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Invited Review

The ubiquitin-proteasome system in cardiac physiology and pathology

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Powell, Saul R. The ubiquitin-proteasome system in cardiac physiology and pathology. *Am J Physiol Heart Circ Physiol* 291: H1–H19, 2006; doi:10.1152/ajpheart.00062.2006.—The ubiquitin-proteasome system (UPS) is the major nonlysosomal pathway for intracellular protein degradation, generally requiring a covalent linkage of one or more chains of polyubiquitins to the protein intended for degradation. It has become clear that the UPS plays major roles in regulating many cellular processes, including the cell cycle, immune responses, apoptosis, cell signaling, and protein turnover under normal and pathological conditions, as well as in protein quality control by removal of damaged, oxidized, and/or misfolded proteins. This review will present an overview of the structure, biochemistry, and physiology of the UPS with emphasis on its role in the heart, if known. In addition, evidence will be presented supporting the role of certain muscle-specific ubiquitin protein ligases, key regulatory components of the UPS, in regulation of sarcomere protein turnover and cardiomyocyte size and how this might play a role in induction of the hypertrophic phenotype. Moreover, this review will present the evidence suggesting that proteasomal dysfunction may play a role in cardiac pathologies such as myocardial ischemia, congestive heart failure, and myofilament-related and idiopathic-dilated cardiomyopathies, as well as cardiomyocyte loss in the aging heart. Finally, certain pitfalls of proteasome studies will be described with the intent of providing investigators with enough information to avoid these problems. This review should provide current investigators in the field with an up-to-date analysis of the literature and at the same time provide an impetus for new investigators to enter this important and rapidly changing area of research.

heart; protein degradation

CIECHANOVER, HOD, AND HERSHKO (37) presented in 1978 the first description of a heat-stable polypeptide that associated with an ATP-dependent proteolytic system in reticulocytes that had been previously described by Etlinger and Goldberg (57) in 1977. Initially, this polypeptide was called ATP-dependent proteolysis factor-1 (APF-1), but it was subsequently identified as ubiquitin by Wilkinson and colleagues (226). In the following year, Rose, Warms, and Hershko (182) made the seminal discovery of a high-molecular-weight protease most active in cytosol derived from liver but also present in mouse kidney, heart, brain, and spleen. This proteolytic complex has been known by several names, including macroxyproteinase, multicatalytic proteinase complex, prosome, and, most commonly, the proteasome (8) or ubiquitin-proteasome system (UPS). In 2004, Ciechanover, Hershko, and Rose were awarded the Nobel Prize in Chemistry for their description of ubiquitin-mediated degradation of proteins. In the years since the original discoveries, it has become clear that ubiquitin-mediated degradation of proteins plays a major role in regulating many cellular processes, including the cell cycle (27, 103), immune response and antigen presentation via the immunoproteasome (80, 81, 125), apoptosis (86, 112, 169), cell signaling (36, 49, 69, 92), and protein turnover under normal and pathological conditions (34, 94, 177, 210, 214). In addition, the UPS plays key roles in protein quality by removal of damaged, oxidized, and/or misfolded proteins (19, 44, 79, 107). In the ensuing review, the reader will be introduced to the role of the proteasome in cardiac physiology and pathology with these topics subjected to an in-depth review. To completely appreciate some of the nuances of UPS function, the reader needs to be aware of basic proteasome nomenclature, structure, assembly. and function. These topics will be reviewed in a general manner with the intent of providing an up-to-date synopsis of the literature. Where appropriate, the reader will be referred to excellent topical reviews.

PROTEASOME NOMENCLATURE AND STRUCTURE

Proteasomes have highly conserved architecture and are found in one form or another in all domains of life (221). The complete eukaryotic proteasome is composed of two complexes of proteins: the proteolytic core or 20S proteasome (on the basis of sedimentation value), containing 28 subunits consisting of duplicates of 14 different proteins and having a molecular mass in excess of 700 kDa; and one or two regulatory complexes, also known as the 19S regulatory complex or proteasome activator of 700 kDa (PA700), consisting of at least 17 additional proteins and having a molecular mass of almost 900 kDa (reviewed in Refs. 46, 238). Association of the proteolytic core with the regulatory complex results in the formation of a macromolecular structure that has become known as the 26S proteasome, which has a molecular mass in excess of 1,500 kDa if associated with one 19S complex (the

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Table 1. *Common nomenclatures of the 20S proteasome*

Nomenclature as Described By				
Baumeister et al. (14)	Groll et al. (88)	Coux et al. (41)	*Homo sapiens* (Human) (14, 88)	Accession No. (Human)
α-Type subunits				
α1	α1_sc	Pro-α6	C2	P25786
α2	α2_sc	Pro-α2	C3	P25787
α3	α3_sc	Pro-α4	C9	P25788
α4	α4_sc	Pro-α3	C6/XAPC-7	P25789
α5	α5_sc	Pro-α1	zeta	P28066
α6	α6_sc	Pro-α5	iota	P60900
α7	α7_sc	Pro-α7	C8	Q8TAA3
β-Type subunits				
β1	β1_sc	Pro-β3	Y/delta/C5	P20618
β1i	β1i_hs	Pro-β3	LMP2/RING12	P28065
β2	β2_sc	Pro-β2	Z	P49721
β2i	β2_hs	Pro-β2	MECL1	P40306 (precursor)
β3	β3_sc	Pro-β6	C10	P49720
β4	β4_sc	Pro-β4	C7	P28070 (precursor)
β5	β5_sc	Pro-β1	X/MB1/epsilon	P28074 (precursor)
β5i	β5i_hs	Pro-β1	LMP7/RING10/Y2/C13	P28062 (precursor)
β6	β6_sc	Pro-β5	C5/delta/subunit Y	P28072 (precursor)
β7	β7_sc	Pro-β7	N3/beta/subunit Z	Q99436 (precursor)

mushroom configuration; sedimentation value of 26) or 2,500 kDa if associated with two 19S complexes (one at each end or the dumbbell configuration; sedimentation value of 30) (46, 191, 238).

20S Proteasome

The proteolytic core of the proteasome is a cylindrical barrel-shaped structure containing four stacked rings each containing seven subunits and has become known as the 20S proteasome. One of the more confusing aspects of proteasome studies is the myriad of nomenclatures for naming these subunits, some dating back to the early 1980s. In Table 1, the more common nomenclatures, as well as primary accession numbers, are summarized. Most recent studies of mammalian proteasome now use the Baumeister et al. (14) nomenclature, which classifies the subunits into α- and β-subtypes depending on the rings. The inner two rings contain homologous β-subunits, which are designated β1–β7 (see Fig. 1). The subunits on these two rings are aligned counter to each other such that β1 on the top ring lies roughly above β7 on the bottom ring. The outer two rings contain homologous α-subunits, which are designated α1–α7 and are aligned over their corresponding β-sub-

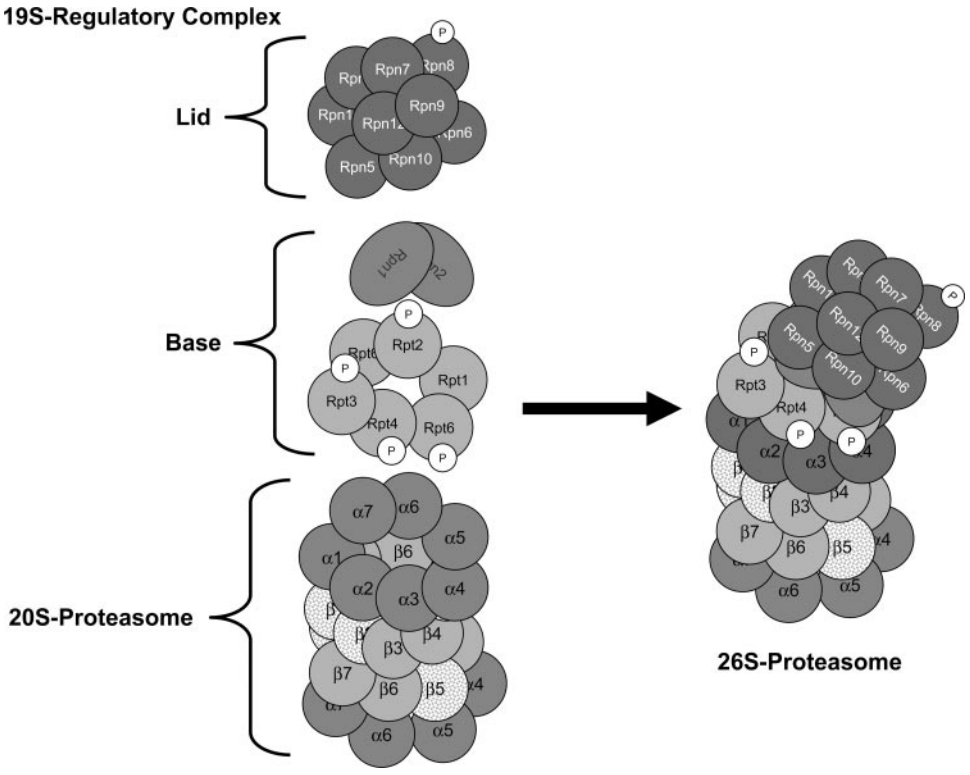

Fig. 1. Structure of the mammalian 26S proteasome. The mammalian 26S proteasome has as its core catalytic unit the 20S proteasome, which is a cylindrical-shaped structure composed of 4 rings, each consisting of 7 subunits. Capping the 20S proteasome at each end is the 19S regulatory complex, consisting of an additional 17 proteins. The entire structure has become known as the 26S proteasome, which is somewhat of a misnomer because the 20S proteasome capped on both ends by the 19S complex is actually 30S and when capped on one end is only 26S. For the sake of clarity, shown here is capping on only one end. Note that subunits β1, β2, and β5 are depicted in a different shade to indicate that these can exist in the immunoforms. Rpt, regulatory particle triple A; Rpn, regulatory particle non-ATPase.

units such that the α1-subunit lies roughly over the β1-subunit (Fig. 1).

The proteolytic activity of the proteasome resides within the inner β-subunits. In general, three main activities have been ascribed to the proteasome: chymotryptic (large hydrophobic groups), tryptic (basic groups), and post-glutamyl hydrolase (acidic groups, e.g., glutamic acid), which is a misnomer because cleavage is faster after aspartic acid; thus the newer term, caspaselike, is more appropriate. On the basis of site-directed mutagenesis studies targeting the active NH_2-terminal Thr1 nucleophile, the chymotryptic activity has been assigned to the β5-subunit, the tryptic activity to the β2-subunit, and the caspaselike activity to the β1-subunit (reviewed in Refs. 14, 98, 168). The β1-, β2-, and β5-subunits can be replaced by immunoforms in response to exposure to the cytokine γ-interferon and are designated β1i, β2i, and β5i (see Table 1), and the transformed 20S proteasome becomes part of the immunoproteasome (reviewed in Ref. 73). Replacement of these subunits with their immunoforms favors formation of peptide fragments consistent with the major histocompatibility class I antigens. Reports of additional proteolytic activities, such as BrAAP activity (cleavage after branched chain amino acids) and SNAAP activity (cleavage after small neutral amino acids), have been ascribed to replacement of these subunits and formation of the immunoproteasome (reviewed in Ref. 168). At least with respect to the β5i-subunit, the response to γ-interferon appears to be rapid, but transient, thus allowing the mammalian cell to rapidly return to normal once immunoproteasome function is no longer required (99). The function of the other four β-subunits in higher eukaryotic proteasomes is unclear at this time, and these have been described as being inactive (89) although at least one study suggests that the mammalian β7-subunit may possess NH_2-terminal nucleophile hydrolase activity (216). In the eukaryotic proteasome, the α-subunits have no direct proteolytic activity but play an important gating role in preventing access of folded and unfolded proteins to the central proteolytic chamber when proteasome is in the nonactivated state (reviewed in Ref. 90). Crystallographic structural analysis of yeast 20S proteasome indicates that the NH_2 termini of subunits α1, α2, α3, α6, and α7 project into the openings at either end of the cylinder, effectively sealing it and preventing access to the central chamber. On activation of the proteasome, these subunits rearrange, allowing access to the proteolytic core (87, 157). Activation of the proteasome is generally considered to be a function of binding of the 19S regulatory complex to the 20S proteasome.

19S Regulatory Complex

The mammalian 19S regulatory complex consists of at least 17 proteins arranged into two distinct subcomplexes, the base and the lid (see Fig. 1). Like the 20S proteasome, there have been several nomenclatures to identify these different subunits. The more common of these are summarized in Table 2. The nomenclature most commonly used by most investigators is that described by Finley et al. (62), who separated the subunits on the basis of whether they have ATPase activity. Of the 17 proteins, 6 are ATPases of the AAA family and are designated regulatory particle triple A (Rpt) 1–Rpt6. The remaining subunits are designated regulatory particle non-ATPase (Rpn) 1–Rpn12, with Rpn4 apparently not tightly associated with the 19S regulatory particle. The base of the 19S complex is composed of the six ATPase subunits (Rpt1–Rpt6) and the two largest of the non-ATPase subunits, Rpn1 and Rpn2 (Fig. 1). The six ATPase subunits assemble into an oligomeric ring, which sits on top of the outer α-ring with at least Rpt2 and Rpt6 having been shown to interact with subunits on the α- and/or β-rings (76, 87, 185, 190). Association of the base is sufficient to activate the 20S proteasome to degrade peptides or nonubiquitinated proteins (78). The primary functions of the base are to act as an interface between the 19S regulatory complex and the 20S proteasome core; to utilize ATP to unfold substrate proteins before translocation to the regulated gate; to activate the 20S proteasome, resulting in rearrangement of the NH_2 termini of α-subunits to allow access to the catalytic chamber; and to act as a point of attachment for lid subunits (reviewed in Refs. 14, 46, 238).

Table 2. *Nomenclatures and functions of the 19S regulatory complex (PA700)*

Nomenclature as Described By				
Finley et al. (62)	Dubiel and Gordon(55)	Previous designations(62)	Accession No.	Function [From Demartino (46)]
Rpt1	S7	Cim5/Yta3	P15531 (mouse)	ATPase
Rpt2	S4	Yta5/Yhs4	P40327 (yeast)	ATPase
Rpt3	S6b	Yta2/Ynt1	P33298 (yeast)	ATPase
Rpt4	S10b	Crl13/Sug2/Pos1	P53549 (yeast)	ATPase
Rpt5	S6a	Yta1	P33297 (yeast)	ATPase/polyubiquitin recognition (133)
Rpt6	S8	Sug1/Cim3/Crl3	Q01939 (yeast)	ATPase
Rpn1	S2	Hrd2/Nas1	Q13200 (human)	UBL binding
Rpn2	S1	Sen3	Q99460 (human)	UBL binding
Rpn3	S3	Sun2	P40016 (yeast)	
Rpn4		Son1/Ufd5	Q03465 (yeast)	
Rpn5		Nas5	Q12250 (yeast)	Proteasome assembly (235)
Rpn6	S9	Nas4	Q12377 (yeast)	
Rpn7	S10a		Q06103 (yeast)	
Rpn8	S12	p40	P51665 (human)	
Rpn9	S11	Nas7	Q04062 (yeast)	
Rpn10	S5a	Mcb1/Sun1	P55036 (human)	Ubiquitin binding domain
Rpn11	S13	Mpr1	O00487 (human)	Deubiquitination
Rpn12	S14	Nin1	P32496 (yeast)	

UBL, ubiquitin-like domains; Rpt, regulatory particle triple A; Rpn, regulator particle non-ATPase; PA700, proteasome activator of 700 kDa.

The remaining nine (or 10) subunits make up the lid complex, most of whose functions are not clear. A recent review by DeMartino (46) summarizes the function of these subunits as follows: polyubiquitin chain recognition through Rpn10, which confers ubiquitin specificity; and intrinsic substrate deubiquitination via Rpn11. The reader is referred to this review (46) for additional details and to Table 2, which also lists known function(s) of the different 19S subunits.

Assembly of the Proteasome

Because variations in proteasome subunit assembly may alter proteasome selectivity and specificity (82, 121), investigators should have a basic understanding of the steps and factors regulating this process. Most of the earlier studies in yeast focused on assembly of the 20S proteasome and, specifically, assembly of the β-rings and the role of the proteasome maturation factor Ump1 (ubiquitin-mediated proteolysis). These studies have postulated a two-step process in which an inactive half-proteasome, consisting of one α- and one β-ring, is formed, followed by dimerization to the latent four heptameric ring structure associated with the 20S proteasome (160, 179, 193, 231). These models all supposed that the α-rings formed spontaneously after processing of β-ring subunit propeptides. However, a recent study (106) has shed new light on initial processing of the α-subunits and has presented evidence for the existence of two chaperone proteins, proteasome assembly chaperone 1 (PAC1) and PAC2, which form a heterodimer that associates with certain of the α-subunits. Accordingly, the following multistep process for assembly of the 20S proteasome can be described. In the initial step, PAC1:PAC2 heterodimer associates with free α5- and α7-subunits, creating a scaffold around which the remaining α-subunits can polymerize and apparently direct their order of incorporation (106). This is followed by association of Ump1 [human analog hUmp1, or proteasome maturation protein (POMP)] with a subset of β-subunits that are synthesized as inactive propeptides to prevent proteolytic activity (30, 231). Studies in human cell lines indicate that the initial intermediate formed is a complex of the complete α-ring plus the β2-, β3-, and β4-propeptides (160, 194). This intermediate then directs incorporation of the remaining four β-subunit propeptides to form the inactive half-proteasome with the PAC1:PAC2 heterodimer and the Ump1 maturation factor still associated (106, 179, 231). At this point, two half-proteasomes undergo rapid dimerization to form the latent 20S proteasome, inducing rapid cleavage of the β-subunit prosequences catalyzed by Ump1, and thereby exposing the active proteolytic sites within the complete catalytic chamber (30, 160, 179, 231). At least in the archaebacterium *Archaeoglobus fulgidus*, this step is preceded by a conformational change that reorients the β-subunits upward and toward an approaching half-proteasome, allowing them to make contact (158). Ump1, PAC1:PAC2, and the β-subunit prosequence cleavage fragments are then released and become substrates for proteolytic degradation by the mature proteasome (106, 179) (see Fig. 2 for model).

Although it is possible to postulate a sequence of events for 20S proteasome assembly, the same is not true for assembly of the 19S regulatory complex as very little is known about this process. What is known suggests that the six ATPase (Rpt1–Rpt6) subunits assemble first as a hexameric ring, which along with the other two "base" subunits, Rpn1 and Rpn2, associate with the 20S proteasome (84). Binding to 20S proteasome appears to be regulated by phosphorylation of at least one of the ATPases, in this case, Rpt6, which directly associates with the α2-subunit (190). Beyond this, little is known about how the remaining lid subunits come together, although certain of these are known to interact with each other (68, 235). A better understanding of the processes involved in proteasome assembly becomes essential in light of a recent report (33) demonstrating that overexpression of the β5-subunits in human embryonic cells upregulates overall proteasome expression and confers enhanced resistance to oxidative stress. Whether this strategy has practical therapeutic application remains to be elucidated.

Cardiac 26S Proteasome

Little is known about how the architecture of the cardiac 26S proteasome may differ from other tissue types. Most investigators in this area have based their hypotheses on studies of noncardiac mammalian proteasome or proteasome in pro-

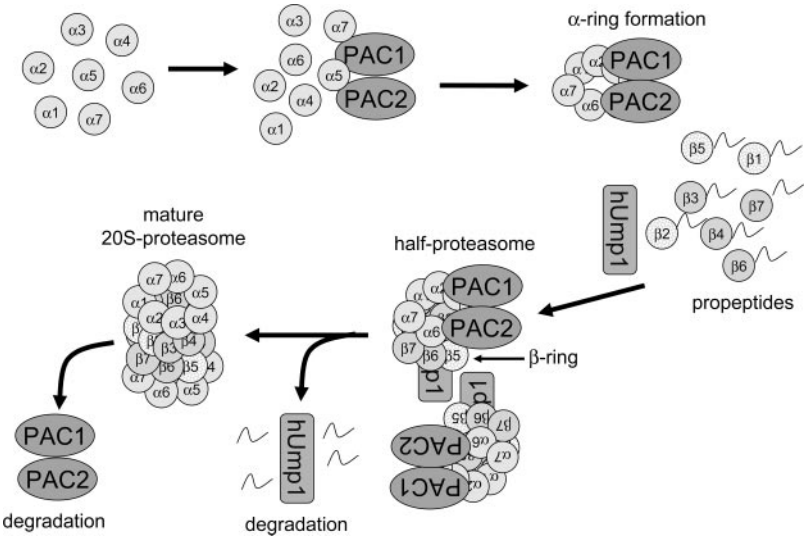

Fig. 2. Multistep model for assembly of the human 20S proteasome. Newly synthesized α-subunits associate with the proteasome assembly chaperone 1 (PAC1):PAC2 heterodimer, which creates a scaffold for formation of the α-ring; β-subunit propeptides, chaperoned by hUmp1 (human analog of ubiquitin-mediated proteolysis), associate with the α-chain/PAC1:PAC2 complex, leading to formation of the inactive half-proteasome. Two half-proteasomes dimerize forming a 4-ring heptamer that undergoes an autocatalytic cleavage of the β-subunit prosequences, revealing the active proteolytic sites. PAC1:PAC2, hUmp1, and the β-subunit prosequence cleavage fragments become substrates for the mature proteasome and undergo proteolytic degradation. [Adapted from Hirano et al. (106) from *Nature* with permission from Macmillan Publishers Ltd., copyright 2005.]

karyotes and lower eukaryotes. A recent report by Gomes et al. (82) detailing preliminary proteomics studies of the murine cardiac 26S proteasome has yielded some rather novel information. These investigators have proposed a model in which the cardiac 26S proteasome contains a variable 20S complex assembled with different proportions of β-subunits in addition to the 19S complex. According to this hypothesis, the β1-, β2-, and β5-subunits may be replaced by the immunoform on one β-ring, but not the other, or perhaps even a mixture of immunoform vs. nonimmunoform subunits (see Fig. 3 for examples). This hypothesis predicts that alterations in proteasome subunit composition would alter specificity and selectivity of the proteasome for various substrates, thus playing a role in regulation of intracellular proteolysis. This is an exciting hypothesis that is supported by reports of altered proteasome function and subunit composition in aged muscle (61, 109) and by studies of immunoproteasome assembly suggesting that differences between the regular and immunoform β-subunit (in particular, β5 and β5i) propeptides may influence and possibly direct assembly of variable subsets of proteasome, promoting diversity of antigenic peptides (120, 121, 160). Whether other proteasome subunits can also assemble in variable proportions or compositions, or what factors would control this, is unclear at this time and is the subject of active study. If this should be the case, it presents the possibility of directing proteasome assembly to direct selective degradation (or not) of a protein or class of proteins, thereby altering the outcome of a disease process.

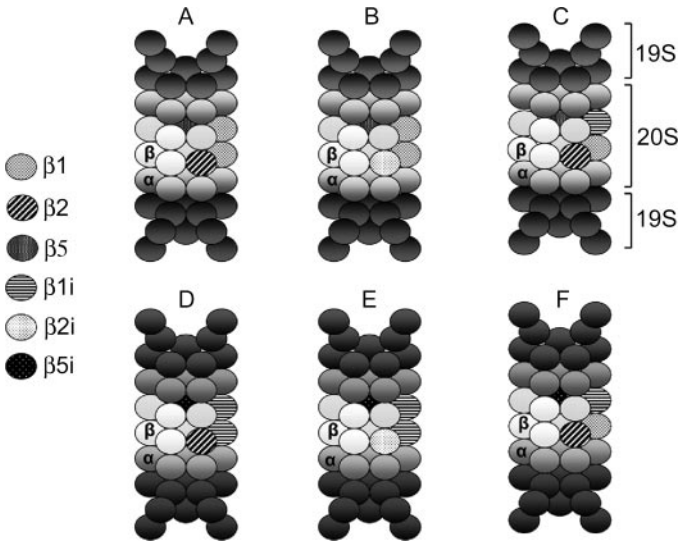

Fig. 3. Models of variable 26S proteasome assembly. Each 20S subunit occurs in duplicate with the possible exception of three subunits, β1, β2, and β5, which may be replaced by 3 immunoforms (β1i, β2i, and β5i). On the basis of preliminary data, Gomes et al. (82) have proposed that the cardiac proteasome system contains multiple complexes with distinct molecular compositions. *A*: the 20S proteasome complex contains two β1-subunits, two β2-subunits, and two β5-subunits. *B*: in contrast to the 20S proteasome shown in *A*, this 20S proteasome contains one β2-subunit and one β2i-subunit. *C*: in contrast to the 20S proteasome shown in *A*, this 20S proteasome contains one β1i-subunit and one β1-subunit. *D*: in contrast to the 20S proteasome shown in *A*, this 20S proteasome contains two β1i-subunits and one β5i-subunit (β5i is shown in the back of the β-ring), respectively. *E*: in contrast to the 20S proteasome shown in *A*, this 26S proteasome contains two β1i-, one β2i-, and one β5i-subunit, respectively. *F*: in contrast to the 20S proteasome shown in *A*, this 20S proteasome contains one β1i-subunit, one β2i-subunit, and one β5i- subunit, respectively. [Reprinted from Gomes et al. (82) with permission from Blackwell Publishing.]

UBIQUITIN-MEDIATED DEGRADATION OF PROTEINS

The 26S proteasome is the major nonlysosomal pathway for intracellular protein degradation. In general, targeting to the proteasome requires a covalent linkage of one or more chains of polyubiquitins to the protein intended for degradation. As this area has been the subject of several recent excellent reviews, what follows is a general review of the literature. Where appropriate, discussions will be supplemented with cardiac-specific information.

Ubiquitination (Ubiquitylation)

Ubiquitination refers to the conjugation of free ubiquitin with some substrate protein. Ubiquitin is a highly conserved compact globular protein consisting of 76 amino acids that is expressed in all eukaryotes but very few prokaryotes. Ubiquitination proceeds along a three-step cascade (see Fig. 4). In the first step, the ubiquitin-activating enzyme, E1, uses ATP to activate ubiquitin to a higher energy state by forming a thiol-ester linkage with the protruding COOH-terminal glycine (G76). The activated ubiquitin is then transferred onto one of several ubiquitin-conjugating proteins, E2, by the formation of an additional high-energy thiol-ester bond, and then covalently linked, generally at the ϵ-NH_2 of a Lys residue, to a protein substrate that is bound to a specific ubiquitin protein ligase, E3. There are multiple classes of E3s, and overall these number in the 1,000s, each one recognizing a specific motif on the substrate. Because specificity of the UPS resides with the E3s and there are specific classes of muscle-specific E3s, these are discussed in detail in a subsequent section. In general, if the substrate is to be degraded by the 26S proteasome, it must be polyubiquitinated, which occurs via successive addition of activated ubiquitins to internal Lys residues of the previously conjugated ubiquitin. Ubiquitin can be ubiquitinated at any one of its seven Lys residues (Fig. 5) with the specific residue linked to various functions. For example, ubiquitination at Lys48 is most common and typically targets the protein substrate to the 26S proteasome. Ubiquitination at Lys63 can target a protein to the 26S proteasome but may also be associated with DNA repair mechanisms, endocytosis, and vesicle transport. Although the other five Lys residues may be ubiquitinated, their function is not clear. In some instances, polyubiquitin chain formation is facilitated by a multiubiquitin elongation factor, E4 (reviewed in Ref. 108). For additional detail on ubiquitin and the ubiquitination process, the reader is referred to Refs. 59, 77, 108, and 173.

Once a substrate is polyubiquitinated, it is either recognized directly by the proteasome or bound to some shuttling protein, which transports it to the proteasome. Recognition is via ubiquitin binding domains contained in the Rpn10 subunit or possibly contained within a shuttling protein (reviewed in Ref. 104). It is now becoming clear that polyubiquitination is not the only type of ubiquitination and that others can occur, each one associated with specific functions (see Fig. 6). Monoubiquitination refers to a single ubiquitin conjugated to a Lys residue of a substrate protein. This type of modification appears to be important in regulating protein activity and function; cargo trafficking, such as receptor internalization through

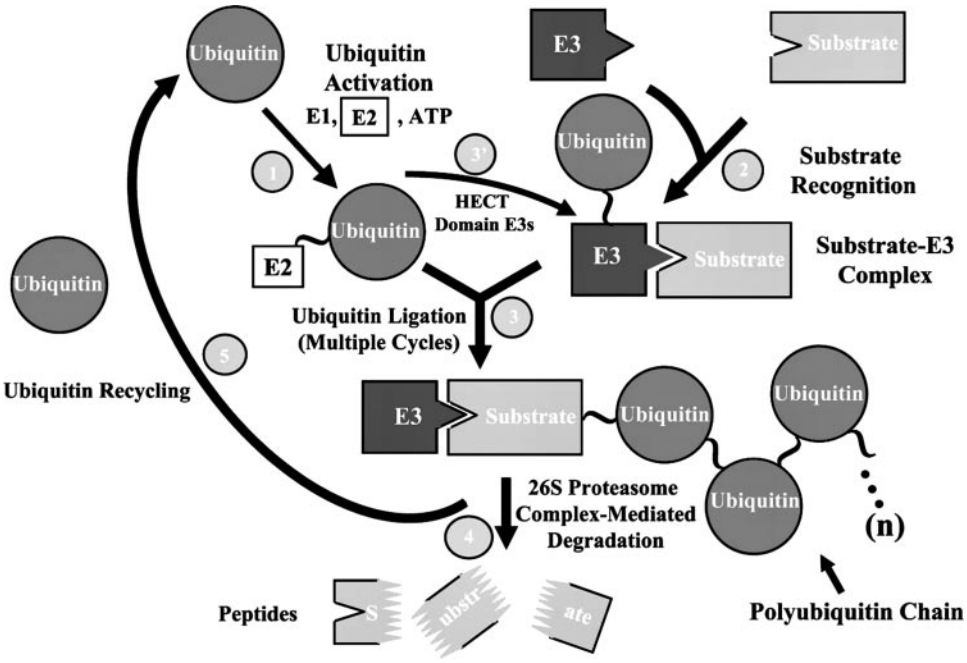

Fig. 4. The ubiquitin-proteasome pathway. *1*: activation of ubiquitin by the ubiquitin-activating enzyme E1, a ubiquitin-carrier protein, E2, and ATP, producing a high-energy E2~ubiquitin thiol ester intermediate. *2*: binding of the protein substrate to a specific ubiquitin-protein ligase, E3. *3*: multiple (*n*) cycles of ubiquitin conjugation, resulting in a polyubiquitin chain. *4*: degradation of the substrate by the 26S proteasome complex with release of short peptides. *5*: recycling of ubiquitin is recycled via deubiquitinating enzymes (DUBs). HECT, homologous to E6-AP carboxy terminus. [Reprinted from Glickman and Ciechanover (77) with permission of the American Physiological Society.]

endocytosis; tagging and sorting of proteins to multivesicular bodies; DNA repair; and regulation of transcription. Some proteins or receptors require monoubiquitination at multiple sites (multimonoubiquitination) to ensure proper function or endocytosis (reviewed in Ref. 92). An additional form of ubiquitination is NH_2-terminal ubiquitination, first described for the cell cycle regulator p21 (17). NH_2-terminal ubiquitination does not follow the canonical three-step conjugation of ubiquitin to a Lys residue of a protein substrate but rather is conjugated to the α-NH_2 group on the NH_2-terminal residue of the substrate. This type of ubiquitination seems to affect stability of a protein, in some cases blocking degradation through a mechanism that is still unclear (reviewed in Ref. 35).

Regardless of the type or extent of ubiquitination, at some point the ubiquitin is going to be removed for the purposes of recycling or to prevent the degradation of a previously ubiquitinated protein. This process is under the control of the deubiquitinating enzymes or DUBs. DUBs are found throughout the cell associated mainly with subcellular structures or large

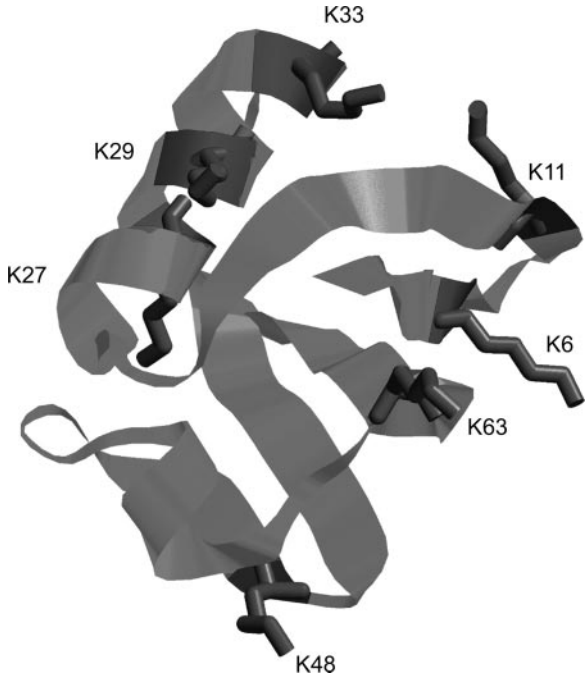

Fig. 5. Ubiquitin ribbon diagram illustrating the 7 lysines available for ubiquitination. Ubiquitination at individual lysines plays a role in the different functions of ubiquitin (see text for details). {Reprinted with permission from *Nature Reviews Molecular Cell Biology* [www.nature.com/reviews; diagram from poster based on review by Welchman et al. (225)], copyright 2005, Macmillan Magazines Ltd.}

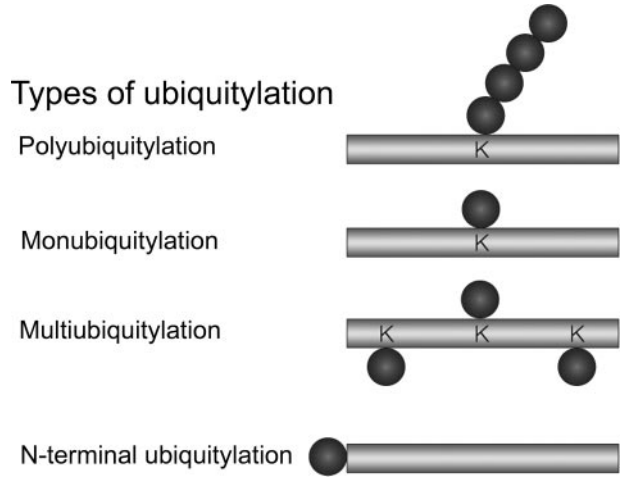

Fig. 6. Types of ubiquitination (ubiquitylation). Proteins can be polyubiquitinated on one or more lysine residues with a ubiquitin chain; monoubiquitinated by a single ubiquitin on a single lysine; multimonoubiquitinated on several lysines by a single ubiquitin; or can undergo NH_2-terminal ubiquitination, which refers to fusion of ubiquitin to the α-NH_2 group of the NH_2-terminal residue. {Reprinted with permission from *Nature Reviews Molecular Cell Biology* [www.nature.com/reviews; from Hicke et al. (104)] copyright 2005, Macmillan Magazines Ltd.}

molecular complexes. In the case of the 26S proteasome, deubiquitination is associated with the Rpn11 subunit in the 19S regulatory complex, which deubiquitinates a polyubiquitinated substrate before unfolding. Deubiquitination is thought to provide another layer of regulation in diverse processes such as cell cycle/cell growth, organism development, and gene transcription (reviewed in Refs. 4, 228).

Ubiquitin-like Proteins (Ubiquitons)

Ubiquitons are small protein modifiers that can be covalently linked to substrates and are related to ubiquitin in that they possess the ubiquitin superfold, which is a β-grasp region. There are numerous ubiquitons, most having little homology with ubiquitin. These small modifiers can have many functions either by themselves or in conjunction with ubiquitin. For example, the RAD proteins assist in DNA repair and may act as a shuttle to chaperone polyubiquitinated proteins to the proteasome for degradation (reviewed in Ref. 218). The small ubiquitin-like modifier (SUMO) family appears to play a role in regulation of traffic into and out of the nucleus (reviewed in Ref. 147). The Atg (or Apg) family plays an important role in initiating, regulating, and targeting of proteins and organelles for autophagy (reviewed in Ref. 155). The neuronal precursor cell expressed developmentally downregulated (Nedd) 8 family plays major roles in regulation of the cullin-based E3s (reviewed in Ref. 227). For a more in-depth review of ubiquitons, the reader is referred to Ref. 225 [a poster is available for download at http://www.nature.com/nrm/poster/ubiquitin/index.html in the online August 2005 issue of *Nature Rev Mol Cell Biol* 6 (8)].

Ubiquitin Protein Ligases or E3s

E3 or ubiquitin protein ligases represent the specificity of the ubiquitin conjugation cascade. Specificity is conferred by the sheer number of ubiquitin ligases, probably in the 1000s, each one acting alone with a single E2 to recognize specific amino acid sequences (reviewed in Ref. 77). Ubiquitin ligases can basically be separated into three groups: those that contain homologous to E6-AP carboxy terminus (HECT) domains; those that contain RING fingers; and the Skp1-Cullin-F-box (SCF) family (reviewed in Refs. 24, 25, 48) (see Fig. 7). Members of the HECT-domain ubiquitin ligases accept activated ubiquitin from the E2 conjugating enzyme, forming a thioester linkage between the COOH-terminal glycine of ubiquitin and an internal Cys residue. The ubiquitin is then transferred directly to the free NH_2 of a lysine on the specific substrate recognized by the ligase (77, 162, 192). The RING finger ubiquitin ligases contain zinc-binding motifs containing cysteine and histidine amino acids in particular sequences (48). Lorick et al. (143) have suggested that unlike the HECT domain, the RING motif associates with the E2-conjugating enzyme while somehow providing an environment conducive for transfer of an activated ubiquitin to a protein substrate lysine. The last group of ligases, the SCF family, is characterized by formation of a complex containing a cullin protein (Cul), SKP1, a RING protein, and an F-box protein or domain. Because they contain a RING protein, the SCF E3s are considered by some to be a subset of the RING finger E3s. In the SCF complex, the cullin protein is thought to sort substrates and provide interaction sites for Skp1 and the RING finger

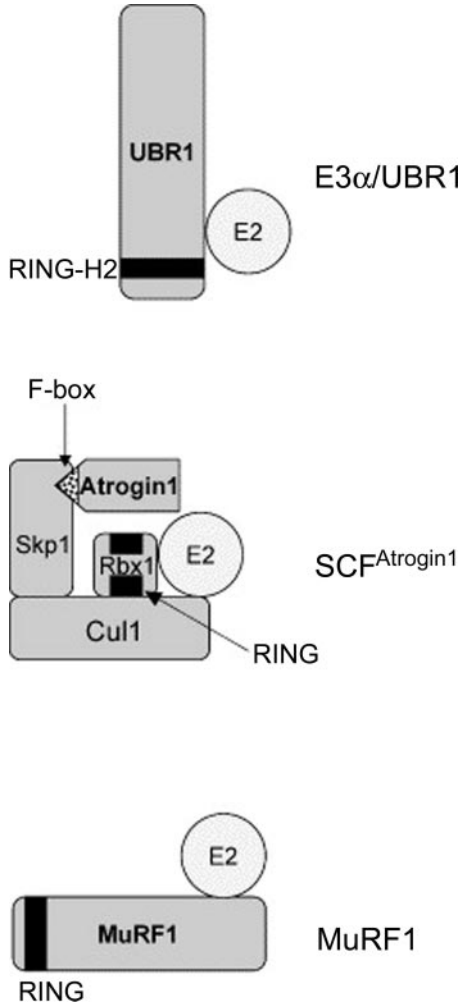

Fig. 7. Structure of E3s (ubiquitin protein ligases) involved in regulation of muscle protein turnover. Note the 4 components comprising the Skp1-Cullin-F-box (SCF) ligase. The RING finger motifs of each E3 is indicated in black. E2, ubiquitin carrier protein; MURF, muscle-specific RING finger. [Reprinted from Cao et al. (24) with permission from Elsevier Publishing.]

protein that associates with a specific E2 conjugating enzyme, while the F-box protein, which binds to Skp1, represents the substrate recognition domain (24, 25, 48) (Fig. 7). In general, the RING finger-containing E3s are thought to act as scaffolding, bringing the activated ubiquitin and substrate together on the same platform (77).

An additional group of proteins with ubiquitin ligase activity are certain atypical RING finger proteins, designated U-box proteins, that lack the characteristic Cys residues that make up part of the Zn-binding domain (6). These proteins appear to facilitate ubiquitin chain elongation (formation of polyubiquitin) and have been referred to as E4 (126). Because some E4s can elongate ubiquitin chains once a substrate is monoubiquitinated, independent of a specific E3, they are said to have ubiquitin protein ligase activity (addition of ubiquitin to ubiquitin) and are considered by many to be a subset of E3s (95, 96). One example is carboxy terminus of Hsp70 interacting protein (CHIP), which is known to interact with both Hsp70 and Hsp90 to target certain proteins for ubiquitin-mediated degradation (11, 40, 45). CHIP is highly expressed in the heart,

and it is of significance to this discussion that a recent study (236) has demonstrated that CHIP-deficient mice had decreased survival, a larger myocardial infarct, and greater incidence of cardiomyocyte and vascular endothelial apoptosis during reperfusion after left anterior descending coronary artery (LAD) occlusion. CHIP and many of the other E3 ligases are expressed in multiple tissue types representing a conservative means for processing of common substrates. However, it is now clear that some ubiquitin ligases are expressed in a tissue-specific manner, presumably as a means for processing of substrates unique to these tissues. Several E3s that specifically target muscle proteins found in the sarcomere have now been described.

Muscle-specific E3-ligases. Much of what is known about the muscle-specific E3s comes from the muscle atrophy literature. Perhaps the earliest indication that the UPS plays a role in muscle protein catabolism were studies by Goldberg and coworkers (58), who showed that skeletal muscle contains this proteolytic complex. Subsequently, this group showed that degradation of muscle protein requires ATP (54) and that multiple conditions that lead to increased muscle proteolysis, such as starvation, fasting, denervation, and cachexia, all have in common increased levels of muscle ubiquitin and polyubiquitinated proteins, as well as increased ubiquitin and proteasome subunit mRNAs (13, 118, 151, 152, 229). Proof of concept was provided by studies with proteasome inhibitors which reduced protein breakdown of atrophying muscle (209), indicating that much of muscle proteolysis is mediated by upregulation of the UPS. Shortly thereafter, Solomon and Goldberg (200) demonstrated that dissociation of certain myofibrillar proteins from the myofibril makes them targets for UPS and suggested that this process is regulated by "ubiquitin conjugating enzymes," which may recognize these dissociated proteins. The first muscle-specific E3 to be associated with muscle atrophy was E3α (or UBR1), which is assisted by the ubiquitin conjugating protein $E2_{14k}$ and recognizes proteins according to the N-end rule (NH_2 terminus destabilizing hydrophobic or basic amino acids) (63, 135, 199, 201).

Other muscle-specific E3s include Atrogin-1 (MaFbx), which is an F-box protein that binds to Skp1 and thereby Cul1 and the ring finger, Roc1, and is an example of an SCF family E3 (83). This protein is selectively expressed in cardiac and striated muscle and has been shown to be strongly induced in fasting and other catabolic states before onset of muscle atrophy (83, 134). Also described is muscle-specific RING finger (MURF)-1, MURF-2, and MURF-3, members of the MURF family of RING E3s, which are also upregulated in catabolic states (18, 28). All MURFs are characterized by an amino-terminal RING finger domain followed by a highly conserved 35 residue motif, the MURF family conserved (MFC) domain, which is specific for this family. The MFC domain is followed by a second Zn-binding motif of the B-Box type followed by a more divergent carboxy-end terminus. In addition, all MURFs are capable of forming dimers or heterodimers (65, 202, 215). Since the initial description of these muscle-specific E3s, studies have focused on examining their critical roles in regulating sarcomere protein turnover and cardiomyocyte size.

Role of muscle-specific ubiquitin ligases in sarcomere protein turnover. MURF-3 was the first member of this family identified and was characterized as a microtubule-associated protein, developmentally upregulated and specifically expressed in cardiac and skeletal muscle, and required for skeletal myoblast differentiation and development of cellular microtubular networks (202). MURF-1 was identified as a myofibrillar-associated protein that localizes to the M-line region in close proximity to the active kinase domain of the giant protein, titin, binding to the adjacent A168/A169 repeats. MURF-1 is also found within the Z-line lattice; in soluble form in the cytoplasm; as well as in the nuclei (28, 148). Expression of MURF-1-encoding fusion proteins in cardiac myocytes results in disruption of titin's M-line region and thick filament structure (148). In mice, conditional expression of truncated titins lacking the kinase and MURF-1 binding domains leads to a myopathy characterized by sarcomere disassembly (85). Taken together, these studies indicate a regulatory role for MURF-1 in maintenance and turnover of myofibrils and specifically the M-line region.

MURF-2 is thought to act as a link between the microtubular MURF-3 and the myofibrillar MURF-1 because this protein colocalizes to both the M-line region of titin and within a subgroup of cardiac microtubules, as well as associating with the intermediate filaments vimentin and desmin (149). Knockdown of MURF-2 in neonatal cardiomyocytes results in loss of integrity of stable detyrosinated microtubules, perturbations in vimentin and desmin structure, and dramatic disruptions in M-line structure (149). Recent studies have identified troponin I, troponin T, myosin light chain-2, nebulin, the nebulin-related protein NRAP, myotilin, and T-cap as additional potential substrates for MURF-1 and MURF-2 (115, 232), suggesting that these two ubiquitin ligases may act in a redundant fashion to regulate proteasome-mediated turnover of myofibril proteins. Significantly, Witt et al. (232) have recently shown that MURF-1 interacts with several other cardiac enzymes required for energy production, suggesting a link between cardiac metabolism and muscle turnover, possibly regulated by stretch through titin-mediated signaling processes.

From the above discussion it is clear that muscle-specific E3s have major roles in myofibrillar protein degradation even though actual mechanisms and interactions may not have been totally elucidated. Given that cardiomyocyte size is determined by a balance between protein synthesis and protein degradation, muscle-specific E3s, and thereby the proteasome, have become the subject of intense study. These studies have provided clues as to the signaling pathways that may regulate certain of the muscle-specific E3s.

Role of muscle-specific ubiquitin ligases in regulation of cardiomyocyte size and induction of cardiac hypertrophy. Cardiomyocyte size is a tightly regulated process representing a dynamic balance between muscle anabolism and catabolism. Because muscle-specific E3s play major roles in muscle protein breakdown, they became logical targets for study in regulation of cardiomyocyte size and in induction of the hypertrophic phenotype. Studies of this type represent a departure from past studies of cardiac hypertrophy, which concentrated on increased protein synthesis in response to hypertrophic stimuli. Perhaps the earliest indication that E3s play a role in induction of the hypertrophic phenotype is a study demonstrating that overexpression of Atrogin-1 in hearts of transgenic mice reduces hypertrophy and fetal gene expression in the thoracic aortic banding model (137). This study demonstrated that Atrogin-1 promotes ubiquitination and degradation of calcineurin A, suggesting repression of this hypertrophic pro-

tein (156, 207) as a primary mechanism. However, studies of IGF-mediated skeletal muscle growth have now suggested an additional mechanism. IGF is know to induce muscle growth through activation of the Akt/mammalian target of rapamycin-dependent (mTOR) pathway (reviewed in Ref. 213), a mechanism it shares with some stimuli shown to be hypertrophic in cardiomyocytes (reviewed in Ref. 171). A recent study has shown that IGF-1 activation of the Akt/mTOR pathway decreases dexamethasone-induced muscle protein breakdown by suppression of Atrogin-1 and MURF-1 expression (186). The link between Akt and expression of ubiquitin ligases was not readily apparent until additional studies demonstrated that Atrogin-1 expression is under the control of the FOXO proteins, a subgroup of the Forkhead family of transcription factors (188, 205). The FOXO proteins are negatively regulated by the PI3k/Akt signaling pathway. Akt can phosphorylate FOXO transcription factors at multiple sites, resulting in exclusion of the protein from the nucleus (reviewed in Ref. 7), thus downregulating expression of the E3s. A recent study (197) has shown that not only is the Akt/FOXO signaling axis present in cardiomyocytes but is also responsive to insulin, IGF-1, and ANG II stimulation, as well as stretch and pressure overload. Moreover, transfer of the FOXO3a gene to neonatal cardiomyocytes upregulates the Atrogin-1 gene and renders these cells resistant to IGF and stretch-induced hypertrophy, while similar transduction of mouse hearts results in shrinkage of cardiomyocytes in vivo. These results suggest that Akt plays a dual role in induction of the hypertrophic phenotype, not only by stimulation of protein synthesis (171) but also by suppressing E3 expression, the one system that could antagonize or reverse myocyte hypertrophy (see Fig. 8). The story does not end here, however, as decreased expression of these E3s could have serious downstream effects. One of the events that may result in cardiac hypertrophy is activation of the protein kinase C (PKC) signaling pathway (reviewed in Ref. 53). A recent study has shown that MURF-1 overexpression inhibits agonist-induced PKC-ϵ-mediated responses in neonatal cardiomyocytes (9). Although it was not clear from this study that MURF-1 promoted ubiquitination of PKC-ϵ, it nonetheless suggests that this ligase may exert an antihypertrophic effect through modulation of different pathways. Taken in total, these results suggest that certain ubiquitin ligases may play a role in cross talk between multiple signaling pathways and that perturbations could have profound effects on cardiomyocyte function. Furthermore, it is quite possible that additional defects in the UPS might be present in end-stage heart failure because at least two studies (128, 224) have demonstrated increased presence of ubiquitinated proteins in explants from failing human hearts, suggesting impaired proteasome function.

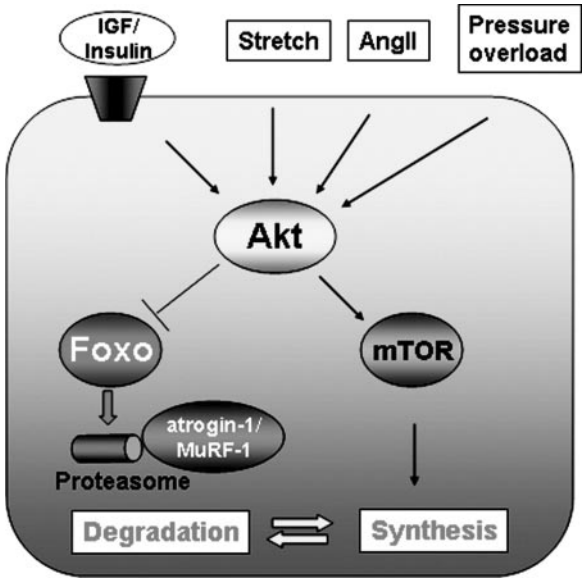

Fig. 8. Proposed scheme for Akt/FOXO-mediated regulation of cardiac myocyte size. Hypertrophic stimuli lead to Akt phosphorylation, which induces hypertrophy in cardiac myocytes by increasing protein synthesis. At the same time, Akt negatively regulates the FOXO transcription factors through phosphorylation, thereby preventing activation of muscle-specific ubiquitin ligases, which would promote muscle degradation. mTOR, mammalian target of rapamycin. [Reprinted from Skurk et al. (197) with permission from the American Society for Biochemistry and Molecular Biology.]

Functions of other E3s. REGULATION OF THE VOLTAGE-GATED CARDIAC SODIUM CHANNEL $Na_v1.5$. Voltage-gated Na^+ channels (Na_v) are integral membrane proteins that initiate the action potential and thus underlie the spread of excitation in the heart muscle (66). The cardiac isoform $Na_v1.5$ is a heteromultimer with four heterogeneous domains each containing six transmembrane spanning segments (reviewed in Ref. 38). Mutations or variations in the gene SCN5A encoding this channel have been linked to several conduction defects, including congenital long-QT syndromes and Brugada syndrome (reviewed in Ref. 75). Recent studies indicate that the Nedd4-like family of E3s, members of the HECT-domain group, play a major role in regulation of this channel. Nedd4-like E3s have two to four WW domains that can target proteins with specific PY motifs (PPXY), such as those found in the carboxy termini of all voltage-gated Na^+ channels (50, 64, 183, 184). In this regard, Nedd4–2 downregulates $Na_v1.5$ by ubiquitination, leading to its internalization and decreased surface channel density (1, 217). Thus far, ubiquitination of the $Na_v1.5$ is regarded as a normal regulatory process, and its significance in cardiac disease is not clear (for a more complete review on this topic, see Ref. 2).

UPS AND APOPTOSIS

One of the earliest indications that the UPS in some way regulates apoptotic cell death was a study in TNF-treated U937 monoblasts in which treatment with several proteasome inhibitors enhanced CPP32 (caspase 3) activity (70). This effect of proteasome inhibitors was subsequently confirmed in numerous proliferating cancer cell lines; however, there was some controversy as opposite effects were observed in quiescent terminally differentiated cell lines (reviewed in Ref. 169). It is now generally accepted that rapidly proliferating or metabolically active cells are more prone than quiescent cells to this effect of proteasome inhibitors and in fact has led to the development of other inhibitors, such as Bortizemib (PS-341), for treatment of cancer (reviewed in Ref. 181).

The relationship between the UPS and apoptosis, and thus the mechanism of proteasome inhibitor-mediated apoptosis, is one that has received intense study. Earlier studies suggested that simple deregulation of proteasome-regulated proapoptotic proteins, such as bax, mdm, and p53, were responsible (29). Later studies suggested that interference with progression of the cell cycle was responsible, as this resulted in accumulation

of cells arrested in the G_1 or G_2/M phase owing to accumulation of various cyclins, and $p27^{kip1}$, substrates for the UPS (reviewed in Refs. 86 and 169). It is now recognized that many pro- and antiapoptotic proteins are regulated by the UPS and include nuclear factor (NF)-κB, which has been extensively studied and is known to be regulated at multiple levels (reviewed in Ref. 129), the JNK and JAK-STAT pathways (reviewed in Ref. 69), the Bcl-2 family (146), and Smac/DIABLO, an inhibitor of apoptosis (IAP)-binding protein (145). Several members of the IAP proteins contain RING finger domains that can act as E3s and ubiquitinate numerous interacting proteins and are themselves ubiquitinated (reviewed in Ref. 219). Recent studies indicate that proteasome inhibitors can activate downstream caspases, possibly by stabilization of Smac/DIABLO (101), which in turn can feed back and cleave the proteasome (3, 206). Thus, rather than being simple deregulation, a very complicated picture is emerging in which inhibition of the proteasome both initiates and amplifies apoptotic cascades through a feedforward amplification loop. A further in-depth discussion on regulation of apoptosis by the proteasome is beyond the scope of this review, and readers are referred to the numerous reviews on this topic (see Refs. 112, 234, and 237 for examples). Suffice it to say that proteasome inhibition does cause apoptosis in myocardial tissue. This was originally shown by us (174) in a rather crude study in which isolated hearts were perfused with the inhibitor, MG132, and subsequently confirmed by others (16, 122) in cardiomyocyte preparations. That inhibition of the proteasome can cause cardiomyocyte apoptosis sets the stage for the ensuing discussions on proteasome dysfunction in cardiac pathologies, many of which have an apoptosis component.

PROTEASOME DYSFUNCTION IN CARDIAC PATHOLOGY

In the following section, the literature suggesting that dysfunction of the UPS plays a role in cardiac pathologies will be reviewed. Evidence has been mounting that decreased proteasome function is present during myocardial ischemia, several cardiomyopathies, and possibly during cardiomyocyte senescence. In addition, studies have suggested a role for the UPS in progression of atherosclerosis, a topic that has recently been reviewed (102) and will not be covered here. Much of this evidence is based on reports of increased ubiquitinated, misfolded, or oxidatively modified proteins, in the presence of diminished proteasome activity. Although these studies present substantial evidence of proteasome dysfunction, in virtually all of these cases, cause and effect is still lacking. Nonetheless, if proteasome dysfunction can be established as an underlying cause of cardiac dysfunction or cell death, there is great potential for therapeutic intervention, thus warranting additional studies.

Proteasome Dysfunction in Myocardial Ischemia

In the discussion that follows, evidence that proteasome is dysfunctional during myocardial ischemia will be examined. This evidence has raised several questions with regard to mechanism and consequences, if any. In addition, some investigators have advocated the use of proteasome inhibitors in the ischemic myocardium, which may seem counterintuitive; thus the rationale, if any, for this treatment modality will be examined.

Proteasome inhibition during ischemia. The first evidence for dysfunction of the proteasome during ischemia is derived from the neurology literature. At least three studies demonstrate inhibition of the 20S proteasome in stroke models associated with accumulation of oxidized and ubiquitinated proteins (10, 114, 119). The earliest evidence of decreased proteasome function in myocardial ischemia was presented by Bulteau et al. (21), who showed loss of non-ATP dependent (20S proteasome) trypsinlike activity after 30 min of in vivo LAD occlusion. Loss of activity was correlated with oxidative modification (4-hydroxy-nonenalyation) of several proteasome subunits as well as increases in myocardial content of ubiquitinated proteins. Recently, we (176) confirmed this observation in the isolated perfused heart preparation but also demonstrated that the ATP-dependent proteasome activity was also decreased, suggesting defects in 26S proteasome function, which was consistent with increases in myocardial ubiquitinated proteins.

Exactly how proteasome is inhibited by ischemia is not clear. The study by Bulteau et al. (21) suggests that proteasome subunits are sensitive to oxidative inactivation, which is consistent with in vitro studies showing that exposure of purified proteasome preparations to oxidants, such as 4-hydroxy-nonenal (67, 166), peroxynitrite (5, 170), hypochlorite, and hydrogen peroxide (180), leads to inactivation. Indeed, a recent study (110) has identified the 19S-subunit ATPase, Rpt6, as exceptionally sensitive to oxidative inactivation. With regard to cardiac ischemia, we (43) have shown that pretreatment of isolated hearts with α-tocotrienol, a vitamin E analog, preserves postischemic proteasome function. Although all of these studies support the notion that proteasome is sensitive to oxidative injury, it must not be forgotten that protein ubiquitination and unfolding are ATP dependent (77) and that during ischemia, ATP can be depleted (150, 208) and thus may be a contributing factor.

Given that the UPS degrades numerous proteins and regulates multiple signaling pathways, it is a reasonable assumption that dysfunction of this complex during ischemia could have profound effects on myocardial function. We (176) have observed that degree of proteasome inhibition during reperfusion is dependent on length of ischemia and seems to correlate with levels of oxidized and ubiquitinated proteins and have suggested that the process known as dysregulation is occurring in which there is failure to degrade normal substrates of the proteasome. In support of this hypothesis, we (43) have recently shown that preservation of proteasome function with tocotrienol pretreatment decreases postischemic levels of phosphorylated c-SRC, which signals for ubiquitination and degradation by proteasome (93), and which is also known to be upregulated during ischemia and associated with poor outcomes (97). Further, we (51) have recently demonstrated that pretreatment of isolated hearts with the proteasome inhibitor lactacystin results in a greater accumulation of oxidized proteins and diminished degradation of oxidized actin in the postischemic heart, implying a mediatory role for proteasome. On the basis of these studies, we (176) have proposed a model (see Fig. 9) whereby conditions that foster excessive inhibition of the proteasome impede removal of oxidized proteins by the 20S proteasome, hindering recovery, and numerous proteins regulated by the 26S proteasome, some of which may be

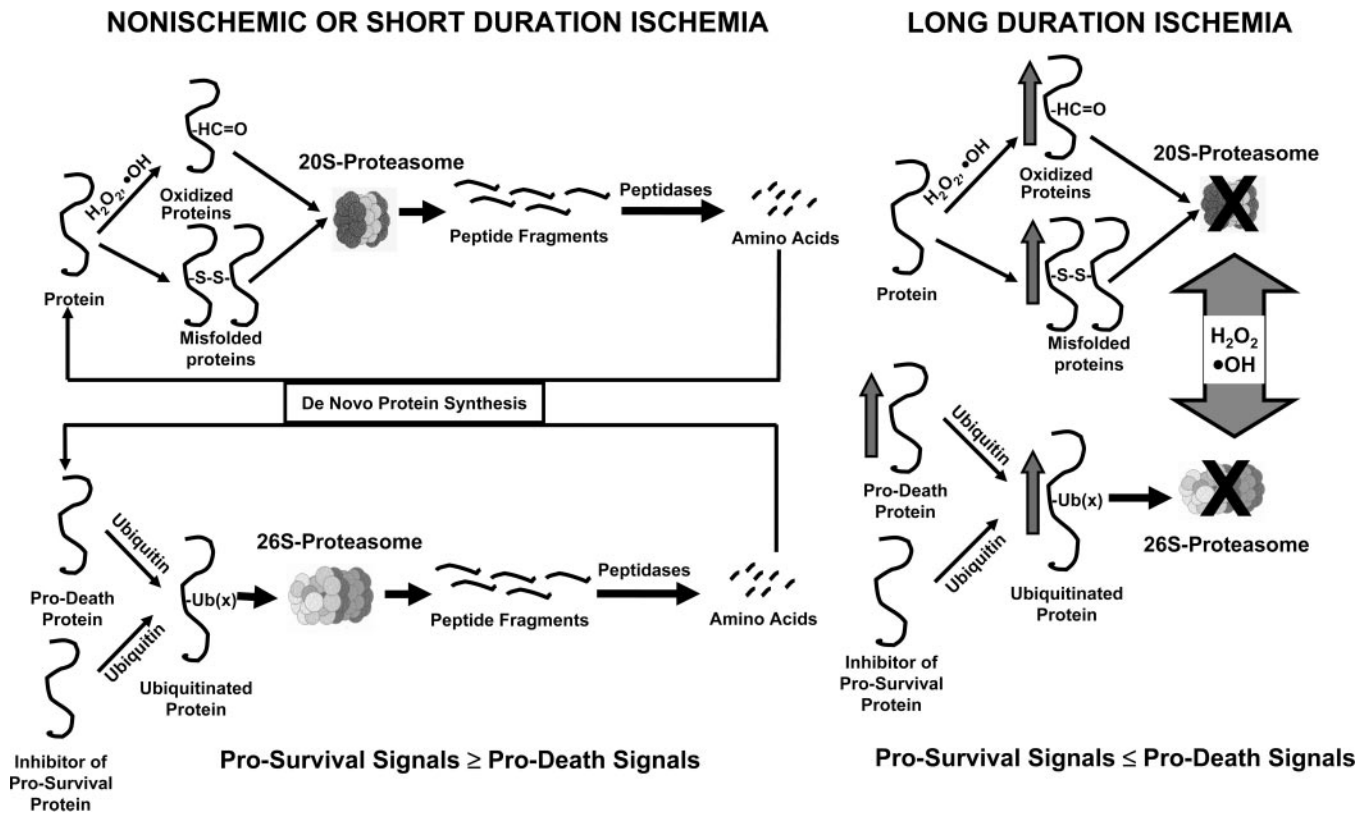

Fig. 9. Scheme illustrating potential roles for 20S and 26S proteasomes in short- and long-duration ischemia. In the nonischemic heart, oxidized, misfolded, and ubiquitinated proteins are degraded through both ubiquitin- and nonubiquitin-mediated pathways, recycling the constituent amino acids, and maintaining a dynamic balance between prosurvival and prodeath signals. During an ischemic insult resulting in cell death or dysfunction, proteasome function is inhibited, leading to accumulation of oxidized and ubiquitinated proteins. In addition, a condition known as dysregulation may occur in which normal proteasome-mediated degradation of prodeath proteins is depressed. Ub (x), multiubiquitin. [Reprinted from Powell et al. (176) with permission from Mary Ellen Liebert Publishers.]

prodeath, would accumulate, thus pushing the cell toward death.

Proteasome inhibitors during myocardial ischemia: a controversy visited. The preceding discussion would appear to preclude the use of proteasome inhibitors in the ischemic heart, yet there is a handful of studies in the literature that suggest that this strategy may actually be beneficial. Two studies (23, 178) have tested the proteasome inhibitor PS-519 (Millennium Pharmaceuticals), with the rationale being that the inhibitor would decrease leukocyte adhesion to endothelial cells, thus limiting the inflammatory response. One of these studies (23) used the leukocyte-supplemented perfused heart preparation but failed to observe any effect, positive or negative, in the absence of the leukocytes, raising the possibility that the effect of the inhibitor was extracardiac. In both studies, peripheral leukocyte, but not myocardial, proteasome activity was determined, and thus it is not clear if the beneficial effect had any relation to myocardial proteasome. An additional study (204) has shown that preperfusion of isolated hearts with the proteasome inhibitor MG132 improves posthypoxic function of excised isolated papillary muscles. However, this was after a prehypoxic delay of at least 30 min with demonstrable increases in heat shock proteins but no determination of myocardial proteasome activity. This same group has recently shown (153) that incubation of vascular smooth muscle cells with low concentrations of proteasome inhibitors results in upregulation of proteasome subunit transcription and translation, raising the possibility that the previous results (204) were related to increases in proteasome activity. Conversely, we (176) have observed that preischemic treatment of isolated rat hearts with MG132 results in a dose-dependent decrease in postischemic function but increased levels of ubiquitinated proteins. In addition, pretreatment with the more specific inhibitor, lactacystin, according to a protocol that decreased preischemic proteasome activity by 40%, failed to have any effects on postischemic function, beneficial or otherwise (51).

How then can these dichotomies be explained? The ability of proteasome inhibitors to decrease the inflammatory response has been well documented (56) and has been attributed to dysregulation of the NF-κB pathways that are regulated by the UPS at multiple levels (reviewed in Ref. 129). Anti-inflammatory or proapoptotic effects of proteasome inhibitors are notorious for their dose dependence (139). After brief ischemia, when proteasome may be minimally dysfunctional (176), decreasing the inflammatory response with a specific peripherally acting proteasome inhibitor may be beneficial. However, when the possibility exists that proteasome may already be significantly dysfunctional, caution is advisable as additional inhibition may tilt the cell toward death.

Is there then any rationale for use of proteasome inhibitors in myocardial ischemia? Maybe, if it can be shown that the inhibitor specifically targets the inflammatory or other deleterious processes. PR39 is a basic proline/arginine-rich antibacterial peptide originally derived from porcine intestine. This

peptide is a noncompetitive and reversible allosteric inhibitor that acts by perturbing the conformation of the noncatalytic α7-subunit such that cleavage of certain substrates, such as inhibitor κB (IκB) and hypoxia-inducible factor-1α is inhibited, yet overall activity of the proteasome is not affected (72, 74). PR39 has been shown to decrease postischemic inflammatory responses in intestine (127) and to improve postischemic cardiac function and decrease infarct size in a rat LAD occlusion model (12) by interfering with NF-κB signaling through inhibition of IκB degradation. Thus it seems that the strategy of targeting degradation of specific proteasome substrates may be a viable therapeutic option warranting additional study.

Proteasome Dysfunction in Cardiomyopathy

The UPS plays a major role in protein quality control by degrading misfolded, unassembled, or otherwise damaged proteins that could form potentially toxic aggregates. Abnormal misfolded proteins are rapidly removed from the endoplasmic reticulum (ER), transported to the cytosol, polyubiquitinated, and degraded by the proteasome in a process known as ER-associated degradation (ERAD), which is one component of the overall unfolded protein response (UPR) (reviewed in Ref. 107). Cells that fail to remove these abnormal proteins and maintain ER homeostasis are subject to programmed cell death through a process that has become known as ER stress (159). ER stress-mediated cell death has been associated with numerous pathological conditions linked with accumulation of aggregates of the misfolded proteins, including Alzheimer's disease (amyloid), Parkinson's disease (15), amyotrophic lateral sclerosis, Huntington's disease, and also islet cell death in diabetes (reviewed in Ref. 233). αB-Crystallin is a small heat shock protein that possesses molecular chaperone activity. A missense mutation of this gene is associated with desmin-related myopathies characterized by accumulation of aggregates of desmin in skeletal and cardiac muscle (187, 220). Upregulation of desmin and/or accumulation of desmin-containing aggregates has been associated with congestive heart failure (100) and desmin-related cardiomyopathy (187, 223), while a missense mutation of the desmin gene has been associated with idiopathic dilated cardiomyopathy (136). Furthermore, animal studies have associated ER stress with hypertrophic cardiomyopathy in the transverse aortic constriction model (165). Other mutations, such as in the *MYBPC3* gene, which encodes for cardiac myosin binding protein C (163), as well as several involving myosin (167) and the troponins (138), are associated with familial hypertrophic cardiomyopathy, although whether these are associated with ER stress is not clear.

Nonetheless, evidence is accumulating that proteasome dysfunction might be associated with these cardiomyopathies. Recent studies have shown that in vitro treatment of cells or in vivo treatment of mice with the ER stress-inducing agent thapsigargin results in significant impairment of proteasome function (154). Likewise, treatment of neuronal cells with amyloid-β peptides has been shown to impair proteasome function (164). With regard to the heart, rat neonatal cardiomyocytes induced to express truncated cardiac myosin binding protein C mutants formed hyperubiquitinated aggregates of these mutants associated with impaired proteasome function (189). Chen et al. (31) have shown that transgenic mice with cardiac-specific overexpression of a missense mutation of αB-crystallin exhibit accumulation of ubiquitinated proteins associated with loss of key subunits of the 19S regulatory complex and impaired UPS function, which was dependent on formation of aberrant protein aggregates. Indeed, very recent studies (141, 142) by this same group utilizing cardiomyocytes expressing a mutant desmin indicate that this abnormal protein impairs UPS function by decreasing delivery of substrates to the catalytic chamber via a process requiring protein aggregation. Furthermore, reports of increased ubiquitinated proteins in explants from failing human hearts (128, 224) support the notion of a dysfunctional proteasome in these pathologies. Taken as a whole, these studies suggest that ER stress-related cardiomyopathies are multifaceted disorders in which the mutated protein, probably by aggregate formation, impairs proteasome function, leading to a progressive impairment of clearance of these misfolded or mutated proteins, allowing them to accumulate within the cell and leading to further proteasome dysfunction. The mechanism of this inhibition is not clear but may be related to a physical plugging of the proteasome core as suggested by Sitte et al. (196) for lipofuscin/ceroid (see next section). Regardless, these studies underscore the importance of proteasome function in maintaining normal cardiac size and structure, which if impaired can lead to hypertrophy and ultimately failure.

Proteasome Dysfunction in Senescent Cardiomyocyte Loss

Decreased function, loss of cardiomyocytes, and increased vulnerability to injury is a phenomena commonly associated with the aging myocardium. Apoptosis has been proposed as one mechanism to account for the loss of cardiomyocytes (113, 161) and is a feature common to many senescent organs (reviewed in Ref. 105). That dysfunction of the UPS might in some way account for cardiomyocyte apoptotic cell death is suggested by the numerous reports demonstrating decreased activity with age in a variety of tissue types, including muscle (61), neuronal (117), retinal (144), lens (39, 195), epidermal cells (172), renal, liver, and lung (116), and of course, heart (22, 116). The mechanism of age-related loss of activity is not clear, and various processes have been proposed. Many studies have shown downregulation of some proteasome subunits in aging heart (22), epidermis (172), and spinal cord (117). Indeed, a recent study (47) suggests that mRNA expression of the E3, MURF-1, and the ubiquitin conjugating enzyme, $E2_{14k}$, are decreased in soleus muscle from aging rat. Other studies have suggested that various subunits of the proteasome are subject to posttranslational modifications that alter their activity (reviewed in Refs. 26 and 32).

An alternative hypothesis is suggested by the studies of Sitte et al. (196), who observed that when human lung fibroblasts were exposed to synthetic lipofuscin/ceroid in culture, cytoplasmic proteasome activity was decreased. Lipofuscins are deposits of large insoluble aggregates of cross-linked oxidized proteins that accumulate with age generally in the lysosomes of cells, their presence considered to be a hallmark of aging. The mechanism for formation or accumulation of these aggregates is not clear but is thought to involve oxidative processes, reduced clearance by lysosomes, or enhanced autophagocytosis (reviewed in Refs. 20 and 212). Numerous studies report accumulation of lipofuscin-like aggregates in postmitotic se-

nescent tissues, many of which also have diminished proteasome function, such as heart (203), retina (60), and neurons (230). In all cases, senescence was associated with accumulations of oxidized and/or ubiquitinated proteins, substrates for the proteasome (22, 116, 144). Moreover, it has been shown that when fibroblasts (211, 222) and cardiomyocytes (198) are either loaded with or induced to form lipofuscin, they assume a senescent phenotype and are more susceptible to oxidative injury.

An attractive hypothesis is that accumulation of lipofuscins in cardiomyocytes over a lifetime leads to proteasome dysfunction and subsequent cell death. We (175) have recently shown that incubation of rat neonatal cardiomyocytes with synthetic lipofuscin/ceroid results in uptake and internalization of the aggregates and rapid induction of apoptotic cell death such that >50% of the cells are dead within 48 h. Moreover, cardiomyocyte apoptosis was associated with significant proteasome dysfunction, increases in proapoptotic proteins degraded by this complex, and accumulation of ubiquitinated homologues of Bax, suggesting that lipofuscin accumulation can lead to dysregulation of proteasome-regulated proteins. Exactly how lipofuscins inhibit proteasome is unknown and is the subject of additional studies, but the studies of Sitte et al. (196) suggest a direct action, possibly by physical plugging of the core channels. In total, these studies suggest that age-related dysfunction of the proteasome may result from multiple mechanisms and that the consequence in the cardiomyocyte may be apoptotic cell death.

PITFALLS OF PROTEASOME STUDIES

As with any area of research, there are certain pitfalls that can be associated with studies of proteasome, the more common of which can be divided into two groups: misinterpretation of results from studies using pharmacological inhibitors of proteasome, and inability to accurately assess proteasome activity. Perhaps the most serious of these is the first one. Numerous studies, including our own (51, 174, 176), have used inhibitors to implicate proteasome in some facet of cardiac function. Many of these studies have utilized MG132, which besides being reversible, has rather low specificity (reviewed in Ref. 123), also inhibiting serine proteases, such as calpain and cathepsins, at concentrations not much different from that which inhibit the proteasome. Even lactacystin, which is much more specific than MG132, does have other intracellular targets. It is incumbent on individual investigators to design their experiments in such a way as to minimize the nonproteasome effects of these compounds either by concentration adjustment or appropriate controls and to take these other activities into account when interpreting results. The danger is that proteasome will be implicated in some process simply because MG132, or some other inhibitor, has an effect when that might not be the case. Although it is understood that MG132 is more economical than many of the more specific inhibitors, this author is of the opinion that this inhibitor should not be considered as a first choice, if it can at all be avoided. In Table 3, characteristics and other intracellular targets of some of the common and uncommon proteasome inhibitors are presented. The reader should note that proteasome inhibition may be a consequence of treatment with some chemicals, such as curcumin (111), used to inhibit other pathways, so this needs to be considered as well. For a more complete review on proteasome inhibitors, readers are referred to Refs. 71, 91, and 123.

The second pitfall involves the methods used to assess proteasome activity. The most common of these techniques follows cleavage of fluorogenic peptide substrates, such as Suc LLVY-amc, to monitor 26S proteasome activity. As pointed out by Kissalev and Goldberg (124) in a recent review, interpretation of results obtained with the use of these peptides can be misleading. For one, many investigators make the incorrect assumption that simply assaying 20S proteasome activity will accurately reflect the state of the 26S proteasome. Activity of the 26S proteasome is dependent on the presence of ATP,

Table 3. *Characteristics of proteasome inhibitors*

Inhibitor Class	Example	Mechanism	Reversibility	Activity	Specificity	Other Intracellular Targets
Peptide aldehydes	MG132	Hemiacetal formation → catalytic β-subunits	Reversible	CT>>>C>T	Low	Calpains, cathepsins
Peptide boronates	MG262, PS341	Tetrahedral adduct → β5	Slowly reversible	CT	High	None known
Peptide vinyl sulfones	NLVS	Covalent adduct formation → catalytic β-subunits	Irreversible	CT>>T>C	Moderate	Cathepsins S and B
Peptide epoxyketones	Epoximicin	Morpholino formation → catalytic β-subunits	Irreversible	CT>>>T>C	High	None known
Lactacystin (spontaneous transition → clasto-lactacystin-β-lactone)		Acylation → all catalytic β-subunits	Very slowly reversible	CT>>T>C	Moderate	Cathepsin A, tripeptidyl peptidase II
Cathelicidins	PR-39	Allosteric at α7 (72)	Reversible	HIF-1α, IκB, possibly others		
Other	Green tea polyphenols [e.g., (−)-epigallocatechin-3-gallate]			CT	Unknown	Unknown (131)
	Curcumin	Unknown	Unknown	Unknown	Unknown	Unknown (111)
	Gliotoxin	Mixed disulfide → β5(?)	Reversible by DTT	CT	Unknown	Unknown (130)

Characteristics of proteasomes are updated and modified from Kissalev and Goldberg (123) and Hermann et al. (102). CT, chymotrypsin-like; T, trypin-like; C, caspase-like; HIF-1α, hypoxia-inducible factor-1α; IκB, inhibitor-κB; DTT, dithiothreitol.

which is necessary for the 19S-regulatory complex ATPases to open the channels in the α-subunit ring, thus allowing free access to the catalytic core. On the other hand, many factors, such as presence of detergents, repetitive freeze-thaw cycles, and even certain fluorogenic peptides, may trigger channel opening and lead to spurious, inconsistent measurement of 20S proteasome activity (124). We routinely measure activity of proteasome in the presence and absence of ATP, and Kissalev and Goldberg (124) suggest determining multiple peptidase activities of the proteasome by using multiple fluorogenic peptides that target different activities. For animal studies, one possible means of working around these issues might be the use of transgenic animals expressing a green fluorescent protein (GFP)-CL1 degron fusion protein (also known as GFP^u), which is a substrate for the proteasome (52). This technique is based on proteasome-mediated degradation of the GFP-fusion protein, which is monitored using fluorescence microscopy (42, 140) and has been suggested to dynamically monitor in vivo proteolytic activity (52). Wang and coworkers (31, 132) have recently used these mice to show changes in cardiac proteasome function after doxorubicin treatment and in a model of intrasarcoplasmic amyloidosis. The development of these transgenics is an exciting innovation that warrants additional studies to determine potential research applications. It may turn out that using a combination of the transgenic model and the fluorogenic peptides is the best strategy for assessing proteasome function in experimental models.

FUTURE DIRECTIONS AND CONCLUSIONS

The role of the UPS in cardiac physiology and pathology is a relatively new and exciting field of research. Investigators are defining roles for the UPS in maintaining normal cardiac function through regulation of signaling pathways and maintenance of normal sarcomere structure. Other studies are beginning to show that downregulation of ubiquitin protein ligases may play pivotal roles in the response of the myocardium to hypertrophic stimuli, and still others suggest that dysfunction of the proteasome may be important in cardiac pathologies, such as ischemic injury and cell death, certain cardiomyopathies, and even senescent cardiomyocyte loss. Although this area is rapidly changing and evolving, progress could be hampered without additional studies that further our understanding of basic proteasome biochemistry and physiology, particularly with regard to the heart. More information defining molecular events that regulate assembly and architecture of the cardiac proteasome is a necessity with the possibility of targeting specific substrates for degradation. A more accurate means for consistent assessment of UPS function would aid our understanding of how proteasome dysfunction may cause cardiac pathologies. A better understanding of the cardiac-specific E3 ubiquitin protein ligases, the specificity and rate-limiting step of the UPS, are necessary as this might be the one step most amenable to therapeutic intervention. From this review it should be apparent that the UPS plays a significant role in regulating normal cardiac function and is a critical regulator of cell life and death. Ongoing research in this challenging area has the potential to lead to development of therapeutic interventions that could significantly impact clinical care.

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