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Protein Quality Control and Degradation in Cardiomyocytes

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Abstract

The heart is constantly under stress and cardiomyocytes face enormous challenges to correctly fold nascent polypeptides and keep mature proteins from denaturing. To meet the challenge, cardiomyocytes have developed multi-layered protein quality control (PQC) mechanisms which are carried out primarily by chaperones and ubiquitin-proteasome system mediated proteolysis. Autophagy may also participate in PQC in cardiomyocytes, especially under pathological conditions. Cardiac PQC often becomes inadequate in heart disease, which may play an important role in the development of congestive heart failure.

Keywords

chaperones; ubiquitin; proteasome; autophagy; proteases; signal transduction

1. Introduction

A polypeptide must assume a proper conformation via folding to fulfill its obligations in the cell. Being the sole pump to drive blood circulation in the body, the heart must perform vigorous mechanical work continuously for an entire lifetime, which arguably makes a beating heart the most stressful organ in the body even at normal conditions, let alone when the heart is afflicted by various types of illness. In such a stressful environment, a polypeptide in cardiomyocytes has a much harder time than those in many other cell types to attain as well as maintain its proper conformation. Consequently, protein misfolding, unfolding, and damaging are inevitable but deployment of misfolded proteins can be catastrophic to the cell. To offset this risk, cardiomyocytes have evolved quite sophisticated sets of mechanisms for protein quality control (PQC) [1–3].

PQC is in fact a multitude of intricate biochemical reactions which support protein (re)folding, prevent nascent or unfolded polypeptides from aggregating, and remove selectively polypeptides that are terminally misfolded. Membrane proteins and proteins for secretion are folded in the endoplasmic reticulum (ER). Quality control of these proteins is done by ER-associated PQC [4]. Molecular mechanisms underpinning ER-associated PQC are more extensively studied than the ER-independent PQC but the latter is responsible for the quality control of the majority of cellular proteins, especially in cardiomyocytes where myofibrillar proteins occupying more than 80% of the cell volume are not processed by the ER.

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Nevertheless, both ER-associated and ER-independent PQC are performed by the highly cooperative systems comprised of molecular chaperones and targeted proteolysis. The latter is primarily accomplished by the ubiquitin-proteasome system (UPS) [1]. A current view on the general PQC process is illustrated in Figure 1. Due to hydrophobic interactions, the misfolded proteins tend to aggregate with each other and with other vulnerable proteins if molecular chaperones and/or UPS proteolytic function are inadequate. In addition to the physical disruption to the cell, the formation of the aggregates can yield other severe impacts on the cell including impairment of UPS proteolytic function which can in turn further damage the ability of the cell to timely remove abnormal proteins and result in more aberrant protein aggregation, thereby forming a vicious cycle. Autophagy may be activated by UPS malfunction and/or aberrant protein aggregation to help remove aggregates [5], thereby playing at least a supplemental role in PQC.

In the past several years, significant advances were achieved in the research into PQC in general and in cardiomyocytes. Multiple lines of evidence suggest that PQC in cardiomyocytes is inadequate in a number of pathological conditions. Here, we submit a hypothesis that *inadequacy in PQC in cardiomyocytes plays an important role in the development of congestive heart failure (CHF)*. After a very brief review of essential elements of PQC, this article will focus on discussing the latest progresses in PQC in cardiomyocytes, with an emphasis on examining emerging evidence that either supports or questions the hypothesis.

2. Chaperones

Post-translational PQC starts at assisting nascent or unfolded polypeptides in folding correctly, which is done largely by chaperones. Different sets of resident chaperones are found in various sub-cellular compartments (e.g., mitochondria, the ER, the nucleus, and the cytosol). They help fold and refold the polypeptides that have been either synthesized onsite (e.g., some mitochondrial proteins, all cytosolic proteins) or imported from the cytosol (e.g., the majority of mitochondrial proteins, all nuclear and ER proteins, and proteins passing through the ER). Since protein translocation across the membrane usually involves sequentially unfolding and refolding, many molecular chaperones play an essential role in this process [6].

Chaperones often serve as the sensor for misfolded polypeptides, bind them and prevent them from aggregating, thereby allowing the misfolded proteins to be either repaired or degraded by proteases when repairing fails. Under conditions of stress, the synthesis of chaperones in cardiomyocytes is increased in an attempt to handle increased protein misfolding. This is best exemplified by the heat shock response in which the expression of a family of peptides, known as heat shock proteins (HSP), is induced. Most HSPs are chaperones and some of them are capable of refolding the proteins that have been denatured under stress while many HSPs also function to escort terminally misfolded proteins for degradation. HSP90, HSP70, carboxyl terminus of HSP70-interacting protein (CHIP), HSP20, and α B-crystallin (CryAB) represent arguably the most studied chaperones that play critical roles in PQC in the heart. Gain- and/or loss-of-function studies have demonstrated a protective role of these chaperones in myocardial ischemia and reperfusion injury [1,7]. Notably, besides being a (co)chaperone for a number of proteins [8,9], CHIP can also function as a ubiquitin E3 ligase (*more in Section 3.1*) and may play a critical role in sorting damaged proteins between repair and degradation [10–12]. Both human genetic studies and experimentations using mouse transgenics have demonstrated that expression of an R120G mutation of CryAB (CryAB^{R120G}) is sufficient to cause aberrant protein aggregation and heart failure *in vivo* [13,14]. Compromised PQC as well as increased reductive stress were proposed as important pathogenic mechanisms for CryAB^{R120G} in the heart [14,15].

3. The UPS

For the safety as well as normal functioning of the cell, both irreparable misfolded polypeptides and some normal proteins that are no longer needed at a given time and location must be permanently removed from the cell. This protein degradation must be highly specific and is accomplished by the UPS [3,12]. Hence, besides PQC, the UPS also regulates a wide variety of cellular processes, for instance, the development and regression of cardiomyocyte hypertrophy [16–21]. Two major steps constitute UPS-mediated proteolysis: ubiquitination of target protein molecules and degradation of the ubiquitinated proteins by the 26S proteasome.

3.1 Ubiquitination

Ubiquitination is ATP-dependent covalent attachment of a ubiquitin protein molecule to a target protein molecule via a cascade of biochemical reactions involving ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), ubiquitin ligases (E3), and occasionally, the polyubiquitin chain assembly factor (E4) [22]. A recent study demonstrates that polyubiquitination can be achieved either by attaching one ubiquitin molecule at a time and multiple rounds of attachments or by transferring a preassembled ubiquitin chain from an E2 [23].

Both the length of the ubiquitin chain formed and the specific site of lysine residues in ubiquitin used for linkage appear to play a role in signaling distinct fates of the target protein [24]. For example, a chain of 4 or more ubiquitin molecules linked via Lys48 usually signal for degradation by the proteasome whereas monoubiquitination or ubiquitination at Lys63 may signal for a non-proteolytic fate for the modified proteins [25]. The counter-process of ubiquitination is de-ubiquitination, which acts as a proof-reading mechanism in proteins inappropriately targeted for degradation. De-ubiquitination adds a layer of regulation on UPS-mediated proteolysis and recycles ubiquitin [26].

Ubiquitin E3 ligases play the central role in attaching ubiquitin moiety to target protein molecules and determine the specificity of ubiquitination and thereby the specificity of UPS-mediated proteolysis. E3s usually carry one of the 3 domains: HECT domain, RING finger, and U-box. Notably, exciting progresses were made in demonstrating the (patho)physiological significance of E3 ligases in the heart in the past 2 years. Muscle-specific ring finger proteins (MuRFs) and atrogin-1 are among a few described ubiquitin E3s that are responsible for the degradation of muscle proteins in cardiomyocytes. They also play important roles in proteolysis in skeletal myocytes [27].

MuRFs are an important subfamily of RING-finger E3s that are specifically expressed in myocytes of striated muscle [28]. In the heart, MuRF1 regulates the induction of cardiac hypertrophy, likely by directing troponin I and PKC ϵ for proteasomal degradation and also its interaction with serum response factor (SRF) [17,29,30]. MuRF2 is sarcomere-associated and may play a role in transducing mechanical signals through its effects on SRF activity [31]. MuRF3 was first identified to be associated with microtubules and regulate the microtubule assembly and disassembly [32]. MuRF3 may also function as an E3 ligase for four and a half LIM domain (FHL2) protein and γ -filament. Mice lacking MuRF3 are prone to ventricular rupture after acute myocardial infarction (MI) [27]. MuRF1 and MuRF3 seem to function redundantly as E3s for UPS-dependent turnover of β /slow myosin heavy chain (MHC) and MHCIIa [28].

As an F-box protein in myocytes, atrogin-1 participates in an SCF (Skp1-Cullin1-F-box) ubiquitin ligase complex in which it recruits specific substrate proteins for ubiquitination [33]. Atrogin-1 can target calcineurin for degradation [16]. Both atrogin-1 and MuRF1 were upregulated in pressure overload hypertrophied hearts and hypoxic hearts [34]. A similar up-

regulation was also observed in MI-induced CHF in rats, which was associated with TNF α production and might be responsible for troponin I degradation [35]. Surprisingly, myocardial transcript levels of atrogin-1 and MuRF1 were decreased during cardiac atrophy induced by heterotopic transplantation of rat hearts [34]. Activation of the FOXO family of transcription factors was known to mediate the transcription of atrogin-1 upon PI3K/AKT inactivation during muscle atrophy [36,37]. Very interestingly, Li et al recently reported that atrogin-1 can suppress AKT-dependent cardiac hypertrophy in mice through co-activating FOXO transcription factors in a polyubiquitination-dependent manner [18], suggesting that atrogin-1 exerts a feed-forward regulation over the activation of atrophic genes including itself and that stimulating atrogin-1 might conceivably be a powerful approach to suppress cardiac hypertrophy [21].

Several other E3s are also of significance to cardiomyocytes. MDM2 is a RING finger protein and is the best characterized E3 ligase for p53 [38]. Based on its activity towards p53, MDM2 is logically necessary for the cardiomyocyte response to hypoxia and ischemic injury [39]. Overexpression of MDM2 showed an anti-hypertrophic effect upon adrenergic stimulation [40]. MDM2 is one of the selectively translated mRNAs and a significantly up-regulated E3 ligase in myocardium in response to acute pressure overload [41,42]. Interestingly, CHIP was recently revealed to directly interact with wild type p53 and protect its transactivation activity [9]; but it ubiquitinates mutant p53 for degradation [43]. The significance of CHIP regulation of p53 in the heart remains to be determined. Nevertheless, accumulating evidence suggests that CHIP ubiquitinates a number of abnormal proteins recognized by chaperones and directs them for degradation [44–46]; thus, CHIP may be an important E3 for PQC in the heart. ARC (Apoptosis Repressor with Caspase recruitment domain) is ubiquitinated by MDM2 and the decrease in ARC resulting from UPS-mediated degradation was shown as a trigger for cardiomyocyte apoptosis upon apoptotic stimulation [47,48]. The inhibitors of apoptosis (IAP) family proteins are RING finger proteins and inhibit apoptosis by targeting caspases for proteasomal degradation [49].

Ubiquitin or ubiquitin conjugates are always associated with aberrant protein aggregates where ubiquitin conjugation has been automatically presumed as a step for the cell's attempt to remove the aggregates. Although this presumption might remain to be true, new evidence emerging from studies on neurodegenerative disease reveals global changes in the ubiquitination in conformational disease and suggests that certain types of ubiquitination (e.g., mono- or diubiquitination) may promote the aggregation of disease-causing proteins [50–52]. Consistently, increasing ubiquitin hydrolase Uch-L1 activity was found to alleviate synaptic dysfunction and memory loss associated with a mouse model of Alzheimer's disease [53]. Since both ubiquitinated proteins and pre-amyloid oligomers were significantly increased in failing human hearts [54,55], it will be important to determine whether ubiquitination is dysfunctional and plays a role in abnormal protein aggregation in disease hearts.

3.2 Proteasomes

In general, the degradation of polyubiquitinated proteins is carried out by the 26S proteasome which consists of a barrel-shaped core particle (the 20S proteasome) and the activation complex (often the 19S proteasome) at one or both ends of the core particle. The cylindrical structure of the 20S proteasome is formed by an axial stack of 4 heptameric rings: 2 opposing identical β rings sandwiched by 2 identical outer α rings. Each α or β ring contains 7 unique subunits (α 1 through α 7, β 1 through β 7). Polyubiquitinated proteins are usually preprocessed by the 19S proteasome which recognizes and removes the ubiquitin chain on the targeted protein molecules, unfolds them, and channels the unfolded polypeptides into the proteolytic chamber of the 20S. Three major peptidase activities: chymotrypsin-like, trypsin-like, and caspase-like (also known as peptidylglutamyl-peptide hydrolase) activities, have been identified and

respectively assigned to the $\beta 5$, $\beta 2$, and $\beta 1$ subunits (*for recent reviews, see [1~3] and references therein*). In addition to gating the entry of polypeptides into the proteolytic cavity and regulating the exit of proteolytic products from the 20S, the α -ring may directly regulate the assembly and peptidase activities of the β -rings [56].

In many types of cells, including cardiomyocytes, it is not uncommon to find 20S proteasomes that are also associated with the 11S proteasome. The latter is also known as proteasome activator 28 (PA28) or REG [57,58]. The 11S proteasome can be formed either by hetero-polymerization between PA28 α and PA28 β ($\alpha 3\beta 4$ or $\alpha 4\beta 3$) or by homo-polymerization of 7 PA28 γ molecules [57]. In addition to being capped by the 19S or the 11S at both ends, a 20S proteasome can simultaneously complex with a 19S at one end and an 11S at the other, forming a hybrid proteasome. The association of the 11S with the 20S proteasome was generally believed to be important for antigen processing but spleen cells isolated from PA28 $\alpha\beta$ or PA28 γ null mice only displayed defects in the processing of selected antigens [59]. Recent studies reveal interestingly that PA28 γ mediates the ATP- and ubiquitin- independent degradation of the steroid receptor coactivator SRC-3 and the cell cycle regulator p21 by the 20S proteasome [60–62]. A significant amount of 20S proteasomes are associated with the 11S proteasome in native murine myocardial proteins and interestingly, the 11S proteasome was significantly up-regulated in mouse hearts overexpressing CryAB^{R120G} (Wang, unpublished data). Powell et al reported that myocardial PA28 α and PA28 β protein levels, 20S proteasome subunits abundance and activities, as well as protein carbonyls and ubiquitinated proteins were markedly increased in rats with pharmacologically induced diabetes, suggesting that 11S associated 20S proteasomes may be involved in the removal of proteins damaged by hyperglycemia [63]. Hence, the 11S proteasome may be important for PQC in the heart but its role remains to be defined.

3.3 Assessing UPS proteolytic function

3.3.1 In vitro peptidase assays using synthetic fluorogenic peptides as substrates—The use of small synthetic fluorogenic peptides as substrates allows rapid assessment of proteasome peptidase activities. The results are generally quite reliable when purified proteasomes are used. However, proteasome purification is considerably labor-intensive, time-consuming, and costly. Hence, crude protein extracts from cultured cells or animal tissue samples are often directly used for these assays; but this adds a host of variables to the assays and sometimes makes them less informative in probing intrinsic proteasome activities [64]. Non-proteasomal peptidases in the crude protein extracts can also cleave the substrates such that only the portion of peptidase activities inhibited by a proteasome inhibitor is attributable to the proteasome. Unfortunately, the specificity of virtually all proteasome inhibitors is questionable, especially when inappropriately high concentrations are used [64]. The alternative to the use of proteasome inhibitors in these assays is to remove or reduce non-proteasome peptidases from the crude protein extracts. Two relatively simple methods were reported to achieve this. The first one takes advantage of the difference in the particle size between proteasome complexes (larger) and non-proteasome peptidases (usually smaller) to remove the latter by using a filter with defined pore size (e.g., 500kDa cut-off) [64]. The second method uses affinity binding matrices to fish proteasomes out of the homogenates or lysates on the basis of the ability of 26S proteasomes to bind a ubiquitin-like domain that has been fused to the matrix [65].

Because the binding of 19S proteasomes to the 20S is ATP-dependent, addition of ATP into the assay allows the 19S to bind to the 20S and this indeed usually increases the assessed peptidase activities, if an optimal amount of ATP is used [66]. The difference between the activities assessed with and without ATP is sometimes considered to reflect the activity of the 26S proteasome [66]. Such interpretation is debatable because the ATP-induced increase in

proteasomal peptidase activities may just reflect an allosteric effect of the binding of the 19S to the 20S on the peptidases in the 20S but not the intrinsic function of the 19S proteasome.

3.3.2 Assessments using a fluorescence probe that selectively binds active proteasome peptidase subunits—A fluorescent, broad-spectrum, and cell-permeable proteasome inhibitor has recently been developed and validated for in-gel simultaneous detection of the activity of individual proteasome peptidase subunits, including $\beta 5$, $\beta 2$, $\beta 1$, $\beta 5i$, $\beta 2i$, and $\beta 1i$ [67]. In this new method, the fluorescent probe applied directly to crude protein extracts or cultured live cells or injected to animals selectively and irreversibly binds to the activated peptidase subunits of the proteasome, allowing the probe-bound proteasome subunits to be visualized in gel after the cell lysates or tissue homogenates are fractionated by, for instance, conventional SDS-polyacrylamide gel electrophoresis. Since the probe is also a proteasome inhibitor, the *in vivo* use of this method is conceivably limited to a short time window of the final stage of an experiment to minimize the inevitable impact of proteasome inhibition from the probe on the experimental system. Remaining to be more extensively tested, this new method is expected to benefit a number of applications, such as profiling proteasome activities of clinical samples, biochemical testing of subunit specificity of new proteasome inhibitors, and monitoring proteasome function and dynamics in living cells [67].

3.3.3 In vivo assessments using surrogate fluorescence protein substrates—The assays discussed in the earlier sections allow relatively rapid assessment of the catalytic activity of the 20S proteasome. However, the results of these assays very often do not reflect *in vivo* status of UPS proteolytic function [15,68]. More importantly, changes detected by assays described in Section 3.3.1 and Section 3.3.2 do not necessarily reveal whether UPS proteolytic function is adequate or not in terms of meeting the cell's needs to maintain protein homeostasis and PQC under a given pathological condition. Increased proteasome peptidase activities may well be secondary responses to inadequate UPS proteolytic function. To address this critical issue, a series of full-length protein reporters were developed through fusion of ubiquitination signals (e.g., the CL1 degron) to proteins that are biologically inert and relatively easy to be detected, such as green fluorescence protein (GFP) [1,69,70]. Stable cell lines, recombinant replication-deficient adenoviruses, and stable transgenic mouse lines that express a GFP modified by carboxyl fusion of degron CL1 sequence (GFP^u or GFPdgn) have been created, validated, and extensively used in experimental investigations into the involvement of UPS dysfunction in cardiac pathogenesis [15,68,70–72].

One report indicated that GFP^u formed aggregates in the cell when it was overexpressed at extremely high levels in *C. elegans* or in cultured human cell lines [73], suggesting that fusion of CL1 causes GFP to misfold. However, neither was this observed in a fruit fly model nor in GFPdgn mice even during severe systemic proteasomal inhibition (Figure 2) [74]. This discrepancy is likely caused by the difference in the level of overexpression. In absence of proteasomal inhibition, much greater overexpression of GFP^u than that of conventional GFP is required to achieve a comparable steady-state protein level between GFP^u and conventional GFP as shown in the report by Link et al. [73], because GFP^u has a much shorter half-life than conventional GFP [75].

3.4 Regulation of UPS proteolytic function

Conceivably, the degradation of a protein is as highly regulated as its synthesis. The activity of protein translation machinery is closely regulated by mechanisms such as post-translational modifications (PTMs), so that protein synthesis is opted for cellular activities at a given time and location. However, much less is known about whether and how the major protein degradation machinery: the UPS is regulated to adjust its activity to cell functions. Excitingly, studies to address this deficit are emerging.

Logically, UPS-mediated degradation of unneeded normal proteins should be regulated at the ubiquitination step where the specificity resides. Many, if not all, known examples have shown that this appears to be the case. However, the speed-limiting step to degrade abnormal proteins likely lies in the proteasome, particularly the delivery into the 20S proteasome, as some evidence suggests [15,68]. Hence, understanding of the regulation of ubiquitination of a given protein will help find new ways to manipulate the stability of the protein while the studies on the regulation of proteasome activities would facilitate searches for novel approaches to enhance removal of abnormal proteins in the cell.

3.4.1 Regulations on Ubiquitination—Ubiquitination of a specific protein substrate is controlled by the maturation of the ubiquitination signal on the substrate, the availability and activity of its specific E3 ligase(s), and the physical interaction between the substrate and the E3 [33].

PTMs on a substrate protein molecule can regulate its ubiquitination likely through changing the conformation of the substrate to expose or mask its ubiquitination signal. For example, phosphorylation of β -catenin at its N-terminal end by GSK-3 β triggers its ubiquitination whereas tyrosine phosphorylation within the PPXY motif of c-Jun prevents E3 from binding and inhibits its ubiquitination [76,77].

The expression of E3 ligases is regulated at both synthesis and degradation ends. Atrogin-1 and MuRF-1 are largely responsible for degradation of muscle proteins during skeletal muscle atrophy. Their expression is stimulated by FOXO transcription factors [36]. The IGF-1/PI3K/Akt pathway can prevent atrogin-1 and MuRF-1 expression by inhibiting FOXO transcription factors [37]. In cardiomyocytes, FOXO3a which activates atrogin-1 transcription and retards or prevents hypertrophy, is down-regulated by multiple physiological and pathological stimuli of myocyte growth [78,79]. E3s can be self-ubiquitinated or ubiquitinated by other E3s, thereby being degraded by the proteasome. The dynamics of assembly and disassembly of an E3 complex is regulated to control its self-destruction [1]. As the most commonly modified enzyme, the activity of E3s can also be regulated by PTMs, such as phosphorylation, neddylation, and ubiquitination [24]. For cullin-based E3s (e.g., the SCF family), the binding of substrate and the substrate recognition subunit increases cullin neddylation and activation of the E3 [33].

Ubiquitination can be regulated by the cellular location of ubiquitinating enzymes. The localization of ubiquitinating enzymes can be controlled by binding to auxiliary proteins. Smad 7 is an auxiliary protein that recruits E3 ligases to the TGF-beta receptor, which stimulates protein degradation [24]. Little is known about regulation on the ubiquitination of misfolded proteins. However, it is generally believed that exposure of a patch of hydrophobic residues is the ubiquitination signal for misfolded polypeptides. Hence, the signal for ubiquitination is always on for misfolded proteins and their ubiquitination is conceivably regulated by the availability and accessibility of their E3's. As discussed earlier, chaperones are thought to play a critical role here.

3.4.2 Regulations on the Proteasome—Because ubiquitination is the well accepted regulation point in UPS-mediated proteolysis, it was assumed that the proteasome should behave like a uniformed automatic “garbage grinder” without little variation in its structure and activity among different tissues and under various physiological conditions. However, this assumption has now proven to be untrue by recent studies on the roles of proteasome accessory proteins and by compositional and functional analyses of proteasome subpopulations [26,56]. Functional proteomics has revealed the composition diversity and striking variations in the activities of proteasomes among different organs of mice [80–82]. Proteasome activities appear

to be well adjusted by the cell to harmonize with its energy metabolism and to accommodate the needs of various cellular processes and responses.

UPS-mediated proteolysis is ATP-dependent. Thus, energy metabolisms may yield effects on proteasomal function through ATP-production. Emerging evidence suggests that energy metabolisms can also regulate the proteasome by means beyond ATP. A glucose sensor, hexokinase1 (HXK1) that plays well-defined enzymatic roles in the first step of glycolysis, has proved recently in *Arabidopsis* to form a signaling complex with Rpt5B, a subunit of the 19S proteasome [83]. Addition or removal of O-linked *N*-acetylglucosamine (O-GlcNAc) on proteins alters their function. O-GlcNAc modification of Rpt2, one of the AAA ATPases in the 19 S proteasome, shuts off the proteasome through the inhibition of ATPase activity [84]. Thus, the function of the proteasome can be coupled to glucose metabolism through control of the flux of glucose into O-GlcNAc.

Proteomic studies have revealed a variety of PTMs in many subunits of the proteasome, including cardiac proteasomes [80–82,85]. However, the molecular events responsible for and signaling pathways regulating the PTMs as well as the physiological significance of the PTMs have just begun to be explored. Protein phosphatase 2A (PP2A) and protein kinase A (PKA) were co-purified with intact murine cardiac 20S proteasomes [85]. Under certain conditions, PP2A and PKA may modify multiple subunits of the 20S (e.g., α 1 and β 2) because inhibition of PP2A or the addition of PKA clearly altered the phosphorylation profiles of the proteasomes. It seems that hyper-phosphorylated 20S proteasomes are more active than hypo-phosphorylated ones [82,85]. In a non-myocyte system, PKA was found to activate proteasomes through inducing phosphorylation at Ser120 of an AAA ATPase subunit, Rpt6 [86]. Mutation of Ser120 to Ala of this 19S subunit blocked proteasome function. The stimulatory effect of PKA and the phosphorylation of Rpt6 were reversible by PP1 γ [86]. It will be extremely important to determine whether and how signaling pathways including the PKA pathway, regulate the proteolytic function of the proteasome in the heart. In addition to PTMs, some subunits of cardiac proteasomes exist as different isoforms [80]. Using a newly developed in-solution isoelectric focusing of multi-protein complexes in a laminar flow to separate the subpopulations of native 20S proteasomes purified from murine livers and hearts, Drews et al have elegantly demonstrated for the first time that employment of different isoforms may also confer a layer of regulation on cardiac proteasome activities [81]. As they speculated [81], the identification of individual subpopulations of cardiac proteasomes with distinct subunit compositions and activities may provide more selective targets for developing new measures to intervene cardiac proteolysis with greater precision and less unintended effects.

An overall increase in the synthesis of proteasomal subunits as a secondary response to proteasome inhibition was observed in cultured cells and implicated in the heart [15,87]. Forced overexpression of the β 5 subunit of the 20S proteasome in cultured human lens epithelial cells was shown to up-regulate all three proteasomal peptidase activities but this remains to be confirmed in intact animals [88]. The expression of proteasomal subunit S5a (Rpn10) can be regulated by Src [89]. S5a rescued Saos-2 cells from apoptosis induced by a Src inhibitor. S5a mRNA and protein levels were down-regulated as a result of Src inhibition, either by siRNA or by pharmacological inhibitors [89]. These findings suggest that the cell may regulate overall proteasomal function by controlling the synthesis of proteasome subunits.

3.5 Interaction of the UPS with other proteases in cardiomyocytes

Besides the UPS and autophagy, calpains and caspases are other major families of proteases participating in proteolysis in cardiomyocytes [90,91]. Caspases are the executor for apoptosis and can also cleave proteins in non-apoptotic settings. As mentioned earlier, many caspase inhibitors are ubiquitin E3 ligases. Proteasome inhibition activates caspases, resulting in

apoptosis; meanwhile, activation of caspases can lead to cleavage critical components of the proteasome [92].

Calpains are intracellular Ca^{2+} -dependent cysteine proteases that are ubiquitously expressed in animal tissues [91]. In the heart, calpain I can break down some myofibril proteins such as troponin I, desmin, and vimentin, leading to the impairment of interactions between the actin and myosin [91]. This process may release the myofilament proteins from the contractile apparatus and initiates the ubiquitination and subsequent proteasomal degradation of myofibril proteins [93,94]. Indeed, cardiac-specific overexpression of calpain I in mice enhanced the cleavage of troponin I and desmin, accumulated ubiquitinated proteins, and accelerated protein turnover [91], suggesting that calpains and the UPS may collaboratively control the turnover of myofibril proteins.

4. Autophagy in PQC

PQC-linked proteolysis has long been thought to depend solely upon the UPS but most recent data show that autophagy (macroautophagy) also plays a crucial role in PQC, especially in pathological conditions [5,74,95].

4.1 Biochemistry and detection of autophagy

Eukaryotic cells primarily use both UPS-mediated proteolysis and autophagy for large scale protein degradation; but only autophagy is known to be capable of degrading whole organelles. The cell uses autophagy to recycle cytoplasm and dispose of excess or defective organelles. Autophagy is classified into microautophagy, chaperon-mediated autophagy (CMA), and macroautophagy [96]. Both CMA and macroautophagy may participate in PQC but here we only discuss macroautophagy which is commonly referred to as autophagy.

Autophagy is a basic cellular process by which a portion of cytoplasm is sequestered and delivered for the degradation by lysosomes. From the morphogenesis point of view, autophagy can be arbitrarily divided into 5 steps: (1) *vesicle nucleation*: formation of an isolation membrane (also known as a phagophore); (2) *vesicle elongation*: expansion of the phagophore; (3) *vesicle completion*: enclosure of the edges of the phagophore and forming an autophagosome, a double-membraned vesicle that encloses cytoplasmic materials including organelles; (4) *docking and fusion*: the fusion of the autophagosome either with an endosome to form amphisome or with a lysosome to form an autolysosome; and (5) *degradation*: digestion of the luminal content along with the inner layer of membrane by the lysosome hydrolases [96]. The double membrane structure is an important feature for electron microscopic (EM) identification of autophagosomes although later staged autophagic vacuoles such as amphisomes and autolysosomes may only have single membrane due to the digestion of the inner membrane [97].

Recent delineation of the genetic program executing and signaling pathways regulating autophagy has rejuvenated the research into the (patho)physiology of this protein degradation mechanism which was initially described long before the UPS [98]. A series of evolutionarily conserved genes, the Atg (autophagy-specific gene) genes control the step-wise progression of autophagic vesicle formation [96]. Two ubiquitination-like conjugation systems participate in the formation and maturation of autophagic vesicles. The first system includes the covalent conjugation of Atg12 to Atg5, which is catalyzed by the E1-like enzyme Atg7 and the E2-like enzyme Atg10. The second pathway mediates the conjugation of phosphatidylethanolamine (PE) to LC3/Atg8 by the collaboration among the protease Atg4, the E1-like enzyme Atg7, and the E2-like enzyme Atg3. This lipid conjugation is essential to the conversion of the soluble LC3 (termed LC3-I) diffusely distributed in the cytosol to the autophagic-vesicle-associated form (LC3-II). Since this conversion occurs in the early stage of autophagosome formation

and the association of LC3-II with autophagosomes does not end until the final degradation step, LC3-II marks autophagosomes in relatively high fidelity. Also because the lipidation of LC3 increases the electrophoretic mobility on gels (i.e., LC3-II runs faster than LC3-I in gel electrophoresis) and specific recruitment of LC3-II to autophagosomes constitutes a shift from diffuse to punctate distribution of LC3 proteins, LC3-II and fluorescent protein fused LC3-II (e.g., GFP-LC3 and mCherry-LC3) have been widely used as a marker of autophagy [99]. Notably, the newly developed transgenic mouse lines with cardiomyocyte-restricted expression of mCherry-LC3 is expected to facilitate the research into the (patho)physiological significance of autophagy in the heart [100]. A recently published comprehensive guideline has recommended a set of assays and methods for assessing autophagy in higher eukaryotes and depicted the limitations associated with some of the approaches [97].

Two related pathways are now known to regulate the induction of autophagy. The first one involving class I phosphatidylinositol 3-kinase (PI3K) suppresses autophagy [96], in which mammalian target of rapamycin (mTOR) proves to be a central downstream effector kinase [101]. Therefore, rapamycin is commonly utilized to increase autophagy in experimental settings. But the mechanisms by which mTOR suppresses autophagy remain to be fully elucidated [101]. More recent studies reveal that activation of FoxO3 can positively regulate autophagy by inducing the transcription of many autophagy-related genes, suggesting that the IGF-1/PI3K/AKT pathway can coordinately suppress both UPS- and autophagy- mediated proteolysis through its regulation on transcription [102,103]. The second pathway involving class III PI3K activates autophagy. Beclin 1, the mammalian homolog of yeast Atg6 and a Bcl-2-interacting protein, plays an important role in engaging class III PI3K to positively regulate autophagy [104].

In contrast to the UPS, which usually targets specific monomeric protein molecules for degradation due to the narrow pore of the proteasome barrel, autophagy mediates the bulk degradation of long-lived proteins, multi-protein complexes, oligomers, protein aggregates, and organelles. This generates metabolic substrates for recycling and providing energy to meet the biosynthetic requirement and eliminates surplus and/or damaged organelles [96,105].

The clearance of aggregation-prone proteins depends heavily on autophagy in cell models. The clearance is delayed by either autophagy inhibitors [106,107] or the knockdown of Atg genes [108], whereas induction of autophagy enhances the clearance [106,107]. Indeed, tissue-specific deletion of Atg7 or Atg5 in the mouse brain and liver leads to focal increases of ubiquitinated proteins [109,110]. Consistently, cardiac-specific knockout of Atg5 in adult mice also accumulates polyubiquitinated proteins [111]. Moreover, recent studies even link autophagy with ER associated degradation (ERAD), which has long been connected only to the UPS. A mutant of the Z variant of human α -1 proteinase inhibitor increases its aggregated form in the ER when autophagy is disrupted [112]. Also efficient degradation of misfolded mutant Pma1 by ERAD involves autophagy in yeast [113]. Similarly, a dysferlin mutant, which causes myopathy, spontaneously formed aggregates in the ER and induced conversion of LC3-II. Furthermore, inhibition of autophagy by either lysosome inhibitors or knockdown of Atg5 stimulated, whereas rapamycin diminished, the ER-associated aggregation [114]. The dependency of the degradation of a protein on autophagy seems to correlate with its propensity to aggregation. For example, blocking autophagy showed much less, if any, effect on the clearance of wild-type huntingtin fragments or wild-type α -synuclein than on the clearance of the mutant aggregation-prone species [115,116].

4.2 Interactions between the UPS and autophagy

The UPS and autophagy have long been considered two parallel degradation systems without intersections [98]. However, this view is challenged by recent studies. On one hand, UPS impairment triggers autophagy both *in vitro* and *in vivo* [74,108,117]; on the other hand, tissue-

specific knockout of Atg7 or Atg5 in mouse brain, liver, and heart all increased the polyubiquitinated proteins and led to formation of ubiquitin-positive protein aggregates [109–111]. Both suggest that autophagy may be a compensatory degradation system when the UPS is impaired; but it remains to be tested whether this is the case in the heart. Histone deacetylase 6 (HDAC6) is a microtubule-associated deacetylase capable of interacting with polyubiquitinated proteins [118]. In a *Drosophila* model, HDAC6 may serve as an essential link between the UPS and autophagy. Genetically induced proteasome inhibition sufficed to induce autophagy in an HDAC6-dependent manner and overexpression of HDAC6 rescued degeneration associated with UPS malfunction in an autophagy-dependent fashion [74]. In addition, Ding et al proposed that proteasome inhibition-induced autophagy may be mediated by ER stress because suppression of autophagy enhances the proteasome inhibition-induced ER stress [119]. It is well recognized that proteasome inhibition accumulates misfolded proteins in both the ER and the cytosol, which could be sufficient to induce ER stress [120]. ER stress may then trigger autophagy to relieve ER overload. Consistent with this notion, classic ER stress inducers, such as A23187, thapsigargin, and tunicamycin, can all induce autophagy in mammalian cells [114,121].

It has also been proposed that abnormal protein aggregates serve as the trigger for autophagy through p62/sequestosome-1 (P62/SQTM1). P62 has both an LC3-binding domain and a ubiquitin-binding domain, which can theoretically mediate the association of ubiquitinated proteins with the autophagosome membranes [122]. Surprisingly, genetic ablation of p62 in mice diminished Atg7 deficiency-induced formation of ubiquitin-positive protein aggregates in both hepatocytes and neurons, suggesting that p62 plays a critical role in the aggregation of ubiquitinated proteins during autophagy deficiency [123]. Taken together, the UPS and autophagy appear to be compensatory for each other in PQC. Because aberrant protein aggregation in the form of pre-amyloid oligomers has been observed in failing human hearts [55], elucidation of molecular details underpinning the interplay among proteasomal malfunction, autophagy, and protein aggregation in cardiomyocytes will be extremely important to CHF research.

Loss-of-function studies suggest that basal constitutive autophagy is required to maintain cardiac function at the baseline and during hemodynamic overload [111,124]. However, autophagic vacuoles are often increased in disease hearts [125–129], indicating that autophagy is either up-regulated or defective at its late stage. Assessment of autophagic flux should help clarify the ambiguity but has not been applied to *in vivo* models of heart disease [99,130]. A recently reported method using monodansylcadaverine (MDC) to label autophagic vacuoles and chloroquine to inhibit lysosomes [100], is expected to “democratize” *in vivo* autophagic flux assessment. However, it should be noted that MDC was also shown to stain endosomes and lysosomes and therefore MDC related data should be interpreted with caution [131]. It remains controversial, but a majority of the studies seem to suggest that increased autophagy is maladaptive to the heart under pathological conditions [105,132,133].

5. ER Stress and ER-associated PQC

The ER is a critical site for the modification and folding of proteins destined to the membrane and the secretory pathway. ER chaperones help and monitor the (re)folding of polypeptides that pass through the ER. Terminally misfolded polypeptides are sensed by ER chaperones and are cleared from the ER by a mechanism termed ERAD [4]. During ERAD, unfolded or misfolded proteins are trapped by ERAD machinery and retrotranslocated to the cytoplasm where they are degraded by the UPS immediately. The process is recently reviewed in detail by Hebert et al [4]. Overload of misfolded or unfolded proteins in the ER, known as ER stress, activates a defense mechanism referred to as the unfolded protein response (UPR), which attenuates protein synthesis to prevent further accumulation of unfolded proteins, induces the

transcription and synthesis of ER chaperones to enhance the folding capacity, and increases the expression of ERAD components to enhance ERAD ability. As the final resort for PQC, prolonged ER stress triggers apoptosis to safely dispose of cells injured by ER stress to ensure likely the survival of the organism [134].

The differential signaling events underlying the two opposing outcomes (life and death) of the UPR have begun to be delineated [135]. Regardless the outcome of the UPR, the initial signaling events in response to ER stress involve all the three arms of the UPR that are respectively sensed by three ER transmembrane proteins: inositol-requiring enzyme-1 (IRE1), Protein kinase R-like ER Kinase (PERK), and activating transcription factor 6 (ATF6) [4]. IRE1 is a kinase/endoribonuclease (RNase) and its activation initiates the non-conventional splicing of X-box binding protein 1 (XBP-1) mRNA. The spliced *Xbp-1* encodes a transcription factor that activates a series of genes including ER chaperones, whose products are responsible for ER protein folding and ER-associated PQC. IRE1 can also selectively degrade existing transcripts that encode secretory proteins to reduce ER load [1]. Activation of PERK reduces general protein synthesis and thereby ameliorates ER protein overload through phosphorylating the eukaryotic translation initiation factor 2 α (eIF2 α). During ER stress, ATF6 is translocated from the ER to the Golgi where its cytoplasmic domain (ATF6f) is liberated and allowed to enter the nucleus to activate the expression of UPR target genes [1]. It appears that all the three branches of UPR signaling collaborate to counter ER stress and re-establish the homeostasis in the ER, thereby protecting the cell. However, it seems that the very same UPR paradoxically activates cell death pathway when ER stress sustains. This paradox of UPR signaling had long been an unsolved puzzle until a recent report which demonstrates that the three signaling arms of the UPR are terminated at different phases in relation to the outcome of the UPR during sustained ER stress [135]. The responses set in motion by IRE1-mediated signaling attenuate fairly quickly even though the stress persists; the termination of the ATF6 arm falls slightly behind IRE1; whereas PERK signaling remains active throughout both the protection and cell death phases of the UPR [135]. A forced delay in shutting down the IRE1 arm significantly attenuates cell death induced by prolonged ER stress [135]. These new findings indicate that the termination of IRE1 signaling activity is a critical factor allowing cell death during the UPR [135].

Another notable advance is that the molecular signaling events underlying UPR-induced cell death have become better understood. Activation of IRE1 was previously shown to cleave caspase-12, which was considered an important mechanism for ER stress-induced apoptosis [136]. However, this was challenged by the caspase-12 deletion study [137]. During the UPR, phosphorylation of eIF2 α by PERK also leads to an increase in the translation of ATF4 [138]. ATF4 activates, in turn, the transcription of CCAAT/enhancer-binding protein-homologous protein (CHOP) [138]. CHOP is a transcription factor that can work with its partner C/EBP α to activate the transcription of Bim [134], a pro-apoptotic BH3-only member of the Bcl-2 family. Bim is required for ER stress to induce apoptosis in diverse cell types. Notably, compared with increases in synthesis due to increased transcription level by the PERK/ATF4/CHOP pathway, stabilization of Bim protein by PP2A-mediated dephosphorylation plays a greater role in ER stress-induced upregulation of Bim protein. PP2A-mediated dephosphorylation prevents Bim from being degraded by the UPS [134]. The link between the ER stress sensors and PP2A activation remains to be established. A recent report suggests that active δ PKC is translocated into the ER and mediates ER stress induced cardiomyocyte death during myocardial I/R [139].

Prolonged ER stress has been shown as a contributor to cardiomyocyte apoptosis during progression of cardiac hypertrophy to failure [140]. The role of ER stress and the UPR in cardiac physiology and pathology was comprehensively reviewed by Glembotski [141].

In cardiomyocytes, the ER appears to be a critical site for both calcium cycling and ER-associated PQC, both of which are essential in maintaining homeostasis in cardiomyocytes. However, it has rarely been explored whether there is an interplay between these two critical processes and if so, how the two interact with each other in cardiac (patho)physiology. Studies along this line will be not only interesting but also of potential importance because both persistent ER stress and dysregulation of calcium cycling are associated with cardiac malfunction and cell death [140,142].

6. PQC is Inadequate in Cardiomyocytes of Failing Hearts

6.1 Production of abnormal proteins is often increased in a disease heart

CHF of various etiologies is often preceded and accompanied by cardiac hypertrophy which is featured by increased protein synthesis in individual cardiomyocytes. Therefore, co-translational production of abnormal proteins is inevitably increased in cardiac hypertrophy. This is because by estimate, approximately one-third of the nascent peptides never make to mature proteins. They are co-translationally degraded by the UPS [143]. Consistent with this postulate, the UPR is activated in pressure overload cardiac hypertrophy in mice as well as in angiotensin II-treated cardiomyocytes. The UPR is activated less when myocyte hypertrophy is attenuated by antagonizing angiotensin II type 1 receptor [140].

Ischemic heart disease has become the number one primary etiological disease of CHF. Increased oxidative stress during myocardial ischemia and I/R injury can conceivably be disruptive to the folding and assembly of nascent polypeptides and damage more mature proteins, resulting in increased production of abnormal proteins. It is well known that oxidized proteins are significantly increased in ischemic and I/R hearts. Hypoxia and global I/R activate the UPR in cardiomyocytes [144,145], while sustained ER stress has recently been shown to inhibit UPS proteolytic function [146]. Induction of ER stress-related genes by cardiomyocyte-restricted overexpression of ATF6 protects against I/R injury in *ex vivo* mouse heart preparations [145], indicating that the demand for ER-associated PQC is significantly increased by myocardial I/R.

A significant portion of cardiomyopathy is caused by genetic mutations in sarcomeric or associated proteins and these are usually very abundant proteins in the heart [7]. Expression of proteins with a mutation, especially the ones causing misfolding, will invariably increase the burden on PQC. Besides, increased mechanical stress in diseased hearts likely damages more matured proteins. Therefore, all the above analyses and mounting evidence support that the burden on PQC in cardiomyocytes is significantly increased in disease hearts.

6.2 Removal of abnormal proteins by proteasomes appears to be inadequate in heart failure

Clearance of abnormal proteins is an important responsibility of PQC and relies primarily on the collaboration between chaperones and the UPS. At least one important molecular chaperone CryAB was significantly decreased in failing human hearts [147]. Multiple lines of evidence point to UPS dysfunction in heart failure.

6.2.1 UPS proteolytic function is inadequate in mouse models of DRC—As revealed by the accumulation of the GFPdgn reporter, proteasomal proteolytic function is severely inadequate in the heart of mouse models of desmin-related cardiomyopathy (DRC) caused by cardiomyocyte-restricted expression of either CryAB^{R120G} or a 7-amino-acid (R172 through E178) deletion mutation of the desmin gene (D7-des) [15,68], both of which were linked to human DRC [1]. In both DRC models, the inadequacy appears mainly to reside in the delivery of ubiquitinated proteins into the 20S proteasome although aberrant ubiquitination has not been ruled out [1].

6.2.2 Proteasomal dysfunction in animal models of pressure-overload and myocardial I/R

—Unlike DRC which does not seem to be common, ischemic heart disease and hypertension are the most common causes of CHF. In thoracic aortic constriction (TAC) induced pressure overload mice, Tsukamoto et al revealed that all the 3 peptidase activities of proteasomes started to decrease significantly at 2 weeks after TAC when cardiac malfunction was not discernible and the decrease became more substantial at 4 weeks when cardiac failure occurred. They also found progressive increases in ubiquitinated proteins in the heart between 2 and 4 weeks after TAC [148], suggesting an inadequacy in proteasomal removal of ubiquitinated proteins. Very interestingly, Depre et al revealed that subunit abundance and peptidase activities of the 26S proteasome, as well as ubiquitinated proteins were significantly increased in the sub-endomyocardium of canine hearts that underwent chronic pressure overload hypertrophy [149]. Furthermore, myocardial chymotrypsin-like activity measured at 5 days after TAC was also significantly increased. Proteasomal inhibition by daily intraperitoneal injections of epoxomicin (0.5 mg/kg/day, started at 1 day before TAC) completely prevented TAC-induced cardiac hypertrophy without significantly affecting cardiac function [149]. More recently, Meiners et al reported that increases in RNA and protein synthesis as well as in the cell profile area induced by various pharmacological stimuli in cultured neonatal rat cardiomyocytes were reversibly suppressed by treatment of proteasome inhibitors in a dose-dependent manner [19]. They further demonstrated that chronic mild proteasome inhibition achieved by intraperitoneal injections of *Valcade* (50 µg/kg body weight, twice weekly for 8 weeks) did not significantly affect the body weight but attenuated the rise in both the blood pressure and the heart weight to tibial length ratio respectively by 7.5% and 6.0% in Dahl-salt sensitive rats with high salt-induced hypertension [19]. Using the isoproterenol-induced mouse cardiac hypertrophy model, Stansfield et al demonstrated that co-administration of a proteasome inhibitor (PS-519) via daily intraperitoneal injection (1mg/kg body weight/day) significantly attenuated cardiac growth and the re-activation of the fetal gene program induced by one and two weeks of infusion of isoproterenol. The attenuation was associated with inhibition of NFκB nuclear translocation [20]. These *in vitro* and *in vivo* tests using pharmacological inhibitors suggest that systemic proteasome inhibition might be an effective approach to inhibit cardiac hypertrophy. Interestingly, ageing-associated reduction of proteasome activities (*see 6.2.3 for details*) does not appear to protect against pressure overload hypertrophy in the elderly. The reason behind this seeming paradox remains unclear but difference in the length and the extent of proteasome inhibition might be important factors.

Using an *in vivo* rat model, Bulteau et al showed that myocardial I/R could cause decreases in myocardial proteasomal peptidase activities. Their further analyses of purified 20S proteasomes revealed that the decreases in activities were accompanied by oxidative modifications of the components of the 20S proteasome [150]. By measuring changes in endogenous UPS substrates during myocardial I/R, Gurusamy et al revealed that oxidative stress caused selective rather than global inhibition of proteasomal activities [151]. However, both detrimental and beneficial effects of proteasome inhibition achieved by use of proteasome inhibitors have been reported on experimental myocardial I/R injury [3]. Consistent with a critical role of UPS-mediated PQC in at least the vasculature compartment of the heart, chronic proteasome inhibition was recently shown to increase coronary artery oxidative stress and contribute to coronary atherosclerosis in pigs [152].

Apparently, the pathophysiological significance of altered UPS function in either pressure overloaded cardiomyopathy or I/R injury has not been established. It should also be noted that the findings from studies using systemic proteasome inhibition are of very limited value in terms of understanding the impact of proteasome functional inadequacy in the cardiomyocyte compartment on the heart because inhibition of proteasomes in non-cardiomyocyte compartments within the heart as well as in other organs may yield indirect effects on the behavior of cardiomyocytes as well as impact the heart directly. To this end, cardiomyocyte-

restricted proteasome inhibition via genetic approaches will be extremely important in determining the effect of proteasome functional insufficiency in cardiomyocytes on cardiac growth and heart function.

6.2.3 Cardiac proteasomal function decreases with ageing—To a large extent, CHF is an ageing related syndrome. Thus, ageing related changes may contribute to the genesis of a subset of CHF. The activities of the ubiquitination enzymes E1, E2, and E3 do not show any consistent change with age. However, declines in proteasome activities were observed in various ageing tissues including myocardium [153]. In parallel, the accumulation of oxidized proteins and ubiquitinated proteins is also evident [153]. The mechanisms that lead to the decreases in proteasome activities are not very clear. Some studies proposed that down-regulations of UPS components (either proteasome subunits, or ubiquitination enzymes) contributed to the loss [154]. Intriguingly, forced expression of catalytic subunits ($\beta 1$ and $\beta 2$) was shown to restore the impaired proteasome activities in aged human fibroblasts. Other studies have suggested that alterations in proteasome structure, instead of decreases in its abundance, might be responsible for the compromised proteasome function. For instance, the decreased proteasome activity was accompanied by reduced proteasome content and altered PTMs of proteasome subunits in aged rat myocardium [154]. Lipofuscin, a complex mixture of oxidized proteins, lipid degradation residues, carbohydrates, and metals, is considered as an indicator for the age of senescent cells such as neurons [155]. Lipofuscin is also present in the heart or cardiomyocytes [156]. Powell et al. revealed that the incubation of lipofuscin with rat cardiomyocytes impaired the proteasome function and induced apoptosis, suggesting that proteasome malfunction may result in loss of cardiomyocytes in aged myocardium [155]. It remains to be determined whether the impaired proteasome activity plays a causative role in diminished cardiac function during ageing.

6.2.4 UPS malfunction is implicated in failing human hearts—

Immunohistopathology revealed marked increases in ubiquitin conjugates in human hearts with end-stage CHF due to dilated cardiomyopathy. And the increases often co-existed with increased autophagosomes in cardiomyocytes [157]. Western blot analysis also showed significant increases in the levels of ubiquitinated proteins in the heart with dilated or ischemic cardiomyopathies [54]. Furthermore, aberrant protein aggregation in the form of formation of pre-amyloid oligomers was frequently observed in the cardiomyocytes of failing human hearts [55]. Taken together, these lines of evidence are strongly indicative of proteasome functional insufficiency in failing human hearts.

7. Pathogenic Significance of PQC Inadequacy in the Heart

The stoichiometry among different sarcomeric proteins in the sarcomere is vigorously maintained; therefore, the synthesis and incorporation of a new constituent protein molecule into the sarcomere to replace an existing aged counterpart must be accurately coupled with the degradation of the existing protein. The degradation of all myofibrillar proteins appears to rely largely on the UPS. Therefore, the normal functioning of the UPS is essential to the quality control of sarcomeres and maintaining its mechanical performance. Insufficiency in molecular chaperone and/or UPS proteolytic function would affect the efficient removal of aged proteins, accumulate abnormal proteins, and cause aberrant protein aggregation. The latter in turn would further compromise the UPS and PQC, forming a vicious cycle. Consequently, dysfunction of the sarcomeres and various organelles as well as deregulation of metabolism and cell signaling would occur and ultimately cardiomyocyte death would ensue (Figure 3).

Multiple lines of evidence support the hypothesis that PQC inadequacy may be a major pathogenic factor in CHF.

7.1 Expression of misfolded proteins causes heart failure in human and mice

PQC inadequacy will inevitably result in accumulation of misfolded proteins and aberrant protein aggregation which have proven to be sufficient to cause cardiomyopathy and CHF in both humans and mice, as exemplified by DRC [158,159].

7.2 Aberrant protein aggregation impairs UPS proteolytic function in cardiomyocytes

In both cultured cardiomyocytes and intact mouse hearts, aberrant protein aggregation caused by expression of a number of mutant proteins has been shown to impair proteasome proteolytic function [15,68,160–162].

It should be pointed out that the term abnormal protein aggregation refers the entire process by which misfolded proteins form initially soluble oligomers and eventually microscopically visible protein aggregates. The insoluble aggregates (also described as inclusion bodies or aggresomes) are currently believed to be potentially protective and may not be the direct cause of UPS impairment. The small, soluble, oligomeric intermediates formed during aberrant protein aggregation are likely the toxic species that impair the UPS via mechanisms currently unknown. Using cell compartment-restricted (cytoplasmic vs. nuclear) GFP-based UPS reporters in cultured cells, Bennett *et al* demonstrated global impairment of the UPS by nuclear or cytoplasmic protein aggregates and they further revealed that the significant UPS impairment occurs prior to the coalescence of aggregated proteins into inclusion bodies [163]. Evidence for direct interaction and inhibition of proteasome proteolytic subunits by aggregation-prone proteins was recently reported [164,165].

7.3 Mitochondrial malfunction and apoptosis in CryAB^{R120G} transgenic mouse hearts

Mitochondrial dysfunction and cell death are often associated with CHF. Mitochondrial malfunction and increased apoptosis were observed in CryAB^{R120G} transgenic mouse hearts which display severe proteasomal malfunction at early age [15,166]. Due to the chaperone property of CryAB which may directly protect mitochondria and prevent caspase activation [167,168], it remains to be investigated whether these derangements is resulted from aberrant protein aggregation and proteasomal malfunction. Nevertheless, pharmacological proteasomal inhibition has been shown to induce cell death in cultured cardiomyocytes [71,148].

7.4 Genetic perturbations of the UPS compromise the heart in mice

Although no animal models with cardiomyocyte-restricted proteasomal inhibition have been reported, several studies demonstrated severe cardiac abnormalities in mice with ubiquitous knockout of ubiquitin E3 ligases. Fielitz et al showed that loss of both MuRF 1 and 3 causes striated muscle myopathy with pathological changes including myosin accumulation, myofiber fragmentation, and diminished performance [28]. The same group reported predisposition of the heart to cardiac rupture after MI by loss of MuRF 3 [27]. Mice deficient of CHIP were defective in their ability to cope with MI [169].

7.5 Proteasome inhibition causes human heart failure

Although studies using experimental animals have suggested that systemic proteasome inhibition can suppress cardiac hypertrophy and may even reduce myocardial I/R injury possibly through preventing NFκB activation [19,20,170,171], emerging clinical evidence supports that chronic proteasome inhibition compromises human heart function. A synthetic proteasome inhibitor (bortezomib or PS341) is clinically used to treat certain types of hematological malignancy and has been experimentally tested in combination with other anti-tumor drugs to treat solid tumors. Recent clinical reports reveal cardiotoxicity in cancer patients receiving proteasome inhibitor bortezomib (Velcade) [172–174]. Nevertheless, no study has

been reported to examine the effects of chronic cardiomyocyte-restricted proteasomal inhibition on the heart.

8. A Summary and Future Directions

In summary, PQC and protein degradation are extremely important for maintaining normal cardiac function. PQC in cardiomyocytes appears to be inadequate in the progression of a range of cardiovascular disease to CHF, as reflected by decreased levels of key HSP (e.g., CryAB), sustained UPR, likely proteasomal malfunction, and accumulation of pre-amyloid oligomers in failing human hearts and several animal models of CHF. Inadequacy in PQC can lead to accumulation of misfolded and damaged proteins which in turn further impairs PQC in the heart, forming a vicious cycle that deteriorates cardiomyocyte function and fate (Figure 3). Therefore, PQC and protein degradation in cardiomyocytes represent an extremely important area that warrants more intensive investigations.

Our understanding of molecular mechanisms governing PQC and protein degradation in cardiomyocytes is far from being complete. Elucidation of the regulation of UPS proteolytic function and its interaction with other proteolytic pathways will be essential to the search for means to manipulate proteolysis in the cell to more effectively treat disease. The role of PQC inadequacy in cardiac remodeling and failure remains to be established. Temporally controlled tissue-specific genetic manipulations will be extremely helpful in addressing whether and how cardiomyocyte-restricted UPS inhibition causes heart failure and/or increases the propensity toward heart failure in adult animals, whether acute or chronic manipulation of proteasomal activity is a viable therapeutic approach, and the necessity of PQC inadequacy in the genesis of CHF.

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Figure 1.
An illustration of PQC and consequence of PQC inadequacy in cardiomyocytes.

Figure 2.

Direct green fluorescence confocal micrographs of cardiac and skeletal muscles (m.). Tissue samples were collected from Line 3 GFPdgn transgenic (TG) mice 20 hours after an intravenous injection of MG-262 (5 μmol/kg body weight) or vehicle control (DMSO). No GFP protein aggregates are detected in either myocardium or skeletal muscle before or after MG-262 induced proteasomal inhibition. The latter uniformly increases GFPdgn fluorescence. Scale bar=50 μm.

Figure 3.

An illustration of the hypothesis on the role of PQC inadequacy in CHF. UPR: unfolded protein response; mito: mitochondrial.