

Autophagy activation reduces renal tubular injury induced by urinary proteins

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Abbreviations: ALB/HSA, albumin; *BECN1*, Beclin 1, autophagy related; C-BSA, cationic bovine serum albumin; HAVCR1/KIM-1, hepatitis A virus cellular receptor 1/kidney injury molecule-1; LAMP, lysosomal-associated membrane protein; LCN2/NGAL, lipocalin 2/neutrophil gelatinase-associated lipocalin; LDH, lactate dehydrogenase; LPS, lipopolysaccharides; MAPILC3 (LC3), microtubule-associated protein 1 light chain 3; MCNS, minimal change nephrotic syndrome; MTT, methyl thiazolyl tetrazolium; ROS, reactive oxygen species; siRNA, small interfering RNA; SQSTM1/p62, sequestosome 1; TECs, tubular epithelial cells; TEM, transmission electron microscopy; tflc3, mRFP-GFP tandem fluorescent-tagged LC3; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

Autophagy is shown to be beneficial for renal tubular injury caused by nephrotoxic drugs. To investigate whether autophagy could protect renal tubular epithelial cells (TECs) from injury induced by urinary proteins, we studied the activity and action of autophagy in TECs after urinary protein overload in vivo and in vitro. We found that autophagic vacuoles increased in TECs from patients with minimal change nephrotic syndrome (MCNS) and rat models with severe proteinuria induced by cationic BSA. In HK-2 cells, exposure to urinary proteins extracted from patients with MCNS led to a significant increase in autophagosome and autolysosome formation and decrease in SQSTM1/p62 protein level. Urinary protein addition also induced lysosomal turnover of LC3-II and perinuclear clustering of lysosomes. These changes were mediated by a reactive oxygen species (ROS)-dependent mechanism. Furthermore, pretreatment of HK-2 cells with rapamycin reduced the production of LCN2/NGAL and HAVCR1/KIM-1 and the level of apoptosis induced by urinary proteins. In contrast, blocking autophagy with chloroquine or *BECN1* siRNAs exerted an opposite effect. Similar results were also observed in animal models with proteinuria after treatments with rapamycin and chloroquine. Taken together, our results indicated an increase in autophagic flux, which mounts an adaptive response in TECs after urinary protein overload.

Introduction

Autophagy is a cellular process that engulfs, digests, and recycles long-lived or aggregated proteins, defective organelles, and various soluble molecules to sustain cellular metabolism.¹ Autophagy machinery is activated under certain circumstances such as starvation, hypoxia, ischemia/reperfusion, stress, and infection.^{2,3} Previous studies have shown that autophagy is involved in the pathophysiologic processes of many human diseases, such as cancer, neurodegeneration, and cardiomyopathy.⁴⁻⁶ Recently, it was also reported that autophagy is activated in acute kidney injury induced by cyclosporine and might be an important protective mechanism for renal tubular-epithelial cell survival.⁷ In addition, in response to cisplatin, autophagy induction is exerted as an adaptive response, resulting in delayed apoptosis in renal TECs.⁸⁻¹⁰ This new evidence suggests that autophagy might be cytoprotective during nephrotoxic injuries.

Proteinuria, a common clinical feature in many glomerular diseases, can result in progression of renal tubular atrophy and interstitial fibrosis.^{11,12} Therefore, urinary proteins should be considered not only as a marker of glomerular injury, but also as a nephrotoxic factor to renal tubuli. The cytotoxic effects of urinary proteins include elevation of ROS production,¹³ impairment of mitochondrial function,¹⁴ induction of endoplasmic reticulum stress,¹⁵ etc., all of which could probably trigger autophagy in order to clean damaged organelles or misfolded proteins in TECs.^{16,17} Thus, in the present study we tested the hypothesis that the autophagic pathway was activated in response to urinary protein overload in renal tubuli and autophagy activation could protect TECs from urinary protein-induced injury.

Results

Autophagic vacuoles are increased in TECs from patients with minimal change nephrotic syndrome

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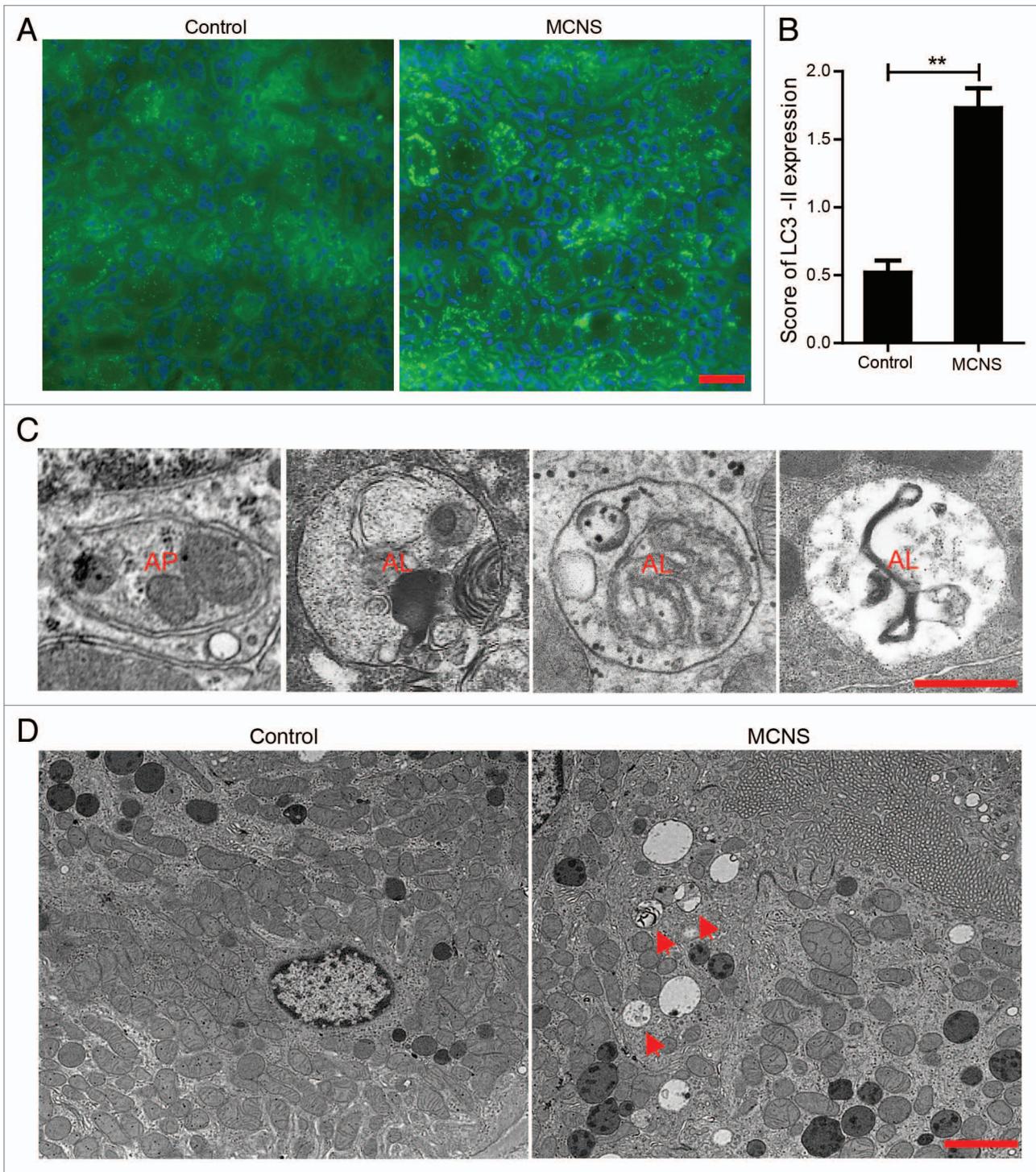


Figure 1. Quantitative change of autophagic vacuoles in TECs from MCNS patients. (**A and B**) Immunofluorescent staining of LC3 and the score of LC3-II expression in renal tubuli of MCNS patients or controls. Scale bar: 50 μm . (**C and D**) Ultrastructural images of autophagic vacuoles in MCNS patients and the controls. TEM images showed a representative initial/early autophagosome (AP) and degradative/late autolysosomes (ALs) (**C**). There were more autophagic vacuoles in TECs from MCNS patients than controls (**D**). Red arrows indicate autophagic vacuoles. Scale bar: 500 nm (**C**) and 2 μm (**D**), respectively. ****** $P < 0.01$.

In this study, we first utilized immunofluorescent technology to examine the expression of microtubule-associated protein 1 light chain 3 (LC3)-II (a key marker of autophagy) in TECs. As shown in **Figure 1A and B**, compared with the control, there were significantly more LC3-II positive dots seen in renal TECs from patients with minimal change nephrotic syndrome ($P < 0.01$). Next, we evaluated the autophagic vacuoles by transmission electron microscopy (TEM), the gold standard technique for detecting autophagy. Under TEM visualization, the autophagosomes (AP, formerly called the initial/early autophagic vacuoles) containing intact cytosol or organelles and the autolysosomes (AL, formerly called the degradative/late autophagic vacuoles) containing partially degraded cytoplasmic material, were clearly detected (**Fig. 1C**). Similar to LC3-II immunostaining, the number of autophagic vacuoles was also increased significantly in renal proximal TECs of MCNS patients when compared with the controls (**Fig. 1D**).

Urinary proteins trigger autophagic response in cultured TECs

We then employed a protein overload model of cultured TECs to explore the quantitative change of autophagic response in vitro. To mimic the pathological process of kidney disease with proteinuria clinically, we used urinary proteins extracted from MCNS patients. In untreated control cells, only a few LC3-II puncta were detected by immunofluorescence staining (**Fig. 2A and B**). Exposure to 0.5 to 1 mg/ml of urinary proteins slightly increased the number of LC3-II positive puncta, which significantly increased in cells treated with 2 to 8 mg/ml of urinary proteins (**Fig. 2A and B**). Similar patterns of LC3-II biosynthesis and SQSTM1 turnover were observed by western blot assay (**Fig. 2D**). Meanwhile, a time-dependent increase of LC3-II biosynthesis was evidenced when cells were treated with 8 mg/ml of urinary protein extracts, and the peak of increase was seen at 8 h post-treatment (**Fig. 2F**). In parallel, TEM assay also confirmed the increase of autophagic vacuoles in cells with 8 mg/ml urinary protein treatment at 8 h compared with the control (**Fig. 2C**). In addition, we tested if addition of ALB/HSA (albumin/human serum albumin) also causes autophagic response. In contrast to urinary protein extracts, only a high dose of ALB at 8 mg/ml induced a significant increase of LC3-II puncta in HK-2 cells but not in 0.1 to 4 mg/ml ALB by immunofluorescent assay (**Fig. 2A and B**). Similar patterns for LC3-II biosynthesis and SQSTM1 turnover were also found by western blot assay (**Fig. 2E**). These data indicate a dose- and time-dependent autophagic response induced by urinary protein extracts that contain a complex mixture of proteins. The different effect observed from urinary protein extracts in comparison to ALB might be due to the more complex components and properties of urinary proteins, such as transferrin and immunoglobulin, that are largely different from ALB.

To rule out the possibility that the increase in autophagic vacuoles was induced by possible endotoxin contamination in urinary protein extracts, we treated cells with lipopolysaccharides (LPS) at various concentrations. The results revealed that LPS exposure did not affect LC3-II biosynthesis even at a relatively high dose that was about 20-fold higher than the measurable

threshold of 0.025 EU/ml, while the LPS level in urinary protein extracts was lower than the measurable threshold in our samples (**Fig. 2G**). These data suggest that LPS is not a major factor in urinary protein-induced autophagic response in the present study.

Then, we determined the effect of urinary proteins on autophagosome and autolysosome formation.¹⁸ An mRFP-GFP tandem fluorescent-tagged LC3 (tfLC3) construct was transfected into HK-2 cells. As shown in **Figure 3A and B**, some green and red puncta were clearly shown after transfection. The numbers of both GFP (green) and mRFP (red) dots were significantly increased after exposure to urinary proteins for 8 h. There were more yellow and red dots in cells after urinary protein exposure than the controls, indicating an increase in the formation of both autophagosomes and autolysosomes. Next, we treated cells with chloroquine, which efficiently inhibited the fusion of autophagosomes and lysosomes.^{19,20} We found that the number of yellow puncta was obviously increased, while free red puncta were significantly decreased in urinary protein plus chloroquine-treated cells compared with urinary protein treated alone (**Fig. 3A and C**), suggesting that urinary protein induced the fusion of autophagosomes and lysosomes to form autolysosomes.

LC3-II turnover was regarded as one of the principal methods to monitor autophagic flux by using lysosomal inhibitor.²¹ Thus, we next examined if urinary protein-induced LC3-II accumulation could be enhanced by chloroquine or leupeptin. Similar to the results shown in **Figures 1 and 2**, LC3-II puncta and protein level in TECs were increased after exposure to urinary protein, which were further elevated by chloroquine addition (**Fig. 4A–C**). Also, leupeptin addition further increased urinary protein overload-induced LC3-II elevation (**Fig. S1**). These data indicate that more amount of LC3-II is delivered to lysosomes for degradation and autophagic flux is increased when exposure of HK-2 cells to urinary proteins.

Last, we tested if urinary protein exposure induced any changes on lysosomal function. Lysosome localization was visualized by immunofluorescence staining of the lysosomal marker lysosomal-associated membrane protein (LAMP) 1 or LAMP2. In TECs, exposure to urinary proteins resulted in lysosome-clustering at the perinuclear region at 8 h (**Fig. 4A; Fig. S2**). Interestingly, perinuclear clustering of lysosomes disappeared at 16 h after urinary protein exposure (**Fig. S2**) in parallel to a decline in LC3-II level. It has been shown that lysosome-clustering at the perinuclear region provides more acceptor sites for autophagosomes to fuse with and thus accelerates degradation of autophagosome.²²

Oxidative stress but not proteasome inhibition is involved in urinary protein-induced autophagy activation

It was reported that ROS production is involved in autophagy activation.¹⁶ Therefore, we assessed if ROS production was elevated in cells after urinary protein exposure. As shown in **Figure 5A**, flow cytometry assay revealed that exposing the cells to urinary proteins induced an obvious ROS response in a dose-dependent manner. Consistently, addition of antioxidant tiron or catalase suppressed urinary protein-induced upregulation of LC3-II and *BECN1* (**Fig. 5C–E**). These data indicate that

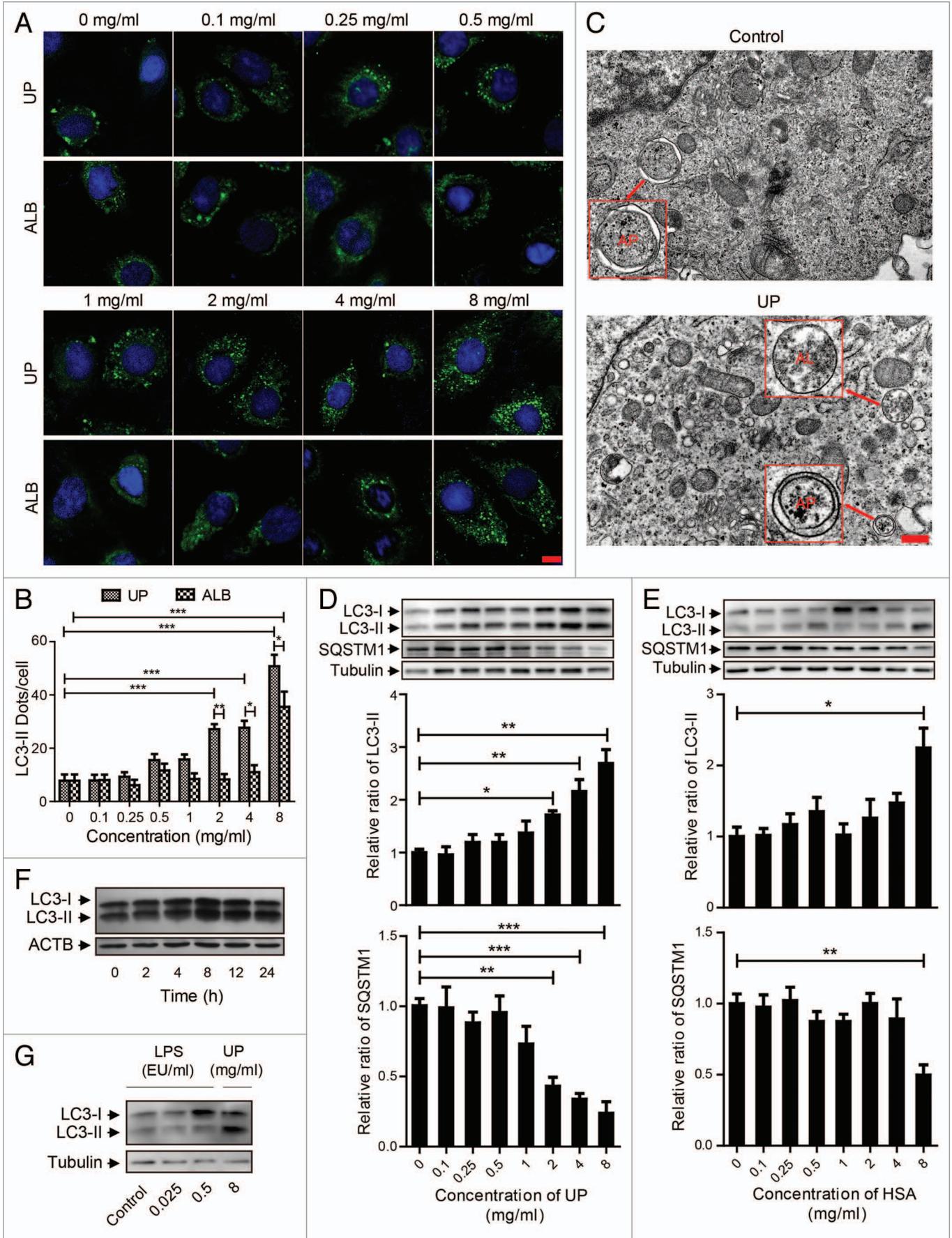


Figure 2 (See opposite page). Changes in autophagic vacuoles and autophagy substrate in HK-2 cells after exposure to urinary proteins. **(A and B)** Immunofluorescence staining and quantitative change of LC3-II (green) after exposure to different concentrations of urinary proteins (UP) or albumin (ALB) for 8 h. The nucleus was counter-stained by DAPI (blue). Scale bar: 10 μ m. **(C)** Quantitative changes of autophagic vacuoles under transmission electron microscope (TEM) in HK-2 cells after exposure to vehicle or 8 mg/ml UP at 8 h. The typical images of the APs and AL are shown at higher magnification. Scale bar: 500 nm. **(D)** Western blot analysis of LC3 or SQSTM1 level after exposure to different concentrations of UP for 8 h. Densitometry was performed for quantification and the ratio of LC3-II or SQSTM1 to tubulin was expressed as fold of control. **(E)** Western blot analysis of LC3 or SQSTM1 level after exposure to different concentrations of ALB for 8 h. Densitometry was performed for quantification and the ratio of LC3-II or SQSTM1 to tubulin was expressed as fold of control. **(F)** Western blot analysis of LC3 level at various time points after exposure to 8 mg/ml UP. **(G)** Western blot analysis of LC3 level after treatment with LPS (0.025 and 0.5 EU/ml) or UP (8 mg/ml) for 8 h. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

urinary protein-induced ROS response is an important factor in triggering autophagy response.

The ubiquitin-proteasome and autophagy-lysosome systems were long considered as independent pathways. However, recent observations suggest that there were interactions between these routes and autophagy could be induced in the state of proteasome inhibition.²³ Therefore, we assessed if proteasome inhibition was involved in urinary protein-induced autophagy response. As shown in **Figure 5B**, exposure of HK-2 cells to urinary proteins did not result in proteasome inhibition nor did it enhance the inhibitory effect of proteasome inhibitor MG132 on proteasome activity, as measured by the chymotrypsin-like and trypsin-like activities of the 26S proteasome. Nonetheless, MG132 addition augmented urinary protein-induced LC3-II increase and SQSTM1 reduction (**Fig. 5F**). These data suggest that proteasome inhibition is not involved in urinary protein overload-induced autophagy induction.

Autophagy activation protects TECs from urinary protein-induced injury

It is thought that rapamycin, the inhibitor of mechanistic target of rapamycin (mTOR), increases autophagic flux by promoting autophagosome formation, while chloroquine impairs autophagic flux by blocking autophagic degradation.²⁴ Thus, we used these 2 drugs to determine the functional role of autophagy activation in urinary protein overload-induced cell injury. Addition of rapamycin to HK-2 cells further increased urinary protein-induced LC3-II puncta and protein levels in comparison with urinary proteins alone, but the protein level of SQSTM1 did not change (**Fig. 4A–C**). In contrast, chloroquine addition not only increased LC3-II puncta and protein levels of LC3-II and LAMP2, but also induced SQSTM1 accumulation compared with urinary proteins alone. These data suggest that rapamycin and chloroquine are functional in modulating autophagy response induced by urinary protein exposure.

We then examined whether rapamycin or chloroquine affected urinary protein overload-induced cell injury. Exposure to urinary proteins significantly increased cellular apoptosis (**Fig. 6A and B**), enhanced the levels of LCN2 and HAVCR1 secretion (**Fig. 6C and D**), as well as inhibited cell viability (**Fig. 6E**). Such effects were attenuated by rapamycin but enhanced by chloroquine.

Next, we further demonstrated the critical role of autophagy on HK-2 survival after exposure to urinary proteins by knocking down *BECN1* expression. As expected, transfection with gene-specific small interfering RNAs successfully reduced *BECN1* protein level and suppressed urinary protein-induced

LC3-II biosynthesis (**Fig. 7A**). Similar to the effect induced by chloroquine, *BECN1* siRNAs also enhanced HK-2 cell apoptosis (**Fig. 7B**), growth inhibition (**Fig. 7E**) and LCN2 and HAVCR1 release induced by urinary protein overload (**Fig. 7C and D**). These results indicated that autophagy activation might be a protective mechanism in cells exposed to urinary proteins.

Finally, we used a rat model with high-grade proteinuria to assess the effect of urinary protein overload on TEC survival in vivo. The rat model was developed by cationic bovine serum albumin (C-BSA) injection, a causative factor of nephropathy with less renal tubular toxicity. As revealed by immunohistochemistry assay, the amount of LC3-II puncta was significantly higher in rat models than the controls (**Fig. 8A**). Western blot assays also showed a significant increase in LC3-II and *BECN1* protein levels and a decrease in SQSTM1 level in TECs of model rats compared with the control rats (**Fig. 8B**). Administration of chloroquine or rapamycin induced the similar effect on LC3-II, SQSTM1, and *BECN1*, as seen in cell-based experiments. These data indicate that high-grade proteinuria induced autophagy response, which could be modulated by rapamycin and chloroquine in model rats.

Morphologically, in rat model, TEC exhibited swelling, as well as vacuolar and granular degeneration. These morphological alterations were aggravated by chloroquine but ameliorated by rapamycin (**Fig. 9A**). Rapamycin treatment significantly reduced TEC apoptosis as assessed by TUNEL assay (**Fig. 9B and C**) and the release of renal tubular injury markers such as LCN2, HAVCR1, and lactate dehydrogenase (LDH) in urine (**Fig. 9D–F**). In contrast, chloroquine treatment increased TEC apoptosis and the urinary levels of LCN2, HAVCR1 and LDH (**Fig. 9B–F**). These animal experiments clearly demonstrated that autophagy response plays a preventive role in TEC injury induced by urinary protein overload.

Discussion

In the present study, we found that urinary protein exposure resulted in the accumulation of autophagic vacuoles in TECs in vivo and in vitro. However, the increase in autophagic vacuoles could be due to the increased formation and/or decreased clearance of autophagic vacuoles.²⁴ Therefore, we examined subsequently a serial process of autophagy. We first found that urinary proteins induced *BECN1* upregulation, which was likely a sign of autophagy induction in the upstream of the autophagic pathway.^{25,26} Next, we showed that the accumulation of autophagosomes accompanied a dramatic increase in autolysosomes, indicating the efficient

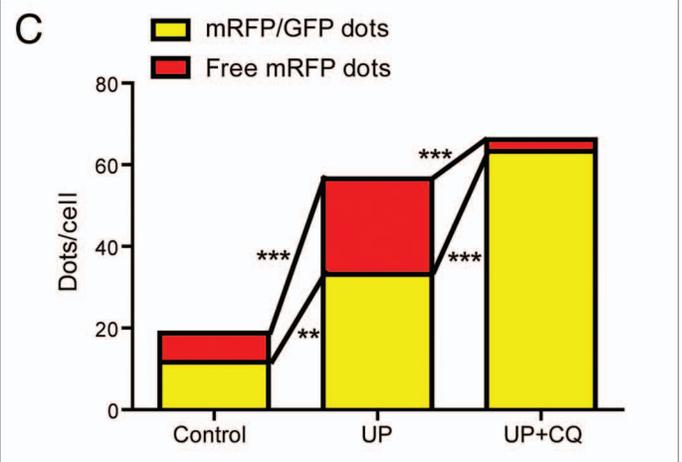
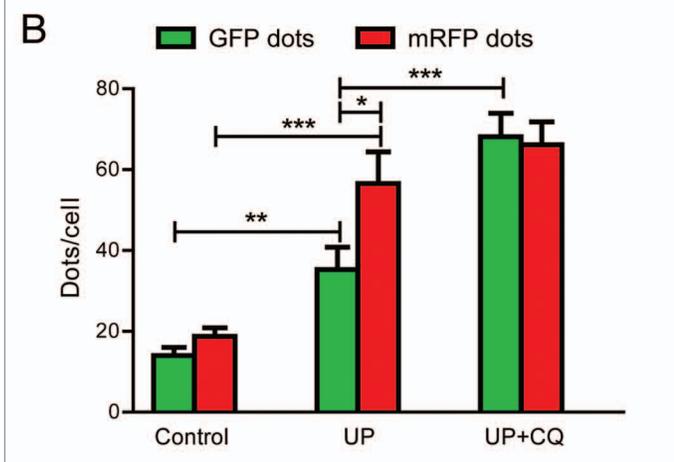
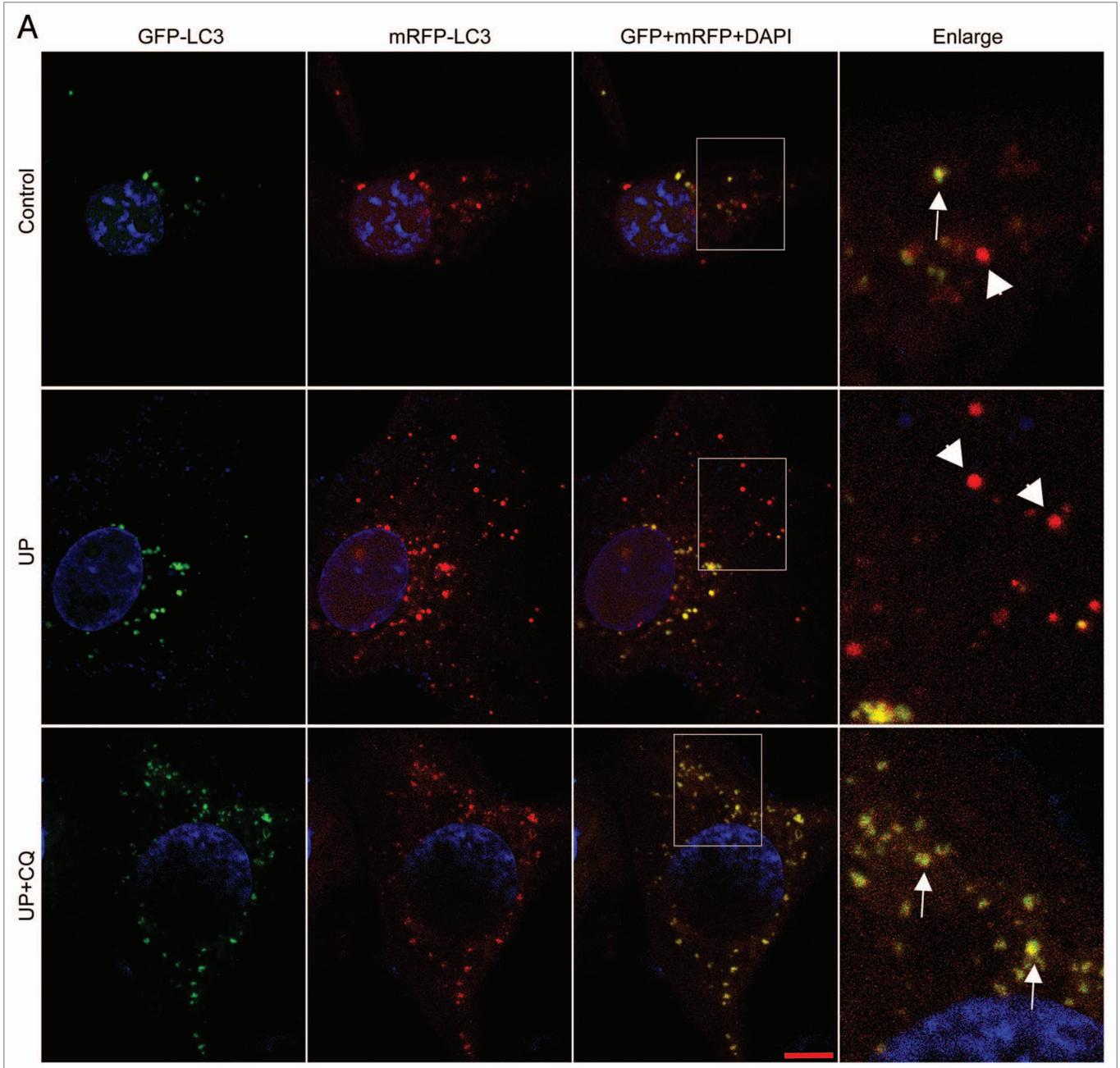


Figure 3 (See opposite page). Quantitative changes of autophagosomes and autolysosomes in HK-2 cells after exposure to urinary proteins. **(A)** Fluorescent microscopic analysis of HK-2 cells transfected with plasmid constructs harboring LC3 fused with tandem mRFP-GFP tag (tFLC3), which were treated with vehicle, urinary proteins (UP, 8 mg/ml), UP (8 mg/ml) plus chloroquine (CQ, 10 μ M) for 8 h. The puncta with yellow color indicate autophagosomes (arrows). The puncta with free red color indicate autolysosomes (arrowheads). Scale bar: 10 μ m. **(B)** Quantitative data for green or red puncta per cell. **(C)** Quantitative data of yellow puncta or free red puncta per cell. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

fusion of autophagosomes and lysosomes. When assessing the transit of LC3-II in the presence and absence of lysosomal inhibitor, we found that urinary protein overload induced the obvious LC3-II turnover, suggesting an efficient lysosomal degradation.¹⁹ Finally, we showed that exposure to urinary protein for up to 8 h resulted in accumulation of lysosomes in the perinuclear region. The perinuclear clustering of lysosomes could result in inactivation of MTOR and could initiate the fusing process of autophagosome with lysosome since there are more acceptor sites provided in the perinuclear lysosomes to fuse with autophagosomes, as reported in previous studies.^{22,27} Interestingly, after exposure to urinary proteins for 16 h, the number of autophagic vacuoles decreased and the lysosome-clustering phenomenon disappeared, indicating a completion of autophagic flux. Taken together, all these data provided strong evidence for autophagy activation in response to urinary proteins. ROS was shown to act as a critical signal in tubulointerstitial damage irritated by proteinuria.¹³ In our study, we also found that urinary protein-induced autophagic activation, at least partially, via oxidative stress, which was also supported by previous study.¹⁶ Therefore, ROS likely functions as a messenger in both tubulointerstitial damage and autophagy activation after exposure to urinary proteins.

Although autophagy was shown to be involved in the development of kidney diseases, it remains controversial as to whether autophagy activation protects renal cell from injury. It is reported that autophagy activation is protective against TEC injury induced by aristolochic acid, cisplatin, or cyclosporine A.^{7,28,29} In contrast, increased autophagic activity contributes to TEC death in ischemia/reperfusion or tunicamycin-induced renal injury.^{30,31} Therefore, whether autophagy is protective or detrimental for TECs might depend on the specific state of the kidney or the stress factors.³²

The role of autophagy in TEC injury induced by urinary proteins remains to be elucidated. In the present study, we found that TEC injury, as assessed by cellular apoptosis, release of renal tubular injury markers HAVCR1 and LCN2, and inhibition of cell growth, were attenuated by rapamycin addition but enhanced by chloroquine or *BECNI* siRNAs. It was reported that excessive apoptosis of TECs led to tubular atrophy and low proliferation of TECs contributed to impairment of tubular repair, all of which were basic characteristics of tubulointerstitial damage.^{33,34} Urinary HAVCR1 and LCN2 have been increasingly recognized as sensitive biomarkers in the assessment of the extent of renal tubular injury.³⁵ Therefore, our results suggest that autophagy activation is involved in urinary protein-induced adaptive response that protects TECs from urinary protein-induced nephrotoxicity.

Proteinuria can accelerate kidney disease progression to end-stage renal failure via multiple mechanisms, including

apoptotic response, inflammatory induction, or fibrogenesis in renal tubulointerstitium.^{36,37} Currently, there is little means to prevent tubulointerstitial from injury induced by excessive urinary proteins. Previous studies have reported that rapamycin could ameliorate proteinuria-associated tubulointerstitial injury and fibrosis.³⁸ Although the specific mechanism is not clear, it is possible that promoting autophagy response, other than suppression of the immune system, might play an important role in rapamycin-mediated protection.³⁹ If this is the case, enhancing autophagic activity by rapamycin treatment might offer a novel therapeutic opportunity in almost all the primary and secondary nephropathies with excessive proteinuria, especially in refractory glomerulonephritis and diabetic nephropathy.

In conclusion, our results indicate that autophagy activation in response to urinary protein exposure represents a protective response, which alleviates TEC injury. Promoting autophagic flux in TECs might be beneficial in clinical management of nephropathies with refractory proteinuria.

Materials and Methods

Patients

This study was approved by the Institutional Review Board of the Affiliated Hospital of Guangdong Medical College. Kidney tissue specimens were obtained from biopsy-proven, untreated MCNS patients ($n = 27$) with the disease course less than 1 mo. Normal kidney specimens ($n = 5$), far away from tumor margin and proven to be normal renal tissue by pathology examination, were obtained from patients with renal cell carcinoma after unilateral nephrectomy.

Extraction of urinary proteins

Urinary proteins were extracted from urine of patients with untreated, biopsy-proven, and uncomplicated idiopathic MCNS using an ammonium sulfate precipitation method as described previously.⁴⁰

Cell culture and treatments

Human proximal tubular HK-2 cells (ATCC) were maintained and exposed to 0, 0.1, 0.25, 0.5, 1, 2, 4, and 8 mg/ml urinary proteins or ALB/HSA (Sigma, A1653) for 0, 2, 4, 8, 16, and 24 h. Western blot or immunofluorescence staining were performed to examine the expression of LC3-II, SQSTM1 or LAMP1. Subsequently, the cells were incubated with 5 mM tiron (Sigma, 172553), 2000 U/ml catalase (Millipore, 219261-100KU), 40 mM MG132 (Sigma, M7449), 10 μ M rapamycin (Calbiochem, 553210), 10 μ M chloroquine (Sigma, C6628) and 200 μ g/ml leupeptin (Sigma, L9783) with or without 8 mg/ml urinary proteins, the production of ROS was assayed post 2 h, the activity of proteasome and the expression of LC3-II, SQSTM1, *BECNI* or LAMP2 was measured post 8 h,

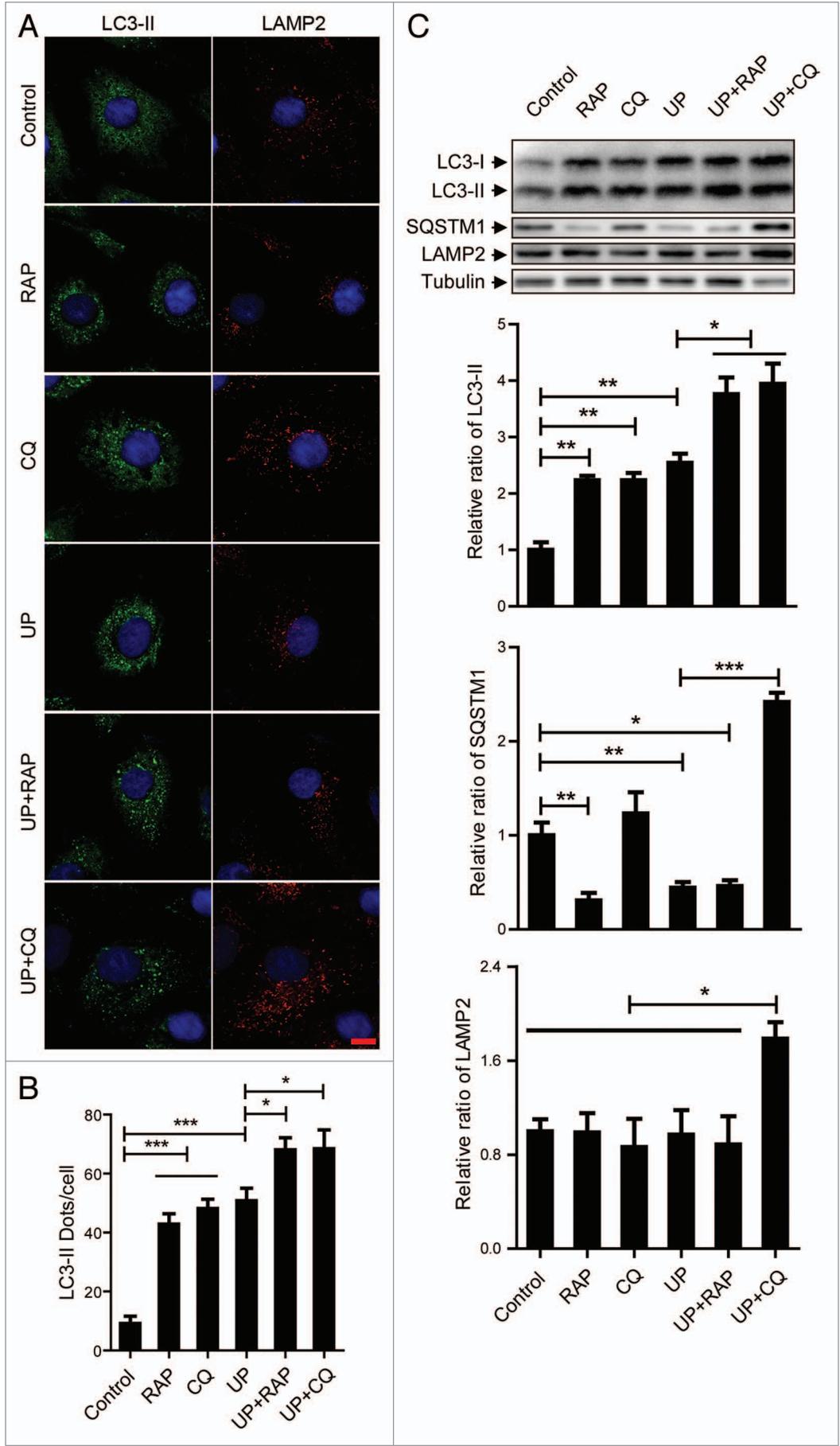
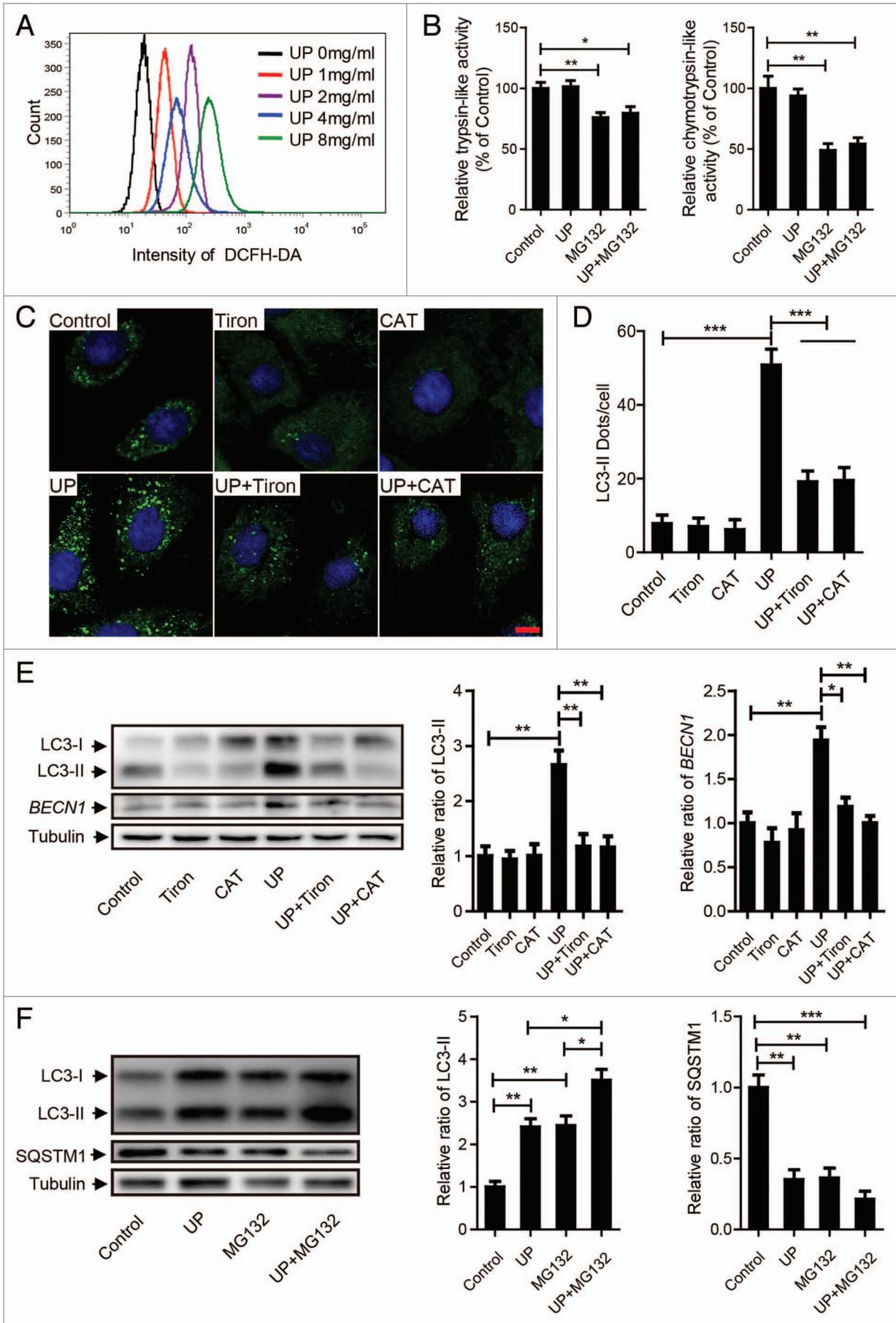


Figure 4. Effects of rapamycin or chloroquine on autophagic activity in HK-2 cells after exposure to urinary proteins. **(A)** Immunofluorescent staining of LC3-II (green) and LAMP2 (red) in HK-2 cells after exposure to vehicle and urinary proteins (UP, 8 mg/ml), with or without rapamycin (RAP, 10 μ M) or chloroquine (CQ, 10 μ M) for 8 h. Scale bar: 10 μ m. **(B)** Quantitative data of LC3-II dots in HK-2 cells treated as described in **(A)**. **(C)** Western blot analysis of LC3, SQSTM1 or LAMP2 level in HK-2 cells treated as in **(A)**. Densitometry was performed for quantification and the ratio of LC3-II, SQSTM1 or LAMP2 to tubulin was expressed as fold of control. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 5 (See opposite page). Effect of urinary proteins on ROS production, proteasome activity, and *BECN1* expression in HK-2 cells. **(A)** ROS levels in HK-2 cells after treatment with various concentrations of urinary proteins (UP, 8 mg/ml) by flow cytometric analysis. **(B)** Activity of different proteasomal subunits of the 26S complex in HK-2 cells after exposure to vehicle and urinary proteins (UP, 8 mg/ml) with or without MG132 (40 μ M) for 8 h. **(C and D)** Immunofluorescence of LC3-II (green) in HK-2 cells after exposure to vehicle or urinary proteins (UP, 8 mg/ml), with or without tiron (5 mM) or catalase (CAT, 2000 U/ml) for 8 h. The nucleus was counter-stained by DAPI (blue). Scale bar: 10 μ m. **(E)** Western blot analysis of LC3 or *BECN1* level in HK-2 cells treated as in **(C)**. Densitometry was performed for quantification and the ratio of LC3-II or *BECN1* to tubulin was expressed as fold of control. **(F)** Western blot analysis of LC3 or SQSTM1 level in HK-2 cells treated as in **(B)**. Densitometry was performed for quantification and the ratio of LC3-II or SQSTM1 to tubulin was expressed as fold of control. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.



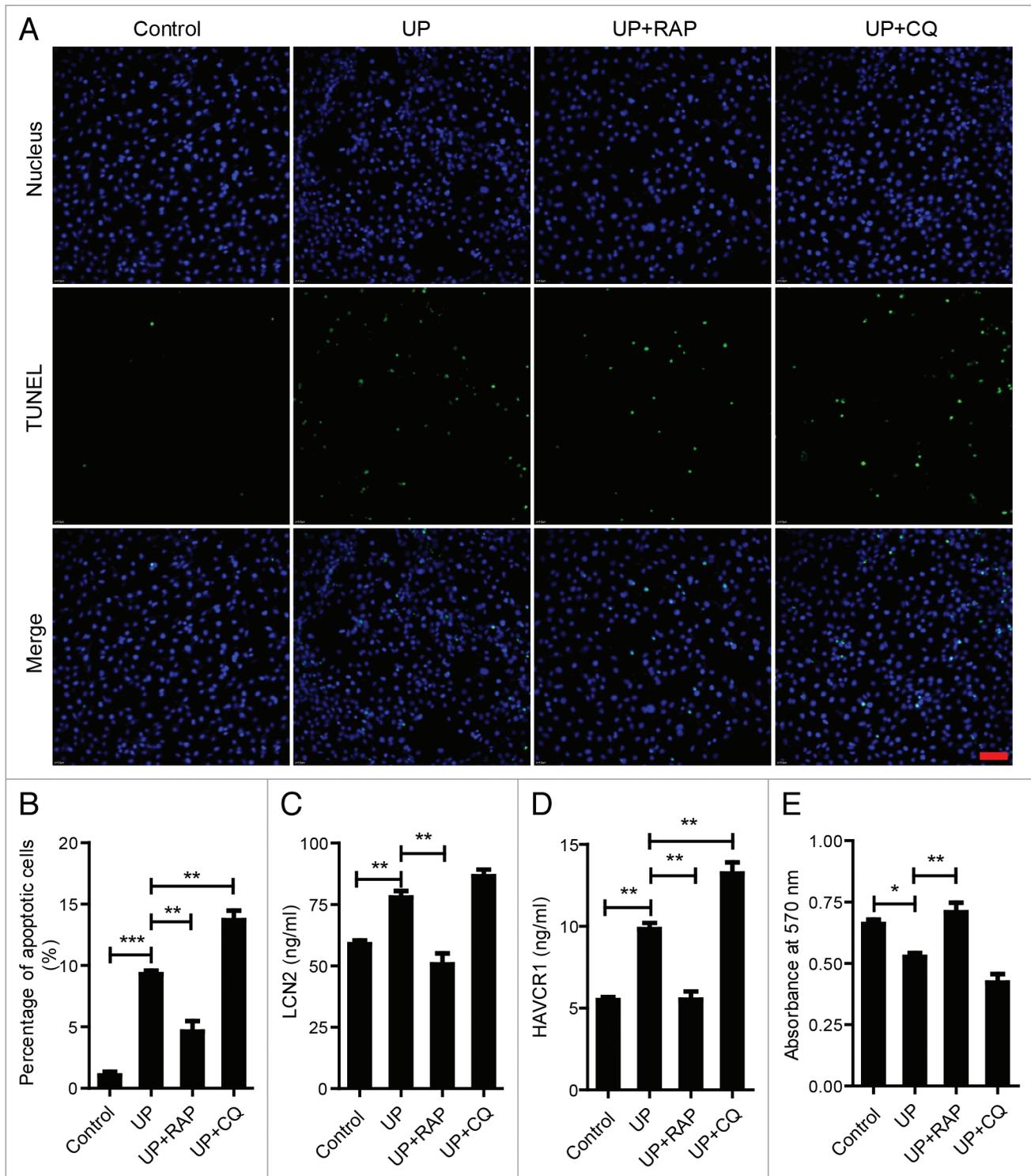


Figure 6. Effects of rapamycin and chloroquine on HK-2 cell injury induced by urinary proteins. **(A and B)** Apoptosis was assessed by TUNEL in HK2 cells after exposure to vehicle, urinary proteins (UP, 8 mg/ml), UP (8 mg/ml) plus rapamycin (RAP, 10 μ M) or UP (8 mg/ml) plus chloroquine (CQ, 10 μ M) for 8 h. Scale bar: 100 μ m. **(C and D)** The levels of supernatant LCN2 and HAVCR1 were measured by ELISA as described in **(A)**. **(E)** Cell viability was assessed by MTT assay as described in **(A)**. * $P < 0.05$ and ** $P < 0.01$.

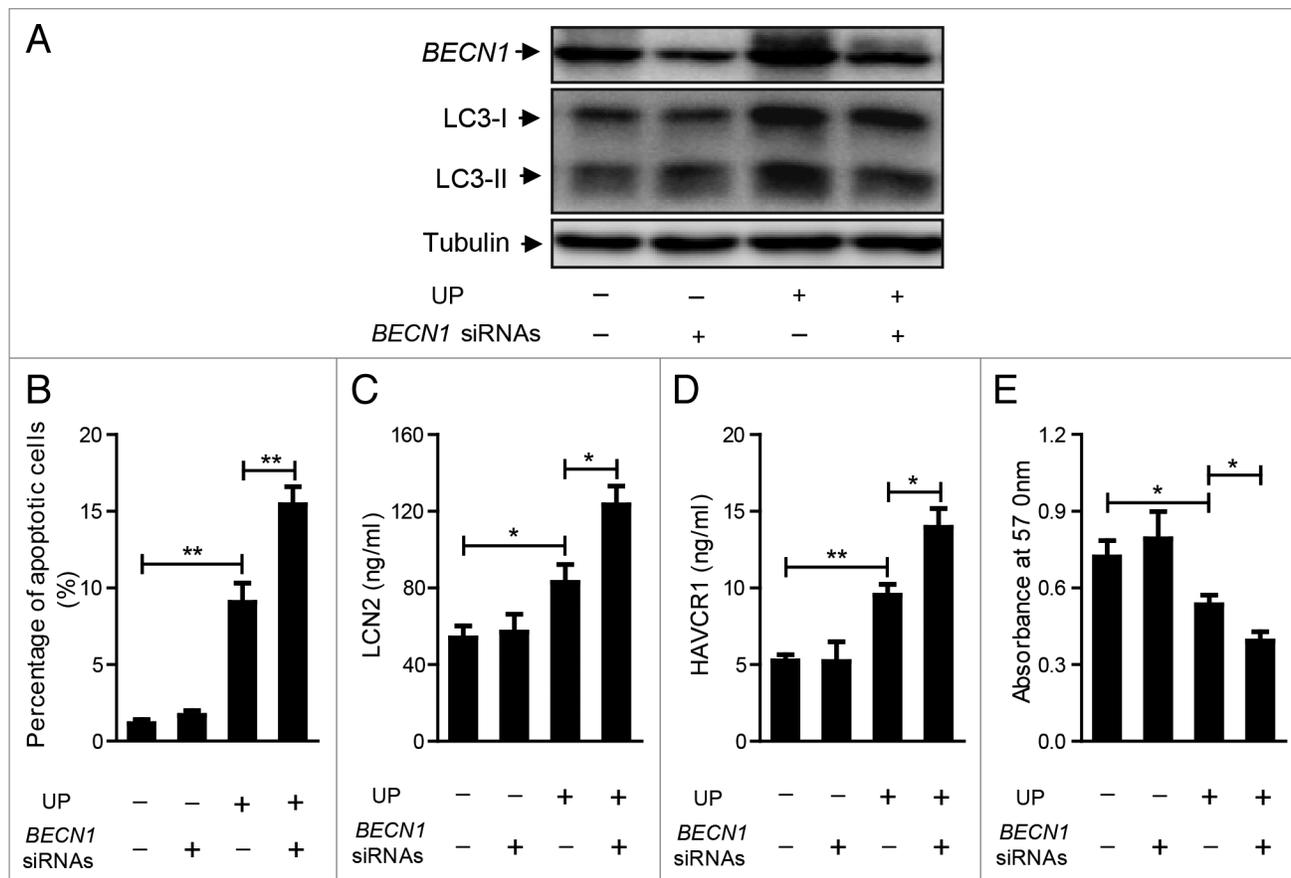


Figure 7. Effects of *BECN1* silencing on HK-2 cell injury induced by urinary proteins. (A) Western blot analysis of *BECN1* or LC3-II level after transfected with the negative control or *BECN1* siRNAs. (B) Apoptosis of HK-2 cell was detected by TUNEL after exposure to vehicle, urinary proteins (UP, 8 mg/ml), UP (8 mg/ml) plus rapamycin (RAP, 10 μ M) or UP (8 mg/ml) plus chloroquine (CQ, 10 μ M) for 8 h. (C and D) The levels of LCN2 and HAVCR1 in cell culture supernatant were measured by ELISA as described in (B). (E) Cell viability was assessed by the MTT assay as described in (B). * $P < 0.05$ and ** $P < 0.01$.

the levels of LCN2 and HAVCR1 in culture supernatant were assessed using Quantikine™ kits (R&D Systems, DLCN20 and DKM100).

Plasmid transfection and small interfering RNAs

HK-2 cells were transfected with t_{LC3} plasmid or siRNA-*BECN1* via Lipofectamine 2000 (Invitrogen, 1153277) according to the manufacturer's instructions. The t_{LC3} plasmid was obtained from Addgene (Plasmid 21074). The negative control and *BECN1* siRNA were purchased from Invitrogen. HK-2 cells transfected with *BECN1* siRNA were then incubated with 8 mg/ml urinary proteins for 24 h prior to LCN2 or HAVCR1 assays, and 36 h prior to apoptosis or cell viability assays. After t_{LC3} transfection cells were treated with 8 mg/ml urinary proteins plus or minus chloroquine (10 μ M) for another 8 h to assess autophagosome and autolysosome formation as described previously.¹⁸

Animal experiments

Forty male Sprague-Dawley rats weighing 160 to 250 g were randomly divided into 4 groups, including control group, model group, and groups of treatment with rapamycin or chloroquine, respectively. Rat models with high-grade proteinuria were established by injection of cationic bovine serum albumin (C-BSA) as described previously.⁴¹ Briefly, rats were injected

subcutaneously with 1 mg C-BSA emulsified in an equal volume of incomplete Freund's adjuvant on day 0. Then, animals received 16 mg/kg C-BSA intravenously from week 2 to 5 (3 times/wk). Rats in control group were injected with saline. Rat models were defined as high-grade proteinuria when 24 h urinary protein excretion was higher than 100 mg. Rats in treatment groups were received rapamycin (0.25 mg/kg/d) or chloroquine (60 mg/kg/d) for 4 wk, respectively. After a 4-wk treatment, urine samples were collected to measure LCN2, HAVCR1, and LDH levels using an automatic biochemical analyzer (Olympus, AU2700). At sacrifice, renal cortex specimens were processed for morphological study or western blot analysis. All the procedures were approved by the Institutional Animal Care and Use Committee of Guangdong Medical College.

Transmission electron microscopy

Kidney tissue specimens from human or rat, as well as HK-2 cells were fixed, embedded and stained as described previously.⁴² Ultrathin sections were cut using a Philips CM100 electron microscope. The AP was defined as a double-membrane structure surrounding undigested cytoplasmic constituents and the AL was defined as single-membrane structures containing cytoplasmic components at various stages of degradation.⁴³

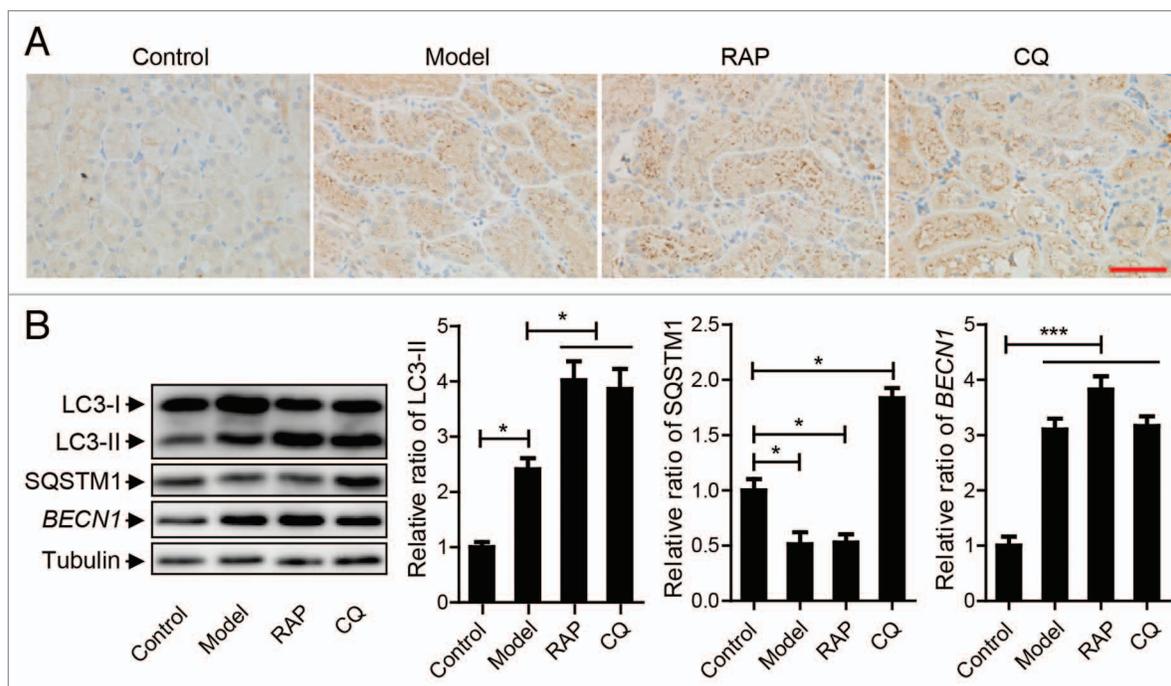


Figure 8. Different effects of rapamycin and chloroquine on autophagic activity in TECs in rat models with proteinuria. **(A)** Immunohistochemical staining of LC3 in renal tissues from the controls and proteinuria model rats treated with or without rapamycin (RAP) or chloroquine (CQ). Scale bar: 50 μ m. **(B)** Western blot analysis of LC3, SQSTM1 or *BECN1* level in TECs from the controls and model rats treated with or without RAP or CQ. Densitometry was performed for quantification and the ratio of LC3, SQSTM1 or *BECN1* to tubulin was expressed as fold of control. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Immunofluorescence and immunohistochemical study

Immunostaining analysis for tissues or cells was conducted as described previously.⁴² Rabbit anti-LC3B (ab51520 for cell), rabbit anti-LAMP1 (ab24170) and mouse anti-LAMP2 (ab25631) antibodies were obtained from Abcam. Rabbit anti-LC3B (Sigma, L7543 for tissue), FITC-labeled goat anti-rabbit IgG (Santa Cruz, sc-2012), Alexa Fluor[®] 594 goat anti-mouse IgG (Invitrogen, A11005) and HRP labeled anti-rabbit IgG antibodies (Zhongshan Goldenbridge Biotechnology, PV-6001) were used for tissue immunostaining assays. Images were taken under TCS SP5 II confocal microscope (Leica Microsystems). Apoptosis was assessed using a TUNEL apoptosis assay kit (Promega, G3250) according to the manufacturer's instructions. Expression levels of LC3-II in 40 to 50 proximal renal tubuli for each human were first graded on a scale of 0 to 4, and the average of the scores was subsequently calculated. LC3-II dots were counted in individual HK-2 cells and the average of dots in at least 30 cells was presented in the figures.

Cell viability assay

Cells were incubated with 5 mg/ml methyl thiazolyl tetrazolium (MTT) solution (Calbiochem, 475989) for 4 h at 37 °C. The formazan crystals were dissolved in dimethylsulfoxide. Optical density was determined at 570 nm with a plate reader (Thermo Labsystems, Multiskan MK3).

Proteasome activity assay

Proteasome activity was measured in living HK-2 cells as previously described.⁴⁴ Briefly, fluorogenic proteasomal peptide substrates specific for the chymotrypsin-like (Millipore, APT280) and trypsin-like (Millipore, 539149) were used at

a final concentration of 20 μ M to assess activity of different proteasomal subunits of the 26S complex. Released AMC was measured using a multi-mode reader at an excitation wavelength of 390 nm and an emission wavelength of 460 nm.

Flow cytometry analysis

After treatment cells were harvested by centrifugation for 5 min and resuspended in PBS. The fluorescence was determined by FACS Calibur flow cytometer (BD, FACSCanto II), with excitation at 495 nm and emission at 525 nm.

Western blot analysis

Western blot analysis was performed as described previously.⁴² The primary antibodies against SQSTM1/p62 protein (Santa Cruz, sc-28359), *BECN1* (Abcam, ab114071), LAMP1 (Abcam, ab24170), LAMP2 (Abcam, ab25631), LC3B (Sigma, L7543) and ACTB/ β -Actin (Santa Cruz, sc-47778), Tubulin (Abcam, ab59680) and the HRP-conjugated secondary antibodies (Beyotime Institute of Biotechnology, A0216 and A0208) were used.

Statistical analysis

All statistical tests were performed with SPSS 16.0. All data are expressed as the means \pm standard error of the mean (S.E.M.). Two group comparisons were carried using our independent-sample *t* test. Multiple group comparison was performed using ANOVA, followed by Bonferroni or Dunnett post-hoc tests. *P* value was considered as statistically significant if it is less than 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

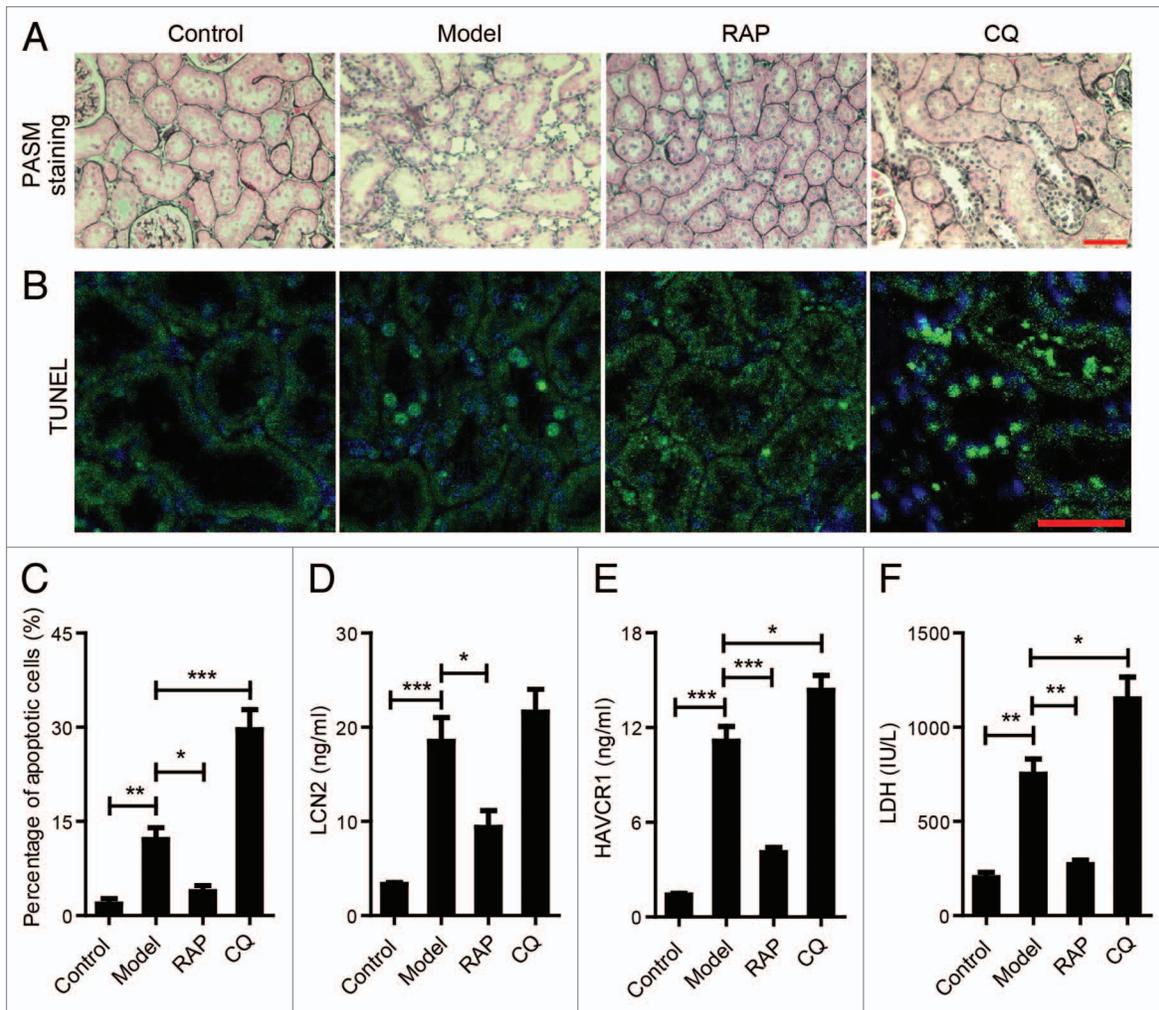


Figure 9. Effects of rapamycin or chloroquine on TEC injury in rat models with proteinuria. **(A)** Representative photomicrographs of PASM-stained renal tubuli of the controls and proteinuria model rats treated with or without rapamycin (RAP) or chloroquine (CQ). Scale bar: 50 μ m. **(B and C)** Apoptosis of TECs was detected by TUNEL in renal tubuli of the controls and model rats treated as in **(A)**. **(D and E)** The urinary LCN2 and HAVCR1 levels were measured by ELISA in controls and model rats treated as in **(A)**. **(F)** The urinary LDH level was assayed by a colorimetric method in controls and model rats treated as in **(A)**. * $P < 0.05$ and ** $P < 0.01$.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/autophagy/article/27004

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