Welcome to the Machine

A Cardiologist's Introduction to Protein Folding and Degradation

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The price paid by the cardiovascular system for maintaining circulation is being a target of chronic forces that contribute to wear and tear. Cardiovascular tissues are in constant peril of damage from these stresses, and are dependent on highly conserved cellular machines for protection. The cellular environment is adapted to detect, repair and, if necessary, dispose of damaged proteins, in part because they are toxic to the cell. The molecular chaperone and ubiquitin-proteasome systems are the machinery that repair and degrade damaged proteins, yet little attention has been given to these systems in cardiovascular pathophysiology. Recent advances have brought protein folding and degradation closer to the forefront of cardiovascular biology. This review discusses these advances and presents new models for consideration of cardiovascular pathophysiology and therapeutics as problems of protein homeostasis.

Principles of Protein Folding and Degradation: Building Up and Tearing Down

In most cases, all the information necessary for a protein to fold is contained in its primary amino acid sequence. The environment within a cell is particularly unfavorable for protein folding, however, because of constraints that occur during protein synthesis and the likelihood of intermolecular interactions that impede folding in a crowded cellular environment. An evolutionarily ancient system exists to prevent damaged or newly synthesized peptides from aggregating and to provide a microenvironment that facilitates their proper folding to a thermodynamically favorable active conformation. This system comprises the molecular chaperones, which are present in every cellular compartment to buffer and repair damaged proteins (the Table provides a partial list of mammalian chaperones). Mechanisms for removing proteins are equally critical to cellular function, and the machinery of protein degradation is similarly archaic. Although several pathways exist for protein destruction in eukaryotes, the ubiquitin-proteasome system is responsible for the majority of protein degradation and is the most tightly regulated pathway.

The chaperone and ubiquitin-proteasome machinery bear the brunt of folding, protecting, and removing intracellular proteins. Together, these related events are referred to as quality control.[1] The premise of quality control is that protein folding in vivo is inefficient, and proteins are routinely subjected to events that favor their misfolding or unfolding. Highly efficient systems exist within the cytosol and the endoplasmic reticulum to identify misfolded proteins and to either assist their refolding or rapidly target them for degradation. Quality control is probably the most primitive function of the chaperone and ubiquitin-proteasome machinery, and it explains their close co-evolution. Additional functions have accrued for these systems, however, many of which are also of enormous consequence to cardiovascular biology.

Molecular Chaperones: Not Just for Folding

Molecular chaperones are defined as "a functionally related collection of highly conserved and ubiquitous proteins that specifically recognize non-native proteins."[2] Although they are an diverse group of proteins, the most abundant cytoplasmic chaperones are also the ones that are most closely associated with cardiovascular events. In general, there are 3 broad families of cytoplasmic chaperones: the heat shock protein (Hsp)90 family, the Hsp70 family, and the small heat shock proteins (Hsps).

Folding and Aggregation

The classic function of chaperones is to facilitate protein folding and to prevent aggregation. These folding events are regulated by interactions between chaperones and ancillary proteins, the co-chaperones, which assist in cycling unfolded substrate proteins on and off the chaperone complex. The Hsp70 proteins bind to misfolded proteins promiscuously during translation or after stress-mediated protein damage and provide a hydrophobic microenvironment that favors peptide folding. In contrast, the Hsp90 family members have a more limited range of client proteins; most of these are signaling molecules or transcription factors. The small Hsps (Hsp27 and αB-crystallin) have limited roles in folding and inhibit aggregation of misfolded proteins so that they can either be disposed of or refolded by other members of the chaperone machinery. Aggregation of misfolded proteins is implicated as a pathogenic mechanism in chronic degenerative diseases of the nervous system; a role for protein aggregates in cardiovascular diseases has not been carefully considered, although it is plausible based on the amount of chronic stress imposed on cardiovascular tissues.

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Examples of Mammalian Chaperones and Their Functions

Folding Factors	Substrates	Cellular Functions
Cytoplasmic chaperones		
Hsp70 (inducible)	Nascent and unfolded proteins	Protein folding, stress tolerance, anti-apoptosis
Hsc70 (constitutive)	Nascent and unfolded proteins	Protein folding, stress tolerance, anti-apoptosis
Hsp90	Signal transduction/transcription factors	Signaling events
Hsp105	Aggregated proteins	Protein disaggregation
Small heat shock proteins		
Hsp27	Cytoskeletal proteins	Modulates actin polymerization
αB-crystallin	Cytoskeletal proteins	Cytoskeletal integrity
Endoplasmic reticulum chaperones		
BiP/Grp78	Glycosylated proteins	Folding along the ER pathway
Hsp47	Collagens	Collagen processing and anti-apoptosis
Co-chaperones		
Hsp40/Hdj1	Substrates of Hsp70	Enhanced Hsp70-dependent folding
Hip	Substrates of Hsp70	Enhanced Hsp70-dependent folding
BAG-1	Substrates of Hsp70	Nucleotide exchange, anti-apoptosis
CHIP	Substrates of Hsp70 and Hsp90	Degradation of chaperone substrates
p23	Substrates of Hsp90	Required for Hsp90-dependent folding
cdc37	Substrates of Hsp90	Modulates Hsp90 function

ER indicates endoplasmic reticulum.

Signaling and Activation

It is evident from evolutionary considerations that the first function of molecular chaperones has been to solve the folding problem. However, the ability of Hsp90 to form multiprotein complexes with a prolific number of co-chaperones has allowed it to assume an additional responsibility as a regulator of signaling molecules and transcription factors (including heat shock factor-1, which itself is a critical regulator of Hsp70 and Hsp90 expression). Several Hsp90-dependent signaling events bear special importance in cardiovascular physiology; among them, the regulation of endothelial nitric oxide synthase (eNOS) function is informative. Hsp90 is a binding partner of eNOS, and generation of NO by eNOS is blocked by the Hsp90 inhibitor geldanamycin, indicating that eNOS function is dependent on interactions with Hsp90.[3] Rather than acting as a folding factor in this context, Hsp90 is a molecular scaffold to facilitate the interaction between Hsp90 and AKT, the kinase required for NO generation by eNOS. Factors such as vascular endothelial growth factor (VEGF) that stimulate NO do so by recruiting AKT and eNOS to a common domain within Hsp90.[4] This and other examples of chaperone interactions with signaling pathways indicate a central and rather complicated role for chaperones in cellular pathways that are relevant to cardiovascular function.

Apoptosis

The involvement of chaperones in apoptotic pathways raises the possibility that the chaperone system and apoptotic events have co-evolved to serve a specific function. Chaperones are powerful modulators of the cell death program, and almost exclusively serve as negative regulators of apoptosis. Anti-apoptotic activity is a common function of cytoplasmic chaperones; Hsp70, Hsp90, and Hsp27 all block cell death under appropriate circumstances. A variety of cell death events are inhibited by the chaperones, including cell death phosphorylation cascades, inhibition of caspase 9-apoptotic protease-activating factor apoptosome assembly,[5] and suppression of caspase-independent apoptotic events.[6] Interestingly, the effects of the heat shock proteins are, in some cases, independent of their chaperone functions, suggesting that regulation of apoptosis is a later adaptation of the chaperone machinery to thwart cell death pathways, in coordination with their folding and anti-aggregation functions.

Ubiquitin Tagging: A Pathway to Degradation (and More)

Ubiquitin is a 76-amino acid molecule that is covalently tagged to proteins, usually in homopolymeric chains, in a reaction catalyzed by 3 sets of enzymes. Ubiquitin-activating enzyme, which is ubiquitous, attaches to ubiquitin via a thiolester linkage. The ubiquitin moiety is then transferred to an ubiquitin-conjugating enzyme, of which there are a dozen or so in each cell. Finally, the ubiquitin molecule is attached to a substrate with the assistance of one of the hundreds of ubiquitin ligases, which serve the primary substrate recognition function of the ubiquitylation machinery. For the most part, ubiquitylation provides a recognition signal that targets proteins to the proteasome, although ubiquitylation can trigger other events, such as endocytosis, signaling, and membrane trafficking.

Protein Degradation

The classic function of ubiquitylation is to render substrates susceptible to degradation by the proteasome. The proteasome contains a cylindrical core comprising 28 proteins and 2 regulatory caps on either end of the core. Several events are necessary for degradation of ubiquitylated proteins, and specific activities within the proteasome machinery are necessary for each. Ubiquitylated proteins must first be recognized by the proteasome, and this requires the ubiquitin chain recognition

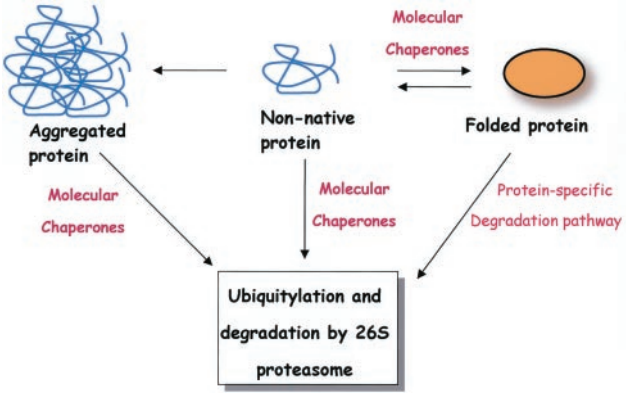

Figure 1. Interactions between the molecular chaperone and ubiquitin-proteasome systems in regulating protein quality control.

particle Rpt5 within the regulatory cap.[7] These proteins must then be deubiquitylated and unfolded by an intrinsic antichaperone activity of the proteasome. These events occur in the regulatory cap, and the unfolded substrate is translocated to the core particle, which degrades substrates into short peptide fragments.

There are 2 major reasons for a protein to be degraded: when it is damaged and when it is no longer needed. The degradation of damaged proteins falls under the auspices of quality control, a function shared by chaperones and the ubiquitin-proteasome system (Figure 1). This link is provided in part through the function of a protein called CHIP (carboxyl terminus of Hsp70-interacting protein), a co-chaperone for Hsp70 and Hsp90 that also contains ubiquitin ligase activity.[8] CHIP assists the chaperones in recognizing terminally misfolded proteins and partitions these damaged proteins to the proteasome by tagging them with ubiquitin. The removal of damaged proteins by factors such as CHIP (perhaps assisted by adaptor molecules such as cdc48 that assist in the transfer of ubiquitylated proteins to the proteasome) is expected to be of enormous consequence to cells subjected to chronic stress; otherwise, damaged proteins that can aggregate or gain toxic functions would accumulate. Damage to a protein, however, is not the only reason for it to be degraded. Many proteins serve functions that are temporally restrained; degradation of these proteins provides a means to rapidly turn off a cellular event. The involvement of the ubiquitin-proteasome system in regulation of signaling and cell cycle events provides an example of the importance of this pathway to cardiovascular processes.

Signaling

Intracellular signaling events must be activated and terminated with precision and speed. Ubiquitylation can terminate signaling by triggering proteosome-dependent degradation of a signaling intermediate in an activatable fashion. This relatively simple method is used by activation protein (AP)-1 family members, which are degraded to arrest mitogenic signaling downstream of mitogen-activated protein kinase activation.[9] This simple mechanism is used frequently, but it is not the only way that ubiquitylation regulates signaling. In fact, the events leading to activation of the proinflammatory transcription factor nuclear factor (NF)-κB provide a lesson in how many ways ubiquitylation can affect signaling (Figure 2). NF-κB consists of dimers of Rel family proteins that exist in the cytoplasm bound to an inhibitor, IκB (α or β). Activation of the IκB kinases IKKα or IKKβ results in phosphorylation, ubiquitylation, and degradation of IκB by the proteasome. IκB degradation allows NF-κB to translocate to the nucleus, where it transactivates the expression of genes, many of which are involved in inflammation, stress responses, and cardiovascular processes. IκB is therefore the "master switch" for NF-κB activation. The ubiquitin-proteasome system is linked to NF-κB activation via at least 3 steps. First, the p50 subunit of NF-κB undergoes ubiquitin-dependent limited processing from its precursor, p105[10] (which is remarkable because it is so unlike the majority of proteasome substrates, which are completely degraded by the proteasome). Second, the degradation of IκB is ubiquitin-dependent. Lastly, cytokine-stimulated IκB kinase activation occurs in an ubiquitin-dependent but proteasome-independent fashion by an activation complex that contains the ubiquitin ligase TRAF6, which decorates IκB kinase with atypical ubiquitin chains that are required for its activation.[11]

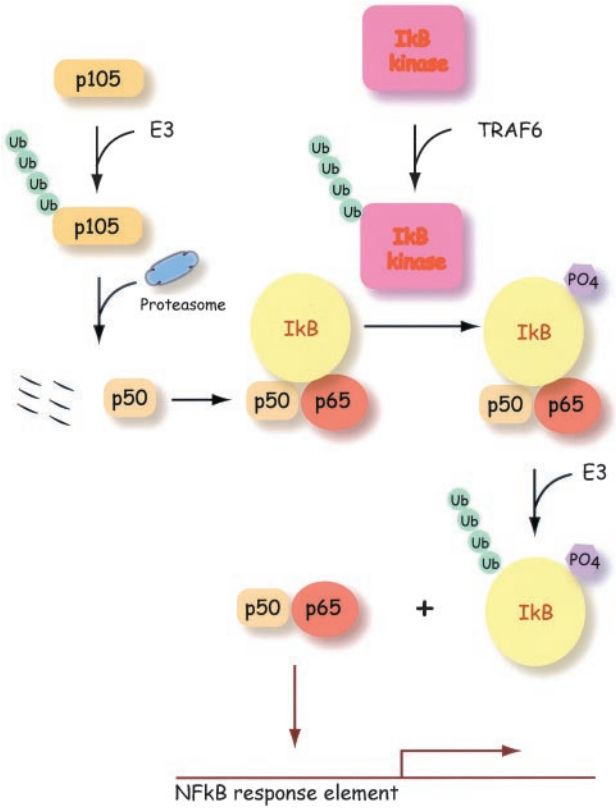

Figure 2. Multiple roles of the ubiquitin-proteasome pathway in NFκB activation. The p105 precursor undergoes limited proteasome-dependent processing to release the NFκB subunit p50. Subunit dimers, such as p50–p65, are held in the cytoplasm by IκB, where they are inactive. The IκB kinase regulates the IκB-NFκB complex, and activation of the IκB kinase is dependent on ubiquitylation of IκB kinase by TRAF6. The activated IκB complex phosphorylates IκB, which serves as a signal for its rapid ubiquitylation and degradation, freeing NFκB to enter the nucleus and transactivate NFκB-responsive genes.

Cell Cycle

Proliferation of cells requires the rapid and sequential synthesis and degradation of proteins for DNA replication and cell

division. Ubiquitin-dependent proteolysis is the major mechanism for removal of effector proteins as they are no longer needed. Interestingly, the transcription of effector proteins that participate in cell proliferation is itself determined by a master group of proteins that guard the cell cycle, the cyclins and their counterpart cyclin-dependent kinase inhibitors. The up- or downregulation of these proteins determines the place of a cell within the cell cycle or, conversely, a cell's arrest in or exit from the proliferative cycle. For example, cyclin A is degraded by the anaphase-promoting complex as cells enter S phase,[12] whereas the cyclin-dependent kinase inhibitor p27Kip1 is ubiquitylated and degraded to allow passage through the G1 phase of the cell cycle.[13] These events have obvious consequences for cardiovascular events that have a proliferative component such as atherosclerosis and restenosis.

Molecular Chaperones and Cardiovascular Diseases

Although the critical cellular functions of chaperones are well established, their importance in cardiovascular diseases is often overlooked. Indeed, the importance of the protein folding machinery in protection against a variety of cellular stresses, as well as their ability to regulate signaling pathways and apoptotic events in cardiovascular tissues, gives them a central role in the most common pathologies of the heart and blood vessels.

Myocardial Protection

The role of molecular chaperones in protection against ischemia is now well established. This is logical, because heat stress and ischemia have many common features and both result in substantial damage to cellular peptides. The abundant constitutive expression of small Hsps in cardiac myocytes is notable, and they may serve as an important early buffer against ischemia-induced damage in these cells. Chaperones are upregulated by ischemia, and overexpression of Hsp70 and the small Hsps αB-crystallin and Hsp27 protect cultured myocytes from ischemic damage.[14] Several groups have now demonstrated that overexpression of Hsp70 improves myocardial function and protects against infarction in the setting of induced myocardial ischemia.[15,16]

Protection of donor hearts after explantation is a promising practical application of molecular chaperones in this context. The damage that occurs during the explant period is a critical determinant of myocardial engraftment and function after transplantation, and this damage occurs predominantly because of changes in temperature, pH, and oxygen delivery that are toxic to heart cells and their resident proteins. Chaperone therapy may have additional benefits in the context of transplantation therapy, as emerging data indicate a role for Hsp70 and Hsp90 in antigen processing. Delivery of Hsp70 by gene therapy techniques has been successful in protection of the myocardium in animal models of heart transplantation.[17] Temporal and delivery considerations make explant chaperone therapy highly practical; extending myocardial preservation during explantation would have enormous repercussions with respect to increasing the donor pool and improving donor–recipient matches, 2 of the major limitations to heart transplantation at the present time.

Cardiomyopathy

αB-crystallin is a chaperone that is abundantly expressed in the heart and skeletal muscle. Genetic studies have determined that a substitution of glycine for arginine at position 120 in αB-crystallin causes an autosomal dominant disorder that results in cataracts, distal and proximal myopathy, and premature death from intractable congestive heart failure and lethal arrhythmias.[18] Pathologically, the cardiomyopathy is characterized by granulofilamentous deposits of αB-crystallin and phosphorylated desmin, indicating that the consequences of this mutation are due to altered chaperone function of the mutant crystallin. Although to date this is the only example of a chaperone mutation resulting in cardiomyopathy, this observation provides proof of principle that defective chaperone activity leads to cardiac dysfunction and raises the possibility that subtle defects in chaperone expression or activity may contribute to congestive heart failure in other circumstances.

Atherosclerosis

It is logical to speculate that molecular chaperones may participate in the cellular events that determine the progression of atherosclerotic lesions; these proteins are expressed in all cell types that contribute to lesion formation and are inducible after balloon injury in humans. Within human neointimal lesions, the expression of Hsp70 is exclusive to activation of apoptotic pathways; it is postulated that plaque destabilization occurs because of insufficient induction of Hsp70 near necrotic zones in atherosclerotic plaques.[19] In atherosclerosis-prone apolipoprotein E $^{-/-}$ mice, Hsp70 is expressed at sites prone to lesion formation before events such as macrophage infiltration have occurred, and subsequently Hsp70 is downregulated in advanced necrotic lesions,[20] perhaps as a result of changes in macrophage content as lesions progress. Hsp47, a small collagen-binding chaperone, is also highly expressed in atherosclerotic lesions, in particular within the fibrous cap,[21] where it may enhance lesion stability.

The selective and localized expression of chaperones within vascular lesions is provocative and argues that a role exists for these proteins in the course of lesion formation; however, misexpression studies have not been performed to define definitive roles for the important chaperones. On the basis of their temporal and spatial expression during lesion formation and known cellular functions, it is logical to expect that chaperone expression would enhance the early stages of lesion formation by promoting smooth muscle proliferation and inhibiting apoptosis. However, expression of chaperones such as Hsp70 and Hsp47 in advanced lesions, particularly within the fibrous cap, may have a stabilizing function. Loss of chaperone expression could increase the likelihood that cells overlying the necrotic lesion core will undergo apoptosis. Thus, chaperone expression may be deleterious in the early stages of lesion formation by promoting lesion growth, but may be protective against plaque rupture late in the course of lesion progression.

Ubiquitin–Proteasome Machinery and Cardiovascular Diseases

The past decade has seen an explosion in our understanding of the importance of the ubiquitin-proteasome pathway and an appreciation for tight regulation of protein degradation. With this

foundation laid, it is now time to consider the impact of protein degradation events in the context of cardiovascular biology.

Myopathy

Both heart and skeletal muscle cells are subject to atrophy or hypertrophy, depending on load and other factors. Changes in myocyte size are dependent foremost on protein synthesis or degradation. Atrophy of skeletal muscle primarily occurs by proteasome-dependent protein degradation through pathways that are activated in response to caloric restriction, unloading, and other factors. This effect is mediated at least in part through activation of the N-end rule ubiquitylation pathway, which is activated by unmasking of specific amino-terminus degradation sequences within proteins.[22] Myofibrillar proteins are major targets of the proteasome in muscle,[23] which may account for the exaggerated loss of contractile function in the setting of atrophy. Surprisingly, much less is known about proteasome pathways in cardiac myocytes, although it is logical to expect the general mechanisms to be similar.

Recently, 2 ubiquitylation factors have been identified that are striated muscle-specific and are upregulated in response to atrophic stimuli.[24,25] One of these, MAFbx/atrogin-1, is an F-box protein; F-box proteins are substrate-recognition modules of some multicomponent ubiquitin ligases. The other, MuRF1, is a RING finger ubiquitin ligase. The muscle-specific substrates of these ubiquitylation factors are not known, but studies suggest that they are essential components of the muscle atrophic response. Interestingly, these proteins are present in both skeletal and cardiac muscle, raising the possibility that they may have a role in cardiomyopathic changes.

Restenosis

Lesion formation after vascular injury is a multistep process that includes components of smooth muscle cell proliferation, remodeling, and apoptosis. In some cases, these same processes are involved in tumor growth and metastasis, and because proteasome inhibitors have proven effective in early-phase cancer trials, it is not surprising that this approach has also been applied to prevent vascular proliferation. Indeed, proteasome inhibitors block smooth muscle cell proliferation and induce a synthetic phenotype in cultured cells, and have pro-apoptotic and antiproliferative effects after balloon injury.[26] Although the diverse effects of proteasome inhibition are likely to limit their use as systemic agents, the ability of proteasome inhibitors to block several relevant steps in the response to vascular injury makes them promising candidates for local delivery after percutaneous coronary interventions.

Angiogenesis

Angiogenesis is regulated through coordinated actions of a number of growth factors, including members of the VEGF family, which together control the cellular events that lead to new blood vessel formation in response to stimuli such as hypoxia. Hypoxic induction of VEGF is determined primarily by the transcription factor hypoxia-inducible factor-1α (HIF-1α). Both the levels and activity of HIF-1α are regulated through a unique ubiquitin-proteasome–dependent mechanism. HIF-1α interacts physically with the von Hippel-Lindau (VHL) tumor promoter, which is now known to be an essential component of an ubiquitin ligase complex, and this interaction triggers ubiquitin-dependent degradation of HIF-1α. The HIF-1α–VHL interaction requires hydroxylation of a proline residue in the oxygen-sensitive domain of HIF-1α.[27,28] Proline hydroxylation is oxygen dependent, so that the proline residue is hydroxylated and HIF-1α binds to VHL and is degraded under normal oxygen tensions. In contrast, when oxygen levels are low, VHL cannot associate with HIF-1α, leading to accumulation of HIF-1α and increased expression of growth factors such as VEGF that stimulate angiogenesis. It is interesting to note that SM-20, a smooth muscle-specific protein, is a part of the prolyl hydroxylation machinery in this pathway.[29] This unique proteasome-dependent oxygen-sensing mechanism explains the major clinical manifestations in patients with von Hippel-Lindau disease (congenital angiomatosis and angiogenesis-dependent tumors).

Hyperlipidemia

Surprisingly, the ubiquitin-proteasome system has a significant impact on lipid metabolism in humans. Apolipoprotein B is a core component of the low-density lipoprotein (LDL) particle that serves as a recognition target for the LDL receptor; thus, functional apolipoprotein B levels are a major determinant of plasma lipid levels. Apolipoprotein B undergoes extensive quality control surveillance within the secretory pathway, and a major determinant of apolipoprotein B secretion is the extent to which it is captured and degraded by the ubiquitin-proteasome system.[30] Modulation of the extent to which nascent apolipoprotein B is diverted for degradation has important clinical consequences. Hydroxymethyl glutaryl coenzyme A reductase inhibitors may influence lipid levels partly through decreasing apolipoprotein B stability by increasing its proteasomal degradation.[31] Conversely, the hyperlipidemia associated with protease inhibitors used in the treatment of HIV infection may be due to concomitant inhibition of the proteasome and enhanced apolipoprotein B secretion.[32]

Emerging Concepts

The chaperone and proteasome systems have acquired surprising diversity in function, and in many instances have acquired activities during evolution that are not strictly linked to their folding or degradation activities. One example is the ability of Hsp70 to inhibit apoptosis, which is at least partly independent of the chaperone function of Hsp70. Diverse functions for ubiquitin modifications and ubiquitin-like molecules are also increasingly recognized. In addition to typical ubiquitin chain assembly, which occurs through covalent ubiquitin-ubiquitin linkages that require a lysine residue at position 48, atypical ubiquitin chains formed via lysine 29 and lysine 63 residues also occur. Instead of targeting tagged proteins for degradation via the proteasome, however, these linkages participate in processes such as signaling and DNA repair.

In addition, 2 groups of proteins related in sequence to ubiquitin have recently been identified. The ubiquitin-like modifiers are small molecules that, like ubiquitin, can be covalently attached to other proteins via conjugation pathways that closely resemble those used for ubiquitin. These modifiers include members of the SUMO/Sentrin and RUB1/NEDD8 family. Unlike ubiquitin, these modifiers do not target proteins for proteasome-dependent degradation. Instead, other functions

have been assigned to these modifications, including translocation, protein stabilization (via competitive interference with ubiquitylation), and transcriptional activation. One interesting example of SUMO modification occurs on IκB, which when modified by SUMO is resistant to ubiquitin modification; SUMO modification of IκB thus prevents NF-κB activation and its ensuing proinflammatory response.[33]

Ubiquitin domain proteins are a second group of ubiquitin-like proteins. These proteins all contain one or more domains with homology to ubiquitin. Many of these proteins associate with the proteasome and probably use their ubiquitin-homology domains to implement this interaction. The BAG proteins are members of this group; BAG-1 is the most completely characterized. In addition to associating with the proteasome, BAG-1 also interacts with both heat shock cognate (Hsc)70 and the upstream mitogen-activated protein kinase activator Raf-1. Association of BAG-1 with Raf-1 occurs preferentially during stress and provides a protective anti-apoptotic signal in the face of dire cellular events.[34]

Summary

Advances continue to occur in our understanding of the biochemical steps required for protein folding and degradation. However, cardiovascular science is only beginning to connect these processes with specific pathophysiologic events. In many cases, these associations are likely to be surprising and may allow for the better understanding of cardiovascular disease and potential new targets for therapy.

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