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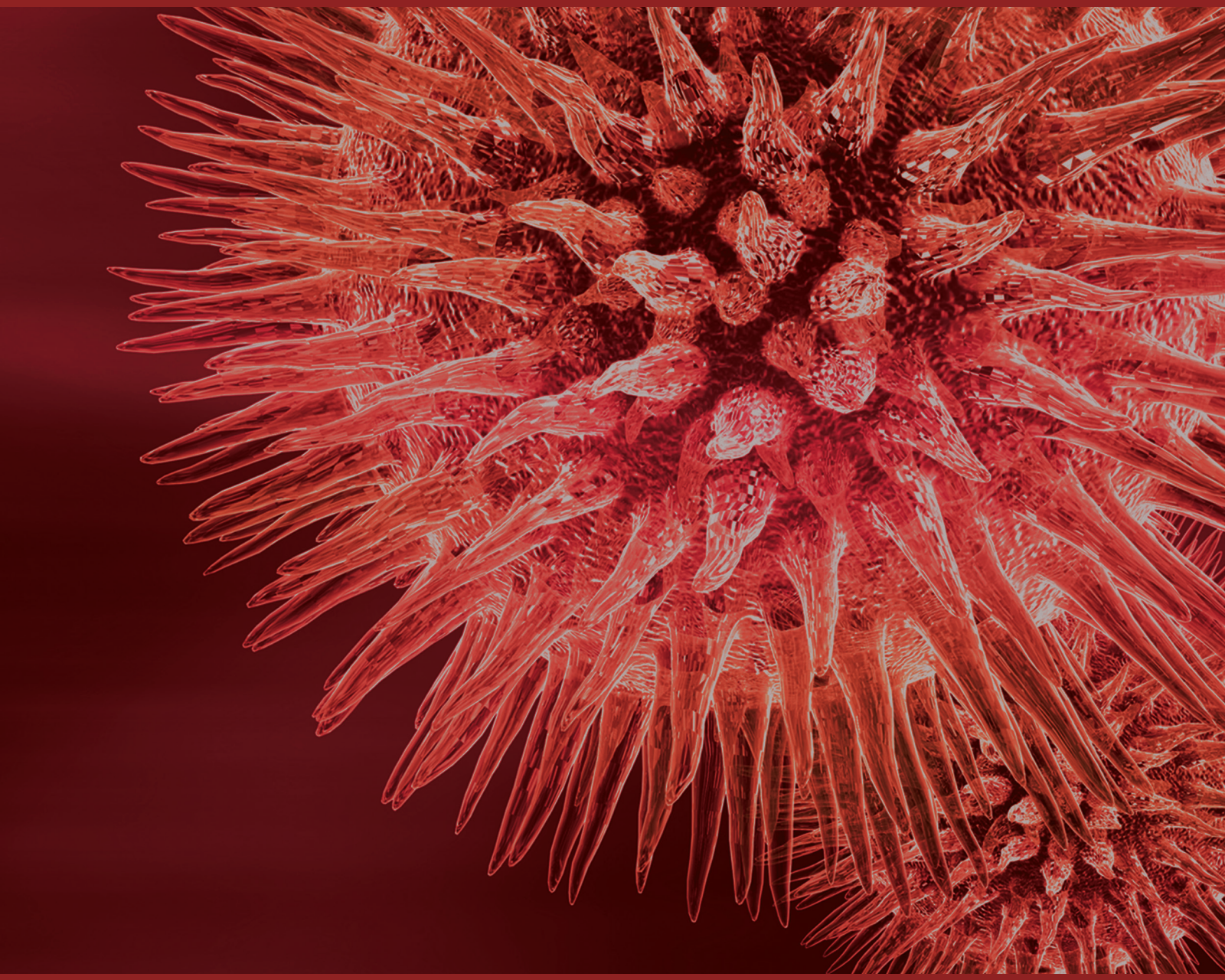
Guest Editors: Raffaele Cursio, Pascal Colosetti, Patrice Codogno, Ana Maria Cuervo, and Han-Ming Shen



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Editorial

The Role of Autophagy in Liver Diseases: Mechanisms and Potential Therapeutic Targets

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Autophagy, or cellular self-digestion, is an orchestrated cellular pathway crucial for development, differentiation, homeostasis, and survival of cells. The autophagic process is used to eliminate unwanted proteins and damaged organelles and to remove intracellular microbial pathogens. Normal liver function requires hepatocellular basal autophagy. In fact, due to their high biosynthetic activity and role in protein turnover and carbohydrate storage, hepatocytes may be particularly dependent on basal autophagy for their normal physiological functions.

Autophagy seems to play an important role not only in normal liver physiology, but also in the pathogenesis of liver diseases such as nonalcoholic and alcoholic fatty liver, drug-induced liver injury, protein conformational liver diseases, viral hepatitis, fibrosis, aging, liver cancer, and liver ischemia-reperfusion injury.

In this special issue, original research and review articles have focused on the role of autophagy in the pathogenesis of the above mentioned liver diseases, bringing new knowledge and suggesting modulation of autophagy as basis for possible treatments for these pathologies.

The liver is one of the principle regulators of lipid in the body. Obesity is closely associated with an increased risk of metabolic diseases including nonalcoholic fatty liver disease (NAFLD). NAFLD ranges from isolated steatosis to nonalcoholic steatohepatitis (NASH) and steatofibrosis, which sometimes leads to cirrhosis and to hepatocellular carcinoma (HCC). Under physiological conditions, autophagy participates in the basal turnover of lipids by engulfing and degrading lipid droplets. In obesity, a decrease of autophagy levels in hepatocytes has been described. As illustrated by V. J. Lavallard and P. Gual in their review, several mechanisms may account for this decline. The authors describe the role of autophagy in specific cells, including hepatocytes, macrophages, hepatic stellate cells (HSCs), and cancer cells and outline its role in the evolution of hepatic complications associated with obesity, from steatosis to HCC. They suggest that activation of autophagy in hepatocytes could constitute a therapeutic approach against hepatic complications of obesity.

In the pathogenesis of NASH, the mitochondrial dysfunction participates in different levels since it impairs fatty liver homeostasis and induces overproduction of reactive oxygen species (ROS) that in turn triggers lipid peroxidation, cytokines release, and cell death. Mitochondrial uncoupling protein 2 (UCP2) seems to have a role in the development of NASH. In their original study J. Lou et al. investigate the relationship between UCP2 and hepatoma cells autophagy

in palmitic acid- (PA-) induced lipotoxicity. They provide evidence that increasing UCP2 expression in hepatoma cells contributes to autophagy. Moreover, UCP2 is a proliferative factor that also has an antiapoptotic role during PA-induced liver injury. These results provide insights into potential NASH therapies.

As outlined in the review of Y. Li et al., autophagy plays significant roles in preserving hepatocyte homeostasis and viability in alcohol consumption-induced multiple tissue/organ injuries including hepatic steatosis and liver injury, pancreatitis, impaired heart function, brain damage, and loss of muscle mass. Although autophagy serves as a cellular protective mechanism against alcohol-induced tissue injury in most tissues, it can be detrimental in heart and muscle. In the liver, it seems that alcohol metabolism through alcohol dehydrogenase and cytochrome P450 family 2, subfamily E, polypeptide 1 (Cyp2E1), is required for autophagy activation. Acute alcohol treatment also induces forkhead box-containing protein class O (FoxO) family of DAF-16 like transcription factor 3-mediated autophagy. Finally, autophagy seems to selectively remove damaged mitochondria and excess lipid droplets and in turn attenuates alcohol-induced steatosis and liver injury.

Liver fibrosis is a common wound healing response to chronic liver injury of all causes. Its end-stage cirrhosis is responsible for high morbidity and mortality worldwide. As a multifaceted partner in liver fibrosis, autophagy elicits divergent and cell-specific effects during chronic liver injury as outlined in the review by A. Mallat et al. In fact, autophagy enhances fibrogenic properties in HSCs and liver fibrosis. In contrast, through its anti-inflammatory effects in macrophages and hepatoprotective effects in hepatocytes, autophagy limits the development of liver fibrosis.

The dual role played by autophagy in inflammation and lipid metabolism in hepatitis C virus- (HCV-) infected liver cells raises an important question regarding the contribution of autophagy defects in disease progression towards steatohepatitis, fibrosis, cirrhosis, and HCC in patients with chronic HCV infection. In their review, T. Vescovo et al. discuss the molecular mechanisms that link the HCV life cycle with the autophagy machinery. In particular, the authors outline the role of HCV/autophagy interaction in dysregulating inflammation and lipid homeostasis and the potential applications in the treatment of HCV-infected patients.

Alpha-1-antitrypsin deficiency (ATD) is one of the most common genetic causes of liver disease. ATD is the prototype of liver disease caused by pathologic accumulation of aggregated mutant alpha-1-antitrypsin Z (ATZ) within liver cells. Accumulation of ATZ in the liver specifically activates autophagy. In their review, A. S. Chu et al. summarize research advances in autophagy and genetic liver diseases. They discuss autophagy enhancing strategies for liver disease due to ATD and to other genetic liver diseases, for example, inherited hypofibrinogenemia, caused by the proteotoxic effects of a misfolded protein. On the basis of recent evidence that autophagy plays a role in cellular lipid degradation, the authors also speculate about autophagy enhancing strategies for treatment of hepatic lipid storage diseases such as cholesterol ester storage disease.

As outlined by M. Kheloufi et al. in their review, increasing evidence demonstrates that autophagy plays a critical role in acute liver injury related to severe anorexia nervosa (AN) and to drug overdose. AN has the highest rate of mortality among eating disorders and can be associated with severe liver insufficiency. Overdose of acetaminophen (APAP), a widely used antipyretic and analgesic drug, is the first cause of acute liver failure in humans. Efavirenz, a nonnucleoside reverse transcriptase inhibitor widely used to treat human immunodeficiency virus (HIV) infections, can be hepatotoxic in some patients. Increased liver autophagy levels are a common feature of these disorders. Autophagy is mainly hepatoprotective. In AN, during the first phase of weight loss, liver blood test abnormalities are moderate suggesting that autophagy can cope with nutrient deprivation. During that period, autophagy is protective and prevents cell death. When starvation continues and body mass index reaches a critically low level, excessive activation of autophagy leads to hepatocyte cell death and liver insufficiency. After APAP or Efavirenz exposure, autophagy removes damaged mitochondria, and liver injury appears only when this process is either blocked by other factors or overwhelmed.

Liver ischemia-reperfusion (I-R) injury which occurs during liver resection, liver transplantation, and hemorrhagic shock can induce liver dysfunction and can increase patient morbidity and mortality after liver surgery, particularly liver transplantation and hemorrhagic shock. Whether autophagy protects from or promotes liver injury following warm and/or cold I-R remains to be further elucidated. R. Cursio et al. summarize in their review the current knowledge on liver I-R injury focusing on both the beneficial and detrimental effects of liver autophagy following warm and/or cold liver I-R. The autophagic cell response to warm and/or cold liver I-R may delay apoptosis and necrosis and thus ultimately increases the possibility for novel therapeutic intervention to diminish the extent of warm/cold liver I-R injury.

The liver is an organ of great complexity with multiple types of cells, parenchymal and nonparenchymal, and with multiple functions too. Dysregulation of liver autophagy functions has an impact on liver physiology, but also on pathologies of the liver. Dysregulation or decrease of autophagy in alcoholic liver diseases can lead to liver cell death, steatohepatitis, and HCC. In liver I-R injury, autophagy has mainly prosurvival effects, while, in hepatitis C infection, increased autophagy supports virus replication. In some diseases, autophagy may have opposite effects; for example, in liver fibrosis, autophagy may be profibrotic as well as antifibrotic. This dual role played by autophagy is one of the main challenges for the establishment of future therapeutic approaches to these liver diseases.

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Review Article

Autophagy and Liver Ischemia-Reperfusion Injury

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Liver ischemia-reperfusion (I-R) injury occurs during liver resection, liver transplantation, and hemorrhagic shock. The main mode of liver cell death after warm and/or cold liver I-R is necrosis, but other modes of cell death, as apoptosis and autophagy, are also involved. Autophagy is an intracellular self-digesting pathway responsible for removal of long-lived proteins, damaged organelles, and malformed proteins during biosynthesis by lysosomes. Autophagy is found in normal and diseased liver. Although depending on the type of ischemia, warm and/or cold, the dynamic process of liver I-R results mainly in adenosine triphosphate depletion and in production of reactive oxygen species (ROS), leads to both, a local ischemic insult and an acute inflammatory-mediated reperfusion injury, and results finally in cell death. This process can induce liver dysfunction and can increase patient morbidity and mortality after liver surgery and hemorrhagic shock. Whether autophagy protects from or promotes liver injury following warm and/or cold I-R remains to be elucidated. The present review aims to summarize the current knowledge in liver I-R injury focusing on both the beneficial and the detrimental effects of liver autophagy following warm and/or cold liver I-R.

1. Introduction

Partial or complete interruption of the liver blood flow and, consequently, interruption of its oxygen supply, followed by reperfusion and reestablishment of blood flow and oxygen supply, characterizes the liver ischemia-reperfusion (I-R) process. The cellular injuries caused by the ischemic period are aggravated by reperfusion [1–4]. Not only liver transplantation or resection but also liver injury following blunt or penetrating abdominal trauma [5] and hemorrhagic shock [6] may cause low liver blood flow resulting in insufficient perfusion. Finally after reperfusion liver I-R injury occurs. Liver I-R injury following hemorrhagic shock remains a major cause of morbidity and mortality after trauma [6]. Liver I-R rapidly leads to an acute inflammatory response, causing significant hepatocellular damage and organ dysfunction. The severity of injury ranges from moderately raised levels of serum aminotransferases to posthepatectomy insufficiency after liver resection or to primary nonfunction or initial poor graft function after liver transplantation [7–13]. Under extreme circumstances, multiple organ failure and death may occur. More precisely, in liver transplantation, up to 10% of early transplant failures are caused by I-R injury with a higher incidence of both acute and chronic graft rejection increasing the need for retransplantation [9–13]. Liver I-R may also be responsible for ischemic-type biliary lesions (ITBls) [14–16] and late allograft failure [17, 18]. The use of marginal liver grafts from non-heart-beating donors, older and/or steatotic organs, and of organs that have been subjected to prolonged periods of warm ischemia and cold storage, has increased in the last years due to organ shortage [19]. These grafts are more vulnerable to warm/cold I-R injury [19] underlining the need of therapeutic strategies to reduce liver I-R injury in order to improve graft viability [20].

Necrosis represents the main mode of liver cell death following warm/cold I-R [21–23], but other modes of cell death, namely, apoptosis [2, 24, 25] and autophagy [26–48], also play an important role.

Mammalian autophagy is an intracellular self-digesting pathway responsible for the removal of long-lived proteins, damaged organelles, and malformed proteins during biosynthesis by lysosomes [49]. Autophagy is divided into three

main types according to the different pathway by which cargo is delivered to the lysosome or vacuole: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy [50]. The last type can be divided into six principal steps: initiation, nucleation, elongation, closure, maturation, and degradation or extrusion [49]. Through these steps cytoplasmic materials, such as protein aggregates and organelles, are sequestered by the phagophore, a preautophagosomal membrane structure, which thereafter expands and encloses its cargo to form a double-membrane vesicle, the autophagosome [51]. By its fusion with a lysosome, it forms an autolysosome, in which the enclosed cargo is degraded by acid hydrolases into biologically active monomers such as amino acids that are subsequently reused to maintain cellular metabolic turnover and homeostasis [51].

Autophagy is involved in normal and diseased liver [52–55]. Studies on autophagy in liver tissue following warm/cold I-R remain controversial. Some show defective and/or decreased autophagy [27–30, 32, 34–37, 39–41, 45, 47]; in others hepatocellular autophagy is increased [26, 31, 33, 38, 42–44, 46, 48]. Whether autophagy protects from or promotes liver injury following warm and/or cold I-R remains to be elucidated.

2. Pathomechanisms of Warm and/or Cold Liver I-R Injury

Warm ischemia of the liver is due to oxygen deficiency caused by vascular occlusion of the liver during liver resection or hemorrhagic shock [2, 10]. Cold ischemia is observed during liver transplantation in which the graft is subjected to warm and cold ischemia followed by a warm reperfusion phase; the warm/cold ischemia sequence is due to vascular occlusion of the liver graft during its procurement from the donor and to graft storage in cold preservation solutions before liver transplantation [2, 10]. Graft implantation into the recipient represents the warm reperfusion phase; all these phases lead to I-R injury of the liver [2, 10]. Pathomechanisms associated with warm I-R seem to differ from those after cold I-R in liver transplantation [57]. Unlike warm liver I-R, which results in primary damage to the hepatocytes, cold I-R is mainly characterized by injury to the sinusoidal lining cells [58–60].

The introduction of oxygenated blood during reperfusion aggravates the ischemic insult, which itself is mainly characterized by cellular adenosine triphosphate (ATP) depletion and results in perturbation of the cellular energy-dependent metabolic and transport processes [1, 61]. Although the graft metabolism is reduced during hypothermia, with a prolonged time period in which the anoxic cells can retain essential metabolic functions, hypothermia may induce liver injury by dysfunction of the Na/K ATPase membrane pump [61, 62]. The resulting intracellular influx and accumulation of sodium and chloride lead to a perturbation of calcium homeostasis and to cell swelling [61, 62]. The inflammatory response to liver I-R involves neutrophils, cytokines, chemokines, complement, monocytes, and macrophages [63–67]. The reperfusion process consists of two phases: in the initial phase, activated resident macrophages of the liver, the Kupffer cells, induce oxidative stress mainly by reactive oxygen species (ROS) generation and, in the later phase, 6–24 hours following reperfusion, recruited neutrophils release inflammatory mediators which can cause direct tissue damage [1]. Kupffer cells play a central role in the pathophysiological mechanisms of liver I-R. Activated Kupffer cells release both ROS and cytokines, including tumor necrosis factor alpha (TNFα), Interleukin-1 (IL-1), and Interleukin-6 (IL-6), leading to granulocytes accumulation in the sinusoidal space and causing microcirculatory disturbances [68]. Accumulation of activated neutrophils through release of oxidants and proteases leads to hepatocyte damage. ROS stimulate endothelial cells to secrete platelet activating factor (PAF) [69]. Monocytes and Kupffer cells-derived ROS activate redox-sensitive transcription factors AP-1 and nuclear factor kappa-B (NF-κB) in endothelial cells and hepatocytes [65]. Complement activation products activate Kupffer cells during the initial phase of liver injury and contribute to tissue inflammation as a membrane-attacking complex that stimulates the production of proinflammatory cytokines and chemotactic agents, which occur immediately after reperfusion [63]. Complement can also regulate the adaptative immunity [70, 71]. In fact, the inflammation occurring during liver reperfusion is predominantly an innate-immune-dominated response, which might induce I-R injury in both parenchymal and nonparenchymal cells, *in situ* and in liver transplants [66, 67, 72, 73]. However, the cold preservation injury of the graft causes a likewise strong adaptative immune response characterized by an early and massive T-cell influx into the ischemic liver graft [73–75]. The conventional T lymphocytes, CD4+ cells, accumulate in the liver within 1 hour after reperfusion preceding any neutrophil accumulation [76]. CD4+ cells are activated by various Kupffer cell-derived products and lead to hepatocytes and sinusoidal endothelial cells damage [76] and finally liver cells necrosis [22, 23, 77]. Apoptosis seems to be a relevant death mechanism during warm/cold liver I-R injury too [2, 24, 25] even if the studies are controversial on this issue [21, 24, 25]. Recently, the role of liver autophagy following warm and/or cold liver I-R has been highlighted [78].

3. Autophagy and Warm and/or Cold Liver I-R Injury

Beside necrosis [21–23] there are other modes of cell death, as apoptosis [2, 24, 25] and autophagy, that may occur simultaneously and or sequentially [26–48, 78] following warm and/or cold liver I-R. While some studies have shown a defective autophagy in hepatocytes following anoxia/reoxygenation and in liver tissue following I-R [27–30, 32, 34–37, 39–41, 45, 47], in others an increase of autophagy has been observed [26, 31, 33, 38, 42–44, 46, 48] (Table 1) with different effects on warm and/or cold liver I-R injury (Figure 1).

3.1. Protective Role of Autophagy against Warm and/or Cold Liver I-R Injury

3.1.1. In Vitro and In Vivo Animal Studies. The mammalian orthologue of yeast autophagy-related gene 6 (Atg6), Beclin 1,

Table 1: Autophagy and warm and/or cold liver I-R injury: *in vitro* and *in vivo* animal and human studies.

Species	Preexisting liver disease	Experimental liver I-R model	Effects on autophagy	Modulation of autophagy by inhibitors	Modulation of autophagy by stimulators	Effects on I-R injury	Authors and references
Rat	No	Partial warm 60 min	Decreased	(a) Hemin + Chloroquine (b) Hemin + Wortmannin		Detrimental Detrimental	Yun et al. 2014 [47]
Mice	No	Partial warm 60 min	Increased	Melatonin		Protective	Kang et al. 2014 [48]
Mice	No	Partial warm 45 min	Increased	Ethyl pyruvate		Protective	Shen et al. 2013 [46]
Mice	No	Total warm 45 min	Decreased		Carbamazepine	Protective	Kim et al. 2013 [45]
Rat	No	Partial warm 60 and 90 min	Increased	Chloroquine		Protective (early reperfusion phase) Detrimental (late reperfusion phase)	Fang et al. 2013 [44]
Rat	No	Partial warm 90 min	Increased	Chloroquine Chloroquine + NAC		Detrimental Protective	Sun et al. 2013 [43]
Rat	No	Partial warm 60 min	Increased		Lithium	Protective	Liu et al. 2013 [42]
Rat	Steatosis	Cold ischemia 24 h	Decreased		Melatonin + Trimetazidine	Protective	Zaouali et al. 2013 [41]
Rat	Steatosis	Cold ischemia 16 h/30 min warm reperfusion *in situ*	Decreased			Detrimental	Gracia-Sancho et al. 2013 [40]
Human	No	Hypoxia/reoxygenation of hepatocytes	Decreased	3-MA		Protective	Bhogal et al. 2012 [39]
Mice	No	(a) Anoxia/reoxygenation of hepatocytes (b) Partial warm 90 min	Increased Increased		Rapamycin Rapamycin	Protective Protective	Wang et al. 2012 [38]
Calcium/ Calmodulin-kinase IV KO mice	No	Partial warm 60 min	Decreased		Rapamycin	Protective	Evankovich et al. 2012 [37]
Human	No	Liver transplantation	Decreased		Ischemic preconditioning	Protective	Degli Esposti et al. 2011 [35]
Old mice	No	Total warm 20 min	Decreased		(a) ALLM Calpain 2 inhibitor (b) Adenoviral overexpression of Atg4B and Beclin 1	Protective	Wang et al. 2011 [34]
Pig	No	Liver transplantation	Decreased		Hypothermic reconditioning by gaseous oxygen	Protective	Minor et al. 2011 [36]
Rat	Steatosis	Cold ischemia 20 h	Decreased		Hypothermic reconditioning by gaseous oxygen	Protective	Minor et al. 2009 [30]
Mice	No	Partial warm 60 min	Decreased		Cisplatin	Protective	Cardinal et al. 2009 [32]

Table 1: Continued.

Species	Preexisting liver disease	Experimental liver I-R model	Effects on autophagy	Modulation of autophagy by inhibitors	Modulation of autophagy by stimulators	Effects on I-R injury	Authors and references
Rat	No	Liver transplantation	Increased	(a) Wortmannin (b) LY294002		Protective	Gotoh et al. 2009 [33]
Human	Postchemotherapy steatosis	Total Warm	Decreased		Ischemic preconditioning	Protective	Domart et al. 2009 [29]
Old mice	No	Partial warm 30, 60, and 90 min	Decreased		Rosiglitazone	Protective?	Shin et al. 2008 [28]
Rat	No	(a) Total warm 45 min (b) Anoxia/reoxygenation of hepatocytes	Decreased Decreased		(a) Nutrient depletion (b) Adenoviral overexpression of Atg7 and Beclin 1 (c) ALLM Calpain 2 inhibitor	Protective	Kim et al. 2008 [27]
Rat	No	Partial warm 120 min	Increased			Protective?	Cursio et al. 2010 [31]
Rat	No	Liver transplantation	Increased			Detrimental	Lu et al. 2005 [26]

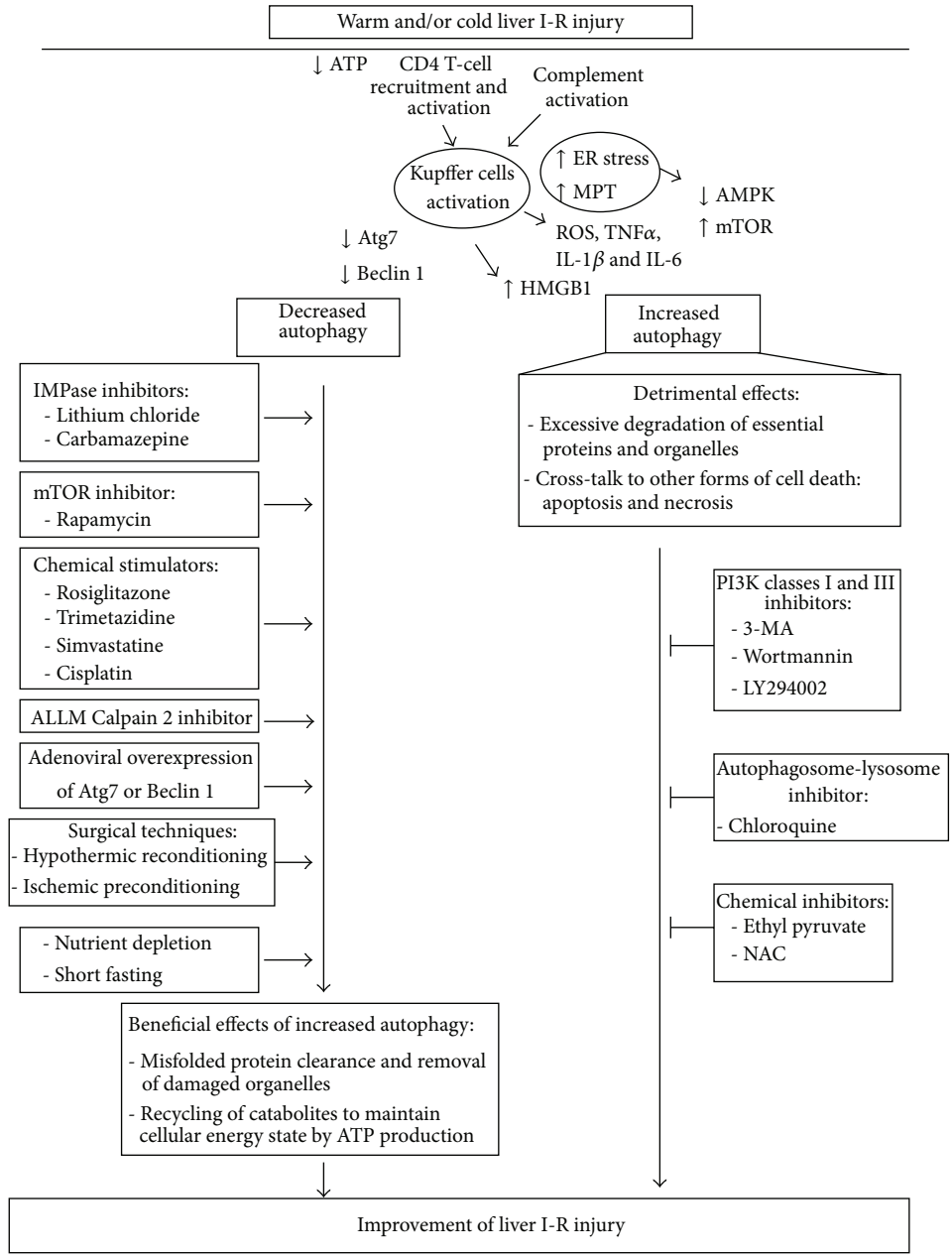

Figure 1: Pathomechanisms of warm and/or cold liver I-R injury and effects of modulation of autophagy. Pharmacological and/or surgical modulation of decreased or excessive autophagy during warm and/or cold liver I-R may improve liver injury. Arrow: stimulation; horizontal T: inhibition.

has an important role in autophagosome formation as a component of multiprotein class III phosphatidyl inositol-3 kinase (PI3K) complex [79]. Beclin 1 is important for localization of autophagic proteins to preautophagosomal membrane structure during the nucleation step of autophagy [80]. Reduced Beclin 1 levels have been observed in hepatocytes during anoxia/reoxygenation in mice [37] and following 45 min warm liver I-R in rats [27]. Increased expression of the autophagic protein Beclin 1 by nutrient deprivation, by its pharmacologic induction, and by its adenoviral overexpression during anoxia/reoxygenation in mouse hepatocytes *in vitro* [37] and in rat livers following I-R injury *in vivo* [32] protects hepatocytes from cell death and reduces liver I-R injury [27, 32, 37].

Impaired liver autophagy seems to be mediated by calcium overload and consequent Calpain 1 and Calpain 2 activation which mediates the proteolytic clivage of the autophagic protein Beclin 1 and/or of Atg7 in anoxic hepatocytes [27] and in livers following total warm I-R [27, 45]. Defective autophagy may culminate in onset of mitochondrial permeability transition (MPT) and hepatocyte death after reoxygenation [27]. MPT results in either, necrosis by uncoupling of oxidative phosphorylation and apoptosis by releasing proapoptotic factors that are normally sequestered in the

mitochondrial intermembrane space [77, 81]. MPT acts also as a molecular signal initiating the autophagic degradation of mitochondria, the mitophagy [82]. Impaired mitophagy following liver I-R fails to remove dysfunctional mitochondria, the mitochondria loaded with ROS undergo MPT, which in turn leads to uncoupling of oxidative phosphorylation, energetic failure, ATP depletion, and ultimately cell death [27, 82]. During liver I-R, Kupffer cells, neutrophils, and platelets are activated. Their activation results in a generation and release of ROS and in a cascade of inflammatory events including release of proinflammatory cytokines such as TNFα, IL-2, IL-6, IL-1, and high mobility group box 1 (HMGB1) protein [83]. HMGB1 is a DNA binding protein, which, when secreted actively by nonparenchymal liver cells (Kupffer cells and endothelial cells) and by neutrophils or when passively released by necrotic liver cells [84, 85], may induce an inflammatory signaling cascade [86]. HMGB1 acts as an alarmin, an alarm protein signal that initiates the inflammatory response resulting from liver I-R [87]. In normal rat liver, HMGB1 is mainly present in the nuclei of hepatocytes [87]. HMGB1 was released into the effluent collected from the infrahepatic vena cava during prolonged cold saline preservation of isolated rat liver grafts [88]. After cold liver graft preservation for 6 hours and transplantation in rats, serum levels of HMGB1 were increased in the early reperfusion phase [89]. Following 60 warm partial liver I-R in mice [86] and following 90 min warm partial liver I-R in rats [90], HMGB1 translocated from the nucleus to the cytoplasm of hepatocytes and was released into the blood circulation within 1 hour after reperfusion. During warm liver I-R, tissue levels of HMGB1 increase with its innate immune activation requiring toll-like receptor 4- (TLR4-) dependent signaling already 1 hour following reperfusion and continue to increase for up to 24 h later [87]. In human liver transplantation, the peak of HMGB1 serum levels was observed 10 min after reperfusion; thereafter, it started to decrease progressively within 1-2 hours [91]. *In vitro*, nontoxic concentrations of Cisplatin, a platinating chemotherapeutic, can sequester HMGB1 inside the nucleus of hypoxic rat hepatocytes, can increase Beclin 1 expression, can modulate liver I-R-induced MAPK activation, and can induce autophagy [32]. These abilities of Cisplatin had beneficial effects on warm liver I-R injury and were also observed *in vivo* in mice [32]. In fact, *in vivo* administration of nontoxic concentrations of Cisplatin prevented HMGB1 release induced by 60 min partial warm liver I-R and reduced subsequent liver injury in mice [32]. Liver I-R alone increased Beclin 1 and Atg8/microtubule-associated protein 1 light chain 3 (LC3) expression; LC3 is also involved in the formation and expansion of the autophagosome; however, after Cisplatin administration, this increase was more pronounced and associated to mitophagy and finally proved to be protective against liver I-R injury [32].

The processing and degradation of LC3, from the unconjugated form microtubule-associated protein 1 light chain 1 (LC3-I) to the conjugated form microtubule-associated protein 1 light chain 2 (LC3-II), which remains associated to the autophagolysosome, indicate an increase in autophagy [49–51].

In rats, 60 min partial warm liver ischemia resulted in increased liver LC3-II. LC3-II is associated with the autophagosomal membrane allowing the closure of the autophagic vacuole and increases Atg5 expression which, when associated in a protein complex with Atg12 and Atg16, leads to autophagosome formation and finally to increased autophagy during reperfusion [42]. This liver I-R-induced LC3-II and Atg5 expression was more pronounced after chronic Lithium pretreatment of rats [42]. In fact, Lithium can induce autophagy by inhibiting inositol monophosphatase (IMPase) and leads to free inositol depletion which in turn may decrease myo-inositol-1,4,5-triphosphate (IP3) levels [92]. Induction of liver autophagy by chronic Lithium treatment before induction of 60 min partial warm ischemia was associated with reduced I-R liver injury, lower hepatic inflammatory cytokines levels, less liver neutrophil infiltration, and lower liver HMGB1 expression and serum HMGB1 levels [42].

It has been shown that the serine/threonine kinase Akt, also known as protein kinase B (PKB), plays a key role in cell survival and proliferation and may protect against liver I-R injury [93–96]. Hepatocytic anoxia/reoxygenation and 90 min partial warm liver I-R in mice resulted in a moderate but significant increase of hepatocellular levels of autophagy [38]. Hydrogen sulphide (H_2S) pretreatment of mice exerted a protective effect in both hepatocytic anoxia/reoxygenation and 90 min partial warm liver I-R injuries through Akt1 activation [38]. However, H_2S pretreatment suppressed the moderate but significant increase of liver autophagy levels occurring during hepatocyte reoxygenation and during warm liver reperfusion. In both experimental conditions, pretreatment with the autophagic inducer Rapamycin, which induces autophagy by binding with cochaperone immunophilin FKBP1 to specifically inhibit the mammalian target of Rapamycin (mTOR) complex 1, can reverse the autophagic inhibition of H_2S and enhances its hepatoprotective effects [38].

Reducing ROS-induced hepatocellular necrosis seems to be another protective role of autophagy in 90 min partial warm liver ischemia injury in rats [43]. Following 90 min partial warm liver ischemia without reperfusion, both increased levels of LC3-II and increased number of autophagosomes were observed [43]. These increases were associated with generation of mitochondrial ROS and liver injury [43]. When autophagy was inhibited by the autophagy inhibitor Chloroquine, the increase of mitochondrial oxidative stress, of ROS production, and of mitochondrial damage was even more pronounced [43]. The consequent accumulation of damaged mitochondria, which are normally sequestered and degraded through autophagy, leads to an enhanced ROS production with a subsequent acceleration of ischemia-induced liver injury and an increase of hepatocellular necrosis [97].

Decreased autophagy is a physiological consequence of aging [98]. *In vitro* hepatocyte I-R and *in vivo* warm liver I-R injury associated with autophagy seems to be age-dependent [28, 34]. *In vitro* old mice hepatocytes subjected to 2 hours of hypoxia followed by 12 hours of reoxygenation and *in vivo* 20 min total warm liver ischemia followed by 40 min reperfusion showed an increase in Calpain 2 activity,

which hydrolyzed Atg4B and led to impaired autophagosome formation, impaired autophagic flux and mitophagy, and promoted the onset of the MPT and subsequent cell death [34]. The activation of peroxisome proliferator-activated receptor gamma (PPARγ), which belongs to the hormone nuclear receptor superfamily, is downregulated during liver ischemia aggravating liver injury [99]. PPARγ activation was lost in old mice after 30 min partial warm liver ischemia [28]. This suppression of PPARγ activation was accompanied by a reduced liver autophagy, which was more important in correlation with the duration of ischemia. Pretreatment of mice with the autophagy inducer Rosiglitazone, activated PPRAγ and increased liver autophagy after warm ischemia [28].

Steatotic livers have an increased risk of postoperative complications after liver resection [100] and liver transplantation [101]. They are particularly susceptible to mitochondrial alterations after storage in cold preservation solutions for transplantation [102, 103]. Steatosis may provide a substrate that promotes not only oxidative stress but indirectly also oxidant injury by decreasing the autophagic function [104, 105]. An impaired autophagy was observed during cold I-R of steatotic rat livers [40, 41]. Pretreatment with some autophagic stimulators, as well as Trimetazidine and Simvastatine, increased liver autophagy and improved I-R injuries of steatotic rat livers submitted to cold I-R [40, 41].

The use of hypothermic reconditioning (HR), a surgical technique consisting of temporary hypothermic oxygenation of the grafts by using an oxygenated machine perfusion or gaseous oxygen persufflation, may improve graft function and viability [106, 107]. HR of steatotic livers by insufflation of gaseous oxygen via the cava vein during the last 90 minutes of cold preservation of the graft has limited mitochondrial dysfunction and restored basal rates of hepatocellular autophagy in rats [30]. The beneficial effects of HR on liver grafts have also been shown in transplantation of nonsteatotic pig livers, where hepatocellular autophagy was preserved, mitigating the activation of innate immunity and leading to an improved survival of recipients [36].

3.1.2. In Vitro and In Vivo Human Studies. In primary isolated human hepatocytes, autophagy is a cell survival mechanism during oxidative stress [39]. Isolated primary human hepatocytes, which were exposed *ex vivo* to hypoxia and hypoxia-reoxygenation, showed an increase of autophagy within the mitochondria [39]. Inhibition of autophagy by 3-methyladenine (3-MA) in these stressed hepatocytes resulted in the lowering of MPT and onset of cell death by apoptosis [39]. During warm and cold liver I-R, ROS are responsible for lipid peroxidation, protease activation, cytokine release, adhesion molecule expression, microcirculatory failure, and finally apoptosis and necrosis [63]. However, ROS are also critical mediators of autophagy [108] and, during hypoxia-reoxygenation of primary human hepatocytes, inhibition of their production by N-acetylcysteine (NAC), Rotenone, and Diphenyliodonium suppressed autophagy and led to reduced levels of apoptosis and necrosis [39]. Thus, ROS seem to be key mediators of autophagy during oxidative stress and, depending on either the absolute level of intracellular ROS, the type of ROS subspecies generated, or the duration of ROS generation, they may be critical factors in determining cell death by apoptosis or necrosis [22, 23, 39].

Ischemic preconditioning (IP) of the liver is a surgical procedure consisting of a short period of liver ischemia (10 min) followed by reperfusion (10 min). Then the prolonged period of ischemia by clamping the hepatic artery and the portal vein on the hepatic pedicule (Pringle maneuver) is better supported by the liver [109]. After promising results in animal models, IP was efficiently used in clinical studies [110, 111]. However, its benefit to protect the liver from I-R injury in liver resection and transplantation in humans remains controversial [112–115]. A recent meta-analysis of IP for liver resection in patients with and without chronic liver diseases failed to find a significant benefit of IP in liver resection [115]. Also, in liver transplantation, there were no clear benefits of IP [112, 113]. Hepatosteatosis and vascular injury induced by chemotherapy can reduce tolerance of the liver to reperfusion injury and increase the risk of subsequent liver failure [116, 117]. In steatotic human livers formerly treated by chemotherapy, the use of IP before prolonged ischemia required by liver resection resulted in limited hepatocyte necrosis and was associated with an activation of liver autophagy [29]. The beneficial effects of IP on liver I-R injury seem to be a consequence of autophagy onset leading to preserved ATP levels and avoiding hepatocellular necrosis by delaying proapoptotic effects [29]. In liver transplantation IP of steatotic grafts (preconditioning in the donor before graft removal) induced autophagy, limited necrosis in human recipients, and decreased the incidence of rejection episodes [35]. In the ischemic preconditioned steatotic graft, a cellular increase of Beclin 1 and LC3 was observed, compared with non-IP steatotic liver grafts [35]. In addition, there was an inverse correlation between the number of LC3-positive cells and the necrotic index in IP steatotic liver grafts [35]. IP decreased the incidence of both acute and chronic liver rejection in recipients of steatotic grafts compared to recipients of non-IP steatotic grafts [35].

Overall, these studies showed that restoration or enhancement of autophagy may improve liver I-R injury by providing cells with the energy derived from lysosomal degradation of cellular materials [118].

3.2. Detrimental Role of Autophagy in Warm and/or Cold Liver I-R Injury

3.2.1. In Vitro and In Vivo Animal Studies. Several studies have shown increased levels of autophagy in hepatocytes following anoxia/reoxygenation and in livers following warm or cold/warm I-R [26, 31, 33, 38, 42–44, 46, 48]. These increased levels of autophagy appeared, either detrimental [26], probably protective [31], or both protective in the early phase of reperfusion and detrimental in the late phase of liver reperfusion [44]. Furthermore, when pharmacologically modulated, inhibited [33, 46, 48] or stimulated [38, 42, 43] autophagy improved warm and/or cold liver I-R injury in both cases.

Increased autophagy was observed following partial 120 min warm liver ischemia in rats 6 hours after reperfusion [31]. The hepatocytes of the ischemic liver lobes, 6 hours

after reperfusion, had dense bodies and various autophagosomes as well as oval and rounded mitochondria [31]. The number of hepatocytes with punctate LC3 staining in the cytoplasm was markedly increased in ischemic compared to nonischemic liver lobes [31]. During orthotopic LT in rat, autophagosomes/autolysosomes were observed in graft hepatocytes in both cold preservation and reperfusion phases [26]. Induction of autophagy was more pronounced in graft hepatocytes after 30 to 60 min of warm reperfusion than in hepatocytes after cold preservation [26]. Abundant autophagosomes/autolysosomes were associated with dying hepatocytes within 2 hours of warm reperfusion [26]. Warm reperfusion phase may facilitate autophagosome formation in hepatocytes under ATP exhaustion as a stress response [26]. About 15 minutes after the start of warm reperfusion, small masses of hepatocytes with abundant autophagosomes/autolysosomes frequently dissociated from the hepatic cords and were extruded into the sinusoidal lumen [33]. Occlusion of the sinusoidal stream contributed to a massive necrosis of hepatocytes within 2 hours and led to liver dysfunction [33]. The hepatocytes containing numerous vacuolar/lysosomal structures often underwent degeneration and were phagocytosed by Kupffer cells late in the reperfusion phase [26]. The inhibition of autophagosome formation and maturation by adding the autophagic inhibitor Wortmannin and LY294002, a specific inhibitor of PI3K/Akt kinase pathway, to the cold preservation solution attenuated liver dysfunction and recipient mortality rates [33].

The beneficial effects of Rapamycin by reversing the autophagic inhibition of H_2S during hepatocytic anoxia/reoxygenation and 90 min partial warm liver I-R in mice [38] are in contrast with the detrimental effects of Rapamycin on reperfused livers following partial warm 60 min liver ischemia in mice shown in a recent study [48]. In fact the increased levels of autophagy induced by warm reperfusion were even higher after Rapamycin pretreatment and this excessive activation of autophagy aggravated liver I-R injury [48]. Furthermore, in the same study, Rapamycin reversed the beneficial effects of Melatonin administration, a lipophilic indole secreted by pineal and nonpineal cells, which seems to protect against liver I-R injury by inhibiting oxidative stress and by improving both mitochondrial respiration and ATP synthesis after cold storage of the liver [119, 120]. In this study, Melatonin downregulated autophagy via activation of mTOR signaling and resulted in improvement of liver I-R injury [48].

In contrast, in moderate and advanced steatotic cold-stored and warm-reperfused livers in rats, in which autophagy was impaired [40], Melatonin associated with Trimetazidine, induced liver autophagy, and improved liver injury [41]. When adding Simvastatine, a statin possessing vasoprotective properties, to the cold-storage solution, the bioavailability of the vasoprotector NO was maintained and led to autophagy induction [40]. Simvastatin treatment prevented hepatic endothelial dysfunction not only in steatotic [40] but also in nonsteatotic [121] livers and resulted in improvement of liver injury.

An increased level of autophagy was observed also in rat liver following 60 and 90 min partial warm liver I-R [44]. LC3-II protein was increased at 6 hours after liver reperfusion; this increase was more pronounced when rats were pretreated with the antimalaria drug Chloroquine [44]. Chloroquine seems to have a dual role in warm liver I-R; in fact, it improved liver injury in the early phase of reperfusion by reducing inflammatory cytokines, as well as IL-6, IL-1, and TNFα; it diminished HMGB1 release, and it modified I-R-induced MAP kinase activation [44]. In the late phase of reperfusion, however, Chloroquine inhibited autophagy and induced apoptosis aggravating liver I-R injuries [44]. By contrast, in another study, levels of autophagy were decreased earlier in hepatocytic anoxia/reoxygenation and at 1 and 4 hours following partial warm 60 min liver I-R *in vivo* in rats [47] and, after Chloroquine treatment, liver I-R injuries were aggravated at these time points studied [47].

HMGB1 has also an important functional role in cross-regulating apoptosis and autophagy [80]. Actively secreted by nonparenchymal liver cells, Kupffer, and sinusoidal endothelial cells and/or passively released by necrotic liver cells [84, 85], the stress sensor with redox activity HMGB1 acts as protein signal that initiates the inflammatory response resulting from liver I-R [87]. For HMGB1 translocation from nuclei to cytoplasm and for enhanced autophagy, ROS signals are required [122]. Following 45 min warm partial liver I-R injury in mice, HMGB1 translocated from the nucleus to the cytoplasm of hepatocytes, competitively combined with Beclin 1 and promoted the levels of autophagy through representing the site of the antiapoptotic Bcl-2 protein, which normally maintains the inactive status of autophagy, and caused warm liver I-R injury in rat [46].

Ethyl-Pyruvate, a lipophilic ester derived from the endogenous metabolite pyruvate, seems to improve liver injury by inhibiting the intrinsic pathway of apoptosis and autophagy [46]. Ethyl-Pyruvate might decrease separately both apoptosis through the downregulation of the HMGB1/TLR4/NF-κB axis and autophagy through competitive interaction with Beclin 1 [46]. In fact, following 45 min warm partial liver I-R injury in mice, stressed hepatocytes released HMGB1 [46]. When animals were treated with Ethyl-Pyruvate, liver injury, apoptosis, and necrosis were decreased as a result of HMGB1 downregulation and autophagy was inhibited through the competitive interaction of HMGB1 with Beclin 1 [46].

Nevertheless, a recent study suggested that HMGB1 is not required for ATP production, cellular respiration, mitochondrial architecture, or autophagy in liver and heart [123]. In mice with conditional HMGB1 deletion, mitochondrial function and liver and heart autophagy were not affected [123]. Other studies again outline the key role of HMGB1 in mitochondrial quality control and autophagy [124–126]. In particular, an aggravation of liver I-R injury after HMGB1 depletion has been reported. In fact, genetic deletion of HMGB1 from hepatocytes resulted in enhanced inflammatory signaling, led to nuclear instability with increased DNA damage and histone release, led to mitochondria damage by exhausting nicotinamide adenine (NAD) and ATP stores, exhibited increased ROS production, and finally increased cell death [126]. In mice, fasting for one day protected from 60 min warm liver I-R injury via Sirt1-dependent down-regulation of circulating HMGB1 [127]. The reduced levels

of circulating HGMB1 damped the activation and self-propagation of Kupffer cells and hence protected from liver I-R [127].

3.3. Protective or Detrimental Role of Autophagy against Warm and/or Cold Liver I-R Injury: Various Drugs and Different Methods for Monitoring Autophagy in Different Animal and Human Experimental Liver Warm and/or Cold I-R Models. How pharmacological and surgical modulation of liver autophagy could protect from or promote liver injury following I-R remains to be clarified, as the studies on this subject report either downregulated or excessive levels of liver autophagy. At this point, it is important to mention that the methods used for measuring autophagy were mainly steady state methods, the drugs used to modulate liver autophagy were not entirely specific for inhibition or stimulation of autophagy, and the animal and human experimental liver I-R models differed considerably. In order to study the effects of autophagy modulation on warm and/or cold liver I-R injury both animal experimental liver I-R models and human experimental I-R models are used. The common length of partial warm ischemia in rodent models was usually 30, 45, 60 or 90 min [128]. In agreement with this, as shown in Table 2, in most of studies, the role of autophagy on liver I-R injury in rodents has been evaluated by using 60 min partial lobar (70%) liver warm ischemia model [28, 32, 37, 42, 44, 47, 48]. The model of partial lobar (70%) liver ischemia includes interruption of blood flow to the left lateral and median liver lobes leaving the right lobe for decompression [129]. As animal studies overall mimic real clinic conditions in which liver surgery and liver transplantation are performed in humans, they permit to draw conclusions with certain relevance for the human physiopathology.

3.3.1. Methods for Monitoring Autophagy. Accumulation of autophagosomes may be due to both the induction of autophagy and the blockage of a late step of the autophagy process, including impaired autophagosome-lysosome fusion and compromised lysosomal activity [130]. Induction of autophagy, assessed by steady state methods, does not allow a determination of whether the autophagic process goes to completion [56]. Incomplete autophagy, which would lead to the accumulation of autophagosomes, may contribute to cellular and organ dysfunction, whereas complete autophagy will generally exert a cytoprotective effect [56]. As steady state methods evaluate autophagy only at a certain time point [56], they may not reflect properly the autophagic activity [56]. Actually, Table 2 shows that most studies on warm and/or cold liver I-R [26, 28–33, 35–42, 44, 46] used steady state methods: electron microscopy, Atg8/LC3 western blotting and ubiquitin-like protein conjugation systems, fluorescence microscopy for monitoring phagophore, and autophagosome formation.

Electron Microscopy. The autophagosome is a transient organelle existing for less than 10 min before fusing with the lysosome, resulting in the appearance of autophagolysosomes at various stages of degradation [131]. Electron microscopy can visualize early-stage autophagosomes but is less sensitive for the visualization of late-stage autophagosomes [132]. So the isolated approach with electron microscopy is not sufficient to evaluate autophagy levels [56].

LC3 Western Blotting. LC3-II is present in most of the autophagic steps and reliably associated with phagophores, sealed autophagosomes and mature autophagosomes/autolysosomes [133]. It is widely used to monitor autophagy. Immunoblot analysis detects the conversion of LC3-I to LC3-II; the amount of LC3-II is clearly correlated with the number of autophagosomes [134]. However, LC3-II itself is degraded by autophagy, and the amount of LC3-II at a certain time point does not necessarily indicate autophagic flux. Simple comparison of LC3-I and LC3-II or summation of LC3-II and LC3-II for ratio determination may not be appropriate to correctly evaluate autophagy [134]. An increased number of autophagosomes can occur despite later steps of autophagy being blocked; the quantification of LC3-II [134] before and after the inhibition of autophagosome-lysosome fusion by using lysosomal inhibitors may indicate more accurately the autophagic flux [134]. Chloroquine and hydroxychloroquine increase the pH of the lysosome; Bafilomycin A1 inhibits the lysosomal Na^+H^+ ATPase; in this way, they prevent the activity of lysosomal acid proteases and cause autophagosomes to accumulate. Similar effects are induced by treatment with specific inhibitors of lysosomal proteases, such as Pepstatin A and/or E64d [134]. In this case, the real autophagic flux is represented by the different amounts of LC3-II in the samples in the absence or presence of lysosomal proteases inhibitors. LC3-II levels proportionally increase in treated versus untreated samples [134].

Ubiquitin-Like Protein Conjugation Systems. The p62 (SQSTM1/sequestosome 1) is a ubiquitin-binding scaffold protein that can bind LC3 [135]. This protein accumulates when autophagy is inhibited and decreases when autophagy is induced [136]. In some studies on warm or cold liver I-R injury in rats, autophagy was monitored by degradation of p62 using Western blot method [41, 42, 44]. However, p62 is regulated at the transcriptional level by oxidative stress and by Ras oncogene and also feeds back to regulate NF-κB activity [136]. As p62 levels may be changed independently from autophagy; additional methods to validate changes in protein aggregate turnover by autophagy are necessary [136–138].

Fluorescence Microscopy. The fluorescent-based method with the green fluorescent protein- (GFP-) LC3 counting the GFP-LC3 puncta uses the fact that, after autophagy induction, LC3B becomes part of the newly formed autophagosomes and that GFP-LC3 changes its cellular localization from a diffuse cytosolic pattern to a punctate pattern. Once again, as steady state measurement, this method is not sufficient to measure autophagy, when used as an isolated approach [56].

Autophagy is a dynamic process of bulk degradation of cellular proteins and organelles in lysosomes [139]. Autophagic substrates need to be monitored to verify that they have reached these organelles and eventually degraded

Table 2: Autophagy and warm and/or cold liver I-R injury. Methods for monitoring autophagy and additional beneficial effects of drugs and surgical techniques on liver I-R injury other than modulation of autophagy.

Authors and references	Monitoring autophagy by flux measurements [56]	Monitoring phagophore and autophagosome formation by steady state methods [56]	Additional beneficial effects of drugs and surgical techniques on liver I-R injury other than modulation of autophagy
Yun et al. 2014 [47]	(+)		HO-1 induction and Calpain 2 inhibition by Hemin
Kang et al. 2014 [48]	(+)		Decrease of apoptosis
Shen et al. 2013 [46]		(+)	Inhibits HMGB1/TLR4/NF-κB axis inducing apoptosis
Kim et al. 2013 [45]	(+)		Suppression of calcium overloading Suppression of uncontrolled Calpain activation
Fang et al. 2013 [44]		(+)	Decrease of HMGB1 and proinflammatory cytokines levels Modulation of MAPK activation
Sun et al. 2013 [43]	(+)		Decreased mitochondrial ROS-inducing necrosis by NAC
Liu et al. 2013 [42]		(+)	Modulation of MAPK activation Inhibition of Caspase-3 and -7 activation Decrease of HMGB1 and proinflammatory cytokines levels
Zaouali et al. 2013 [41]		(+)	Decrease of apoptosis
Gracia-Sancho et al. 2013 [40]		(+)	Decrease of the oxidative stress and Caspase-3 activation by Simvastatine
Bhogal et al. 2012 [39]		(+)	
Wang et al. 2012 [38]		(+)	Akt1 activation and decrease of apoptosis by H_2S
Evankovich et al. 2012 [37]		(+)	
Degli Esposti et al. 2011 [35]		(+)	
Wang et al. 2011 [34]	(+)		
Minor et al. 2011 [36]		(+)	Decrease of HMGB1 and IFN beta levels
Minor et al. 2009 [30]		(+)	ROS decrease ATP increase
Cardinal et al. 2009 [32]		(+)	Decrease of HMGB1 and proinflammatory cytokines levels Modulation of MAPK activation
Gotoh et al. 2009 [33]		(+)	
Domart et al. 2009 [29]		(+)	Bcl-2 increase
Shin et al. 2008 [28]		(+)	ATP increase Inhibition of Caspase-3 activation
Kim et al. 2008 [27]	(+)		
Cursio et al. 2010 [31]		(+)	
Lu et al. 2005 [26]		(+)	

[56]. Evaluation of the autophagic flux, a complete process of autophagy including the delivery of cargo to lysosomes, via its fusion with autophagosomes or amphisomes and its subsequent breakdown and recycling, [56] is important to determine whether drugs and/or surgical techniques truly affect autophagy. As shown in Table 2, only a few studies have monitored the autophagic flux in order to evaluate the extent of autophagy [27, 34, 43, 45, 47, 48].

3.3.2. Side Effects of Chemical Autophagy Stimulators or Inhibitors. The chemical stimulators and inhibitors of autophagy used actually are not specific and may have a series of additional effects on liver I-R apart from their action on autophagy.

(1) Chemical Stimulators of Autophagy. Inducing, increasing, or restoring basal autophagic activity in certain cell types as the hepatocytes, following warm and/or cold liver I-R, might be of therapeutic benefit. As shown in Table 2, the modulation of autophagy by some stimulators protected against liver I-R injury [27, 28, 32, 34, 37, 38, 41, 42, 45]. However, the unspecificity of the used drugs renders the interpretation of the results difficult.

Rapamycin, an autophagy inducer by inhibiting the mTOR pathway, plays a central role in several important cellular processes other than autophagy [140]. Interfering with the translation of HIF-1α, it has an antiangiogenic effect, and inhibiting the phosphorylation of BAD by S6 K1 Rapamycin may promote apoptosis [140]. So its protective effects in 90 min warm liver I-R injury in mice may be partly due to Akt1 activation and decreased apoptosis by H_2S associated copretreatment [38]. Similarly Rosiglitazone, another autophagy stimulator, has shown additional protective effects (increase of ATP levels and the inhibition of Caspase-3 activation) in 30, 60, and 90 min liver I-R injury in old mice that cannot be accounted to the increase of autophagy [28].

The IMPase inhibitors, Lithium chloride and Carbamazepine, can induce autophagy [92]. They have also shown additional protective effects against liver I-R injury other than autophagy induction [42, 45]. Lithium chloride showed multiple additional effects, the decrease of HMGB1 and proinflammatory cytokines levels, the modulation of MAPK activation, and the inhibition of Caspase-3 and Caspase-7 activation [42]. The second, Carbamazepine, suppressed both calcium overloading and uncontrolled Calpain activation [45]. In a murine model of 60 min liver I-R Cisplatin treatment increased liver autophagy and protected against I-R injury [32]. Although the beneficial effects of Cisplatin are not only the result of autophagy stimulation but also of decreased HMGB1 and proinflammatory cytokines levels and of the modulation of MAPK activation [32].

In steatotic rat livers that were preserved in cold solution for 24 hours, autophagy was decreased [41]. A close relationship between 5′ AMP-activated protein kinase (AMPK) activation and endoplasmic reticulum (ER) stress and autophagy has been observed in these livers [41]. The addition of a Melatonin and Trimetazidine cocktail to the cold preservation solution improved steatotic liver graft preservation through AMPK activation, which in turn reduced ER stress and increased autophagy [41]. However, these beneficial effects of the Melatonin and Trimetazidine cocktail may be due in part to a reduction of apoptotic liver cells mainly observed in periportal and midzonal areas of the liver [41].

In human liver surgery and transplantation, the improvement of clinical outcomes by the use of IP of the liver was associated to higher levels of autophagy. However, the decrease of ER stress [35] and the increase of ATP and Beclin 1 levels observed after IP of the liver may have been responsible too for the beneficial effects of this surgical strategy [29].

(2) Chemical Inhibitors of Autophagy. Similar to autophagy stimulators, autophagy inhibitors are not specific [33, 39, 43, 44] and additional effects of these drugs may play an important role [43, 44, 46–48].

Autophagy inhibition by 3-MA in human hypoxemic/reoxygenated hepatocytes resulted in MPT lowering and onset of apoptosis [39]. Autophagy inhibition by LY294002 and Wortmannin during liver transplantation in rats reduced liver graft dysfunction and mortality rate of transplanted animals [33].

The PI3K inhibitors, 3-MA, LY294002, and Wortmannin, typically block Class III PI3Ks, which act as downstream of the negative regulatory Class I kinase. However, their inhibitory action on autophagy differs [141]. In fact 3-MA may promote or suppress autophagic flux [142], whereas Wortmannin seems to have inhibitory effects opposite to those of 3-MA. It has persistent effects on class III PI3K, an autophagy activator, but also it has transient effects on class I PI3K, which is an autophagy inhibitor [142]. The autophagic inhibitor LY294002 plays also a dual role in the regulation of autophagy, as it may activate autophagy by inhibiting the class I PI3K [143] and as it modulates calcium overloading, Calpain activation, and MPT, all implicated in the development of warm liver I-R injury [77, 81].

Also Melatonin, Ethyl-Pyruvate, and Chloroquine, all three autophagy inhibitors, present additional protective effects against liver I-R injury. Melatonin in fact may decrease liver apoptosis [48], Ethyl-Pyruvate may inhibit HMGB1/TLR4/NF-κB axis inducing apoptosis [46] and Chloroquine, which has protective effects in the early reperfusion phase after 60 and 90 min warm liver ischemia, may modulate MAPK activation [44].

Autophagy inhibition by Chloroquine pretreatment increased mitochondrial oxidative stress and hepatocellular necrosis following 90 min warm liver I-R in rats [43]. Antioxidant NAC pretreatment again diminished the ischemia-induced liver injury of these rats, which received also Chloroquine treatment. However, the beneficial effects against liver injury by NAC seem to be due also to decreased mitochondrial ROS-inducing necrosis [43].

4. Conclusion

The pathogenesis of warm and/or cold liver I-R injury represents a complex interplay between necrosis, apoptosis, and autophagy. It seems that stimulation of autophagy plays a more important role during liver reperfusion than

ischemia. Depending on the context, induction or impairment of autophagy during warm and/or cold liver I-R can be protective or detrimental for liver cells. Stimulation of impaired autophagy following warm and/or cold I-R may promote hepatocyte survival by degradation of intracellular contents to maintain ATP production and removal of damaged organelles and protein aggregates. Excessive and long-term upregulation of autophagy, as it occurs during severe ischemic insult of the liver, may lead to destruction of essential proteins and organelles resulting in hepatocellular apoptosis and necrosis.

However, the results of the studies on autophagy during warm and/or cold liver I-R remain discordant. This may be due to several factors, namely, the lack of drugs which exert specific and exclusive autophagic stimulation or inhibition, the different experimental liver I-R models used, and the different methods of autophagy evaluation. So how pharmacological and/or surgical modulation of liver autophagy could protect from or promote liver injury following warm and/or cold I-R remains to be clarified. Large animal studies on liver I-R, also at a genetic level with knockout models, which provide a very specific targeted disruption of a particular autophagic protein and will therefore be more informative than the use of not entirely specific chemical stimulators or inhibitors of the same autophagic protein, are needed. Last but not least the methods for monitoring autophagy should preferentially measure the autophagic flux.

In any way, the autophagic cell response to warm and/or cold liver I-R may provide an additional time to the cell death processes, delaying apoptosis and necrosis, and thus ultimately increasing the possibility for novel therapeutic intervention to diminish the extent of warm/cold liver I-R injury.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Autophagy and Non-Alcoholic Fatty Liver Disease

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Autophagy, or cellular self-digestion, is a catabolic process that targets cell constituents including damaged organelles, unfolded proteins, and intracellular pathogens to lysosomes for degradation. Autophagy is crucial for development, differentiation, survival, and homeostasis. Important links between the regulation of autophagy and liver complications associated with obesity, non-alcoholic fatty liver disease (NAFLD), have been reported. The spectrum of these hepatic abnormalities extends from isolated steatosis to non-alcoholic steatohepatitis (NASH), steatofibrosis, which sometimes leads to cirrhosis, and hepatocellular carcinoma. NAFLD is one of the three main causes of cirrhosis and increases the risk of liver-related death and hepatocellular carcinoma. The pathophysiological mechanisms of the progression of a normal liver to steatosis and then more severe disease are complex and still unclear. The regulation of the autophagic flux, a dynamic response, and the knowledge of the role of autophagy in specific cells including hepatocytes, hepatic stellate cells, immune cells, and hepatic cancer cells have been extensively studied these last years. This review will provide insight into the current understanding of autophagy and its role in the evolution of the hepatic complications associated with obesity, from steatosis to hepatocellular carcinoma.

1. Introduction

Autophagy, or cellular self-digestion, is a catabolic process that targets cell constituents, such as damaged organelles, unfolded proteins, and intracellular pathogens, to lysosomes for degradation [1, 2]. Under basal conditions, autophagy is involved in the degradation of long-lived proteins, whereas the ubiquitin-proteasome pathway, another catabolic process, is responsible for the degradation of short-lived proteins [3, 4]. In response to cellular stress such as nutrient deprivation, an increase in autophagic turnover maintains the cellular energy homeostasis. Three types of autophagy have been identified: macroautophagy, chaperone-mediated autophagy, and microautophagy. Macroautophagy, hereafter referred to as autophagy, involves the formation of a small vesicular sac called the isolation membrane or phagophore. The phagophore encloses a portion of cytoplasm resulting in the formation of a double-membraned structure termed an autophagosome. The autophagosome then fuses with a lysosome leading to the degradation of the cellular constituents sequestered into the autophagosome. Amino acids and other compounds generated by autophagic degradation of macromolecules are released into the cytoplasm for recycling or for energy production (Figure 1(a)) [1]. The origin of the membranes involved in the formation of autophagosomes could be the endoplasmic reticulum (ER), mitochondria, and golgi [5–8]. However, it is still not clear which is/are the major contributor(s).

Microautophagy also involves the sequestration of cellular constituents within lysosomes but in this case through the invagination of the lysosomal membrane. Chaperone-mediated autophagy concerns the sequestration of polypeptides and soluble proteins containing a KFERQ motif in their amino acids sequence. These proteins are bound to a chaperone protein for translocation to lysosomes where binding to the lysosome-associated membrane protein type 2A receptor leads to protein internalization and degradation [9].

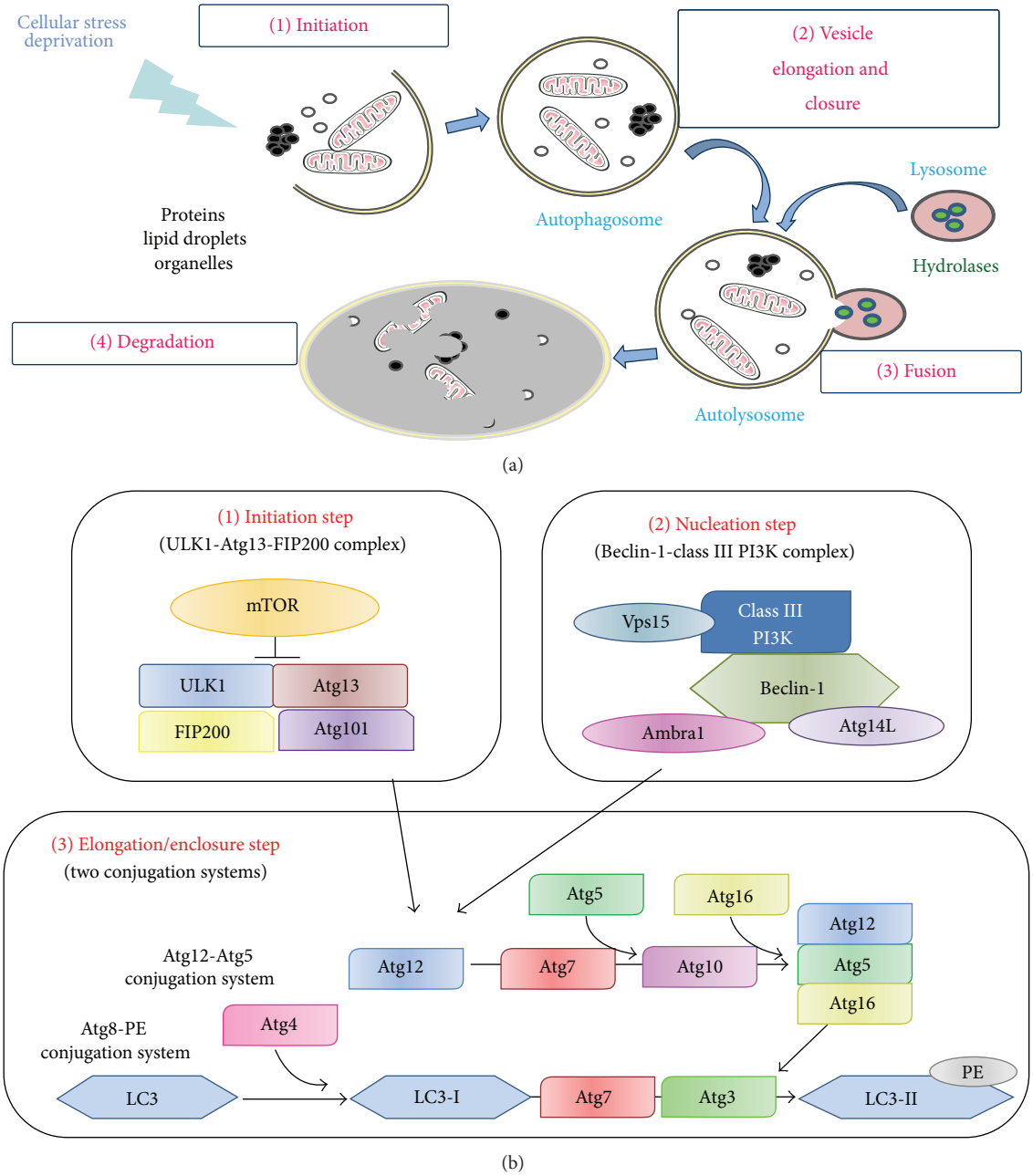

Figure 1: Macroautophagy. (a) Macroautophagy involves the formation of a double-membrane vesicle called the autophagosome. This structure sequesters damaged organelles and misfolded proteins that are degraded by lysosomal enzymes. (b) The *Initiation step* is controlled by the ULK1-Atg13-FIP200 complex, which also contains Atg101. mTOR interacts with and inactivates ULK1 by phosphorylation. Under starvation conditions, mTOR is inactivated leading to ULK1 activation, phosphorylation of Atg13 and FIP200, and consequently the induction of autophagy. The *Nucleation step* requires the Beclin-1-class III PI3K complex including Beclin-1, class III PI3K, Vps15, Atg14L, and Ambra1. The ULK1-Atg13-FIP200 and Beclin-1-class III PI3K complexes recruit two conjugation systems essential for the *elongation and enclosure step*: the Atg12-Atg5 and the Atg8-PE conjugation systems. PE: phosphatidylethanolamine.

In autophagy, the formation of phagophores and autophagosomes requires 18 different autophagy-related proteins, Atg, which were initially identified in yeast [10]. The process of autophagosome formation involves three major steps: initiation, nucleation, and elongation/enclosure (Figure 1(b)). The initiation step is controlled by the ULK1-Atg13-FIP200 complex [11, 12]. The serine/threonine kinase mammalian target of rapamycin, mTOR, a component of the mTORC1 complex, is the main inhibitor of autophagy. The nucleation step requires the Beclin-1-class III phosphatidylinositol 3-Kinase (PI3K) complex that includes Beclin-1, Vps34 (class III PI3K), Vps15, Atg14L/Barkor, and Ambra-1 [13]. The involvement of mTOR and the Beclin-1-class III PI3K complex in the regulation of autophagy are discussed below. Two

conjugation systems are involved in the elongation/enclosure step. The first is the conjugation of Atg12 to Atg5 mediated by two ligases Atg7 and Atg10. Atg5 also associates with Atg16 to form the Atg12-Atg5-Atg16 complex. The second involves the cleavage of LC3/Atg8 by Atg4 leading to the soluble form LC3-I, which is then conjugated to phosphatidylethanolamine, PE, via the participation of Atg7 and Atg3. This lipid conjugation forms the autophagic double-membrane-associated LC3-II protein allowing the closure of the autophagic vacuole [14, 15]. LC3-II is used as a marker of autophagosomes [16].

The last phases of autophagic process mediate the autophagy degradation. A large number of factors/actors regulate the autophagosome-lysosome fusion and the lysosomal biogenesis, activation, reformation, and turnover [17]. In the autophagosome-lysosome fusion process, soluble N-ethylmaleimide-sensitive factor attachment protein, cytoskeleton proteins, and small GTPases are involved, for example. This mechanism is sensitive to changes in the membrane lipid composition of autophagosomes and lysosomes that can regulate their fusogenic capacity as it has been reported in lipid-enriched diets [18]. Other important regulator of lysosomal biogenesis, function, and autophagy is the transcription factors EB, TFEB [19, 20]. TFEB coordinates the cellular responses to different stresses, including nutrient starvation, metabolic stress, and lysosomal stress, to maintain cellular homeostasis. Indeed, TFEB regulates the expression of genes involved in lipid metabolism and in the pathways of autophagy and lysosome [19, 20].

Autophagy is a cellular pathway that is crucial for the maintenance of cellular homeostasis, normal mammalian physiology and could play a protective or deleterious role in a range of diseases. Recent studies have reported its role and its regulation in the complications associated with obesity.

2. Hepatic Complications Associated with Obesity

The incidence of overweight and obesity is rapidly increasing in many Western countries. Obesity leads to numerous adverse metabolic disorders such as dyslipidemia, hypertension, reduced HDL cholesterol, and glucose intolerance. This cluster of metabolic abnormalities is grouped into the so-call metabolic syndrome that increases the risk of cardiovascular diseases, type 2 diabetes and liver complications, the non-alcoholic fatty liver disease (NAFLD). The spectrum of these hepatic abnormalities extends from isolated steatosis with triglyceride accumulation to non-alcoholic steatohepatitis (NASH), steatofibrosis, which sometimes leads to cirrhosis and hepatocellular carcinoma (Figure 2). NAFLD is one of the three main causes of cirrhosis [21]. Despite this major public health concern, apart from lifestyle changes, NAFLD is still difficult to treat as no large study has shown any efficacy of pharmacological treatments for NAFLD.

Insulin resistance is at the core of the pathophysiology of the metabolic syndrome and type 2 diabetes. Insulin resistance is characterized by a decrease in insulin signaling and action. Adipose tissue plays a central role in the control of glucose and lipid metabolism through its ability to control glucose transport, lipid storage, and adipokines secretion. In obesity, the excessive gain of adipose tissue, in particular visceral adipose tissue, causes its dysfunction, which could participate in the development of insulin resistance and other obesity-linked complications such as the NAFLD. Adipose tissue expansion is associated with chronic low-grade inflammation with infiltration of the tissue by immune cells such as dendritic cells, macrophages, and T lymphocytes. Consequently, adipose tissue produces inflammatory cytokines and free fatty acids (FFA) in excess, and adipokine secretion is perturbed. Inflammatory cytokines and FFA antagonize local insulin signaling in adipocytes and also in muscles and liver. In liver, combined hyperglycemia and hyperinsulinemia promote *de novo* lipid synthesis and mitochondrial structural defects within hepatocytes. Moreover, insulin resistance of adipose tissue leads to an enhanced FFA flux to the liver, for example, in contribution to insulin resistance and steatosis. Oxidative and ER stresses play an important role in the alteration of insulin signaling and in the development of liver complications [22]. NASH is characterized by a fatty liver, hepatic inflammation, and substantial death of hepatocytes. Hepatocyte apoptosis is important in the progression of the severity of liver complications. Apoptotic hepatocytes are engulfed by kupffer cells, which results in their activation and inflammation. The activation of stellate cells by apoptotic bodies or by TGFβ from activated kupffer cells then leads to liver fibrosis [22]. Among the factors involved in this process, upregulation of cell death receptors, such as Fas and the TNFα receptor, and of TNFα has been reported in the NASH liver. Saturated FFA, sustained ER stress, cytokines, and adipokines could also be involved [22].

Interestingly, it has been recently reported that autophagy regulates food intake, adipose tissue development, hepatic complications, and insulin resistance and plays a protective role against lipotoxicity in β cells [23]. Here, we will provide insight into the current understanding of the role of autophagy in the liver complications associated with obesity.

3. Hepatic Autophagy in Obesity

Under physiological conditions, autophagy participates in the basal turnover of lipids by engulfing and degrading lipid droplets. Autophagy is inhibited by the insulin-, amino acid-mTOR signaling pathway *via* both short- and long-term mechanisms of regulation. Short-term inhibition can be produced by the mTOR complex. Long-term regulation occurs *via* the transcription factors FoxO and TFEB, which control the transcription of autophagic genes and are inhibited by insulin-induced activation of Akt/PKB and mTOR, respectively [23]. In obesity, the level of autophagy could be decreased in hepatocytes. Several mechanisms may account for this decline (Figure 3).

(i) An obesity-induced increase in the calcium-dependent protease calpain-2 leads to the degradation of Atg7 and then to a defective autophagy. Acute inhibition of calpain is able to restore Atg7 expression [24]. How obesity enhances the activity or expression of the hepatic calpain 2 has not yet been elucidated.

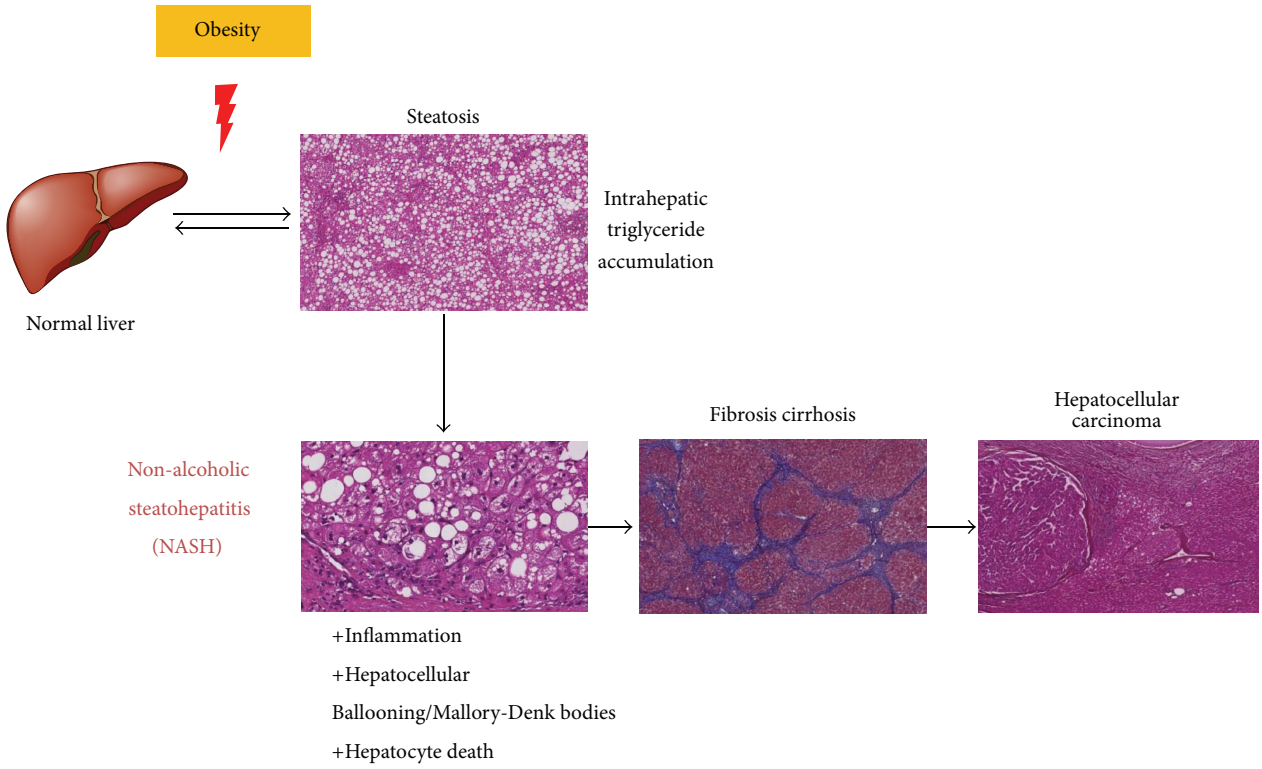

Figure 2: NAFLD. The spectrum of non-alcoholic fatty liver diseases (NAFLD) extends from isolated steatosis (triglyceride accumulation) to steatohepatitis (steatosis with inflammation) (non-alcoholic steatohepatitis [NASH]), steatofibrosis, which sometimes leads to cirrhosis, and hepatocellular carcinoma. Peripheral insulin resistance may represent the "first hit" in the pathogenesis of NAFLD, which leads to hepatic steatosis. Combined hyperglycemia and hyperinsulinemia promote *de novo* lipid synthesis and structural defects in mitochondria within hepatocytes. Moreover, insulin resistance of adipose tissue leads to an enhanced free fatty acid flux to the liver that contributes to steatosis. Steatotic hepatocytes may be vulnerable to a "second hit" induced by cytokines (such as TNFα) and oxidative/ER stresses, which lead to the development of steatohepatitis and fibrosis. Apoptotic hepatocytes are engulfed by kupffer cells, which results in their activation and inflammation. The activation of stellate cells by apoptotic bodies or by TGFβ from activated kupffer cells then leads to liver fibrosis [22].

(ii) In obese mice with hepatic steatosis, the autophagy inhibitor mTOR is overactivated in the liver, presumably as a result of an increased amino acid concentration following overnutrition. Indeed, it has been previously shown that the overactivation of mTOR by infusion of an amino acid mixture can result in liver and muscle insulin resistance because of phosphorylation and inhibition of IRS1 by S6 kinase, a downstream target of mTOR [25, 26].

(iii) Although controversial, hyperinsulinemia may also contribute to downregulation of autophagy in obese mice. Indeed, Akt/PKB, a key molecule in the insulin pathway, decreases autophagy in the liver of obese mice [27]. However, destruction of insulin production by β cells with streptozotocin does not increase autophagy in the liver of obese mice [24], in contrast to lean mice [27]. The reasons for these discrepancies are unclear.

(iv) A defect in lysosomal acidification and a reduction in cathepsin L that impaired substrate degradation in autolysosomes have also been reported for obese ob/ob mice. This is associated with an increased autophagosome number and normal fusion of autophagosomes to lysosomes [28]. The same team recently reported that cathepsin B, D, and L expression was significantly decreased in the liver from NAFLD patients [29].

(v) Defective autophagosome-lysosome fusion has also been reported in livers from high fat diet- (HFD-) induced obese mice. This defect was attributed to HFD-induced changes in the membrane lipid composition [18]. A defect in hepatic autophagy and its associated decrease in the rate of lysosomal degradation contribute to a further increase in the ER stress. This could be induced by nutrient overload in an inflammatory milieu [24, 30]. Together, a decline in autophagy and an increase in ER stress lead to insulin resistance [24].

4. Hepatic Steatosis

In response to a moderate increase in lipid availability or during nutrient deprivation, hepatic autophagy degrades lipid droplets to provide FFA for ATP production. In contrast, a sustained availability of lipids, induced by a long-lasting HFD

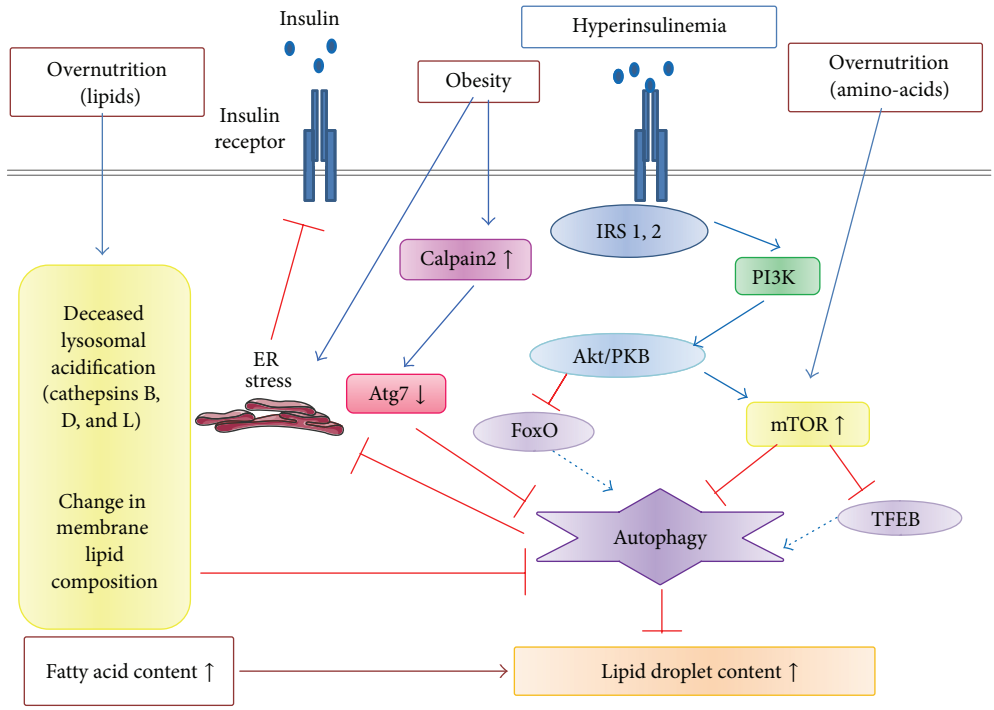

Figure 3: Molecular mechanisms of the impairment of hepatic autophagy in obesity. Short-term inhibition can be produced through the mTOR complex. Long-term regulation could occur *via* the transcription factors FoxO and TFEB, which control the transcription of autophagic genes and are inhibited by insulin-induced activation of Akt/PKB and mTOR, respectively. mTOR could be overactivated in the liver, presumably as a result of an increased amino acid concentration following overnutrition and/or hyperinsulinemia. An obesity-induced increase in the calcium-dependent protease calpain-2 could also lead to the degradation of Atg7 and then to defective autophagy. A defect in lysosomal acidification and a reduction in cathepsins B, D, and L expression, which impaired substrate degradation in autolysosomes, have also been reported. Finally, a defect in fusion in organelles including autophagosome-lysosome fusion attributed to HFD-induced changes in the membrane lipid composition. A defect in hepatic autophagy and the associated decrease in the rate of lysosomal degradation contribute to a further increase in the ER stress induced by nutrient overload and insulin resistance.

challenge, inhibits hepatic autophagic turnover [31]. This ability of autophagy to degrade lipid droplets in hepatocytes has been termed lipophagy. Singh et al. reported for the first time that autophagy regulates lipid metabolism by eliminating triglycerides and by preventing development of steatosis. Inhibition of macroautophagy by genetic knockdown of the autophagy gene *atg5*, or pharmacological inhibition with 3-methyladenine in cultured hepatocytes challenged with a lipid load, significantly increased the cellular triglyceride content. Excessive triglycerides and cholesterol were retained in lipid droplets because of a decreased rate of lipolysis and the resultant reduction in fatty acid β-oxidation in cells in which macroautophagy was inhibited. Lipid movement through the autophagic pathway was confirmed by fluorescence microscopy demonstrating colocalization of lipid with autophagosomes and lysosomes, electron microscopic evidence of lipid in the autophagic vacuoles, and immunogold staining demonstrated an association of the autophagosome-associated protein microtubule-associated LC3 protein with lipid droplets. The LC3 protein directly interacts with lipid droplets before autophagosome formation. Lysosomes merge only with autophagosome-associated lipid droplets [31]. These results have been confirmed [32].

Differences in the activation of autophagy, p53, damage-regulated autophagy modulator (DRAM), and BAX expression have also been observed in function to the severity of the hepatic steatosis. In the mouse model of mild (20 weeks of HFD) and severe hepatosteatosis (40 weeks of HFD), p53 expression increased in both mild and severe hepatic steatosis, and increased DRAM expression and autophagy were identified in mild hepatosteatosis, whereas higher BAX expression was observed in severe hepatosteatosis [33]. From *in vitro* approaches, the authors proposed that mild steatosis induced autophagy and apoptosis mostly via a p53/DRAM pathway. In severe steatosis, apoptosis was mainly dependent on p53-induced expression of BAX, which also localized to mitochondria [33]. Since discrepancies between *in vivo* and *in vitro* approaches exist, future investigations are necessary to confirm this potential mechanism.

HFD-fed mice (16 weeks) show impairment in the hepatic autophagic function, as demonstrated by the decreased mobilization of lipid into the autophagic compartment [31]. Lipid accumulation altered the membrane structure, and a resultant decrease in the efficiency of fusion between autophagosomes and lysosomes may explain the inhibitory effect on macroautophagy of lipid accumulation induced by a HFD.

However, in livers with a deficiency in autophagy, an alternative protective mechanism could then take place. Indeed, Kim et al. have recently reported that mice with a deficiency in hepatic autophagy displayed induction of fibroblast growth factor 21 (FGF21), resistance to diet-induced obesity, and amelioration in insulin resistance. The deficiency in autophagy and subsequent mitochondrial dysfunction could promote FGF21 expression, which in turn protects from diet-induced obesity and insulin resistance [34]. In NAFLD patients, the serum level of FGF21 is also modified. In 146 overweight patients, it was reported that the serum levels of FGF21 were significantly higher in NASH patients. FGF21 also correlated positively with the triglyceride level, metabolic syndrome, steatosis grade, lobular inflammation, and fibrosis [35]. Interestingly, the effects of LY2405319 (LY), an engineered FGF21 variant [36, 37], in a randomized, placebo-controlled, double-blind proof-of-concept trial on 46 patients with obesity and type 2 diabetes has been recently reported. Patients received placebo or 3, 10, or 20 mg of LY daily for 28 days. LY treatment produced a significant improvement in dyslipidemia. Favorable effects on body weight, fasting insulin, and adiponectin were also detected. While only a trend toward glucose lowering was observed, FGF21-based therapies may be effective for the treatment of selected metabolic disorders [38]. Additional exploration would be necessary in order to assess the full range of LY effects and the potential to achieve significant antidiabetic efficacy. Further, the effect of LY administration on liver complications (hepatic steatosis) should be evaluated. The improvement of insulin sensitivity by LY could reduce the free fatty acid flux to the liver that contributes to steatosis.

5. Activation of Hepatic Autophagy Decreases Liver Steatosis

Activation of autophagy in hepatocytes could constitute a therapeutic approach against hepatic complications. To illustrate this, it has been reported that hepatic overexpression of Atg7 in HFD-fed mice or ob/ob mice improved the condition of the fatty liver and insulin resistance [24].

Starvation induces hepatic autophagy and increases delivery to the liver of FFA from adipose tissue. The liver of starved mice displayed an increase in the number of lipid droplets, autophagosomes, lysosomes, and autophagolysosomes [31]. Hepatocyte-specific Atg7-deficient mice are characterized by hepatomegaly and accumulation of poly-ubiquitinylated proteins, as previously reported by Komatsu et al. [39]. Hepatic triglycerides and the cholesterol content are also increased in these mice, which confirm the crucial role of autophagy in the regulation of lipid storage. The activation of autophagy by starvation is a complex mechanism. A new actor has been identified: acetyl-coenzyme A (AcCoA). AcCoA is a major integrator of the nutritional status at the crossroads of fat, sugar, and protein catabolism and cytosolic AcCoA functions as a central metabolic regulator of autophagy. Nutrient starvation causes rapid depletion of AcCoA and induction of autophagy *via* the reduction in the activity of acetyltransferase EP300, a suppressor of autophagy, by high AcCoA levels [40].

Enhancers of autophagy such as carbamazepine and rapamycin have been recently tested in HFD-obese mice and both had protective effects in reducing steatosis and in improving insulin sensitivity. The agents were given two or three times in the last week of a 12-week feeding scheme. This short-term treatment could significantly reduce hepatic steatosis and hepatic and blood triglyceride levels. The plasma ALT level was also noticeably, although not statistically significantly, reduced. Interestingly, insulin resistance was improved as evaluated by the level of blood glucose and insulin [41]. While rapamycin and carbamazepine are already approved for other human clinical uses, the lack of specificity and the absence of organ or cell selectivity are the major limitations of these compounds.

The beneficial effects of coffee on hepatic steatosis and the link with autophagic flux have been recently tested. The recent evidence of the beneficial effects of coffee on the liver came from epidemiologic studies that revealed a strong association of drinking coffee with decreased serum hepatic enzymes, including GGT, AST, and ALT, in persons with a high risk of liver injury, such as in alcoholic, diabetic, and in viral infections [42]. Recent epidemiologic studies further support the finding that drinking coffee reduces the risk for fatty liver, fibrosis, and hepatocellular carcinoma in NAFLD patients [43, 44]. Sinha et al. recently reported that mice given a HFD for 4 weeks, then continued HFD with 0.05% (w/v) caffeine in the drinking water for the next 4 weeks displayed an induction of hepatic autophagy (lipophagy) with a decrease in hepatic steatosis [45]. This treatment was nevertheless associated with a decrease in HFD-induced obesity. Using genetic, pharmacological, and metabolomic approaches on hepatic cells and on the liver, the authors showed that caffeine induced lipophagy and mitochondria β-oxidation leading to a reduction in the intra-hepatic lipid content. Caffeine may inhibit PI3K-AKT and, in turn, inhibit mTOR to trigger autophagy by activating the ULK1 complex. The later includes ULK1, Atg13, FIP200, and Atg101. Autophagy selectively removes excess lipid droplets to generate FFA. Decreased mTOR levels induced TFEB nuclear translocation by decreasing TFEB phosphorylation. TFEB upregulates expression of autophagy and lysosomal genes, as well as PGC-1α and PPARα, which burn FFAs by increasing mitochondria β-oxidation. Thus, caffeine protects against fatty liver by coordinating the induction of lipophagy and mitochondrial β-oxidation [45, 46].

6. Hepatic Insulin Resistance

Recent studies have suggested that ER stress could be the link between obesity, insulin resistance, and type 2 diabetes [47–49]. The inhibition of hepatic ER stress reduced liver steatosis [50]. Defective hepatic autophagy in obesity could promote ER stress and cause insulin resistance [47]. Since autophagy is known to eliminate mis-/unfolded proteins, and impairment in hepatic autophagy could lead to accumulation of mis-/unfolded proteins and induction of ER stress. In the liver of obese mice, it has been reported that a decrease in autophagy promotes ER stress leading to insulin resistance [24, 51]. Overexpression of Atg7 in the liver of obese mice significantly

reduced ER stress, decreased the triglyceride content, and improved glucose tolerance and insulin sensitivity [24]. Thus, a vicious cycle takes place: hyper-insulinemia negatively regulates hepatic autophagy in the steatotic liver and the decline in hepatic autophagy enhances ER stress and insulin resistance (Figure 3).

7. Steatohepatitis (NASH)

The possible contribution of autophagy to the evolution of steatosis to NASH has not yet been fully elucidated [52]. The diagnosis of NASH still requires a liver biopsy and is defined by histological features including steatosis, lobular inflammation, and hepatocellular ballooning [53, 54]. Evaluating hepatic fibrosis using a histopathological algorithm and scoring systems to diagnosis NASH is still under debate [55]. Hepatic cell injury including hepatocyte death and hepatocellular ballooning are key features of NASH. Inhibition of autophagy and the accumulation of p62 could be involved in the formation of the Mallory-Denk bodies (MDB). MDB are mainly found in ballooned hepatocytes, one of the components used for NASH diagnosis and for the scoring system [53, 54]. In contrast, activation of autophagy by rapamycin leads to MDB resolution in mice [56].

A recent report has evaluated autophagic markers in obese patients with hepatic steatosis ($n = 26$), patients with steatosis with NASH, and fibrosis ($n = 23$) versus normal liver ($n = 34$). A significant increase in the LC3-II/LC3-I ratio was detected in both patients with steatosis and NASH compared with subjects with a normal liver. A progressive increase in the amount of p62 was observed in obese patients with steatosis and in obese patients with NASH compared to control patients. The accumulation of p62 and the LC3II/LC3I ratio could indicate that autophagy was decreased in both patients with hepatic steatosis and NASH [57]. The level of autophagic markers in patients with the same grade of steatosis and fibrosis with or without NASH remains to be investigated, to determine if a discrepancy exists between steatosis and NASH. In contrast, NASH patients had a significant increase in hepatic ER stress (ATF4 at the mRNA level; CHOP and GRP78 at the mRNA and protein levels) compared with patients with steatosis [57]. In a mouse model of NASH (methionine/choline-deficient diet), the authors also demonstrated that the autophagic flux was impaired in the liver [57]. We would like to underline the fact that the evaluation of autophagic flux *in vivo* in mice is still difficult and even more difficult in human samples. Further, assessing autophagy by the LC3-II/LC3-I ratio and p62 level need always caution. The levels of LC3-I can also vary. In addition, p62 levels are enhanced by other factors such as stress, or proteasome inhibition, which are mostly independent of changes in autophagy.

The immune system plays an important role in the evolution of NAFLD [22]. For example, the dysregulation of the balance in M1 (pro-inflammatory) versus M2 (anti-inflammatory) macrophages is emerging as a central mechanism governing the pathogenesis of NAFLD [58, 59]. In the last few years, the regulation of the immune system by autophagy has been reported [60–63]. The evaluation of the autophagic flux and its role in specific cells such as macrophages, T cells, and neutrophils in the NAFLD liver remains to be fully investigated. To illustrate this, the role of autophagy in tumor-associated macrophages in hepatocellular carcinoma is discussed below.

8. Hepatic Fibrosis

Hepatocyte apoptosis and inflammation are key players in the progression of the severity of liver complications. With the aim of simplifying hepatic fibrogenesis [64], hepatic stellate cells (HSCs) in response to damage (hepatocyte death…) and inflammation (TGFβ) differentiate into myofibroblast-like cells, which produce most extracellular matrix components. Prevention of hepatocyte death can prevent fibrosis. For example, pan-caspase inhibitors reduced hepatic fibrosis in db/db mice feed with a methionine/choline-deficient diet [65]. Circulating levels of markers of hepatocyte death and/or apoptosis also increased with the severity of hepatic fibrosis in obese or alcoholic patients [66, 67].

Since autophagy plays a hepatoprotective role, the steatotic liver with decreased hepatocyte autophagy is more vulnerable to injury induced by inflammation (TNFα, FASL) and stresses (ER and oxidative) and is therefore more prompted to develop fibrosis. Furthermore, activation of autophagy in HSCs has recently emerged as an additional mechanism involved in their activation (Figure 4) [64, 68, 69]. The increase in autophagic flux in activated HSCs leads to the loss of perinuclear lipid droplets (containing retinyl esters) associated with the transdifferentiation of quiescent HSC towards the activated phenotype [68, 69]. It has been hypothesized that the metabolism of lipid droplets mainly due to autophagy (probably by lipophagy) may provide the cellular energy that is critical for fueling catabolic pathways of HSCs activation. Under conditions of stress, ER stress (IRE1α-Xbp1-p38 pathway) could play an important role in the activation of autophagy and in turn fibrogenic activity of HSCs [70]. In a mouse model of liver fibrosis (carbon tetrachloride or thioacetamide), hepatic autophagy is activated and the loss of autophagic function in HSCs ($Atg7^{F/F}$—glial fibrillary acidic protein—Cre mice) reduced their activation, fibrogenesis, and matrix accumulation. This inhibition of autophagy in HSCs also prevented the loss of perinuclear lipid droplets *in vivo* [68]. Unfortunately, this has not yet been evaluated in a mouse model of NAFLD.

Thus, in addition to selective activation of autophagy in hepatocytes to decrease cell death, selective reduction of autophagic activity in fibrogenic cells in the liver unveils a potential new therapeutic strategy for liver fibrosis. It seems thus important to well targeting the liver cells and disease (severe fibrosis versus steatosis/NASH) for the treatment. Activation of autophagy in hepatocyte in early stages of NAFLD (steatosis/NASH) could prevent their progression to fibrosis. Indeed, limiting liver injury could be a therapeutic way to prevent the progression of hepatic complications [71]. It has been reported that a pan-caspase inhibitor or overexpression of the anti-apoptotic Bcl2 protein reduced fibrosis in an animal model of NAFLD and fibrosis, respectively [65, 72]. Furthermore, two years of treatment with

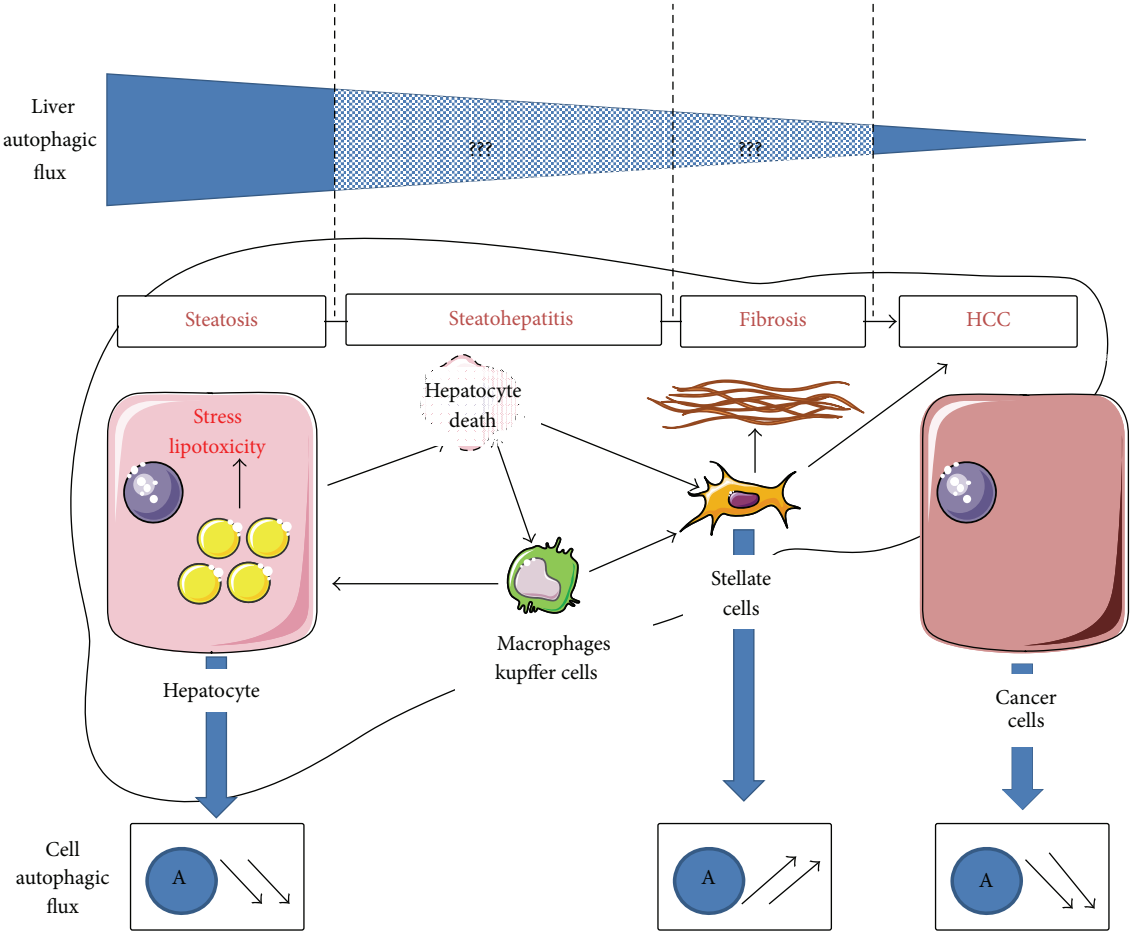

Figure 4: Autophagy and NAFLD. The evolution of NAFLD could be associated with dynamic regulation of autophagy. In the steatotic liver and hepatocytes, the autophagic flux is decreased and is associated with an alteration in the metabolic homeostasis and vulnerability of the liver. The low number of reports concerning the NASH liver does not allow one to clearly establish if an additional alteration in the autophagic flux occurs with inflammation. In the fibrotic liver, activation of autophagy in HCSs regulates their activation. In HCC, a decrease in autophagy in cancer cells and tumor-associated macrophages (M2 phenotype) facilitate tumor initiation and progression, respectively. Furthermore, liver complications increase with age and it is well established that hepatic autophagy is impaired in aging. It is thus difficult to obtain a clear picture of the level of autophagy in liver diseases in reinforcing the notion of dynamic processes. Additional studies are required to better understand the role of autophagy in the liver and in specific liver cells in NAFLD. However, it is obvious that important deregulation of hepatic autophagy facilitates the development of NAFLD. (A: autophagy.)

ursodeoxycholic in combination with vitamin E improved the level of hepatic enzymes AST/ALT and hepatic steatosis of patients with NASH [73]. Ursodeoxycholic is commonly used in the treatment of cholestatic liver disorders but its potential ability to prevent hepatocyte apoptosis has been evaluated in NAFLD [74]. Larger trials are warranted.

9. Hepatocellular Carcinoma, HCC

Autophagy is also involved in hepatocarcinogenesis, while its role remains controversial. Autophagy could play a dual role in cancer initiation and in cancer survival. First, autophagy eliminates senescent and injured cells, thereby limiting chromosomal instability and suppresses tumor initiation. Second, autophagy could provide energy by recycling damaged organelles, DNA, aggregated proteins, and pathogens to maintain energy balance, which promotes cancer cell survival [75]. In addition, the regulation of autophagy in liver macrophages and more specifically in tumor-associated macrophages could also play an important role in the development of HCC.

To illustrate the tumor suppressor role of autophagy, it has been reported that mice with heterozygous disruption of beclin-1 have a high frequency of spontaneous hepatocellular carcinoma [76]. Similarly, the deletion of Atg5 or Atg7 in liver resulted in the increasing incidence of benign liver adenomas [77]. Further, the expression of several autophagy related genes (*atg5*, *Atg7,* and *Atg6*/*beclin-1*) and their corresponding autophagic activity is decreased in HCC cell lines compared to normal hepatic cell lines [77, 78]. Similarly, beclin-1 mRNA and protein levels are lower in HCC tissue samples than in adjacent nontumor tissues from the same patients [78]. In 300 patients with HCC, the expression of Beclin-1 correlated with disease-free survival and overall survival only in the

Bcl-xL$^+$ patients. Multivariate analyses revealed that Beclin-1 expression was an independent predictor for disease-free survival and overall survival in Bcl-xL$^+$ patients. Further, Beclin-1 expression correlated with tumor differentiation in Bcl-xL$^+$ but not in Bcl-xL$^-$ HCC patients. These data suggest that a defect in autophagy synergizes with altered apoptotic activity and facilitates tumor progression and poor prognosis of HCC [78].

Since autophagy negatively regulates stresses and prevents cell death, its activation could also be involved in the survival of cancer. Indeed, LC3 was highly expressed in HCC compared with noncancerous tissues and correlated with tumor size. In addition, LC3 was an independent predictor of HCC recurrence after surgery only in the context of large tumors [79]. Furthermore, autophagy induced by oncogenic K-Ras mediates functional loss of mitochondria during cell transformation to overcome an energy deficit resulting from glucose deficiency [80].

In addition, autophagy could also act *via* the regulation of the function of tumor-associated macrophages. It is well established that the tumor-associated macrophage density in human cancer correlates with poor prognosis in most human cancers and the inhibition or enhancement of the macrophage density in tumors by genetic and pharmacological approaches, respectively, inhibits or promotes tumor angiogenesis, growth, and progression [62, 81, 82]. Further, the polarization of macrophages into the M2 (anti-inflammatory) phenotype favors tumor progression, while M1 (proinflammatory) macrophages exert an anticancer activity. Tumor-associated macrophages could sense factors from the tumor microenvironment that lead to their polarization. Autophagy is involved in this process [62, 81]. For example, the TLR2 deficiency causes a reduction in macrophage infiltration but also induces significant suppression of autophagy associated with a decrease in the hepatic expression of tumor necrosis factor, interferon gamma, and [C-X-C motif] ligand 2. This enrichment in M2 macrophages in turn promotes hepatocarcinogenesis [83].

A specific enhancement in autophagy in tumor-associated macrophages could enhance polarization into the M1 phenotype and could have a beneficial effect. The activation of the mTOR-TSC2 pathway, a key negative regulator of autophagy, is critical for macrophage polarization toward the M2 phenotype to promote tumor angiogenesis and growth in mouse hepatocellular carcinoma models. In contrast, inhibition of this pathway by for example rapamycin/silorimus exerts the opposite effects [84]. Interestingly, the use of rapamycin/silorimus as an immunosuppressor displays beneficial responses in patients. Indeed, survival after liver transplantation has been evaluated according to the immunosuppression protocol applied to 2491 adult recipients of isolated liver transplantation for HCC and 12,167 for non-HCC. These patients remained on stable maintenance of immunosuppression protocols for at least 6 months after transplant. Treatment with rapamycin was associated with improved survival after transplantation for HCC. Interestingly, rapamycin showed a trend toward lower rates of survival in non-HCC recipients, confirming the specificity of its beneficial impact for cancer patients [85].

Activation of autophagy in HCC targeting tumor-associated macrophages may represent a promising and effective strategy for liver cancer therapy. In contrast, the inhibition of autophagy in malignant cells may be a novel strategy to improve the efficacy of anticancer therapy. However, further investigations are required to determine the role of autophagy as a function of the type of tumor, stage, and genetic context.

10. Aging

The prevalence of type 2 diabetes and liver complications increases with age and it is well established that autophagy and chaperone-mediated autophagy are impaired in aging [86, 87]. This decline is associated with an increase in the lipid content in different organs such as the liver. As seen above, excessive accumulation of lipids alters further autophagic turnover and its protective role against accumulation of lipid droplets and insulin resistance. A link between aging, loss of mitochondrial function, and ROS production has also been reported [86, 88]. Calorie restriction enhances longevity and this may be due, at least in part, to activation of autophagy, in particular lipophagy and mitophagy leading to a decrease in the lipid content and in oxidative stress [89–91]. Aging, dysfunctional mitochondria, and oxidative stress contribute to the development of type 2 diabetes and liver complications [92, 93]. The increase in autophagic turnover could enhance mitochondrial turnover and biogenesis, and, consequently, decrease ROS production. This may contribute to life span extension and prevent or delay the development of complications associated with obesity [94]. The development of NAFLD has recently been examined in mice of different ages (2, 8, and 18 months) in response to a fixed period 16 weeks of HFD. Weight gain, insulin resistance, and hepatic steatosis were equivalent for the three ages. In contrast, liver injury occurred exclusively in the two older ages and older mice had an elevated innate immune response with more M1 (proinflammatory) macrophages. Aged hepatocytes were further selectively sensitized to the Fas death pathway *in vitro*. Aging thus leads to increased hepatocellular injury and inflammation upon HFD challenge [95]. Unfortunately, the level of autophagy was not evaluated in this study.

11. Conclusions

Autophagy is a crucial physiological process in providing nutrients to maintain vital cellular functions during fasting, but also to purge the cell of superfluous or damaged organelles, lipids, and misfolded proteins in obesity. Obesity is a complex multifactorial chronic disease affecting multiorgans and physiological responses [22]. It is clear that modifications in the autophagic turnover during obesity mediate protective or deleterious responses depending on the cell/organ [23]. This review summarized the knowledge of the role of autophagy in specific cells, including hepatocytes, macrophages, HSCs and cancer cells, and liver diseases from steatosis to HCC in the context of obesity (Figure 4). Some drugs that modulate autophagy are already approved for other human clinical: rapamycin, carbamazepine, cisplatin,

and metformin promote autophagy in some cells, while the antimalaria compound chloroquine (which increases the intralysosomal pH) inhibits autophagic turnover. However, the lack of specificity and the absence of organ or cell selectivity are the major limitations of these compounds. Better understanding of the molecular mechanisms of autophagy and its implication in specific liver cells, such as hepatocyte, HSCs, macrophages but also endothelial and other immune cells, during the evolution of the liver complications will highlight new potential therapeutic targets.

Abbreviations

AcCoA: Acetyl-coenzyme A
Akt/PKB: Protein kinase B
ALT: Alanine transaminase
AST: Aspartate transaminase
ATF4: Activating transcription factor 4
Atg: Autophagy-related protein
BAX: Bcl-2-associated X protein
Bcl-2: B-cell lymphoma 2
Bcl-xL: B-cell lymphoma extra-large
CHOP: C/EBP-homologous protein
DRAM: Damage-regulated autophagy modulator
ER: Endoplasmic reticulum
FASL: Fas ligand
FFA: Free fatty acids
FGF21: Fibroblast growth factor 21
FIP200: Focal adhesion kinase family interacting protein of 200 kD
FoxO: Forkhead box protein O
GGT: Gamma-glutamyl transferase
GRP78: Glucose regulate protein 78
HCC: Hepatocellular carcinoma
HDL: High density lipoprotein
HFD: High fat diet
HSCs: Hepatic stellate cells
IFN: Interferon
IRE1: Inositol requiring enzyme 1
LAMP-2A: Lysosome-associated membrane protein type 2A
LC3: Microtubule-associated protein 1A/1B-light chain 3
MDB: Mallory-Denk Bodies
mTOR: Mammalian target of rapamycin
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
OA: Oleic acid
PA: Palmitic acid
PGC-1: Peroxisome proliferator-activated receptor gamma coactivator 1
PI3K: Phospho-inositide 3-kinase
PPAR: Peroxisome proliferator-activated receptor
TFEB: Transcription factor EB
TGF: Transforming growth factor
TLR: Toll-like receptor
TNF: Tumor necrosis factor
Xbp1: X-box binding protein 1.

Conflict of Interests

Authors declare no conflict of interests.

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Review Article

Autophagy: A Multifaceted Partner in Liver Fibrosis

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Liver fibrosis is a common wound healing response to chronic liver injury of all causes, and its end-stage cirrhosis is responsible for high morbidity and mortality worldwide. Fibrosis results from prolonged parenchymal cell apoptosis and necrosis associated with an inflammatory reaction that leads to recruitment of immune cells, activation and accumulation of fibrogenic cells, and extracellular matrix accumulation. The fibrogenic process is driven by hepatic myofibroblasts, that mainly derive from hepatic stellate cells undergoing a transdifferentiation from a quiescent, lipid-rich into a fibrogenic myofibroblastic phenotype, in response to paracrine/autocrine signals produced by neighbouring inflammatory and parenchymal cells. Autophagy is an important regulator of liver homeostasis under physiological and pathological conditions. This review focuses on recent findings showing that autophagy is a novel, but complex, regulatory pathway in liver fibrosis, with profibrogenic effects relying on its direct contribution to the process of hepatic stellate cell activation, but with antifibrogenic properties via indirect hepatoprotective and anti-inflammatory properties. Therefore, cell-specific delivery of drugs that exploit autophagic pathways is a prerequisite to further consider autophagy as a potential target for antifibrotic therapy.

1. Liver Fibrosis

Liver fibrosis is defined by the excessive accumulation of extracellular matrix in response to chronic injury regardless of the cause. The condition arises from an altered wound-healing reaction designed in an attempt to reduce hepatic damage. Scar accumulation is the result of a bidirectional process combining increased synthesis and deposition of extracellular matrix proteins within the liver, and a parallel failure of physiological mechanisms underlying matrix turnover [1, 2]. Progression of fibrosis upon sustained liver insult is associated with expansion of fibrotic septa, ultimately leading to cirrhosis, which is a condition defined by fibrotic septa surrounding regenerating nodules and marked alterations of hepatic vascularisation. Whereas early stages of fibrosis do not generate any significant morbidity, cirrhosis carries a high risk of morbimortality, owing to severe complications of liver failure and portal hypertension (i.e., ascites, variceal bleeding, bacterial infections, hepatic encephalopathy, hepatorenal syndrome, acute-on-chronic liver failure, etc.) and to the high incidence of hepatocellular carcinoma in the cirrhotic liver [1, 2]. Given the high prevalence of several causes of liver diseases worldwide (e.g., alcohol, hepatitis B and C viruses, nonalcoholic fatty liver disease, etc.), cirrhosis is regarded as a high public health burden worldwide, representing the most common nonneoplastic cause of death among diseases of the gastrointestinal tract in Europe and the USA. Therefore, efficient antifibrotic therapeutic approaches are a high

priority goal for hepatologists. In this respect, recent data have conclusively established, both in experimental models and in cohort studies, that eradication or efficient control of the cause of liver disease may be associated with regression of fibrosis and early stage cirrhosis [2]. However, this goal cannot be achieved in several instances, which justifies past and ongoing massive efforts to identify potential therapeutic antifibrotic targets.

2. Autophagy

Autophagy covers three catabolic processes (i.e., macroautophagy, microautophagy, and chaperone-mediated autophagy) responsible for the degradation of cell components in the lysosome [3, 4]. Macroautophagy (hereafter referred to as autophagy) is the most well char-acterized mechanism in eukaryotic cells and requires a vacuolar transport of cytoplasmic material to the lysosome. Autophagy starts with the formation of a double-membrane surrounded vacuole, known as the autophagosome, which ultimately fuses with the lysosomal compartment where autophagic cargoes are degraded. The autophagosome originates from the phagophore, a membrane that is nucleated and elongated by a family of autophagy-related (*ATG*) genes conserved between yeast and humans [5]. The phagophore formation is initiated by the UNC-51-like kinase 1 ULK1 (ATG1) complex in the omegasome, an endoplasmic reticulum (ER) based structure. The activity of this complex is controlled by the mammalian target of rapamycin complex 1 (mTORC1), which integrates diverse signals such as amino acids, glucose, and growth factors [6]. Upon mTOR inhibition by starvation, ULK1/2 dissociates from the complex and drives autophagosome formation, in a coordinated manner with the Beclin 1 (ATG6): vacuolar protein sorting 34 (Vps34, class III phosphatidylinositol 3-kinase) complex I. The synthesis of PtdIns3P by vacuolar protein sorting 34 (Vps34) is an important trigger for the elongation and closure of the autophagosome by two ubiquitin-like conjugation systems, ATG5-ATG12 and LC3 (ATG8)-PE (phosphatidylethanolamine).

Autophagy is an important regulator of liver homeostasis under physiological conditions [7–9]. The basal rate of autophagy is required to maintain liver homeostasis by elimination of aggregate-prone proteins and damaged mitochondria and by counteracting hepatocyte swelling [7–9]. The sequestration of mitochondria and protein aggregates mainly relies on the selective recognition of cargoes by autophagy adaptors, such as SQSTM1/p62, that bridge the cargoes to the autophagic machinery [3, 4]. SQSTM1/p62 contains a LC3-interacting region (LIR) that interacts with both LC3 and a UBA (ubiquitin-associated) domain, leading to the selective degradation of the ubiquitinated cargo by autophagy. SQSTM1/p62 also interacts with several signaling components, such as ERK1, αPKC, TRAF6, Keap1, and mTORC1 [3, 4]. Thus, regulation of cellular levels of SQSTM1/p62 by autophagy controls antioxidant defense, inflammatory response, cell growth, and apoptosis. Additional physiological functions of autophagy in the liver include regulation of metabolic pathways such as gluconeogenesis during fasting, β-oxidation of fatty acids, and ketone body formation. Amino acids used for gluconeogenesis are produced by proteolysis through bulk autophagy [10], whereas fatty acids are mainly produced by selective autophagy of triglycerides stored in lipid droplets (lipophagy) [11]. Autophagy probably also controls the level of very-low-density lipoprotein (VLDL) particles through lipophagy, which releases fatty acids and degrades apolipoprotein B. Moreover, liver autophagy plays a key role in restoring plasma glucose concentrations in neonates during fasting [12]. Finally, hepatocyte autophagy promotes liver regeneration after partial hepatectomy, by preserving the integrity of mitochondria and protecting hepatocytes from senescence [13].

Mounting evidence also indicates that alterations in the autophagic process in parenchymal and nonparenchymal liver cells drive or control the progression of various liver diseases, including alcoholic and nonalcoholic fatty liver disease, viral hepatitis, drug and ischemia-reperfusion injury, and hepatocellular carcinoma [7–9]. Novel findings also implicate autophagy in the control of liver fibrosis.

3. Cellular Effectors of Liver Fibrogenesis

3.1. Hepatic Myofibroblasts as Fibrogenic Cells of the Liver. Extracellular matrix accumulation during chronic liver injury is driven by a heterogenous population of myofibroblasts that migrate and accumulate at sites of liver injury in response to a wide variety of paracrine/autocrine signals produced by neighbouring inflammatory and parenchymal cells [1, 2]. Hepatic myofibroblasts display a fibrogenic phenotype (Figure 1(a)) characterized by (i) the secretion of an array of extracellular matrix proteins (ECM) predominating in fibrillar collagens, (ii) a high proliferative capacity and relative resistance to apoptosis, (iii) production of a wide range of ECM degrading enzymes (metalloproteinases, MMP) that modulate ECM remodeling and specific tissue inhibitors of the metalloproteinase family (TIMPs), and (iv) release of cytokines and growth factors that maintain a sustained inflammatory reaction and assist liver regeneration and angiogenesis. Several studies have shown that hepatic myofibroblasts of diverse origins coexist in the injured liver, with a large preponderance of cells derived from hepatic stellate cells and to a minor extent from resident portal fibroblasts [1, 2].

Hepatic stellate cells (HSC) represent the main source of liver fibrogenic cells. In the normal liver, HSC reside in the perisinusoidal space between endothelial cells and hepatocytes and display a quiescent phenotype characterized by the expression of a large panel of adipogenic genes and neural markers [1, 2]. A characteristic feature of quiescent HSC is the presence of cytoplasmic lipid vacuoles loaded with retinoids stored as retinyl esters and triglycerides. Upon acute or chronic liver injury, parenchymal injury and the resulting inflammatory reaction generate a large panel of signals that promote induction of specific sets of transcription factors and morphogens (Hedgehog ligands, Wnt) in quiescent HSC, thereby triggering the activation program and the acquisition of fibrogenic and proinflammatory properties [1, 2, 14–16]. Upon activation, quiescent HSC lose their retinyl ester-containing lipid droplets and the expression

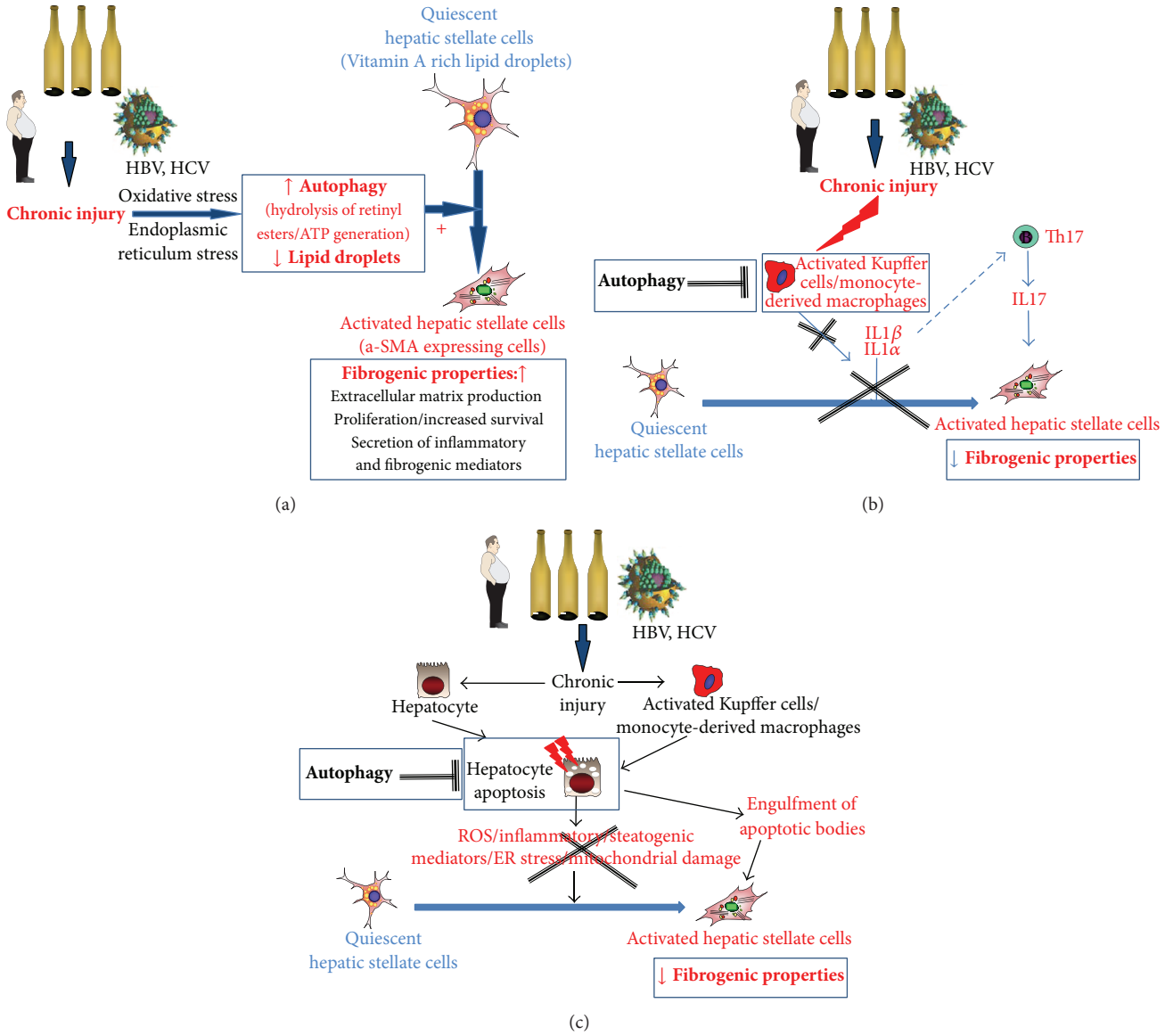

Figure 1: Impact of autophagy on the cellular effectors of liver fibrogenesis. (a) Autophagy drives hepatic stellate cell activation from a quiescent, lipid-rich to a myofibroblastic, fibrogenic phenotype. In response to chronic liver injury, quiescent hepatic stellate cells lose their retinyl ester-containing lipid droplets and acquire myofibroblastic features, associated with fibrogenic properties. Autophagy in hepatic stellate cells is stimulated by oxidative and endoplasmic reticulum stress and may serve to provide free fatty acids from retinyl esters, thereby supplying the energy required for initiating and perpetuating the fibrogenic phenotype. (b) Autophagy as a protective anti-inflammatory process with antifibrogenic properties. Hepatic macrophage autophagy stimulates an anti-inflammatory pathway, that reuses the production of IL1 alpha and IL1 beta, resulting in inhibition of liver fibrogenesis. In addition, inhibition of Th17 polarisation by IL1 alpha and beta may also contribute to the antifibrogenic effects of macrophagic autophagy. (c) Hepatoprotective properties of autophagy may contribute to inhibition of fibrogenesis. Autophagy is generally considered as a survival pathway for hepatocytes, therefore limiting oxidative stress profibrogenic pathways for hepatic stellate cells such as ER stress and mitochondrial damage.

of adipogenic/lipogenic factors. In parallel, they acquire myofibroblastic-like features, including the expression of smooth muscle alpha actin, and *de novo* expression of receptors for fibrogenic, chemotactic, and mitogenic factors [1, 2, 14–16]. The activation process occurs in response to classical signals including lipid peroxides reactive oxygen species, proinflammatory and mitogenic cytokines and growth factors, and the matrix itself via integrin-mediated pathways activated by ECM molecules, matrix stiffness, and the degree of collagen crosslinking [1, 2, 14–16]. More recently, reprogramming of HSC metabolic program and epigenetic events have been identified as additional mechanisms driving HSC activation/deactivation program.

3.2. Hepatocytes. Hepatocyte apoptosis and/or necroapoptosis are key contributors of the fibrogenic process. Indeed, injured hepatocytes display enhanced oxidative stress, ER stress, and mitochondrial damage that are potent stimuli

for hepatic stellate cell activation. Moreover, activated HSC display phagocytic properties towards hepatocyte-derived apoptotic bodies. Engulfment of apoptotic bodies results in enhanced resistance of HSC to apoptosis and increased profibrogenic properties [17] (Figure 1(b)).

3.3. Immune Cells. As described in other organs, sustained hepatic inflammation resulting from parenchymal liver injury is a major driving force of both fibrosis progression and fibrosis resolution, depending on cell type and activation state. Selective depletion of individual inflammatory cells has allowed characterizing the complex interactions and impact of innate (macrophages) and adaptive (T lymphocyte subsets) immune cells on fibrosis accumulation and regression.

3.3.1. Innate Immune Cells. Activation of Kupffer cells and recruitment of monocyte/macrophages are a key event governing initiation, perpetuation, and resolution of fibrosis and has been extensively characterized, using pharmacological or conditional genetic ablation of monocytes/macrophages, in mice with ongoing liver injury [18, 19]. These studies have been corroborated by *in vitro* data showing that Kupffer cells promote activation and survival of HSC [19, 20]. However, macrophages harboring a distinct phenotype induce hepatic stellate cell apoptosis and produce active metalloproteinases that drive resolution of fibrosis [21]. Moreover, other innate immune cells have also been implicated. In particular, dendritic cells may orchestrate the inflammatory response during both progression and resolution of liver fibrosis [22–24]. NK cells reduce fibrogenesis by inducing apoptosis of early activated and senescent hepatic stellate cells via TRAIL [25, 26].

3.3.2. Adaptive Immune Cells. $CD4^+$ T lymphocytes (Th1, Th2, Th17, and Treg) control the fibrogenic process with positive or negative outcome depending on their phenotype [27]. Indeed, whereas Th1 effector T cells reduce liver fibrogenesis via the release of IFN-gamma, Th2 polarization promotes liver fibrosis via production of IL-13. T helper 17 (Th17) lymphocytes have also more recently emerged as critical enhancers of profibrogenic properties of hepatic myofibroblasts via secretion of IL17 [28–31]. The role of regulatory T cells has not been investigated as yet, but antifibrogenic properties might be anticipated from data obtained in cardiac and pulmonary fibrosis [27].

4. Autophagy and Liver Fibrosis

Currently recognized antifibrotic strategies include targeting of several steps leading to liver fibrogenesis, that is, inhibition of hepatocyte apoptosis, liver inflammation, and/or promotion of fibrogenic cell apoptosis or reversion of fibrogenic cell phenotype to a quiescent state. Autophagy has recently emerged as a novel but complex regulator of liver fibrosis, with profibrogenic effects relying on its direct contribution to the process of HSC activation but antifibrogenic properties via indirect hepatoprotective and anti-inflammatory properties.

4.1. Autophagy in Hepatic Stellate Cells: A Profibrogenic Process (Figure 1(a)). A number of cells maintain energy homeostasis through autophagic digestion of intracellular lipids (lipophagy); this process has been well characterized in hepatocytes [11].

Because the progressive loss of retinoid-containing lipid droplets is a feature of hepatic stellate cell activation, autophagy has been hypothesized to govern the activation process by digesting lipid droplets. Two groups recently independently reported that autophagy contributes to hepatic stellate cell activation *in vitro*, both in mice and human cells, and confirmed these findings in cells isolated from mice acutely exposed to either thioacetamide or carbon tetrachloride [32, 33]. These conclusions were drawn on the basis of an increase in LC3-II and a decrease in p62/SQSTM1 expressions upon hepatic stellate cell activation, associated with enhanced autophagic flux and the presence of a high number of autophagic vacuoles. Conversely, pharmacological inhibition of autophagy or downregulation by small interfering RNAs against *Atg5* or *Atg7* reduced the number of lipid droplets within HSC [32, 33]. The findings were also supported in mice and fibrotic liver samples from patients with hepatitis B, which showed increased levels of autophagy in HSC upon liver injury [32]. Further experiments allowed establishing a link between autophagy, elimination of lipid droplets, and myofibroblastic differentiation of HSC. Indeed, as previously shown in hepatocytes, autophagy enables catabolism of retinyl esters by lipases, thereby providing free fatty acids that increase generation of ATP following mitochondrial β-oxidation [32]. Potential signals triggering autophagy in hepatic stellate cell have been recently identified and they include oxidative stress and endoplasmic reticulum stress [34], which are recognized signals for hepatic stellate cell activation *in vitro* and in the injured liver. Altogether these data identify lipophagy of retinyl esters as a mandatory driver of the initiation and perpetuation of the activated phenotype of liver fibrogenic cells.

Another major finding of these studies was the demonstration of the profibrogenic consequences of autophagy activation in hepatic stellate cells. Indeed, when autophagy was blunted with pharmacological inhibitors or following genetic invalidation of the autophagic genes *Atg7* or *Atg5*, downregulation of the fibrogenic properties of cultured hepatic stellate cells was observed, as illustrated by HSC growth inhibition, reduced expression of fibrogenic genes and of the activation marker alpha SMA [32, 33]. These data were further confirmed *in vivo,* in mice harboring a specific deletion of the autophagic gene *Atg7* in hepatic stellate cells ($Atg7^{F/F}$-GFAP-cre mice) that showed decreased hepatic stellate cell activation and reduced liver fibrogenesis and matrix accumulation upon chronic administration of carbon tetrachloride or thioacetamide [32]. These results demonstrated that autophagy in hepatic stellate cells contributes to the liver fibrogenic process. Importantly, inactivation of autophagy in kidney and lung fibrogenic cells also reduced their capacity to drive a fibrogenic response [32], identifying autophagy as a potential core pathway of fibrogenesis.

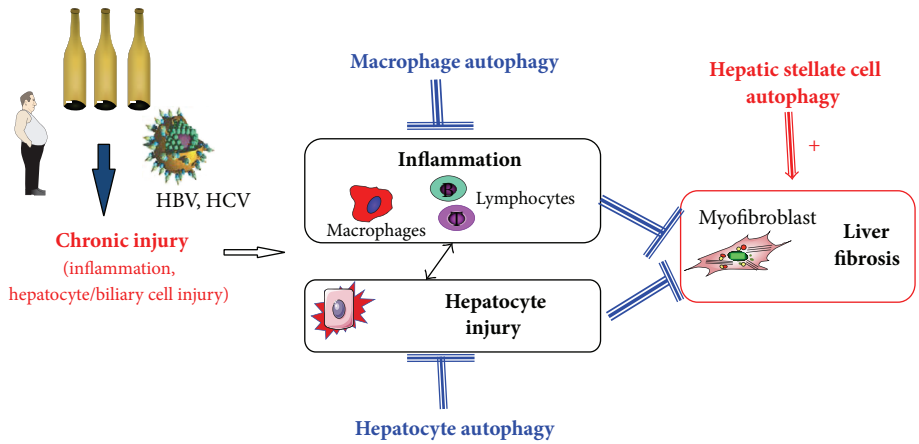

FIGURE 2: Autophagy in the liver: a pathway with divergent cell-specific effects? Autophagy enhances fibrogenic properties in hepatic stellate cells. In contrast, anti-inflammatory effects in macrophages and hepatoprotective effects in hepatocytes limit the development of liver fibrosis.

However, a more complex scheme is emerging, as recent data indicate that, in other hepatic cell types, autophagy reduces profibrogenic signals, by protecting hepatocytes from apoptosis [35] and eliciting anti-inflammatory effects in Kupffer cells [36].

4.2. Autophagy: A Protective Anti-Inflammatory Process with Antifibrogenic Properties (Figure 1(b)). Several studies have conclusively demonstrated the role of autophagy in the control of proinflammatory signaling [37]. In macrophages, autophagy regulates phagocytosis of pathogens and is critically involved in monocyte differentiation into macrophages and acquisition of phagocytic functions [38]. Interestingly, macrophages exposed to an autophagy inhibitor or lacking one of the autophagic components (Atg16L1, ATG5, ATG7, Beclin 1, or LC3B) display a proinflammatory phenotype, characterized by enhanced IL1β secretion, that results from ROS-mediated activation of the NRLP3 inflammasome pathway [39–41]. In addition, autophagy-defective (*Atg5*fl/fl *LysM*-Cre+) macrophages secrete high levels of IL1α through a ROS/calpain-dependent but inflammasome-independent pathway [42]. The central role of autophagic genes in the anti-inflammatory response of macrophages suggests that in the context of liver fibrosis, macrophage autophagy may be a protective pathway that prevents excessive release of inflammatory mediators during chronic liver injury. We recently addressed this hypothesis in mice lacking the autophagic gene *Atg5* in myeloid cells (*Atg5*fl/fl *LysM*-Cre+ mice) and uncovered the beneficial consequences of macrophage autophagy on liver fibrosis [36]. Indeed, these mice were more susceptible to liver inflammation and liver injury when exposed to carbon tetrachloride and showed higher hepatic secretion of IL1α and -β, increased recruitment of neutrophils and monocytes into the liver, and enhanced hepatocyte apoptosis. Administration of carbon tetrachloride to *Atg5*fl/fl *LysM*-Cre+ mice was also associated with exacerbated fibrosis accumulation in the liver and accumulation of fibrogenic cells [36]. In keeping with *in vivo* data, mechanistic studies confirmed the higher fibrogenic potential of hepatic myofibroblasts exposed to the conditioned medium of *Atg5*fl/fl *LysM*-Cre+ macrophages. This effect resulted from an increased release of IL1α and -β from *Atg5*fl/fl *LysM*-Cre macrophages, since it was blunted by IL1α and -β neutralizing antibodies. Overall, these data identify liver macrophage autophagy as an anti-inflammatory pathway, with protective antifibrogenic effects by paracrine interactions with hepatic myofibroblasts (Figure 1(b)) [36].

Autophagy also controls T cell activation, in part by regulating the inflammatory response of macrophages and dentritic cells. Thus, autophagy-deficient macrophages show excessive secretion of IL1α and IL1β, two cytokines that function together with IL-6 and TGF-β to promote Th17 differentiation and responses [42]; similarly, pharmacological inhibition of autophagy in dendritic cells enhances the production of inflammatory mediators from $\gamma\delta$ T cells, including IL17 [43]. Concordantly, mice with selective deletion of autophagy protein in myeloid cells demonstrate enhanced inflammatory responses, including increased secretion/release of IL-1 and IL-17 in response to mycobacterium tuberculosis [42]. Given the reported profibrogenic role of IL-17 in the liver, whether macrophage and/or dentritic cell autophagy may also indirectly inhibit liver fibrosis via limitation of IL-17 release is an important issue that deserves further investigation.

4.3. Hepatoprotective Properties of Autophagy May Contribute to Inhibition of Fibrogenesis (Figure 1(c)). Although both survival and apoptotic properties of autophagy have been described, recent studies using more specific tools have established that autophagy is mainly a prosurvival pathway that removes misfolded proteins, accumulated lipids (lipophagy), and/or damaged mitochondria (mitophagy) to reduce oxidative stress and lipid peroxidation and supply nutrients to maintain cellular energy homeostasis under injured conditions.

In the liver, autophagy behaves as a protective pathway in the face of various forms of injury. Thus, in the context of nonalcoholic fatty liver disease, free fatty acids inhibit autophagy in hepatocytes, thereby inducing hepatocyte apoptosis; conversely, autophagy underlies resistance of hepatocytes to the apoptotic effects of free fatty acids

[35]. Similarly, autophagy is a survival pathway against acute alcohol-induced hepatocyte apoptosis [44]. In response to acetaminophen intoxication, autophagy serves to remove damaged mitochondria (mitophagy) thereby providing regulatory loop protecting against hepatocyte necrosis [45]. Moreover, during ischemia/reperfusion injury suppression of autophagy triggers hepatocyte death [46]. Finally, in alpha-1 antitrypsin deficiency, pharmacological induction of autophagy limits the cellular aggregation of mutant α1-antitrypsin, thereby reducing liver fibrogenesis in rodents [47]. Interestingly, in this setting, autophagy induction provides protection towards both hepatocellular damage and liver fibrosis, in sharp contrast with the profibrogenic effects of autophagy in hepatic stellate cells. Moreover, we recently showed that *Atg5*fl/fl *LysM*-Cre+ mice with exacerbated liver fibrosis in response to CCl_4 also display enhanced hepatocyte apoptosis [36], suggesting that macrophage autophagy might provide an additional hepatoprotective mechanism contributing to its antifibrogenic effects.

5. Autophagy in Liver Fibrosis: Friend or Foe?

Autophagy has initially been viewed as a hepatoprotective and anti-inflammatory pathway during liver injury. However, a more complex paradigm is emerging with the identification of the profibrogenic effect of autophagy in fibrogenic cells (Figure 2). Therefore, because autophagy elicits divergent and cell-specific effects during chronic liver injury, manipulation of autophagy for therapeutic antifibrogenic purposes should only be considered by means of cell-specific delivery approaches.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Liver Autophagy in Anorexia Nervosa and Acute Liver Injury

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Autophagy, a lysosomal catabolic pathway for long-lived proteins and damaged organelles, is crucial for cell homeostasis, and survival under stressful conditions. During starvation, autophagy is induced in numerous organisms ranging from yeast to mammals, and promotes survival by supplying nutrients and energy. In the early neonatal period, when transplacental nutrients supply is interrupted, starvation-induced autophagy is crucial for neonates' survival. In adult animals, autophagy provides amino acids and participates in glucose metabolism following starvation. In patients with anorexia nervosa, autophagy appears initially protective, allowing cells to copes with nutrient deprivation. However, when starvation is critically prolonged and when body mass index reaches 13 kg/m^2 or lower, acute liver insufficiency occurs with features of autophagic cell death, which can be observed by electron microscopy analysis of liver biopsy samples. In acetaminophen overdose, a classic cause of severe liver injury, autophagy is induced as a protective mechanism. Pharmacological enhancement of autophagy protects against acetaminophen-induced necrosis. Autophagy is also activated as a rescue mechanism in response to Efavirenz-induced mitochondrial dysfunction. However, Efavirenz overdose blocks autophagy leading to liver cell death. In conclusion, in acute liver injury, autophagy appears as a protective mechanism that can be however blocked or overwhelmed.

1. Introduction

Autophagy (literally "self-eating") is a cellular process responsible for the degradation of excess or aberrant long-lived cytosolic proteins and organelles within lysosomes in order to remove and eventually recycle the resulting macromolecules [1]. It has an important role in various biological events such as cellular remodeling during development and differentiation, adaptation to stress conditions, and extension of lifespan [2]. Depending on physiological functions and mode of cargo delivery to the lysosome, three forms of autophagy have been identified: chaperone-mediated autophagy, microautophagy, and macroautophagy [3]. In this review we will focus on macroautophagy, hereafter referred to as "autophagy."

Autophagy consists of several sequential steps by which a portion of the cytoplasm, including organelles, is engulfed by a phagophore to form an autophagosome. The autophagosome subsequently fuses with a lysosome to form an autolysosome, and the internal material is degraded by lysosomal hydrolases and recycled to the cytoplasm [4].

The initial studies that led to the identification of autophagy were conducted in the liver [5]. Afterward, extensive work has been carried out on this organ to dissect the regulation and the roles of autophagy. Notably, a remarkable work by Mortimore's group led to the discovery that amino acids as well as insulin and glucagon were crucial regulators of starvation-induced autophagy [6]. Subsequently, the implication of autophagy has been highlighted in various chronic liver diseases, including alcoholic liver disease, viral hepatitis, alpha1-antitrypsin deficiency, and hepatocellular carcinoma [7, 8]. Recently, several works have also pointed out the involvement of autophagy in several acute liver diseases. This review aims to summarize current knowledge on this last

topic, with a particular focus on acute liver injury associated with severe anorexia nervosa.

2. Regulation of Starvation-Induced Autophagy

Starvation or food restriction is one of the best-known inducers of autophagy. Thus, extensive work has been carried out under this condition to study autophagy. In this stressful context, intracellular material is degraded and the resulting breakdown products are released into the cytoplasm to be used by cell metabolism [4]. In 1983, Mortimore et al. observed that mice lose about 40% of their liver protein content within 48 hrs of starvation [9]. Similarly, 4 to 5% of total protein content of isolated rat hepatocytes cultured under amino acid free conditions is degraded each hour [10]. Schworer et al. showed in rats that starvation shifts basal liver protein degradation from about 1.5%/hour (basal) to 4.5%/hour (starvation induced) [11], which correlated with autophagy kinetics determined by quantitative electron microscopy [11], leading to the concept that autophagy mediates protein degradation under nutrient deprivation [12]. Although these proteolysis rates seemed to reflect autophagic degradation, the definitive demonstration of the implication of this process was only confirmed more than 2 decades later by the use of autophagy-deficient cell models. Indeed, in isolated Atg7-deficient hepatocytes, starvation-induced proteolysis is almost completely lost [13].

Starvation-induced autophagy is regulated by several metabolic parameters including amino acid, insulin, and glucagon levels.

Experiments performed using isolated perfused liver in the absence of the potent autophagy regulators present *in vivo*, including insulin and glucagon, showed that amino acids are strong inhibitors of autophagy [12]. Indeed, half normal plasma level concentration of complete amino acid mixtures suppresses autophagy. Further investigations identified a group of 8 amino acids (leucine, tyrosine, phenylalanine, glutamine, proline, histidine, tryptophan, and methionine), including 5 essential amino acids (leucine, phenylalanine, histidine, tryptophan, and methionine), which were as effective as complete plasma mixtures for autophagy inhibition, in isolated perfused rat livers [14]. Similar results were obtained on isolated rat hepatocytes *in vitro* wherecombination of high concentrations of leucine with either histidine or glutamine inducedeffective inhibition of autophagy [15]. Leucine is by far the most efficient autophagy inhibitor and alanine, which does not have an inhibitory effect by itself, displayes a coregulatory effect [16]. Although there is evidence that most of the inhibitory effect of amino acids on autophagy occurs at the initiation step (sequestration) [6, 17], an effect on the late step (autophagosome and lysosome fusion) cannot be ruled out, since leucine at high concentration can modify lysosomal pH [18], and this might interfere with the fusion between autophagosomes and lysosomes. Furthermore, asparagine is also able to inhibit the fusion between autophagosomes and lysosomes [19]. Autophagy regulation by glutamine is indirect. A glutamine transporter, SLC1A5, is responsible for the uptake of glutamine from extracellular compartment into the cell. Glutamine is thereafter transported outside the cell by SLC7A5/SLC3A2 in exchange of essential amino acids such as L-leucine that elicit mTOR activation and subsequent autophagy inhibition [20]. The mammalian target of rapamycin (mTOR) is a central cellular metabolism protein on which several signaling pathways converge in response to changes in energy/nutritional status. mTOR stimulates protein synthesis by inducing translation of mRNA and inhibits protein catabolism by decreasing autophagy [21]. How amino acids activate mTOR is not fully understood. However, recent lines of evidence show that these molecules, when present in sufficient amounts, accumulate in lysosomes and elicit mTORC1 recruitment and activation through a lysosomal v-ATPase-Ragulator-Rag GTPase complex [22, 23]. In addition to these posttranslational effects, amino acids also modulate autophagy at the transcriptional level. During starvation, the transcription factor EB (TFEB), a master regulator of lysosomal biogenesis and autophagy, is activated, translocates into the nucleus, and drives the transcription of autophagy and lipid metabolism genes. Activity and localization of TFEB is regulated by the extracellular signal-regulated kinase 1/2 (ERK-1/2), a sensor of nutrients status [24, 25], and by mTORC1. In the presence of sufficient nutrients, TFEB interacts with a complex nutrient sensing machinery at the lysosome surface, including mTORC1 that phosphorylates TFEB at Ser211 [26, 27]. Phosphorylated TFEB is sequestered in the cytosol and is thus inactive as a transcription factor. However, during starvation, mTOR is no longer recruited at the lysosomal surface and unphosphorylated TFEB translocates to the nucleus [27].

In addition to amino acids, liver autophagy is tightly controlled by hormones. Plasma glucagon levels are increased during fasting in humans [54]. Glucagon stimulates autophagy [5]. In the presence of normal concentrations of amino acids, activation of autophagy by glucagon is maximal, whereas higher concentrations of amino acids abolish this effect [55]. Schworer et al. suggested that the stimulation of proteolysis by glucagon was a manifestation of starvation-induced autophagy. Indeed, glucagon stimulation elicits amino acids utilization for gluconeogenesis, leading to a decrease in amino acids pool. This decrease may trigger autophagy, as it mimics the effect of amino acids deprivation [55]. Although glucagon is known to activate AMPK which positively regulates autophagy [56], the mechanism of autophagy activation by glucagon remains unclear.

Insulin also plays a critical role in starvation-induced autophagy [12]. Insulin is known to activate mTOR via a class I phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway, which inhibits autophagy [57]. During fasting, plasma insulin level drops by 50% between 12 hrs and 72 hrs of fasting in humans [54, 58]. Mice also show a significant decrease in plasma insulin level after 24 hrs of starvation, while their plasma glucagon level remains relatively stable [33]. This suggests that the role of insulin level in the control of starvation-induced autophagy might be more important than that of glucagon.

Table 1: Starvation-induced autophagy in different experimental models.

Author, year	Model	Techniques used	Time point
Takeshige et al., 1992 [28]	*S. cerevisiae*	Electron microscopy: increase in the number of autophagosomes and delivery to the vacuole	1 hrs–3 hrs
Scott et al., 2004 [29]	Drosophila fat body	Electron microscopy: increase in the number of autophagosomes	3 hrs
Mizushima et al., 2004 [30]	GFP-LC3 transgenic mice	Fluorescence microscopy: increase in the number of LC3-GFP dots (24 h) and then return to basal level (48 h)	24 hrs–48 hrs
Komatsu et al., 2005 [13]	Atg7F/+:Mx1-Cre mice	Electron microscopy: increase in the number of autophagosomes Western blot: increase in LC3II/I ratio	24 hrs
Martinet et al., 2006 [31]	GFP-LC3 transgenic mice	Fluorescence microscopy: increase in the number of LC3-GFP dots	24 hrs–48 hrs
Hansen et al., 2008 [32]	LGG-1-GFP transgenic *C. Elegans* Eat-2 mutants	Fluorescence microscopy: increase in the number of LGG-1-GFP foci	60 hrs
Ezaki et al., 2011 [33]	C57BL/6J mice	Electron microscopy: increase in the number of autophagosomes Western blot: increase in LC3II/I ratio	24 hrs 3 hrs–18 hrs
Uddin et al., 2011 [34]	C57BL/6 mice	Western blot: increase in LC3II/I ratio	12 hrs–24 hrs–36 hrs–48 hrs
Skop et al., 2012 [35]	Wistar rats	Western blot: increase in LC3II/I ratio	24 hrs

GFP: green fluorescent protein; LC3: microtubule-associated protein 1 light chain 3 alpha; LGG-1: LC3, GABARAP, and GATE-16 family; Mx-1: myxovirus (influenza virus) resistance 1.

3. Physiological Significance of Starvation-Induced Autophagy

Studies in yeast showed that autophagy is efficiently induced following 1 hour nitrogen starvation and reaches a maximal level at 3 hrs. Yeast autophagy is also induced by starvation of other nutrients such as carbon sources, sulfate, or auxotrophic amino acids [28]. Autophagy-deficient yeast cells have a loss of viability and most of them (more than 80%) die within 5 days of nitrogen starvation, indicating that starvation-induced autophagy is essential for cell viability under this stressful condition [59]. Moreover, autophagy-deficient yeasts were unable to maintain physiological levels of amino acids and to synthetize important proteins for surviving nitrogen starvation [60]. Similarly, in the eukaryote *C. Elegans,* autophagy was induced in response to nutrients shortage [32]. In the *Drosophila* larval fat body, a nutrient storage organ analogous to the vertebrate liver, starvation induced a robust autophagic response in the first 3 hrs [29].

Starvation-induced autophagy is critical during the early neonatal period in response to the sudden arrest of the transplacental supply and subsequent nutrient deprivation [61]. After birth, autophagy is immediately upregulated in various tissues, including the liver, heart, lung, diaphragm, pancreas, and the gastrocnemius muscle, and is maintained at high levels for 3–12 hrs before returning to basal levels within 1-2 days. Mice deficient for Atg5, an essential autophagy gene, die within the first day of delivery, although they appear normal at birth. Forced milk feeding of Atg5 knockout mice delayed neonates' death. This shows that autophagy is critical for survival during neonatal starvation in mammals.

Identification of key proteins regulating the autophagy machinery and the development of molecular tools to monitor autophagy *in vivo* led to a better understanding of the response of organisms to starvation. In rats as well as in mice, 24 hrs starvation increases both liver LC3II/I ratio and the number of autophagosomes assessed using electron microscopy [13, 33–35] (Table 1). Studies using GFP-LC3 transgenic mice in which the number of LC3 puncta reflects the number of autophagosomes gave similar results [30, 31] (Table 1). This model also provided evidence for differential induction patterns in several other tissues. Indeed, starvation induces autophagosome formation in the liver, skeletal muscle, heart, pancreatic acinar cells, seminal gland cells, and kidney podocytes. In most tissues, the autophagic activity reaches maximal levels within 24 hrs and then progressively decreases, whereas it further increases after 48 hrs in the heart and the soleus muscle [30]. In contrast, induction of autophagy in the brain was not observed even after 48 hrs of

Table 2: Studies assessing liver blood tests in patients with anorexia nervosa.

Author, year	Body mass index (kg/m^2)	Percentage of patients with increased serum transaminases levels
Cravario et al., 1974 [36]	14.4	4% ($N = 27$)
Kanis et al., 1974 [37]	15	0% ($N = 24$)
Milner et al., 1985 [38]	—	45% ($N = 42$)
Mira et al., 1987 [39]	15.9	9% ($N = 22$)
Palla and Litt, 1988 [40]	—	33% ($N = 24$)
Umeki, 1988 [41]	—	59% ($N = 27$)
Hall et al., 1989 [42]	—	32% ($N = 31$)
Waldholtz and Andersen, 1990 [43]	—	0% ($N = 13$)
Sherman et al., 1994 [44]	—	26% ($N = 19$)
Mickley et al., 1996 [45]	—	7% ($N = 282$)
Ozawa et al., 1998 [46]	13.2	29% ($N = 101$)
Miller et al., 2005 [47]	16.8	12% ($N = 214$)
Montagnese et al., 2007 [48]	15.6	14% ($N = 97$)
Rautou et al., 2008 [49]	11.3	66.6% ($N = 12$)
Fong et al., 2008 [50]	18	26% ($N = 53$)
Tsukamoto et al., 2008 [51]	15.2	52% ($N = 25$)
Gaudiani et al., 2012 [52]	13.1	76% ($N = 25$)
Hanachi et al., 2013 [53]	12	56% ($N = 126$)
Cumulated (mean)	14.6	24% (278/1158)

starvation. This might be explained by the fact that the brain is a metabolically privileged site that is supplied with glucose and ketone bodies from the liver and other tissues [62], even though brain cells are autophagy competent [63–65].

Moreover, the use of liver specific knockout models for autophagy genes unraveled a pivotal role of basal and stress-induced autophagy in the maintenance of liver cell homeostasis. Whereas starvation transiently elevates amino acid levels in the liver and the blood for 24 hrs in wild type animals, mice with liver Atg7 deficiency exhibit an impaired response to fasting, including an absence of decrease in liver protein levels and of increase in blood amino acid levels [13]. Fasting blood glucose level is also decreased in these Atg7-deficient mice [33]; this may be due to the lack of amino acids supply by autophagy for gluconeogenesis, further supporting a role of autophagy in the maintenance of blood glucose level upon starvation. In humans, although liver autophagy kinetics following starvation has not been assessed, one could speculate that autophagy is rapidly increased during fasting as in mice or rats, since plasma levels of insulin start to decrease, and those of glucagon start to increase in the first hours of fasting [54, 58].

4. Liver Autophagy and Anorexia Nervosa

Anorexia nervosa (AN) is characterized by a distorted perspective of body image with an intense fear of gaining weight manifesting through self-induced starvation. AN has the highest rate of mortality among eating disorders [66]. Two main subtypes of AN are recognized: restricting type and binge-eating/purging type. Average prevalence of AN is of 0.3% in young women [67] and might be up to 4% with a broader definition, close to DSM-5 criteria [68]. AN can be associated with several medical complications, including cardiovascular complications (bradycardia and hypotension), gastrointestinal problems (lack of food intake induces reflex hypofunctioning of the colon and subsequent constipation), endocrine and electrolytes abnormalities, amenorrhea in women [69, 70], and liver blood tests abnormalities [36–53] (Table 2). Mild increase in serum transaminases levels (<200 IU/L) is observed in up to 75% of AN patients [52]. Marked increases (>200 IU/L) are less common (Table 2) [46, 71–78]. Interestingly, several independent groups observed that serum transaminases levels inversely correlate with body mass index (BMI) [46, 51, 53], suggesting a role of nutritional status in the liver changes of these patients. However, understanding of the mechanisms of these abnormalities is hampered by the absence of available description of liver histological or ultrastructural changes.

Although much less common, severe liver insufficiency associated with AN has been better investigated [49]. A series of 12 patients with acute liver insufficiency (prothrombin index <50% and/or an international normalized ratio >1.7) and AN as the only cause for acute liver injury has been analyzed. All patients had severe AN attested by a BMI systematically equal to or less than 13 kg/m^2 and by severe hypoglycemia and coma at admission in half of them. Serum transaminases levels were highly increased in all patients (average 2000 IU/L) suggesting severe liver injury. Liver biopsies were available in all patients. Surprisingly, liver histological analysis as well as TUNEL staining disclosed no or rare features of necrosis or apoptosis. On electron microscopy, hepatocytes showed numerous autophagosomes, as well as a low density of organelles and of glycogen. Moreover, some hepatocytes presented morphological characteristics of autophagic cell death (also called type II cell death). This aspect was not observed in patients with other causes of acute liver insufficiency. These results support the view that hepatocytes autophagic death was the leading pathway of acute liver injury in patients with severe AN. This may explain the increase in aminotransferases levels in the absence of hepatocytes necrosis on histology, since autophagic cell death is associated with cytoplasmic membrane permeability, allowing the release of transaminases in the blood [49]. Patients management with controlled enteral supplementation, plasma glucose, and electrolytes correction led to rapid improvement in liver function. None of them developed hepatic encephalopathy, and all patients with initial cardiac dysfunction recovered within one month. This beneficial effect of refeeding further supports the role of severe starvation and subsequent autophagic cell death in acute liver injury in these patients.

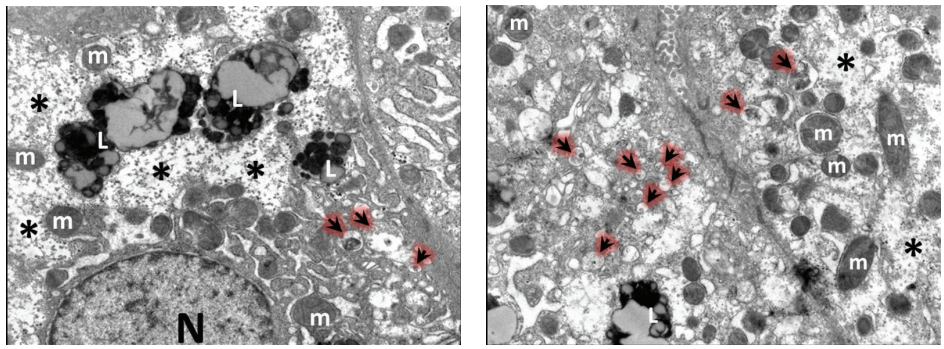
Altogether, we can speculate that starvation-induced autophagy in AN plays a dual role. During the first phase of weight loss, liver blood tests abnormalities are moderate suggesting that autophagy can cope with nutrient deprivation. During that period, autophagy is protective and prevents cell death. When starvation continues and BMI reaches a critical level equal or less than 13 kg/m^2, excessive activation of autophagy leads to hepatocyte cell death and liver insufficiency (Figure 1).

5. Autophagy in Acute Liver Injury

Recent studies highlighted the involvement of autophagy in drug-induced hepatotoxicity. Overdose of acetaminophen (APAP), a widely used antipyretic and analgesic drug, is the first cause of acute liver failure in humans [79]. The mechanisms leading to APAP-induced liver injury are well documented. In the liver, therapeutic doses of APAP are mainly excreted into the bile or the blood after glucuronic acid and sulfate conjugation. A small amount of the drug is metabolized to N-acetyl-p-benzoquinone imine (NAPQI) by cytochrome P450 enzymes, mainly via CYP2E1 isoform. NAPQI, which is highly electrophilic, reacts with glutathione (GSH) to form a GSH adduct. In case of APAP overdose, GSH stores are exhausted and NAPQI binds to cellular, including mitochondrial, proteins leading to mitochondrial damages and necrotic cell death [80]. As a defense mechanism against necrosis, APAP induces autophagy to remove damaged mitochondria [81]. Interestingly, mitochondria are frequently seen within APAP-induced autophagosomes, and expression level of mitochondrial proteins is decreased, supporting the role of mitophagy in the removal of damaged mitochondria. Moreover, autophagy inhibition by chloroquine or 3-methyladenine exacerbates APAP-induced necrosis, whereas induction of autophagy with rapamycin completely blocks it, further supporting a protective role of autophagy in APAP-induced liver injury [81] (Figure 2). Consistent with these data, studies performed by Igusa et al. using inducible liver Atg7-deficient mice indicated that loss of autophagy promoted APAP-induced reactive oxygen species, mitochondrial damage, and subsequent liver injury [82]. However, mice with a constitutive hepatocyte specific deletion in Atg5 displayed resistance to APAP overdose [83]. In these constitutive Atg5 deficient mice, compensatory increase in hepatocytes proliferation and in basal GSH levels as well as faster recovery of GSH content after APAP insult mediated by persistent activation of Nrf2 could account for this apparent discrepancy. Indeed, prolonged loss of autophagy increases levels of p62 leading to stabilization of Nrf2 and in turn to transcriptional activation of Nrf2 target genes, including antioxidant proteins and detoxifying enzymes [84]. These discrepancies between inducible and constitutive genetic deletions indicate that caution should be taken when working with genetic models of autophagy deficiency, as discussed elsewhere [85, 86]. There is to date no data on autophagy level in the liver of patients with APAP overdose. Electron microscopy analysis of liver samples from patients could be useful to confirm what has been observed in mice [81]. Chronic exposure to alcohol decreases autophagic flux by inhibiting the fusion of autophagosomes with lysosomes [87]. This may explain why chronic consumption to alcohol favors APAP hepatotoxicity [88, 89]. Besides induction of autophagy, APAP also induces the formation of mitochondrial spheroids *in vivo* [90], which are ring-like spherical structures with lumen surrounded by mitochondrial membranes that can contain cytoplasmic material. Formation of mitochondrial spheroids in response to oxidative stress is inversely correlated with Parkin expression and requires mitofusins [90]. However, the exact mechanisms by which APAP induces mitochondrial spheroids remain to be elucidated. Ni et al. suggested that posttranslational modifications of Parkin due to increased nitric oxide (NO) and reactive nitrogen species by APAP may promote mitofusin-mediated formation of mitochondrial spheroids [91]. Although the physiological significance of mitochondrial spheroids formation in response to APAP is not clear, this mechanism may represent an alternative defense route against APAP-induced liver injury. Further work is needed to address this issue.

Efavirenz, a nonnucleoside reverse transcriptase inhibitor widely used to treat HIV infections can be hepatotoxic in some patients [92]. The molecular pathogenesis of this effect involves mitochondrial dysfunction and subsequent decrease in ATP production and mitochondrial membrane potential and increase in reactive oxygen species generation [93]. At clinically relevant concentrations, Efavirenz induces mitochondrial damage and triggers mitophagy as a rescue mechanism. The beneficial effect of mitophagy is supported by the fact that pharmacological inhibition of autophagy enhances Efavirenz-induced cell death [94]. At higher concentrations, corresponding to those observed in slow metabolizing patients [95], Efavirenz blocks autophagic flux, leading to an increase in mitochondrial damage and eventually to cell death [94] (Figure 3). This complex concentration-dependent dual effect of Efavirenz on hepatocytes autophagy may be involved in other hepatotoxic drugs mechanisms that interfere with mitochondrial function.

The role of autophagy has been investigated in two other models of acute liver injury, namely, the concanavalin A (Con-A) and the lipopolysaccharide/D-galactosamine models. Con-A induces hepatitis by T cell-dependent and T cell-independent mechanisms. The former mechanism induces hepatocyte apoptosis whereas the latter leads to hepatocyte autophagic cell death [96]. Indeed, intravenous injection of Con-A in SCID/NOD mice, that is, mice with a defect in lymphocytes function, induced an acute hepatitis associated with an increased autophagy as demonstrated by the increased LC3I conversion to LC3II [96]. Con-A also induces cell death in hepatoma cell line by a mechanism involving mitochondrial membrane permeability, BNIP3 induction, and LC3-II generation. Concanavalin A-induced cell death could be partially inhibited by either 3-methyladenine or knockdown of BNIP3 and LC3 by siRNA, suggesting that autophagy is involved in its effect [97]. Not only hepatocytes, but also liver endothelial cells can undergo autophagic cell death following Con-A exposure *in vitro* and in mice [98]. Altogether, these data highlight a deleterious effect of Con-A-induced autophagy on hepatic cells. By contrast, induction of



(a) (b)

Figure 1: Electron microscopy pictures of hepatocytes from patients with severe anorexia nervosa. Hepatocytes show low density of organelles in the cytoplasm, glycogen depletion (∗), and autophagosomes sequestering cytoplasmic material (arrows), N: nucleus; m: mitochondria; L: mature lysosomes.

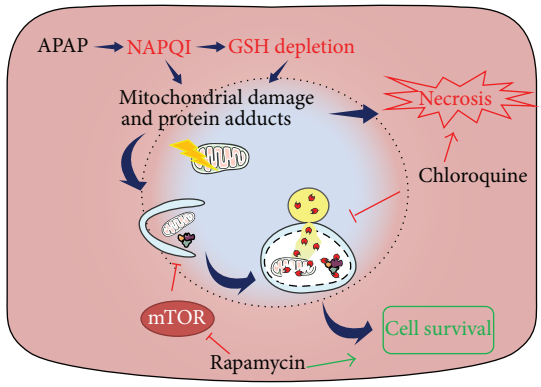


Figure 2: Protective role of autophagy in APAP-induced hepatotoxicity. APAP is metabolized in hepatocytes to generate NAPQI, which depletes GSH stores and induces mitochondrial damage by generating protein adducts, leading to hepatic necrosis. Autophagy is induced as a defense mechanism and promotes cell survival by removing damaged mitochondria and decreasing oxidative stress. Pharmacological activation of autophagy promotes cell survival while its inhibition favors cell death, APAP: acetaminophen; NAPQI: N-acetyl-p-benzoquinone imine; GSH: glutathione; mTOR: mammalian target of rapamycin.

liver autophagy in the lipopolysaccharide/D-galactosamine model seems to be hepatoprotective. Indeed, autophagy was rapidly induced in both wild type and pregnane X receptor (PXR) knockout mice after lipopolysaccharide/D-galactosamine insult. However, this increase was only transient in the latter group, and autophagy level rapidly dropped. This significant reduction of autophagy in PXR knockout mice was associated with a greater liver injury, characterized by increased alanine aminotransferase, hepatocyte apoptosis, necrosis, and hemorrhagic liver injury [99].

6. Conclusion

Increasing evidence demonstrates that autophagy plays a critical role in acute liver injury related to severe anorexia nervosa and to drug overdose. Increased liver autophagy level is a common feature of these diseases. Autophagy is mainly hepatoprotective. In anorexia nervosa, autophagic cell death occurs only when body mass index reaches a critically low level. After APAP or Efavirenz exposure, autophagy removes damaged mitochondria, and liver injury appears only when this process is either blocked by other factors or overwhelmed. Whether molecules stimulating autophagic flux are beneficial in acute liver injury remains to be determined.

Abbreviations and Acronyms

Akt:	Protein kinase B
AN:	Anorexia nervosa
APAP:	Acetaminophen
Atg:	Autophagy-related gene
BMI:	Body mass index
BNIP3:	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
Con-A:	Concanavalin A
CYP2E1:	Cytochrome P450 2E1
DSM:	Diagnostic and statistical manual of mental disorders
ERK 1/2:	Extracellular signal-regulated kinases 1/2
GFP:	Green fluorescent protein
GSH:	Glutathione
LC3:	Microtubule-associated protein 1 light chain 3 alpha
LGG-1:	LC3, GABARAP, and GATE-16 family
mTOR:	Mammalian target of rapamycin
mTORC1:	Mammalian target of rapamycin complex 1
Mx-1:	Myxovirus (influenza virus) resistance 1
NAPQI:	N-acetyl-p-benzoquinone imine
Nrf-2:	Nuclear factor erythroid 2-related factor 2
PI3K:	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PXR:	Pregnane X receptor
SCL1A5:	Solute carrier family 1 (neutral amino acid transporter), member 5

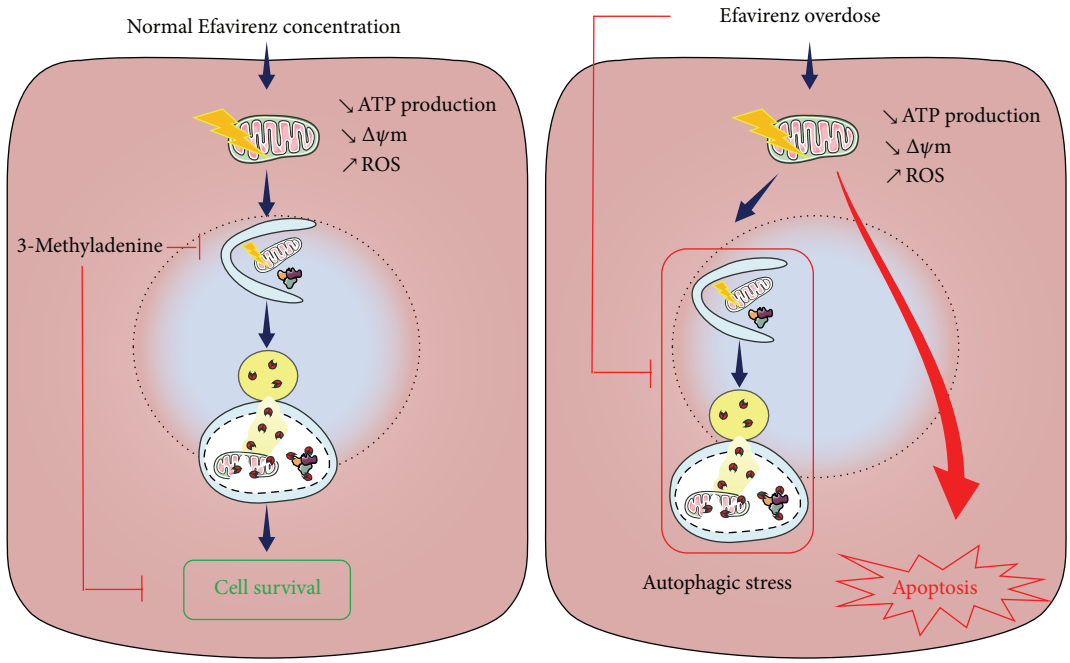


Figure 3: Role of autophagy in Efavirenz-induced hepatotoxicity. Clinically relevant concentration of Efavirenz induces mitochondrial dysfunction, which in turn induces autophagy, thereby promoting cell survival. However, higher concentration of Efavirenz is associated with inhibition of autophagic flux, which seriously compromises cell survival, ATP: adenosine triphosphate; Δψm: mitochondrial membrane potential; ROS: reactive oxygen species.

SCL3A2: Solute carrier family 3 (amino acid transporter heavy chain), member 2
SCL7A5: Solute carrier family 7 (amino acid transporter light chain, L system), member 5
TFEB: Transcription factor EB
v-ATPase: Vacuolar—type H+—ATPase.

Data Sources and Searches

The authors searched PUBMED (1960–2014) for studies on autophagy and liver physiology and pathology by using combinations of the terms: autophagy, autophagosome, liver, starvation, acute liver injury, anorexia nervosa, acetaminophen, and Efavirenz. Relevant papers were acquired as abstracts or full text. The authors also reviewed publications in personal reference lists and citation sections of the recovered articles.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Autophagy in HCV Infection: Keeping Fat and Inflammation at Bay

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Hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease. Viral persistence and pathogenesis rely mainly on the ability of HCV to deregulate specific host processes, including lipid metabolism and innate immunity. Recently, autophagy has emerged as a cellular pathway, playing a role in several aspects of HCV infection. This review summarizes current knowledge on the molecular mechanisms that link the HCV life cycle with autophagy machinery. In particular, we discuss the role of HCV/autophagy interaction in dysregulating inflammation and lipid homeostasis and its potential for translational applications in the treatment of HCV-infected patients.

1. Introduction

Hepatitis C virus (HCV) infection is a major global health problem. Almost 200 million people are infected worldwide, with 3/4 million new infections and 350,000 deaths per year [1, 2]. HCV has a marked ability to establish chronic infection in the liver. Acute infection is often asymptomatic, with spontaneous immune-mediated clearance occurring in only 15%–30% of individuals [3]. Over a time span of years, chronic infection causes a series of liver damages, that is, steatosis, inflammation, fibrosis, and cirrhosis, which significantly increase the risk of developing hepatocellular carcinoma [4].

The HCV world is facing a revolution in terms of therapeutic approaches. First generation direct-acting antivirals (DDAs) are now available in clinics and a large series of new DDAs and host-targeted agents are in advanced clinical trials, with the expectation of curing more than 90% of infections [5]. However, understanding the mechanisms by which chronic HCV infection interferes with host metabolic and immune systems will provide important information to identify new host-targeted antiviral strategies for treating patients who are resistant to DDAs, as well as monitoring the evolution of morbidities in long-term infected HCV patients upon viral clearance.

HCV does not cause a direct cytopathic effect on host cells; most of the related liver dysfunctions are considered a consequence of the virus's ability to dysregulate host processes [6]. In particular, to establish chronic infection, HCV mainly interferes with two important cellular processes, lipid metabolism [7, 8] and immune response [9], by not fully characterized molecular mechanisms. Recent evidence has indicated direct involvement of the autophagy pathway in mediating both positive and negative effects on these processes [10–12]. Here, we will summarize recent advances in understanding the role of autophagy in HCV infection and discuss their translational potential for developing novel therapeutic strategies in order to potentiate viral clearance and prevent HCV-related diseases.

2. HCV Life Cycle

HCV is a blood-borne virus of the Flaviviridae family that replicates primarily in hepatocytes. The HCV genome is composed of a positive, single-stranded RNA of approximately 9.6 Kb, containing a long, open reading frame (ORF) flanked

by nontranslated regions (NTRs) at 5′ and 3′ ends that regulate RNA translation and replication [13]. HCV RNA encodes a single polyprotein precursor of about 3,000 amino acids, which is cotranslationally and posttranslationally processed by cellular and viral proteases. Polyprotein cleavage products include the structural proteins (the nucleocapsid protein core and the envelope glycoproteins E1 and E2) and nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) involved in various processes which are essential for completion of the HCV life cycle [14].

HCV entry in hepatocytes is mediated by the interaction of the viral envelope proteins with several cell surface receptors and entry cofactors including glycosaminoglycans, LDL receptor, tetraspanin CD81, scavenger receptor class B member I, tight junction proteins claudin1 and occludin, and the cholesterol uptake receptor Niemann-Pick C1-like 1 [15]. HCV particles are then internalized by clathrin-mediated endocytosis [16] and viral and endosomal membrane fuse, in a pH-dependent manner, to release viral nucleocapsid into the cytoplasm where the uncoating occurs [17].

HCV RNA is delivered to endoplasmic reticulum, where viral translation is mediated by an internal ribosomal entry site located in the 5′ NTR. Among nonstructural proteins, NS4B and NS5A are able to induce ER membrane alteration, called membranous web, that provide a scaffold for the assembly of the HCV replication complex and protection from host immune defenses [18, 19]. HCV replication is catalyzed by the viral RNA-dependent-RNA polymerase NS5B that allows for the synthesis of new positive strand RNAs, in cooperation with the other nonstructural proteins, through the formation of negative strand replicative intermediates [20]. The formation of new virus assembly is then triggered by the relocalization of core protein and NS5A from ER membranes to cytoplasmic lipid droplets where viral particles are assembled. Maturation and release of HCV particles are closely linked to the very low density lipoprotein (VLDL) synthesis and secretion pathways [21]. Indeed nascent virions associate with pre-VLDL particles to form lipoviroparticles (LVPs) containing apolipoproteins (ApoB, apoE, apoC, and apoA-I), [22, 23]. LVPs then pass through the Golgi and are released from the cells by the secretory pathway [13].

3. Autophagy and HCV Infection

The term autophagy refers to a series of processes that allow for the degradation of intracellular components by delivering them into lysosomes [24]. In most cases, double-membrane vesicles, termed autophagosomes, engulf the target material and fuse with lysosomes (macroautophagy) [25]. Alternatively, substrates are directly entrapped in endosomal/lysosomal membrane invaginations (microautophagy) [26, 27] or internalized upon binding to lysosomal receptors (chaperone-mediated autophagy) [28]. Each step of the autophagy process is regulated by specific genes (ATG genes), originally identified in yeast and mostly conserved in higher eukaryotes [29]. The Ulk1 kinase is the first protein in the autophagy cascade into which both positive and negative pathways converge, such as those mediated by the AMPK and mTOR kinases, respectively [30, 31]. Ulk1 activates the Beclin 1/Vps34 complex, which generates PI3P-rich membrane domains allowing for the nucleation of autophagosome precursor structures, known as phagophores [32–34]. Subsequently, two ubiquitin-like conjugation systems mediate the recruitment of ATG12-ATG5 and LC3 proteins to the phagophore allowing for its expansion and closure to form the mature autophagosome [24]. Finally, autophagosomes fuse to endocytic vesicles to form multivesicular bodies and eventually to lysosomes, where their content is degraded by a variety of hydrolases and monomers released for recycling [35].

Although autophagy was originally described as a nonspecific process induced in response to nutrient deprivation, it is now evident that many selective forms have evolved to recognize and degrade damaged, supernumerary, or unwanted substrates. Consistently, signaling pathways triggering selective autophagy as well as substrate receptors specific for unique targets have been identified [36]. For example, removal of damaged mitochondria is activated by the recruitment of Pink1 and Parkin proteins on the outer membrane [37, 38]. Moreover, ubiquitinated protein aggregates are recognized by adaptor proteins SLR (sequestosome 1/p62-like receptors) that share the ability to bridge ubiquitin chains to the autophagosome protein LC3 [39, 40].

The emerging complexity in the field of selective autophagy provides new hints for understanding how autophagy alterations could be associated to the development of a wide range of human diseases [41–43]. In this regard, an important example is represented by the multifaceted role played by autophagy in infectious diseases. Indeed, autophagy plays a crucial role both in the innate and adaptive immunity [44]. On one hand, it acts as a defense mechanism by directly recognizing and degrading pathogens [11]. On the other hand, autophagy participates in the activation of intracellular and systemic immune responses, primarily by making pathogen antigens accessible to immune receptors [11]. Conversely, pathogens have developed many strategies to usurp autophagy to favor persistent infection [45, 46].

HCV is highly successful in establishing chronic infection. Since 2008, several studies have been conducted to clarify the contribution of autophagy alterations in establishing chronic HCV infection and in the onset of associated diseases [47–49]. In particular, two aspects of the autophagy process have been extensively investigated in the context of HCV infection: (1) whether autophagy is induced or inhibited by HCV, (2) whether basal or induced autophagy plays a role in HCV infection.

The emerging scenario, not devoid of conflicting results, indicates the presence of a delicate balance between proviral and prohost functions of autophagy depending on the activation of selective types of this process.

4. Is Autophagy Induced or Inhibited in HCV Infected Cells?

The presence of an increased number of autophagosomes in HCV-infected cells has been reported by several groups,

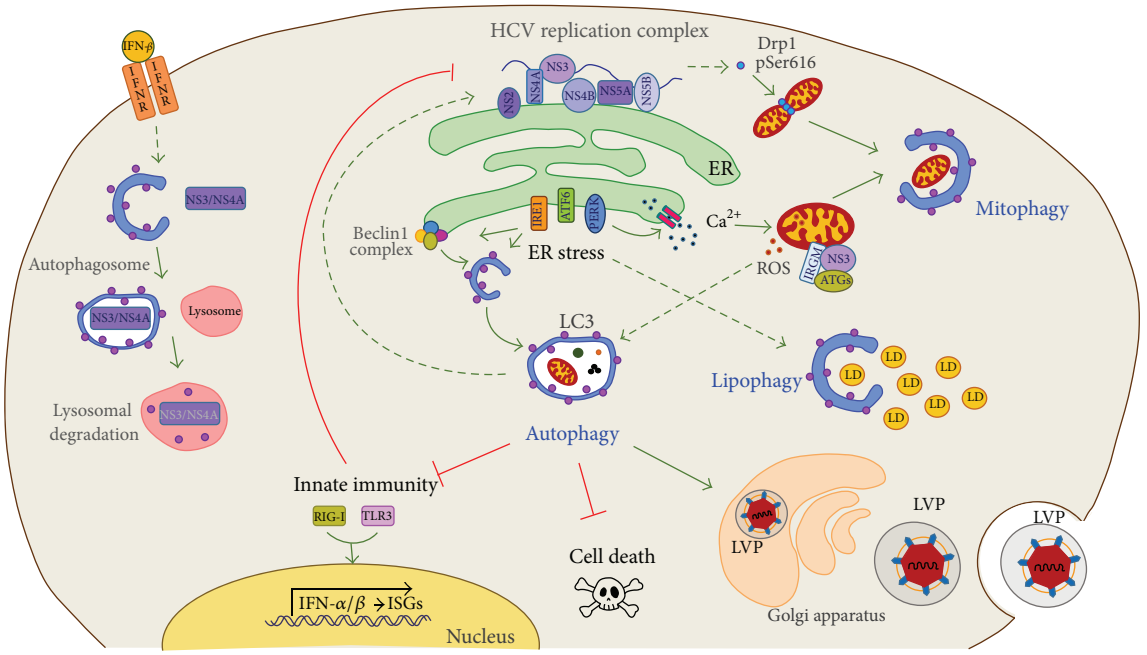

FIGURE 1: A schematic drawing showing the interplay between HCV and autophagy. HCV nonstructural (NS) proteins assemble with the viral genome on the endoplasmic reticulum (ER) membranes to form the replication complex. Viral replication alters ER homeostasis leading to ER stress, which, in turn, activates the unfolded protein response (UPR) through ATF6, IRE1, and PERK proteins. UPR plays a major role in the induction of autophagy, either directly or indirectly by affecting cellular redox balance. In fact, ER stress causes calcium release from ER that results in impaired mitochondrial activity coupled to excessive production of reactive oxygen species (ROS). Damaged mitochondria are thus targeted for degradation via mitophagy. Mitophagy is also stimulated by HCV by activating the mitochondrial fission protein Drp1. Notably, the mitochondrial protein IRGM interacts with both viral and autophagy proteins (ATGs) and is required for autophagy induction. HCV exploits the autophagic process to accomplish different steps of its life cycle such as translation, replication, assembly, and release of lipoviroparticles (LVP). These effects are, at least in part, indirect since autophagy represses the intracellular innate immune pathways, thus inhibiting IFN production. On the other hand, autophagy could also act as defense mechanism against HCV, since when induced by IFN-β causes the degradation of the viral protein NS3/NS4A. Importantly, autophagy is crucial for preventing pathogenesis induced by HCV. Indeed, a type of autophagy selective for lipids (lipophagy) protects cells from an excessive lipid accumulation triggered by HCV (LD: lipid droplets). Moreover, infected cells are more prone to death when autophagy is inhibited. Mitophagy is selectively involved in the control of HCV infection, innate immunity, and cell death (not shown in the figure, see text for details).

based on observations in either cell culture infection systems or liver samples from patients with chronic HCV infection [50–55]. However, whether HCV induces a functional or incomplete autophagy process remains controversial. Initial works reported an accumulation of autophagosomes in the absence of increased turnover of long-lived proteins and degradation of the autophagic cargo p62 [51]. Conversely, using similar *in vitro* infection or replication systems, proper fusion between autophagosomes and lysosomes has been reported, as well as the occurrence of LC3 protein degradation within the lysosome, indicating that a functional autophagy flux occurs in the presence of HCV [54]. A possible explanation for these contradictory results came from the observations that degradation of specific autophagic targets is increased in HCV-infected cells, such as damaged mitochondria and lipid deposits, suggesting that HCV induces selective rather than bulk autophagy [56, 57] (Figure 1). Moreover, it has recently been proposed that the ability of HCV to inhibit the autophagic flux may depend on the viral genotype. In fact, HCV replicon cells carrying a genotype 1b strain Con1 exhibit an incomplete acidification of autolysosomes, while those with the genotype 2a strain JFH1 have no alterations [58].

5. How Does HCV Modulate Autophagy?

Multiple interactions between HCV and autophagy proteins have been identified. By yeast two-hybrid assays, NS5B, NS5A, and p7 were found to interact with ATG5, ATG12, and FIP200, respectively [59, 60]. Moreover, immunoprecipitation assays revealed the ability of NS4B to copurify with the Beclin 1-associated lipid kinase Vps34, as well as p7 with Beclin 1 [61, 62]. Interestingly, in most cases these interactions result in autophagy induction [60, 63].

Many evidences point to the fact that autophagy is indirectly induced by cellular stress activated by HCV infection. In particular, the modulation of autophagy by HCV has been primarily linked to the activation of endoplasmic reticulum (ER) stress (Figure 1). HCV infection induces chronic ER stress in hepatocytes, which has been functionally linked to presence of clinical dysfunctions observed in patients, including steatosis, cell death, and immune escape [64]. ER

stress is known to activate a signaling network, called the unfolded protein response (UPR), to cope with the damaging agents. UPR consists of three distinct pathways, identified by their main regulatory factors, namely, the activating transcription factor 6 (ATF6), the inositol-requiring enzyme 1 (IRE1), and the double-stranded RNA-activated protein kinase-like ER kinase (PERK) [65] (Figure 1). HCV-induced ER stress activates all three UPR pathways and the inhibition of each of them was shown to decrease autophagy levels in infected cells [51, 54, 66], although conflicting results have been reported on the contribution of IRE1 to UPR-induced autophagy [67] How HCV, ER stress, and autophagy are functionally linked has begun to be clarified. The core protein has been shown to activate both ATF6 and PERK pathways that, in turn, increase the expression of ATG12 and LC3 genes [63]. HCV is also able to transcriptionally upregulate Beclin 1 expression [68]; however, whether this is dependent on UPR or not has not been investigated. Moreover, the role of Beclin 1 complex remains unclear with reports showing that HCV-induced autophagy is either dependent or independent of its activity [52, 53, 69, 70].

The activation of signaling pathways downstream to ER stress may play a role in the induction of autophagy by HCV. For example, it has been proposed that the induction of autophagy by HCV depends on the inhibition of the AKT/mTOR pathway by UPR [71]. However, discordant results have been reported on the status of mTOR activity in HCV-infected cells [68, 71–73].

An important consequence of ER stress induced by HCV is the alteration of ER calcium homeostasis (Figure 1). Release of calcium from ER to cytosol is known to impair mitochondrial activity, resulting in the accumulation of mitochondrial reactive oxygen species (ROS), which are potent autophagy inducers [74, 75]. In line with this model, induction of mitophagy, a selective form of autophagy for removal of damaged mitochondria, has been observed in HCV-infected cells [56] (Figure 1). Moreover, modulation of the cellular redox state by catalase expression is able to reduce autophagy levels in HCV replicon cells [76]. Interestingly, full-length HCV replicon cells show a decreased antioxidant response when compared to those expressing only the nonstructural proteins, suggesting that autophagy is induced by ROS as a compensative mechanism to the inhibition of host antioxidant pathways by structural proteins [76]. Recently, it has been reported that HCV is able to directly promote mitophagy by inducing mitochondrial fission via phosphorylation of the dynamin-related protein 1(Drp1) [77].

A central role of mitochondria in the induction of autophagy has been also confirmed by a recent study aimed at characterizing common mechanisms of autophagy modulation by different RNA viruses [60, 78]. The authors identify the immunity-associated GTPase family M (IRGM) protein as a potential hub to convey autophagy and viral proteins. IRGM is a mitochondria-associated factor playing a crucial role in the regulation of autophagy in a variety of infections [79, 80]. By two-hybrid screening, IRGM was found to directly interact with NS3/4A and several autophagy proteins, that is, ATG5, ATG10, LC3, and BIF1, and the downregulation of IRGM expression reduces autophagy levels in HCV-infected cells NS5B [60]. Interestingly, also BNIP3, an important regulator of mitochondrial selective autophagy, is able to interact with different HCV proteins, that is, NS2, NS4A, NS5A, and NS5B [60].

It is therefore likely that a combination of different pathways, not all yet characterized, may induce autophagy at different stages of HCV infection. HCV infection activates different pattern recognition receptors [81], which are able to trigger autophagy [11]; however, their contribution in the induction of autophagy during HCV infection remains poorly characterized. Another important yet unexplored aspect is whether HCV could control autophagy by modulating the activity of the cytosolic RNA-sensing protein kinase PKR, which has been reported to regulate virus- and starvation-induced autophagy [82–85]. Intriguingly, HCV is either able to activate (via RNA IRES and core protein) or to inhibit PKR (via NS5A and E2 proteins) at different steps of the viral life cycle [82], which could possibly account for a dual regulation of autophagy activity in the course of infection.

In this regard, an integrated analysis of how autophagy is modulated during different steps of infection will provide useful information to elucidate how HCV intersects the autophagy pathway.

6. Is Autophagy Required for HCV Infection?

Several reports have highlighted a strict interdependence between the execution of the HCV life cycle and autophagy activity or the expression of autophagy proteins. It is recognized that inhibition of the autophagy machinery affects the production of new infective particles [53, 69]. However, at which steps autophagy is required for the HCV life cycle remains controversial.

HCV replication was shown to occur either normally or drastically inhibited in autophagy-deficient cells [52, 53, 58, 59, 69]. Discrepancies were also reported with regard to the presence of HCV replication complexes on autophagosomal membranes. Confocal microscopy and immunoelectron microscopy studies showed that HCV NS4B, NS5A, and NS5B proteins and nascent HCV RNA colocalize with autophagy markers and/or autophagosomal structures [59, 70, 86]. In contrast, several other studies failed to detect any significant colocalization [51, 53, 67].

The highly heterogeneous nature of Huh7 cells and derivative clones and the different strategies of HCV expression (RNA transfection versus infection) may account, at least in part for the observed discrepancies. In addition, the interaction between HCV and the autophagy machinery may be regulated in a temporally regulated manner, as suggested by a recent study showing that the colocalization of ATG5 and NS5B is detected at 2 days postinfection, while it is absent at 5 days [59]. This hypothesis is substantiated by data from The Chisari Lab, underlining the involvement of the autophagy machinery in the translation and/or delivery of incoming viral RNA to the translation apparatus, while it becomes dispensable for HCV RNA progeny once replication is established [52]. The authors proposed that the autophagy

pathway may provide an initial membranous support for translation of incoming RNA, before accumulation of viral proteins and establishment of the replication complex within the ER-associated membranous web. At these early stages of infection, basal rather than stress-induced autophagy is likely to contribute to HCV infection, since autophagy increase was reported to occur at a relatively late time, when replication has been established [71]. Experimental evidences support the idea that late induction of autophagy, as well as mitophagy, is required to sustain the survival of infected cells, a crucial aspect for a virus able to establish a chronic infection [69, 77].

7. Autophagy and the Innate Immune Response in HCV-Infected Cells

HCV has evolved multiple mechanisms to evade innate immunity. In particular, HCV inhibits the interferon (IFN) response, acting both upstream and downstream to IFN production [87]. For example, the viral protease NS3/4A inhibits the retinoic acid-inducible gene I (RIG-I) and toll-like receptor 3 (TLR3) antiviral pathways by proteolytically cleaving the MAVS and TRIF factors [88–91]. Moreover, different HCV proteins are able to interfere with the JAK-STAT pathway activated by IFNs [92–94].

A turning point in elucidating the relationship between autophagy and HCV infection is represented by three recent works demonstrating the involvement of autophagy in repressing the anti-HCV innate immune response (Figure 1). Indeed, the impairment of autophagy, either by targeting ATG genes or the upstream ER stress process, leads to a significant upregulation of the innate immune response to HCV [54, 69, 95]. The suppression of innate immunity during HCV infection relies on the catabolic activity of autophagy, since it was also prevented by interfering with lysosome function [54]. This is different from what reported in cells infected with vesicular stomatitis virus, where the inhibition of IFN-β production by autophagy was dependent exclusively on the interaction between the Atg5-Atg12 complex and the RIG-I and IFN-β promoter stimulator 1 (IPS-1) proteins [96].

Although the relationship between autophagy and innate immunity in HCV infected cells has been well established, it remains unclear whether the exacerbation of innate immune response is the main cause of the inhibition of HCV infection in autophagy-defective cells. To answer this question, the impact of autophagy inhibition on HCV infection should be tested in cells defective for the major viral sensing pathways.

ER stress and autophagy are also involved in the inhibition of the antiviral response downstream to IFN production by downregulating the expression of type I IFN (IFN-α) receptors but not type II (IFN-γ) or type III (IFN-λ) [95]. In the same context, the expression of the nucleoside transporters ENT1 and CNT1, which are involved in the transport of the antiviral drug Ribavirin (RBV), is also decreased, thus suggesting a possible contribution of autophagy in the partial resistance to type I IFN/RBV-based therapy *in vivo* [95]. On the other hand, it should be underlined that the IFN signaling pathway is preactivated in nonresponsive patients [97, 98], which leads to the hypothesis that interfering with the crosstalk between autophagy and innate immunity may actually represent an advantage for HCV persistence.

Recent data highlighted a further level of complexity in the crosstalk between the interferon pathway and autophagy in the context of HCV infection. By means of transgenic mice expressing HCV NS3/NS4A protein in the liver, it was shown that IFN-β is able to trigger autophagy-mediated degradation of the viral protein [99] (Figure 1). Degradation is dependent on the mitochondria-associated antiviral signaling protein MAVS and is specific for IFN-β, since it is not observed when mice are treated with IFN-α. If confirmed in the context of HCV infection, these results represent an important example of how autophagy may play both positive and negative roles in the innate immune response to HCV infection. In relation to these results, it has been reported that HCV clearance can be induced by treating infected cells with AICAR, an AMP analog capable of activating AMPK, a protein kinase with pleiotropic functions including direct stimulation of the autophagy process [100]. However, the contribution of autophagy in HCV clearance mediated by AMPK remains to be assessed.

HCV infection is not only impaired by the general inhibition of the autophagy process but also by interfering with the induction of mitophagy, a selective type of autophagy activated by the PINK1/Parkin pathway [56, 77].

In light of the autophagy-innate immunity relation, it would be important to test whether mitophagy is also able to hamper the innate immune response. Interestingly, aberrant stimulation of mitochondrial fission by HCV, which leads to mitophagy, results in the repression of interferon response [77]. Alternatively, the inhibition of HCV infection could be a consequence of a dysregulated production of ROS by unremoved damaged mitochondria, which may directly affect the HCV replication machinery or indirectly, the host metabolic pathways required for viral life cycle.

8. Autophagy and the Alteration of Lipid Metabolism during HCV Infection

The HCV life cycle is tightly coupled to the lipid metabolism of host cells [7, 19]. HCV virions are bound to lipoproteins, called "lipoviroparticles," wherein the circulating virus hides and mediates its entry into the hepatocytes through lipoprotein receptors [13]. Moreover, ER-associated lipid droplets are the viral assembly sites where the nucleocapsid protein core and the replication complex interact to initiate capsid assembly [101]. NS5A also interacts with apolipoproteins A and E that are required for both HCV replication and the release of viral particles via VLDL secretion [102–104].

HCV not only associates with intracellular lipid compartments but also induces profound alteration in lipid metabolism leading to steatosis [105]. The core and NS5A expression results in the accumulation of lipid droplets (LD), and polymorphisms in the LD-binding domain of Core correlate with the different steatogenic properties of HCV strains, which are more accentuated in the HCV genotype 3 [106–108]. Core and NS5A also inhibit the microsomal triglyceride

transfer protein, a key protein in VLDL assembly, and increase lipogenesis through the activation of sterol regulatory element-binding protein 1, peroxisome proliferator-activated receptor, and retinoid X receptor [8, 109, 110].

Lipophagy has been recently described as a selective type of autophagy dedicated to the degradation of intracellular lipid stores [111, 112]. The contribution of defective autophagy in the onset of lipid dysmetabolic diseases, such as steatosis and atherosclerosis, has recently emerged [10]. Notably, when we analyzed the levels of autophagy in the liver of HCV-infected patients with respect to clinical symptoms, we found an inverse correlation with the presence of microsteatosis [57]. The role of autophagy in the catabolism of lipid stores during HCV infection was confirmed by *in vitro* studies using both HCV-replicon and HCV-infected cells. In these systems, we found that a large fraction of autophagosomes selectively colocalize with lipid deposits, whose levels are increased by viral replication [57] (Figure 1). Moreover, inhibition of autophagy by pharmacological and genetic approaches leads to a substantial increase of intracellular cholesterol deposits, while preventing cholesterol synthesis by statins significantly decreases autophagy levels as well as viral replication [57]. Taken together, these data suggest that inhibition of lipid selective autophagy may contribute to the onset of steatosis in chronically HCV-infected patients.

Many aspects of the role of lipid-selective autophagy in HCV-infected cells require further investigation. For example, it will be interesting to test whether the interaction of core protein with lipid droplets, in particular from HCV genotype 3 with a higher steatogenic potential, is able to interfere with the access of these structures to the autophagic machinery. Moreover, being cholesterol essential for HCV virion assembly and release [8], it is conceivable that lipophagy inhibition may account for the observed defects in HCV infectivity in autophagy-deficient cells by decreasing the availability of recycled cholesterol for the synthesis of novel lipoproteins.

9. Perspectives

The dual role played by autophagy in inflammation and lipid metabolism in HCV-infected liver cells raises an important question regarding the contribution of autophagy defects in disease progression towards steatohepatitis, fibrosis, cirrhosis, and hepatocarcinoma in patients with chronic HCV infection. Recent studies not focused on HCV infection show that the onset of steatohepatitis in nonalcoholic fatty liver patients is associated to lower levels of "bulk" autophagy and, in particular, to the accumulation of the autophagy cargo protein p62 [113–115]. In agreement with this observation, hepatocyte-specific atg7 knockout mice show liver damage associated with increases in hepatic TGs and cholesterol [111, 116]. Notably, in these mice, accumulation of p62 accelerates liver damage, which leads to the development of hepatic cancer via persistent activation of the Nrf2 pathway [117–119].

Autophagy has been proposed as a potential target for tackling HCV infection. However, the emerging scenario indicates that many types of selective autophagies are playing a role in the liver homeostasis of both healthy and HCV-infected individuals. Full elucidation of the molecular basis of this selectivity is therefore needed to provide a framework for the rational design of feasible anti-HCV drugs or intervening applications.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Uncoupling Protein 2 Regulates Palmitic Acid-Induced Hepatoma Cell Autophagy

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Mitochondrial uncoupling protein 2 (UCP2) is suggested to have a role in the development of nonalcoholic steatohepatitis (NASH). However, the mechanism remains unclear. Autophagy is an important mediator of many pathological responses. This study aims to investigate the relationship between UCP2 and hepatoma cells autophagy in palmitic acid- (PA-) induced lipotoxicity. H4IIE cells were treated with palmitic acid (PA), and cell autophagy and apoptosis were examined. UCP2 expression, in association with LC3-II and caspase-3, which are indicators of cell autophagy and apoptosis, respectively,was measured. Results demonstrated that UCP2 was associated with autophagy during PA-induced hepatic carcinoma cells injury. Tests on reactive oxygen species (ROS) showed that UCP2 overexpression strongly decreases PA-induced ROS production and apoptosis. Conversely, UCP2 inhibition by genipin or UCP2 mRNA silencing enhances PA-induced ROS production and apoptosis. Autophagy partially participates in this progress. Moreover, UCP2 was associated with ATP synthesis during PA-induced autophagy. In conclusion, increasing UCP2 expression in hepatoma cells may contribute to cell autophagy and antiapoptotic as result of fatty acid injury. Our results may bring new insights for potential NASH therapies.

1. Introduction

The uncoupling proteins (UCPs) belong to the mitochondrial anion transporter superfamily in the inner mitochondrial membrane [1, 2], and UCP2 is believed to play a role in adaptive thermogenesis and lipid metabolism [3]. Moreover, the promoter region of UCP2 contains Sp1, double E-box, and sterol response elements, which could explain why UCP2 is upregulated in response to high levels of fatty acids, obesity, fasting, leptin, and other conditions [4, 5]. Although normal healthy hepatocytes do not express UCP2, there is an increase in expression of this protein following oxidative stress and steatosis [6]. It has been suggested that UCP2 has a role in the development of nonalcoholic fatty liver disease (NAFLD). However, the mechanism remains unclear. UCP2 has been reported to play a role in antioxidant defense [7], as inhibition with the specific inhibitor genipin or inhibition by UCP2 siRNA increases mitochondrial ROS levels, while overexpression of UCP2 diminishes ROS production [8]. These findings suggest that UCP2 might influence the development of NAFLD by regulating ROS production.

Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic fatty liver disease in Western countries. NAFLD refers to a wide spectrum of liver damage from steatosis to nonalcoholic steatohepatitis (NASH) and, lastly, cirrhosis. NASH is known to be a significant cause of cryptogenic cirrhosis, and therefore it has attracted more attention in the past few years. Studies have shown that FFAs-induced lipotoxicity has been documented to play an essential role in the pathogenesis of NASH [9], and our latest study has shown that palmitic acid- (PA-) induced apoptosis plays an important role in the pathogenesis and development of NASH [10]. At present there is accumulating evidence suggesting that autophagy is involved in the physiological and pathological responses of cells to lipid stimulation [4, 5]. With the evidence that autophagy could regulate hepatic lipid stores [11], and with loss of autophagic function, white adipocyte differentiation was blocked *in vitro* and white adipose tissue mass was markedly decreased *in vivo*. Decreased lipid storage occurred with these changes, and this effect along with an increase in the mass of normal brown adipose

tissue led to a significantly increased rate of fatty acid β-oxidation [12]. A previous study has shown that PA also triggers autophagy responses following hepatic lipotoxicity [13], which implies that autophagy may be involved in the etiology of NASH.

Autophagy is a lysosome-mediated degradation process for nonessential or damaged cellular constituents. It has a multistep process, including the formation of double-membrane vesicles known as autophagosomes [14]. Moreover, autophagy has been shown to have a critical role in the regulation of hepatocellular lipid accumulation and liver injury during oxidative stress [15]. *In vivo* studies have indicated that starvation-stimulated macroautophagy provides the liver with a lipid challenge in the form of increased serum FFAs and that the autophagic pathway selectively targets lipids for breakdown in response to this physiological stimulus [11]. Moreover, UCP2 has been shown to trigger ROS-dependent autophagic cell death in pancreatic adenocarcinoma cells [16]. These studies prove that UCP2 probably mediates FFA-induced hepatocyte autophagy.

Although the mechanism of how did UCP2 mediate PA-induced autophagy in hepatocytes remains unclear, insights into these mechanisms may be useful in designing effective management strategies in dealing with NASH. Therefore, in the present study, we tested the autophagic responses and the underlying mechanisms following PA-induced injury. We observed the induction of autophagy by PA and UCP2 overexpression decreased ROS production. Moreover, UCP2 could enhance hepatoma autophagy, such that UCP2-mediated autophagy induced by PA was first found to serve as an antiapoptotic mechanism to oppose the lipotoxic effects. These results indicate that modulation of UCP2 can serve as a potential experimental therapy for NASH caused by the lipotoxic effects of PA.

2. Materials and Methods

2.1. Cell Culture and PA Treatment. H4IIE cells, a rat hepatoma cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% (v/v) fetal bovine serum (Hyclone, Rockford, IL, USA), penicillin (100 UI/mL), and streptomycin (100 UI/mL). All cell cultures were maintained in a 37°C incubator with 5% (v/v) CO_2. To induce cellular damage, 250 μM PA (Sigma, St. Louis, MO, USA) was added to serum-free DMEM medium after the cells grew to ~70–80% confluence. PA-BSA (bovine serum albumin) conjugate was prepared as described previously [17]. In brief, a 100 mM solution of PA in 0.1 M NaOH was incubated at 80°C, and fatty acid soaps were then complexed with 10% (w/v) BSA in phosphate buffered saline (PBS) at a 3.5 : 1 molar ratio of PA to fatty acid free BSA (Wako, Japan). The BSA was used as a vehicle control. CQ (Sigma, USA) was used to block lysosomal function, and the later degradation stage of autophagy was used to measure autophagic flux in cells.

2.2. Electron Microscopy. H4IIE cells were seeded on 100 mm plates at a density of 10^6 cells/plate. After the respective treatments for 6 h, cells were fixed with 3% (v/v) glutaraldehyde and washed three times with 0.1 M phosphate buffer (pH 7.4). Cells were postfixed with 1% (v/v) osmic acid followed by dehydration with an ascending series of alcohol before being embedded in araldite for 24 h. After dehydration, thin sections were cut and stained with uranyl acetate and lead citrate. Digital images were obtained using a JEM 1016CX electron microscope. Random images were obtained.

2.3. Cell Proliferation Assay. Cells were seeded in 96-well plates (10^5 cells/well). After 24 h, cells were treated with various compounds and further incubated for the indicated times (see legends to Figure 1(a)). At the end of the treatments, cells were stained with the cell proliferation reagent WST-1 (Roche, Germany). The dye was solubilized in serum-free DMEM medium and measured photometrically at 450 nm to determine cell growth. Three independent experiments were performed for each assay condition.Cell proliferation was measured using a WST-1 kit according to manufacturer's instruction (Roche, Germany).

2.4. Analysis of Intracellular Reactive Oxygen Species. Intracellular reactive oxygen species (ROS) generation was measured with diacetylated 2′,7′-dichlorofluorescein (DCFH-DA, Nanjing Jiancheng Bioengineering Institute, China). In brief, 10^6 cells were incubated in 60 mm plates and, 24 h later, treated with various compounds, as indicated in the legends to figures. Cells were incubated with 10 μM of DCFH-DA for 20 min at 37°C, and the DCF fluorescence was measured by flow cytometry (Becton Dickinson FACScan, USA) as previously described in detail [18]. Data were analyzed using Cellquest software (Becton Dickinson). All data are presented as the mean of three independent experiments.

2.5. Hepatic ATP Level. Cells were seeded in 96-well plates (10^5 cells/well). After 24 h, cells were treated as indicated. At the end of the treatments, protein concentrations of the lysates were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA), and ATP content was measured using a CellTiter-Glo2.0 assay kit (Promega, USA).

2.6. Real-Time PCR. Total RNA from cells was extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA). The first strand of cDNA was synthesized from 5 μg RNA (Superscript III cDNA Synthesis Kit, Invitrogen). The mRNA for UCP2 and 18S was estimated by quantitative real-time PCR using a SYBR Green PCR Kit (Roche). Quantitative RT-PCR was performed with a Prism 7300 Sequence Detecting System (Applied Biosystems). UCP2 primer sequences were as follows: forward primer 5′-AGCAGTTCTACACCAAGGGC-3′, reverse primer 5′-TGGAAGCGGACCTTTACCAC-3′and 18s primer sequences were as follows: forward primer 5′-GTAACCCGTTGAACCCCATT-3′, reverse primer 5′-CCATCCAATCGGTAGTAGCG-3′.

2.7. Immunoblot Analysis. Cells were washed in PBS, and whole-cell extracts were prepared in lysis buffer (Tris-HCl (20 mM), pH 7.4, NaCl (150 mM), and glycerol (10% (v/v)),

Nonidet P-40 (0.2%), EGTA (1 mM), EDTA (1 mM), PMSF (1 mM), NaF (10 mM), leupeptin (20 mM), aprotinin (5 mg/mL), and sodium orthovanadate (1 mM)) and centrifuged at 8,000 ×g for 15 min. Protein concentrations were measured using the BCA assay (Pierce). Protein (50 μg) was separated on a 15% (w/v) sodium dodecyl sulphate polyacrylamide gel and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were incubated overnight with primary antibodies specific to UCP2 (1 : 1000, Biolegend), p-AMPK (1 : 2000, Cell Signaling Technology), caspase-3 (1 : 1000, Cell Signaling Technology, Beverly MA, USA), and LC3 (1 : 2000, Cell Signaling Technology) at 4°C overnight. The positive reaction against these antibodies was visualized by enhanced chemiluminescence (ECL, Santa Cruz) reagent, followed by exposure to Kodak X-Omat X-ray film. After rinsing the membranes with acetonitrile for 10 min, the membranes were rehybridized with antibodies against β-actin (1 : 2000, Cell Signaling Technology) as the loading control. Relative density of protein bands was determined using ImageJ software (National Institutes of Health, USA).

2.8. Overexpression and Silencing of UCP2. UCP2 overexpression experiments were performed using a pcDNA3.1+ expression vector containing the rat cDNA of UCP2 that we designed previously. The cells were transfected with 5 μg of either the UCP2-bearing vector or control vector with Lipofectamine 2000 (Roche Diagnostic) following the manufacturer's recommendation. Eight hours after transfection, cells were selected using G418 sulphate (600 μg/mL) for 21 days. The cell colonies resistant to G418 were harvested. Stably expressing UCP2-transfected cells were cultured for further studies. UCP2 silencing experiments were carried out with specific small interfering (si) (5′-GUGGUCAAGACG-AGAUAUATTUAUAUCUGUCUUGACCACTT-3′) RNA targeting UCP2 mRNA and a nontargeting (NT) siRNA (5′-UUCUCCGAACGUGUCACGUTTACGUGACACGUU-CGGAGAATT-3′) purchased from Invitrogen Technologies (Shang Hai, China). Cells were transiently transfected with siRNA according to the manufacturer's protocol (Invitrogen Technologies).

2.9. Fluorescence Microscopy. For fluorescence microscopy, cells were cultured in 24-well plates with microscope cover glass. After the designated treatments, cells were fixed with 3% (w/v) paraformaldehyde in PBS. For quantification of autophagic cells, LC3 punctate dots were determined from triplicates by counting at least 60 cells. DAPI (1 μg/mL) was used to detect fragmented and condensed nuclei. Images were acquired with a laser scanning confocal microscope (LEICA TCS SP5). Intracellular lipid droplets were stained as previously described [10]. In brief, cells were stained with Nile red at room temperature; then lipid droplets were obtained using an inverted Olympus fluorescence microscope.

2.10. Apoptotic Analysis. Cells were plated in 60 mm plates. After attachment, cells were incubated with 250 μM of PA for 24 h. Cell apoptosis was analyzed using the Annexin V-FITC/PI Apoptosis Kit (Biosea, Beijing, China) according to the instructions and measured by flow cytometry. Data are presented as the mean of three determinations. Cells were seeded in 24-well plates. After being treated as indicated, cells were fixed using 4% paraformaldehyde, and the TUNEL (Promega, USA) manufacturer's protocol was followed. Cells were observed under confocal microscopy and then averaged by the number of TUNEL positive cells/100 cells.

2.11. Statistical Analysis. Data are presented as the mean ± SD. Analyses were performed using SPSS 13.0 software, and graphs were performed using Prism 5 software. P values < 0.05 or 0.01 are indicated as $(*)$ or $(**)$, respectively.

3. Results

3.1. PA Induces Autophagy Activation. To evaluate the effects of PA on intracellular autophagy in H4IIE cells, we first performed WST-1 assays on H4IIE cells treated with PA at different concentrations and different times. Figure 1(a) shows that treatment with PA resulted in a decrease in the levels of cell growth for up to 24 h when compared to control cells treated with fatty acid-free BSA (Wako, Japan). As shown in Figure 1(b), cells were stained by Nile red. We observed an increased number of intracellular lipid droplets in H4IIE cells treated with PA compared to BSA. Western blotting revealed a significant increase in the levels of LC3-II for up to 24 h in comparison to control cells. It is indicated that that PA could increase the levels of LC3-II upon a time course (Figure 1(c)). After inhibition of the late phase of autophagic process by CQ, PA treatment also increased the LC3-II level. However, western blotting revealed that treatment with OA could increase the levels of LC3-II upon a time course (see Supplementary SFigure 4 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/810401), but far more less when compared with the PA-treated cells.

To further confirm the observation that PA treatment does indeed induce autophagy in H4IIE cells, transmission electron microscopy studies were performed under PA treatment (Figure 1(d)). Autophagosome-like vacuoles were hardly seen in BSA-treated control cells. In contrast, we observed an increase in the formation of autophagosome-like structures and lysosomes in PA-treated cells.

3.2. UCP2 Partially Mediated PA-Induced Autophagy. To evaluate if PA treatment could also impact UCP2, we monitored UCP2 mRNA and protein expression by real-time PCR and western blotting. Results revealed that the UCP2 mRNA and protein levels were both increased in H4IIE cells treated with 250 μM PA for 6 h compared to the vehicle control (Figure 2), suggesting that the upregulation of UCP2 expression is mediated by PA in hepatoma cells.

Given that a marked increase in autophagy was observed, we tried to increase intracellular UCP2 expression by UCP2-bearing plasmid transfection to ascertain whether antiautophagy effects could be induced following PA treatment. We first performed WST-1 assays on UCP2 overexpression

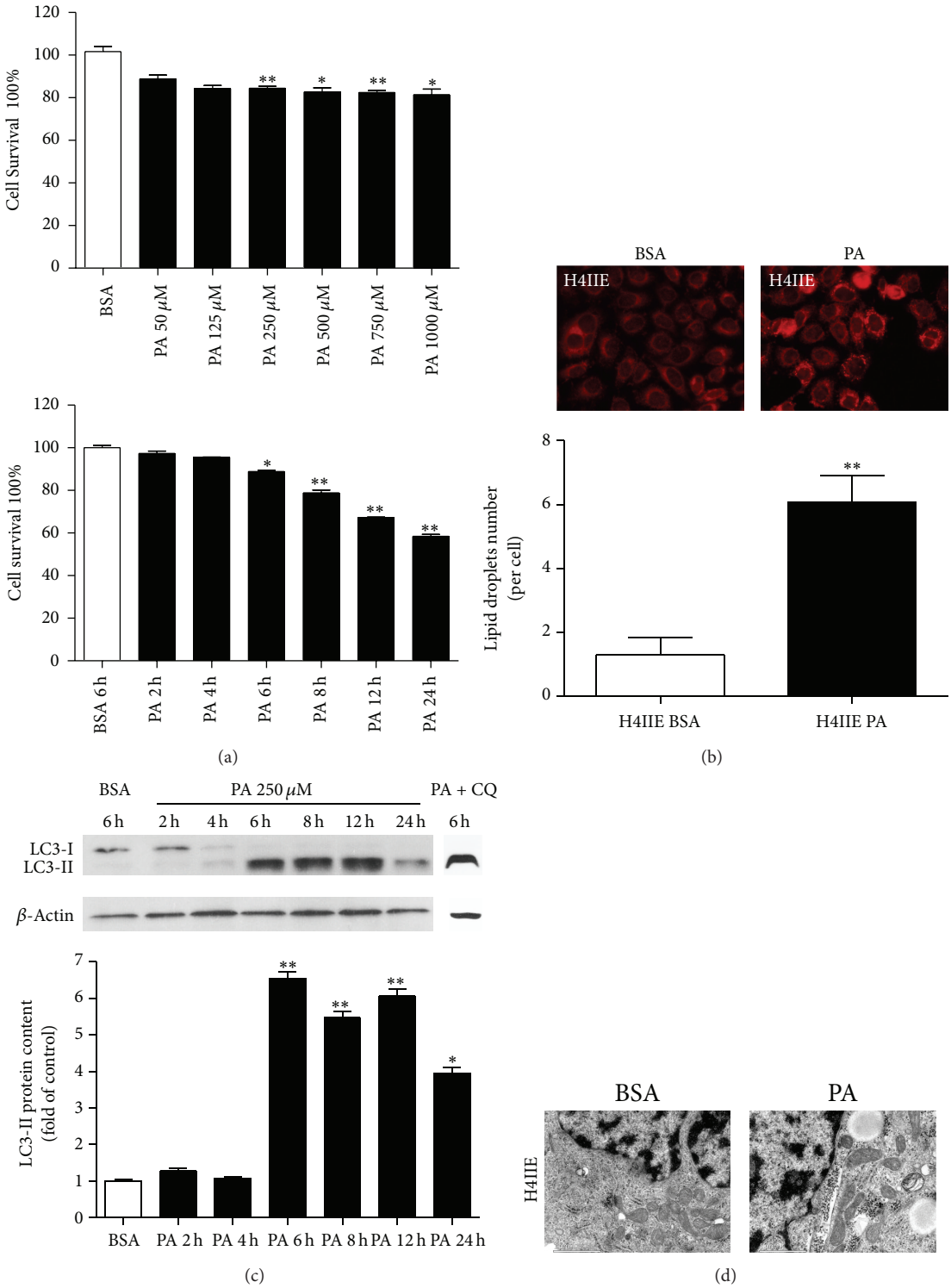


Figure 1: PA induces autophagy in H4IIE cells. (a) H4IIE cells were treated with PA (6 h) conjugated to fatty acid-free BSA at different concentrations, or H4IIE cells were treated with PA (250 μM) conjugated to fatty acid-free BSA at different time points. H4IIE cells treated with BSA acted as a control. After treatments, cells were stained and subjected to the WST-1 assay. (b) Intracellular lipid accumulation was assessed with Nile red staining. PA-induced autophagy (250 μM) of H4IIE cells exhibited numerous small discrete bodies distributed throughout the cytoplasm (objective lens, ×40). (c) H4IIE cells were treated with PA (250 μM) conjugated to fatty acid-free BSA for 2, 4, 6, 8, 12, and 24 h as indicated. Cells treated with BSA acted as a control. After the treatment, cell lysates were collected and subjected to western blotting. (d) H4IIE cells were treated with BSA, PA (250 μM), or PA + CQ (10 μM) for 6 hours before being processed; then electron microscope was performed at 40,000x magnification. All values are the means ± SD of three independent experiments each performed in triplicate.

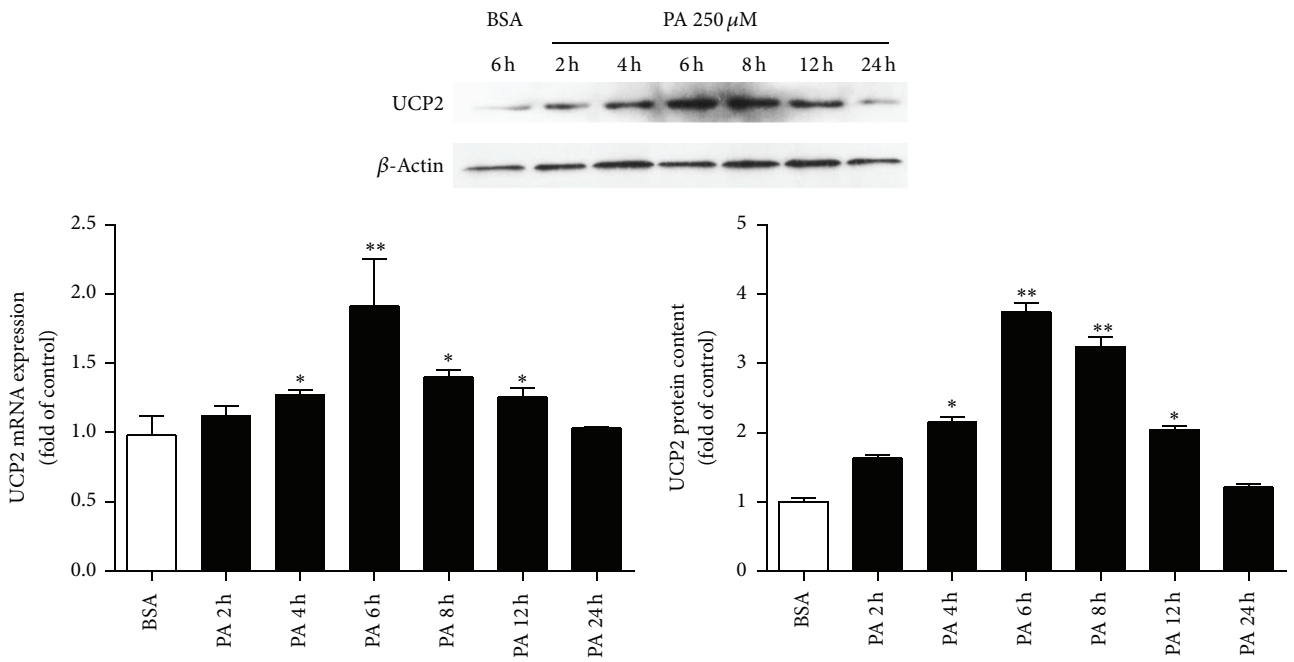


Figure 2: The effects of PA on UCP2 expression. H4IIE cells were treated with PA (250 μM) conjugated to fatty acid-free BSA for 2, 4, 6, 8, 12, and 24 h as indicated. The mRNA level of UCP2 was normalized to 18 s. This ratio was set as 100% with respect to the BSA control. The protein level of UCP2 was normalized to β-actin, and this ratio was set as 100% with respect to the BSA control. Data are expressed as the mean ± SD of three independent experiments each performed in triplicate.

cells treated with PA at different concentrations and different times. It demonstrated that treatment with PA resulted in a decrease in the levels of cell growth for up to 24 h when compared to BSA-treated cells (Supplementary SFigure 2). UCP2 expression in cells transfected with the UCP2-bearing plasmid was much higher as compared with cells treated with the control vector (Figure 3(a) and Supplementary SFigure 1). First, UCP2 overexpression significantly increased LC3-II levels in cells treated with PA with or without CQ (Figure 3(b)), suggesting that overexpression of UCP2 increased PA-induced autophagy. Furthermore, using inverted fluorescence microscopy (Figure 3(c)), we observed a marked increase of LC3 puncta in PA-treated cells. After all, to investigate autophagosome, electron microscopy analysis was carried out in PA-treated H4IIE cells transfected with the UCP2-bearing plasmid and cells transfected with vector-bearing plasmid acted as a control. As shown in Figure 3(d), more autophagosome structures were observed in UCP2 overexpression cells as compared with control cells by PA treatment under electron microscopy analysis.

To further demonstrate that induction of UCP2 levels is one of the major factors that lead to autophagy following PA treatment, and to confirm the above observations, we next investigated if UCP2 was associated with autophagy in PA-treated cells following UCP2-siRNA transfection. UCP2-siRNA markedly decreased UCP2 mRNA and protein levels in cells after being transfected with UCP2-siRNA for 72 hours (Figure 4(a)). It seems that UCP2-siRNA partially decreased LC3-II levels in cells treated with PA (Figure 4(b)), suggesting that inhibition of UCP2 interferes in PA-induced autophagy. These results indicated that deletion of UCP2 could decrease the effect of PA on LC3 puncta formation, suggesting a positive effect of UCP2 in PA-induced hepatic carcinoma cells autophagy.

3.3. PA-Mediated ROS Production Is Partially UCP2 Dependent. To evaluate the effect of UCP2 on intracellular ROS production, we performed ROS assays on H4IIE cells that were overexpressed UCP2 or inhibited by UCP2 siRNA and genipin. We observed that treatment with PA up to 6 h resulted in a significant increase in ROS production as compared with the BSA-treated control cells using the DCFH-DA assay by flow cytometry (Figures 5(a)–5(c)). This observation was further validated under fluorescence microscopy as shown in Figures 5(d)–5(f). As shown in Figure 5, UCP2 overexpression cells treated with PA resulted in a significant decrease (about 65%) in ROS production as compared with the vector-Tr cells, although overexpressed UCP2 decreases ROS levels in BSA treatments. After inhibiting UCP2 expression, the ROS production of H4IIE cells that were treated with PA increased 60% as compared with the scramble cells. But in BSA treatments the increased ratio is only 40%. Notably, the addition of CQ markedly enhanced ROS production induced by PA in three treatments (overexpression, siRNA, and genipin). However, the addition of CQ to the BSA control cells for up to 6 h did not induce any significant decrease in cell viability, suggesting that CQ alone is not cytotoxic to the H4IIE cells (Figures 5(a)–5(c)).

3.4. PA and UCP2 Mediate ATP Synthesis. To evaluate the effects of UCP2 on AMPK activation and ATP synthesis, we

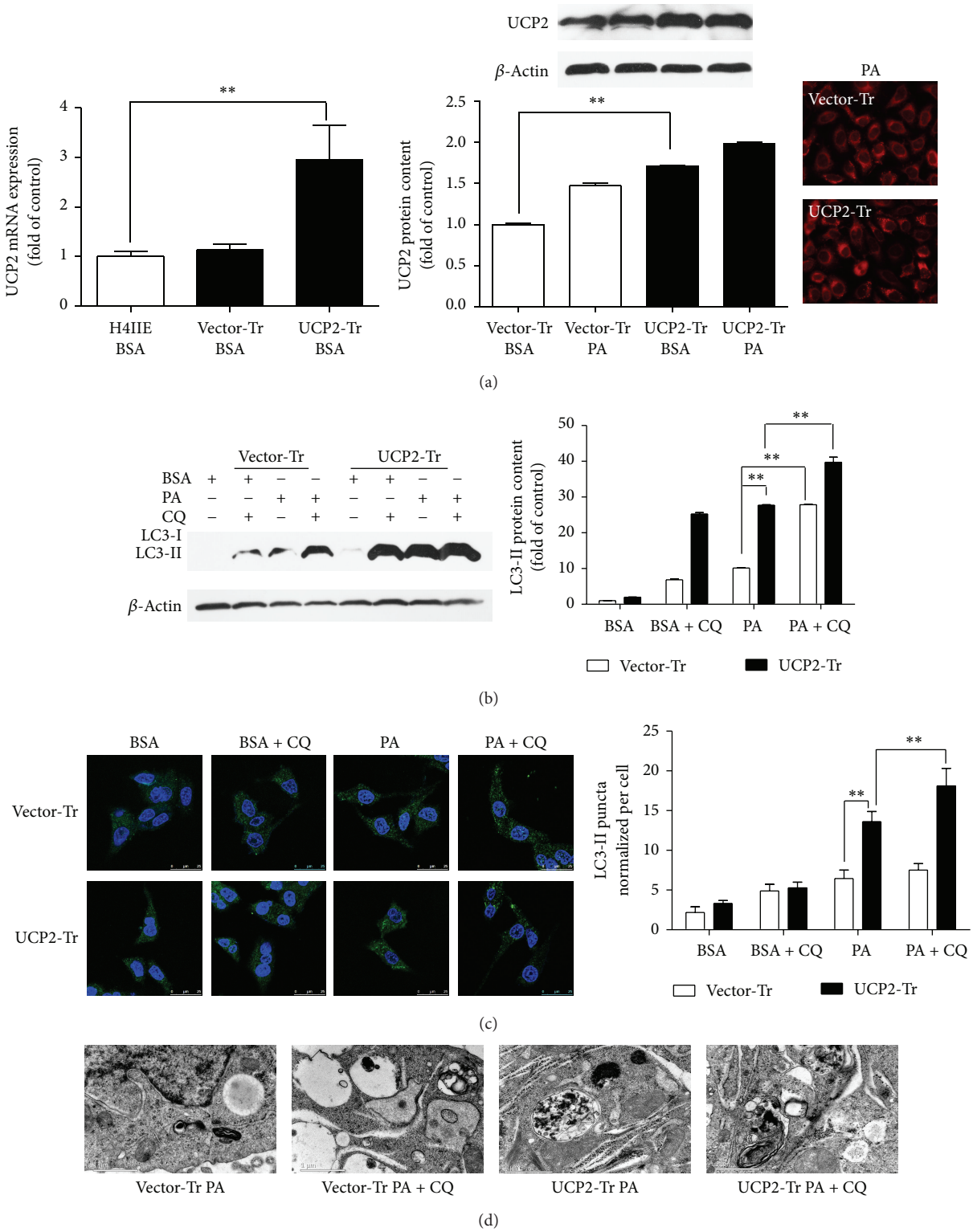


Figure 3: UCP2 overexpression enhanced PA-mediated autophagy. H4IIE cells were transfected with UCP2-bearing plasmid and control vector plasmid and then treated with 250 μM PA for 6 h, with or without CQ (10 μM). (a) The level of UCP2 mRNA was normalized to 18 s, and UCP2 protein was normalized to β-actin. This ratio was set to 100% in the control of BSA. (b) The level of LC3 protein was normalized to β-actin, and this ratio was set to 100% in the control of BSA. (c) H4IIE cells were treated with 250 μM PA for 6 h. Then LC3 puncta formation was observed using an inverted fluorescence microscope. The numbers of LC3 puncta/cell were counted from ≥100 cells. (d) Cells were treated with PA (250 μM) for 6 h, with or without CQ before being processed; then electron microscope was performed at 40,000x magnification. Data are expressed as the mean ± SD for each experiment. All data presented are representative of three separate experiments with consistent results.

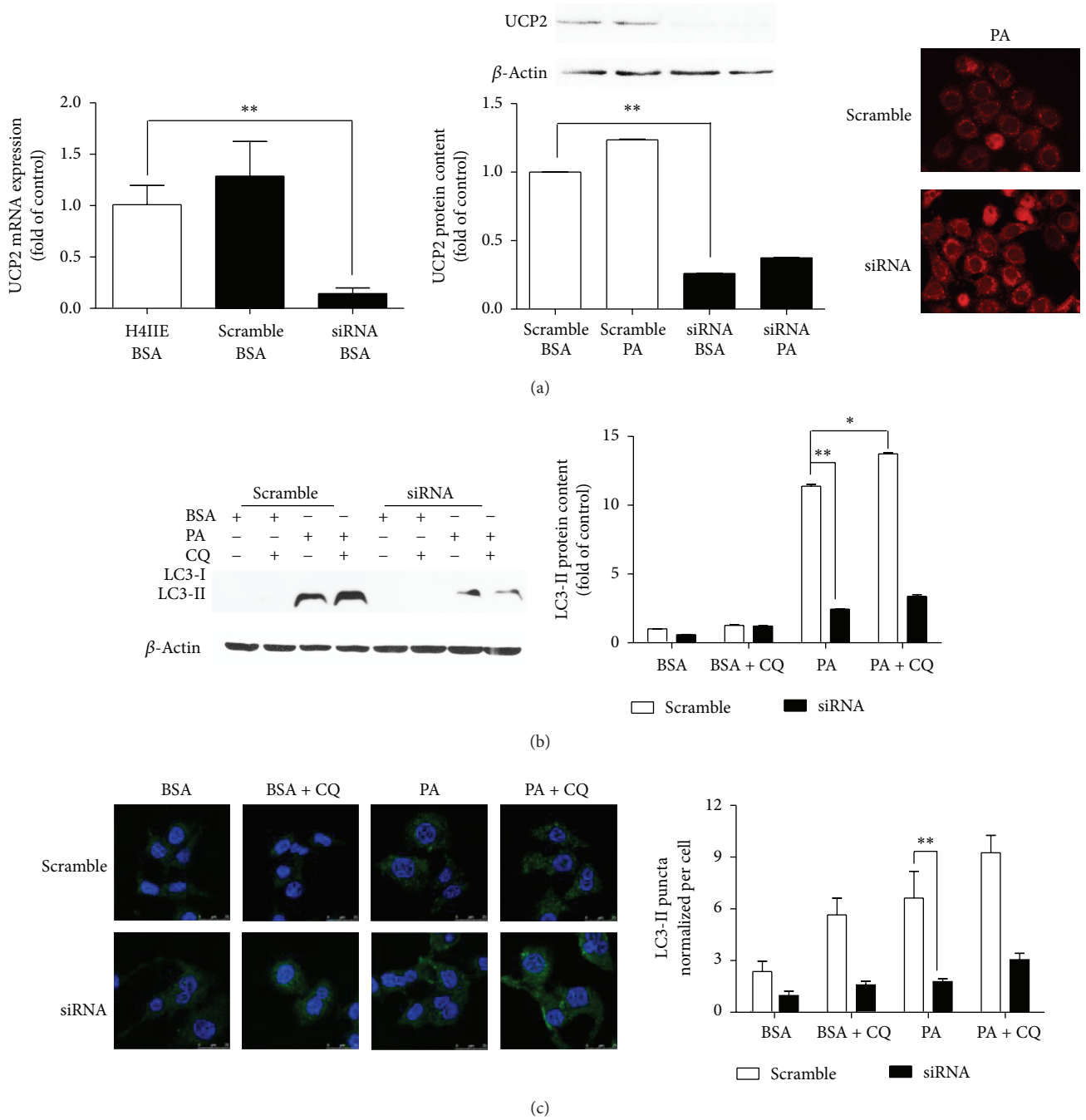


Figure 4: Inhibition of UCP2 decreases PA-mediated autophagy. H4IIE cells were transfected with UCP2-siRNA for 72 h to inhibit the expression of UCP2, followed by 250 μM PA for 6 h, with or without CQ (10 μM). (a) The level of UCP2 mRNA was normalized to 18 s, and UCP2 protein was normalized to β-actin. This ratio was set to 100% in the control of BSA. (b) The level of LC3 protein was normalized to β-actin, and this ratio was set to 100% in the control of BSA. (c) H4IIE cells were transfected with UCP2 siRNA for 72 h, followed by 250 μM PA for 6 h. Then LC3 puncta formation was observed using an inverted fluorescence microscope. The numbers of LC3 puncta/cell were counted from ≥100 cells. (d) Cells were treated with PA (250 μM) for 6 h, with or without CQ before being processed; then electron microscope was performed at 40,000x magnification. Data are expressed as the mean ± SD for each experiment. All data presented are representative of three separate experiments with consistent results.

performed ATP assays. Figure 6(a) indicated that overexpression of UCP2 could decrease intracellular ATP synthesis. In addition, Figure 6(b) demonstrated that ATP synthesis was inc-reased in UCP2 silencing H4IIE cells. Figure 6 showed that PA treatment could also decrease intracellular ATP synthesis. UCP2 overexpression or silencing could mediate AMPK activation to some level, but this phenomenon seemed so complicated that needs to be further studied.

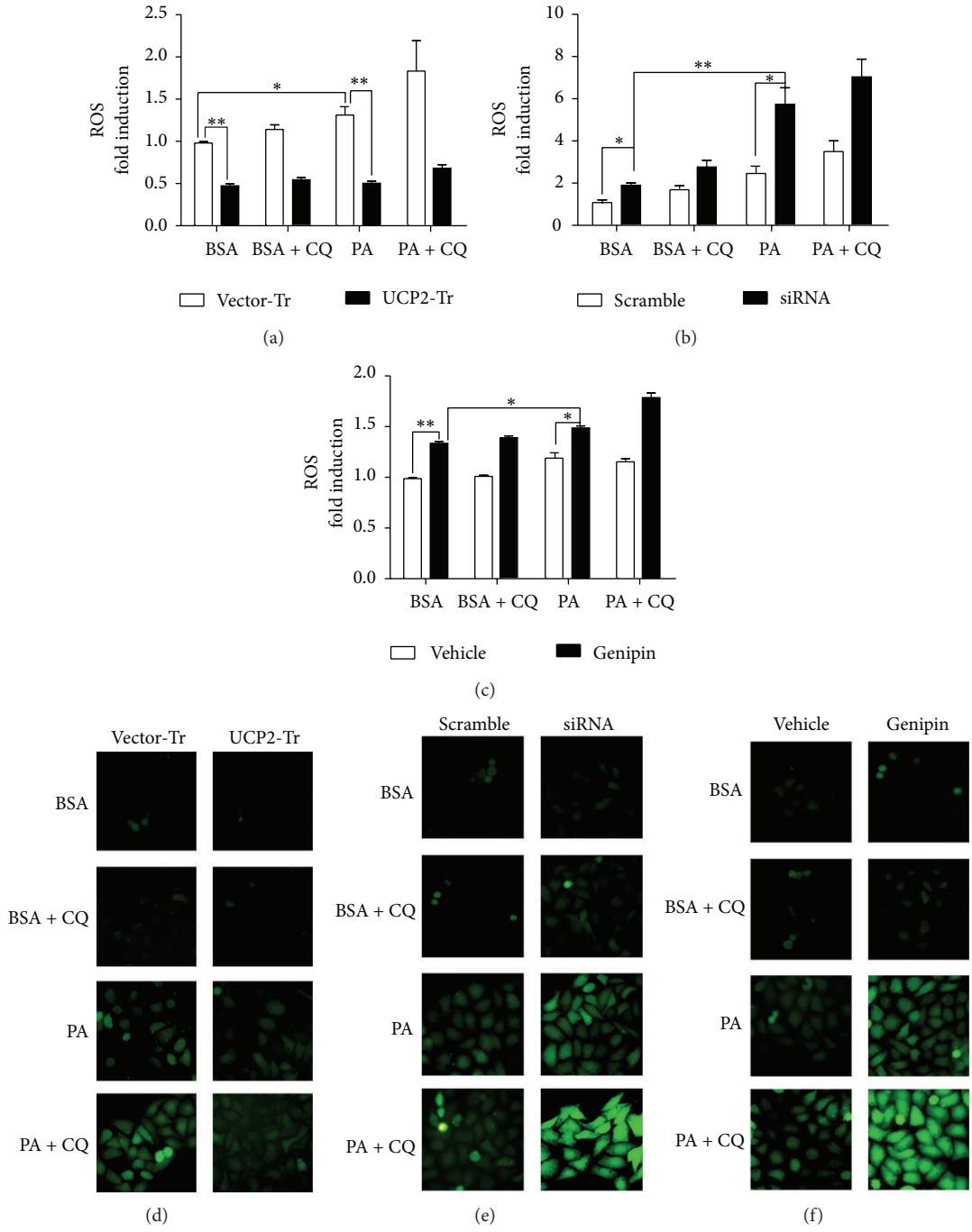


Figure 5: PA-mediated intracellular ROS production is UCP2 mediated. (a) H4IIE cells were transfected with UCP2 plasmid (UCP2-Tr) or control vector (Vector-Tr). Intracellular ROS production induced by 250 μM PA for 6 h was assessed using DCFH-DA and analyzed by flow cytometry. (b) H4IIE cells were transfected with UCP2 siRNA (UCP2-siRNA) or scrambled siRNA (Scramble) for 72 h to inhibit the expression of UCP2 and treated with PA. (c) H4IIE cells were treated with 50 μM genipin for 24 h, followed by PA, with or without CQ (10 μM). (d)–(f) Intracellular ROS was assessed using DCFH-DA staining and microscopy. Data are expressed as the mean ± SD for each experiment. All data presented are representative of three separate experiments with consistent results.

3.5. UCP2 Has an Antiapoptotic Effect on PA-Induced Apoptosis. Recent studies have shown that PA possesses cytotoxic properties [19, 20]. Our latest study has also shown marked cell apoptosis in livers during NASH progression [10]. To further investigate the physiological relevance of UCP2 in the progress of NASH, we treated H4IIE cells with 250 μM PA for up to 24 h. The results showed a significant increase in the number of apoptotic cells when treated with PA compared to BSA-treated cells (Figure 7(a)). It suggested that UCP2 expression may serve as a protective mechanism against lipotoxicity. To assess the relationship between cellular apoptosis and UCP2, we performed the TUNEL assay.

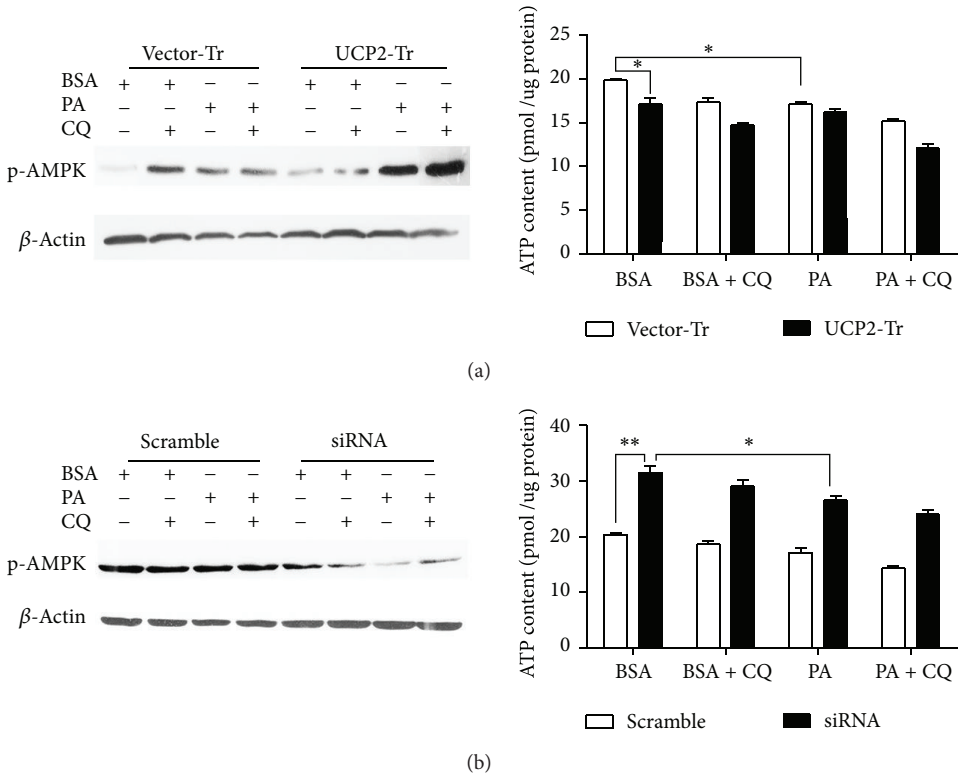


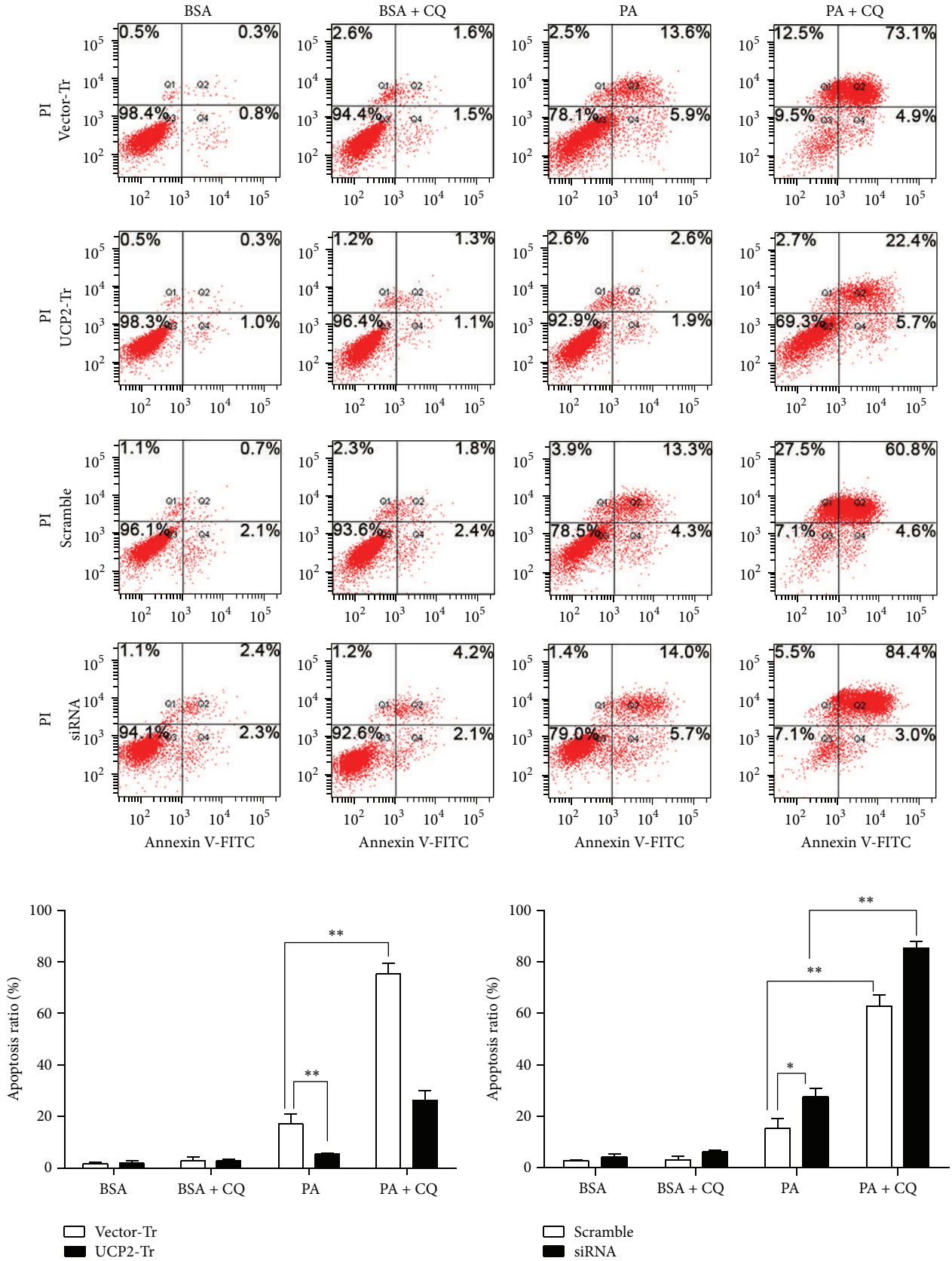
Figure 6: UCP2- and PA-mediated ATP synthesis. (a) H4IIE cells were transfected with UCP2 plasmid (UCP2-Tr) or control vector (Vector-Tr). ATP induced by 250 μM PA for 6 h was assessed using CellTiter-Glo2.0 assay kit, and p-AMPK was normalized to β-actin. This ratio was set to 100% in the control of BSA. (b) H4IIE cells were transfected with UCP2 siRNA (UCP2-siRNA) or scrambled siRNA (scramble) for 72 h to inhibit the expression of UCP2 and treated with PA. ATP induced by 250 μM PA for 6 h was assessed using CellTiter-Glo2.0 assay kit, and p-AMPK was normalized to β-actin. This ratio was set to 100% in the control of BSA. Data are expressed as the mean ± SD for each experiment. All data presented are representative of three separate experiments with consistent results.

PA treatment significantly promoted apoptosis in H4IIE cells, as the numbers of TUNEL-positive cells increased at PA treatments (Figure 7(b)). The same results further confirm that UCP2 plays an antiapoptotic role in PA stimulus. As shown in Figure 7(c), there were higher levels of cleaved caspase-3 in vehicle control cells, which indicated that UCP2 may play an antiapoptotic role and inhibit lipotoxic stress.

As shown in Figure 7, the addition of CQ also enhanced cell death. The addition of CQ enhanced cell death induced by PA. As shown in the BSA control cells, the addition of CQ induced an increase in cell apoptosis. It suggests that autophagy may act as a protective mechanism against apoptosis. This possibility has been reported in a previous study [21], and our data demonstrated a similar mechanism in hepatic carcinoma cells. We observed that treatment with PA resulted in a significant increase in apoptosis as compared with the BSA-treated control cells (Figure 7(a)). This observation was further validated under TUNEL assay as shown in Figure 7(b). As shown in Figure 7, UCP2 overexpression cells treated with PA resulted in a significant decrease (about 200%) in apoptotic cells as compared with the Vector-Tr cells. After inhibiting UCP2 expression, the apoptosis ratio of H4IIE cells that were treated with PA increased 40% as compared with the scramble cells. Notably, the addition of CQ markedly enhanced apoptosis ratio induced by PA in both treatments (overexpression and siRNA). However, the addition of CQ to the BSA control cells did not induce any significant decrease in cell viability, suggesting that CQ alone is not cytotoxic to the H4IIE cells (Figures 5(a)–5(c)).

4. Discussion

Lipotoxicity has been thought to be the main contributor to the progression of various diseases associated with excess lipid accumulation in the body, such as obesity and steatohepatitis [22]. The autophagic process has been well documented as a cell survival mechanism and has been implicated in several diseases such as cancer and neurodegenerative diseases [23, 24]. At present, autophagy has been shown to have a role in regulating lipid metabolism. The inhibition of autophagy in cultured hepatocytes and the mouse liver has been shown to increase triglyceride storage in lipid droplets [11], and it is known that PA can regulate autophagic activity in hepatocytes [25]. In this study, we reproved evidence that autophagy can be induced by the saturated fatty acid PA in H4IIe cells. Meanwhile, we showed that autophagy induction by PA is dependent on UCP2 activity. Furthermore, we also present evidence that autophagy plays a prosurvival



(a)

Figure 7: Continued.

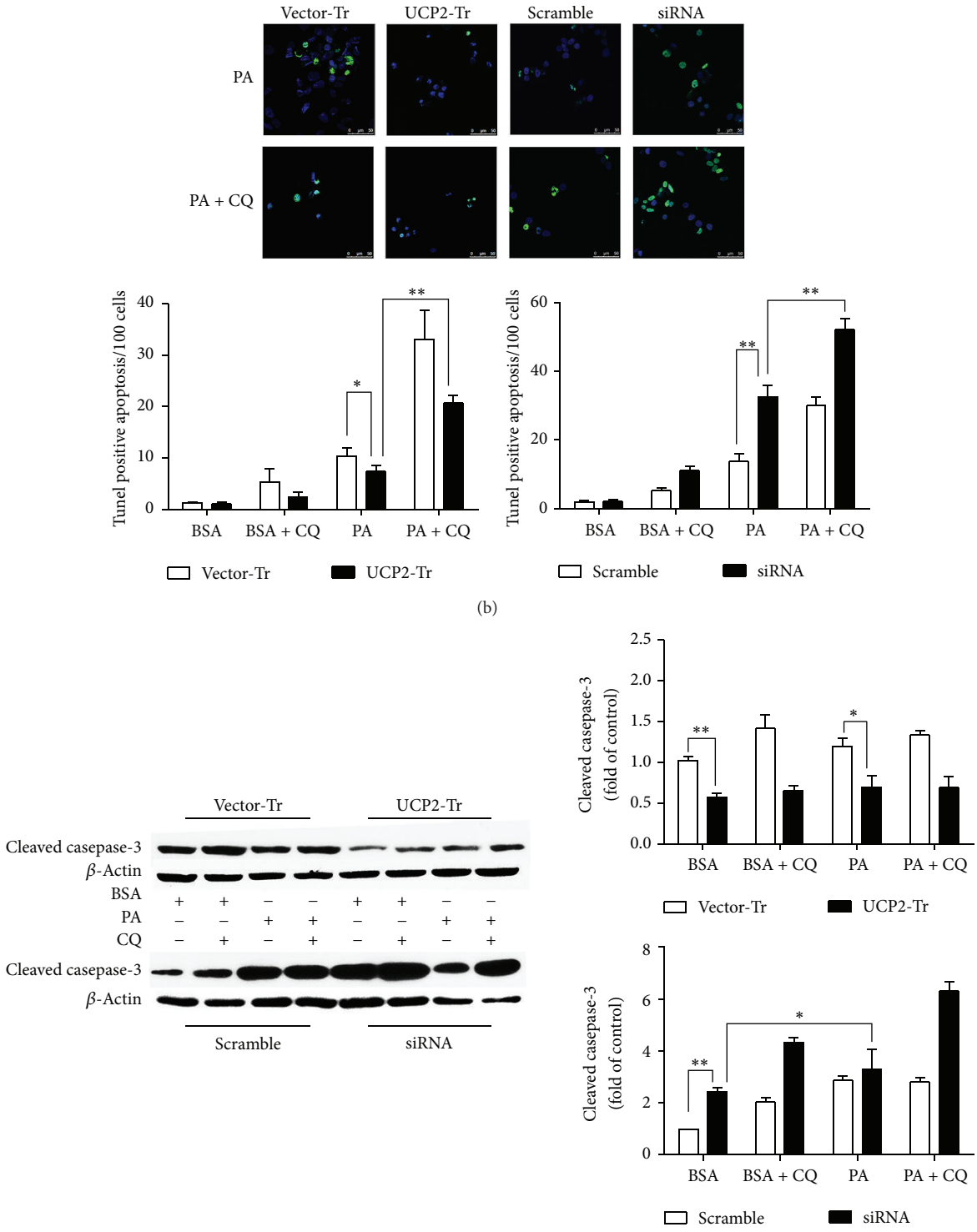


Figure 7: UCP2 has an antiapoptotic effect in PA-induced apoptosis. H4IIE cells were transfected with UCP2-bearing plasmid and UCP2-siRNA and then treated with 250 μM PA for 24 h. (a) Cellular apoptosis induced by 250 μM PA was analyzed by flow cytometry. Data are expressed as the mean ± SD for each experiment. (b) TUNEL-positive cells that were green under fluorescence microscopy were quantified from 100 cells at ×400 magnifications. Data were expressed as the mean ± SD for each experiment. (c) Representative of cleaved caspase-3 and β-actin proteins expression by western blot. Data were expressed as the mean ± SD. All data presented are representative of three separate experiments with consistent results.

function to protect against PA-induced lipotoxicity. Our findings are generally consistent with earlier reports that PA is capable of inducing autophagy in pancreatic β-cells [26, 27] and embryonic fibroblasts [21]. In a recent study, it has been reported that autophagy can be induced by PA, but not by OA [21]. On the other hand, it is believed that only OA but not PA was capable of inducing autophagy in hepatocytes [28]. One study has also reported that PA prevented fusion of autophagosomes and lysosomes and thus inhibited autophagy [29]. It is believed that cell type, concentration, duration of FFA treatment, and the ratio of conjugated BSA to FFA used could be attributed to these conflicting results.

UCP2 is one of the mitochondrial transporters that are located in the inner mitochondrial membrane and belong to a family of mitochondrial anion carriers, which includes adenine nucleotide transporters. Mild uncoupling of respiration has been reported to diminish mitochondrial reactive oxygen species (ROS) formation [30]. It has been demonstrated that upregulation of UCP2 by AMPK activation attenuates oxidative stress [31]. A recent study also shows that AMPK is an upstream kinase for UCP2 [32]. Our results indicate that UCP2 could decrease intracellular ATP synthesis, and PA stimuli may partially decrease the level of intracellular ATP synthesis. UCP2 is rather an upstream kinase for AMPK than feedback to AMPK activation.

It is well known that UCP2 is an antioxidant mitochondrial protein and that inhibition of UCP2 induces oxidative stress favoring the formation of mitochondrial superoxide ions [33]. Recently, it has been demonstrated that UCP2 is a key redox-sensitive protein [34]. Overexpression of UCP2 decreases cell death following downregulation of ROS production [35]. This aspect of UCP2 function further strengthens the proposition that UCP2 can modulate mitochondrial ROS production and activity. In the present study, we clearly elucidate for the first time the role of UCP2-mediated mitochondrial uncoupling on autophagy regulation in hepatocyte ROS production. The results showed that high UCP2-expressing H4IIE cells have more enhanced adaptive abilities to PA-induced lipotoxicity partly through diminishing ROS production than low UCP2-expressing cells. Because CQ is known to block autophagy by suppressing the lysosomal function, our findings thus indicate that UCP2 may protect against PA-mediated autophagy on ROS production. This aspect of UCP2 function further strengthens the proposition that UCP2 can modulate mitochondrial ROS production and activity [36]. Here, we confirmed that ROS production is mediated by UCP2 after PA-induced hepatocyte lipotoxicity.

Autophagy is a critical intracellular pathway that targets cell constituents to the lysosome for degradation. Recent studies showed that established functions for both macroautophagy and chaperone-mediated autophagy in hepatic lipid metabolism, insulin sensitivity, and cellular injury suggest a number of potential mechanistic roles for autophagy in NASH [15]. Decreased autophagic function in particular may promote the initial development of hepatic steatosis and progression of steatosis to liver injury [15]. In the present study, we observed that UCP2 overexpression also significantly increased autophagy in PA-treated cells, while inhibition of UCP2 resulted in a decrease in PA-induced autophagy. Furthermore, with loss of autophagic function, decreased lipid storage occurred, and an increased rate of fatty acid β-oxidation was observed [12]. We suspect that PA-induced autophagy occurred partly through increased UCP2 upregulation.

In this study, we have shown that UCP2 is associated with apoptosis induced by fatty acids *in vitro*. Our previous study has shown that PA was able to induce liver damage that resembles NAFLD in humans and was characterized by increasing caspase-3 activity and prominent apoptosis [10]. To further confirm these results, we altered UCP2 expression levels in H4IIE hepatoma cells by transfection with either an UCP2 mRNA interference (siRNA) plasmid or a UCP2-overexpressing plasmid. UCP2 overexpression caused significantly decreased apoptosis rates and caspase-3 activity in the PA-treated cells while UCP2 siRNA resulted in an increase in apoptosis rates and caspase-3 activity. Overall, we have demonstrated that UCP2 protects hepatic carcinoma cells from PA-induced apoptosis *in vitro* by increasing hepatocyte autophagy. We predict that the antiapoptotic effect of UCP2 most likely relates to its preventative role in its inductive effect of hepatoma autophagy.

5. Conclusion

Our present study shows that UCP2 was upregulated and that hepatocellular autophagy was increased during PA treatment. Increasing UCP2 expression in hepatoma cells may contribute to cell autophagy. Hepatic autophagy play a protective role in hepatocyte lipoapoptosis. The results provide evidence that UCP2 is a proliferative factor that also has an antiapoptotic role during PA-induced liver injury. The current data obtained from our experiments may provide useful information regarding potential molecular targets for NASH prevention and treatment.

Abbreviations

ROS: Reactive oxygen species
UCP2: Uncoupling protein 2
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
FFA: Free fatty acid
PA: Palmitic acid
CQ: Chloroquine diphosphate.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Autophagy in Alcohol-Induced Multiorgan Injury: Mechanisms and Potential Therapeutic Targets

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Autophagy is a genetically programmed, evolutionarily conserved intracellular degradation pathway involved in the trafficking of long-lived proteins and cellular organelles to the lysosome for degradation to maintain cellular homeostasis. Alcohol consumption leads to injury in various tissues and organs including liver, pancreas, heart, brain, and muscle. Emerging evidence suggests that autophagy is involved in alcohol-induced tissue injury. Autophagy serves as a cellular protective mechanism against alcohol-induced tissue injury in most tissues but could be detrimental in heart and muscle. This review summarizes current knowledge about the role of autophagy in alcohol-induced injury in different tissues/organs and its potential molecular mechanisms as well as possible therapeutic targets based on modulation of autophagy.

1. Introduction

Chronic or acute alcohol abuse often leads to liver injury associated with alcoholic hepatitis, liver fibrosis, cirrhosis, and liver cancer [1]. In addition to the liver, alcohol abuse also induces a variety of other tissue injuries including pancreatitis [2, 3], cardiomyopathy [4, 5], neurotoxicity [6], muscle loss [7], impaired immune functions [1], endocrine and fetal abnormalities [8], and osteoporosis [9]. According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), more than 18 million adults are affected by alcoholism in the United States, which costs 27 billion dollars for treating alcohol-attributable diseases.

The mechanisms for alcohol-induced detrimental effects in various tissues/organs have been extensively studied, which involves genetic and environmental factors as well as altering multiple cellular signaling pathways. These mechanisms involve ethanol and its metabolites that induce reactive oxygen species (ROS) generation, lipid peroxidation, cytokine expression/inflammation, organelle damage, and stress and activate both apoptotic and necrotic cell death pathways [10]. However, the full picture especially the cellular adaptive and protective mechanisms against ethanol-induced stress and tissue injury has not been well depicted yet. Currently, the treatment for chronic alcohol diseases is not very effective owing largely to our incomplete understanding of the cellular adaptive response to ethanol toxicity.

Macroautophagy (hereafter referred to as autophagy) is a genetically programmed, evolutionarily conserved intracellular degradation pathway in response to stress. It is involved in the trafficking of long-lived proteins and cellular organelles to the lysosome for degradation to maintain cellular homeostasis. It is tightly controlled by over 30 autophagy-related (Atg) genes [11]. Autophagy is generally considered as a cell survival mechanism in response to various stress conditions and plays a critical role in human physiology and diseases [12].

Accumulating evidence has shown that altered autophagy is implicated in the pathogenesis and protection of ethanol-induced tissue injury [13–15]. This review aims to summarize

current knowledge about the role of autophagy in alcohol-induced injury in multiple tissues/organs and their underlying molecular mechanisms as well as potential therapeutic targets based on modulation of autophagy.

2. Ethanol Metabolism and Its Effect on Tissue Injury

Ethanol is metabolized through several pathways. Predominantly alcohol is metabolized by alcohol dehydrogenase (ADH) into acetaldehyde, a highly reactive byproduct, and acetaldehyde is further metabolized by aldehyde dehydrogenase (ALDH) into acetate, a more harmless substance. The most important two isoforms of ALDH are the cytosolic ALDH1 and the mitochondrial ALDH2 [16]. It has been reported that there are functional polymorphisms among ADH and ALDH that can influence the susceptibility of humans to alcoholism [17]. The oxidation of ethanol mainly not only occurs in liver [18], but also occurs in pancreas [19], heart [5], and other organs [20], accompanied by conversion of nicotinamide adenine dinucleotide (NAD^+) into its reduced form, NADH, which plays critical roles in cellular redox status. Besides, ethanol could also be metabolized by cytochrome P450 family 2, subfamily E, polypeptide 1 (Cyp2E1), and catalase. Excessive ethanol exposure largely induces Cyp2E1, which not only mediates and activates reactions of many toxicological substrates, but also generates ROS leading to cellular damage [21]. Additionally, a minimal amount of ethanol can also be metabolized via two nonoxidative pathways. In the first pathway, ethanol interacts with fatty acid and generates fatty acid ethyl ester (FAEE), which is catalyzed by FAEE synthase in many tissues and organs [22]. FAEE was thought to have minor effect and mainly considered as a diagnostic marker but accumulated evidence shows that FAEE exacerbates injury after ethanol exposure especially in pancreas [23, 24], liver [25], and heart [23, 25, 26] and is facilitated by ADH deficiency [27]. One explanation of cytotoxicity of FAEE is that FAEE binds to mitochondria membrane and with its hydrolysis products, fatty acids, causes damage by uncoupling oxidative phosphorylation [28]. In the second pathway, phospholipase D (PLD), which normally breaks down phospholipids to generate phosphatidic acid (PA), reacts with ethanol to generate phosphatidyl ethanol. Following chronic consumption of large amounts of alcohol, phosphatidyl ethanol may accumulate to detectable levels because it is poorly metabolized. However, the effects of phosphatidyl ethanol on cellular functions remain to be further studied [20].

Alcohol is easily absorbed and could be metabolized and impact almost all over the body. Matured brain is less affected compared with developing brain due to the blood-brain barrier, but excessive oxidative stress and intracellular Ca(2+) release induced by ethanol could impair the barrier function [29]. Dysregulated metabolites lead to changes in carbohydrate metabolism, cell death signaling, mitochondria damage, and epigenetic regulation [30]. Apoptosis and necrosis induced or exacerbated by alcohol metabolism have been studied in liver [31, 32], pancreas [33, 34], heart, brain [35, 36], developing brain [37], and skeletal muscle [38]. The important mechanisms that are thought to be involved in alcohol-induced liver injury include (1) oxidative stress and lipid peroxidation [39, 40]; (2) liver hypoxia [41, 42]; (3) endoplasmic reticulum (ER) stress and activation of mitogen activated protein kinases (MAPK) [43, 44]; and (4) inhibition of proteasome and lysosomal functions that causes hepatomegaly and Mallory inclusion bodies [45–47]. Recent evidence has also suggested that chronic alcohol consumption induces necroptosis in mouse livers, which is dependent on the receptor interacting protein 3 (RIP 3) [48]. The effect of ethanol metabolites in different tissue/organ autophagy and cell injury will be further discussed below.

3. Role of Autophagy in Alcoholic Liver Disease

Liver is one of the most active organs, which plays a central role in regulating the overall organism energy balance by controlling carbohydrate and lipid metabolism. Under physiological conditions, liver also serves as a major buffering system to ensure other tissues to function normally by maintaining the homeostasis of macro- and micronutrient. To accomplish the vital missions, liver may rely on autophagy, the cellular catabolic process to breakdown macromolecules, lipids, and damaged/excess organelles. Indeed, liver-specific autophagy gene knockout (KO) mice have disrupted metabolism of proteins, glucose, and lipids, accumulated damaged and excess organelles such as mitochondria and peroxisomes, and resulted in increased cell death, inflammation, and liver tumorigenesis [49–52]. Alcoholic liver disease (ALD) also involves the disruption of cellular metabolism of proteins and lipids and homeostasis of organelles such as mitochondria and endoplasmic reticulum (ER) resulting in increased cell death that contributes to alcoholic hepatitis, liver fibrosis, cirrhosis, and liver cancer [1, 53]. All these pathogenic events are intimately related to the autophagic process, and modulating autophagy may thus affect the ALD pathogenesis. Indeed, accumulating evidence now indicates that autophagy plays a critical role in the pathogenesis of ALD [13–15].

In the past several years, many animal models as well as *in vitro* cell culture models have been developed to study ALD. While baboons, pigs, and rats have been used to study ALD, mice have been predominantly used in current ALD research. The animal models for ALD include acute alcohol gavage, *ad libitum* oral alcohol in drinking water, intragastric infusion (Tsukamoto-French model), chronic Lieber-DeCarli diet ethanol feeding, and the most recent Gao-binge (chronic + binge) models [53, 54]. In addition to *in vivo* models, primary cultured hepatocytes and engineered HepG2 cells that are stably expressing Cyp2E1 and ADH are also used to study alcohol-induced pathophysiological changes in hepatocytes [55, 56]. Unfortunately, all these current models can only capitulate some of the phenotypes of ALD, which do not progress beyond liver steatosis, inflammation, and injury. Owing to the complexity of the models to study ALD, lack of reliable markers, and the difficulty to monitor autophagic flux in chronic ALD models, controversial results regarding the autophagy status (activated or impaired) during the pathogenesis of ALD have been reported. Some possible

explanations for these contradictory results will be discussed in detail below.

Autophagy was first described by de Duve and Wattiaux in the rat liver that was challenged with glucagon in the 1960s [57], but the molecular characterization of autophagy began in the 1990s by Ohsumi and Wolf's laboratories using yeast genetic screens that led to the discovery of a group of essential Atg genes in yeast [58, 59]. These Atg genes were later found to be highly conserved in mammals. Autophagy is a highly dynamic process involving several key steps: (1) the first step is to activate the preinitiation complex that is composed of Unc-51 like kinase 1- (ULK1-) FAK family-interacting protein of 200 kDa (FIP200)-Atg13, which is negatively regulated by the upstream nutrient sensor the mammalian target of rapamycin complex 1 (mTORC1) and positively regulated by the energy sensor Amp-activated protein kinase (AMPK), leading to the initiation of autophagosome biogenesis [60–62]; (2) the preinitiation complex and the ER-resident SNARE protein syntaxin 17 (STX17) then recruit Atg14L to the rough ER or ER-mitochondria contact site, which further recruits Beclin-1 and VPS34 to the autophagosome initiation site on the rough ER [63, 64]. VPS34 then promotes the generation of phosphatidylinositol 3-phosphate- (PI3-P-) enriched autophagosome initiation sites that further recruit PI3-P effectors including double FYVE domain-containing protein 1 (DFCP1), WD-repeat interacting protein with phosphoinosides 1 (WIPI1), and WIPI2 to initiate the biogenesis of autophagosomes [65–67]. This complex is positively regulated by activating molecule in Beclin-1-regulated autophagy (Ambra-1) [68], UV irradiation resistance-associated gene (UVRAG) [69], Bif-1/Endophilin B1 [70], and AMPK and negatively regulated by binding to Bcl-2 [71], Bcl-xL, run domain protein as Beclin-1 interacting and cysteine-rich containing (Rubicon) [72, 73], AKT, and epidermal growth factor receptor (EGFR); (3) next, two ubiquitin-like conjugation systems, Atg7-Atg3-microtubule-associated light chain (LC3) and the Atg12-Atg5-Atg16L1 complex, regulate conjugation of phosphatidylethanolamine with LC3 (called LC3-II), which expands the autophagosome membrane [74–76]. Atg9 also delivers membranes from *trans*-Golgi network/endosomes to the site of autophagosome biogenesis in an ULK1- and VPS34-dependent manner to promote the expansion of the autophagosome membrane [77]. The Atg12-Atg5-Atg16L complex only transiently attaches to the autophagosomal membranes and is later dissociated from the autophagosomal membranes [78], and PI3-P is also dephosphorylated locally by the phosphatases myotubularin-related protein 3 (MTMR3, also called Jumpy) upon closure of the autophagosomes [79, 80]; (4) finally, autophagosomes fuse with lysosomes/endosomes to form autolysosomes, which is mediated by Rab7, Lamp1/2, and the SNARE protein STX17 [81–83]. After fusion, the outer membrane of LC3-II is dissociated from the autolysosomal membrane through a deconjugation process mediated by Atg4B, and inner membrane LC3-II is degraded together with autophagosome cargos [84, 85].

Due to the complex dynamic nature of the autophagic process, it is very challenging to monitor autophagy in a quantitative way, in particular *in vivo* in whole animal tissues/organs. While LC3-II is widely used to monitor the autophagic process, LC3-II itself is also degraded in the autolysosomes. Thus an autophagic flux assay, which monitors LC3-II levels with or without a lysosomal inhibitor such as chloroquine or bafilomycin A1, has been recommended to determine autophagy status by the autophagy research community [86]. In addition, the level of p62/sequestosome 1 (SQSTM1) has also been suggested to use another marker for autophagic flux because p62/SQSTM1 is normally degraded in response to starvation and accumulated in genetic autophagy gene deleted mouse livers [50, 87]. However, the levels of p62/SQSTM1 may not always be suitable to monitor autophagic flux because its levels are also regulated at the transcriptional level, which is often induced in many experimental autophagy models including prolonged starvation conditions [88]. As discussed above, there are some controversial reports regarding the autophagy status in ALD research. The reasons behind these controversial reports are likely due to the complexity of autophagy assays and the use of many different ALD models. It is generally agreed that acute ethanol (binge) treatment increases autophagy in mouse livers and in primary cultured murine hepatocytes, a conclusion that is supported by autophagic flux data in these studies [89, 90]. In contrast, impairment of autophagy has also been reported in ethanol-treated Cyp2E1 overexpressing HepG2 cells or in Cyp2E1 knock-in mice that were given acute alcohol twice a day for four days [91, 92]. However, only a decreased LC3-II/I ratio was observed and no autophagic flux assays were conducted in these studies. Using HepG2 cells that stably overexpressed both Cyp2E1 and ADH, Thomes et al. [93] reported that ethanol treatment not only increased autophagosome synthesis but also impaired lysosomal degradation. However, the autophagic flux data in this study actually supported an increase in autophagy [93]. Early works from the same group showed that ethanol treatment may increase lysosomal pH and impair cathepsin maturation [94, 95]. Thus it is possible that increased autophagy by ethanol treatment may serve as a compensatory mechanism in response to ethanol-induced mild impaired lysosomal functions. In contrast to acute alcohol treatment, it has been generally thought that chronic alcohol consumption may have impaired autophagy in the liver because it has long been shown that chronic alcohol administration leads to hepatomegaly and protein accumulation in liver [96–98]. In a recent chronic ethanol feeding study, Lin et al. performed autophagic flux studies in mice that were fed an ethanol diet for 4 weeks and increased autophagic flux was found in that study [99]. However, we should interpret these data cautiously since the autophagic flux assay was only performed at one time point after the 4-week feeding, while ideally, autophagic flux assays should be applied to multiple time points owing to the dynamic nature of autophagy. In addition, the autophagy status in other ALD models, such as the Tsukamoto-French model and Gao-binge model, has not been reported. Moreover, in addition to hepatocytes, whether and how ethanol would affect autophagy on other liver cells, such as hepatic stellate and Kupffer cells, and their impacts on ALD are largely unknown. Regardless of the controversies on autophagy status, it has been unanimously shown that activating

autophagy is beneficial against ALD in various ALD models [89, 90, 99, 100].

4. Possible Mechanisms Affecting the Autophagy Process Induced by Alcohol

As discussed above, autophagy is a dynamic multistep process that is tightly regulated by many signaling pathways involving nutrients, energy, and stress response. Below we discuss the regulating pathways of autophagy that have been shown to be affected by alcohol.

4.1. Class I PI3K-Akt-mTOR. As discussed above, mTORC1 is a negative regulator at the preinitiation complex to regulate the initiation of autophagosome biogenesis [101]. mTOR is part of two structurally and functionally different complexes, mTORC1 and mTORC2. The former complex is sensitive to rapamycin and plays a major role in regulation of cell growth and autophagy. mTORC1 is also a sensor of various signals including growth factors, insulin, nutrients, energy status, and cellular stressors. In nutrient-sufficient condition, growth factors activate the class I phosphoinositol-3-kinase (PI3K) to catalyze PIP3 and subsequently activate Akt, which then further activates downstream mTORC1 and inhibits autophagy. mTORC2 may also negatively regulate autophagy because it is required for full activation of Akt [102, 103]. Suppression of Akt and mTOR are common mechanisms of autophagy induction, which is also affected by ethanol treatment. It has been demonstrated that acute ethanol-treated mouse liver and chronic ethanol-treated rat liver had increased expression of phosphatase and tensin homolog (PTEN) resulting in the suppression of Akt [104, 105]. We also found that acute ethanol treatment decreased the level of phosphorylated Akt in mouse liver [89]. Besides, ethanol treatment also inhibited mTORC1 activity in primary cultured mouse hepatocytes [90]. More importantly, pharmacological inhibition of mTOR by either rapamycin or Torin 1 significantly suppressed acute ethanol-induced liver steatosis and injury [15, 90]. More future studies are needed to further determine the detailed time-course changes of mTOR and their associations with autophagy during chronic alcohol feeding.

PI3K/Akt activation is known to enhance sterol regulatory element-binding protein-1 (SREBP-1) [106]. In addition, a system biology-based integrative computational analysis also suggests that SREBP-1 may coordinate autophagy-lysosomal activities and lipid metabolism [107]. Interestingly, application of wortmannin, a PI3K/Akt inhibitor, showed dual effects on acute ethanol-induced fatty liver depending on dose [108]. Low dose wortmannin inhibited whereas high dose of wortmannin exacerbated acute ethanol-induced steatosis in mouse livers. It has been suggested by the authors that high dose of wortmannin might inhibit hepatic autophagy whereas low dose of wortmannin alleviated the rise of hepatic triglycerol possibly by inhibiting SREBP-1 via PI3K/Akt inhibition [108]. These findings suggest that special attention should be paid to the use of PI3K/Akt inhibitor in alcohol-induced fatty liver studies.

4.2. AMPK. AMPK is a key energy sensor that regulates cellular metabolism and energy homeostasis. AMPK can directly inhibit mTOR through increased phosphorylation of TSC2 [109, 110] and Raptor [111], which activates autophagy. AMPK also promotes autophagy by phosphorylating ULK1 [109, 112], VPS34, and Beclin-1 [113]. However, it has been shown that administration of an AMPK activator adenosine, 5-amino-4-imidazole carboxamide riboside (AICAR), suppresses autophagy in hepatocytes [114]. Moreover, administration of compound C, an AMPK inhibitor, activates autophagy via AMPK-independent blockade of the Akt/mTOR pathway, which overcomes the expected inhibitory effect on autophagy via AMPK inhibition in cancer cells [115]. Several lines of evidence show that AMPK activity is reduced in liver by ethanol consumption, which is believed to promote fatty liver through activation of SREBP-1 and upregulation of lipin-1 expression [116, 117]. The exact role of AMPK in ethanol-induced autophagy is not clear but it is possible that ethanol induces autophagy independent of AMPK activation.

4.3. ADH, Cyp2E1, and ROS. In liver, ethanol is mainly metabolized by ADH and Cyp2E1, which promotes the generation of ROS and other reactive toxic metabolites. Interestingly, ethanol-induced autophagy requires its metabolism and ROS production because autophagic flux was only induced in HepG2 cells stably expressing ADH and Cyp2E1 but not in parental HepG2 cells [90, 93]. Moreover, blocking ADH and Cyp2E1 by 4-methylpyrazole or inhibiting ROS by antioxidants also reversed the inhibition of mTOR and diminished increased GFP-LC3 puncta [90, 93]. It seems that alcohol oxidation by Cyp2E1 is also important for alcohol-induced inhibition of cellular proteasome activity and increased autophagosome numbers [118]. It has been proposed that ROS may activate autophagy through modulating the oxidization of Atg4, an autophagy machinery protein important for generating and recycling of LC3-II [119]. As for the importance of Cyp2E1-mediated ethanol metabolism on ethanol-induced autophagy changes, several studies have reported that ethanol-treated HepG2 cells that are overexpressing Cyp2E1 and ethanol-treated *Cyp2E1* KO or knock-in mice showed decreased LC3-II levels [91, 92, 100, 120]. While the authors concluded that Cyp2E1-mediated metabolism of ethanol may lead to inhibition of autophagy in these studies, autophagic flux assays were not conducted in these studies. Future studies are needed to further confirm the autophagy status in ethanol-treated *Cyp2E1* KO or knock-in mice by performing an autophagic flux assay.

4.4. FoxO3 and SIRT1. Forkhead box-containing protein class O (FoxO) family of DAF-16 like transcription factors are evolutionarily conserved transcriptional factors that regulate the expression of genes involved in multiple cellular functions including oxidative stress, glucose metabolism, apoptosis, cell cycle transition, and DNA repair [121, 122]. Four FoxO proteins including FoxO1, FoxO3, FoxO4, and FoxO6 are found in mammals, which have redundant yet distinctive roles in regulating gene expression. While FoxO1, FoxO3, and FoxO4 are ubiquitously expressed in most tissues, FoxO6 is mainly expressed in neurons. Studies from gene KO mice show that *FoxO1* KO mice are embryonically lethal due to impaired

angiogenesis but *FoxO4* KO mice are viable with decreased migration of vascular smooth muscle cells [123]. While *FoxO3* KO mice are also viable, female mice are infertile due to ovarian activation and they also have spontaneous T cell activation and lymphoproliferation with time [124]. FoxO3 mainly regulates the expression of genes responsible for oxidative stress, apoptosis, cell cycle transition, and DNA repair but FoxO1 is more important in regulating glucose and lipid metabolism. All the FoxO family proteins are subjected to multiple posttranslational modifications, including phosphorylation, acetylation, methylation, and ubiquitination [122]. Akt-mediated phosphorylation of FoxO3 causes its nuclear exclusion and thus inactivates FoxO3. It seems that the acetylation of FoxO3 mainly regulates the specificity of a subset of FoxO3 target genes by increasing the expression of antioxidant genes and suppressing the expression of apoptosis genes in response to oxidative stress [125], whereas methylation of FoxO3 at K270 results in loss of DNA binding [126].

Increasing evidence now suggests that FoxO family proteins can also regulate autophagy by three distinctive mechanisms: direct transcriptional regulation of Atg gene expression [127, 128], transcriptional regulation of glutamine synthetase expression and increasing intracellular glutamine levels [129], and interaction of cytosolic FoxO1 with Atg7 independent of its transcription activity [130].

Sirtuin 1 (Sirt1) belongs to the evolutionarily conserved sirtuin family, which are NAD-dependent class III protein deacetylases. There are 7 sirtuins (Sirt1–7) that have been identified in mammals, which have distinct cellular locations. Sirt1, 6, and 7 are mainly in the nucleus, Sirt2 is mainly in the cytosol, and Sirt3, 4, and 5 are found in the mitochondria [131, 132]. Sirt1 has a broad range of physiological functions including the control of aging, metabolism, and gene expression by promoting the deacetylation of a variety of substrates from histones to nonhistone proteins. Increasing evidence suggests that sirtuins also play roles in regulating autophagy. *Sirt1* KO mouse embryonic fibroblasts have decreased autophagy in response to starvation, which is accompanied by increased acetylated Atg5, Atg7, and LC3 proteins, although it remains unclear how increased acetylation of these proteins affects their functions on autophagy [133]. In response to stress, cytosolic FoxO1 is dissociated from Sirt2 resulting in an increase of acetylated FoxO1. Acetylated FoxO1 then binds to Atg7 and promotes autophagy in some human cancer cells [130]. Adult-onset and long-term calorie restriction in mice increased Sirt1 expression in aged kidney and attenuated hypoxia-associated mitochondrial and renal damage by enhancing Bcl2/adenovirus E1B 19-kDa interacting protein 3-(Bnip3-) dependent autophagy. This increased autophagy was found to be regulated by Sirt1-mediated FoxO3 deacetylation resulting in increased expression of Bnip3 under hypoxia conditions [134].

We recently demonstrated that acute ethanol treatment increased the expression of Atg genes in mouse liver and in primary cultured mouse and human hepatocytes, which was accompanied by increased hepatic nuclear accumulation of FoxO3 [89]. Acute ethanol treatment decreased the level of phosphorylated Akt, causing decreased FoxO3 phosphorylation at Ser253, which could account for increased nuclear FoxO3. Resveratrol increases Sirt1 activity by promoting its binding with both NAD^+ and the acetylated substrate through allosteric interaction [135]. Indeed, we found that activation of Sirt1 by resveratrol increased deacetylation of FoxO3 and enhanced ethanol-induced expression of Atg genes [89]. Moreover, we found that *FoxO3* KO mice had decreased expression of Atg genes and had increased steatosis and liver injury compared to wild type mice after acute ethanol treatment [89]. These findings indicate that FoxO3-mediated autophagy plays a protective role against alcohol-induced steatosis and liver injury. It has been suggested that ethanol consumption may inhibit Sirt1 via increased $NADH/NAD^+$ ratio through its metabolism. This may lead to the inhibition of autophagy in the liver either through FoxO3-dependent or independent mechanisms. However, ethanol consumption can also inhibit Akt phosphorylation, which can lead to increased nuclear retention of FoxO3 and ultimately increased expression of autophagy genes. Thus it is possible that Akt-mediated FoxO3 nuclear retention would be more important or dominant in regulating the expression of Atg genes than Sirt1-mediated acetylation of FoxO3. Nevertheless, our results suggest that activation of Sirt1, such as by using resveratrol, can further enhance ethanol-induced FoxO3-mediated expression of Atg genes. More studies are definitely needed to determine whether other FoxO and sirtuin family proteins are also involved in autophagy in alcohol-induced liver injury.

Nepal and Park recently also reported a link between AMPK/FoxO3 and autophagy when they studied the protective effect of globular adiponectin (gAcrp) on ethanol-treated HepG2 cells [136, 137]. The fat-derived hormone adiponectin is known to be protective in ALD [138]. Greer et al. showed that gAcrp restored ethanol-induced suppression of autophagy genes including *Atg5* and autophagosome formation, which was accompanied by FoxO3 translocation in HepG2 cells. Silencing FoxO3 or its upstream regulator AMPK [139] abrogated the restoration, indicating the importance of FoxO3 and AMPK in ethanol-mediated expression of autophagy genes. However, since HepG2 cells are human hepatoma cells and can barely metabolize ethanol, the interpretation of these results needs to be cautious.

4.5. Methionine, SAM, and Methylation. Methionine is a sulfur-containing essential amino acid that is important in biogenesis of cysteine, carnitine, taurine, lecithin, phosphatidylcholine, and other phospholipids. Catalyzed by methionine adenosyltransferase, a liver-specific enzyme, methionine is metabolized into S-adenosylmethionine (SAM), which is a universal methyl donor. After transferring the methyl group, SAM becomes S-adenosylhomocysteine, which can be further converted to adenosine and homocysteine via S-adenosylhomocysteine hydrolase. Homocysteine is a source for generation of methionine through methionine synthase (MS) and glutathione through cystathionine b-synthase [140]. Aberrant methionine metabolism has been well documented in ALD [140–142]. Ethanol exposure inhibits MS activity resulting in decreased hepatic methionine levels [140]. Cells activate a compensatory pathway in methionine

metabolism by increasing betaine homocysteine methyltransferase activity, but this pathway is compromised under extended chronic alcohol exposure resulting in a general decrease of hepatic SAM and essential methylation reactions [141].

Emerging evidence shows that posttranslational modification of proteins with methylation may play important roles in regulating autophagy in at least three aspects: methylation of protein phosphatase 2A (PP2A) to negatively regulate autophagy through modulating target of rapamycin (TOR) [143], epigenetic regulation of autophagy gene transcription through the methyltransferase G9a [144], and arginine methylation in selective autophagy [145]. In a recent yeast study, it was found that methionine and SAM inhibited autophagy and promoted growth through the protein phosphatase methyltransferase 1 (Ppm1p), which increases PP2A methylation. Methylated PP2A promoted the dephosphorylation of natriuretic peptide receptor B (Npr2), a yeast phosphoprotein that negatively regulates TORC1, resulting in TORC1 activation and autophagy inhibition [143]. However, whether methionine and SAM would also inhibit autophagy in mammals through similar mechanisms remains to be studied. The H3K9 methyltransferase G9a was also reported to inhibit autophagy by inducing an increase of dimethylated H3K9 (H3Kme2), which repressed the expression of several essential Atg genes including LC3B, WIPI1, and diabetes and obesity-regulated (DOR). Upon autophagy induction, G9a leaves the promoter region of *LC3B* to release its repression on the expression of *LC3B* and other Atg genes to promote autophagy [144]. For selective autophagy, it is known that the autophagy receptor complex is important for mediating recognition of cargos (such as ubiquitinated mitochondria) and also binds with autophagy machinery proteins (such as LC3), which allows the cargo to be selectively removed [146–149]. Phosphorylation of p62/SQSTM1 and Atg32, two important autophagy receptor proteins, has been shown to play important roles in selective removal of protein aggregates and mitochondria [150–152]. Optineurin, another autophagy receptor protein, is also phosphorylated by the protein kinase TBK1, which enhances its binding with LC3 resulting in selective autophagic clearance of cytosolic *Salmonella enterica* [153]. In addition to phosphorylation, arginine methylation is another major type of protein posttranslational modification and is catalyzed by protein arginine methyltransferases (PRMT). Interestingly, a recent study showed that mutations in *C. elegans epg-1I*, a homologue of mammalian *PRMT1*, led to the defective removal of P granule components phenolic glycolipid-1 (PGL-1) and PGL-3. Furthermore, mutating the methylated arginine residues on PGL-1 and PGL-3 resulted in impaired degradation of PGL-1 and PGL-3 [145]. These results indicate that modification of autophagic cargo proteins by arginine methylation may provide a regulatory mechanism for modulating autophagic degradation efficiency during selective autophagy. As discussed above, alcohol consumption impairs methionine metabolism and methylation reactions. It will be interesting to determine how these methylation changes would affect selective autophagy (such as mitophagy and lipophagy) and general autophagy in alcohol-induced liver disease in the future. The possible autophagic signaling pathways or targets modulated by ethanol are summarized in Figure 1.

5. Autophagy in Alcohol-Induced Pancreatitis

The pancreas is a glandular organ that has both endocrine and exocrine functions in vertebrates. In response to fasting or feeding, the pancreas secretes insulin or glucagon through its endocrine system (islet β or α cells) to maintain blood glucose levels. After a meal, the exocrine pancreas acinar cells release digestive enzymes into the pancreatic duct and ultimately the duodenal space. The exocrine acinar cells of the pancreas play a critical role in pancreatitis, and the hallmark changes of the acinar cells during pancreatitis include the intracellular activation of digestive enzymes, intracellular vacuolization, apoptosis, necrosis, edema, and inflammation [154, 155]. Acute and chronic pancreatitis are common gastrointestinal diseases that are potentially lethal with considerable morbidity and reduced life expectancy. Chronic pancreatitis is also associated with a high risk for the development of pancreatic adenocarcinoma, for which no treatment is currently available [156, 157].

While the molecular mechanisms for induction of acute pancreatitis (AP) are still poorly understood, several mechanisms have been identified that may play critical roles in the development of AP. Among them, premature activation of trypsin from trypsinogen within acinar cells to further trigger activation of the cascade of other pancreatic digestive zymogens has been considered a key pathogenic mechanism [154, 156]. This notion is also supported by the genetic evidence that mutations in cationic trypsinogen (*PRSS1*), pancreatic secretory trypsin inhibitor (*SPINK1*), and chymotrypsinogen C (*CTRC*) are associated with the susceptibility of pancreatitis [157]. However, the importance of the intra-acinar activation of trypsin has been challenged recently because it has been found that *trypsinogen isoform 7* (*T7*) KO mice have decreased trypsin activity but still develop pancreatitis upon cerulein-induced pancreatitis [158]. Furthermore, it has also been shown that intra-acinar trypsinogen activation leads to induction of acinar cell apoptosis resulting in the resolution of acute inflammation without causing chronic pancreatitis accompanied by fibrosis [159]. Therefore, other pathways may also be critical in the development of AP. Induction of inflammatory mediators is also a key feature in AP, and nuclear factor-κB (NF-κB) has been shown to play a critical role in AP. However, both activation and inhibition of NF-κB have been shown to exacerbate acinar cell injury from experimental animal pancreatitis models, and clearly more studies are needed to further clarify the exact role of NF-κB in the pathogenesis of AP [160–164]. In addition, acinar cells have an extensive network of ER to produce large amount of digestive enzymes, and thus acinar cells are more susceptible to ER stress. Mice with an acinar cell-specific deletion of X-box binding protein 1 (XBP-1), one of the three important unfolded protein response (UPR) proteins in response to ER stress, have extensive acinar cell apoptosis followed by pancreas regeneration [165]. Chronic ethanol feeding induces ER stress and the UPR response in mouse acinar cells and ethanol fed XBP1$^{+/-}$ mice show loss of enzymogen granules

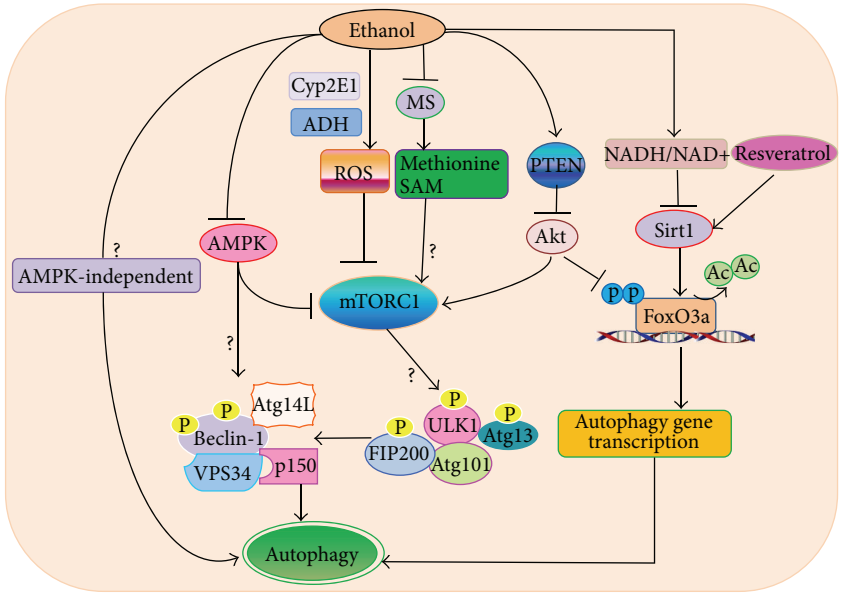

Figure 1: The major molecular pathways and targets in alcohol-induced autophagy changes in hepatocytes. Ethanol modulates autophagy through multiple mechanisms. (1) Ethanol-induced autophagy requires ethanol metabolism and ROS production. ROS may activate autophagy by further suppressing mTOR. (2) Alcohol (ethanol) consumption inhibits methionine synthase (MS) resulting in decreased methionine and S-adenosylmethionine (SAM) levels. Methionine and SAM inhibit autophagy by activating mTORC1. Thus it is possible that ethanol-induced decreased methionine and SAM will inhibit mTORC1 resulting in autophagy activation although this has not been directly tested in the alcohol model (?). (3) Ethanol may also suppress Akt through the upregulation of PTEN and in turn inhibits mTORC1 to induce autophagy. (4) Ethanol-induced impaired AMPK and Akt may counteract each other on mTOR, and impaired Akt plays a dominant role toward the inhibition of mTOR. (5) Decreased Akt can also trigger autophagy through the activation of FoxO3 by promoting the dephosphorylation and nuclear retention of FoxO3. Increased NADH/NAD^+ ratio through ethanol metabolism inhibits Sirt1 activity resulting in increased acetylated FoxO3. Increased acetylated FoxO3 may decrease FoxO3-mediated expression of autophagy genes, which can be abolished by resveratrol that activates Sirt1. (6) Other AMPK-independent pathways remain to be determined in alcohol-induced autophagy (?). (7) mTORC1 negatively regulates autophagy through direct phosphorylation of ULK1 to inactivate ULK1 complex activity. ULK1 directly phosphorylates Beclin-1 and enhances VPS34 kinase activity to promote autophagy. AMPK positively regulates autophagy by suppressing mTORC1 activity through phosphorylation of TSC2 and raptor and by promoting VPS34 kinase activity through phosphorylation of Beclin-1. Activated VPS34 increases the production of phosphatidylinositol 3-phosphate (PI3P), which promotes the biogenesis of autophagosomes although the activities of ULK1 and VPS34 after alcohol exposure still remain to be determined (?).

and increased acinar cell death [166]. These findings suggest that ER stress and defective UPR may contribute to acinar cell death and pancreatitis. As an inflammatory disorder disease, acinar cell death is a key event in AP and both apoptotic and necrotic acinar cell death have been observed in experimental models of AP. It seems that acinar cell apoptosis can attenuate cerulein-induced acinar cell necrosis and protects against cerulein-induced AP by promoting caspase-mediated RIP cleavage [167]. Necrosis, which was initially thought of as a nonprogrammed cell death, has recently been shown to be highly regulated through formation of the necrosome. This programmed necrosis is also called necroptosis and is mainly mediated by RIP1–RIP3 signaling pathways. RIP1 and RIP3 are serine/threonine kinases, and their kinase activities are necessary for the formation of the necrosome, which further recruits downstream mixed lineage kinase domain-like protein (MLKL) and phosphoglycerate mutase family member 5 (PGAM5) [168–171]. PGAM5 is a mitochondrial phosphoglycerate mutase, which can dephosphorylate dynamin-related protein 1 (Drp1) resulting in Drp1 mitochondrial translocation and mitochondrial fragmentation to trigger necroptosis [171]. RIP3 can also directly phosphorylate MLKL to cause the translocation of MLKL to the plasma membrane and subsequent membrane rupture and necrosis [172–174]. *RIP3* or *MLKL* KO mice are resistant to cerulein-induced AP, suggesting that necroptosis plays a critical role in animal experimental AP, but its relevance to human AP is not clear.

Although prolonged alcohol abuse is correlated with the clinical symptoms of a vast array of pancreatic diseases, alcohol alone does not cause severe pancreatic damage in human. Only a minority of subjects (~10%) who abuse alcohol develop clinical pancreatitis, indicating that other cofactors like environmental and genetic elements also contribute to disease development [175]. Cigarette smoking [175–177] and dietary habits [178] may be involved in the progress of alcoholic pancreatitis. Smoking accelerates the deterioration of pancreatitis because it significantly increases the risk of pancreatic calcifications [179]. While high fat and protein diets appear to exacerbate the course of chronic pancreatitis, saturated fatty acids and vitamins, especially vitamin E, may play a protective role against the detrimental effects on the pancreas caused by alcohol [180, 181]. Moreover, it seems that gender and ethnicity may also be factors for alcoholic pancreatitis; that is, men have a higher risk than women, and

African Americans have a greater chance for development of chronic alcoholic pancreatitis than other ethnic groups.

There are several widely used rodent models for nonalcoholic pancreatitis. Reliable AP animal models should reproduce the clinical pathophysiology, symptomatology, and etiology, such as a significant activation of serum pancreatic enzymes, remarkable histological changes, and pancreatitis-associated complications [182]. Choline-deficient, ethionine-supplemented diet is a widely used model for inducing pancreatitis since a synergistic action of choline deficiency with the basic toxicity of ethionine on acinar cells leads to intraparenchymal activation of zymogens [183]. Young female mice fed with this diet developed acute hemorrhagic pancreatitis with massive fat necrosis throughout the peritoneal cavity. However, the nonselective effects of this model hamper its ability to study pancreatitis-induced multiply organ dysfunction syndromes as it can directly affect liver and brain. The intraperitoneal injection of L-arginine can induce acute necrotizing pancreatitis in rats and mice [184, 185]. This model has high specificity and flexibility in controlling the extent of pancreatic severity, which makes it suitable for studying extrapancreatic organ damage, but the precise mechanism is not fully understood. Accumulated evidence suggests that nitric oxide (NO) [186], oxygen free radicals [187, 188], and inflammatory mediators [189, 190] are all involved in the progression of the disease. Treatment with supramaximal cholecystokinin (CCK) or its analogue cerulein induces pancreatitis in rodents, which has been studied extensively since the pathological and histological presentation of this model is similar to the early phase of AP in human [191]. Furthermore, both CCK and cerulein can be used to initiate hyperstimulation-induced pancreatitis in primary cultured acinar cells, which makes it a valuable tool for studying the pathophysiology and mechanisms of secretagogue-induced pancreatitis [192]. In addition to these noninvasive models, a closed duodenal loop-induced pancreatitis model is utilized for studying duodenal reflux-induced AP [193], and the duct obstruction model is used to mimic gallstone obstruction-induced AP in the clinical setting [194, 195]. Moreover, duct infusion-induced pancreatitis is also used in combination with bile acids, like taurocholate or glycodeoxycholic acid to trigger necrotizing AP [196].

Similar to the animal models for alcohol-induced liver injury, neither acute nor chronic administration of ethanol alone in rodents leads to pancreatitis. Alcohol-induced pancreatitis requires other additional factors such as a viral infection, a high fat diet or submaximal postprandial dose of CCK or cerulein or cholinergic stimulation (such as by carbachol). Though the precise mechanisms by which alcohol induces pancreatic damage remain vague, several mechanisms have been suggested. A series of elegant published works from Gaisano's group showed that alcohol consumption may alter apical and basolateral exocytosis in pancreatic acinar cells [197, 198]. Mechanistically, it has been shown that alcohol induced protein kinase Cα, which phosphorylated Munc18c and displaced it from binding to basolateral plasma membrane syntaxin 4 (Syn-4), which results in formation of the Syn4/synaptosomal-associated protein 23 (SNAP23)/vesicle-associated membrane protein 8 (VAMP8) fusion complex. The Syn4/SNAP23/VAMP8 fusion complex then redirected the zymogen from apical exocytosis to basolateral exocytosis, which causes pancreatitis [197–199]. Chronic ethanol feeding promotes a shift of acinar cell apoptosis to necrosis, but little is known about the mechanisms involved. In pancreas, ethanol is metabolized through both oxidative and nonoxidative pathways. Oxidative metabolism of ethanol is mediated by ADH in cytosol and ALDH2 in mitochondria, which generates acetaldehyde and acetate, respectively. Nonoxidative metabolism converts ethanol to FAEE via fatty acyl responsive regulator (FarR) synthase. It has been shown that oxidative metabolism of ethanol causes mitochondrial failure by activating mitochondrial permeability transition, a key event in regulating cell death [200]. However, whether ethanol feeding would affect other necrotic proteins such as RIP3 in pancreas is not known although RIP3 has been reported to be important in mediating ethanol feeding-induced liver injury in mice [48]. In addition, as discussed above, ethanol feeding may also induce ER stress in acinar cells to trigger cell death [166]. Alcohol consumption can increase gut permeability, which causes bacterial translocation across the mucosal barrier, and leads to the elevation of lipopolysaccharide (LPS) levels. Alcohol-fed rats that were further treated with LPS had increased expression of TGF-β, which led to subsequent pancreatic fibrosis [3].

Increasing evidence now supports the role of autophagy in both alcoholic and nonalcoholic pancreatitis, and it is generally thought that lysosomal/autophagic dysfunction can initiate pancreatitis. It has long been noted that there is an increased accumulation of large intracellular vacuoles in acinar cells in both experimental and human pancreatitis, and recent evidence indicates that the nature of these vacuoles is autophagic and lysosomal origin because these structures have double-membrane and are positive for LC3-II. In cerulein-induced acute AP, there was an increase in autophagosome numbers but autophagic flux was impaired due to lysosomal dysfunction [201]. Decreased Lamp-2 proteins and possible fusion of autophagosomes with lysosomes were also found in alcohol- and LPS-induced AP [202]. Moreover, $XBP1^{+/-}$ mice fed with chronic ethanol had increased acinar cell death with loss of enzymogen granules. These mice also had increased LC3-II levels in acinar cells although autophagic flux assay was not performed in this study [166]. Mechanistically, it has been suggested that autophagy may help remove zymogens through a selective process termed zymophagy, which is regulated by the vacuole membrane protein- (VMP1-) USP9x-p62/SQSTM1 complex and attenuates intra-acinar trypsinogen activation. VMP1 interacts with Beclin-1 to promote the formation of autophagosomes, and it also interacts with the ubiquitin-protease USP9x to induce selective zymophagy, which prevents acinar cell death [203, 204]. In addition, results from Gukovskaya's group suggest that inefficient autophagic degradation of zymogens due to defective lysosomal proteolytic activity may promote pancreatitis. They also proposed that an imbalance between cathepsin B (CatB) and cathepsin L (CatL) may result in decreased degradation of trypsin, which leads to pancreatitis [155, 192]. Moreover, mice with pancreas-specific deletion of IKK-α, an essential component for NF-κB activation, develop

spontaneous pancreatitis. Interestingly, decreased autophagic flux has been found in the mouse pancreas with specific deletion of IKK-α. Similar to the autophagy-deficient liver, increased p62/SQSTM1 levels were also found in the IKK-α-deficient pancreas, and further deletion of p62/SQSTM1 in the pancreas attenuated pancreatitis in pancreas-specific IKK-α-deficient mice [160]. Taken together, it seems that all of the above evidence supports that impaired autophagy may contribute to pancreatitis. Nevertheless, an early study using pancreas acinar cell-specific-*Atg5* KO mice showed decreased acinar cell vacuolization and pancreatitis after cerulein treatment, and the authors proposed that autophagy machinery may be required for the trypsinogen activation to induce pancreatitis [205]. These results from acinar cell-specific *Atg5*-KO mice seem to be contradictory to the above other findings that suggest impaired autophagy promotes pancreatitis. However, Atg5 mainly regulates the upstream formation of autophagosomes, and it is possible that upstream autophagy (autophagosome biogenesis) and downstream of autophagy (autolysosome degradation) could play different roles in pancreatitis. Trypsinogen may use autophagosomes as vehicles for transport to lysosomes where trypsinogen is activated. Indeed, inhibition of the early phase of autophagy by 3-methyladenine (3-MA) completely blocked trypsinogen activation [201]. In contrast, impaired functions of downstream autolysosomes also led to trypsinogen activation and pancreatitis [155, 206]. Therefore, it is possible that targeting different phases of autophagy may lead to different outcomes of pancreatitis. Suppression of early phase autophagosome formation and improvement of late autolysosome functions may attenuate pancreatitis, but future experiments are needed to test this hypothesis.

6. Autophagy in Other Tissue Injury Induced by Alcohol

6.1. Heart. Heart is mainly comprised of long-lived and postmitotic cardiomyocytes. Increasing evidence indicates that autophagy plays an important role in maintaining the function and viability of cardiomyocytes by controlling the homeostasis of intracellular proteins, energy, and organelles [207]. Studies from genetic KO animal models, such as using the cardiomyocyte-specific *Atg5* KO mice, revealed that basal autophagy plays a vital housekeeping role in removing damaged organelles and proteins in cardiomyocytes to maintain their normal functions [208]. In contrast, both protective and detrimental roles of autophagy have been reported in "stressed" or "diseased" heart. For example, induction of autophagy is protective against ischemia-induced heart injury, whereas autophagy could be detrimental in pressure overload-induced heart failure and during reperfusion [209, 210].

Low to moderate alcohol consumption is beneficial to patients with cardiovascular events [211]. In contrast, heavy alcohol consumption impairs cardiac geometry and function [5] and increases the incidence of sudden cardiac death and ventricular arrhythmias [212]. Whether autophagy plays a protective or detrimental role in alcoholic heart disease is not fully understood. Jun Ren's team has conducted a series of studies on ethanol-induced cardiac dysfunction with a focus on autophagy [213–217]. In both binge [213] and chronic [214] alcohol models, heart LC3-II levels were increased in an AMPK-dependent manner. Furthermore results from this group's studies tend to suggest that autophagy may contribute to alcohol-induced malfunction of cardiomyocytes. Acute ethanol treatment led to compromised heart functions with decreased fractional shortening, peak shortening, and an intracellular Ca^{2+} rise in mouse cardiomyocytes. Acute ethanol exposure also increased LC3-II level, which was accompanied by increased phosphorylation of AMPK and Raptor and decreased phosphorylation of mTOR and ULK1 in mouse cardiomyocytes. Interestingly, pharmacological or genetic inhibition of AMPK attenuated ethanol-induced autophagosome formation and cardiomyocyte apoptosis. Moreover, 3-MA reversed ethanol-induced cardiomyocyte contractile defects [215]. Similar to acute ethanol treatment, chronic ethanol feeding also led to increased autophagosome formation in mouse cardiomyocytes with heart hypertrophy and cardiomyocyte contractile anomalies, and 3-MA treatment also ablated this ethanol-induced cardiomyocyte malfunction [216]. Moreover, mice with cardiac-specific overexpression of ADH, which metabolizes alcohol to acetaldehyde, were more susceptible to ethanol-induced autophagy changes and ethanol-induced damage of cardiomyocytes [216]. In contrast, transgenic mice overexpressing ALDH2, which converts acetaldehyde to acetate during alcohol metabolism, blunted chronic alcohol-induced mTOR inhibition and increased LC3-II levels resulting in improved cardiac geometry and function in alcohol-treated mice [214]. These findings suggest that the ethanol metabolite acetaldehyde may account for ethanol-mediated autophagy changes and impaired cardiac functions.

While these data generally suggest that either acute or chronic ethanol treatment may induce autophagy and contribute to ethanol-induced malfunction of cardiomyocytes, no clear autophagic flux data were shown in these studies. It is intriguing that acute ethanol treatment increased both LC3-II and p62/SQSTM1 in mouse cardiomyocytes [215], although it is not clear whether the increased p62/SQSTM1 was due to decreased degradation or increased transcription. More studies, in particular the use of genetic autophagy-deficient animal models, are definitely needed to further clarify the autophagy status after alcohol exposure and the exact role of autophagy in alcohol-induced heart dysfunction.

6.2. Brain. It is well established that excessive ethanol intake results in regional brain damage and cognitive dysfunction [6, 218]. Potential mechanisms that are responsible for alcohol-induced brain injury include higher sensitivity for excitotoxicity, impaired catabolism of homocysteine, reduced neurotrophic factors, failure to repair damaged DNA, acetaldehyde adduct formation, and so on [218]. It is generally thought that, in the mature mammalian brain, autophagy is hard to detect even under nutrient deprivation conditions [219]. This is probably due to the vital functions of brain that need to be protected from even systemic nutrient deprivation. However, neural cell-specific *Atg5* KO mice have increased accumulation of cytoplasmic inclusion bodies in neurons and

develop progressive deficits in motor function, suggesting that basal autophagy in the brain is important for preventing the accumulation of abnormal proteins to preserve neural function and protects against neurodegeneration [220]. Moreover, both increased and impaired autophagy have also been observed in various acute brain injuries including those induced by alcohol [221, 222].

Ethanol treatment increased autophagic flux in SH-SY5Y neuroblastoma cells and in the developing mouse brain through inhibition of mTOR. More importantly, induction of autophagy by rapamycin attenuated ethanol-induced ROS production and neuronal cell death in SH-SY5Y cells and in the mouse developing brain. In contrast, inhibition of autophagy either by wortmannin or shRNA knockdown of Beclin-1 exacerbated ethanol-induced neurotoxicity [222]. Moreover, hypoxic preconditioning activated autophagy and protected against ethanol-induced neurotoxicity, which was abolished when autophagy was inhibited by either bafilomycin A 1 or wortmannin [223]. These results suggest that autophagy protects against ethanol-induced neuronal injury. Fetal alcohol spectrum disorder (FASD) results from prenatal exposure to alcohol, which is the leading cause of mental retardation. Children with FASD often have neuropsychological and behavioral problems and develop secondary disabilities including depression and anxiety disorder. Alimov et al. [224] found that subcutaneous injection with ethanol induced neuroapoptosis in postnatal day 4 mice but not in postnatal day 12 mice. Interestingly, they further found that the expression of genes that regulate autophagy and the UPR was lower whereas the expression of proapoptotic genes was higher in postnatal day 4 mice than postnatal day 12 mice. These results imply that decreased autophagy activity may contribute to the vulnerability of the immature brain to alcohol exposure. However, a more recent study found that administration with 10% (v/v) ethanol for 4 month led to an accumulation of polyubiquitinated proteins in the mouse cerebral cortex likely due to an impaired ubiquitin-proteasome system and autophagy. Specifically, it was found that ethanol treatment increased mTOR activity and decreased expression of several Atg genes including *Atg12*, *Atg5*, *p62/SQSTM1,* and *LC3*. Ethanol treatment also increased brain inflammatory mediators such as IFN-γ. Interestingly, these ethanol-induced changes were attenuated in *toll-like receptor 4* (*TLR4*) KO mice, which were protected against chronic ethanol exposure-induced brain injury [225]. These results suggest that ethanol-induced impairment of the ubiquitin proteasome system and autophagy could be due to the activation of TLR4 by inflammatory mediators. In the future, more studies are needed to determine whether autophagy is activated or impaired after alcohol consumption using different animal models such as Gao-binge model. Moreover, it will also be interesting to determine whether the metabolism of ethanol is required for ethanol-induced changes on autophagy in brain. Nevertheless, it seems that activation of autophagy is beneficial for alcohol-induced brain injury.

6.3. Muscle. Skeletal muscles are composed of myofibers that control our body's motion. Myofibers are composed of myofibrils that form highly organized units called sarcomeres, which contain repeated actin and myosin filaments. Approximately 40% of our body mass is from skeletal muscle, which also plays a critical role in regulating metabolism by providing amino acids through breaking proteins and organelles to meet the energy needs of the body [226]. Thus, it is not surprising that emerging evidence suggests that autophagy is important for controlling muscle mass. Modulating muscle autophagy also influences exercise and energy and lipid metabolism [226, 227]. Both beneficial and deleterious roles of autophagy in regulating muscle mass/wasting have been proposed. Activation of FoxO3 led to increased expression of Atg genes and activation of autophagy, which resulted in muscle atrophy [127, 128]. MTMR14 is a lipid phosphatase that antagonizes VPS34 to dephosphorylate PI3-P to phosphatidylinositol (PI) and thus inhibits autophagy. Increased autophagy and muscle atrophy have been reported in *MTMR14* knockdown zebrafish. Moreover, centronuclear myopathy was also found in humans that have *MTMR14* mutations [228]. Paradoxically, muscle-specific *Atg7* KO mice also developed myofiber degeneration and muscle atrophy accompanied with increased accumulation of protein aggregates, abnormal mitochondria, sarcoplasmic reticulum distension, vacuolization, increased oxidative stress, and apoptosis [229]. It is possible that autophagic degradation of proteins may lead to muscle atrophy whereas the muscle atrophy observed in the muscle autophagy-deficient mice is a maladaptive response due to the chronic loss of autophagy.

In addition to regulating muscle mass, autophagy in muscle also regulates body glucose and lipid metabolism. It has been shown that exercise induces autophagy in multiple organs involved in metabolic regulation including muscle, liver, pancreas, and adipose tissue [230]. Exercise increases Bcl-2 phosphorylation resulting in its dissociation from Beclin-1, which leads to the initiation of autophagy. Nonphosphorylatable mutation in Bcl-2 (Thr69Ala, Ser70Ala and Ser84Ala, Bcl2 AAA) knock-in mice causes them to be defective in exercise- and starvation-induced autophagy, and they show decreased exercise endurance. These defects are due to impaired exercise-induced skeletal muscle glucose uptake because of a loss in glucose transporter 4 (GLUT4) translocation [230, 231]. These findings suggest that autophagy may be beneficial for glucose homeostasis during exercise. Moreover, studies from muscle-specific *Atg7* KO mice also reveal that autophagy in muscle may regulate glucose and lipid homeostasis [232]. *Atg7* muscle-specific KO mice have decreased fat mass and are resistant to high fat diet-induced obesity and insulin resistance. Mechanistically, it has been suggested that loss of *Atg7* may lead to accumulation of damaged mitochondria, which induces an Atf4-dependent production of fibroblast growth factor 21 (Fgf21) that increases fatty acid oxidation and browning of white adipose tissue (WAT) [232]. Thus, these seemingly beneficial effects of loss of muscle autophagy on glucose and lipid metabolism could be a secondary adaptive response in response to organelle damage induced by the loss of autophagy. It is not clear how long these adaptive responses would last and whether maladaptive responses would develop after long-term loss of muscle autophagy.

Table 1: Summary of *in vivo* studies on autophagy in alcohol-induced tissue injury.

	Model	Level of autophagy	Role of autophagy	References
Liver	Acute	Activated	Protective	Ding et al., 2010 [90]; Ni et al., 2013 [89]; Thomes et al., 2013 [93]
		Impaired*	Protective	Wu et al., 2012 [91]; Yang et al., 2014 [100]; Zeng et al., 2012 [108]
	Chronic	Activated	Protective	Lin et al., 2013 [99]
Pancreas	Chronic	Impaired	Protective	Fortunato et al., 2009 [202]
Heart	Acute	Activated*	Detrimental	Ge et al., 2011 [213]; Guo and Ren, 2012 [215]; Kandadi et al., 2013 [217]
	Chronic	Activated*	Detrimental	Ge and Ren, 2012 [214]; Guo et al., 2012 [216]
Brain	Acute	Activated	Protective	Chen et al., 2012 [222], Alimov et al., 2013 [224]; Wang et al., 2013 [223]
	Chronic	Impaired	Protective	Pla et al., 2014 [225]
Skeletal muscle	Chronic	Activated	Detrimental	Thapaliya et al., 2014 [235]

Note: *autophagy flux assay is lacked.

It is well known that chronic alcoholics have severe muscle loss and myopathy. Both *in vivo* and *in vitro* studies show that ethanol can inhibit skeletal muscle protein synthesis, which is likely mediated by increased expression of insulin-like growth factor binding protein-1 and myostatin (a TGFβ superfamily member) resulting in the inhibition of mTOR and limitation of translational efficiency [7, 233, 234]. Using skeletal muscle biopsies from alcoholic cirrhotics, gastrocnemius from ethanol and pair-fed mice, and ethanol-exposed murine myotubes, Thapaliya et al. [235] provided evidence that autophagy contributes to alcohol-induced skeletal muscle loss. Using a standard CT imaging technique, it was found that alcoholic cirrhotics had lower muscle mass than controls. Interestingly, proteasome components and activity were decreased in alcoholic biopsy samples, suggesting that decreased skeletal mass in alcoholic cirrhotics is less likely mediated by the proteasome. Indeed, they found that the expression of several essential Atg genes and autophagic flux were increased in alcoholic biopsy samples, ethanol-fed mice, and ethanol-treated C2C12 murine myotubes. Alcohol-induced autophagy was mediated by acetaldehyde, the metabolite of ethanol, rather than ethanol *per se*. More importantly, pharmacological or genetic inhibition of autophagy mitigated the proteolysis of myotubes and the reduction of muscle mass [235]. However, most of the results were obtained from short term alcohol exposure experiments. It is uncertain whether long-term blockage of autophagy would be beneficial for alcohol-induced muscle loss. More studies are needed to further dissect the underlying mechanisms by which autophagy regulates skeletal muscle mass in alcoholics.

7. Concluding Remarks and Future Perspective

Recent rapid research progress has significantly enriched our knowledge on the molecular mechanisms regulating autophagy and its impact on human diseases. As outlined in this review, autophagy plays significant roles in alcohol consumption-induced multiple tissue/organ injuries including hepatic steatosis and liver injury, pancreatitis, impaired heart function, brain damage, and loss of muscle mass. While autophagy has been generally considered as a cell survival mechanism, both beneficial and detrimental effects of autophagy have been reported in alcohol-induced multiple tissue/organ injuries (Table 1). As a critical cellular mechanism sentinel for the homeostasis of proteins, energy, and organelles, autophagy may be beneficial for alcohol-induced liver injury through removing damaged mitochondria and lipid droplets, for AP through preventing zymogen activation and for brain injury through inhibiting ROS generation. However, autophagy seems to be detrimental for alcohol-induced heart malfunction and muscle atrophy, although more studies are needed to further confirm these concepts due to limited research and lack of clear autophagic flux data in these two areas (Figure 2). Given the dynamic nature of autophagy and the chronic alcohol consumption process, we are still facing great challenges to monitor the autophagy status *in vivo* for chronic diseases induced by alcohol consumption. Similarly, it is also difficult to monitor the autophagy status *in vivo* after chronic modulation of autophagy using either pharmacological autophagy inducers or inhibitors. More reliable *in vivo* autophagic flux assays are urgently needed to help further assess the therapeutic potential of pharmacological modulation of autophagy as a means to treat alcohol-induced tissue injuries.

Abbreviations

ADH: Alcohol dehydrogenase
AICAR: 5-Amino-4-imidazole carboxamide riboside
Akt/PKB: Protein kinase B
ALD: Alcoholic liver disease
ALDH: Aldehyde dehydrogenase
Ambra1: Activating molecule in Beclin-1-regulated autophagy protein 1
AMPK: Amp-activated protein kinase
AP: Acute pancreatitis
Atg: Autophagy-related
Bcl-2: B-cell lymphoma 2
Bcl-xL: B-cell lymphoma-extra large
Bnip3: Bcl-2/adenovirus E1B 19 kDa interacting protein 3
CatB: Cathepsin B
CatL: Cathepsin L
CBS: Cystathionine b-synthase
CCK: Cholecystokinin

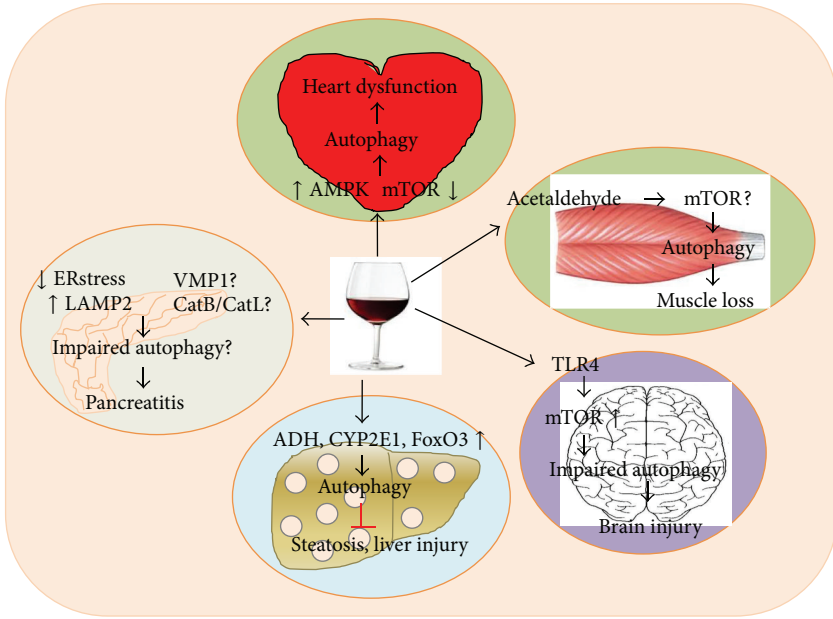

Figure 2: Differential roles of autophagy in alcohol-induced multitissue injury. Emerging evidence now indicates that alcohol consumption can either activate or impair autophagy as either a cellular adaptive/compensatory protective mechanism or as a detrimental factor contributing to alcohol-induced injury in various tissues/organs. In liver, it seems that alcohol metabolism through ADH and Cyp2E1 is required for autophagy activation. Acute alcohol treatment also induces FoxO3-mediated autophagy. Autophagy seems to selectively remove damaged mitochondria and excess lipid droplets and in turn attenuate alcohol-induced steatosis and liver injury. In pancreas, alcohol can induce ER stress and also decrease LAMP2 in the presence of endotoxin LPS, which leads to impaired autophagy resulting in pancreatitis. It is not known whether alcohol consumption would affect VMP1 and the ratio of CatB (cathepsin B)/CatL (cathepsin L), two important factors that regulate autophagy and pancreatitis, respectively. In heart, alcohol may activate autophagy through activating AMPK and inactivating mTOR. Autophagy activation seems to contribute to alcohol-induced heart dysfunction. In muscle, metabolism of alcohol to acetaldehyde activates autophagy resulting in muscle loss. Whether alcohol-induced autophagy in muscle is mediated by mTOR is not clear. In brain, alcohol increases mTOR and impairs autophagy in the mouse cerebral cortex resulting in brain injury, which is TLR4 dependent. Together, it is clear that alcohol can affect the autophagy process and in turn regulate tissue injury in various tissues/organs.

CP: Chronic pancreatitis
CTRC: Chymotrypsinogen C
Cyp2E1: Cytochrome P450, family 2, subfamily E, polypeptide 1
DFCP1: Double FYVE-containing protein 1
Drp1: Dynamin-related protein 1
EGFR: Epidermal growth factor receptor
ER: Endoplasmic reticulum
FarR: Fatty acyl responsive regulator
FIP200: FAK family-interacting protein of 200 kDa
FoxO: Forkhead box-containing protein, class O
gAcrp: Globular adiponectin
KO: Knockout
LAMP: Lysosomal-associated membrane protein
LC3: Microtubule-associated protein 1 light chain 3
LPS: Lipopolysaccharide
MLKL: Mixed lineage kinase domain-like protein
MTMR: Myotubularin-related protein
3-MA: 3-Methyladenine
MS: Methionine synthase
mTOR: Mammalian target of rapamycin
mTORC1: Mammalian target of rapamycin complex 1
NAD^+/NADH: Nicotinamide adenine dinucleotide
NIAAA: National Institute on Alcohol Abuse and Alcoholism
NF-κB: Nuclear factor-Kb
Npr2: Natriuretic peptide receptor B
PGAM5: Phosphoglycerate mutase family member 5
PGL: Phenolic glycolipid
PI3K: Phosphoinositide 3-kinase
PI3P: Phosphatidylinositol 3-phosphate
PIP3: Phosphatidylinositol-3,4,5-trisphosphate
PP2A: Protein phosphatase 2A
Ppm1p: Protein phosphatase methyltransferase 1
PRMT: Protein arginine methyltransferases
PRSS1: Cationic trypsinogen
PTEN: Phosphatase and tensin homolog
RIP: Receptor interacting protein kinase
ROS: Reactive oxygen species
Rubicon: Run domain protein as Beclin-1 interacting and cysteine-rich containing
SAM: S-Adenosylmethionine
SIRT: Sirtuin
SNAP23: Synaptosomal-associated protein 23
SPINK1: Pancreatic secretory trypsin inhibitor
SQSTM1: Sequestosome 1 (p62)

SREBP-1: Sterol regulatory element-binding protein-1
STX17: Syntaxin 17
Syn-4: Syntaxin 4
T7: Trypsinogen isoform 7
TLR: Toll-like receptor
TOR: Target of rapamycin
TORC: Target of rapamycin complex
ULK1: Unc-51 like kinase 1
UPR: Unfolded protein response
UVRAG: UV irradiation resistance-associated gene
VAMP8: Vesicle-associated membrane protein 8
VMP1: Vacuole membrane protein 1
WIPI: WD-repeat domain phosphoinoside-interacting protein
XBP1: x-box binding protein 1.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Capitalizing on the Autophagic Response for Treatment of Liver Disease Caused by Alpha-1-Antitrypsin Deficiency and Other Genetic Diseases

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Alpha-1-antitrypsin deficiency (ATD) is one of the most common genetic causes of liver disease and is a prototype of liver diseases caused by the pathologic accumulation of aggregated mutant alpha-1-antitrypsin Z (ATZ) within liver cells. In the case of ATD-associated liver disease, the resulting "gain-of-function" toxicity can lead to serious clinical manifestations, including cirrhosis and hepatocellular carcinoma. Currently, the only definitive therapy for ATD-associated liver disease is liver transplantation, but recent efforts have demonstrated the exciting potential for novel therapies that target disposal of the mutant protein aggregates by harnessing a cellular homeostasis mechanism called autophagy. In this review, we will summarize research advances on autophagy and genetic liver diseases. We will discuss autophagy enhancer strategies for liver disease due to ATD and another genetic liver disease, inherited hypofibrinogenemia, caused by the proteotoxic effects of a misfolded protein. On the basis of recent evidence that autophagy plays a role in cellular lipid degradation, we also speculate about autophagy enhancer strategies for treatment of hepatic lipid storage diseases such as cholesterol ester storage disease.

1. Introduction

Alpha-1-antitrypsin deficiency (ATD) is one of the most common genetic causes of liver disease. It is characterized by accumulation of a misfolded secretory protein in the endoplasmic reticulum of liver cells. In some affected homozygotes this "proteotoxic" state leads to hepatic fibrosis/cirrhosis and hepatocellular carcinoma. Investigations in a variety of model systems have shown that macroautophagy is activated when the mutant α1-antitrypsin molecule, α1-antitrypsin Z (ATZ), accumulates in cells and autophagy plays a key role in intracellular degradation of ATZ. Therapeutic strategies that enhance autophagy, using either drugs or gene transfer with a transcriptional activator of autophagy, have recently been shown to reverse hepatic accumulation of misfolded protein and hepatic fibrosis in a mouse model of ATD. In this review we will discuss autophagy enhancer strategies for liver disease due to ATD and another genetic liver disease, inherited hypofibrinogenemia, caused by the proteotoxic effects of a misfolded protein. On the basis of recent evidence that autophagy plays a role in cellular lipid degradation, we also speculate about autophagy enhancer strategies for treatment of hepatic lipid storage diseases such as cholesterol ester storage disease.

2. Mechanisms of Liver Disease in ATD

α1-Antitrypsin (AT) is an abundant serum glycoprotein predominantly synthesized by liver parenchymal cells. It is a prototype member of the serine protease inhibitor family, known as serpins, with a particular strong profile for inhibiting neutrophil elastase, cathepsin G, and proteinase 3. The classical form of ATD (referred to as PIZZ) results

from a point mutation in the AT gene that leads to a lysine to glutamate substitution at residue 342 of the protein that renders the mutant AT protein (termed ATZ) prone to misfolding. A number of studies have shown that ATZ is also prone to polymerization and aggregation, and it is likely that this aggregation-prone tendency plays a distinct role in the nature of the pathology of the disease [1]. The characteristic histologic finding in the liver is periodic acid-Schiff- (PAS-) positive, diastase-resistant globules in hepatocytes [2], representing the accumulation of ATZ within the early compartments of the secretory pathway. Because of misfolding, the mutant form of AT is inefficient in traversing the secretory pathway, and so there is a substantial reduction of AT in the systemic circulation, such that serum AT levels are approximately 10–15% of normal. Over the years, studies of this deficiency have led to the conclusion that the two consequences of misfolding are responsible for the two major clinical manifestations. Decreased serum levels of AT are primarily responsible for lung damage because there is loss of antineutrophil elastase function and neutrophil elastase can destroy the extracellular matrix of the lung. Intracellular accumulation of ATZ in the liver is primarily responsible for liver damage by gain-of-function proteotoxicity. The gain-of-function mechanism for liver disease is best demonstrated by transgenic mouse models [3, 4] that strongly recapitulate many of the features of the human liver disease even though they have normal levels of endogenous murine AT, eliminating the possibility that loss of AT function contributes to liver damage [5, 6].

The clinical presentation of ATD-associated liver disease is highly variable and may become apparent in distinct forms during infancy, childhood years, adolescence, or at 50–65 years of age [1]. Patients who exhibit features of liver disease during infancy can present within the first two months of life with persistent jaundice, hepatomegaly, and elevated serum conjugated bilirubin and transaminase levels. A prospective Swedish national screening study initiated in the 1970s provides important insights into the natural history of ATD [7]. In that study, Sveger et al. screened 200,000 newborns, of whom 120 were ZZ homozygotes. A follow-up study of a subset of 26-year-old individuals from this cohort determined that less than ten percent of the cohort had experienced clinically significant liver disease [8]. A limitation of this cohort study is the lack of liver biopsy data, which makes it difficult to assess whether asymptomatic homozygotes can have subclinical pathology for decades that eventually presents as "adult" ATD-associated liver disease. However, the study clearly demonstrated that only a subgroup of affected homozygotes are susceptible to clinically significant liver disease in the first three decades of life and led to the recognition of the importance of putative genetic and environmental modifiers in liver disease pathogenesis.

In adults, ATD-associated liver disease may be recognized either because of portal hypertension or because of the detection of hepatocellular carcinoma (HCC). HCC is an important manifestation that is likely caused by chronic proteotoxicity that drives liver fibrosis and hyperproliferation of hepatocytes. Support for this hypothesis comes from experimental studies that have found that ATZ accumulation in hepatocytes is associated with impaired cell proliferation and chronic regenerative signaling [9]. A Swedish autopsy study conducted by Eriksson et al. revealed a highly significant association of ATD with HCC beyond what would be expected with cirrhosis alone [10]. In the past two decades, there has been increased recognition of "adult" ATD-associated liver disease. Over the last ten years, 85 to 90 percent of liver transplants for ATD performed in the US were for adults, most commonly around 50 to 65 years of age (United Network of Organ Sharing, personal communication).

3. Role of Autophagy in ATD Liver Disease

To understand the mechanism of the liver disease associated with ATD, our laboratory focused initially on mechanisms by which mutant ATZ is degraded when it accumulates within cells. Early studies using yeast and mammalian cell lines showed that the proteasomal pathway participated in intracellular degradation of mutant ATZ [11–13]. Indeed mutant ATZ was one of the first identified substrates of the pathway that is now known as ER-associated degradation (ERAD). Nevertheless, there was evidence from these studies that the proteasomal pathway could not completely account for the disposal of ATZ. Studies in human cell line models of ATD first led to the recognition that autophagy was activated by intracellular accumulation of ATZ, and later it was shown that autophagy participated as a second pathway for disposal of mutant ATZ [14, 15]. Autophagy refers to distinct intracellular catabolic pathways that mediate degradation of unnecessary or dysfunctional cellular components through the machinery of lysosomes. These pathways act, at least in part, as protective and survival mechanisms under the condition of nutrient starvation or other stress states. Autophagy also appears to play critical roles in homeostasis, cell growth, and differentiation. According to the mechanism by which the substrates are delivered to the lysosomes, autophagy can be divided into three subtypes: macroautophagy, microautophagy, and chaperone-mediated autophagy. The studies investigating autophagy in ATD have focused exclusively on macroautophagy. Macroautophagy is characterized by the formation of double membrane vacuoles in the cytoplasm, also called autophagosomes, which then fuse with lysosomes for degradation of the internal constituents and generation/recycling of new amino acids for cell survival.

Autophagy was firstly implicated in ATD when increased autophagosomes were observed in human fibroblast cell lines engineered for expression of ATZ [14]. Increased autophagic vacuoles were also observed in the liver of PiZ transgenic mice and in liver biopsy specimens from patients with ATD [14]. This led to the conclusion that autophagy was activated by intracellular accumulation of ATZ in model systems and in affected tissues from the disease and also raised the possibility that autophagy could participate in degrading mutant ATZ. In initial studies, we showed that degradation of ATZ in human cell line models was partially abrogated by chemical inhibitors of autophagy, such as 3-methyladenine,

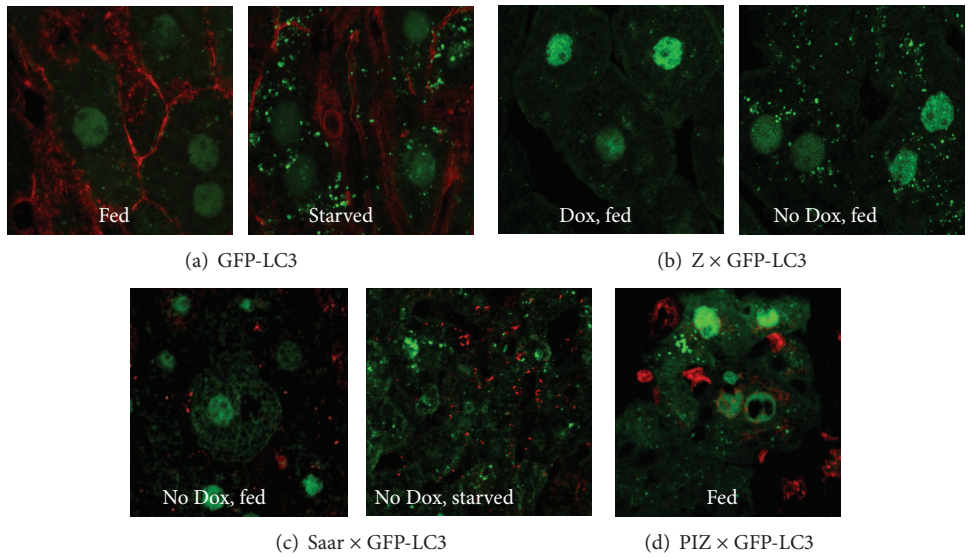

Figure 1: Accumulation of ATZ in liver specifically activates autophagy. Liver sections are stained with anti-GFP to enhance the fluorescent signal. (a) Sections from the GFP-LC3 mouse have GFP+ autophagosomes only in the starved state (right panel—starved for 24 h). (b) Sections from the Z × GFP-LC3 mouse have GFP+ autophagosomes; however, the mouse is fed, but only after doxycycline (Dox) is removed from the drinking water so that the ATZ gene is expressed (right panel). (c) Sections from the Saar × GFP-LC3 mouse (lower right) do not show autophagosomes when the AT Saar variant is expressed following withdrawal of Dox in the fed state, but starvation does lead to GFP+ autophagosomes (right panel). (d) GFP+ autophagosomes are present in the PiZ × GFP-LC3 mouse in the fed state, as this mouse has constitutive expression of ATZ. Reprint from reference [1] with permission.

wortmannin, and LY-294002 [14]. Later we provided definitive genetic evidence using an embryonic fibroblast cell line (MEF) from an ATG5-null mouse engineered for expression of ATZ [15]. The results from this study showed that the degradation rate of ATZ was attenuated in ATG5-null cells compared to the wild-type MEFs. Furthermore, we observed massive accumulation of ATZ with very large inclusions in the ATG5-null cell line. Thus, in addition to providing definitive evidence that autophagy participates in the degradation of ATZ, these data suggest that it also plays a homeostatic role in ATD state by preventing the toxic cytoplasmic accumulation of ATZ through piecemeal digestion of insoluble aggregates [15]. Further, by breeding a mouse model with liver-specific inducible expression of ATZ onto the GFP-LC3 mouse background that is characterized by GFP+ autophagosomes, we found that accumulation of ATZ in liver cells is sufficient to activate autophagy [15]. The effect of ATZ accumulation that leads to activation of autophagy is also specific and probably reflects the aggregation-prone properties of ATZ because accumulation of the nonpolymerogenic variant, AT Saar, does not activate autophagy (Figure 1).

Another series of studies using a completely different system for modeling ATD, yeast expressing human ATZ, also showed the importance of autophagy [16]. These studies showed marked delay in degradation of human ATZ in autophagy-deficient yeast strains. Interestingly, the delay in ATZ degradation was most apparent when ATZ was expressed at high levels [16]. These results suggested that when ATZ is predominantly soluble at lower levels of expression, it could be degraded by proteasome, whereas, at higher levels of expression, it is more likely to be associated with chronic accumulation of insoluble polymers or aggregates, in which case autophagy is needed. Recent studies have also demonstrated the role of autophagy in a novel *C. elegans* model of ATD [17].

In addition to the proteasomal and autophagic pathways for disposal of ATZ, a recent study showed that a pathway from Golgi complex to lysosome that involves the endosomal protein sorting receptor sortilin plays a role in degrading intracellular ATZ [18]. We suspect that there are still other mechanisms by which cells degrade mutant ATZ that have not yet been identified.

To understand the molecular mechanism by which the cells mitigate the proteotoxicity of intracellular ATZ accumulation and presumably protect ATD hosts from liver disease, we also focused on the signaling pathways that modulate proteostasis. Using mammalian cell line and mouse models with inducible expression of ATZ and genomic analysis, we found that the NF-κB and TGF-β signaling pathways are part of the distinct gene expression profile associated with intracellular accumulation of ATZ, and these pathways are likely central to the hyperproliferation and fibrosis, respectively, which characterizes the hepatic pathology of ATD [19, 20]. Genomic analysis also demonstrated upregulation of regulator of G signaling 16 (RGS16), and we have subsequently found that RGS16 is a specific marker for the proteotoxic state created by intracellular accumulation of ATZ and may be one of the mediators by which autophagy is activated in the liver [21].

4. New Autophagy Enhancer Strategies for Treatment of ATD Liver Disease

Because autophagy is activated when mutant ATZ accumulates in cells and participates in intracellular disposal of ATZ, we reasoned that it was a potentially attractive target for drug therapy to mitigate the proteotoxicity that causes liver damage in ATD. From a list of drugs that have been purported to have autophagy enhancer properties, we investigated the effect of carbamazepine (CBZ) in cell line and mouse models of ATD. We selected CBZ because it is approved by the US Food and Drug Administration (FDA) with an extensive safety profile from its use as anticonvulsant and mood stabilizer. We found that CBZ enhanced autophagy and mediated a marked increase on the degradation of mutant ATZ in cell line models [6]. More importantly, CBZ mediated a reduction on hepatic ATZ load and ameliorated hepatic fibrosis in the PiZ mouse model of ATD [6]. These results validated the concept of using autophagy enhancer drugs that target an endogenous presumably protective proteostasis mechanism as a therapeutic strategy. Furthermore, because it is already FDA-approved, this drug could be moved immediately into a phase II/III trial for treatment of severe liver disease due to ATD. Although the lowest effective dose of CBZ (200 mg/kg/day) in mice was considerably higher than the doses used in humans (10–20 mg/kg/day) effective doses of drugs can be 10 to 20 times as high in mice because of the higher ratio of surface area to body weight when compared to humans. The current Phase II/III trial for use of CBZ in severe liver disease due to ATD already ongoing uses doses of CBZ commonly used in clinical medicine, 1200 mg/day for subjects over 15 years of age.

The concept that autophagy enhancer drugs counteract the accumulation and proteotoxicity of misfolded proteins has been further substantiated by the results from an automated high-content screening using a novel *C. elegans* model of ATD [17]. The initial screen of 1280 compounds in the Library of Pharmacologically Active Compounds (LOPAC) identified 5 hit compounds that mediate dose-dependent reduction of ATZ load in this worm model. Interestingly, four of these five hits showed the property of enhancing autophagy. Another interesting observation from these results is that two of these compounds (fluphenazine and pimozide) are from the phenothiazine family, which is structurally related to the tricyclic antidepressants family of which CBZ is a member. We have further validated the effect of fluphenazine (Flu) in mammalian cellular and mouse models of ATD [22]. In nanomolar concentrations, Flu enhanced the degradation of ATZ and reduced the cellular ATZ load in human cellular models of ATD. In PiZ mouse model, Flu reduced the proteotoxicity of ATZ accumulation *in vivo* and mediated a decrease in hepatic fibrosis [22]. As Flu is a FDA-approved drug that is in active clinical use, like CBZ, it could be immediately tested in clinical trials, "repurposing" for treating ATD-associated liver disease. In addition, several other autophagy enhancer drugs, including overlapping hits, have been identified from two independent high-throughput screenings [23, 24]. The identified compounds from these screenings showed enhanced autophagic degradation of aggregation-prone protein *huntingtin* in different models of Huntington's disease. Furthermore, several compounds identified in these screenings are in the phenothiazine family, for example, fluphenazine, pimozide, and fluspirilene [17, 23, 24]. Thus the results from these screenings provide further evidence for the potential therapeutic application of drugs that target endogenous proteostasis mechanisms and identify two new strategies for chemical- and computation-based drug discovery using the autophagy enhancer drug paradigm and the phenothiazine structure.

The specific molecular target of CBZ and the phenothiazine drugs that leads to enhanced autophagy of ATZ remains largely unknown. Because the classical mTOR antagonist rapamycin had minimal effects on ATZ disposal in cell line models and did not alter hepatic ATZ levels in the PiZ mouse model in our studies [6], we have surmised that the effects of CBZon autophagic degradation of ATZ act through an mTOR-independent mechanism.Previous work on the mood- stabilizing effects of CBZsuggests that it is similar to lithium and valproic acid, andall three compounds appear to have autophagyenhancer properties [6, 24, 25]. The mood-stabilizing effects of lithium are thought to involve inhibition of inositol monophosphatase(IMPase) and prevention of inositol recycling, while CBZ andvalproic acid appear to act on inositol (1,4,5)-trisphosphate (Ins) [24, 25]. The inhibition of IMPase or Ins leads to reducedintracellular inositol levels and inositol-1,4,5-trisphosphate (IP3) levels, which negatively regulate autophagy (Figure 2). The phenothiazines are also thought to act on autophagy by mTOR-independent mechanism(s). One of the phenothiazines that have beeninvestigated, fluspirilene, is thought to induce autophagy by reducing intracellular Ca^{2+} and preventing calpain-1-mediated cleavage ofautophagy gene ATG5 [26]. Nevertheless, further work on themolecular targets of these drugs that mediate activation of autophagy and whether these targets are truly independent of TOR is needed.

Several other drugs have recently been shown to induce autophagy. Glucosamine and N-acetylglucosamine increased autophagy in mammalian cell lines [27]. Although the mechanism of this effect was not elucidated, it was independent of mTOR pathway. Another drug that has recently been shown to activate autophagy specifically is the cholesterol-lowering agentezetimibe [28]. This drug was investigated by Yamamura et al. because cholesterol depletion had an effect on autophagy. These authorsshowed that ezetimibe activates autophagy only in hepatocytes and intestinal epithelial cells, consistent with the presumed mechanismof action of the drug which involves the inhibition of cholesterol efflux Niemann-Pick-type C1-like 1 (NPC1L1) [29]. The studies suggest that, by inhibitingNPC1L1, ezetimibe reduces the recruitment of mTOR to the lysosome andtherein inhibits mTORC1 activity to activate autophagy. Mostexciting, the authors found that ezetimibe reduces cellular accumulationof ATZ in primary cultures of human hepatocytes engineered for expression of ATZ [28].

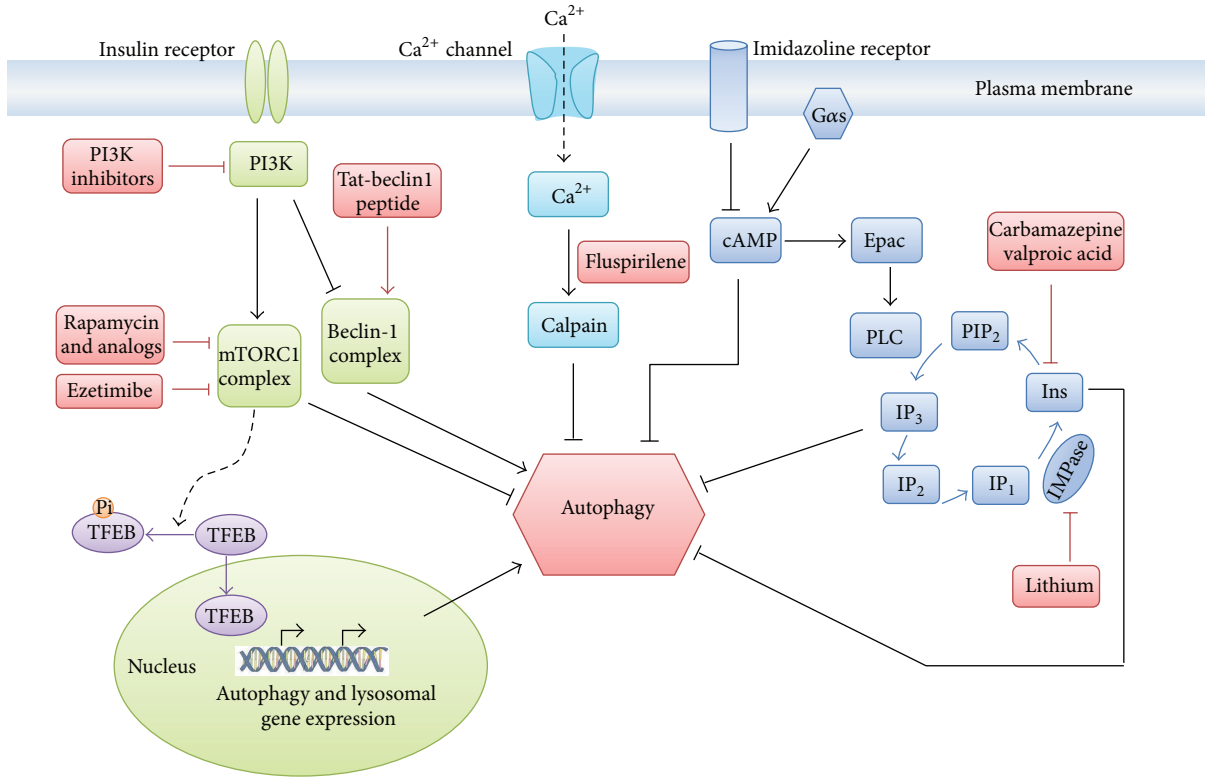

Figure 2: Regulation of autophagy and possible targets of the autophagy enhancer drugs. Drugs that modulate autophagy can be divided into two categories depending on whether or not they act through mTOR, a master negative regulator of autophagy that functions through the formation of mTORC1 complex. This complex is suppressed by specific inhibitors such as rapamycin and its analogs, which therefore enhance autophagy [43]. A recent study showed that ezetimibe perturbed the cholesterol homeostasis and decreased mTOR recruitment to late endosome/lysosome; thus ezetimibe is thought to induce autophagy by suppressing mTORC1 [28]. The mTOR signaling pathway can also be regulated by specific inhibitors target to the upstream of mTOR, for example, PI3K inhibitor [44]. In addition, nuclear translocation of TFEB promoted autophagic degradation of ATZ in PiZ mouse model [33]. There is evidence showing that TFEB interacts with mTORC1 on the lysosomal membrane; because it promotes phosphorylation of TFEB, mTORC1 is now considered an antagonist of TFEB activity [32]. Tat-beclin 1 peptide is identified as a potent inducer of autophagy and enhances the degradation of mutant huntingtin and several invasive bacterial and viral pathogens [30]. Several autophagy enhancer drugs identified from the recent drug screenings, including the phenothiazines, are thought to act on autophagy by mTOR-independent mechanism(s) [17, 23, 24]. One of the phenothiazines that have been investigated, fluspirilene, is thought to induce autophagy by reducing intracellular Ca^{2+} and preventing calpain-1-mediated cleavage of autophagy gene ATG5 [26]. The mood-stabilizing effects of lithium are thought to involve inhibition of IMPase and prevention of inositol recycling, while CBZ and valproic acid appear to act on Ins [24, 25]. The inhibition of IMPase or Ins leads to reduced intracellular inositol and IP3 levels, which therefore induce autophagy. However, the precise mechanism by which autophagy is regulated by the calcium-related signaling pathway or the phosphatidylinositol signaling pathway has not been elucidated. In addition, further work on whether these targets are truly independent of mTOR is needed.

Studies by Shoji-Kawata et al. have identified a potent autophagy-inducing peptide which could potentially be utilized for drug development. Building on prior observations that the HIV protein Nef inhibits autophagy by directly interacting with the autophagy regulatory factor beclin 1 [30, 31], these authors succeeded in identifying an 18-amino acid Nef-interacting domain of beclin 1 and linked it to the Tat sequence to increase cell uptake. They went on to show that Tat-beclin 1 peptide is a potent inducer of autophagy and enhances the degradation of mutant huntingtin and several invasive bacterial and viral pathogens. Their findings suggest that Tat-beclin 1 may potentially be considered for diseases like ATD, other diseases caused by aggregation-prone proteins, and also possibly for certain infectious diseases.

A recent study showed that activation of autophagy using gene transfer of the transcription factor TFEB can ameliorate the proteotoxicity of ATZ. TFEB is a master gene that regulates autophagy and lysosomal gene expression (Figure 2). It appears to interact with mTORC1 on the lysosomal membrane and is negatively regulated by mTORC1 [32]. It was recently reported that TFEB induced autophagy-dependent ATZ clearance in a mammalian cellular model of ATD; using adenovirus-mediated gene transfer of TFEB in the PiZ mouse model, this strategy significantly promoted autophagic degradation of ATZ and reduced liver fibrosis *in vivo* [33]. These results provide further validation for the therapeutic strategy of enhancing autophagy for the liver disease caused by ATD and suggest that gene therapy of this

type may also be possible to treat ATD liver disease in the future.

5. Potential Role for Autophagy-Enhancing Strategies in Other Genetic Liver Diseases

ATD serves as a paradigm for gain-of-toxic function liver disease resulting from the pathologic intracellular accumulation of misfolded proteins. Similar phenomena occur in two other rare liver diseases—fibrinogen storage disease and cholesterol ester storage disease. The defects in each of these conditions lead to abnormal intracellular accumulation of substrates that are hepatotoxic and can result in significant clinical liver disease. These disorders illustrate the essential role of autophagy in liver disease and provide insights into the potential efficacy of therapeutic strategies designed to enhance autophagic function.

Fibrinogen Storage Disease. Fibrinogen is a 340 kD dimeric plasma protein produced by hepatocytes that is a central component of the coagulation cascade. Each half of the dimer is composed of three polypeptide chains (termed Aα, Bβ, and γ) that are assembled in the ER. Several mutations in the fibrinogen gamma chain have been reported (fibrinogen Brescia, Aguadilla, AI DuPont, and Angers) that are associated with fibrinogen storage disease, an autosomal-dominant disorder that is characterized by low circulating plasma fibrinogen levels and abnormal accumulation of fibrinogen components within the hepatocyte ER [34, 35]. Reminiscent of ATD-associated liver disease, patients with fibrinogen storage disease display characteristics of both loss of function (hypofibrinogenemia) and gain-of-toxic function (accumulation of fibrinogen components in ER). In addition to hematologic morbidity such as bleeding and thrombosis, patients may develop chronic liver injury that progresses to cirrhosis. Histologic examination of liver biopsy specimens reveals rounded, eosinophilic inclusions within hepatocytes that are weakly positive for phosphotungstic acid hematoxylin but are PAS-negative when pretreated with diastase. At the electron microscopic level these inclusions have a crystalline lattice organization and are now known to be composed of partially assembled fibrinogen molecules [34].

Experimental studies in yeast models of Aguadilla-variant fibrinogen storage disease by Kruse et al. demonstrated that disposal of mutant fibrinogen from the ER is mediated by two proteostasis mechanisms: endoplasmic reticulum-associated degradation (ERAD) and autophagy [36]. When the mutant γD chain was expressed in yeast, initial clearance of the protein from the ER was performed by ERAD. However, once ERAD was saturated, γD aggregates accumulated within the ER, and clearance of these aggregates was dependent on autophagy. These results suggest that the mutant fibrinogen Aguadilla behaves similarly to mutant ATZ in the fact that it accumulates within the secretory pathway, has a tendency to polymerize/aggregate, and is degraded by proteasomal and autophagic mechanisms. Based on these findings as well as work by the Perlmutter group that demonstrated amelioration of experimental ATD-associated liver disease by the autophagy-enhancing drug carbamazepine, Puls et al. recently conducted a small study of two patients with fibrinogen storage disease in which they found that administration of carbamazepine at antiepileptic doses was associated with normalization of alanine-aminotransferase and reduction of markers of apoptosis and necrosis [35].

Cholesterol Ester Storage Disease. Cholesterol ester storage disease (CESD) is an autosomal recessive condition that results from mutations in the *LIPA* gene, which encodes lysosomal acid lipase (LAL). LAL is a critical component of the low-density lipoprotein (LDL) receptor pathway that is responsible for hydrolyzing cholesterol esters that have been taken up into lysosomes. *LIPA* mutations associated with deficient LAL activity lead to intracellular accumulation of cholesterol esters and triglycerides [37]. The most severe form of CESD, known as Wolman disease, results from *LIPA* mutations that cause nearly complete absence of LAL activity and presents during infancy with hepatosplenomegaly, cholestasis, intestinal malabsorption, adrenal calcifications, and diffuse xanthomatosis [37, 38]. Less severe mutations result in a spectrum of disease collectively referred to as CESD, with age of presentation ranging from childhood to late adulthood. Liver manifestations include hepatomegaly and elevated transaminases occurring in combination with elevated serum cholesterol and triglyceride levels, low high-density lipoprotein (HDL) levels, and nonspecific gastrointestinal symptoms. In late-onset disease, adults may present with evidence of chronic liver disease and micronodular cirrhosis along with atherosclerosis. Liver biopsies may exhibit birefringent cholesterol ester crystals that are pathognomonic for this condition, as well as microvesicular steatosis that can be confused with nonalcoholic fatty liver disease [38, 39].

Lysosomal function is critical for efficient autophagic degradation of substrates, and recent experimental studies have provided evidence for the key role of autophagy in lipid metabolism. In a *C. elegans* system, the lysosomal lipases *lipl-1* and *lipl-3* (worm homologues of human LAL) are important to the mobilization of cytosolic fat through a specific autophagic process termed as lipophagy. Larvae that were double mutants for *lipl-1* and *lipl-3* accumulated three times the cytosolic fat as wild-type larvae, an observation that is reminiscent of cholesterol ester accumulation seen in CESD [40]. Another series of studies that examined lipid droplet mobilization in macrophages demonstrated that cholesterol efflux from macrophage foam cells is mediated by lipophagy and this mechanism is dependent on LAL function. Lipid-loaded macrophages treated with the specific LAL inhibitor Lalistat 1 demonstrated increased intracellular cholesterol ester load. Furthermore, autophagy-deficient Atg5-knockout macrophages and Atg5-knockout mice exhibited a significantly decreased ability to clear ^{3}H-labeled cholesterol [41].

Severe CESD disease may lead to the need for liver transplantation, underscoring the need for novel therapies that can ameliorate the natural course of this condition. Lipid-lowering therapies including statins and cholestyramine have not been shown to reverse liver disease. More recently, efforts

have focused on enzyme replacement therapy using recombinant human LAL (sebelipase alfa), which in experimental mouse studies appeared to reduce hepatic cholesterol ester load and reduced the presence of foamy macrophages in the liver, spleen, and intestines of these mice. This agent is currently in clinical trials [38, 39]. Due to theoretical concerns over the ability of enzyme replacement therapy to fully access target tissues in lysosomal storage disorders, an alternative approach has targeted TFEB. Spampanato et al. recently demonstrated in cell and mouse models of Pompe disease (a lysosomal storage disorder characterized by pathologic accumulation of intracellular glycogen) that adenoviral overexpression of TFEB induced lysosomal exocytosis and ameliorated excessive glycogen burden. These effects were blunted in autophagy-deficient cells [42]. Given the lysosomal dysfunction inherent to CESD and Pompe disease, we wonder whether CESD might be a novel target for autophagy-enhancing therapies, particularly TFEB-mediated clearance of aggregated substrates via lysosomal exocytosis and aggregate expulsion. Indeed, because TFEB activates multiple genes within the autophagolysosomal system, it is possible that its overexpression could potentially overcome the compromised lysosomal function in patients with partial enzyme deficiencies.

6. Conclusions

Because autophagy mediates turnover of damaged organelles and degradation of denatured and misfolded proteins and also mediates disposal of stored lipids, it represents a critical cytoprotective mechanism in homeostasis and during stress states. It appears to be particularly important in diseases caused by misfolded proteins and this may be because it is specialized for degradation of insoluble and soluble proteins. Recent studies have shown that strategies which enhance autophagy can reverse the consequences of proteotoxicity in the liver. These strategies include drugs and viral-mediated gene transfer approaches using the transcriptional activator of the autophagolysosomal system, TFEB. Because some of the drugs are FDA-approved and have been extensively used in clinical medicine, we should see results from clinical trials in the next several years.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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