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Renalase attenuates mitochondrial fission in cisplatin-induced acute kidney injury *via* modulating sirtuin-3

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ABSTRACT

Aims: Acute kidney injury (AKI) can limit the clinical use of cisplatin in cancer treatment. The drivers of cisplatin-induced AKI include oxidative stress, mitochondrial dysfunction and apoptosis. Previous studies showed renalase protected cultured human renal proximal tubular cell (HK-2) against cisplatin induced necrosis, and renalase-knockout mice subjected to cisplatin showed exacerbated kidney injury. Therefore, it is necessary to determine the exact mechanisms of renalase in cisplatin-induced nephrotoxicity.

Main methods: To study the protective effect of renalase on cell viability, renal function, apoptosis, reactive oxygen species (ROS) production and mitochondrial dynamics, cultured HK-2 cells and male mice were subjected to cisplatin. Signaling proteins related to apoptosis, survival, and mitochondrial fission were analyzed by Western blot.

Key findings: In this study, we showed that the protective effect of recombinant renalase in cisplatin-induced AKI was associated with the regulation of ROS production, mitochondrial dynamics and sirtuin-3 (Sirt3) levels *in vivo* and *in vitro*. After cisplatin treatment, recombinant renalase restored Sirt3 expression, reduced mitochondrial fission and ROS generation. In HK-2 cells, downregulation of endogenous Sirt3 expression by siRNA transfection abrogated the renalase cytoprotection.

Significance: Our study suggests that renalase protects against cisplatin-induced AKI by improving mitochondrial function and inhibiting oxidative stress, and *in vitro*, it functions in a Sirt3-dependent manner.

1. Introduction

Cisplatin is a widely used antitumor drug for solid tumors, yet its application in chemotherapy is limited due to its nephrotoxicity [1]. Acute kidney injury (AKI), which can be induced by cisplatin, is an urgent condition with a high mortality. AKI also contributes to the progression of chronic kidney disease (CKD) and can even lead to renal replacement [2]. One common histopathological feature of AKI is renal tubular cell death, and therefore targeting tubular cell injury may ameliorate cisplatin nephrotoxicity.

Central to tubular cell injury is mitochondrial dysfunction [1], which is manifested by the subsequent oxidative stress, inflammation, and finally cell damage [3]. Recent studies have highlighted the role of mitochondrial structural integrity in mitochondrial function. Mitochondria exist in a dynamic fashion: they constantly remodel through fission and fusion to meet the energy demands of cells [4]. Increased mitochondrial fission promotes cell apoptosis through the release of apoptogenic factors and the overproduction of reactive oxygen species (ROS). Sirtuin3 (Sirt3) is a deacetylase of the sirtuin family located in the mitochondrial matrix. Previous studies showed that Sirt3 was a key regulator of mitochondrial dynamics, antioxidant defenses and cell survival, suggesting it could be a new target for organ damage improvement [5]. Sirt3 was also proved to be protective in cisplatin-induced AKI by reducing mitochondrial fragmentation [6].

Renalase, first identified in 2005 [7], is an enzyme made of 342 amino acids with oxidase activity. Its high expression in kidney tissues and secretion into circulation suggest that it participates in catecholamine metabolism. Recently, most studies of renalase focus on its mammalian physiology (*i.e.* its ability to promote cell survival as a signaling molecule) and are uncoupled to its enzymatic function [8]. It was documented that cisplatin-induced AKI was exacerbated in renalase-deficient mice compared with wild type ones [9]. This effect was related to its capacity to activate MAPK signaling and prevent cisplatin-induced apoptosis [10]. Recent studies showed renalase protein

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decreased the levels of oxidative stress that could induce nephropathy [11,12]. However, the mechanisms related to the protective effects of renalase in cisplatin-induced AKI have not been elucidated.

Therefore, we examined the effects of exogenous renalase in the cisplatin-induced AKI both *in vivo* and *in vitro*. In addition, we explored whether Sirt3 and mitochondrial dynamics participated in this process.

2. Materials and methods

2.1. Reagents and antibodies

Cisplatin (catalog number P4394), dihydroethidium (DHE, catalog number D7008) and 2′,7′-dichlorofluorescein diacetate (DCFDA, catalog number D6883) were purchased from Sigma-Aldrich (St. Louis, MO). Renalase (catalog number ab134535), anti-Sirt3 (catalog number ab86671), anti-dynamin-related protein 1 (Drp1, catalog number ab184247), phosphor anti-Drp1 (Ser637) (catalog number ab193216), anti-Bcl-2 (catalog number ab32124), anti-Bcl-2 associated X (Bax, catalog number ab32503) antibodies were obtained from Abcam (Cambridge, MA, USA). Anti-Cleaved Caspase3 (catalog number 9661), anti-Voltage-dependent anion channel (VDAC, catalog number 4866), anti-beta actin (catalog number 4970) antibodies were from Cell Signaling (Danvers, MA, USA). Anti-glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was from Sanying biotechnology (Wuhan, China, catalog number 10494-1-AP). All secondary antibodies for immunoblot analysis were obtained from Zhongshan Golden Bridge Biotechnology (Beijing, China, ZB-2301). Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (catalog number ab150077) was from Abcam (Cambridge, MA, USA). MitoTracker (catalog number M7512), MitoSOX (catalog number M36008) and FITC-conjugated wheat germ agglutinin (WGA) (catalog number W11261) were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Animals

Male C57BL/6J mice (2-month old) were purchased from the Experimental Animal Center of Nanjing Medical University (Nanjing, China). Mice were randomly assigned into 4 groups: Control (Cntl), received normal saline (n = 5); Renalase (Ren), received a single intraperitoneal injection of renalase (1.5 mg/kg) (n = 5); Cisplatin (Cis), given a single intraperitoneal injection of cisplatin (20 mg/kg) to induce nephrotoxicity (n = 6); Cisplatin + Renalase (Cis + Ren), received recombinant renalase (1.5 mg/kg, intraperitoneally) 30 min prior to cisplatin administration (n = 6). The animals were subsequently sacrificed under anesthesia at 72 h after cisplatin injection. Blood and kidneys were collected for experiments. The animal protocol was approved by the Nanjing Medical University Institutional Animal Care and Use Committee.

2.3. Renal function and histology

Blood was collected from mice *via* cardiac puncture into a heparinized syringe and centrifuged (4 °C, 800 × *g*, 5 min) to separate plasma. Blood urea nitrogen (BUN) was assessed by a kit from Stanbio (Boerne, TX) and creatinine was measured by a kit from Jian Cheng (Nanjing, China), according to the manufacturer's instructions. Harvested kidney tissues from mice were fixed with 4% paraformaldehyde paraffin sections (2 μm/slice) and stained with periodic acid-Schiff (PAS). Tubular injury scores were calculated based on the percentage of tubules affected (0: < 10%; 1: 10–25%; 2: 26–50%; 3: 51–75%; 4: > 75%), as previously described [13]. The scores of AKI-associated tubular injury (tubular epithelial cell loss, necrosis, tubular epithelial simplification, intratubular debris, and casts) were assessed by a pathologist blinded to the experimental groups (using > 5 random fields/section, 5–6 mice/group).

2.4. Electron microscopy

Kidney cortex was fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide. After solidification, ultrathin sections were stained with uranyl acetate and lead citrate, and then examined using an electron microscope (JEM-1010, Tokyo, Japan). The aspect ratio as mitochondrial morphology assessment was calculated according to the previously published methods [14–16].

2.5. Cell culture and treatment

Human renal proximal tubular cells (HK-2), kindly provided by Ding Wei(Shanghai Jiaotong University, Shanghai, China), were cultured in DMEM/F12 media (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml) in 5% CO_2 at 37 °C. Cells were exposed to cisplatin (20 μM) for 16 h in the presence or absence of renalase (5 μg/ml). Sirt3 specific small interfering RNAs (siRNAs) (Santa Cruz, USA) and negative controls (Santa Cruz, USA) were transfected into HK-2 cells by Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. The sequences of siRNA were as follows: Sirt3 siRNA sense 5′-CCAGUGGCAUUCCAGACUUTT-3′, Sirt3 siRNA antisense 5′-AAGUCUGGAAUGCCACUGGTT-3′, scramble siRNA sense 5′-UUCUCCGAACGUGUCACGUTT-3′, scramble siRNA antisense 5′-ACGUGACACGUUCGGAGAATT-3′.

2.6. Western blot analysis

The kidney tissues were put in liquid nitrogen followed by storing at − 80 °C for immunoblotting. Cells and renal tissues were harvested in RIPA lysis (Beyotime, Shanghai, P0013C) supplemented with a protease and phosphatase inhibitor (Beyotime, Shanghai, P1046). Protein concentration was determined using a BCA assay. Equal amounts of 30 μg proteins were separated by SDS-PAGE and transferred to PVDF membranes. After one hour blocking, the blots were incubated with the primary antibodies as anti-Bcl-2(1:1000), anti-Bax(1:2000), anti-Caspase3(1:500), anti-Sirt3(1:1000), anti-Drp1(phospho Ser637) (1:500), anti-Drp1(1:1000) anti-GAPDH(1:5000), anti-β-actin(1:5000) and anti-VDAC(1:1000), respectively. The bands were visualized by corresponding horseradish peroxidase-conjugated secondary antibodies, enhanced using chemiluminescence (ECL, Thermo Fisher Scientific, Rockford, IL, USA, catalog number 32106), and finally quantified by densitometric analysis using Quantity One Software (Bio-Rad, Hercules, CA, USA).

2.7. Cell viability

Cell viability was analyzed using the cell counting kit-8 (CCK-8) assay (Beyotime, Shanghai, China) following the manufacturer's instructions.

2.8. Mitochondrial morphology

Living HK-2 cells were incubated with the 200 nM MitoTracker Red at 37 °C for 30 min and Hoechst 33342 (Beyotime Biotechnology, Shanghai, China) at 37 °C for 10 min. After incubation, the visualization and quantification of mitochondrial fission of cells were examined under a confocal microscope. Numerical percentage of mitochondrial fragmentation was estimated according to Craig Brooks et al. [17]. For each sample, > 100 cells per dish were evaluated for mitochondrial morphology. The mitochondria within a cell were often either fragmented or filamentous. The cells were classified based on the majority (> 70%) of mitochondria in cases of mixed mitochondrial morphology.

2.9. *Assessment of ROS levels*

ROS levels in kidney tissues were detected by staining the fresh frozen sections (4 μm/slice) with DHE (10 μM) as previously described [18]. In *in vitro* assays, HK-2 cells were incubated with DCFDA (10 μM) for 30 min at 37 °C in the dark, and then visualized under the fluorescence microscope. We also measured the mitochondrial ROS production by MitoSOX, a mitochondrial superoxide indicator. Cells were incubated with MitoSOX (5 μM) at 37 °C for 10 min before fluorescence measurement with a microplate reader from Eppendorf.

2.10. *Drp1 translocation*

The mitochondria fraction from cells was isolated using a cell mitochondria isolation kit (Beyotime, Shanghai, catalog number C3601) according to the protocol. Mitochondrial proteins were collected to detect Drp1 translocation by immunoblotting. VDAC was used as a sample loading control.

2.11. *Apoptosis analysis*

After treatment, HK-2 cells were seeded into 6-well plates and gathered by trypsinization. According to the manufacturer's instructions, apoptosis was analyzed by flow cytometry after incubation with annexin V-fluorescein isothiocyanate and propidium iodide double staining (BD Biosciences, San Diego, CA, USA). FITC and PI double positive cells (Q2; upper-right) were late apoptotic cells, and FITC-positive and PI-negative cells (Q3; lower-right) were early apoptotic cells. Percentage of apoptotic cells was calculated from early apoptotic cells and late apoptotic cells (Q2 + Q3).Kidney apoptosis was determined using a fluorescein isothiocyanate-labelled *in situ* TdT-mediated dUTP Nick-End Labeling (TUNEL) assay (Roche Molecular Biochemicals). FITC-conjugated WGA (10 μg/ml) was applied to the 4 μm tissue sections for 30 min at room temperature to display the renal structure. After incubation, the slides were washed in Hank's balanced salt solution to remove unbound lectin, and subsequently cell apoptosis was detected following the instruction of the TUNEL assay. Tissue sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and then examined by a fluorescence microscope. Apoptosis rates calculated by TUNEL-positive cells per 100 cells in each higher power field. Moreover, HK-2 cells were incubated with the TUNEL reaction mixture as described in the manufacturer's instructions. The apoptotic cells were counted and expressed as the number of TUNEL-positive cells per 100 cells.

2.12. *Statistical analysis*

Data were expressed as mean ± SEM. Comparisons were made using one way-ANOVA and Bonferroni tests. The definition of significance is $P < 0.05$.

3. Results

3.1. *Renalase protected against cisplatin-induced AKI*

To assess the effects of renalase on cisplatin-induced AKI, we examined renal function and structure. As shown in Fig. 1A and B, the blood urea nitrogen and plasma creatinine significantly decreased in the renalase group compared with the cisplatin group. Moreover, PAS staining of kidney tissue revealed casts, renal tubular epithelial cell edema and detachment in the cisplatin group. Treatment of renalase reduced renal histologic injury induced by cisplatin (Fig. 1C, D). The number of apoptotic renal cells increased in cisplatin mice, which was ameliorated by pretreatment with renalase (Fig. 1E, F). Likewise, western blot analysis showed that renalase reduced the cisplatin-induced upregulation of Bax and Cleaved Caspase 3 and the cisplatin-induced downregulation of Bcl-2 (Fig. 1G, H). These results suggested renalase inhibited cisplatin-induced kidney injury.

3.2. *Renalase increased Sirt3 and inhibited mitochondrial dysfunction in cisplatin mice*

Western blotting revealed that renalase pretreatment markedly prevented the reduction of Sirt3 expression induced by cisplatin (Fig. 2A). As Sirt3 is a regulator of mitochondrial functional integrity [6], we observed mitochondrial fragmentation at the ultrastructural level and ROS production in the kidney. As shown in Fig. 2B and C, renalase treatment reduced mitochondrial fragmentation in cisplatin mice. It also inhibited ROS production in cisplatin-induced kidney injury (Fig. 2D, E).

To detect whether mitochondrial fragmentation was induced by activated fission, we assessed Drp1, a key mediator of mitochondrial fission [17]. When dephosphorylated at serine-637, Drp1 can be translocated to mitochondria from the cytoplasm and result in the fragmentation of mitochondria [19]. As shown in Fig. 2A, cisplatin reduced the phosphorylation of Drp1 at serine 637 compared with the control group. In contrast, renalase treatment blocked the effects of cisplatin. Taken together, these results indicated that renalase could increase Sirt3 level and decrease mitochondrial fission and dysfunction in cisplatin mice.

3.3. *Renalase had a protective role in vitro model of cisplatin toxicity*

In vitro, CCK8 assay showed renalase inhibited the cisplatin-induced cytotoxicity in HK-2 cells (Fig. 3A). Effects of renalase on cellular apoptosis were detected by TUNEL staining and flow cytometry. We observed the cells pretreated with renalase were more resistant to cisplatin-induced apoptosis (Fig. 3B, C, D and E). The protective effect of renalase on apoptosis prompted us to assess the apoptotic proteins. As shown in Fig. 3F and G, the pro-apoptotic protein Bax and Cleaved Caspase 3 increased and the anti-apoptotic protein Bcl-2 decreased after exposure to cisplatin, while renalase blocked these changes. These findings supported the idea that renalase protected HK-2 cells from cisplatin-induced apoptosis.

3.4. *Renalase increased Sirt3 and inhibited mitochondrial dysfunction in vitro*

Given that the increased level of Sirt3 was accompanied with renalase *in vivo*, we then observed the effect of renalase on Sirt3 expression *in vitro*. Consistent with the *in vivo* findings, Sirt3 expression was reduced by cisplatin-induced toxicity, whereas renalase abrogated the changes (Fig. 4A). A previous study showed ROS levels were tightly linked with Sirt3 [20]. We found by DCFDA staining that the cisplatin-induced H_2O_2 production was abolished following pretreatment with renalase (Fig. 4B). Furthermore, renalase inhibited cisplatin induced mitochondrial ROS generation measured by MitoSOX (Fig. 4C).

We further sought supports for the effect of renalase on cisplatin-induced mitochondrial fragmentation. After pretreatment with renalase, mitochondrial fragmentation was reduced as visualized by MitoTracker Red staining (Figs. 4D, E, S1). Moreover, renalase significantly increased phosphorylation of Drp1 at serine 637 in cisplatin-induced cell injury (Fig. 4A). As expected, western blot (Fig. 5A, B) showed Drp1 protein in HK-2 cells accumulated in mitochondria in cisplatin group, and significantly reduced by renalase treatment. We demonstrated that renalase prevented cisplatin-induced reduction of Sirt3 and mitochondrial dysfunction.

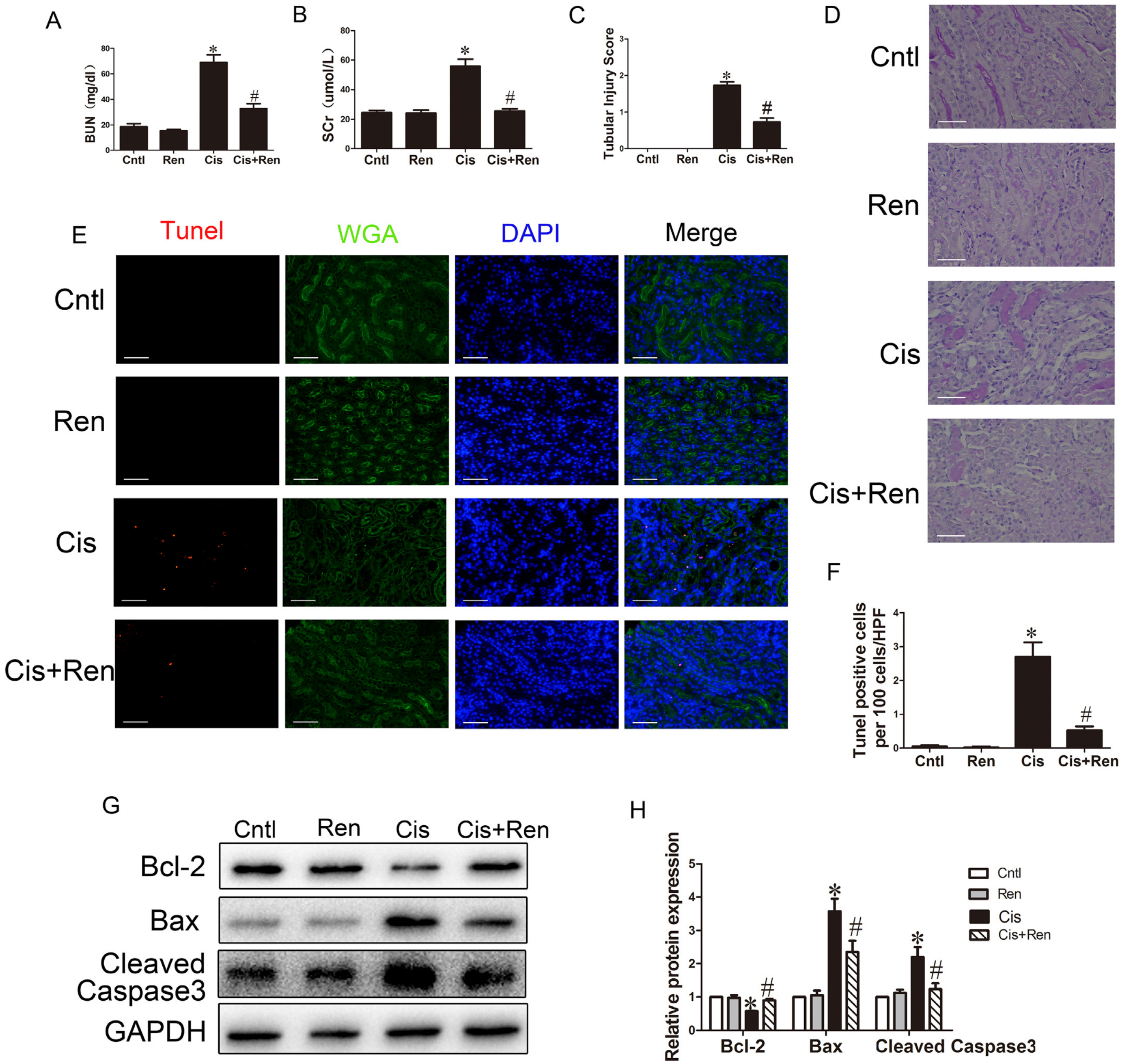

Fig. 1. Effect of renalase on cisplatin-induced AKI. Male C57BL/6J mice were separated into control (Cntl) group (n = 5), renalase (Ren) group (n = 5), cisplatin (Cis) group (n = 6), cisplatin pretreatment with renalase (Cis + Ren) group (n = 6). (A, B) Blood urea nitrogen and creatinine levels of mice 72 h after administration. (C) Tubular injury scores (range 0–4), based on the percentage of tubules affected (0: < 10%; 1: 10–25%; 2: 26–50%; 3: 51–75%; 4: > 75%). (D) Histological examination of periodic acid-Schiff (PAS) staining. (E) Representative photomicrographs of kidney TUNEL staining (red) in each group, and WGA co-staining (green). Nuclei were counterstained with DAPI (blue). (F) Numbers of apoptotic nuclei per 100 cells in each high power field. (G) Expression of Bcl-2, Bax and Cleaved Caspase3 measured by Western blot. (H) Quantification of Western blot results by densitometry. Scale bar, 50 μm. Values are presented as means ± SEM. $^*P < 0.05$ *vs.* control group, $\#P < 0.05$ *vs.* mice treated with cisplatin alone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Suppression of endogenous Sirt3 expression abrogated the effects of renalase on cisplatin-induced mitochondrial fission and cell apoptosis in vitro

Given the observation that the protection of renalase *in vivo* and *in vitro* was accompanied by an increase of Sirt3 expression, we investigated the role of Sirt3 in this process. Sirt3 expression was inhibited by Sirt3 siRNA (Fig. 6A). As shown in Fig. 6B, Bcl-2 decreased while Cleaved Caspase 3 increased in the Sirt3 siRNA transfected cells compared with scrambled RNA transfected cells in renalase and cisplatin treatment. Sirt3 siRNA transfected cells showed significantly higher mitochondrial fragmentation than the scramble siRNA transfected cells in response to renalase and cisplatin treatment (Fig. 6C, D). Furthermore, the flow cytometry (Fig. 6E, F) revealed that renalase reduced the number of apoptotic cells among the scramble RNA transfected cells treated with cisplatin, but Sirt3 siRNA abrogated the protective effect. We concluded that renalase was unable to protect against cisplatin-induced HK-2 cell injury in Sirt3 silencing group, whereas it performed a protective role in the negative control group.

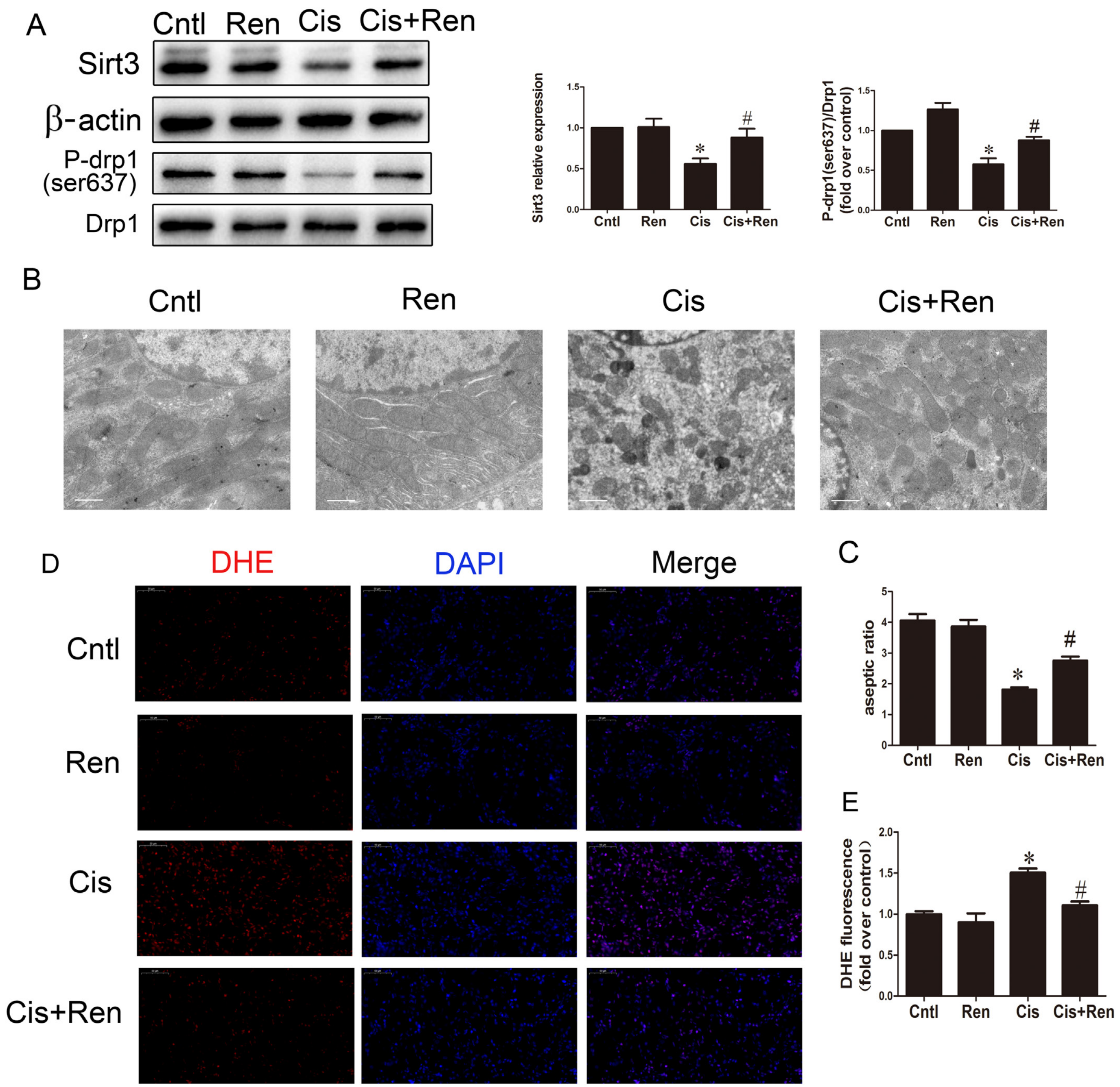

Fig. 2. Effect of renalase on mitochondrial dysfunction after 3-day exposure to cisplatin *in vivo*. (A) Western blot for Sirt3 and Drp-1 phosphorylation on ser637 expression. Quantification of protein levels was performed by densitometry. (B) Representative electron micrographs of the ultrastructure of proximal tubular cells from each group (scale bar, 1 μm). (C) Average aspect ratios in each group. (D) Representative images of DHE staining (red) to detect ROS production and DAPI staining (blue) to detect nuclei in the renal tissues (scale bar, 50 μm). (E) DHE fluorescence intensity analysis. Values from at least three independent experiments are presented as means ± SEM. $^*P < 0.05$ *vs.* Control group, $\#P < 0.05$ *vs.* Mice treated with cisplatin alone. Cntl, control group; Ren, renalase group; Cis, cisplatin group; Cis + Ren, cisplatin and renalase group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The present study confirmed that renalase prevented cisplatin-induced AKI. Renalase-deficient mice subjected to cisplatin suffered more severe renal injury [9], suggesting that endogenous renalase played a protective role in cisplatin-induced AKI. In this study, the assessments of plasma creatinine, blood urea nitrogen and renal tubular cell apoptosis revealed that pretreatment with exogenous renalase ameliorated the renal injury. As expected, our results demonstrated that *in vitro*, renalase had a protective ability to ameliorate cisplatin-induced apoptosis of HK-2 cells through promoting cell viability and reducing the apoptotic cell rate. Moreover, renalase inhibited cisplatin induced upregulation of Cleaved Caspase-3 expression and downregulation of Bcl-2 expression.

The production of ROS is responsible for cisplatin-induced renal injury [21]. Supplementation with antioxidants has been reported by a number of studies as a renoprotective strategy [22–25]. Renalase is protective in ischemic AKI [9], contrast-induced nephropathy [12], and subtotal nephrectomy [26]. Renalase can protect against renal fibrosis *via* inhibiting oxidative stress in unilateral ureteral obstruction rats

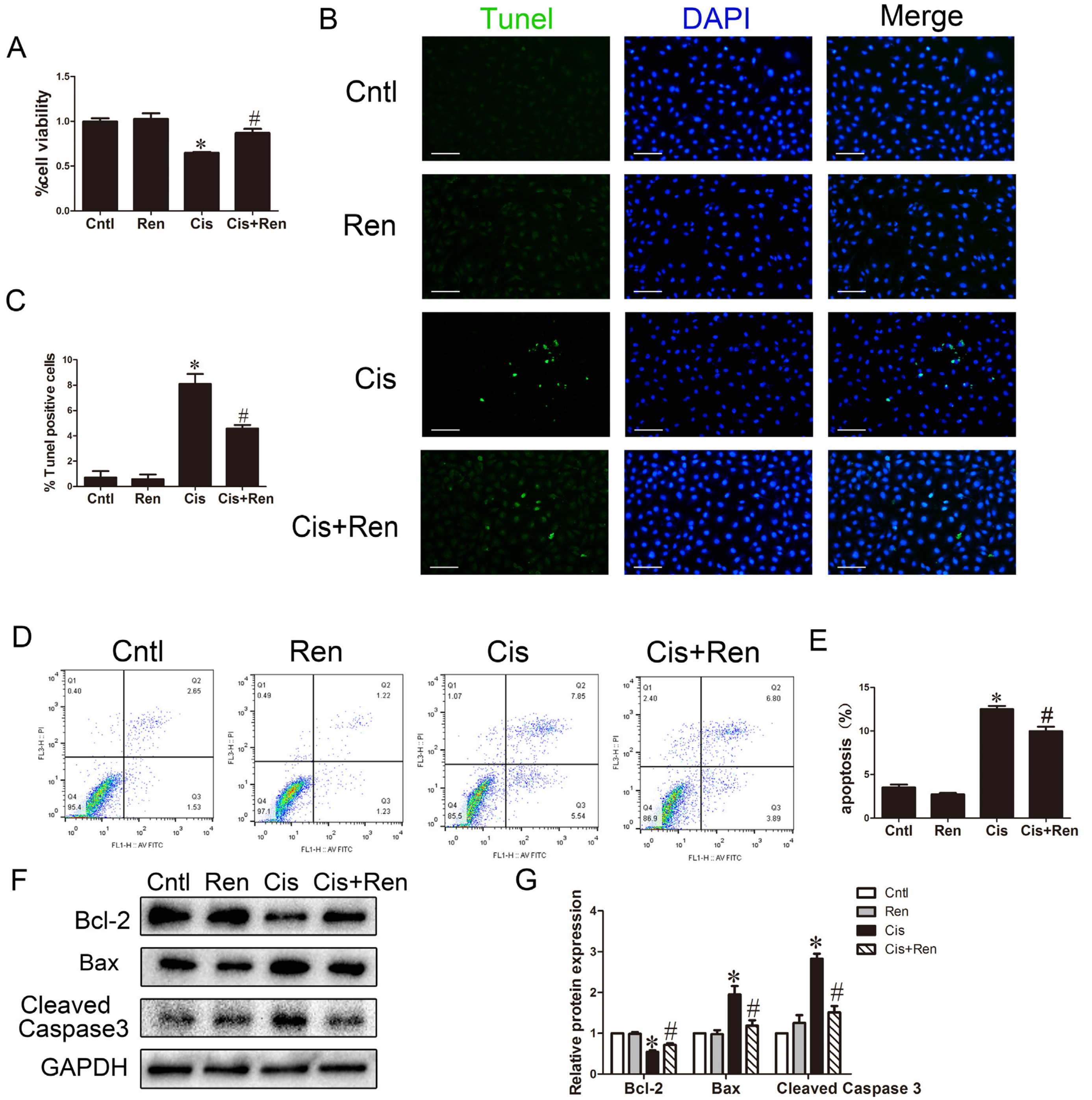

Fig. 3. Effect of renalase on HK-2 cells exposed to cisplatin. HK-2 cells were treated with 20 μM cisplatin for 16 h. (A) Cell viability detected by the CCK8 assay. (B) Representative photomicrograph of TUNEL staining (green) of HK-2 cells. Nuclei were counterstained with DAPI (blue). Scale bar 50 μm. (C) Numbers of apoptotic nuclei per 100 cells. (D) Representative pictures of apoptosis examined by flow cytometry. (E) Quantification of apoptotic cells. Percentage of apoptotic cells was the sum of (Q2 + Q3). (F) Expression levels of Bcl-2, Bax and Cleaved Caspase3 detected by immunoblotting. (G) Densitometry analysis for western blots. Data are expressed as means ± SEM (n = 3–6). $*P < 0.05$ *vs.* control group, $\#P < 0.05$ *vs.* cisplatin treatment group. Cntl, control group; Ren, renalase group; Cis, cisplatin group; Cis + Ren, cisplatin and renalase group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[27]. Furthermore, in contrast-induced rats with nephropathy, pretreatment with renalase can decrease renal MDA level, increase renal SOD level, and decrease ROS production *in vivo* [12]. Endogenous renalase can protect against contrast-induced nephropathy by limb ischemia preconditioning [11]. The reduced oxidative stress is abolished by silencing renalase with siRNA *in vivo*. In our present study, cisplatin-induced ROS production was inhibited by renalase both *in vivo* and *in vitro*. Moreover, renalase alleviated cisplatin-induced mitochondrial ROS production *in vitro*.

It has been demonstrated that mitochondrial damage is associated with ROS production and apoptosis [28,29]. Cisplatin can disrupt mitochondrial dynamics, resulting in mitochondrial fragmentation, cell injury and even death. Drp1, a mitochondrial fission protein, is translocated to mitochondria during tubular cell injury [17].

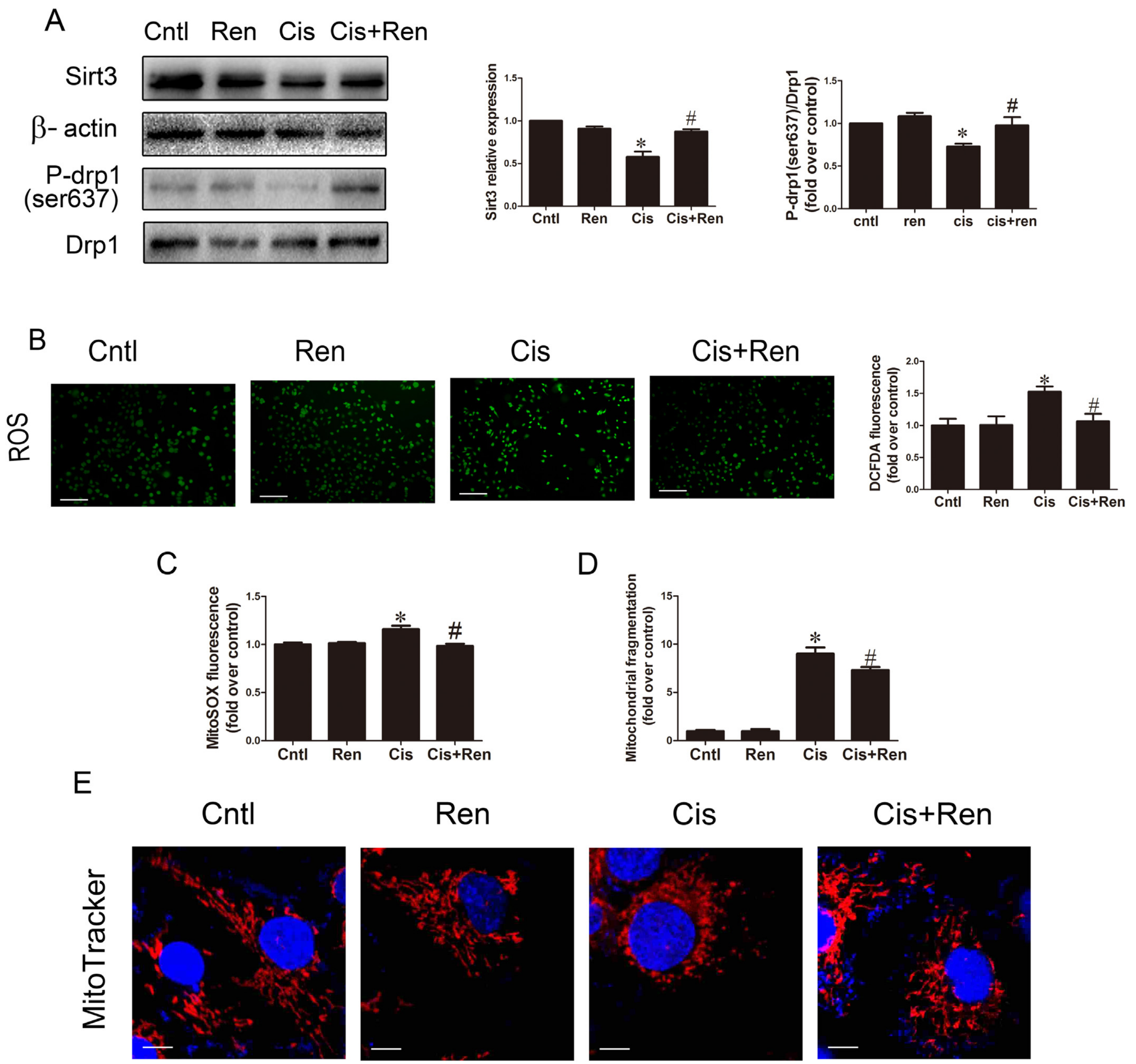

Fig. 4. Effect of renalase on cisplatin-induced mitochondrial dysfunction in HK-2 cells. (A) Sirt3 and Drp-1 phosphorylation on ser637 expression measured by Western blot. Left panel: representative blots. Right panel: qualification by densitometry. (B) HK-2 cells stained with DCFDA to detect (ROS production. Left panel: representative images by fluorescence microscope (scale bar 50 μm). Right panel: DCFDA fluorescence intensity analysis. (C) Measurement of mitochondrial superoxide with MitoSOX. (D) Percentage of cells with mitochondrial fragmentation. (E) Representative images of HK-2 cells mitochondrial morphology by using MitoTracker Red staining. Nuclei were counterstained with Hoechst 33342 (blue) (scale bar, 10 μm). The results from at least three independent cell experiments are presented as means ± SEM. *$P < 0.05$ *vs.* control group, #$P < 0.05$ *vs.* cisplatin treatment group. Cntl, control group; Ren, renalase group; Cis, cisplatin group; Cis + Ren, cisplatin and renalase group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Dephosphorylation of Drp1, especially in serine 637, regulates its translocation to mitochondria [30]. Our results further confirmed the significance of mitochondrial dynamics in the cisplatin-induced tubular injury. By assessing MitoTracker Red staining in the cisplatin-induced cell injury and electron microscopy in cisplatin-treated mice, we evidenced that renalase could ameliorate mitochondrial fission. Moreover, the increased phosphorylation of Drp1 at serine 637 and decreased Drp1 translocation to mitochondria confirmed the modulatory effect of renalase on mitochondrial dynamics.

Several lines of evidence have highlighted the role of Sirt3 in maintaining mitochondrial morphology *via* modulating mitochondrial fission and fusion [6,31]. Moreover, silencing Sirt3 increased tubular apoptosis and resulted in aggravated cisplatin nephrotoxicity [32]. Consistent with these observations, studies in renalase-deficient mice showed the number of apoptotic renal cells increased in cisplatin-treated knockout mice compared with wild-type ones [9]. Our study also showed that Sirt3 expression was reduced in the cisplatin group, while the expression level was restored in the group pretreated with renalase. In agreement with this result, mitochondrial fragmentation was also reduced when the restoration of Sirt3 was observed following the renalase treatment. *In vitro*, knockdown of Sirt3 almost had no beneficial effect in cisplatin-induced HK-2 cells pretreated with

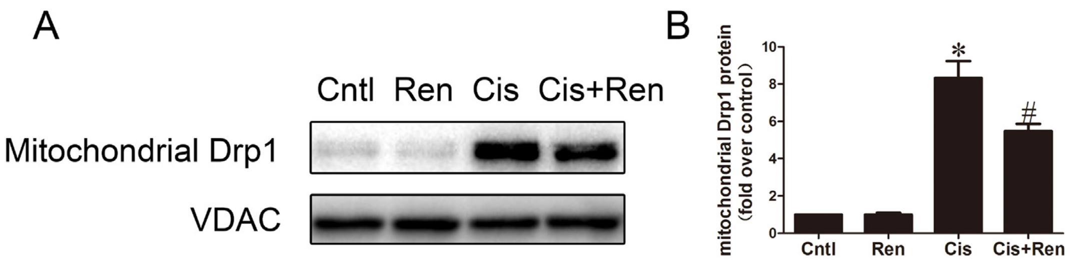

Fig. 5. Effect of renalase on Drp1 translocation. (A) Immunoblot analysis of Drp1 in mitochondrial fraction. (B) Densitometry analysis of (A). Each bar represents the means ± SEM of three independent cell experiments. *$P < 0.05$ *vs.* control group, #$P < 0.05$ *vs.* cisplatin treatment group. Cntl, control group; Ren, renalase group; Cis, cisplatin group; Cis + Ren, cisplatin and renalase group.

renalase. The results suggested renalase might protect against cisplatin-induced kidney injury by reducing mitochondrial fission and tubular epithelial cell apoptosis in a Sirt3-dependent manner.

Renalase in this study contains 18 to 342 amino acid with His-tag N-Terminus. Amino acid 220–239, which promotes cell survival [9], is conserved in the isoform of renalase in this study. It is similar to the renalase protein that exerts anti-fibrotic renal protection in the TGF-β1-stimulated HK-2 cells and partly inhibits oxidative stress [27,33], which ensures that the protective role discussed in this study is related to the role of cytokine. Ling Wang et al. identified that the extracellular renalase receptor PMCA4b [10], which was characterized as a plasma ATPase, could regulate local calcium concentration [34]. Calcium gain was a typical symptom of cisplatin-induced renal epithelial cell injury [35]. Additionally, Calcium participated in mitochondrial function and ROS production [36]. Sirt3 was shown to be associated with mitochondrial calcium in cortical neurons [37]. Early MAPK signal

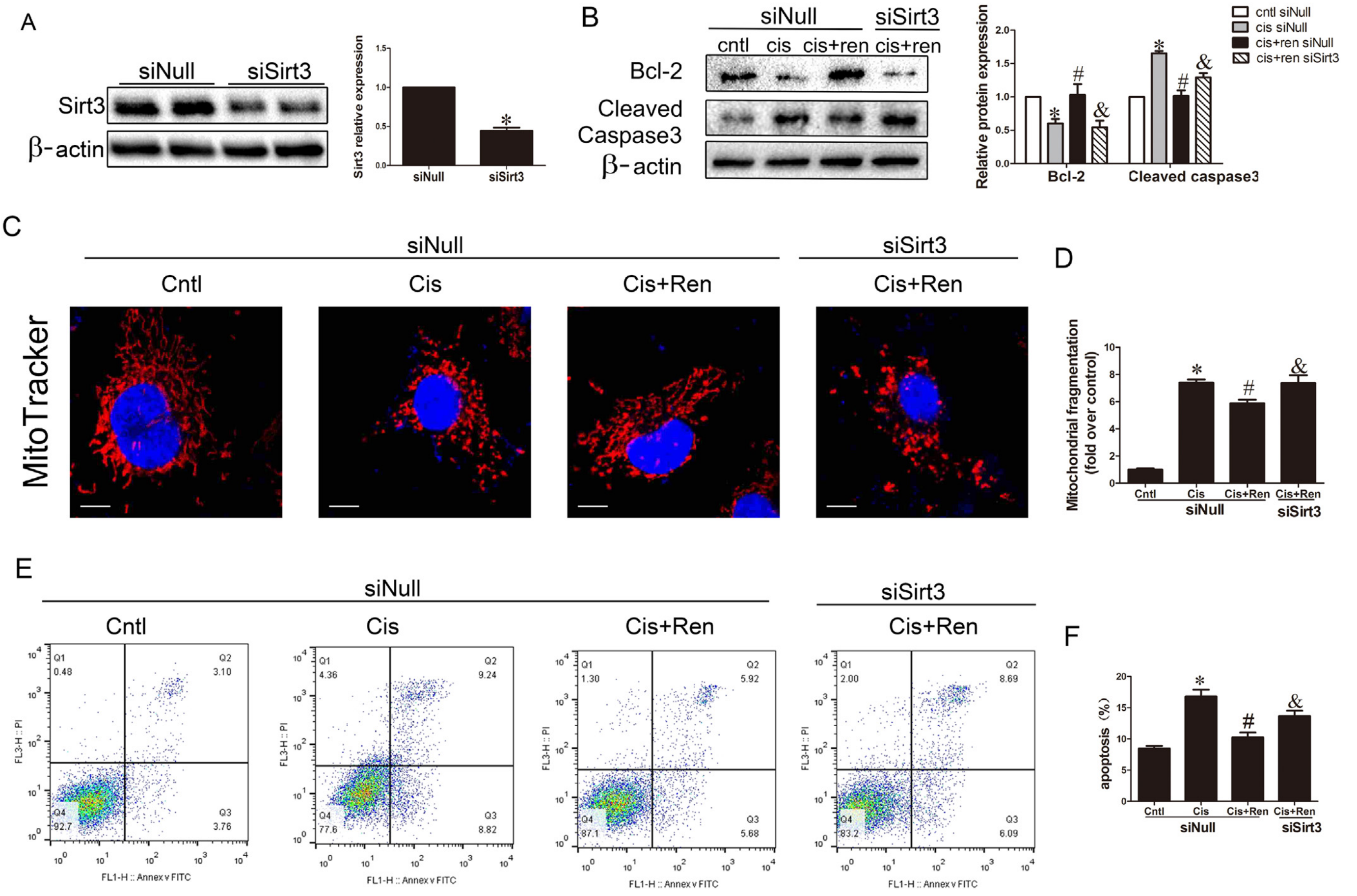

Fig. 6. Role of Sirt3 in renalase protection against cisplatin-induced HK-2 cells injury. HK-2 cells transfected with Sirt3 siRNA (siSirt3) or scrambled siRNA (siNull). (A) Western blot (left panel) and densitometric analysis (right panel) of Sirt3 protein expression after transfection for 24 h. (B) Exposure of transfected cells to medium or 20 μM cisplatin for 16 h in the presence or absence of renalase (5 μg/ml), Bcl-2 and cleaved casepase3 expressions analyzed by western blot (left panel) and densitometric analysis (right panel). (C) Representative images of HK-2 cells stained with MitoTracker Red of the transfected cells. Nuclei were counterstained with Hoechst 33342 (blue) (scale bar, 10 μm). (D) Quantification of mitochondrial morphology. (E) Representative graphs showing results of the transfected cells apoptosis by annexin-V-FITC and PI staining. (F) Quantification of apoptotic cells by flow cytometry. Percentage of apoptotic cells was calculated by the sum of (Q2 + Q3). Data from at least three independent cell experiments are presented as means ± SEM. *$P < 0.05$ *vs.* control group transfected with scramble siRNA, #$P < 0.05$ *vs.* cisplatin treatment group transfected with scramble siRNA. & $P < 0.05$ *vs.* scramble transfected cells exposed to cisplatin in the presence of renalase. Cntl, control group; Ren, renalase group; Cis, cisplatin group; Cis + Ren, cisplatin and renalase group; siNull, scramble siRNA; siSirt3, Sirt3 siRNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

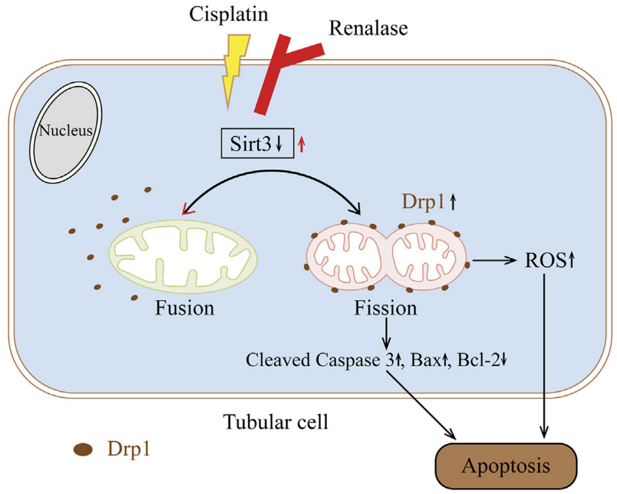

Fig. 7. Molecular mechanism of renalase's protection in cisplatin-induced acute kidney injury.

activation was a key factor in protecting renalase peptide against cisplatin toxicity [10]. However, the mechanism underlying early MAPK signal activation and mitochondrial function is not yet clear and needs further study (Fig. 7).

5. Conclusion

In conclusion, this study shows that renalase can protect renal epithelial cells in cisplatin-induced AKI by promoting mitochondrial dynamics and inhibiting ROS production in a Sirt3-dependent manner.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.02.042.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Author contributions

Zhimin Huang, Qing Li, Chengning Zhang, Lin Wu, Xi Liu, Wei Cao performed the experiments; Honglei Guo, Suyan Duan, Xueqiang Xu analyzed the data; Yanggang Yuan and Bo Zhang drafted the manuscript; Changying Xing edited and revised the manuscript; All approved the final version of the manuscript.

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