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RESEARCH ARTICLE

Salvianolic acid A ameliorates renal ischemia/reperfusion injury by activating Akt/mTOR/4EBP1 signaling pathway

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Song Y, Liu W, Ding Y, Jia Y, Zhao J, Wang F, Bai J, Cheng L, Gao K, Liu M, Yao M, Li L, Zhang Y, Wen A, He L. Salvianolic acid A ameliorates renal ischemia/reperfusion injury by activating Akt/mTOR/4EBP1 signaling pathway. *Am J Physiol Renal Physiol* 315: F254–F262, 2018. First published January 31, 2018; doi:10.1152/ajprenal.00508.2017.—Salvianolic acid A (Sal A) has been shown to prevent and treat ischemic cardiovascular, as well as cerebral vascular diseases. However, little is known about Sal A in renal ischemia/reperfusion (I/R) injury. In this study, a renal I/R injury model in rats and a hypoxia/reoxygenation (H/R) model to damage proximal renal tubular cells (HK-2) were used to assess whether Sal A halts the development and progression of renal I/R injury. As compared with vehicle treatment, Sal A significantly attenuated kidney injury after renal I/R injury, accompanied by decreases in plasma creatinine, blood urea nitrogen levels, the number of apoptosis-positive tubular cells, and kidney oxidative stress. Sal A also activated phosphorylated protein kinase B (p-Akt) and phosphorylated-mammalian target of rapamycin (p-mTOR) compared with vehicle-treated I/R injury rats. In H/R-injured HK-2 cells, Sal A can reduce the levels of reactive oxygen species in a dose-related manner. Similar to the results from in vivo experiments, in vitro Sal A also increased the protein expression of phosphorylated-eukaryotic initiation factor 4E binding protein 1 (p-4EBP1) compared with vehicle. Furthermore, the cytoprotective activity of Sal A was inhibited by LY294002 and rapamycin. These findings indicate that Sal A can ameliorate renal I/R injury and promote tubular cell survival partly via the Akt/mTOR/4EBP1pathway. Sal A could be a candidate compound to prevent ischemic tissue damage.

INTRODUCTION

Renal ischemia/reperfusion injury is a leading cause of acute kidney injury, which is still associated with high morbidity, mortality, and increased costs of treatment in both the adult and pediatric population (15, 16). The pathogenic mechanism underlying renal ischemia/reperfusion (I/R) injury is complicated, involving free radicals, inflammatory medium, calcium overload, cell apoptosis, as well as energy metabolism dysfunction (33). Because of this complex pathogenesis and the lack of effective drugs, it is imperative to investigate novel drug therapies to attenuate the development and progression of renal I/R injury.

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Several epidemiological studies have shown a correlation between a diet rich in polyphenols and the prevention of various human diseases (8, 26, 27). Polyphenols, a large group of natural compounds ubiquitously found in plants, are current research hotspots. Polyphenols possess many biological activities, including antioxidant, antiapoptotic, anti-inflammatory, antimicrobial, antiviral, and anticancer properties. Reperfusion damage is thought to be partly due to oxidative stress, which could be partially prevented by antioxidants and free radical scavengers (7). Apoptosis is a significant cellular mechanism responsible for I/R injury in the myocardium, and oxidative stress is a well-known factor in promoting apoptosis (5, 40). Therefore, a reduction of oxidative stress could be an effective therapy for the attenuation of renal I/R injury, making polyphenols an ideal candidate, as they possess antioxidant properties and are ubiquitously found in plants.

Salvianolic acid A (Sal A) (Fig. 1*A*), a polyphenolic compound, is one of the active components found in the aqueous extract of the *Salvia miltiorrhiza* root (also known as DanShen) (22), an herbal medicine commonly used to I/R-related disease. Studies have shown that Sal A possesses a variety of pharmacological actions for the treatment of I/R, including antioxidant (9, 24), anti-inflammatory (4, 28), antiplatelet (11), antifibrotic (25), and antiapoptotic effects (4). In a rat model, Sal A had significant protective effects against I/R-induced myocardial injury through its antioxidant activity (39). Sal A was also found to upregulate B-cell lymphoma 2 (Bcl-2) expression and increase the phosphorylation of proteins such as Akt to protect against myocardial I/R injury (10). In vivo, Sal A can protect retinal pigment epithelial cells against oxidative stress via activation of the Akt/mammalian target of rapamycin (mTOR) signaling pathway (38). However, its effect and mechanism in renal I/R injury have not been clearly investigated.

The aim of the present study was to investigate the effects of Sal A in a renal I/R model to elucidate whether pretreatment with Sal A before ischemia would attenuate renal I/R injury and to elucidate possible underlying mechanism(s) of action.

MATERIALS AND METHODS

Animals. The animal experiments in this study were approved by the Animal Care and Ethics Committee of Fourth Military Medical University. Male adult Sprague-Dawley rats (Department of Laboratory Animal Science, Fourth Military Medical University, Xi'an, China), weighing 250–300 g, were used in the present study. All animal welfare and experimental procedures were carried out in strict

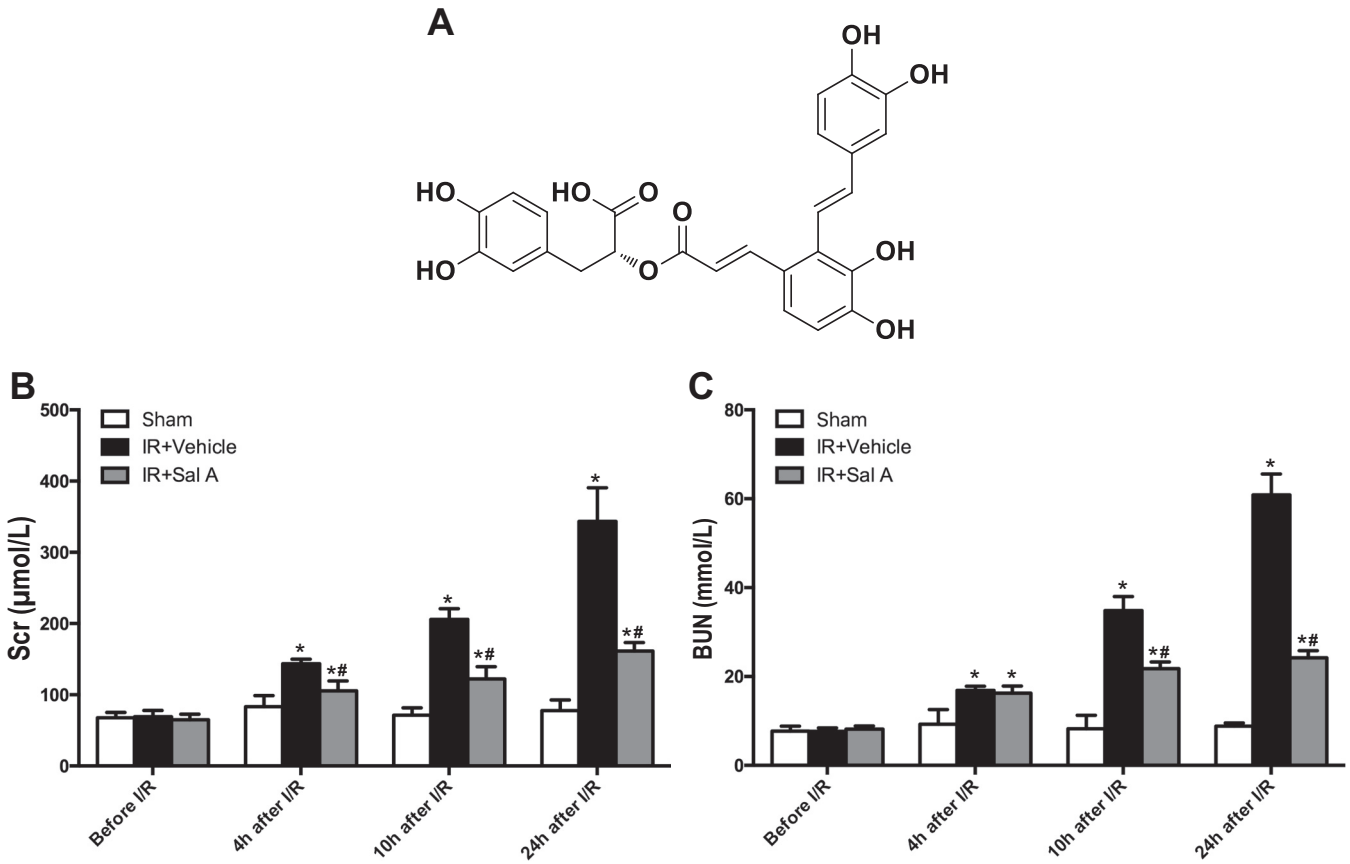

Fig. 1. Effects of Sal A on ischemia/reperfusion (I/R)-induced renal injury. *A*: chemical structure of Sal A. *B*: level of serum creatinine (Scr) levels at 4, 10, and 24 h after I/R was abrogated by Sal A (40 mg/kg ip) ($n = 10$ rats/group). *C*: level of blood urea nitrogen (BUN) at 4, 10, and 24 h after reperfusion after I/R was abrogated by Sal A (40 mg/kg ip) ($n = 10$ rats/group). *$P < 0.05$ vs. Sham group. #$P < 0.05$ vs. I/R + Vehicle group.

accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85–23, National Academy Press, Washington, DC, rev. 1996). All experimental rats were kept in an environmentally controlled breeding room for 5 days before experiments and fed with standard laboratory food and water.

Chemicals. Sal A (97599) (≥98% by HPLC) and 4′,6-diamidino-2-phenylindole (DAPI) (9542) were obtained from Sigma (St. Louis, MO). CM-H2DCFDA (C6827) and the MTT assay kit were procured from Thermo Fisher Scientific (Waltham, MA). Malondialdehyde (MDA) (S0131) and superoxide dismutase (SOD) (S0060) kits were obtained from Beyotime Bio-technology (Jiangsu, China). A terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) assay kit (ApoBrdu DNA fragmentation assay kit) (K403-50) was procured from Biovision (Milpitas, CA). Rapamycin (no. 9904), LY294002 (no. 9901), and monoclonal antibodies specific for phosphorylated-Akt (p-Akt, Ser-473; no. 4060), Akt (no. 4685), phosphorylated-mTOR (p-mTOR, Ser-2448; no. 5536), mTOR (no. 2983), eukaryotic initiation factor 4E binding protein 1 (4EBP1; no. 9644), phosphorylated-4EBP1 (p-4EBP1, Thr-70; no. 9455), and GAPDH (no. 5174) were obtained from Cell Signaling Technology (Danvers, MA). Bcl-2 (sc-7382) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while anti-active caspase-3 (ab2302) was purchased from Abcam (Cambridge, UK). Secondary antibodies goat anti-rabbit (621140380011730) and goat anti-mouse (621140680011730) were procured from Merck Genei (Karnataka, India). HK-2 cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). DMEM and other cell culture supplies were purchased from Gibco (Grand Island, NY). All materials for SDS-PAGE were obtained from Bio-Rad Laboratories (Hercules, CA). Hypoxic/ischemic chambers were obtained from Billups-Rothenberg (San Diego, CA). All other chemicals used were of analytical grade and were purchased from Sigma.

Rat model of renal I/R injury. All rats were anesthetized with sodium chloral hydrate (85 mg/kg ip) and placed in a prone position on a warming pad at 37°C to perform surgical procedures. The sham-operated groups (sham group) were only subjected to the removal of the right kidney, whereas rats in I/R, I/R+Sal A, and I/R+Rapa+Sal A groups were also subjected to acute I/R injury to the left kidney, which was induced by clamping the renal artery for 45 min using nontraumatic vascular clips. The clamp was then removed for reperfusion. Blood was collected from the eye socket at 4, 10, and 24 h postreperfusion, and the left kidney was removed at 24 h. The rats were euthanized by decapitation and exsanguination at 24 h after the I/R procedure. The kidney and blood were collected for further analyses.

Rat experimental design. Forty rats were randomly divided into four groups: *1*) a sham-operated group (sham group: $n = 10$) treated with tail vein injection of saline; *2*) an I/R group treated with tail vein injection of saline (I/R+Vehicle group, $n = 10$); *3*) an I/R group treated with tail vein injection of Sal A (I/R+Sal A group, $n = 10$); and *4*) an I/R group (I/R+Rapa+Sal A group: $n = 10$) were administered intraperitoneally with rapamycin (Rapa: 1.5 mg/kg body wt) 24 h before the I/R procedure. Sal A was administered to animals in the same manner as the I/R+Sal A group. Sal A (40 mg/kg, dissolved in normal saline) or the same volume of saline was injected intravenously into the tail vein 30 min before surgery. The rat model of renal I/R injury and the surgical procedures involved were similar to those previously described (1, 14).

To clarify the effect of Sal A on non-I/R injury rats, we performed an experiment in which Sal A was injected into the tail vein of the

sham group ($n = 10$). And the protein expression (Akt and mTOR) in the renal tissue was compared with the sham group ($n = 10$) treated with saline at 24 h after injection.

In addition, we performed another experiment in which Sal A was injected into the tail vein after I/R ($n = 10$), and renal function (plasma creatinine and urea nitrogen) was assessed and compared with the I/R group ($n = 10$) at 24 h of reperfusion.

Cell culture and treatment. HK-2 cells, a human proximal tubular cell line, were cultured in DMEM/F12 (1:1) and were passaged every 2–3 days in 100-mm dishes supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were grown at 37°C in a humidified 5% CO_2 atmosphere. To mimic ischemia-like conditions in vitro, HK-2 cells were exposed to ischemia by replacing the medium with an "ischemic buffer" (5 mM HEPES, 137 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, and 1.5 mM $CaCl_2$, pH 7.0). The cells were then incubated in a hypoxic/ischemic chamber at 37°C for 60 min in a humidified atmosphere of 5% CO_2 and 95% N_2. Finally, the cells were incubated again in the culture medium in an incubator with 95% air and 5% CO_2 for an additional 24 h, as previously described (14, 18, 20, 21). For all experiments, cells were plated at an appropriate density, according to the experimental design and grown for 24 h before treatment. Five sets of experiments were performed: *1*) for the Control Group, cells were treated with PBS; *2*) for the Hypoxia/Reoxygenation (H/R) Group, cells were treated as described above to mimic ischemia-like conditions; *3*) for the Sal A+H/R Group, cells were treated with 40 μM Sal A for 1 h and then treated with the H/R condition; *4*) for the RA+Sal A+H/R group, cells were treated with rapamycin (100 nM) for 1 h and 40 μM Sal A for 1 h and then treated with the H/R condition; and *5*) for the LY+Sal A+H/R group, cells were treated with an inhibiter LY294002 (20 μM) for 1 h and 40 μM Sal A for 1 h, and then treated with the H/R condition.

Assessments of biochemical parameters. Blood was collected using retro-orbital puncture at 4, 10, and 24 h following reperfusion. Serum was separated by centrifugation at 2,700 *g* and at 4°C, and serum creatinine (Scr) and blood urea nitrogen (BUN) levels were determined by staff at the Clinical Laboratory of Xijng Hospital, who were blinded to the treatments given. Blood samples were stored at −80°C for analyses.

Histological analyses. Staff at the Pathology Department of Xijng Hospital, who were blinded to the treatments given, performed the morphological assessments. The left kidney was fixed and embedded, and each paraffin-embedded kidney was cut into 3-μm sections and subjected to hematoxylin-and-eosin staining, as previously reported (14). The histopathological changes in the cortex and medulla were evaluated by a pathologist in a blinded fashion on a scale from 0 to 5: 0, none; 1: 0–10%; 2: 11–25%; 3: 26–45%; 4: 46– 75%; and 5: 76–100%, as previously described (35).

Determination of apoptosis. Renal cell apoptosis was detected using a TUNEL assay with tissue paraffin blocks. Renal slides were incubated with TUNEL reaction mixture in a humidified chamber for 60 min at 37°C in the dark. The renal sections were then rinsed three times in PBS, and the nuclei were mounted with DAPI (300 nM). Apoptosis was quantified by calculating the percentage of TUNEL-positive nuclei out of the total nuclei in an average of 20 high-power fields for each section in a blinded manner.

Measurements of oxidative stress markers. Dissected kidneys were immediately rinsed in ice-cold PBS. Tissues were homogenized in 10% 150 mM phosphate buffer (pH 7.4) (1/10 wt/vol). The homogenate was centrifuged at 6,000 *g* for 10 min at 4°C. The total protein level in the supernatant was measured using a BCA protein assay reagent kit. The concentrations of MDA and SOD in renal tissue were measured using commercial kits, according to the manufacturer's instruction. The final levels of MDA and SOD were normalized to the protein concentration of kidney tissue homogenate, as previously described (34).

Assessment of cell viability. Cell viability was measured using the cell viability assay kit (MTT) assay. HK-2 cells were seeded in 96-well plates (5×10^3 cells/well) at 37°C in a 5% CO_2 incubator in DMEM/F12. Following overnight incubation, the cells were incubated in the presence or absence of rapamycin or LY294002 and subsequently treated Sal A for 1 h before exposure to the H/R condition. The final incubation of the cells with 10 μl of kit reagent was performed for 45 min at 37°C. The absorbance was measured at 460 nm using a microplate reader (Bio-Rad Laboratories). Cell viability was calculated and averaged. The cells from the control group were treated in the same manner without exposure to the H/R condition, and cell viability was expressed as a percentage of the untreated controls.

Assessment of reactive oxygen species production in HK2 cell. CM-H_2DCFCA, a reactive oxygen species (ROS)-sensitive fluorescent dye, was used to measure ROS levels. To evaluate the effect of Sal A on I/R injury-induced ROS production, HK-2 cells were cultured in 96-well plates. Cells were washed twice with PBS, incubated with 5 mM CM-H_2DCFCA for 30 min in Dulbecco's PBS, and again washed three times with PBS. Fluorescence intensities were measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a microplate fluorescence reader.

Western blot analysis. Protein extraction and Western blot analysis were done as previously described (14). Cell or renal lysates (50 μg) were separated on polyacrylamide SDS gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk for 1 h and then incubated with antibodies with optimal dilutions against Bcl-2, cleaved caspase-3, p-Akt (Ser-308), Akt, p-mTOR (Ser-2448), mTOR, p-4EBP1 (Thr-70), or 4EBP1 overnight at 4°C. After six washes with PBS-Tween (each for 5 min), the membranes were probed with an horseradish peroxidase-conjugated secondary antibody. Blots were developed using enhanced chemiluminescent reagents (Thermo Fisher Scientific), and blotting signals were visualized by a ChemiDoc MP System (Bio-Rad Laboratories). GAPDH was used for normalization, and the intensity of each band was analyzed using the densitometry plugin on ImageJ (https://imagej.nih.gov/ij/) as instructed.

Statistical analysis. Data are expressed as means ± SD. Numerical data are presented as means ± SD from at least three individual experiments. All statistical analyses were performed using the SPSS 16.0 software package. Comparisons between the two groups were performed using an independent-sample *t*-test. Experiments with more than two groups were compared by ANOVA followed by the Tukey post-hoc test. P values <0.05 were considered statistically significant.

RESULTS

Sal A provides protection against renal I/R injury in vivo. To assess the effect of Sal A pretreatment on renal I/R injury, rats were administered with Sal A, followed by I/R insult (I/R+Sal A Group). In addition, rats were pretreated with vehicle (I/R Group) or had undergone similar surgical processes absent of I/R insult (sham group). I/R insult led to a significant impairment of renal function. Renal I/R injury led to a significant increase in the levels of Scr and BUN in a time-dependent manner following reperfusion (Fig. 1, *B* and *C*). Sal A-pretreated rats displayed well-preserved renal function, as manifested by significantly lower levels of serum BUN (Fig. 1*B*) and Scr (Fig. 1*C*), as compared with rats from the I/R+Vehicle Group. Twenty-four hours after renal reperfusion, rats in the I/R+Vehicle group developed significant renal dysfunction indicated by an increase in Scr (343.45 ± 47.08) and BUN (60.85 ± 4.73) levels. Rats pretreated with Sal A did not

exhibit such a significant increase in Scr (161.36 ± 11.90) and BUN (24.21 ± 1.58) levels.

In addition, to confirm these observations, histological analyses of renal sections were performed. Compared with the sham group, rats in the I/R group showed significant pathological changes, as evidenced by severe tubular necrosis, dilation, and cast formation (Fig. 2*A*). Meanwhile, the pathological score of histological lesions was significantly lower ($P < 0.05$) in Sal A-treated I/R rats (2.53 ± 0.68) than untreated I/R rats (3.87 ± 0.54) at 24 h after reperfusion. These histopathological alterations were moderately reduced in I/R+Rapa+Sal A group. The renal histopathological score of all the rats are presented in Fig. 2*B*.

In the Sal A treatment after I/R experiment, the Sal A-treated groups also showed lower Scr (I/R: 343.3 ± 47.1; Sal A after I/R: 285.6 ± 76.8 μmol/l), and BUN (I/R: 60.3 ± 4.5; Sal A after I/R: 45.6 ± 9.7 mmol/l).

HK-2 cells were also pretreated with Sal A (10, 20, and 40 μM) for 1 h, after which, they were exposed to the H/R condition to mimic ischemic-like conditions in vitro. Exposure to the H/R condition resulted in 43.03 ± 4.21% cell death. However, cell viability was significantly increased (85.12 ± 5.37%) when cells were pretreated with Sal A (40 μM) (Fig. 2*C*). Altogether, the data support that administration of Sal A may attenuate I/R-induced renal damage.

Sal A prevents I/R-induced tubular apoptosis. Tubular apoptosis is a crucial event in I/R-induced renal injury and drives the severity of tissue damage (6, 31). To determine whether the beneficial effects of Sal A on renal I/R injury were associated with the inhibition of apoptosis, TUNEL assays were performed on kidney sections. Similar to immunohistochemistry results, TUNEL-positive cells were almost absent in the sections of mice from the sham group, while a substantial increase of apoptotic cells was noted in I/R+Vehicle rats (Fig. 3*A*). However, I/R+Sal A rats manifested a threefold reduction of apoptotic cells as compared with I/R+Vehicle rats (Fig. 3*B*), indicating that Sal A pretreatment prevented I/R-induced tubular apoptosis. Furthermore, the expression of Bcl-2 and cleaved caspase-3 was also examined. Similarly, I/R led to a substantial decrease in the expression of Bcl-2 and an increase in active caspase-3 activity (Fig. 3*C*). Our data show that Bcl-2 expression was restored to nearly normal levels by Sal A preconditioning, while cleaved caspase-3 expression was dramatically suppressed. In vitro, Sal A pretreatment in HK-2 also suppressed H/R-induced activation of caspase-3 (Fig. 3*D*). Taken together, Sal A administration mitigated renal cell apoptosis in I/R injury rats.

Sal A reduces I/R-induced ROS accumulation. To explore whether the renoprotection conferred by Sal A in I/R injury was associated with oxidative stress, related markers of oxida-

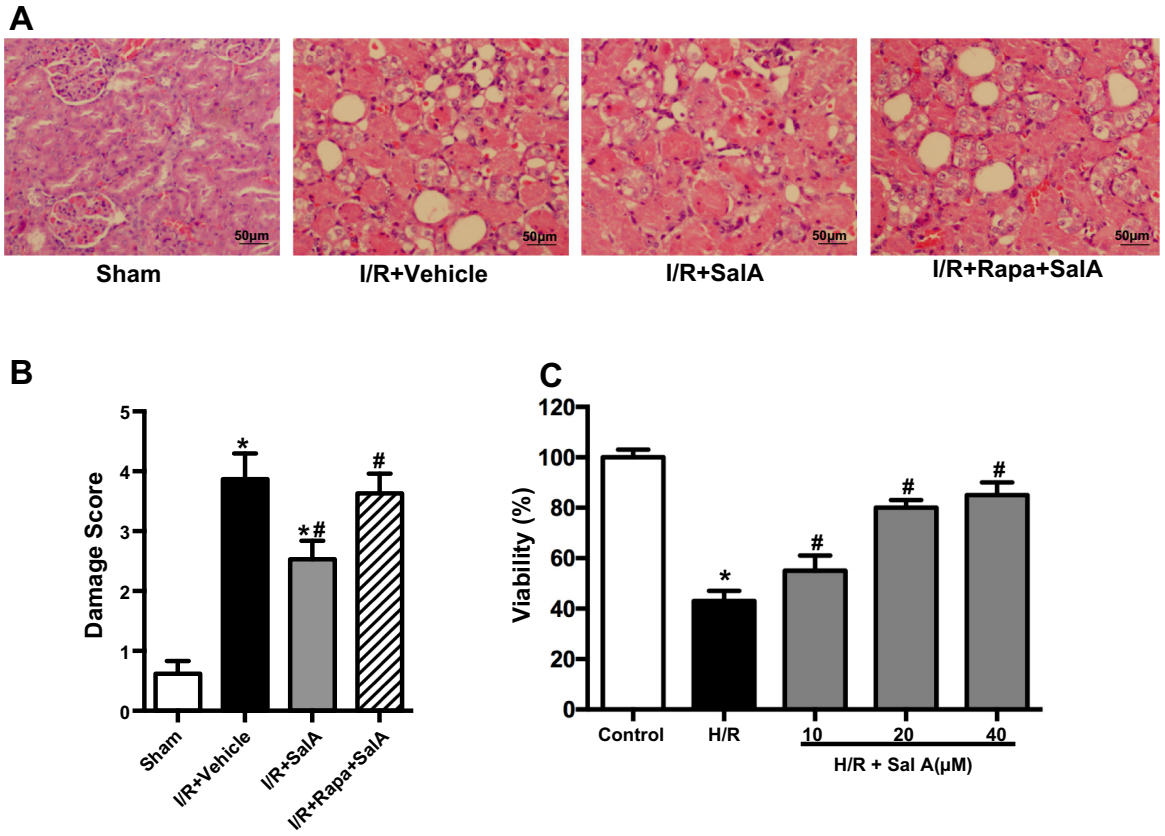

Fig. 2. Sal A pretreatment mitigated renal histological injury in I/R injury rats. Rats were challenged with a sham operation or 45 min of renal ischemia, respectively. Kidney tissues were harvested at 24 h after reperfusion. Periodic acid-Schiff (PAS) staining and a semiquantitative scoring system were used to evaluate the severity of tubular injury. *A*: representative photomicrographs (hematoxylin and eosin staining; magnification, ×200) of renal sections from rats under various experimental conditions. *B*: semiquantitative assessment of tubular injury. All data were presented as means ± SD ($n = 6$); *$P < 0.05$ vs. Sham; #$P < 0.05$ vs. I/R+Vehicle. *C*: protective effect of Sal A (10, 20, and 40 μM) against the renal H/R-induced loss of cell viability. Cells in the control group were considered 100% viable. Data are presented as the means ± SD ($n = 3$). *$P < 0.01$ vs. Control group. #$P < 0.01$ vs. H/R group.

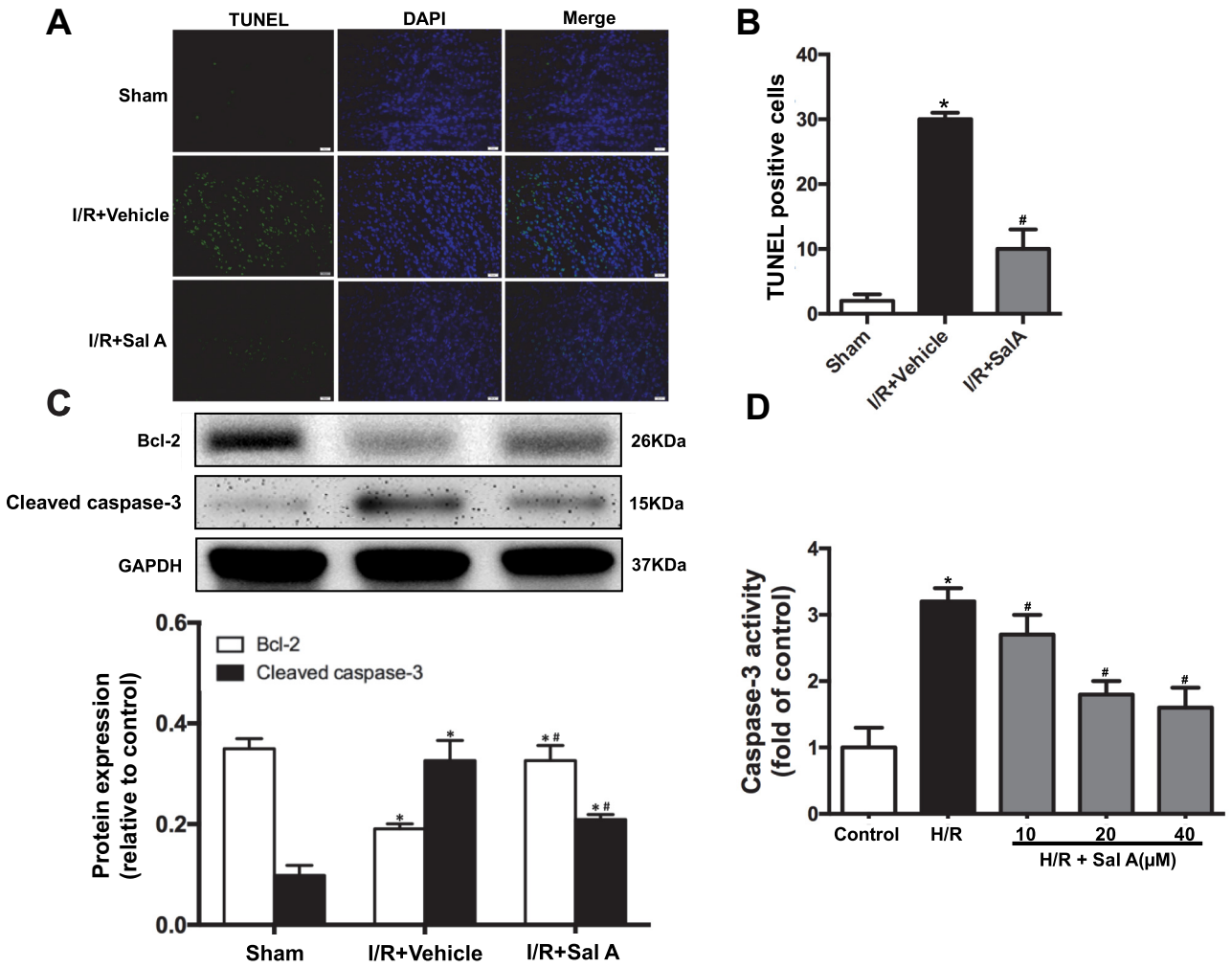

Fig. 3. Sal A ameliorated tubular apoptosis induced by renal I/R. *A*: representative results for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays of renal sections (magnification ×400). *B*: quantitative analysis of TUNEL-positive cells as means ± SD of 10 rats analyzed for each group. *C*: renal Bcl-2 and active caspase-3 protein expression. *$P < 0.05$ vs. Sham. #$P < 0.05$ vs. I/R + Vehicle. *D*: caspase-3 activity in HK-2 cells. Data are presented as means ± SD and are representative of three independent experiments; *$P < 0.01$ vs. Control. #$P < 0.01$ vs. H/R.

tive stress were examined. MDA is a naturally occurring product of lipid peroxidation and an indicator of ROS production. MDA levels were increased in I/R injury rats compared with the sham group. Pretreatment with Sal A decreased renal MDA levels. The activity of SOD, a critical enzyme responsible for the detoxification of ROS, was then analyzed. Surprisingly, I/R injury resulted in a significant reduction of SOD activity, and pretreatment with Sal A restored renal SOD levels (Fig. 4*B*). To determine whether Sal A reduces I/R-induced ROS production, HK-2 cells were incubated with increasing concentrations of Sal A in the presence or absence of the H/R condition. Subsequently, ROS were detected quantitatively using a fluorescence microplate reader. The H/R condition resulted in a significant increase in intracellular ROS production, which was reduced by Sal A pretreatment (Fig. 4*C*), suggesting that Sal A can attenuate oxidative stress from two directions in rats with I/R injury.

Sal A activates the Akt/mTOR/4EBP1 pathway in renal I/R injury. Cellular proliferation and survival require the involvement of mTOR and phosphoinositide 3-kinase (PI3-K)/Akt pathways (30). Therefore, whether the Akt/mTOR pathway is involved in the renoprotection provided by Sal A was investigated. Interestingly, I/R injury significantly suppressed p-Akt and p-mTOR proteins, while Sal A pretreatment significantly attenuated this phenomenon. The higher levels of p-Akt and p-mTOR (Fig. 5*A*) were also noted in Sal A-pretreated rats compared with I/R+Vehicle rats.

In the presence of Rapa (an inhibitor of mTORC1), phosphorylation of mTOR, 4EBP1 was significantly lower compared with I/R+Sal A group. (Fig. 5*B*). In addition, Sal A did not affect significantly the protein expression of p-Akt and p-mTOR on non-I/R-injured rats (data not shown).

To confirm the above results, HK-2 cells were cultured under the H/R condition in the presence or absence of Sal A, as described earlier. As is known, mTOR phosphorylates and activates 4EBP1, which serves as a marker for mTOR activity. The expression of phosphorylated mTOR and its downstream target p-4EBP1 were assessed. Induction of the H/R condition almost blocked the expression of p-mTOR and p-4EBP1, while Sal A (40 μM) significantly restored the expression of these proteins in the presence of the H/R condition (Fig. 5*C*). We hypothesized that Akt/mTOR/4EBP1 activation is responsi-

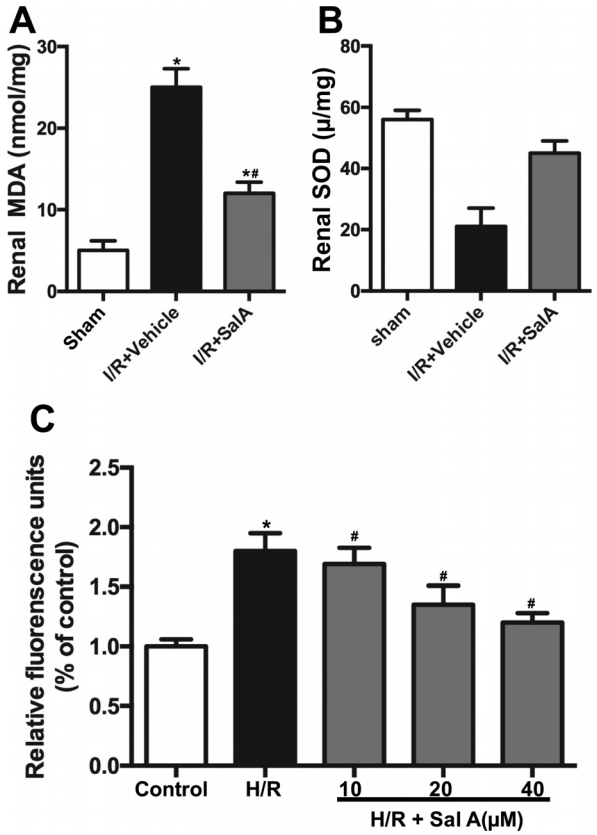

Fig. 4. Effects of Sal A on oxidative stress level in renal tissues and a human proximal tubular (HK2) cells. Kidney tissues were harvested at 24 h after reperfusion. *A*: renal malondialdehyde (MDA) levels. *B*: renal superoxide dismutase (SOD) levels. All data were presented as means ± SD ($n = 3$). *$P < 0.01$ vs. Sham. #$P < 0.01$ vs. I/R+Vehicle. *C*: ROS production. HK-2 cells were pretreated with Sal A (10, 20, 40 μg/ml) 1 h before exposure to hypoxia for 60 min, and then the cells underwent 30 min of reoxygenation. Data are presented as means ± SD and are representative of three independent experiments; *$P < 0.01$ vs. Control; #$P < 0.05$ vs. H/R.

ble for the increased cell survival in response to the H/R condition and, thus, performed inhibitory experiments using rapamycin. Rapamycin completely reversed Sal A-mediated mTOR/4EBP1 phosphorylation in response to the H/R condition (Fig. 5*C*). Furthermore, increased expression of p-mTOR and p-4EBP1 by Sal A during exposure to the H/R condition was blocked following the application of the inhibitor LY294002 (20 μM) (Fig. 5*D*). In addition, a considerable decrease in cell survival was found when rapamycin (77.8% of Sal A+H/R group, $P < 0.01$) or LY294002 (75.6% of Sal A+H/R group, $P < 0.01$) was applied in combination with Sal A (Fig. 5*E*). These data indicate that Sal A protects the kidney from I/R-induced injury, at least partially via activation of the Akt/mTOR/4EBP1 pathway.

DISCUSSION

Here, we used a renal I/R injury in rats and a hypoxia/reoxygenation (H/R) model to damage proximal renal tubular cells (HK-2) as the disease model to investigate the effects of Sal A on renal I/R injury. Our results suggest that Sal A protects against I/R injury by attenuating renal dysfunction and histopathology alteration, and reducing oxidative stress, as well as apoptosis, which is potentially mediated by promoting tubular cell survival through the Akt/mTOR/4EBP1 pathway.

In the present study, the occlusion of renal blood flow was accomplished by clamping the renal artery, and after 45 min, kidney reperfusion was initiated. The sham operation did not alter the renal parameters (serum creatinine, BUN, and histological features) compared with rats in the I/R+Vehicle group. By contrast, renal I/R worsened renal dysfunction and histopathological features in rats. Sal A protected the rats against renal I/R injury, as manifested by the attenuation of renal dysfunction and histopathologic alteration. In vivo, under H/R conditions, cell viability was significantly increased in cells pretreated with Sal A. Our results are in accordance with a previously published study, which had shown that Sal A administration improved renal function in doxorubicin-induced toxicity in rats (12).

There is substantial evidence from previous reports that oxidative stress is involved in the pathogenesis of I/R injury (7). In the present study, we observed that in the I/R+Vehicle Group, there was increased ROS generation, as evidenced by increased levels of MDA and decreased SOD activity. Sal A administration reduced ROS generation and restored antioxidant status, and these effects were confirmed by in vitro studies. Altogether, this suggested that I/R-induced ROS accumulation is, at least in part, caused by the impaired capability for ROS detoxification. This is in accordance with the previous studies, which demonstrated that Sal A has antioxidant properties (37, 38).

Given that oxidative stress is caused by a burst of ROS generation and a decrease of antioxidants is a critical feature in I/R-induced tubular apoptosis (17), ROS accumulation triggers apoptosis. Apoptosis of tubular cells is another critical characteristic relevant to I/R-induced renal injury (23). The result of our study is that I/R insult results in the activation of apoptosis in tubular cells along with the upregulation of caspase-3 activity and downregulation of Bcl-2, which supports previous studies where proapoptotic proteins increased and antiapoptotic proteins decreased in the I/R group (13). Treatment with Sal A (40 mg/kg) reversed these changes with a near-complete reversal, and pretreatment with Sal A prevented tubular cells from I/R-induced apoptosis, as evidenced by the significant reduction of TUNEL-positive tubular cells. The results support the antiapoptotic effects of Sal A that have been documented in previous studies (4, 13, 29).

mTOR operates as a multichannel processor in the cellular nutrient-sensing network by receiving multiple inputs derived from distinct environmental cues and directing different outputs to appropriate downstream effectors. Prior studies have shown that oxidative stress can block the activity of mTOR signaling pathways to reverse cell metabolism and longevity leading to cell death (3). The Akt/mTOR signaling is well known in regulating a variety of essential cellular functions, such as protein synthesis, cell proliferation, apoptosis, and survival (2, 19, 32). There is strong evidence supporting the pivotal role of the Akt/mTOR pathway in I/R injury (36, 41). Recent data from studies suggest that Sal A protects against oxidative stress by activating Akt/mTOR signaling (38). Consistent with these previous studies, our results showed that Sal A manifested significantly attenuated levels for phosphorylated Akt and mTOR, while total Akt and mTOR levels were not changed in an I/R rat model. Furthermore, in vitro blocking of

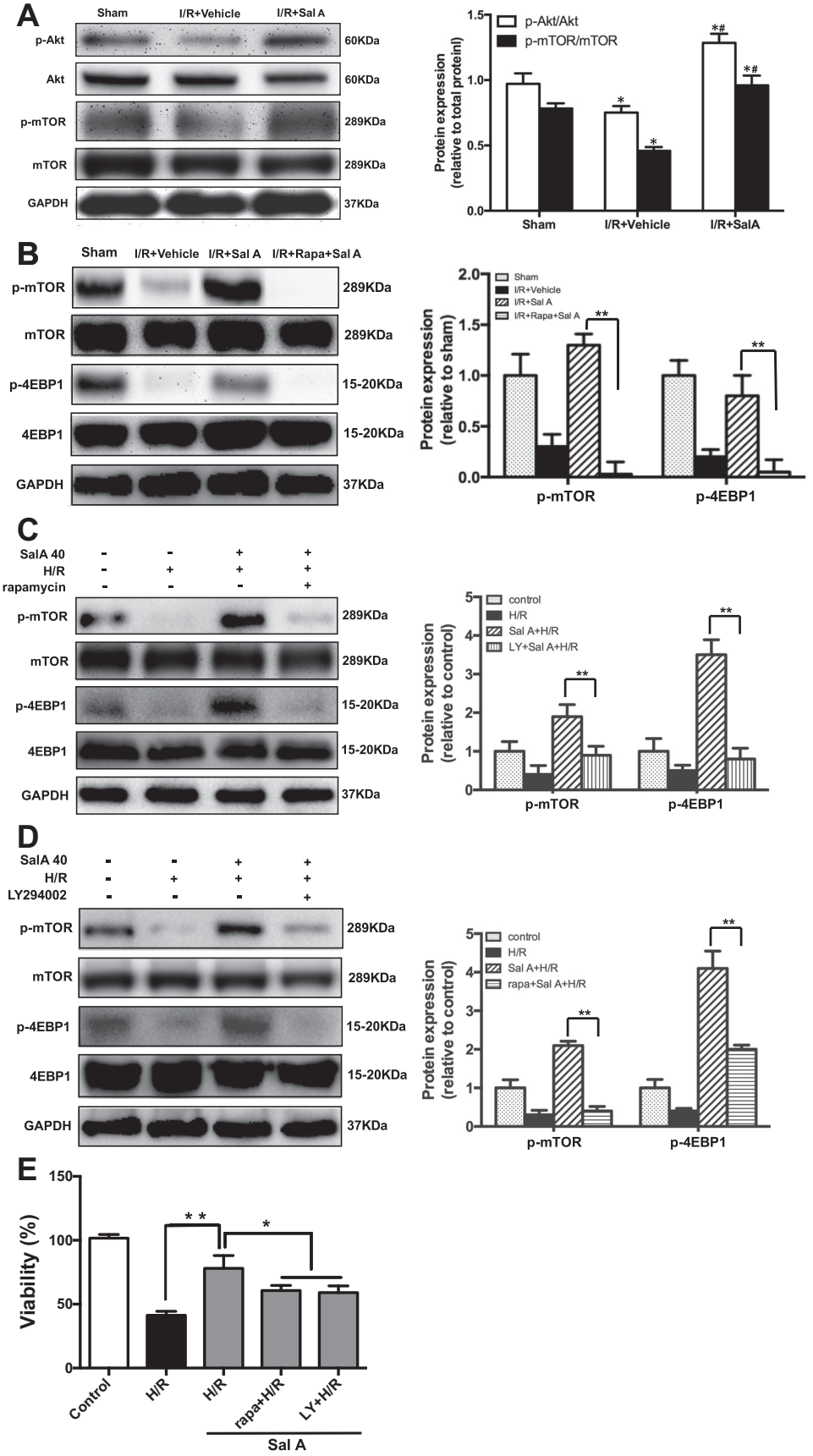

Fig. 5. The cellular protection of Sal A via activating Akt/mTOR/4EBP1 pathway during renal I/R injury. *A* and *B*: representative immunoblots and statistical data of phosphorylated and total Akt, mTOR, and 4EBP1 in the renal lysates from kidney tissues. The kidney tissues were harvested at 24 h after reperfusion. $*P < 0.05$ vs. Sham. $\#P < 0.05$ vs. I/R+Vehicle. $**P < 0.01$ vs. I/R+Sal A. *C* and *D*: immunoblot analysis of p-mTOR and p-4EBP1. Sal A (40 μM) alone or combined with rapamycin (100 nM) or LY294002 (20 μM) were applied to HK-2 cells before H/R condition treatment and Western blot analysis were performed. All Western blot band intensities were normalized to the total proteins or GAPDH. $*P < 0.05$. $**P < 0.01$. rapa, rapamycin; LY, LY294002. *E*: viability of HK-2 cells. Cells were pretreated with rapamycin or LY294002 for 1 h and then stimulated by H/R condition. Cellular survival rates were determined by MTT, $n = 6$. All data are expressed as mean ± SD. $*P < 0.05$. $**P < 0.01$.

the mTOR pathway during rapamycin and LY294002 application significantly prevented cellular protection by Sal A, illustrating that Sal A relies upon the activation of Akt/mTOR to protect HK-2 cells against oxidative stress. In addition, mTOR is a major downstream target of the PI3K/Akt pathway and activates protein synthesis.

mTOR depends upon the modulation of 4EBP1 to prevent cell death during apoptosis. 4EBP1 binds to eIF4E, preventing its assembly into the EIF4F complex and inhibiting cap-dependent mRNA translation. Phosphorylation of 4EBP1 disrupts this binding, activating cap-dependent mRNA translation. In the characterization of mTOR-controlled downstream targets, HK-2 cells were cultured under hypoxic conditions in the presence of Sal A, and a distinct increase in the phosphorylation of mTOR and 4EBP1 was observed following Sal A stimulation. These findings highlight the crucial role of the Akt/mTOR/4EBP1 signaling pathway in cellular protection against oxidative stress during I/R renal injury.

In summary, we have demonstrated that pretreatment of rats with Sal A provides protection against I/R-induced renal injury. Sal A can alleviate renal I/R injury through its antioxidant function and reduce apoptosis to preserve renal function. Mechanistic studies revealed that Sal A activated Akt/mTOR/4EBP1 signaling, indicating that Sal A may be a potential drug for I/R injury prevention and treatment. However, whether prophylactic and therapeutic administration of Sal A can effectively prevent I/R injury incidence and improve clinical outcome in patients remains to be determined in the future.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.S. and A.W. conceived and designed research; Y.S.,W.L., J.Z., F.W., J.B., L.C., and L.L performed experiments; Y.S., Y.D., Y.J., and A.D. analyzed data; Y.S. and A.D. interpreted results of experiments; Y.S. prepared figures; Y.S. drafted manuscript; Y.S., Y.J., A.D and L.H. edited and revised manuscript; L.H. approved final version of manuscript.

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