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Forum Review

Autophagy, Redox Signaling, and Ventricular Remodeling

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Abstract

Autophagy is a catabolic process through which damaged or long-lived proteins, macromolecules, or organelles are recycled by using lysosomal degradation machinery. Although the occurrence of autophagy in several cardiac diseases including ischemic or dilated cardiomyopathy, heart failure, hypertrophy, and during ischemia/reperfusion injury have been reported, the exact role of autophagy in these diseases is not known. Emerging studies indicate that oxidative stress in cellular system could induce autophagy, and oxidatively modified macromolecules and organelles can be selectively removed by autophagy. Mild oxidative stress–induced autophagy could provide the first line of protection against major damage like apoptosis and necrosis. Cardiac-specific loss of *Atg5*, an autophagic gene involved in the formation of autophagosome, causes cardiac hypertrophy, left ventricular dilation, and contractile dysfunction. Recently, it was revealed that *Atg4*, another autophagic gene involved in the formation of autophagosomes, is controlled through redox regulation under the condition of starvation-induced autophagy. In this review, we discuss the function of autophagy in association with oxidative stress and redox signaling in the remodeling of cardiac myocardium. Further research is needed to explore the possibilities of redox regulation of other autophagic genes and the role of redox signaling–mediated autophagy in the heart. *Antioxid. Redox Signal.* 11, 1975–1988.

Introduction

In response to intrinsic or external stress, cardiac myocytes adopt a complex process involving molecular modifications and remodeling. Cardiac myocytes have long been considered terminally differentiated cells that cannot be replaced, unlike other cells such as intestinal epithelium, epidermal keratinocytes, or mature blood cells, which constantly undergo renewed cell division and differentiation. A cellular-degradation mechanism plays an important role in the homeostasis of cardiac cells (87, 90, 109).

Two major pathways exist for the degradation of cellular materials: the ubiquitin-proteasomal pathway and autophagy. Most long-lived proteins, macromolecules, biologic membranes, and organelles, including mitochondria, ribosomes, endoplasmic reticulum, and peroxisomes, are turned over through autophagy, a process involving cellular lysosomal degradation machinery (16, 38, 139). Autophagy can promote cell survival by generating free amino acids and fatty acids required to maintain function during nutrient-limiting conditions, or by removing damaged organelles and intracellular pathogens (11, 37, 131). However, autophagy might also promote cell death through excessive self-digestion and degradation of essential cellular constituents (3, 15, 118). Irreversibly damaged or diseased cells can initiate a self-killing program in the form of either apoptotic or autophagic cell death, followed by phagocytosis of cellular remains by neighboring cells, macrophages, or other scavengers (19, 37, 79).

A variety of cellular stresses, such as endoplasmic reticulum stress or mitochondrial dysfunction, can induce autophagy (43, 49, 51, 141). Several reports indicate that oxidative stress mediated *via* reactive oxygen species (ROS) plays an important role in the myocardial repair/remodeling (43, 141).

Oxidative stress occurs when excess ROS are generated that cannot be adequately counteracted by the intrinsic antioxidant systems. Autophagy occurs at low levels under normal conditions and is important for the turnover of organelles (12, 157). Studies have shown that autophagy is upregulated in the heart in response to stress, such as ischemia/reperfusion (13, 41, 43, 111, 131), and in many cardiovascular diseases, such as cardiac hypertrophy and heart failure (22, 29, 37, 38, 133, 139, 157). Oxidative stress can induce autophagy under

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starvation and ischemia/reperfusion conditions (14, 62, 77, 119–121). The amino acid cysteine plays an essential role in the redox regulation of proteins *via* the thiol group present in cysteine (34). Thus, cysteine proteins can act as redox switches, to sense concentrations of oxidative stressors and to take part in important regulatory and signaling pathways (33, 34).

Recently, Scherz-Shouval *et al.* (120, 121) demonstrated that ROS act as signaling molecules in starvation-induced autophagy, in which ROS directly oxidize a cysteine residue in the cysteine protease Atg4, an autophagic gene regulating the formation of autophagosomes. Mild oxidative stress–induced soluble oxidized proteins are selectively degraded by chaperone-mediated autophagy, which is mediated *via* the heat-shock protein, Hsc73 (16, 21, 114). The purpose of this review is to discuss the role of autophagy and its association with oxidative stress–mediated redox signaling in ventricular remodeling.

Autophagy

To maintain cellular homeostasis, the eukaryotic cell system possesses two major degradation mechanisms, ubiquitin-proteasomal degradation and autophagy. Autophagy (in Greek: self-digestion) under normal conditions occurs for the recycling of long-lived macromolecules and organelles *via* a lysosomal degradative pathway (70). Three types of autophagy exist: macroautophagy, microautophagy, and chaperon-mediated autophagy.

Macroautophagy is the most active form of autophagy and is known to play a major role in intracellular degradation (16). Macroautophagy (hereafter referred to as autophagy) is initiated by the formation of an isolation membrane, a single-membrane structure possibly derived from the sarcoplasmic/endoplasmic reticulum (53). Fusion of the tips of the isolation membrane results in the formation of a double-membrane structure known as the autophagosome, which surrounds portions of the cytoplasm and organelles such as mitochondria (16, 140), selectively targeted to the toxic protein aggregates (110) or intracellular pathogens (40). Autophagosomes undergo a series of maturation steps and finally fuse with the lysosome (this combined structure is known as the autophagolysosome), in which the sequestered contents and the inner membranes of autophagosomes are degraded by the lysosomal hydrolases (16, 106). The lifetime of autophagosomes is as short as $\sim$8 min.

In microautophagy, cytosolic macromolecules are locally taken into lysosome *via* invagination of the lysosomal membrane (16, 97). During chaperone-mediated autophagy, delivery of modified proteins to lysosome occurs *via* heat-shock protein 73 and the receptor lysosome-associated membrane protein type 2a (LAMP2a) (16, 17, 59, 84).

Formation of autophagosomes

Several autophagic genes are found to be regulated during the induction of autophagy. Autophagic genes are denoted as Atg, and they are further numbered (71). Atg12 is a small hydrophilic modifier protein, which is covalently conjugated to Atg5, independent of autophagy induction (95). Atg12 is activated by Atg7 in an ATP-dependent manner, resulting in the formation of thioester bond between the C-terminal glycine in Atg12 and a cysteine residue in Atg7 (95). The carboxy-terminal glycine of Atg12 is transferred to Atg10, leading to the formation of a new thioester bond, and Atg7 is released. Atg10 activates the conjugation of carboxy-terminal glycine in Atg12 with lysine 149 in Atg5 (154). The formation of conjugation between Atg12 and Atg5 is irreversible and, at the same time, is essential for the formation of the autophagosome (154). Atg16 self-dimerizes and forms a complex with a pair of Atg12-Atg5 (32). Atg16 is found to bind only with Atg5, but not with Atg12 (32). This new complex, Atg12-Atg5-Atg16, is vital for the elongation of the isolation membrane (Fig. 1) (96). At the end of autophagosome formation, the Atg12-Atg5-Atg16 complexes dissociate from the membrane and are not present in the mature autophagosome (16, 96).

The development of the isolation membrane (phagophore) into the autophagosome requires the participation of microtubule-associated protein light-chain 3 (LC3) (135). LC3, the mammalian orthologue of Atg8, is synthesized as a cytosolic full-length precursor protein. Precursor LC3 is converted into LC3-I *via* cleavage by the cysteine protease Atg4 (autophagin), resulting in the exposure of glycine residue in the carboxy terminal of LC3-I (Fig. 1) (67). Atg7 activates the glycine residue of LC3-I in an ATP-dependent manner and finally is conjugated to phosphatidylethanolamine (PE) *via* an amide bond (83). The complex LC3-PE (or LC3-II) is then transferred to the outer autophagosomal membrane in an Atg5-dependent manner (96). LC3-II, in contrast to Atg12-Atg5, is present both in the early phagophores and in the mature autophagosomes. Atg4 also converts LC3-II back to LC3-I *via* deconjugation of PE from LC3-II (54). Lysosomal proteases ultimately degrade most of the LC3-II found in autophagosomes (136).

In addition to LC3, another two mammalian homologues of yeast Atg8 are known: γ-aminobutyric acid type A receptor–associated protein (GABARAP) and Golgi-associated ATPase enhancer of 16 kDa (GATE-16) (112, 145). GABARAP and GATE-16 also have been found to be associated with an autophagosomal membrane (54, 93). All these three mammalian Atg8 homologues possess a conserved glycine residue near their carboxy termini, which corresponds to the PE-acceptor site of yeast Atg8 (54). Four human homologues of Atg4 have been identified: autophagin 1 (HsAtg4B), autophagin 2 (HsAtg4A), autophagin 3, and autophagin 4 (83). GABARAP and GATE16 can be cleaved by either HsAtg4A or HsAtg4B, but LC3 is preferentially cleaved by HsAtg4B (54). Like LC3, it was suggested that GATE16 and GABARAP are also converted into form II (135).

In F9 teratocarcinoma cells, both GATE16 and GABARAP are detected in two forms (I and II); form I of both proteins is primarily cytosolic, whereas form II is associated in the membrane fraction (54, 55). The formation of both GATE-16-II and GABARAP-II has been increased under starvation, and further, they have been associated with the autophagosomal membrane (54, 55).

Fusion of autophagosomes

On completion of autophagic sequestration, the autophagosome undergoes multistep maturation, leading to fusion with endosomal vesicles and protease-containing late endosomal compartments and finally with lysosomes (Fig. 1) (36).

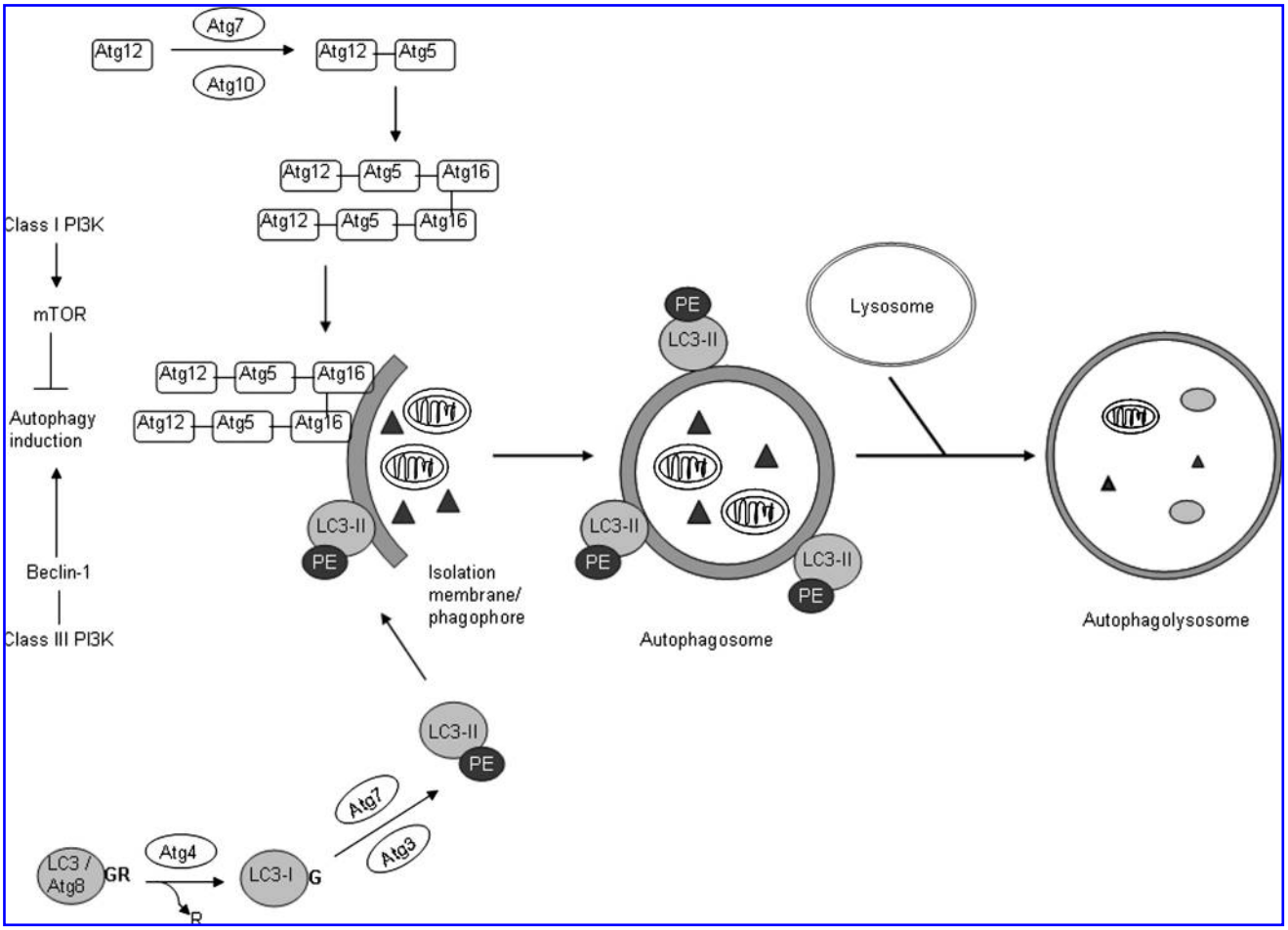

FIG. 1. Process of macroautophagy. First step in the process of macroautophagy involves the formation of a double-membrane structure called the isolation membrane or phagophore, composed of the complex Atg12-Atg-5-Atg16 and LC3-II. Atg12 is activated by Atg7 and Atg10, resulting in the conjugation with Atg5. Homodimers of Atg16 bind a pair of Atg12-Atg5, and the complex Atg12-Atg5-Atg16 then associates with the autophagosomal membrane. Atg4 cleaves LC3/Atg8, generating LC3-I, with exposure of the C-terminal glycine residue. LC3-I is then activated by Atg7 and Atg3, and conjugated to phosphatidylethanolamine (PE), forming LC3-II, which is then bound to the autophagosomal membrane. An isolation membrane engulfs a portion of cytosol containing damaged proteins or organelles, forming the structure known as the autophagosome. The autophagosome fuses with a lysosome, forming an autophagolysosome, in which the damaged proteins or organelles are degraded by lysosomal proteases.

The fusion events are mediated in part by a family of Rab guanosine triphosphatases, known mediators of vesicular trafficking. Rab7 is shown to be a key component in the regulation of autophagosomal maturation (39). Lysosomal-receptor proteins Lamp1 and Lamp2 are involved in Rab7-mediated fusion of autophagosomes and lysosomes (52). Through fusion with a lysosome, an autophagosome acquires the milieu and enzymes necessary for the degradation of its cargo.

Lysosomes contain a cocktail of predominantly hydrolytic enzymes that are maximally activated at low pH (*e.g.*, cathepsins B and D, which degrade proteins, lipids, nucleic acids, and polysaccharides) (147). Autophagy-mediated recycling of degraded products (amino acids, sugars, and nucleotides) is achieved *via* lysosomal transport proteins such as cystinosin, which transfer the degraded products from the lysosome back to the cytosol (147), and thus the catabolic process is completed.

Detection of autophagy

Formation of double-layered autophagosomes can be monitored by electron microscopy (92), but lack of morphologic expertise can lead to misidentification of other cellular structures, such as swollen endoplasmic reticulum (in dying cells) as autophagosomes. In some cases, monodansylcadaverine has been used as a fluorescent marker for autophagic vacuoles (7); however, this method is limited to *in vivo* conditions. LAMP-2a has been used as a marker to study chaperone-mediated autophagy (20). However, at present, only LC3 is the credible marker to study autophagosome formation. LC3 is found to be present in both the isolation membrane and the autophagosome (55); the conversion of LC3 to LC3-I and LC3-II can be studied with Western blotting. The generation of transgenic mice expressing green fluorescent protein fused to LC3 becomes a useful tool to study autophagic activity *in vivo* (94).

Regulation of autophagy

A great number of extracellular stimuli (starvation, hormone, or therapeutic treatment) as well as intracellular stimuli (accumulation of misfolded proteins, invasion of microorganisms) are able to modulate the autophagic response (15). Nutrient deprivation is the most generalized stimuli for macroautophagy. However, a decrease in specific regulator amino acids and increased levels of glucocorticosteroids and thyroid hormone also stimulate macroautophagy (153). Insulin, various growth factors, cyclic adenosine monophosphate (AMP), and cyclic guanosine monophosphate (GMP) as well as conditions that lead to reduced intracellular levels of adenosine triphosphate (ATP) are known as physiologic inhibitors of autophagy (153). However, glucagon and β-adrenergic agonists inhibit rather than stimulate autophagy in cardiac and skeletal muscle (139).

Amino acids that are the final products of autophagic protein degradation act as negative-feedback regulators for the process. A combination of leucine and a few other amino acids is very effective in inhibiting autophagy (6). Addition of amino acids has been shown to phosphorylate ribosomal protein S6 kinase (S6K) and 4E-binding protein 1 (4E-BP1) (6). Amino acids and insulin act synergistically on both processes to inhibit autophagy (8). In the presence of amino acids and growth factors, the mammalian target of rapamycin (mTOR) activates the p70 S6K, which phosphorylates the ribosomal protein S6, resulting in the upregulation of the translational machinery (6). 4E-BP1 is an inhibitor of translation that can be directly phosphorylated by mTOR, leading to the deactivation of 4E-BP1 (15). Deactivated 4E-BP1 then dissociates from the initiation factor eIF4E, which is the rate-limiting step in protein synthesis. Free eIF4E binds to the 5′ terminal cap structure of RNAs and promotes the progress of translation (15).

Inhibiting mTOR activity with the immunosuppressant rapamycin increases the catabolic process of autophagy (8). p70 S6K is needed for the entire process of autophagy activation, and it must be activated first for maximal activation of autophagy (123).

Activation of TOR signaling negatively regulates the association between Atg13 and Atg1. Under nutrient-rich conditions, active TOR causes hyperphosphorylation of Atg13, inhibiting its association with Atg1 (56, 124). Starvation or rapamycin treatment inactivates TOR, leading to rapid dephosphorylation of Atg13, which promotes the binding with Atg1. This association promotes autophosphorylation and activation of Atg1, leading to the induction of autophagy (56, 124).

Depletion of ATP is associated with an increase in AMP. AMP-activated protein kinase (AMPK), which serves as a general integrator of metabolic responses to changes in energy availability, is activated in response to elevations of the AMP/ATP ratio (115). TOR can act not only as an amino acid sensor, but also as an ATP sensor (101) Recently, it was shown that glucose deprivation in cardiac cells and cardiac ischemia activate AMPK, leading to the activation of autophagy (85, 132).

Autophagy was shown to be regulated through class III and class I phosphatidylinositol 3-kinases (PI3K) pathways. Class I PI3-K suppresses autophagy, whereas class III PI3-K activates autophagy. Beclin1/Atg6, the first mammalian protein described to mediate autophagy (81), by forming a complex with Vps34, a class III PI3-K, positively regulates autophagy (65, 156). The Beclin 1/PI3K-III complex is involved in the formation of autophagosomes and the initiation of autophagy (156). PI3K-III inhibitors like 3-methyl adenine, wortmannin, and LY294002 interfere with this pathway (9). Beclin 1 has been shown to colocalize with the sarcoplasmic/endoplasmic reticulum, mitochondria (80), and Golgi (65). Furthermore, the yeast homologues of Beclin 1 and Vps34 have been shown to mediate intracellular vesicle trafficking, including endosomes, and transport of lysosomal proteases from the Golgi to the lysosome (64, 102).

ROS and Redox Signaling in Autophagy

As mentioned earlier, autophagy is a unique catabolic pathway in eukaryotic cells, inducible by a variety of intracellular and extracellular stimuli such as starvation, environmental stress, damaged protein or organelles, and pathogenic infections for the degradation and recycling of macromolecules and long-living organelles. However, the exact molecular mechanism that induces autophagy under these stimuli is not known.

Recently, Scherz-Shouval *et al.* (121) showed that ROS generated during starvation act as signaling molecules to initiate autophagosome formation and autophagic degradation. Oxidative stress can induce autophagy under starvation and ischemia/reperfusion conditions (14, 62, 77, 119–121). Increasing evidence suggests that ROS play an important role in the induction of autophagy. For example, ROS are involved in the induction of neuronal autophagic cell death after withdrawal of nerve growth factor (68, 150). In Parkinson disease, oxidation of dopamine induces autophagy (35). In yeast, ROS-mediated lipid oxidation of mitochondria has been shown to induce autophagy (69). Tumor necrosis factor-α induces autophagy in an ROS-dependent mechanism (23). In plant cells, oxidative stress–induced oxidized proteins are removed through autophagy (148).

ROS are highly reactive oxygen-based chemical species, which include free radicals (superoxide and hydroxyl radicals, which have one or more unpaired electrons in the outer orbit) and nonradicals capable of generating free radicals, such as hydrogen peroxide. ROS are normally produced in cells during oxidative phosphorylation and play a role in redox control of physiologic signaling pathways (98). Excessive ROS can induce oxidation and damage to DNA, lipids, proteins, and other macromolecules (98). Intracellular ROS can be produced either through electron leakage during mitochondrial oxidative phosphorylation or through the activation of several cellular enzymes, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, and nitric oxide synthase (117). Superoxide is formed intracellularly by the activation of either NADPH oxidase or xanthine oxidase (98, 117), and it can rapidly react with nitric oxide to form $ONOO^-$ or convert into H_2O_2 to form hydroxyl radical (57).

The intracellular redox state is characterized by the balance of oxidant production and the antioxidant capacity of the cell, based on a variety of antioxidant enzymes such as superoxide dismutase (which reduces superoxide into H_2O_2), catalase, as well as glutathione peroxidase (which reduces H_2O_2 to H_2O). The nonenzymatic antioxidant molecules include vitamin E,

vitamin C, β-carotene, ubiquinone, lipoic acid, and urate (30). The thioredoxin system, including thioredoxin, thioredoxin reductase, and NADPH, forms an additional integrated antioxidant defense system, which operates as a powerful protein–disulfide oxidoreductase (60). At lower concentrations, ROS serve as second messengers that transmit biologic information, which is known as redox signaling. Redox signaling processes are involved in the activation of many signal-transduction protein kinases and transcription factors, the stimulation of DNA synthesis, and expression of growth-related genes (48).

Redox regulation by cysteine

The amino acid cysteine plays an essential role in the redox regulation of proteins through the thiol group present in cysteine. A number of cysteine-containing proteins, such as the thioredoxins, and enzymes, such as glutathione reductase, require redox-active cysteine residues for their activity (1, 105). Oxidative modification of cysteine residues in proteins influences the activity of many proteins (149) and has been implicated in cellular signaling and regulatory pathways (27). Cysteines can undergo various redox-dependent modifications, such as formation of disulfide bonds, S-nitrosylation, and the formation of sulfenic, sulfinic, and sulfonic acid, and sulfur-centered radicals (34). The low redox potential of cysteine allows rapid electron transfer from cysteine, resulting in thiyl radical and disulfide formation (33, 34).

The reverse reaction, for example, occurs in proteins such as human glutathione disulfide reductase and thioredoxin reductase, in which a two-electron transfer from the reduced form of flavin adenine dinucleotide reduces a cystine disulfide bond to two cysteine thiols (99). Changes in the cellular redox balance trigger signal-transduction pathways by modifying the oxidation state of cysteine residues in participating proteins. Redox modifications can exert a variety of effects on proteins ranging from inactivation of a catalytic residue to extensive structural changes (28, 33). Cysteine-harboring proteins serve as redox sensors, and they can undergo rapid and reversible posttranslational modifications in response to changes in the oxidative stress of the environment (33). Although many cysteine modifications irreversibly abolish protein function, the reversible (*e.g.*, *S*-thiolation, sulfenic acid formation) and some of the irreversible (*e.g.*, sulfinic and sulfonic acid formation) modifications have been recently considered to play a regulatory role during normal cell function, apoptosis, aging processes, and oxidative stress (27, 34).

Redox regulation in autophagy

Atg4s are cysteine proteases that share several conserved cysteine residues. Atg4s cleave Atg8s near the C-terminus, downstream of a conserved glycine (82). This cleavage allows the conjugation of Atg8 (LC3) to PE through the exposed glycine, a process mediated through a ubiquitination-like mechanism (67). Atg8-PE serves as another substrate for Atg4, which cleaves Atg8 and releases it from the autophagosomal membrane (67, 137). In the process of autophagy, after initial cleavage of Atg8-like proteins, Atg4 must become inactive to ensure the conjugation of Atg8 to the autophagosomal membrane. Later, as the autophagosome fuses with lysosome, Atg4 must be locally reactivated to delipidate and recycle Atg8.

In mammalian cells, amino-acid deprivation induces lipidation of LC3 as well as of GATE-16 and GABARAP (54, 55). Lipidated Atg8s associate with phagophores and autophagosomes and remain there until fusion with lysosomes, at which point intraautophagosomal Atg8 is probably degraded (54, 55). Recently, Atg4 was shown to be regulated by a redox-dependent mechanism during starvation-induced autophagy, in which the conjugation (lipidation) reaction of Atg4 is not being affected, but the deconjugation (delipidation) activity of Atg4 is redox regulated (121). Through *in vitro* studies, it has been demonstrated that a reducing environment activates the delipidation reaction of Atg4, but the oxidizing environment reversibly inactivates the delipidation reaction of Atg4 (121).

Starvation induces the formation of H_2O_2, a process which is partially dependent on class III PI3 kinase, leading to the inactivation of Atg4 at the site of autophagosome formation, and inactivation of Atg4 causes the accumulation of lipidated form of Atg8 (121). Redox regulation of several cysteine proteases, including cathepsin D, cathepsin B, and the cytosolic caspase-3, as well as calpains, has been shown to occur through a direct modification of the active site (34). Thus, redox regulations of Atg4 can cause rapid activation and inactivation of this protease, leading to induction of the autophagic process.

At least four Atg4 mammalian homologues have been reported. Two of the homologues, HsAtg4A and HsAtg4B, were shown to cleave the three mammalian Atg8s with different efficiencies: HsAtg4A cleaves mainly GATE-16, whereas HsAtg4B cleaves all three homologues (GATE-16, GABARAP, and LC3), with the highest efficiency for LC3 (54). Atg4A contains 12 cysteines, seven of which are highly conserved among tetrapod homologues of Atg4A and Atg4B. *In vitro* experiments show that cysteine 81, but not cysteine 77, in Atg4 is important for redox regulation, in which mutation of cysteine 81 to serine significantly impaired the redox activity of Atg4A and abolished the formation of GATE-16–labeled autophagosomes in cells (121). This study (121) indicated that oxidative conditions are essential for autophagy, as treatment with antioxidative agents abolished the formation of autophagosomes. Furthermore, the cysteine protease HsAtg4 is a direct target for oxidation by H_2O_2, and a cysteine residue located near the HsAtg4 catalytic site acts as a critical regulator (121). Atg4 shares a high level of structural homology with the papain superfamily (129), in which the cysteine is oxidized to sulfenic acid (107). In the same manner, Atg4 can be oxidized to sulfenic acid by H_2O_2. Although the formation of a disulfide bridge was not detected in the study of Scherz-Shouval *et al.* (121), the authors suggested the possibility of formation of a disulfide bridge between the catalytic cysteine (Cys77 or Cys74 of HsAtg4A or HsAtg4B, respectively) and the regulatory cysteine residue (Cys81 or Cys78 of HsAtg4A or HsAtg4B, respectively).

Oxidative stress and autophagy

Oxidatively modified macromolecules often crosslink with other proteins, lipids, carbohydrates, or metals, forming a brownish pigment known as lipofuscin (138). Lipofuscin, a biomarker of aging, is an autofluorescent pigment that accumulates during aging in the lysosomal lumen of postmitotic cells (138). The formation of lipofuscin requires the presence of H_2O_2 and ferrous iron in the lysosomal lumen. H_2O_2

generated by mitochondria and other organelles permeates into the lumen of secondary lysosomes (138). These lysosomes contain iron derived from cellular structures undergoing autophagic degradation. Diffusion of hydrogen peroxide into the lysosomes results in Fenton-type reactions with the formation of hydroxyl radicals and ensuing peroxidation of lysosomal contents with formation of lipofuscin (16).

Increased levels of oxidative stress also were shown to accumulate another autofluorescent pigment known as ceroid (58). The normal accumulation of lipofuscin in lysosomes seems to reduce the autophagic capacity of senescent postmitotic cells, which resulted in an insufficient autophagy, causing accumulation of damaged cellular components (16).

Oxidative stress enhanced the formation of hydroxyl radicals within lysosomes that risks the membrane stability of iron-rich lysosomes and autophagolysosomes (16, 62). Cells experimentally loaded with lipofuscin showed an increased susceptibility to oxidative stress, leading to lysosomal membrane breakage (16, 62). Nutrient deprivation in cells induces autophagy, which causes an increase in the availability of ferric iron pool, resulting in the increased sensitivity of cells to oxidative stress (103). The rupture of a limited number of lysosomes has been recognized as an early upstream event in many cases of apoptosis, particularly oxidative stress–induced apoptosis, whereas necrosis results from a major lysosomal break (10). Thus, the regulation of the lysosomal content of redox-active iron seems to be essential for the survival of cells in both the short and the long term. Oxidative stress can affect the permeability of the lysosomal membrane by inducing the crosslinking of lysosomal membrane proteins *via* disulphide bonds; it results in increased lysosomal proton permeability, luminal pH, and membrane potential (144). These results show that the redox status of the lysosomal membrane thiol groups influence its permeability (144).

Although autophagy is nonselective under oxidative conditions, it is an efficient mechanism in removing oxidatively damaged mitochondria, peroxisomes, and endoplasmic reticulum (66). Oxidative damage increases the permeability and depolarization of the mitochondrial membrane and results in leakage of intramitochondrial components such as cytochrome *c*, which initiates programmed cell death (2). Macroautophagy protects cells from major harm by removing the damaged mitochondria at an early stage (78). This protective macroautophagy is considered to be the crossroad between cell death and survival (125). Death-associated protein kinase and tumor necrosis factor–related apoptosis-inducing ligand are the two molecules that have been shown to be involved in the activation of both autophagy and apoptosis in a caspase-dependent or -independent manner (104). At low levels, these factors may induce macroautophagy; however, if the activated autophagy is insufficient to remove the cellular damage, the intracellular level of the two death factors would remain high to induce cell death. It is clear from these results that protective macroautophagy truly draws a borderline between cell survival and cell death, when autophagy and apoptosis are governed by the extent of oxidative stress.

Chaperone-mediated autophagy participates under mild oxidative-stress conditions by the removal of soluble and oxidatively modified proteins from the cytosol (63). During chaperone-mediated autophagy, oxidized substrate proteins are directly translocated into the lysosomal lumen (63). Oxidative modification of proteins facilitates the exposure of the lysosomal targeting motif, KFERQ, which is recognized through Hsc73 (20, 21). The substrate/chaperone complex is translocated into the lysosomal lumen *via* interaction with LAMP2a, a receptor protein present in the lysosomal membrane (26). Studies have shown that the lysosomal ability to internalize substrates is enhanced during mild oxidative stress in cultured cells and in animals (63).

Microautophagy refers to the formation of lysosomal membrane invaginations, which sequester the contents of cytoplasm and degrade them intralysosomally (16). Microautophagy is a constitutive mechanism for the continuous degradation of long-lived proteins inside many types of cells (16). However, the molecular mechanisms of microautophagy have not been well characterized. Although starvation has been shown to induce both macroautophagy and chaperone-mediated autophagy (16), the molecular events directing the autophagic route toward macroautophagy, microautophagy, and chaperone-mediated autophagy are not well understood. It is possible that the extent of oxidative stress may play a role in this process.

A genomic study by Thorpe *et al.* (142) showed that autophagic genes have been upregulated in response to protection against reactive oxygen species in *Saccharomyces cerevisiae*. Recently, Chen *et al.* (14) demonstrated that oxidative stress caused by treatment with H_2O_2 induces autophagic cell death in transformed and cancer cell lines. Inhibition of autophagosome accumulation through treatment with 3-methyl adenine and knocking down the genes involved in autophagy, such Beclin-1, *Atg5*, and *Atg7*, by treatment with respective siRNAs effectively blocked the oxidative stress–induced autophagic cell death. Inhibiting ROS generation by treatment with the ROS scavenger tiron inhibited autophagic cell death. Further, it was demonstrated that H_2O_2-induced autophagic cell death is independent of apoptosis (14). Yu *et al.* (155) showed that inhibition of caspase induces the degradation of catalase, leading to the accumulation of ROS and autophagic cell death. Taken together, these studies clearly indicate the role of ROS in the induction of autophagy.

Autophagy in Ventricular Remodeling

Autophagy in cardiac myocytes was first reported by Sybers *et al.* in 1976 (130), approximately one decade after the initial description of autophagy in mammalian cells (18). In the study of Sybers *et al.* (130), the formation of autophagic vacuoles was revealed through electron microscopy, which showed the presence of damaged organelles within autophagic vacuoles. This process was accelerated by transient deprivation of oxygen and glucose, followed by resupply of oxygen and glucose (130).

A disturbed balance between a high rate of ubiquitination and inadequate degradation of ubiquitin/protein conjugates contributes to autophagic cell death in failing human hearts (73). Autophagic cell death in cardiac tissue of patients in the terminal stage of heart failure, as a consequence of either ischemic cardiomyopathy or dilated cardiomyopathy (DCM), was demonstrated by granular cytoplasmic ubiquitin inclusions, an established marker of autophagocytosis (72). Interestingly, these autophagic cardiomyocytes were not only terminal dUTP nick-end labeling (TUNEL) and activated caspase-3 negative but also were negative for C9, a marker for

necrosis (72). The results of this study reveal that cardiomyocytes in heart failure show caspase-independent autophagic cell death rather than apoptotic cell death (72). Recently, dead and dying cardiomyocytes showing characteristics of autophagy were reported in heart failure caused by DCM and valvular and hypertensive heart disease (22).

Hein *et al.* (46, 47) showed that progression from compensated hypertrophy to heart failure and left ventricular systolic dysfunction in the pressure-overloaded human heart involves cell loss, mainly by autophagy and oncosis. In the myocardium of patients with DCM, autophagy appears to be associated not only with degradation of damaged intracellular organelles but also with progressive destruction of cardiomyocytes (126). Strong staining for LAMP-1 and cathepsin D indicated increased activity of lysosomal functions in atrophic and degenerated cardiomyocytes (126). These degenerated cardiomyocytes were mostly adjacent to the area with massive replacement fibrosis and fatty tissue, where cardiomyocyte loss was highly indicative. Both electron microscopy and immunohistochemistry indicated that autophagic and lysosomal functions may contribute to cellular atrophy and the destruction leading to cell death, as well as the degradation of damaged intracellular organelles (126).

Yan *et al*. (151, 152) found that in chronically ischemic myocardium, a significant increase is seen in the expression of autophagic marker proteins, including cathepsin D, cathepsin B, heat-shock cognate protein Hsc73 (a key protein marker for chaperone-mediated autophagy), Beclin 1, and LC3. It was shown that the myocytes containing autophagic vacuoles are observed in chronically ischemic myocardium in live, but not in lysed cells. In contrast, autophagic markers are downregulated in infarcted myocardium. Apoptosis is maximal after three episodes of ischemia, but declined markedly after six episodes (152). The appearance of autophagy becomes evident after three episodes of ischemia and is fully manifest after six episodes of ischemia. Immunostaining of cathepsin B is much more abundant in myocytes in nonapoptotic regions, but myocytes in apoptotic regions rarely stained positive for cathepsin B (152). Such evidence suggests that autophagy triggered by ischemia could be a homeostatic mechanism, by which apoptosis is inhibited and the deleterious effects of chronic ischemia are limited (151, 152).

In human hibernating myocardium, ubiquitin-related autophagic cell death was identified by the occurrence of autophagic vacuoles, cellular degeneration, and nuclear disassembly. In addition to ubiquitin-related autophagic cell death, loss of myocytes by apoptosis also was observed in hibernating myocardium (24, 25). In a case of unclassified cardiomyopathy, prominent autophagic degeneration was accompanied by an elevated level of plasma brain natriuretic peptide (600 μg/ml) despite the lack of overt heart failure. Immunohistochemical and electron-microscopic analysis revealed the staining of BNP and the presence of autophagosomes, respectively (113). In a hamster model of human dilated cardiomyopathy, autophagic degeneration of cardiomyocytes led to cell death (133). Treatment with granulocyte colony-stimulating factor (G-CSF) significantly improved survival and remodeling, which was accompanied by a reduction in autophagy (133). G-CSF treatment induced the activation of Akt and signal transducer and activator of transcription-3, followed by a reduction in the level of myocardial tumor necrosis factor-α (133). Recently, it was shown that pressure overload, a major risk factor for cardiac hypertrophy and heart failure, triggers basal autophagy, particularly in the basal septum (157).

Autophagy plays a crucial role in normal heart function and serves as a catabolic energy source during starvation. Autophagy in cardiac myocytes has been suggested to provide a necessary source of energy between birth and suckling (76). Cardiac myocytes from starved animals display high numbers of autophagosomes, which aid in cell survival in the adverse conditions of nutrient deprivation (94). Apart from cardiomyocytes, autophagy also occurs in interstitial cells of the aortic valves of patients with severe aortic-valve stenosis (91, 128), in which cell death in the calcification of aortic leaflets is shown to be caused by autophagy rather than apoptosis. The relation between cell growth and autophagic vacuole volume during cardiac hypertrophy induced by supravalvular aortic constriction indicates that the degradation of cytoplasmic components by autophagy is inhibited in cardiomyocytes undergoing hypertrophy (91, 128). Such an anticatabolic reaction seems to play an important role in establishing the positive balance of cellular metabolism generally required for growth processes.

Cardiac-specific loss of *Atg5* in mice led to cardiac hypertrophy, left ventricular dilatation, and contractile dysfunction, accompanied by increased levels of ubiquitination (100). Moreover, *Atg5*-deficient hearts showed disorganized sarcomere structure and mitochondrial aggregation. Under baseline conditions, cardiac-specific deficiency of *Atg5* in early cardiogenesis does not show any abnormalities but develops cardiac dysfunction and left ventricular dilatation 1 week after pressure overload (100). These results indicate that, under baseline conditions, constitutive autophagy in the heart is a homeostatic mechanism for maintaining cardiomyocyte size and global cardiac structure and function, and that upregulation of autophagy in failing hearts is an adaptive response for protecting cells from hemodynamic stress (100). The embryonic *Atg5*-knockout mice are viable and live to adulthood without any detectable heart abnormalities (100), presumably owing to compensatory mechanisms that also perform cellular maintenance. Defective autophagic degradation owing to the deficiency of LAMP-2 in mice manifested a vacuolar cardioskeletal myopathy leading to severe cardiac dysfunction, a disorder also known as Danon disease (134).

Cardiac ischemia and glucose deprivation in cardiac myocytes induce autophagy *via* activation of AMPK, a sensitive nutrient sensor (85, 86). In contrast, autophagy induced during the reperfusion phase is accompanied by the upregulation of Beclin 1, but not by AMPK activation (85, 86). Glucose deprivation has been shown to increase AMPK and eEF2 phosphorylation and to decrease p70S6K and mTOR phosphorylation (85). Apart from being a sensor of amino acids, mTOR also can sense changes in the cellular energy state *via* AMPK (88). In yeast and mammalian cells, activation of AMPK was shown to stimulate autophagy (127, 146). Activation of AMPK inhibits mTOR-dependent signaling and protein synthesis, (88) which are consistent with the AMPK function of switching off ATP-dependent processes (45). In contrast, activation of AMPK by addition of the cell-permeable nucleotide analogue 5-amino-4-imidazole carboxamide riboside and N6-mercaptopurine in hepatocytes strongly inhibits autophagy (115, 116). Insulin signaling is known to activate mTOR leading to the suppression of

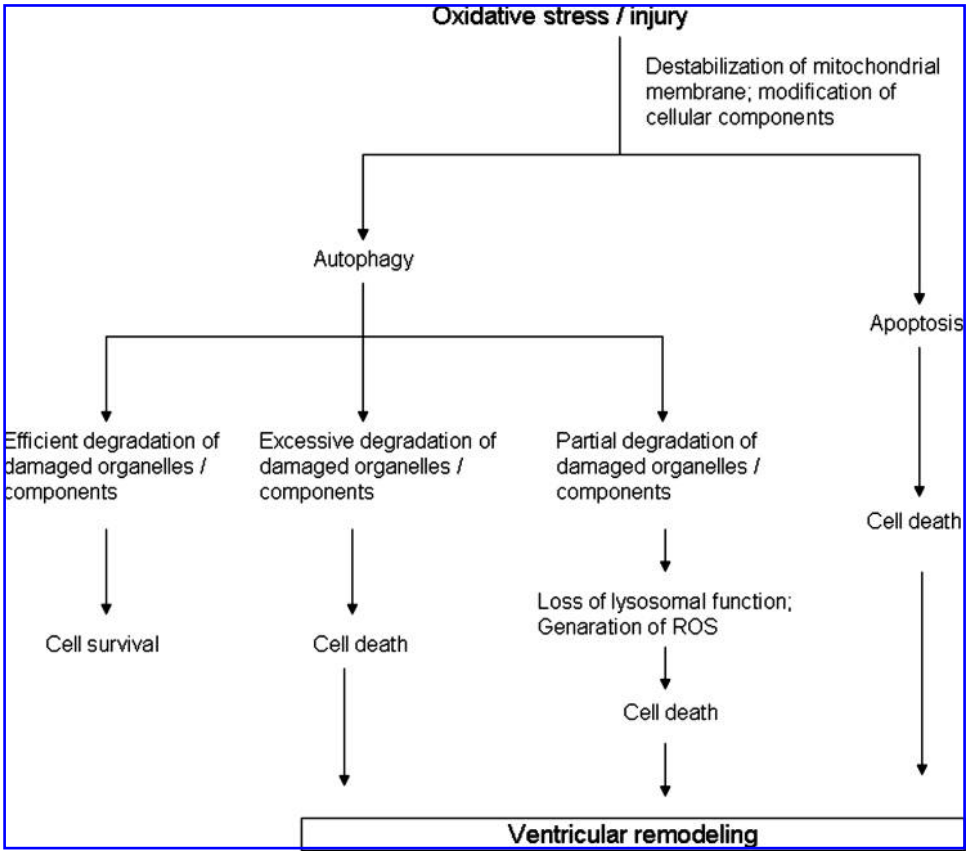

FIG. 2. Schematic diagram showing the putative role of autophagy in ventricular remodeling.

autophagy (89). In cardiac muscle, insulin antagonizes the activation of AMPK (5, 31), and this appears to involve activation of the protein kinase Akt (74). Phosphorylation of AMPK subunits on Ser485 or Ser491 by Akt antagonizes AMPK activation *via* phosphorylation at Thr172 by LKB1 kinase (50). mTOR was shown to be associated with the mitochondrial outer membrane (122); thus, mTOR could sense changes in the ATP/AMP ratio (88) and control autophagy of individual mitochondria. These results suggest that AMPK may be required for the autophagy of mitochondria in particular (88).

The reperfusion-induced cardiac injury and autophagy were significantly attenuated in *Beclin* $1^{+/-}$ mice (84). These results indicate that autophagy plays distinct roles during ischemia and reperfusion (*i.e.*, autophagy elicited during ischemia is protective; however, the autophagy induced during the reperfusion phase may be detrimental). Ischemia/reperfusion injury in cardiac HL-1 cells impaired the formation and downstream lysosomal degradation of autophagosomes (42). Overexpression of *Beclin 1* enhanced autophagic flux after ischemia/reperfusion (I/R) and significantly reduced the activation of proapoptotic Bax, whereas knockdown of *Beclin 1* by RNA interference increased Bax activation (42). These results demonstrate that autophagic flux is impaired at the level of both induction and degradation during stimulated ischemia/reperfusion, and enhancing autophagy stimulates a powerful protective mechanism against ischemia/reperfusion injury in cardiac cells (42). Bnip3 is a mitochondrial proapoptotic Bcl-2 protein expressed in the adult myocardium (44). Hamacher-Brady *et al.* (43, 44) demonstrated that Bnip3 contributes to cardiac I/R injury in a Bnip3-dependent manner. Bnip3 causes disruption of mitochondrial integrity, leading to enhanced superoxide production and the release of proapoptotic factors, such as cytochrome *c* and apoptosis-inducing factor (43, 44). These results imply that Bnip3 contributes to myocardial injury, which triggers a protective stress response *via* upregulation of autophagy, which in turn helps with the removal of damaged mitochondria (43, 44).

Taken together, it is assumed that autophagy functions in the heart predominantly as a prosurvival mechanism during nutrient deprivation and other forms of cellular stress (Fig. 2). However, when autophagy is induced rigorously, the autophagic machinery might also be used for self-destruction. In this way, autophagic cell death can occur in a detectable number of cardiac cells and may lead finally to ventricular remodeling and heart failure (Fig. 2). Macroautophagy was shown to protect cells from apoptosis by removing the damaged mitochondria at an early stage (78). This protective macroautophagy is considered to be the crossroad between cell death and survival (125). Both autophagy and apoptosis are induced by death-associated protein kinase and tumor necrosis factor–related apoptosis-inducing ligand (104). At low levels of these factors, autophagy may be induced; however, if the activated autophagy is insufficient to remove the cellular damage, the intracellular level of the two death factors would remain high to induce cell death. Insufficient autophagy or partial degradation of damaged organelles or components causes the generation of ROS and loss of lysosomal function, leading to cell death and cardiac remodeling (75) (Fig. 2).

Therapeutic Implications

Therapeutic advances for the modulation of autophagy in cardiovascular disease are very limited with the identification of many genes, and the intracellular regulators of autophagy are revealed to some extent. Because class III PI3 kinases are known to induce autophagy, several studies have implicated the use of inhibitors of the class III PI3 kinase pathway like wortmannin and 3-methyl adenine (41). However, these drugs are not suitable for therapeutic applications because they are highly toxic and are known to have multiple targets *in vivo*.

The serine/threonine kinase TOR is a key molecule in nutrient-sensing signal pathways in response to nutrients, amino acids, and mitogens; it participates in the regulation of autophagy. Rapamycin, an inhibitor of mTOR, was shown to confer preconditioning-like protection against I/R injury in isolated mouse heart through the opening of mitochondrial K_{ATP} channels (61). Treatment with rapamycin reduced cardiac infarct size, although the infarct-limiting effect of rapamycin was not associated with improved recovery of ventricular function. Further, at low doses (25–100 n*M*), rapamycin reduces necrosis as well as apoptosis after simulated ischemia-reoxygenation in adult cardiomyocytes (61).

In a hamster model of human dilated cardiomyopathy, treatment with G-CSF significantly improved the autophagic degeneration of cardiomyocytes and cardiac remodeling, which is accompanied by the activation of Akt and signal transducer and activator of transcription-3, followed by a reduction in the level of myocardial tumor necrosis factor-α (133). Treatment with urocortin, an endogenous cardiac peptide, was shown to reduce autophagic and apoptotic cell death induced by I/R (143). The inhibition of autophagy by urocortin is mediated in part by inhibition of *Beclin 1* expression, an effect mediated by activation of the PI3 kinase/Akt pathway but which does not involve activation of p42/p44 MAPK (143).

In addition, treatment with propranolol, a nonselective beta blocker, and verapamil, calcium-channel blocker, has been shown to induce autophagy (4). However, treatment with β-adrenoreceptor agonist isoproterenol inhibited the formation of autophagic vacuoles (108). Because verapamil affects neither the β-adrenoreceptors nor the intracellular levels of the second-messenger cyclic AMP, the induction of cellular autophagy is suggested to be an early regulatory step in the adaptation of heart muscle mass to reduced work load (108).

Although autophagy has been shown to play both protective and detrimental roles in cardiac remodeling, therapeutic implications for the regulation of autophagy in ventricular remodeling induce skepticism, which is accentuated by our limited knowledge of the genes and intracellular regulators of autophagy.

Conclusions

Although autophagy is induced with several cardiac diseases like ischemic cardiomyopathy, dilated cardiomyopathy, cardiac hypertrophy, and ischemia/reperfusion injury, the role of autophagy under those conditions is controversial (*i.e.*, whether the effect of autophagy is protective or detrimental for the heart). Although autophagy is nonselective, under oxidative conditions, it was shown to be an efficient mechanism in removing oxidatively damaged mitochondria, peroxisomes, and endoplasmic reticulum (66). Oxidative modification of cysteine residues in proteins influences the biologic activity of many proteins (149), and recently, Atg4 (specifically, cysteine 81 of Atg4) was shown to be regulated by a redox-dependent mechanism during starvation-induced autophagy (121). *In vitro* cell-free assays show that a reducing environment activates Atg4, whereas an oxidizing condition inhibits the activity of Atg4. Starvation induces the production of H_2O_2, which could inhibit the cleavage activity of Atg4 (121). Autophagy is considered an energy-efficient recycling system, as it supplies amino acids for the synthesis of essential proteins during starvation and thus helps to extend cell survival. This indicates that ROS produced during starvation-induced autophagy play an important role in the regulation of autophagy and, in turn, extend the life of cell. Further studies are required to explore the possibilities of redox regulation of other autophagic genes and the role of redox signaling–mediated autophagy in the heart.

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Abbreviations

4E-BP1, 4E-binding protein 1; AMP, adenosine monophosphate; AMPK, adenosine monophosphate–activated protein kinase; ATP, adenosine triphosphate; DCM, dilated cardiomyopathy; GABARAP, γ-aminobutyric acid type A receptor–associated protein; GATE-16, Golgi-associated ATPase enhancer of 16 kDa; G-CSF, granulocyte colony-stimulating factor; GMP, guanosine monophosphate; HsAtg4A, human homologues of Atg4 or autophagin 2; HsAtg4B, human homologues of Atg4 or autophagin 1; Hsc73, heat-shock protein; I/R, ischemia/reperfusion; LAMP2a, lysosome-associated membrane protein type 2a; LC3, microtubule-associated protein light-chain 3; LC3-II, complex of LC3-I and PE; mTOR, mammalian target of rapamycin; NADPH, nicotinamide adenine dinucleotide phosphate; PE, phosphatidylethanolamine; PI3-K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; S6K, S6 kinase; TUNEL, terminal dUTP nick-end labeling.

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