Targets, trafficking, and timing of cardiac autophagy

David Rotter and Beverly A. Rothermel
The University of Texas Southwestern Medical Center, Dallas, TX 75390

Abstract

Heart failure is the major case of death in developed countries, and its prevalence is growing worldwide. Autophagy is a fundamental cellular mechanism through which intracellular components can be removed, recycled and repaired. Studies in humans and animal models demonstrate a marked increase in cardiac autophagic activity under a wide range of disease states and in response to diverse stimuli. Recently, autophagy has been widely promoted as a potential therapeutic target for the treatment of cardiovascular disease and heart failure. An important challenge to achieving this goal is the dual nature of cardiac autophagy, sometimes acting to help preserve cardiac function, other times appearing to promote cardiac decline. Numerous control points regulating autophagic activity and cargo selection provide a diversity of opportunities for drug targeting. In addition there is an innate circadian rhythm to the systemic regulation of autophagy that is often overlooked but provides potential opportunities to target and optimize pharmacological intervention.

1. Introduction

The heart is a highly plastic organ, adapting size and morphology in response to changes in cardiac demand and undergoing pathological remodeling during heart failure. An association between autophagic activity and heart disease has been noted for almost 40 years (13, 40), however, it wasn’t until a gene central to the control of autophagy, Beclin 1, was identified as important in human cancer (44) that research in cardiology turned its attention toward understanding the impact of autophagy on the heart and cardiovascular system (38, 69, 74). In postmitotic cells, such as cardiomyocytes, autophagy is essential for the continual process of repairing, removing, and replacing damaged cellular components. Numerous animal studies using targeted disruption of autophagy in specific tissues have demonstrated the importance of autophagic activity in almost every tissue or cell type of the body (53). Here we will focus on autophagy specifically in cardiac myocytes, however, it is important to keep in mind that myocytes comprise only about 40% of the cells in the heart and that cardiovascular health depends on other organs in addition to the heart. Consequently, attempts to alter autophagic flux beneficially in one tissue or cell type may have detrimental results in another.
A recurring paradox in the study of autophagy is its dual nature. Whereas, autophagic activity can be protective and essential for myocyte survival, there are clear indications that under certain disease settings it may actually contribute to functional decline or cardiac failure. Whether autophagic activity is beneficial or pathological may ultimately be determined by the identity of the cellular components being degraded as well as the timing and magnitude of autophagic activity relative to other essential cellular processes. Circadian rhythms are self-sustaining, 24-hour cycles in molecular, biochemical, and behavioral parameters that help an organism prepare for anticipated changes in physiological demand. In humans the incidence of adverse cardiac events, such as myocardial infarction, ventricular tachycardia, and death from ischemic heart disease, vary according to the time of day (25, 46, 52, 82), and forced changes in circadian rhythm, such as shift work or sleep apnea, are associated with increased risk for heart failure. Many tissues, including heart, display a circadian rhythm in autophagic activity (47, 63).

This review will outline the basic molecular mechanism of autophagy and highlight key regulatory steps at which autophagic flux can be controlled. Autophagic trafficking of specific cardiomyocyte structures will be addressed in the context of its contribution to cardiac remodeling. The central regulatory role of the kinase mammalian target of rapamycin (mTOR) and the fundamental paradox of apparent simultaneous activation of both growth and atrophy in hypertrophic heart failure will be discussed. Finally, we will address the importance of innate circadian control of autophagy and how it might act to provide temporal separation of apparently conflicting processes.

We will highlight fundamental challenges and opportunities to developing autophagy as a therapeutic target in heart disease. A comprehensive review of specific drugs and their modes of action is beyond the scope of this article, however, a number of recent reviews that catalogue the diversity of potential pharmacological interventions are available (32, 43, 59). Importantly, many drugs that are potent regulators of autophagy, such as rapamycin, are already in clinical use and therefore might be readily adapted for use in the setting of cardiovascular disease. A better understanding of the targets, trafficking, and timing of cardiac autophagy will help optimize therapeutic intervention.

2. Mechanisms of autophagy and its regulation

2.1. There are three basic types of autophagy

Autophagy refers to the processes through which intracellular components or invading pathogens are identified and delivered to the lysosome for degradation by acid hydrolases capable of degrading proteins, nucleic acids, lipids and carbohydrates. There are three basic types of autophagy. In chaperone-mediated autophagy, proteins carrying a KFERQ-motif are recognized by a complex of chaperone proteins and fed directly into lysosomes with the assistance of the receptor lysosomal-associated membrane protein 2A (LAMP-2A). In microautophagy, substrates are engulfed directly by lysosomal membranes. In Macroautophagy, cytoplasmic components are engulfed in an autophagosome, which then fuses with a lysosome to deposit its contents for degradation in the mature autolysosome (Figure 1). Ultrastructurally autophagosomes are distinctive because they are composed of a double membrane, a consequence of the way in which they are formed. This feature is a hallmark of macroautophagy and aids in distinguishing autophagosomes from other vesicular structures in a cell. For the remainder of this review “macroautophagy” will be referred to simply as “autophagy” because this pathway is the focus of the vast majority of current studies, however, it is important to keep in mind that chaperone-mediated autophagy and microautophagy also carry out significant functions. For instance, it is estimated that as many as 30% of cytoplasmic proteins contain a putative KFERQ motif (81) and LAMP-2 deficient mice develop cardiomyopathy (79).
2.2. Macroautophagy has diverse functions

Autophagy carries out a variety of functions. (A) It is induced in response to starvation and promotes survival by allowing a cell to break down non-essential or excess cellular components, thus releasing high-energy substrates for the generation of ATP and recycling molecules that the cell itself can’t manufacture de novo such as essential amino acids. (B) Autophagy is the primary route for the removal of damaged or excess organelles. It is also capable of engulfing and degrading complex aggregates of misfolded or damaged proteins that are too large to be handled by the proteasome (41). (C) Autophagy is involved in both innate and adaptive immunity by degrading invading pathogens (xenophagy) and delivering self-antigens for MHC-class-II loading (39). (D) Autophagy participates in a number of important developmental steps such as the elimination of mitochondria from red blood cells (70). (E) Autophagy may also contribute to cell death. This will be discussed further in section 4.

2.3. Numerous points of control regulate autophagic flux

From yeast to mammals there is remarkable conservation of the proteins and pathways that carry out and regulate autophagy. For the sake of identifying pivotal control points it is useful to divide autophagosome processing into three discrete steps, each of which requires a specific set of proteins. These steps are nucleation, elongation and fusion (Figure 1). In addition, three critical control points to be considered are induction, substrate targeting and degradation. We will discuss each of these steps in broad conceptual terms, as opportunities for controlling flux through the system. Several excellent recent reviews can be consulted for additional details of the molecular mechanisms involved at each step (8, 86, 87).

2.3.1. Nucleation—The initial nucleation of an autophagic isolation membrane requires activation of a class III phosphoinositide 3-kinase (PI3K), called Vps34, by its binding partner Beclin-1. Class III PI3 Kinases phosphorylate the three position hydroxyl group of the inositol ring of Phosphatidylinositol (PI), a negatively charged membrane phospholipid, to produce PI(3)P. PI(3)P then recruits additional proteins necessary for isolation membrane formation (7). There are two well characterized PI(3)P binding domains, the cysteine-rich Zn\(^{2+}\) FYVE binding domain and the Phox homology (Px) domain. Numerous FYVE and Px domain proteins are involved in vesicular trafficking throughout the cell and PI(3)P provides a recruitment platform for these trafficking events. The role of Vps34 is not limited to autophagy as it is thought to be the primary source of PI(3)P in the cell. Specificity for its regulatory role in autophagy is mediated through its participation in the nucleation complex and interaction with Beclin-1 (18). Nucleation is suppressed by competition for Beclin-1 by an endoplasmic reticulum (ER) -localized pool of the anti-apoptotic protein Bcl2 (B-cell lymphoma 2). Phosphorylation of Bcl2 and Beclin-1 by the MAP Kinase JNK and the death-associated protein kinase respectively weakens their interaction and releases Beclin-1 from the ER to participate in the nucleation complex activating Vps34 (83, 91). Beclin-1 protein abundance is rate limiting for initiation as Beclin-1\(^{+/−}\) mice, haploinsufficient for Beclin-1, display reduced rates of starvation-induced cardiac autophagy (95). The availability of Beclin-1 therefore provides a titratable control point for manipulating autophagic activity.

2.3.2. Elongation—Growth of autophagic membranes requires two cascades of “ubiquitin-like” conjugation events in which the ultimate product is covalent linkage of a phospholipid phosphatidylethanolamine to the microtubule-associated protein light chain 3 (LC3). Lipidation of LC3 converts it from the LC3-I form to the LC3-II form and allows insertion of the protein into the growing autophagic membrane. The free cytoplasmic protein tail of LC3-II identifies the membrane as an autophagosome and often aids in the targeting of selective substrates. LC3-II therefore presents a potential avenue for targeting active autophagosomes prior to their fusion with lysosomes. The most common methods for
monitoring changes in autophagic activity are monitoring conversion of LC3-I to LC3-II by western blot and the use of a green fluorescent protein fused to LC3 (GFP-LC3) to visualize vesicle formation.

2.3.3. Fusion—A mature autophagosome can fuse directly to a lysosome or first fuse with an endosome prior to trafficking to the lysosome. Many of the components required for this step are involved in vesicular trafficking in general and are not specific for autophagy. This is probably why genetic screens in yeast have not identified mutant alleles involved in this process. It also suggests strongly that pharmacological compounds aimed at this step would not be specific for autophagy and are therefore likely to demonstrate broad range toxicity.

2.3.4. Induction—A central control point for the induction of autophagy is the kinase mammalian target of rapamycin (mTOR). mTOR lies downstream of insulin signaling and class I PI3K. It phosphorylates inhibitory sites on the unc-51-like kinase 1 (ULK1 or ULK2) in the induction complex (34). Indeed the most common pharmacological inducer of autophagy is the mTOR inhibitor rapamycin. Many of the mechanisms that promote or inhibit autophagy converge in some way on mTOR and the induction complex. Mechanisms that detect low energy stores, such as AMP-activated protein kinase (AMPK) (2), or amino acid deficiency, such as leucyl-tRNA synthetase (23), use mTOR and the ULK1 complex as a key regulatory switch.

In contrast to class III PI3K, class I PI3K generates PI(3,4)P₂ and PI(3,4,5)P₃ in addition to PI(3)P from PI. Binding of insulin or other growth factors to receptors in the plasma membrane activate Class I PI3K. PI(3,4)P₂ and PI(3,4,5)P₃, formed at the plasma membrane, then recruit phosphoinositide-dependent kinase-1 and protein kinase B (Akt) to the membrane via their pleckstrin homology domains resulting in sequential activation (17). The cascade of events activated by class I PI3K at the plasma membrane leads to activation of mTOR and inhibition of autophagy. Thus, class I and class III PI3 Kinases have opposing actions on autophagic flux. Drugs specific for class I PI3K could be used to over-ride insulin-dependent inhibition of autophagy. However, a broad spectrum PI3K inhibitor would also inhibit Vps34 in the nucleation complex and block flux at the level of initial phagophore formation.

There are two distinct mTOR complexes, which act upon different downstream targets (84). The mTORC1 complex contains the regulatory protein raptor and is sensitive to rapamycin. The mTORC2 complex contains the regulatory protein rictor. The mTORC1 complex promotes cell growth both by blocking autophagy through ULK1 phosphorylation and by increasing protein translation through phosphorylation of ribosomal protein S6 kinase, eukaryotic elongation factor 2 kinase and 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1). Thus, activation of mTOR promotes cellular catabolism, whereas, inhibition of mTOR promotes anabolism. This creates a paradox in hypertrophic heart failure where robust increases in both cardiomyocyte cell size and autophagic activity are observed. Is it possible for the two to occur simultaneously (58)? Section 5 of this review will discuss how circadian control of mTOR activity may provide temporal separation of opposing catabolic and anabolic processes, while allowing increased flux through both.

2.3.5. Substrate targeting—Starvation induced autophagy is relatively nonselective, carrying out the degradation of a diversity of cellular components at one time to preserve cellular homeostasis. However, autophagy can also act selectively to remove damaged or excess cellular components. Although a diversity of organelles and structures can be singled out for selective autophagic degradation, cells appear to use a recurring suite of tools and adaptors proteins to achieve this specificity. Selected substrates are often tagged for degradation by ubiquitination (33). For example, polyubiquitination of misfolded protein

*Pharmacol Res. Author manuscript; available in PMC 2013 December 01.*
aggregates or ubiquitination of a protein in the outer membrane of a damaged organelle provides essential targeting signals to attract adaptor proteins, such as p62, that contain both a ubiquitin binding domain and an LC3 interacting region (LIR). The adaptor proteins help recruit ubiquitinated targets to the nascent phagophore via binding of their LIR domain to the cytoplasmic tail of LC3-II in the membrane of the autophagosome. Variations on this theme are LIR-only proteins, such as NIX (BCL2/adenovirus E1B interacting protein 3-like), that interact directly with the target cargo without the need for tagging by ubiquitin. Selective specificity depends upon the transmission of an appropriate “damage signal” from the target cargo to recruit the appropriate ubiquitin ligase, or cell-type specific expression of cargo-specific adaptor proteins. These selective adaptors provide a potential target for the development of small molecules that can alter trafficking of a specific substrate while sparing desirable cellular components.

2.3.6. Degradation—The final step controlling autophagic flux is degradation of substrates in the autolysosome and subsequent release by permeases to the cytoplasm. The internal pH of a lysosome is around 4.5. A pH at which acid hydrolases are designed to work optimally. This helps protect the cell from self digestion because lytic enzymes that leak out of the lysosome would quickly be inactivated in the neutral environment of the cytoplasm. It also suggests that acidification of the cytoplasm, such as occurs during hypoxia, could render cytoplasmic constituents more vulnerable to lysosomal leakage. Drugs that interfere with proton pumps or chloride ion channels responsible for acidification of the lysosome will block all lysosomal-mediated turnover including those occurring via an autophagic pathway.

2.4. Measuring changes in autophagic activity can be challenging

For years the gold standard for evaluating autophagic activity was noting the presence of double membrane bound autophagosomes on an electron photomicrograph. The limitation of this approach is that it is a static snapshot in time and does not provide insight into the underlying cause of a change in the abundance of autophagosomes. An increase in autophagosomes could reflect an increase in autophagosome formation or a decrease in lysosomal processing of autophagosomes. In other words, ultrastructural changes resulting from an increase in autophagic activity can be indistinguishable from those caused by blocking autophagy downstream of autophagosome formation. An excellent illustration of this issue is the large increase in autophagic vesicles seen in histology samples from the brains patients with Alzheimers. Initially this was taken as an indication of a massive increase in autophagic activity, however, it is now clear that a primary component of the Alzheimer’s pathology is actually a defect in autophagy and protein degradation (9).

There are established techniques for assessing changes in autophagic flux in cultured cells that involve the addition of lysosomal inhibitors to the media allowing a build up of components fluxing through the system that can then be quantified. Unfortunately rigorous assessment of changes in autophagic flux in live animals or humans is not as straightforward (54). Changes in levels of adapter proteins such as p62 may be useful in assessing changes in autophagic flux in vivo: with inhibition of autophagy resulting an accumulation of p62 and increases in autophagic flux resulting in a decrease in p62 levels. However, there are many examples of investigators interpreting a parallel increase in LC3-II and p62 as an increase in autophagic activity. New techniques currently being developed for assessing autophagic flux in vivo will be important for verification or reinterpretation of studies that have addressed the role of autophagy in the heart and other organs.

Assessing autophagic activity in the heart also comes with some major challenges unique to striate muscle. The utility of GFP-LC3 fusions to monitor autophagosome formation in the
heart is limited by the high level of autofluorescence of cardiac tissue, due to its high oxidative capacity, in a spectral range directly overlapping the emission spectrum of GFP (31). Using a marker protein with a different emission wavelength, such as mCherry, can overcome this conflict, however, a majority of studies have relied upon GFP-fusions. Another challenge to molecular and biochemical analysis of protein extracts from the heart is the innate insolubility of the sarcomere under standard protein extraction conditions. In many studies autophagic components fractionating with the sarcomere have been thrown away, resulting in an incomplete picture of autophagic activity.

3. Autophagy in the heart

The structure, composition, and function of cardiac myocytes pose some interesting challenges to autophagic flux. Cardiac myocytes are extremely long-lived as their capacity to regenerate in an adult heart is limited. Therefore, there must be a continual process of cellular repair involving removal and replacement of damaged structures. The tightly packed structure of sarcomeres creates topological challenges for vesicle trafficking. Furthermore, autophagy needs to occur in the context of continuous cardiac contraction and large cytoplasmic calcium transients without compromising cardiac function. In this section we will discuss autophagic catabolism of specific cardiomyocyte structures.

3.1. Autophagic remodeling of the sarcomere

Until recently it was believed that turnover of the sarcomere in striated muscle was carried out primarily by cytoplasmic proteases and the proteasome. It is now clear that autophagy and the proteasome work in concert to repair and maintain striated muscle. Interestingly, there are indications that both these catabolic systems are activated not only during cardiac atrophy but also during cardiac growth (62, 85). In response to increased cardiac demand, individual myocytes increase in size, primarily through concentric hypertrophy where new sarcomeres are laid down in parallel with existing sarcomeres. This compensatory form of hypertrophy helps increase cardiac output. In contrast, under sustained pressure overload, such as might occur in a patient with uncontrolled hypertension, the heart undergoes decompensation where eccentric hypertrophic growth predominates and new sarcomeres are laid down in series with existing sarcomeres. This leads to elongation and thinning of individual myocytes and the ventricular wall, culminating in remodeling of the ventricular chamber to a dilated failure phenotype. It is easy to imagine that the tasks performed by autophagy might be different in concentric versus eccentric remodeling.

3.1.1. Sarcomeric remodeling in cardiac pressure overload—Transverse aortic constriction (TAC) is a commonly used surgical procedure to model cardiac pressure overload in rodents. In TAC the aorta is constricted to reduce flow, thus creating pressure on the left ventricle. The rate of progression through hypertrophy to decompensated failure can depend on the severity of constriction, the duration of constriction and the genetic background of the animal (67). Many groups have reported evidence of increased cardiac autophagy in response to TAC. Most studies suggest that autophagic activity is detrimental in this context. Mice with cardiomyocyte-specific expression of a Beclin-1 transgene (!MHC-Beclin1) have normal cardiac function but progress more rapidly to failure in response to TAC (95). Despite a greater increase in both stress and starvation-induced cardiac autophagy in !MHC-Beclin1 mice, their increase in heart weight body weight ratio (HW/BW) in response to TAC is also greater than that of a wild type mouse. This suggests that although autophagy is a catabolic process, increased autophagy somehow contributes to pathological hypertrophic growth. Exactly how autophagy promotes myocyte growth is not clear but the process may be analogous to remodeling a house, structures need to be torn down prior to adding new structures. Consistent with autophagy promoting pathological...
remodeling in pressure overload, Beclin-1+/− mice have reduced autophagic activity and improved cardiac function in response to severe TAC compared to wild type littermates (95). Likewise, mice with an increased capacity for inhibiting autophagy, due to cardiac-specific expression of an mTOR transgene, have reduced hypertrophy, fibrosis, and inflammation in response to TAC (76).

Supporting the hypothesis that unrestrained cardiac autophagy promotes pathological cardiac remodeling, cardiac-specific loss of mTORC function, either due to deletion of mTOR itself or the mTORC1 regulatory subunit Raptor, leads directly to dilated failure with increased autophagic activity and no adaptive hypertrophy (73, 93). In addition to lacking a primary break on autophagy, the cardiac-specific mTOR and Raptor deficient mice can’t activate mTOR-mediated growth signals. Interestingly, deletion of 4E-BP1, to directly restore downstream translational activity, improves cardiac function in the mice with a cardiac-specific disruption of mTOR, suggesting that pro-growth functions of mTOR provide an important counterbalance to autophagic remodeling, without which, compensatory concentric hypertrophy does not occur (93). Taken as a whole, the genetic models suggest that cardiac autophagy is maladaptive in the setting of pressure overload and approaches to blunt autophagy could be beneficial. It is important to note, however, that a complete loss of cardiac autophagy is clearly not beneficial as mice with a cardiac-specific disruption of Atg5 progress rapidly to failure in response to TAC (56).

3.2. Autophagic turnover of mitochondria

The heart has one of the highest rates of oxidative metabolism of any tissue and mitochondria are estimated to comprise up to 40% of an adult cardiomyocyte. Declines in metabolism and mitochondrial function are key features of cardiovascular disease (30). In striated muscle there are distinct pools of mitochondria that can respond differently to cardiac stress. The subsarcolemmal population is comparatively easy to isolate and study on a biochemical level, whereas intermyofibrillar mitochondria are arranged in rows between sarcomeric bundles, often span from Z-band to Z-band, and are extremely difficult to purify free of sarcomeric and nuclear contamination. During compensatory hypertrophy the abundance of mitochondria increases. During decompensated failure mitochondrial abundance and function decline.

3.2.1. Mechanisms of selective mitophagy—Autophagy is the primary avenue for disposing of mitochondria (42). They can be engulfed non-selectively during non-selective, starvation-induced autophagy or they can be selectively autophagized via a process termed mitophagy (36). The importance of mitophagy to human health was brought to the forefront over the past three years with the discovery that many of the human mutations causally linked to Parkinson disease are in genes encoding proteins involved in mitophagy (57). Briefly, loss of mitochondrial membrane potential (MMP) triggers fission of mitochondria and recruitment of Parkin, a ubiquitin ligase. Parkin ubiquitinates proteins in the outer mitochondrial membrane, tagging the effected mitochondrion for autophagic degradation (19). The heart has the highest levels of Parkin protein of any tissue in the body, suggesting that Parkin-mediated mitophagy could play a prominent role in cardiac physiology.

3.2.2. Reactive oxygen species as inducers of mitophagy—The heart generates high levels of mitochondrial reactive oxygen species (ROS), which promote loss of MMP through opening of the mitochondrial permeability transition pore (MPTP). MPTP opening is a fundamental intracellular trigger of apoptotic cell death, and indeed mitophagy can be triggered by many of the same cellular stresses that trigger apoptosis (36, 42). Whether a cardiomyocyte undergoes apoptosis or mitophagy in response to MPTP opening may depend upon the availability of the cellular components required to carry out the respective...
processes. For instance, adult cardiomyocytes have high levels of Parkin protein, yet are relatively deficient for Apaf-1 (37), a required component of the apoptosome. This may help cardiomyocytes evade apoptotic death, activating mitophagy instead to remove damaged mitochondria. If less efficient, damaged mitochondria are not removed they may continue to generate high levels of ROS that could cause further damage to cellular proteins, lipids and DNA.

3.2.3. BH3-only proteins that promote mitophagy—BNIP3 (BCL2/adenovirus E1B interacting protein 3) and NIX are two BH3-only pro-apoptotic proteins involved in promoting mitophagy. BNIP3 expression is induced by cardiac hypoxia but also requires posttranslational regulation to translocate to mitochondria and disrupt MMP, whereas, NIX can promote mitophagy when localized to either mitochondria or the endoplasmic reticulum (ER) (94). The contribution of these proteins to cardiac homeostasis is demonstrated by the observation that the hearts of mice with a double knockout of both proteins develop advanced dilated heart failure by 30 weeks of age (15). It is also important to note that hearts deficient for BNIP3 are resistant to damage from ischemia reperfusion. Thus, like many other proteins involved in autophagy BNIP3 activities can be both adaptive and maladaptive (14).

3.2.4. Calcium regulation of mitophagy—Calcium is an important regulator of both mitochondrial metabolism and turn over (45). Mitochondria are actively involved in the control of cellular Ca\(^{2+}\) levels, providing a buffer to protect against potentially lethal systolic cytoplasmic Ca\(^{2+}\) loads. Under normal working conditions, mitochondrial Ca\(^{2+}\) enhances mitochondrial metabolism by increasing substrate uptake, the activity of several TCA cycle enzymes, and ATP production. However, mitochondrial Ca\(^{2+}\) overload can induce MPTP opening, setting in motion autophagic, apoptotic or necrotic mechanisms. Cytoplasmic Ca\(^{2+}\) enters the mitochondrial matrix primarily via the Ca\(^{2+}\) uniporter, driven by the negative potential across the inner mitochondrial membrane. A major source for stored Ca\(^{2+}\) is the sarcoplasmic reticulum/endoplasmic reticulum (SR/ER). Close physical coupling between SR/ER and mitochondria allows rapid transfer of SR/ER Ca\(^{2+}\) stores to mitochondria, particularly in response to ischemia/reperfusion (I/R) (11). Mitofusin 2 (Mfn2), a protein involved in the dynamic cycle of mitochondrial fission and fusion, is also involved in tethering at mitochondrial SR/ER junctions. Mfn2-deficient myocytes are more resistant to oxidative stress and Ca\(^{2+}\)-mediated MPTP opening (61). Furthermore, mice with Mfn2-deficient hearts are resistant to I/R injury. A variety of mechanisms help protect against mitochondrial Ca\(^{2+}\) overload. For instance, transient opening of the MPTP, known as flickering, may provide an avenue for rapid Ca\(^{2+}\) release with out causing irreversible commitment to MPTP (65).

Ca\(^{2+}\) is also an important regulator of mitochondrial dynamics. Mitochondrial fission prior to mitophagy helps to generate a mitochondrion of the appropriate size for engulfment by the autophagosome. It also provides a mechanism through which damaged components of a larger mitochondrial network can be partitioned for degradation. During fission, the dynamin-related protein-1 (DRP1) is recruited from the cytoplasm to mitochondria. DRP1 forms a large homo-multimeric ring encircling the mitochondrion, which then constricts in an energy-dependent manner until fission occurs. Ca\(^{2+}\) controls DRP1 translocation via several mechanisms. Phosphorylation at Ser600 by calmodulin-dependent protein kinase 1 (CaMK1) promotes translocation to mitochondria as does dephosphorylation of DRP1 at Ser656 by calcineurin. Thus, two calcium-dependent signaling cascades prominently involved in pathological cardiac remodeling can promote mitochondrial fission, subsequent loss of MMP, and activation of selective autophagy (29).
3.2.5. Mitophagy in I/R—During cardiac I/R there are dramatic changes in the character of autophagic activity and the propensity of mitochondria for MPTP opening. During a myocardial infarction (MI), when blood flow to the myocardium is disrupted, the affected region of the heart switches to anaerobic glycolysis, producing lactate that acidifies the cytoplasm (88). Both cytoplasmic and mitochondrial Ca\(^{2+}\) levels increase (21), however, in this context, Ca\(^{2+}\) does not promote MPTP opening because the influx of hydrogen ions into mitochondria both dissipates inner membrane potential and acts to inhibit MPTP opening. Under hypoxia, starvation induced autophagy is likely protective, helping to maintain ATP levels and promote myocyte survival. If left ischemic, the region of the heart affected will eventually die so it is imperative to restore blood flow as quickly as possible. Paradoxically, reperfusion of the myocardium is even more damaging than hypoxia and increases the rate of cell death. Upon reperfusion several events happen that promote MPTP opening. The rapid release of Ca\(^{2+}\) from ER increases mitochondrial Ca\(^{2+}\) load dramatically, the switch back to oxidative phosphorylation increases mitochondrial pH, and there is a burst of ROS generation that exceeds the cell’s antioxidant capacity. The increase in autophagic activity seen in response to reperfusion is likely focused on selective removal of ROS damaged proteins and organelles.

There is widespread agreement that I/R increases cardiac autophagy, however, the available evidence as to whether autophagy is damaging or beneficial in the setting of I/R is conflicting. Studies manipulating autophagic flux in cultured myocytes suggest that autophagy is protective in I/R (22), however, the results in animal models are not as clear cut. Beclin-1\(^{+/−}\) mice or mice with an mTOR transgene have smaller infarcts after I/R (3, 51) suggesting that reducing autophagic activity in the setting of I/R may be beneficial. Conversely, activation of autophagy by rapamycin (35, 64) or prior starvation (78) decreases infarct size suggesting instead that increasing autophagy may be beneficial. The cardioprotective properties of chloramphenicol succinate and sulfaphenazole during I/R have been attributed to an increase in autophagy, as has the protection from I/R damage in mice with a deletion of the transcription factor STAT1. Experiments examining autophagic flux in vivo suggest that autophagosome clearance is impaired after long periods of ischemia prior to reperfusion (49). Further studies will be required to better define the role of autophagy and selective mitophagy in this setting.

The evidence regarding the role of autophagy during ischemic preconditioning of the heart is more compelling. Ischemic preconditioning occurs as a result of brief periods of cardiac ischemia that are not sufficient to damage the heart but confer protection against a subsequent larger ischemic insult. Many factors contribute to preconditioning including an increase in mitochondrial uncoupling. Autophagy is induced during ischemic preconditioning. Expression of a dominant negative ATG5 to block autophagy reduces preconditioning, leaving the heart susceptible to damage from I/R (28). During ischemic preconditioning Parkin has been shown to translocate to mitochondria indicating activation of selective mitophagy during preconditioning (27). Furthermore, the capacity for preconditioning is reduced in Parkin\(^{−/−}\) mice, suggesting that Parking-mediated mitophagy is protective. However, pharmacological inhibition of mitochondrial fission by mdivi, an inhibitor of Drp1, reduces ROS generation and cardiac damage from I/R (20). Thus, it may be that inhibition of mitophagy upstream of initiation events, prior to the release of ROS, can be protective, whereas inhibition later in the process increases damage, perhaps by allowing the persistence of damaged organelles that continue to generate damaging levels of ROS.
3.3. Autophagy of protein aggregates

Proteinopathies are diseases caused by accumulation of misfolded, mutant, or damaged proteins. Neurons and striated muscle are particularly susceptible to these disorders. Poly-ubiquitinated protein aggregates that are too large for removal by the proteasome can be degraded via autophagy. Inhibition of neuronal autophagy has been shown to increase the pathology of Huntington aggregates in mouse models, whereas treatment with rapamycin to increase autophagic activity can be protective (66). The desmin-related myopathies are caused by mutations in the intermediate filament protein desmin or associated proteins and lead to the accumulation of aberrant sarcoplasmic protein aggregates in the heart. As in the neurological disorders decreasing autophagy increases the rate of cardiac decline in mouse models of desmin-related myopathies suggesting that autophagy plays a critical role in the degradation of toxic protein aggregates in the heart (80).

3.4. Autophagy in remodeling of gap junctions

Coordinated depolarization of cardiac muscle requires maintenance of an ordered array of connexins at Gap junctions located at opposing ends of adult cardiomyocytes. Connexin 43 is the major ventricular cardiac connexin. In heart failure, connexin 43 levels are reduced and the protein is often found mislocalized to lateral regions of the cell. Remarkably, these complex integral membrane proteins have a very short half-life of only a few hours even in the adult heart (4). Recent studies have demonstrated that autophagy is involved in the internalization and rapid degradation of connexin 43 (1). Similar to other forms of targeted autophagy, connexin 43 is tagged by ubiquitination and associates with LC3 via the intermediary protein p62. In addition, endosomes carrying a variety of cell surface cargos often fuse with autophagosomes before trafficking to lysosomes. Therefore disruptions in normal autophagic flux may directly or indirectly alter the turnover of many critical receptors and channels at the plasma membrane.

3.5. Autophagic activity and inflammation

Autophagy is involved in diverse immune processes ranging from active degradation of invading pathogens to antigen presentation and self-tolerance. Chronic inflammation is an important underlying factor in cardiovascular disease. Elevated circulating levels of tumor necrosis factor alpha and monocyte chemotactic protein-1 are associated with chronic inflammation. Whereas, in cultured cardiomyocytes, treatment with either of these cytokines can induce autophagy and autophagic-dependent cell death (89, 90), the increase in autophagy in response to these cytokines in vivo may be cardio-protective via a preconditioning-like mechanism.

Recent findings from the Otsu lab have opened a remarkable new paradigm in our understanding of the role of autophagy in cardiac inflammation and failure (60). Although the vast majority of mitochondrial proteins are encoded by the nuclear genome, mitochondria contain their own DNA (mtDNA) that codes for a handful of proteins primarily involved in electron transport. Evolutionarily, mtDNA is derived from an ancient endosymbiont and has similarities to bacteria DNA. Like bacterial DNA, mtDNA contains unmethylated CpG motifs, which are inflammatogenic. Lysosomal deoxyribonuclease II (Dnase2a) degrades the genomes of invading bacteria and other pathogens. It is also responsible for the degradation of autophagosed mtDNA. Otsu and colleagues propose that mtDNA escaping from autophagy causes a Toll-like receptor inflammatory response that contributes to heart failure. Mice with a cardiac-specific deletion of Dnase2a subjected to TAC have increased mortality, severe myocarditis, and progress more rapidly to a dilated cardiomyopathy when compared to wild type controls subjected to TAC (60). It is interesting to speculate whether this mechanism could contribute to many chronic non-
infectious inflammatory diseases, particularly those associated with aging such as atherosclerosis and type II diabetes.

3.6. Lipophagy

The heart relies primarily on \( \beta \)-oxidation of long chain fatty acids to meet its demand for energy. Fatty acids are stored as triglyceride in lipid droplets that are actually membrane bound organelles distributed throughout the sarcoplasm. Despite the extremely high rate of lipolytic turnover in the heart, triglycerides reserves are low compared to other tissues. In fact there is a direct association between increased triglycerides content and decreased cardiac function in humans and rodents. The primary route for triglycerides catabolism is through adipose triglyceride lipase (ATGL) and mutations in ATGL lead to myocardial steatosis in humans (71). In addition to this traditional view of triglycerides turnover, recent studies demonstrate that triglycerides can also be mobilized from lipid droplets via a targeted autophagic mechanism called lipophagy (75). The extent to which lipophagy contributes to lipolysis in the heart is not yet known, but evidence suggests that it contributes at least a basal rate of turnover in most tissues. Increasing lipophagic flux may prove a viable approach to the treatment or prevention of cardiac steatosis.

4. Can autophagy kill?

The ability of autophagy to improve cell survival during nutrient and energy deprivation is undisputed, as is its involvement in cellular repair. The question of whether excessive autophagy can kill a cell is still debated in the field (72). Autophagy-mediated death has been termed “type II” programmed cell death (PCD) in contrast to “type I” apoptotic PCD. Both processes are ATP-dependent, but autophagic death is caspase-independent. Certainly in many disease contexts, particularly in neurodegenerative diseases, there is a clear correlation between cell death and the abundance of autophagic vesicles. However, cells may have died not as a consequence of autophagy but because the autophagic response was insufficient to preserve cell viability. Furthermore, as discussed earlier, vesicles may accumulate not because of an increase in autophagy, but because of a deficiency in the ability to process autophagic vesicles. In this case the cells may be dying because of an insufficiency in autophagy rather than a surplus.

The strongest evidence for autophagic cell death comes from studies of salivary gland removal and recycling during Drosophila development. Both autophagy and caspase activity increase during this process, but glands from mutant flies lacking caspsases still undergo PCD, whereas, flies with mutations in genes required for autophagy display incomplete degradation of salivary glands (5). Thus, the abundance of autophagic vesicles in this tissue does not simply reflect activation of a failed survival mechanism, but suggests that autophagy actively participates in complete loss of a cell.

Can autophagy carryout similar cell death functions in adult mammals? A scenario with similarities to that of the Drosophila salivary gland occurs during involution of the mammary gland in cows. After weaning there is an almost complete loss of alveoli and duct structures concurrent with a massive increase in caspase and autophagic activity that precedes PCD (92). However, no definitive studies have as yet shown that autophagic activity is required for the death of cells during mammary gland involution. Unlike the mammary gland, adult cardiomyocytes do not undergo cycles of proliferation and death and therefore long-term survival requires that the human heart be relatively unresponsive to signals that could trigger a cascade leading to a programmed cell death pathway of any kind, although, it is clear that under catastrophic injury cardiomyocytes will die via necrosis.
Under conditions of chronic stress, the appearance of fibrotic patches suggests that adult cardiac myocytes do die and can be replaced by fibroblasts, but this process has not yet been definitively attributed to a specific mode of death. In the proper context excess autophagy could contribute to cell death, perhaps by removing essential organelles or structures below a viable threshold, however, in vivo it is not trivial determining whether a cell has died because of autophagic excess or because of autophagic insufficiency. This will be an extremely important question to answer if autophagy is to become a viable therapeutic target in cardiovascular disease. The issue of whether cardiac autophagy is beneficial or damaging may depend entirely upon the extent of the initial insult, the duration of the autophagic response and the cargo being degraded. We would like to propose that the time of day a cardiac insult occurs is also a critical factor in the both the susceptibility of the heart to damage and the ability of the heart to mount a productive autophagic response.

5. Tapping into innate circadian control

Many components of the cardiovascular system show circadian rhythmicity, including metabolism, heart rate, blood pressure and hormone release. In humans, the risk for having an adverse cardiovascular event is highest in the early waking hours (55). Furthermore, studies in both humans and animal models indicate that susceptibility to damage from I/R is also greatest in this time period (16, 77). Cycling of some cardiovascular factors may be under the direct control of the transcriptional circadian clock mechanism, while others clearly respond to circadian changes in physical activity or feeding and are therefore only indirectly tied to a true free-running circadian cycle. Autophagic activity is circadian in many tissues, particularly liver and striated muscle where it increases during fasting to help maintain homeostatic blood glucose and amino acid levels. During waking hours, food consumption increases circulating insulin levels activating the insulin/Akt/mTOR-signaling pathway in target tissues including heart. This inherent daily rhythm in mTOR activity in the heart translates to an innate circadian rhythm of autophagy coinciding with the period of waking, the time of greatest cardiac demand. Ultrastructural studies of rat heart reveal a strong peak in the abundance of autophagic vesicles during the daytime hours of fasting (63). In addition to the circadian rhythm driven by a response to feeding, there is a feeding-independent circadian rhythm in autophagic activity in the liver driven by the transcription factor C/EBP! Knockdown of C/EBP! in the liver abolishes the diurnal autophagy rhythm (48). It is not known if C/EBP! plays a similar coordinating role in the heart.

In skeletal muscle, sarcomere composition and mitochondrial content remodel in response to exercise. In general, remodeling doesn't occur during muscle contraction but begins afterwards in appropriate response to the type of exercise. Unlike skeletal muscle, the heart does not have the luxury of inactivity and must continue to beat 24 hours a day. mTOR-mediated inhibition of autophagy during waking could help to focus autophagy-driven repair and remodeling during sleep when cardiac demand is lowest. This would provide temporal separation of processes that optimize cardiac output and those involved in repair and remodeling (Figure 2).

In addition to the feeding-driven rhythm in mTOR activity, adrenergic drive is also greatest during waking hours. This rhythm results in a circadian pattern of activation of cyclic AMP dependent kinase (PKA). PKA phosphorylates a number of target proteins that act in concert to increase cardiac contractility. There is a peak in the phosphorylation of phospholamban and inhibitor-1 in the mouse heart early in its waking hours consistent with circadian activation of PKA activity (68). PKA also phosphorylates the mitochondrial fission protein DRP1 at Ser656. Phosphorylation of this site inhibits translocation of DRP1 to mitochondria (10). Thus, daily increases in !-adrenergic activity may set the stage for a time of day-dependent inhibition of mitophagy, that is synchronous with a daily rhythm in
mTOR-mediated inhibition of non-selective autophagy, although this remains to be demonstrated experimentally.

Consistent with the concept that in a healthy heart there are innate circadian rhythms providing temporal separation of signaling pathways that increase cardiac output and those that facilitate cardiac remodeling and repair, the mouse heart displays a circadian rhythm in the activity of the protein phosphatase calcineurin (68). Despite the fact that activation of calcineurin has primarily been associated with pathological cardiac remodeling, there is over a ten-fold change in activity of calcineurin in the heart of a healthy mouse over the course of 24 hours without pathological consequences. Furthermore, mice with a cardiac-specific deletion of calcineurin develop a lethal cardiomyopathy indicating that, in addition to its role in pathological remodeling, calcineurin signaling carries out beneficial housekeeping functions (50). We’ve postulated that calcineurin controls essential functions at the time of day when its activity peaks in the heart that are incompatible with equally important processes that occur at the trough of calcineurin activity.

Could the need to preserve circadian control of autophagic activity explain why reduction of autophagic activity in a Beclin-1+/− mouse is protective in the setting of pressure overload (95), whereas, complete loss of autophagic activity in a cardiac-specific Atg5 KO is not (56)? In the heterozygous Beclin-1+/− mouse, autophagic activity would be reduced but remain under circadian control, thus, the important day-to-day housekeeping functions it carries would not be lost. Likewise, the chronically active Akt cardiac-specific transgene would override circadian rhythms in insulin signaling and inhibit autophagy 24 hours, leading to the observed decline in cardiac function (26), whereas, a cardiac-specific mTOR transgene that is still able to respond to circadian signals is protective in the setting of pressure overload (76). Given that the susceptibility of the heart to damage from I/R is time of day dependent, and the innate circadian nature of mTOR activity it is interesting to speculate whether some of the conflicting data regarding the beneficial or damaging nature of autophagic activity during I/R could stem from the interplay between these cycles and the differences in timing of experiments between labs.

Chronotherapy is the concept of administering medical treatment or drugs on a schedule that corresponds to a person’s daily biological clock, in order to maximize the health benefits and minimize adverse side effects. This approach has been effective in the control of hypertension where clinical studies demonstrate that night-time dosing of blood pressure-lowering medications verses conventional dosing at the time of waking achieves better blood pressure control and significantly reduces morbidity and mortality (24). Chronotherapy is also proving beneficial in cancer patients to maximize efficacy of a drug against malignant tumor cells and minimize damage to healthy cells (6). Likewise chronotherapeutical management of long-term low-dose glucocorticoid treatment in rheumatoid arthritis is effective in optimizing the risk-benefit ratio (12). We predict that the strong circadian control over cardiac autophagy and its central regulator mTOR will be both a powerful and essential tool for the development and implementation of therapeutics aimed at altering autophagy in the cardiovascular system.

6. Conclusion

Despite aggressive medical intervention, most patients with a diagnosis of heart failure continue a relentless decline. There is an urgent need to develop new approaches to prevention and management of this devastating disease. Autophagy as a therapeutic target holds both great promise and substantial challenges. Basel levels of autophagy are essential for cardiac maintenance and its role in trafficking mitochondria and sarcomere give it prominence in the repair and turnover of the majority of the content of a cardiac myocyte.
Therefore, any approach to either increase or decrease autophagic flux must do so in a way that preserves these vital functions. Experimental data suggest that the increase in cardiac autophagic activity seen across a wide range of cardiac pathologies may be beneficial in some contexts and harmful in others. The inherent circadian control of mTOR activity, a fundamental gatekeeper of autophagic activity, provides a potentially powerful tool for tuning up or down autophagy while still maintaining autophagy’s vital housekeeping functions. For instance, in a context where autophagy is maladaptive, such as pressure overload, one might take an approach that inhibits autophagy when the heart is under greatest demand, while releasing inhibition during sleep, allowing essential repair processes to continue. Conversely, in a disease context where autophagy is beneficial, such as the desmin-related myopathies, one might choose to stimulate autophagy primarily during rest to help preserve opposing, but essential, processes that predominate when the heart is most active. We are only just beginning to appreciate the complexity and importance of the processes carried out by autophagy in the heart. An integrated view of autophagic flux, the structures being processed, and the time of day at which it occurs relative to other cardiac functions will provide an important platform for capitalizing on its therapeutic potential.

Acknowledgments

This work was supported by the National Institutes of Health Heart, Lung and Blood Institute [Grants HL072016 and HL09776801 to BR], the American Heart Association [Grant 0655202Y to BR and 11POST7950051 to DR], and the American Heart Association Jon Holden DeHaan Cardiac Myogenesis Research Center.

Nonstandard abbreviations

<table>
<thead>
<tr>
<th>Akh</th>
<th>AMPK</th>
<th>ATGL</th>
<th>Bcl-2</th>
<th>BNIP3</th>
<th>Dnase2a</th>
<th>DRP1</th>
<th>4E-BP1</th>
<th>GFP</th>
<th>I/R</th>
<th>LAMP-2</th>
<th>LC3</th>
<th>LIR</th>
<th>Mfn2</th>
<th>MMP</th>
<th>MMP</th>
<th>MPTP</th>
<th>mtDNA</th>
<th>mTOR</th>
<th>NIX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
<td>ATGL</td>
<td>adipose triglyceride lipase</td>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
<td>BNIP3</td>
<td>BCL2/adenovirus E1B interacting protein 3</td>
<td>Dnase2a</td>
<td>lysosomal deoxyribonuclease II</td>
<td>DRP1</td>
<td>dynamin-related protein-1</td>
<td>4E-BP1</td>
<td>eukaryotic translation initiation factor 4E -binding protein 1</td>
<td>GFP</td>
<td>green fluorescent protein</td>
<td>I/R</td>
<td>ischemia/reperfusion</td>
</tr>
</tbody>
</table>
P62  adaptor protein
PCD programmed cell death
PI Phosphatidylinositol
PI3K phosphoinositide 3-kinase
PKA cyclic AMP dependent kinase
ROS reactive oxygen species
SR/ER sarcoplasmic reticulum/endoplasmic reticulum
TAC transverse aortic constriction
ULK1 unc-51-like kinase 1

References

11. Durgan DJ, Pulinilkunnil T, Villegas-Montoya C, Garvey ME, Frangogiannis NG, Michael LH, Chow CW, Dyck JR, Young ME. Short communication: ischemia/reperfusion tolerance is time-of-


Figure 1.
Schematic depicting autophagic flux from nucleation of a phagophore to a mature autolysosome. Multi protein complexes are involved at each step. Only proteins referred to directly in the text have been identified. Ub indicates ubiquitin. PE indicates the phospholipid phosphatidylethanolamine.
Figure 2.
Proposed model for the temporal separation of processes that optimize cardiac output and those involved in repair and remodeling. During waking hours mTOR suppresses autophagic activity. Likewise, increased adrenergic drive during waking provides additional inhibition of selective mitophagy via PKA activation. During sleep these two points of repression are reduced. The figure depicts day and night relative to a nocturnal rodent. In humans the circadian timing of these events would be reversed.