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Autophagy During Cardiac Stress: Joys and Frustrations of Autophagy

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Abstract

The study of autophagy has been transformed by the cloning of most genes in the pathway and the introduction of GFP-LC3 as a reporter to allow visual assessment of autophagy. The field of cardiac biology is not alone in attempting to understand the implications of autophagy. The purpose of this review is to address some of the controversies and conundrums associated with the evolving studies of autophagy in the heart. Autophagy is a cellular process involving a complex orchestration of regulatory gene products as well as machinery for assembly, selective targeting, and degradation of autophagosomes and their contents. Our understanding of the role of autophagy in human disease is rapidly evolving as investigators examine the process in different tissues and different pathophysiological contexts. In the field of heart disease, autophagy has been examined in the settings of ischemia and reperfusion, preconditioning, cardiac hypertrophy, and heart failure. This review addresses the role of autophagy in cardioprotection, the balance of catabolism and anabolism, the concept of mitochondrial quality control, and the implications of impaired autophagic flux or frustrated autophagy.

Keywords

autophagosome; autophagy; bafilomycin A1; calcium overload; cardioprotection; catabolism/anabolism balance; chloroquine; exosome; flux; frustrated autophagy; glutathione; ischemia/reperfusion; ischemic preconditioning; lysosome; mitochondria; mitochondrial quality control; mitophagy; phagophore; preconditioning

AUTOPHAGY MACHINERY

Autophagy is initiated in response to nutrient limitation, cellular stress, reactive oxygen species, or accumulation of protein aggregates or damaged organelles. Autophagy is essential to withstand starvation, particularly during the fasting interval between birth and the beginning of nursing. The key sensor of nutrient status of the cell is mammalian target of rapamycin (mTOR), which when activated stimulates protein synthesis and suppresses autophagic degradation. Conversely, inhibition of mTOR suppresses protein synthesis and activates autophagy. However, mTOR signaling is not the only regulator of autophagy, and other signals, such as AMP-activated protein kinase (AMPK), can override mTOR suppression (1). Once initiated, Beclin1 and vacuolar protein sorting gene34 (VPS34), a class I PI3 kinase, trigger downstream events leading to activation of the first of two

DISCLOSURE STATEMENT

R.A.G. is majority owner and CEO of Radical Therapeutics, Inc. (RX*), a drug discovery company developing cardiotherapeutics related to autophagy. R.M.M. is on the scientific advisory board of RX*. R.A.G. is an inventor on two patents related to autophagy and cardioprotection.

ubiquitin-like pathways. First, autophagy-related protein (Atg)12 is activated similarly to ubiquitin by Atg7, an E1-like enzyme, which then transfers Atg12 to Atg10, an E2-like enzyme, which then conjugates Atg12 to Lys 130 of Atg5. The Atg5/Atg12 conjugate then complexes with a homodimer of Atg16, and the complex assembles on the forming membrane structure termed the phagophore. This event is a prerequisite for the second ubiquitin-like pathway, which involves the cleavage of the terminal Cys residue of Atg8 (LC3) by Atg4, a cysteine protease, which exposes a terminal glycine residue. Atg4 is redox regulated and appears to be responsible for upregulating autophagy in the face of oxidative stress (2). The glycine-exposed form of LC3 is known as LC3-I. Atg7 activates LC3 and then transfers it to Atg3, an E2-like enzyme, which finally conjugates LC3 onto phosphatidylethanolamine. The lipidated LC3 is known as LC3-II and has a slightly faster mobility on SDS-PAGE than does LC3-I. The incorporation of these Atg proteins supports elongation of the phagophore and attachment to its target (e.g., a protein aggregate or damaged organelle) followed by closure, giving rise to the double-membrane autophagosome. Recent reviews have described the details of the structural machinery (3, 4).

Although the removal of organelles by autophagy (organellophagy, mitophagy) is selective, the mechanism for recognition of targets is incompletely understood. It is thought to involve specific ubiquitin ligases and adaptor proteins. The next important step in the life of the autophagosome involves fusion with a lysosome, and in cells in which autophagy is upregulated, the number of lysosomes is also increased, along with mRNA and protein corresponding to lysosomal hydrolases such as cathepsin D. However, detecting an increase in lysosomal markers does not necessarily indicate increased autophagosome-lysosome fusion (flux). Fusion depends upon lysosomal acidification and can be prevented by addition of a weak base such as chloroquine or bafilomycin A1, the potent and specific inhibitor of the vacuolar proton ATPase. Subsequent to degradation of the contents, amino acids, free fatty acids, and carbohydrates are exported from the lysosome by various permeases, and the lysosome shrinks. The fate of the excess membrane is unclear, but in some cases the excess lysosomal membrane may be inserted into the plasma membrane. This interpretation is based on the fact that MHC class II proteins can be loaded with peptides derived from proteolytic degradation of autophagosome cargo (5). Bafilomycin A1 disrupts endosome cycling, which is thought to require an acidification step to dissociate ligands bound to receptors before returning the receptors to the cell surface. Endosome acidification may involve insertion of VPATPase (vacuolar proton ATPase, or v-type [H^+] ATPase) into preexisting endosomes but more likely involves fusion with other vesicles containing VPATPase (e.g., lysosomes). Because the key function of autophagy is catabolic, it is important for the cell to be competent in the late steps in autophagy, specifically fusion with lysosomes, subsequent degradation of contents, and export of amino acids and other components back into the cytosol. Given that the cell has a limited supply of membrane, any obstruction to membrane cycling is likely to disrupt many processes, including endosomal trafficking, receptor-mediated signaling, and plasmalemmal substrate transport by glucose transporters (6).

MEASUREMENT OF AUTOPHAGY

Autophagy was first observed by electron microscopy, and until recently, tools for its study were quite limited. Although cloning most of the pathway’s components has led to significant advances (3), existing research tools are still limited and limiting. Autophagy is commonly measured in cell culture or in transgenic mice expressing the fusion protein green fluorescent protein (GFP)-LC3, in which one can visualize autophagy as GFP-tagged autophagosomes (7, 8). The presence of numerous autophagosomes is generally assumed to be synonymous with increased autophagic activity. Because autophagy is a dynamic process that reflects both formation of autophagosomes and their clearance subsequent to lysosomal

fusion, the presence of numerous autophagosomes can reflect an increase in formation or a decrease in clearance.

Many groups also assess autophagy using Western blotting to measure the ratio of LC3-II to LC3-I (9, 10). LC3 is a labile protein that can undergo spontaneous proteolysis when frozen and thawed in SDS sample buffer, thus contributing a potential artifact to the analysis. The conversion of LC3-I to LC3-II is interpreted as an indication of active autophagy. However, this assumption is not necessarily correct, as blocking lysosomal degradation will result in an increase in the LC3-II:LC3-I ratio. For these reasons, it is essential to be able to measure flux.

A number of approaches have been developed for use in cell culture using various reporter constructs, but the only method that can be applied to tissue is analysis of the degradation of p62 (also known as Sequestosome1, or SQSTM1) (11). P62 is an adapter protein that binds ubiquitinated protein aggregates and recruits the LC3-decorated phagophore to engulf and remove the complex. P62 is removed along with the ubiquitinated protein aggregates and therefore is a good reflection of clearance of protein aggregates. Accumulation of p62 implies a defect in clearance, whereas low p62 levels imply active flux through the autophagosome/lysosome pathway. However, changes in p62 levels relate primarily to clearance of ubiquitinated protein aggregates, and if aggregates are not present in the first place, then p62 levels may be low despite minimal flux. It is not clear whether clearance of p62 is an accurate reflection of clearance of other autophagic cargo.

An alternative approach is to assess autophagosome abundance and LC3-II:LC3-I ratios in the presence and absence of lysosomal inhibitors such as bafilomycin A1 or chloroquine (12). This can provide an estimate of flux but requires paired samples (e.g., two animals per data point). A compromise has been to verify that flux is occurring (by using chloroquine) in a particular setting and then to assume that an increase in autophagosomes represents an increase in flux when the system is perturbed by experimental interventions.

Many published studies that report an increase in autophagy, on the basis of an increase in the number of autophagosomes or LC3-II:LC3-I ratio, must be interpreted cautiously. In fact, some studies should be reevaluated in light of subsequent work demonstrating impaired clearance of autophagosomes in some settings where autophagy was reported to be increased. For instance, an increase in the number of autophagosomes has been noted in hearts subjected to ischemia/reperfusion (I/R) (13). However, it is not clear whether this finding represents increased autophagosome formation or decreased clearance. Studies in HL-1 cells subjected to simulated I/R indicate that during ischemia autophagosome formation is suppressed and that during reperfusion clearance is impaired, resulting in an accumulation of autophagosomes. Clearance may also be impaired in the ischemic heart. The presence of autophagosomes does not provide insight into the pathological significance of autophagy: Is it part of a salvage/repair pathway, or does it mediate cell death after I/R?

PERTURBATIONS OF AUTOPHAGY

The best approach to understanding the role of autophagy in a particular setting is to inhibit or upregulate autophagy and evaluate the functional outcome. Here again, our understanding is limited by the available research tools. Agents to inhibit autophagy are limited to broad-spectrum PI3 kinase inhibitors 3-methyladenine (3-MA) and wortmannin. Because these agents also abrogate activation of Akt, the results must be interpreted with caution. Furthermore, 3-MA also has complex effects on intermediary metabolism that may confuse the picture. To examine the role of autophagy in vivo, genetic models have been employed. Atg5 and Atg7 knockout mice develop normally but die in the early postnatal period because they cannot tolerate the first episode of postnatal starvation unless they are fed

promptly. However, they subsequently develop dilated cardiomyopathy, revealing an essential role for autophagy in cardiac homeostasis. Beclin1 may have more complex roles beyond autophagy, as the knockout is embryonic lethal. Beclin1 interacts with Bcl-2 and Bcl-X_L, thus hinting at a potential interconnection with apoptosis. We and others have used a point mutation of Atg5(K130R) to disrupt autophagosome formation. Given that the function of Atg5 is restricted to nucleation of the phagophore, Atg5(K130R) is a highly specific inhibitor. We developed Tat-Atg5(K130R), a cell-permeable recombinant protein that functions as a dominant-negative protein that can suppress autophagy when perfused into the heart in the Langendorff model (14).

Autophagy is regulated in part by AMPK, the central energy-sensing protein kinase in the heart. A number of studies have altered AMPK activity to modulate autophagy. Although the use of dominant-negative and constitutively active forms of AMPK may be informative, the use of the drug AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) may be more problematic. AICAR is a potent activator of AMPK but paradoxically inhibits autophagy (15, 16). Therefore, experiments involving AICAR must be interpreted carefully. Other agents that activate AMPK are also problematic, as they interfere with mitochondrial respiration (e.g., oligomycin) or glucose metabolism (e.g., deoxyglucose, metformin).

CONUNDRUM AND CONTROVERSY

Until relatively recently, it has been difficult to discern whether autophagy is protective or deleterious in the setting of I/R injury in the heart. Vatner's group (17) observed an increase in the number of autophagosomes and upregulation of Atg proteins, including Beclin1, in hearts subjected to chronic low-flow ischemia in a hibernating myocardium preparation. One hint that autophagy was induced as a protective response in that context was the observation that cells that underwent apoptosis did not have autophagosomes. However, a study by Sadoshima's group (18) suggests that autophagy increases injury in the setting of I/R because infarct size and apoptosis are diminished in Beclin1 (+/−) mice. Beclin1, however, induces not only autophagosome formation but also lysosomal fusion. Interaction between Beclin1 and a protein called Rubicon slows down autophagosome-lysosome fusion (18). Therefore, the Beclin1 (+/−) mice may fare better because they clear autophagosomes more efficiently through enhanced lysosomal fusion. This scenario would also be consistent with the observation of fewer autophagosomes after I/R. Thus, the explanation for the Beclin1 (+/−) findings may be due to improved flux rather than to a reduction in autophagosome formation. The broader implications of this possibility is discussed in the section titled Frustrated Autophagy (below).

The above raises the question, however, as to whether numerous autophagosomes reflect increased autophagy or decreased clearance, and if Beclin1 slows down lysosomal fusion (a conjecture), then one must ask what the significance of autophagosome accumulation is. If the function of autophagosomes is merely to sequester damaged organelles such as proapoptotic mitochondria, then does it matter if the autophagosomes do not deliver their contents to lysosomes? Apparently so. Danon cardiomyopathy, a genetic disease arising from mutation of lysosome-associated membrane protein 2 (LAMP2), is characterized by a failure of autophagosomes to fuse with lysosomes (19). In cell-based studies, LAMP2 deficiency in the setting of cellular stress results in ATP depletion and necrotic cell death (20). These findings suggest that autophagy and lysosomal clearance are part of an important compensatory pathway.

That autophagy is beneficial in the setting of I/R is supported by the finding that autophagy is upregulated during pharmacological and ischemic preconditioning. Gurusamy et al. (21) reported that Bag-1 mediates a protected state that is associated with increased autophagy,

and Vatner's group (22) reported increased autophagy in preconditioned hibernating myocardium. Using a cell culture model, we showed that activation of autophagy is protective against simulated I/R in HL-1 cells (23). We have also shown in neonatal rat cardiomyocytes and in vivo in αMHC-mCherry-LC3 transgenic mice that the A1 adenosine receptor agonist 2-chloro-*N*(6)-cyclopentyladenosine (CCPA), a potent preconditioning agent, causes an increase in the number of autophagosomes within 10 min of treatment. Importantly, we were able to block the protective effect with Tat-Atg5(K130R) (14). Interestingly, other stimuli that confer cardioprotection but are not known as classical preconditioners also induce autophagy. Examples include rapamycin, statins, chloramphenicol, lipopolysaccharide (LPS), exercise, and starvation (reviewed in Reference 24). If, then, autophagy plays an important role in conferring protection or represents some form of a final common pathway, what are the processes supported by autophagy that protect the heart during I/R? To address this question, it is important to review the important cellular changes that occur during I/R (below).

INJURY DURING ISCHEMIA/REPERFUSION

At the onset of ischemia, oxygen deprivation results in an abrupt cessation of oxidative phosphorylation and a decline in intracellular ATP and creatine phosphate: the resulting levels of these critical energy components are insufficient to meet the needs of the contractile elements (25). Cell function is progressively compromised by the accumulation of Pi, ADP, lactic acid, and a rapid decline in intracellular pH (pH_i). Acidosis suppresses ATP generation from glycolysis. The increase in intracellular H^+ ($[H^+]_i$) activates the Na^+/H^+ antiporter to restore the pH but also results in a concomitant increase in the intracellular Na^+ concentration ($[Na^+]_i$). The increased $[Na^+]_i$ slows or reverses the direction of the Na^+/Ca^{2+} exchanger and ultimately results in a deleterious increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), resulting in Ca^{2+} overload. This transient increase in $[Ca^{2+}]_i$ can activate numerous degradative enzymes, including proteases such as calpain, as well as phospholipases and endonucleases. Calpain activation and its subsequent action on contractile proteins have been implicated in the reduction in myofilament sensitivity, cleavage of proapoptotic Bid, and spectrin cleavage, contributing to destabilization of the plasma membrane.

Simultaneously, there is an increase in reactive oxygen species (ROS) production from mitochondrial electron transfer complexes I and III. ROS production increases with the duration of ischemia and may sensitize the cell to the stresses that accompany reperfusion. The generation of ROS during early reperfusion in conjunction with Ca^{2+} overload may be responsible for the opening of the mitochondrial permeability transition pore (mPTP), which triggers necrotic cell death (26–28).

The metabolic changes that occur during I/R also impair the endogenous antioxidant defense systems of cardiomyocytes. The first line of defense against mitochondrial ROS formation and their deleterious effects is the GSH (reduced glutathione)/GSSG (oxidized glutathione) system, which is directly linked to the NADPH:$NADP^+$ ratio via glutathione reductase. The depletion of glutathione increases ROS formation, oxidative stress, and $[Ca^{2+}]_i$. Mitochondrial membrane depolarization and the mPTP are sensitive to decreased GSH and NADPH levels (29, 30). Because NADPH is not produced during ischemia, the normal metabolic mechanism for regenerating GSH reductase does not function. Thus, the formation of ROS during reperfusion occurs when the heart cell's endogenous defense mechanisms are depressed.

In short, the primary mediators of I/R injury appear to arise from oxidation of glutathione and disruption of ionic homeostasis, leading to $[Ca^{2+}]_i$ overload and oxidative stress (31).

These factors contribute to damage of mitochondrial oxidative phosphorylation machinery, especially of complexes I and III (which in turn can lead to further mitochondrial ROS production), the ATP synthase, and adenine nucleotide translocase. Bid-mediated release of proapoptotic mitochondrial proteins into the cytosol leads to caspase activation, which in turn may further damage mitochondrial complex I (32). All these insults contribute to a perfect storm, triggering opening of the mPTP, which appears to play an important role in mediating irreversible I/R injury (33). Importantly, ischemic preconditioning results in a population of mitochondria that are more resistant to mPTP triggers (34).

BENEFITS OF AUTOPHAGY

Why might autophagy be protective in I/R? What are the processes supported by autophagy that protect the heart during I/R?

Insoluble intracellular protein aggregates are normally cleared by autophagy. When autophagy is diminished, aggregates accumulate, leading to cellular dysfunction and eventual cell death. Certain mutations in sarcomere or cytoskeletal proteins can increase the tendency of proteins to misfold, leading to early-onset cardiomyopathies and heart failure. Environmental stresses can also contribute to protein misfolding. Mutations in desmin (intermediate filament protein) and its chaperone αB-crystallin (small heat shock–like protein) genes result in desmin-related myopathy. Upregulation of autophagy in cardiac myocytes is an adaptive response to the disorder, whereas suppressing the pathway accelerates heart failure (35). To the extent that protein misfolding is exacerbated during I/R (36), autophagy may mitigate the damage.

Mitochondria are removed by autophagy during starvation and I/R (37–39). If the most damaged mitochondria are selectively removed by autophagy, the remaining mitochondria will likely have a higher threshold for mPTP opening (40), greater resistance to cytochrome *c* release and diminished ROS production and may also exhibit an altered protein composition, including fewer oxidatively modified proteins (41, 42). Removal of ROS-producing mitochondria through autophagy will also decrease the overall oxidative stress experienced by a cell, which may explain in part why caloric restriction is associated with less oxidative damage (43). Conceivably, any stimulus that results in enhanced mitochondrial quality control through autophagy will have benefits with respect to organ function and aging. Mitochondria damaged during hypoxic stress and associated ROS may be removed through autophagy in a process mediated by Bnip3 (BCL2/adenovirus E1B 19 kD–interacting protein 3) (44, 45). Mitochondrial biogenesis may follow a bout of mitophagy (46, 47).

The energetic stress of I/R injury should be a potent stimulus for autophagy (13, 15, 48). But our studies in HL-1 cells indicate that autophagy is suppressed during ischemia, and flux is impaired during reperfusion (23). However, restoration of autophagic flux allows degradation of proteins to amino acids, which can be transported out of the autophagolysosome and used as substrates for nonoxidative ATP production (49).

Amino acids liberated by lysosomal proteolysis may also support glutathione biosynthesis. A proteomic study of hepatic autophagosomes identified numerous enzymes involved in sulfhydryl repair (50), leading us to hypothesize that the autophagolysosomal membrane serves as a scaffold for repair of cytoplasmic proteins. Because autophagy is potently induced by ROS, it may be part of a cellular homeostatic response enabling repair of oxidatively modified proteins.

Ischemia results in substantial intracellular acidification (51). During early reperfusion, protons are eliminated via the Na^+/H^+ exchanger. The increased intracellular Na^+ competes

with Ca^{2+} for extrusion via the Na^{+}/Ca^{2+} exchanger, resulting in Ca^{2+} overload and hence in activation of a number of destructive pathways, including calpain activation and triggering of mPTP opening. Ischemic preconditioning limits intracellular acidification, which depends upon the activity of VPATPase, the proton pump responsible for lysosomal acidification (52, 53).

FRUSTRATED AUTOPHAGY (OR, AUTOPHAGUS INTERRUPTUS)

The earliest studies of autophagy in the heart noted the appearance of autophagosomes early in the course of ischemia. With prolonged ischemia, the structures became dilated and more numerous, and Sybers et al. (54) suggested that these dilated vacuoles were incapable of productive degradation because their appearance coincided with the onset of hypercontracture, an indication of profound ATP depletion. Impaired autophagic flux interferes with all forms of membrane traffic. Our studies of autophagy in LPS-exposed hearts revealed an upregulation of lysosomes in parallel with autophagosomes (cathepsin D staining) (55). The upregulation and expansion of lysosomes may be like nuclear weapons proliferation: Sooner or later a terrorist is likely to hijack one for evil purposes. This process occurs in hepatocytes, where the BH3-only protein truncated Bid can permeabilize the lysosomal membrane, causing catastrophic leakage of lysosomal proteases and cell death.

What else can go wrong if autophagosome-lysosome fusion is impaired? Beyond loss of the physiological functions of autophagic degradation and potential lysosomal leakage, impaired flux may lead to exosome extrusion. These events are depicted in Figure 1. Exosomes are tiny membrane-enclosed particles that contain cytosolic contents and are derived from multivesicular bodies. When autophagy is competent, multivesicular bodies undergo fusion with autophagosomes; however, if autophagy is impaired, multivesicular bodies may be redirected toward extrusion, resulting in the release of these highly inflammatory particles. This process would undoubtedly contribute to the inflammatory response during reperfusion. *N*-ethylmaleimide-sensitive factor (NSF) is a protein that is involved in vesicle fusion in conjunction with the SNARE (soluble NSF attachment protein receptor) complex (56). NSF is required for exosome secretion and inflammation, and a peptide inhibitor of NSF decreases infarct size, suggesting that exosome secretion after I/R increases inflammation and injury (57). Confusingly, however, NSF is extremely sensitive to oxidative stress and is inactivated in I/R (57).

These considerations lead us to propose the concept of frustrated autophagy, in which autophagosome-lysosome fusion is impaired. The best-known example of such a situation is a myopathy arising in patients receiving chloroquine: a myopathic condition characterized by numerous vacuoles (autophagosomes). Chloroquine, a weak base, accumulates in lysosomes and neutralizes their pH, preventing autophagosome clearance. Studies from our lab and others indicate that during ischemia, autophagic flux is impaired and recovers only partially during reperfusion. Reinspection of our studies of CCPA reveal that the number of autophagosomes decreases in cells treated with CCPA and I/R. Although we initially attributed this decrease to a diminished need to remove damaged organelles at reperfusion, it is also consistent with enhanced removal. Because we neglected to measure flux in this body of work, we cannot distinguish between enhanced removal and diminished formation, but work is under way to evaluate this potentially important possibility. We suggest that an important aspect of preconditioning may be to enhance autophagic flux. One tantalizing hint is that phosphorylation regulates subunit C of the VPATPase, thus stimulating activity of the holoenzyme (58–60). Importantly, ischemic preconditioning is abolished if bafilomycin A1, the VPATPase inhibitor, is concurrently administered (52, 53).

Although autophagy is in general a protective response, frustrated autophagy may be bad news. No autophagy may be preferable to frustrated autophagy. This possibility may explain why Beclin1 haploinsufficient mice have better survival after I/R (13). However, another interpretation arises from the fact that Beclin1 functions in both autophagosome formation and clearance (autophagosome-lysosome fusion) (61, 62). Recent work has revealed that Beclin1 exists in two different complexes: one with Atg14L and the other with Rubicon. When Beclin1 is complexed with Rubicon, the effect is to suppress autophagosome-lysosome fusion (18, 63). If Beclin1 is dispensable for autophagosome formation, as suggested by Kim et al. (64), then its absence may allow for more rapid clearance of autophagosomes (by releasing the Rubicon brake). This possibility is consistent with existing observations: Beclin1-deficient mice show fewer autophagosomes (could be fewer formed but could also be due to more rapid flux), and Beclin1-deficient mice had better survival after infarction (could be due to less autophagy but could also be due to improved flux and less frustrated autophagy). The mortality was due to cardiac rupture, which is often associated with activation of matrix metalloproteinase (MMP)9 (65). MMPs can be released via exosome secretion (66). We suggest that impaired autophagic flux (frustrated autophagy) may lead to exosome secretion with release of MMPs and activation of inflammation, which would promote cardiac rupture. Further work is needed to assess the interplay between Beclin1, exosome secretion, inflammation, and postinfarct cardiac remodeling.

MITOCHONDRIAL QUALITY CONTROL, BIOGENESIS, AND AUTOPHAGY

Mitochondria contain excellent protein quality-control systems in the form of the Lon protease and other proteases. However, the only way to eliminate an entire organelle is via macroautophagy. Over the circadian cycle, when activity is low, excess mitochondria may be hyperpolarized and may generate excess ROS. Therefore, it may be useful to remove them entirely. However, Mother Nature is thrifty. Why eliminate perfectly good mitochondria when it would be possible to selectively remove the most decrepit ones? We propose, therefore, that the circadian cycle (which also implies a feed/fasting cycle) provides a mechanism for selective removal of deteriorating mitochondria. The mitochondrial quality-control hypothesis was first advanced by Shirihai's group (67), who showed that mitochondrial fusion is followed by fission, resulting in two daughter mitochondria: one with high membrane potential that can proceed to successive rounds of fusion and fission and one with low membrane potential that is targeted for autophagic destruction. If autophagy is suppressed, decrepit mitochondria will accumulate; these mitochondria will emit more ROS, contain more oxidized proteins and lipids, and be more susceptible to triggers of apoptosis or mPTP opening. Moreover, suppression of mitophagy (mitochondrial autophagy) may interfere with renewed mitochondrial biogenesis, as a number of reports have linked the two processes (44, 46, 68). AMPK, which activates autophagy, also activates PGC1α (peroxisome proliferator–activated receptor γ coactivator α), which is the key transcriptional regulator of mitochondrial biogenesis (69). How fission results in asymmetric mitochondrial daughters is the next mystery, but recent work by Rosca & Hoppel (70) may offer a possible mechanism. They showed that respiratory dysfunction in heart failure is associated with a depletion of supercomplexes (respirasomes) and an accumulation of free complexes (particularly complex IV). We hypothesize that supercomplex assembly and disassembly are a dynamic process, resulting in exclusion of damaged and dysfunctional components. This process may allow spatial segregation of supercomplexes and damaged isolated subunits or subcomplexes and may explain the basis for asymmetric fission. More work will be required to address this possibility.

AUTOPHAGY AND INSULIN SIGNALING

Two key regulators of autophagy are mTOR and AMPK, which integrate signals related to nutrient status and metabolic demand. Under conditions of nutrient abundance (signaled in part through the insulin receptor and Akt), mTOR stimulates protein synthesis and suppresses autophagy. Conversely, when energy supply is limited, AMPK stimulates autophagy as well as signaling to the nucleus to drive mitochondrial biogenesis to increase ATP production. At the intracellular level, type I and type II diabetes may have opposite effects: In the case of insulin deficiency, mTOR will be inhibited, and autophagy will increase. In the case of the CIRKO (cardiac insulin receptor knockout) mouse, insulin deficiency leads to cardiac dysfunction because constitutively upregulated autophagy will eventually result in an imbalance in which protein degradation outstrips resynthesis (71). Conversely, overstimulation through the insulin receptor will suppress autophagy (72) and will interfere with the housekeeping levels of protein degradation, resulting in an accumulation of protein aggregates and impaired mitochondrial quality control. Global loss of autophagy, as in the case of the Atg5-null mouse, results in cardiomyopathy and heart failure (73). Akt overexpression initially supports cardiac hypertrophy but eventually leads to heart failure. In that model, however, the picture may be more complex, as other drivers of autophagy [such as hypoxia at the core of the hypertrophied cell leading to histone deacetylase (HDAC) activation] may eventually override the Akt-mediated suppression. In this case, the simultaneous signals driving protein synthesis and degradation may precipitate a metabolic crisis for the cell. We propose that this crisis is responsible for the transition from hypertrophy to heart failure.

What this proposed series of events suggests is that chronic caloric excess will suppress autophagy and may accelerate age-related diseases including neurodegeneration and heart failure. One unanswered question is whether all fuel sources suppress autophagy to a similar extent. Certain amino acids are known to be potent suppressors of autophagy (74), but less information is available regarding the effects of fats and carbohydrates. With respect to carbohydrates, there is little information available as to whether glucose (from glycolysis plus glucose oxidation), fructose, or galactose (from preferential oxidation) has a differential effect on autophagy. A more significant open question is whether autophagy induced by caloric restriction or alternate-day fasting is responsible for life span extension. If that is the case, then one wonders whether pharmacological stimulation of autophagy will be beneficial. One hint supporting this idea is the recent report that treatment with rapamycin was able to extend the life span in mice (75, 76). What remains to be explored is whether less frequent fasting (e.g., once a week) will be sufficient to facilitate removal of protein aggregates and to maintain mitochondrial quality control. The link between sirtuins, caloric restriction, and autophagy represents an exciting new area of investigation (see sidebar on sirtuins).

DISCUSSION

Given these considerations, we can begin to put the current controversial state of the literature into context. This discussion represents the authors' opinions, based on their interpretation of published work. We hope that these conjectures will drive further discussion and experimentation. Autophagy serves an essential homeostatic function in long-lived cells such as cardiomyocytes and neurons. It is important for removal of protein aggregates and organelles including mitochondria and serves an essential role in mitochondrial quality control. Life span extension has been linked to caloric restriction and more recently to autophagy, yet autophagic capacity diminishes with age, perhaps explaining the acceleration of neurodegenerative (protein aggregate) diseases and heart failure (mitochondrial dysfunction) with age. In the presence of caloric excess and insulin

signaling, which also suppress autophagy, the normal homeostatic function of autophagy would be suppressed, again contributing to acceleration of age-related diseases. (In the normal homeostatic setting, autophagy is episodic: It is upregulated during sleep and fasting and downregulated during feeding.)

As opposed to episodic autophagy, constitutively upregulated autophagy may not be such a good thing, as it would allow no opportunity for the replacement of organelles removed by autophagy, or replacement would occur at considerable metabolic cost, not unlike running the air conditioner and the heater at the same time. When protein and organelle destruction exceeds replacement for a sustained period of time, cellular dysfunction is likely to ensue. We suggest that this process may take place in the transition from hypertrophy to heart failure and may also be a feature of postinfarct remodeling. Autophagy is upregulated by inflammatory mediators including tumor necrosis factor α (TNFα) and LPS (44, 55), which may explain in part why chronic exposure to these mediators can exacerbate heart failure.

Despite the potential deleterious effects of sustained upregulation of autophagy, its acute induction has been linked to myocardial preconditioning (14, 21, 22). It is likely that enhanced removal of unstable mitochondria lowers cellular ROS production, decreases likelihood of mPTP opening, and decreases cytochrome *c* release. Autophagic induction may also enhance proton sequestration, thereby limiting Na^+/H^+ and Na^+/Ca^{2+} exchange and thus limiting Ca^{2+} accumulation. Finally, induction may add to nonoxidative energy production and provide the driving force for GSH synthesis through amino acid efflux from the lysosomes. Not only have several preconditioning agents (including ischemic preconditioning) been shown to upregulate autophagy, but several agents that upregulate autophagy can also give rise to a cardioprotected state.

The final concept that we have introduced is that of frustrated autophagy, or autophagus interruptus. In this setting, autophagosomes form and engulf targets but cannot fuse with lysosomes and clear their contents. The cell may respond by ejecting the autophagosomes from the cell, thereby eliciting an acute and significant inflammatory response. Moreover, activated lysosomes that are unable to fuse with autophagosomes may eventually self-digest, spilling activated cathepsins and lipases into the cytoplasm and resulting in uncontrolled autodigestion and cell death. In this context, no autophagy may be preferable to frustrated autophagy. Frustrated autophagy may be relevant to postischemic injury, in which inflammation contributes significantly to remodeling. Further work, in which flux is measured, will be important to understand the significance of this concept. Moreover, we suggest that exosome release may be related to impaired autophagic flux and may offer insights into new therapeutic targets. Further investigation into the role of autophagy in additional contexts will yield a clearer understanding of its significance. At present we can conclude that it serves an essential homeostatic function in long-lived cells, but dysregulation (excessive autophagy) or dysfunction (frustrated autophagy) can have deleterious consequences.

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Glossary

mTOR	mammalian target of rapamycin
AMPK	AMP-activated protein kinase

Vacuolar protein sorting gene34 (VPS34)	a class III phosphatidylinositol 3-kinase
Autophagy-related protein 8 (Atg8)	also known as LC3 (microtubule-associated light chain 3)
VPATPase	vacuolar proton ATPase (v-type [H^+] ATPase)
Sequestosome1/p62 (SQSTM1/p62)	a polyubiquitin chain–binding protein)
I/R	ischemia/reperfusion
3-Methyladenine (3-MA)	an inhibitor of autophagosome formation
2-Chloro-*N*(6)-cyclopentyladenosine (CCPA)	an adenosine A1 receptor agonist
LPS	lipopolysaccharide
ROS	reactive oxygen species
mPTP	mitochondrial permeability transition pore
NSF	*N*-ethylmaleimide-sensitive factor
HDAC	histone deacetylase

LITERATURE CITED

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SIRTUINS

Sirtuins are HDACs that are important to life span extension. Sirtuins have also been linked to cardioprotection (77). SIRT1, which is upregulated by caloric restriction, regulates autophagy through deacetylation of several of the autophagy proteins, including Atg5, Atg7, and Atg8. SIRT1 also regulates mitochondrial biogenesis through FoxO1 and PGC1α. Resveratrol activates SIRT1 and AMPK, triggering autophagy. Current evidence suggests that autophagy may be the fundamental process by which caloric restriction, resveratrol, and sirtuins extend life span. For a recent review, see Reference 78.

SUMMARY POINTS

1. Autophagy is a complex system for homeostatic protein and organelle degradation and is tightly regulated by nutrient status and redox poise.
2. Autophagy is an essential process in long-lived cells, contributing to optimal cell viability through mitochondrial quality control and removal of protein aggregates.
3. Suppression of autophagy (e.g., through excessive insulin signaling) may lead to the accumulation of protein aggregates and dysfunctional mitochondria and therefore contributes to age-related diseases.
4. Upregulation of autophagy is part of a cardioprotective response to ischemic injury or aggregopathy accompanying heart failure.
5. Proposed mechanisms for the beneficial effects of autophagy include the removal of proapoptotic damaged mitochondria, proton sequestration to limit Ca^{2+} overload, regeneration of amino acids for ATP production and new protein synthesis, and support of protein disulfide repair.
6. When autophagy exceeds the cellular capacity to replace lost components, the resulting energetic crisis may culminate in cell death.
7. Frustrated autophagy, in which autophagosome-lysosome fusion is impaired, may be worse than no autophagy, as it may lead to exosome release and local inflammation.

FUTURE ISSUES

1. Beclin1 and flux: Beclin1 plays a role in initiation of autophagy but may also regulate fusion with lysosomes. More work is required to understand the complex functions of Beclin1.
2. Frustrated autophagy: To what extent does impaired autophagic flux contribute to cell injury? What is the connection between autophagy, exosome release, and inflammation? Why is flux impaired after ischemia/reperfusion?
3. Which of the many proposed mechanisms by which autophagy may protect the cell are truly important?
4. What is the signal transduction pathway between preconditioning and autophagy?
5. Mitochondrial quality control: What governs selective mitophagy, and how do mitochondria accomplish the sorting that can lead to asymmetric fission?
6. The balance of catabolism and anabolism: How do cells regulate the balance between protein synthesis and degradation? To what extent does intermittent fasting (overnight or longer) with resulting autophagy contribute to optimal cellular function?

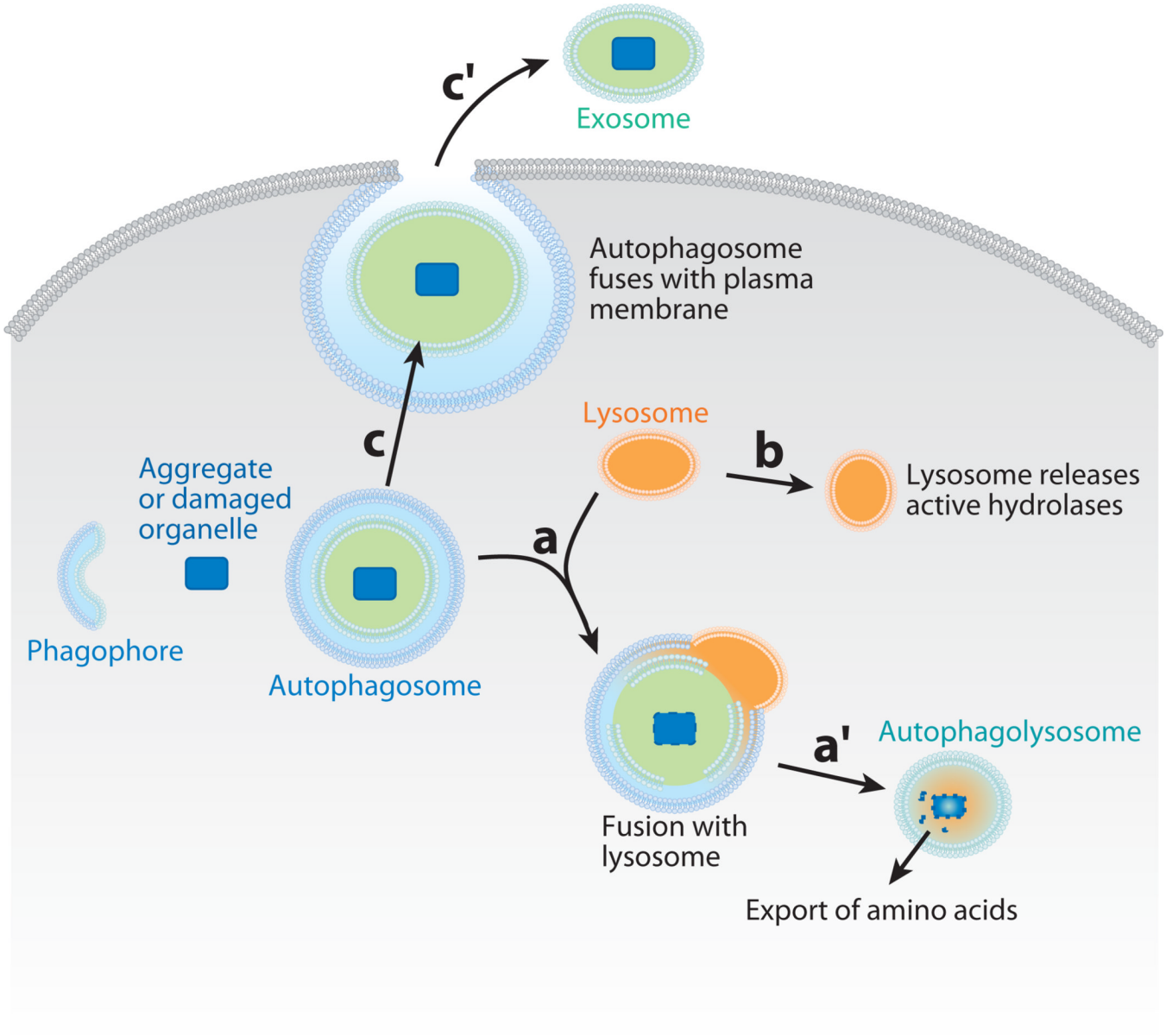

Figure 1.
Fates of autophagosomes and lysosomes. The phagophore forms and engulfs a target, typically a damaged organelle or protein aggregate. Under normal circumstances, the autophagosome fuses with a lysosome, which degrades the autophagosome's inner membrane and contents (*a*). As digestion proceeds, the amino acids and other hydrolyzed components are exported to the cytosol (*a′*). Amino acid export may support glutathione biosynthesis and sulfhydryl repair by enzymes associated with the autophagolysosome membrane. Lysosomes that are unable to fuse with autophagosomes may become leaky (*b*). In the setting of frustrated autophagy, in which lysosomal fusion is prevented, autophagosomes may accumulate and eventually fuse with the plasma membrane (*c*). Autophagosome contents may be ejected from the cell as exosomes (*c′*).