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Nuclear factor erythroid-2 related factor 2 (Nrf2)-mediated protein quality control in cardiomyocytes

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1. ABSTRACT

Protein quality control (PQC) acts to minimize the level and toxicity of malfolded proteins in the cell. It is performed by an elaborate network of molecular chaperones and targeted protein degradation pathways. PQC monitors and maintains protein homeostasis or proteostasis in the cells. Whilst chaperones may actively promote refolding of malfolded proteins, the malfolded proteins which cannot be correctly refolded are degraded by the ubiquitin proteasome system (UPS) and the autophagic-lysosome pathway (ALP). The UPS degrades individual misfolded protein molecules, whereas the ALP removes large and less soluble protein aggregates and organelles. Emerging evidence indicates that dysregulated and inadequate PQC play an important role in the pathogenesis of not only classic conformational disease but more common forms of cardiac pathology such as cardiac pathological hypertrophy and heart failure. Nuclear factor erythroid 2-related factor 2 (Nrf2), a master transcription factor of cellular defense, appears to regulate the USP and the ALP by directly controlling the expression of UPS- and ALP- related genes. This article highlights an emerging role of Nrf2 in the regulation of intracellular PQC as well as its potential involvement in cardiac pathology.

2. INTRODUCTION

Normal function of proteins is essential for virtually every aspect of life. A cellular protein exerts its normal functions only when it is in its native conformation, a 3-dimensional structure with the polypeptide chain properly folded. Therefore, it is important for a polypeptide to attain and maintain its native conformation. The native conformation that a protein acquires during its biogenesis is attained through folding of the polypeptide chain spontaneously or with the help of molecular chaperones (1). However, the biogenesis of native proteins is not a very efficient process because studies have shown that approximately one third of newly synthesized polypeptides never make to mature proteins; they are co-translationally degraded presumably due to errors in translation and/or folding (2). Genetic defects and environmental stress can disrupt proper folding of a protein and even cause unfolding and misfolding of already properly folded proteins in the cell (3). It has been recently suggested that misfolded and unfolded proteins be collectively referred to as “malfolded proteins” (4). As misfolded proteins or more accurately malfolded proteins can be highly toxic if they are left unattended, the cell has developed multi-layered mechanisms to constantly monitor the level of malfolded proteins, to keep the level low, and to minimize the toxicity of malfolded proteins. These mechanisms are referred to as protein quality control (PQC) (5). PQC is carried out by an elaborate network of molecular chaperones and protein degradation pathways. The precise mechanism by which the cell distinguishes a terminally misfolded protein is unclear; however, the misfolded proteins are targeted for degradation by two proteolytic pathways: the ubiquitin-proteasome system (UPS) and the autophagic-lysosome pathway (ALP) (Figure 1) (6,7).

UPS-mediated protein degradation consists generally of two main steps: (a) covalent attachment of a chain of ubiquitin (Ub) protein molecules to a target protein molecule via a process known as ubiquitination and (b) degradation of the ubiquitinated protein molecule by the 26S proteasome. Hence, the UPS degrades proteins in a highly selective fashion. Soluble misfolded proteins in the cell are primarily degraded by

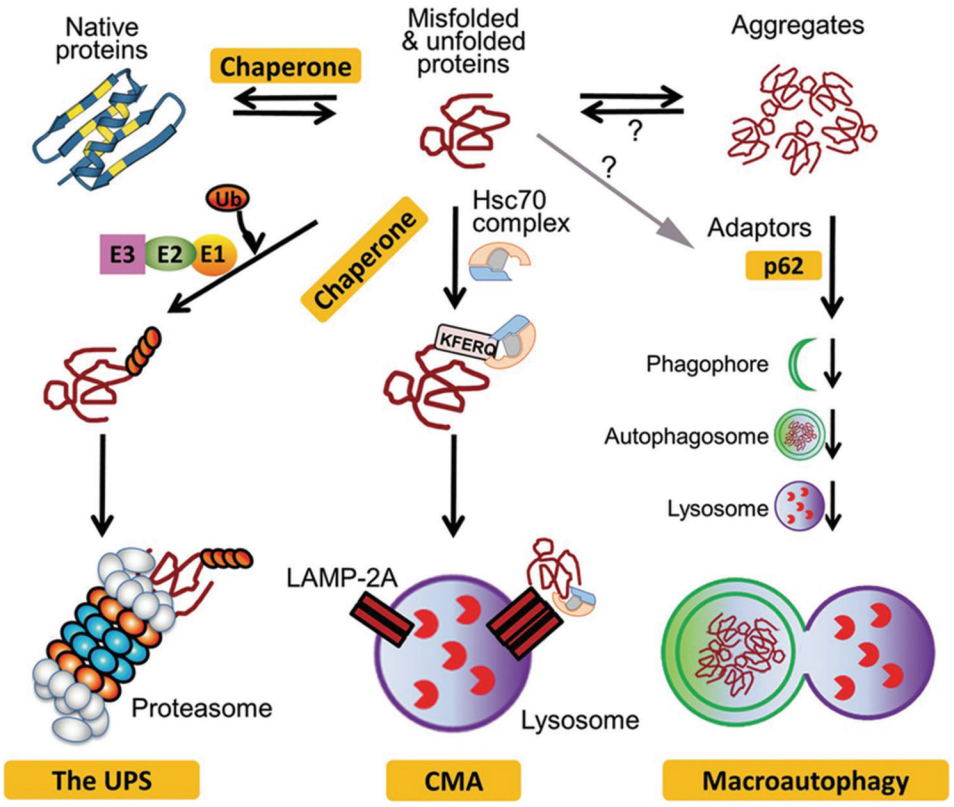

Figure 1. A Scheme of protein quality control in the cell. Chaperones aid folding nascent polypeptides, unfolding misfolded proteins and refolding them, and channeling terminally misfolded proteins for degradation by the ubiquitin–proteasome system (UPS) or chaperone-mediated autophagy (CMA). When escaped from targeted degradation, misfolded proteins form aggregates via hydrophobic interactions. Aggregated proteins can be selectively targeted by macroautophagy to, and degraded by, the lysosome. Reproduced with permission from Wang *et al*. (5).

the UPS (6,8). Ub is the first and best studied modifier protein that can be covalently linked to other proteins via an isopeptide bond. Ubiquitination is accomplished via a series of chemical reactions catalyzed sequentially by the Ub-activating enzyme (E1), Ub-conjugating enzymes (E2s), and Ub ligases (E3). In addition to targeting proteins for degradation, ubiquitination, both mono- and poly-ubiquitination, regulates a variety of cellular functions via a non-proteolytic fate. The proteasome which is found in both cytoplasm and the nucleus is an ATP-dependent multi-subunits protein complex with its protease activity residing in the core particle (the 20S proteasome). Proteasome subunit expression and proteasome assembly appear to be regulated by metabolic stresses although the precise underlying mechanism of the regulation remains obscure. The vast majority of normal proteins in the cell when they become no longer needed are also degraded by the UPS. Hence, the UPS plays pivotal role in controlling both quality and quantity of cellular proteins, thereby touching virtually every corner of the cell. Therefore, it is not surprising that dysregulation of UPS components and the overall UPS-proteolytic function has been implicated in the pathogenesis of numerous human diseases including a large subset of cardiovascular diseases. For example, proteasome functional insufficiency is implicated in the majority of failing human hearts and has been experimentally demonstrated to be a major pathogenic factor in not only cardiac conformational disease but ischemia-reperfusion injury (7,9,10). Hence, it is important to better understand how the UPS, especially proteasome function, is regulated in cardiac health and disease.

Autophagy (Greek for “self-eating”) is an evolutionarily conserved pathway that targets cytoplasmic contents (e.g., protein aggregates and organelles) to the lysosome for degradation; it was coined by Belgian biochemist Christian de Duve based on his discovery of lysosomes (11-13). Autophagy has been classified into three different types depending on the means by which the target is delivered into lysosomes for final degradation: (a) macroautophagy that is mediated by formation of double-membrane bound vesicles termed the autophagosome which engulfs a portion of cytoplasm or cytoplasmic organelles and delivers its content to lysosomes by fusion with the lysosome; (b) microautophagy by which the cytosolic materials is directly engulfed and

internalized by lysosomes; (c) chaperone-mediated autophagy (CMA) that involves the direct translocation of cytosolic proteins across the lysosomal membrane by chaperone proteins (14). Both macroautophagy (hereafter referred to as autophagy) and CMA may participate in PQC-associated protein degradation, with removal of protein aggregates or defective organelles by the former and individual malfolded proteins by the latter (Figure 1) (5,14). Dysregulation in the ALP has been linked to a variety of human diseases, including infectious diseases, Crohn's disease, neurodegenerative diseases, pancreatitis, diabetes, cancer, myopathy, and cardiovascular diseases (7,12,15). Furthermore, emerging evidence suggest that pharmacological intervention of the ALP pathway may represent a new therapeutic strategy to treat many forms of common and precipitating diseases, such as cancer, liver disease, neurodegenerative diseases, and heart diseases (16-18).

The UPS and the ALP may function distinctly but they appear to take the responsibility of degrading misfolded proteins in PQC in a complementary manner. It is generally accepted that for the purpose of PQC, the UPS degrades soluble misfolded or damaged proteins before they form aggregates, whereas the ALP clears large and less soluble or insoluble protein aggregates or defective organelles such as damaged mitochondria. Moreover, UPS impairment results in compensatory activation of autophagy for clearing damaged proteins (19-21), whereas autophagy malfunction may impair UPS performance (22,23). The function and regulation of the UPS and the ALP as well as their interplay in cardiac diseases have been recently reviewed (1,5,7). Herein we will summarize the latest literature regarding an emerging role of Nuclear factor erythroid-2 related factor 2 (Nrf2) in the regulation of intracellular PQC as well as its potential involvement in cardiac pathology.

3. Nrf2 IN THE UPS

Nuclear factor erythroid-2 related factor 2 (Nrf2) is a member of the Cap 'n' Collar (CNC) family of basic leucine zipper (bZip) transcription factor (24-27). There are also two additional Nrf proteins: Nrf1 and Nrf3. Nrf1-3 proteins are ubiquitously expressed, with each showing distinct but overlapping expression profiles. Nrf2 is the most studied. Activity of Nrf2 is mainly regulated by its interaction with an inhibitory protein called Keap1. Keap1 contains two known protein-interacting domains: the bric-a-brac, tramtrack, broad-complex domain (BTB domain) in the N terminal region, and the Kelch repeats in the C-terminal region homologous to Drosophila actin-binding protein Kelch (Kelch repeat, double glycine repeat (DGR) domain). Keap1 binds Nrf2 through its C-terminal Kelch domain, which contains six copies of the evolutionarily conserved kelch repeat sequence motif. BTB mediates homodimerization and binding of Keap1 to Cullin (Cul) 3, a scaffold protein of Nrf2 Ub ligase (E3). Thus, Keap1 acts as a substrate adaptor to bring Nrf2 into the Ub E3 complex. RING box protein 1 recruits the catalytic function of ubiquitin-conjugating enzyme (E2) by binding to Cul3 C terminal region. E2 catalyzes polyubiquitination of Nrf2 protein on the lysine residues of the Neh2 domain. As a result, Nrf2 is ubiquitinated and degraded by the proteasome. Under basal conditions, Nrf2 is rapidly degraded by the UPS, which affords Nrf2 a half-life of only 20 minutes, resulting in a low protein level of Nrf2 in various types of cells.

Nrf2 forms a heterodimer with its obligator partner Maf, thereby binding to a cis-acting enhancer sequence known as the antioxidant response element (ARE) with a core nucleotide sequence of 5-RTGACNNNGC-3 to control the basal and inducible expression of more than 200 genes that can be grouped into several categories including antioxidant genes, phase II detoxifying enzymes, transcriptional factors, transporters, scavenger receptors, and chaperone proteins (24-27). Thus the functions of Nrf2 spread rather broadly from antioxidant defense to cell cycle regulation and PQC. A critical role of Nrf2 in the control of a battery of antioxidant and detoxifying genes such as heme oxygenase-1 (HO-1), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferases (GST), NAD(P) H:quinone oxidoreductase-1 (NQO-1), NQO2, γ-glutamylcysteine synthase (γ-GCS), and glucuronosyltransferas (UGT), has been well established in diverse cell types (24-27); however, the specificity and functional significance of Nrf2-mediated expression of the other genes remain to be further studied.

It is worthy to note that Nrf1 is crucial in liver erythropoiesis, whereas the role of Nrf3 is unclear (28). One critical evolutionary divergence is that Nrf1 functions independently of Keap1 and is targeted to the endoplasmic reticulum (ER), which is markedly different than the subcellular localization of Nrf2 (29). While the Nrf2 subcellular targeting might specifically aid in protection against ER stress (30), the Nrf2-driven antioxidants seem to be essential for liver development (31). It has been proposed that Nrf3 may be a negative regulator of Nrf2 by interfering with ARE-mediated transcription (32); however, the physiological relevance remains to be elucidated.

Importantly, several studies have revealed that Nrf2 is capable of upregulating a group of proteasome genes (Table 1) (33-36). Microarray analysis has shown that 19 genes coding for 19S and 20S proteasome subunits are Nrf2-dependently upregulated by dithiolethione 3-H-1,2-dithiole-3-thione (D3T) in liver tissue (33,34). However, the basal expression of the proteasome subunit genes including proteasome alpha-subunit (PSMA)1, PSMA4, proteasome beta-subunit (PSMB) 3, PSMB5, PSMB6, PSMB8, (proteasome 26S subunit ATPase 1) PSMC1, PSMC3, and PSMD14 (proteasome 26S subunit

Table 1. The list of proteasome subunits upregulated by Nrf2

Proteasomes	Proteasome Subunits	Tissues or Cells	References
19S	PSMC1, PSMC3, PSMD1, PSMD5, PSMD7, PSMD11, PSMD12, PSMD13, PSMD14	Mouse liver	(33)
	PSMD4	Human colon cancer cells	(35)
20S	PSMA1, PSMA4, PSMA5, PSMA6, PSMB1, PSMB2, PSMB3, PSMB4, PSMB5, PSMB6	Mouse liver	(33)
	PSMB5	MEFs	(33)
	PSMB1	MEFs	(36)
	PSMA5	Human colon cancer cells	(35)
11S	(None)	Mouse liver	(33)
	PA28αβ/PSME1	MEFs	(36)
Immuno-proteasome	(None)	Mouse liver	(33)
	β1i/PSMB9/LMP2	MEFs	(36)
Others	POMP	Human embryonic stem cells	(38)
MEFs, mouse embryonic fibroblasts			

non-ATPase 14) may be independent of Nrf2 in normal mouse liver or embryonic fibroblasts (33). In addition, the Nrf2-dependent upregulation of 20S subunits such as PSMB5 is most likely dependent on the nature of Nrf2-activating small molecules because Nrf2-driven transcriptional activity was activated by sulforaphane (SFN) and D3T but not by butylhydroxytoluene (BHT) (33). Consistent with its induction of proteasome synthesis, Nrf2 inducer SFN was recently shown to increase proteasomal peptidase activities and proteolytic function in not only cell cultures but also intact mice, promoting the degradation of mutant Huntingtin and reducing its cytotoxicity (37). Nrf2 is also essential for H_2O_2-induced increases in proteasomal activity and upregulation of 20S subunit PSMB1 and 11S subunit PSME1 in mouse embryonic fibroblasts (36). In human colon cancer cells, however, Nrf2 is an essential mediator for both basal and inducible expression of PSMD4 and PSMA5 and the Nrf2-dependent PSMD4 and PSMA5 expression confers protection against apoptosis (35). These results indicate that there are hitherto unappreciated factors that are required for the stimuli- and cell type-dependent Nrf2-mediated upregulation of proteasome genes. A recent study showed that Nrf2 plays a key role in self-renewal and pluripotency of human embryonic stem cells partly via proteasome maturation protein (POMP)-mediated maintenance and regulation of proteasome activity (38). Collectively, these results suggest that Nrf2 appears to play an important role in PQC through a positive regulation of proteasome expression, thereby maintaining proteostasis in the cells. Nevertheless, the role of the Nrf2-proteasome axis in the heart remains to be investigated. Notably, the related Nrf1 also appears to be able to regulate the transcription of proteasome genes in mammalian cells (39-41); several studies have demonstrated that Nrf1meidates the expression of proteasome subunits during proteasome rebound and recovery after proteasome inhibition (41,42). It will be important to sort out how Nrf1 and Nrf2 work together to regulate proteasome expression and proteasome function.

4. Nrf2 IN AUTOPHAGY

Nrf2 appears to be involved in the regulation of protein aggregation in autophagic substrate selection (43) as well as autophagy per se (44-47). Although it remains unclear whether the observed increased steady state levels of autophagosomes in Nrf2-deficient breast cancer cells, endothelial cells, and airway epithelial cells are caused by an impairment of autophagosome clearance (44,46,47), it has been demonstrated that Nrf2 is a critical mediator of autophagic clearance of ubiquitinated protein aggregates in macrophages (45). In addition, a recent study showed that Nrf2 is capable of clearing phosphorylated tau by induction of nuclear dot protein 52 (NDP52), an autophagy adaptor protein (also known as CALCOCO2) in the presence of autophagy stimulator thereby potentially contributing to brain protection (48). Given the well-documented protective role of Nrf2 signaling (26,27) and the ALP pathway (49,50) in diverse pathological settings, it is likely that Nrf2 may act as a modulator to ALP-mediated cellular defense.

On the other hand, the functional integrity of the ALP may have great impact on biological and pathophysiological consequences of Nrf2 activation. In this context, there is a complex interplay between Keap-Nrf2 and autophagy pathways. For example, the phosphorylation of the autophagy-adaptor protein p62

increases p62 binding affinity for Keap1 thus activating Nrf2 and the persistent activation of Nrf2 through accumulation of phosphorylated p62 contributes to the growth of human hepatocellular carcinomas (51). While Keap1 is constitutively degraded through the ALP, autophagic deficiency leads to p62 accumulation which disrupts the Keap1-Nrf2 association and provokes Nrf2 stabilization and accumulation, resulting in liver damage (52). This Nrf2-mediated liver damage is, however, independent of p62 (52). Interestingly, defective proteasome function in the liver leads to damage associated with the activation of autophagy and Nrf2, in which Nrf2 activation serves as a physiological adaption (21). These results suggest that Nrf2 activation is cytoprotective when autophagy function is intact, whereas it becomes detrimental if autophagy is impaired. However, the precise underlying mechanisms remain to be defined.

5. Nrf2-MEDIATED PQC IN CARDIOMYOCYTES

Despite the increasing evidence that Nrf2 is capable of regulating PQC in the cells aforementioned, a link between Nrf2-mediated PQC and cardiac function has not been reported until recently. Wang *et al*. have recently demonstrated Nrf2-mediated clearance of ubiquitinated protein aggregates in the heart (53,54). Consistent with previous observations that Nrf2 deficiency leads to an earlier onset of cardiac pathological remodeling and the subsequent development of heart failure in response to hemodynamic overload or pharmacologically induced cardiac stress (55,56), Wang *et al.* found that the cardiomyocyte-specific overexpression of Nrf2 suppressed myocardial oxidative stress as well as cardiac apoptosis, fibrosis, hypertrophy, and dysfunction in a setting of sustained pressure overload produced by transverse aortic constriction (53). Interestingly, the constitutive activation of Nrf2 increased the steady level of autophagosomes while decreasing ubiquitinated protein levels and ubiquitin-positive protein aggregates in the pressure overloaded heart. Nrf2 gene gain- and loss-of-function approaches revealed that Nrf2 enhances autophagosome formation and autophagic flux in cultured cardiomyocytes. Unexpectedly, while Nrf2 minimally regulated apoptosis, it significantly suppressed the proteotoxic necrosis of cardiomyocytes. Moreover, Nrf2 attenuated the proteocytotoxicity presumably via enhancing autophagy-mediated clearance of ubiquitinated protein aggregates in cardiomyocytes. Therefore, cardiac-specific activation of Nrf2 suppresses cardiac maladaptive remodeling and dysfunction most likely by enhancing autophagic clearance of toxic protein aggregates in the heart. Since the pressure overload-induced cardiac dysfunction has been situated in the category of proteinopathies (57), these results have not only demonstrated that Nrf2 is a critical regulator of myocardial PQC but also argue against the previous claim that a sustained activation of Nrf2 in the heart is harmful due to the reductive stress (58,59). More recently, Li *et al*. have also demonstrated that Nrf2 is capable of ameliorating doxorubicin-induced impairment of autophagic flux and accumulation of ubiquitinated protein aggregates, and suppressing doxorubicin-induced cytotoxicity in cardiomyocytes (54). These results suggest that Nrf2 has an enormous impact on cardiac PQC under stress conditions, which may contribute to its protection against cardiac adverse remodeling and dysfunction.

In the past 15 years, a missense (R120G) mutation of alpha B-crystallin (CryAB^{R120G}), a *bona fide* misfolded protein linked to human disease (60), has been extensively used to study cardiac cytosolic PQC (10,20,61-65). Inadequate PQC, as manifested by UPS and ALP functional insufficiency (10,20,61,62,65), has been experimentally demonstrated to play a major pathogenic role in CryAB^{R120G}-induced cardiomyopathy in both cardiomyocyte cultures and transgenic mice (7). Notably, a reductive stress hypothesis had been proposed by Rajasekaran and colleague to explain pertubed PQC and resultant cardiac pathology caused by increased expresion of a misfolded cytosolic chaperone protein, based on their studies on a transgenic mouse model of cardiac overexpression of a human CryAB^{R120G} (hCryAB^{R120G}) (66). More recently, they suggest that sustained Nrf2 activation mediates this reductive stress because they observed that ubiquitous knockout of Nrf2 prevented reductive stress and attenuated aberrant protein aggregation, accumulation of ubiquitinated proteins, and pathological cardiac hypertrophy and heart failure in the aged hCryAB^{R120G} transgenic mice (59). The authors proposed that Nrf2-mediated reductive stress is the contributing mechanism for hCryAB^{R120G}-induced cardiomyopathy. It should be pointed out that the murine CryAB^{R120G} (mCryAB^{R120G}) used earlier by Wang *et al*. and the hCryAB^{R120G} used later by Rajasekaran *et al*. have virtually identical amino acid sequences because CryAB is one of the exquisitely conserved proteins between mice and humans (64,66). Hence, here we propose an alternative model which takes the findings by Kannan *et al*. into consideration along with most, if not all, other important findings from studying a similar mCryAB^{R120G} mouse model. First, although both proteasome activity and macroautophagy are adaptively upregulated in CryAB^{R120G} overexpressing hearts (62,67), these upregulations are inadequate and both proteasome functional insufficiency and ALP insufficiency have been demonstrated to play an essential role in mCryAB^{R120G}-based cardiomyopathy (10,65). Second, the ALP insufficiency can in turn impede the degradation of ubiquitinated proteins by proteasome via accumulation of an ALP substrate protein p62 (22,23). Third, in both cultured cardiomyocytes and intact mouse hearts, expression of mCryAB^{R120G} led to significant increases in both the mRNA and protein levels of p62, which in turn promotes aggresome formation (20); although it is unclear whether the increased p62 transcription is

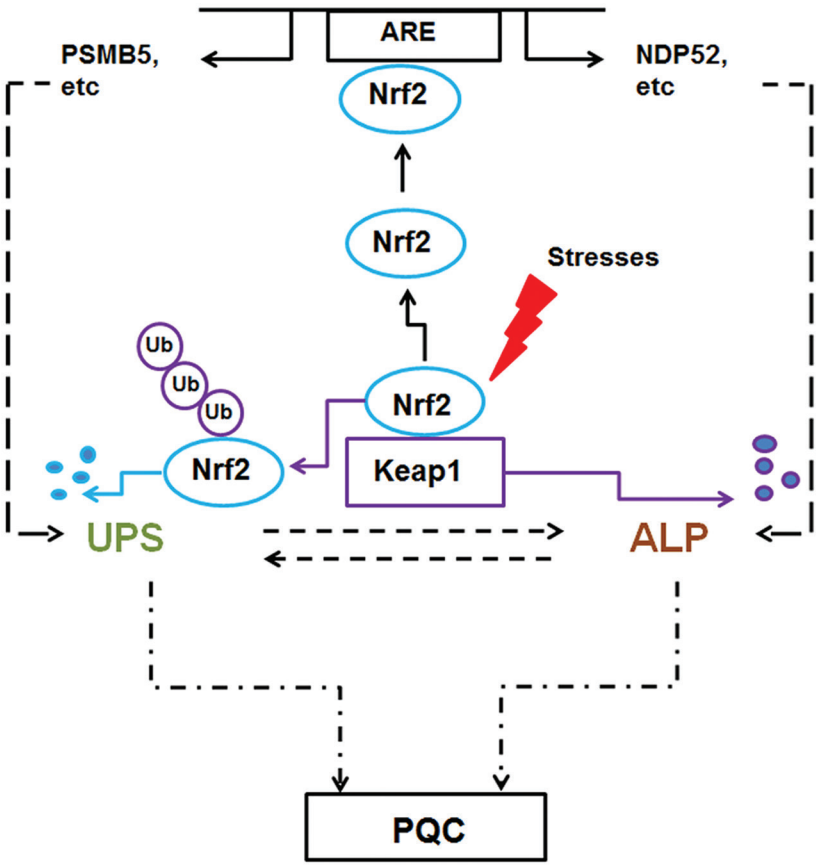

Figure 2. A hypothesis for Nrf2-mediated PQC in the heart. Nrf2 is constitutively degraded via the ubiquitin-proteasome system (UPS) by Keap1-mediated ubiquitination, while Keap1 is degraded by the autophagic-lysosome pathway (APL). Upon external or internal stresses which interrupt Keap1-mediated Nrf2 degradation, Nrf2 is released from Keap1-Nrf2 complex and newly synthesized Nrf2 is accumulated thereby triggering nuclear translocation of Nrf2 to activate its downstream genes, such as PSMB5 and NDP52. As a result, the UPS and the ALP are enhanced for clearing malfolded proteins in the cardiomyocytes.

mediated by Nrf2, Nrf2 activation is capable of driving p62 expression and thereby leads to hepatotoxicity in autophagy deficient liver aforementioned (52). Hence, in aged hCryAB^{R120G} transgenic mouse hearts which are likely with ALP insufficiency, a constitutive activation of Nrf2-mediated p62 expression may result in the accumulation of p62-protein aggregate complexes, which, in turn, compromises myocardial UPS performance in the setting of autophagy impairment in the heart as recently reported (22). Lastly, as described earlier, cardiomyocyte-specific Nrf2 transgenic mice displayed normal cardiac function as well as cardiac protective phenotype (53), arguing against the notion that the Nrf2-mediated antioxidant signalling causes pathogenic reductive stress. Therefore, it is very possible that the rescue effect of Nrf2 knockout on the aged hCryAB^{R120G} transgenic mice be mediated by p62 down-regulation and improvement of UPS performance, not necessarily requiring diminution of reductive stress.

Taken together, it is apparent that the contribution of Nrf2-mediated regulation of both the UPS and autophagy to its cardioprotective effects has not been well established; the precise mechanism by which Nrf2 regulates cardiac PQC also needs to be further investigated.

6. A SUMMARY AND FUTURE DIRECTIONS

Protein quality control (PQC) is a tightly-controlled orchestra of multiple proteins in multiple pathways that ultimately serve to ensure proteostasis in cells. The UPS and the ALP degradation are the two major pathways for clearing damaged proteins in the cell. The substrate selection of the UPS is largely specified by Ub E3's; however, the mechanism determining the specificity for substrate selection of autophagy is not well understood. Moreover, the precise mechanism by which the UPS and the ALP interact in clearing damaged proteins remains to be unveiled. In this context, Nrf2 appears to be a novel regulator of the UPS and the ALP (Figure 2). While the UPS downregulates Nrf2 activity by Keap1-mediated degradation of Nrf2, the ALP seems to increase Nrf2 activity via autophagic clearance of Keap1.

In contrast, Nrf2 is capable of augmenting both USP and ALP activities via the upregulation of subunits of proteasome and autophagy adaptor protein NDP52. The Nrf2-driven NDP52 in selectively facilitating degradation of phosphorylated tau via autophagy highlights a potential role of Nrf2 in activating selectively autophagic clearance of target proteins by upregulating NDP52.

Because of the wealth of evidence that has accumulated showing that Nrf2 is the major regulator of cellular defense against various pathological stresses in different organs including lungs, liver, gastrointestinal tract, bladder, kidneys, brain, skin, ovary, and the heart, Nrf2 has evolved to be an attractive drug target for the treatment or prevention of human diseases (26). Of note, Nrf2 is downregulated in at least a subset of human failing hearts (68); however, inappropriate activation of Nrf2 may cause heart failure in a setting of diabetes in humans (69). Given the emerging role of Nrf2 in coordinating the UPS and the ALP, further investigation of Nrf2-operated signaling in orchestrating the PQC by the UPS and the ALP in a variety of proteinopathies will not only provide novel insights into understanding the nature of USP and/or APL in the PQC involved in human disease, but also unveil the molecular mechanisms underlying controversial observations of Nrf2-mediated seemingly opposing actions associated with dysregulated UPS and/or ALP functions. Importantly, there are a large number of different Nrf2 activators in the pipelines for drug development (70,71); the outcome will pave the way for developing a new class of drugs for the treatment of human disease including heart failure.

7. ACKNOWLEDGEMENTS

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