Introduction

Since its discovery in 1979 (65), the ubiquitin proteasome system (UPS) has been the subject of intensive research and witnessed some exciting developments. Researchers have been investigating the UPS and defining its importance in normal physiology and in pathological conditions. Studies have described roles for the UPS in cell-cycle control (19, 64), cell signaling (22, 106), apoptosis (76, 154), immune response and antigen presentation (34, 85, 101), transcription regulation (22, 106), protein quality control (157, 163, 164), and protein turnover under normal and pathological conditions (21, 61, 117, 145, 147). In regard to cardiovascular disease, the majority of studies suggest that at some point the UPS becomes dysfunctional in several pathologies including familial and idiopathic cardiomyopathies (17, 116, 139, 156), atherosclerosis, and myocardial ischemia. This review will focus in on the role of the UPS in myocardial ischemia. As a necessity, short descriptions of the structure, organization, and regulation of the UPS with a focus on the cardiac proteasome will be presented. These will be followed by a synopsis of the current knowledge of how the UPS is affected by myocardial ischemia and its potential roles in ischemic preconditioning (IPC). As part of each topic, any controversies or new hypotheses will be presented with a discussion of potential new directions.

Structure and Organization of the UPS

The four components of the UPS include the proteasome (in its various forms), ubiquitin, the ubiquitination machinery, and the deubiquitinases (DUBs).

The 20S proteasome. The 20S proteasome is the central proteolytic structure of the UPS and is a barrel-shaped structure consisting of two pairs of rings each containing seven subunits (Fig. 1). The constitutive proteolytic activity resides in the inner two rings that contain the β-type subunits, designated β1 through β7. Three subunits are responsible for this activity: the β1 subunit which has “caspase-like” activity, the β2 subunit which has trypsin-like activity, and the β5 subunit which has chymotrypsin-like activity. These three subunits can be replaced by isoforms which are designated βi1, βi2, and βi5 (38). Earlier studies indicated that the isoforms were upregulated and incorporated into the 20S proteasome in response to exposure to γ-interferon primarily in immune cells. Replace-ment with these isoforms confers two additional proteolytic activities to the 20S proteasome: BrAAP, which is hydrolysis of peptide bonds after branched chain amino acids, and...
SNAAP, which is hydrolysis after small neutral amino acids. These changes favor the formation of peptides consistent with the major histocompatibility complex class I antigens; thus, proteasomes containing these isoforms are known as “immunoproteasomes” (102). However, this might be a misnomer because in depth proteomic analysis of the cardiac proteasome has also identified these isoforms in the constitutive proteasome in nonimmune cells. Once considered to be homogenous, in fact the β-subunit containing rings are quite heterogeneous and exist as a dynamic mixture of both constitutive and immunofrom catalytic β-type subunits (33, 47). Sitting atop and aligned counter to the inner β-rings are the two outer rings containing the α-type subunits, designated α1 through α7 (Fig. 1). In the eukaryotic proteasome these subunits have no direct proteolytic activity, but subunits α1, α2, α3, α6, and α7 project into the openings at either end of the proteasome, sealing it to prevent access to the central chamber. When activated, the NH2-termini of these subunits retract and allow access of substrate into the proteolytic chamber (52). Molecular modeling studies have revealed that the 20S proteasome is arranged such that three chambers are present: two outer antechambers and one inner chamber containing the exposed active sites of the six catalytic subunits.

**Different proteasome isoforms (zomes) and associated regulatory particles.** Proteasomes can be defined by the regulatory particles that sit at either end of the barrel. The process of mating of a regulatory particle at one or both ends of the 20S proteasome is called “docking.” There are at least three proteasome regulators described in eukaryotes that can be loosely classified according to whether or not they use ATP. Those that do not use ATP include the 11S activator ring and the PA200 activator. The ATP-utilizing activator is the 19S regulatory particle (135). The PA200 activator appears to be associated with DNA repair and genomic stability (8, 93, 149). Although subunits of this regulatory particle have been detected in heart (47), little else is known about its function. The following is a discussion of the 19S regulatory particle and associated ubiquitination machinery and the 11S activator ring.

**The 19S regulatory particle and associated ubiquitination machinery.** The 19S regulatory particle is a large macromolecular structure containing an additional 18 subunits arranged in two distinct subcomplexes, the “base” and the “lid” (Fig. 1). The base contains six subunits, regulatory peptide triple A 1–6 (Rpt1 through Rpt6), that have ATPase activity and are arranged in a ring. Also included are the two largest of the regulatory particle non-ATPase subunits, Rpn1 and Rpn2. Some investigators include Rpn10, the ubiquitin recognition domain, as part of the base, and others do not. The base ATPase subunits use ATP to unfold the substrate and induce a conformational change in the α-subunits to open up the entrance channel to the catalytic chamber and thus activate the 20S core (121, 135). The Rpt2, Rpt5, and Rpn2 subunits play important roles in the attachment of the “base” to 20S proteasome α-rings and binding of the 19S particle “lid” to the “base” (26, 43, 49). The “lid” contains the remaining non-ATPase subunits, Rpn3 through Rpn12. As indicated, Rpn10 contains the main ubiquitin binding domain. Rpn11 is one of the intrinsic deubiquitinating enzymes. The functions of the remaining subunits are somewhat obscure. If just one 19S regulatory particle is docked at one end (mushroom configuration), the entire complex is known as the 26S proteasome. If two particles are docked, one at each end (dumbbell configuration), the complex is known as the 30S proteasome. The 19S regulatory particle confers selectivity for ubiquitin-conjugated proteins. The 26S or 30S proteasomes mediate the process known as ubiquitin-mediated degradation of proteins.

**Ubiquitin-mediated degradation of proteins and associated ubiquitination machinery.** Over the past several years, there have been many advances in understanding the process of ubiquitination, and there are numerous excellent recent reviews on this topic; many cited throughout this section (12, 27, 50, 51, 68, 69, 81, 82, 126, 128, 130, 131, 150, 158). What follows is a brief description representing the currently accepted sequence of events that lead to the ubiquitination of a protein. The ubiquitination of a protein is an energy-requiring multistep E1-E2-E3 cascade that results in addition of ubiquitin to the...
e-NH$_2$ of a lysine residue (Fig. 2). Proteins can be either monoubiquitinated at a single substrate lysine, multubi-quitinated at several substrate lysines, or polyubiquitinated in which chains of ubiquitin are added to one or more substrate lysines. Ubiquitin itself contains seven lysines, all of which have the potential to be ubiquitinated or polyubiquitinated, although the most common appear to be Lys48 and Lys63. It is generally accepted that polyubiquitination through ubiquitins’ Lys48 targets the substrate for proteasomal degradation. In the first step, a ubiquitin-activating enzyme (E1) binds ATP·Mg$^{2+}$ and ubiquitin (or a ubiquitin-like protein) and then catalyzes ubiquitin COOH-terminus adenyla- tion. This is followed by attack- ing of the catalytic E1-Cys on the ubiquitin-adenylate complex to form the activated E1-ubiquitin thioester complex. In hu- mans, eight E1s that initiate ubiquitin or ubiquitin-like conjugation have been identified (50, 131). In the second step, the E1 transfers the activated ubiquitin (or ubiquitin-like protein) to a family of proteins that are called ubiquitin-conjugating en- zymes (E2). These form a ubiquitin-E2 high-energetic conjugate through a highly conserved E2-Cys residue. The E2s play an essential role in conjugation and, by influencing which of the ubiquitins’ seven lysines is conjugated with the substrate, can also play a role in determining the ultimate fate of the sub- strate. There are at least 35 E2s that have been described in humans, and these are classified into one of four classes based on the presence of extensions to the NH$_2$- or COOH-terminus of the catalytic core (150, 158). The third and final step is the selective interaction of the loaded E2 with a specific ubiquitin protein ligase (E3). E3s selectively recruit specific substrates and act as scaffolds, essentially bringing the activated ubiquitin into close proximity with the protein substrate. E3s represent the specificity of the UPS with over 600 having been identified at the time of this writing (27). E3s are generally classified into two basic groups: homologous to E6-AP COOH-terminus (HECT) and really interesting new gene (RING), which includes the U-box containing proteins, such as carboxyl terminus of Hsc70-interacting protein (CHIP). The basic differences between them is that HECT E3s use a conserved Cys residue to form a transient thioester linkage with the activated ubiquitin COOH-terminus before substrate ubiquitination. The RING E3s act as true scaffolds, binding both ubiquitin and substrate, providing an optimal conformation for conjugation of the ubiquitin COOH-terminus with the ε-amino group of one or more substrate lysines (69, 126, 128, 130, 175).

Deubiquitinases. DUBs are isopeptidases that belong to a superfamily of ~80 ubiquitin-specific proteases. These main- tain cellular ubiquitin homeostasis and counter the ubiquitina- tion process by removing ubiquitin from substrates and by disassembling polyubiquitin chains. DUBs are classified into one of five structurally unrelated categories. Of these, the ubiquitin COOH-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumour proteases (OTUs), and the Josephins are cysteine proteases. The JAB1/MPN/MOV34 family are zinc metalloproteases. DUBs have three main ac- tivities. The first is to generate free ubiquitin molecules from polyubiquitin expressed as a linear fusion protein. The second is to reverse ubiquitin signaling by removing ubiquitin chains from ubiquitinated substrates, which may lead to protein stabilization. If the protein is committed to degradation, the DUB can recycle the ubiquitin. Lastly, DUBs can trim ubiquitin chains and thus alter the form of ubiquitin modification to exchange one type of ubiquitin signal for another (i.e., receptor polyubiquitination degradation signal for monoubiquitination internalization signal). DUBs have considerable specificity for substrates and types of ubiquitin chains and are themselves subject to regulation by post-translational modifications, such as phosphorylation, and subcellular localization (12, 68, 82). With the exception of one study (23) that suggests that the DUB enzyme breast cancer-1 (BRCA-1)/BRCA-2-containing complex subunit 36 (BRCC36)-containing isopeptidase complex (BRISC), which deubiquitates Lys63-linked polyubiquitin, has a protective role in myocardial ischemia-reperfusion injury, little else is known about the role of this class of ubiquitin associated proteins in the heart.

The 11S-activated proteasome. The 11S-activated protea- some is a different proteasome configuration (“zones”) that uses the 11S activator ring as its regulatory particle. The 11S-activated proteasome consists of a 20S proteasome that can be docked with one or two 11S activator rings or one 19S reg- ulatory particle on one end and an 11S activator ring on the other end, called the hybrid proteasome. The 11S activator ring is a heterohexamer or heteroheptamer consisting of three protease activator 28α (PA28α) and three protease activator 28β (PA28β) subunits or three PA28α and four PA28β subunits, respectively, arranged in an alternating pattern (Fig. 3) (134, 170). When an 11S activator ring docks with a 20S protea- some, the carboxy-terminus tails of the PA28 subunits insert into pockets created by the α-subunits, resulting in a confor- mational shift that opens the access channel to a greater degree, enhancing substrate access to the catalytic chamber. This re-

![Fig. 2. Ubiquitination of a protein. In step 1, a ubiquitin-activating enzyme (E1) binds ATP-Mg$^{2+}$ and ubiquitin and then catalyzes ubiquitin COOH-terminus adenylation followed by attack of the catalytic E1-Cys on the ubiquitin-adenylate complex to form the activated E1-ubiquitin thioester complex. In step 2, ubiquitin is transferred to a ubiquitin-conjugating protein E2, producing a high-energy E2-ubiquitin thiol ester intermediate. In step 3, the intermediate is ligated to a protein substrate bound to a specific ubiquitin protein ligase E3. Modified from Powell and Divald (110) with permission from Oxford University Press.](image-url)
Proteomic studies have demonstrated a high variability in the distribution of subunits in the murine heart (155) have also been reported. The presence of multiple proteasome subpopulations may regulate degradation of a particular protein or class of proteins, which in turn may alter the outcome of a particular disease.

**Post-translational modification as a basis for regulation.** Proteasomes can be regulated by both reversible and irreversible post-translational modifications. Examples of reversible modifications include phosphorylation, ubiquitination, sumoylation, and a 19S regulatory particle docked at the end that, if it contains β-subunit immunoforms, has also been called the immunoproteasome. Modified from Powell and Divald (110) with permission from Oxford University Press.

The following discussion focuses on the cardiac proteasome but may be applicable to proteasomes found in other tissues.

**Heterogeneity as a basis for regulation.** For many years, the prevailing hypothesis was that the two β-rings of the catalytic core of the 20S proteasome consisted of seven homologous β-subunits. The distribution of subunits β1, β2, and β5 were believed to be all constitutive or all replaced by the immunoform subunits β1i, β2i, and β5i. Several relatively recent studies have challenged this view and have provided novel evidence of heterogeneity within the β-rings (32, 45–47). Proteomic studies have demonstrated a high variability in cardiac proteasomes with regard to the distributions of the β-subunits. In addition to proteasomes having all constitutive or immunoform subunits, proteasomes may also contain one β-ring of constitutive subunits and one β-ring of immunoform subunits. Proteasomes may also be composed of a mixture of constitutive and immunoform subunits within each individual β-ring. Consistent with these varying structures, these subpopulations of 20S proteasomes, it is not surprising, exhibit varying degrees of proteolytic function and substrate specificity (32, 45, 166) (Fig. 4). In regard to the 19S regulatory particle, heterogeneity in the form of alternate splicing of the Rpn10 subunit (47) and two different populations of 19S regulatory particles in the murine heart (155) have also been reported. The presence of multiple proteasome subpopulations may regulate degradation of a particular protein or class of proteins, which in turn may alter the outcome of a particular disease.

Fig. 3. The 11S-activated proteasome and the related hybrid proteasome. 11S-activated proteasomes are formed when an 11S activator ring docks at one end of a 20S proteasome. The 11S activator ring is composed of either 3 protease activator (PA) 28α and 3 PA28β subunits (shown) or 3 PA28α and 4 PA28β subunits and may be induced by interferon-γ. One form of an 11S-activated proteasome has been called the immunoproteasome and contains one or more of the immunoforms of the catalytic β-type subunits. Another form of an 11S-activated proteasome does not contain β-subunit immunoforms. Also shown is the hybrid proteasome which has a 11S activator ring docked at one end of the 20S proteasome and a 19S regulatory particle docked at the end that, if it contains β-subunit immunoforms (shown), has also been called the immunoproteasome. Modified from Powell and Divald (110) with permission from Oxford University Press.
lation, O-linked N-acetylglucosamination, and possibly some oxidative modifications. Irreversible modifications may include NH$_2$-terminus acetylation and NH$_2$-terminus myristoylation, as well as some oxidative modifications. For additional information, the reader is referred to an excellent review (132) on post-translational modification of the proteasome that has recently been published as part of the American Journal of Physiology-Heart Circulatory Physiology review collection on the “Role of the Ubiquitin Proteasome System in Cardiac Disease.”

**Associating partners.** In general, the associating partner of the proteasome is often responsible for the post-translational modification discussed above. The best example of this is phosphorylation which can be mediated by several associating kinases, such as cAMP-dependent protein kinase A (PKA), calcium/calmodulin-dependent protein kinase II (CaMKII), and casein kinase II, which may phosphorylate multiple proteasome subunits including the α2, α3, α5, α7, β1, β2, β3, β5, β6, and β7 on the 20S proteasome and Rpt6 on the 19S. In general, phosphorylation of proteasome subunits tends to stabilize the proteasome and increase activity (10, 18, 31, 47, 90, 129, 141, 155, 167, 169, 176, 177). Phosphorylation of Rpt6, in particular, appears to enhance docking and stabilize the 26S (or 30S) proteasome (129, 169). The known exception to this is apoptosis signal-regulating kinase 1, which phosphorylates Rpt5, decreasing its ATPase activity and overall proteasome activity (148). In contrast, the associating serine threonine protein phosphatases PP2A and PP1 dephosphorylate various proteasome subunits and, in general, decrease activity (47, 90, 176, 177). With regard to the 19S regulatory particle, a study has reported that heat shock protein 90 can associate with this regulatory particle and in doing so decreases functional potency (155). Inhibiting the associating heat shock protein 90 enhanced activity of the proteasome. We have proposed that the cardiac proteasome is actually part of and regulated by signal transduction pathways (107) (see Fig. 5), which is relevant to UPS dysfunction during myocardial ischemia as these pathways may become dysregulated.

**UPS Dysfunction in Ischemia**

Ischemia is reduced blood flow causing oxygen deprivation, reduced substrate supply, and decreased metabolite removal. Most studies have observed that the UPS becomes dysfunc-

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**Fig. 4. Regulation of 20S proteasomes by assembly of heterogeneous configurations.** Under the influence of γ-interferon, select β-subunits are replaced by their immunoforms, which are directed by the complete α-rings to form half-proteasomes with specific configurations. In the example shown above, the top half-proteasome has constitutive β2 and β5 subunits and the β1 subunit has been replaced by the immunoform. The bottom half-proteasome has a constitutive β1 subunit, but the β2 and β5 subunits have been replaced by their immunoforms. These two distinctly different half-proteasomes mate to form a mature 20S proteasome that has a heterogeneous configuration. For the sake of clarity, the involvement of the proteasome assembly chaperones PAC1, PAC2, PAC3, and PAC4 and the proteasome maturation protein factor POMP are not depicted here. Modified from Powell (107) with the permission of Walters Kluwer Health.

**Fig. 5. Regulation of proteasome activity by kinase cascades.** In this scenario, binding of an agonist with a cell surface receptor initiates a phosphorylation cascade with an end effector protein kinase kinase kinase (PKKK) phosphorylating some transcription factor that can then enter the nucleus and interact with DNA resulting in gene transcription. Phosphorylation of signaling intermediates, in this case the transcription factor, is often a signal for ubiquitination and targeting to the 26S-proteasome for degradation. To amplify or dampen the effect of the signaling intermediate, any one of the protein kinases (PKs) within in the cascade might phosphorylate proteasome subunits, resulting in increased or decreased peptidase activity that would lessen or enhance availability of the signaling molecule accordingly. Another possibility would be for the gene product to activate a different protein kinase (PK1), which would phosphorylate proteasome resulting in activation and thus decreased availability of the transcription factor, essentially a form of feedback control. Reproduced from Powell (107) with the permission of Walters Kluwer Health.
ional during myocardial ischemia (11, 114). For the purposes of this discussion, dysfunction means an impaired ability of the UPS to process ubiquitinated proteins. This is usually observed as diminished proteolytic activities in tissue extracts as measured by cleavage of small peptides linked to a fluorogen in combination with increases in total ubiquitinated or misfolded proteins (11, 114). In one study, this has been observed using a UPS reporter mouse expressing a green fluorescent protein fused with the CL1 degron sequence of amino acids (146). In theory, dysfunction of the UPS could involve any one of the components described earlier. While roles for some of the E3 ligases in ischemia have been proposed, they have not been shown to be dysfunctional. As this topic has been reviewed it will not be discussed here (125). The only component shown to be dysfunctional as a consequence of ischemia is the proteasome itself and this will be discussed in detail. Understanding how proteasome dysfunction occurs and how it effects myocardial ischemia is important as underscored by a recent study (146) which shows that even moderate knockdown (50%) of the β5 subunit is sufficient to worsen postischemic function and infarct size. The ensuing discussion starts with the cerebral proteasome since dysfunction was first described in this organ.

Cerebral ischemia. Proteasome dysfunction, first described in 1992, was suggested by an increase in insoluble ubiquitin-conjugates in gerbil cortex and hippocampus following 5 min of transient forebrain ischemia (62). Formation of ubiquitin conjugates appears to be a more a function of reperfusion rather than ischemia and may not be related to proteasome dysfunction (66). Activity of the proteasome has been measured in several models of cerebral ischemia in gerbil, rat, and mouse and in general was found to be decreased in several areas. The degree of dysfunction was generally found to be related to length of ischemia and was reversible with short durations of ischemia (2, 40, 66, 77, 79). Subsequent reports have suggested that proteasome dysfunction is secondary to oxidative damage (79) and disassembly of mature proteasome with deposition of subunits into protein aggregates (40). A very recent report (91) suggests that not only is cerebral proteasome dysfunctional but that a different form of proteasome containing immunofroms may predominate.

Myocardial ischemia. Proteasome dysfunction in myocardial ischemia was first described by Bulteau et al. (11) in 2001 who reported that following 30 min of in vivo left anterior descending coronary artery occlusion, proteasomes exhibit decreased chymotryptic and caspase- and trypsin-like activities coupled with an increase in ubiquitinated proteins. However, following purification, only the decrease in trypsin-like activity was observed. This observation was confirmed in the isolated perfused heart in studies by Powell et al. (109, 114) who also demonstrate that ATP-dependent proteasome activity is preferentially affected. This is consistent with the suggestion that 26S proteasomal insufficiency can result in increased myocardial ubiquitinated proteins. A later study that examined turnover of signaling proteins known to be degraded by the UPS as a means of assessing proteasome function suggested that shorter periods of ischemia may result in selective proteasome dysfunction which may in turn affect turnover of specific proteins (54). In consideration of the presence of multiple proteasome subpopulations and regulation of the cardiac UPS as previously discussed, this is an intriguing observation.

Potential mechanisms involved in proteasome dysfunction. Various mechanisms have been proposed but only two have received experimental support.

1. ISCHEMIA INDUCED ATP DEPLETION. Docking of the 19S regulatory particle to the 20S proteasome, activation of the proteasome, and protein ubiquitination all require ATP (44, 67). Intracellular ATP levels are decreased during ischemia (137). One study has suggested that proteasome activities are regulated by intracellular ATP levels according to a biphasic relationship (increasing concentrations activate up to maxima and thereafter inhibit activity) (70). Therefore, it is reasonable to suggest that ATP depletion during ischemia could be partially responsible for decreased cardiac proteasome activity. In fact, two studies (3, 20) using two different animal models of ischemia, an in vivo canine model and an ex vivo isolated rat heart model, have suggested just this mechanism. Yet, when measuring lyase proteasome activity, ex vivo, adding back the ATP did not reverse the dysfunction. We have shown that lysate postischemic proteasome activity is still diminished following an addition of ATP at concentrations that evoke maximal activation (109). Another rather intriguing study suggests that some proteasomes may actually be activated by low ATP concentrations (41). While these studies may suggest that ATP depletion can be a contributing factor to proteasome dysfunction during ischemia, direct evidence is still lacking. What the ex vivo studies of proteasome activity in lysate do suggest is a defect in ATP utilization by postischemic proteasomes. This has led us and others to postulate that the subunits of the 20S proteasome and its regulatory particles may be targets for oxidative damage, certainly conceivable, given the numerous reports of oxidative stress and oxidized cytosolic, myofibrillar, and mitochondrial proteins in the postischemic heart (16, 80, 103, 111).

2. OXIDATIVE DAMAGE TO PROTEASOME AND/OR REGULATORY SUBUNITS. Oxidative modifications to proteins may lead to unfolding and loss of function because of changes in secondary or tertiary structures. These modifications may also lead to exposure of hydrophobic patches and increased susceptibility to degradation (24, 25). Studies that have examined purified 20S and 26S proteasomes have shown that the 26S proteasome configuration is more vulnerable to oxidative damage (122). The initial report that cardiac proteasome was dysfunctional following ischemia suggested that several of the α-subunits of the 20S proteasome were 4-hydroxynonylated (11). However, it was not clear that the proteasome dysfunction was related to these modified subunits as it was later reported that concentrations of 4-hydroxynonanol in excess of 100 μM are required inactivate purified proteasome from rat hearts, suggesting relative resistance to this type of oxidative modification (37). We have developed evidence that postischemic ATP-dependent proteasome function may be preserved by pretreatment with α-tocotrienol, a vitamin E analog (Table 1) (unpublished data). Taking into consideration the effect of ischemia-reperfusion on ATP-dependent proteasome activity (20, 109) and the reported increased vulnerability of the 26S proteasome to oxidative inactivation (123) would lead one to suggest that perhaps the ATP-utilizing subunits of the 19S regulatory particle are subject to oxidative damage. In fact, the S6 ATPase subunit, Rpt3, has been reported to be sensitive to carbonylation in SH-SY5Y cells exposed to an oxidizing environment (75). This led us to examine the 19S regulatory particle for oxidative damage.
Table 1. Pretreatment of hearts with tocotrienol-rich fraction preserves postischemic proteasome function

<table>
<thead>
<tr>
<th>Treatment</th>
<th>20S</th>
<th>26S</th>
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</thead>
<tbody>
<tr>
<td>PreIschemic</td>
<td>100 ± 17</td>
<td>100 ± 15</td>
</tr>
<tr>
<td>Ischemia (30 min)</td>
<td>55 ± 10*</td>
<td>54 ± 7*</td>
</tr>
<tr>
<td>+ Reperfusion (120 min)</td>
<td>78 ± 16</td>
<td>42 ± 9*</td>
</tr>
<tr>
<td>+ Tocotrienol-rich fraction</td>
<td>144 ± 22</td>
<td>152 ± 15</td>
</tr>
<tr>
<td>+ Reperfusion (120 min)</td>
<td>102 ± 26</td>
<td>112 ± 45</td>
</tr>
</tbody>
</table>

Isolated hearts perfused in the working mode were perfused with buffer containing tocotrienol-rich fraction (0.035%) for 15 min and subjected to 30 min normothermic global ischemia, followed by 120 min reperfusion. Hearts were harvested and 20S and 26S proteasome activities determined as previously described (114). The values are expressed as the percentage of baseline (20S protease, 1,261 ± 210; 26S protease, 612 ± 95 fluorescence U·h⁻¹·mg⁻¹ protein) and represent means ± SE of 5 to 11 hearts. *P < 0.05 (ANOVA) when compared with the preischemic group.

following myocardial ischemia and has identified significant carbonylation of subunits Rpt3 and Rpt5 of the 26S proteasome (29). These were the same subunits that appeared to be oxidized in explants from human hearts from patients with end-stage heart failure (116). These latter studies present the best evidence for a role of oxidative damage to proteasome subunits but do not provide cause and effect for a relationship between the dysfunction and the damage. This required an intervention, in this case IPC, which is described in a later section.

The continuing controversy: can proteasome inhibitors improve outcomes following myocardial ischemia? The use of proteasome inhibitors as a strategy to treat myocardial ischemia is an ongoing controversy that requires some discussion. Several studies have suggested that the use of proteasome inhibitors in myocardial ischemia is beneficial and have examined the proteasome inhibitor PS-519 (Millennium Pharmaceuticals). PS-519 is a lactacystin derivative originally tested and found to have some protective effects in experimental models of cerebral ischemia (7, 160–162). The rationale for testing this inhibitor is that proteasome inhibitors, in general, do have a well-documented anti-inflammatory effect (35) because of interference with UPS-mediated activation of the NF-κB pathways [reviewed in Krappmann and Scheidereit (84)]. The initial study (15) used a leukocyte-supplemented perfused heart preparation and did observe improved postischemic function when hearts were treated with PS-519. However, in the absence of the leukocytes, no effect of the inhibitor was observed. In a subsequent study (120) a single dose of PS-519 administered at time of reperfusion in an in vivo porcine model demonstrated reduced activation of myocardial NF-κB, release of creatine kinase and troponin I, and improved segmental contractile function. However, a more recent study (98) in an in vivo murine model, while showing that PS-519 administered at time of reperfusion decreased infarct size, failed to have any effect on NF-κB activation. When administered 2 h postreperfusion, PS-519 had no effect at all, even though an IκB inhibitor was effective, thus suggesting that any protective effects may not be related to NF-κB signaling.

These types of studies have not been limited to PS-519. Pretreatment of isolated hearts with the proteasome inhibitor MG132 has been shown to improve posthypoxic function of excised isolated papillary muscles (136). However, this was after a delay of at least 30 min in the presence of increased heat shock proteins with no analysis of myocardial proteasome activity. This is in contrast to our studies (30, 114), which demonstrate that preischemic treatment of isolated rat hearts with MG132 results in a dose-dependent decrease in postischemic function and that the inhibitor lactacystin failed to have any effects on postischemic function even though preischemic proteasome activity was decreased by 40%. Other studies have examined bortezomib, a reversible nonselective proteasome inhibitor, in a canine model of left anterior descending coronary artery occlusion and have observed decreased arrhythmias and infarct size when the drug was administered 1 h preligation and then repeated 5 h postligation (168). A follow-up study showed similar effects when bortezomib was administered 1 h postligation (71). In both studies, the protective effect was associated with decreased loss of G protein receptor kinase 2 in the epicardial border zone and decreased proteasome activity in whole blood. Because proteasome activity was not determined in the corresponding epicardial border zone tissue, while an attractive hypothesis, it is unclear that the lack of change in the kinase was related to the inhibitory effect of bortezomib. Nonproteasome targets of bortezomib is an issue as a recent study indicates that this drug inhibits other proteolytic enzymes at potencies near that of the proteasome (1).

Whether a proteasome inhibitor has beneficial or detrimental effects is notoriously dose and time dependent (88, 96). Low nontoxic doses have been observed to upregulate several anti-inflammatory enzymes and confer protection against H₂O₂-induced endothelial cell damage (95). In addition, sublethal doses of proteasome inhibitors may result in upregulation of proteasome subunits (94). It is quite possible that in the presence of mild UPS dysfunction, a low nontoxic dose of an inhibitor that upregulates antioxidant enzymes and subunits of the proteasome and is anti-inflammatory without effecting either UPS-mediated turnover of signaling proteins or protein quality control could be beneficial. From a clinical perspective, this is a tall order that requires specific doses and dosing intervals just not possible at this time with first generation proteasome inhibitors. Given the degree of proteasome dysfunction generally present during myocardial ischemia, there are serious concerns with the use of these inhibitors (108). This is best illustrated by the fact that the only clinically available proteasome inhibitors bortezomib (Velcade, Millennium Pharmaceuticals) and carfilzomib (Kyprolis, ONYX Pharmaceuticals) are indicated for treatment of multiple myeloma. While experience with carfilzomib is limited, bortezomib has been associated with several reports of “unexpected” cardiovascular toxicity, including heart failure and myocardial ischemia (36, 58, 144, 153). This raises the concern about nonselectivity of the first generation proteasome inhibitors. In this instance, nonspecificity refers to their ability to differentiate between constitutive subunits versus the immunofoms. As indicated earlier, proteasomes can be defined as to whether they contain the constitutive catalytic subunits β1, β2, or β5 or the immunofoms β1i, β2i, or β5i, originally observed to be upregulated in the presence of γ-interferon. The vast majority of the first generation inhibitors cannot discriminate between these subunits. Even the newest, carfilzomib, which is selective for the β5 and β5i subunits, does not discriminate between them (174). The importance of subunit selectivity is best illustrated by a very recent study that demonstrates that selective cardiomyocyte-
specific knockdown of the constitutive β5 subunit exacerbates myocardial ischemia-reperfusion injury (146). Does this mean that there is no role for proteasome inhibition as a strategy to decrease myocardial ischemia-reperfusion injury? No, rather, more selective inhibitors that selectively target the different subunits need to be developed. The recent literature suggests that it is a proteasome containing immunofoms, and specifically β1i and β5i, that is responsible for activating the NF-κB inflammatory signaling pathways through processing of IkB (48, 152). ONX-0914 is a second generation proteasome inhibitor that is 20–40 times more selective for the β5i subunit (72, 99). This inhibitor has protective effects and blocks inflammatory cytokine production in models of experimental arthritis (99), lupus-induced nephritis (73), and experimental colitis (6). At the time of this writing, ONX-0914 has not been examined in models of myocardial ischemia; thus it is unclear whether a strategy of specific inhibition of the immunoproteasome would be effective. Clearly, though, this is an area of research requiring additional development.

The UPS in IPC

IPC is an experimental technique that decreases vulnerability to ischemia-reperfusion injury. The current thinking is that IPC involves signaling changes that cause the inward mitochondrial ATP-sensitive K⁺ channel channels to open and prevent opening of the mitochondrial permeability transition pore (9, 53, 83). Several studies suggest that the UPS plays a role in IPC by facilitating some of the pre- and postischemic signaling changes (3, 20, 29). In order for this to occur, it is necessary that IPC preserve postischemic proteasome function in some way. Like the ischemia studies, the earliest investigations suggesting this possibility are found in the brain literature that examined IPC as a means of protection against cerebral ischemia. Most of these studies did not directly assess proteasome function but rather relied on decreased accumulation of proapoptotic proteins and protein aggregates in the postischemic brain as surrogate markers (89, 97, 115). One rather intriguing study suggests that myocardial IPC is prevented in a mouse deficient in the β1i subunit (14). Perhaps the earliest study in the heart to suggest that postischemic function of the proteasome is protected to some extent by preconditioning is a study that used nicorandil to mimic IPC (114). All three of the aforementioned studies in the heart (3, 20, 29) agree that IPC is associated with improved proteasome peptidase activities and decreased accumulation of ubiquitinated or misfolded proteins. Where they differ is in the proposed mechanisms.

IPC preserves UPS function by preventing degradation of ePKC. The heart has been reported to be protected from ischemic injury when the δ-isofrom of protein kinase (δPKC), a prodeath kinase, is inhibited (74). As a result of IPC, the prosurvival kinase εPKC is activated and translocated to cardiac mitochondria (5). Churchill et al. (20) have shown that IPC alters the ratios of these two kinases and improves postischemic UPS function in turn favoring tissue survival. Considering that a proteasome inhibitor prevented the effects of IPC on postischemic function and the ratios of δPKC/εPKC, the authors proposed that the UPS regulates the ratio between these two kinases. This is an attractive hypothesis; however, IPC alters levels of many prosurvival and prodeath proteins thought to be regulated by the UPS, including PTEN (13), 1kB (54), Bax (29), PKC, and Akt (protein kinase B) (146). Many diverse pathways are regulated by the UPS, and it is likely that UPS-mediated changes in δPKC account for only a portion of the protective effects of IPC.

IPC increases protein kinase A (PKA)-mediated activation of proteasome. This theory is highly credible given the regulation of the cardiac proteasome by PKA (see Regulation of the Cardiac Proteasome). Proteasome peptidase activity is activated by PKA-induced phosphorylation of several subunits of the 20S proteasome and the 19S regulatory particle (169, 176). Evidence that PKA can enhance docking of 20S proteasomes and 19S regulatory particles was presented by Asai et al. (3) who suggest that assembly of intact 26S proteasome is enhanced by IPC-mediated transient postischemic increases in PKA, which would explain the higher peptidase activity observed in the immediate postischemic period. It is of interest that PKN (protein kinase C-related kinase 1) has also been shown to stimulate proteasome activity and possibly play a protective role in the heart during myocardial ischemia (142).

IPC decreases postischemic oxidation of 19S regulatory particle subunits. As described above, the 19S regulatory particle subunit Rpt5 was observed to be susceptible to oxidative modifications during myocardial ischemia (29). In this study, IPC was used as an intervention to show cause and effect between changes in proteasome activity and 19S regulatory particle subunit oxidation, in this case decreased postischemic carbonylation of the Rpt5 subunit (29). The Rpt5 subunit has two roles: attachment of the “base” of the 19S regulatory particle to 20S proteasome α-rings (43) and binding of the 19S regulatory particle “lid” to the base (43). In consideration of these roles, it is conceivable that by decreasing oxidation of this subunit, IPC can improve the docking of these two complexes. However, this was not examined, and it is not clear that improved proteasome activity is related to decreased oxidation of this sole subunit. IPC has a high degree of complexity, and it is unlikely that a single mechanism accounts for the positive outcome of this procedure on postischemic proteasome function.

Roles for other Regulatory Particles in Myocardial Ischemia

The studies thus far have examined the role of ubiquitin-mediated degradation of proteins, or more specifically, dysfunction of 26S proteasome in myocardial ischemia. However, as described above, there are other forms of the proteasome. Recent studies have begun to define a role for the 11S-activated proteasome more related to protein quality control and removal of damaged proteins during myocardial ischemia.

The 11S-activated proteasome in myocardial ischemia. As described above, if the 20S proteasome is docked with the 11S activator ring and does not contain immunofoms, it is called the 11S-activated proteasome. Studies have suggested this proteasome functions in the removal of oxidized proteins (104, 105). In consideration of the relationship between myocardial ischemia, oxidative stress, and protein damage (111, 112), as well as the reported upregulation of the 11S-activated proteasome in a related cardiomyopathy associated with oxidative stress (113), it is conceivable that the 11S-activated proteasome plays a role in removal of proteins oxidized during myocardial ischemia. We (30) have previously shown that the proteasome is responsible for the removal of proteins that have
been oxidized during myocardial ischemia and that this appeared to occur in an ubiquitin-independent manner, suggesting involvement of a proteasome other than the 26S proteasome. A recent report that has shown that in cardiomyocytes, forced overexpression of the PA28α subunit of the 11S activator ring results in increased proteasome activity and an enhanced resistance to oxidative stress (87). Furthermore, it has been shown that posttranslational function is improved and infarct size is decreased with overexpression of PA28α in a transgenic mouse model, providing strong evidence for a role of this zyme in myocardial ischemia (86).

**Autophagy and Ischemia**

A major function of the UPS is protein quality control and the prevention of proteotoxic stress. However, this is not the only responsible system; thus this review would be incomplete without some discussion of autophagy, which is a parallel system also playing a major role in prevention of proteotoxic stress (55, 56). Discussion will be limited since there are excellent recent reviews on autophagy, in general, and in ischemia (4, 28, 55–57, 118, 143, 173). Also, whereas these two systems are related in function, their targets tend to be different with autophagy removing macromolecular structures and organelles. Unlike the UPS that tends to become dysfunctional during ischemia-reperfusion, most studies indicate that autophagic flux is increased during ischemia (60, 92, 127, 143, 165). In general, agents that enhance autophagy have a beneficial effect on ischemic injury (119, 127). It has been suggested that the enhanced flux is actually in response to the diminished capacity of the UPS in ischemic tissue and thus is an attempt to maintain cellular protein quality control. In fact, a recent study indicates that simply inhibiting the proteasome with an inhibitor is enough to result in upregulation of autophagy in cardiomyocytes (172) and that the protein p62/SQSTM1 may act as a proteotoxic sensor that connects the two systems (140, 171). This type of inverse relationship between UPS and autophagic flux is not limited to the ischemic myocardium but has also been observed in heart failure (78, 173).

**Summary and Future Directions**

The UPS has often been described as simply a means for the removal of unwanted, damaged, or otherwise unneeded proteins. Research over the past 20 years has shown there is much more to this concept. By removing proteins, some highly reactive, the UPS plays multiple critical roles in regulating many of the intracellular processes necessary for cell function and survival. The evidence to date is quite conclusive that the UPS is dysfunctional during myocardial ischemia-reperfusion, at least in experimental models. Given the absolute requirement for the UPS, it seemed reasonable that UPS dysfunction, particularly when a cell is being exposed to a stress such as ischemia, would be detrimental as it would result in defects in removal of highly reactive signaling molecules, for example, Bax (29) and proteins damaged by oxidative stress (30). Figure 6 presents a scheme describing the proposed functions of the 26S- and 11S-activated proteasomes and consequences of dysfunction. There have been multiple mechanisms suggested for the dysfunction, but the actual mechanism(s) is still not known, nor is it clear whether dysfunction is a primary or secondary process. As part of our discussion, we reviewed the evidence for and against the suggested use of proteasome inhibitors in treating myocardial ischemia. Part of this discussion suggests that perhaps new second generation proteasome inhibitors targeting specific catalytic subunit immunoforms might present a viable clinical option for decreasing the inflammatory response associated with myocardial ischemia, certainly an area requiring additional studies. Lastly, we discussed the potential role that the UPS might play in IPC and suggested that improved function of the UPS in the preconditioned heart might facilitate degradation of proapoptotic proteins in the postischemic period, although this story is also far from complete. The recent development of genetic models that either enhance or diminish proteasome function should help to
sheds some light on these issues as well as demonstrating the various roles of the different zomes and proteasome subunits in cardiac (patho)physiology. Hopefully, this review will spur research to expand our knowledge of the how the UPS functions in the normal heart and how its dysfunction could lead to the worsening of cardiac pathologies.

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AUTHOR CONTRIBUTIONS

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REFERENCES


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