Review Series: The Role of Autophagy During Ischemia/Reperfusion

Eat your heart out

Role of autophagy in myocardial ischemia/reperfusion

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Autophagy is an important process in the heart which is responsible for the normal turnover of long-lived proteins and organelles. Inhibition of autophagy leads to the accumulation of protein aggregates and dysfunctional organelles which can cause cell death. Autophagy occurs at low basal levels under normal conditions in the heart, but is rapidly upregulated in response to stress such as nutrient deprivation, hypoxia and pressure overload. Autophagy is a prominent feature of myocardial ischemia and reperfusion. Although enhanced autophagy is often seen in dying cardiac myocytes, the functional significance of autophagy under these conditions is not clear. Upregulation of autophagy has been reported to protect cardiac cells against death as well as being the cause of it. Here, we review the evidence that autophagy can have both beneficial and detrimental roles in the myocardium, and discuss potential mechanisms by which autophagy provides protection in cells.

Introduction

Autophagy is an evolutionary conserved process involved in degradation of long-lived proteins and excess or dysfunctional organelles. In this process, the cytoplasmic contents in the cell are sequestered within double membrane vacuoles called autophagosomes and subsequently delivered to the lysosome for degradation. Autophagy occurs constitutively at low levels under normal conditions in most cells, including cardiac myocytes, and is an important housekeeping process in the cell. Defective autophagy results in the accumulation of protein aggregates and abnormal organelles which are toxic to the cell and lead to cell death. Autophagy is usually thought of as a survival mechanism that is rapidly activated during starvation when the cell needs to catabolize amino acids and fatty acids from proteins and lipids. Starvation has been shown to cause substantial upregulation of autophagy in mouse heart. However, increasing evidence suggests that autophagy is also upregulated in response to other stresses in the heart and is a prominent feature of cardiovascular diseases including cardiac hypertrophy and heart failure. Many studies have reported that autophagy is upregulated during myocardial ischemia/reperfusion (I/R). The functional contribution of autophagy to cell survival and death in the heart is currently a subject of controversy and it is unclear whether autophagy plays a beneficial or detrimental role. Recently, several recent loss-of-function studies of autophagy (Atg) genes have begun to address the roles of autophagy in the heart. In this review, we discuss the emerging evidence of the dual roles of autophagy in the heart, discuss the possible functions that autophagy might play in cells, and identify relevant issues for future investigation.

Overview of Autophagy

When autophagy is activated, a pre-autophagosomal double membrane structure surrounds a portion of cytoplasm and then closes to form the autophagosome. The outer membrane of the autophagosome then fuses with the lysosome to form a single membrane-surrounded vesicle called the autolysosome. The inner membrane of the autophagosome and its contents are subsequently degraded by lysosomal proteases. Beclin 1 (Atg6) and two evolutionarily conserved protein conjugation systems [Atg12-Atg5 and the microtubule associated protein light chain 3-phosphatidylethanolamine (LC3-PE)] are necessary for the formation of the autophagosome. Atg5 plays an essential role in the development of the autophagosomal membrane in mammalian cells and deletion of the Atg5 gene results in disruption of the autophagosome. Atg5 is conjugated to the ubiquitin-like molecule Atg12 by the E1-like protein Atg7 and the E2-like protein Atg5. This complex then localizes to the bowl-shaped autophagosome precursor membrane called the isolation membrane or phagophore which is essential for recruitment of LC3 and maturation of the membrane into an autophagosome. While the membrane elongates and matures into a cup-shaped isolation membrane, LC3 is proteolytically cleaved by the cysteine protease Atg4 which exposes a glycine residue at the C-terminus. LC3 is then activated by Atg7, the E1-like protein, and transferred to Atg3, an E2-like protein specific for LC3, which conjugates LC3 to PE. LC3-PE is subsequently recruited to the membrane in an Atg5-dependent manner. Beclin 1 is part of a class III phosphoinositide 3-kinase (PI3-K) complex which participates in the early stages of autophagosome formation. The complex plays a role in increasing the size of isolation membrane and its synthesis by recruiting proteins from the cytosol. The PI3-K complex does not influence the formation of Atg5-Atg12 and LC3-PE, but is needed for their proper localization in the isolation membranes.
Assessment of Autophagy

The early studies on autophagy in the heart were carried out using electron microscopy to assess whether autophagy was occurring in the heart. However, interpreting electron microscopy is subjective and it can be difficult to distinguish autophagosomes from lysosomes and endosomes. Another drawback of electron microscopy is the difficulty to accurately quantify autophagy in vivo since the area of the heart that is analyzed is very small. Monodansylcadaverine (MDC) has been widely used as a specific marker for autophagic vacuoles. However, it was recently demonstrated that late endosomes and lysosomes also stain positively with MDC and therefore studies of autophagy using MDC should be interpreted with care. The recent identification of the Atg genes in mammalian cells and the characterization of LC3 (Atg8 homolog) as a tool to study autophagy have resulted in a dramatic increase in autophagy research. Most studies today are using LC3 as a molecular marker for induction of autophagy. Upon induction of autophagy, cytosolic LC3-I gets cleaved as well as covalently conjugated to PE to form LC3-II which then translocates to the forming autophagosomal membrane. When LC3 is tagged with a fluorescent protein such as GFP, the formation of autophagosomes can be visualized by fluorescent microscopy, and modification of LC3-I to the more rapidly-migrating form of LC3-II can be detected on SDS-PAGE. The development of transgenic mice expressing GFP-LC3 systemically or specifically targeted to the heart has made it possible to study autophagy in vivo.

Most studies of autophagy are based on the observation of an increase in the number of autophagosomes using LC3 as a marker. However, it is important to note that using LC3 as a marker alone to study autophagic activity is not sufficient. Autophagy is a highly dynamic process and an increase in the number of autophagosomes at a fixed time point doesn’t necessarily correlate with increased autophagic activity in response to a particular stimulus. Rather it could indicate that the fusion process with lysosomes is impaired which would cause an accumulation of autophagic vesicles in the cytosol without an increase in autophagic activity. For instance, Danon’s cardiomyopathy is characterized by extensive accumulation of autophagic vacuoles in cardiac cells due to impaired fusion with lysosomes. Using an approach based on the inhibition of downstream lysosomal degradation of autophagic vesicles coupled with analysis of GFP-LC3-II accumulation in cells, our laboratory has been able to distinguish between an increase in autophagic activity or an impairment of autophagosome-lysosome fusion process. An increase in the number of autophagosomes in response to a stimulus with and without the presence of the inhibitors suggests an increase in autophagic activity (flux). In contrast, if there is an increase in autophagosomes without the inhibitors, but not in the presence of the inhibitors, it suggests that autophagosomes are accumulating due to impaired fusion and not an increase in flux. Another approach has been described by Bampton et al. who used RFP-LC3 to follow autophagy by fluorescence microscopy while labeling lysosomes with GFP-CD63. This approach allows the investigator to monitor induction of autophagy and autophagosome-lysosome fusion in real time. In addition, Kimura et al. noted that GFP was sensitive to the acidic environment in the autolysosome and that GFP-LC3 rapidly lost its fluorescence when the autophagosome became acidified upon fusion with the lysosome. In contrast, RFP was resistant to the acidic environment and mRFP-LC3 did not lose its fluorescence. Based on this observation, this group made an mRFP-GFP-LC3 reporter protein that was used to study the fusion between autophagosomes and lysosomes. A more recently identified marker of autophagy is p62/SQSTM1, which binds both ubiquitin and LC3. p62 was recently shown to regulate formation of protein aggregates in cells and knockout of the p62 gene correlated with reduced appearance of protein aggregates in mice during defective autophagy. For instance, inhibiting fusion between the autophagosome and lysosome with bafilomycin A1 resulted in accumulation of p62/SQSTM1 in cells. Thus, it is possible to use LC3 together with p62 to measure autophagic activity, where an increase in LC3-II correlates with a reduction in p62 levels.

Autophagy in Myocardial I/R

Autophagy has been shown to be upregulated in response to ischemia and reperfusion. In 1976, Sybers et al. first reported that autophagy was upregulated in response to glucose deprivation and hypoxia with subsequent reoxygenation plus glucose in fetal mouse hearts in organ cultures. A few years later, Decker and Wildenthal observed induction of autophagy in Langendorff perfused rabbit hearts subjected to ischemia and reperfusion. They found that 20 min of ischemia did not induce autophagy, but the number of autophagosomes increased when reperfusion was initiated. However, 40 min of ischemia alone caused an increase in autophagy which was further enhanced during reperfusion. When ischemia was extended to 60 min, the authors observed the presence of large and likely dysfunctional lysosomes during reperfusion, suggesting that the prolonged ischemia impaired the autophagic-lysosomal pathway. A swine model of stunning, in which hearts were subjected to six episodes of ischemia and reperfusion, resulted in upregulation of autophagy and increased lysosomal activity. Autophagy has also been studied in isolated cardiac myocytes using models of simulated I/R (sI/R). Myocardial ischemia is characterized by limited availability of glucose and a buildup of metabolic by-products such as lactic acid which leads to intracellular acidosis. To simulate ischemia, cultured cells are subjected to hypoxia in a low pH buffer (usually around pH 6.6) containing lactate and 2-deoxyglucose to inhibit glucose metabolism. Reperfusion is subsequently initiated by removing cells from the hypoxic chamber and changing to a pH 7.4 buffer containing glucose. It has been reported that exposure of isolated neonatal and adult cardiac myocytes to sI/R caused an increase in the number of autophagic vesicles. Our lab found that when HL-1 myocytes were subjected to sI/R, autophagic activity was inhibited during ischemia, but was re-activated during reperfusion. Although it is clear that autophagy is enhanced during I/R, the functional significance of autophagy in the heart remains controversial.

Protective Effects of Autophagy in the Heart

Autophagy is important for the normal turnover of cellular components including organelles. A deficiency in the autophagic process has been associated with a variety of cardiac pathologies including cardiac hypertrophy and cardiomyopathy. A study by Nakai and colleagues recently demonstrated that autophagy is essential for cellular housekeeping under normal conditions. They found that
Inhibition of autophagy is also associated with accumulation of oxidized proteins but it is unclear whether this is due to impaired redox systems or removal of oxidized proteins and organelles.

**Mitochondrial Autophagy**

It has been proposed that autophagy can provide protection by removing damaged and dysfunctional mitochondria. Many studies have observed mitochondria sequestered inside autophagosomes in the myocardium after stress such as I/R. For instance, when Sybers and colleges first reported upregulation of autophagy in fetal hearts in organ culture after hypoxia/reoxygenation, they noted that many cells contained autophagosomes with clearly visible mitochondria inside of them. The study by Decker and Wildenthal also noted that numerous autophagic vacuoles contained damaged mitochondria during reperfusion and suggested that upregulation of autophagy was a repair process responsible for removal of damaged, nonfunctional organelles. In addition, we reported that autophagy was present in the adult rat myocardium after I/R and that many of the autophagosomes contained mitochondria. Damaged mitochondria release proapoptotic factors such as cytochrome c which can activate the intrinsic apoptotic pathway. Thus, upregulation of autophagy in response to stress may serve as a protective response by removing harmful and leaky mitochondria, thus preventing activation of apoptosis.

Autophagy has been thought of as a non-selective process when sequestering materials for degradation, but there is evidence that a distinct mechanism exists that targets mitochondria for degradation by autophagy. It is not clear exactly how autophagy protects cells against I/R injury, and several potential mechanisms have been proposed. First, it is possible that autophagy promotes survival by maintaining energy homeostasis during ischemia. Under normal conditions, ATP is produced by mitochondria via oxidative phosphorylation in cardiac myocytes. However, during ischemia, oxidative phosphorylation ceases and there is a decrease in the levels of ATP. Autophagy has been reported to be induced in response to reduced levels of ATP. Since degradation of lipids and proteins by autophagy generates free fatty acids and amino acids, these can be reused to maintain mitochondrial ATP production and protein synthesis, and promote survival of cardiac cells. Consistent with this idea, glucose deprivation significantly reduced the cellular ATP content which correlated with upregulation of autophagy in cardiac myocytes. Moreover, they found that inhibiting autophagy with 3-MA caused a further reduction in the ATP levels and resulted in enhanced cell death in response to glucose deprivation, suggesting that autophagy may promote survival by maintaining ATP production during stress. Inhibition of autophagy is also associated with accumulation of oxidized proteins but it is unclear whether this is due to impaired redox systems or removal of oxidized proteins and organelles.

**Cross-talk between Autophagy and the Ubiquitin-Proteasome System (UPS)**

It is possible that the protective effects of autophagy may be at the level of protein clearance. Intracellular proteins can be degraded by either the ubiquitin-proteasome system or the autophagy-lysosome pathway. The UPS regulates levels of short-lived proteins which are targeted for degradation by linkage of ubiquitin molecules to lysine residues and subsequent delivery to the proteasome for degradation. Autophagy is responsible for the degradation of long-lived proteins and organelles. The UPS and autophagy have long been considered to be two distinct degradation systems with no cross-talk, but recent studies have challenged this belief. For instance, conditional deletion of autophagy in the mouse brain led to accumulation of polyubiquitinated proteins in neurons. In addition, several
studies have found that inhibition of the proteasome resulted in activation of autophagy, whereas suppression of autophagy promoted accumulation of polyubiquitinated protein aggregates, suggesting that the two are functionally coupled. Nakai et al. reported that polyubiquitinated protein levels and proteasome activity increased in heart with cardiac specific deletion of Atg5. Also, induction of autophagy with rapamycin increased clearance of aggregate-prone proteins and reduced the appearance of protein aggregates in vitro and in vivo. Inhibition of the proteasome is toxic to cells as it leads to the accumulation of many pro-apoptotic proteins as well as aggregates of misfolded proteins. Induction of autophagy has been reported to attenuate the toxicity induced by proteasome inhibition, suggesting that autophagy is protective by removing protein aggregates. It will be interesting to examine if there is cross-talk between the UPS and autophagy pathway in the heart and whether upregulation of autophagy in the heart protects against cell death mediated by a dysfunctional UPS by clearing proteins that would normally have been removed by the proteasome.

Autophagy and Cell Death

The functional contribution of autophagy to cell death has been a subject of great controversy. The question that always comes up is whether increased autophagy in dying cells is the cause of cell death or a failed attempt to prevent it. Extensive autophagy is often seen in dying cells and it is quite possible that constitutive and excessive autophagy could cause cell death. In most studies, reports of autophagic cell death have been based on the observation of increased number of autophagosomes in the cell, but have failed to show that upregulation of autophagy is the actual cause of cell death. However, recent studies involving manipulation of genes essential for autophagy have provided increased insight into the role of autophagy in cell death. For instance, downregulation of the autophagy genes, Atg7 or Beclin 1 using siRNA suppressed death in mouse L929 fibroblastic cells treated with the caspase inhibitor, zVAD-fmk. Bax/Bak-deficient mouse embryonic fibroblasts (MEFs) which are resistant to apoptosis mediated through the intrinsic pathway undergo autophagic cell death in response to etoposide or staurosporine where siRNA against Atg5 or Beclin 1 suppressed death. The same study also found that overexpression of anti-apoptotic Bcl-2 or Bcl-Xl in wild type MEFs blocked apoptosis but not autophagic cell death in response to etoposide treatment. Moreover, Pyo et al. reported that downregulation of Atg5 expression or ectopic expression of the dominant-negative Atg5 mutant ATG5K130R blocked IFNγ-induced death in HeLa cells.

Increased levels of autophagy have also been reported to contribute to cell death in the heart. Recently, Zhu et al. showed that pressure overload-induced heart failure correlated with increased levels of autophagy in the heart. The most convincing evidence that autophagy contributed to cell death was the fact that mice with heterozygous disruption of Beclin 1 (Beclin 1+/−), which exhibit reduced levels of autophagy, also had diminished cardiac remodeling induced by pressure overload. In contrast, transgenic mice overexpressing Beclin 1 in the heart had increased autophagy and enhanced pathological remodeling in response to stress compared to wild type mice. Autophagy has also been shown to contribute to cell death in I/R. For instance, Valentim et al. found that blocking autophagy with 3-MA or by downregulating Beclin 1 using siRNA in isolated cardiac myocytes correlated with reduced cell death in response to I/R.

Moreover, Matsui et al. found that autophagy was protective during ischemia, but that it switched to a detrimental role during reperfusion in the heart. They found that Beclin 1+/− mice had reduced induction of autophagy during reperfusion which correlated with decreased apoptosis and reduced infarct size. However, hidden within Beclin 1 is a pro-apoptotic BH3 domain that has been shown to bind and inhibit Bcl-Xl, suggesting that Beclin 1 may be able to directly activate apoptosis. It is possible that this domain may be exposed if Beclin 1 is proteolyzed by proteases such as calpain. Under conditions of calpain activation, Beclin 1 might be converted to a pro-apoptotic protein that will predominate over the protective effects of autophagy. In support of this, fewer TUNEL-positive cells are seen in hearts from Beclin 1+/− mice after I/R compared to wild type, although it has been shown that defects in autophagy results in accumulation of apoptotic bodies. Clearly, more work is needed to understand the significance of the findings in the Beclin 1+/− mice and the role of autophagy in the heart. These studies demonstrate that autophagy can have dual roles in the heart. Although it is not known what factor determines whether autophagy will be protective or detrimental to the cell, it is likely that the level and duration of autophagy plays a role. For instance, low levels of autophagy during ischemia and early reperfusion might protect against cell death by providing the cell with free fatty acids and amino acids and removing damaged organelles, whereas high levels or long-term upregulation of autophagy during reperfusion can trigger cell death by excess degradation of essential proteins and organelles. Clearly, the relationship between the survival and death functions of autophagy in the heart needs to be further elucidated.

Conclusion

Enhanced levels of autophagy in cardiac myocytes are observed in many cardiovascular diseases, but the functional role of autophagy in these settings is not clear. Increasing evidence from in vitro and in vivo studies using genetic and pharmacologic manipulation of autophagy suggest that autophagy can play a dual role in the heart. Basal levels are important for maintaining cellular homeostasis and for protecting cells against damaged or dysfunctional organelles. Enhancing autophagy under certain conditions can promote survival in response to stress, whereas excessive and long-term upregulation of autophagy may promote cell death. Therefore, modulation of the autophagic pathway may represent a potential future therapeutic target to treat or prevent a variety of cardiovascular diseases. However, many questions regarding its role in the heart remain unclear and need to be further investigated. The molecular basis and the regulation of autophagy need to be further clarified since it is possible that different pathways trigger death-associated and survival-associated autophagy. Thus, it is important to elucidate exactly how and under what conditions autophagy contributes to survival or cell death in the heart.

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References