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Involvement of autophagy in the pharmacological effects of the mTOR inhibitor everolimus in acute kidney injury

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ABSTRACT

Inhibitors of mammalian target of rapamycin (mTOR) have immunosuppressive and anti-cancer effects, but their effects on the progression of kidney disease are not fully understood. Using cells from normal kidney epithelial cell lines, we found that the antiproliferative effects of mTOR inhibitor everolimus accompanied the accumulation of a marker for cellular autophagic activity, the phosphatidylethanolamine-conjugated form of microtubule-associated protein 1 light chain 3 (LC3-II) in cells. We also showed that the primary autophagy factor UNC-51-like kinase 1 was involved in the antiproliferative effects of everolimus. Levels of LC3-II decreased in the kidneys of rats treated with ischemia-reperfusion or cisplatin; however, renal LC3-II levels increased after administration of everolimus to rats subjected to ischemia-reperfusion or cisplatin treatment. Simultaneously, increased signals for kidney injury molecule-1 and single-stranded DNA and decreased signals for Ki-67 in the proximal tubules were observed after treatment with everolimus, indicating that everolimus diminished renal function after acute tubular injury. We also found leakage of LC3 protein into rat urine after treatment with everolimus, and urinary LC3 protein was successfully measured between 0.1 and 500 ng/mL by using an enzyme-linked immunosorbent assay. Urinary LC3 levels were increased after administration of everolimus to rats subjected to ischemia-reperfusion or cisplatin treatment, suggesting that renal LC3-II and urinary LC3 protein are new biomarkers for autophagy in acute kidney injury. Taken together, our results demonstrated that the induction of autophagy by everolimus aggravates tubular dysfunction during recovery from kidney injury.

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1. Introduction

Everolimus is as an immunosuppressive macrolide derived from sirolimus (rapamycin) that inhibits the kinase activity of mammalian target of rapamycin (mTOR) (Wullschleger et al., 2006). The renal toxicity of everolimus is thought to be lower than that of other agents, such as calcineurin inhibitors (e.g., cyclosporine or tacrolimus), which are often used as primary immunosuppressants after organ transplantation (Lehmkuhl et al., 2005; Schweiger et al., 2006). However, de novo use of mTOR inhibitors after renal transplantation has been reported to cause decreased renal graft survival (Ekberg et al., 2007) and delayed wound healing (Kahn et al., 2005).

Previous studies in models of progressive kidney disease have shown that sirolimus and everolimus have preventive effects on renal hypertrophy and fibrosis (Huber et al., 2011). Recently, we demonstrated that the mTOR pathway is activated in the proximal tubular cells of rat kidneys after subtotal nephrectomy (Nakagawa et al., 2010). We found that treatment with everolimus in rats 8 weeks after subtotal nephrectomy, an animal model of end-stage renal disease, had restorative effects on the tubular reabsorption of albumin and the expression levels of membrane transporters in the proximal tubules. However, mTOR inhibitors also display adverse effects on the kidneys. Treatment with mTOR inhibitors in ischemia-reperfusion injury deteriorates the renal function of rats and mice by preventing recovery (Esposito et al., 2011; Lieberthal et al., 2001). In addition, we found that everolimus treatment in rats 2 weeks after subtotal nephrectomy, an animal model of compensative chronic renal failure, prevented cellular hyperplasia of the proximal tubules, resulting in severe renal damage (Nishihara et al., 2010). These findings suggest that mTOR inhibitors could contribute to renal toxicity by inhibiting the adaptive response of tubular cells during acute kidney injury, and therefore, biomarkers are needed to predict treatment outcomes with mTOR inhibitors during kidney injury.

The mTOR pathway is known to regulate autophagy (Wullschleger et al., 2006). Recent studies have shown that the active mTOR pathway suppresses autophagy by preventing UNC-51-like kinase 1 (ULK1) activity (Chan et al., 2007). Autophagy is an evolutionarily conserved process through which organelles or long-lived proteins are sequestered in a double-membrane vesicle...
“autophagosome” and subsequently degraded in lysosomes (Levine and Kroemer, 2008). Because the cytosolic form of microtubule-associated protein 1 light chain 3 (LC3-I) is modified into its phosphatidylethanolamine-conjugated form, LC3-II, which is associated with autophagosomes, LC3-II has been examined as a marker for autophagic activity (Mizushima et al., 2010). Recent studies have focused on the roles of autophagy in kidney disease; however, the influence of mTOR activation on the LC3-II levels in the kidneys remains to be delineated.

Given these findings, we examined the association between the mTOR pathway and autophagy in the kidney. Our results demonstrated that mTOR inhibition with everolimus induced the renal accumulation of LC3-II in acute kidney injury, preventing tubular cell proliferation after injury. We also identified the presence of LC3 protein in rat urine and found that the quantification of urinary LC3 was beneficial in the evaluation of the pharmacological activity of mTOR inhibitors in the kidney.

2. Materials and methods

2.1. Animals

Wistar/ST rats were purchased from SLC Animal Research Laboratories (Shizuoka, Japan) and cared for in accordance with
the Guidelines for Animal Experiments of Kyoto University. All protocols were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University. For studies on the effects of starvation, 8-week-old rats were deprived of food for 0 or 48 h. These rats had free access to drinking water. To investigate the effects of ischemia-reperfusion, we subjected 8-weeks-old rats to ischemia-reperfusion following a protocol published elsewhere (Goncálves et al., 2006). In brief, both renal pedicles were cross-clamped for 40 min. During the procedure, animals were well hydrated with saline and maintained at a constant temperature (37°C) using a heating pad. Sham-operated rats at 2 days after surgery were used as controls. To induce nephrotoxicity with cisplatin, we intraperitoneally administered cisplatin (5 mg/kg; Randol: Nippon Kayaku Co., Ltd., Tokyo, Japan) to 8-week-old rats. Control rats were administered the same volume of saline. Kidneys were collected 1, 2, 4, and 7 days after ischemia-reperfusion or cisplatin treatment. The levels of blood urea nitrogen and plasma creatinine (PCR) were measured to examine renal function by using assay kits from...
Wako Pure Chemical Industries (Osaka, Japan). The urinary protein concentration was determined using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

Rats were subcutaneously administered everolimus (2 mg/kg body weight per day; LC Laboratories, Woburn, MA) or vehicle (90% saline, 5% ethanol, 5% Cremophor EL, [Nacalai Tesque, Kyoto, Japan]). The rats were administered everolimus for 5 days to obtain a stable everolimus blood concentration (Fig. 1B). On the sixth day, the rats were subjected to ischemia-reperfusion or treated with cisplatin.

2.2. Cell culture

Cells from kidney epithelial cell lines (human kidney 2 [HK-2], ATCC CRL-2190; Madin–Darby canine kidney [MDCK], ATCC CCL-34; normal rat kidney epithelial [NRK-52E], ATCC CRL-1571; American Type Culture Collection, Manassas, VA) were cultured in complete medium consisting of Dulbecco’s modified Eagle’s medium (Wako) with 10% fetal bovine serum (Whittaker Bioproducts Inc., St. Louis, MO) in an atmosphere of 5% CO2–95% air at 37 °C. The effects of everolimus on viable cell number and LC3-II were measured after exposure to everolimus for 48 h.

The effect of everolimus on cell proliferation was determined by adapting a published previously protocol (Iwamaru et al., 2007). Cell viability was determined using a WST-8 cell proliferation reagent kit (Nacalai Tesque) according to the manufacturer’s instructions. The cells were seeded into 24 wells at 2 × 104 cells per well and incubated overnight in an atmosphere of 5% CO2–95% air at 37 °C. After exposure to everolimus (100 nM) for 48 h, the cells were incubated with cell culture medium containing 10% WST-8 for 1 h, and the absorbance of the samples against a background control was measured. The viability of the cells treated with vehicle alone was set as 1.0. For the RNA interference system, the sequence of small interfering RNA (siRNA) targeting rat Ulk1 was as follows: 5′-GGUCUCAGAGUUGCUACCCUCGUU-3′. The siRNA was synthesized by Invitrogen Life Technologies (Carlsbad, CA). We used Stealth RNAi Negative Control Duplexes (Invitrogen) as the negative control. On the day before transfection, the cells were seeded into 48-well plates at a density of 7.5 × 103 cells per well. The cells were transfected with 8 pmol per well siRNA by using 0.4 μL Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were treated with vehicle alone or everolimus (1–100 nM) for 48 h.

2.3. Western blotting

Cells, rat skeletal muscles and rat whole kidneys were homogenized in lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM NaF, 1 mM Na2P2O7, 1 mM Na2VO4, 1% NP-40, and 1% protease inhibitor cocktail). To solubilize LC3-II completely, we boiled and subsequently separated fresh samples using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred them to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). Membranes were blocked, washed, and incubated overnight at 4 °C with primary antibodies to total S6 (Cell Signaling Technology #2317, Tokyo, Japan), phosphorylated ribosomal protein S6 (p-S6; Cell Signaling Technology #2211), LC3 (Novus Biologicals NB100-2331, Littleton, CO), gliceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology sc-59540, Avenue, CA), Ulk1 (Santa Cruz Biotechnology sc-33182), and phosphorylated Ulk1 (p-Ulk1; Cell Signaling Technology #6888). The bound antibodies were detected on X-ray film by using enhanced chemiluminescence with horseradish peroxidase (HRP)-conjugated secondary antibodies and cyclic diacyldiacylhydrazides (Mercck Millipore). The relative densities of the bands in each lane were determined using NIH Image J 1.39 (National Institutes of Health, Bethesda, MD).

For the urine analysis, urine samples collected from the bladders of the rats were concentrated using ultrafiltration membrane units (Amicon Ultra 3 kDa, Merck Millipore) and diluted with 20 mM Tris–HCl. Concentrated urine was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. Two antibodies specific for LC3 (Novus Biologicals NB100-2331B and Cell Signaling Technology #2775) were used as primary antibodies.

2.4. Measurement of urinary LC3

Urine was collected from the bladders of the rats and stored at −80 °C until use. Urinary LC3 was measured using a sandwich enzyme-linked immunosorbent assay (ELISA). One antibody specific

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for LC3 (Cell Signaling Technology #2775) served as a capture antibody. Another biotinylated LC3 antibody (Novus Biologicals NB100-2331B) was used as a detection antibody. The wells of a 96-well plate (ELISA Starter Accessory Kit, Bethyl Laboratories, Montgomery, TX) were coated with capture antibody (diluted 1:250) and blocked with 1% bovine serum albumin in phosphate-buffered saline. Standard LC3 protein (abcam ab87823, Cambridge, UK) in serial dilutions (0–500 ng/mL) or urine samples were then added to the plate in duplicate. All of the dilutions of LC3 standards, urine samples, antibodies, and HRP-conjugated streptavidin (R&D Systems, Minneapolis, MN) were performed in the blocking solution. After the washing, detection antibody and then HRP-conjugated streptavidin were added. Color was developed by adding the 3,3,5,5-tetramethylbenzidine substrate (Bethyl Laboratories), and the reaction was stopped by adding 2N H2SO4. Absorbance was measured at 450 nm and corrected for plate artifacts at 570 nm. Urinary LC3 concentration was calculated based on the standard curve and expressed in ng/mg creatinine.

2.5. Fluorescence microscopy

After perfusion with 4% paraformaldehyde in phosphate-buffered saline, the kidneys were embedded in Tissue-Tek® O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) and frozen rapidly in liquid nitrogen. To detect Ulk1, LC3, kidney injury molecule-1 (Kim-1), and Ki-67, we cut 5-μm-thick sections of the rat kidney and covered them with 5% bovine serum albumin containing 0.3% Triton X-100 at 37 °C for 60 min. The covered sections were incubated overnight at 4 °C with primary antibodies specific for Ulk1 (Merck Millipore ST1521), LC3 (Novus Biologicals NB100-2331), Kim-1 (Nakagawa et al., 2010), and Ki-67 (abcam ab16667), washed 3 times, and incubated with secondary antibodies (Alexa 546-labeled goat anti-rabbit immunoglobulin G [IgG] or Alexa 546-labeled goat anti-mouse IgG; Life Technologies, Tokyo, Japan), Alexa 488-phalloidin (Life Technologies) and 4',6-diamidino-2-phenylindole (Wako) at 37 °C for 60 min. As a negative control for Ulk1, primary antibody against Ulk1 was incubated after preabsorption with recombinant full-length Ulk1 protein (abcam ab95322). To detect single-stranded DNA (ssDNA), we treated 5-μm-thick sections with proteinase K and heated them in 50% formamide. The sections were then covered with 3% non-fat dry milk at 37 °C for 20 min. The covered sections were incubated for 30 min at room temperature with a primary antibody specific for ssDNA (Merck Millipore MAB3299), washed 3 times, and incubated with secondary antibodies at room temperature for 30 min. Images for immunofluorescent analysis were captured and examined with a BZ-9000 (Keyence, Osaka, Japan). To evaluate the levels of LC3, Kim-1, Ki-67, and ssDNA, we took 20 independent photographs of the renal cortex (at 1000× magnification for LC3; at 100× magnification for Kim-1, Ki-67, and ssDNA) and counted the dot structures in the proximal tubules (for LC3), the stained proximal tubules (for Kim-1), or nuclei (for Ki-67 and ssDNA).

![Fig. 6. Effects of everolimus in rat kidneys after treatment with ischemia-reperfusion. Rats were administered everolimus (E+, 2 mg/kg per day) or vehicle (E−) for 7 days. On day 5 of administration, rats were also subjected to ischemia-reperfusion. Kidneys were collected on day 2 post-treatment. In a control group, rats were sham-operated, and kidneys were collected on day 2 after surgery. The expression levels of LC3, total Ulk1, p-Ulk1, total S6, p-S6, and GAPDH were examined using Western blot analysis (A). The relative densities of the bands in each lane were determined (B)–(D). *P < 0.05, **P < 0.01, †P < 0.05, ††P < 0.01 show significant difference.](image-url)
2.6. Statistical analysis

Data are expressed as mean ± S.E.M. Data were statistically analyzed using the unpaired t test or multiple comparisons after one-way analysis of variance. Probability values of less than 0.05 were considered statistically significant. Statistical analysis was performed using Prism Version 4.0 software (GraphPad, San Diego, CA).

3. Results

3.1. Localization of Ulk1 in rat kidneys

First, renal localization of Ulk1 was examined using immunofluorescent analysis (Fig. 2). Signals for Ulk1 were predominantly observed in the cortex (Fig. 2A–C). The serial sections were stained with phalloidin and antibodies against Ulk1. Phalloidin conjugated with F-actin, making signals for phalloidin abundant at glomeruli and the luminal side of proximal tubules (Fig. 2D and G). The results showed that the intensities of the signals for Ulk1 were relatively higher in proximal tubular epithelial cells (Fig. 2D–F). The preabsorption of antibody with Ulk1 protein abolished these positive signals (Fig. 2G–I), demonstrating the presence of Ulk1 protein in proximal tubular epithelial cells. Furthermore, the signals for LC3 were observed in the proximal tubular epithelial cells (Fig. 2J–L).

3.2. Effects of everolimus on renal tubular epithelial cell lines

To characterize the effects of everolimus on tubular epithelial cells, we carried out in vitro experimentation with the renal tubular epithelial cell lines HK-2, MDCK, and NRK-52E. LC3-II levels in the cells were determined using recommended methods in mammalian autophagy research (Klionsky et al., 2008; Mizushima et al., 2010). In the renal tubular epithelial cell lines, the LC3-II levels were significantly increased in cells exposed to everolimus for 48 h compared with controls. This increase was associated with a decrease in Ki-67 staining, indicating suppression of cell proliferation.

Fig. 7. Effects of everolimus on renal functions after ischemia-reperfusion. Levels of PCr (A), kidney weight (B), LC3 (C)–(E), kidney injury molecule-1 (Kim-1; (F)–(H)), single-stranded DNA (ssDNA) (I)–(K), and Ki-67 (L)–(N) were examined after concomitant treatment with everolimus and ischemia-reperfusion. Green signals for phalloidin are merged with red signals for LC3 (C), (D); Kim-1 (F), (G); ssDNA (I), (J); or Ki-67 (L), (M). Magnification, 400 ×; scale bars, 50 μm. (⁎), glomeruli. The numbers of dot signals (for LC3, (E)); stained proximal tubules (for Kim-1, (H)); or stained nuclei (for ssDNA, (K); Ki-67, (N)) were counted. Each column represents the mean ± S.E.M. (⁎P < 0.05, ⁎⁎P < 0.01, ⁎⁎⁎P < 0.005, and ⁎⁎⁎⁎P < 0.001 show significant differences).
with those treated with vehicle (Fig. 3A). Simultaneously, the treatment of everolimus decreased the number of viable cells (Fig. 3B). To determine the involvement of Ulk1 in the effect of everolimus on cell proliferation, we used siRNA-targeting rat Ulk1 (siUlk1). The protein expression levels of Ulk1 in NRK-52E cells were markedly decreased after treatment with siUlk1 (Fig. 3C). Transfection with siUlk1 significantly decreased the cell viability at 24, 36, and 48 h after treatment with vehicle alone (Fig. 3D). While the treatment with everolimus (1, 10, and 100 nM) markedly inhibited cell proliferation of control cells, transfection with siUlk1 attenuated the anti-proliferative effect of everolimus (Fig. 3E–G).

3.3. Effects of ischemia-reperfusion on the mTOR pathway in the kidney

Before assessing LC3-II levels in vivo, we validated our methods based on a previous report that the levels of LC3-II in muscle tissue increases after nutrient starvation (Mizushima et al., 2004). The levels of LC3-II in rat skeletal muscles after fasting for 0 or 48 h was examined. As shown in Fig. 4, fasting for 48 h significantly increased LC3-II levels in muscles.

Next, we examined the effects of ischemia-reperfusion on the levels of LC3-II, p-Ulk1, and p-S6. Ischemia-reperfusion injury was used as a model of acute tubular necrosis. A reduction in renal blood flow during ischemia and subsequent reperfusion caused pronounced tubular necrosis, endothelial injury, and leukocyte activation (Bonventre and Weinberg, 2003). The levels of LC3-II 1, 2, and 4 days after ischemia-reperfusion were significantly lower than those in normal rat kidneys (Fig. 5A and B). Recent studies showed that mTOR phosphorylates Ulk1 at Ser 757. We found that phosphorylation of Ulk1 in the kidneys increased significantly 1, 2, and 4 days after ischemia-reperfusion (Fig. 5A and C). Levels of p-S6 have been used to distinguish the activation of the mTOR pathway in mice and rat kidneys. Phosphorylated S6 and total S6 levels significantly increased the day after administration (Fig. 5A, D, and E). These results clearly showed that the mTOR pathway was activated after ischemia-reperfusion. In this model, the levels of Pcr and blood urea nitrogen were significantly increased 1 and 2 days after ischemia-reperfusion (Fig. 5F and G).

3.4. Effects of everolimus in ischemia-reperfusion injury

We defined the effects of everolimus on the levels of LC3-II, p-Ulk1, and p-S6 in the kidneys after treatment with ischemia-reperfusion. Ischemia-reperfusion decreased the levels of LC3-II and, conversely, increased those of p-Ulk1 and p-S6; however, treatment with everolimus restored the levels of LC3-II (Fig. 6A and B) and increased phosphorylation of Ulk1 1 and 2 days after administration (Fig. 7C–E). The day after administration. Cisplatin was used as a typical model of drug-induced nephrotoxicity; it was taken into renal proximal tubular cells via membrane transporters (Yonezawa and Inui, 2011) such that it exerted its cytotoxicity specifically in proximal tubular cells. The levels of LC3-II were significantly lower in kidneys treated with cisplatin than in normal rat kidneys (Fig. 8A and B). In addition, we observed increased phosphorylation of Ulk1 and Ki-67 in ischemia-reperfusion regulated LC3-II levels in the kidney. We also explored the effects of everolimus on renal function. The Pcr levels after ischemia-reperfusion were markedly increased by pretreatment with everolimus (Fig. 7A). In addition, everolimus pretreatment prevented renal hypertrophy in cisplatin-induced nephrotoxicity. Cisplatin was taken into renal proximal tubular cells via membrane transporters (Yonezawa and Inui, 2011) such that it exerted its cytotoxicity specifically in proximal tubular cells. The levels of LC3-II were significantly lower in kidneys treated with cisplatin than in normal rat kidneys (Fig. 8A and B). In addition, we observed increased phosphorylation of Ulk1 and Ki-67 in ischemia-reperfusion regulated LC3-II levels in the kidney.

3.5. Effects of cisplatin on the mTOR pathway in the kidney

To clarify the effects of cisplatin on the mTOR pathway in the kidney, we examined LC3-II and pS6 levels 1, 2, 4, and 7 days after administration. Cisplatin was used as a typical model of drug-induced nephrotoxicity; it was taken into renal proximal tubular cells via membrane transporters (Yonezawa and Inui, 2011) such that it exerted its cytotoxicity specifically in proximal tubular cells. The levels of LC3-II were significantly lower in kidneys treated with cisplatin than in normal rat kidneys (Fig. 8A and B). In addition, we observed increased phosphorylation of Ulk1 and Ki-67 in ischemia-reperfusion regulated LC3-II levels in the kidney. We also explored the effects of everolimus on renal function. The Pcr levels after ischemia-reperfusion were markedly increased by pretreatment with everolimus (Fig. 7A). In addition, everolimus pretreatment prevented renal hypertrophy in cisplatin-induced nephrotoxicity. Cisplatin was taken into renal proximal tubular cells via membrane transporters (Yonezawa and Inui, 2011) such that it exerted its cytotoxicity specifically in proximal tubular cells. The levels of LC3-II were significantly lower in kidneys treated with cisplatin than in normal rat kidneys (Fig. 8A and B). In addition, we observed increased phosphorylation of Ulk1 and Ki-67 in ischemia-reperfusion regulated LC3-II levels in the kidney.

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blood urea nitrogen levels significantly increased 4 days after administration (Fig. 8F and G).

3.6. Effects of everolimus in cisplatin-induced nephropathy

Next, we examined the effects of everolimus on the levels of LC3-II, p-Ulk1, and p-S6 in the kidney after treatment with cisplatin. Exposure to cisplatin markedly decreased the levels of LC3-II and increased the levels of p-Ulk1 and p-S6 in the kidneys; however, everolimus treatment resulted in the accumulation of LC3-II (Fig. 9A and B and Fig. 10C–E) and dephosphorylation of Ulk1 and S6 (Fig. 9A, C and D), which indicated that the mTOR pathway regulated the levels of LC3-II in the kidney after cisplatin treatment.

After co-treatment with everolimus and cisplatin, the PCr levels were significantly higher than those in control rats (Fig. 10A), suggesting that mTOR inhibition with everolimus worsened renal function. Furthermore, kidney weight tended to be altered by everolimus treatment (Fig. 10B). We also explored the effects of everolimus on the levels of Kim-1 and apoptosis. Although not statistically significant, the number of proximal tubules with Kim-1 immunostaining nearly doubled after co-treatment with everolimus and cisplatin (Fig. 10F–H). Conversely, the number of apoptotic cells was comparable in the treatment and control groups (Fig. 10I–K). However, everolimus treatment significantly decreased the number of Ki-67–positive nuclei in tubular epithelial cells (Fig. 10L–N).

3.7. Quantification of urinary LC3

We next examined whether LC3 protein was present in rat urine. After co-treatment with everolimus and cisplatin, LC3-I and LC3-II were detected in the urine (Fig. 11A). In addition, we confirmed that two antibodies produced using different epitopes of LC3 could detect both LC3-I and LC3-II in the urine. To quantify the concentration of LC3 in rat urine after everolimus treatment, an LC3 sandwich ELISA was constructed using these two antibodies. A standard curve was generated with serial dilutions of a synthetic peptide of LC3 starting with 500 ng/mL (Fig. 11B). The lower limit of detection – defined as the lowest LC3 peptide concentration that was significantly differentiated from 0 (assay blank) by an unpaired t test ($n = 3, P < 0.001$) – was 0.122 ng/mL. The coefficient of variation obtained for each standard from 0.122 to 500 ng/mL was $< 10\%$.

Next, urinary LC3 concentration was measured in rats taken from the ischemia-reperfusion and cisplatin groups described above. The mean urinary LC3 concentration in the everolimus-treated group was significantly higher than that in the vehicle-treated group (Fig. 11C and D).

4. Discussion

Recent findings have demonstrated that mTOR inhibitors have therapeutic effects in progressive kidney diseases, including polycystic kidney disease and diabetic nephropathy (Huber et al., 2011).
but mTOR inhibitors can also cause renal toxicity (Lieberthal et al., 2001; Nishihara et al., 2010). Therefore, the details of the action of mTOR inhibitors in the kidney need to be clarified to aid the development of targeted therapy against kidney disease.

Ulk1 has been shown to be necessary for the induction of autophagy by mTOR inhibitors (Chan et al., 2007); however, the roles of Ulk1 in the antiproliferative effects of everolimus remain unclear. Immunofluorescence analysis showed expression of Ulk1 and LC3 proteins in the proximal tubular epithelial cells of rat kidneys (Fig. 2). The results suggested that the mTOR-Ulk1 pathway had active roles in the proximal tubular epithelial cells. They also indicated that the monitoring of LC3-II levels in the kidneys was useful for evaluating mTOR activity and the efficacy of mTOR inhibition with everolimus in proximal tubular cells. We found that the accumulation of LC3-II resulting from everolimus treatment accompanied the prevention of cell proliferation in renal tubular epithelial cell lines (Fig. 3A and B). Notably, treatment with siUlk1 reduced the antiproliferative effects of everolimus in NRK-52E cells (Fig. 3D–G). These results showed that Ulk1 and its downstream cellular events, such as autophagy, had key roles in the antiproliferative effects of everolimus.

After ischemia-reperfusion injury, renal tubular epithelial cells rapidly enter the cell cycle to adapt to the loss of neighboring cells by proliferating and replacing the dead cells (Venkatachalam et al., 1978). Previous studies in mouse and rat kidneys have suggested that the mTOR pathway is involved in the repair of renal tubular cells after acute injury (Gonçalves et al., 2006; Lieberthal et al., 2001). To characterize roles of the mTOR pathway after ischemia-reperfusion injury, we examined influences of mTOR activation on autophagy in ischemia-reperfusion injury. We found that the levels of LC3-II in the kidneys decreased when the mTOR pathway was activated (Fig. 5). In contrast, co-treatment with everolimus and ischemia-reperfusion resulted in the accumulation of renal LC3-II (Fig. 6). These results suggested that the activated mTOR pathway after ischemia-reperfusion was involved in decreasing LC3-II levels in the kidney. We also found that Kim-1 levels and the number of apoptotic cells in the renal cortex increased after everolimus treatment (Fig. 7). The results from tubular cell lines (Fig. 3)
suggested that mTOR inhibition prevented cell proliferation in the proximal tubules after ischemia-reperfusion by inducing LC3-II accumulation. These results suggest that the proximal tubular cells underwent autophagy-mediated cell death after treatment with everolimus and ischemia-reperfusion. Shimizu et al. demonstrated autophagy-dependent programmed cell death in embryonic fibroblasts from Bax/Bak double-knockout mice (Shimizu et al., 2004). Furthermore, it was reported that cytotoxicity of temozolomide and sirolimus against human malignant glioma cells was caused by induction of autophagy (Iwamaru et al., 2007; Kanzawa et al., 2004). These findings suggest that the use of mTOR inhibitors in acute kidney injury could mediate cellular demise of proximal tubules via the direct self-destructive potential of excessive autophagy.

Recent studies have shown that mTOR and AMP-activated protein kinase (AMPK) are the most important regulators of Ulk1-mediated autophagy (Egan et al., 2011; Kim et al., 2011). mTOR inhibits the phosphorylation-mediated activation of Ulk1 at Ser 757 and disrupts the interaction between Ulk1 and AMPK. We found significant increase in Ulk1 phosphorylation (Ser 757) in the rat kidneys after ischemia-reperfusion injury and treatment with cisplatin (Figs. 5, 6, 8, and 9); however, we found a decrease in the total protein expression levels of Ulk1 in the kidneys after acute injury. Although further studies are required to have a comprehensive understanding of Ulk1 regulation in the kidney, and our results showed direct interaction between mTOR and Ulk1 in the kidney.

Interestingly, ischemia-reperfusion treatment induced significant increase in the total levels of S6 in the kidneys (Fig. 5), but cisplatin treatment did not (Fig. 8). Ribosomal protein S6 plays important roles in cell growth and proliferation (Ruvinsky and Meyuhas, 2006). Previous studies showed that the expression level of S6 protein increased in serum-stimulated cells (Tushinski and Warner, 1982). In this study, a higher number of tubular epithelial cells underwent the cell cycle progression after ischemia-reperfusion (Fig. 7) than after cisplatin treatment (Fig. 10). Therefore, not only the phosphorylation status but also the expression levels of total S6 protein may be the key factors in the progression of tubular cell proliferation.

The profiles of renal LC3-II levels suggested that autophagic activity in the kidneys was suppressed during the recovery phase of ischemia-reperfusion injury and cisplatin-induced nephropathy.
cisplatin treatment. These results suggest the usefulness of additional olimus in response to acute proximal tubular damage such as indicate renal tubular injury through mTOR inhibition with ever-
olimus (dead cells), our results showed that the presence of LC3 in urine could 
excretion of LC3-II or LC3-I from epithelial cells into urine was unclear (i.e., from living cells or from 
precise role of AMPK in Ulk1 activation should be addressed in future 
studies. Although the origin of the excretion of LC3-II from 
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Fig. 12. The hypothesized mechanisms of mammalian target of rapamycin (mTOR) inhibitor action in proximal tubular epithelial cells. (A) During the recovery phase of nephrotoxins-induced tubular injury, the mTOR pathway in proximal tubular cells is activated to promote cell proliferation. (B) Everolimus treatment exerts antiproliferative effects by blocking cell survival pathways. The quantification of urinary LC3 protein could be beneficial in predicting outcomes of treatment with mTOR inhibitors.

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