Autophagy in the Heart

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The autophagic machinery is a well-conserved degradation system in eukaryotes from yeast to mammals. Autophagy has been thought of as a nonselective degradation process in which cytoplasmic proteins and organelles are degraded by fusion with lysosome. Recent studies have revealed selective forms of autophagy, such as mitochondria-specific autophagy, termed “mitophagy”. Research over the past decade has revealed that autophagy in cardiomyocytes plays a protective role, not only during hemodynamic stress but in homeostasis during aging. Hemodynamic stress and aging induce mitochondrial damage, leading to increased oxidative stress and decreased ATP production. Damaged mitochondria are generally degraded through mitophagy, which might be the main protective function of autophagy in the heart. Complete digestion of mitochondrial DNA through mitophagy is important to avoid inflammatory responses that can induce heart failure. A polyamine, spermidine, is reported to bring about an extension of lifespan and to protect the heart from age-related cardiac dysfunction, both of which are mediated through induction of autophagy. Therefore, appropriate induction of autophagy could be a novel therapeutic target for cardiovascular diseases, including heart failure. However, precise evaluation of autophagic activity in the human heart is difficult at this time, but exploitation of the novel technique of autophagy evaluation is expected for both drug discovery and clinical application.

Key Words: Autophagy; Heart failure; Inflammation; Mitochondria; Mitophagy

The 2016 Nobel Prize in physiology and medicine was awarded to Dr. Yoshinori Ohsumi for his discovery of the molecular mechanism for autophagy. Recent studies have shown that autophagy is involved in various phenomena in tissues and cells, and is also thought to play a pivotal role in maintaining physiological function in cardiomyocytes and the heart, making it important for cardiologists to understand the autophagic process.

Dr. Christian de Duve was the first to use the term “autophagy”, which means “self-eating” in Greek, at the Ciba Foundation Symposium on Lysosome in 1963. Autophagy via lysosomes is a major degradation system for intracellular components such as cytosolic protein and organelles. Three types of autophagy, macroautophagy, microautophagy, and chaperone-mediated autophagy, utilize distinct molecular pathways to deliver each substrate to lysosomes.¹ The molecular mechanism and function of macroautophagy have been well studied, and will hereafter be referred to as autophagy in this review, as in other articles.

In autophagy, cytosolic proteins and organelles such as mitochondria are sequestered in a double-membrane vacuole, the isolation membrane, that fuses with the lysosome to form an autolysosome. The contents of the autolysosomes are then degraded for recycling, synthesis, and generation of ATP² (Figure 1). Autophagy is a widely conserved system in eukaryotes from yeasts to mammals and is regulated by autophagy-related (*Atg*) genes, which were first

identified in yeasts. Dr. Ohsumi discovered the molecular mechanism of autophagy by using a mutant yeast that could not induce autophagy.³ Most of the *Atg* genes are conserved in mammals; however, some *Atg* homologs in mammals have not been identified yet. Autophagy is considered to be a nonselective degradation system, with one of its principal roles being to provide nutrients for survival during starvation. Starvation is one of the strongest stimuli to induce autophagy. When wild-type mice are starved for 24 h, most organs, except the nervous system, show significantly increased levels of autophagy. However, autophagy occurs not only under starvation conditions but also in basal physiological conditions. Gene-targeting of the *Atg* family in various organs has revealed that basal constitutive autophagy is important for clearance of proteins and organelles in order to maintain homeostasis and the functions of cell and organs.^{4,5} Autophagy is essential during the early neonatal period,⁶ and its deficiency in the central nervous system causes neurodegenerative diseases.^{7,8} Autophagy is involved in many more process, such as the turnover of mitochondria, the regulation of lipid metabolism, degradation of intracellular bacteria and viruses, and antigen presentation.^{9,10} Through these functions, autophagy can affect the pathophysiology of multiple human diseases such as neurodegenerative diseases,¹¹ cancer,¹² inflammatory bowel disease,¹³ and cardiac diseases.

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Figure 1. Summary schematic of the autophagic machinery. Cytosolic protein and organelles are engulfed by the isolation membrane to form an autophagosome, and then fused with a lysosome to become an autolysosome. The cargo is degraded for energy production and recycling. (Modified from Oka T, Yamaguchi O. *Shinshu ON-SITE* 2014; 9: 12–13.)

Molecular Mechanism of Autophagy

Formation of autophagosomes comprises 3 steps,^{14,15} although the initiation site of autophagosome formation is controversial.¹⁶ Recently, it was reported that the endoplasmic reticulum (ER)-mitochondria contact site is important for autophagosome formation.¹⁷ The Unc-51-like kinase (ULK) complex initiates formation of autophagic double-membrane vesicles. The ULK complex contains ULK1 or 2, Atg13, Atg101, and focal adhesion kinase family interacting protein of 200 kDa (FIP200).⁵ The mammalian homolog of the rapamycin (mTOR) complex reduces autophagic activity thorough suppression of the ULK complex. The class III phosphoinositide 3-kinase (PI3K) complex, comprising beclin 1, Atg14L, Vps34, and Vps15, is also an essential component in the initiation of autophagy. Starvation or AMP-activated protein kinase (AMPK) can activate the ULK complex, which regulates the PI3K complex. After initiation, the isolation membrane is elongated by a lipid kinase signaling complex, and ubiquitin-like protein conjugation pathways are required for vesicle expansion and completion. Two ubiquitin-like conjugation systems, Atg5, Atg12 and Atg16L1 complex and microtubule-associated protein 1 light chain 3 (LC3), play an essential role in these steps. The conjugation induces LC3-I to convert to LC3-II, a phosphatidylethanolamine-conjugated form. LC3-II is located on the autophagosome membrane and is widely used as a marker of autophagosome formation in experimental assays. In the final step, the autophagosome fuses with the lysosome to form an autolysosome, in which the sequestered materials and inner membrane of the autophagosome are degraded by acid hydrolases from the lysosome. These contents can be recycled for ATP production or protein synthesis for cell survival. The interaction between the autophagosome and lysosome is mediated by syntaxin 17 (Stx17) and SNAREs,

such as synaptosome-associated protein 29 (SNAP29) and vesicle-associated membrane protein 8 (VAMP8).¹⁸

Quality Control of Mitochondria by Autophagy

Mitochondria are essential organelles that produce ATP through oxidative phosphorylation for cell survival. Extrinsic or intrinsic agents induced by hemodynamic stress can impair the mitochondria, and these damaged mitochondria can generate reactive oxygen species (ROS) as byproducts, leading to cellular dysfunction and cell death. Accumulation of damaged mitochondria is often observed in various diseases, including heart failure, neurodegenerative diseases, and aging-related organ disorders. Thus quality control of mitochondria is important for maintenance of cellular homeostasis, particularly in cardiomyocytes. Because cardiomyocytes are terminally differentiated cells, they cannot dilute ROS or other harmful agents by cell division. Recent studies have revealed selective autophagy, which targets specific proteins or organelles such as mitochondria to lysosomes for degradation, termed “mitophagy”. Dysregulation of mitophagy is implicated in the development of many diseases and disorders. The precise molecular machinery of mitophagy was first unveiled in yeasts.^{19,20} By genome-wide screening, Atg32 was found to be an essential mitophagy receptor protein on the outer mitochondrial membrane in yeasts.^{21,22} Atg32 binds to Atg8, a yeast homolog of LC3, through its WXXI motif to induce mitophagy.

Similar to Atg32 in yeast, mitophagy receptors in mammalian cells are expected to localize to the outer mitochondrial membrane and interact with autophagy-related proteins such as LC3. Several mitophagy-related factors have been identified in mammalian cells, including phosphatase and tensin homolog-induced putative protein kinase 1 (PINK1)/Parkin, NIX/BNIP3L, FUNDC1, and

BCL2L13 (Bcl-rambo).^{23–26}

PINK1 and E3 ubiquitin ligase Parkin play important roles in the selective elimination of damaged mitochondria that have lost their membrane potential.^{26–29} The role of Parkin in mitophagy is well established. Mitofusins (Mfns), located in the outer mitochondrial membrane, act as key regulators of mitochondrial fusion.³⁰ Mfn2 is also a Parkin ubiquitination substrate,³¹ and acts as a receptor for Parkin on damaged mitochondria, thereby facilitating mitophagy.²⁸ Parkin binds to Mfn2, and the association is enhanced in a PINK1-dependent manner. In the absence of Mfn2, translocation of Parkin to the mitochondria and the subsequent mitophagic pathway mediated through the PINK1-Parkin axis are inhibited. Another pathway to delivering mitochondria to the lysosomes was recently reported. Mitochondria that have been ubiquitinated by Parkin are sequestered inside Rab5-positive early endosomes mediated through the endosomal sorting complexes required for transport (ESCRT) machinery, and then early endosomes, including mitochondria, mature into Rab7-positive late endosomes to be delivered to the lysosomes for degradation.³²

Cardiac-specific deletion of Parkin in the perinatal period induces lethal cardiomyopathy.³³ Elimination of fetal mitochondria in a Parkin-dependent manner through replacement by adult mitochondria in cardiomyocytes soon after birth is necessary for metabolic transitioning from carbohydrates to fatty acids. However, cardiac-specific deletion of Parkin in adult mice showed no significant cardiac phenotypes,³⁴ which indicates that Parkin-mediated mitophagy is not the main pathway for cardiac homeostasis in the adult heart. Furthermore, Parkin is expressed in most adult tissues, but some fetal tissues and cell lines that have little or no endogenous expression of Parkin can induce mitophagy.^{27,35} It is reasonable to assume, therefore, that there might be other cell- or tissue-specific molecules involved in mitophagy.

FUNDC1, which is localized on a mitochondrial outer-membrane, acts as a receptor for hypoxia-induced mitophagy.²³ The mitophagic activity induced by FUNDC1 is inhibited through Src kinase, casein kinase-2, or Bcl-xL.^{36,37}

NIX is a BH-3-only family protein, which induces cell death and autophagy.³⁸ NIX eliminates mitochondria from erythrocytes to develop red blood cells by its interaction with LC3 and GABARAP through its N-terminal LIR motif.^{25,39} In cardiomyocytes, NIX plays an important role during necrotic cell death induced by mitochondrial permeability transition. NIX also has an essential function in “mitochondrial pruning”,⁴⁰ which restricts mitochondrial proliferation and prevents accumulation of abnormal mitochondria.

Using the molecular profile of Atg32 as a search tool, we identified BCL2L13 (also known as Bcl-rambo) as a novel mitophagy receptor.²⁴ BCL2L13 protein contains a single transmembrane domain at its C-terminus and 4 BCL2 homology domains (BH1–4).⁴¹ Overexpression of BCL2L13 induces mitochondrial fragmentation, whereas the knock-down of BCL2L13 induces mitochondrial elongation, indicating that BCL2L13 plays an important role in mitochondrial fission. Mitochondria change their morphology continuously during the process of fusion and fission, termed “mitochondrial dynamics”. Mitochondrial dynamics are closely involved in the process of mitochondrial quality control by mitophagy. Mitochondrial fusion is mediated by Mfn1 and 2, and optic atrophy 1 (Opa1). Mitochondrial

fission is mainly mediated by dynamin-related protein 1 (Drp1). Mitochondrial fragmentation by fission commonly precedes mitophagy.²⁴ In yeasts, Atg32 can induce mitophagy, but not mitochondrial fission. Expression of BCL2L13 in *atg32*-deficient yeast can rescue mitophagy deficiency. Additionally, BCL2L13-dependent mitophagy is mediated through the canonical autophagy pathway. Phosphorylation of BCL2L13 is speculated to regulate its mitophagic activity, but the responsible kinase has not been elucidated. There is little understanding of the role of BCL2L13 in the heart and other organs. Analysis of gene-targeting animal models, such as cardiac-specific BCL2L13-deficient mice, will reveal its *in vivo* role in the heart under physiological and pathological conditions.

Assessment of Autophagic Activity

The 3rd guideline for the use and interpretation of assays for monitoring autophagy states “no individual assay is guaranteed to be the most appropriate one in every situation, and we strongly recommend the use of multiple assays to monitor autophagy”.⁴² The gold standard for assessing the volume or number of autophagosomes is electron microscopy. However, they are not easy to identify. Immunoelectron microscopic analysis using LC3-antibody is better for identifying autophagosome in cells or tissues. The expression level of LC3-II on the autophagosome has been used as a specific marker of autophagy, which can be assessed by assays such as western blotting.⁴³ The accumulation of autophagosomes, as seen by electron microscopy, or an increase in LC3-II expression does not necessarily mean the upregulation of autophagic activity or autophagic flux. These specific observations would also be seen without an increase in autophagic activity, if the fusion step between the autophagosome and lysosome was inhibited. The further increase in LC3-II expression by inhibition of the fusion step through bafilomycin A1 strengthens the notion that autophagic flux is upregulated.⁴⁴ Recently, a novel fluorescent probe was developed to evaluate autophagic flux,⁴⁵ and will be useful for screening or evaluating autophagy inducers or inhibitors. Keima is a coral-derived lysosomal proteases-resistant fluorescent protein that exhibits pH-dependent excitation.⁴⁶ A mitochondria-targeting form of Keima (mtKeima) can detect the delivery of mitochondria to lysosomes. A model using transgenic mice expressing mtKeima is a promising tool for detecting mitophagy *in vivo*.⁴⁷ However, we cannot utilize any of these methods in the human body to assess autophagic flux. Thus there is a need for a novel evaluation system for autophagic flux within the human body or specific organs.

Role of Cardiac Autophagy Under Physiological Conditions

To elucidate the role of autophagy under baseline conditions, we generated tamoxifen-induced cardiac-specific Atg5-deficient mice using the MERCreMER-loxP system.⁴⁸ Atg5 is an essential protein during autophagosome formation. Cardiac-specific rapid ablation of Atg5 induced severe cardiac dysfunction, accompanied by heart failure.⁴⁹ The autophagy-deficient heart exhibited accumulation of ubiquitinated protein and increased ER stress. Pathological analyses revealed a disorganized sarcomere structure, misalignment and aggregation of mitochondria, and apoptotic cardiomyocyte death in the Atg5-deficient

hearts. On the other hand, cardiac-specific ablation of Atg5 in the embryo did not induce cardiac dysfunction at 10 weeks of age. It has been reported that Atg5/Atg7-independent autophagosomes are generated in a Rab9-dependent manner. Atg5/Atg7-independent autophagy may compensate for deficiency of Atg5.⁵⁰

Age-related reduction of autophagic activity has been reported.^{51,52} Cardiac-specific deletion of Atg5 also induces age-related cardiac dysfunction. Atg5-deficient mice begin to die of heart failure at 6 months of age.⁵³ Atg5-deficient mice have a disorganized sarcomere structure and collapsed mitochondria with decreased mitochondrial respiratory functions. These observations indicate that constitutive autophagy under baseline conditions and during aging plays a pivotal role in maintaining cardiac homeostasis, such as the size of cardiomyocytes, cardiac function, global structure, and mitochondrial morphology. Quality control of mitochondria is a major role of autophagy in cardiomyocytes under baseline conditions. It has recently been reported that Rab9-dependent alternative autophagy plays a crucial role in mediating mitophagy in cardiomyocytes during ischemia.⁵⁴

Role of Autophagy in Cardiac Hypertrophy

Cardiac hypertrophy is a compensatory adaptation to hemodynamic stress in order to decrease wall stress and maintain cardiac output. If the heart is exposed to excessive load, the hypertrophic response can cause collapse leading to heart failure. Knockdown of Atg7 in isolated rat neonatal cardiomyocytes induced significant hypertrophy.⁴⁹ Autophagic activity evaluated by LC3-II indicated that autophagic activity was downregulated in the hypertrophic heart 1 week after pressure overload induced by thoracic aortic constriction (TAC).⁴⁹ However, the reduction in autophagic activity during the cardiac hypertrophic period is controversial. Heart weights of cardiac-specific Atg5-deficient

mice and their controls were similar even after continuous infusion of angiotensin II or mild TAC.⁵⁵ These observations indicate that Atg5-dependent autophagy in cardiomyocytes is not necessary for cardiac hypertrophy induced by hemodynamic stress. On the other hand, cardiac autophagy is an essential step in inducing reverse remodeling, which is characterized by regression of hypertrophy.⁵⁶ Reverse remodeling is observed after removal of hemodynamic stress. Regression of cardiac hypertrophy after unloading of neurohumoral or hemodynamic stress was significantly attenuated in cardiac-specific Atg5-deficient mice.⁵⁵

Role of Autophagy During Heart Failure

Autophagic vacuoles are observed in the failing heart⁵⁷ and autophagic activity was upregulated in a wild-type failing heart, induced by 4 weeks' TAC.⁴⁹ To elucidate the role of autophagy in the failing heart, cardiac-specific Atg5-deficient mice were subjected to TAC or isoproterenol infusion. The mice showed a significant increase in left ventricular dimensions and decreased fractional shortening of the left ventricle compared with the control mice, indicating that upregulation of autophagy induced by hemodynamic stress is a cardioprotective mechanism (Figure 2).⁴⁹ Accumulation of ubiquitinated protein, increased ER-stress, and apoptotic cardiomyocyte death were also observed in Atg5-deficient hearts. Deletion of Mst1, which inhibits autophagy, has a protective role in cardiac remodeling after myocardial infarction.⁵⁸ Autophagosome formation in patients with dilated cardiomyopathy has a positive correlation with better prognosis, highlighting the protective role of autophagy in heart failure.⁵⁹ However, heterozygous deletion of beclin 1 improved cardiac function after TAC.⁶⁰ Excessive autophagy could be maladaptive through degradation of necessary proteins and mitochondria for cell survival.⁶¹

An R120G missense mutation in the alphaB-crystallin chaperone gene (CryAB^{R120G}) causes an autosomal dominant desmin-related myopathy. Accumulation of misfolded protein is observed in skeletal and cardiac muscle.⁶² Cardiac dysfunction induced by overexpression of CryAB^{R120G} in mice was attenuated by cardiac-specific overexpression of Atg7, which normally increases autophagic activity.⁶³ This suggests that autophagic degradation can protect the heart from protein aggregation. In a subset of hypertrophic cardiomyopathy (HCM) patients, the expression level of Vps34 is reduced.⁶⁴ Muscle-specific deletion of Vps34 results in cardiac hypertrophy and sudden death in mice.⁶⁵ Accumulation of alphaB-crystallin is observed in both the Vps34-deficient mouse heart and myocardium from HCM patients whose Vps34 expression is decreased.

An X-linked mutant of lysosome-associated membrane protein 2 (LAMP 2) causes Danon disease, which exhibits lysosomal glycogen storage to induce HCM and neural disorders, accompanied by accumulation of autophagic vacuoles. LAMP2 deficiency disrupts the fusion between autophagosome and lysosome, leading to decreased autophagy flux and increased accumulation of autophagic vacuoles.^{66,67}

Inflammatory Responses Induced by Mitochondrial DNA

Hemodynamic stress induces mitochondrial damage and

Table. Potential Cardioprotective Drugs in the Heart Through Induction of Autophagy		
Agent	Model	Effects
Spermidine ⁷⁴	Aging	Improved cardiac function
Carvedilol ⁴⁵	In vitro	Induced autophagy
Trehalose ⁷⁸	TSC2-deficiency	Attenuated cardiac dysfunction
Resveratrol ^{82,83}	MI	Attenuated cardiac remodeling
	Doxorubicin	Attenuated cardiac remodeling
Metformin ⁸⁵	Diabetes mellitus	Improved cardiac function
Simvastatin ⁸⁶	IR	Reduced infarct size
Suberoylanilide hydroxamic acid ⁸⁷	IR	Reduced infarct size
Everolimus ⁸⁸	MI	Attenuated cardiac remodeling

IR, ischemia-reperfusion; MI, myocardial infarction.

damaged mitochondria are degraded through mitophagy. During the mitophagic process, mitochondrial DNA is degraded by the DNaseII in lysosomes. Mitochondrial DNA resembles bacterial and viral DNA, which contains unmethylated CpG motifs that can promote severe inflammatory responses.⁶⁸ Incomplete digestion of mitochondrial DNA because of a DNaseII deficiency induces infiltration of inflammatory cells and production of proinflammatory cytokines in the TAC-operated heart, and causes rapid cardiac dysfunction and heart failure.⁶⁹ Toll-like receptor 9, which is an innate immune response-related protein, recognizes dsDNA with unmethylated CpG motifs and mediates this inflammatory response.⁷⁰ Ablation of TLR9 or inhibition of TLR9 by inhibitory oligodeoxynucleotides (ODNs) can ameliorate cardiac inflammation and heart failure, even in the wild-type mouse, induced by severe pressure overload by means of TAC. Thus, mitochondrial DNA is a candidate responsible for sterile inflammation, which is often observed in heart failure patients, further emphasizing that complete digestion of mitochondria during mitophagy is important for cardiac protection (Figure 3).

Autophagy as a Target of Treatment for Cardiac Disease

As described, induction of autophagy could be a therapeutic target for cardiac diseases. Pharmacological modulators of autophagy may be beneficial for treatment and prevention. There are many agents known to induce or reduce autophagic activity^{71,72} (Table).

A polyamine, spermidine, is reported to delay age-associated memory impairment in flies,⁷³ and extend lifespan and protect the heart from age-related cardiac diastolic dysfunction in mice. Both of these effects were mediated through induction of autophagy.⁷⁴ Spermidine increases autophagic and mitophagic activity, thus improving mitochondrial respiratory function. These beneficial effects were not observed in cardiac-specific Atg5-deficient mice, which could not induce autophagy in the heart. Surprisingly, a high intake of dietary spermidine in humans, which was confirmed by food questionnaires, correlated with a reduction in the incidence of cardiovascular diseases. Spermidine also inhibited kidney damage and fibrosis. The effect of spermidine in improving cardiac function is thought to be

mediated through promotion of autophagy and mitophagy in the heart, and by reducing systemic chronic inflammatory responses. Clinical trials to evaluate the effects of spermidine on reducing cardiovascular diseases are expected.

Recently, a high-throughput screening of a drug library identified some drugs that could enhance or inhibit autophagic activity.⁴⁵ Among 1,054 approved drugs, 47 were identified as autophagy inducers, which included carvedilol used for treating heart failure. The cardioprotective role of carvedilol might be partially mediated through this autophagy-related function.

A natural disaccharide, trehalose, is known to protect cells against various stresses. One of the protective roles of trehalose is mediated by its function as an mTOR-independent autophagy inducer.⁷⁵ Trehalose delays the progression of amyotrophic lateral sclerosis in mice,⁷⁶ and ameliorates podocyte injury.⁷⁷ Tuberous sclerosis complex 2 (TSC2)-deficiency induces mTOR hyperactivation and downregulation of autophagy, which leads to cardiac dysfunction. The upregulation of autophagic flux by trehalose can attenuated cardiac dysfunction in the TSC2-deficient heart.⁷⁸ Trehalose also improves cardiac remodeling, fibrosis, and apoptosis after myocardial infarction.⁷⁹ The cardioprotective effect of trehalose was not observed in heterozygous knockout of beclin 1 in mice, indicating that these protective effects are mediated through autophagy.

Resveratrol is a polyphenol found in red wine, which activates sirtuins and extends the lifespan of both *Saccharomyces cerevisiae*⁸⁰ and mice.⁸¹ It also induces autophagy and protects the heart from cardiac remodeling induced by myocardial infarction, doxorubicin-induced toxicity, and oxidative stress from diabetes.^{82–84}

Perspective

I have described some of the recent advances in the understanding of the molecular mechanism and cardioprotective roles of autophagy. Upregulation of autophagic activity is a promising therapeutic target for cardiovascular diseases.^{85–88} Over the past decade, our comprehension of autophagy in the heart has dramatically improved. However, there remain many issues to be elucidated, the most important being the detection and examination of autophagy flux in the human body. Technical advances that may allow this would significantly promote our knowledge about autophagy in human physiology and pathology involving cardiac diseases.

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