Ammonia and autophagy: An emerging relationship with implications for disorders with hyperammonemia

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Abstract
(Macro)autophagy/autophagy is a highly regulated lysosomal degradative process by which cells recycle their own nutrients, such as amino acids and other metabolites, to be reused in different biosynthetic pathways. Ammonia is a diffusible compound generated daily from catabolism of nitrogen-containing molecules and from gastrointestinal microbiome. Ammonia homeostasis is tightly controlled in humans and ammonia is efficiently converted by the healthy liver into non-toxic urea (through ureagenesis) and glutamine (through glutamine synthetase). Impaired ammonia detoxification leads to systemic hyperammonemia, a life-threatening condition resulting in detrimental effects on central nervous system. Here, we review current understanding on the role of ammonia in modulation of autophagy and the potential implications in the pathogenesis and treatment of disorders with hyperammonemia.

KEYWORDS
autophagy inducers, hyperammonemia, liver, mTORC1, TFEB, ureagenesis

1 INTRODUCTION

The term autophagy (from the Greek: auto-self; phagy-eating) was first introduced by the Nobel laureate Christian De Duve in the 1960s to describe the process resulting in lysosomal degradation of intracellular components.1 However, the mechanisms underlying autophagy and its role in health and diseases have been elucidated only several decades later. In recognition of the importance of this process, a Nobel Prize has been awarded to cell biologist Yoshinori Ohsumi in 2016.2,3 Autophagy-mediated degradation of proteins and organelles generates amino acids, sugars, and lipids that can be recycled for synthesis of new cellular components and for energy production.4 Autophagy is now recognized as a key process in cell homeostasis and plays an important role in multiple cellular processes, such as resistance to stress due to lack of nutrients or oxygen, immune response, aging, cancer, and neurodegeneration.

Recent studies revealed that ammonia activates autophagy in several disease conditions.5–7 Ammonia is generated as a bioproduct of the catabolism of nitrogen-containing molecules, is highly diffusible and neurotoxic at high concentrations. In acquired liver diseases or inherited deficiencies of urea cycle enzymes, ammonia is poorly removed from the circulation resulting in hyperammonemia, a life-threatening condition with potentially irreversible brain damage.
In this review, we provide a brief description of autophagy functions in liver and we review recent data on the interplay between autophagy and ammonia.

2 | AUTOPHAGY: GENERAL CONSIDERATIONS

Autophagy is a highly conserved and regulated cellular process resulting in delivery of cytoplasmic components, such as proteins and organelles to lysosomes for their degradation. Three main types of autophagy have been defined: macro-autophagy/autophagy (hereafter referred to as autophagy), chaperone-mediated autophagy, and endosomal (micro)autophagy. Autophagy begins with the dynamic formation of a membrane vesicle called phagophore or isolation membrane from autophagy-related (ATG) proteins and lipids derived from plasma membrane and organelles including mitochondria, endoplasmic reticulum (ER), Golgi complex, and endosomes [for detailed review on phagophore biogenesis see10]. Next, the isolation membrane expands engulfing cytosolic components and macromolecules such as glycogen, proteins, and lipid droplets and finally, it seals into a double membrane organelle named autophagosome that is the distinctive intracellular structure of autophagy (Figure 1A). Autophagy substrates can be sequestered either in bulk or selectively. Selective autophagy depends on specific recognition by ligand receptors or scaffold proteins acting as bridges between cargos targeted for degradation and ATG8 proteins, including the light-chain 3 (LC3) and gamma-aminobutyric acid receptor-associated proteins (GABARAP) on the autophagosome.11 Following fusion of autophagosomes with lysosomes, degradation of cargos and their autophagosome membranes occurs in the autolysosome by means of acidic hydrolases. Amino acids, sugars, and lipids are then transported out of the autolysosomes for cellular re-cycling by permeases located on the autolysosome membrane12 (Figure 1A).

All organisms and cell types have a constitutive basal level of autophagy. Nevertheless, autophagy is finely regulated and is strongly induced by stress conditions including lack of nutrients and energy, low oxygen levels, dysfunctional (or excessive number of) organelles, accumulation of unfolded protein aggregates, oxidative stress, DNA lesions, and pathogen infections.13–15 Several signaling pathways regulate autophagy16–18, including the mechanistic target of rapamycin kinase complex 1 (mTORC1) and the energy and glucose sensor adenosine monophosphate-activated protein kinase (AMPK) that result in inhibition or stimulation of autophagy, respectively.19 Moreover, autophagy is controlled by extracellular factors, mainly hormones such as insulin, glucagon, thyroid hormones, and several growth factors,20 and cytokines including tumor necrosis factor alpha (TNF-α) and several interleukins.21

The major regulator of cell growth and metabolism mTORC1 inhibits autophagosome biogenesis, maturation, and fusion with lysosomes by phosphorylation of Unc-51 like autophagy activating kinase 1 (ULK1) initiation complex,22 UV radiation resistance associated gene protein (UVRAG),23 and beclin-1-associated autophagy-related key regulator (BARKOR/ATG14).24 In addition, mTORC1 regulates autophagy through inhibition of the nuclear translocation/export of transcription factors EB (TFEB) and E3 (TFE3), two master regulators of autophagy and lysosomal biogenesis.25–29

Because autophagy has been implicated in a wide range of human disorders from neurodegenerative conditions to hepatic and metabolic disorders, cardiovascular diseases, infectious diseases and cancer, there is strong effort towards the development of drugs enhancing autophagy with the goal of increasing lysosomal degradation of accumulated toxic cargos including protein aggregates, dysfunctional organelles, lipid droplets, and invading microorganisms.30,31

![FIGURE 1](image-url) (A) Schematic representation of (macro) autophagy/autophagy pathway. (B) Known mechanisms of ammonia-mediated induction of autophagy (arrows indicate activation). (C) Ammonia-induced autophagy according to different cell/tissue contexts.
3 | LIVER AUTOPHagy

Being a central organ in metabolism, it is not surprising that autophagy is highly active in liver.\(^9,32\) Liver autophagy is inhibited under conditions of nutrient abundance but strongly activated by fasting, mainly in response to reduced blood amino acids.\(^33\) By autophagy, hepatocytes sustain acute interruptions of trans-placental nutrient supply occurring at birth, allowing survival of neonates facing severe starvation until supply can be restored through milk nutrients.\(^34,35\) Proteins, glycogen, and lipid droplets are degraded to release amino acids, glucose, and free fatty acids which can either be reutilized for synthesis of new proteins and macromolecules, or enter the tricarboxylic acid cycle to generate ATP in mitochondria.\(^12\) Moreover, autophagy regulates hepatic metabolism by controlling the energetic balance through fine-tuned modulation of the number and quality of hepatic mitochondria and peroxisomes.\(^36,37\) Autophagy also protects liver from various insults including damaged or misfolded/aggregated proteins, dysfunctional organelles, excessive nutrient load, and high fat diet.\(^32\) Defective autophagy has been involved in the pathogenesis of several congenital and acquired liver diseases, such as alpha-1 antitrypsin deficiency, acetaminophen (APAP) overdose, alcohol-induced injury, and nonalcoholic fatty liver disease (NAFLD).\(^9,32,38\)

Autophagy functions as protective response against genotoxic and proteotoxic insults preventing the development of hepatocellular carcinoma (HCC).\(^39\) However, after tumor formation, autophagy favors cancer progression and resistance to chemotherapy by supplying tumor cells with a powerful machinery to overcome intracellular and environmental stress (i.e. low nutrients, hypoxia and cytotoxic drugs).\(^40\) Therefore, drugs blocking autophagy are thought to be valuable for cancer only at advanced stages.

4 | AMMONIA AND AUTOPHagy

Ammonia is a highly diffusible weak base found in body fluids either in its neutral (NH\(_3\)) or ionized (NH\(_4^+\)) form. However, at physiologic pH approximately 99% of ammonia is present as NH\(_4^+\). In mammals, free ammonia is continuously released by the catabolism of amino acids and other nitrogen-containing molecules, such as nucleotides and amines. In addition, ammonia is generated from the gut microbiome.\(^41\) Waste ammonia is efficiently converted into non-toxic urea and glutamine.\(^42\) Therefore, plasma ammonia concentrations are maintained below 40 to 50 μM under physiologic conditions.\(^43\) Liver failure\(^44\) or inherited deficiencies of urea cycle enzymes result in hyperammonemia with plasma ammonia levels ranging from 0.5 to 5 mM, causing neuronal dysfunction, increased intracerebral pressure and death due to cerebral edema if left untreated.\(^45,46\)

Besides its toxic and life-threatening effects on brain cells, ammonia induces multiple alterations in several other cell types, such as pH changes, electrolyte imbalance and metabolic dysfunction.\(^47\)

Ammonia has a dual effect on autophagy in vitro: inhibition at high concentrations and activation at lower concentrations. At elevated concentrations (above 20 mM), ammonia impairs lysosomal function because it increases lysosomal pH and water influx which secondarily result in defective substrate degradation and lysosome swelling, respectively.\(^48,49\) In contrast, ammonia strongly activates autophagy at lower concentrations (from 0.2 to 10 mM) both in vitro and in vivo.\(^5,7,50–53\) Ammonia derived from glutamine catabolism also strongly induces autophagy in cancer cells.\(^54\)

Ammonia induces autophagy by several mechanisms (Figure 1B). It was initially reported that ammonia-induced autophagy was independent of mTORC1 but dependent on ULK1 kinase.\(^54\) Another study showed that ammonia can directly engage the autophagy machinery in an mTORC1 and ULK1/2 independent-manner requiring ATG5.\(^50\) However, ammonia-dependent inhibition of mTORC1 and autophagy activation were observed in multiple cell lines.\(^52,55\) Studies in cells suggested that ammonia also promotes autophagy through activation of dopamine receptor D3 (DRD3) and subsequent mTORC1 inhibition.\(^52\) Moreover, activation of AMPK and components of the unfolded protein response (UPR) have been involved in ammonia-mediated induction of autophagy.\(^51\) In addition, induced autophagy in vivo was observed in livers and skeletal muscles of mice with hyperammonemia.\(^5,7,56\) The reasons underlying such differences in mTORC1 response to ammonia are unknown but they could depend on cell and tissue contexts.

mTORC1 is regulated by several signals including growth factors, hypoxia, energy and nutrients such as amino acids and alpha-ketoglutarate (α-KG), a tricarboxylic acid cycle intermediate.\(^57,58\) During hyperammonemia, liver autophagy is activated by inhibition of mTORC1 due to depletion of hepatic α-KG without affecting AMPK signaling.\(^7\) Depletion of α-KG is indeed known to activate mTORC1 on the lysosome surface.\(^59,60\) Interestingly, increased amino acid flux activates AMPK through increased AMP generated by the urea cycle enzyme argininosuccinate synthetase (ASS).\(^61\) Therefore, both ammonia through mTORC1 and amino acids through AMPK converge towards activation of autophagy.

In skeletal muscle, impairment of mTORC1 signaling during hyperammonemia can occur as consequence of: (a) elevated levels of myostatin\(^62\); (b) increased phosphorylation and activation of either the amino acid deficiency sensor general control non-pressible 2 kinase (GCN2) or the eukaryotic initiation factor 2 alpha (eIF2α), an essential
factor for protein synthesis\cite{56}; and (c) an impaired production of energy with excessive cataplerosis of $\alpha$-KG\cite{63,64}. The consequences of ammonia-induced autophagy vary depending on the target cell or tissue (Figure 1C). Ammonia was found to activate liver autophagy supporting ureagenesis.\cite{7} In skeletal muscle, ammonia-induced autophagy plays a key role in sarcopenia induced by chronic hyperammonemia during liver cirrhosis and is involved in degradation of abnormally tyrosine-nitratred proteins.\cite{6,65} Noteworthy, two established ammonia-lowering strategies, namely rifaximin and $L$-ornithine-$L$-aspartate, reduced autophagy and improved the sarcopenia.\cite{5} Although the deleterious consequences of hyperammonemia on brain cells and particularly astrocytes have been extensively investigated,\cite{45-47} the consequence of hyperammonemia on autophagy flux in cells of the central nervous system has been not addressed so far. Nevertheless, ammonia has been proposed as a possible etiological factor in neurodegenerative disorders such as Alzheimer disease,\cite{66} a condition in which autophagy has been heavily implicated.\cite{67}

In cancer cells, induction of autophagy by ammonia is detrimental because it is cytoprotective thus permitting cell survival under adverse conditions. In interstitial fluids from human tumor xenografts, higher concentrations of ammonia (2-5 mM) stimulate autophagy both in an autocrine and paracrine manner, favoring cancer development and progression in the face of reduced nutrient and/or oxygen availability, particularly under cytotoxic chemotherapies.\cite{54,68}

5 | GLUTAMINE AND AUTOPHAGY

Glutamate-ammonia ligase also known as glutamine synthetase (GS) catalyzes the incorporation of ammonia into glutamine and is another major ammonia detoxification system in humans besides the urea cycle.\cite{69,70} Glutamine is the most abundant free amino acid in the human body and plays a major role in metabolic pathways, cell signaling, proliferation, and autophagy. Glutamine-dependent regulation of autophagy is especially relevant in cancer cells which support their proliferation by increased uptake and catabolism of glutamine required as source of nitrogen and for anaplerosis.\cite{58,71} Glutamine generated by GS inhibits mTORC1 thus activating autophagy in cancer cells\cite{72} whereas sequential reactions catalyzed by glutaminase (GLS) and glutamate dehydrogenase (GDH) enzymes generate $\alpha$-KG from glutamine that stimulates mTORC1 and inhibits autophagy.\cite{60,73,74} Therefore, enhanced oxidative/proteotoxic stress induced by simultaneous inhibition of GLS (a source of glutathione) and heat shock protein 90 (HSP90) strongly induces programmed cell death in models of cancer driven by mTORC1 hyper-activation.\cite{75} Hence, selective GLS inhibition has been investigated as anti-cancer target for mTORC1-driven cancers.\cite{73} Interestingly, glutaminolysis was also reported to strongly activate autophagy upon ammonia release.\cite{53,54}

6 | UREAGENESIS AND AUTOPHAGY

In mammals, under physiologic conditions the flux through the urea cycle is estimated to be 20% to 50% of its full capacity\cite{43} and increases during starvation or with intake of high amounts of proteins. When liver is facing high ammonia, intra-hepatic protein catabolism provides aspartate to fuel the urea cycle.\cite{76,77} Previous studies have also shown that exogenous supply of urea cycle intermediates prevented ammonia toxicity after an acute nitrogen load in wild-type\cite{78,79} and ornithine transcarbamylase (OTC)-deficient rodents.\cite{80,81} Consistent with these studies, hepatic autophagy was recently found to be involved in ammonia detoxification under conditions of excessive ammonia levels by furnishing the urea cycle with intermediates and energy that increase urea cycle flux.\cite{7} Liver-specific deficiency of autophagy impaired ammonia detoxification whereas its enhancement resulted in increased urea synthesis and protection against hyperammonemia in various mouse models, including mice with OTC deficiency.\cite{7} In agreement with these data, hepatic activation of autophagy by everolimus, a derivative of rapamycin, resulted in reduced serum ammonia levels in a mouse model of ischemia-reperfusion injury, although urea production was not directly evaluated.\cite{82} Therefore, drugs enhancing autophagy have potential for treatment of urea cycle disorders.\cite{83} Furthermore, activation of autophagy should be considered as a mechanism of action of drugs under investigation for hyperammonemia. Notably, sodium phenylbutyrate used as ammonia scavenger in urea cycle disorders has been also reported to activate autophagy in liver cells.\cite{84}

Hepatic autophagy is under the control of nutrients and hormones. Interestingly, there is an overlap in signaling pathways which stimulate liver autophagy and ureagenesis. One of the first autophagy-inducing stimuli discovered by De Duve was glucagon\cite{65} that also activates ureagenesis. To maintain systemic glucose and amino acids homeostasis during starvation, glucagon induces hepatic autophagy to supply substrates for gluconeogenesis and ketogenesis.\cite{86,87} Glucagon regulates ureagenesis\cite{88} by increasing N-acetylglutamate (NAG) that activates the urea cycle in the short term\cite{89,90} and by inducing expression of urea cycle enzymes\cite{91,92} and a mitochondrial ammonia transporter in the long term.\cite{93} Several actions elicited by glucagon during starvation are mediated by the cAMP response element binding protein
(CREB)\textsuperscript{94} that is involved in transcriptional regulation of autophagy together with the farnesoid X receptor (FXR).\textsuperscript{95} While FXR acts as a physiological repressor of autophagy in the fed-state, CREB upregulates autophagy genes under fasting condition. Upon glucagon signaling, CREB also stimulates urea synthesis by transcriptional induction of carbamoyl-phosphate synthetase 1 (CPS1), ASS, and N-acetylglutamate synthase (NAGS).\textsuperscript{96–98} In summary, ureagenesis and hepatic autophagy are both activated by CREB.

Sirtuins (SIRTs) are protein deacetylases which have also been involved in both autophagy and ureagenesis.\textsuperscript{99} Mitochondrial SIRT5 is involved in ammonia-induced autophagy and mitophagy\textsuperscript{53} as well as urea synthesis via activation of CPS1.\textsuperscript{100–102} Moreover, mitochondrial SIRT3 has been implicated in ureagenesis by deacetylation and activation of OTC\textsuperscript{103} and regulation of liver autophagy by modulation of AMPK-mTORC1 signaling pathways.\textsuperscript{104} In conclusion, autophagy and ureagenesis are regulated by overlapping pathways supporting the concept that they cooperate in ammonia detoxification.

7 | CONCLUDING REMARKS

Depending on the target tissue, hyperammonemia-induced autophagy can have either beneficial or detrimental consequences. In liver, evidence from in vivo studies suggests that autophagy cooperates with the urea cycle in ammonia homeostasis and, importantly, its enhancement protects against hyperammonemia. These findings also suggest that selective activation of hepatic autophagy can be exploited to treat hyperammonemia due to acquired or inherited diseases. In muscle, ammonia-induced autophagy contributes to muscle mass depletion due to cirrhosis. In cancer cells, the activation of autophagy by ammonia likely acts as a cell protective process promoting survival under adverse conditions. Whether autophagy is also involved in damage induced by hyperammonemia in brain and other tissues remains to be addressed.

In conclusion, emerging evidence indicates that modulation of autophagy by ammonia has implications in the pathogenesis of various disorders presenting with hyperammonemia. While detrimental in liver cancer and in sarcopenia due to cirrhosis, activation of autophagy in liver has been identified as a potential therapeutic target for inherited urea cycle disorders and other liver disorders causing hyperammonemia.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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