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Albumin Overload and PINK1/Parkin Signaling-Related Mitophagy in Renal Tubular Epithelial Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Albumin, as a major urinary protein component, is a risk factor for chronic kidney disease progression. Mitochondrial dysfunction is one of the main causes of albumin-induced proximal tubule cells injury. Mitophagy is considered as a pivotal protective mechanism for the elimination of dysfunctional mitochondria. The objective of this research was to determine whether albumin overload-induced mitochondrial dysfunction can activate PINK1/Parkin-mediated mitophagy in renal tubular epithelial cells (TECs).

Material/Methods: Immunofluorescence assay and Western blot assay were used to detect the effects of albumin overload on autophagy marker protein LC3. Transmission electron microscopy and Western blot assay were used to investigate the role of albumin in mitochondrial injury. Western blot assay and co-localization of acidic lysosomes and mitochondria assay were employed to detect the activation of mitophagy induced by albumin. Finally, we explored the role of PINK1/Parkin signaling in albumin-induced mitophagy by inhibiting mitophagy by knockdown of PARK2 (Parkin) level.

Results: Immunofluorescence and Western blot results showed that the expression level of LC3-II increased, and the maximum increase point was observed after 8 h of albumin treatment. Transmission electron microscopy results demonstrated that albumin overload-induced mitochondrial injury and quantity of autophagosomes increased. Additionally, expression of PINK1 and cytosolic cytochrome C increased and mitochondria cytochrome C decreased in the albumin group. The co-localization of acidic lysosomes and mitochondria demonstrated that the number of albumin overload-induced mitophagy-positive dots increased. The transient transfection of PARK2 siRNA result showed knockdown of the expression level of PARK2 can inhibit mitophagy induced by albumin.

Conclusions: In conclusion, our study suggests that mitochondrial dysfunction activates the PINK1/Parkin signaling and mitophagy in renal tubular epithelial cells under albumin overload condition.

MeSH Keywords: Albuminuria • Autophagy • Mitochondrial Degradation • Proteinuria • Renal Insufficiency, Chronic

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Background

Proteinuria, a universal feature in many renal glomerular diseases, can promote glomerular sclerosis and interstitial fibrosis [1–3]. Therefore, urinary proteins should not only be regarded as indicating glomerular injury, but also as a nephrotoxic factor for renal tubular cells [4,5]. Continuous high levels of proteinuria are an independent cause of progressive renal damage and play an important part in the progression from chronic kidney disease (CKD) to end-stage renal disease (ESRD) [6].

Albumin, as the main component of the protein in nephrotic urine, is normally filtered in the glomeruli and reabsorbed by Megalin and Cubilin receptors, which are co-localized in the renal proximal tubule [7,8]. Previous studies have shown that excessive albumin can induce apoptosis of renal tubular epithelial cells (TECs) [9,10]. Further studies have shown that mitochondrial damage is an important element in albumin overload-induced tubular apoptosis [11,12].

Mitophagy is a highly protected mechanism of lysosomal degradation for removing impaired mitochondria and preserving mitochondria in the appropriate condition [13,14]. Recent studies suggested that the Pink1/Parkin-mediated mitophagy pathway is one of the best studied mechanisms for mitophagy in mammalian cells. Parkin is a cytosolic E3 ubiquitin ligase which is encoded by the PARK2 gene. Pink1 can promote Parkin E3 ligase activity and recruit Parkin to mitochondria, then it promotes mitophagy [13,14]. It is closely related to many diseases, such as neurodegenerative diseases, blood diseases, tumors, and other diseases [14,15]. Mitophagy is also associated with renal injury. Chuanyan Zhao et al. showed that Pink1/Parkin-mediated mitophagy plays a protective role in cisplatin-induced renal tubular epithelial cell injury [16]. However, whether Pink1/Parkin-mediated mitophagy is involved in albumin overload-induced renal TEC injury has yet to be determined. Therefore, our study investigated the possible mechanism of mitophagy by observing the effects of albumin overload on autophagy, mitochondrial damage, and Pink1/Parkin-mediated mitophagy in renal TECs.

Material and Methods

Reagents and antibodies

The human proximal tubular epithelial HK-2 cell line was supplied by the China Center for Type Culture Collection (Wuhan, China). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium/F12 (DMEM/F12) were obtained from Gibco (Grand Island, NY, USA). Human serum albumin (HAS/ALB) was acquired from Sigma-Aldrich (St. Louis, MO, USA). LysoTracker Red and MitoTracker Green were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The antibodies used are described in Table 1.

Cell culture

Human proximal tubular epithelial HK-2 cells were cultured using DMEM/F12 medium containing 10% fetal calf serum, penicillin, and streptomycin in a 5% CO_2 incubator at 37°C. The medium was changed to serum-free medium at 24 h before incubation with endotoxin-free human serum albumin at 80% confluence.

Western blot analysis

The total soluble proteins, cytosolic fraction, and mitochondrial fraction were subjected to Western blot analysis. Total proteins were extracted with a protein extraction kit (Promega, USA) in accordance with the instructions. Isolation of mitochondria from HK-2 cells was performed using the Mitochondria/Cytosol Fractionation Kit (Beyotime, China). The cytosolic and mitochondrial fraction proteins were collected according to the manufacturer's instruction. We used a BCA assay kit (Thermo, USA) to measure protein concentrations. We separated 20 μg

Table 1. Primary and secondary antibodies used for Western blot.

Antibody	Host	Dilution	Company
LC3 (primary)	Rabbit	1: 1000	CST (2775s)
Cyt.c (primary)	Rabbit	1: 1000	CST (4272s)
GAPDH (primary)	Rabbit	1: 1000	CST (2118s)
P62(primary)	Rabbit	1: 1000	CST (88588S)
PARK2/Parkin (primary)	Rabbit	1: 1000	Abcam (ab15954)
PINK1 (primary)	Rabbit	1: 1000	Abcam (ab23707)
COX IV(primary)	Rabbit	1: 1000	Abcam (ab16056)
HRP (secondary)	Goat	1: 1000	Beyotime (A0208)

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of protein per sample on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred it to a 0.22-μm polyvinylidene difluoride (PVDF) membrane.

Membranes were blocked on a shaker at room temperature with 5% non-fat milk for 2 h, followed by incubated with primary antibodies described in Table1 overnight in TBS-T at 4°C. The membranes were washed 3 times with TBS-T for 5 min on the shaker, then incubated with secondary antibody (Table 1) on the shaker at room temperature for 1 h. Chemiluminescence was imaged in a Molecular Imager Chemi DocTM XRS + WB with Image LabTM software (Bio-Rad, CA, USA), and band intensity was measured by ImageJ software.

Detection of mitophagy

Mitophagy was determined by the co-localization of mitochondria with lysosome. The cells were co-incubated with red-fluorescing LysoTracker Red (1 μM, Molecular Probes, Beyotime, China) and green-fluorescing MitoTracker Green (200 nM, Molecular Probes, Beyotime, China) for 20 min. Images were acquired with a Zeiss AXIO Observer D1 (Carl Zeiss, Oberkochen, Germany). The number of MitoTracker- and LysoTracker-positive dots was determined by manual counting of fluorescent puncta in more than 60 cells for each group.

Immunofluorescence staining for microtubule-associated LC3 and DAPI

After treatment with albumin, the HK-2 cells were observed by optical microscopy and photographed. The cells were fixed in 4% paraformaldehyde for 15 min and washed 3 times with phosphate-buffered saline (PBS) for 5 min each time. Then, we added 0.1% Triton X-100 in PBS for permeabilization for 10 min and rinsed 3 times with PBS for 5 min each time. Then, the samples were blocked with BSA for 30 min at room temperature. Next, the HK-2 cells were incubated in a wet box at 4°C overnight with the anti-light chain 3B (LC3B) antibody (1: 50, Cell Signaling Technology, USA). After being rinsed 3 times in PBS for 5 min each time, the cells were incubated with an appropriate secondary antibody for 1 h in the dark at room temperature. Lastly, 4,6-diamidino-2-phenylindole (DAPI) (Beyotime, C1002) was used as a counterstain. Results were evaluated by use of an OLYMPUS microscope (cellSens system) and an OLYMPUS confocal microscope (FV 1200). LC3-II dots were counted in individual HK-2 cells and the average of dots in at least 30 cells is presented in the figures.

Electron microscopy (EM)

HK-2 cells were fixed in 2.5% PBS-buffered glutaraldehyde at 4°C for 1 h after washing 3 times in PBS. Cells were fixed in 2.5% PBS-buffered glutaraldehyde at 4°C for 1 h after washing 3 times in PBS. Postfixation was performed in 1% osmium tetroxide/0.1 M phosphate buffer (pH 7.4), and dehydrated with an ethanol gradient. Then, we embedded the fixed HK-2 in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate. The sections were observed under a transmission electron microscope (Hitachi, Tokyo, Japan).

The small interfering RNA (siRNA) and transfection

Small interfering RNA (siRNA) targeting PARK2 was purchased from Shanghai Gene Pharma Co., Ltd. (Shanghai, China). The PARK2 siRNA was transfected into cells with 5 μl/well of LipofectamineTM3000 in a 6-well plate following the manufacturer's instructions. After a total of 48 h of transfection, the maximum inhibitory effect of PARK2 expression was observed at 24 h. Untransfected cells were considered as a blank control, and a scrambled sequence siRNA was considered as the negative control.

Statistical analysis

Statistical analyses were performed with SPSS 16.0 (SPSS Inc., USA). ImageJ software was used to analyze the immunostaining. The *t* test and one-way ANOVA were used for comparing with baseline values and the variance between groups, respectively. Quantitative data are expressed as mean ± standard deviation (SD). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of albumin overload on LC3 expression in HK-2 cells

We investigated the effects of albumin on LC3 expression by immunofluorescence and Western blot analysis. Cells were incubated with albumin (8 mg/ml) for various durations. The concentration of 8 mg/ml albumin was selected based on our previous studies. The time-related increase of LC3-II biosynthesis was evident. As shown in Figure 1A and 1C, in the 0 h group, only a few LC3-II dots were detected by immunofluorescence staining. Exposure to albumin (8 mg/ml) for 4 h or 12 h, the number of LC3-II-positive puncta significantly increased in cells treated with albumin for 8 h to 24 h ($P<0.05$), and the peak of the increase was at 8 h post-treatment. Similar patterns of LC3-II biosynthesis were observed by Western blot assay (Figure 1B, 1D). These results suggest that albumin overload can induce autophagy. The expression level of LC3-II was markedly increased by treatment with 8 mg/ml albumin for 8 h, which is the most suitable concentration for studying the autophagy of renal TECs.

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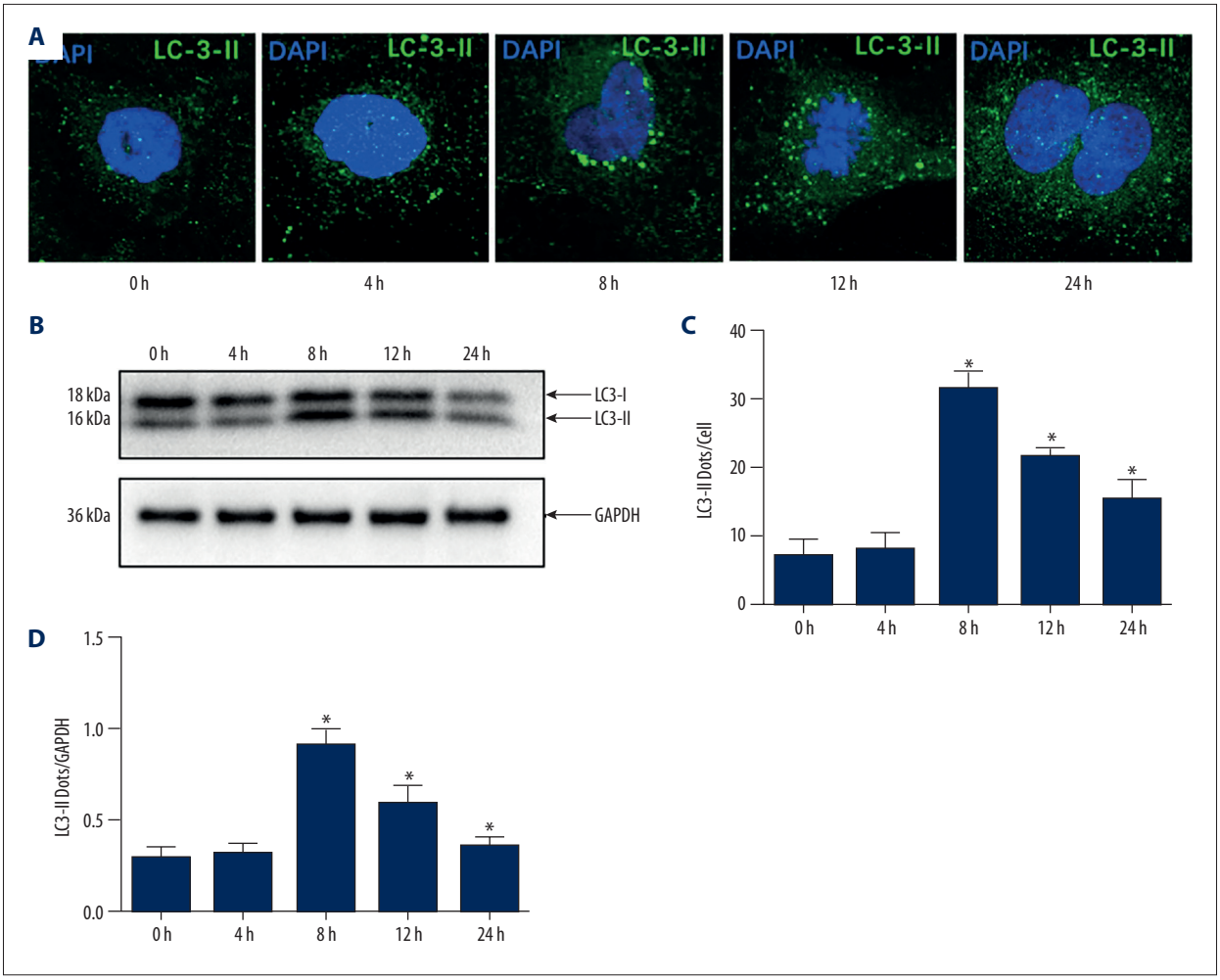

Figure 1. Effect of albumin on LC3 expression in HK-2 cells. The cells were treated with albumin 8 mg/ml for 4, 8, 12, and 24 h. (**A, C**) Western blot analysis of LC3-II level. Densitometry was performed for quantification and the ratio of LC3-II to GAPDH was expressed as fold of control. (**B, D**) Immunofluorescence staining and quantitative change of LC3-II (green). The nucleus was counter-stained by DAPI (blue). (** Significantly different from 0 h group * $P<0.05$). Scale bar: 10 μm.

Effects of albumin overload on morphology of mitochondria

We observed the morphology of mitochondria by optical microscopy after the albumin treatment. The intact structure of mitochondria suffered severe damage in the albumin group compared with the control group (Figure 2). We observed mitochondrial swelling, vacuolization, crista disruption, and disappearance of normal morphology in the albumin group (Figure 2B), and the autophagosomes containing damaged mitochondria were also clearly evident (Figure 2B–2D). These results suggest that autophagy activation is related with mitochondrial injury induced by albumin overload in HK-2 cells.

Effects of albumin on mitochondrial injury and mitophagy in HK-2 cells

To analyze the effects of albumin on mitochondrial injury, we examined the expression level of cytochrome C. Under normal conditions, cytochrome C resides in the mitochondrial membrane. With mitochondrial injury, cytochrome C translocates from the mitochondrial membrane to the cytoplasm. As shown in Figure 3, the level of cytosolic cytochrome C was notably increased by treatment with albumin ($P<0.05$), while mitochondrial cytochrome C was observably decreased ($P<0.05$) (Figure 3A, 3B). These results indicate that albumin overload induced mitochondrial dysfunction and led to cytochrome C release from mitochondria to cytosol.

Albumin resulted in mitochondrial damage, so we next explored whether mitophagy was activated by albumin in HK-2

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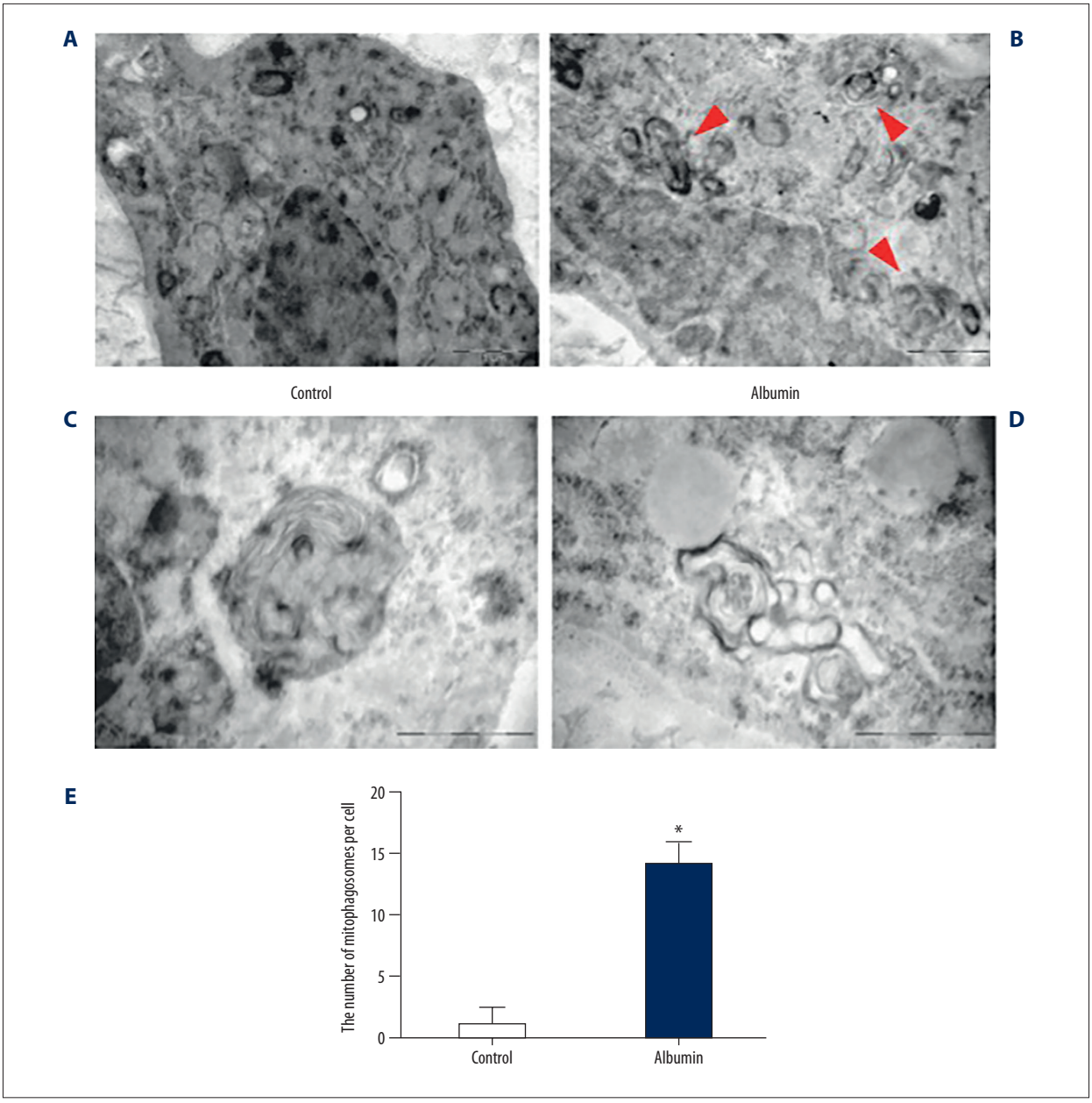

Figure 2. Quantitative changes of mitophagy vacuoles under transmission electron microscopy (TEM) in HK-2 cells. (**A, B**) Ultrastructural images of mitophagy vacuoles in the albumin group and the controls. (**C, D**) The typical images of the mitophagy vacuoles are shown at higher magnification. Red arrows indicate mitophagy vacuoles. (* Significantly different from control * $P<0.05$) Scale bar: 1 μm.

cells. We detected the expression level of PINK1 by Western blot and the results showed the HK-2 cells treated with albumin the expression level of PINK1 was greatly increased when compared to control cells ($P<0.05$) (Figure 3C).

To further analyze the effect of albumin overload on mitophagy, we examined the quantification for dots of mitophagy by use of MitoTracker and LysoTracker co-staining. We observed that lysosomes and mitochondria were separated in the control group cells; however, the co-localization of lysosomes and mitochondria formed yellow fluorescence in the albumin-treated group cells, demonstrating that mitophagy was activated. The quantitative analysis of mitophagy-positive dots (yellow fluorescent dots) showed there were significantly more dots in the albumin-treated group compared with the control group ($P<0.05$) (Figure 3D). These observations indicate albumin can induce mitophagy.

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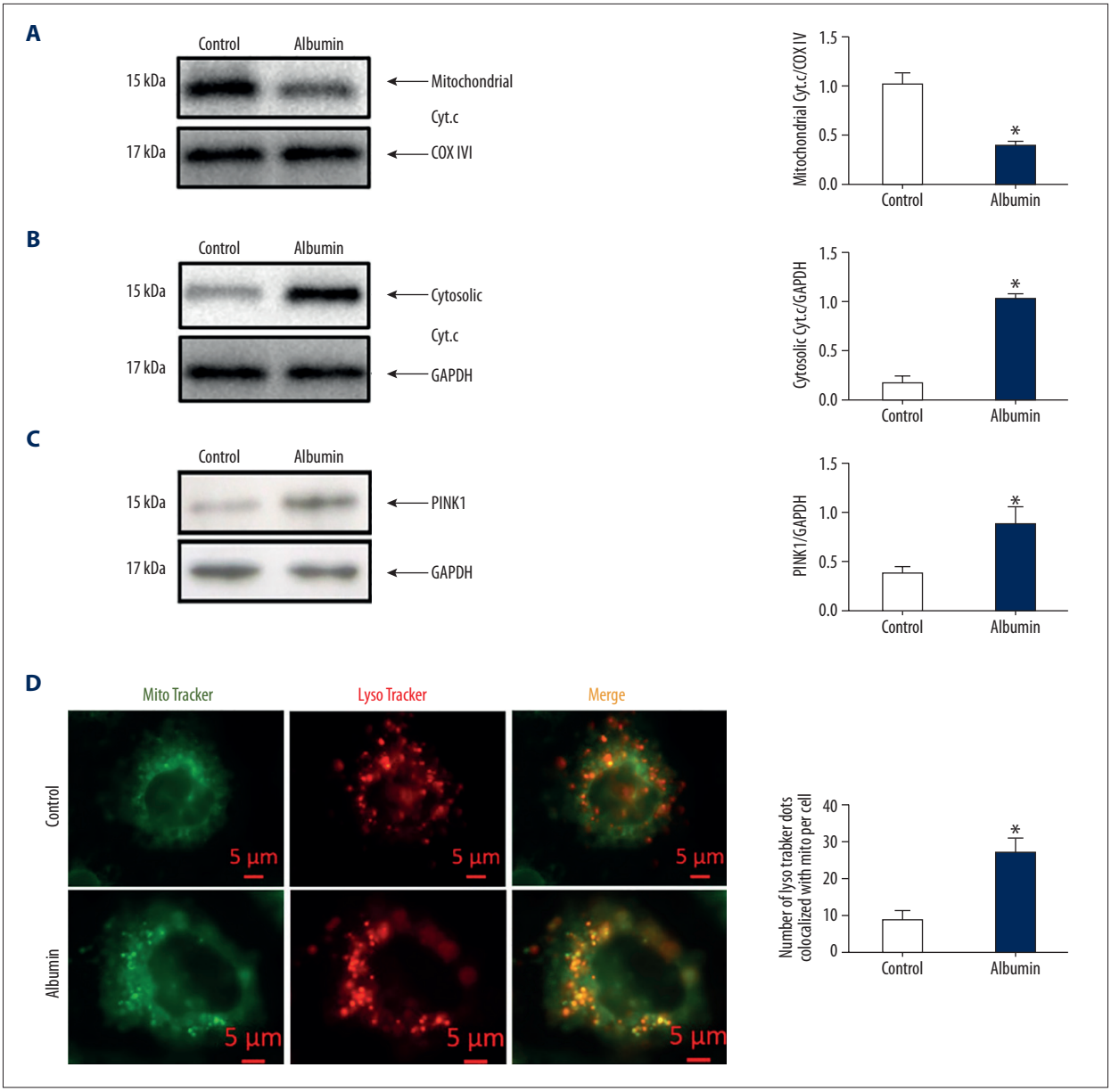

Figure 3. Effects of albumin on mitochondria injury and mitophagy in HK-2 cells. HK-2 cells were treated with albumin at 8 mg/ml for 8 h. (**A**) Western blot analysis of mitochondrial cytochrome C. Densitometry was performed for quantification and the ratio of mitochondrial cytochrome C to COX V was expressed as fold of control. (**B**) Western blot analysis of cytosolic cytochrome C. Densitometry was performed for quantification and the ratio of cytosolic cytochrome C to GAPDH was expressed as fold of control. (**C**) Western blot analysis of PINK1. Densitometry was performed for quantification and the ratio of PINK1 to GAPDH was expressed as fold of control. (**D**) Mitophagy was detected using MitoTracker Red and LysoTracker green staining and is displayed in the merged image as yellow fluorescence dots. (* Significantly different from control * $P<0.05$) Scale bar: 5 μm.

Effects of PARK2 knockdown on the mitophagy induced by albumin in HK-2 cells

We further investigated whether the PINK1/Parkin pathway was activated in albumin-induced mitophagy. First, we transfected the small interference RNA (siRNA) into HK-2 cells to knock down the level of PARK2. As shown in Figure 4A, 4B, 4D, and 4E, at 24 h after transfection, its normal levels were successfully reduced in 3# Parkin siRNA compared with the control group, so we chose 3# PARK2 siRNA for the following experiments.

Western blot analysis showed that, when compared to the control group, in the HK-2 cells treated with albumin, the expression levels of PARK2 (Parkin) was increased conspicuously,

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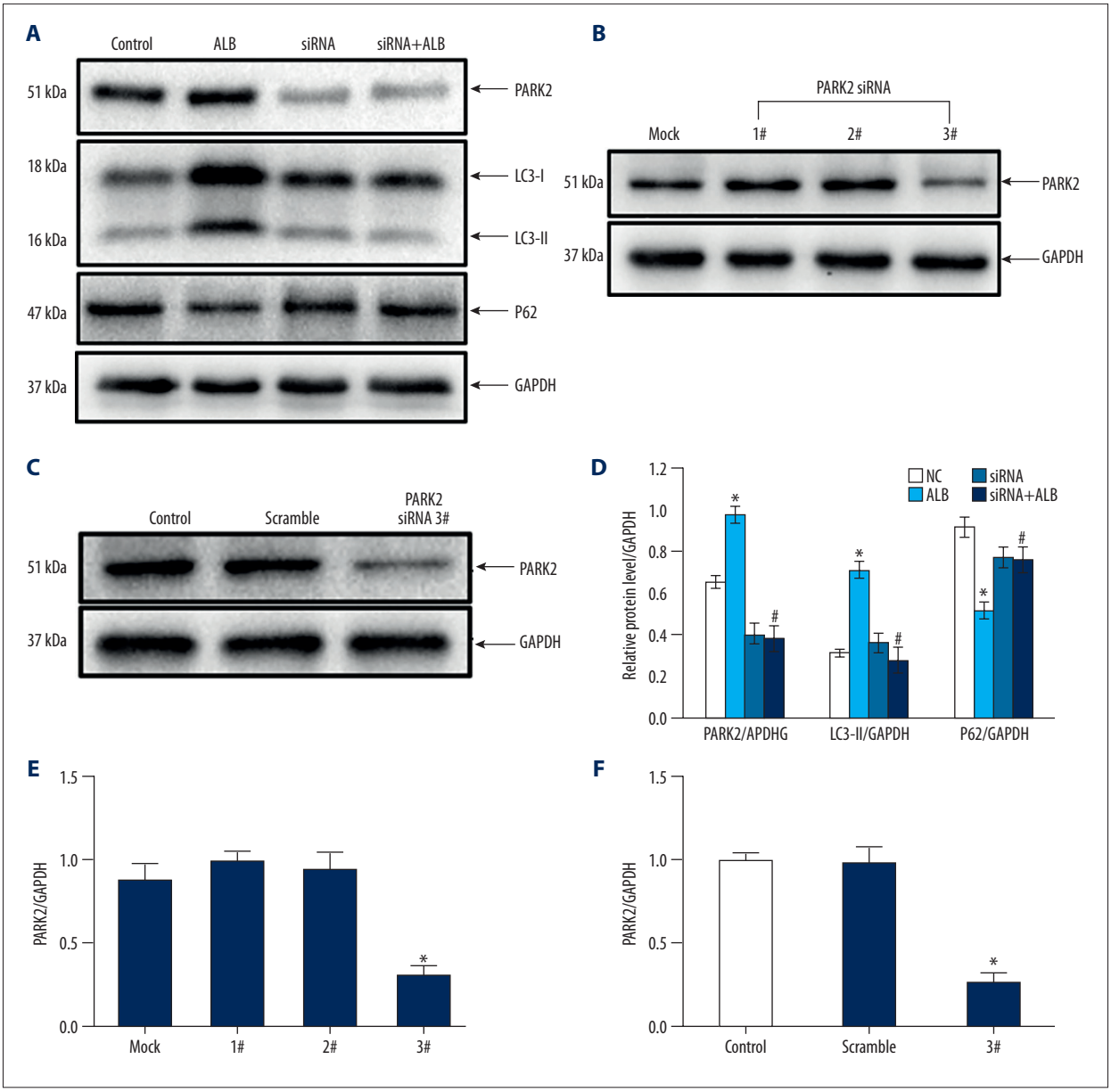

Figure 4. The effect of PARK2 siRNA. HK-2 cells were transfected with PARK2 siRNA or scrambled siRNA followed by exposure to albumin at 8 mg/ml. (**A, B, D, E**) Decreased endogenous PARK2 (Parkin) expression in PARK2 siRNA-transfected HK-2 cells. As shown, 3# Parkin siRNA was successfully reduced to its normal levels at 24 h after transfection. (**C, F**) Western blot analysis of PARK2 (Parkin), LC3-II, or p62 level in the control group, albumin-treated group, siRNA group, and siRNA+ albumin group. Densitometry was performed for quantification and the ratio of PARK2 (Parkin), LC3-II, or p62 to GAPDH was expressed as fold of control. (* Significantly different from control * $P<0.05$; # Significantly different from albumin group # $P<0.05$).

which occurred concurrently with the change of autophagy markers, increases of LC3-II, and decrease of p62 level (Figure 4C, 4F). Our findings indicated that the PINK1/Parkin pathway was activated in mitophagy induced by albumin, and silencing PARK2 reduced albumin-induced increase in the expressions of PARK2 (Parkin) and LC3-II. Simultaneously, after PARK2 siRNA transfection, albumin-induced mitophagy was remarkably inhibited in HK-2 cells, as indicated by MitoTracker and LysoTracker co-staining, showing that the number of mitophagy-positive dots was decreased in the PARK2 siRNA transfected group ($P<0.05$) (Figure 5). These results suggested that knockdown of PARK2 inhibited the PINK1/Parkin-mediated mitophagy induced by albumin.

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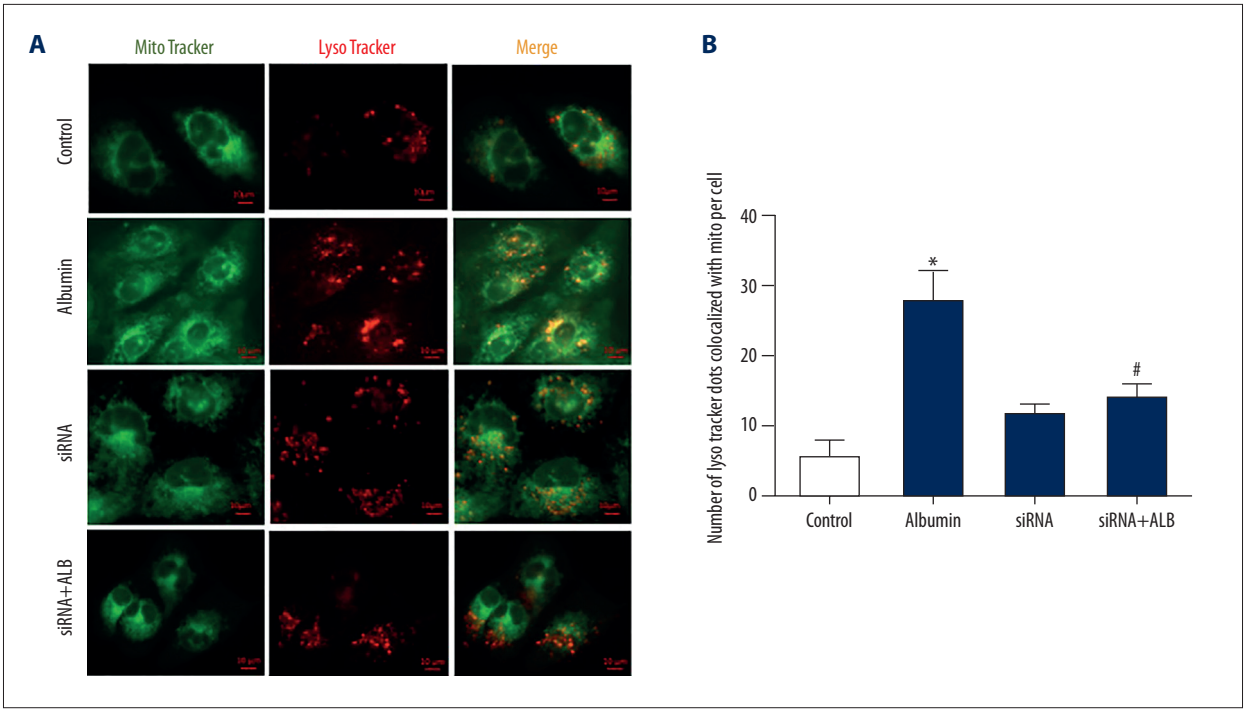

Figure 5. Effects of PARK2 siRNA on mitophagy. HK-2 cells were transfected with PARK2 siRNA, followed by exposure to albumin at 8 mg/ml. (**A, B**) Mitophagy was detected by using MitoTracker Green and LysoTracker Red staining and is displayed in the merged image as yellow fluorescence dots. Compared to the control group, quantification of mitophagy was higher in the albumin overload treatment group. Compared to the albumin group, quantification of mitophagy was lower in the siRNA+albumin group. (* Significantly different from control * $P<0.05$; # Significantly different from albumin group # $P<0.05$). Scale bar: 10 μm.

Discussion

Albumin, as the majority component of urinary protein, can induce TECs injury such as apoptosis, necrosis, or transdifferentiation, and can activate autophagy in TECs [17,18]. Growing evidence suggests that mitochondria are targets of albumin-induced renal TECs injury [11,19]. Mitophagy plays a pivotal role in elimination of damaged mitochondria, which is mainly a process of sequestrating autophagic mitochondria and delivering them to lysosomes for hydrolytic degradation [20]. In the present research, we studied the effects of albumin overload on autophagy, mitochondrial damage, and mitophagy in renal proximal tubular (HK-2) cells *in vitro*.

Albumin overload triggers autophagic response in cultured TECs. We investigated the effects of various durations of albumin treatment on autophagy in HK-2, showing that the level of LC3-II was not markedly increased in the 4 h group, but it was significantly increased in the 8-h, 12-h, and the 24-h groups, especially in the 8-h albumin-treated group. As in our previous study, we found that 8 mg/ml is the optimal concentration for studying the damage and autophagy of TECs [17,18]. In brief, the albumin overload model using 8 mg/ml for 8 h may be the best condition for researching autophagy in HK-2 cells.

The albumin overload model can induce mitochondrial damage. We observed the morphology of mitochondria before and after albumin treatment. In this study, we found the manifestation of mitochondrial damage after albumin treatment, such as swelling, vacuolization, crista disruption, and normal morphological disappearance. At the same time, we observed the formation of double-membrane autophagosomes that contain damaged mitochondria, which suggests that autophagy activation is related with mitochondrial injury induced by albumin overload. In the 1950s, autophagy was first detected by transmission electron microscopy (TEM). Even now, TEM is still one of the best techniques for assessing autophagy by providing direct evidence, but TEM is problematic in quantitative studies for cell numbers/sections [20]. Thus, we examined the expression level of mitophagy marker PINK1 and mitochondrial injury marker cytochrome C, and found that the level of PINK1 was increased and cytochrome C was released from mitochondria to cytosol in the albumin group. As previously shown, mitochondrial depolarization accompanies cytochrome C release [21]. These results indicate that albumin overload induced mitochondrial damaged and activation of mitophagy.

Dysfunctional mitochondria can trigger their degeneration by mitophagy [14]. Several distinct mechanisms for mitophagy

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have been described in some recent papers. However, in mammalian cells, the Pink1-Parkin signaling pathway in mitophagy is the most documented [13,16,22]. The level of Pink1 is normally undetectable in most cells because mitochondrial Pink1 is cleaved by PARL and then degraded by mitochondrial peptidases [20,23]. However, when mitochondria are depolarized, Pink1 is no longer cleaved and becomes stabilized on the outer mitochondrial membrane [24]. PARK2 encodes for the E3 ubiquitin ligase Parkin. Pink1 promotes Parkin-mediated mitophagy by recruiting Parkin on mitochondria and promoting Parkin E3 ligase activity [24]. Subsequently, Parkin mediates the formation of 2 distinct poly-ubiquitin chains, linked through Lys 63 and Lys 27. Then, the adaptor protein p62 binds ubiquitinated mitochondrial proteins and LC3 on autophagosomes, recruiting autophagic membranes for mitochondrial clearance [24]. In the present study, we found that the expression level of PINK1 in the albumin group was significantly increased, and our quantitative analysis showed increased co-localization between mitochondria and lysosomes in the albumin group. Furthermore, in we found that knockdown of the PARK2 inhibited albumin-induced mitophagy, and the levels of autophagy- and mitophagy-related molecules were changed, including downregulation of LC3 and PARK2(Parkin) and upregulation of p62. Those results indicate that albumin overload can induce mitophagy activation in HK-2 cells, and the mitophagy was associated with activation of the PINK1/Parkin signaling pathway. Mitophagy is an important protective mechanism that removes damaged mitochondria and preserves a healthy mitochondrial population. Impaired mitophagy in kidney cells can accelerate CKD progression, and has been implicated in several kidney disease models. Zhan et al. showed that upregulation of myo-inositol oxygenase (MIOX) reduces Pink1 expression and inhibits mitochondrial autophagy, which can contribute to the pathogenesis of diabetic nephropathy [25]. However, the specific mechanism by which the PINK1/Parkin pathway is involved in albumin-induced mitophagy is still unclear, and the role of mitophagy in renal tubular interstitial injury caused by urinary protein needs further study.

Conclusions

In summary, our results show that albumin overload-induced mitochondrial dysfunction can activate the PINK1/Parkin-mediated mitophagy in renal tubular epithelial cells. Albumin overload-induced mitophagy was increased in TECs, which suggests that the autophagy activated by urinary proteins may be a selective mitochondrial autophagy, and is mediated by the PINK1/Parkin pathway. This study further verified our previous research findings that, under urinary protein overload condition, autophagy activation can add adaptive reaction through eliminating injured mitochondria (mitophagy) in TECs.

Conflict of interest

None.

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