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Research Paper

Oxidative stress and autophagy: Crucial modulators of kidney injury

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ABSTRACT

Both acute kidney injury (AKI) and chronic kidney disease (CKD) that lead to diminished kidney function are interdependent risk factors for increased mortality. If untreated over time, end stage renal disease (ESRD) is an inevitable outcome. Acute and chronic kidney diseases occur partly due to imbalance between the molecular mechanisms that govern oxidative stress, inflammation, autophagy and cell death. Oxidative stress refers to the cumulative effects of highly reactive oxidizing molecules that cause cellular damage. Autophagy removes damaged organelles, protein aggregates and pathogens by recruiting these substrates into double membrane vesicles called autophagosomes which subsequently fuse with lysosomes. Mounting evidence suggests that both oxidative stress and autophagy are significantly involved in kidney health and disease. However, very little is known about the signaling processes that link them. This review is focused on understanding the role of oxidative stress and autophagy in kidney diseases. In this review, we also discuss the potential relationships between oxidative stress and autophagy that may enable the development of better therapeutic intervention to halt the progression of kidney disease and promote its repair and resolution.

Introduction

Kidneys are remarkable organs because they perform several functions essential for healthy living such as regulation of body fluids and blood pressure, waste products excretion, and production of red blood cells. Human kidneys receive approximately 25% of cardiac output and consume 7% of daily energy expenditure to support their diverse functions. Kidney diseases pose a worldwide health problem and lead to significant morbidity and mortality amongst adults, especially older adults. In a recent National Health interview survey, out of 234,921 adults aged 18 and over, 3882 (approximately 2%) adults reported that in the past 12 months they had been diagnosed with kidney disease. Kidney diseases are mainly classified into two types, either acute kidney injury (AKI) or chronic kidney disease (CKD). AKI is characterized by sudden and sometimes fatal loss of kidney function resulting in the accumulation of end products of nitrogen metabolism (urea) and creatinine, or decreased urine output, or both [1]. The number of hospital stays associated with AKI has increased from 3942 in 1996 to 23,052 in 2008 [2]. Reduction in kidney function over a period of time results in chronic kidney disease. CKD is characterized by a glomerular filtration rate below 60 mls per minute for more than 3 months or urine albumin-to-creatinine ratio over 30 mg of albumin for each gram of creatinine. According to the Center for Disease Control and Prevention "1 in 10 American adults, more than 20 million individuals, have some level of chronic kidney disease" [2]. The prevalence of CKD is increasing most rapidly in adults 60 years old and above, with the incidence of CKD increasing most rapidly in individuals of age 65 and above [2]. Both AKI and CKD are closely interconnected syndromes and each disease serves as a risk factor for the other [3].

Autophagy: the basics

Autophagy is a highly dynamic multi-step biological process that involves breakdown and recycling of intracellular components and serves as a pro-survival mechanism amongst all eukaryotes ranging from yeasts to plants to mammals. This process occurs constitutively at a basal level and acts as a housekeeping mechanism to remove damaged or long-lived macromolecules or

Abbreviations: AKI, acute kidney injury; Atg, autophagy-related protein; CKD, chronic kidney disease; CLP, cecal ligation and puncture; CMA, chaperone mediated autophagy; DN, diabetic nephropathy; ESRD, end stage renal disease; FSGS, focal segmental glomerulosclerosis; I/R, ischemia–reperfusion; LAMP, lysosomal-associated membrane protein; LC3, microtubule-associated protein 1 light chain 3; LPS, lipopolysaccharide; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; RNS, reactive nitrogen species; ROS, reactive oxygen species; TGF-β1, transforming growth factor-beta 1; UUO, unilateral ureteral obstruction

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organelles. However, the implication that autophagy may be linked to a plethora of human diseases has led to intense interest in the autophagy field over the past decade. In the year 1963, Christian de Duve was the first to coin the term “autophagy” for the observation of organelle degradation within lysosomes [4]. Nearly after 30 years, the scientific world has begun to elucidate the molecular events of autophagy. Autophagy is categorized into three types: macroautophagy, microautophagy and chaperone mediated autophagy (CMA). Macroautophagy (hereafter referred as autophagy) refers to an evolutionarily-conserved lysosomal-mediated intracellular degradation pathway that is activated in response to diverse stressful conditions [5]. In microautophagy, cytosolic components are directly assimilated into the surface membrane of the lysosome or late endosome through invagination. In contrast, CMA targets only single proteins in which KFERQ-motif bearing proteins are identified by a cytosolic chaperone heat shock cognate (hsc70) that delivers them to the surface of the lysosomes for internalization through multimerization of the CMA receptor protein and lysosomal-associated membrane protein (LAMP)-2A.

Molecular aspects of autophagy

Autophagy proceeds through a series of biochemical reactions catalyzed by a core set of proteins termed autophagy-related proteins (Atg). The mechanism of autophagy is best understood in the context of nutrient starvation. To date, nearly 36 Atg proteins have been identified in yeast, and many of their mammalian homologs have also been identified [6,7].

The execution of autophagy involves five major steps (Fig. 1). 1. The formation of the isolation membrane or phagophore 2. Elongation of the phagophore and cargo recruitment 3. Closure of the mature autophagosome 4. Fusion between autophagosome and lysosome 5. Termination or degradation of the autolysosome. The process of autophagy begins with the formation of an isolation membrane or phagophore membrane. The phagophore assembly site (PAS) requires the recruitment of a core set of Atg proteins and several phosphorylation events (Fig. 1). This process is initiated by the activation of the Unc-51-like kinase-1 (ULK1)-Atg13-FIP200 complex upon stimulation with metabolic sensors, for example ATP depletion. At the molecular level, activation of the 5′-adenosine monophosphate-regulated protein kinase (AMPK) phosphorylates ULK1/2, which in turn phosphorylates Atg13 and FIP200 [8]. Recent studies suggest that AMPK-dependent ULK1 phosphorylation regulates the trafficking of mAtg9, a transmembrane protein responsible for membrane vesicle delivery to the PAS [9]. A key report has identified Atg1, Atg2 and Atg9 as Atg1 kinase substrates *in vivo*. Furthermore, a recent report also demonstrated that Atg1 directly phosphorylates Atg9 essential for the autophagosome formation, and added this ULK1/Atg1 signaling mechanism to the pathway of autophagy [10]. The ULK1/Atg1 kinase complex, the class III PI3-kinase Beclin1 complex and PI3P effectors and their related proteins are important for the autophagosome initiation step. A molecular link between ULK1 and the regulation of the Beclin1 complex through direct phosphorylation of Vps34 has recently been described [11,12].

Two ubiquitin-like conjugation systems Atg5-12 and the microtubule-associated protein 1 light chain 3 (LC3)/Atg8 catalyze the elongation of the phagophore membrane. Ubiquitin-like protein Atg5 conjugates with Atg12 with the help of E1-like enzyme Atg7 and E2-like enzyme Atg10 respectively. LC3/Atg8 is processed by Atg4 to generate the cytosolic LC3-I, which then conjugated to phosphatidylethanolamine (PE) in a reaction that requires Atg7 and the E2-like enzyme Atg3 respectively. LC3-II remains incorporated in the autophagosomal membrane until the autophagosome-lysosome fusion step. During the late stages of autophagy, LC3B-II associated with the outer autophagosomal membrane is recycled by Atg4B, whereas LC3B-II at the inner membrane is degraded by lysosomal activity [13]. Termination is the least well-studied step of autophagy, but some evidence suggests that mTOR activity is reactivated after a prolonged period of stress and hence may regulate the termination step [14]. Further investigations are required to elucidate the molecular events regulating the termination process of autophagy.

Selective autophagy/mitophagy

Mitophagy is involved in the selective removal of dysfunctional mitochondria using the autophagy machinery. To date, two types of mitophagy pathways have been described. Activated PINK1 translocates Parkin from the cytoplasm to defective mitochondria followed by polyubiquitination of mitochondrial substrates, which

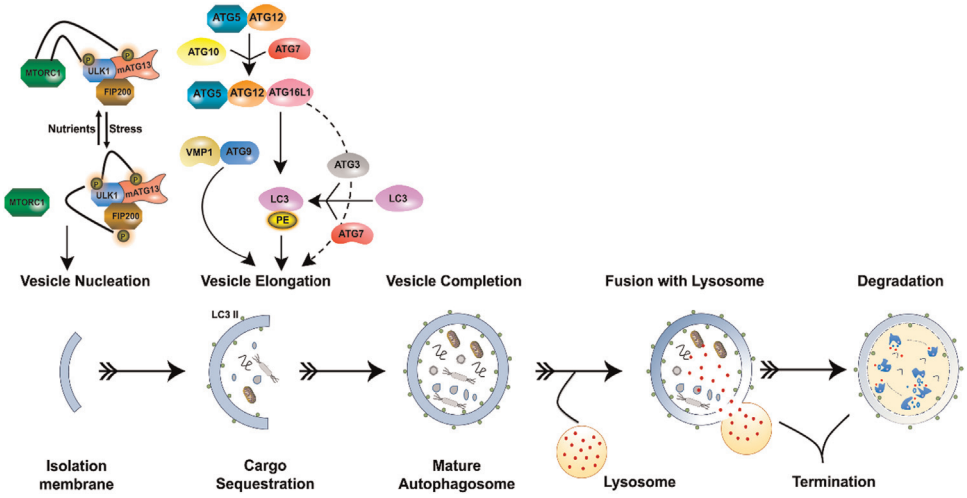

Fig. 1. Schematic depiction illustrating the molecular machinery of autophagy with the major autophagy-related proteins in the autophagy signaling pathway. Molecular components that include the ULK1/2-mAtg13-FIP200 complex are necessary for the induction of the autophagy pathway. Under a nutrient-rich environment, mTORC1 phosphorylates ULK1/2 and mAtg13 and leads to association and subsequent inhibition. During stress or starvation, mTORC1 dephosphorylates ULK1/2 and leads to the phosphorylation of FIP200, resulting in downstream activation of the autophagy pathway. E1-like ATG7 and E2-like ATG3 enzymes mediate the lipidation of ubiquitin-like enzyme LC3 with phosphatidylethanolamine (PE) to form LC3-PE. ATG5-ATG12-ATG16L1 complex and VMP1-ATG9 and LC3-PE systems function in cargo sequestration.

ultimately lead to mitophagy [15]. Another pathway executes the removal of healthy mitochondria via direct interaction of Nix/Bnip3 with LC3 [16]. Induction of Parkin-dependent mitophagy through mitochondrial Reactive Oxygen Species (ROS) has been reported using a mitochondrial-targeted photosensitizer [17]. In line with this observation, dynamin-related protein (DRP-1) dependent type of mitophagy can be induced by mild oxidative stress in both human and mouse cells [15,18]. Both of these reports suggest that mitochondrial degradation by autophagy and oxidative stress are interrelated.

Oxidative stress in kidney diseases

There is general consensus that oxidative stress contributes to the development of several diseases including AKI and CKD. Oxidative stress occurs when the generation of pro-oxidants or reactive oxygen species (ROS) exceeds endogenous antioxidant capacity. Both free radical derivatives (*e.g.*, ROS) and non-radical derivatives (*e.g.*, H_2O_2) have the ability to oxidize cellular biomolecules and ultimately leading to cellular demise. The fact that the kidney organ is rich in mitochondria indicates that it is highly vulnerable to damage caused by oxidative stress. In kidney, mitochondrial respiratory chain and NADPH oxidases (NOX) are the major common sources of ROS. To lessen the deleterious effects of free radicals, antioxidant enzymes such as superoxide dismutase and catalase have evolved as cellular defenses. It is documented that ESRD patients have higher rates of pro-oxidant activity and lower rates of anti-oxidant activity [19,20]. Peroxynitrite ($ONOO^-$) and hydroxyl (OH^-) radicals are considered highly reactive oxidants as they are capable of extensive modification of biomolecules including lipids, DNA and proteins [21–23].

Oxidative stress in AKI

Oxidative stress is a predominant component involved in the pathogenesis of AKI. Other components include inflammatory responses and tubular and vascular damage. Ischemia–reperfusion (I/R) injury and sepsis are two of the most common pathologies leading to AKI. Numerous studies suggest oxidative stress and its systemic effects play a pivotal role in the development of AKI. For example, in a mouse model of renal I/R injury, heme oxygenase-1 knockout ($HO\text{-}1^{-/-}$) mice were found to be sensitive to kidney I/R injury, with subsequent increased kidney injury, inflammation and increased mortality as compared to wild type counterparts [24]. When subjected to kidney ischemic injury, mice deficient in transient receptor potential cation channel, subfamily M, member 2 (TRPM2) were found to be resistant to oxidative stress and apoptosis [25]. A recent study demonstrated an increased urinary thioredoxin 1 (TRX1) expression as an oxidative stress biomarker with respect to I/R-induced renal injury [26]. Moreover, deficiency of CCAAT-enhancer-binding protein homologous protein (CHOP) prevented oxidative damage and suppressed inflammation in renal tubular cells subjected to I/R injury [27].

Oxidative stress in CKD

Diabetic nephropathy (DN) is a devastating complication of diabetes and a major cause of CKD. In DN, early structural changes consist of glomerular and tubuloepithelial hypertrophy. This is followed by progressive thickening of the glomerular and tubular basement membranes, which becomes evident over a period of years [28]. As with most progressive chronic kidney diseases, tubulointerstitial fibrosis develops in advanced stages of DN. In CKD, NOX1, NOX2 and NOX4 are all demonstrated to produce oxidative stress by enhancing vascular dysfunction and fibrosis [29,30]. Interestingly, NOX2 and NOX4 expressions were found to be elevated in I/R injury [31]. Moreover, NOX5 expression was found to be increased in human biopsy specimens from patients diagnosed with DN [32]. Different drugs and small peptides have been explored to reduce oxidative stress and ameliorate acute and chronic kidney diseases (Table 1).

Inflammation

Inflammation is the most common outcome of oxidative stress. ROS directly or indirectly elevate inflammation, and trigger the expression of pro-inflammatory cytokines and chemokines. Although considered an important part of host defense mechanisms, inflammation is excessively activated in many pathological conditions. Oxidative stress is well known to induce inflammation and both processes are deeply interrelated in kidney diseases [33,34]. Pro-inflammatory cytokines and chemokines generated by tubular epithelial cells act as a stress response and a mechanism to undergo repair and restoration. However, unresolved renal inflammation results in acute and chronic kidney diseases. Celastrol treatment has been shown to prevent I/R injury by reducing tubular injury and renal inflammation [35]. In the context of systemic diseases such as sepsis, tubular epithelial cells contribute to the excessive synthesis and release of inflammatory mediators that ultimately result in injury of local and distant organs.

Autophagy in kidney diseases

To date, evidence from numerous studies corroborate that autophagy has an important renoprotective function in tubular epithelial cells and in podocytes [28,36]. Although autophagy plays a protective role in acute and chronic kidney diseases, further

Table 1
Drugs and synthetic peptides that modulate oxidative stress-mediated acute and chronic kidney diseases.

Drug/peptide treatment	Injury/disease	Effects	Reference
Mangiferin	Cecal ligation and puncture (CLP)-induced sepsis	Inhibits oxidative stress and decreases apoptosis *in vitro* and *in vivo*	[56]
Sialic acid	Lipopolysaccharide (LPS)-induced sepsis	Reduces oxidative stress, inflammation and apoptosis in rat kidneys	[57]
Lutein	I/R injury	Increases antioxidant capacity in rat kidneys	[58]
C-type natriuretic peptide	I/R injury	Inhibits oxidative stress and apoptosis	[59]
Fluoxetine	I/R injury	Reduces oxidative stress and inflammation	[60]
Celastrol	I/R injury	Reduces inflammation and tubular injury	[35]
PMVE/MA-SOD & CMC-SOD	Diabetes	Reduces oxidative stress	[61]
Curcumin	Maleate-induced Nephropathy	Prevents oxidative stress and preserves mitochondrial function	[62]
Angiotensin [1–7]	Diabetic Nephropathy	Reduces glucose-induced oxidative stress and cell proliferation	[63]
Febuxostat	Diabetic Nephropathy and Albuminuria	Prevents oxidative stress and inflammation via inhibition of XO and XDH activities	[64]

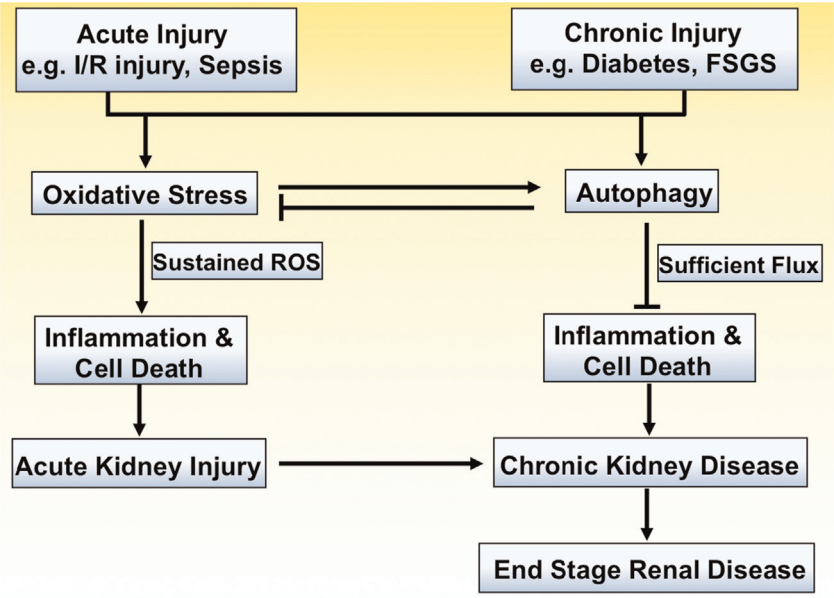

Fig. 2. Interplay of redox signaling and autophagy in acute and chronic kidney injury. In general, both acute and chronic kidney injuries increase oxidative stress and autophagic flux. The proposed mechanism by which oxidative stress contributes to inflammation and cell death involves sustained levels of ROS generation. This excessive oxidative stress, depending upon intensity and/or duration, induces cellular dysfunction and the pathogenesis of AKI and CKD, and ultimately kidney failure and ESRD. Autophagy protects renal tissue by clearing damaged organelles such as mitochondria and thereby indirectly inhibits excessive ROS production, inflammation and cell death.

research to elucidate the molecular mechanisms, signaling pathways and consequences of autophagy related to the regulation of inflammation and cell death is required (Fig. 2). Genetically modified animal models that are deficient in autophagy pathway served as valuable tools to advance the understanding of the function of autophagy in a variety of kidney diseases.

Autophagy in AKI

To date, the effects of autophagy in AKI have been studied mostly in tubular epithelial cells and podocytes. The first evidence came from a renal I/R rat model, where Atg proteins such as Beclin1 and LC3 were found increased in proximal and distal epithelial cells. Furthermore, it was shown that augmented expression of Bcl-X_L in the kidney was sufficient to inhibit autophagy induction and apoptosis [37]. In line with this observation, autophagosomes were identified in murine renal tubular cells in response to I/R injury [38]. It was also documented that autophagic flux was increased during the reperfusion period [39]. Similarly, kidney tubule-specific autophagy deficient mice models have added more robust conclusions. In response to I/R injury, tubular cell specific Atg5 and Atg7 knockout mice displayed dramatically increased tissue damage and apoptosis [40,41]. However, the mechanism by which I/R injury promotes autophagic induction and its consequences in terms of inflammation and cell death is unclear.

As aforementioned, autophagy was also documented to have a renoprotective role in response to septic insults. In a rat model of cecal ligation and puncture (CLP)-induced sepsis, autophagy was increased in the early stages followed by decline with the later stages of kidney injury in sepsis. In line with this observation, Atg7 siRNA treatment enhanced tumor necrosis factor-α-induced cell death in renal tubular cells *in vitro* [42]. In a recent study, older mice showed diminished autophagy as compared to younger counterparts during endotoxemia [43]. Therefore, these data suggest that induction of autophagy protects younger mice whereas restoration of autophagy protects older mice in sepsis-induced AKI. Since sepsis is a highly complex pathophysiological disease, it may be clinically-relevant to target autophagy in combination with other multiple mechanistic pathways that prove beneficial therapy.

Autophagy in CKD

An emerging body of evidence suggests that autophagy plays a critical role in chronic kidney diseases. Targeting fibrogenic components in tubular epithelial cells and fibroblasts or myofibroblasts as a therapeutic approach is currently under active research. Transforming growth factor-beta 1 (TGF-β1) is the most potent profibrogenic factor for the development of renal fibrosis [36]. Recently, our group has demonstrated induction of autophagy in renal tubular epithelial cells of obstructed kidneys after unilateral ureteral obstruction (UUO), an *in vivo* experimental model of renal fibrosis. LC3 knockout mice and Beclin1 heterozygous knockout mice exhibited increased collagen deposition and increased mature TGF-β1 levels in obstructed kidneys after UUO. These data suggest that in renal proximal tubular epithelial cells, mature TGF-β1 levels are regulated through autophagic degradation, which suppresses kidney fibrosis induced by UUO [44].

In podocytes, high basal levels of autophagy infer its requirement for normal cellular homeostasis [45]. Autophagy-specific Atg5 or Atg7 conditional knockout mice exhibited enhanced vacuolization in podocytes and tubular cells and ultimately resulted in Focal Segmental Glomerular Sclerosis (FSGS) and organ failure [46]. Furthermore, Atg5 deficient proximal tubular epithelial cells showed enhanced mitochondrial dysfunction and elevated ROS production [46]. These data suggest that autophagy deficiency in the kidney epithelium can recapitulate the characteristic features of FSGS observed in the kidneys of patients with idiopathic FSGS.

Autophagy and oxidative stress

In the past few years, there is growing consensus that oxidative stress and autophagy are intricately connected (Fig. 3). It is well known that basal levels of redox signaling and autophagy

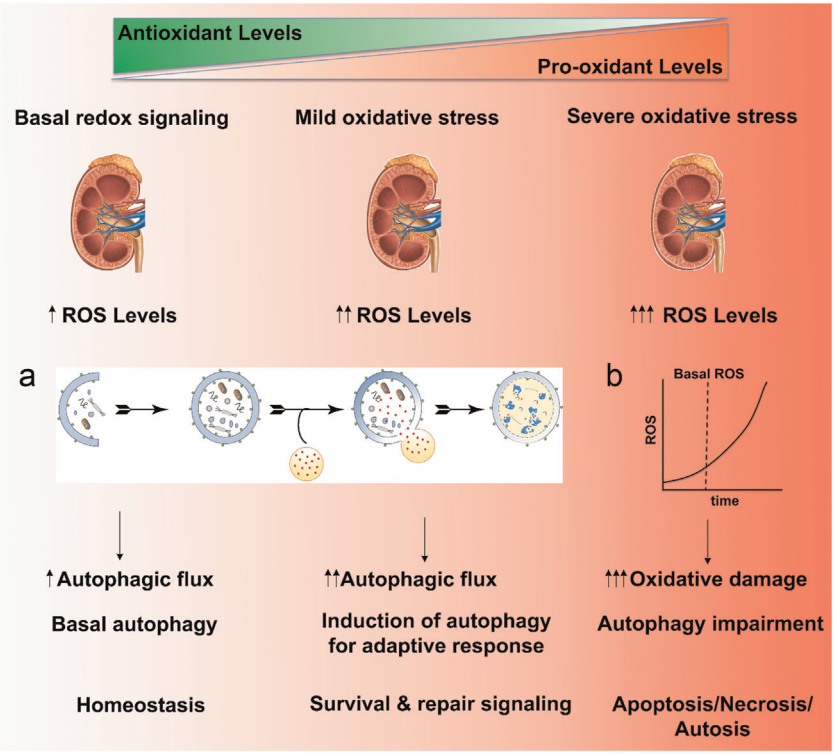

Fig. 3. Diagrammatic representation illustrating the induction of oxidative stress and autophagy leading to normal cell growth or cell death at various levels of stress in the kidney. Basal levels of redox signaling and autophagy are necessary for the homeostasis. Mild oxidative stress triggers cell survival and repair mechanisms such as the autophagy pathway. a) Schematic overview of the autophagy pathway b) increased ROS levels above basal values for a prolonged period. In the case of severe oxidative stress, ROS/RNS levels are excessive for a prolonged period leading to oxidative damage and ultimately cell demise. For example, lysosomal permeabilization occurs due to excessive ROS levels can contribute to autophagic impairment and autosis.

signaling are necessary to maintain cellular homeostasis and also mediate cytoprotective mechanisms. Under distinct circumstances, changes in autophagic flux have been shown to regulate ROS formation and redox signaling. Numerous lines of evidence suggest that ROS and reactive nitrogen species (RNS) act as upstream modulators of autophagy induction [47]. In line with this, a few studies have demonstrated that ROS act as inducers of autophagy with respect to nutrient deprivation [48]. Therefore, oxidative stress can affect autophagy and vice-versa. In a key report, p62 dependent degradation of Keap1 has been shown to regulate Nrf2 signaling and protect against oxidative stress [49–51]. However, there remains a significant amount of work that needs to be done in the field of kidney diseases. Although the concepts of oxidative stress and autophagy have been extensively studied individually, the emerging links between these processes in kidney diseases have not been examined in detail.

Autophagy and cell death

Another issue to be considered is how autophagy and cell death are related in kidney diseases. The functional relationship between autophagy and cell death in renal pathophysiology is somewhat controversial. Thus far, the majority of evidence suggests that autophagy functions as a renoprotective process, yet some evidence suggests that autophagy contributes to cell death (Fig. 3). Although there is much debate on the definition of autophagic cell death, the following criteria specified by the nomenclature committee on cell death should be met: 1) occurrence of cell death without caspase activation and chromatin condensation, 2) dying cells should demonstrate increased autophagic flux, and 3) genetic blockade of at least two molecules of the autophagy pathway that can delay or prevent cell death [52,53]. Inhibition of mitochondrial electron transport chain complexes I and/or II induces autophagic cell death which is reduced by knockdown of major autophagy related proteins Atg5, Beclin1 and/or Atg7 in human embryonic kidney cells [54]. In contrast, a study has reported that inhibition of autophagy can lead to podocyte apoptosis by activating endoplasmic reticulum stress [55].

Concluding remarks

Both acute and chronic kidney diseases remain major contributors to morbidity and mortality in hospitalized patients. To date, limited information is available for understanding the roles of autophagy and oxidative stress in kidney diseases as interlinked pathways. This implies that there are important questions that still need to be answered by the scientific world. In this regard, for example, implications of defective redox signaling and autophagy signaling in kidney diseases remain to be uncovered. Another important question would be to identify specific signals that regulate the molecular interactions between oxidative stress and autophagy. Therefore, a detailed understanding of the molecular regulation of oxidative stress by autophagy signaling will answer key questions about this bilateral relationship in kidney health and disease. Nevertheless, genetically modified animal models that are deficient in oxidative stress-related and autophagy processes may serve as valuable tools and provide new insights into the mechanisms that need to be elucidated. Equally, specific therapeutic agents that regulate both oxidative stress signaling and autophagy signaling are needed. Advances such as these will be likely to improve therapeutic approaches and hold promise to benefit patients with kidney diseases.

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