

Roles of organelle-specific autophagy in hepatocytes in the development and treatment of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a disorder of lipid metabolism. The lipotoxic intermediates of lipid metabolism cause mitochondrial dysfunction and endoplasmic reticulum stress. Organelle-specific autophagy is responsible for the removal of dysfunctional organelles to maintain intracellular homeostasis. Lipophagy contributes to lipid turnover by degrading lipid droplets. The level of autophagy changes during the course of NAFLD, and the activation of hepatocyte autophagy might represent a method of treating NAFLD.

Keywords: Autophagy; Lipophagy; Mitophagy; Reticulophagy; Non-alcoholic fatty liver disease

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become one of the most common chronic liver diseases around the world and is associated with heavy economic and social burdens. The pathology of this hepatic condition ranges from simple steatosis non-alcoholic fatty liver (NAFL) to the more severe steatohepatitis non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and hepatocellular carcinoma.^[1,2] Hepatic steatosis represents an early stage of NAFLD and is characterized by the accumulation of lipid droplets (LDs) in hepatocytes. The dysregulation of several metabolic pathways may account for the development of hepatic steatosis, including greater free fatty acid (FFA) uptake, *de novo* lipogenesis, triglyceride (TG) synthesis; and lower β -oxidation of FFAs and very-low-density lipoprotein (VLDL) secretion. The accumulation of lipids induces lipid peroxidation and inflammation, which mediates the transition from NAFL to NASH.^[3] When the fat accumulation exceeds the hepatic capacity for storage, secretion, or oxidation, steatohepatitis and liver fibrosis develop.^[4] Necrotizing inflammation and fibrosis may occur in this lipotoxic environment, which is created by specific lipid species, especially lysophospholipids and diacylglycerol.^[5] Therefore, the control of systemic

hepatic lipid accumulation is important in the prevention or reversal of the progression of NAFLD.^[6]

Autophagy is a mechanism for the removal of dysfunctional cytoplasmic cargo that is highly conserved in eukaryotic cells.^[6] Autophagy can be classified as macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA).^[7] Macroautophagy is the most active and common form of autophagy in mammalian cells. The *de novo* formation of autophagosomes occurs when soluble macromolecular substances and denatured organelles in the cytoplasm are enveloped by endoplasmic reticulum (ER)/mitochondrially derived mono- or bilayer membranes. The autophagosomes formed then fuse with the lysosomes to form autophagic lysosomes, in which substrates are degraded by the hydrolytic enzymes within. In microautophagy, the lysosomal membrane itself collapses, instead of forming an autophagosome, and substrates are directly enveloped, phagocytosed, and degraded in the lysosomes. In CMA, soluble proteins carrying specific amino acid sequences are recognized and bound by molecular chaperones and then transported into lysosomes via the lysosomal-associated membrane protein 2a (LAMP2A) receptor on the lysosomal membranes, where they are degraded by hydrolase. Therefore, this

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process represents selective protein degradation and differs from macroautophagy and microautophagy.

The process of autophagy can be divided into the following five stages: induction, vesicle nucleation, elongation, maturation of the autophagosome, and cleavage of the autophagosome. The amino acids and proteins produced by the degradative process can provide raw materials and energy for cells. The mammalian target of rapamycin (mTOR) complex can inhibit autophagy in the short term and the transcription factors Forkhead Box O (FOXO) and transcription factor EB (TFEB) can regulate autophagy in the long term.^[8] A number of assays can be used to monitor autophagy in experimental systems, such as transmission electron microscopic analysis of the accumulation of autophagosomes, the identification of green fluorescent protein-labeled microtubule-associated protein 1 light chain 3 puncta on fluorescence microscopy, or changes in the amount of lipidated LC3 on western blots. The correct interpretation of the results requires the complementary use of markers of autophagy to estimate overall autophagic flux, for example, by measuring the rate of general protein breakdown by autophagy.^[9]

Autophagy and NAFLD

A number of studies have investigated the role of autophagy and its relationship with the development and progression of NAFLD. NAFLD represents a hepatic complication of metabolic syndrome, in which the level of autophagy is low in hepatocytes.^[10] The examination of liver sections from individuals with severe steatosis showed an accumulation of the autophagy substrate p62/SQSTM1.^[11,12] The proposed mechanisms for this are as follows: (1) an obesity-induced increase in the expression of the calcium-dependent protease calpain II, resulting in Atg7 degradation and autophagy defects;^[13] (2) overactivation of the autophagic inhibitor mTOR in the liver; (3) hyperinsulinemia;^[14] (4) a defect in lysosomal acidification and a decrease in cathepsin L expression, impairing the degradation of lysosomal substrates;^[15] (5) a defect in the fusion of autophagosomes and lysosomes;^[16] and (6) lipotoxicity induced by excessive triglyceride and FFA accumulation, which occurs in the context of insulin resistance and oxidative stress, and inhibits autophagic activity.^[11,17] Notably, insulin can inhibit autophagy through the activation of mTORC1, leading to UNC-51-like kinase 1 (ULK1) phosphorylation and the inactivation of FOXO transcription factors.^[18] Consistent with this, hepatic autophagy is compromised in patients with obesity, NAFLD, and insulin resistance.^[19]

Autophagy not only regulates lipid metabolism and insulin resistance but also protects hepatocytes against injury and death.^[20] Therefore, the activation of hepatocyte autophagy might represent a means of treating NAFLD. In addition, weight loss achieved through bariatric surgery ameliorates liver steatosis and upregulates the autophagy-related genes Atg5 and Atg7 and the LC3B-II/I ratio, suggesting that the post-surgical restoration of autophagy improves hepatic lipid clearance via lipophagy.^[21]

The regulation of autophagy in hepatocytes is complex. mTOR is a crucial negative regulator of autophagy that

potently inhibits autophagy by the direct phosphorylation and inhibition of ULK1. The upstream regulators of mTOR are phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), AMP-activated protein kinase (AMPK),^[22] and p53.^[23,24] AMPK also promotes autophagy by directly activating ULK1 [Figure 1]. Therefore, in recent years, many studies have aimed to determine whether activating the AMPK–mTOR pathway would improve NAFLD by promoting autophagy. Natural products, such as resveratrol, acetylshikonin, anthocyanin, and trehalose, have been reported to enhance autophagy by activating the AMPK–mTOR signaling pathway,^[25-28] and a substantial amount of research is currently being performed regarding the prevention and treatment of hepatic steatosis using these molecules, which may cause fewer side effects.

The most important roles of autophagy are in cell quality control and energy turnover.^[29,30] In the context of NAFLD, the accumulation of toxic lipids modifies intracellular organelles, especially the ER and mitochondria. The net result of autophagy is the removal of damaged or dysfunctional mitochondria and ER, and the degradation of LDs.^[31,32]

Role of Lipophagy in Liver Lipid Metabolism

In hepatocytes, lipophagy results in the release of lipids stored in LDs,^[33] and this is considered to be the fifth pathway of lipid metabolism, in addition to the four classical lipid metabolism pathways of FFA uptake, *de novo* lipogenesis, fatty acid oxidation, and triglyceride secretion [Figure 2].^[6]

Under physiological conditions, autophagy participates in the turnover of lipids through phagocytosis and the degradation of LDs.^[18] The four principal pathways of the lipid metabolism that contribute to the level of storage of TG are as follows: (1) the uptake of FFAs from the circulation, derived from the diet or adipose tissue; (2) hepatic fatty acid synthesis, also known as *de novo* lipogenesis; (3) secretion in the form of VLDL particles; and (4) fatty acid oxidation.^[34] The process of intracellular lipid transport to lysosomes in autophagosomes is termed lipophagy. Lipophagy leads to the turnover of LDs, which represents a novel pathway with relevance to NAFLD.

As a means of reducing their toxic effects, FFAs can be esterified to form TGs and cholesterol esters in cells.^[35,36] This anabolic process occurs in the ER and leads to the deposition of neutral lipids in a lenticular microdomain

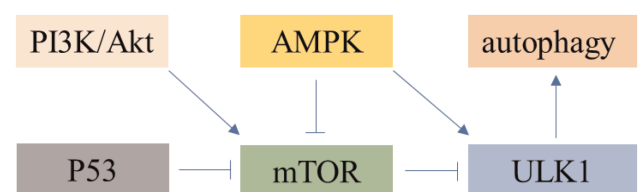


Figure 1: Regulation of autophagy. AKT: Protein kinase B; AMPK: AMP-activated protein kinase; PI3K: Phosphatidylinositol 3-kinase; ULK1: UNC-51-like kinase 1.

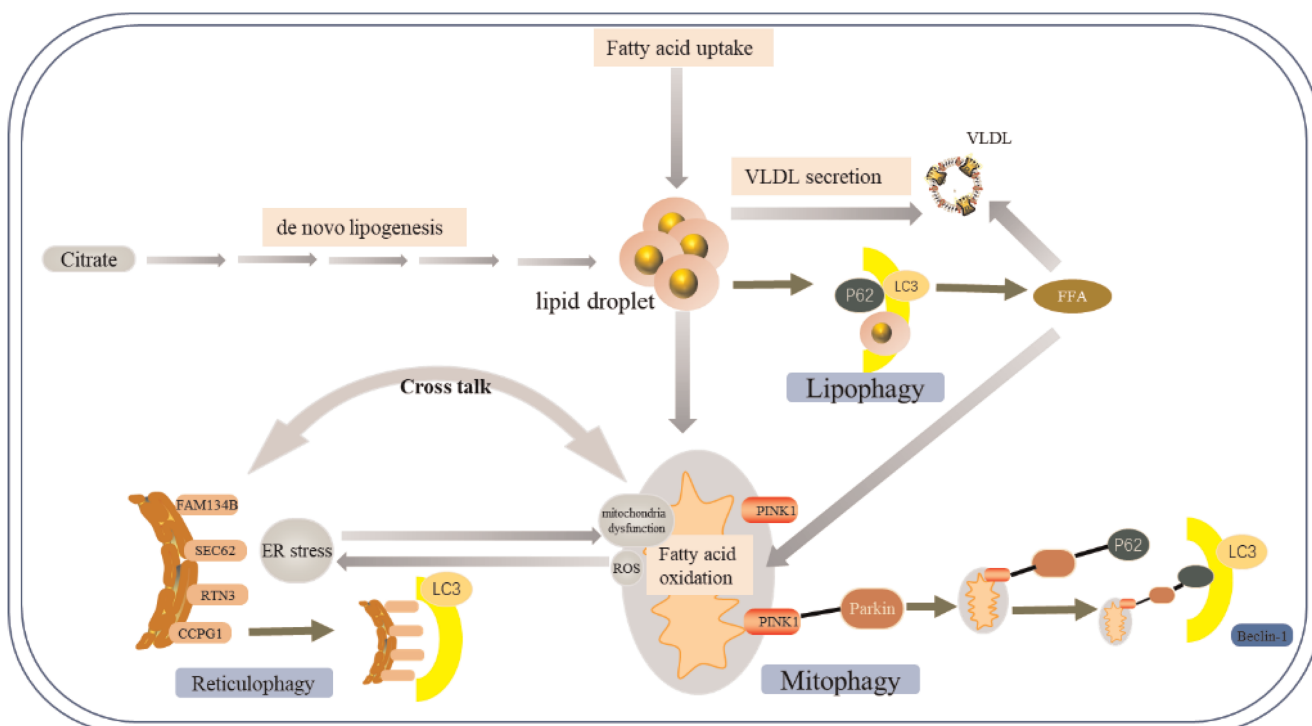


Figure 2: Schematic of the four main pathways involved in hepatic lipid metabolism, and the processes of lipophagy, mitophagy, and reticulophagy. ER: Endoplasmic reticulum; FFA: Free fatty acid; ROS: Reactive oxygen species; VLDL: Very low-density lipoprotein.

between the two layers of the ER, which ultimately leads to the expansion of the outer leaves of the ER bilayer to form a unique spherical organelle: an LD.^[37] LDs are complex subcellular organelles that have a unique and dynamic proteome.^[38] The formation of LDs is a means of preventing lipid toxicity in the liver and other organs.^[39] One feature of steatosis is the abnormal accumulation of LDs in hepatocytes.

Singh first proposed the link between autophagy and liver lipid metabolism in 2009.^[40] The inhibition of the autophagy regulatory factor ATG5 using the autophagy inhibitor 3-methyladenine (3-MA) or small interfering RNA results in an increase in liver TG content and affects mitochondrial β -oxidation. However, the increase in the number of LDs caused by a deficiency of autophagy is not the result of an increase in TG synthesis, but a decrease in lipid turnover. In this study, it was found that a lipid load, such as the addition of oleic acid (OA) to hepatocytes, activates lipophagy, which may represent an adaptive response to excess lipid. However, long-term lipid-loading such as in the form of high-fat diet feeding inhibits lipophagy. In a mouse model of high-fat diet-induced obesity, there was a lower hepatic expression of Atg7 and an inhibition of autophagy.^[41] The mechanism involved is unclear, but it may be related to poor autophagic lysosomal fusion, secondary to the change in lysosomal membrane lipid composition, resulting from changes in lysosomal pH, ATP supply, and diet.^[16,42] This study updated the general view that lipid is catabolized in hepatocytes only through the action of cytoplasmic hormone-sensitive lipases, and that FFAs are then sent to mitochondria for oxidation. In fact, the proteins that

cover the surface of LDs (mainly perilipin 2, Plin2) have a “shielding effect,” reducing the accessibility of TGs to lipases. Therefore, they must be removed to expose the lipid core of the LDs, which can be achieved through CMA.^[43,44] AMPK mediates the binding of Plin2 to LAMP2A, which results in greater catabolism of Plin2 and the consequent exposure of LDs to lipohydrolases, reducing the amount of lipid stored in hepatocytes.^[45]

The lipophagic degradation of LDs is principally in the form of macroautophagy. First, the formation of autophagosomes is initiated through a complex array of signaling pathways. Then, autophagy receptors on LDs, such as p62, optic nerve protein, NBR1, NDP52, and Huntingtin protein, are recognized by microtubule-associated protein 1 light chain 3 on the autophagosome membrane,^[43] which connects the membranes of the organelles. During this process, the proteins on the surface of the organelles are polyubiquitinated and act as recruitment signals, and then the isolated bilayer membrane gradually extends and closes to form autophagosomes. The outer membrane (OMM) of these autophagosomes fuses with lysosomes to form autophagosomes. In the lysosomes, acid lipase degrades TGs to liberate FFAs and glycerol. The autophagy pathway can rapidly eliminate a large amount of stored cellular lipid, and therefore impaired lipophagy contributes to the development of NAFLD.^[40] Previous studies have shown that rats fed a high-fat diet for 4 months develop hepatosteatosis and exhibit defective hepatic autophagy. However, they do not show changes in the expression of the autophagy-related factors Atg5 and Atg7 or the formation of autophagosomes, evidenced by normal LC3B-II/I conversion and p62 accumulation, despite the hepatic lipid

overload. Thus, hepatic lipophagy might be influenced by the degree of hepatic steatosis, with changes in autophagosome formation during the progression of NAFLD.^[121]

In hepatocytes, the abnormal accumulation of LDs is a marker of steatosis and an important pathological feature of the early stages of NAFLD. At present, the most effective available means of treating NAFLD are exercise and caloric restriction, which can induce autophagy in various tissues, including the liver, thereby reducing steatosis.^[46-48] Liver autophagy induced directly by rapamycin or carbamazepine protects against high-fat diet-induced NAFLD in mice.^[49] In addition, the induction of lipophagy by caffeine,^[50] plant-derived substances such as resveratrol, diosgenin, and capsaicin,^[51,52] quercetin (a flavonoid),^[53] and anthocyanin^[54] also ameliorates NAFLD. Thus, the drug-induced upregulation of lipophagy may represent a novel method of treating NAFLD.

Mitophagy

Mitochondria are subject to stress-induced dysfunction. Mitochondrial damage results in two main responses: (1) the mitochondrial unfolded protein response (UPR^{mt}) promotes cell recovery and survival of the mitochondrial network; and (2) dysfunctional mitochondria are eliminated by mitophagy,^[55] as first described by LeMasters in 1998.^[56,57] Each type of stimulus is dealt with by a unique mitophagic effector and receptor, but they share an LC3 region that promotes mitochondrial sequestration.^[58] However, mitophagy and macroautophagy share a number of signaling pathways and key regulatory proteins, such as LC3 and p62.

Mitophagy is a form of targeted phagocytosis, and the destruction of mitochondria by autophagy represents a key mechanism of mitochondrial quality control. The classical pathways mediating mitophagy are the PINK1-parkin-dependent pathway and the BNIP3/NIX pathway. Narendra *et al*^[59] identified parkin as an E3 ubiquitin ligase that is encoded by the *PARK2* gene in 2008. PINK1 is strongly anchored to the mitochondrial OMM and promotes the recruitment of parkin under conditions of stress. Parkin then ubiquitinates OMM proteins and promotes their binding of mitophagy adaptors, such as p62. Both of them contain a ubiquitin-binding region and an LC3 interaction region, and when LC3 is recruited by an adaptor protein, mitophagy occurs. However, mitochondria can also be degraded by autophagy independent of parkin. The BNIP3 and NIX receptors are also mitochondrial resident proteins that contain BH3 domains, which interact with LC3, causing the recruitment of autophagosomes to mitochondria [Figure 2].

Mitochondria are vital for liver lipid metabolism because they perform FFA oxidation. Thus, mitophagy plays an important role in the maintenance of mitochondrial and intracellular homeostasis during stress, and a reduction in mitophagy permits chronic mitochondrial dysfunction and may lead to liver failure.^[60] Indeed, an impairment in mitochondrial function is part of the “second hit” of lipotoxicity in the etiology of NAFLD.^[60,61] In the early

stages of NAFLD, mitochondrial activity is upregulated to compensate for the excessive lipid influx. However, sustained excessive lipid supply causes defects in mitochondrial structure and function,^[62] characterized by a loss of the circular shape of the mitochondria and of the folding of the mitochondrial membranes, especially during NASH,^[63-65] and defective mitochondrial respiratory chain activity and ATP production.^[66-68] In addition, the greater lipid influx leads to a large amount of incomplete mitochondrial oxidation, which results in the greater production of reactive oxygen species (ROS) and toxic lipid intermediates.^[69] The decrease in cellular antioxidant concentrations also contributes to the accumulation of ROS in mitochondria. Mitochondrial coenzyme Q10 acts as an antioxidant on the mitochondrial membrane and is less abundant in patients with NASH.^[70] One study has shown that mitochondrial biogenesis and quality are regulated in patients with NAFLD, but that mitophagy is defective.^[71] In addition, the expression of PINK1 and parkin is lower in a high-fat diet-fed mouse model of NAFLD, which implies that mitophagy is downregulated.^[72]

The removal of damaged mitochondria by mitophagy is an adaptive mechanism in chronic NAFLD. A series of NAFLD-related phenotypes, such as defects in mitophagy and greater fat accumulation, oxidative stress, and inflammation, have been identified in high-fat diet-fed animal models and cells cultured in the presence of OA or palmitic acid (PA).^[61] Although most studies have shown that mitophagy is inhibited in NAFLD, others have shown that continuous exposure to lipids induces mitophagy, but the mechanisms remain to be determined.^[73] However, efforts have been targeted at increasing mitophagy to ameliorate NAFLD. Yu *et al*^[74] found that liraglutide reduces lipid accumulation and the production of ROS, ameliorates mitochondrial dysfunction, and enhances mitophagy in a HepG2 model of NASH created by incubation with both PA and lipopolysaccharide. Furthermore, the inhibition of mitophagy by 3-MA or PINK1-directed siRNA attenuates the anti-inflammatory effect of liraglutide. The anthocyanin cation cyanidin-3-O-glucoside has also been shown to ameliorate NAFLD by promoting PINK1-mediated mitophagy in mice.^[75] Quercetin, which is a plant flavonoid, has been shown to induce mitophagy through a PINK1/parkin-dependent pathway, thereby ameliorating hepatic steatosis.^[76] In addition, the inhibition of macrophage-stimulating 1 (Mst1) ameliorates NAFLD by improving parkin-related mitophagy.^[77] The high-fat diet-induced liver injury involves the downregulation of SIRT3 and the inhibition of mitophagy mediated by BNIP3, resulting in the activation of a mitochondrial-dependent death pathway in hepatocytes.^[78] Akebia saponin D has also been shown to ameliorate hepatic steatosis by inducing BNIP3-mediated mitophagy.^[79] Finally, wolfberry enhances mitophagy and promotes mitochondrial biogenesis, thereby preventing hepatic steatosis in obese mice.^[80]

Reticulophagy

The concept of reticulophagy was first proposed in 2007 and referred to as ER stress inducing selective autophagy

of ER in yeast.^[81] The main function of reticulophagy is to degrade excess ER membrane to control the volume of the ER and maintain cellular homeostasis. In recent years, it has been shown that ER stress not only can trigger non-selective cellular macroautophagy but also can directly induce reticulophagy. ER components are sequestered into double-membrane-enclosed autophagosomes and delivered to vacuoles for degradation.^[82,83]

An appropriate volume and activity of the ER are maintained under physiological conditions, and the structure expands under ER stress, but subsequently returns to its original dimensions. An ER quality control system is used to monitor the folding of membrane and secretory proteins by ER-associated protein degradation (ERAD), the unfolded protein response (UPR), and autophagy. The homeostasis of the ER depends on appropriate quality control of the organelle, with the key objective being to prevent the aggregation of unfolded proteins in the lumen. ERAD refers to the translocation of unfolded proteins to the cytoplasm, which is followed by their 26S proteasomal degradation.^[84] However, not all unfolded proteins are suitable for ERAD, and reticulophagy is required to fully resolve the ER stress. Thus, reticulophagy is considered to be a basic regulatory pathway of the ER quality control system. It eliminates excess ER components by delivering fragments to lysosomes, thereby maintaining ER and protein homeostasis.^[85,86]

The receptors that mediate mammalian reticulophagy include FAM134B, SEC62, RTN3, and CCPG1^[85,87-91] [Figure 2], all of which contain an LC3-interacting region that binds LC3 on autophagic vesicles. The ER fragments are then encapsulated by autophagosomes and sent to lysosomes for degradation. However, different receptors mediate the autophagic degradation of different components of the ER during the various stages of ER stress.^[92] FAM134B and RTN3 both promote reticulophagy by increasing membrane bending and ER rupture, whereas FAM134B mainly mediates the remodeling and fracture of ER sheets, RTN3 causes the fragmentation of ER tubules.^[89] After ER stress, SEC62 promotes recovery reticulophagy, which eliminates the excess ER membrane produced during the acute phase of the UPR.^[90]

Reticulophagy occurs at a low level to maintain ER homeostasis under normal physiological conditions, but it is significantly upregulated during persistent ER stress, nutritional deficiency, unfolded protein aggregation, and pathogen infection.^[93] If reticulophagy is dysregulated, the accumulation of unfolded proteins triggers the UPR, which can result in inflammation, cell death, and carcinoma.^[94-100]

Severe ER stress and impaired autophagy characterize NAFLD^[11,101] because the ER not only is the principal site of LD synthesis but also provides the enzymes required for drug and nutrient metabolism.^[102] Many studies have shown that liver lipid overload induces ER stress, which promotes hepatic lipogenesis and steatosis.^[103] In addition, the UPR signaling cascade may be involved in the inflammation that characterizes NASH.^[104] Autophagy can alleviate ER stress by eliminating the excess lipid and dysfunctional ER,

thereby ameliorating NAFLD.^[105] The UPR, which is an ER stress response that restores protein homeostasis, is one of the causes of the chronic hepatic inflammation that characterizes obesity-associated NAFLD. Interestingly, weight loss induced by bariatric surgery ameliorates ER stress, which likely contributes to the reduction in NAFLD following surgery.^[106,107]

FFA-induced lipotoxicity impairs ER function by increasing protein misfolding and inducing ER stress, thereby aggravating NAFLD.^[108] Cui *et al*^[109] found that reticulophagy is upregulated to protect HepG2 cells treated with OA, and Pang *et al*^[73] found that reticulophagy was induced instead of lipophagy in HepG2 cells treated with 400 $\mu\text{mol/L}$ OA, thereby preventing lipotoxicity-induced apoptosis. In fact, OA treatment induces mitophagy once reticulophagy is inadequate, an effect that is mediated by DRAM, and leads to cell death. Pang *et al* also found that autophagy is occurring in liver samples from patients with mild or severe steatosis. However, the activation of the PI3K/AKT pathway could only be detected in the liver tissue of patients with mild steatosis, and the expression of DRAM could only be detected in the liver tissue of patients with severe steatosis. The results of this clinical study are consistent with our *in vitro* findings, and the findings together imply that reticulophagy and mitophagy may be independently involved in the progression of NAFLD, having both anti-apoptotic and pro-apoptotic effects in hepatocytes. However, the mechanism of the transition from reticulophagy to mitophagy is unclear.

ER-Mitochondrial Crosstalk

There is strictly regulated communication under physiological conditions between the mitochondria and ER in hepatocytes, involving the shuttling of lipid and Ca^{2+} across organelles^[110] [Figure 2]. Previous studies have shown that FFAs, ROS, and insulin resistance can trigger ER stress, and the production of the transcription factors and kinases that mediate ER stress further aggravates mitochondrial dysfunction. The production of lipid peroxides because of lipid overload impairs ER homeostasis and leads to ER stress, which involves the activation of caspase-12, caspase-3, and other factors, leading to apoptosis. ER stress is also characterized by the outflow of stored Ca^{2+} , and excessive mitochondrial Ca^{2+} accumulation can lead to cell death by changing the mitochondrial membrane potential and opening the mitochondrial permeability transition pore^[111,112]. Dying mitochondria release further Ca^{2+} , forming a vicious circle that leads to apoptosis^[112,113]. However, it remains unclear whether changes in inflammation, mitochondrial dysfunction, and ER stress precede or are consequences of the development of hepatic steatosis^[114]. The mechanism by which the accumulation of saturated FFAs in hepatocytes affects ER-mitochondrial communication requires further exploration.

Conclusions and Future Perspectives

In this review, we have described the three organelle-specific forms of autophagy that are involved in NAFLD and discussed the crosstalk between the ER and

mitochondria. We have summarized the findings of studies that demonstrate that substances that target lipophagy, mitophagy, or reticulophagy help restore hepatic lipid homeostasis and cell quality control in models of NAFLD. Organelle-specific autophagy is a complex process, and any impediments may contribute to the development of NAFLD. In addition, NAFLD itself might lead to impairments in organelle-specific autophagy, resulting in a vicious cycle that would promote disease progression.

Autophagy has two functions: to participate in catabolism to generate substrates to maintain cellular energy homeostasis during malnutrition; and to remove unwanted cell components.^[115-119] Damaged organelles, such as mitochondria, ER, and peroxisomes, undergo autophagy to preserve cell function, and lipids are degraded in an attempt to maintain energy balance during NAFLD. However, in general, autophagy is inhibited and its substrates accumulate during NAFLD. Lipophagy, mitophagy, and ER-phagy are responsible for the degradation of LDs, mitochondria, and the ER, respectively; participate in lipid turnover; compensate for excessive lipid turnover; and maintain intracellular homeostasis. A number of drugs that target autophagy have considerable potential for the treatment of NAFLD, but the importance of the various types of autophagy at the various stages of disease progression, the relationship between autophagy and cellular lipid load, and the communication between mitochondria and the ER require further study.

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Conflicts of interest

None.

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