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Participation of autophagy in renal ischemia/reperfusion injury

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Abstract

Renal ischemia–reperfusion (I/R) injury is inevitable in transplantation, and it results in renal tubular epithelial cells undergoing cell death. We observed an increase in autophagosomes in the tubular epithelial cells of I/R-injured mouse models, and in biopsy specimens from human transplanted kidney. However, it remains unclear whether autophagy functions as a protective pathway, or contributes to I/R-induced cell death. Here, we employed the human renal proximal tubular epithelial cell line HK-2 in order to explore the role of autophagy under hypoxia (1% O_2) or activation of reactive oxygen species (500 μM H_2O_2). When compared to normoxic conditions, 48 h of hypoxia slightly increased LC3-labeled autophagic vacuoles and markedly increased LAMP2-labeled lysosomes. We observed similar changes in the mouse IR-injury model. We then assessed autophagic generation and degradation by inhibiting the downstream lysosomal degradation of autophagic vacuoles using lysosomal protease inhibitor. We found that autophagosomes increased markedly under hypoxia in the presence of lysosomal protease inhibitors, thus suggesting that hypoxia induces high turnover of autophagic generation and degradation. Furthermore, inhibition of autophagy significantly inhibited H_2O_2-induced cell death. In conclusion, high turnover of autophagy may lead to autophagic cell death during I/R injury.

Keywords: Transplantation; ischemia–reperfusion; Autophagy; Lysosome

Renal ischemia–reperfusion (I/R) injury, which is unavoidable in renal transplantation and is frequently associated with shock or surgery, is a major cause of acute renal failure [1]. The long-term graft function in renal transplantation is associated with the initial intensity of I/R injury [2]. Furthermore, renal hypoxia and/or I/R injury lead to chronic allograft nephropathy (CAN), the most important cause of graft failure after the first year following kidney transplantation [3].

Although ischemic injury is a major cause of acute renal failure, reperfusion itself causes additional cellular injury, resulting in the eventual death of renal cells in a sequential combination of programmed cell death [4]. Autophagy is thought to be an essential function for cell homeostasis, as well as cell defense and adaptation to adverse environments [5,6]. In autophagy, which is typically activated by starvation [7,8], cytoplasmic proteins or dysfunctional organelles are sequestrated in double membrane-bound vesicles, termed autophagosomes, delivered to the lysosome by fusion, and then degraded. Autophagy allows the cell not only to recycle amino acids but also to remove damaged organelles, thereby eliminating oxidative stress and allowing cellular remodeling for survival [9,10]. It can also prevent accumulation of misfolded and aggregated proteins in Parkinson's, Huntington's and Alzheimer's diseases [11]. It should be noted, however, that formation of autophagosomes and degradation of the bulk of cytoplasm are also observed in cells undergoing cell death.

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Increasing lines of evidence suggest that autophagy is activated by various types of stress, such as ischemia and inflammation in mammals [12–16]. Our observations in transplanted kidney specimens suggest that autophagy is involved in the progression of CAN. Under hypoxic or ischemic conditions, autophagy not only degrades unnecessary proteins but also extracts amino acids and fatty acids as energy sources for cell survival, while inappropriate activation of autophagy might facilitate cell death, termed autophagic cell death or type II programmed cell death [17,18]. Whether autophagy is protective or detrimental against stress in mammalian cells is not fully understood.

Here, we employed the human renal proximal tubular epithelial cell line HK-2 in order to explore the role of autophagy under hypoxia (1% O_2) and activation of reactive oxygen species (500 μM H_2O_2) in an *in vitro* model of I/R injury, and examined the effects of I/R injury on autophagic cell death.

Materials and methods

Allograft biopsy. Routine allograft biopsies were performed at one month after renal transplantation. All biopsy specimens were examined by light and electron microscopy. All patients gave written consent to undergo biopsy.

Cell Culture and in vitro I/R injury. Human renal proximal tubular epithelial cells (human kidney-2, HK-2; ATCC, Manassas, VA) were cultured in DMEM (Invitrogen Corp., Carlsbad, CA) with 10% fetal bovine serum under a 5% CO_2 and 95% air atmosphere at 37 °C.

In order to mimic ischemic injury *in vitro*, HK-2 cells were incubated under low-oxygen conditions. Oxygen concentrations were maintained at 1% using the compact gas oxygen controller APM-36 (ASTEC, Fukuoka, Japan) with a residual gas mixture composed of 94% N_2 and 5% CO_2 for 6–24 h. As an *in vitro* model of reperfusion injury, HK-2 cells were treated with 500 μM of H_2O_2 for 8–12 h. To examine the effects of autophagy or apoptosis on I/R-induced cell death, 5 mM 3-methyladenine (3MA; Sigma, St. Louis, MO), an inhibitor of autophagy, 10 μg/ml E64d (Peptide Institute, Osaka, Japan) with 10 μg/ml pepstatin A (Peptide Institute, Osaka, Japan), a lysosomal protease inhibitor, and 100 μM zVAD (Peptide Institute, Osaka, Japan), a caspase inhibitor, were added to the medium 1 h before each experiment.

I/R injury. In order to examine the effects of I/R injury on the formation of autophagosomes, 8- to -10-week-old male C57BL/6J mice were used. All studies were performed in accordance with the principles of the Guidelines on Animal Experimentation of Osaka University. All mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg). Right kidneys were removed at 1 week before the induction of I/R injury, and the left renal artery and vein were clamped for 30 min with a small vascular clip. Reperfusion was initiated by removal of the clamp. Left kidneys were harvested 0–48 h after reperfusion, and were perfused with phosphate buffered saline (PBS).

Immunostaining. Treated HK-2 cells were washed with PBS-T (0.1% Tween 20 in PBS), and fixed with 4% of buffered paraformaldehyde (PFA) at room temperature for 15 min. Fixed cells were washed with PBS-T, incubated with 2% goat serum, followed by incubation with anti-LC3 antibody (kindly provided by Prof. Uchiyama) [19] and labeling with an anti-rabbit fluor conjugate Alexa 488 (Cell Signaling Technology) or fluorescein isothiocyanate (FITC)-labeled anti-LAMP2 antibody (Abcam, Cambridge, UK) in 2% goat serum for 12 h. Cells were also stained with a nucleic acid dye, 0.1 μM 4′6′-diamino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR).

In order to confirm the effects of I/R injury on autophagosome formation *in vivo*, renal tissues were fixed in ice-cold neutral-buffered 4% PFA solution and embedded in O.C.T. compound (Tissue-Tek; Sakura Finetek, USA, Inc., Torrance, CA, USA). Cryostat sections (4 μm) were stained with anti-LC3 and anti-LAMP-1 antibody (The Developmental Studied Hybridoma Bank, Iowa City, IA), followed by labeling with immunoglobulin Alexa-fluor conjugates (Alexa 488 and Alexa 594, respectively; Cell Signaling Technology). For quantification of the autophagic response of individually cultured cells, 30 representative cells under each condition were employed, and total number of LC3-positive autophagosomes per cell was determined.

Electron microscopy. Renal samples for electron microscopy were fixed with 2% OsO_4, dehydrated in a graded series of alcohol, and embedded in Epon 812. Ultrathin sections were cut with an ultramicrotome (Ultract N; Reichert-Nissei, Tokyo Japan), followed by addition of 2% uranyl acetate and lead citrate, and were then photographed under an electron microscope.

Atg7 knockdown by small interfering RNA. In order to examine the effects of autophagy on reperfusion-induced cell death, Atg7-knockdown was accomplished by transfecting HK-2 cells with small interfering RNA (siRNA). The sense strands of siRNA for Atg7 (siAtg7) were: 5′-CCAACACACUCGAGUCUUU-3′ [20]. As a control, scrambled siRNA (siSCR, sense; 5′-ATCCGCGCGATAGTACGTA-3′) was used. siRNAs were purchased from B-bridge Mountain View, CA, USA. siRNA was transfected using Lipofectamine™ 2000 (Invitrogen Corp., Carlsbad, CA), as recommended by the manufacturer. Briefly, HK-2 cells (1.5×10^3 cells/well) were plated in 24-well plates. One day later, 50 μM siRNA and 5 μl of lipofectamine were suspended in 500 μl of Opti-MEM I medium, and were added to each well, followed by incubation at 37 °C in a 5% CO_2 incubator. After 4 h, medium was replaced with DMEM.

LDH assay. Cell viability was evaluated by measuring lactate dehydrogenase (LDH) activity in cell culture medium and cell lysates using the MTX "LDH" assay kit (Kyokuto, Japan) according to the manufacturer's instructions. Cell viability was assessed based, on thr total LDH activity released into the medium relative to total LDH (cellular + medium).

Statistical analysis. Data are expressed as means ± SD. Statistical analysis was performed by unpaired *t*-test or ANOVA for multiple comparisons, followed by Scheffe's *F*-test.

Results

Allograft biopsy specimens

We observed numerous PAS-positive vesicles in the cytoplasm of tubular epithelial cells from allograft biopsy specimens obtained from 1 month after renal transplantation (Fig. 1A), which were rarely observed in zero-hour biopsy specimens (data not shown). Electron microscopy showed vacuolar structures containing cytoplasm and that were clearly encircled by double membrane structures, resembling autophagosomes, in tubular epithelial cells, suggesting that some of these PAS-positive vesicles are autophagosomes (Fig. 1B–D).

Effects of hypoxia on autophagosomes in tubular epithelial cells

We employed HK-2 cells in order to explore the role of autophagy during I/R injury. First, we determined whether autophagic activity is modulated in response to ischemia. We characterized changes in cellular autophagosomal content using anti-LC3 and anti-LAMP antibodies, which are markers of autophagosomes and lysosomes, respectively. Under normoxic conditions, LC3 was diffusely distributed throughout the cell, with a few detectable vacuoles

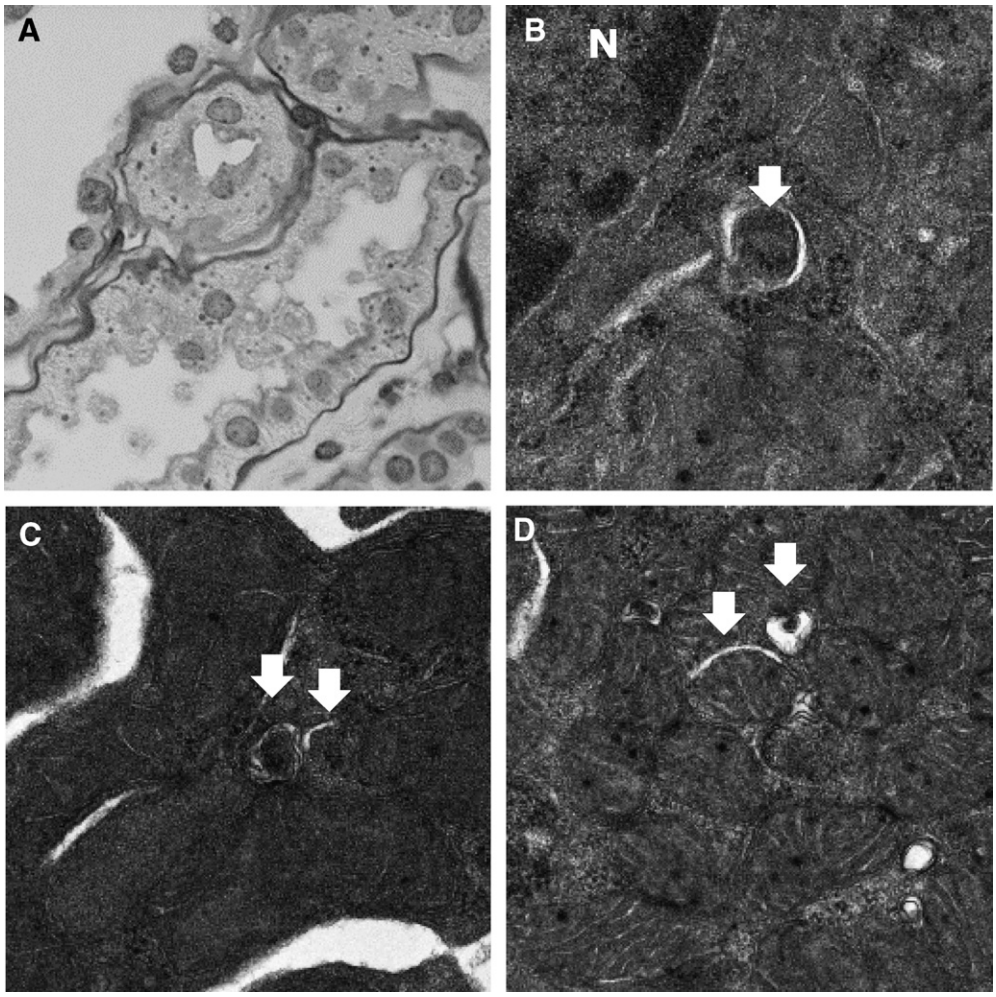

Fig. 1. Autophagosomes in tubules of allograft. Representative PAS-stained light micrograph (A) and electron micrographs (B–D) from allograft biopsy specimens obtained from 1 month after renal transplantation. White arrows; autophagosomes, N; nucleus.

(Fig. 2A). HK-2 cells subjected to hypoxia (1% O_2) for 6 h and 24 h, however, showed slight increases in the number of LC3-positive vacuoles. LAMP-2-positive lysosomes were not observed under normoxic conditions or under hypoxia for 6 h, while 24 h of hypoxia induced marked increases in lysosomes.

Our results demonstrate that cellular autophagosomal content was slightly increased; however, the marked increases in lysosomes suggest that slight increases in autophagosomal content reflect the enhanced degradation of autophagosomes by lysosomes. Therefore, we employed an approach based on inhibiting the downstream lysosomal degradation of autophagosomal vacuoles. HK-2 cells were treated with a mixture of lysosomal inhibitors to lysosomal protease activities (E64D and pepstatin A, E/P), and were subjected to normoxic or hypoxic conditions (Fig. 2B). When compared with cells unexposed to E/P (5.6 ± 4.8 per cell), treatment with E/P did not increase the number of LC3-positive autophagosomes after 6 h (9.1 ± 4.1 per cell) or 24 h (6.9 ± 4.1 per cell) under normoxia. In contrast, autophagosomal vacuoles increased after 6 h of hypoxia (16.5 ± 5.8 per cell, $p < 0.05$ vs. normoxia), and further increased after 24 h of hypoxia (25.7 ± 9.0 per cell, $p < 0.01$ vs. normoxia) (Fig. 2C).

Effects of I/R injury on autophagosomes

We investigated the effects of I/R injury on autophagosome generation in tubular epithelial cells *in vivo* (Fig. 3). Weak immunoreactivity for LC3 was diffusely observed before the induction of I/R injury, and LAMP-positive lysosomes were not detected. PAS-positive vesicles were also undetectable. The LC3 and LAMP staining patterns were similar to those observed in cultured HK-2 cells under normoxia. In contrast, kidney specimens at 6 h after I/R injury showed abundant LC3-positive autophagic vacuoles in tubular epithelial cells. LAMP-positive lysosomes appeared in tubular cells. Of interest is that numerous PAS-positive vesicles were seen in the cytoplasm of tubular epithelial cells at 6 h after I/R injury prior to tubular cell death. At 24 h after I/R injury, however, LC3-positive autophagosomes had decreased when compared with 6 h. In contrast, LAMP-positive lysosomes accumulated in

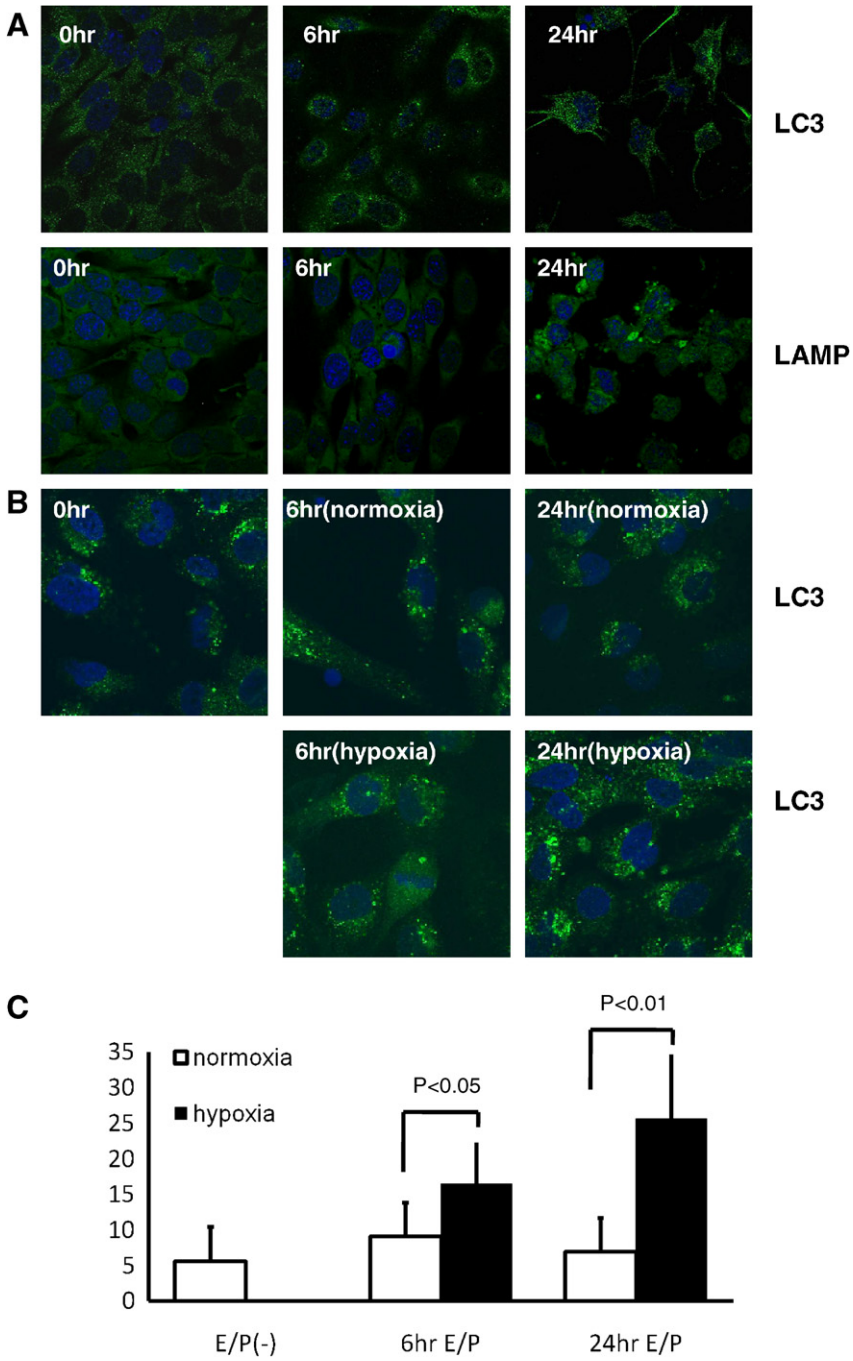

Fig. 2. Autophagosomal turnover under hypoxia. (A) HK-2 cells were incubated under hypoxia for 6 h or 24 h. Expression of autophagosomes and lysosomes were detected using anti-LC3 and anti-LAMP antibodies, respectively. (B) Lysosomal inhibitor-treated HK-2 cells were also incubated under normoxia or hypoxia for 6 h or 24 h, and the total flux of autophagosomes were examined using anti-LC3 antibody. (C) Autophagosomes had increased at 6 h of hypoxia, and had further increased at 24 h when compared with normoxic conditions.

tubular epithelial cells. PAS staining of the kidney sections obtained from rats at 24 h after I/R injury revealed marked disruption of normal tubular morphology, including widespread degeneration of tubular architecture, tubular dilatation, swelling, and luminal congestion with loss of the brush border and abundant PAS-positive vesicles. A fraction of LC3-II protein was shown to be trapped within autolysosomes and was degraded, as LC3-positive autophagosomes were merged with LAMP-positive lysosomes at 24 h after I/R injury.

Effects of autophagy on reperfusion injury in tubular epithelial cells

Although ischemic events alone may lead to tubular cell necrosis in kidney, reperfusion occurs upon restoration of

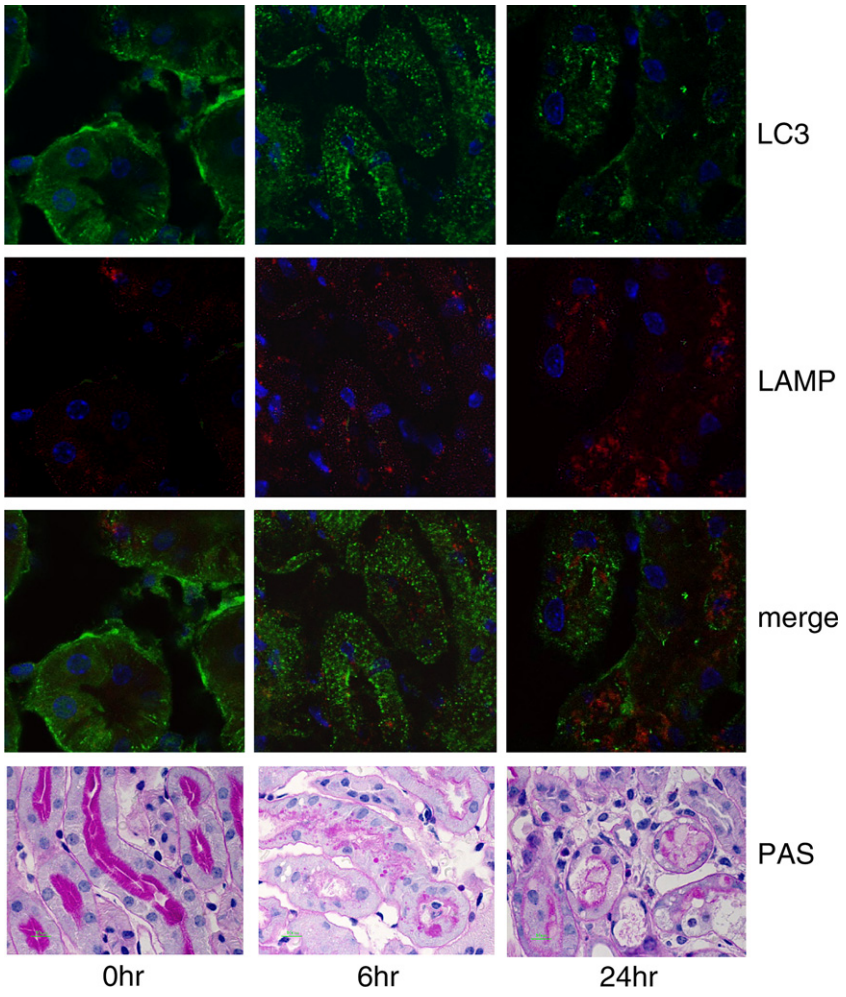

Fig. 3. Colocalization of autophagosomal and lysosomal markers. We examined autophagosomal (LC3) and lysosomal (LAMP) expression in tubular epithelial cells in vivo at 6 h and 24 h after I/R injury in mice. We also investigated the relationship between autophagosomal expression and tubular lesions stained with PAS (magnification, 400×).

blood flow and is associated with production of reactive oxygen species (ROS), which are considered to play an important role in reperfusion injury [21]. To investigate whether reperfusion-induced ROS contributes to autophagic cell death, we examined the effects of autophagic inhibition on cell death in HK-2 cells after ROS treatment. Cultured HK-2 cells were treated with 3-MA, E/P or zVAD, and were then subjected to 500 μM H_2O_2 (*in vitro* chemical reperfusion model). Exposure of untreated HK-2 cells to H_2O_2 resulted in $26.1 \pm 11.8\%$ and $63.9 \pm 18.8\%$ cell death at 8 h and 12 h, and this was largely prevented by treatment with 3-MA (4.9 ± 2.6 and $11.3 \pm 10.6\%$ at 8 h and 12 h, respectively), E/P (2.2 ± 3.1 and $10.6 \pm 3.7\%$ at 8 h and 12 h, respectively), or zVAD ($4.2 \pm 4.0\%$ and $12.8 \pm 1.8\%$ at 8 h and 12 h, respectively), (Fig. 4A). In addition, transfection of siAtg7 also prevented ROS-induced cell death of HK-2 cells ($18.3 \pm 0.8\%$ at 10 h), while transfection with scrambled siRNA (siSCR) was ineffective ($33.9 \pm 6.1\%$ at 10 h, Fig. 4B).

Discussion

Data presented in this study indicate that autophagosomes are present in human renal tubular epithelial cells from allograft biopsy specimens and in cultured human proximal tubular epithelial cells, where I/R injury may lead to autophagosomal cell death. This notion is supported by several lines of experimental evidence: (1) Electron microscopy from allograft biopsy specimens obtained at 1 month after renal transplantation showed increased autophagosomes in tubular epithelial cells; (2) In an I/R mouse model, tubular epithelial cells showed abundant LC3-positive autophagic vacuoles at 6 h, these cells exhibited cell death at 24 h. (3) Under hypoxic conditions, cultured human tubular epithelial cells exhibited the enhanced generation of autophagosomes and accelerated degradation in lysosomes; (4) Inhibition of autophagy by 3-MA or treatment with a mixture of lysosomal inhibitors protected tubular epithelial cells from ROS-induced cell death; (5) Similar protective effects against

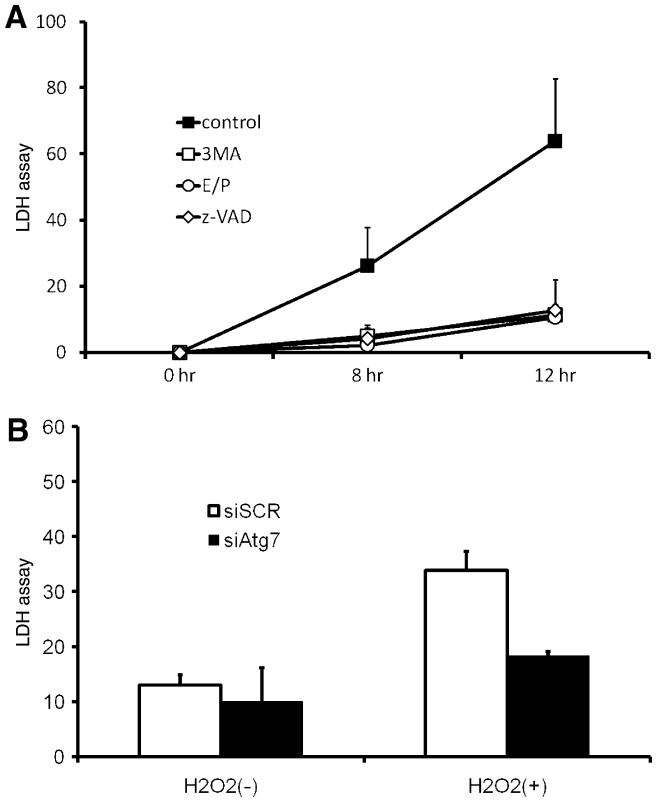

Fig. 4. Effects of autophagy on ROS-induced cell death. (A) HK-2 cells were treated with 3-MA, E/P or zVAD, and were then subjected to 500 μM of H_2O_2 for 12 h. Cell viability was assessed by LDH assay. (B) siAtg7- or siSCR-transfected HK-2 cells were also treated with 500 μM of H_2O_2 for 10 h.

ROS injury were observed in HK-2 cells by blockade of Atg-7 by siRNA silencing.

Until recently, there was a common notion that the autophagic activity in the kidney is high in podocytes in glomeruli, but not in tubular epithelial cells [22]. This notion arose from the observation that GFP-LC3 dots were present in podocytes, and were further enhanced by starvation [22], while those in tubules were not clear, because autofluorescent signals in tubules disturbed the analysis of expression levels of GFP-LC3 dots in tubules. Immunofluorescence using a specific antibody for LC3, however, demonstrated that weak immunoreactivity for LC3 was diffusely present in normal tubular epithelial cells, but that it became abundant LC3-positive dots at 6 h after I/R injury.

In addition, cultured human tubular epithelial cells exhibited enhanced generation of autophagosomes and accelerated degradation into lysosomes under hypoxia. As LC3-II-associated autophagosomes are transient structures, LC3-II levels only indicate the amount of autophagosomes at a given moment, rather than the magnitude of total flux through the autophagic pathway. If autophagosomes have a shorter half-life in tubular epithelial cells (i.e., faster degradation into autophagolysosomes), it is expected that less LC3-II will accumulate, even if the influx to the autophagic pathway occurs under physiological or pathophysiological conditions. Our observations using lysosomal inhibitors demonstrated that hypoxia induced enhanced influx to the autophagic pathway in cultured human tubular epithelial cells when compared with normoxia. Furthermore, a fraction of LC3-II protein may be trapped within autolysosomes and degraded, as some LC3-positive autophagosomes merged with LAMP-positive lysosomes at 24 h after I/R injury.

The precise mechanism by which autophagic inhibition confers a cytoprotective role against I/R-induced renal tubular injury needs to be investigated further. Increased production of ROS and activation of apoptotic pathways have been shown to contribute to the pathogenesis of renal I/R injury [23,24]. Proximal tubular epithelial cells are more sensitive to I/R injury than distal tubules, because they tend to produce more ROS and receive more severe oxidative damage than distal tubules. In addition, levels of the cytoprotective bcl-2 were sustained at low levels in proximal tubules after I/R injury, while inhibition of autophagosomes protected ROS-injured human proximal tubular epithelial cells, as did treatment with the pan-caspase inhibitor zVAD.

Autophagy is active at basal levels in most cells in the body, and this probably reflects its role in regulating the turnover of long-lived proteins, and disposing of damaged structures. Both proteasomes and autophagosomes are able to degrade proteins, but only autophagy can degrade other macromolecules and even entire organelles. Although autophagy can be stimulated by nutrient depletion [8], it is also implicated in various diseases, including cancer, neurodegenerative diseases, pathogen invasion, and muscle and liver disorders [11]. In most of these situations, autophagy has both favorable and damaging effects. This is reflected by the dual role of autophagy, which can be tissue protective, but also leads to programmed cell death. During apoptosis, for example, stimulation of autophagy can be either a protective mechanism or a process that contributes to cell death [25,26]. In the absence of apoptosis, autophagy can trigger a form of cell death known as autophagic cell death [27,28].

The most challenging question remains: does autophagy function as a protective pathway, or contribute to I/R induced-cell death? We speculate that autophagy may have the following effects on tubular epithelial cells in I/R injury. First, autophagy may be triggered by renal ischemia or reperfusion injury to eliminate damaged mitochondria and abort apoptosis. Previous studies have shown that mitochondria are selectively eliminated from eukaryotic cells after the blockade of caspases during apoptosis [29]. Moreover, mitochondrial disappearance is partially prevented by bafilomycin A1, an inhibitor of autophagosome fusion with lysosomes, suggesting that autophagy contributes to this process [29]. This is further supported by data indicating that inhibition of autophagy in starving cells activates apoptosis [30].

However, these results do not exclude the possibility that autophagy also has an active role in cell death. We demonstrated that inhibition of autophagy with 3-MA

and siAtg7 protected tubular epithelial cells from ROS-induced cell death. Cell death in this manner may be achieved by several mechanisms, including autophagic digestion of a crucial threshold of cytoplasmic factors necessary for cell survival, or selective degradation of regulatory molecules or organelles that are essential for survival.

In conclusion, our results demonstrate that autophagy is part of the physiologic response to I/R injury, for example in renal transplantation, and that high turnover of autophagy may lead to autophagic cell death during I/R injury. In addition, inhibition of autophagy is potentially compatible with therapeutic interventions for I/R injury. Further experiments are necessary to determine whether inhibition of autophagy represents a means to reduce renal I/R injury *in vivo*.

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