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Mitophagy: Therapeutic Potentials for Liver Disease and Beyond

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Mitochondrial integrity is critical for maintaining proper cellular functions. A key aspect of regulating mitochondrial homeostasis is removing damaged mitochondria through autophagy, a process called mitophagy. Autophagy dysfunction in various disease states can inactivate mitophagy and cause cell death, and defects in mitophagy are becoming increasingly recognized in a wide range of diseases from liver injuries to neurodegenerative diseases. Here we highlight our current knowledge on the mechanisms of mitophagy, and discuss how alterations in mitophagy contribute to disease pathogenesis. We also discuss mitochondrial dynamics and potential interactions between mitochondrial fusion, fission and mitophagy.

Key words: Autophagy, Mitochondria, Mitophagy, Liver, Brain

INTRODUCTION

Macroauotphagy (referred to as autophagy hereafter) is a major catabolic pathway to remove surplus or unnecessary cytoplasmic contents and dysfunctional organelles, and defects in this pathway negatively impact the cellular environment by accumulating potentially toxic moieties. Autophagy is essential for sustaining cells during various physiological and pathological stresses, including nutrient deprivation, oxidative stress, ischemia/reperfusion (I/R) and pathogen invasion. However, depletion of essential cellular components due to excessive autophagy could also trigger autophagic cell death. In cancer, the role of autophagy is more complicated, as autophagy can either prevent or promote cancer based on the developmental stage of the disease. During initial stages of tumorigenesis when cells undergo hyperproliferation, activating autophagy inhibits cell division and suppresses tumor growth (1). However, in more established tumors at later stages, cancer cells depend on autophagy to supply essential nutrients and thus inhibiting autophagy has a therapeutic potential to suppress metastasis and malignancy of cancer (2,3).

How does autophagy impact mitochondrial health? Mito-

chondria are dynamic organelles that generate ATP through a proton motive force across inner membranes and are responsible for supplying the majority of energy needed by cells. Mitochondria are also the central regulators for apoptosis and the major source for the production of reactive oxygen species (ROS). Environmental toxins as well as chemotherapeutic drugs can damage mitochondria. Mitochondrial dysfunction is also a key causative factor inducing cell death after I/R, aging and many neurodegenerative conditions such as Alzheimer's and Parkinson's disease. Therefore, it is important to timely eliminate abnormal, aged and dysfunctional mitochondria to sustain cell viability. The process of eliminating mitochondria through a selective autophagy is termed mitophagy, which not only facilitates turnover of normal mitochondria but also removes unnecessary or damaged mitochondria to prevent the accumulation of dysfunctional mitochondria and potentially cytotoxic mitochondrial byproducts in the cytosol. De Duve first observed this process of mitochondrial sequestration into lysosomes in hepatocytes, after stimulating autophagy with glucagon (4). A strong positive link between autophagic capacity and mitochondrial integrity was observed in autophagy-deficient transgenic mice that exhibit swelling and loss of structural integrity in mitochondria (5).

To date, mitophagy is the only known mechanism to remove aged or damaged mitochondria. Depending on the tissue, normal mitochondria are turned over with a half-life range of 9~25 days, with livers having a relatively faster turnover than brain, heart or kidney (6). Active mitophagy in livers may be attributable to the high content of mitochondria in this tissue that reflects the high energy demand for operating multiple hepatic functions. For the same rea-

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son, hepatocytes are frequently used to characterize the features of mitophagy.

MITOPHAGY MECHANISMS

Although mitophagy is evolutionary conserved in all types of cells, the mechanisms triggering the onset of mitophagy differ depending on the type of stresses and cells. Using a fluorescent marker for the autophagosome [GFP-labeled microtubule-associated protein-1 light chain-3 (GFP-LC3)] and a mitochondrial fluorophore, activation of mitophagy can be visualized upon nutrient starvation of hepatocytes; whereby small pre-autophagic structures near the mitochondria grow into a cup-shaped phagophore that engulfs individual mitochondrion within 5 min after nutrient depletion (7,8) (Fig. 1). Mitochondria maintain their membrane potential during sequestration, as evidenced by red fluorescing tetramethylrhodamine methylester (TMRM), but become depolarized after either onset of the mitochondrial permeability transition (MPT) or acidification of luminal pH in the mitophagosome. Sequestration of polarized mitochondria also occurs to ischemic hepatocytes after reperfusion (9). Additional features of this type of mitophagy include the requirement for phosphatidylinositol-3-kinase (PI3K), and coordination with mitochondrial fission (7).

In contrast to PI3K-dependent mitophagy, mitochondrial depolarization can induce a different type of mitophagy in a PI3K-independent manner (Fig. 2). Global mitochondrial injury from widespread depolarization induces a strong autophagy response (10,11). For instance, photodamage to hepatocytes has demonstrated a sequential chain of mitochondrial depolarization and subsequent mitophagy (7). The specific proteins that have known roles in mitophagy are summarized in Table 1. In yeast, ATG32 is a mitophagy receptor that specifically targets mitochondria for autophagy and has no impact on macroautophagy (12). For mammalian cells, FUN14 domain-containing protein-1 (FUNDC1) was recently identified as a mitochondrial autophagy recep-
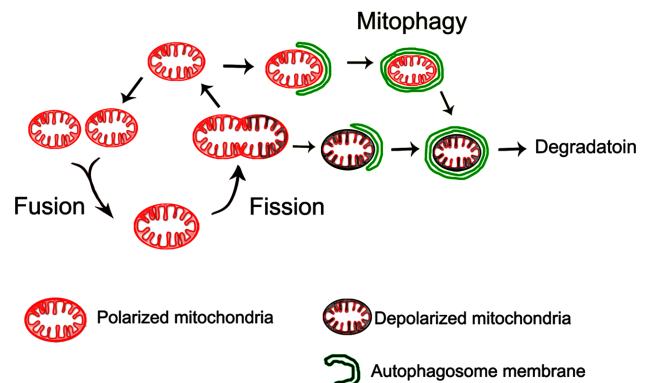
Fig. 2. Scheme of mitochondrial dynamics and mitophagy. Under the conditions of nutrient depletion and I/R in the liver, a polarized mitochondrion is initially recognized by a U-shaped phagophore and later sequestered completely by an autophagosome. The mitochondrion entrapped in the autophagosome loses its membrane potential, presumably due to onset of the MPT or luminal acidification after fusion with a lysosome. When mitochondria undergo fission, a mitochondrion is divided into two daughter mitochondria with disparate membrane potential. Whereas the mitochondrion with the higher membrane potential returns to the cycle of fusion, the other with the lower or depolarized membrane potential is engulfed into an autophagosome and subsequently cleared by an autolysosome.

tor, whose exogenous expression induces mitophagy, in the absence of any mitochondrial damage or general autophagy-stimulating conditions (13). Activating mitophagy through elevated FUNDC1 expression has been reported to reduce the total amount of both outer and inner mitochondrial membrane proteins, without reducing other organelle markers. Following mitochondrial damage by hypoxia or mitochondrial uncoupler treatment, FUNDC1 is phosphorylated by ULK1, and promotes mitophagy (14).

In mammalian cells, mitophagy of depolarized mitochondria has also been shown to involve BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3)/BNIP3L (also known as NIX), and PTEN-induced putative kinase protein
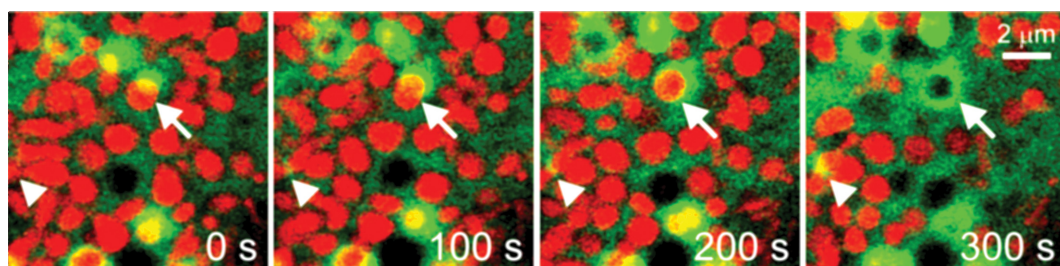
Fig. 1. Visualization of mitophagy in primary mouse hepatocytes. Time-lapse images of confocal microscopy with GFP-LC3 and TMRM. Cells were maintained in amino acid- and serum-free Krebs-Ringer-HEPES (pH 7.4) solution for 2 hrs to stimulate autophagy. Arrow indicates a progress of mitophagy wherein polarized mitochondrion (red) is surrounding by an elongating autophagic membrane (green). Note a depolarized mitochondrion in the lumen of autophagosome at 300 sec. Arrowhead displays an initiation of new mitophagy.

Table 1. Summary of mitophagy mechanisms

Protein	Function	Reference
ATG32	• Yeast mitochondrial protein for recognition as autophagy cargo. • Interacts with Atg8 and Atg11.	(12)
FUNDC1	• Regulates Ulk1 recruitment to damaged mitochondria. • Interaction with LC3 is regulated by phosphorylation.	(13,14)
PINK1	• Normally undergoes rapid processing/degradation on mitochondria • Full length protein recruits Parkin	(11,16,17,22)
Parkin	• E3 ubiquitin ligase • Endogenous Parkin ubiquitinates MFN1 and MFN2, overepxressed Parkin ubiquitinates many other outer membrane proteins	(11,16-18,22)
BNIP3/NIX	• Mediates mitophagy during red blood cell maturation • Activates mitophagy induced by hypoxia or mitochondria uncoupling. • Homodimerization required for mitophagy • Interacts with LC3 and OPA1	(15,20,21)

1 (PINK1)/Parkin (13,15). Among these two systems, PINK1 and Parkin have been the most actively researched. PINK1 and Parkin are proteins in which mutations are linked to familial forms of Parkinson's disease. Pink1 recruits Parkin to the outer membrane of dysfunctional mitochondria and subsequent ubiquitination of proteins on the mitochondrial outer membrane induces mitophagy (11,16). The mitochondrial accumulation of Parkin is voltage-dependent, and does not depend on changes in pH or ATP levels (17), and suggests that Parkin may act as a sensor for mitochondrial integrity and trigger mitophagy upon dysfunction. Parkin-mediated ubiquitination is opposed by the deubiquitinase USP30, whereby reducing USP30 activity enhances mitophagy (18). Parkin also participates in the maturation of mitophagosomes through the endosomal-lysosomal protein CLEC16A, which interacts with the E3 ligase NRDP1 to regulate Parkin levels. Reduced CLEC16A expression elevates Parkin levels and increases mitophagosomes, however, the mitophagosomes fail to undergo fusion with lysosomes, which results in the net increase of abnormal and dysfunctional mitochondria. In hepatitis C virus, its core protein was found to bind directly to Parkin, and suppress its translocation to mitochondria, thereby inhibiting mitophagy (19).

BNIP3 and BNIP3L are proteins with homology to BCL2 in the BH3 domain, which induce both cell death and autophagy. In erythrocytes, the elimination of mitochondria during maturation requires BNIP3L (15). BNIP3L is also involved in mitochondrial depolarization-induced mitophagy. Upon treatment with a mitochondrial uncoupler [carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)] to induce a short circuit between electron transfer and ATP generation, BNIP3L is required to activate mitophagy through mitochondrial depolarization and ROS generation (20). BNIP3 also regulates mitophagy in response to hypoxia. In cardiac myocytes, I/R leads to BNIP3-mediated upregula-

tion of autophagy. This serves as a protective response to remove damaged mitochondria after hypoxia and reoxygenation (21).

MITOPHAGY IN LIVER DISEASE

Regarding systemic metabolism, the hepatocytes of livers serve a major role in regulating many integral metabolic functions. Hepatocytes also contain numerous mitochondria to meet the high energy demands of these metabolic activities. Thus, maintaining healthy mitochondria and, at the same time, clearing unhealthy mitochondria through efficient autophagy are critical for supporting liver functions. Indeed, in livers of autophagy-deficient mice, mitochondrial damage is evident such as swelling and ROS formation (5). Impaired autophagy and mitophagy can affect multiple aspects of hepatic functions and underlie many liver injuries including steatosis, hepatocellular carcinoma (HCC), and liver injuries caused by toxins, drugs and I/R (22). In HCC, autophagy may prevent or promote cancer process, depending on the disease stage itself as in other cancers. Autophagy initially functions as a tumor suppressor. On the contrary, in established tumors, autophagy fuels tumor survival by supplying its metabolic demands. Thus, the loss of autophagy can increase or decrease hepatic tumors at the early or late stage of HCC, respectively (5,23). In fatty liver disease, reduced autophagy can be causative in the accumulation of lipids, as autophagy degrades intracellular lipids through lipophagy (24). Moreover, the accumulation of excess lipids further depresses autophagy function by affecting autophagy protein expression and autophagy maturation (25). This perpetual worsening of autophagy function and lipid accumulation is implicated in the progression from simple steatosis to non-alcoholic steatohepatitis (NASH). In toxin-induced liver injuries including alcohol and acetaminophen (APAP), the

disease process involves the metabolism of toxins that increase ROS production that damages mitochondria. In experimental models of alcohol and APAP-induced liver injury, enhancing autophagy and mitophagy has been shown to counteract insufficient autophagy and to improve liver function and mitochondrial bioenergetics (22,26,27).

ISCHEMIA/REPERFUSION (I/R) INJURY

Cellular respiration requires a continuous oxygen supply. For livers, hypoxic or anoxic stress can occur during hepatectomy, liver preservation for transplantation, cardiac failure and, hemorrhagic shock. Similar to the liver, organs such as the brain and the heart have a high metabolic demand and are also highly susceptible to ischemia-induced cellular damage. The pathogenic events during ischemia include loss of oxygen or anoxia, decreased ATP production, and acidosis. Acidosis results from the combination of high-energy phosphate hydrolysis, lactate buildup from anaerobic respiration, and proton release from acidic compartments. Interestingly, acidosis itself is not the culprit for cellular damage, as it may confer cytoprotection against ischemic necrosis (28,29). Instead, cellular damage paradoxically occurs upon reperfusion, when the oxygen supply is restored and cellular pH is recovered to normal.

Multiple events during reperfusion are likely to contribute to cell death, including ROS formation and oxidative damage to mitochondrial components, calcium overloading and activation of cytosolic calpains and other injurious proteases, impaired autophagy and loss of mitochondrial energy production (30). These events are likely to converge on the MPT. When ischemic cells undergo reperfusion, high conductance permeability transition pores in the mitochondrial inner membranes are opened, which triggers the onset of the MPT. These pores normally restrict the passage to particles less than 1,500 Da, and proper function of these inner membrane pores are critical to maintain mitochondrial polarization, oxidative phosphorylation cycle, and ATP production. Opening of the inner membrane pores leads to non-specific diffusion of proteins across the inner membrane, leading to mitochondrial swelling and subsequent loss of mitochondrial membrane integrity. The MPT also allows pro-apoptotic proteins such as cytochrome C, which is normally confined in the mitochondrial intermembrane space, to release into the cytosol and to activate caspases. ATP depletion that follows from uncoupling of oxidative phosphorylation, ultimately leads to either necrotic or apoptotic cell death, depending on glycolytic ATP availability (31,32).

During ischemia, anoxia depletes the liver of ATP. Since the execution of autophagy needs a large amount of ATP, the formation of autophagic vesicles is halted in ischemic cells. However, at the stage of reperfusion, the capacity to carry out autophagy and mitophagy are critical for cell survival. Upon restoration of oxygen and nutrients, a brief period of mitochondrial polarization is followed by the MPT and consequent depolarization of mitochondrial membrane potential. Importantly, key autophagy proteins, including ATG 7, BECN1, and ATG12-5, decrease over the periods of prolonged ischemia and further reduce at the early stage of reperfusion when the MPT has not yet to occur. Depletion of these autophagy proteins is partially attributable to calpain-mediated degradation (33). Loss of autophagy-related proteins and subsequent impaired autophagy leads to or potentiates a sequential chain of the MPT onset, widespread mitochondria failure and ultimately cell death. Conversely, activating autophagy either by pharmacological activators or by overexpression of key autophagy proteins is cytoprotective against reperfusion injury and restores the autophagic process (33,34) Recovering autophagy also suppresses the spread of toxic insults to neighboring healthy mitochondria through enhanced mitophagy, as the sequestration of damaged mitochondria prevents the propagation of injury signals that can originate from a small subset of mitochondria.

MITOCHONDRIA AND AUTOPHAGY DEFECTS

In response to persistent proteotoxic stress such as protein misfolding diseases, the autophagic machinery may become activated in attempts to clear aggregate proteins. Under such conditions, activating autophagy, or mitochondrial biogenesis, or both appears to be therapeutic. In Huntington's disease, the mutant Huntingtin protein is associated with the formation of toxic aggregates, mitochondrial damage and oxidative stress. Interestingly, enhancing mitochondrial biogenesis by activating the peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α) ameliorates neuronal damage caused by mutant Huntingtin, through activating autophagy-regulating transcription factor EB (TFEB) (35), which assumes to be a master regulator of autophagy and lysosomal genes. Alternatively, disease-causing proteins may interfere with transcriptional regulators of autophagy to inhibit autophagy activation. In the polyglutamine expansion of the androgen receptor of x-linked spinal and muscular dystrophy, polyglutamine-expanded mutant protein binds directly to TFEB and interferes with its nuclear transactivation (36). Additionally, prolonged autophagy impairment may result in the gradual buildup of damaged mitochondria. An example for this type of secondary mitochondria damage is the antitrypsin disease (ATD), a relatively common liver disease that is caused by a point mutation in the secretory protein alpha 1-antitrypsin. Whereas this protein is normally secreted by hepatocytes, the mutant protein is misfolded, and causes endoplasmic reticulum (ER) retention and proteotoxic stress (37). Using a genetic model for autophagy inhibition (ATG5 knockouts), the critical role of autophagy in the dis-

posal of the disease protein has been demonstrated, as the turnover of mutant antitrypsin is significantly delayed when autophagosome synthesis is genetically impaired (38). The pivotal role of autophagy for antitrypsin clearance is confirmed in the yeast, as the yeast homolog of ATG6 or ATG16 was identified in a mutant library screening, and found that absence of either gene causes a marked delay in anti-trypsin clearance (39). This hypothesis that ineffective or defective autophagy contributes to ATD disease is further supported by studies where activating autophagy by pharmacological agents or liver-directed gene transfer of TFEB promotes degradation of the mutant protein and ameliorates hepatic pathologies (40,41). While the primary defect in this disease is a mutation of secretory proteins, extensive mitochondrial defect and accumulation of mitophagosomes have been reported in patient liver tissues (42). Thus, primary autophagy defects can, in turn, precipitate in mitochondrial damage.

MITOCHONDRIA DYNAMICS, AUTOPHAGY AND MITOPHAGY

Mitochondria are dynamic organelles that continuously undergo fusion and fission. Not only do fusion and fission regulate mitochondrial morphology, but also modulate intermixing mitochondrial contents. Mitochondrial fusion is mediated by three proteins: mitofusin-1 (MFN1) and mitofusin-2 (MFN2) for the outer membranes, and optic atrophy-1 (OPA1) for the inner membranes (43,44). Among them, MFN2 localizes at the contact site between outer mitochondrial and ER membranes (45). Mitochondrial fusion facilitates intra-mitochondrial repair and exchange of mitochondrial DNA (mtDNA) (46), whereas fission can serve to temporarily isolate defective segments and/or promote their autophagic clearance (47,48). Growing evidence is indicating that mitophagy and mitochondrial dynamics are intimately connected, as fusion and fission maintain a healthy pool while segregating mitochondria that are destined for mitophagy (Fig. 2). In both yeast and mammalian cells, a certain type of mitophagy is preceded by mitochondrial fission, which divides elongated mitochondria into pieces of manageable size for encapsulation. Furthermore, the reduction of mitochondrial volume by fission can also assist mitochondrial quality control since smaller mitochondria can be readily cleared by mitophagy.

Mitochondria may also be a source of autophagic membranes. In starved mammalian cells, it was shown that autophagosomes are formed in the vicinity of mitochondria (49,50), especially at mitochondria-associated ER membranes, and that mitochondria supply phospholipids to growing autophagosomes via a transient interaction (50). Intriguingly, autophagosome formation is impaired by depletion of either MFN2 or phosphofurin acidic cluster sorting protein-2 (PACS-2) (49), later of which is a cytosolic protein implicated in ER–mitochondrion communication.

Evidence has been accumulating that either MFN1 or MFN2 or both can directly regulate mitophagy. Both MFN1 and MFN2 are targets for ubiquitination by Parkin (16), thus the ubiquitination status of these proteins likely acts as receptors for mitophagy. In cardiac myocytes, genetic ablation of MFN2 prevents depolarization-induced translocation of Parkin to the mitochondria, leading to suppression of mitophagy (51). In addition, mutations in MFN2 have been reported in patients of Charcot-Marie-Tooth neuropathy type 2A (CMT2A) (52,53), which causes degeneration of sensory neurons. Interestingly, both dopaminergic neurons in Parkinson's disease and sensory neurons in CMT2A lack myelination, which makes electrical impulse propagation energetically more demanding. This suggests that cells with a higher energy demand are likely to be more reliant on efficient mitochondrial dynamics and mitophagy. Supporting this hypothesis, reduced MFN2 expression is observed in skeletal muscle of type 2 diabetes (54,55). Patients with this disease demonstrate characteristic muscle fatigue and weakness. In the liver, loss of MFN2 disrupts metabolic functions and displays characteristics of enhanced hepatic glucose production by gluconeogenesis and increased expression of gluconeogenesis (56). We have also observed that silencing of MFN2 abolishes both basal autophagy and sirtuin 1-dependent cytoprotection against I/R in the liver (57).

Besides the role in initiating mitophagy, the levels of PINK1 or Parkin regulate mitochondrial morphology, implying that PINK1/Parkin could regulate both mitochondrial dynamics and mitophagy. In hippocampal neurons, overexpression of either PINK1 or Parkin causes an overall smaller size of mitochondria due to prevailing fission, whereas inactivation of PINK1 produces elongated mitochondria or more incidences of mitochondrial fusion (58). Similarly, flies expressing PINK1 or Parkin mutants display the loss of mitochondrial integrity, which is derived directly from reduced mitochondrial fission (59). Thus mutations in PINK1/Parkin may impact autophagy or mitophagy through alterations in mitochondrial dynamics.

CLINICAL IMPLICATIONS OF MITOPHAGY

I/R injury is a causative factor of morbidity and mortality during liver resection, hemorrhagic shock, cardiac arrest and transplantation. During hepatectomy, ischemia is induced through clamping of the portal venous and hepatic arterial blood flow in an attempt to minimize blood loss. However, recovery of blood flow after the inflow occlusion causes reperfusion injury and severely damages the liver and other organs. Importantly, livers with preexisting diseases such as aging, steatosis and fibrosis from alcohol or hepatitis B or C infection tolerate I/R poorly. With increasing prevalence of obesity, diabetes, and metabolic syndrome, hepatic steato-

sis has become a major cause of chronic liver diseases and the prevalence of hepatic steatosis would substantially increase the likelihood of hepatectomy or liver transplantation and the associated I/R injury (22). At present, efforts to improve liver function after I/R have not been successful mostly due to an incomplete understanding of the pathogenesis of I/R injury. For instance, pharmacological treatment of mitochondria-related diseases with MPT blockers such as cyclosporin A (CSA) and their derivatives has been disappointing due to its narrow range of therapeutic efficacy and the onset of CSA-insensitive MPT (60). Gene targeting therapy has been heralded as a promising therapy, but technical and ethical issues must be addressed prior to its clinical application. A novel and previously unexplored avenue to the treatment of I/R injury to the liver may be up-regulation of endogenous cytoprotective events. Since mitophagy selectively targets and timely removes damaged or abnormal mitochondria, active enhancement of mitophagy could have a therapeutic potential for mitochondria-related diseases.

CONCLUSIONS

Mitophagy functions as an early protective response, favoring adaptation to stress by removing damaged mitochondria. Despite its importance and therapeutic potentials, our understanding of mitophagy remains limited. Future studies are warranted to identify new signaling proteins of mitophagy and unravel its interaction with other signaling pathways.

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