Proteotoxicity and Cardiac Dysfunction

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Abstract: Baseline physiological function of the mammalian heart is under the constant threat of environmental or intrinsic pathological insults. Cardiomyocyte proteins are thus subject to unremitting pressure to function optimally, and this depends on them assuming and maintaining proper conformation. This review explores the multiple defenses a cell may use for its proteins to assume and maintain correct protein folding and conformation. There are multiple quality control mechanisms to ensure that nascent polypeptides are properly folded and mature proteins maintain their functional conformation. When proteins do misfold, either in the face of normal or pathological stimuli or because of intrinsic mutations or post-translational modifications, they must either be refolded correctly or recycled. In the absence of these corrective processes, they may become toxic to the cell. Herein, we explore some of the underlying mechanisms that lead to proteotoxicity. The continued presence and chronic accumulation of misfolded or unfolded proteins can be disastrous in cardiomyocytes because these misfolded proteins can lead to aggregation or the formation of soluble peptides that are proteotoxic. This in turn leads to compromised protein quality control and precipitating a downward spiral of the cell’s ability to maintain protein homeostasis. Some underlying mechanisms are discussed and the therapeutic potential of interfering with proteotoxicity in the heart is explored. (Circ Res. 2015;116:1863-1882. DOI: 10.1161/CIRCRESAHA.116.305372.)

Key Words: autophagy ■ proteasome ■ protein

Introduction: Proteotoxicity

Protein homeostasis, or proteostasis, plays an essential role in maintaining overall cellular health. Perturbations to proteostasis must be adeptly controlled to avoid pathological consequences. In the absence of external or cell autonomous compensatory mechanisms, alterations to proteostasis, the cell’s overall health may rapidly deteriorate, leading to necrosis, apoptosis, or caspase-independent cell death, all of which seem to involve regulated cellular and molecular programs.1,2

Many factors can affect protein folding and misfolding, but the cellular pathogenic aspects of protein misfolding and aggregation are collectively termed proteotoxicity. The term is relatively recent, with the first references in the cited literature occurring in a 1991 meeting report and was coined to describe “damage to proteins caused by chemical and physical agents.”3 The concept of proteotoxicity was firmly established over the next 10 to 20 years; broadly defined, it is any impairment of cellular function as a result of protein misfolding or aggregation. Its importance has been emphasized and remains a particular focus of study for various neurodegenerative diseases in which protein misfolding and the occurrence of proteinaceous aggregates is a recurring theme.4-8 Proteotoxicity is also a normal occurrence during the aging process (a universal risk factor), and indeed, many of the proteins that function in maintaining proteostasis4 have been implicated in cellular aging,9 offering an intriguing rationale for the age-dependent onset of many protein conformation-dependent diseases, including some of the neurodegenerative pathologies.10

The general importance and prevalence of protein misfolding and its impact on human disease was illustrated by the first mechanistic understanding of an inherited disease, sickle cell anemia, when, in 1957 to 1959, Ingram and Hunt showed that a glutamate to valine mutation in hemoglobin’s β-globulin chain resulted in a conformational change in the protein and aberrant polymerization.11,12 However, it rapidly became apparent that there is no single resultant pathogenic mechanism that occurs as a result of protein folding.13 Rather, the resultant pathologies are protein-specific: (1) improper folding or maintenance of structure can lead to a protein’s improper degradation, such as occurs in cystic fibrosis (cystic fibrosis transmembrane conductance regulator protein) and Gaucher disease (β-glucosidase)14,15; (2) a dominant negative mutation that leads to a hypo-functional peptide, which occurs in epidermolysis bullosa simplex (keratin)16; (3) a gain of toxicity function, such as occurs with certain variants of APOE4 in Alzheimer disease (AD)17; and (4) the accumulation of multiple, misfolded species, resulting in formation of toxic poly peptides and aggregates, such as occurs with many of the neurodegenerative diseases.18,19
Thus, protein misfolding disorders are complex, multifactorial, and can be cell autonomous or not with neighboring cells affected. In this review, we necessarily focus on a subset of processes that underlie an important part of proteostasis in the heart.

**Network of Cellular Defense**

Cells possess innate mechanisms to sense protein unfolding/misfolding and restore normal conformations. The mechanisms that comprise cellular protein quality control (PQC) are complex and multifaceted, consisting not only of constitutive and inducible chaperones (discussed below), but also engaging compartment-specific mechanisms. But even PQC represents only a small part of the overall process of proteostasis, which can be portrayed as an entire landscape, or all-encompassing network, that takes into account all aspects of the processes that underlie the cell’s stable or unstable protein complements. Thus, transcriptional, translational, and post-translational regulation all form a part of this network as do the dynamic processes of folding, unfolding, post-translational modifications, trafficking, and finally, degradation. Rather than being static, proteostasis itself changes over time and is thought to break down as the organism ages. Although the network can often handle processes that lead to damaged proteins by subjecting these peptides to various facets of the PQC, such as degradation via the proteasome, eventually the cell can be overwhelmed, either as part of the normal aging process or, earlier in life, as a part of a disease process. Thus, an aberrantly misfolded protein that is competent to provoke pathogenesis may be tolerated for years or even decades, but as the overall competence of the proteostasis network declines as a result of age or some other extrinsic or intrinsic stimulus, the pathology begins to present clinically. Consistent with this view is the compromised ability of aged cells to maintain proteins in their proper conformation, as well as compromised PQC. Thus, an aging cell, particularly one that does not divide, may be faced with an ever-increasing burden of protein misfolding in the face of compromised systems for decreasing the pathogenic load.

That protein misfolding can lead to cardiovascular disease has been known for many years; transthyretin amyloidosis, caused by the by-product of transthyretin tetramer disassembly and the subsequent extracellular deposition of the resulting amyloid fibrils, has been well characterized and extensively reviewed. However, only recently have cardiomyocyte-based proteotoxic entities become a focus of mechanistic studies, which has led to the realization that proteotoxicity may represent a convergent pathway in heart disease and the development of heart failure. In this review, we will consider the particular sensitivity of the cardiomyocyte cell population to proteotoxic insult and introduce the relevant pathways that constitute aspects of the cardiomyocyte’s PQC response. Some specific proteotoxic stimuli will be considered and the cardiomyocyte’s ability to respond to these insults will be outlined, as well as the pathogenic sequelae that can ensue. Finally, we will consider whether and how our developing understanding of these processes might be translated into new therapeutic initiatives for decreasing cardiac morbidity and improving clinical outcomes.

**Protective Pathways**

Preventing the accumulation of misfolded proteins is the job of every cell in an organism. An estimated 30% of translated proteins never reach their final cellular destination, and these newly synthesized nascent proteins are recognized and degraded almost immediately after translation; degradation of some proteins occurs cotranslationally. This requires constant monitoring of protein fidelity and has led to evolutionarily conserved, efficient mechanisms of protein degradation to keep misfolded proteins from exerting cytotoxic effects. However, the risk of proteotoxic insult is not equal among all cell types. Not surprisingly, postmitotic cells seem to be particularly susceptible to proteotoxic effects. When faced with a proteotoxic insult, mitotic cells can asymmetrically divide the misfolded protein load, thus protecting one daughter cell while sacrificing the other. Post-mitotic cells do not have this luxury; the relative inability of these cells to regenerate puts the entire organ at increased risk of proteotoxic threats because the cells cannot readily divide to replace cells that are lost to injury. In this respect, the cardiomyocyte is similar to another postmitotic cell in a vital organ: the neuron. These 2 cell types are the most susceptible to proteotoxic insults; neuronal cell death can result in neurologological dysfunction and neurodegeneration, just as loss of cardiomyocytes as a result of cell death leads to cardiomyopathy and heart failure. Indeed, recent data have strengthened the idea that cardiomyocyte proliferation and regeneration of the adult heart is a rare occurrence as is the case for human neocortical neurons.

Proteotoxic insults can lead to cell death and dysfunction and give rise to severe phenotypes in tissues, such as the brain and heart. The inability of a cell to divide its way out of trouble has led to the evolutionary development of sophisticated, multi-tiered, and interconnected mechanisms of PQC; these have evolved to maintain protein fidelity in the face of intrinsic and extrinsic insults and prevent misfolded...
proteins from accumulating. Among these are the chaperones, which include the heat shock proteins (HSP), the ubiquitin proteasome system (UPS), the autophagy–lysosome pathway, and the endoplasmic reticulum (ER)–associated degradation stress response (Figure 1). These 4 pathways comprise the cellular PQC response. Together, they normally ensure that the balance of protein synthesis to protein degradation is maintained, functioning independently and in concert with one another to recognize and eliminate proteotoxic threats.

Heat Shock Response
Chaperones represent a first line of defense in the post-translational processes that conserve protein tertiary and quaternary structure and conformation. Chaperones are constitutively expressed, but the expression of a small subset can also be upregulated in response to unfolded or misfolded protein accumulations; this response represents part of the heat shock response (HSR). The molecular identities, functions, and genetics of HSPs were first rigorously explored using the power of fly genetics. As the field has grown and matured over the past 35 years, the importance of the chaperones and their essential role in the correct folding of multiple proteins has become well established. The chaperones are represented by multiple classes and have diverse natures. Their many functional roles are underscored by changes they may undergo as a result of post-translational modification, the potential for their transcellular activities and the role they play in a wide variety of diseases, including but not limited to Pompe disease, Huntington disease, cancer, and pulmonary and cardiovascular disease.

The HSR is the most evolutionarily ancient of the PQC pathways, enlisting molecular chaperones (Figure 1, top panel), such as the HSPs, to manage two primary functions within the cell: transiently interact with nascent unfolded polypeptides to guide them toward their proper folded conformation and target terminally misfolded proteins for degradation. Many or even a majority of proteins cannot spontaneously fold to assume a functional conformation; during or immediately after translation on the polysomes, exposed hydrophobic stretches are prone to aggregate in the aqueous environment of the cytoplasm. HSPs bind to these hydrophobic stretches and prevent them from aggregating before the proper folded conformation can be attained. A primary driver of the HSR is heat shock factor-1, a powerful transcription factor that is normally bound in an inactive complex in the cytosol containing other stress-responsive proteins, such as Hsp90 and histone deacetylase (HDAC). Misfolded protein stress facilitates dissolution of this complex, and heat shock factor-1 is translocated into the nucleus and induces expression of other HSPs, such as Hsp70 and Hsp40. In the heart, chaperones are critical for ensuring that proteins, such as those of the contractile apparatus, arrive at their proper folded conformation and location within the cell. For example, αB-crystallin (CryAB), a member of the small HSP family, ensures proper folding of desmin, an intermediate filament protein with critical roles in cardiomyocyte structure and function.

Ubiquitin–Proteasome System
The second line of defense in the PQC response is the UPS (Figure 1, panel 2). The UPS mediates the turnover of most cellular proteins, as well as degrading terminally misfolded proteins, via proteasomal proteolysis. The proteasome is a large, multi-subunit complex tasked with the degradation of monomeric proteins; the majority of misfolded protein species are eliminated from the cell by way of the proteasome. Proteins destined for degradation via the proteasome are poly-ubiquitinated by a cadre of enzymes (E1, E2, and E3 enzymes), which engage free ubiquitin and covalently conjugate ubiquitin via a K48 linkage to the target protein substrate. Multiple rounds of this process occur until a polyubiquitin chain is attached to the substrate, which is then recognized by transport proteins and trafficked to the proteasome. The proteasome contains a cavity, or bone, that is equipped with a variety of proteases, which have cleavage activities similar to trypsin, chymotrypsin, and caspaspe, providing the ability to nonselectively degrade most cellular proteins. The fidelity of this process is essential to cellular homeostasis; malfunction of proteasomal proteolysis will lead to misfolded protein accumulation and cellular dysfunction. In fact, proteasomal inhibition as a therapy for selected cancers is being intensively studied, with the goal of inducing proteotoxicity-induced cell death in the metastasizing cell population. However, the efficacy toward cancer cells comes at the expense of the heart, possibly because of the same induced proteotoxicity.

Autophagy–Lysosome Pathway
Recent work suggests that the autophagy–lysosome pathway plays a major role in the cardiac stress response, and here we consider it in some detail. Macroautophagy (hereafter referred to as autophagy) involves a budding membrane called a phagophore, which envelopes cargo destined for degradation into a maturing double-membrane vesicle, the autophagosome. The autophagosome travels along microtubules and fuses with lysosomes, forming a distinct structure, the autolysosome. There, the vesicle is acidified and lysosomal enzymes degrade the enclosed cargo. Membrane components to form these vesicles come from diverse cellular locations, such as the plasma membrane, mitochondria, golgi, and the ER, with multiple proteins precisely controlling all aspects of the process. For a complete description of the autophagic process and its relevance in cardiovascular biology, we direct the reader to a recent comprehensive set of reviews on the topic.

Under basal conditions, autophagy functions at low levels, helping the cell to remodel when necessary and generally functions to maintain a healthy homeostatic balance. However, autophagy can be quickly induced under conditions of cellular stress. For example, subjecting cardiomyocytes to starvation, via either calorie deprivation or fasting in mice or serum/glucose deprivation in vitro, results in a rapid upregulation of autophagic flux. This allows cellular components to be degraded and maintains the pool of free amino acids so that the nutrient and metabolic demands of the cell continue to be satisfied. Indeed, autophagy has been shown to be necessary in all stages of life; for example, autophagy is markedly upregulated in neonates during the latent period between birth and establishment of the maternal
milk supply. Ablation of the autophagy gene aut5, which encodes an E3 ubiquitin ligase that plays an essential role in autophagosome formation, resulted in neonatal lethality within one day after birth, an effect that could be prevented by forced milk feeding. Autophagy is also reduced in aging, suggesting that a deficit in the breakdown of damaged...
proteins and organelles may contribute to the general aging process. 

Autophagy is essential for normal heart maintenance and function, and defects in any part of the autophagic process can lead to cardiomyopathy. Deletion of arg5 in the adult mouse led to increased pathological hypertrophic signaling, the development of hypertrophy and cardiac dysfunction, and increased cell death. 

Induction of parallel PQC mechanisms, such as proteasomal activity and ER stress markers, was also observed. These pathways may be induced to compensate for loss of autophagy, although the response clearly could not fully mitigate the damage. These data are underscored by deficits in the autophagic pathways being shown to directly cause human cardiac disease. For example, a deficiency in the lysosomal membrane protein Lamp2 causes Danon disease, a severe cardiomyopathy. Lam2 knockout mice show significant accumulations of autophagic vacuoles, implying a deficit in lysosomal degradation of autophagic cargo, a phenotype that leads to increased mortality and cardiac dysfunction.

In addition to the role played in degrading organelles and pathogens, autophagy is the primary pathway for degrading and recycling long-lived proteins, and particularly those that form larger aggregates that cannot be processed through the proteasomal core. In some cases, these aggregates can actively form by a process that was well characterized in the neurodegenerative diseases, in which small proto-aggregates or misfolded polypeptides are actively transported on dynein motors retrogradely via microtubules to a perinuclear location to form proteinaceous bodies known as aggresomes (Figure 1, panel 3). As autophagy is the only direct mechanism that can degrade these large, misfolded protein aggregates within cells, this pathway is particularly consequential in combating proteotoxic phenotypes.

Endoplasmic Reticulum–Associated Degradation and Stress Response

The ER stress response, also referred to as the unfolded protein response, is another important PQC mechanism in the heart. This pathway plays a critical role in maintaining proteostasis because it serves as a cellular monitor of misfolded protein load and, through induction of ER stress genes, tailors the cellular homeostatic balance in response to increased levels of misfolded protein in the ER. On sensing changes to the misfolded protein load in the ER/SR microenvironment, 3 transmembrane ER proteins (PERK, IRE1, and ATF6) are activated. Activation stimulates nuclear translocation of transcription factors ATF4, ATF6, and XBP1, which upregulate the proteins that comprise the ER stress response, including chaperones and other proteins involved in misfolded protein stress. When a protein misfolds and cannot be rescued, these ER stress response genes efficiently target aberrantly folded proteins for degradation through the mechanism of ER-associated degradation (Figure 1, panel 4). In which multiple ER lumen or ER membrane–associated proteins bind to the misfolded protein, the end result being export of the peptide into the cytoplasm where it is ubiquitinated and targeted to the proteasome or for chaperone-assisted refolding. This process is crucial for dealing with terminally misfolded proteins and preventing their accumulation. Unsurprisingly, ER stress is induced with cardiac injury, including ischemia, pathological hypertrophy, and heart failure, and helps to manage the cellular protein imbalance induced by these conditions in an attempt to mitigate cellular damage.

Recent data have linked ER stress to autophagy activation, suggesting the 2 pathways might work in concert in a cardioprotective mechanism designed to restore homeostasis. However, the intersections, interplay, and coordinating control mechanisms between these pathways remain largely obscure. These pathways are indispensable to ensure that the degradative capacity of the cardiomyocyte is met. However, it is important to note that more protein degradation is not protective in all cases. To the contrary, these pathways are only protective if they maintain the appropriate balance of protein synthesis and degradation such that proteostasis is achieved. Unconstrained or inappropriate catabolism can be equally damaging to the heart. For example, the mechanistic target of rapamycin (mTOR) is a negative regulator of autophagy, and the use of mTOR inhibitors can induce autophagy. However, ablation of mTOR leads to unrestrained autophagy and severe cardiac dysfunction. Similarly, the chemotherapeutic drug, doxorubicin, can be cardiotoxic. Intriguingly, recent data show that doxorubicin can augment proteasomal activity, suggesting that increased proteasomal degradation can also be deleterious to the heart.

Choices: Selective Autophagy

The cell must have efficient mechanisms for selecting the correct, misfolded proteins for either degradation or sequestration. In this section, we focus on the process of autophagy and discuss mechanisms that make it more selective. Autophagy was first thought to be a relatively nonspecific process, but we now know that it is highly regulated and cargo-directed. In fact, the process can be selective, with specialized pathways existing for specific degradation of old or damaged organelles, such as mitochondria, peroxisomes, and ribosomes, although the specific players and processes of these pathways are just beginning to be understood. This process, known as selective autophagy, uses specific cargo proteins, such as p62 and Nbr1, which bind ubiquitinated proteins and bring them into contact with the protein LC3, which is associated with the autophagosomal membrane, thus targeting ubiquitinated proteins for autophagic degradation (Figure 2). For example, upon polyubiquitination of mitochondrial membrane proteins by the E3 ligase, parkin, p62 binding facilitates degradation of the mitochondria by mitophagy. As damaged mitochondria can quickly begin to produce cellular oxidative stress and other inducers of cell death, the fidelity of these processes is crucial to overall cell health. Indeed, similar autophagic mechanisms can also be induced to quickly recognize and eliminate cellular pathogens, thus playing a critical role in the host response to viral infection.

Cargo adapter proteins, such as p62, may hold the key to selective autophagy: p62 colocalizes with ubiquitin in punctate structures and is found in both membrane-bound autophagosomes and protein aggregates and is degraded by autophagy. Interestingly, when p62 levels are reduced, LC3 puncta, indicative of autophagosomes and aggresomal structures, are not formed, suggesting that a cargo adapter like p62...
is necessary to bind misfolded ubiquitinated structures and transport them for autophagic and aggresomal sequestration (Figure 2).88

Zheng et al probed the role of p62 in cardiac proteinopathy and found increased p62 levels in 2 mouse models that exhibit proteotoxicity as a result of cardiomyocyte-specific expression of either a mutated desmin or an associated protein, a mutated α-B crystallin (CryABR120G).89 This compensatory induction is likely an attempt to bind ubiquitinated substrates for autophagic degradation because the aggregates in CryABR120G contain ubiquitinated proteins.90 Indeed, knockdown of p62 not only reduced protein levels of the autophagic marker, LC3-II, but also reduced aggresome formation in a manner that was cytotoxic to the cardiomyocytes.89 These data suggest that p62 is a necessary factor in inducing aggregate formation and autophagic degradation of these aggregates. Indeed, on Atg7-mediated autophagy induction in CryABR120G hearts, p62 levels increased, which correlated with reduced aggregates and improved cardiac function.91 We speculate this increase in p62 allows more ubiquitinated misfolded protein to be sequestered in autophagosomes by a selective autophagic mechanism, consistent with a hypothesis that aggregate formation process may, in some cases, be protective, which is discussed in detail in the following sections.

In addition to p62, HDAC6 may also be a central player in the cellular response to proteotoxic stress. HDAC6 regulates many aspects of PQC;57 for example, HDAC6 deacetylation of Hsp90 helps to maintain a complex between HDAC6, Hsp90, and heat shock factor-1, which serves to repress the HSR; this complex is dissociated in response to proteasomal inhibition.52,92 HDAC6 is central to aggresome formation and, in its absence, aggregate-prone proteins have a reduced ability to form aggresomes, which increases cell death.93 HDAC6 expression rescued neurodegenerative phenotypes in a Drosophila model of polyglutamine expansion in an autophagy-dependent manner. The rescue was abolished in autophagy-deficient flies,94 consistent with a role for HDAC6 in autophagic degradation.95

Not coincidentally, some of the same proteins involved in selective autophagy seem to contribute to aggresome formation as well,95,96 suggesting that process and autophagic degradation of large aggregates may directly intersect (Figure 2). Recent data bear this out: small aggregates can be degraded by both basal (ie, that which occurs under normal cellular conditions) and induced autophagy by starvation, whereas aggresomal proteins are only degraded under conditions of induced autophagy and not by autophagy that occurs at the basal state.97

Cardiac Proteotoxicity

In light of the potential for varying etiologies and mechanisms, it is not surprising that a unifying mechanism of cardiac proteotoxicity is not yet apparent. At this point, it is clear that protein misfolding in the heart can be either extracellular, as is the case for transthyretin amyloidosis,24,98 or intracellular, as occurs in the desmin-related (cardio)myopathies (DRM).99

Figure 2. Selective cargo recognition and degradation by autophagy. Step 1, Ubiquitin binding is necessary for most modes of selective autophagy. Polyubiquitin conjugation onto a substrate protein is accomplished via an E3 ubiquitin ligase. Many of these proteins are ubiquitinated via a K63 linkage, which signals lysosomal degradation rather than the K48 linkage that is typical for proteasomal targeting. Step 2, Ubiquitinated proteins can be recognized by cargo receptors, such as p62, NBR1, or histone deacetylase (HDAC)6 for transport towards the lysosome or aggresome. p62 (icon) binds ubiquitinated proteins for interaction with LC3 in a growing autophagosome, thus selectively targeting the protein for autophagic degradation after fusion with lysosomes. In addition, HDAC6 can bind ubiquitinated proteins through its ubiquitin-binding domain (icon) and transport them in a retrograde fashion along microtubules by binding to the motor protein dynein through the dynein motor–binding domain (icon). These can be single misfolded species or soluble proto-aggregates. This event can happen many times, causing the proteins to coalesce and form aggresomes in the perinuclear region of the cell. Aggresomal proteins can also be degraded by autophagy, and these cargo transport proteins may be involved in this process as well, particularly HDAC6 with known roles in mediating the autophagosome–lysosome fusion event.
Diverse pathogenic insults can also lead to proteotoxicity, including, but not limited to, chronic synthesis of a compromised protein, organ-specific pathogenic stimuli, such as cardiac infarction, pressure overload–induced hypertrophy, or the production of reactive oxygen species, and generalized systemic insults, such as diabetes or aging. These different primary etiologies can have multiple effects on either the production of misfolded proteins, their accumulation in critical cellular compartments or the cell’s machinery for decreasing the misfolded protein/protein aggregate load.

**Mechanistic Basis for Protein Aggregation Toxicity**

Amyloidosis remains the classic manifestation of protein aggregate–based disease but, as many proteins have amyloidogenic potential, its diagnosis is dependent on general histological criteria, including positive staining with Congo Red, which produce an apple-green characteristic appearance when viewed under birefringent microscopy. Early on, the discovery that neurodegenerative diseases, such as AD, were invariably accompanied by the accumulation of amyloid aggregates or plaques prompted the conclusion that the aggregates themselves were toxic but, as murine models of the disease were developed in the recent past, a more nuanced view has emerged.

As noted earlier, amyloidosis in the cardiac system is well-characterized, with multiple proteins such as transthyretin, the immunoglobulin light and heavy chains, serum amyloid A, atrial natriuretic factor, and a subset of the apolipoproteins confirmed as being amyloidogenic in the heart. However, the study of other cardiac diseases that are characterized by the intracellular deposition of proteinaceous aggregates has been instructive as well in determining the pathogenic sequelae associated with abnormal peptide aggregates in the cardiomyocyte. Approximately a decade ago, others and we began an intensive study of an aggregation-prone form of the small HSP-like CryAB and its expression in the heart. Originally identified as disease-causing through the genetic analysis of a French family, disease causation of the mutated protein, CryABR120G, was confirmed by its cardiomyocyte-directed expression in transgenic animals. As is the case in human patients, expression of CryABR120G in the mice led to the gradual development of restrictive, hypertrophic, or dilated cardiomyopathy and the formation of large, granulofilamentous protein aggregates in the myoplasm that were easily visible by both light and electron microscopy (Figure 3). Interestingly, the aggresomal and autophagic machinery and transport mechanisms have many common players, and we are beginning to understand a sophisticated mechanism that can recognize and degrade protein aggregates by autophagy, termed aggrephagy (Figure 1, panel 3). Similar to other forms of selective autophagy, aggrephagy involves recognition of ubiquitinated proteins by cargo adaptors, such as p62 and NBR1, which recruit other autophagic proteins, such as Atg5-Atg12 and Alfy, a proposed component of the specific aggrephagic response.

Are aggresomes intrinsically toxic? Like many of the amyloid diseases, DRM is caused by a toxic gain-of-function protein, in the sense that it is dominant over the wild-type protein and causes aggregates to form even in the presence of near normal amounts of wild-type CryAB. For many years, these protein deposits in general, and amyloid plaques in particular, were thought to be the toxic species that caused the clinical disease as, in the different neurodegenerative diseases, they were invariably present in high concentrations on postmortem assessment of the affected tissues. But closer examination in animal models that appeared to contain benign amyloid deposits, or even deposits that were posited to have positive phenotypic effects, remained a nagging contradiction to this hypothesis. A variety of experimental avenues from different neurodegenerative diseases, including AD and the polyglutamine expansion-based diseases, indicated that the visible aggregates were, in fact, not the toxic entity; rather, a soluble, oligomeric precursor that assumed a characteristic conformation might be the primary culprit (Figure 4). Development of an antibody capable of recognizing the conformer, which forms as a result of a folding process that many diverse proteins can undergo, revealed the presence of the preamyloid oligomers (PAO) in many protein aggregation-based diseases, including DRM. Although the toxic oligomer, as opposed to the insoluble plaques, is now widely thought of as being the causative agent in many neurodegenerative diseases, the concept is not universally accepted. That, and the lack of an agreed upon structure for the oligomer (which can apparently be formed by many diverse proteins through an as yet ill-defined process), continue to hamper progress toward a coherent therapeutic approach and a final determination as to whether the toxic oligomer truly is the pathogenic species.

Although often associated with the same misfolding phenomena, aggresome formation (Figure 1) is fundamentally different from PAO production (Figure 4), even though the same protein or proteins may or may not be involved. In a mouse model of AD, cellular toxicity is correlated with the presence of PAO, not amyloid fibrils. Cellular toxicity in Huntington disease, which is caused by the accumulation of mutant huntingtin protein aggregates within intranuclear inclusion bodies, is associated not with these large aggregates but with the presence of a soluble form of the mutant protein. Similarly, the large, neuronal intranuclear inclusions in spinobulbar muscular atrophy, which is caused by polyglutamine expansion (CAG repeat expansion) in exon 1 of the androgen receptor, consist of proteinaceous aggregates that do not correlate well with cellular toxicity. Instead, the morbidity in this degenerative disease of the lower motor neurons and skeletal muscle correlated with high levels of the soluble intermediates. In fact, cellular toxicity presented in the absence of visible aggregates when androgen receptor containing expanded CAG repeats was expressed in a neuroblastoma cell line. Similar data concerning the toxicity of the soluble oligomer were obtained in an AD mouse model in which an oligomer-capable amyloid β (Aβ) species was synthesized and the animals developed neurodegenerative processes, even the absence of visible fibrils. These data were also recapitulated in human and mouse models of Type 2 diabetes mellitus, which is characterized by a loss of β cell mass...
and extracellular accumulations of islet amyloid derived from the islet amyloid polypeptide, expressed by the β cells along with insulin. When islet amyloid polypeptide accumulates as oligomers, it is a potent cytotoxin, but its accumulation within inclusion bodies in the form of inert fibrils seems to be largely benign. Cardiac data are consistent with the hypothesis that aggresomes are not necessarily cytotoxic because, in inducible PAO-gene models, rescue of the animal was accompanied by decreased PAO levels, although aggresome concentrations appeared to be unaffected. To determine the relevancy of proteotoxicity in cardiac muscle, an analysis of normal and diseased human hearts for the presence of PAO was undertaken to determine whether PAO was present in diseased cardiomyocytes. Although 3 nondiseased hearts showed no or minimal traces of PAO, samples from 9 hypertrophic or dilated cardiomyopathic hearts showed high, but variable, levels of PAO internal in the cardiomyocytes. The presence of PAO in diseased hearts having presumably different primary causes implies that PAO formation may be a general and potentially widespread phenomenon.

Figure 3. Anatomy of aggregates. A, A typical, mature aggregate in a αB-crystallin (CryABR120G) cardiomyocyte derived from a 4-month-old, symptomatic heart. Note the fenestrated organization and the mitochondria trapped within the aggregate. B, Immunogold staining (dark grains) showing the strong presence of CryAB within both the large, well-defined aggregates and the smaller, more amorphous masses, some of which are mitochondria (mit). C and D, High magnification, immunogold staining using cardiomyocytes derived from 6-week-old CryABR120G hearts showing the internalization of CryAB, but also (D) the presence of desmin. Photomicrographs courtesy of H. Osinska. Mice, fixation techniques and details of the transmission electron microscopy were performed as described.

Figure 4. Proteotoxic peptides. Shown is the formation of what are thought to be the toxic entities in a wide variety of proteotoxic diseases: small, soluble preamyloid oligomers. These are metastable entities in slow or rapid equilibriums with one another and, at least for some entities, the dimers and trimers are the most toxic form. Enhanced autophagy is correlated with reduction in toxic preamyloid levels, but the mechanism of clearance remains obscure (see text for details).
in several different heart failure presentations. However, this remains to be formally demonstrated in a rigorous, carefully controlled manner, and sample collection becomes a key factor in quantitating the degree of pathology as characterized by PAO levels.

**PAO Causality in Heart Failure**

Are the soluble PAOs observed in diseased hearts sufficient to cause heart disease and failure? To address this question, ectopic expression of a PAO-genic polypeptide specifically in cardiomyocytes was performed using transgenesis. Expression of a PAO-genic CAG repeat at low concentrations in the cardiomyocytes of transgenic mice was invariably toxic, with all mice dying of heart failure by 7 months. Although aggresomes were also present, they appeared to be frequently engulfed by autophagic vesicles, consistent with the hypothesis that the aggresomes were in the process of being degraded. This experiment confirmed that synthesis of a PAO-genic polypeptide in cardiomyocytes was sufficient to cause death by heart failure, and causality for the PAO was thus established.

**Preamyloid Structure and Toxicity**

The nature of these soluble oligomers has been extensively studied but, as alluded to above, the processes leading to their generation and structure or structures remain elusive because they are detected by either polyclonal or monoclonal antibodies that recognize conformers that do not depend on a specific linear amino acid sequence. This diversity has led to an unsettled literature as to the toxic mechanism or mechanisms by which their pathogenic effects are mediated. Although the oligomerization pathways (Figure 4) are not straightforward because of the diversity of primary sequences in the many proteins with amyloidogenic potential, there do seem to be some commonalities. Many of the oligomers show, on biophysical examination, ordered assemblies with a well-defined pattern of intermolecular contacts and at least some of these contacts involve regions that form a β structure. This is partially mediated via their action on specific receptors. 

It is not clear whether this is caused by direct binding or to some other action of the oligomer on one or more signaling pathways, but binding to multiple receptors has been observed. The propensity of oligomers to be sticky and exhibit detergent-like qualities has been remarked upon by several groups, and the oligomer’s ability to interact with different membrane systems has been proposed to be intrinsic to their toxicity.

In the heart, membrane integrity is obviously critical for the maintenance of cell viability. Disruption of either the plasma membrane or the other vital membrane-enclosed organelles, such as mitochondria or T-tubules, would clearly affect cardiomyocyte function. Data from model membrane systems demonstrate the ability of oligomers to form cation-specific channels that destroy calcium homeostasis with concomitant cytosolic elevations of the ion. Consistent with this hypothesis, the mutant form of CryAB, CryAB<sup>G120G</sup>, preferentially associates with mitochondrial membrane proteins (Figure 3, panel B), and the early ablation of mitochondrial membrane integrity occurred almost immediately on expression of the CryAB<sup>G120G</sup> in cardiomyocytes, with cytotoxicity quickly following despite abundant mitochondrial biogenesis. On the basis of all these observations, we can conclude that PAO interactions at the membrane can clearly impair ion and redox homeostasis in the affected cells, but the specific interactions that result in cytotoxicity will differ depending on the membrane system and, to some extent, the oligomeric form assumed by the amyloidogenic polypeptide.

In cardiomyocytes, inducible expression of PAO-genic CryAB<sup>G120G</sup> was used to determine whether the cardiomyocyte had the capacity to clear PAO after it accumulated. Pharmacological intervention via doxycycline treatment effectively terminated expression of the mutant CryAB in symptomatic mice (11–12 weeks of CryAB<sup>G120G</sup> expression), and PAO was subsequently measured after 1 month. Decreases of ~40% were observed, implying that either the PAO is intrinsically unstable during this time period or the cardiomyocyte is able to clear the toxic entity.

As the earlier discussion makes clear, soluble oligomers can interact with membrane systems, and it was therefore reasonable to test whether autophagy could play a role in PAO clearance because the process involves interaction with and...
engulfment by membrane vesicles.\(^65,72\) Upregulating autophagy by either exercise or Atg7 overexpression in cells did, in fact, lead to decreased PAO accumulation.\(^28,112,148\) Similarly, loss of function for autophagy led to increased PAO accumulation,\(^28,148\) although a direct demonstration of toxic PAO trafficking through autophagosomes was lacking.

### Are Aggresomes Also Toxic?

The toxic role that aggresomes play may be more nuanced because they represent the end result of a process by which misfolded proteins and protein aggregates are sequestered from the general cytoplasm (Figure 1, panel 3). If the misfolded protein is indeed toxic, then within limits, aggresome formation might be thought of as a protective mechanism for isolating a potentially toxic entity, as noted above for the islet amyloid polypeptide.\(^127\) Indeed, inclusion body formation in the α-synuclein accumulation diseases, such as Parkinson disease, does seem to be cytotoxic.\(^140,150\) Aggresomes can, in many cases, successfully protect the cell against proteotoxicity, likely by sequestering the toxic species until the aggresome or inclusion body can be cleared by autophagy.\(^7,38\) Sequestration of toxic oligomers by the chaperone, HspB1, resulted in removal of the Aβ oligomers from the soluble fraction into large, nontoxic aggregates.\(^151\) However, one could envision these garbageumps becoming cytotoxic if PAO was not sufficiently sequestered in the aggregate interior or the aggregate becomes excessively large. This is particularly relevant in cardiomyocytes because function depends on coordinated, compliant contraction over the entire cell. Generation of ever-increasing amounts of large proteinaceous aggregates could also compromise the mechanical characteristics of the cytoplasm. Indeed, in a cell-based study in which cardiomyocytes rapidly expressed high levels of CryAB\(^R120G\) and large aggregates formed, determination of compliance in 3 dimensions by 2-photon microscopy\(^172\) and magnetic bead microrheology at the single cardiomyocyte level showed significant changes in the cytoskeleton’s mechanical characteristics and deficits in passive cytoskeletal stiffness.\(^153\) Additionally, although some commonalities in protein content across different aggresomes occur,\(^8\) there seem to be distinct morphologies as well, with some larger aggregates showing a fenestrated appearance and other, smaller aggregates showing a less-defined morphology (Figure 3, panels A and B). In the CryAB\(^R120G\) model of proteotoxicity, the aggresomes contain high concentrations of CryAB. The intermediate filament protein, desmin, is also present (Figure 3, panel D), a result consistent with data obtained from diverse systems in which intermediate filament proteins, including vimentin and keratin,\(^154\) form a cage around the aggresome, presumably functioning to sequester off the potentially proteotoxic aggregated protein species.

### Restoration of Cardiac PQC Reduces Proteotoxicity

The soluble and insoluble inclusions present in proteotoxic diseases will invariably invoke the cellular PQC response. However, the dysfunction of these processes is as much a part of the proteotoxic phenotype as the oligomers and aggregates themselves. To understand how failure of the proteolytic pathways can cause proteotoxic cardiac disease, it is useful to examine known proteotoxic disease models and understand the specific defects in the cardiac PQC response and, the subsequent, functional consequences.

Generally speaking, there are 2 somewhat predictable responses to PQC pathways as a result of increased proteotoxicity: compensatory induction or inhibition. In the CryAB\(^R120G\) model, both of these effects are observed when examining proteasomal function. Proteasomal activity is targeted to small peptides and smaller misfolded proteins that have not yet formed oligomeric quaternary structures because the small proteasomal bore dimensions (\(≈13\) Å) disallow entry and degradation of larger structures (Figure 1, panel 2).\(^155\) Using a green fluorescent protein (GFP)-tagged proteasomal degron sequence (GFPdgn)\(^156\) as a reporter of proteasomal degradation, Chen et al showed increased levels of GFPdgn in the hearts of 2 DRM models, as well as increased levels of ubiquitinated proteins, both of which point to decreased degradation of proteasomal substrates.\(^155,157\) In addition, they noted dysregulation of the expression of individual proteasomal subunits, suggesting abnormalities in the proteasome itself. Despite this, the specific enzymatic activities of the proteasome were increased, suggesting a functional proteasome that was simply unable to degrade the misfolded protein load. To test this concept, they treated cardiomyocytes infected with CryAB\(^R120G\) with Congo Red, a compound that can prevent aggregate formation by binding misfolded proteins and preventing increased intermolecular interaction. After this treatment, they found reduced aggregate formation and a concomitant decrease in proteasomal degradation, shown by decreased levels of GFPu (a GFPdgn analog). This study demonstrated a deficit in proteasomal degradation in cardiac proteotoxicity that appeared to be the direct result of misfolded protein accumulation.\(^157\) This situation, coined proteasome functional insufficiency,\(^158\) can activate the pathological calcineurin-nuclear factor of activated T-cells pathway,\(^159\) a deleterious pathway in the heart.\(^160\) Of note, GFPu accumulation was also observed in the brain of an AD mouse model,\(^161\) providing further evidence of the similarities in PQC dysfunction, particularly proteasome functional insufficiency, in different proteinopathies.

These data imply that proteasomal degradation is critical in preventing proteotoxicity and that increased misfolded protein load in the sarcoplasm can negatively affect the proteasome from efficiently degrading proteins. Increasing proteasomal function may be beneficial to keep misfolded protein accumulation in check and prevent pathogenesis. To test this hypothesis, Wang and colleagues used transgenesis to induce overexpression of proteasomal components. As expected, overexpression of PA28τ, a component of the 11S proteasome, increased proteasomal proteolytic function as measured by reduced GFPdgn levels. When crossed with CryAB\(^R120G\) mice, they observed reduced protein aggregation and hypertrophy, which led to an extension of lifespan.\(^162\) Further, when they subjected PA28τ mice to ischemia/reperfusion, they noted reductions in infarct size. Consistent with these data, they noted that activation of protein kinase G could increase proteasomal activity and degradation of a proteasomal substrate and reduce pathological aggregates in the CryAB\(^R120G\) mouse model.\(^163\) Recently, Gupta et al demonstrated that increased...
levels of Ubc9, an E2-like ligase in the SUMOylation cascade, also enhanced proteasomal function and degradation, leading to reduced levels of both soluble and insoluble CryAB\textsuperscript{R120G} protein aggregates. Convincingly, this protection was abolished on proteasomal inhibition, confirming that increased proteasomal degradation was causative in the protection.\textsuperscript{164} These data show an important role for the proteasome in reducing misfolded protein accumulation and imply that inducing proteasomal function might be a legitimate therapeutic avenue for the cardiac proteinopathies.

### Autophagy and Cardiac Disease

Autophagic degradation is required for normal cardiac development and function; however, whether autophagy is protective or detrimental during the development and progression of cardiac disease is still a matter of some debate. As with most cardiac PQC mechanisms, autophagic function can be altered under disease conditions, which can lead to an induction or inhibition of autophagic degradation. Some of the first genetic studies of autophagy in the heart focused on \textit{beclin1}, a gene involved in autophagosome formation. Reduction of \textit{beclin1} by heterozygous deletion reduced levels of autophagy and led to a reduction in infarct size in mice after reperfusion injury. Reduced cell death was also noted, and so it is not completely clear that the reduction in autophagy was necessarily the driving force behind reduced ischemic injury.\textsuperscript{70} Similarly, \textit{beclin1} deletion improved hypertrophic phenotypes.\textsuperscript{166} To determine the importance of autophagy in cardioprotection after IPC, Huang et al inhibited autophagy using a dominant-negative Atg5, \textit{atg}5\textsuperscript{K130R}. They found that the cardioprotection afforded by IPC was almost completely abolished when autophagy was disrupted.\textsuperscript{167} Indeed, mTOR inhibition and autophagy stimulation were observed in both chronic and acute IPC.\textsuperscript{168} In addition, recent data in a large animal model showed beneficial effects of pharmacological autophagy induction after reperfusion injury.\textsuperscript{169}

Although some groups have reported that autophagy is activated during a myocardial infarction and upregulating autophagy can be cardioprotective in this context by limiting pathological remodeling and cellular injury,\textsuperscript{170} others have published conflicting data, and this issue has not yet been completely resolved.\textsuperscript{171} Indeed, the apparent discrepancies between different groups’ data may be both apparent and real, with the net effect of autophagy being dependent on the exact environmental insult, its duration, the particular mouse strain, when the autophagic stimulus is induced, and the methodology used to induce it. Similar conflicting data have been obtained concerning the role of autophagy in mediating pressure overload–induced hypertrophy,\textsuperscript{172} and the controversies have recently been reviewed.\textsuperscript{173} Carefully controlled studies are needed to resolve these apparent conflicts, and the use of pure mouse strains and standardized protocols across the different laboratories will be needed to bring much needed clarity to the questions being asked. Although the effects of autophagy may not be universally protective, a consensus is beginning to be reached that autophagy is a protective mechanism in many cardiac pathophysiologies, particularly in those phenotypes with a proteotoxic component. There are multiple steps in autophagy, and the process may be compromised at a single or multiple sites. If only markers of the early autophagic processes are measured, an investigator might be misled, and before any firm conclusions about increased or decreased autophagy are reached, it is important to measure the overall activity of the entire process.\textsuperscript{67}

The effects of autophagy on cardiac proteotoxicity have been comprehensively studied in the CryAB\textsuperscript{R120G} model. As misfolded proteins begin to accumulate and aggregates coalesce into aggresomes (Figure 1, panel 3), one might expect compensatory induction of autophagy as the cardiomyocyte adapts to the insult. Indeed, in proteotoxicity induced by pressure overload and CryAB\textsuperscript{R120G} expression, compensatory induction of autophagy was observed.\textsuperscript{174} However, as the disease progressed, the autophagic system became impaired, leading to decreased autophagic flux and an inability to clear newly formed and preexisting aggregates.\textsuperscript{28,175} These data suggest that autophagic activity is necessary for aggregate clearance, and impairment is a significant aspect of the proteotoxic phenotype.

The hypothesis that increased autophagy might be beneficial in the setting of cardiac proteotoxic disease was tested using the DRM model. Taking advantage of the known pathways that underlie macroautophagy, Pattison et al were able to increase autophagic flux in isolated cardiomyocytes by expressing high levels of Atg7, a critical, ubiquitin-like modifier-activating enzyme (E1) that functions in the initial stages of vesicle elongation, a necessary step for vesicular engulfment of the aggregates or damaged organelles destined for degradation and recycling.\textsuperscript{176,177} When Atg7 was expressed either in cultured cardiomyocytes\textsuperscript{28} or subsequently in transgenic mouse hearts during the proteotoxic insult of CryAB\textsuperscript{R120G} expression,\textsuperscript{178} increased autophagic flux resulted in decreased aggregate accumulation, decreased levels of toxic PAO, and in the whole animals, improved cardiac function and decreased early mortality. Thus, increasing compromised levels of autophagy to basal or even greater than basal activities can be cardioprotective during proteotoxic disease. These data unambiguously demonstrate a beneficial effect of autophagy stimulation on cardiac proteotoxicity in the DRM model and are considered below in terms of potential therapies.

### Mitophagy

Mechanistically, autophagy seems to be protective in part through degradation of damaged organelles. The process of mitochondrial turnover by autophagy, known as mitophagy, is particularly important in the heart because cardiomyocyte function absolutely requires steady production and supply of ATP. When damaged, mitochondria can become tagged for...
degradation through a variety of pathways. The best understood signaling cascade for mitophagy involves PINK1/Parkin, wherein PINK1, a serine/threonine kinase, phosphorylates proteins on the mitochondrial outer membrane and recruits parkin, an E3 ubiquitin ligase, to ubiquitinate these proteins through K63 linkages, which are thought to signal lysosomal rather than proteasomal degradation. Adaptor proteins, such as p62, bind and signal autophagosomal recruitment and engulfment of the mitochondrion by binding to LC3. On ischemic insult or IPC, parkin translocates to mitochondria, which in turn signals the autophagic adaptor p62 to the mitochondria, suggesting that ischemic insult is inducing mitophagy. That similar signaling cascades are activated in both ischemic injury and IPC imply this pathway is cardioprotective. Indeed, the recruitment of p62 to mitochondria after IPC is abolished in parkin-ablated mice, suggesting that the PINK1/parkin route is essential for cardioprotective autophagy postischemia.

**Pathway Coordination**

The above data confirm that the UPS and autophagic pathways are instrumental in trying to manage proteotoxic phenotypes in the cardiomyocyte. From a therapeutic perspective, factors that can modulate both pathways would be of particular interest. Carboxy terminus of Hsc70-interacting protein (CHIP) is one such protein and functions as a molecular chaperone or E3 ubiquitin ligase. Interestingly, CHIP is highly expressed in tissues that require an intensive proteotoxic response, such as the heart and brain: CHIP plays a major role in the cardiac stress response. Recently, in addition to these functions with the UPS, Schisler et al showed that CHIP is required for activation of AMP kinase (AMPK) in response to pressure overload. They identified CHIP as a constituent of the AMPK complex and found decreased AMPK phosphorylation in the absence of CHIP, leading to an exacerbated hypertrophic phenotype. AMPK is a known regulator of autophagy because activated AMPK inhibits mTOR, allowing autophagy to proceed. Proteins, such as CHIP, which mediate crosstalk among multiple protective pathways, may prove especially useful for treating proteotoxic diseases of the heart.

Although modulation of the UPS and autophagic degradation have clear benefits toward modulating cardiac proteotoxicity, other strategies, such as manipulation of the oxidative-reductive or ER stress pathways, also show promise. In the CryABR120G model, abnormalities in the HSR have been observed, including induction of chaperone proteins and sequestration of HSPs inside aggregates. In transgenic mice expressing human CryABR120G in the cardiomyocytes, increased levels of Hsp25, glucose-6-phosphate dehydrogenase, catalase, and glutathione reductase led to the hypothesis that reductive stress via overcompensation of the oxidative stress response was pathogenic. Indeed, reducing the levels of glucose-6-phosphate dehydrogenase by crossing human CryABR120G mice with glucose-6-phosphate dehydrogenase mutant mice, which have reduced glucose-6-phosphate dehydrogenase activity, reduced hypertrophy and aggregate formation, although the exact mechanism by which this occurs was not determined.

**Therapeutic Modulation of PQC to Curtail Proteotoxicity**

Taken together, the above data demonstrate that there are several protective mechanisms that could potentially be targeted for therapeutic intervention in cardiac proteotoxicity. The specifics of potential therapeutic targets differ for the different proteotoxic diseases, but may broadly be divided into 2 approaches: enhancing the intrinsic clearance mechanisms capable of degrading and recycling the proteinaceous aggregates or preventing their formation. Herein, we explore a few promising candidates, illustrating their actions on autophagy and underscoring the therapeutic potential of interfering with these proteotoxic pathways.

It is becoming clear that different clearance mechanisms are affected in several protein misfolding diseases. For example, the UPS is compromised in both the neurodegenerative diseases and in DRM. Conceptually, it is rather straightforward to appreciate that large aggregates of ubiquitinated proteins may negatively affect a subset of the cell’s clearance pathways as they are trafficked to the proteasome, but are too large to pass through the constraints of the proteasomal core (Figure 1, panel 2). Indeed, impairment of the UPS by protein aggregation in the heart has been well documented, both here and elsewhere. The proteasome is already a target in treatment of different blood cancers, with inhibitors of the 20S proteasome, such as bortezomib or marizomib, validated in clinical trials. However, for cardiac disorders, inhibition of the proteasomal activity is clearly detrimental in the context of proteotoxicity. Therefore, efforts to augment proteasomal activity are underway to determine whether cardiac function can be maintained in the face of an acute or chronic proteotoxic insult.

The modulation of another major clearance pathway, autophagy, is also being explored as a potential therapeutic target. As outlined in detail earlier, in the face of a proteotoxic insult, autophagy can serve as the primary clearance mechanism for proteinaceous aggregates that are too large for proteasomal degradation. Continuing the analogy to the proteotoxic neurodegenerative diseases, accumulating evidence suggests that the autophagic pathways are significantly compromised in cardiac proteotoxic environments. Unambiguous data show autophagy is reduced in Parkinson disease, with decreased autophagic flux resulting in α-synuclein accumulation, mitochondrial dysfunction, and neuronal cell death. By analogy, compromised autophagy in the context of proteotoxic heart disease might well serve as an increased pathogenic insult and lead to increased morbidity and hasten heart failure. Decreased autophagic flux has been noted in many disease processes, and restoration of normal or even enhanced autophagy seems to be beneficial in a wide spectrum of proteotoxic diseases ranging from the neurodegenerative diseases to Type 2 diabetes mellitus.

Because of the diverse roles that autophagy can play in both normal and disease metabolism, it should be emphasized that these data cannot be broadly applied to cardiac disease in general or even throughout the natural history of the same disease. But, if upregulation of autophagy is beneficial in certain proteotoxic contexts, how might it be upregulated as part of
a directed therapeutic approach? There are many small molecule effectors of autophagy, with some FDA-approved drugs having potent effects on autophagy in the cardiovascular system. However, nonpharmacological approaches may also be possible. The beneficial effects of exercise are far-reaching and well-documented across human physiology, and there are extensive data concerning the impact of physical activity on certain neurodegenerative diseases, such as AD. In different AD mouse models, environmental enrichment or voluntary exercise was beneficial, resulting in either the delayed onset of overt symptoms or progression of the disease. The physical component of environmental enrichment had particular impact, with voluntary exercise delaying the onset of neurological deficits in a mouse model of Huntington disease and long-term exercise in a mouse model of AD decreasing Aβ and amyloid deposition levels. A recent computational analysis involving genome-wide expression data from Parkinson and Huntington disease brain tissue was entirely consistent with the importance of autophagy in these disease processes.

On the basis of these data and the parallels between the neurodegenerative disorders and proteotoxicity-induced cardiomyopathy, we tested the effects of long-term voluntary exercise in the CryAB DRM model. Voluntary exercise was quantitated with the use of running wheels with which both duration and distance could be continuously monitored in an isolated setting, free from any overt stress over a period of 6 months. The results were striking: mice that were not placed in cages where they could voluntarily exercise were all dead by 7 months, whereas the mice that were exercising showed 100% survival at that time point. Although those mice did eventually succumb to heart disease, lifespan increased by ~30% in later trials. It is well established that exercise results in increased autophagy, and, indeed, exercise does result in numerous autophagic markers being upregulated (Figure 5). In contrast, when autophagy is induced by Atg7 expression in cardiomyocytes, a general upregulation of genes associated with autophagy does not occur. This raised the intriguing possibility that, although both interventions (voluntary exercise and Atg7 overexpression) led to the same outcome in terms of increased autophagic flux, the 2 interventions might be synergistic. This appeared to be the case, and when the Atg7 mice were allowed to voluntarily exercise, survival was prolonged as compared with either the Atg7 overexpressors or the exercise-only DRM animals.

**Pharmacological Therapy**

As outlined previously, HDAC inhibition can also be cardioprotective, due in part to antihypertrophic effects. Several laboratories have now shown a positive effect on cardiac autophagy when using the FDA-approved pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA). Hill and colleagues administered SAHA to rabbits before and during the course of ischemia/reperfusion injury. SAHA-treated rabbits showed a reduction in infarct size, which led to an improvement in cardiac fractional shortening. They noted LC3-II, a reliable autophagic marker, was significantly increased in the infarct border zone of SAHA-treated mice compared with controls, which led to a reduction in cell death in this region of the myocardium. Indeed, studies in cardiomyocytes corroborate that SAHA could increase autophagic flux under normal and ischemic conditions.

![Figure 5. Autophagy polymerase chain reaction (PCR) array in voluntary exercised CryABxAtg7xTtTA hearts.](https://example.com/image)

**Figure 5.** Autophagy polymerase chain reaction (PCR) array in voluntary exercised CryABxAtg7xTtTA hearts. Graph representing direct group-wise comparison of fold change in mRNA levels in male voluntary exercise CryABxAtg7xTtTA and control nonexercised CryABxAtg7xTtTA hearts at 4.5 months (fold change versus nonexercised CryABxAtg7xTtTA control; n=3 per group). All values are reported as mean±SEM. P<0.05 and P<0.01 by Student’s t test. Mice and experimental treatments were performed as described previously. CryAB indicates αB-crystallin.
SAHA had similar effects when applied to a pure model of proteotoxicity. Chronic SAHA administration to CryAB\textsuperscript{R120G} mice showed attenuation in sarcoplasmic aggregates and a concomitant improvement in cardiac function compared with animals treated with vehicle.\textsuperscript{210} Similarly, it was found that SAHA increased autophagic flux in CryAB\textsuperscript{R120G} expressing cardiomyocytes, suggesting that increased autophagy plays an important role in instigating the observed cardioprotective effects. However, further study is needed to ascertain which HDACs are directly involved in the autophagic response.\textsuperscript{210}

HDAC6 knockout mice displayed improved systolic function after chronic stimulation with angiotensin II,\textsuperscript{211} underscoring that HDAC6 function may be deleterious in the heart under some stress conditions, but the protective mechanisms are unclear. To determine the role of HDAC6 in a proteotoxic disease model, we assessed the role of HDAC6 function in DRM. We found that, despite increases in HDAC6 protein and catalytic activity, deacetylation of its microtubule substrate was decreased,\textsuperscript{210} underscoring that it is essential to measure the downstream signaling effects of the protein’s activity rather than merely quantitating protein levels. As per its canonical function, HDAC6 mediated aggresome formation in cardiomyocytes. Chronic treatment with SAHA improved cardiac function and reduced cellular aggregates while also increasing acetylation of the HDAC6 substrate \(\alpha\)-tubulin. Interestingly, in this case, inhibiting HDAC6-mediated tubulin deacetylation led to increased autophagic flux. Although seemingly at odds with HDAC6’s reported function, reduction in tubulin deacetylation may be necessary for autophagic activity.\textsuperscript{212}

Indeed, increased levels of tubulin acetylation were found in
CryAB<sup>R120G</sup> mice after 5 months of voluntary exercise, despite no change in HDAC6 catalytic activity. Although more data are needed to assess the precise roles of HDAC6 in cardiac physiology and pathobiology, it remains an intriguing therapeutic target.

**Final Thoughts: A Next Step**

Currently, there exist no available therapeutics that will effectively dissolve preexisting protein inclusions and reverse a proteotoxic disease state. The existence of protein inclusions continues to pose a constant and progressive threat. This reality is a particularly distressing one: many proteotoxic phenotypes do not present in patients until significant tissue damage has occurred. Thus, new therapeutics that can fully reverse proteotoxic phenotypes are needed.

Disease progression seems to proceed through successive accumulation of newly misfolded protein. As outlined earlier, this likely occurs because of reduced protein clearance through the PQC mechanisms, which leads to aggregate buildup, cellular dysfunction, and ultimately cell death. However, data suggest that blunting the expression of the aggregate-prone protein can halt the disease progression. In fact, reversal of disease and functional improvement on clearance suggests that the continued production of the aggregate-prone protein serves as a feed-forward loop that may be necessary for disease progression and an ever-worsening prognosis. Looking at this situation optimistically, therapeutic opportunities could be directed at several points along the pathogenic sequence: removing aggregated species, targeting the soluble PAO, or reducing causative protein expression.

A striking feature of protein aggregate-based disorders is the remarkable similarity between the aggregates, despite myriad differences in cell type and affected protein. Therapeutically, this may be a fortuitous feature: if the cell biology governing aggregate formation is conserved, then disaggregation mechanisms that work for one proteotoxic model might translate to another. Hypothesis-driven science has provided several promising strategies for combating aggregate formation, but none have yet reached fruition. Our current understanding of the protein aggregation process is still limited, underscoring the broad need for discovery-driven science that can identify new factors and potentially intersecting and novel pathways that could effectively reduce or reverse protein aggregation.

High-throughput screening is an advantageous and relatively unbiased method to find novel effectors for a given molecular and cellular process. Screens can take many forms but usually consist of an appropriate cellular or organismal model being probed with either a large pharmacological-based compound library or via genetic tools, with a defined cellular phenotype or response (readout) being measured. The quality of the data in such a screen is reliant on the robustness of the model used, the intrinsic noise of the system, and the strength of the signal constituting the readout. In the case of proteotoxic phenotypes, the readout may be relatively straightforward: augmented or decreased protein aggregates. For the proteotoxic diseases, laboratories are beginning to publish some nascent high-throughput screening aimed at identifying new pathways or finding new drugs to reduce aggregation and cellular toxicity.

With the goal of finding novel pathways that affect cardiac proteotoxicity, we have developed a cellular model of protein aggregation in primary mouse cardiomyocytes. Using a fluorescent, aggregate-prone protein (CryAB<sup>R120G</sup>), we were able to produce a cellular model with a robust cardiac protein aggregation phenotype and a high signal-to-noise ratio, such that phenotypic changes could be easily quantified. To find novel effectors of cardiac protein aggregation, we systematically knocked down each gene in the mouse genome using a library of short hairpin RNAs (shRNA) expressed via lentiviral transduction, and the phenotypic changes were measured using high-content imaging (Figure 6). Preliminary data from the screen have yielded several promising new inhibitors of cardiac protein aggregation that, once independently validated, will help to uncover novel pathways involved in protein aggregation in the heart.

These technology platforms are especially valuable because of the potential to uncover information about a disease state that is unattainable by more conventional methods. For example, as patient-derived cells become more widely used in medicine, they will provide unprecedented potential for probing modulators in the pathobiology of a specific patient. This was recently achieved in amyotrophic lateral sclerosis patients, in which patient fibroblasts were transformed via induced pluripotent stem cells into motor neurons that could then be used in a high-content drug screen to find appropriate compounds to reduce aggregate content. We are hopeful that ongoing and future screens may enable unbiased, discovery science that will not only find new therapeutic targets, but will generate testable hypotheses that will uncover the underlying mechanisms of proteotoxic disease in the heart.

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**Disclosures**

None.

**References**

Rapid degradation of a large fraction of newly synthesized proteins by...


