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Autophagy as a regulator of cardiovascular redox homeostasis

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Abstract

Autophagy is a highly regulated process involving the removal of damaged proteins and organelles from cells and tissues through a lysosomal-mediated pathway. Accumulating evidence suggests that autophagy is necessary to maintain redox homeostasis. Here, we explore the connection between autophagy and reactive oxygen species (ROS). In particular, we discuss how oxidant-dependent signaling can modulate autophagic flux and how autophagy can, in turn, modulate ROS levels. Finally, we discuss how a decline or disruption of autophagy might contribute to redox-dependent cardiovascular pathology and help fuel the age-dependent decline in cardiovascular function.

Keywords

autophagy; ROS; cardiovascular disease; mitophagy

Introduction to Autophagy

Autophagy, literally “self-eating” is a bulk process by which intracellular components such as macromolecules (e.g. long lived proteins) and organelles (e.g. mitochondria) are targeted to the lysosome for degradation. Autophagy and the ubiquitin-proteasome system (UPS), the latter serving as the major pathway for degradation of specific short-lived proteins, are critical regulators of cellular homeostasis with links to cell survival, chronic diseases, as well as, normal physiological aging [1]. There are three classes of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. In microautophagy, the lysosome/late endosome engulf small volumes of cytosol by inward invagination of their membrane [2]. In chaperone-mediated autophagy, substrate proteins harboring a KFERQ-like pentapeptide motif are recognized by the chaperon protein HSP70 and other co-chaperones, and directly targeted to the lysosome by binding to the lysosome-associated membrane protein (LAMP)-2a receptor [3]. Macroautophagy (hereafter refer to as autophagy) is the major form of autophagy and has been the most extensively characterized. The process of autophagy can be divided into sequential steps. The first step is the initiation of a

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phagophore assembly site (PAS in yeast, omegasomes in mammals) and nucleation of isolated membranes to form a phagopore. The second step is the expansion of this membrane to sequester cytoplasmic components such as proteins, organelles, eventually forming a double membrane vesicle called the autophagosome. Finally, in the last step, the outer membrane of the autophagosome fuses with the vacuole (yeast)/lysosome (mammals) and the lysosomal machinery then degrades the materials that the autophagosome has delivered [4] (Figure 1).

The identification of autophagy related (*Atg*) genes (to date, more than 35 essential *Atg* genes have been identified in yeast) provided the basis for our understanding of the molecular mechanism underlying autophagy [5]. There are 19 core ATG proteins (highly conserved in eukaryotes, including mammals) that govern the different steps of autophagy [6]. Autophagy is often activated by nutrient deprivation with the mTOR kinase acting as a critical regulator. Both PI3K-I/Akt and MAPK/Erk1/2 signaling activate mTOR which then suppress autophagy, while AMPK and p53 signaling inhibit mTOR thereby promoting autophagy [7]. In general, when nutrients are abundant, mTOR is activated and autophagy is suppressed, while when nutrients are scarce, mTOR is inhibited and autophagy is stimulated. Similar to yeast ATG1, in mammals, UNC-51-like kinase (ULK1/ULK2) forms a complex with ATG13, and FIP200 (an ortholog of yeast ATG17) to function downstream of the mTOR complex and initiate phagophore assembly [8]. This regulation is achieved, at least in part, by a direct phosphorylation of ULK proteins by mTOR, leading to an inhibition of ULK activity [9]. Further nucleation involves the class III PI3K complex, which consists of vacuolar protein sorting 34 (VPS34) PI3K, ATG14L, p150 (VPS15 in yeast) and Beclin 1 (Atg6 in yeast) [10]. Next, phagophore membrane elongation and autophagosome closure requires two ubiquitin-like conjugation systems, the ATG12–ATG5–ATG16L1 complex and the LC3 (mammalian homolog of yeast Atg8)–phosphatidylethanolamine (PE) machinery. In the first system, ATG12 associates with the E1-like enzyme ATG7, is transferred to E2-like enzyme ATG10, and is finally conjugated to ATG5. The ATG12-ATG5 conjugate then interacts with ATG16L to form a large (~350kD) multimeric complex, which acts as structural support for membrane elongation [11]. In the second system, the LC3/ATG8 is cleaved by the ATG4 protease to generate the cytosolic LC3-I. LC3-I associates with E1-like ATG7, is then transferred to E2-like ATG3, and is subsequently conjugated to PE via the E3 like action of the ATG12-ATG5 complex [12]. The lipidated form of LC3 (LC3-II), is attached to the autophagosome membrane, which is required for the fusion of autophagosomes with lysosomes, ultimately leading to formation of the autolysosome (see Figure 1) [13].

ROS trigger autophagy

One of the first links between redox biology and autophagy came nearly a decade ago, when investigators began closely analyzing a classic inducer of autophagic flux, namely starvation. In this case, external nutrients are absent or extremely limited and the cell turns to autophagy as a means to supply biosynthetic intermediates. The overall cellular response to starvation is complex and involves the activation and suppression of various signaling pathways. One response, however, is a rise in ROS levels which appears to derive primarily from a mitochondrial source [14]. Moreover, the relevance of this ROS increase to autophagy was

demonstrated by the observation that addition of the antioxidant N-acetylcysteine was able to blunt the starvation-induced increase in autophagic flux [14]. The generation of ROS appears to be upstream of autophagy, as cell deficient in essential autophagy genes appear to have increased basal levels of ROS, as well as having increased ROS levels when starved [14, 15]. Further analysis revealed that one molecular target of starvation-induced ROS was the essential autophagy gene ATG4. This gene product has been previously demonstrated to function as a cysteine protease involved in the processing of ATG8 [16]. Starvation-induced ROS oxidize a critical cysteine residue on ATG4 near its catalytic site, inactivating the enzyme and thereby shifting ATG8 to a state that favors autophagosome formation (Figure 2). Thus, in this experimental paradigm, ROS appear to act as signaling molecules to regulate autophagy. While there is general agreement that in most cases, the mitochondria are the primary source of starvation-induced ROS, there is some disagreement as to whether the critical molecular species is hydrogen peroxide [14] or superoxide anion [17]. Finally, while the initial trigger of ROS production concentrated on oxidants generated during starvation, this phenomenon appears to be more general. For instance, both hypoxia and exercise can also induce autophagy through a similar redox-dependent pathway involving the regulation of ATG4 activity. Evidence suggests that with these stimuli, a protein complex involving the mTORC1 inhibitor REDD1 and the thioredoxin inhibitor TXNIP regulates ROS levels [18]. This suggests that for a wide variety of stresses that induce autophagy, ROS may serve as an intracellular messenger to regulate autophagosome formation.

While the previous discussion centered on the redox regulation of ATG4, this cysteine protease is not the sole target of intracellular ROS. Several recent examples suggest that in fact induction of autophagic flux by ROS is likely to be quite complex and multifaceted. For instance, a recent study identified the lysosomal calcium channel TRPML1 as a target of mitochondrial ROS [19]. In this study, pharmacological uncoupling of the mitochondria with agents such as carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; often used as a stimulus for mitophagy) resulted in increased autophagy, as well as increased ROS levels. These mitochondrial ROS appeared to activate TRPML1, a lysosomal calcium channel, leading to release of lysosomal calcium stores (Figure 3). This calcium release triggered raised cytosolic calcium levels and stimulated the nuclear localization of the transcription factor TFEB, known to regulate a host of gene products involved in autophagosome and lysosomal biogenesis. This transcriptional network thereby increased autophagy, removed damaged ROS-producing mitochondria and thereby acted as feedback to restore redox homeostasis. It remains unclear, however, whether TRPML1 is a direct or indirect target of ROS in this system. Adding to the complexity, it appears as if this CCCP-triggered pathway leading to TFEB nuclear accumulation that involves lysosomes and mitochondrial ROS appears to differ from the TFEB nuclear localization that occurs when the stimulus is starvation [19].

Another organelle that appears to be involved in ROS-induced autophagy is the peroxisome, the site of β -oxidation of very long chain fatty acids. The process of β -oxidation is known to produce intracellular ROS [20]. The ataxia-telangiectasia mutated (ATM) kinase, linked to nuclear DNA damage signaling, has previously been shown to localize to peroxisomes [21]. It is also known that ATM can be activated by ROS resulting in a disulfide-crosslinked dimer [22]. Interestingly, a recent study suggested that in the peroxisome, ATM could be activated by ROS leading to the phosphorylation of the peroxisome import receptor PEX5. This ATM-

dependent phosphorylation leads to subsequent PEX5 ubiquitylation and recognition by the autophagy adaptor p62. Thus, a presumably homeostatic loop exists whereby a rise in peroxisome-induced ROS triggers ATM activation, PEX5 phosphorylation and ubiquitination and subsequent pexophagy (Figure 3). The removal of these oxidatively-stressed peroxisomes would presumably help restore redox balance and serves as another example of how organelles signal distress through pathways that involve ROS-dependent autophagic stimulation. A similar pathway involving mitochondrial ROS and Parkin-dependent ubiquitination appears to exist for mitochondria [23].

Autophagy regulates redox homeostasis

The previous examples involved cases in which a rise in ROS resulted in an augmentation in autophagy implying that intracellular oxidants regulate autophagic flux. While these examples suggest that autophagy is downstream of ROS, there are many other examples placing autophagy upstream of oxidants. One early example involved an analysis of cell death triggered paradoxically by caspase inhibition [24]. In this analysis, cell death involved an increase in ROS levels triggered by the autophagic clearance of the antioxidant protein catalase. A more recent example involved analysis of neural stem cells. Previous results have demonstrated that deletion of FIP200, a ULK-interacting protein required for autophagosome induction, resulted in a rise in ROS and a defect in neural stem cell (NSC) function [25]. Interestingly, this defect in

NSC maintenance and differentiation was not seen when other essential autophagy genes were deleted such as *Atg5* or *Atg7* [26]. Further analysis revealed that only Fip200 deletion resulted in cytoplasmic aggregates of p62. These aggregates were subsequently shown to bind and inactivate the antioxidant protein superoxide dismutase (SOD1). Interestingly, the defects in FIP200^{-/-} NSCs could be rescued genetically by p62 deletion or pharmacologically by treating with SOD mimetics [26]. The notion that p62 aggregates could inactivate SOD1 and hence alter the redox balance is reminiscent of another set of observations involving the Nrf2 transcription factor. This factor induces expression of a host of cytoprotective enzymes that modulate the levels of ROS. In general, Nrf2 regulation is determined by protein stability and subcellular localization. Under resting conditions, Nrf2 binds to the Kelch-like ECH-associated protein 1 (Keap1). This interaction facilitates the ubiquitination and ultimate degradation of Nrf2. Under oxidative stress, critical cysteine residues on Keap1 are oxidized, disabling its ability to stimulate ubiquitination and resulting in stabilization and subsequent nuclear accumulation of Nrf2. Interestingly, while Nrf2 levels are regulated by ubiquitination and the proteasomal pathway, levels of Keap1 are regulated by autophagy [27]. This is due to a specific interaction between phosphorylated p62 and Keap1. In the setting of a defect in autophagic flux, this p62/Keap1 interaction ultimately leads to augmented Nrf2 activity [28–30]. For instance, in genetic models that results in the disruption of hepatic autophagy, much of the subsequent pathology derives from the inappropriate and persistent activation of Nrf2 targets. This continuous activation appears to result in significant Nrf2-dependent liver injury and helps to promote hepatic tumor growth [28, 30].

In addition to directly regulating antioxidant pathways such as SOD1 or Nrf2, autophagy can regulate key metabolic pathways that have important consequences for redox homeostasis. One recent example involves the ULK1 and ULK2 (ULK1/2) kinases. These kinases are activated in response to stresses such as starvation and are critical for the subsequent induction of autophagy (see Figure 1). Recently, the substrates of ULK1/2 have expanded and appear to include a host of cytoplasmic glycolytic enzymes [31]. One effect of ULK1/2 activation is to shift more carbon flux down the pentose phosphate pathway (PPP), resulting in increased NADPH levels and hence decreased oxidative stress. Thus, under starved conditions ULK1/2 is responsible for simultaneously activating autophagy and also re-directing the flow of glucose, thereby helping to maintain redox homeostasis in two distinct ways. A related effect has been seen through analysis of the p53-inducible gene TIGAR. Enzymatically, TIGAR resembles the glycolytic enzyme 6-phosphofructose-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2). Indeed, expression of TIGAR results in decreased levels of Fru-2,6-P₂, with decreased glycolytic flux and increased diversion of carbon through the PPP. In the absence of TIGAR, levels of both ROS and autophagy rise dramatically [32]. Thus, both ULK1/2 and TIGAR provide tantalizing links between glucose metabolism, ROS levels and autophagic flux.

Autophagy, redox and cardiovascular disease

Given the role of autophagy in redox homeostasis, it seems reasonable to assume that disruption in autophagic flux could lead to altered ROS levels in tissue and organ and thereby contribute to various pathological conditions. There is certainly some evidence to support this. For instance, deletion of the essential autophagy gene *Atg7* in the pancreatic β -cell leads to oxidative stress within this cell type and subsequent impairment in insulin secretion. Treatment of these animals with the antioxidant N-acetylcysteine can markedly attenuate the diabetic phenotype [33]. Growing evidence suggests that similar examples are found within the context of the cardiovascular system. Perhaps the strongest case can be made for the specialized form of autophagy involving mitochondria, termed mitophagy. Mitochondria are thought to be the source of >90% of intracellular ROS [34] and so it would seem logical that impaired removal of damaged or dysfunctional mitochondria could lead to a tonic increase in oxidative stress. In this regard, the recent demonstration that mitophagy declines as animals age represents a potential link between normal aging, ROS levels and various disease states characterized by oxidative stress [35]. The removal of mitochondria is largely achieved through the macroautophagy machinery, although it is increasingly clear that fragments of mitochondria, termed mitochondrial-derived vesicles, can be directed to the lysosome by methods that are independent of classical autophagy pathways [36, 37]. Interestingly, the formation of mitochondrial-derived vesicles appears to be triggered by a rise of ROS within the mitochondria [37]. The best studied system to trigger mitophagic removal in cell culture is the addition of mitochondrial membrane depolarizing agents such as CCCP. Such interventions act to stabilize PINK1 on the outer mitochondrial membrane surface [38]. This stabilization of PINK1 allows for the mitochondrial recruitment and activation of the normally cytosolic E3 ubiquitin ligase Parkin [39–41]. Both PINK1 and Parkin are known susceptibility genes for early-onset Parkinson's disease, and as such, the biochemical interaction between these two proteins supports the well-documented genetic

interaction [42, 43]. Once Parkin is recruited to the outer mitochondrial membrane it appears to catalyze the ubiquitination of a wide range of mitochondrial proteins [44], thereby tagging this now heavily ubiquitinated mitochondria for subsequent mitophagic removal.

Based on this analysis, it would appear that Parkin-dependent or independent mitophagy might be critical for maintaining cardiovascular homeostasis. Consistent with such notion, Parkin-deficient flies develop dilated hearts that are filled with dysmorphic and depolarized mitochondria that generate high levels of ROS [45]. In contrast, mice with a germline-deletion of Parkin have relatively subtle mitochondrial alterations and no defect in basal cardiac function, at least when assessed up until one year of age [46]. The differences between flies and mice may be due to existence of multiple Parkin-independent mitophagy pathways in mammals. Analysis of cardiac-specific PINK1 deletion in mice revealed that these animals developed an age-dependent decline in cardiac function that was accompanied by increased oxidative stress and impaired mitochondrial function [47]. Interestingly, analysis of human heart failure samples revealed that PINK1 was dramatically reduced in expression in end-stage heart failure patients [47]. There is also an important relationship between mitochondrial dynamics, i.e. fusion and fission, and mitophagy [48]. In that context, it is of interest to note that cardiac-specific deletion of mouse mitofusin 2 (Mfn2) leads to a dilated cardiomyopathy due to a defect in mitophagy [49]. The link between mitochondrial fusion and mitophagy appears to be in part due to the observation that Mfn2 is a target of PINK1-dependent phosphorylation which can then serve as a docking site for Parkin recruitment to the mitochondria [49]. Interestingly, expression of the hydrogen peroxide scavenging protein catalase improved mitochondrial morphology and rescued structural defects in the Mfn2-deficient mice [50]. Again, these observations strengthen the mechanistic link between a disruption in mitophagy, a corresponding alteration in redox homeostasis and the subsequent development of cardiovascular pathology.

While the above discussion has centered primarily on the mitochondria, it is important to realize that other intracellular ROS generating systems also connect with the autophagy machinery. The family of NADPH oxidases known as NOX enzymes are critically important for multiple signaling pathways implicated in cardiovascular disease [51]. Connections between NOX enzymes and autophagy have been made in several notable studies. For instance, in cultured rat cardiomyocytes, removing extracellular glucose triggers an increase in ROS that was dependent on NOX4 activity within the endoplasmic reticulum [52]. This NOX4-dependent increase in ROS induced a corresponding increase in autophagic flux. Interestingly, rather than proceeding through the redox-dependent modification of ATG4 that was discussed previously, in this context, NOX4-derived ROS appeared to trigger a transcriptional program mediated through a pathway involving protein kinase RNA-activated-like ER kinase (PERK) [53]. A similar relationship between NOX activity and autophagy appears to occur in endothelial cells. For instance, pulmonary artery endothelial cells from lambs with persistent pulmonary hypertension (PPH) exhibit increased NOX activity, as well as increased autophagy [54]. In this context, antioxidants, or inhibition of NOX activity results in a reduction of autophagy and improvement in the angiogenesis potential of these PPH-derived endothelial cells [54]. In contrast to these observations where NOX activity promoted autophagy, there are also examples where the opposite occurs. To date, these appear to involve other NOX isoforms. In high fat-fed mice, increased NOX2

activity appears to result in impaired lysosomal acidification and reduced autophagic flux [55]. In this experimental paradigm, inhibition of NOX2 restored autophagy. A similar relationship between activated NOX2 and impaired autophagy was also observed in mouse models of Duchenne's muscular dystrophy [56].

Finally, another important link between autophagy, ROS and cardiovascular disease might revolve around normal aging. It is clear that aging represent the most potent risk factor for most diseases including atherosclerosis and heart failure. Indeed, over 80% of those individuals who die from cardiovascular disease are over 65 years of age [57]. Evidence suggest that levels of ROS increase as we age and this might contribute to age-related disease [58]. Similarly, as mentioned, rates of autophagy and mitophagy decline as a function of age [35]. Furthermore, this decline appears to be important in driving a senescent phenotype in the stem and progenitor pool in a redox-dependent fashion [59]. As such, it is tempting to speculate that the age-dependent decline in autophagy (currently incompletely understood) leads to lack of mitophagy and the accumulation of damaged and ROS-producing mitochondria (Figure 4). This bioenergetics and redox insult, in turn, predisposes to age-related pathologies in the cardiovascular system. In that sense, it is of interest that cardiac-specific deletion of *Atg5* has been shown to lead to an age-dependent cardiomyopathy [60]. Similarly, there is evidence that diminished autophagy can contribute to arterial aging and accelerate atherogenesis [61, 62]. All of this would suggest that strategies that augment autophagy would provide benefit by reducing oxidative stress and inhibiting disease progression. There is some evidence that this is the case. For instance, the natural agent spermidine, known to increase autophagy, was associated with improved vascular redox parameters and a reversal of age-related stiffness [63]. More recently, spermidine was noted to extend the overall lifespan of mice, at least in part, by preventing the age-dependent decline in cardiac function [64]. Similarly, the mTOR inhibitor rapamycin appears to reverse aspects of cardiac aging [65].

Conclusions

In summary, there are multiple links between the cellular redox state and autophagy. In many cases, evidence suggests that stresses, such as starvation, stimulate an increase in mitochondrial ROS or ROS generated through NOX enzymes that signal to increase autophagic flux. Similarly, the induction of autophagy appears to result in a diminishment of oxidative stress, either through a change in metabolism or through the removal of ROS-producing organelles (e.g. mitochondria or peroxisomes). This suggests that there is an important homeostatic link between oxidative stress and autophagy, whereby a rise in ROS triggers and increase in autophagy, which in turn, functions to lower oxidant levels. In disease states, or with normal aging, this homeostatic loop appears to be impaired and levels of oxidants rise as autophagic flux inappropriately declines. It is tempting to speculate that restoration of autophagic flux through pharmacological approaches might therefore be of benefit in a wide range of cardiovascular condition previously characterized to have derangements in redox homeostasis [66]. Such strategies might prove more beneficial than direct scavenging of ROS, which while capable of inhibiting damage, might also diminish the normal signals that act to augment autophagy. In this sense, autophagy or mitophagy

activators might prove significantly more efficacious than antioxidant therapies. It is likely that the next decade will test this hypothesis.

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Highlights

- ROS acting as signaling molecules can regulate autophagy
- Autophagic flux helps establish redox homeostasis
- A decline in autophagy may underlie redox-fueled, age-dependent diseases

Figure 1. Molecular regulation of autophagy. The initiation of autophagy is modulated by positive (e.g. AMPK) and negative (mTOR) regulators. Essential autophagy genes (Atg1, Atg2, etc.) participate in multiple steps including the formation of the pre-initiation complex and processing of LC3 to its final proteolytically cleaved and lipidated (phosphatidylethanolamine, PE) form (shown as pink ball). Some of the autophagy gene products resemble ubiquitin-conjugating enzymes (e.g. E1, E2, etc.).

Figure 2.

Regulation of autophagy through mitochondrial ROS. Various stimuli (e.g. starvation, hypoxia, exercise) can trigger an increase in mitochondrial ROS. This rise in oxidants appears to modify a reactive cysteine(s) in ATG4, inactivating its enzymatic activity. This leads to augmented ATG8 activity and increased autophagosome formation. See text for details. REDD1- regulated in development and DNA damage responses 1, TXNIP- Thioredoxin Interacting Protein.

Figure 3. Organelle stress, ROS and autophagy. Two different examples involving subcellular organelle stress (e.g. lysosomes and peroxisomes). In each case, a rise in ROS triggers a molecular event, be it lysosomal calcium release or ATM-dependent phosphorylation that ultimately results in increased autophagy. TRPML1- Transient Receptor Potential Channel Mucolipin-1, TFEB- Transcription Factor EB, ATM- ataxia-telangiectasia mutated kinase, PEX5-Peroxisomal Biogenesis Factor 5.

Figure 4. Interaction between aging, autophagy and redox homeostasis. A theoretical model whereby an age-dependent decline in autophagy results in a rise in ROS that helps increase cellular/organ damage and ultimately helps fuel disease.