Autophagy Is a Renoprotective Mechanism During *in Vitro* Hypoxia and *in Vivo* Ischemia-Reperfusion Injury

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Autophagy mediates bulk degradation and recycling of cytoplasmic constituents to maintain cellular homeostasis. In response to stress, autophagy is induced and may either contribute to cell death or serve as a cell survival mechanism. Very little is known about autophagy in renal pathophysiology. This study examined autophagy and its pathological role in renal cell injury using *in vitro* and *in vivo* models of ischemia–reperfusion. We found that hypoxia (1% O₂) induced autophagy in cultured renal proximal tubular cells. Blockade of autophagy by 3-methyladenine or small-interfering RNA knockdown of Beclin-1 and ATG5 (two key autophagic genes) sensitized the tubular cells to hypoxia-induced apoptosis. In an *in vitro* model of ischemia–reperfusion, autophagy was not induced by anoxic (0% O₂) incubation in glucose-free buffer, but was induced during subsequent recovery/reperfusion period. In this model, suppression of autophagy also enhanced apoptosis. *In vivo*, autophagy was induced in kidney tissues during renal ischemia–reperfusion in mice. Autophagy was not obvious during the ischemia period, but was significantly enhanced during reperfusion. Inhibition of autophagy by chloroquine and 3-methyladenine worsened renal ischemia/reperfusion injury, as indicated by renal function, histology, and tubular apoptosis. Together, the results demonstrated autophagy induction during hypoxic and ischemic renal injury. Under these pathological conditions, autophagy may provide a protective mechanism for cell survival. (Am J Pathol 2010, 176:1181–1192; DOI: 10.2353/ajpath.2010.090594)

Autophagy is a cellular process of “self-eating” wherein various cytoplasmic constituents are broken down and recycled through the lysosomal degradation pathway.¹ This process consists of several sequential steps, including sequestration of cytoplasmic portions by isolation membrane to form autophagosome, fusion of the autophagosome with lysosome to create an autolysosome, and degradation of the engulfed material to generate monomeric units such as amino acids.² Identification of the autophagy-related genes (ATG) in yeast and their orthologs in other organisms including mammals demonstrates that autophagy is evolutionarily conserved in all eukaryotic cells. The ATG genes constitute the core molecular machinery of autophagy and function at the different levels to regulate autophagy induction, progression, and completion.¹

Autophagy occurs at basal level in most cells and contributes to the turnover of long-lived proteins and organelles to maintain intracellular homeostasis. In response to cellular stress, autophagy is up-regulated and can provide an adaptive strategy for cell survival, but may also directly or indirectly lead to cell demise.^{3–6} With the dual role in life and death, autophagy is involved in various physiological processes, and more importantly, linked to the pathogenesis of a wide array of diseases, such as neurodegeneration, cancer, heart disease, aging, and infections.^{1,2,6,7} However, it remains largely unknown how autophagy makes the life and death decisions of a stressed cell. Moreover, the conundrum is further complicated by the cross talk and coordinated regulation between autophagy and apoptosis.^{4,5,8}

Despite rapid progress of autophagy research in other organ systems, the role of autophagy in the pathogenesis of renal diseases was not recognized until very recently. In 2007, Chien et al⁹ suggested the first evidence of

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autophagy during renal ischemia–reperfusion in rats. Subsequent work by Suzuki et al¹⁰ further showed autophagy in ischemic mouse kidneys and notably, in transplanted human kidneys. In nephrotoxic models of acute kidney injury, we and others have demonstrated autophagy during cisplatin nephrotoxicity and have suggested a role for autophagy in renoprotection.^{11,12} A prosurvival role of autophagy was also shown in tubular cells during cyclosporine A nephrotoxicity.¹³ In contrast, Gozuacik et al¹⁴ suggested that autophagy may serve as a second cell killing mechanism that acts in concert with apoptosis to trigger kidney damage in tunicamycin-treated mice. A cell killing role for autophagy was also suggested by Suzuki et al¹⁰ during H₂O₂-induced renal tubular cell injury. As a result, whether autophagy is a mechanism of cell death or survival in renal pathology remains unclear.

In this study, we have determined the role of autophagy in renal tubular cell injury using *in vitro* and *in vivo* models of renal ischemia–reperfusion. We show that autophagy is induced in these models. Importantly, blockade of autophagy sensitizes renal cells and tissues to injury by hypoxia and ischemia–reperfusion, suggesting a prosurvival role for autophagy.

Materials and Methods

Cells, Antibodies, and Reagents

Immortalized rat kidney proximal tubular cell line (RPTC) was originally obtained from Dr. Ulrich Hopfer (Case Western Reserve University, Cleveland, OH) and maintained for experiments as described previously.^{15–17} Isolation and primary culture of proximal tubular cells from mice were described in our recent work.^{18–20} Antibodies in the study were from the following sources: anti-LC3 from Dr. Tamotsu Yoshimori and Dr. Noboru Mizushima,²¹ anti-Becclin-1 from Santa Cruz Biotechnology (Santa Cruz, CA), anti-ATG5 and anti- β -actin from Sigma (St. Louis, MO), all secondary antibodies from Jackson ImmunoResearch Laboratories Inc (West Grove, PA). Carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD.AFC) and 7-amino-4-trifluoromethyl coumarin (AFC) were from Enzyme Systems Products (Livermore, CA). Lipofectamine transfection reagents were from Invitrogen (Carlsbad, CA). Unless indicated, other reagents including 3-methyladenine (3-MA) and chloroquine were from Sigma (St. Louis, MO).

Plasmids and Transient Transfection

The GFP-LC3 fusion plasmid was generously provided by Dr. Tamotsu Yoshimori and Dr. Noboru Mizushima.²¹ Green fluorescent protein (GFP)-tagged plasmids for the short hairpin RNA (shRNA) of Becclin-1, ATG5 and their negative control shRNA were purchased from SuperArray (Frederick, MD). Transient transfection of RPTC cells and primary proximal tubular cells was described in our recent work.²² Briefly, cells were plated on a coverslip at approximately 50% confluence and then transfected with 1.0 μ g plasmid DNA using Lipofectamine PLUS reagents

for RPTC cells or Lipofectamine 2000 reagents for primary cells. After incubation in serum-free medium for 4 to 5 hours, the cells were transferred into full culture medium and incubated for ~24 hours to reach 80 to 90% confluence before experiment. The transfection efficiency for both RPTC and primary cells was around 20%.

Hypoxic Incubation and *in Vitro* Ischemia-Reperfusion Treatment of Cells

Cells were plated in 35-mm dishes at a density of $\sim 1.0 \times 10^6$ cells/dish for RPTC cells or $\sim 0.3 \times 10^6$ cells/dish for primary tubular cells and reached $\sim 90\%$ confluence by next day for experiment. Hypoxia treatment was conducted in a hypoxia chamber as before.²³ Briefly, cells were incubated in a hypoxia chamber (COY Laboratory Products, Ann Arbor, MI) with a compact gas oxygen controller to maintain oxygen concentration at 1% by injecting a gas mixture of 95% N₂ and 5% CO₂. For *in vitro* ischemia, RPTC cells were washed with phosphate-buffered saline and incubated for 2 hours in a glucose-free Krebs-Ringer bicarbonate buffer in an anaerobic chamber equilibrated with 5% CO₂, 5% H₂ and 90% N₂. After ischemic treatment, the cells were transferred back to full culture medium with oxygen for reperfusion. The incubation medium used for hypoxia or ischemia treatment was pre-equilibrated overnight in the respective chambers. Control cells were incubated in a regular cell culture incubator with 21% oxygen. At the end of treatment, cells were monitored morphologically or harvested with indicated buffers to collect cell lysates for biochemical analyses. For cell lysis, both floating and adherent cells were collected.

Analysis of Autophagy by GFP-LC3 Redistribution and LC3 Immunoblot

The two commonly used methods for autophagy analysis were described in our recent study.¹¹ To monitor the formation of GFP-LC3 puncta, RPTC or primary tubular cells were transiently transfected with 1.0 μ g GFP-LC3 plasmid and then treated with hypoxia as described above. After treatment, the cells were fixed with 4% paraformaldehyde for fluorescence microscopic examination. Twenty fields of $\times 600$ magnification with 20 to 30 GFP-labeled green cells per field were counted in each condition. The following criteria were used to determine the cells with punctuate GFP-LC3 (positive cells): 1, with uneven, ring-shaped dots in the cytoplasm; 2, with more than 10 dots per cell. The percentage of such positive cells was recorded for quantification. For LC3 immunoblot analysis, whole cell or tissue lysates were extracted in 2% SDS buffer and protein concentration was determined with bicinchoninic acid reagent from Pierce (Rockford, IL). Equal amounts of protein were loaded in each lane and resolved in 12% SDS-polyacrylamide electrophoresis gel. After transferred onto polyvinylidene difluoride membrane, the blots were subsequently incubated with 5% milk, anti-LC3 primary antibody and horseradish peroxidase-conjugated anti-rabbit secondary antibody.

Antigens on the blots were revealed using the enhanced chemiluminescence kit from Pierce (Rockford, IL). Same blots were also probed with anti- β -actin to monitor protein loading and transferring.

Apoptosis Determination

Apoptosis was determined by morphological and biochemical methods as described in our previous work.^{16–18} Morphologically, after treatment, cells were stained with 10 μ g/ml Hoechst 33342. Cellular and nuclear morphology was examined by phase contrast and fluorescence microscopy, respectively. Typical apoptotic cells were identified by their morphology including cellular shrinkage, nuclear condensation and fragmentation, and formation of apoptotic bodies. Four fields with $\sim$ 200 cells per field were examined in each condition to estimate the apoptosis percentage. Biochemically, the enzymatic activity of caspases was measured using DEVD.AFC, a fluorogenic peptide substrate. Briefly, cells were extracted with 1% Triton X-100. The lysates of 25 μ g protein were added to enzymatic reactions containing 50 μ mol/L DEVD.AFC. After 1 hour incubation at 37°C, fluorescence was measured at excitation 360 nm/emission 530 nm. For each measurement, a standard curve was constructed using free AFC. Based on the standard curve, the fluorescence reading from each enzymatic reaction was converted into the nanomolar amount of liberated AFC per mg protein to indicate caspase activity.

Animals and Renal Ischemia–Reperfusion

C57BL/6 mice were originally purchased from Jackson Laboratory and maintained in the animal facility of Charlie Norwood VA Medical Center under a 12-hour light/12-hour dark pattern with free access to food and water. All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Charlie Norwood VA Medical Center. Both littermate and age/sex-matched nonlittermate male mice of 8 to 10 weeks were used for renal ischemia–reperfusion surgery as described in our recent studies.^{22,24} Briefly, after anesthetized with pentobarbital (50 mg/kg, i.p.), the mice were kept on a Homeothermic Blanket Control Unit (Harvard Apparatus Ltd, UK) with a rectal probe to monitor and maintain body temperature at $\sim$ 36.4°C. Flank incisions were made to expose both renal pedicles for bilateral clamping to induce 30 or 28 minutes of renal ischemia. The clamps were then released for reperfusion. Kidneys and blood were collected after indicated durations of reperfusion for the following examinations. Color changes of kidneys during the initiation of clamping and after removal of clamps were monitored to indicate sufficient renal ischemia and reperfusion. Control animals were subjected to sham operation without renal pedicle clamping. To detect the effects of chloroquine, the mice were treated with chloroquine (60 mg/kg, i.p.) 1 hour before renal ischemia, and were continuously subjected to daily chloroquine injection for up to 2 days through reperfusion. To examine the effects of 3-MA, the

mice were injected with one dose (30 mg/kg) of 3-MA 1 hour before ischemia–reperfusion.

Examination of Autophagic Vacuoles in Renal Tissue by Electron Microscopy

Renal tissue electron microscopy assay was described in our recent work.^{11,22} Briefly, after indicated treatment, the mice were sacrificed and perfused with 10 ml (10 units/ml) heparin, followed by 50 ml fixative (100 mmol/L sodium cacodylate, 2 mmol/L CaCl_2 , 4 mmol/L MgSO_4 , 4% paraformaldehyde, and 2.5% glutaraldehyde). Kidneys were then harvested and postfixed in the same fixative. An approximately 1 mm³ of tissue cube was collected from each kidney, including a portion of renal cortex and outer medulla for standard electron microscopy processing. According to their morphology, various autophagic structures including phagophore, autophagosome, and autolysosome in proximal tubular cells were revealed at high magnification ($\times$ 10,000). For quantification, 20 to 30 fields of low magnification ($\times$ 1000) were randomly selected from each kidney and digital images with scale bars were taken. Using Axio-Vision 4 software, the amount of autophagic vacuoles per unit cytoplasmic area of 100 μ m was evaluated.

Renal Function and Histology

Renal function was monitored by blood urea nitrogen (BUN) and serum creatinine as described before.^{19,24} Briefly, blood samples were collected and coagulated at room temperature, followed by centrifugation to have serum. BUN was measured with a kit from Biotron Diagnostics Inc (Hemet, CA) and absorbance at 540 nm was recorded at the end of reaction. Serum creatinine was determined using a kit from Stanbio Laboratory (Boerne, TX) and kinetic absorbance at 510 nm was monitored at 20 and 80 second of reaction. BUN and creatinine levels (mg/dl) were then calculated based on standard curves. For histology, kidneys were fixed with 4% paraformaldehyde and embedded in paraffin. The tissues were then sectioned at 4 μ m for H&E staining. As described previously,^{19,20,22} histopathological changes, including loss of brush border, tubular dilation, cast formation, and cell lysis, were evaluated. Tissue damage was examined in a blind manner and scored according to the percentage of damaged tubules: 0, no damage; 1, <25%; 2, 25 to 50%; 3, 50 to 75%; 4, >75%.

TUNEL Assay

As shown in our recent studies,^{19,20,22,24} apoptosis in renal tissue was identified by TdT-mediated dUTP nick-end labeling (TUNEL) assay using an *in situ* cell death detection kit (Roche Applied Science, Indianapolis, IN). Briefly, paraffin-embedded renal tissue sections of 4 μ m were deparaffinized and permeabilized with 0.1 mol/L sodium citrate, pH 6.0 at 65°C for 2 hours. The sections were then exposed to a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and nucleotides including tetramethylrhodamine-labeled dUTP. After 1 hour incubation at 37°C in

Figure 1. Hypoxia induces autophagy in renal tubular cells. **A** and **B**: RPTC cells were transiently transfected with GFP-LC3. After 0 to 24 hours of hypoxic incubation, the cells were fixed to analyze the formation and distribution of GFP-LC3 puncta by fluorescence microscopy. **A**: Representative images ($\times 600$). **B**: Percentage of cells with GFP-LC3 puncta. **C** and **D**: RPTC cells were exposed to hypoxia for 0 to 24 hours to collect whole cell lysates for immunoblot analysis of LC3 and β -actin. **C**: Representative blots. **D**: Densitometry of LC3II signals. For densitometry, the protein expression level of control (0 hours) group was arbitrarily set as 1 in each blot, and the signals of other conditions in the same blot were normalized with the control to indicate their protein expression levels. **E**: RPTC cells were incubated with hypoxia for 0 to 24 hours in the absence (–) or presence (+) of lysosomal protease inhibitors (10 μ g/ml E64d + 10 μ g/ml pepstatin A). Whole cell lysates were collected for immunoblot analysis of LC3 and β -actin. **F**: Apoptosis in RPTC cells assessed by morphological methods. Data in panels **B**, **D**, and **F** are expressed as mean $\pm$ SD ($n = 6$). * $P < 0.05$, significantly different from the control (0 hours) group.

a humidified atmosphere, positive staining with nuclear DNA fragmentation was detected by fluorescence microscopy. For quantification, 10 representative fields were selected from each tissue section and the amount of TUNEL-positive cells per 100 mm^2 was evaluated.

Statistics

Qualitative data including immunoblots and cell images are representatives of at least three experiments. Quantitative data were expressed as means $\pm$ SD. Statistical analysis was conducted using the GraphPad Prism software. Statistical differences in multiple groups were determined by multiple comparisons with analysis of variance followed by Tukey's post-tests. Statistical differences between two groups were determined by two-tailed unpaired Student's *t*-test. $P < 0.05$ was considered significantly different.

Results

Autophagy Is Induced Early in Response to Hypoxia, before Tubular Cell Apoptosis

Accumulation of LC3 (also called ATG8) in autophagosomes and lipidation of LC3 to form LC3-II are two hallmarks of autophagy and are commonly used for autophagy detection.^{25,26} Thus we initially examined autophagy

by analyzing the formation of fluorescent puncta or autophagosomes in GFP-LC3-transfected cells. As shown in Figure 1A, most control RPTC cells had an even and diffused GFP-LC3 staining with occasional puncta. On hypoxic incubation, some cells showed numerous unevenly distributed, cup- or ring-shaped green dots of various sizes. Cell counting indicated that 6 to 12 hours of hypoxia increased GFP-LC3 punctuate cells from the basal level of 15 to 34%, which decreased thereafter to 23% at the end of 24 hours (Figure 1B). We further examined LC3-II formation by immunoblot analysis. As shown in Figure 1C, hypoxic incubation induced a time-dependent accumulation of LC3-II in RPTC cells, starting at 6 hours and increasing markedly after 12 to 24 hours of treatment. The results were confirmed by densitometry of immunoblots from separate experiments (Figure 1D). Of note, the formation of GFP-LC3 puncta seemed to occur earlier than LC3-II (Figure 1, B and D), suggesting that LC3 may first accumulate to autophagic vesicles and then undergo lipidation.

Autophagy is a dynamic, multistep process, and an accumulation of autophagosome content may reflect either increased autophagic activity or reduced autophagic flux and lysosomal degradation.^{25,26} Did hypoxia induce autophagy or block autophagic flux to lysosomal degradation? To address this question, we tested the effects of E64d and pepstatin A, two lysosomal protease inhibitors used to study autophagic flux.²⁷ As shown in

Figure 2. Inhibition of autophagy by 3-MA increases apoptosis during hypoxic incubation of RPTC cells. **A, B:** RPTC cells were transiently transfected with GFP-LC3 and then incubated with hypoxia for 12 hours in the absence (–) or presence (+) of 1 hour of 10 mmol/L 3-MA pretreatment. After fixation, the formation of GFP-LC3 puncta was examined by fluorescence microscopy. **A:** Representative images ($\times 600$). **B:** Percentage of cells with punctuate GFP-LC3. **C, D:** RPTC cells were treated with hypoxia for 0 to 24 hours in the absence or presence of 1 hour of 10 mmol/L 3-MA pretreatment. After incubation, whole cell lysates were collected for immunoblot analysis of LC3. The blots were re probed for β -actin. **C:** Representative blots. **D:** Densitometric analysis of LC3II. The protein expression level of control (0 hours) group was arbitrarily set as 1 in each blot, and the signals of other conditions in the same blot were normalized with the control to indicate their protein expression levels. **E–G:** Cells were incubated with hypoxia for 24 hours in the absence or presence of 1 hour of 10 mmol/L 3-MA pretreatment. Apoptosis was assessed by morphology and caspase activation. **E:** Apoptosis percentage. **F:** Representative images showing apoptotic cells ($\times 400$). **G:** Caspase activity measured by enzymatic assays using DEVD-AFC as substrates. Data in panels B, E and G are expressed as: mean $\pm$ SD ($n = 4$). * $P < 0.05$, significantly different from the control group. ** $P < 0.05$, significantly different from the hypoxia-only group.

Figure 1E, the lysosomal inhibitors significantly increased LC3-II accumulation during hypoxic incubation of RPTC cells at each time point (lanes 6–9 vs. 2–5). The results suggest that hypoxia did not block autophagic flux; rather the autophagic activity was induced in these cells. Of note, hypoxia did not induce significant apoptosis in RPTC until 24 hours of incubation (Figure 1F). We further showed autophagy during hypoxic incubation of primary proximal tubular cells that were isolated from C57BL/6 mice (data not shown). In these cells, apoptosis or cell death was not induced even after 72 hours of hypoxic incubation (data not shown), further suggesting that autophagy is an early response to hypoxic stress whereas apoptosis is a late outcome.

Inhibition of Hypoxia-Induced Autophagy by 3-MA Increases Apoptosis in RPTC Cells

Autophagy induction under cellular stress may either contribute to cell death or act as a mechanism for cell survival.^{3–6} In renal cells and tissues, whether autophagy is cell killing or cytoprotective remains unclear. To address the role of autophagy in hypoxia-induced renal cell injury, we tested the effect of 3-MA, a pharmacological inhibitor of autophagy.^{28,29} We first titrated the condition of 3-MA treatment and found that one hour pretreatment with 10 mmol/L 3-MA could effectively block autophagy without significant cytotoxicity. As shown in Figures 2A

Figure 3. Knockdown of Beclin-1 or ATG5 sensitizes RPTC cells to hypoxia-induced apoptosis. RPTC cells were transiently transfected with GFP-tagged control shRNA, Beclin-1 shRNA, or ATG5 shRNA. The transfected cells were then untreated or treated with hypoxia for 24 hours. After treatment, the cells were stained with Hoechst33342 and apoptosis in transfected (green) cells was determined by nuclear morphology. **A:** Representative images ($\times 400$). **B** and **C:** Apoptosis percentage. Open bars: normoxia; filled bars: hypoxia-treated. Data are expressed as: mean $\pm$ SD ($n = 4$). * $P < 0.05$, significantly different from the control group. ** $P < 0.05$, significantly different from the hypoxia-treated control shRNA group. **D:** Immunoblots showing Beclin-1 and ATG5 knockdown by specific shRNAs. RPTC cells were transfected with a control shRNA or four shRNAs specific for Beclin-1 or ATG5 to collect whole cell lysate for immunoblot analysis.

and 2B, 3-MA pretreatment attenuated the formation of GFP-LC3 puncta during hypoxic incubation of RPTC cells. Consistently, hypoxia-induced LC3-II accumulation was also abrogated by 3-MA pretreatment (Figure 2C: lanes 4 vs. 5; 6 vs. 7). Densitometry of the immunoblots further confirmed the inhibitory effects of 3-MA on LC3-II accumulation during hypoxic incubation (Figure 2D). We then determined the effects of 3-MA on apoptosis during hypoxic incubation of RPTC cells. By morphology, hypoxia (1% O_2) induced $\sim 10\%$ apoptosis within 24 hours, which was increased to $\sim 20\%$ by 3-MA pretreatment (Figure 2E). The apoptotic cells assumed a shrunken configuration with apoptotic bodies and condensed and fragmented nuclei (Figure 2F). The morphological observation was confirmed by biochemical analysis of caspase activation. As shown in Figure 2G, 24 hours of hypoxic incubation increased caspase activity to 17 nmol/mg/h, which was further increased to 24 nmol/mg/h by 3-MA. Together, the results showed that inhibition of autophagy could increase hypoxic injury, suggesting that autophagy might be a cytoprotective mechanism in renal tubular cells.

Knockdown of Beclin-1 and ATG5 Sensitizes RPTC Cells to Apoptosis During Hypoxia Treatment

To confirm the pharmacological results of 3-MA, we further examined the effects of Beclin-1 knockdown on hy-

poxia-induced apoptosis in RPTC cells. Beclin-1 is an essential autophagy gene that contributes to vesicle nucleation, an initial step for autophagosome formation.³⁰ We transfected RPTC cells with GFP-tagged shRNA of Beclin-1 or a nontargeting control shRNA. The cells were then subjected to 24 hours of hypoxic incubation. Apoptosis was examined by cellular and nuclear morphology. Since the transfection efficiency in RPTC cells was not very high, apoptosis evaluation was focused on the transfected cells that expressed green fluorescent GFP. As shown in Figure 3A, hypoxia induced apoptosis in some of the control shRNA transfected cells and obviously more apoptotic cells were induced in the Beclin-1 shRNA-transfected group. The results were confirmed by cell counting. As shown in Figure 3B, regardless the transfection with targeting or nontargeting shRNA, the cells under normoxia had a similar low level of apoptosis (6 to 7%); after hypoxia treatment, the cells transfected with control shRNA had 26% apoptosis, which was increased to 45% in Beclin-1 shRNA transfected cells. We confirmed the results with two more Beclin-1 shRNAs, which increased apoptosis to 63% and 44% during 24 hours of hypoxia, respectively (not shown). In addition, we determined the effect of RNA interference knockdown of ATG5, which participates in autophagic vesicle elongation and completion.^{1,2} As shown in Figure 3C, 24 hours of hypoxia induced 36% apoptosis in control shRNA transfected cells, which was increased to 61%

Figure 4. Induction of autophagy and its cytoprotective effect against cell apoptosis in response to *in vitro* ischemia–reperfusion. **A** and **B**: RPTC cells were transiently transfected with GFP-LC3. For *in vitro* ischemia, the transfected cells were incubated in a glucose-free buffer in an anaerobic chamber for 2 hours (I2 hours); for reperfusion, the cells were transferred back to full culture medium with oxygen for another 2 hours (I2 hours + R2 hours). The formation of GFP-LC3 puncta was analyzed immediately after ischemia or following reperfusion. **A**: Representative images (×600). **B**: Percentage of cells with punctate GFP-LC3. Data are expressed as mean $\pm$ SD ($n = 3$). * $P < 0.05$, significantly different from the control group. **C**: RPTC cells were transiently transfected with GFP-tagged control shRNA, Beclin-1 shRNA, or ATG5 shRNA. The transfected cells were then untreated (control) or treated by *in vitro* ischemia–reperfusion described above (I2 hours + R2 hours). After treatment, the cells were stained with Hoechst33342 and apoptosis percentage in transfected (green) cells was determined by nuclear morphology. Data are expressed as mean $\pm$ SD ($n = 4$). * $P < 0.05$, significantly different from the control group. ** $P < 0.05$, significantly different from the ischemia–reperfusion treated control shRNA group.

in ATG5 shRNA transfected cells. Knockdown of Beclin-1 and ATG5 by shRNAs was verified by immunoblot analysis (Figure 3D). These results further suggest that the early autophagic response during hypoxia may play a protective role for cell survival.

Induction of Autophagy and Its Cytoprotective Effect against Tubular Cell Apoptosis during *In Vitro* Ischemia–Reperfusion

In 2006, Gottlieb and colleagues³¹ demonstrated an autophagic response to *in vitro* ischemia–reperfusion injury in a cardiac cell line and interestingly, autophagy was shown to occur during the reperfusion but not ischemia period. To follow up this finding, we examined autophagy using an *in vitro* ischemia–reperfusion model. As shown in Figure 4A, after 2 hours of ischemic incubation, GFP-LC3 was still diffusely distributed throughout the cells, with occasionally detectable puncta. In contrast, numerous GFP-LC3 puncta were formed in the cells after 2 hours of reperfusion. Cell counting confirmed the morphological observation (Figure 4B). The control group had punctate GFP-LC3 in 10% cells, which was not increased during ischemia (8%) but significantly increased to 36% after reperfusion. To determine the role of autophagy in this injury model, we transfected RPTC cells with shRNAs of Beclin-1, ATG5 or control sequence and examined apoptosis after ischemia–reperfusion treatment. As shown in Figure 4C, *in vitro* ischemia–reperfusion induced 30% apoptosis in control shRNA transfected cells, which was increased to ~50% by either knockdown of Beclin-1 or ATG5. Together with the previous study,³¹ these results indicate that autophagy is not induced by ischemia but significantly enhanced by subsequent reperfusion. Under this condition, autophagy may protect against apoptosis.

Autophagy Induction during Renal Ischemia–Reperfusion in Mice

To extend the *in vitro* findings to *in vivo* situation, we determined autophagy induction in a characterized model of renal ischemia–reperfusion injury.^{22,24} C57BL/6 mice were subjected to sham operation or 30 minutes of bilateral renal ischemia followed by reperfusion. Kidneys were harvested at various time points for following analyses. First, we examined LC3-II accumulation in renal cortical and outer medulla tissues by immunoblotting. As shown in Figure 5A, a basal level of LC3-II was shown in the sham control lysate (lane 1), which was reduced during renal ischemia (lane 2). However, on reperfusion, a significant amount of LC3-II accumulated in renal tissues in a time-dependent manner, starting at 6 hours and further increasing after 24 and 48 hours (lanes 3–5). Densitometry of immunoblots from separate experiments confirmed LC3-II accumulation during renal ischemia–reperfusion injury (Figure 5B). By 24 and 48 hours of reperfusion, LC3-II was increased to 1.9- and 2.6-fold over control, respectively. We further examined autophagy by electron microscopy. The appearance of autophagosomes and related autophagic vacuoles was monitored. Consistent with the timeline of LC3-II accumulation in renal tissues, no obvious autophagic vacuoles were shown during 30 minutes of ischemia. In contrast, numerous autophagic vacuoles appeared in proximal tubular cells during the subsequent 6 to 48 hours of reperfusion (Figure 5C). The structures of autophagic vacuoles or vesicles were further revealed by high magnification (×10,000) electron microscopy. While the autophagosomes were identified as double or multiple membrane structures containing cytoplasm (Figure 5D, upper left panel, arrows) or undigested organelles such as mitochondria (Figure 5D, upper right panel, arrow), the autolysosomes appeared to be single membrane structures with remnants of cytoplasmic components (Figure 5D, lower two panels, arrow heads). By mor-

Figure 5. Autophagy induction in renal tissue during ischemia-reperfusion injury *in vivo*. C57BL/6 mice (male, 8 to 10 weeks old) were subjected to sham operation or 30 minutes of bilateral renal ischemia, followed by 0 to 48 hours of reperfusion. **A:** Representative immunoblots of LC3 in kidney cortex and outer medulla tissue lysate. The blots were reprobed for β-actin. **B:** Densitometric analysis of LC3II blots. The protein expression level of sham mice was arbitrarily set as 1 in each blot, and the signals of other conditions in the same blot were normalized with the control to indicate their protein expression levels. Data are expressed as mean ± SD ($n = 3$). * $P < 0.05$, significantly different from the sham control. **C:** Representative electron micrographs showing autophagic vacuoles in renal tubular cells (×2500). **D:** High magnification of electron micrographs showing structures of autophagosomes (upper panels, arrowheads indicate vesicles) and autolysosomes (lower panels, vesicles indicated by arrowheads) (×10,000). **E:** Quantification of the number of autophagic vacuoles per 100 μm cytoplasm.

phometric analysis, the amount of autophagic vacuoles per unit cytoplasmic area of 100 μm was evaluated (Figure 5E). Compared with the basal level of 1.52 in the sham control, fewer autophagic vacuoles (1.34) were shown in ischemic tissues. However, autophagic vacuoles were drastically increased following reperfusion, to 4.77, 7.84, and 10.53 at 6, 24, and 48 hours, respectively. Thus, autophagy is induced in a time-dependent manner during renal ischemia–reperfusion in C57BL/6 mice.

Suppression of Autophagy by Chloroquine and 3-MA Worsens Renal Ischemia–Reperfusion Injury

To determine the role of autophagy in renal ischemia–reperfusion injury, we examined the effects of chloroquine, a pharmacological inhibitor of autophagy that has been used in *in vivo* studies.^{32–34} Unlike 3-MA (which is a class III phosphatidylinositol 3-kinase inhibitor that blocks the initial autophagic sequestration and autophagosome formation^{28,29}), chloroquine inhibits autophagy by acting as a lysosomotropic agent that raises lysosomal pH to suppress the activity of lysosomal acid hydrolases and hence prevent the maturation and lysosomal degradation of autophagosomes.^{35–37} To test the effects of chloroquine, we induced moderate renal injury in C57BL/6 mice via 28 minutes of bilateral renal ischemia. We first con-

firmed the effects of chloroquine on autophagy in the *in vivo* model. As shown in Figure 6A, renal ischemia followed by 48 hours of reperfusion led to an increase of LC3-II in renal tissues (Figure 6A, lanes 3 & 4). By blocking the last step of autophagic flux, chloroquine prevented the lysosomal degradation of LC3-II in autophagosomes, resulting in further LC3-II accumulation (Figure 6A, lanes 5 & 6). We then examined the renal damage in the absence or presence of chloroquine. Twenty-eight minutes of ischemia followed by reperfusion induced a moderate renal failure, as indicated by increases of BUN to 132 mg/dl (Figure 6B) and serum creatinine to 0.87 mg/dl (Figure 6C) at the end of 48 hours of reperfusion. Importantly, chloroquine induced more severe loss of renal function, further increasing the values of BUN and serum creatinine to 197 mg/dl and 1.75 mg/dl, respectively (Figure 6, B and C). Histological examination was concentrated on the outer stripe of outer medulla, the main injury site of renal ischemia–reperfusion.³⁸ Consistent with the functional measurements, tubular damage following renal ischemia was aggravated by chloroquine; in this group, more proximal tubules showed dilation and distortion, loss of brush border, cell lysis, and sloughed debris in the lumen space (Figure 6D). These tubular disruptions were then graded and the pathological scores were shown in Figure 6E. Chloroquine increased the tubular damage score from 2 to 3.25. We further analyzed apoptosis in the collected tissues by TUNEL assay. The results of

Figure 6. Effects of chloroquine and 3-MA on renal ischemia-reperfusion injury in mice. To examine the effect of chloroquine, C57BL/6 mice (male, 8 to 10 weeks old) were divided into three groups (4 to 8 mice for each group) and subjected to the following treatments: 1) sham; 2) 28 minutes of bilateral renal ischemia followed by 48 hours of reperfusion (I/R48 hours); 3) 28 minutes of bilateral renal ischemia followed by 48 hours of reperfusion plus daily 60 mg/kg chloroquine intraperitoneal injection (CQ+I/R48 hours). **A:** Representative blots. Whole tissue lysates of kidney cortex and outer medulla were collected for immunoblot analysis of LC3. **B** and **C:** Blood samples were collected for measurements of BUN and serum creatinine. Data are expressed as mean $\pm$ SD. * P < 0.05, significantly different from the sham; ** P < 0.05, significantly different from the I/R48 hours group. **D:** Representative images of renal histology from outer stripe of outer medulla (H&E staining, $\times 200$). **E:** Pathological score of tubular damage in I/R48 hours and CQ+I/R48 hours groups. **F:** Representative images of TUNEL staining ($\times 200$). **G:** Quantification of TUNEL-positive cells in I/R48 hours and CQ+I/R48 hours groups. Data in **(E)** and **(G)** are expressed as mean $\pm$ SD. * P < 0.05, significantly different from the I/R48 hours group. **H** and **I:** C57BL/6 mice (male, 8 to 10 weeks old) were subjected to sham or 28 minutes of bilateral renal ischemia followed by 48 hours of reperfusion in the absence (I/R48 hours) or presence of 3-MA (3-MA+I/R48 hours, single dose of 30 mg/kg, intraperitoneal injection before ischemia). Blood samples were collected for measurements of BUN and serum creatinine. Data are expressed as mean $\pm$ SD (for each group, $n = 5$). * P < 0.05, significantly different from the sham; ** P < 0.05, significantly different from the I/R48 hours group.

representative images and cell counting were shown in Figure 6F and 6G. While no TUNEL-positive cells were detected in the sham control, renal ischemia followed by 48 hours of reperfusion induced 66 apoptotic cells per mm² tissue, which was further increased to 101 by chloroquine. Of note, other than inhibiting autophagy, chloroquine per se at the dose used in our study did not have obvious nephrotoxicity in the mice. For example, in chloroquine-treated sham-operated animals, the values of BUN and serum creatinine were 36 mg/dl and 0.33 mg/dl, respectively; furthermore, no obvious tubular damage was found by renal histological examination (Figure 6D:

CQ sham). Collectively, the results suggest that autophagy during renal ischemia-reperfusion *in vivo* may be a renoprotective mechanism against renal injury. To complement the chloroquine study, we also tested the effects of 3-MA on renal injury during ischemia-reperfusion. It was shown that 3-MA partially but significantly increased renal dysfunction during renal ischemia-reperfusion, increasing BUN from 202 to 240 mg/dl and serum creatinine from 2.01 to 2.64 mg/dl, respectively (Figure 6, H and I). 3-MA per se did not induce obvious nephrotoxicity in control animals (BUN: 28.01 mg/dl; serum creatinine: 0.26 mg/dl). These results provide further support to

the chloroquine study for a renal protective role of autophagy.

Discussion

Despite rapid progress in autophagy research in other organ systems, very limited is known about autophagy in renal pathophysiology.³⁹ Recent studies have demonstrated autophagy in renal cells and tissues during ischemic and nephrotoxic kidney injury; however, the role played by autophagy under these pathological conditions is poorly understood. Our recent work has suggested a renoprotective role for autophagy during cisplatin-induced kidney injury or nephrotoxicity.¹¹ Nevertheless, as pointed out by the accompanying editorial, the extent to which autophagy can ameliorate acute kidney injury caused by other types of renal insults such as ischemia remains to be determined.⁴⁰ The current study has characterized autophagy induction during renal ischemia–reperfusion using *in vitro* and *in vivo* models. Importantly, this study has demonstrated that autophagy is a protective mechanism for cell survival under these pathological conditions.

Chien et al⁹ showed that the expression of Beclin-1 and LC3 was increased in renal tubules during renal ischemia–reperfusion in rats. Moreover, expression of Bcl-xL and Bcl-2 could ameliorate both autophagy and apoptosis, accompanied by the amelioration of ischemic kidney injury. Although the role of autophagy was not directly investigated, it was suggested that autophagy might contribute to tubular cell injury and death.⁹ Suzuki et al¹⁰ further demonstrated the formation of autophagosomes in renal tubular cells during hypoxic incubation and in mice during renal ischemia–reperfusion. Based on the *in vitro* observation that autophagy inhibitors could protect renal tubular HK2 cells from H₂O₂-induced cell death, they concluded that autophagy might play a cell killing role during renal ischemia–reperfusion injury.¹⁰ Our current study has systematically analyzed autophagy and its potential pathogenic role during renal ischemia–reperfusion using both *in vitro* and *in vivo* models.

We have shown the induction of autophagy in renal tubular cells and tissues in response to *in vitro* hypoxic and *in vivo* ischemic stress, as indicated by punctuate GFP-LC3 localization, LC3-II formation, and accumulation of autophagic vacuoles. Autophagy was shown to occur early both in RPTC and primary tubular cells within 3 to 6 hours of hypoxia treatment, and maintains at high level for 12 to 24 hours (Figure 1). In addition, autophagy was also induced in response to *in vitro* ischemia–reperfusion incubation (Figure 4, A and B). In mice, autophagy was not activated by ischemia, but was induced rapidly during reperfusion (Figure 5). We have also evaluated the autophagic flux by using lysosomal protease inhibitors *in vitro* and chloroquine *in vivo* to block lysosomal degradation (Figures 1E and 6A). As autophagy is a dynamic, multi-step process, an accumulation of LC3-II at a given time point (or steady state) may reflect either induction of autophagy or defect of lysosomal degradation.^{25,26} Un-

der this condition, it is important to measure lysosomal degradation by comparing LC3-II levels in the presence and absence of lysosomal protease inhibitors. Turnover of LC3-II in the presence of lysosomal protease inhibitors indicates the delivery of LC3-II to lysosomes for degradation and completion of autophagic flux.²⁶ Therefore, the fact that the LC3-II accumulation during renal cell hypoxia/ischemia was increased by these lysosomal inhibitors suggests that renal injury induces autophagy and does not block autophagic flux.

Importantly, our study has further provided evidence to support a renoprotective role for autophagy during ischemic kidney injury. *In vitro* in cultured RPTC cells, inhibition of autophagy by either 3-MA or siRNA knockdown of Beclin-1 or ATG5 enhanced apoptosis during hypoxic incubation (Figures 2 and 3) and ischemic-reperfusion treatment (Figure 4C). *In vivo* in C57BL/6 mice, inhibition of autophagy by chloroquine exacerbated kidney injury following ischemia–reperfusion (Figure 6, A–G). It is noteworthy that chloroquine has been recently used to inhibit autophagy *in vivo* without noticeable side effects.^{32–34} Iwai-Kanai et al⁴¹ has further suggested to use chloroquine for evaluation of autophagic flux *in vivo*, which provides a reliable method to verify that high autophagosome content observed in animal organs or tissues indeed results from increased autophagic activity rather than decreased lysosomal clearance. In our study, chloroquine blocked autophagic flux as shown by LC3-II accumulation and importantly, it exacerbated ischemic kidney injury, suggesting a renoprotective role for autophagy. Although chloroquine did not show obvious nephrotoxicity in control mice, we recognize that the effects of chloroquine may not be limited to inhibition of autophagy and autophagy-independent effects of chloroquine may contribute to the worsened renal injury during ischemia–reperfusion. To strengthen the chloroquine study, we tested the effects of 3-MA, which inhibits autophagy at the early stage of autophagosome formation. It was shown that 3-MA induced more severe loss of renal function during ischemia–reperfusion (Figure 6, H and I), providing further support to the chloroquine study for a renoprotective role of autophagy. Further investigation should use autophagy gene knockout animal models to determine conclusive evidence for the involvement of autophagy in renal ischemia–reperfusion injury *in vivo*.

The mechanisms of autophagy induction and regulation during hypoxic/ischemic renal injury remain unclear. In heart, brain, or cancer cells, hypoxic stress may activate autophagy via signaling pathways mediated by hypoxia-inducible factor-1 (HIF-1), 5'-AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), or endoplasmic reticulum stress. HIF-1 is a transcription factor activated by low oxygen conditions during hypoxia and ischemia. Zhang et al⁴² showed that during hypoxia, HIF-1 induces Bcl-2 nineteen-kilodalton interacting protein 3 (BNIP3), leading to selective autophagy of mitochondria. A subsequent study extended these observations and suggested that the BH3 domain of BNIP3 is responsible for hypoxia-induced autophagy.⁴³ In contrast, Papandreou et al⁴⁴ reported a HIF-1/BNIP3-independent pathway of autophagy that was mediated by AMPK during hypoxia. As a sensor of energy

stress, AMPK may regulate autophagy through different downstream signals including inhibition of mTOR, phosphorylation of eukaryotic elongation factor-2 kinase, phosphorylation of p27, and direct activation of autophagic genes.⁴⁵ In addition to AMPK, HIF-1-induced REDD also contributes to mTOR inhibition, thus integrating the two O₂-sensing pathways (HIF-1 and mTOR) for autophagy induction.⁴⁶ Recently, endoplasmic reticulum stress through unfolded protein response and intracellular calcium has been implicated in autophagy regulation.⁴⁷ Given that hypoxia and ischemia are potent activators of unfolded protein response,⁴⁸ it would be important to investigate endoplasmic reticulum stress as a mechanism of autophagy induction under these pathological conditions.

Although we have demonstrated a protective role for autophagy during ischemic kidney injury, it is unknown how autophagy protects against cell injury and death. In response to starvation or nutrient deprivation, autophagy can digest cytoplasmic materials to generate essential metabolic substrates and energy to maintain cell viability.^{2,4,40,49} Under other stress conditions, autophagy may work as a cellular housekeeper to eliminate damaged organelles such as mitochondria, peroxisomes, and endoplasmic reticulum, to clear intracellular pathogens, and to remove protein aggregates along with the ubiquitin-proteasome pathway for protein quality control. This housekeeping role is critical for autophagy-dependent protection against neurodegenerative diseases, tumorigenesis, aging, and infection;^{2,4,40,49} however, its contribution to renal pathophysiology including hypoxic/ischemic renal injury remains unclear. Finally, the cross-regulation between autophagy and apoptosis raise an interesting possibility that signaling activated during autophagy may interfere with or compromise cell death pathways.^{4,5,8} Further studies are needed to gain insights into the regulatory mechanisms of autophagy and its function in renal pathophysiology.

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