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Autophagy in Acute Kidney Injury

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Abstract

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, ER 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.

Keywords

acute kidney injury; autophagy; mitophagy; apoptosis; cell death; ischemia reperfusion; cisplatin nephrotoxicity

INTRODUCTION

Basic principle of autophagy and its relevance to acute kidney injury

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-

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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 autophagosomes¹⁻³.

Autophagy is an evolutionarily conserved multi-step process of degradation of intracellular organelles, proteins, and other macromolecules by the hydrolases of lysosomes⁴⁻⁶. The degraded cellular contents are reutilized 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 autophagy⁷⁻⁹. Among these stresses, autophagy induction in response to starvation is extensively studied, well characterized, and is shown to be mediated through mTOR, 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 eliminating 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 diseases¹¹. However, the role and regulation of autophagy induction in AKI is not extensively studied as in other diseases as noted above.

Lysosomal degradation of cellular contents involve three 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 is under current investigation, and some studies have proposed plasma membrane, Golgi complex, ER or mitochondria as possible sources¹²⁻¹⁶. Following 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 (CMA) 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 degradation¹⁷. 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 ULK1/ULK2 (uncoordinated (UNC)-51-like kinase ½) 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 LC3 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}.

(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 (mTORC1) and AMPK are two nutrient- and energy-sensitive kinases²²⁻³⁰ involved in regulating activity of the ULK1/2 complex. mTORC1 negatively regulates autophagy, and this function of mTORC1 is conserved in eukaryotes³⁰. AMPK phosphorylates tuberous sclerosis complex (TSC), which inhibits mTORC1^{26, 31}. AMPK can also phosphorylate the raptor component of mTORC1 when released from the ULK1 complex, resulting in inactivation of mTORC1²⁶.

(ii) Beclin-1/class III phosphatidylinositide-3 kinase (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 1³³, and the active PI3K complex phosphorylates phosphatidyl inositol to form phosphatidyl inositol-3 phosphate (PtdIns3P). PtdIns3P produced at the site of phagophore membranes not only is important for stable phagophore membrane nucleation, further growth, and initial phagophore curvature, but also important to facilitates recruitment of other regulatory PtdIns3P-binding proteins including DFCP1 (double FYVE-containing protein 1) and WIPI (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 formation of two ubiquitin-like conjugation systems: ATG16L1 complex (ATG12-ATG5-ATG16L1) and lipidation of microtubule-associated protein-1 light chain-3 (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 phosphatidylethanolamine (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 WIPI2 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 formation⁴⁰⁻⁴².

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 involved⁴³⁻⁴⁵. A recent study demonstrated that autophagosome-endolysosome fusion requires oligomeric ATG14 that interacts with STX17–SNAP29 binary t-SNARE complex on autophagosomes and primes it for VAMP8 interaction to promote fusion⁴⁶. The inner membrane LC3-II of the autolysosome is degraded along with the rest of the sequestered cargo by lysosomal hydrolases¹⁹.

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), endoplasmic reticulum (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 (UBA), 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 (UBA) 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 NBR1 (neighbor of BRCA1 gene1), NDP52 (nuclear dot protein 52 kDa), optineurin, and TAX1BP1 (Tax1 binding protein 1) and display much similarity to the domain structure of p62 and contain dimerization or polymerization domain, LIR region, and UBA 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 important roles 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 ATP production, all of which are associated with AKI⁵⁶⁻⁵⁸, promote mitochondrial depolarization and dysfunction, which can trigger mitophagy⁵⁹. Parkin, PINK1 (PTEN-induced putative kinase protein 1), NIX, BNIP3, and FUDNC1 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 disease⁶². The underlying mechanism 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 mitochondria⁶³. 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 that may facilitate autophagic clearance of the damaged mitochondria⁶⁷. 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-1⁷⁰. FUNDC1 mediates in hypoxia-induced mitophagy⁷¹.

Little information is known on the induction of mitophagy in renal tubular cells in AKI. Ishihara et al.⁷² demonstrated that BNIP3 and sestrin 2 are upregulated during renal 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 microscopy⁷². The expression of PINK, a marker of mitophagy, was increased during renal ischemia-reperfusion injury⁷³. Another study has shown that mitophagy in the kidneys of a rat fed low-calorie diet was markedly increased and ameliorated oxidative damage compared to that of high-calorie fed rats⁷⁴. Limited information is available on the selective eliminations of other organelles in AKI⁷⁵. 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 ischemia-reperfusion (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 LC3-1 to LC3-II or by 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 utilizing conditional kidney proximal tubule-specific Atg5- or Atg7-knockout (KO) mice. Kimura et al.⁸¹ used Atg5^{flox/flox} Kap-Cre mice under the control of an inducible promoter KAP (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 TUNEL-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 injury⁸¹, 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 to wild-type mice⁷⁹. 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 BUN and

creatinine for up to 16 days after IR injury⁷¹. 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 damage⁷¹. 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 protection⁸⁴.

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 function⁸⁵. 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 inhibitor mTORC1, was able to increase autophagy in rats subjected to IR but was unable to protect from IR-induced renal dysfunction and tubular damage⁸⁷. 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 kidney transplant patients and was shown to prevent IR-induced renal injury⁹⁰. Since 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)-EGFP-LC3 fusion protein under the control of chicken β -actin (CAG) and subjected these mice to IR injury. Since RFP is stable in acidic pH while EGFP is quenched in acidic pH, formation of acidic autolysosomes was distinguished from autophagosomes during the course of IR injury. In this study, basal EGFP and RFP fluorescence did not change when examined at the end of 45 minutes of ischemic injury or 4h post-reperfusion period, suggesting that autophagy was not activated during this time. At the later reperfusion periods, both EGFP and RFP fluorescence reached a peak value at 24h after reperfusion, but at 3d after reperfusion, only RFP fluorescence persisted. Additionally, at 7d after reperfusion, RFP fluorescence returned to basal level. These studies suggest that autophagosome formation is reduced 24 h after reperfusion and thereafter, upon fusion with lysosomes, formation of autolysosomes persist 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 ATP-depletion, up-regulation of autophagy protected LLC-PK1 cells via AMPK-mediated down-regulation of phosphorylation of mTOR⁹². In this model, inhibition of AMPK by small hairpin RNA (shRNA) increased the phosphorylation of mTOR and suppressed autophagy⁹², 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 injury⁹³.

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 prior to 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 nephrotoxicity⁹⁶⁻¹⁰⁰. Inhibition of autophagy either by 3-MA or siRNAs specific to beclin-1 or Atg5 increased cisplatin-induced caspase activation and apoptosis in LLC-PK1 cells⁹⁴, 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 cisplatin⁹⁵. 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 apoptosis¹⁰¹. In addition, taurine enhanced autophagic protection against cisplatin-induced apoptosis by reducing ER stress in NRK-52E cells¹⁰¹. Another study in NRK-52E cells has shown that suppression of autophagy either by autophagy inhibitors or beclin-1 siRNA also prevented apoptosis⁹⁶. 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 to wild-type mice exposed to cisplatin⁹⁹, 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 utilized. 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 to cisplatin-administered wild-type mice. Autophagy-deficient mice exhibited enhanced activation of p53, apoptosis (measured by TUNEL 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 to control cells¹⁰⁰.

Autophagy in response to sepsis

Autophagy in rat proximal tubules was transiently induced at 3 h but declined at 9 h until 18 h during cecal ligation and puncture (CLP) model of sepsis¹⁰². 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 endotoxemia¹⁰³. 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 CLP model, induction of autophagy by AICAR-induced activation of AMPK decreased circulating cytokines, endothelial activation, and improved decline in renal function¹⁰⁴. Contrary to the accepted paradigm, a recent study has reported that CaMKIV-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 injury⁷⁵.

Triggers for induction of autophagy in AKI

A wide range of cellular stresses can trigger autophagy during the pathogenesis acute kidney injury. Production of ROS and subsequent oxidative stress are involved in the development of AKI. ROS are known to trigger autophagy in mammalian cells and tissues^{106, 107}. Mitochondria abundantly present in the kidney are vulnerable to damage by ROS that leads mitochondrial dysfunction, loss of mitochondrial membrane potential initiation of mitophagy¹⁰⁸. ER stress contributes to the development of AKI in humans and in animal models of AKI¹⁰⁹⁻¹¹¹. ER stress is known to induce autophagy in mammalian cells^{111, 112} and in the kidney¹¹³. Tunicamycin-induced ER stress and pharmacological compound Bix that activates UPR afforded protection against renal ischemia-reperfusion (IR) injury^{114, 115} and ER-induced autophagy was shown to be involved in this protection¹¹³. Energy sensitive kinases mTORC1 and AMPK that are important components of AKI, are known to regulate autophagy^{24, 26, 28, 29, 31}. mTORC1, phosphorylates ULK1/2 and Atg13 that inhibit ULK1/2 kinase activity^{23, 28} and therefore negatively regulates autophagy. AMPK acts as an appositive regulator of autophagy by inactivating mTORC1 and activating ULK1/2 kinase^{26, 31}. Other molecules including antiapoptotic members of the Bcl-2 family, BNIP3, HIF, and p53 that play important roles in AKI are known to induce autophagy¹¹⁶. 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 cross-talk between autophagy and apoptosis is mediated by several key molecules including members of Bcl-2 family, autophagy proteins, and apoptosis related proteins including caspases (**Figure 3, Table 1**).

Levine and colleagues first identified that beclin-1 interacts with the Bcl-2 antiapoptotic protein and inhibits autophagy^{117, 118}. 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 bound to beclin-1 retains its antiapoptotic function¹²¹. Under nutrient-rich conditions, beclin-1 is bound to antiapoptotic Bcl-2 proteins and thereby inhibits autophagy¹¹⁸. 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 complex¹²². 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 ischemia-reperfusion model, although Adv-Bcl-xL administration significantly reduced beclin-1 expression⁷⁶ and Bcl-2 augmentation suppressed autophagy⁷⁸, 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-FADD complex with the Atg5-Atg12 complex¹²⁷⁻¹²⁹, 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 (UBD) and is recruited to the autophagosome by binding to LC3-II through the LC3-interacting region (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 following 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 siRNA 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 following inhibition of autophagy by chloroquine or by utilizing proximal 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-1¹³³⁻¹³⁵, Atg5¹³⁶, VPS34¹³⁷, ATG3¹³⁸, ATG4D¹³⁹, Atg16 L¹⁴⁰, and AMBRA1¹⁴¹ that result in suppression of autophagy (**Table 1**). The cleaved fragments of autophagy proteins produced have been shown to have a pro-apoptotic 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 in RTEC *in vitro* and *in vivo* and the pancaspase inhibitor zVAD-fmk prevented cleavage of autophagy proteins⁹⁸. 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 cross-talk 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 cross-talk 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 yet elucidated.

Autophagy and necroptosis

Necroptosis is regulated necrosis or a programmed form of necrotic cell death that is mediated by receptor-interacting protein kinase-1 (RIPK1), RIPK3, and mixed-lineage kinase domain-like protein (MLKL). Necroptosis has morphological features similar to that 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 including death receptors of the TNF- α superfamily^{143, 146}, genotoxic stress^{147, 148}, Toll-like receptors^{149, 150}, interferons¹⁵¹, oxidative stress^{152, 153}, and virus-induced activation of DNA-dependent activator of IFN 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 TRADD (TNF receptor-associated death domain protein), RIP1 (receptor-interacting protein 1), TRAFs (TNF receptor-associated factor proteins), cIAP1 or cIAP2 (cellular inhibitors of apoptosis) and forms complex 1¹⁵⁵. In the complex 1, RIP1 is polyubiquitinated by cIAP1 and cIAP2, and it prevents the activation of caspase-8, and promotes cell survival^{156, 157}. The deubiquitination of RIPK1 by cylindromatosis (CYLD)¹⁵⁸ promotes the formation of the pro-apoptotic complex IIa comprised of TNFR1, TRADD, FADD, RIPK1, RIPK3, and procaspase-8. This complex, also known as death inducing signaling complex (DISC), facilitates activation of caspase-8, which subsequently activates the executioner caspases to promote cell apoptosis¹⁵⁹. 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 necroptosis¹⁶⁰⁻¹⁶². Active caspase-8 has been reported to inhibit necroptosis by cleaving RIPK1, RIPK3, and CYLD¹⁶³⁻¹⁶⁵. RIPK1 associate with RIPK3 through their unique interacting domain known as RIP homotypic interaction motif (RHIM), leading to the formation of supramolecular complex necrosome. RIPK3 in the complex is activated by autophosphorylation that enables RIPK3 to phosphorylate MLKL¹⁶⁶. 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 disrupt membrane integrity^{167, 168} and induce influx of Ca⁺² and Na⁺, causing membrane rupture or necrosis¹⁶⁸⁻¹⁷⁰.

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 IR¹⁷³. Both RIPK3-KO and MLKL-KO mice subjected to cisplatin nephrotoxicity were resistant to cisplatin-induced necroptosis and renal dysfunction¹⁷². 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 necroptosis¹⁷².

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 RIPK1¹⁷⁵ but autophagy provided a protective role in zVAD-induced cell death¹⁷⁷. Inhibition of autophagy has been shown to prevent necroptosis¹⁷⁸ and conversely, inhibition of necroptosis suppressed not only necrosis but also autophagy¹⁷⁹. While insufficient production of bioenergetics or ATP depletion results in induction of both autophagy^{10, 180} and necrosis/necroptosis¹⁸¹⁻¹⁸³, autophagy induction promotes production of ATP and meets energy needs to ensure cell viability^{177, 180, 184, 185}. Some molecular mechanisms that link autophagy with RIPK1-mediated necroptosis have been reported¹²⁸. 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 p62¹⁸⁶. Another study has shown that Atg3 and cFLIP participate in a cross-talk between autophagy and necroptosis. cFLIP 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}. Since autophagy plays a protective role in acute kidney injury, more studies are required to elucidate whether autophagy influences AKI-induced necroptosis⁴⁸.

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. Since efficient flux is critical for survival, more careful studies are required for measurement of autophagic flux *in vivo* during AKI. Since 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 CKD. About 15-20% patients with AKI progress to CKD 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 CKD.

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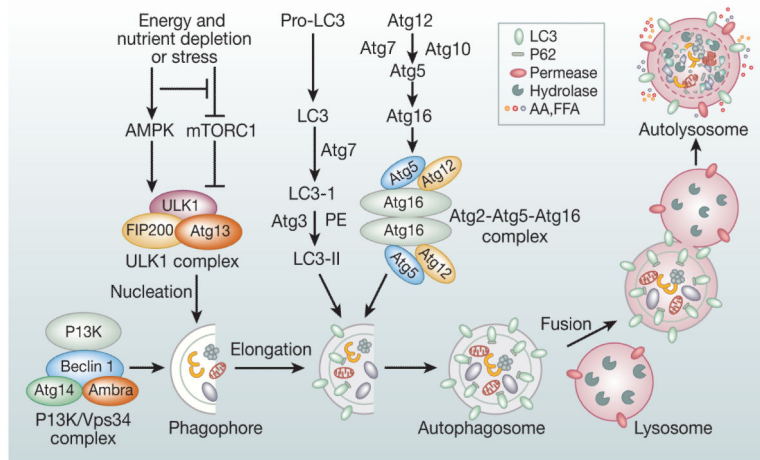


Figure 1.

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 ULK1/2 kinase complex composed of ULK1/2, Atg13, RB1CC1/FIP200, and Atg101 is recruited to the phagophore membranes. It is required for initiation of autophagy and is regulated by mTORC1 and AMPK. (ii) PI3K/Vps34 complex composed of Vps34, Vps 15, 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/LC3-II are two ubiquitin-like conjugation systems that are involved in the elongation and expansion steps in the autophagosome formation. LC3-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.

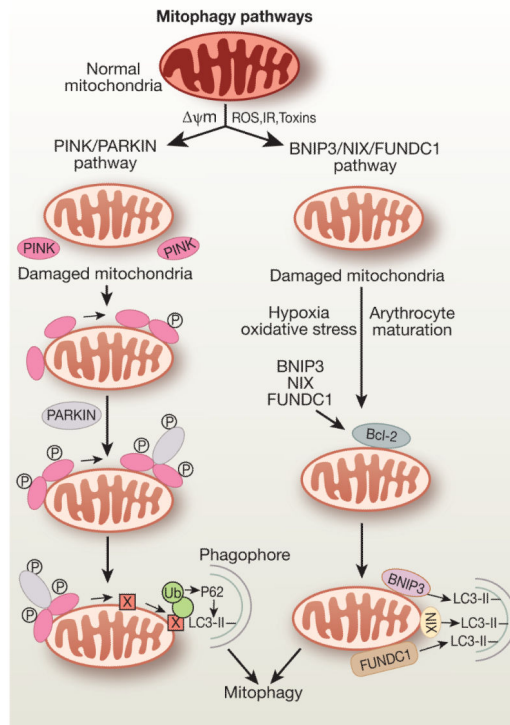


Figure 2.

PINK/PARKIN and BNIP3/NIX/FUNDC1 pathways of mtophagy. In the PINK/PARKIN pathway, mitochondrial depolarization or mitochondrial fission leads to localization of PINK1 in the outer membrane of the depolarized mitochondria. PINK1 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 LC3, thereby targeting the mitochondrion for mitophagy. In the BNIP3/NIX/FUNDC1 pathway, mitochondrial outer membrane proteins BNIP3, NIX, or FUNDC1 bind to LC3-II via their LIR motifs facing the cytosol and promote selective clearance of mitochondria. NIX is involved in the process of mitochondrial clearance during reticulocytes maturation and BNIP3 is upregulated in renal IR injury and overexpression of BNIP3 in NRK-53E cells selectively induced mitophagy⁷².

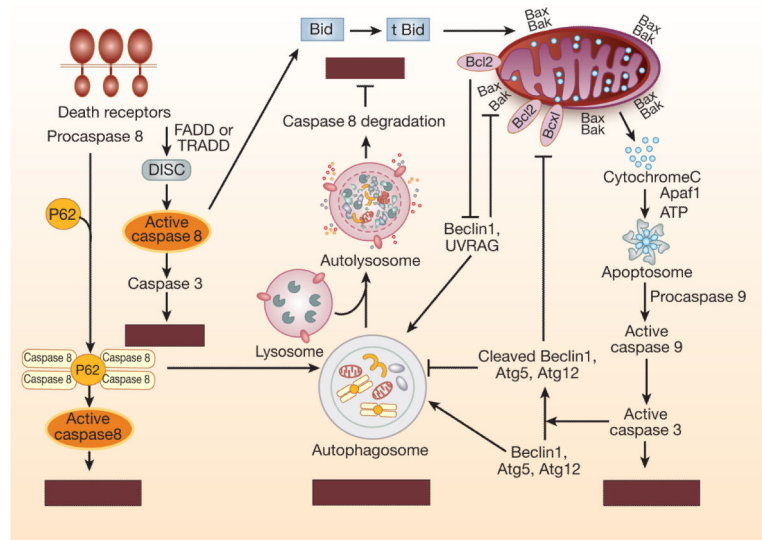


Figure 3. Cross-talk between apoptosis and autophagy. Common proteins in the autophagy and apoptotic pathways are shared and intimately linked in cross-talk between apoptosis and autophagy regulating cell death in mammalian cells. Degradation of key autophagy proteins such as Atg5, Atg3, Atg4D, Beclin-1 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 Beclin-1 for autophagosome formation and suppress autophagy. Autophagy substrate P62 participates in recruitment of caspase-8 and facilitates self-oligomerization of caspase-8 and subsequent activation. Caspase-8 once recruited to the autophagosome can also be degraded that suppresses apoptosis.

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	104,105,106, 107
Bcl-2 and Mcl-1	Atg12	Atg12 enhances mitochondrial-targeted apoptosis by binding and antagonizing Bcl-2 and Mcl-1	178
Caspase-3, -7, -8	Beclin-1, PI3KC3/Vps 34	Cleavage of Beclin-1 and PI3KC3/Vps 34 by caspases suppresses autophagy and cleaved Beclin-1 fragments enhance mitochondrial apoptosis	120,122,124
Caspase-8	Atg5	Caspase-8-mediated cleavage of Atg5 enhances apoptosis	123
Caspase-8-FADD complex	Atg5-Atg12 complex	Caspase-8 activation results in apoptosis	114, 115, 116
Caspase-3	Atg4D	Atg4D cleaved by caspase-3 increases autophagy and mitochondrial-targeted apoptosis	126
Caspase-3, -7	Atg16L1	Caspase degradation of Atg16L1 suppresses autophagy	127
Caspases	Ambra1	Suppression of autophagy	128
Bnip3, Nix, Noxa, Puma, bid, and Bad	Bcl-2 - Beclin-1	Promote autophagy by disruption of interaction between Bcl-2 and Beclin-1	110,111
Bax	Beclin-1 interacting UV radiation-associated gene (UVRAG)	UVRAG prevents translocation of Bax to mitochondria and prevents apoptosis	179
Bim	Beclin-1	Bim sequesters Beclin-1 and suppresses autophagy	180
DAP kinase	Beclin-1	DAPK Phosphorylate BH3 domain of Beclin-1 and promotes dissociation of Beclin-1-BclxL and induction of autophagy	181
DAP kinase	VPS34	DAP kinase activates VPS34 via PKD and upregulates autophagy	182
DAP kinase 2	mTORC1	Interacts with mTORC1 and its partners, raptor and ULK1 and negatively regulates mTORC1	183
cFLIP	Atg3	cFLIP prevents Atg3 conjugation to LC3 and suppresses autophagy	174, 175
Cytoplasmic p53	FIP200	Cytoplasmic p53 suppresses autophagy by interacting with FIP200	184
Nuclear p53	ULK1, sestrin1/2, and DRAM	Nuclear p53 induces autophagy by transactivation of ULK1, sestrin1/2, and DRAM	185-187