subunits (Fig. 3). Thus, each subunit is incorporated twice. A

distinctive feature of the proteolytic subunits is that during assembly, $\beta 1$, $\beta 2$, and $\beta 5$ can be replaced by $\beta 1i$, $\beta 2i$ and $\beta 5i$, respectively (155). Proteasomes cleave polypeptides after acidic ($\beta 1$), alkaline ($\beta 2$), and hydrophobic ($\beta 5$) amino-acid residues, which are widely referred to as caspase-, trypsin-, and chymotrypsin-like activity, respectively (67, 78, 160). Notably, differential incorporation of the proteolytic subunits affects the kinetics for cleaving polypeptides. Increased incorporation of $\beta 1i$ in 20S proteasomes achieved by transfection is associated with reduced caspase-like activity, and conversely increased $\beta 1$ incorporation is associated with increased caspase-like activity (65, 68). The precise activity conferred by $\beta 1i$ incorporation appears controversial in reporter assays, potentially due to co-incorporation of $\beta 2i$ (77). However, a $\beta 1i$ -specific inhibitor reduces chymotrypsin-like activity and hydrophobic substrate preference of $\beta 1i$ is supported by protein crystallography (97, 103). Similar to $\beta 2$ and $\beta 5$, subunits $\beta 2i$ and $\beta 5i$ preferentially cleave polypeptides after alkaline and hydrophobic amino acids, respectively (68, 103). Overall, incorporation of $\beta 1i$, $\beta 2i$, and $\beta 5i$ increases the preferential proteolysis after alkaline and hydrophobic amino-acid residues, which seems to be tailored to cope with specific physiologic and pathophysiologic cellular events (54, 200). Thus, 20S proteasome function is tightly linked to assembly and proteasome subpopulations differ in their proteolytic function (50). Site-specific peptidolytic activities of 20S proteasomes can be measured *via* reporter peptides and differentiated from nonproteasomal activities *via* proteasome inhibitors (34, 66, 199).

Over the years, inhibitors with increasing specificity for 20S proteasomes have been developed (17). Besides their

FIG. 1. The UPS is a multilayered machinery. E1 enzymes activate, E2 conjugate, and E3 ligate ubiquitin proteins to target substrate proteins for proteasomal degradation by 26S proteasomes. If E4 enzymes aid in the process of ubiquitination, multiple ubiquitins are transferred in the form of ubiquitin chains (100). Ubiquitination is counteracted by DUBs, but deubiquitination at the 19S regulatory particle is also required for efficient degradation and ubiquitin recycling. The 26S proteasomes are composed of 19S regulatory and 20S core particles. Association of 19S with 20S proteasomes and substrate unfolding *via* the 19S proteasome are ATP dependent. The numbers in parentheses indicate the number of distinct genes encoding for proteins of the corresponding layer. It should be noted that not all UPS proteins are required simultaneously. A single E3 enzyme or 14 different 20S proteasome subunits are sufficient for ubiquitin ligation or a functional 20S proteasome, respectively. Furthermore, many E3 genes have been identified by homology only. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 2. Proposed role for the ubiquitin-proteasome system in heart disease with focus on oxidative stress. In cardiac tissue, oxidative stress occurs under conditions, such as ischemia/reperfusion, aging, and heart failure (35, 118, 194). At low stress levels, such as ischemic preconditioning or early cardiac remodeling, proteasome function is adjusted presumably in a compensatory manner (5, 29, 40, 45, 49). Protein substrates of the UPS participate in cardio-protective as well as in pathologic signaling (e.g., cardioprotection/PKC ϵ , pro-inflammatory/NF- κ B, pro-hypertrophic/calcineurin; Table 1), but not all substrates are affected by E3 ligases, DUBs, and proteasomal degradation equally. Thus, targeting the UPS in cardiac disease will be dependent on type and stage of the pathogenesis. With increasing stress load, reduced proteasome function is observed while oxidized and ubiquitinated proteins accumulate (46, 169, 170, 219). In turn, aggregation of carbonylated and ubiquitinated proteins (181) as well as 4-hydroxy-2-nonenal modification and carbonylation of 20S and 19S proteasome subunits are considered to inhibit proteasome activities (21, 45, 170), forming a vicious cycle. Inhibition of DUBs due to oxidation promotes the degradation of proteasomal substrates (55, 126), but potentially contributes to protein aggregation in case of insufficient proteasome activities. Progressive oxidative damage may contribute to the higher incidence of cardiac disease with increasing age. Familial cardiomyopathies with a mutation in genes encoding proteasome substrates seem to accelerate the cycle by facilitating aggregate formation (7, 135, 182).

apparent inhibitory effect, induced expression and *de novo* assembly of proteasome complexes is observed after inhibition, but the inhibitory effect prevails (145). Allosteric drugs may have distinct advantages compared with irreversible and competitive proteasome inhibitors, such as higher versatility

in targeting sites, but proteasome allostery is poorly understood (67). For research and clinical purposes, further specialization of inhibitors to differentiate between proteasome subpopulations is an ongoing endeavor with major achievements being accomplished over the past few years (97, 103, 121, 151). Differential targeting of proteasome subpopulations by current proteasome inhibitors is feasible, but lacks precision (117). The challenge remains in the high similarity of active sites of 20S proteasome subunits, resulting in co-targeting of multiple sites at higher inhibitor concentrations (17, 115, 153).

The majority of inhibitors used in cardiac research are most effective against the chymotrypsin-like activity of 20S proteasomes. This bias stems likely from research and development in cancer chemotherapy. The increased cell permeability of inhibitors developed for cancer research makes them suitable for pharmacological *in vivo* studies. However, inhibitors designed for cancer chemotherapy may not be suitable for the heart, because they were developed with the intention to induce apoptosis (43, 160). Notably, mechanisms mediating apoptosis induced by proteasome inhibition include increased formation of reactive oxygen species (ROS), Ca^{2+} release from endoplasmic reticulum, and mitochondrial dysfunction, which are reviewed in a companion review article (160). Thus, negative impacts of proteasome inhibition on the heart are anticipated. Indeed, perfusion of isolated rat hearts with proteasome inhibitors before ischemia is associated with reduced cardiac function (heart rate $\times$ pressure product) and the accumulation of oxidized proteins (Fig. 2) (24, 169). Still, proteasome inhibitors administered in animal models of heart disease also have desirable effects. These include reduction in cardiac remodeling and infarct size, improved cardiac function, and even a decrease in apoptosis (85, 171, 193). As will be discussed later, disease model, type of inhibitor, and submaximal inhibition determine the outcome in animal models of cardiac disease phenotypes.

In patients with multiple myeloma, individual cohorts are associated with higher incidence of cardiac disease after proteasome inhibitor treatment (56). A much larger clinical trial conducted earlier found the incidence comparable to that with dexamethasone treatment (179). The unclear association of bortezomib administration with adverse cardiac events in patients with multiple myeloma contributes to the debate as to whether proteasome inhibition has therapeutic potential in the treatment of cardiac disease. Based on the success of bortezomib in cancer chemotherapy, a second generation of proteasome inhibitors has been developed for clinical therapies with the aims to overcome acquired bortezomib resistance, reduce adverse effects, increase activity in solid tumors, and enable oral administration (42, 90, 116, 150). Resistance is overcome, at least in part, by carfilzomib (90), a peptidyl-epoxyketone that targets similar proteasome activities such as bortezomib, but binds irreversibly to proteolytic sites (122). Lessening adverse effects may be achieved by targeting those proteasome subpopulations that are most abundant in target cells (97, 121, 151). For example, the inhibitors IPSI-001 and PR-957 preferentially target inducible proteasome subunits (122), such as $\beta 5i$ (103). Although the induction of inducible subunits and rearrangement of the composition of 20S proteasome subpopulations in the pathogenesis of heart conditions are described (49, 157, 196), their role in the myocardium remains unknown at this time.

FIG. 3. Proteasomes are a heterogenic group of multi-protein complexes. Cardiac 20S proteasomes incorporate one or two of six different proteolytic subunits with distinct preference of cleavage sites and turnover and, hence, exist in subpopulations in mammalian hearts (50). Mixed assembly with constitutive and inducible proteolytic subunits results in the formation of intermediate subpopulations (...indicates that additional subpopulations exist). Any of these subpopulations can assemble with approximately two regulators (*top and bottom panels*) and the possibility of forming hybrid proteasome complexes (assembly with two different regulators) (26, 74). Post-translational modifications of proteasome subunits cause further heterogeneity (73, 138, 233). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

We predict that in the future, subpopulation-specific inhibition of proteasomes may enable a more differentiated analysis of the UPS and its role in heart disease.

Proteasome regulators

Prevalent models of proteasomal degradation assign the role of substrate recognition to the 19S regulatory proteasome complex. When 19S and 20S proteasome complexes are assembled in a supercomplex called the 26S proteasome, poly-ubiquitinated substrate proteins are recognized, unfolded, and de-ubiquitinated before proteolysis (Fig. 1) (30). Thus, intracellular accumulation of ubiquitinated proteins in the heart may be associated with insufficient capacity of 26S proteasomes to degrade these proteins (214). Other possibilities are increased protein ubiquitination or decreased de-ubiquitination (10, 219). Inhibitors of substrate recognition and de-ubiquitination by 19S proteasome subunits are tools that are available to investigate the corresponding function (116), but have yet to find their way into cardiac research. Proteolytic activities of 26S proteasomes are ATP dependent, and detergents destabilize 26S complexes (49, 158, 162). For cardiac tissue homogenates, optimal ATP concentrations for assaying 26S proteasomes lie between 10 and 100 μ M, depending on the cytosolic protein amount per assay (167). Beyond this concentration, inhibition by ATP can even be observed in cultured cells (101).

Similar to other tissues and cells, cardiac 19S proteasome complexes consist of at least 19 distinct subunits (74, 213). It should be noted that any 20S proteasome subpopulation can be assembled with 19S proteasome complexes. Additional evidence for proteasome heterogeneity exists in, but is not limited to, the heart due to combinations of one or two 19S regulatory particles with 20S core complexes as well as assembly with PA200 or the 11S regulators (Fig. 3) (26, 74). The latter is composed of a heteromeric complex of PA28 α and PA28 β

subunits or a PA28 γ homomer (51). Functional studies on PA200 and 11S in cardiac tissue have not been performed in detail, but both generally enhance proteasome activities. Two distinct forms of 19S complexes observed in hearts contribute to additional heterogeneity due to an interaction with HSP90 (213). Again, their roles in healthy and diseased myocardium have yet to be elucidated. HSP90 interaction with proteasome complexes is not exclusively limited to the heart (106). In the future, refined studies of proteasome interacting proteins may reveal cardiac-specific interactions (109). Altogether, multiple proteins directly regulate proteasome activities, but knowledge of their therapeutic relevance is limited and research would greatly benefit from the development of corresponding cell-permeable inhibitors. All regulatory proteins investigated thus far in the heart are considered to perform their canonical function known from noncardiac tissue.

Impact of post-translational modifications on proteasome activities

A rapidly expanding, but therapeutically unexplored area of proteasome regulation is the modulation of activities *via* post-translational modifications (PTMs). Numerous PTMs of proteasomes have been reported, but only a few are investigated functionally (33). Multiple phosphorylation sites are found in proteasome complexes (some unique in heart), and their modulation *via* protein kinase A (PKA) stimulates cardiac proteasome activities *in vitro* as well as *in vivo* (5, 49, 73, 138, 232, 233). Conversely, protein phosphatase 2A activity is associated with decreasing cardiac proteasome activities (232). Additional kinases affecting proteasome activities in the heart are PKG and PKN (Fig. 4) (173, 197). In contrast to the stimulating effect of protein phosphorylation on proteasome activities, oxidative modification of proteasome subunits, for example, by 4-hydroxy-2-nonenal (a by-product of lipid peroxidation), seems to be associated with

FIG. 4. Mechanisms for enhancing proteasome activities in the myocardium. Ischemic preconditioning preserves proteasome activities during subsequent ischemia/reperfusion treatments and is associated with reduced infarct size, decreased Bax, PKC δ , and PTEN abundance, as well as less accumulation of ubiquitinated and oxidized proteins (5, 23, 29, 45). Preservation and stimulation of 26S proteasome activities is at least, in part, mediated by PKA (5) and PKC ϵ (29) and protection against reactive oxygen species (ROS) (45). *Stimulation of proteasome activities by PKA is observed in other models as well (49, 183, 230, 232). Cardiac expression of constitutive active PKN enhances 26S proteasome activity and assembly, which is associated with hypertrophy (197). It also protects against ischemia/reperfusion injury by reducing the infarct size. Overexpression of the 11S subunit PA28 α increases the turnover of the proteasome reporter substrate GFP/CL1, potentially *via* increased 11S assembly with 26S proteasomes (132, 133). Genetic proteasome enhancement *via* PA28 α reduces infarct size induced by cardiac ischemia/reperfusion as well (132). In a model of desmin-related cardiomyopathy, PA28 α overexpression enhances lifespan and reduces hypertrophy, abundance of mutated CryAB aggregates, as well as ubiquitinated proteins (132). Similarly, decreased GFP/CL1 abundance after PKG activation *via* sildenafil is associated with reduced hypertrophy, less aggregation of mutated CryAB, and preserved cardiac function in the same DRC model (173). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

reduced proteasome activities (Fig. 2) (21, 58). Whether S-glutathionylation contributes to proteasome inhibition under oxidative stress in the heart, as reported for a noncardiac background (38, 231), remains to be investigated.

Oxidative modification of proteasome subunits is suggested to be a mechanism for reduced capacity of proteasome complexes to degrade substrate proteins during aging, reviewed in (57). Age-dependent impairment of the proteasome may contribute to the increasing incidence of impaired cardiac function at a higher age. However, this hypothesis has yet to be proven. A cardiac disease phenotype particularly impacted by oxidative stress is myocardial infarction (161, 194). After ischemia/reperfusion injury of the heart, reduced proteasome activities are indeed associated with 4-hydroxy-2-nonenal modification and carbonylation of 20S and 19S proteasome subunits, respectively (21, 45). Carbonylation of a 19S subunit is also observed in end-stage human heart failure (HF), (Fig. 2) (170). Whether oxidative modification of proteasome subunits and the concomitant reduction in activities are reversible and interfere with activating PTMs, such as phosphorylation, remains to be investigated. Current studies indicate that modulation of specific PTMs may be a valuable molecular target to enhance proteasome activities (Fig. 4). Moreover, differential occurrence of proteasomal PTMs in cardiac tissue in comparison to other tissues is ob-

served (50, 73, 138), which may enable heart-specific therapeutic approaches.

Ubiquitination and deubiquitination in the myocardium

Ubiquitin requires activation (E1), conjugation (E2), and, finally, ligation (E3) to proteins, before substrate proteins are recognized and degraded by proteasome complexes (Fig. 1) (148). Current knowledge indicates that at least four ubiquitin moieties have to be ligated *via* a specific linkage (mostly Lysine 48) to a substrate to provide a sufficient signal for degradation. Thus, for canonical degradation of UPS substrates, E1, E2, and E3 enzymes are required. More than 600 genes exist, encoding an ubiquitin ligase family member with many of them requiring formation of heteromeric protein complexes to perform substrate-specific ubiquitin ligation (41, 148, 154).

Besides ubiquitin, several ubiquitin-like proteins exist (*e.g.*, SUMO1-3, NEDD8, Atg8, and Atg12), which resemble ubiquitin more in their 3D structure than in their amino-acid sequence (89, 207). In contrast to ubiquitin, they do not participate in the canonical pathway for degradation by the UPS and have different roles in cellular signaling, such as autophagy (Atg8 and Atg12) (98, 207). Still, ubiquitin-like modification impacts proteasomal degradation, for example, by competing for the same lysine (*e.g.*, sumoylation of p53)

FIG. 5. Modulation of cardiac tissue mass via the UPS. In mice subjected to transverse aortic constriction (TAC), knock-out of the E3 ligase MuRF1 promotes cardiac hypertrophy (222). After TAC release, MuRF1 promotes cardiac atrophy in wild-type mice (223). MuRF1 also promotes cardiac atrophy in response to fasting (10). Atrogin-1 overexpression (OE) in the TAC model reduces hypertrophic remodeling (130). Administration of 20S proteasome inhibitors in the TAC model, after β -adrenoreceptor (β -AR) stimulation, or in hypertensive Dahl-salt sensitive rats (DSS), reduces hypertrophic remodeling (40, 85, 144, 193), promotes reverse remodeling (85, 193), and improves cardiac function (85). Modulation of cardiac tissue mass via deubiquitinating enzymes (DUBs) may be possible, but to our knowledge has not yet been reported. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(91). The potential impact of ubiquitin-like proteins on cardiovascular disease is discussed in (12, 91).

It has been shown that muscle-specific proteins, which are a part of E3 ligases, have the potential to influence cardiac tissue mass by targeting specific proteins for proteasomal

degradation (Fig. 5). Atrogin-1/MAFbx is an F-box protein that is a part of an SCF complex with ubiquitin ligase activity, and it is involved in muscle atrophy (Table 1) (16, 76). In the myocardium, Atrogin-1/MAFbx influences calcineurin abundance as well as pathways downstream of Akt and interferes with hypertrophic signaling when being overexpressed (130, 131, 166). Interestingly, Atrogin-1/MAFbx knock-out (KO) interferes with the development of cardiac hypertrophy as well (206), which seems to be mediated, at least in part, by $I\kappa B\alpha$ stabilization and inactivation of NF- κ B (Table 1). Intervention with NF- κ B signaling by itself is sufficient to inhibit cardiac remodeling and hypertrophy (63). Atrogin-1/MAFbx KO also interferes with the development of atrophy of the transplanted, unloaded heart through increased calcineurin activity (9). Furthermore, Atrogin-1/MAFbx enhances ischemia/reperfusion-induced apoptosis through MAPK phosphatase-1 degradation and JNK activation (226).

MuRF1 is one of three MuRFs containing a tripartite RING-B-box:coiled-coil domain and targets Troponin I among others (Table 1) (113). Similar to Atrogin-1/MAFbx, MuRF1 expression has an antihypertrophic effect in mice subjected to aortic banding (Fig. 5) (222). In addition, it promotes atrophic remodeling (223). Furthermore, MuRF1 reduces infarct size after 30 min cardiac ischemia and 24 h reperfusion, at least in part, by targeting phospho-c-Jun for proteasomal degradation and interfering with apoptotic signaling (129). Mice deficient for MuRF1 and MuRF3 develop hypertrophic cardiomyopathy (HCM), which is characterized by subsarcolemmal myosin heavy chain accumulation, as they target the sarcomeric protein for proteasomal degradation (60). Lack of MuRF3 is also associated with cardiac rupture after myocardial infarction (61). Additional ubiquitin ligases implicated in cardiac disease phenotypes are MuRF2, CHIP, and Mdm2 (Table 1) (125, 204, 212, 224).

Enhancement of protein ubiquitination is described in the context of cardiac atrophy (10, 176). Interestingly, two distinct mechanisms seem to be involved. While increases in mRNA and protein abundance of Atrogin-1 and MuRF1 are

TABLE 1. SUBSTRATE PROTEINS AND E3 LIGASES DISCUSSED IN THIS ARTICLE IN THE CONTEXT OF HEART DISEASE

E3 target	Pathway(s)	E3 ^a	Degradation ^b	References
AMPK	Energy metabolism		Suggested	(234)
Calcineurin	NFATc4/hypertrophy	Atrogin-1/MAFbx	X	(130)
Foxo1	Akt/hypertrophy	Atrogin-1/MAFbx		(131)
Foxo3a	Akt/hypertrophy	Atrogin-1/MAFbx		(131)
GRK2/ β ARK1	β -AR/contractility, etc.	MDM2	X	(102, 180, 227, 228)
$I\kappa B\alpha$	NF- κ B/inflammation	SCF/ β TrCP	X	(8, 171, 192)
		Atrogin-1/MAFbx	X	(206)
MAPK-Phosphatase 1	JNK, c-Jun/apoptosis	Atrogin-1/MAFbx	X	(226)
β /slow MHC, MHCIIa	Contractility	MuRF1, MuRF3	X	(32, 60)
p53	Apoptosis	MDM2, CHIP	X	(204, 224)
phospho-c-Jun	JNK/apoptosis	MuRF1	X	(129)
PKC δ	Apoptosis/ROS		X	(29, 202)
PKC ϵ	Cardioprotection/hypertrophy	MuRF1		(4)
PTEN	Apoptosis	NEDD4-1, WWP2	X	(23, 141, 202, 217)
Oxidized proteins	Stress/apoptosis	not required?	X	(45, 46, 133, 169)
Serum response transcription factor	Mechanical inactivity	MuRF2	X	(125)
Troponin I	Contractility	MuRF1	X	(113)

^aUbiquitin ligation is not necessarily demonstrated in cardiac tissue.

^bX marks whether the target protein is subject to proteasomal degradation.

measured in cardiac atrophy induced by nutrient deprivation (10), transcript levels of both E3 ligases are decreased in cardiac atrophy after mechanical unloading by heterotopic transplantation of the rat heart (174, 188). Instead, increased transcript and protein levels of the ubiquitin conjugating enzyme UbcH2 are observed in the latter model (176). Reduced expression is attributed to increased IGF-1 transcript levels, because IGF-1 regulates muscle atrophy-induced ubiquitin ligases *via* FOXO3a (191, 195). In the atrophy model induced by nutrient deprivation, stimulation of Atrogin-1 and MuRF1 expression seems to be mediated by MEF2 after AMPK activation (10). Furthermore, lack of MuRF1 decreases starvation-induced protein degradation and preserves systolic function in fasting mice in comparison to wild-type animals (Fig. 5). AMPK is a cellular sensor for the available energy (83, 229). Although AMPK regulates E3 ligases in rodent hearts (10), a reciprocal regulation of AMPK by the UPS seems to exist as well (Table 1) (234). The feedback between AMPK and the UPS under other metabolic stress conditions, such as cardiac ischemia, has yet to be investigated.

Deubiquitinating enzymes (DUBs) oppose the forces of the E1 to E3 cascade (Figs. 1 and 5) (156, 178). For cardiac tissue, much more than expressional data are rarely available. Recently, the impact of ROS on DUBs came into focus (31), which suggests investigating the impact of DUBs in models of myocardial infarction and other cardiac pathogenesis with increased ROS levels. DUBs belonging to the USP, UCH, OTU, and Josephin subfamilies are cysteine proteases that seem to be sensitive to reversible inhibition by cysteine oxidation (Fig. 2) (123, 127). Inactivation of DUBs by ROS has regulatory consequences on intracellular pathways, such as NF- κ B dependent transcription of pro-inflammatory signaling (55). Thus, inactivation of DUBs *via* ROS may contribute to cardiac remodeling due to inflammation. Within this context, a particular interesting DUB is USP14, which is found in association with 26S proteasome complexes, because its inhibition accelerates the degradation of oxidized proteins and confers resistance to oxidative stress (126). In contrast, USP14/UCHL5 inhibition with b-API5 is associated with increased oxidative as well as endoplasmic reticulum stress and apoptosis (20). Thus, the protective effect of USP14 inhibition described earlier may be dose and inhibitor dependent.

Altogether, there are a multitude of molecular mechanisms by which proteasomal degradation in the heart can be either enhanced or inhibited. In the next sections, hypotheses for interfering with the UPS in cardiac disease phenotypes and corresponding results are discussed in detail. Certainly, vascular disease negatively impacts the heart as well and is, in turn, influenced by the UPS. Proteasome regulation and targeting in vascular disease is the focus of a companion review article of this Forum (221).

Proteasome Function and Intervention in Animal Models with Phenotypes of Heart Disease

Cardiac remodeling and hypertrophy with enhanced proteasome function

Conclusive results indicating that proteasomal intervention may benefit heart disease exist for animal models of early cardiac remodeling and HF (40, 49, 85, 86, 193). In human

heart disease, left ventricular remodeling is induced by several pathologic events, including hypertension, myocardial infarction, or aortic stenosis (88). Contributing to cardiac remodeling is the sympathetic response, which aims at maintaining cardiac output and sufficient perfusion of vital organs (18, 104, 128, 136). In mice mimicking this response by continuous β -adrenergic stimulation for 1 week, all three proteolytic 26S proteasome activities are consistently increased in hypertrophied hearts (+50% HW/BW) by 80%–89% (49). Proteasome inhibition *via* a daily intraperitoneal injection of 1 mg/kg PS-519 during β -adrenergic stimulation for 1 week effectively reduces cardiac remodeling (Fig. 5) (193). In animals with increased HW/BW after 1 week, daily treatment with PS-519 in parallel to continuous β -adrenergic stimulation for another week even promotes regression of cardiac hypertrophy (Fig. 5) (193). Similarly, transverse aortic constriction (TAC) in mice for 5 days to 3 weeks increases the chymotrypsin-like 26S proteasome activity 2.5- to 3.5-fold in hypertrophied hearts (+50%–60% LV/TL) (40, 85). Reduced proteasome activities reported in another study on TAC in mice may indicate that the results are dependent on the murine strain or level of constriction (205). A critical difference between the studies is also the concentration of ATP in proteasome assays, which is 2 mM in the latter study and beyond the recommended concentration for assays of cardiac tissue (see Proteasome Regulators section) (167).

Daily intraperitoneal administration of the proteasome inhibitor epoxomicin (0.5 mg/kg) almost completely blocks the development of cardiac hypertrophy during TAC for 5 days (Fig. 5) (40). Neither proteasome inhibition nor the absence of cardiac hypertrophy seems to have a negative impact on cardiac function. Three weeks after TAC in mice, systolic function is reduced despite increasing gain in left ventricular tissue mass (85). Daily treatment with epoxomicin during the third week not only reduces ventricular tissue mass but also preserves systolic function (Fig. 5) (85). Altogether, current evidence suggests that pharmacologic intervention with enhanced 26S proteasome activities reduces early cardiac remodeling and HF in animal models, but so far, only short-term responses for a maximum of 3 weeks have been monitored. A single long-term study, inhibiting proteasome activities by a daily intraperitoneal injection of 0.1 mg/kg MG132 for approximately 16 weeks after TAC, found attenuated ventricular dysfunction with reduced cardiac fibrosis (140).

Two major aspects seem critical for the positive effect of proteasome inhibition on cardiac remodeling and systolic function: (i) 26S proteasome activities are enhanced and (ii) inhibited submaximally (40, 49, 85, 193). In a long-term study of aortic banding and ensuing cardiac remodeling in dogs (+80% LV/BW), trypsin- (+40%) and chymotrypsin-like 26S proteasome activities (+50%) were increased specifically in the sub-endocardium, but not in the sub-epicardium, even after 2 years (40). The long-term increase of proteasome activities suggests that proteasome inhibition may reduce cardiac hypertrophy even after extended periods of remodeling. Within this context, it should be noted that cardiac function (measured as left ventricular dp/dt) in the canine model was elevated after 2 years of TAC, indicating that the hearts were still in a period of functional compensation (40). In contrast, cardiac remodeling in patients is usually noticed because of symptoms mediated by HF (*e.g.*,

dyspnea, fatigue, or peripheral edema). However, at that time, cardiac 26S proteasome activities may be reduced (170) and it seems that further proteasome inhibition in human HF is counterintuitive (see UPS regulation in human heart disease: a call for action? section).

Recently, a few studies on UPS regulation in animal models of right ventricular remodeling and failure were published (59, 114, 172). Similar to left ventricular remodeling, proteasome inhibition during early pathogenesis reduces hypertrophy (59, 114), whereas enhancing proteasome activities attenuates right ventricular failure (172). Similar and unique patterns in UPS regulation and inhibition in right *versus* left ventricular disease are discussed in a separate article (48).

Mechanism for enhanced proteasome function during cardiac remodeling

The underlying mechanism for increased 26S proteasome activities in cardiac remodeling on β -adrenergic stimulation or aortic banding seems to rest in increased abundance of 19S subunits and their assembly with 20S proteasome complexes into 26S proteasomes (Fig. 6) (40, 49). Sympathetic signaling itself does not alter 26S proteasome assembly and activities within the first 24 h, but it increases the abundance of proteolytic subunits of 20S proteasomes (49). Therefore, it seems unlikely that β -adrenergic signaling has an immediate and direct effect on 26S proteasome function. The acute increase of proteolytic subunits is associated with elevated levels of oxidized (carbonylated) proteins (49), potentially induced by the stimulating effect of sympathetic signaling on heart rate and contractility (Fig. 2). Notably, only inducible 20S proteasome subunits are detected at higher levels in assembled proteasome complexes during the development of cardiac hypertrophy (Fig. 6). After 1 week, increased 26S proteasome assembly and function may be the result of overcompensation in response to reduced caspase-like and trypsin-like 20S proteasome activities (Fig. 6). Both 20S proteasome activities are reduced by 40% after chronic β -adrenergic stimulation, despite 26S proteasome activities being increased by more than 80% (49). The role of reduced 20S proteasome activities in cardiac remodeling is still unknown, but they provide an alternative avenue for pharmaceutical therapy, because the activities can be recovered *via* cAMP- and PKA-dependent signaling (49, 232).

Multiple PKA phosphorylation sites were identified on several 20S proteasome subunits derived from healthy hearts (138, 233). After 1 week of continuous β -adrenergic stimulation, a PKA-dependent rescue is specific for reduced 20S proteasome activities and does not further enhance 26S proteasome activities in hypertrophic hearts (49) (Fig. 6). Chronic β -adrenergic stimulation leads to desensitization of the pathway involving adenylate cyclase and PKA in cardiomyocytes (105, 120, 136), which is considered a fundamental basis for pharmacotherapy of HF with β -blockers. Further studies will show whether the benefit of β -blocker therapy includes recovery of 20S proteasome function and potentially equilibration of 26S proteasome function.

Minimizing adverse effects of proteasome inhibition

Investigators reporting beneficial effects of proteasome inhibition on cardiac remodeling and function emphasize the

FIG. 6. Proteasome regulation during hypertrophic remodeling. At least three mechanisms regulate proteasome activities during cardiac remodeling. I. After continuous β -adrenergic stimulation (β -AR), the composition of 20S proteasome subpopulations is altered, due to increasing incorporation of inducible subunits during proteasome assembly, which changes the ratios of proteolytic activities (49). '...' indicates that additional possibilities for subpopulation assembly exist. II. In addition, a decline in 20S proteasome activities occurs, which can be rescued by cAMP or PKA signaling. At the current state of investigations, it is unknown whether the rescue is mediated by phosphorylation (P^-) of 20S proteasomes or an associating partner (depicted in gray, attached to the 20S proteasome). III. Increased 26S proteasome assembly and activities correlate with decreased abundance of ubiquitinated proteins in hypertrophic hearts after continuous β -AR stimulation (49) and transverse aortic constriction (TAC) (40). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

importance of submaximal reduction of proteasome activities (40, 85, 86, 193). One of the studies pursued a detailed analysis on the degree and type of proteolytic activity that was inhibited by epoxomicin in the myocardium (85). As discussed in the section on 20S proteasome inhibition, with increasing concentrations, proteasome inhibitors have the capacity to effectively block more than one proteolytic activity of proteasome complexes, but generally were designed to block one activity in particular (17, 115). In biochemical studies *in vitro*, epoxomicin primarily targets the chymotrypsin-like proteasome activities and at higher concentrations also the trypsin-like proteasome activities (73, 115, 147). *In vivo*, a daily intraperitoneal injection of 0.5 mg/kg epoxomicin for 1 week inhibits the chymotrypsin-like 26S proteasome activity in hearts by approximately 50% without affecting the trypsin-like 26S proteasome activity (85). No change in TUNEL staining of cardiomyocytes indicates that apoptosis is not stimulated by the treatment. Instead, less TUNEL staining is observed in the myocardium of mice at 3 weeks after banding when they are treated with epoxomicin for the last week.

Avoiding the induction of apoptosis should be a primary concern in heart disease to prevent further loss of heart muscle. Pigs treated twice weekly for a total period of 12 weeks with 0.08 mg/kg of the proteasome inhibitor MLN-273 *via* subcutaneous injections exhibit diastolic dysfunction with reduced cardiac output, which is associated with a marked increase in cardiac fibrosis (93). The authors report a 77% decrease in chymotrypsin-like proteasome activity with a two-fold increase in the abundance of ubiquitinated proteins and a six-fold increase in apoptosis (TUNEL positive cells) after 12 weeks of proteasome inhibition (93). Thus, severe proteasome inhibition is associated with diastolic dysfunction.

At first glance, the administered dose of proteasome inhibitor in pigs seems to be lower than the dose utilized in mice (0.08 mg/kg MLN-273 *vs.* 0.5 mg/kg epoxomicin). However, the two compounds are poorly comparable, because they belong to two distinct substance classes and are distinct in their ability to enter cells or tissue (17). In a previous study, the authors point out that MLN-273 is very closely related to bortezomib and that a subcutaneous dose of 0.08 mg/kg MLN-273 inhibits the chymotrypsin-like proteasomal activity in circulating peripheral mononuclear cells by 68% after 1 h and 40% after 24 h (92). Indeed, both inhibitors belong to the substance class of peptide boronates, but MLN-273 is approximately four-fold more potent than bortezomib (ki 0.15 *vs.* 0.6 nM) (17). Multiple myeloma patients treated with bortezomib receive 1.3 mg/m² of body-surface area, which translates roughly to 0.03 mg/kg for a person being 1.8 m tall at 80 kg with a calculated body surface area of 2 m² (179). Proteasome activity in whole blood after treatment with 1.04 mg/m² bortezomib is inhibited by 60% after 1 h and 25% after 24 h (159). Thus, MLN-273 administered in pigs and bortezomib in humans cause quite similar proteasome inhibition in blood, despite MLN-273 being injected subcutaneously, whereas bortezomib is typically injected intravenously (93, 159). Furthermore, the cumulative dose is regarded as a critical factor in toxicity studies, which would be 1.04 mg/kg over 24 weeks reached in multiple myeloma patients with a body surface area of 2 m² and 1.92 mg/kg over 12 weeks reached in the study using pigs (93, 179).

In humans, pharmacodynamics indicate that bortezomib administration at intervals of 72 h does not show a significant increase of proteasome inhibition in whole blood measured at 1 h after the injection throughout 25 days of therapy (159). As mentioned earlier, chymotrypsin-like proteasome activity in hearts of pigs measured 2 ± 1 days after the final injection of 0.08 mg/kg MLN-273 at the end of a 12-week treatment is reduced by 77% (93) and a similar decrease is seen in coronary arteries (92). The possibility of MLN-273 accumulation in blood cells of the porcine model has not been investigated. Therefore, it is unknown whether the pharmacodynamics of MLN-273 in blood of this animal model correlate with the 77% decrease in proteasome activity seen in cardiovascular tissue.

The discussed studies illustrate the challenges in predicting and adjusting the level of proteasome inhibition in cardiovascular tissue, which appears essential to avoid HF. Submaximal inhibition is reportedly also critical for beneficial effects on vascular disease phenotypes (137, 139, 146, 220).

Biphasic regulation of proteasome activities in HCM

The animal models with cardiac disease phenotypes exhibiting increased 26S proteasome activities discussed so

far represent acquired cardiac disease after reaching adulthood. In humans, genetic predisposition contributes substantially to the development of HF. A fitting example is HCM (64, 142). HCM is characterized by a genetic predisposition for progressive cardiac remodeling, which is associated with mutations in sarcomeric proteins (6, 96). One of the proteins frequently mutated in HCM is cardiac myosin binding protein C (185).

Homozygous knock-in (KI) mice carrying a G > A transition on the last nucleotide of exon 6 of the gene encoding cardiac myosin binding protein Mybpc3 have significantly increased cardiac hypertrophy (+108% LV/BW) and reduced systolic function (22% fractional shortening) at the age of 3 months (208). Within the first 3 months after birth, 26S proteasome activities are consistently higher in hypertrophic hearts of Mybpc3 KI compared with those of wild-type mice (184). Furthermore, proteasome function positively correlates with increasing ratios of heart weight to body weight (HW/BW) from week 9 to 13 only in Mybpc3 KI mice. In contrast, chymotrypsin-like 26S proteasome activity is approximately 25% lower in Mybpc3 KI than in wild-type mice by the age of 1 year (184). Similarly, HCM patients, with the majority of them carrying Mybpc3 mutations, have reduced cardiac chymotrypsin-like 26S proteasome activity at an average age of 37 years in comparison to a control group with an average age of 59 years (170). Proteasome activities of HCM patients in early adulthood have not been analyzed so far. However, mutations of *TRIM63*, which encodes MuRF1, have been identified in patients with HCM, impart loss-of-function effects on E3-ligase activity, and are probably causal mutations in HCM (28). Overexpression of mutated Mybpc3 seems to possess cytotoxic potential, in part by reducing proteasome activity (Fig. 2) (7, 182).

The early rise and late decline of proteasome activities in Mybpc3 KI mice suggests that proteasome impairment is not part of the primary mechanism for disease development in this model of HCM (184). Thus, proteasome activities during an early stage of HCM are elevated in a similar manner as in cardiac hypertrophy induced by β -adrenergic stimulation or aortic banding (40, 49, 85). It would be highly interesting to restore 26S proteasome activities in the 3-month-old Mybpc3 KI mice to the level observed in wild-type mice and to determine whether it reduces cardiac hypertrophy or preserves systolic function as observed after acute remodeling (40, 85, 193). Within this context, it is noteworthy that chronic hypertrophy induced by another genetic background is successfully reduced *via* proteasome inhibition by 50% (86). In this model, transgenic mice overexpressing the chaperone H11 Kinase/Hsp22 show approximately a 25% increase in cardiac mass and a two-fold increase in chymotrypsin-like 26S activity (86). A daily intraperitoneal injection of 0.5 mg/kg epoxomicin for 7 consecutive days reduces cardiac hypertrophy by 50% without observable effects on wild-type animals. Similar effects are observed using lactacystin as an alternative proteasome inhibitor. With increasing age of the animals, the HSP22 model is distinct from the Mybpc3 KI phenotype, because proteasome activity remains increased and systolic function is not altered (39, 86).

Overall, proteasome activities in HCM of young animals are consistently enhanced and proteasome inhibition may improve cardiac function. In contrast, NYHA class III and IV patients with HCM show reduced proteasome activities and

accumulate ubiquitinated proteins in cardiac tissue (170). Therefore, proteasome inhibition is counterintuitive in human HF.

Proteasome inhibition during ischemia and reperfusion of the heart

In scenarios of cardiac disease with decreased proteasome function and perhaps increased abundance of ubiquitinated proteins, proteasome inhibition seems an unlikely therapeutic option to restore cardiac function. Still, proteasome inhibition is associated with beneficial effects even under conditions of decreased proteasome function, for example, after myocardial ischemia/reperfusion injury. Isolated perfused rat hearts exhibit preserved left ventricular developed pressure and less polymorphonuclear leukocyte accumulation in coronary vessels after 20 min of ischemia followed by 45 min of reperfusion in a dose-dependent manner of the proteasome inhibitor PS-519 (25). Proteasome activities were not provided in this study, but the chymotrypsin-like 20S as well as 26S activities are decreased in a similar study applying 30 min of ischemia and 60 min of reperfusion in the absence of leukocytes (169). However, proteasome inhibition with MG132 reduces cardiac function (heart rate $\times$ pressure product) in that study. Administration of different proteasome inhibitors is discussed as a potential reason for the disparate outcome of the two studies (24) and may indicate differential targeting of proteasome subpopulations (50, 117). A heterogenic group of proteasome subpopulations exists in the myocardium, which have distinct biochemical properties from those in other tissues and immune cells (see 20S proteasome inhibition section and Fig. 3).

A study focusing on the role of $\beta 5$ -specific proteasome subpopulations in cardiomyocytes in ischemia/reperfusion injury shows exacerbated structural and functional damage in transgenic mice overexpressing catalytically inactive $\beta 5$ (myc-T60A- $\beta 5$) under an mhc6 promoter (202). Under baseline conditions, T60A- $\beta 5$ overexpression results in approximately 60% decrease of myocardial chymotrypsin-like proteasome activity without changes in the abundance of ubiquitinated proteins, suggesting that $\beta 5$ -dependent activity is either excessive or not essential for the UPS under unchallenged conditions. In contrast, the abundance of ubiquitinated proteins is increased by approximately two-fold in transgenic hearts subjected to 30 min of ischemia and 24 h of reperfusion, suggesting that reducing the chymotrypsin-like proteasome activity by 60% is inadequate to maintain degradation *via* the UPS in the stressed myocardium (202).

Another critical difference between the studies discussed concerns the duration of ischemia and perfusion, because 15 min of ischemia has no significant effect on the chymotrypsin-like 20S proteasome activity, even when followed by 60 min of reperfusion (169). A suggested mechanism for decreased proteasome activities during ischemia/reperfusion is deactivation through ROS *via* 4-hydroxy-2-nonenal modification and carbonylation of 20S and 19S proteasome subunits, respectively (Fig. 2, see impact of PTMs on proteasome activities section) (21, 45, 58). Consequently, accumulation of oxidized (carbonylated) proteins correlates with proteasome function (46, 169).

In vivo, coronary administration of 1 mg/kg PS-519 in pigs before 1 h of ischemia and 3 h of reperfusion confers higher

segmental shortening in the infarct region than in control animals (171). Proteasome inhibition reduces the binding of nuclear NF- κ B to DNA, which is suggested as a protective mechanism. NF- κ B translocation to the nucleus occurs in the absence of I κ B α -binding, which is a proteasomal substrate (Table 1) (84). After translocation, NF- κ B acts as a transcription factor, promoting not only the synthesis of proteins participating in inflammation, but also cell survival, differentiation, and proliferation (84). Reduced I κ B α degradation after 30 min of ischemia and 24 h of reperfusion is reported after proteasome inhibition by an intramyocardial injection of 10 nmol/kg PR-11 or PR-39 in rats, which is associated with reduced infarct size and improved systolic function (8). Similarly, proteasome inhibition by an intraperitoneal injection of 1 mg/kg PS-519 in mice reduces I κ B α degradation after 30 min of ischemia and 1 h of reperfusion, which correlates with reduced infarct size and preserved systolic function after 30 min of ischemia and 24 h of reperfusion (192).

Besides preventing NF- κ B translocation, alternative protective mechanisms against ischemia/reperfusion injury are suggested for proteasome inhibition and include prevention of GRK2 degradation, which is associated with suppressing malignant tachyarrhythmias and sudden cardiac death (102, 227, 228). GRK2 degradation is reduced by intravenous administration of 0.0875 mg/kg bortezomib in dogs and depends on the cardiac region, indicating differential proteasome activities or GRK2 targeting by a ubiquitin ligase in the epicardial border zone *versus* the infarcted tissue (102). GRK2 is also known as β ARK1, a proteasome substrate that is involved in beta-adrenergic receptor signaling (Table 1) (105).

Altogether, the outcome of proteasome inhibition during ischemia/reperfusion seems to depend on the duration of ischemia, the type of inhibitor, and likely the inhibitor concentration reached in inflammatory cells and cardiac tissue. Inhibition of inflammation *via* NF- κ B signaling and preservation of beta-adrenergic receptor signaling *via* disruption of GRK2 degradation are suggested pathways supporting proteasome inhibition during cardiac ischemia/reperfusion.

Preservation and stimulation of proteasome function by ischemic preconditioning

Animal models of cardiac ischemia and reperfusion indicate a reduction of proteasome activities and accumulation of ubiquitinated proteins in cardiac tissue (2, 5, 21, 45). Consequently, the question arises as to whether preservation or enhancement of proteasome activities during or before cardiac ischemia/reperfusion may be cardioprotective. Cardioprotection against ischemia/reperfusion injury is an intensely investigated concept that was initially discovered by introducing brief ischemic insults before the main ischemic/reperfusion period and generally called ischemic preconditioning (152). Even today, the underlying mechanisms of ischemic preconditioning are not completely understood. Several studies suggest that the UPS plays an essential role in ischemic preconditioning (5, 29, 45, 168).

Four ischemic insults of 5 min increase the chymotrypsin-like 26S proteasome activity by approximately 50% in a canine model (Fig. 4) (5). Although several studies using proteasome inhibitors in ischemia/reperfusion models suggest that proteasome inhibition may have cardioprotective

potential against ischemia/reperfusion injury, the enhancement of proteasome activities by ischemic preconditioning is associated with reduced infarct size as well (5). Furthermore, proteasome inhibition by an intraperitoneal injection of 2.5 $\mu\text{g/kg}$ epoxomicin during ischemic preconditioning abrogates the cardioprotective effect against 90 min of ischemia/6 h of reperfusion and the infarct size is then comparable to control animals. As a mechanism for increased 26S proteasome activity, increased 26S proteasome assembly *via* PKA-dependent phosphorylation is suggested, because PKA stimulators mimic and the PKA inhibitor H-89 blunts the activating effect of ischemic preconditioning on proteasome complexes (5). In previous studies on noncardiac proteasomes, Rpt6 phosphorylation *via* PKA is suggested to be associated with the stability of 26S proteasome complexes (183, 230). In addition, modulation of cardiac 20S proteasome activities by exogenous and endogenous PKA has been demonstrated (49, 232).

Ischemic preconditioning also prevents the loss of chymotrypsin-like 26S proteasome activity as seen in isolated rat hearts (29). Activity of the 26S proteasome is linked to PKC ϵ (Fig. 4), as suggested by the observation that a PKC ϵ inhibitor disrupts the stimulating effect on 26S proteasomes by ischemic preconditioning and a PKC ϵ activator enhances 26S proteasome activity after 30 min of ischemia. PKC ϵ signaling regulates multiple pathways, which are critical for ischemic preconditioning (163–165). Proteasome inhibition by 20 μM lactacystin interferes with the positive effects of ischemic preconditioning, including decreasing levels of PKC δ (29). PKC δ is a proteasomal substrate and is considered a pro-death kinase (Table 1) (53). Both PKC δ and PKC ϵ are associated with ROS-dependent signaling (107, 110). Taken together, the UPS provides a pathway for regulating the ratio between PKC ϵ and PKC δ under oxidative stress, as observed in ischemia/reperfusion injury.

Preservation of chymotrypsin-like 26S proteasome activity after 30 min of ischemia and 60 min of reperfusion is also achieved by three cycles of 3 min of ischemia followed by 2 min of reperfusion in isolated perfused hearts of mice (45). The same protocol of ischemic preconditioning protects against 30 min of ischemia and 3 h of reperfusion injury *in vivo*, while maintaining the chymotrypsin-like 26S proteasome activity at the level of sham-treated animals. Protection against accumulation of ubiquitinated, oxidized (carbonylated) proteins, as well as the proapoptotic protein Bax, is disrupted by 2 μmol lactacystin and is suggested as a mechanism (Table 1) (45). Remarkably, the authors imply that Rpt5 itself is subject to increased protein carbonylation after ischemia/reperfusion injury and that ischemic preconditioning protects Rpt5 against this modification (Fig. 4). The mechanisms for Rpt5 oxidation as well as for its precise impact on processing proteasome substrates remain elusive.

In noncardiac cells, oxidative stress induced by hydrogen peroxide exposure is associated with temporary reduction of 26S proteasome activities and dissociation of 20S core particles from 19S regulatory particles of 26S proteasomes (79, 177, 218). Since 20S proteasomes degrade oxidized proteins without prior ubiquitination (see 20S proteasome inhibition section and Fig. 2) (80, 189), increasing the pool of free 20S proteasomes was suggested to be important for cellular recovery from oxidative stress despite ubiquitinated proteins accumulating due to reduced 26S proteasome activities (79,

218). It remains to be investigated whether enhancing only 20S proteasome activities is sufficient to protect against ischemia/reperfusion injury. So far, the current state of research suggests that preserving or enhancing 26S proteasome activities is associated with reduced infarct size (Fig. 4). In general, oxidative modification of proteasome complexes seems to restrict their capability to degrade proteins, although protein oxidation itself seems to promote protein degradation (Fig. 2) (21, 57, 108).

A novel aspect for the role of proteasome composition in ischemic preconditioning was introduced by examining the latter in mice lacking the inducible proteasome subunit $\beta 1i$ (23). As discussed in the section on 20S proteasome inhibition, 20S proteasome complexes can incorporate different proteolytic subunits during assembly and reportedly exist as subpopulations with distinct proteolytic function in the mammalian heart (50). The role of 20S proteasome subpopulations in the heart has yet to be determined. In the hearts of $\beta 1i$ KO mice, reduced expression and incorporation of $\beta 2i$ and $\beta 5i$ in proteasome complexes is observed as well and is associated with reduced trypsin- and chymotrypsin-like 20S activities, whereas the corresponding caspase-like activity is increased (23). Interestingly, the functional difference of proteasome complexes in $\beta 1i$ KO mice seems to be organ dependent, because different activity ratios are reported for brain and liver tissue (44). Ischemic preconditioning of mice lacking $\beta 1i$ (10 min of ischemia and 5 min of reperfusion before 30 min of ischemia and 90 min of reperfusion) neither reduces infarct size nor preserves cardiac function (23). Preconditioned wild-type hearts exhibit similarly reduced trypsin- and chymotrypsin-like 20S proteasome activities as preconditioned $\beta 1i$ KO hearts after ischemia/reperfusion injury. In contrast, caspase-like 20S proteasome activity is increased in hearts of wild-type animals, but reduced in hearts of $\beta 1i$ KO mice, although $\beta 1i$ incorporation is associated with increased chymotrypsin-like and decreased caspase-like activity (see 20S proteasome inhibition section) (68, 97, 103). Impaired proteasomal degradation of PTEN has been suggested as a mechanism for the lack of cardioprotection against ischemia/reperfusion injury in $\beta 1i$ KO mice (23). PTEN is ascribed a role in ischemic preconditioning and cardiac survival and is reportedly subject to proteasome degradation (Table 1) (22, 187, 203).

The current state of investigations regarding proteasome activities and ischemic preconditioning (i) emphasizes that the UPS plays an important role in cardioprotection and (ii) reveals mechanisms for the preservation or activation of proteasome activities in the heart. Several mechanisms have been proposed to regulate proteasome activities during ischemic preconditioning, but further studies are needed to determine their therapeutic potential.

Enhancing cardiac proteasome activities by genetic engineering

Besides infarcted hearts, established cardiomyopathies are associated with reduced proteasome activities in comparison to corresponding controls (170, 184). Furthermore, the capacity of 26S proteasomes to degrade ubiquitinated proteins in a murine model of desmin-related cardiomyopathy (DRC) seems insufficient potentially due to reduced 19S proteasome subunit abundance, despite 20S proteasome activities being increased

(134). Under these conditions, enhancing proteasome activities for a longer period than achieved by ischemic preconditioning may appear to be a reasonable aim. From a cell biological perspective, the 19S proteasome complex activates 20S proteasomes and enables degradation of polyubiquitinated proteins. However, stimulating 26S proteasome assembly seems impractical due to the large number of proteins required for complete assembly (Fig. 1) as well as due to the potential disturbance of the protein homeostasis (see proteasome regulators section). Other activators include the 11S complex and PA200 (Fig. 3). The precise cellular role of both activators in the myocardium is still unknown, but their assembly is less laborious than 19S proteasome assembly. Overexpressing the 11S subunit PA28 α in hearts of transgenic mice increases PA28 β abundance as well and decreases an *in vivo* reporter substrate of proteasomes [GFP with CL1 degron (13, 124)] by 80% (132). Subjecting the PA28 α transgenic animals to 30 min of ischemia followed by 24 h of reperfusion reduces the infarct size more than two-fold (Fig. 4) (132). Cardiac function measured at 30 and 45 min after reperfusion is significantly improved as well. Oxidative stress is a major cause of ischemia/reperfusion injury (Fig. 2; see the two previous sections on ischemia, reperfusion and preconditioning) (161, 194). In cultured cardiomyocytes, adenoviral PA28 α overexpression reduces hydrogen peroxide-induced apoptosis in TUNEL and DNA laddering assays (133). The protective effect is associated with increased caspase- and chymotrypsin-like 26S proteasome activities after PA28 α overexpression and reduced abundance of carbonylated proteins after oxidative stress, which suggests that PA28 α overexpression improves degradation of oxidized proteins *via* increasing proteasome activities (Table 1) (133).

In addition to its protective effect against ischemia/reperfusion injury and accumulation of oxidized proteins, PA28 α overexpression attenuates hypertrophy and extends the lifespan of transgenic mice harboring DRC (Fig. 4) (132). Desmin is an intermediate filament protein located in proximity to the Z-disk of cardiomyocytes. Mutations in desmin or associated proteins, such as CryAB, are found in human cardiomyopathy (72, 201). Among others, the cardiac disease phenotype is characterized by misfolded protein aggregation (Fig. 2) (143, 215, 216). Accumulation of aberrant intracellular proteins is inherent with increased abundance of ubiquitinated proteins in DRC mouse models expressing desmin with a 7-amino-acid (R172 through E178) deletion or CryAB with a R120G missense mutation (27, 134). Furthermore, mutated desmin or CryAB impairs degradation of an *in vivo* reporter substrate [GFP with CL1 degron (13, 124)], although 20S proteasome activities are increased (27, 134). Degradation of the reporter substrate requires prior ubiquitination (70, 149) and may be influenced by reduced ubiquitin-ligation, for example, by reduced bioavailability of ubiquitin or E3 ligase activity. In isolated cardiomyocytes, mutated desmin inhibits the UPS in a dose-dependent manner (Fig. 2) (135). In transgenic mice harboring the CryAB R120G missense mutation, PA28 α overexpression reduces the abundance of ubiquitinated proteins and mutated CryAB aggregates in DRC hearts (132). Similarly, autophagy is attenuated in mice with CryAB R120G mutation and enhancing the levels of autophagy ameliorates DRC (14). Reduced function of both autophagy and UPS in the murine DRC model indicates severe impairment of protein degradation, leading to proteotoxicity, which may end in impaired cardio-

myocyte function and cell death (181). Although protein aggregation seems to impair proteasome activities, amelioration of DRC by enhancing proteasomal degradation suggests that the level of proteotoxicity is dependent on the ratio of damaged proteins to their degradation.

Expression of a constitutive active form of PKN in the myocardium of transgenic mice enhances the chymotrypsin-like 26S proteasome activity as well (Fig. 4) (197). Transgenic PKN mice develop cardiac hypertrophy with unaltered left ventricular function. They show reduced infarct size after 45 min of ischemia and 24 h of reperfusion, again with higher chymotrypsin-like 26S proteasome activity compared with nontransgenic animals. Blocking proteasome activity by an intraperitoneal injection of 0.5 mg/kg epoxomicin partially interferes with the cardioprotective effect of PKN. Conversely, transgenic mice expressing a dominant negative form of PKN in the myocardium show increased infarct size with reduced chymotrypsin-like 26S proteasome activity (197). The mechanism for enhancing proteasome activity by PKN remains to be investigated. However, activation of CryAB in PKN transgenic mice provides a link to investigations in DRC, suggesting that a CryAB missense mutation overloads the capacity of regular UPS function (27). Thus, activation of CryAB may relieve the workload of the UPS by its chaperone activity (19).

Altogether, the results suggest that genetic enhancement of proteasome activities protects against ischemia/reperfusion injury as well as DRC. The cardioprotective effect is inherent with improved degradation of oxidized and misfolded proteins without disturbing protein homeostasis (Fig. 2).

UPS Regulation in Human Heart Disease: A Call for Action?

At the current stage of investigation, HF in human patients is associated with markedly reduced proteasome function (Fig. 7). In HF patients, on average 10 years after first diagnosis and with severe systolic dysfunction (15% ejection fraction [EF]), ventricular caspase- and chymotrypsin-like 26S proteasome activities are decreased by 40% compared with those in nonfailing hearts (170). The authors associate the reduced activities with proteasome dysfunction, because significant accumulation of ubiquitinated proteins is observed in ventricular extracts of failing hearts. Twelve times more ubiquitin-positive myocytes are visible in immunostained sections in the left ventricular septum of patients with aortic stenosis and 24% EF compared with those with normal EF averaging at 59% (87). Proteasome activities were not measured. At intermediate pathogenesis with a significant increase in myocyte cross-sectional area and 41% EF, the number of ubiquitin positive myocytes is only two-fold higher, indicating a progressive inverse relation of ubiquitin abundance and systolic function (87). Notably, the abundance of ubiquitinated proteins in ventricular extracts of patients with HCM and increased EF is not significantly different from those in nonfailing hearts, while the caspase- and chymotrypsin-like 26S proteasome activities are similarly decreased as in failing hearts (170). The authors speculate that the duration of proteasome dysfunction may contribute to the different result, which appears plausible when considering the time since diagnosis of the symptomatic disease (5 years at average in HCM *versus* 10 years in HF

FIG. 7. Coupling of cardiac function and the UPS in human cardiac disease. Several investigations report increased abundance of ubiquitinated proteins in human heart disease (87, 170, 205, 219, 225). This is paralleled by increased abundance of a deubiquitinating enzyme in dilated cardiomyopathy (219), decreased 20S proteasome subunit abundance in congestive heart failure (225), decreased assembly of 19S with 20S proteasomes (37), and decreased 26S proteasome activities in hypertrophic cardiomyopathy and heart failure (170). Ventricular unloading after implantation of left ventricular assist devices decreases the abundance of ubiquitinated proteins (112, 225), and stimulates the expression of proteasome subunits (225) as well as proteasome activities (112, 170). Therapeutic proteasome inhibition in patients with multiple myeloma (MM) is associated with the onset and deterioration of cardiac disease in several reports (15, 36, 56, 62, 81, 82, 198, 211), which is reversible after discontinuation of the therapy (15, 81, 82, 211). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

patients). Alternatively, the findings could be interpreted in a way that the abundance of ubiquitinated proteins and proteasome function are not strictly correlated in heart disease and are independently regulated. Comparing the studies on HCM and HF with those on aortic stenosis, another significant difference becomes apparent: With an average of approximately 70 years, the patients in the latter study were 15–20 years older than those in the former. Decreased proteasome activities associated with oxidative damage are observed during aging (35, 57). Thus, aging may play an important role in the susceptibility of the heart to accumulate ubiquitin and its conjugates.

Further evidence that ubiquitinated proteins accumulate in human heart disease is reported for ischemic heart disease (2.5-fold) and dilated cardiomyopathy (DCM; five-fold), (Fig. 7) (205, 219). Not measuring proteasome activities, this phenomenon was interpreted as hyperubiquitination, suggesting increased E3 ligase activities (219). By analyzing the cardiac proteome, the authors identified an 8.5-fold increased abundance of ubiquitin carboxyl-terminal hydrolase (UCH) in DCM samples. UCH is one of approximately 80–90 DUBs that catalytically counteracts the function of E3 ligases and potentially participates in cleaving translated polyubiquitin chains into single ubiquitins (190). Thus, increased UCH abundance in DCM would increase the availability of ubiquitin and may be considered a response to cellular ubiquitin shortage. Since cardiac E3 ligases and DUBs as well as their targets and signaling function are not yet systematically studied, interpretation of their regulation remains speculative.

Studies monitoring proteasome function or ubiquitin accumulation during cardiac disease progression in humans are

usually limited by sampling of a single biopsy in a patient's life. One exception is paired sampling before and after implantation of ventricular assist devices as a bridge to transplantation. At the time of organ transplantation, the explanted heart allows for an additional sample from the same patient. During the period carrying a ventricular assist device, the myocardial structure and function partially recovers due to ventricular unloading (175). After ventricular unloading for an average of 214 days in patients with idiopathic DCM, myocardial chymotrypsin-like 20S proteasome activity is significantly increased (Fig. 7) (112). Similarly, the chymotrypsin-like 26S proteasome activity is increased by approximately 50% in HF patients after an average of 31 weeks postventricular unloading (170). The abundance of ubiquitinated proteins seems to be increased while protein carbonylation is decreased after ventricular unloading, but both are not significant (112, 170). Another study associated ventricular unloading for an average of 158 days in congestive HF patients with increased 20S proteasome subunit and decreased ubiquitin immuno-staining in sarcoplasm of cardiomyocytes (225). Altogether, the studies indicate that proteasome activities reflect some remission of HF, but are not necessarily tightly associated with protein ubiquitination.

The mechanism for reduced proteasome function in cardiac disease is either dependent on the etiology or heterogeneous in its final stage. In HF, reduced caspase- and chymotrypsin-like 26S activities are suggested to result in part from oxidation of the 19S proteasome subunit Rpt5 (Fig. 2) (170). No differences in the abundance of 20S proteasome subunit $\alpha 7$, 19S subunits Rpt1, 3, 4 as well as Rpn10, and 11S subunit PA28 α are observed between nonfailing, HCM, and

failing hearts (170). In contrast, reduced 20S proteasome subunit abundance is reported in paraffin-embedded myocardial sections of patients with congestive HF (225). More recently, reduced 19S particle docking to 20S proteasomes potentially due to reduced $\alpha 7$ phosphorylation was suggested to contribute to reduced 26S proteasome activities in HF (37). Although the results were published in two separate studies, reduced proteasome function in HF may be caused by a series of events affecting PTMs. For example, carbonylation of Rpt5 may interfere with proper phosphorylation of $\alpha 7$, which, in turn, impacts 19S with 20S proteasomal docking.

Identification of the mechanisms for reduced proteasome function in human cardiac disease will be fundamental for deciding on an optimal strategy for targeting the UPS by cardiologists on the one hand, as well as oncologists and immunologists on the other (see also next section).

Cardiac Dysfunction in Patients Undergoing Cancer Chemotherapy: Prevention Versus Eradication

The prevention of cardiac dysfunction and effective cancer chemotherapy become intertwined when proteasome (dys-)regulation is considered a cause for HF, while proteasomes are regarded effective targets in the treatment of cancer. Therefore, when considering proteasome complexes as therapeutic targets in cardiac disease, a very important aspect is the fact that proteasome inhibition is a Food and Drug Administration (FDA) approved strategy to treat multiple myeloma in patients (90, 111). Within this context, the question arises as to whether cancer patients treated with proteasome inhibitors experience a greater predisposition for cardiac dysfunction than others.

In the medical review of the proteasome inhibitor bortezomib issued by the FDA, it is noted that doses of 3.0 mg/m^2 cause acute cardiovascular mortality in monkeys, which is, however, not observed in humans (1). Subsequently, a clinical trial was published, implicating bortezomib treatments in three deaths from cardiac causes (179). According to the trial, the incidence of heart disease during treatment with bortezomib is 15% ($n=331$). A few case reports indicate that systemic treatment with bortezomib is associated with acute HF (EF of 10%–25%) (15, 82, 211). The authors of the reports suggest a causative role for bortezomib in these cases because of two factors: (i) HF is observed after two to four treatment cycles (6–12 weeks), and (ii) the EF increased by 10%–35% within 6–12 months after discontinuing the chemotherapy (Fig. 7). Thus, the reports show that onset and recovery of HF are associated with proteasome inhibition.

Further case reports associating the pathogenesis of cardiac disorders with bortezomib treatment include diagnosed left ventricular midwall fibrosis (62), acute myocardial infarction (198), and bradycardia due to complete atrioventricular block (36). One factor impeding the establishment of a coherent link between cardiac disease and proteasome inhibition is that multiple myeloma patients previously received other chemotherapeutics or are on combination therapy. Furthermore, adverse events of chemotherapy due to anemia, such as dyspnea or fatigue, overlap with symptoms of HF (90, 179). Thus, diagnosis of developing HF in bortezomib-treated patients may be delayed.

A medical history of cardiovascular disease, such as hypertension, may preclude multiple myeloma patients from

bortezomib treatment (15, 81). However, screening for clinical features of cardiac disease may be insufficient for risk stratification before commencement of bortezomib therapy, as increased predisposition through subclinical cardiomyopathy and UPS dysregulation was suggested (211). A study emphasizing absence of cardiovascular abnormalities before bortezomib treatment reports 8 of 69 patients developing cardiac complications with half of them having reduced systolic function after a minimum of four treatment cycles (EF of 20%–35%) (56). Notably, all affected patients in the study were older than 60 years. It is established that the incidence of HF increases with age, but here it is additionally associated with proteasome inhibition. Further investigation will be necessary to demonstrate whether the (in-)ability of the UPS to maintain its proper function during aging may contribute to the increased predisposition.

The remission of HF associated with the discontinuation of proteasome inhibition, described in several case reports (15, 81, 82, 211), mirrors the recovery of proteasome activity through ventricular unloading discussed in the previous section (112, 170). Altogether, the results discussed from proteasome inhibition in anti-cancer therapy support the hypothesis that proteasome activity and cardiac function are linked.

Current research and development in multiple myeloma treatment based on proteasome inhibition are directed toward overcoming the acquired resistance against bortezomib (90). Since the impact of proteasome inhibition on the heart is still not conclusive, adverse events affecting cardiac function are carefully monitored (15, 52, 99). In a clinical trial of the second-generation proteasome inhibitor carfilzomib, HF occurred in 7% of 526 patients and several deaths were caused by cardiac events within hours or a few days after administration (90). More profound knowledge on the adverse effects of pharmaceutical therapies is gained with increasing number of treatments after FDA approval as the aforementioned reports on the cardiotoxicity of bortezomib indicate. Since carfilzomib was approved in 2012, the comparison of its cardiotoxic potential is based on a limited number of recent clinical trials, which reported similar adverse effects while partially overcoming bortezomib resistance (90). Currently, four additional proteasome inhibitors are in clinical trials and the effectiveness of proteasome inhibition is tested in other types of cancers and autoimmune diseases, including those affecting the heart (52, 69, 116). For bortezomib alone, about 700 clinical trials are registered in the NIH database clinicaltrials.gov with 186 listed as open studies and 159 of them currently recruiting patients (July 2014). Therefore, there is urgent need for a better understanding of (i) the association of proteasome targeted cancer chemotherapy with adverse cardiac events and (ii) the role of the UPS in the pathogenesis of heart disease.

Conclusion

After a decade of research on the UPS in the heart, there is no doubt that the UPS plays a critical role in adaptation and maladaptation of the myocardium to pathologic and oxidative stress. Manipulation of the UPS has the potential to improve cardiac function. At the current state of investigations, there is still too little known to intervene with UPS function in human heart disease, mainly because the deviations of UPS expression and activities in diseased hearts are not fully

understood. Early cardiac disease and remodeling seem to be associated with enhanced proteasome activities, whereas decreased proteasome function and accumulation of ubiquitinated as well as oxidized proteins, including proteasome subunit oxidation, is observed in HF. The early rise in proteasome function is absent in acute ischemia/reperfusion injury. Investigators are just beginning to understand whether a gain or loss of proteasome activities is beneficial or detrimental for cardiac function. During early cardiac remodeling, inhibition of proteasome function alleviates the development of pathological hypertrophy. In contrast, accumulation of ubiquitinated and oxidized proteins in chronic cardiac disease phenotypes is counteracted by enhancing proteasome activities, which is accompanied by improved cardiac function. In models of acute myocardial ischemia/reperfusion injury, proteasome enhancement reduces ROS-induced apoptosis in cardiomyocytes, whereas proteasome inhibition reduces leukocyte accumulation. At the current stage, long-term studies of UPS manipulation in heart are required to demonstrate whether the ameliorating effect on cardiac function reported in short-term studies prevails. Targeting proteasomal degradation is clinically performed *via* a growing repertoire of FDA-approved proteasome inhibitors. With regard to their potential to target proteasome complexes differentially, the dynamics and impact of the heterogeneity in proteasome complexes remain to be investigated. Besides using genetic approaches, enhancement may be achieved by modulating PTMs of proteasomes, for example *via* PKA. Furthermore, intervention at the level of E3 enzymes and DUBs may be utilized to promote or prevent proteasomal degradation of substrate, damaged, and oxidized proteins. Last but not the least, in light of an increasing number of clinical trials evaluating novel applications for proteasome inhibitors as therapeutics against cancer and autoimmune disease, full knowledge about their impact on the heart is urgently needed to minimize any adverse effects.

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Abbreviations Used

β -AR	= β -adrenoreceptor
DCM	= dilated cardiomyopathy
DRC	= desmin-related cardiomyopathy
DSS	= dahl-salt sensitive
DUB	= deubiquitinating enzyme
EF	= ejection fraction
FDA	= Food and Drug Administration
FS	= fractional shortening
HCM	= hypertrophic cardiomyopathy
HF	= heart failure
HW/BW	= heart weight to body weight ratio
KI	= knock-in
KO	= knock-out
LV/TL	= left ventricle to tibia length
NYHA	= New York Heart Academy
OE	= overexpression
PKA	= protein kinase A
PTM	= posttranslational modification
ROS	= reactive oxygen species
TAC	= transverse aortic constriction
UCH	= carboxyl-terminal hydrolase
UPS	= ubiquitin-proteasome system