

Autophagy in acute kidney injury



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Autophagy is a conserved multistep pathway that degrades and recycles damaged organelles and macromolecules to maintain intracellular homeostasis. The autophagy pathway is upregulated under stress conditions including cell starvation, hypoxia, nutrient and growth-factor deprivation, endoplasmic reticulum stress, and oxidant injury, most of which are involved in the pathogenesis of acute kidney injury (AKI). Recent studies demonstrate that basal autophagy in the kidney is vital for the normal homeostasis of the proximal tubules. Deletion of key autophagy proteins impaired renal function and increased p62 levels and oxidative stress. In models of AKI, autophagy deletion in proximal tubules worsened tubular injury and renal function, highlighting that autophagy is renoprotective in models of AKI. In addition to nonselective sequestration of autophagic cargo, autophagy can facilitate selective degradation of damaged organelles, particularly mitochondrial degradation through the process of mitophagy. Damaged mitochondria accumulate in autophagy-deficient kidneys of mice subjected to ischemia-reperfusion injury, but the precise mechanisms of regulation of mitophagy in AKI are not yet elucidated. Recent progress in identifying the interplay of autophagy, apoptosis, and regulated necrosis has revived interest in examining shared pathways/molecules in this crosstalk during the pathogenesis of AKI. Autophagy and its associated pathways pose potentially unique targets for therapeutic interventions in AKI.

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Christian De Duve (discoverer of lysosomes and peroxisomes in 1955), while studying the structures of lysosome by electron microscopy in the early 1960s, observed double-membrane vesicles containing degraded intracellular organelles and lysosomal enzymes in the cells. In 1963, De Duve coined the term “autophagy” (meaning “self-eating” in Greek) for the vesicle formation phenomenon, and later on provided evidence that lysosomes are involved in the degradation of sequestered cellular contents enclosed in autophagy vesicles, now known as autophagosomes.^{1–3}

Autophagy is an evolutionarily conserved multi-step process of degradation of intracellular organelles, proteins, and other macromolecules by the hydrolases of lysosomes.^{4–6} The degraded cellular contents are reused for the synthesis of new macromolecules and organelles. Many recent studies have documented the pivotal role of autophagy in physiological processes as well as in pathogenesis of a disease. In normal physiological conditions, a low level of basal or constitutive autophagy occurs to maintain cellular homeostasis by controlling the turnover of damaged proteins and organelles. In pathological conditions, a wide range of cellular stresses including cell starvation, hypoxia, nutrient and growth-factor deprivation, oxidant injury, genotoxic agents, and other damaging insults contribute to the induction of autophagy.^{7–9} Among these stresses, autophagy induction in response to starvation is extensively studied, well characterized, and shown to be mediated through mammalian target of rapamycin (mTOR), adenosine monophosphate kinase (AMPK), and sirtuins.^{5,7,10}

The pathogenesis of acute kidney injury (AKI) also involves multiple stresses including hypoxia, nutrient and growth-factor deprivation, energy depletion, oxidant injury, genotoxic stress, endoplasmic reticulum (ER) stress, and other damaging insults, all of which are known to drive autophagy induction. In response to numerous stresses, autophagy is a mechanism to promote cellular adaptation with cytoprotective effects by the elimination and recycling of damaged macromolecules and organelles.^{5,7} In general, autophagy induction in response to multiple stresses including those induced during AKI is cytoprotective (reviewed in later sections). Therefore, autophagy is important in renal injury and is a potential therapeutic target in the pathogenesis of AKI. Dysregulation of autophagy results in pathophysiologies such as cardiomyopathy, infectious diseases, Crohn’s disease, and neurodegenerative disorders including Alzheimer’s, Huntington’s, and Parkinson’s diseases.¹¹ However, the role and regulation of autophagy

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induction in AKI are not extensively studied as in other diseases as noted above.

Lysosomal degradation of cellular contents involves 3 autophagy pathways

Three processes of lysosomal-mediated degradation of intracellular contents have been identified in mammalian cells and classified into three subtypes of autophagy: macroautophagy, chaperone-mediated autophagy, and microautophagy. The distinct route of delivery of cytoplasmic material to the lysosome distinguishes different types of autophagy. Macroautophagy, generally referred to as “autophagy,” is the main and widely studied pathway to degrade or eliminate damaged cell organelles and proteins. The macroautophagy begins in the cytoplasm by formation of a double-membrane structure known as a phagophore or isolation membrane. The studies on the origin or source of this process are currently ongoing, and some studies have proposed plasma membrane, Golgi complex, ER, or mitochondria as possible sources.^{12–16} After nucleation, the phagophore membrane sequesters the targeted portion of the cytoplasm containing damaged macromolecules and organelles and then further elongates to form a double-membrane vesicle known as the autophagosome. The autophagosome then fuses with a lysosome and forms an autolysosome. The lysosomal hydrolases degrade cytoplasmic constituents, and the resulting breakdown products are recycled to synthesize new proteins, organelles, and energy needs of the cell.^{5,6} Autophagy plays a vital role in eliminating unwanted damaged macromolecules and organelles in the cells. Chaperone-mediated autophagy is a selective form of

autophagy that has been described in mammalian cells only. Cytoplasmic proteins containing the KFERQ (Lys-Phe-Glu-Arg-Gln) pentapeptide recognition sequence are recognized by the chaperone complex hsc70 (heat shock cognate 70 of the Hsp70 family). Hsp8 and other co-chaperones target the substrate to the lysosomal surface, where they bind to the lysosome-associated membrane protein type 2A (LAMP-2A). Lysosomal hsc73 and hsc70 facilitate subsequent translocation and internalization of the substrate for degradation.¹⁷ Microautophagy is a process in which cytoplasmic contents are directly engulfed into the lysosome by lysosomal membrane invagination or protrusion. This process is not well studied.

Molecular machinery of autophagy: sequestration of cytoplasmic contents and their degradation

The hallmark of the autophagy process in the mammalian cell is the formation of autophagosomes and autolysosomes. Formation of an autophagosome involves the coordinated action of several Atg protein complexes including (i) the uncoordinated 51-like kinase (ULK) 1/ULK2 complex; (ii) beclin-1/class III phosphatidylinositide-3 kinase (PI3K) complex; (iii) transmembrane proteins Atg9 and vacuole membrane protein 1 (VMP1); and (iv) Atg12-Atg5-Atg16 ubiquitin-like protein conjugation system and lipidation of light chain (LC)-3 with phosphatidylethanolamine (PE) to form LC3-II^{4,6,18} (Figure 1). The LC3-II levels are generally assessed by western blots and LC3-II-positive dots in the form of punctate staining by immunostaining. Atg proteins were originally identified by genetic screening in yeast.^{4–6,19}

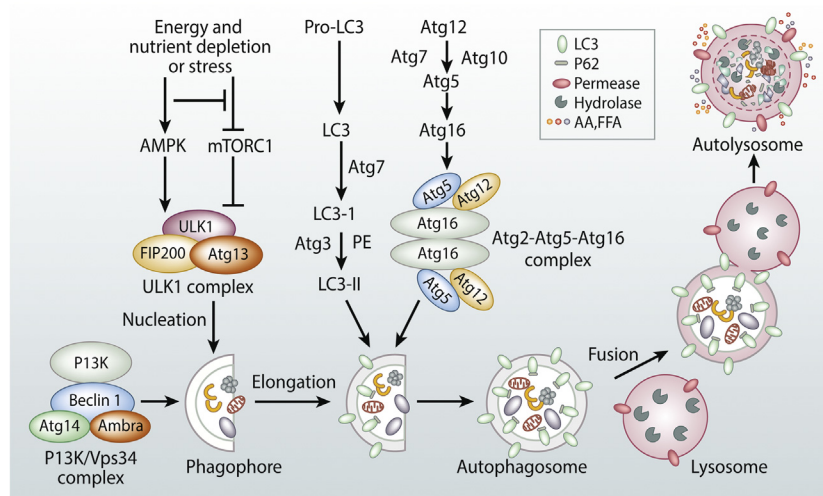


Figure 1 | Overview of the autophagy pathway and its molecular machinery. Formation of autophagosome involves a series of steps mediated by functional complexes containing Atg proteins: (i) The uncoordinated 51-like kinase 1/uncoordinated 51-like kinase 2 complex composed of uncoordinated 51-like kinase 1/uncoordinated 51-like kinase 2, Atg13, RB1CC1/FIP200, and Atg101 is recruited to the phagophore membranes. It is required for initiation of autophagy and is regulated by mammalian target of rapamycin 1 and adenosine monophosphate kinase. (ii) PI3K/Vps34 complex composed of Vps34, Vps15, Beclin-1, Ambra, and Atg14L is required for nucleation of the phagophore membrane. (iii) Atg9 vesicles are required for membrane expansion for the autophagosome assembly. (iv) Atg12-Atg5-Atg16 multimeric complex and Atg8/light chain 3-II are two ubiquitin-like conjugation systems that are involved in the elongation and expansion steps in the autophagosome formation. Light chain C3-II remains present on both the membranes of the autophagosome and can bind to selective substrates including p62. The mature autophagosome then fuses with lysosome to form autolysosome. Upon fusion with lysosome, the cytoplasmic contents sequestered in the autophagosome contents are degraded by the lysosomal hydrolases. AA, amino acids; AMPK, adenosine monophosphate kinase; FFA, free fatty acids; mTOR, mammalian target of rapamycin; ULK, uncoordinated 51-like kinase.

- (i) The ULK1/2 complex composed of ULK1/ULK2 (two homologs of yeast Atg1) serine-threonine kinases, ATG13 (mammalian homolog of yeast Atg13), RB1CC1/FIP200 (homolog of yeast Atg17), and ATG101^{20,21} function upstream of the autophagy pathway and is recruited to the phagophore membranes. mTOR (mTOR complex 1 [mTORC1]) and AMPK are two nutrient- and energy-sensitive kinases^{22–30} involved in regulating activity of the ULK1/2 complex. mTORC1 negatively regulates autophagy, and this function of mTORC1 is conserved in eukaryotes.³⁰ AMPK phosphorylates tuberous sclerosis complex, which inhibits mTORC1.^{26,31} AMPK can also phosphorylate the raptor component of mTORC1 when released from the ULK1 complex, resulting in inactivation of mTORC1.²⁶
- (ii) Beclin-1/class III PI3K complex is recruited to the phagophore membrane to further promote autophagosome nucleation. Activated ULK1 complex and transmembrane protein ATG9 facilitate the recruitment of the autophagy-specific class III PI3K complex to the phagophore membranes. VPS34, VPS15, and Beclin-1 are the core components, and other accessory proteins such as AMBRA, AtG14L, or UVRAG associate with the complex by binding to Beclin-1.^{4,6,19} VPS34 is the catalytic subunit, and other associated components are essential for the catalytic activity of the complex. ULK1/2 complex activates PI3K complex by phosphorylating its components Beclin-1 on Ser 14³² and AMBRA 1,³³ and the active PI3K complex phosphorylates phosphatidyl inositol to form phosphatidyl inositol-3 phosphate. Phosphatidyl inositol-3 phosphate produced at the site of phagophore membranes not only is important for stable phagophore membrane nucleation, further growth, and initial phagophore curvature, but is also important to facilitate recruitment of other regulatory phosphatidyl inositol-3 phosphate-binding proteins including double FYVE-containing protein 1 and WD-repeat domain phosphoinositide-interacting proteins.^{34,35}
- (iii) Atg9 vesicles are translocated to the isolation membrane or phagophore sites and play an important role in membrane expansion in the autophagosome assembly both in yeast and mammalian cells.^{36,37}
- (iv) Expansion and elongation of phagophore membranes involve the formation of two ubiquitin-like conjugation systems: ATG16L1 complex (ATG12-ATG5-ATG16L1) and lipidation of microtubule-associated protein-1 LC3, a mammalian homolog of yeast ATG8. For the formation of ATG12-ATG5-ATG16L1, ATG12 is activated by E1-like conjugating enzyme ATG7 and transferred to the E2-like conjugating enzyme ATG10. Formation of an ATG12-ATG10 intermediate facilitates the transfer of ATG12 to Atg5 to form covalently linked ATG12-ATG5. Further, noncovalent association with ATG16L1 results in the formation of the multimeric complex ATG12-ATG5-ATG16L1.^{4,38} The ATG16L1 complex is recruited to the phagophore membranes and is required for the efficient

execution of the second ubiquitin-like conjugation system for lipidation of LC3 (Atg8) by conjugation with PE.^{38,39} LC3 is first cleaved by ATG4 to produce cytosolic LC3-I. The E1-like conjugating enzyme ATG7 then activates LC3-I and transfers to the E2-like conjugating enzyme ATG3. Formation of an LC3-1-ATG3 intermediate facilitates the transfer of LC3-I to PE to form covalently linked LC3-PE, also known as LC3-II, and it becomes an integral component of the outer and inner membrane of the autophagosome, whereas ATG12-ATG5-ATG16L1, present on the outer membrane, dissociates on completion of the autophagosome¹⁹ (Figure 1). A recent study has demonstrated that WD-repeat domain phosphoinositide-interacting 2 interacts with ATG16L1 and is involved in the recruitment of ATG12-ATG5-ATG16L1 complex to the expanding phagophore membranes, thus linking PI3KC3 complex to LC3 lipidation in the process of autophagosome formation.^{40–42}

Fusion of lysosome with autophagosome and cargo degradation. Once the autophagosome formation is complete, it fuses with the lysosome to generate an autolysosome (Figure 1). The process of fusion is not completely understood in mammalian cells, but the Ypt 7 homolog RAB7 is involved.^{43–45} A recent study demonstrated that autophagosome-endolysosome fusion requires oligomeric ATG14 that interacts with STX17–SNAP29 binary t-SNARE (target-soluble NSF [n-ethylmaleimide-sensitive factor] attachment protein receptor) complex on autophagosomes and primes it for VAMP8 interaction to promote fusion.⁴⁶ The inner membrane LC3-II of the autolysosome is degraded along with the rest of the sequestered cargo by lysosomal hydrolases.¹⁹

Selective degradation of cytoplasmic contents by autophagy

The process of autophagy was traditionally viewed as a nonselective sequestration in autophagosomes of the cytoplasmic contents and their degradation by lysosome. Many studies now have demonstrated that autophagy selectively eliminates intracellular molecules as well as damaged organelles and pathogens. Selective autophagy has been reported for the elimination of damaged mitochondria (mitophagy), peroxisomes (pexophagy), lipids (lipophagy), aggregated proteins (aggrephagy), ER (ER-phagy), pathogens and microorganisms (xenophagy), and cilia (ciliophagy).^{47–50}

One of the selective mammalian autophagy cargo receptors first identified is p62/SQSTM1, also known as a sequestosome-1.^{51,52} p62 is a well-studied multifunctional protein, and among its many structural domains, PB1 domain, ubiquitin-associated domain, and LC3-interacting region are involved in p62-mediated selective autophagy. The N-terminal PB1 domain enables p62 to self-associate and polymerize in the cytoplasm to form aggregates and cytoplasmic inclusion bodies, and these self-aggregates are degraded by autophagy.^{53,54} The C-terminus ubiquitin-binding domain enables p62 to recognize and bind to polyubiquitinated proteins. The presence of LC3-interacting region (LIR) in p62 facilitates binding to LC3-II, and targeting p62 and

p62-bound polyubiquitinated cargo to the autophagosomes for degradation.^{52,54,55} Other related autophagy receptors are neighbor of BRCA1 gene1, nuclear dot protein 52 kDa, optineurin, and Tax1-binding protein 1, and they display much similarity to the domain structure of p62 and contain dimerization or polymerization domain, LIR region, and ubiquitin-binding domain.^{48,55}

Selective degradation of damaged mitochondria (mitophagy) and its induction and role in AKI. Selective removal of damaged and depolarized mitochondria, known as mitophagy, is necessary to maintain quality control of mitochondria and cellular homeostasis. Although initially identified in neurodegenerative diseases, mitophagy is being recognized as playing an important role during AKI. To meet high-energy needs for the processes of electrolyte reabsorption and excretion of waste products, the kidney has the second highest abundance of mitochondria and oxygen consumption next to the heart. Increased oxidative stress, inflammation, and uncoupling of oxygen consumption from adenosine triphosphate production, all of which are associated with AKI,^{56–58} promote mitochondrial depolarization and dysfunction, which can trigger mitophagy.⁵⁹ Parkin, PTEN-induced putative kinase protein 1 (PINK1), NIX, BNIP3, and FUNDC1 are proteins involved in the process of mitophagy in mammalian cells. Mutations in Parkin⁶⁰ and PINK1⁶¹ genes were first identified to cause familial Parkinson's disease, and mitochondrial dysfunction is shown to be associated with this disease.⁶² The underlying mechanism on how the PINK1-Parkin pathway results in removal of damaged mitochondria is well characterized (Figure 2). In normal mitochondria, PINK1 is degraded from the mitochondria by the proteasome-mediated pathway but selectively accumulated in the outer membrane of depolarized and damaged mitochondria.⁶³ At the mitochondrial surface, PINK1 recruits the E3 ubiquitin ligase Parkin and activates Parkin by phosphorylation.^{62,64,65} Active Parkin then promotes ubiquitination of a broad range of outer mitochondrial surface proteins including mitofusins (Mfn1/2), mitochondrial rho GTPase (MIRO), voltage-dependent anion channel (VDAC) TOM70, DRp1, and PARIS (ZNF746).^{49,66} The autophagy receptor optineurin and p62 both contain ubiquitin-binding and LIR structural domains, which may facilitate autophagic clearance of the damaged mitochondria.⁶⁷ Another mechanism for mitophagy has also been reported. Mitophagy receptors including BNIP3 (Bcl-2 and adenovirus E1B 19-kDa-interacting protein 3), BNIP3L (BNIP3-like, also known as NIX), and FUNDC1 (FUN 14 domain-containing protein 1) are mitochondrial proteins and play a role in specific autophagic clearance of the damaged mitochondria. BNIP3/NIX and FUNDC1 interact with LC3 via their LIR domain and promote autophagic clearance of damaged mitochondria.^{66,68,69} BNIP3/NIX may promote mitophagy by directly binding with the mitochondria or through the interaction with Bcl-2/beclin-1.⁷⁰ FUNDC1 mediates in hypoxia-induced mitophagy.⁷¹

Little information is known on the induction of mitophagy in renal tubular cells in AKI. Ishihara *et al.*⁷² demonstrated

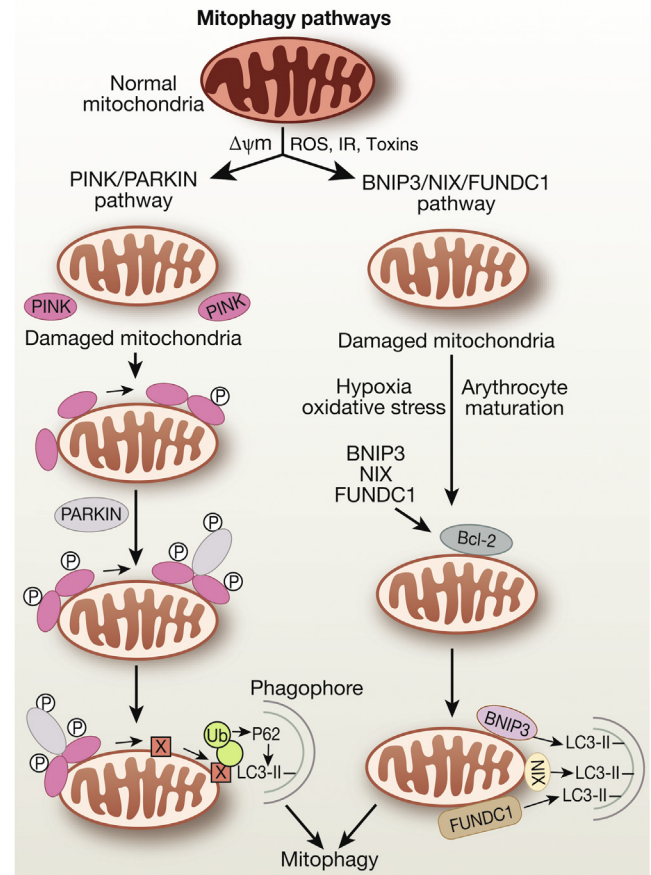


Figure 2 | PTEN-induced putative kinase protein 1/Parkin and BNIP3/NIX/FUNDC1 pathways of mitophagy. In the PTEN-induced putative kinase protein/Parkin pathway, mitochondrial depolarization or mitochondrial fission leads to localization of PTEN-induced putative kinase protein 1 in the outer membrane of the depolarized mitochondria. PTEN-induced putative kinase protein 1 then recruits E3 ubiquitin ligase, Parkin, to the mitochondria and activates Parkin to facilitate ubiquitination of mitochondrial surface proteins for autophagic clearance of the damaged mitochondria. Polyubiquitinated outer mitochondrial proteins interact with p62 bound to autophagosomal light chain 3, thereby targeting the mitochondrion for mitophagy. In the BNIP3/NIX/FUNDC1 pathway, mitochondrial outer membrane proteins BNIP3, NIX, or FUNDC1 bind to light chain 3-III via their light chain 3-interacting region motifs facing the cytosol and promote selective clearance of mitochondria. NIX is involved in the process of mitochondrial clearance during reticulocyte maturation and BNIP3 is upregulated in renal ischemia-reperfusion injury, and overexpression of BNIP3 in NRK-53E cells selectively induces mitophagy.⁷² IR, ischemia reperfusion; ROS, reactive oxygen species.

that BNIP3 and sestrin 2 are upregulated during renal ischemia-reperfusion (IR) *in vivo* and in NRK-52E cells exposed to hypoxia. Although overexpression of both BNIP3 and sestrin 2 induced autophagy as measured by LC3-II formation, only BNIP3 selectively induced mitophagy as visualized by confocal and electron microscopy.⁷² The expression of PINK, a marker of mitophagy, was increased during renal ischemia-reperfusion injury.⁷³ Another study has shown that mitophagy in the kidneys of a rat fed a low-calorie diet was markedly increased and ameliorated oxidative

damage compared with that of high-calorie fed rats.⁷⁴ Limited information is available on the selective eliminations of other organelles in AKI.⁷⁵ The studies on the role and prevalence of other forms of selective autophagy such as lipophagy, aggrephagy, ER-phagy, and ciliophagy have not been yet investigated during AKI.

Autophagy in AKI

Autophagy in IR injury. Autophagy is induced in response to renal IR injury in *in vivo* and *in vitro* models.^{71,76–81} In most of these studies, autophagy induction was revealed by conversion of LC3-I to LC3-II or by green fluorescent protein (GFP)–LC-II punctate formation using LC3-GFP transgenic mice. In a few studies, induction of autophagy has been demonstrated by formation of autophagosomes when visualized by an electron microscope.^{71,77,79} Many studies have reported the role of autophagy during IR-induced AKI. The beneficial effect of autophagy during renal IR was revealed by using conditional kidney proximal tubule-specific Atg5- or Atg7-knockout (KO) mice. Kimura *et al.*⁸¹ used Atg5^{fllox/fllox} Kap-Cre mice under the control of an inducible promoter kidney androgen-regulated protein to specifically delete Atg5 in proximal tubules in response to androgen. The proximal tubule-specific Atg5-KO mice accumulated deformed mitochondria, p62, ubiquitin-positive inclusion bodies, and increased terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labeling-positive cells. These results indicate that basal autophagy is important for the normal homeostasis of proximal tubules. In addition, tubular damage and renal dysfunction worsened in these mice when subjected to IR injury,⁸¹ suggesting that autophagy is renoprotective in IR injury. Similar results were obtained with proximal tubule-specific Atg7-deficient mice, which were more sensitive to IR injury compared with wild-type mice.⁷⁹ Mice with Atg5 deletion in both proximal and distal tubules when subjected to IR injury also had more severe tubular damage and renal dysfunction, with increased levels of blood urea nitrogen and creatinine for up to 16 days after IR injury.⁷¹ These mice accumulated damaged mitochondria, p62, and ubiquitinated proteins, and displayed increased apoptosis (caspase-3 activation)⁷¹ in kidneys. Mice deficient in Atg5 only in distal tubules did not cause renal dysfunction and tubular damage.⁷¹ Pharmacological approaches were also considered to examine the role of autophagy in IR injury.^{76–79,82,83} Currently, effective pharmacological inhibitors that specifically target autophagy are yet to be developed. Caloric restriction that stimulates autophagy has also provided evidence for a protective role of autophagy in IR injury as autophagy inhibition abrogated this protection.⁸⁴

Pitfalls in induction of autophagy by mTORC1 inhibition in IR injury

The underlying mechanisms of how autophagy is regulated in IR injury are not clearly understood. A serine threonine kinase, mTORC1, upon activation negatively regulates

autophagy by controlling phosphorylation of ULK1. mTORC1 also participates in multiple cellular processes and promotes cellular growth, proliferation, survival, and metabolism,^{25,85,86} and is reported to maintain renal tubular homeostasis. mTORC1 deletion in proximal tubules increased susceptibility to IR injury as reflected in more severe tubular damage and decline in renal function.⁸⁵ However, the beneficial effect of enhanced autophagy in response to inhibition of mTORC1 during IR may be offset by the loss of mTOR1-mediated effects on cellular growth and survival. Along these lines, everolimus, a derivative of rapamycin and an inhibitor of mTORC1, was able to increase autophagy in rats subjected to IR but was unable to protect from IR-induced renal dysfunction and tubular damage.⁸⁷ Rapamycin, an inhibitor of the mTOR pathway, was reported to impair tubular proliferation and delay recovery of renal function during IR^{88,89} in accordance with inhibition of mTOR-mediated delayed graft function in patients who had undergone kidney transplant and was shown to prevent IR-induced renal injury.⁹⁰ Because multiple cellular processes of cellular growth and survival impinge on the mTORC1 inhibition, caution is needed in the interpretation of the autophagy induction effects by mTORC1 inhibition.

A recent study evaluated autophagic activity (autophagic flux) during both the injury and recovery phase of IR injury. Li *et al.*⁹¹ generated autophagy reporter mice that express a tandem red fluorescent protein (RFP)–enhanced GFP–LC3 fusion protein under the control of chicken β -actin and subjected these mice to IR injury. Because RFP is stable in acidic pH, whereas enhanced GFP is quenched in acidic pH, formation of acidic autolysosomes was distinguished from autophagosomes during the course of IR injury. In this study, basal enhanced GFP and RFP fluorescence did not change when examined at the end of 45 minutes of ischemic injury or 4 hours post-reperfusion period, suggesting that autophagy was not activated during this time. At the later reperfusion periods, both enhanced GFP and RFP fluorescence reached a peak value at 24 hours after reperfusion, but at 3 days after reperfusion, only RFP fluorescence persisted. Additionally, at 7 days after reperfusion, RFP fluorescence returned to basal level. These studies suggest that autophagosome formation is reduced 24 hours after reperfusion, and thereafter, upon fusion with lysosomes, formation of autolysosomes persists during renal recovery to clear the autophagosomal cargo. However, caution should be exercised in reporting RFP puncta for the autophagosomal vesicles, as RFP protein is reported to accumulate in the lysosome during overexpression, which can lead to incorrect interpretation.

In an *in vitro* model of adenosine triphosphate depletion, upregulation of autophagy protected LLC-PK1 cells via AMPK-mediated downregulation of phosphorylation of mTOR.⁹² In this model, inhibition of AMPK by small hairpin RNA increased the phosphorylation of mTOR and suppressed autophagy,⁹² suggesting that AMPK regulated mTOR pathway in an *in vitro* model of IR. Activation of the AMPK-regulated

autophagy pathway in response to quercetin flavonol provided protection from IR injury.⁹³

Autophagy in response to cisplatin

Autophagy induction and its cytoprotective role have been demonstrated in both *in vitro* and *in vivo* models of cisplatin-induced AKI. Cisplatin induced autophagy before caspase activation and apoptosis *in vitro* in cultured proximal tubular epithelial cells^{94,95} and *in vivo* in renal tubules from a murine model of cisplatin nephrotoxicity.^{96–100} Inhibition of autophagy either by 3-MA or small interfering RNAs specific to beclin-1 or Atg5 increased cisplatin-induced caspase activation and apoptosis in LLC-PK1 cells,⁹⁴ suggesting that autophagy plays a cytoprotective role against cisplatin injury. A similar pro-survival role of autophagy was observed in cultured rat proximal tubular epithelial cells exposed to cisplatin.⁹⁵ In NRK-52E cells, autophagy provided protection from cisplatin injury at a lower dose (10 μ M) of cisplatin, whereas 50 μ M cisplatin resulted in very low induction of autophagy and the autophagy inhibitor did not increase cisplatin-induced apoptosis.¹⁰¹ In addition, taurine enhanced autophagic protection against cisplatin-induced apoptosis by reducing ER stress in NRK-52E cells.¹⁰¹ Another study in NRK-52E cells has shown that suppression of autophagy either by autophagy inhibitors or beclin-1 small interfering RNA also prevented apoptosis.⁹⁶ The discrepancy in this study with other *in vitro* results could be due to differences in the doses of cisplatin or differences in the cell types used. Primary cultures of proximal tubular epithelial cells prepared from proximal tubule-specific Atg7-KO mice (described below) were more susceptible to cisplatin-induced caspase activation and apoptosis compared with wild-type mice exposed to cisplatin,⁹⁹ further supporting a cytoprotective role of autophagy against cisplatin injury to cultured proximal tubular epithelial cells. Nevertheless, further work in *in vivo* studies has confirmed the cytoprotective role of autophagy as reported in the *in vitro* findings.

To evaluate the role of autophagy *in vivo* in cisplatin nephrotoxicity, both pharmacological and genetic approaches were used. Chloroquine, which blocks autophagy flux and impairs cargo clearance, worsened cisplatin-induced tubular damage and decline in renal function,^{98,99} suggesting a renoprotective role of cisplatin-induced autophagy. For a genetic approach, proximal tubule-specific autophagy-deficient mice were used. Cisplatin treatment caused more severe tubular damage and renal dysfunction in proximal tubule-specific Atg5-KO mice¹⁰⁰ as well as in Atg7-KO mice⁹⁹ compared with cisplatin-administered wild-type mice. Autophagy-deficient mice exhibited enhanced activation of p53, apoptosis (measured by terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate nick end-labeling assay) and c-Jun terminal kinase signaling pathways known to cause cisplatin-induced AKI. In addition, cisplatin treatment markedly increased damaged mitochondria in immortalized Atg5-deficient proximal tubular cells compared with control cells.¹⁰⁰

Autophagy in response to sepsis

Autophagy in rat proximal tubules was transiently induced at 3 hours but declined at 9 hours until 18 hours during cecal ligation and puncture model of sepsis.¹⁰² Augmentation of autophagy either by temsirolimus or an inducer of AMP kinase, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), protected proximal tubules and improved renal function in a mouse model of endotoxemia.¹⁰³ In these studies, endotoxemia was associated with activation of mTORC1. Therefore, it is likely that mTORC1 inhibition enhances autophagic flux. The inability of older mice to recover from AKI has been attributed to an age-dependent loss of autophagy. In the cecal ligation and puncture model, induction of autophagy by AICAR-induced activation of AMPK decreased circulating cytokines and endothelial activation, and improved decline in renal function.¹⁰⁴ Contrary to the accepted paradigm, a recent study has reported that calcium/calmodulin-dependent protein kinase type IV-dependent preservation of mTORC1 is indispensable in LPS-induced autophagy in renal tubular cells and macrophages both *in vitro* and *in vivo*.¹⁰⁵ However, additional studies are required to investigate the role of mTORC1-mediated induction of autophagy in sepsis-induced AKI. Pexophagy, a form of selective autophagy, was recently shown to be induced in LPS-induced AKI and lysosomal defect accumulated dysfunctional peroxisomes that promoted oxidative injury.⁷⁵

Triggers for induction of autophagy in AKI

A wide range of cellular stresses can trigger autophagy during the pathogenesis of AKI. Production of reactive oxygen species and subsequent oxidative stress are involved in the development of AKI. Reactive oxygen species are known to trigger autophagy in mammalian cells and tissues.^{106,107} Mitochondria abundantly present in the kidney are vulnerable to damage by reactive oxygen species, which leads to mitochondrial dysfunction, loss of mitochondrial membrane, and potential initiation of mitophagy.¹⁰⁸ ER stress contributes to the development of AKI in humans and in animal models of AKI.^{109–111} ER stress is known to induce autophagy in mammalian cells^{111,112} and in the kidney.¹¹³ Tunicamycin-induced ER stress and pharmacological compound Bix, which activates unfolded protein response, afforded protection against renal IR injury,^{114,115} and ER-induced autophagy was shown to be involved in this protection.¹¹³ Energy-sensitive kinases mTORC1 and AMPK, which are important components of AKI, are known to regulate autophagy.^{24,26,28,29,31} mTORC1 phosphorylates ULK1/2 and Atg13, which inhibit ULK1/2 kinase activity^{23,28} and therefore negatively regulate autophagy. AMPK acts as a positive regulator of autophagy by inactivating mTORC1 and activating ULK1/2 kinase.^{26,31} Other molecules including anti-apoptotic members of the Bcl-2 family, BNIP3, HIF, and p53, which play important roles in AKI, are known to induce autophagy.¹¹⁶ The specific roles of induction of autophagy in response to these triggers are yet to be precisely elucidated in models of AKI.

Interplay of autophagy, apoptosis, and caspases and their interactions in AKI

Both autophagy and apoptosis are generally induced in response to a common stimulus. The regulation of shared pathways between autophagy and apoptosis determines the outcome of cell fate for either cell survival or cell death. The crosstalk between autophagy and apoptosis is mediated by several key molecules including members of the Bcl-2 family, autophagy proteins, and apoptosis-related proteins including caspases (Figure 3, Table 1). Liang and colleagues¹¹⁷ and Pattingre *et al.*¹¹⁸ first identified that beclin-1 interacts with the Bcl-2 antiapoptotic protein and inhibits autophagy. Further studies demonstrated that beclin-1, through its BH3-only domain, is able to bind to other Bcl-2 antiapoptotic proteins including Bcl-2, Bcl-xL, Bcl-x, Bcl-B, and, to a lesser extent, Mcl-1.^{119,120} Bcl-2 that is bound to beclin-1 retains its antiapoptotic function.¹²¹ Under nutrient-rich conditions, beclin-1 is bound to antiapoptotic Bcl-2 proteins and thereby inhibits autophagy.¹¹⁸ Under starvation conditions, JNK1-mediated phosphorylation of Bcl-2 prevents its interaction with beclin-1, enabling free beclin-1 to promote autophagy by binding to the class III PI3K complex.¹²² In addition, other proteins that disrupt interaction between the beclin-1 and antiapoptotic Bcl-2 family to promote positive regulation of autophagy are BH3-only proteins including BNIP3, NIX, NOXA, PUMA, BID, and BAD.^{123,124} Similarly, BH3 mimetics can displace Bcl-2 from beclin-1 and activate a pro-autophagic pathway.^{125,126} In a renal IR model, although Adv-Bcl-xL administration significantly reduced beclin-1 expression⁷⁶ and Bcl-2 augmentation suppressed autophagy,⁷⁸ the

interaction between Bcl-xL and beclin-1 in this model of AKI has not been elucidated.

Caspases also play important roles in the regulation of autophagy, in addition to their role in apoptosis. Caspase-8 has been shown to interact as a caspase-8–fas-associated death domain complex with the Atg5-Atg12 complex,^{127–129} be recruited to the autophagosome¹³⁰ and activated, leading to autophagy-mediated apoptosis (Figure 3, Table 1). p62 has also been shown to participate in recruitment of caspase-8 to the autophagosome. Ubiquitinated caspase-8 binds to p62 through its ubiquitin-binding domain and is recruited to the autophagosome by binding to LC3-II through the LIR of p62.^{130,131} The recruitment to the autophagosome by p62 facilitates self-oligomerization of caspase-8 and subsequent activation.

Both autophagy and caspases are activated in response to cisplatin in the renal tubular cell *in vitro* and *in vivo*. Autophagy induction is an immediate response, whereas caspases are activated after a pre-apoptotic lag phase in response to cisplatin.^{94,95,132} It has been demonstrated that an initial induction of autophagy was responsible for the pre-apoptotic lag phase in cisplatin injury.^{94,95,132} Inhibition of cisplatin-induced autophagy by pharmacological inhibitors 3-MA or Wortmannin or bafilomycin A and by small interfering RNA specific to Atg5 or beclin-1 enhanced caspase-3/7 and -6 activation^{94,132} and apoptosis,^{94,95,132} and overexpression of Atg5 and beclin-1 proteins prevented cisplatin-induced caspase activation and apoptosis⁹⁸ in cultured renal proximal tubular epithelial cells. Cisplatin administration after inhibition of autophagy by chloroquine or by using proximal

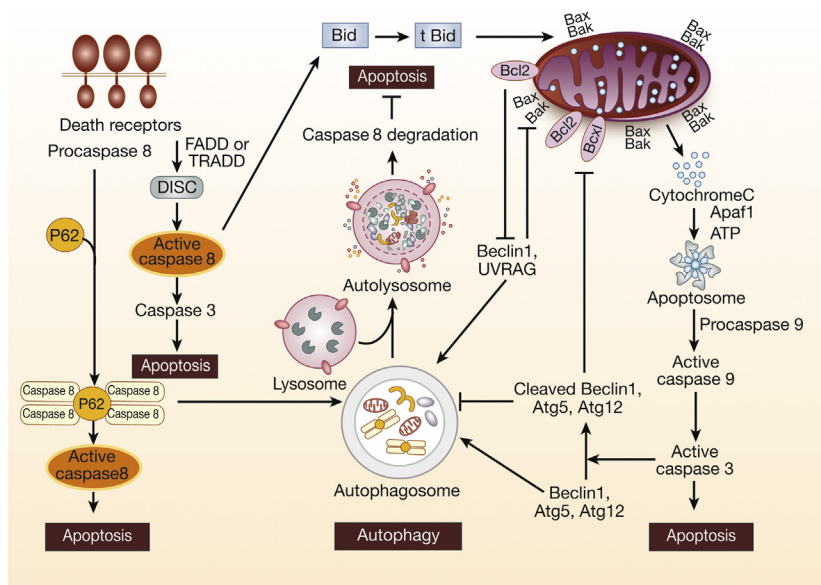


Figure 3 | Crosstalk between apoptosis and autophagy. Common proteins in the autophagy and apoptotic pathways are shared and intimately linked in crosstalk between apoptosis and autophagy-regulating cell death in mammalian cells. Degradation of key autophagy proteins such as Atg5, Atg3, Atg4D, Beclin-1, and Atg12 by active caspases represents one mechanism that limits continuous autophagosome formation. Antiapoptotic Bcl-2 family members bind to Beclin-1 and restrict availability of Beclin-1 for autophagosome formation and suppress autophagy. Autophagy substrate P62 participates in recruitment of caspase-8, which facilitates self-oligomerization of caspase-8 and subsequent activation. Once recruited to the autophagosome, caspase-8 can also be degraded, which suppresses apoptosis. DISC, death-inducing signaling complex; FADD, fas-associated protein with death domain; TRADD, tumor necrosis factor receptor type 1-associated death domain protein.

Table 1 | Molecular interactions between apoptosis and autophagy

Apoptosis-related proteins	Autophagy-related proteins	Effects of interaction	References
Bcl-2, BclxL, Bcl-x, Mcl-1 (antiapoptotic proteins)	Beclin-1	Beclin-1 binding to antiapoptotic proteins suppresses autophagy.	Escobar <i>et al.</i> , 2015 ¹⁰⁴ ; Zhang <i>et al.</i> , 2014 ¹⁰⁵ ; Chen <i>et al.</i> , 2009 ¹⁰⁶ ; Scherz-Shouval and Elazar, 2011 ¹⁰⁷
Bcl-2 and Mcl-1	Atg12	Atg12 enhances mitochondrial-targeted apoptosis by binding and antagonizing Bcl-2 and Mcl-1.	Khan <i>et al.</i> , 2012 ¹⁷⁸
Caspase-3, -7, -8	Beclin-1, PI3KC3/Vps 34	PI3KC3/Vps 34 by caspases suppresses autophagy, and cleaved Beclin-1 fragments enhance mitochondrial apoptosis.	Robert <i>et al.</i> , 2012 ¹²⁰ ; Wei <i>et al.</i> , 2008 ¹²² ; Mazure and Pouyssegur ¹²⁴
Caspase-8 Caspase-8-FADD complex	Atg5 Atg5-Atg12 complex	Caspase-8-mediated cleavage of Atg5 enhances apoptosis. Caspase-8 activation results in apoptosis.	Chang <i>et al.</i> , 2010 ¹²³ Prachasilchai <i>et al.</i> , 2009 ¹¹⁴ ; Prachasilchai <i>et al.</i> , 2008 ¹¹⁵ ; Livingston and Dong, 2014 ¹¹⁶
Caspase-3	Atg4D	Atg4D cleaved by caspase-3 increases autophagy and mitochondrial-targeted apoptosis.	Pedro <i>et al.</i> , 2015 ¹²⁶
Caspase-3, -7 Caspases BNIP3, NIX, NOXA, PUMA, BID, and BAD	Atg16L1 Ambra1 Bcl-2 - Beclin-1	Caspase degradation of Atg16L1 suppresses autophagy. Suppression of autophagy Promote autophagy by disruption of interaction between Bcl-2 and Beclin-1	Pyo <i>et al.</i> , 2005 ¹²⁷ Bell <i>et al.</i> , 2008 ¹²⁸ Kitamura, 2008 ¹¹⁰ ; Kitamura, 2009 ¹¹¹
BAX	Beclin-1 interacting UVRAG	UVRAG prevents translocation of BAX to mitochondria and prevents apoptosis.	Wang <i>et al.</i> , 2012 ¹⁷⁹
BIM DAP kinase	Beclin-1 Beclin-1	BIM sequesters Beclin-1 and suppresses autophagy. DAP kinase phosphorylates BH3 domain of Beclin-1 and promotes dissociation of Beclin-1-BclL and induction of autophagy.	Loos <i>et al.</i> , 2013 ¹⁸⁰ Lemasters, 1998 ¹⁸¹
DAP kinase DAP kinase 2	VPS34 mTORC1	DAP kinase activates VPS34 via PKD and upregulates autophagy. Interacts with mTORC1 and its partners, raptor and ULK1, and negatively regulates mTORC1	Jin <i>et al.</i> , 2007 ¹⁸² Liang <i>et al.</i> , 2014 ¹⁸³
cFLIP	Atg3	cFLIP prevents Atg3 conjugation to LC3 and suppresses autophagy.	Sridevi <i>et al.</i> , 2013 ¹⁷⁴ ; Yu <i>et al.</i> , 2004 ¹⁷⁵
Cytoplasmic p53	FIP200	Cytoplasmic p53 suppresses autophagy by interacting with FIP200.	Amaravadi and Thompson, 2007 ¹⁸⁴ ; Wu <i>et al.</i> , 2009 ¹⁸⁵
Nuclear p53	ULK1, sestrin1/2, and DRAM	Nuclear p53 induces autophagy by transactivation of ULK1, sestrin1/2, and DRAM.	Wu <i>et al.</i> , 2009 ¹⁸⁵ ; Matsuzawa <i>et al.</i> , 2015 ¹⁸⁶ ; Lee <i>et al.</i> , 2009 ¹⁸⁷

cFLIP, cellular fas-associated death domain-like interleukin-1 β -converting enzyme-inhibitory protein; DAP, death-associated protein; DRAM, damage-regulated autophagy modulator; FADD, fas-associated protein with death domain; LC, light chain; mTORC1, mammalian target of rapamycin complex 1; PKD, polycystic kidney disease; ULK1, uncoordinated 51-like kinase 1; UVRAG, ultraviolet radiation-associated gene.

tubule-specific Atg5-KO or Atg7-KO mice enhanced apoptosis and caspase activation.^{4,98,99}

Caspases are able to cleave several key autophagy proteins including beclin-1,^{133–135} Atg5,¹³⁶ VPS34,¹³⁷ ATG3,¹³⁸ ATG4D,¹³⁹ Atg16 L,¹⁴⁰ and AMBRA1,¹⁴¹ which result in suppression of autophagy (Table 1). The cleaved fragments of autophagy proteins produced have been shown to have a proapoptotic function. The protein fragments produced upon cleavage of beclin-1 by caspase-3, -6, or -9, and caspase-3–cleaved fragments of Atg4D, can localize to the mitochondria, resulting in mitochondrial permeabilization leading to cytochrome c release.^{137,139} In cisplatin nephrotoxicity, autophagy proteins beclin-1, Atg5, and Atg12 were cleaved and degraded during the course of renal tubular epithelial cells *in vitro* and *in vivo*, and the pancaspase inhibitor zVAD-fmk prevented cleavage of autophagy proteins.⁹⁸ Thus, autophagy proteins are targets of cisplatin-induced caspase activation, and the degradation of autophagy proteins is responsible for the decrease in autophagy during the course of cisplatin injury.

These studies suggest a crosstalk between autophagy, caspases, and apoptosis in renal injury in *in vitro* and *in vivo* models of AKI. Although not understood completely, some information on the signaling pathways involved in crosstalk between autophagy and apoptosis has been obtained from studies in non-renal cells. The signaling mechanisms in interactions between autophagy and apoptosis in AKI have not been elucidated yet.

Autophagy and necroptosis

Necroptosis is regulated necrosis or a programmed form of necrotic cell death that is mediated by receptor-interacting protein kinase (RIPK)-1, RIPK3, and mixed-lineage kinase domain-like protein (MLKL). Necroptosis has morphological features similar to those of unregulated necrotic cell death and was initially observed in response to inhibition of caspase-8 *in vitro*^{142,143} and in caspase-8–deficient mice *in vivo*.^{144,145} Diverse stimuli of necroptosis include death receptors of the tumor necrosis factor (TNF)- α superfamily,^{143,146} genotoxic

stress,^{147,148} toll-like receptors,^{149,150} interferons,¹⁵¹ oxidative stress,^{152,153} and virus-induced activation of DNA-dependent activator of interferon regulatory factors (DAI).^{133,154} The process of necroptosis is very well studied, initially in the TNF- α signaling pathway.

Upon activation of the death receptor TNFR1 by binding with a TNF- α ligand, the active receptor recruits several proteins including TNF receptor-associated death domain protein, receptor-interacting protein 1, TNF receptor-associated factor proteins, cellular inhibitors of apoptosis 1 or cellular inhibitors of apoptosis 2 and forms complex 1.¹⁵⁵ In complex 1, receptor-interacting protein 1 is polyubiquitinated by cellular inhibitors of apoptosis 1 and cellular inhibitors of apoptosis 2, and it prevents the activation of caspase-8 and promotes cell survival.^{156,157} The deubiquitination of RIPK1 by cylindromatosis¹⁵⁸ promotes the formation of the proapoptotic complex IIa comprised of TNFR1, TNF receptor-associated death domain protein, fas-associated death domain, RIPK1, RIPK3, and procaspase-8. This complex, also known as death-inducing signaling complex, facilitates activation of caspase-8, which subsequently activates the executioner caspases to promote cell apoptosis.¹⁵⁹ When caspase-8 is not fully active or inhibited by viral or chemical inhibitors or the levels of RIPK3 and MLKL are high, a necrosome composed of RIPK1, RIPK2, and MLKL is formed that results in necroptosis.^{160–162} Active caspase-8 has been reported to inhibit necroptosis by cleaving RIPK1, RIPK3, and cylindromatosis.^{163–165} RIPK1 associate with RIPK3 through their unique interacting domain known as RIP homotypic interaction motif, leading to the formation of supramolecular complex necrosome. RIPK3 in the complex is activated by autophosphorylation, which enables RIPK3 to phosphorylate MLKL.¹⁶⁶ The phosphorylation of MLKL leads to the formation of MLKL oligomers that translocate from the cytosol to the plasma membrane. It has been suggested that binding of MLKL oligomers to phosphatidylinositol phosphates in plasma membranes disrupts membrane integrity^{167,168} and induces influx of Ca⁺² and Na⁺, causing membrane rupture or necrosis.^{168–170}

Necrostatin-1 (an inhibitor of RIPK1, RIPK3-KO, and MLKL-KO) mice were recently used to demonstrate the role of necroptosis in IR- and cisplatin-induced AKI. Inhibition of necroptosis by necrostatin-1 provided protection from tubular damage and prevented a decline in renal function during IR-¹⁷¹ and cisplatin-induced¹⁷² AKI. RIPK3-KO mice were protected from tubular necrosis and renal dysfunction during renal IR.¹⁷³ Both RIPK3-KO and MLKL-KO mice subjected to cisplatin nephrotoxicity were resistant to cisplatin-induced necroptosis and renal dysfunction.¹⁷² Inhibition of apoptosis was unable to prevent a cisplatin-induced decline in renal function,^{98,172,174} and it was suggested that cisplatin-induced cell death is predominantly due to necroptosis.¹⁷²

Autophagy induction has been shown to regulate necroptosis in some models.^{128,175,176} Pancaspase-inhibitor zVAD-induced autophagy, as well as caspase-independent

cell death that involved RIPK1,¹⁷⁵ but autophagy provided a protective role in zVAD-induced cell death.¹⁷⁷ Inhibition of autophagy has been shown to prevent necroptosis,¹⁷⁸ and conversely, inhibition of necroptosis suppressed not only necrosis but also autophagy.¹⁷⁹ Although insufficient production of bioenergetics or adenosine triphosphate depletion results in induction of both autophagy^{10,180} and necrosis/necroptosis,^{181–183} autophagy induction promotes production of adenosine triphosphate and meets energy needs to ensure cell viability.^{177,180,184,185} Some molecular mechanisms that link autophagy with RIPK1-mediated necroptosis have been reported.¹²⁸ A recent study has identified that RIPK3 interacts with the selective autophagy substrate p62 and regulates the p62-LC3 complex via caspase-8-dependent cleavage of p62.¹⁸⁶ Another study has shown that Atg3 and cellular fas-associated death domain-like interleukin-1 β -converting enzyme-inhibitory protein participate in a crosstalk between autophagy and necroptosis. Cellular fas-associated death domain-like interleukin-1 β -converting enzyme-inhibitory protein mediates the initiation of necroptosis by inhibiting caspase-8, binds to Atg3 and prevents its conjugation to LC3, and blocks the formation of LC3-II and autophagy.^{187,188} Because autophagy plays a protective role in AKI, more studies are required to elucidate whether autophagy influences AKI-induced necroptosis.⁴⁸

CONCLUSIONS

There is a growing interest in studying the role and regulation of the autophagy pathway in AKI partly because of the notion that autophagy is cytoprotective in response to various stresses. The development of kidney-specific KO mice has provided evidence that deletion of autophagy in proximal tubules worsens tubular injury and renal function. However, direct evidence of the role of overexpression of autophagy in AKI is not yet known. There is a need to specifically induce autophagy in proximal tubules to examine the effect in models of AKI. Also, there is a need to develop effective pharmacological inducers of autophagy that are specifically able to induce autophagy without having side effects on other metabolic pathways. Although pharmacological inhibition of mTOR may upregulate autophagy, the mTOR pathway is also an important regulator of cell growth and proliferation and cellular homeostasis. The pathogenesis of AKI may result in multiple stresses that induce autophagy. Whether signaling pathways transduced from these stresses converge into suppression of the mTOR pathway for the induction of autophagy is yet to be elucidated.

At present, the efficiency of the autophagic flux of the autophagy pathway in AKI is not known. The precise kinetics of autophagy activation during the course of development of AKI in experimental models is not yet established. It is not known whether autophagy is insufficient or the flux is impaired during AKI. Because efficient flux is critical for survival, more careful studies are required for measurement of autophagic flux *in vivo* during AKI. Because lysosomes are involved in autophagy cargo clearance, it may be necessary to

critically assess lysosomal dysfunction in the pathogenesis of AKI. Recent studies have recognized that autophagy is selective in degrading specific targets including damaged organelles. However, more studies are required to understand precise mechanisms of selective autophagy including mitophagy in AKI.

Finally, both in animal models and humans, severity of AKI is linked with the progression of chronic kidney disease. About 15% to 20% of patients with AKI progress to chronic kidney disease stage IV. Considering tubulointerstitial fibrosis as the hallmark of end-stage renal disease and the basic degradative potential of normal autophagic flux, there is a need to explore whether autophagy defect plays a role in the progression of AKI to chronic kidney disease.

DISCLOSURE

All the authors declared no competing interests.

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