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Mitochondrial autophagy in cardiomyopathy

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Abstract

Cardiac mitochondria produce vast amounts of ATP through oxidative phosphorylation to maintain contractile function. They are also the primary source of reactive oxygen species, which contribute to mitochondrial dysfunction, cardiomyocyte death, and heart failure. To protect against mitochondrial damage, cardiomyocytes develop well-coordinated quality control mechanisms that maintain the overall mitochondrial health through mitochondrial biogenesis, mitochondrial dynamics, and mitochondrial autophagy (mitophagy). Mitophagy removes dysfunctional mitochondria in the heart not only under normal physiological conditions, but also in response to pathological stresses. Accumulating evidence suggests that mitophagy dysregulation can induce cardiomyocyte death and cardiomyopathy. In this review, we discuss what is currently known about mitophagic mechanisms, regulatory pathways, and function in the heart.

Keywords

mitochondria; autophagy; mitophagy; cardiomyopathy; heart failure

Maintenance of mitochondrial function and integrity is essential for normal cell physiology and survival. This is particularly true in cells with high-energy demand such as cardiomyocytes, where mitochondria comprise approximately 30% of the total cell volume and generate vast amounts of ATP through oxidative phosphorylation to maintain contractile function[1]. In addition, mitochondria regulate cell survival and death, including apoptosis and necrosis, by integrating cellular signals, including reactive oxygen species (ROS), Ca^{2+}, and NAD^{+}/NADH. To protect against stresses, cells have well-coordinated quality control mechanisms that maintain the overall health of mitochondria, including fusion, fission, mitochondrial autophagy[2] or mitophagy[3], and mitochondrial biogenesis[4,5] (Figure 1). It remains to be clarified whether autophagic removal of damaged mitochondria in cardiomyocytes is mediated by a truly mitochondria-specific form of autophagy, termed mitophagy, or by general autophagy. Morphologically, however, autophagosomes containing only mitochondria can be observed in electron microscopic analyses of heart sections, supporting the presence of mitophagy in cardiomyocytes[6]. Removal of damaged and

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dysfunctional mitochondria through autophagy and mitophagy appears particularly important in terminally differentiated cells such as cardiomyocytes, as evidenced by the high basal level of mitophagic activity[7] and the fact that accumulation of protein aggregates in dysfunctional cardiomyocytes is commonly accompanied by the suppression of autophagy[8]. Mitochondrial DNA is also degraded during the mitophagic process by DNaseII in lysosomes. Incomplete digestion of mitochondrial DNA induces inflammation in the heart and causes heart failure[9].

Mitophagy removes dysfunctional mitochondria in the heart under normal physiological conditions, and during nutrient starvation, ischemic preconditioning (IPC), myocardial infarction (MI), ischemia and reperfusion (I/R), and cardiac hypertrophy[10]. In order to determine the precise role of mitophagy in cardiomyocytes, reliable tools to accurately assess mitophagy are essential. Traditional methods have been successfully used to analyze mitophagy in cardiomyocytes[11], including electron microscopy, which recognizes mitochondrial remnants within autophagosomes, analysis of the mitochondrial protein turnover rate, and dual fluorescence labeling of mitochondria and autophagic markers such as LC3[12], or lysosome markers, such as Lamp1 and Lamp2[13]. In addition, recent innovative methods have been developed for the analysis of mitophagy, such as the novel pH-dependent fluorescent molecule, Keima [14,15]. When Keima is localized in cellular compartments with neutral pH it emits a green color, whereas when it is in lysosomesat acidic pH it emits a red color. Keima directed to mitochondria using a mitochondrial localization signal (Mito-Keima) allows for accurate evaluation of the presence of mitochondrial proteins in lysosomes (pH~4.5). The level of mitophagy can be quantified by the number of red pixels divided by the total number of all pixels[6,15–19]. In Mito-Keima transgenic mice, mitophagy is observed at high rates in the heart and the brain compared to other organs[7]. Another method potentially useful for monitoring mitophagy in the heart is MitoTimer, which is a mitochondria-targeted mutant of the red fluorescent protein DsRed (DsRed1-E5) that changes color during protein maturation; shortly after translation, it emits green light, whereas 12–18 hours after translation it emits red light [20,21]. Mice expressing MitoTimer in the heart are useful for investigating mitochondrial turnover in cardiomyocytes, where the accumulation of red-shifted mitochondria indicates low mitochondrial turnover as a result of low mitophagic flux[22].

Parkin-dependent mitophagy

The most well studied mechanism of mitophagy in cardiomyocytes is that mediated by the cytosolic E3 ubiquitin ligase Parkin[23] and the mitochondrial membrane kinase PTEN-induced putative kinase-1 (PINK1)[24]. When dysfunctional or damaged mitochondria occur, Parkin is recruited from the cytosol to damaged mitochondria[24] and ubiquitinates mitochondrial outer membrane proteins, such as mitofusin 1 (MFN1), mitofusin 2 (MFN2) and voltage dependent anion channel (VDAC)[24]. In healthy mitochondria, PINK1 is unstable and rapidly degraded, but in depolarized mitochondria, PINK1 is stabilized, allowing PINK1 accumulation only in depolarized mitochondria[25]. PINK1 phosphorylates MFN2, which promotes Parkin translocation to mitochondria[26]. PINK1 also phosphorylates ubiquitin and phosphorylated ubiquitin activates Parkin E3 ubiquitin ligase activity[27,28]. In cardiomyocytes, MFN2 deficiency prevents depolarization-induced

recruitment of Parkin to damaged mitochondria, resulting in cardiomyopathy[26]. *Pink1*$^{-/-}$ mice develop left ventricular dysfunction and pathological cardiac hypertrophy[29], which is mediated by increased oxidative stress and dysfunctional mitochondria in cardiomyocytes, and PINK1 protein levels are significantly decreased in heart failure[29]. Interestingly, Parkin can still be recruited to damaged mitochondria after MI in the absence of PINK1 and compensates for PINK1 deficiency[30]. In addition, nitric oxide induces Parkin-dependent mitophagy even in the absence of PINK1 [31]. Conversely, PINK1 recruits autophagy receptors to induce mitophagy even without Parkin[32], indicating the presence of alternative pathways.

Parkin$^{-/-}$ mice exhibited normal myocardial function at baseline[33]. Autophagic clearance of damaged mitochondria is still possible in the absence of Parkin in the heart, most likely through activation of Parkin-independent mitophagy and upregulation of macroautophagy[34]. In fact, there is a compensatory increase of several Parkin-related E3 ubiquitin ligases of the RING families in *Parkin*$^{-/-}$ hearts[35]. TNF-receptor-associated factor 2 (TRAF2), an E3 ubiquitin ligase, is recruited to mitochondria and promotes the removal of ubiquitin-tagged damaged mitochondria during I/R[36]. TRAF2 co-localizes with ubiquitin, p62, and LC3, and deletion of TRAF2 induces accumulation of depolarized mitochondria in cardiomyocytes. Thus, TRAF2 functions independently of Parkin to mediate mitophagy in cardiomyocytes[36]. Smad-ubiquitin regulatory factor 1(SMURF1) is a HECT-domain ubiquitin ligase that is recruited to damaged mitochondria, where it promotes mitophagy[37]. SMURF1 is required for damaged mitochondria to be engulfed by autophagosomes. Cardiomyocytes in *Smurf1*$^{-/-}$ mice showed an accumulation of abnormal mitochondria that were swollen, fragmented and contained abnormal cristae, and an increased number of p62 aggregates[37], suggesting that SMURF1 is involved in cardiac mitochondrial quality control at baseline. It should be noted, however, that *Parkin*$^{-/-}$hearts exhibit altered mitochondrial networks with small mitochondria and are more sensitive to MI[33]. Cardiac-specific *Parkin*$^{-/-}$ mice and MFN2 mutant mice lacking the PINK1 phosphorylation sites necessary for Parkin binding exhibited impaired metabolic maturation in the postnatal hearts, suggesting that Parkin-mediated mitophagy plays an important role in mediating developmental mitochondrial plasticity and metabolic remodeling in the heart [38]. In *Parkin*$^{-/-}$*Drosophila* [35], mitochondria are depolarized and generate more ROS, and the heart is dilated even at baseline, most likely because *Drosophila* has only one *Parkin* gene. These results support the fundamental importance of Parkin in cardiac mitophagy throughout the evolutionary tree.

The role of Drp1 in mitophagy in the heart

Dynamin-related protein 1 (Drp1) is a small GTPase involved in mitochondrial fission. Although Dnm1, a yeast homolog of Drp1, has been identified as an essential mediator of mitophagy in yeast[39], it has not yet been determined whether or not Drp1 also plays an essential role in mediating mitophagy in the mammalian heart. Drp1 is more strongly expressed in the brain and the heart than in other tissues or mouse embryonic fibroblast (MEF) cells[40]. Multiple reports have shown that Drp1 is essential for normal cardiac function at baseline and in response to stress[6,17,40–43]. Furthermore, postnatal cardiac-specific downregulation of Drp1 reproducibly induces dilated cardiomyopathy and rapid

lethality in mice[6,17,40,42]. However, whether Drp1 plays an essential role in mediating mitophagy in the heart remains controversial. Song et al. reported that mitophagy is hyper-activated in Drp1-deficient mouse hearts. Since Parkin is upregulated in Drp1-deficient hearts, and since concomitant ablation of Parkin increases the survival of Drp1-deficient mice, Parkin-mediated hyper-activation of mitophagy may be detrimental for the heart[41]. On the other hand, using Mito-Keima, Ikeda et al.[6] reported that endogenous Drp1 is required to mediate mitophagy in cardiomyocytes in response to glucose deprivation. Furthermore, Drp1 downregulation in the adult heart inhibits mitochondrial autophagy and causes mitochondrial dysfunction and consequent cell death in the heart, both at baseline and under stress conditions. Kageyama et al.[17] reported that Drp1 mediates Parkin-independent mitophagy, whereas Parkin is critical for the maintenance of mitochondrial respiratory function in the absence of Drp1. Interestingly, ubiquitination of mitochondrial proteins was unaffected in Parkin/Drp1 knockout mice, indicating that other E3 ligases may mediate mitochondrial degradation in the absence of Parkin. Cahill et al.[43] reported that C452F mutation in Drp1 causes dilated cardiomyopathy with abnormal mitochondrial morphology and defective mitophagy. In a clinical study, a newborn girl with a Drp1 heterozygous mutation (A395D) displayed a defect in mitochondrial fission with persistently elevated levels of lactate and very-long chain fatty acids in the plasma[44]. In summary, further investigation is required to elucidate the role of Drp1 in mitophagy in the heart, as well as the precise molecular mechanism involved.

Other mechanisms mediating mitophagy in the heart

Damaged mitochondria are engulfed by autophagosomes through interaction between autophagy adaptors or receptors and LC3 or LC3-related proteins. Autophagy adaptors, including p62/SQSTM1, Tax1BP1, NBR1, NDP52, and optineurin, possess ubiquitin binding domains through which they recognize damaged mitochondria ubiquitinated through either Parkin-dependent [45] or –independent mechanisms [32,36], whereas mitochondrial receptors, such as NIX/BNIP3L, mitochondrial pro-apoptotic BH3-only domain protein (BNIP3) and FUNDC1, are located in the outer mitochondrial membrane and do not require ubiquitination of mitochondria to promote mitophagy. BNIP3 is upregulated in myocardia during hypoxia[46], while NIX is upregulated during pathological cardiac hypertrophy[47]. Both BNIP3 and NIX are mitophagy regulators in adult hearts[48]. FUNDC1 is an integral mitochondrial outer-membrane protein that interacts with LC3 to induce mitophagy during hypoxia in HeLa cells[49]. FUNDC1 is dephosphorylated at Serine 13 by phosphoglycerate mutase family member 5 (PGAM5), a Ser/Thr phosphatase, leading to activation of mitophagy[50]. PGAM5 has been shown to play a critical role in mediating PINK1-mediated mitophagy in the heart [51] but the connection between PINK1 and FUNDC1 is currently unknown. In addition, ULK1, a Ser/Thr kinase required for early autophagosome formation, is upregulated and translocates to mitochondria, where it interacts with FUNDC1 and phosphorylates it at Serine 17, thereby enhancing the interaction of FUNDC1 and LC3 in MEF cells[52]. However, it remains unclear whether a similar mechanism exists in the heart. Bcl2-like protein 13(Bcl2-L-13) has been identified as a mammalian mitophagy receptor[53] that is homologous to Atg32 in yeast[54,55]. Bcl2-L-13 was shown to bind to

LC3 and induce mitochondrial fragmentation independently of either Drp1 or Parkin in HEK293A cells[53], providing a novel insight into the mechanism of mitophagy.

Other mechanisms of mitochondrial degradation

Several recent studies in yeast have shown that mitochondria can be degraded by micromitophagy, where the lysosome directly engulfs mitochondria[13,56–58]. Similarly, in the heart, damaged mitochondria can be taken up directly by lysosomes during I/R[13]. This micromitophagy is independent of macroautophagy, which involves the formation of double membranes around organelles targeted for destruction that then fuse with lysosomes, and is regulated by δPKC-mediated phosphorylation of GAPDH[13]. Pharmacological upregulation of GAPDH-driven mitophagy promotes the clearance of damaged mitochondria and inhibits cell death during I/R[13].

Another form of micromitophagy is mediated by mitochondria-derived vesicles (MDVs) that bud off from mitochondria and are engulfed by lysosomes[59,60]. During oxidative stress, MDVs act as an early response, delivering their cargo of dysfunctional proteins and lipids from mitochondria to lysosomes independently of the mitochondrial fission machinery and without requiring mitochondrial depolarization. Although MDV formation and delivery to lysosomes are independent of the macroautophagic machinery, including ATG5 and LC3, the vesicles do require PINK1 and Parkin. However, whether MDVs exist in the heart is currently unknown.

Upstream signaling mechanisms of mitophagy

ULK1, a mammalian homolog of yeast Atg1, is an mTOR substrate. Phosphorylation of ULK1 at Ser757/758 (mouse/human) by mTOR inhibits its activity and autophagy[61,62]. AMP-activated protein kinase (AMPK) is a sensor for the metabolic state activated during starvation. Activation of AMPK inhibits the TORC1 pathway, activates ULK1, and positively regulates mitophagy [63–65].

Hexokinase-II (HK-II), a kinase in the first step of glycolysis, is a predominant isoform in the heart, adipose tissue and skeletal muscle. Reduction in HK-II levels results in decreases in cardiac function during IPC, I/R and cardiac hypertrophy[66–69]. In a recent study, HK-II was shown to positively regulate autophagy in response to glucose starvation in cardiomyocytes through interaction with and inhibition of mTOR[70]. HK-II overexpression enhances glucose deprivation-induced dephosphorylation of downstream targets of TORC1, whereas HK-II deficiency inhibits this response. Furthermore, HK-II overexpression leads to reduced phosphorylation of Ser757 in ULK1 and increased AMPK-mediated phosphorylation of Ser555, thereby stimulating autophagy[70].

Mitogen-activated protein kinase 1 (MAPK1) and MAPK14 and their upstream signaling pathways are essential for mitophagy[19]. Using Mito-Keima, it was demonstrated that only a small portion of mitochondria was degraded through mitophagy during starvation and hypoxia in HeLa cells. This process is ATG5-ATG7-independent, but requires ULK1, Beclin1, and class III PI3K. Knockdown of MAPK1 and MAPK14 efficiently inhibited mitophagy, without affecting macroautophagy. Phosphorylation of MAPK1 and MAPK14 is

significantly increased during starvation and hypoxia, indicating that MAPK1 and MAPK14 are upstream of and regulate the activation of mitophagy, which is mediated through non-conventional mechanisms of autophagy[19].

Lysosomes also regulate mitophagy. TFEB, a transcription factor involved in lysosome biogenesis[71], facilitates the removal of damaged mitochondria and attenuates cardiomyocyte death during I/R[72]. Downregulation of TFEB results in impairment of autophagic flux, accumulation of damaged mitochondria, and increases in myocardial oxidative stress[73], suggesting that endogenous TFEB-mediated stimulation of autophagic flux protects cardiomyocytes against hypoxia-reoxygenation injury in cardiomyocytes[73].

Nutrient- and stress-dependent modification of mitochondrial proteins by acetylation/deacetylation plays a fundamental role in mitochondrial function. Both acetyltransferase and deacetylase enzymes regulate macroautophagy[74,75]. In particular, GCN5L1, a component of the mitochondrial acetyltransferase machinery, has been shown to regulate mitophagy[76]. GCN5L1 deficiency not only increases deacetylation of mitochondrial proteins and initiates mitophagy[76] but also activates the expression of TFEB, with a coordinated increase in mitochondrial biogenesis through increased PGC-1α in MEFs. Thus, GCN5L1 serves as a negative regulator of both mitochondrial biogenesis and mitophagy[5].

In non-cardiomyocytes, starvation induces autophagic degradation of mitochondria later than autophagic and proteasomal degradation of cytosolic proteins, possibly because re-synthesis of intracellular organelles is energetically more costly[77]. During the initial hour of starvation, mitochondria begin to elongate, are spared from autophagic degradation, and try to maintain ATP production[78]. As starvation continues, mitophagy follows. However, since cardiomyocytes have a high energy demand and one third of their volume consists of mitochondria, whether a similar mechanism exists in cardiomyocytes is yet to be determined.

p53/TIGAR (TP53-induced glycolysis and apoptosis regulator)-mediated inhibition of mitophagy causes damage to mitochondrial integrity and apoptosis in cardiomyocytes[79]. Deletion of p53 or its downstream mediator, TIGAR, promotes mitophagy, thereby decreasing abnormal mitochondria and promoting resistance to ischemic injury[79] in the heart. In addition, cytosolic p53 inhibits Parkin-dependent mitophagy through interaction with Parkin[80]. Likewise, ALCAT1, a lysocardiolipin acyltransferase, was potently upregulated by hyperthyroid cardiomyopathy, leading to oxidative stress and mitochondrial dysfunction[81], whereas loss of ALCAT1 increased mitophagy and prevented cardiomyopathy. In contrast, PGAM5 promotes the clearance of dysfunctional mitochondria to maintain mitochondrial homeostasis through mitophagy[82].

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Clinical significance

Mitophagy is essential for cardiac mitochondrial quality control[10]. The regulation of mitophagy is diverse but must be precise. Either insufficient or exacerbated mitophagy can result in cardiomyopathy. For instance, in type 1 diabetes, diminished autophagy is a beneficial adaptive response to protect against diabetic cardiac injury, most likely by upregulating alternative pathways of autophagy and mitophagy[83]. Mainly, however, mitophagy exerts a protective role in cardiac function. IPC induces Parkin translocation to mitochondria and promotes mitophagy. Parkin knockout attenuates IPC-induced p62 translocation to mitochondria and abolishes the cardioprotective effects of IPC[45]. Mitophagy is also important for the clearance of damaged mitochondria during aging. Parkin deficiency results in the accumulation of damaged mitochondria in cardiomyocytes with age[84]. During myogenesis, mitophagy is upregulated by a process mediated by Drp1 and p62/SQSTM1[85]. Differentiation of primitive myoblasts into mature myotubes requires a metabolic switch from glycolysis to oxidative phosphorylation in skeletal muscle. The process requires dramatic remodeling of the mitochondrial network through coordinated actions of both mitochondrial biogenesis and clearance[85]. Whether the same mechanism exists in the heart remains to be shown. Addressing the in vivo importance of mitophagy is challenging, since some mediators of mitophagy, such as Parkin, have multiple functions besides mitophagy[86]. In addition, since multiple layers of back-up mechanisms exist for mitochondrial degradation, loss of function of one mechanism of mitophagy is often compensated for by other mechanisms[87]. Given that mitophagy is essential in mitochondrial quality control but can be harmful when excessively activated, it is of particular interest to investigate the participation of mitophagy in cardiovascular disease caused by distinct mechanisms in a context-dependent manner. In summary, if mitophagy is to emerge as a promising therapeutic target, it will be critical to determine when and how manipulating mitophagy activity can protect patients from cardiovascular diseases.

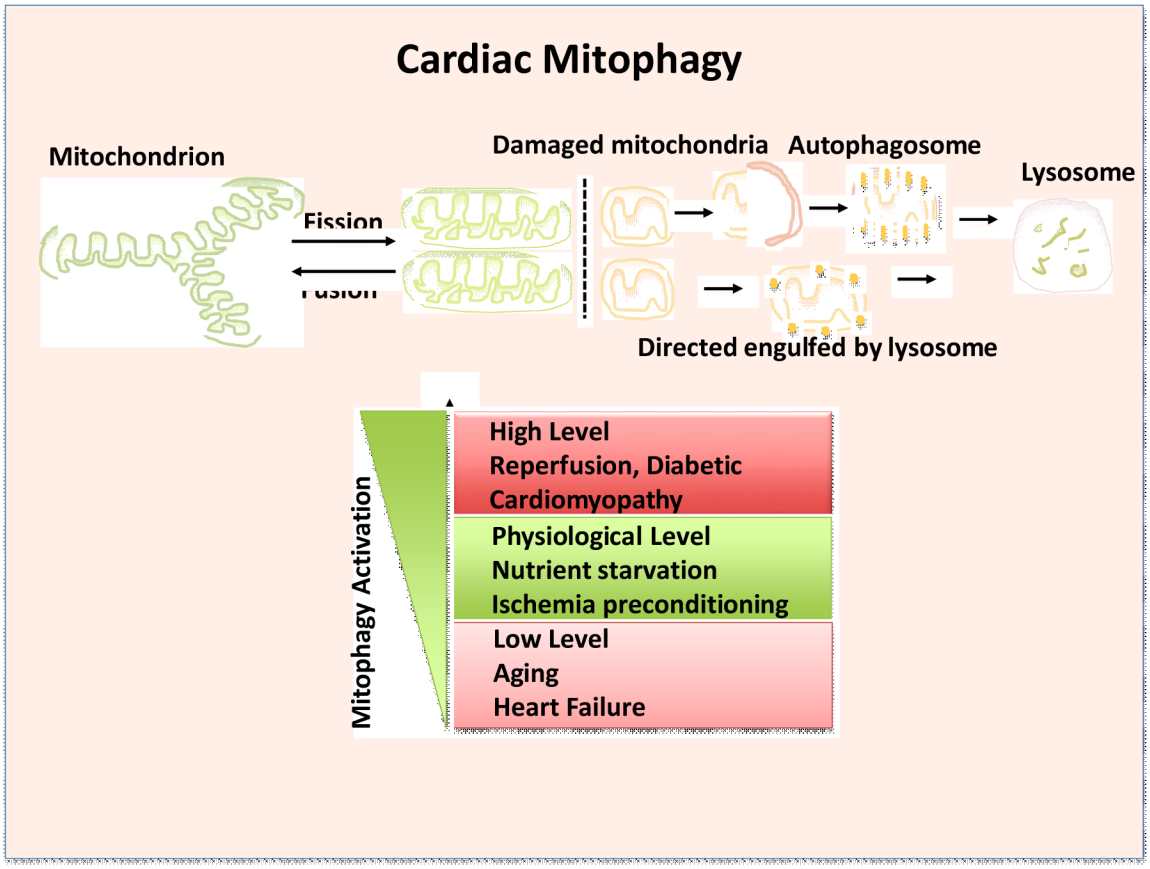

Figure 1. Outline of mitophagy in cardiomyopathy

Mitophagy is essential for cardiac mitochondrial quality control. The regulation of mitophagy must be precise. Insufficient or exacerbated mitophagy results in cardiomyopathy. An appropriate level of mitophagy protects the heart by increasing mitochondrial health. However, mitophagy is suppressed below physiological levels in some cardiac conditions, such as aging and heart failure, which leads to mitochondrial dysfunction and cell death and exacerbates cardiac dysfunction. On the other hand, mitophagy may be stimulated excessively in some conditions, including ischemia/reperfusion and diabetic cardiomyopathy, and this may be harmful for the heart, although whether excessive mitophagy induces death of cardiomyocytes requires more investigation.