Recent Progress in Research on Molecular Mechanisms of Autophagy in the Heart

Running Head: Autophagy and Heart Disease

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Abstract

Dysregulation of autophagy, an evolutionarily conserved process for degradation of long-lived proteins and organelles, has been implicated in the pathogenesis of human disease. Recent research has uncovered pathways that control autophagy in the heart and molecular mechanisms by which alterations in this process affect cardiac structure and function. Although initially thought to be a non-selective degradation process, it has become increasingly clear that autophagy can exhibit specificity in the degradation of molecules and organelles, such as mitochondria. Furthermore, it has been shown that autophagy is involved in a wide variety of previously unrecognized cellular functions, such as cell death and metabolism. A growing body of evidence suggests that deviation from appropriate levels of autophagy causes cellular dysfunction and death, which in turn leads to heart disease. Here, we review recent advances in understanding the role of autophagy in heart disease, highlight unsolved issues, and discuss the therapeutic potential of modulating autophagy in heart disease.

Key Words: Autophagy, Mitophagy, Protein Quality Control, Autosis
Introduction

Autophagy is an evolutionarily conserved cellular process in which proteins, lipids, and organelles are degraded in lysosomes (42). In most cells, several forms of autophagy operate that differ in the means by which cargo is delivered to the lysosome. In macroautophagy, the form that has been most intensively studied, double membrane vesicles called autophagosomes engulf cytoplasm, protein aggregates, pathogens, and organelles (or parts thereof) and traffick the contents to lysosomes. Cargo selection in macroautophagy is often non-specific. However, specificity may be conferred in some instances, when autophagosome formation is directed to a specific target (e.g. the elimination of defective mitochondria during “mitophagy”). A second form of autophagy, chaperone-mediated autophagy (CMA), employs molecular chaperones – rather than autophagosomes – to move soluble cytoplasmic proteins to lysosomes for degradation. In this form of autophagy, the chaperone binds a specific target sequence on the protein to be eliminated. A third form of autophagy, microautophagy, involves the uptake of cargo directly into lysosomes.

Autophagy plays important roles in baseline cellular homeostasis. Examples include the catabolism of certain long-lived proteins and the elimination of denatured/aggregated proteins and damaged/senescent organelles (cellular “quality control”). Moreover, autophagy is a critical adaptive mechanism. It is markedly augmented in response to a wide variety of cellular stresses, and helps cells to cope with these insults. One example is starvation where autophagy-mediated catabolism and recycling of cellular components helps cells survive an energetic crisis. In contrast to
these adaptive roles, evidence suggests that autophagy may sometimes also play
pathological – even lethal – roles in some cellular contexts (46), although the molecular
basis of these maladaptive effects remains poorly understood.

The last decade has witnessed a tremendous growth in research into the
physiological and pathological roles, as well as the molecular mechanisms, of
autophagy (33). Increasing lines of evidence suggest that, besides catabolism and
quality control, autophagy is involved in multiple cellular functions, including cell
death, defense against pathogens, immunity, and metabolism (42). For example,
autophagy degrades lipid droplets, thereby regulating fatty acid metabolism and
lipotoxicity (76). In addition, dysegulation of autophagy can contribute to a wide range
of human disorders, including heart disease (38, 42, 69). Indeed the adult heart, which is
under continuous mechanical and neurohormonal stress, is particularly dependent upon
autophagy to maintain normal function. Here we summarize recent topics and unsolved
issues in autophagy research in the heart.

Techniques for Quantifying Autophagy

Quantification of autophagy is critical to understanding its adaptive and maladaptive
roles. Unfortunately, obtaining accurate information regarding rates of autophagy in
the heart is challenging technically. Since almost all work to date in the heart has
focused on macroautophagy, our comments will be restricted to this form (which will be
referred to simply as “autophagy”), unless otherwise specified. One of the most
common misconceptions is the concept that an increased number of autophagosomes
indicates increased rates of autophagy (34). The number of autophagosomes is determined by the balance between the rate of generation of autophagosomes and that of their conversion to autolysosomes or degradation in lysosomes. Accordingly, increases in autophagosome number may indicate either enhancement of autophagosome formation or inhibition of the autophagic pathway downstream of autophagosome formation. To know whether autophagic rates are increased, it is essential to distinguish these possibilities by assessing “autophagic flux”. Autophagosome quantity in the heart can be monitored conveniently using GFP-LC3 transgenic mice (55). These mice express a GFP reporter fused to LC3, the only autophagy-related protein located on autophagosomes, either systemically (55) or in a heart-restricted manner (86).

Autophagic flux can be evaluated by counting autophagosome number as indicated by GFP-LC3 puncta in the presence or absence of chloroquine, an inhibitor of autophagosome-autolysosome fusion (26). If a given stimulus results in increases in autophagosome number in the presence of chloroquine, then that stimulus is augmenting autophagosome formation or flux. mRFP-GFP-LC3, another LC3 indicator, is convenient in that it allows one to distinguish autolysosomes from autophagosomes (32). This LC3 indicator utilizes the fact that color emission from GFP, but not mRFP, is quenched at a low pH in autolysosomes. Thus, this reporter emits both green and red colors on autophagosomes, while it only emits red color on autolysosomes. By quantifying LC3 puncta showing both green and red, indicating autophagosomes, and those showing only red, indicating autolysosomes, one can assess whether conversion from autophagosomes to autolysosomes (i.e. flux) is stimulated.
Tandem-fluorescent LC3 mice, which express mRFP-GFP-LC3 in a heart-specific manner, are useful for assessing the extent of autophagic flux in response to stress in the heart (18). Even if the laboratory setting does not allow the use of these indicator mice, one should consider at least the following methods to assess autophagic flux in the heart. The first is to measure the level of autophagic substrates, including p62/SQSTM1 (p62) and GFP-LC3 (34). For example, decreases in p62 protein without changes in p62 mRNA expression, or cleavage of GFP from GFP-LC3 may indicate that protein degradation is due to autophagy. It should be noted, however, that accumulation, rather than degradation, of p62 can be observed in some cases, such as when mitochondrial autophagy is stimulated (81). Thus, the absence of a decrease in p62 may not necessarily exclude stimulation of autophagy. Measurement of lysosome-dependent long-lived protein degradation is also useful for evaluating autophagic flux. By combining multiple assays, it may be possible to monitor autophagic flux in the heart in a more reliable manner (56).

Quantification of autophagic flux allows one to correlate changes with a given pathological stimulus. However, to establish a cause-and-effect link between changes in autophagic flux and function, it is necessary to perturb autophagic flux. For example, if one finds that autophagy is stimulated in response to a pathological stress on the heart, one could test how the heart reacts in the absence of autophagy. On the other hand, if one finds that autophagy is inhibited, one could examine how the heart reacts when autophagy is rescued. Traditionally, 3-MA is used to inhibit autophagy whereas rapamycin is used to stimulate autophagy in the heart. However, given the non-specific
autophagy-independent effects associated with these chemicals, the use of more specific
interventions directly acting on the autophagic machinery, such as genetic deletion of
Atg genes (57) and overexpression of Atg7 (62), both in vitro and in vivo, is desirable.
Unfortunately, even if one uses these genetic manipulations, many Atg genes have
autophagy-independent functions. Thus, the results of the interventions may derive from
their autophagy-independent actions. To avoid these limitations, one could employ
multiple genetic and/or pharmacological approaches to modulate autophagy and
evaluate whether those interventions lead to similar effects. Inclusion of autophagic flux
data and mechanistic assessment of the functional significance of changes in autophagic
flux is needed to gain a basic understanding of the role of autophagy in the process
under consideration. The reader is referred to “Guidelines for the use and interpretation
of assays for monitoring autophagy” regarding the current standards in autophagy
research (34).

Mitophagy and Heart Diseases
Increasing lines of evidence suggest that selective forms of autophagy may operate in an
organelle-specific manner, selectively targeting mitochondria (mitophagy), the
endoplasmic reticulum (reticulophagy), ribosomes (ribophagy), peroxisomes
(pexophagy), and portions of the nucleus (nucleophagy) (60). Among various body
tissues, the myocardium contains a particularly high concentration of mitochondria.
While these organelles are critical for the production of ATP, when damaged or
senescent they pose a risk to the internal cellular environment by virtue of their abilities
to produce reactive species and contaminate the mitochondrial pool with mutated DNA.

Accordingly, the heart is particularly susceptible to stresses that damage mitochondria, and defective mitochondria must be efficiently removed (24). This takes place both through generalized autophagy (where mitochondria are captured non-specifically in bulk by autophagosomes) and by mitophagy (a more specialized form of macroautophagy where damaged and senescent mitochondria are specifically targeted for elimination).

Removal of damaged organelles by autophagy can be documented by either immunostaining to show co-localization of organelle-specific markers with LC3 puncta or by direct visualization of the presence of specific organelles within autophagosomes via electron microscopic analyses (22, 39). Lysosomal degradation of mitochondrial proteins can be evaluated using mito-Keima, a fluorescent protein that changes color at acidic pH (29). Although this indicator alone cannot prove that translocation of mitochondrial proteins occurs through mitophagy, inhibition of the translocation by interventions that suppress autophagy or mitophagy would indicate that the mitochondrial clearance is mediated through autophagy or mitophagy (25). Parkin translocation to mitochondria and Parkin-dependent ubiquitination of mitochondrial proteins (see below) are also used to show activation of mitophagy (23, 39). Mitophagy is often accompanied by decreases in mitochondrial inner membrane/matrix proteins and mitochondrial DNA (22, 25). It should be noted, however, that caution should be exercised when interpreting the results obtained from these assays; none of these assays
alone can prove the presence of cargo-specific autophagy such as mitophagy because it is challenging to distinguish cargo-specific autophagy from general autophagy.

There are two signaling pathways involved in targeting damaged mitochondria to autophagosomes, the PINK1-Parkin-mediated pathway and the mitophagy receptor-mediated pathway (Figure 1). PINK1 is a mitochondrially-targeted serine/threonine kinase that is degraded by the presenilin-associated rhomboid-like (PARL) protease under control conditions (27). In damaged mitochondria with depolarized membrane potential PINK1 is stabilized on the OMM, where it then phosphorylates multiple substrates, including the mitochondrial fusion-promoting protein Mfn2 (8). On the other hand, PINK1 is downregulated through posttranscriptional mechanisms in the human end-stage failing heart (5). When phosphorylated, Mfn2 acquires a second function as a mitochondrial receptor for Parkin, an E3 ubiquitin ligase - thereby recruiting Parkin to the OMM (80). PINK1 also phosphorylates Parkin (73) as well as ubiquitin (28, 36). Once Parkin is activated, it promotes ubiquitination of a number of OMM proteins, including Mfn1/2, Hexokinase I, VDAC1, and Miro. Next, the adapter protein p62 binds to ubiquitinated substrates via its ubiquitin-associated domains and to LC3 on the autophagosomal membrane, thereby resulting in selective engulfment of the damaged mitochondrion by an autophagosome and elimination of that mitochondrion (17). Recently, several endogenous inhibitory mechanisms of PINK1-Parkin-mediated mitophagy have been discovered. USP30, a deubiquitinase localized in the OMM, inhibits mitophagy by removing ubiquitin from substrates ubiquitinated by Parkin (6). Clec16a, a membrane-associated endosomal
protein and a product of a disease susceptibility gene for type 1 diabetes, multiple sclerosis and adrenal dysfunction, was also found to be a novel negative regulator of PINK1-Parkin-mediated mitophagy (77). Clec16a physically interacts with Nrdp1, an E3 ubiquitin ligase, thereby promoting proteasomal degradation of Parkin, which in turn inhibits mitophagy. Anti-apoptotic BCL-2 family proteins, including BCL-xL, MCL-1 and BCL-2, on the OMM inhibit mitophagy through inhibition of Parkin translocation to depolarized mitochondria in a Beclin1-independent manner (20). The tumor suppressor p53 also inhibits mitophagy by sequestering Parkin in the cytosol in the mouse heart (22).

Several previous studies suggest that PINK1/Parkin-mediated mitophagy may play a protective role in the heart. A significant increase in mortality and infarct size is observed in Parkin−/− mice compared to in wild-type mice 7 days after myocardial infarction (MI) (37). Conversely, overexpression of Parkin inhibits hypoxia-induced acute cell death in adult rat cardiomyocytes in vitro (26). Similarly, the infarct size after I/R is significantly greater in the hearts of PINK1−/− mice than in wild-type mice in an ex vivo model, and overexpression of PINK1 protects against simulated I/R-mediated cell death in HL-1 cardiac cells (75). Furthermore, the hearts of PINK1−/− mice exhibit ventricular dysfunction and hypertrophy with increased fibrosis, apoptosis, oxidative stress, and reduced mitochondrial respiration at 2 months of age (4, 75). Whether PINK1 and Parkin function exclusively through mitophagy remains to be clarified. The role of PINK1 and Parkin in mitochondrial autophagy and mitophagy has been investigated primarily using stimuli inducing mitochondrial depolarization, such as
cyanide m-chlorophenylhydrazone (CCCP). Whether similar mechanisms mediate the removal of damaged mitochondria through mitophagy in response to more pathologically relevant stimuli, such as I/R and hemodynamic stress, remains to be elucidated.

Damaged mitochondria are also recognized by autophagosomes through BNIP3 and NIX, pro-apoptotic BH3-only family proteins (65, 85). In addition to their cell death functions, these OMM proteins also bind to LC3 on the autophagosome, which, in turn, mediates mitophagy. Ablation of NIX expression results in progressive cardiac dysfunction with age as a consequence of the accumulation of impaired mitochondria (14). The fact that hearts of NIX\textsuperscript{-/-} BNIP3\textsuperscript{-/-} double-knockout mice demonstrate accelerated cardiac hypertrophy and mitochondrial dysfunction compared to the single NIX\textsuperscript{-/-} and BNIP3\textsuperscript{-/-} mice indicates that Nix and Bnip3 have non-overlapping functions (14). Further investigation is required to elucidate the roles of NIX and BNIP3 in mediating mitophagy in the heart in vivo. Recently, other mitochondrial proteins and lipids, including FUNDC1 and cardiolipin, have been found to act as receptors for LC3 and participate in mitophagy (11, 45); their roles in mitophagy in the heart remain to be elucidated.

When mitochondria are degraded by autophagy, lysosomal DNase II digests mitochondrial DNA released from damaged mitochondria. However, as mitochondrial DNA contains inflammagenic unmethylated CpG motifs, mitochondrial DNA that escapes degradation by autophagy promotes inflammation in a Toll-like-receptor 9-dependent manner in cardiomyocytes, which in turn causes chronic inflammation in
failing hearts (59). Thus, coordination between autophagy and lysosomes appears essential to prevent a myocardial inflammatory response caused by mitochondrial DNA. Removal of damaged mitochondria by autophagy appears coupled to mitochondrial fission, a process that asymmetrically divides mitochondria into two fragments. The smaller mitochondrial fragment generated by fission is often depolarized and susceptible to being engulfed by autophagosomes. Mitochondrial fission is regulated by the recruitment of Dynamin-related protein 1 (Drp1), a cytoplasmic GTPase, to mitochondrial fission sites in the outer mitochondrial membrane (OMM) in coordination with mitochondrial fission 1 (Fis1) and mitochondrial fission factor (Mff) (64). In contrast, mitochondrial fusion is regulated by mitofusin1 (Mfn1) and mitofusin2 (Mfn2), also on the OMM (40). A lack of mitochondrial fusion due to downregulation of Mfn1 and Mfn2 promotes cardiac dysfunction at baseline and in response to stress (9). In contrast, suppression of mitochondrial fission by inhibition of Drp1 with Mdivi-1, a chemical inhibitor of Drp1, results in reduction of infarction size in response to ischemia/reperfusion (I/R) (61). Similarly, inhibition of Drp1 with Mdivi-1 or knockdown by Drp1 RNAi prevents cardiomyocyte death after hypoxia/reoxygenation (H/R) (71). However, inhibition of mitochondrial fission using Fis1 RNAi or dominant-negative Drp1 mutant (Drp1K38A) results in suppression of mitophagy, accumulation of damaged mitochondrial proteins and reduced respiration, thereby leading to metabolic dysfunction in pancreatic INS1 cells (79), suggesting that mitochondrial fission is also essential for cell survival. Cardiac specific-heterozygous deletion of Drp1 exacerbated myocardial injury in response to ischemia/reperfusion (25).
Thus, mitochondrial fission appears to affect cell survival both positively and negatively in a context-dependent manner. It should be noted that many conclusions regarding the role of fission and fusion in regulating mitochondrial functions and autophagy have been obtained in non-cardiac cells. Although the function of fission and fusion in the heart has been deduced primarily from genetic studies, the occurrence of fission and fusion has not been demonstrated in an unequivocal manner in adult ventricular cardiomyocytes. In addition, although electron microscopic analyses allow observation of autophagosomes containing mitochondria in the heart and the cardiomyocytes therein, whether they reflect degradation of mitochondria by general autophagy or mitophagy that selectively degrades mitochondria remains to be elucidated. Furthermore, since many interventions affect mitochondrial morphology and autophagy simultaneously, it is challenging to separate the effect of fission and fusion from that of mitochondrial autophagy alone. Thus, careful observation of mitochondrial morphology as well as molecular interventions to distinguish the effect of fission and fusion from that of mitochondrial autophagy are needed to better understand the precise role of these mitochondrial quality control mechanisms in the heart.

**Protein Toxicity in Heart Diseases and Autophagy**

As the endogenous proliferative capacity of mammalian adult cardiomyocytes is limited, vigorous systems for maintaining protein quality control (PQC) are essential to sustain the long-term well-being of cardiomyocytes. In addition to various protein refolding
mechanisms (7), PQC is maintained through degradation via the ubiquitin-proteasome system (UPS) (44) and autophagy (3). Impairment of autophagy causes aggregation of damaged and/or misfolded proteins in cardiomyocytes, thereby damaging the cell and potentially leading to cell death. Suppression of constitutive autophagy by genetic deletion of Atg5 leads to cardiac dysfunction with increased protein aggregation in the heart (57). Desmin-related cardiomyopathies (DRCs), cardiomyopathies caused by inherited or de novo mutations in desmin and accessory proteins, are typified by severe and progressive forms of heart failure. Impairment of interaction between desmin and αB-crystallin (CryAB), a small heat shock protein that functions as a molecular chaperone, causes protein aggregation and myofibrillar disarray, thereby leading to severe cardiac dysfunction. Bhuiyan et al. found that enhanced levels of autophagy ameliorate DRC, by crossing CryABR120G mice, a model of DRC, and cardiac-specific atg7 transgenic mice (3). Accumulation of protein aggregates and ubiquitinated proteins is also seen in cardiomyocytes from hearts with pressure-overload (PO)-induced cardiac hypertrophy, chronic MI and dilated cardiomyopathy (DCM) (49, 78). We have shown recently that aggresomes co-localized with p62 accumulate markedly in cardiomyocytes from chronic MI hearts (49).

What is the cause of protein aggregation in the heart during stress?

Mammalian sterile 20-like kinase 1 (Mst1) is a proapoptotic serine/threonine kinase that is activated in response to stress, including volume overload during cardiac remodeling. Mst1 phosphorylates Thr108 of Beclin1, enhances the interaction between Beclin1 and Bcl-2/Bcl-xL, and induces Beclin1 homodimer formation (Figure 2). In this condition,
Beclin1 cannot activate class III PI3 kinase and, thus, autophagy is suppressed (84).

Increased phosphorylation of Beclin1 by Mst1, inhibition of autophagy, and increased accumulation of aggresomes are also seen in hearts in human end-stage DCM. These results suggest that Mst1 may be one of the endogenous facilitators of protein aggregation in the stressed heart. It has been shown recently that overexpression of Tollip, a new class of ubiquitin-binding CUE-domain protein, causes clearing of Huntington’s-disease-linked poly Q proteins by autophagy (47). Whether endogenous Tollip regulates aggresome formation in the heart is currently unknown.

Can Autophagy Induce Cell Death?

Despite its clearly demonstrated role as an adaptive/survival process, accumulating evidence suggests that, in certain contexts, autophagy may induce cell death. The initial data were merely associative: the presence of autophagic vacuoles in some dying cells (43). But, these observations were unable to differentiate between the possibilities that autophagy was promoting cell death or attempting to rescue the cell. Some insight into the ordering of events has been provided by studies in which autophagy has been genetically manipulated during a pathological process. For example, cardiomyocyte death and pathological remodeling during ischemia/reperfusion and pressure overload in the heart can be reduced by inhibiting the induction of autophagy that takes place in these conditions by depletion of Beclin1, an autophagy gene that is often upregulated in response to oxidative stress (18) and is involved in autophagosome formation through activation of class III PI3 kinase (54, 86). Autophagy suppression through genetic...
inhibition has also been shown to alleviate tissue damage in other systems including cerebral ischemia/reperfusion, neurodegenerative disease, and liver injury (1, 35). Conversely, induction of autophagy can promote caspase-independent cell death in apoptosis-competent cells (15, 66). Taken together, these observations suggest the possibility that autophagy can induce cell death in some contexts. However, the determinants of whether autophagy results in cell survival or death, and the underlying mechanism of autophagy-related cell death remain poorly understood.

Liu Y et al. recently showed that increased levels of autophagy in response to Tat-Beclin1, a cell-penetrating, autophagy-inducing peptide, induce cell death (46). This cell death, termed “autosis” has unique morphological characteristics that are distinct from those of either apoptosis or necroptosis, including increased numbers of autophagosomes/autolysosomes, nuclear convolution at early stages and focal swelling of the perinuclear space at late stages. Autosis is observed in hippocampal neurons of neonatal rats subjected to cerebral hypoxia-ischemia in vivo. Through a chemical screen, this unique form of autophagy-dependent cell death was found to be suppressed effectively by cardiac glycosides, antagonists of Na\(^+/\)K\(^{-}\)-ATPase (46). The molecular mechanisms connecting autophagy, the Na\(^+/\)K\(^{-}\)-ATPase and cell death remain to be elucidated. Interestingly, however, cardiac glycosides attenuate autotic cell death in vivo.

At present, we do not know whether autosis is observed in the heart under conditions in which autophagy has been shown to be detrimental, such as ischemia/reperfusion (18, 53) and acute pressure overload (86). If autosis exists in the heart, it would be interesting to test whether cardiac glycosides offer protection against such insults.
Chaperone-mediated autophagy and alternative autophagy (Figure 3)

In contrast to macroautophagy, CMA (30) has been studied to only a limited extent in the heart. The targets of this form of autophagy are specific soluble cytoplasmic proteins that contain a KFERQ-like motif, although the precise sequence can vary (13). CMA substrates are transported to the lysosome in complex with the chaperone Hsc70, which binds the KFERQ-like motif, rather than in autophagic vacuoles (10). The complex interacts with the lysosomal membrane protein LAMP-2A, the specific alternatively spliced Lamp2 isoform that mediates CMA (12). Following unfolding (68), the substrate is imported into the lysosome for degradation. An estimated ~30% of cytosolic proteins possess KFERQ-like sequences (13) and are, therefore, potential CMA substrates, although which actually undergo CMA remains to be determined for each cell type. Interestingly, the ryanodine receptor (RyR2), an SR Ca\(^{2+}\)-release channel that plays critical roles in excitation-contraction in cardiomyocytes, was recently reported to possess multiple KFERQ-like motifs and to be degraded, in part, through CMA (63). Although the role of CMA in cardiac biology has yet to be defined, early studies showed that starvation induces marked reductions of proteins with KFERQ motifs in heart, but not in soleus or extensor digitorum longus muscles, *in vivo* (82). In the liver, where it has been studied, protein loss by macroautophagy and CMA exhibit distinct kinetics with CMA being delayed. Until recently, little was known about the specific role of CMA in physiological processes *in vivo*. However, using mice with conditional deletion of LAMP-2A, which exhibit selective downregulation of
CMA but not macroautophagy, Schneider et al. found that hepatocyte-specific blockage of CMA induces hepatic glycogen depletion and liver steatosis (70). Interestingly, the mechanism involved coordinate increases in the levels of enzymes involved in carbohydrate and lipid metabolism resulting from inhibition of CMA. This work demonstrates a role for CMA in regulating metabolism in vivo.

Macroautophagy is separated into several specific steps, including induction, recognition and selection of cytoplasmic substrates, formation of the autophagosome around substrates, autophagosome-lysosome fusion, degradation of the autolysosomal contents, and release of the degraded products into the cytoplasm. The canonical, or conventional, autophagic pathway consists of evolutionarily conserved signaling molecules encoded by Atgs, including Atg4, Atg5, Beclin1 (Atg6), Atg7, Atg12, and Atg16, which govern these steps. On the other hand, accumulating lines of evidence suggest that non-canonical autophagic pathways are present. Nishida et al. revealed that Atg5⁻/⁻ Atg7⁻/⁻ double-knockout cells are still able to form autophagosomes and degrade autophagic substrates inside the autolysosomes in response to certain stimuli (58). During the process of this Atg5/Atg7-independent autophagy, termed “alternative autophagy”, the lipidation of LC3 does not occur. Instead, Rab9, a small GTPase involved in membrane trafficking and fusion between the trans-Golgi network (TGN) and late endosomes, plays a critical role in generating autophagosomes in the alternative autophagic pathway by promoting fusion of the phagophore with vesicles derived from the TGN and late endosomes. Recently, Honda S et al. revealed that ULK1-dependent, Atg5-independent macroautophagy is the dominant process for removing mitochondria
from reticulocytes in the final stage of erythrocyte maturation (21). However, the
functional significance of alternative autophagy has not yet been demonstrated in the
heart.

**Autophagy as a therapeutic target for heart diseases?**

Several clinical trials targeting autophagy have been undertaken to treat cancers (52), α1-antitrypsin deficiency liver cirrhosis (19), and amyotrophic lateral sclerosis (16). To our knowledge, clinical trials modulating autophagy have not yet been undertaken for heart disease yet. Recently, a novel agent to induce autophagy was developed. Tat-Beclin1, a peptide derived from an evolutionarily conserved domain of Beclin1 (267-284) binding with human immunodeficiency virus-1 Nef, strongly induces autophagy by interacting with Golgi-associated plant pathogenesis-related protein 1, a newly identified negative regulator of autophagy (74). Tat-Beclin1 attenuates the accumulation of polyglutamine expansion protein aggregates (htt103Q) derived from human mutant Huntingtin protein and the replication of several pathogens *in vitro* and *in vivo*. Since the efficacy of Tat-Beclin1 to induce autophagy is potent, it may normalize the level of autophagy in chronic myocardial infarction hearts where autophagy is suppressed below physiological levels. Further studies are required to test whether Tat-Beclin1 improves cardiac function in the chronic myocardial infarction heart. Suppression of histone deacetylases by trichostatin A and suberoylanilide hydroxamic acid, a drug approved by the US Food and Drug Administration, also
stimulates autophagy in the heart and the cardiomyocytes therein and attenuates myocardial injury in response to ischemia/reperfusion (83).

Mariño G et al. recently discovered a mechanism of autophagy regulation by cytosolic acetyl-coenzyme A (AcCoA), a principle integrator referred to as the “hub of metabolism” (50). Starvation rapidly stimulates rapid depletion of AcCoA, thereby suppressing the activity of the EP300, an acetyltransferase and endogenous negative regulator of autophagy, which, in turn, activates autophagy. Consistently, pharmacological inhibition of EP300 by c646 effectively induced autophagy in mice. Furthermore, maintenance of high AcCoA levels by supplementation of dimethyl-α-ketoglutarate, a cell-permeant precursor of α-ketoglutarate, suppressed maladaptive autophagy induced by pressure overload in the heart. Thus, these results suggest that pharmacological strategies targeting AcCoA may allow for novel therapeutic manipulation of autophagy as well.

Although here we discussed Tat-Beclin1 and AcCoA as examples of interventions to stimulate or inhibit autophagy, respectively, whether autophagy is stimulated or inhibited varies substantially depending upon the nature of stress and the timing/stage of disease. Further studies are required to address these issues.

Perspectives

Increasing lines of evidence suggest that suppression of autophagy below physiological levels disrupts the PQC system in cells, thereby inducing chronic heart failure (49, 57). Conversely, excessive induction of autophagy beyond a physiological range may cause...
cell death, thereby aggravating cardiac function (54, 86). Thus, maintaining autophagy at physiological levels appears indispensable for normal cellular homeostasis (41). If this hypothesis is true, assessment of the current level of autophagy is essential. To this end, development of convenient and non-invasive methods to evaluate the level of autophagic flux in vivo is urgently needed.

Recent evidence suggests that autophagy is involved in the degradation of specific proteins or organelles (67). As selective autophagy is mediated by LC3 receptors, including p62 and Nbr1 or ubiquitin-Atg8 adaptors called CUET proteins (47), it is possible that autophagy has the potential to degrade specific proteins. Although autophagy may promote survival of energy-starved cardiomyocytes by promoting general catabolism, degradation of specific molecules may also play important roles in mediating cell survival. Identifying individual proteins degraded by autophagy should allow us to identify novel functions of autophagy in cardiomyocytes.

Although we did not discuss them in detail in this review, mechanisms mediating lysosomal degradation, the second half of the autophagic flux, are also likely to be affected in heart diseases and would require further investigation (72). For example, TFEB is a basic helix-loop-helix leucine zipper transcription factor regulating genes involved in lysosomal biogenesis and its activity is regulated by phosphorylation by mTORC1, ERK and PKC (48, 51). We have shown recently that the combined loss of RagA and RagB GTPases, key components of a lysosome-based signaling system (2), in cardiomyocytes induces cardiomyopathy mimicking lysosomal storage disease. In these mice, lysosomal acidification is severely compromised due to a decreased
vacuolar-type H(+)‐ATPase level in the lysosomal fraction, suggesting that RagA and
RagB play important roles in regulating the lysosomal function in the heart (31). Further
investigations are required to elucidate how autophagy is regulated by these endogenous
regulators during stress in the heart.

In summary, autophagy critically controls survival and death of
cardiomyocytes during cardiac stress and heart failure. The study of autophagy in the
heart is hampered by the technical difficulty of correctly assessing autophagic flux and
the lack of interventions selectively modulating the activity of autophagy in the heart.
Despite these challenges, modulation of autophagy has therapeutic potential in the
context of heart diseases, and thus, better understanding of autophagy is essential.
Judging from the fundamental importance of autophagy in survival and death of
cardiomyocytes, our effort should lead to the discovery of a new modality for the
treatment of heart disease in the near future.

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Figure legends

Figure 1. Schematic model of the molecular mechanisms regulating mitophagy. There are two signaling pathways for selective activation of mitophagy: the PINK1-Parkin-mediated pathway and the mitophagic-receptor (BNIP3 and NIX)-mediated pathway (See text for details). There are also several systems for suppression of mitophagy. Arrows denote stimulation, and T-shaped indicators denote inhibition.
**Figure 2.** Schematic model of Beclin1, Bcl-2/xL and Mst1 interactions in the regulation of autophagy in the heart. Mst1 phosphorylates Beclin1 at Thr\(^{108}\), leading to enhancement of Beclin1-Bcl-2/xL binding, Beclin1 homodimer formation, and inhibition of Vps34 kinase activity, thereby suppressing autophagosome formation. Activation of Mst1 downregulates autophagy below physiological levels and suppresses protein quality control, contributing to to cardiac dysfunction (See text for details). Arrows denote stimulation, and T-shaped indicators denote inhibition.
Figure 3. Schematic model of the major pathways in the regulation of the autophagic machinery. There are three different types of autophagic pathways: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). In macroautophagy, there are two subtypes of autophagic pathways: conventional and alternative macroautophagy (See text for details).