rhMYDGF Alleviates I/R-induced Kidney Injury by Inhibiting Inflammation and Apoptosis via the Akt Pathway

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Background. Renal ischemia/reperfusion (I/R) injury is one of the crucial factors affecting the outcome of renal transplantation. In recent years, myeloid-derived growth factor (MYDGF) has received a lot of attention for its extensive beneficial effects on cardiac repair and protection of cardiomyocytes from cell death. Therefore, we hypothesized that the recombinant human MYDGF (rhMYDGF) protein might play an essential role in safeguarding renal I/R injury. **Methods.** In vivo experiments were conducted using a mouse unilateral I/R model. Mice were pretreated with rhMYDGF by intraperitoneal injection to study the potential mechanism of renal protection. In vitro, we established hypoxia/reoxygenation and H_2O_2 treatment models to pretreat cells with rhMYDGF. The expression levels of oxidative stress, inflammation, and apoptosis-related factors in tissues and cells were detected. Finally, we explored the role of the protein kinase B (Akt) pathway in the renal protective mechanism of rhMYDGF. **Results.** In this study, we found that intraperitoneal injection of 1.25 µg rhMYDGF could significantly improve renal function of I/R mice, and reduce oxidative stress, inflammation, and apoptosis. For the human proximal tubular epithelial cell line and human kidney cell line, pretreatment with 0.3 µg/mL rhMYDGF for 24 h significantly downregulated oxidative stress, inflammation, and apoptosis via the phosphorylation of Akt, which could be ameliorated by LY294002. **Conclusions.** rhMYDGF protects kidney from I/R injury by attenuating oxidative stress, inflammation, and apoptosis through the activation of the Akt pathway.

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INTRODUCTION

Ischemic acute kidney injury caused by renal ischemia/reperfusion (I/R) injury develops in various ischemic conditions in transplanted kidneys. In the deceased donor kidney transplantation, kidney inevitably experienced cold ischemia, warm ischemia, and reperfusion injury.[1] Previous studies have confirmed that renal I/R can significantly reduce the clinical efficacy of kidney transplantation, leading to delayed graft function and increasing the risk of acute rejection in renal allografts.[2] Therefore, how

to reduce the impact of I/R on kidney allograft function is one of the main problems facing kidney transplantation at present.

Until now, the mechanism underlying I/R injury has not been fully understood. After restoring renal blood flow and oxygenation, high oxidative stress is crucial for the cascade of processes participating in the pathogenesis of I/R.[3] In recent years, studies have confirmed that a large number of reactive oxygen species (ROS) produced by mitochondria are involved in the onset and progression of

renal I/R injury, which in turn can disrupt mitochondrial function, promote inflammation, and accelerate cell apoptosis.[3,4] Therefore, therapeutic strategies to suppress oxidative stress, inflammation, and apoptosis hold promise to alleviate renal I/R injury.

Myeloid-derived growth factor (MYDGF) is a paracrine-acting protein produced by bone marrow–derived monocytes and macrophages.[5] Extensive studies have focused on the antiapoptotic effect of MYDGF. In model of myocardial infarction, compared with wild-type mice, MYDGF-deficient mice developed more extensive infarct scars and severe contractile dysfunction. Treatment with recombinant MYDGF protein significantly reduced infarct size after 24 h reperfusion and attenuated apoptosis of neonatal rat ventricular myocytes cultured in serum starvation.[5,6] Besides, MYDGF can reduce hypoxia/reoxygenation (H/R)-induced apoptosis of cardiac microvascular endothelial cells by regulating oxidative stress in endoplasmic reticulum stress.[7,8] In addition, mucin-fused myeloid-derived growth factor (MYDGF164) can stimulate the proliferation in endothelial and epithelial cells through phosphorylating MAPK1/3, and exert antiapoptotic and angiogenic activities to significantly alleviate fibrosis in chronic kidney disease.[9] However, the functional role of MYDGF and its mechanisms involved in renal I/R injury were not fully understood.

Based on previous studies, we hypothesized that recombinant human MYDGF (rhMYDGF) may also be useful for renal ischemic repair. Here we showed that rhMYDGF significantly attenuates oxidative stress, inflammatory response, and apoptosis levels in response to I/R injury in vivo and in vitro. Mechanistically, we found that rhMYDGF exerts its renal protective effect via the activation of the protein kinase B (Akt) pathway. Therefore, rhMYDGF is a promising strategy to alleviate renal I/R injury.

MATERIALS AND METHODS

Renal I/R Injury Model, rhMYDGF, and LY294002 Administration and Sample Preparation

Male C57Bl/6 mice were obtained from the experimental animal center of Xi'an Jiaotong University, Xi'an. This study was approved by the Xi'an Jiaotong University Committee on Animal Care and was conducted following the guidelines.

Kidney I/R surgery was performed using male C57Bl/6 mice (8–12 wk). Briefly, mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.), injected with heparin (100 U/kg) followed by an abdominal median incision, and then the left ureter and left renal artery and vein were permanently ligated, and a left unilateral nephrectomy performed. The right kidney was visualized and separated from surrounding connective tissue. Renal ischemia was induced by clamping the right renal pedicle for 35 min[10] with microvascular clamps. The body temperature was maintained constantly at 37°C by using a heating pad until awake. Sham-operated control mice also underwent a left nephrectomy, but without the clamping of the right renal pedicle. rhMYDGF was obtained from Novoprotein Scientific Inc. (Shanghai, China). In addition, I/R mice were treated with intraperitoneal injection with 1.25, 2.5, or 5.0 µg rhMYDGF at 1 h before ischemia. rhMYDGF was diluted in 50 µL sterile normal saline. Control animals were given 50 µL of physiological saline. The LY294002 (MCE, Shanghai, China), a PI3K inhibitor that reduces the phosphorylation of Akt, was injected intraperitoneally at 25 mg/kg and pretreated with rhMYDGF at 1 h before ischemia.[11] Twenty-four hours after I/R, animals were again anesthetized, and 0.5 mL of arterial blood was obtained via inferior vena cava puncture as previously described for biochemical measurements.[12] Samples were stored at –80°C until assayed. According to the manufacturer's instructions, the serum creatinine (SCr) and blood urea nitrogen (BUN) levels in the serum samples were measured using commercially available assay kits (Jiancheng Bioengineering Institute, Nanjing, China). As for kidney tissues, we assigned them into 4 parts. The first part was fixed in 4% paraformaldehyde and embedded into tissue wax blocks for histological examinations; the second and third parts were quick-frozen in liquid nitrogen for extracting tissue proteins and tissue RNA, respectively; and the last part was used to obtain tissue homogenates for detecting oxidative stress indicators.

Analysis of Kidney Histology

The kidney tissue was embedded in paraffin and was cut into 4-µm-thick sections. Renal sections were stained with periodic-acid-Schiff and then scanned with a microscope equipped with a digital camera (NikonInstruments, Melville, NY). Using light microscopy at 200× and 400× magnification, histological damage was assessed blinded according to a modified scoring system.[13] Scores ranged from 1 (<10%), 2 (10%–25%), 3 (25%–50%), 4 (50%–75%), and 5 (>75%). Average scores were calculated from 5 random fields in each sample.

Biochemical Assays for Oxidative Stress

In the case of ice water bath, we used a homogenizer to prepare the tissue into 10% homogenate and collected the supernatant after centrifugation at 5000 rpm for 30 min. For human renal tubular epithelial (HK-2) cells, we used radioimmunoprecipitation assay buffer to extract cell lysates, and then centrifuged at 3000 rpm for 10 min to collect the pellet. Superoxide dismutase (SOD), malondialdehyde (MDA) content, and glutathione peroxidase (GSH-Px) activity were measured in tissue homogenates and cell pellets using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the respective manufacturers' protocols.

Dihydroethidium Staining

ROS levels were measured using 10 µM dihydroethidium (DHE; Molecular Probes, Inc., Eugene, OR) as described previously.[14] Tissues were incubated for 60 min in Krebs-HEPES buffer containing DHE and washed twice. DAPI (Invitrogen Corporation) was used to label the nucleus. The intensity of DHE staining was quantified using ImageJ v1.50d (National Institutes of Health).

Enzyme-Linked Immunosorbent Assay

Tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 were quantified from mice serum by enzyme-linked

dUTP Nick End Labeling Assay

Terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assays were performed using the TUNEL assay kit (Beyotime Biotechnology, Jiangsu, China) according to the manufacturer's instructions. The fluorescent photos of cells were captured by a fluorescence microscope (Zeiss, Jena, Germany). To test the apoptotic rate, 5 visual fields were chosen randomly from each section, positive cells and total cells were determined with ImageJ software. And the percentage of TUNEL-positive cells/total cells was calculated for statistical analysis.

Transmission Electron Microscope

Glutaraldehyde (0.3%) was used to prefix the renal cells overnight, and 10 mL/L osmic acid was used to postfix the cells. Cells were observed under a Hitachi HT7700 transmission electron microscope (Hitachi, Japan).

HK-2 and Human Kidney Cells

HK-2 (ATCC, Manassas, VA) and tubular epithelial cell line human kidney cell (HKC) were purchased from the Chinese Academy of Medical Sciences cell bank. These 2 cell lines were both cultured in DMEM/F12 (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (0.1 g/mL). And cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells were free of contamination with bacterial, fungal, and mycoplasma.

When the cells attached with a density of 70%–80%, they were digested with 0.25% trypsin, and then a single-cell suspension was prepared with serum culture medium. For routine cell cultures, T25 culture flasks were used, and cells were seeded in a 96-well culture plate at a density of 5000 cells per/well for CCK8 experiment. As for extracting cell protein, RNA, supernatant, and precipitation, cells were plated at a density of 20 000 cells per well in a 6-well plate. All cell culture flasks and plates were purchased from Corning Company (Corning, NY).

Establishment of H/R Model and H_2O_2 Treatment

To induce the H/R model, based on the Cell Counting Kit-8 (CCK8) assay results, HK-2 cells were cultured in medium without nutrients (glucose-free, serum-free) under (1% O_2, 94% N_2, and 5% CO_2) for different hours. And then, the medium was refreshed again, and cells were cultured in normoxia (5% CO_2 and 95% air) for various hours. The control cells were incubated in normoxia. For the H_2O_2 treatment, HKC were treated with 0.25 mM concentration of H_2O_2 for different hours.

Cell Viability and Injury Assays

For quantitative viability measurements, 10 µL/well CCK8 colorimetric assay (Beyotime Biotechnology, China) was added to the cells to be tested in 96-well plates and incubated for 2 h at 37°C. Absorbance was measured at 450 nm using a microplate reader (BioTeck, CA). The control group (untreated cells) was considered to have viability of 100%. In addition, cell injury was assessed based on the levels of lactate dehydrogenase (LDH) release in the supernatants of cultured cells using the LDH assay kit (Beyotime, China).

Propidium Iodide/Hoechst Staining

Cell nuclei were counterstained by Hoechst staining, and cells were stained with propidium iodide (PI, Sigma-Aldrich) to label dead cells. After H/R, rhMYDGF-pretreated and control cells were washed with PBS 2–3 times, 100 µL binding buffer, 2 µL Hoechst 33324, and 5 µL PI were added for 10 min at room temperature, away from light. Images were taken with a Nikon eclipse fluorescence microscope (Nikon, Tokyo, Japan). The numbers of live cells, dead cells, and condensed nuclei were manually counted in each image. Data were represented as % of dead cells or % of apoptotic cells.

RNA Preparation and Real-time Quantitative Polymerase Chain Reaction

RNA samples were prepared as described previously.[15] Briefly, renal tissues were prepared using (Beyotime Biotechnology, China) and cells were procured using the TRIzol reagent (Tiangen, China) according to the respective manufacturers' protocols. The high-capacity cDNA reverse transcription kit (Roche) was used to reverse-transcribe 1 µg total RNA into cDNA. Quantitative polymerase chain reaction (qPCR) was carried out using quantitative SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) in an ABI 7500 Fast Real-Time (RT) PCR system (Thermo Fisher Scientific) according to the manufacturer's cycling conditions. GAPDH was used as an internal inference.

Primers for mouse and human TNF-α, IL-6, IL-1β, kidney injury molecule 1 (KIM-1), and GAPDH were synthesized by Xi'an Qingke Biological Company and they are shown in **Table S1, SDC**, http://links.lww.com/TP/C664.

Western Blot Analysis

Western blot assays were performed according to standard protocol. Primary antibodies used were: rabbit anti–B-cell lymphoma-2 (Bcl-2) (1:1000, Cell Signaling Technology); rabbit anti–Bcl2-associated X protein (Bax) (1:1000, Cell Signaling Technology); rabbit anti–phospho-Akt (Ser473) (1:1000, Cell Signaling Technology); rabbit anti-Akt (1:1000, Cell Signaling Technology), and mouse anti-GAPDH (1:5000, Proteintech).

Statistical Analyses

Normality was assessed by Shapiro-Wilk W test using SPSS version 19.0. All data were expressed as mean ± standard error of mean. Statistical analysis was performed by ordinary 1-way analysis of variance using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA). $P < 0.05$ were considered statistically significant.

RESULTS

Administration of rhMYDGF Improves Renal Function

At first, we conducted animal experiments according to the design (Figure 1A). SCr and BUN were measured at 24 h after reperfusion to understand whether rhMYDGF
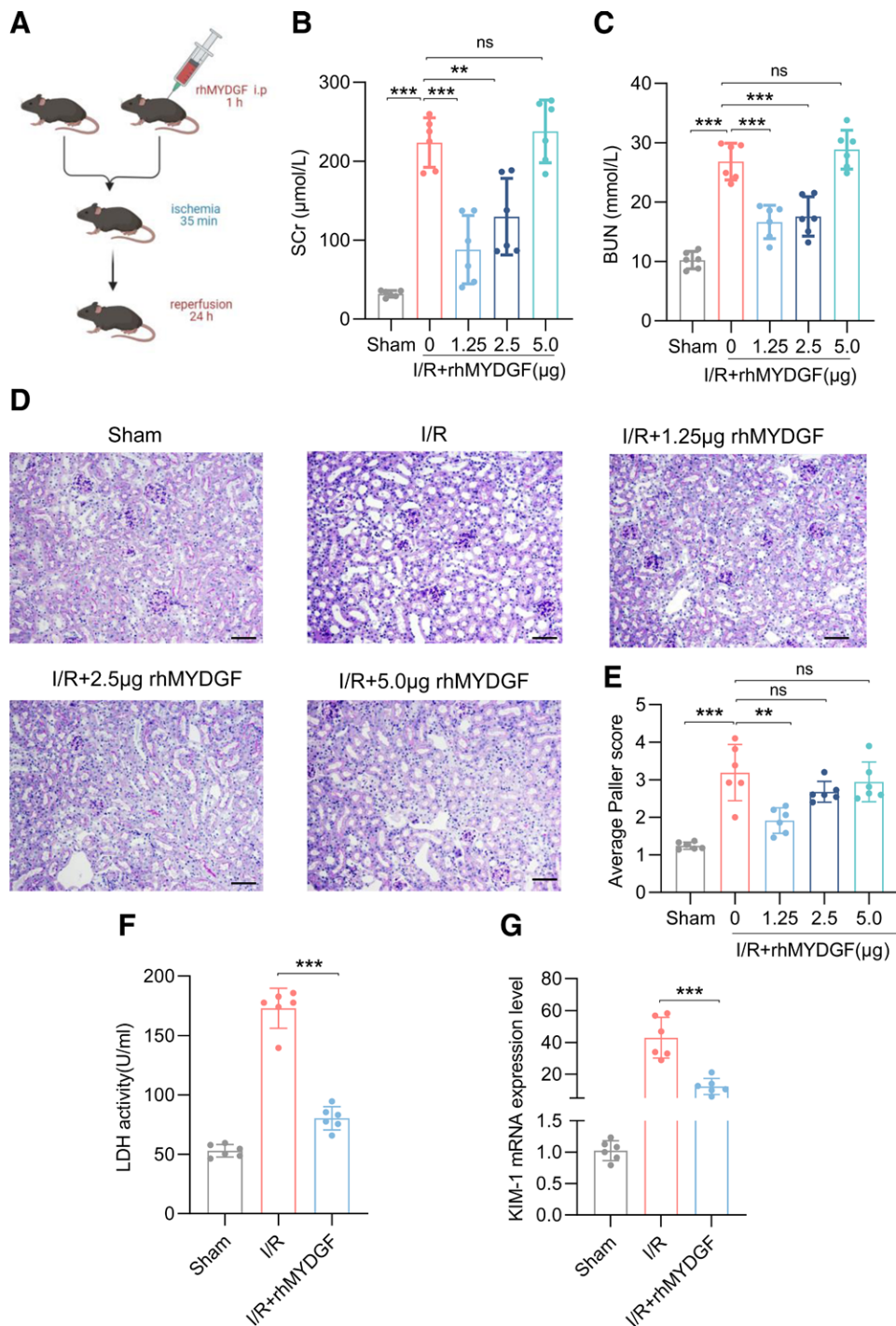
FIGURE 1. Administration of rhMYDGF improves renal function. The animal experiment of our analyses was depicted in (A). The SCr (B) and BUN (C) in 5 groups of mice were detected. D, Representative pictures of PAS in kidney slices, and scores of renal tubule injury based on staining sections. Scale bar: 50 μm. E, Scores of renal tubule injury based on staining sections. Serum LDH activity (F) and KIM-1 mRNA expression level (G) were used to complement the measurement of renal function. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Data are represented as mean ± SEM. BUN, blood urea nitrogen; I/R, ischemia/reperfusion; KIM-1, kidney injury molecule 1; LDH, lactate dehydrogenase; MYDGF, myeloid-derived growth factor; ns, not significant; PAS, periodic-acid-Schiff; rhMYDGF, recombinant human myeloid-derived growth factor; ; SCr, serum levels of creatinine.

treatment can improve the renal function of mice after I/R injury. Compared with the sham group, serum levels of SCr (Figure 1B) and BUN (Figure 1C) were significantly increased in the I/R model group. Furthermore, 1.25 and 2.5 μg rhMYDGF treatment successfully protected

renal function by intraperitoneal injection, and 1.25 μg rhMYDGF showed the best protective effect. Kidneys from all experimental mice were isolated and stained by periodic-acid-Schiff. As shown in Figure 1D and E, 1.25 μg rhMYDGF treatment strikingly ameliorated kidney

tissue damage after I/R injury. Therefore, we chose prophylactic administration of 1.25 µg rhMYDGF for the following study. Moreover, subsequent plasma LDH levels (Figure 1F) and KIM-1 mRNA expression levels (Figure 1G) were used to complement the measurement of renal function, and these data also confirmed the robustness of the above.

RhMYDGF Protects HK-2 and HKC Cells From Cell Death

To investigate the renal I/R injury in vitro, HK-2 cells and HKC were treated with H/R and H_2O_2 separately. At first, we tested the following H/R conditions: H6/R24, H12/R12, H12/R24, and H24/R12, and the CCK8 data showed that the survival rate of HK-2 cells treated with H12/R12 was markedly reduced to 55%. Meanwhile, HKC treated with 0.25 mM H_2O_2 various times (0, 2, 4, and 6 h) showed a significant decrease in cell viability (Figure 2C), suggesting that H_2O_2 damaged HKC in a time-dependent manner. And then, we chose H12/R12 and 0.25 mM H_2O_2 treatment 4 h for the subsequent studies. The HK-2 and HKC cells were pretreated with various concentrations of rhMYDGF (0, 0.1, 0.3,

0.5, 0.7, and 1.0 µg/mL) at 24 h before H/R and H_2O_2 stimulation, respectively, and then subjected to a CCK8 assay to detect the effect of rhMYDGF on cell viability. As shown in Figure 2B and D, the results showed that 0.3 µg/mL rhMYDGF pretreatment for 24 h had the most optimal protective effect on cells. Therefore, we chose the concentration of 0.3 µg/mL to perform the following experiments.

rhMYDGF Alleviates Kidney Oxidative Stress Induced by I/R

To determine the role of rhMYDGF in oxidative stress, we estimated the SOD, GSH-Px activities, and MDA content. At 24 h after I/R, there was a significant decrease in renal tissues SOD and GSH-Px activities. In addition, MDA content showed a distinct increase when compared with those in the sham group. In contrast, pretreatment of rhMYDGF was found to significantly increase the activities of SOD and GSH-Px, whereas it reduced the level of MDA (Figure 3A–C). More importantly, DHE staining was used to monitor ROS production in renal tissue.[16] We found that the ROS intensity was profoundly increased in the I/R group, and the ROS
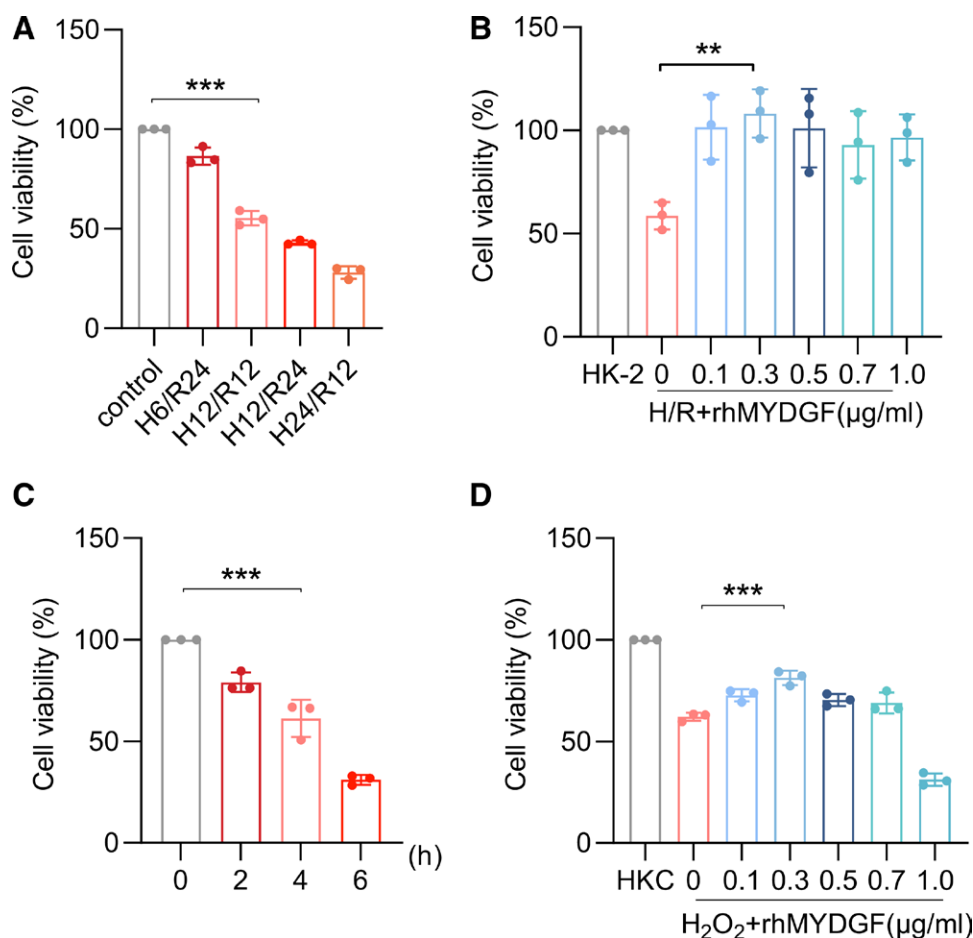
FIGURE 2. rhMYDGF protects HK-2 and HKC from cell death. A, CCK8 assays were used to detect HK-2 cell viability under different hypoxia and reoxygenation conditions. C, HKCs were cultured with 3% H_2O_2 for 0, 2, 4, and 6 h, respectively. The HK-2 (B) and HKC (D) cell lines were pretreated with various rhMYDGF concentrations (0, 0.1, 0.3, 0.5, 0.7, and 1.0 µg/mL) 24 h, after H/R and H_2O_2 stimulation, respectively, whose viabilities were measured using CCK8 kits. **$P < 0.01$ and ***$P < 0.001$. Data are represented as mean ± SEM. CCK8, cell counting kit-8; H/R, hypoxia/reoxygenation; HK-2, human proximaltubular epithelial cell; HKC, human kidney cell; rhMYDGF, recombinant human myeloid-derived growth factor.

FIGURE 3. rhMYDGF alleviates oxidative stress in vitro and in vivo. A–C, Effects of rhMYDGF on the levels of SOD, MDA, and GSH-Px in renal tissues 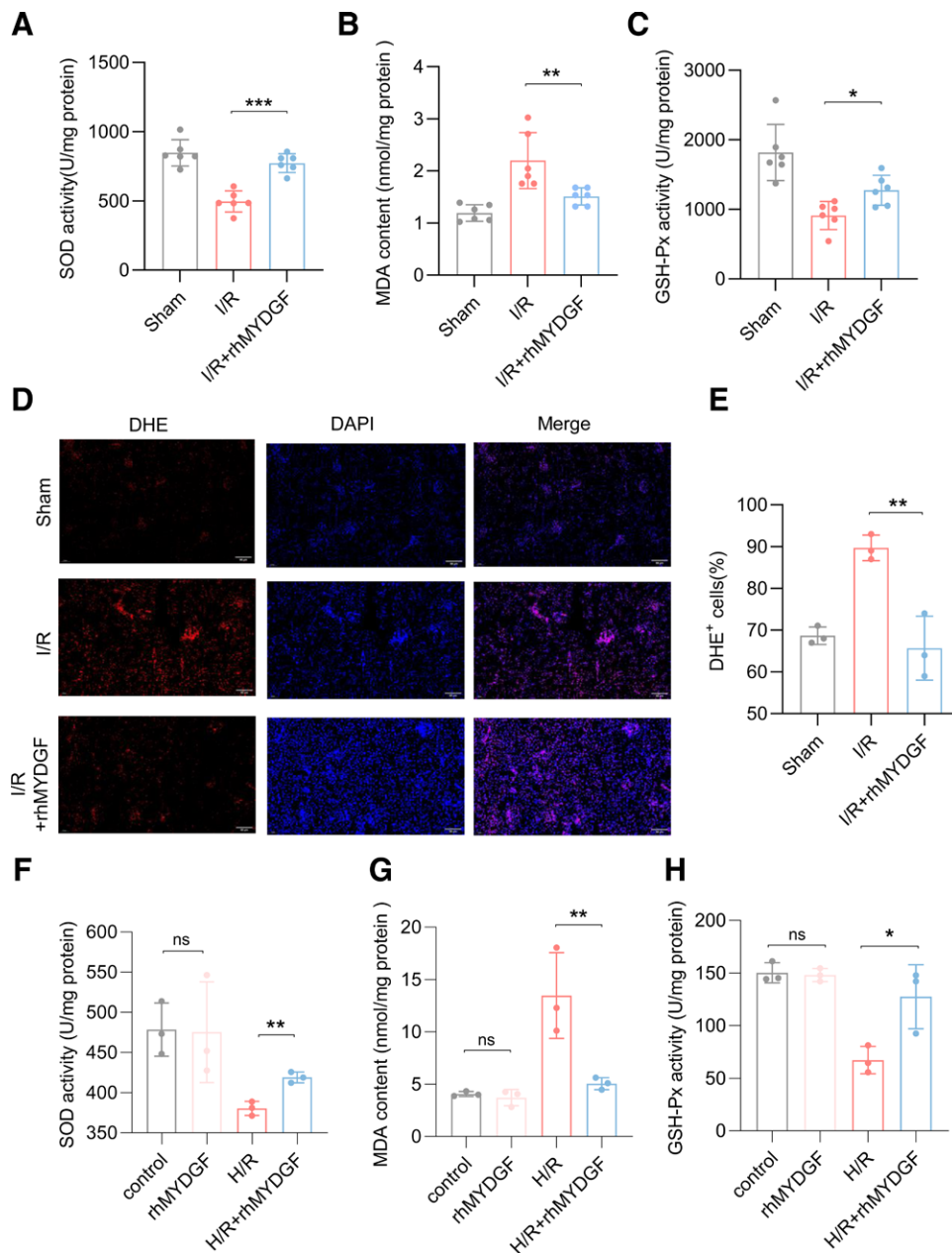of mice caused by I/R injury. D, ROS content of renal tissue in 3 groups was measured by DHE staining. Scale bar: 50 μm. E, Quantitative analysis of the DHE⁺ cells in each group. F–H, Effects of rhMYDGF on the levels of SOD, MDA, and GSH-Px in HK-2 cells caused by H/R injury. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Data are represented as mean ± SEM. DHE, dihydroethidium; GSH-Px, glutathione peroxidase; H/R, hypoxia/reoxygenation; HK-2, human proximaltubular epithelial cell; I/R, ischemia/reperfusion; MDA, malondialdehyde; ns, not significant; rhMYDGF, recombinant human myeloid-derived growth factor; ROS, reactive oxygen species; SOD, superoxide dismutase.

production benefited from rhMYDGF protein therapy (Figure 3D and E).

In HK-2 cells, there were no statistically significant differences between rhMYDGF alone group and the control group. The H/R group exhibited the lowest levels of SOD and GSH-Px, and the highest level of MDA. Moreover, rhMYDGF pretreatment reversed the decreases in SOD and GSH-Px levels ($P < 0.001$, $P < 0.05$) and the increase in MDA content ($P < 0.05$; Figure 3F–H). Collectively, these findings indicate that rhMYDGF attenuates I/R and H/R injury through scavenging ROS and inhibiting oxidative stress.

RhMYDGF Suppresses Inflammation

The present study also investigated by RT-qPCR whether rhMYDGF attenuated the expression levels of proinflammatory cytokines in kidneys. As illustrated in Figure 4A–C, I/R triggered the production of TNF-α, IL-6, and IL-1β. Administration of rhMYDGF inhibited TNF-α expression to 42.4% (Figure 4A), IL-6 to 43.3% (Figure 4B), and IL-1β to 57.5% (Figure 4C) when compared with the expression levels in the I/R group. Similarly, TNF-α, IL-6, and IL-1β expression were increased in H/R cells and rhMYDGF effectively decreased the expression of these inflammatory factors (Figure 4D–F). In summary,
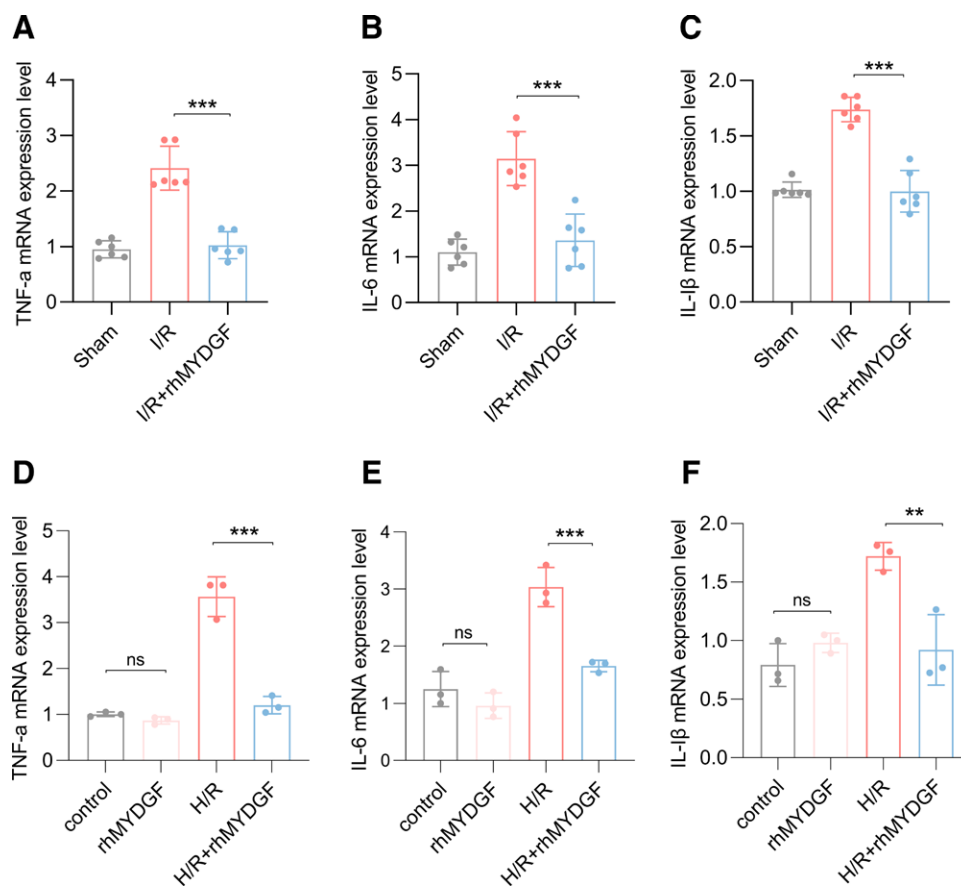
FIGURE 4. rhMYDGF suppresses inflammation. A–C, Inflammatory cytokines, TNF-α, IL-6, and IL-1β, mRNA expression levels in kidney analyzed by real-time qPCR at 24 h after I/R injury. D–F, Gene expression of TNF-α, IL-6, and IL-1β in the HK-2 cells. The expression levels were normalized to the expression of control cells. **P < 0.01 and ***P < 0.001. Data are represented as mean ± SEM. HK-2, human proximal tubular epithelial cell; I/R, ischemia/reperfusion; IL-6, interleukin-6; IL-1β, interleukin-1β; ns, not significant; qPCR, quantitative polymerase chain reaction; rhMYDGF, recombinant human myeloid-derived growth factor; TNF-α, tumor necrosis factor-α.

these results demonstrate that rhMYDGF relieves inflammation in the kidney following I/R and H/R.

rhMYDGF Limits I/R- and H/R-induced Renal Tubular Cell Apoptosis

To investigate whether rhMYDGF had a protective effect on apoptosis of kidney tissues, TUNEL staining was performed. As shown in Figure 5A and B, renal I/R resulted in more TUNEL-positive cells than the sham group, and pretreatment with rhMYDGF significantly decreased kidney tissue apoptosis rate compared with I/R-injured mice. Consistently, Western blot indicated that I/R enhanced the expression of Bax and reduced Bcl-2 expression, but the mice pretreated with rhMYDGF showed an apparent reduction of Bax expression and enhancement of Bcl-2 expression (Figure 5C and D). In addition, after I/R injury, typical apoptotic morphology changes were observed by transmission electron microscopy, including condensed nuclear chromatin and vacuolated cytoplasm (Figure 5E).

Double-stained by Hoechst 33342 and PI, HK-2 cells that underwent H/R treatment had an apoptosis rate of 24.3%. However, the red fluorescence signal was significantly decreased after being pretreated with rhMYDGF, and the apoptosis rate was 14.0% (Figure 5F and G). In addition, H/R and H_2O_2 markedly increased apoptosis as evidenced

by an increase in proapoptotic Bax and a decrease in antiapoptotic Bcl-2 (Figure 5H–K). Furthermore, we pretreated HK-2 and HKC cells with 0.3 µg/mL rhMYDGF and observed a remarkable reduction of apoptosis.

RhMYDGF Improves I/R Injury Dependent on the Akt Pathway

It has been proven that the Akt signaling pathway plays an essential role in renal I/R injury.[17] And the cytoprotective effect of rhMYDGF has been shown to be related to the phosphorylation of Akt and the inhibition of apoptosis.[5] Therefore, we next investigated whether rhMYDGF exerted its protective effect via the activation of Akt signaling pathway. To investigate the contribution of the Akt pathway in mice and cells, the activity of Akt was examined. As shown in Figure 6A–D, the effect of rhMYDGF on Akt was not statistically significant without I/R or H/R injury. And compared with the I/R and H/R group, both mice and cells pretreated with rhMYDGF had markedly higher phosphorylation of Akt (p-Akt)/Akt ratios, indicating that rhMYDGF activates the Akt pathway. Furthermore, LY294002 (LY) significantly decreased p-Akt expression levels but showed no effect on Akt expression (**Figure S1A–D, SDC,** http://links.lww.com/TP/C664). We further treated I/R-injured mice with LY to see whether activation of the Akt pathway was necessary for rhMYDGF to exercise its
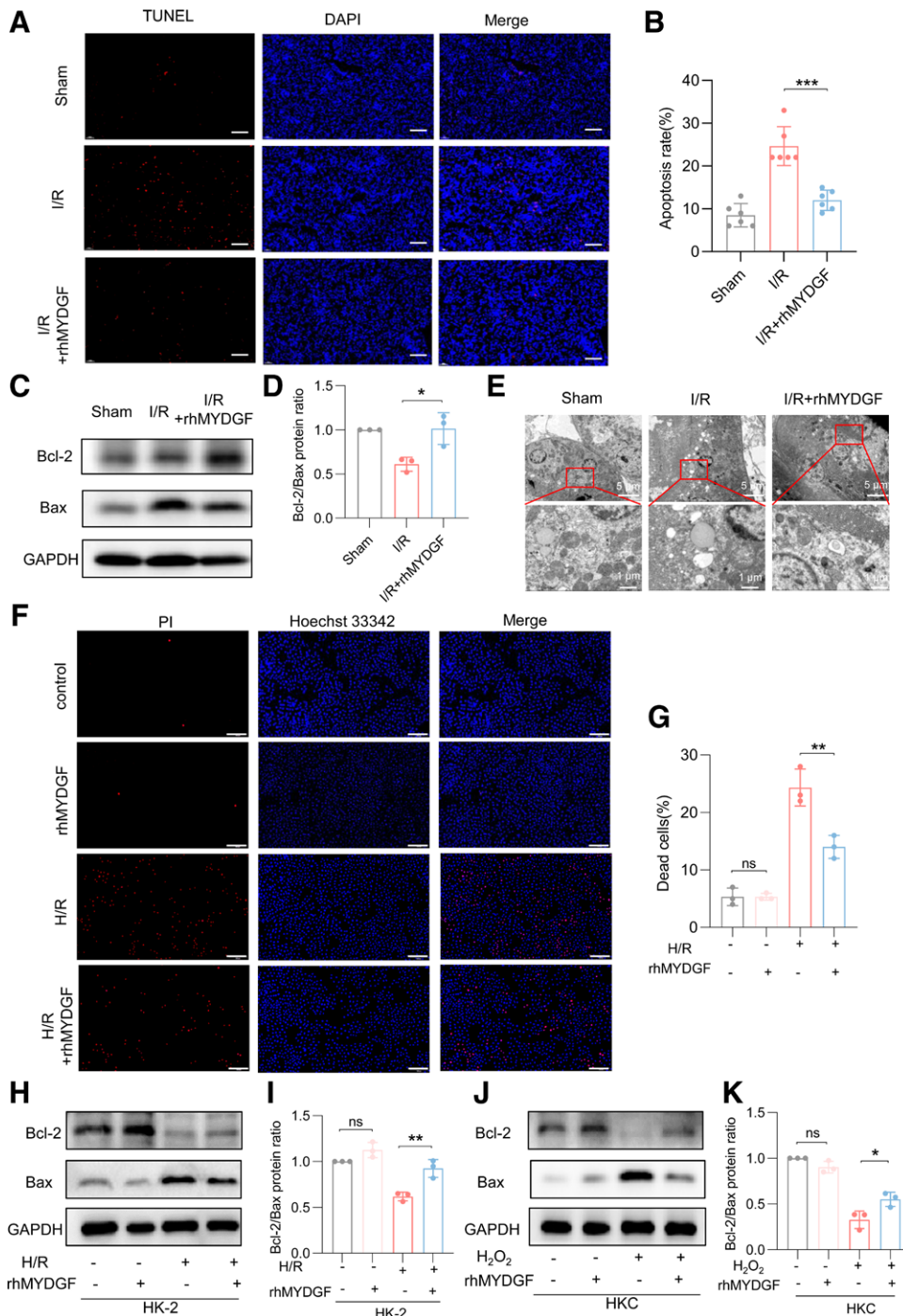
FIGURE 5. rhMYDGF limits I/R- and H/R-induced renal tubular cell apoptosis. A, TUNEL staining to measure the apoptosis of renal tubular epithelial cells in each group at 24 h after I/R injury (scale bar, 100 μm). B, Quantitative analysis of the number of apoptotic cells in each group. C, Expression levels of antiapoptotic gene Bcl-2 and the proapoptotic gene Bax were measured by Western blot. D, The intensity of the Bcl-2 and Bax protein bands was quantified by densitometry analysis. E, Typical apoptosis characteristics of renal tubular epithelial cells in each group were observed under transmission electron microscopy. The HK-2 cells were double-stained by Hoechst 33342 and PI (scale bar, 100 μm). Representative fluorescence images (F) and quantification of apoptosis rates (G) in 4 groups were obtained after staining. H, The representative Western blot and quantitative analysis (I) of protein levels of Bcl-2, and Bax in the control, rhMYDGF, H/R, and H/R + rhMYDGF-treated cultured HK-2 cells. J, Western blots were performed and quantitatively analyzed (K) to detect protein levels of Bcl-2, and Bax in HKC pretreated with rhMYDGF or not. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Data are presented as mean ± SEM. Bax, Bcl2-associated X protein; BCl-2, B-cell lymphoma-2; H/R, hypoxia/reoxygenation; HK-2, human proximaltubular epithelial cell; HKC, human kidney cell; I/R, ischemia/reperfusion; ns, not significant; PI, propidium iodide; rhMYDGF, recombinant human myeloid-derived growth factor; TUNEL, terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling.
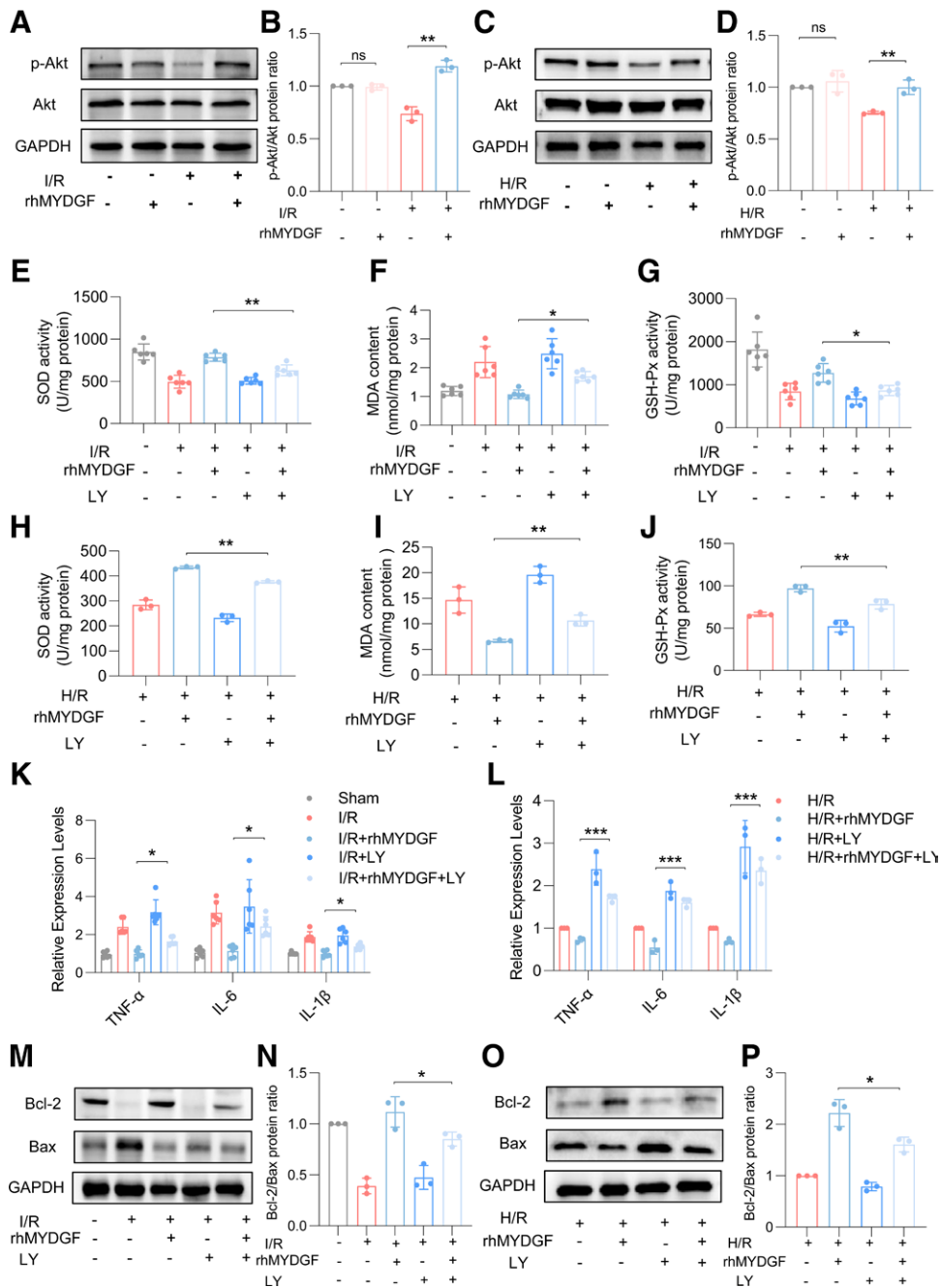
FIGURE 6. rhMYDGF improves I/R and H/R injury via activating the Akt pathway. A–D, Western blot was performed and quantitatively analyzed to determine the protein levels of p-Akt at Ser473 and Akt in the different groups. E–G, Effects of rhMYDGF on the levels of SOD, MDA, and GSH-Px in renal tissues from rhMYDGF-treated I/R mice with or without coadmistration of LY294002 (LY, 25 mg/kg). H–J, Effects of rhMYDGF on the levels of SOD, MDA, and GSH-Px in HK-2 cells among H/R, H/R+rhMYDGF, H/R+LY294002 (LY, 10 μM/mL) and H/R+rhMYDGF+LY294002 groups. K and L, RT-qPCR was utilized to evaluate the mRNA expression of TNF-α, IL-6, and IL-1β in different groups in vivo and vitro. K, The protein expressions of Bcl-2 and Bax in the different groups. L, Western blot images of Bcl-2 and Bax after H/R of HK-2 cells among 4 groups. N and P, Densitometric analysis of the Western blots shown in (K) and (L). *P < 0.05, **P < 0.01, and ***P < 0.001. Data are presented as mean ± SEM. Akt, protein kinase B; Bax, Bcl2-associated X protein; BCl-2, B-cell lymphoma-2; GSH-Px, glutathione peroxidase; H/R, hypoxia/reoxygenation; HK-2, human proximaltubular epithelial cell; HKC, human kidney cell; I/R, ischemia/reperfusion; IL, interleukin; MDA, malondialdehyde; ns, no significance; p-AKT, phosphorylation of Akt; rhMYDGF, recombinant human myeloid-derived growth factor; RT-qPCR, real time quantitative polymerase chain reaction; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.

protective effects. SCr (**Figure S2A, SDC,** http://links.lww.com/TP/C664) and BUN (**Figure S2B, SDC,** http://links.lww.com/TP/C664) levels from rhMYDGF-treated mice

with coadmistration of LY were significantly increased (*P* < 0.05). Next, our CCK8 assay data showed that LY significantly reduced the protective effect of rhMYDGF

on HK-2 and HKC cells (**Figure S2C and D, SDC**, http://links.lww.com/TP/C664). Furthermore, the result of LDH in the supernatant (**Figure S2E, SDC**, http://links.lww.com/TP/C664) was consistent with that of CCK8 experiment. As shown in Figure 6E–J, pretreatment with LY effectively reversed the protective effect of rhMYDGF on oxidative stress in vivo and in vitro. Meanwhile, we assessed if the pretreatment with LY would influence the effect of rhMYDGF on inhibiting inflammatory responses. In vivo enzyme-linked immunosorbent assay (**Figure S2F–H, SDC**, http://links.lww.com/TP/C664) and RT-qPCR (Figure 6K) results showed that the TNF-α, IL-6, and IL-1β expression levels were increased in the I/R+rhMYDGF+LY compared with the I/R+rhMYDGF group, and the differences were statistically significant. Similar changes were obtained in HK-2 cells (Figure 6L). In addition, to investigate whether rhMYDGF exerts its antiapoptotic effect through the Akt pathway, we observed the changes in the levels of apoptosis-related proteins after adding LY. And our results showed that Bcl-2/Bax ratio increased significantly both in vivo and in vitro (Figure 6M–P). Taken together, our data demonstrate that rhMYDGF may prevent renal I/R injury through the Akt pathway.

DISCUSSION

Kidney transplantation is a life-saving treatment for those with end-stage renal disease.[18] Renal I/R injury is an inevitable consequence of kidney procurement for kidney transplantation, which is associated with an increased risk of death in transplant patients. It has become one of the leading causes of delayed graft function, graft rejection, chronic graft dysfunction, and progressive interstitial fibrosis.[19-21] Although the mechanism of renal I/R injury has made some progress in recent years,[22] such as autophagy,[23] ferroptosis,[24] necroptosis,[25] and other aspects, its pathophysiological mechanism has not been fully elucidated so far. At present, the treatment of I/R injury mainly focuses on removing ROS, reducing inflammation and promoting cell survival.[26]

Renal transplantation storage,[27] ischemic preconditioning treatment,[28] stem cell and stem cell–derived extracellular vesicles therapy,[29,30] and protein therapy have been proved to be interventions to reduce renal I/R injury. Drug development targeting renal I/R mainly focuses on scavenging ROS, anti-inflammatory, and antiapoptosis.[31,32] Previous basic research has tested recombinant MYDGF as a therapy for acute MI.[5] The data showed that MYDGF protein therapy reduced infarct size and had antiapoptotic, infarct-sparing, and angiogenic effects. Also, MYDGF is reported to alleviate podocyte apoptosis through its regulation of Runx2.[33] In addition, the anti-inflammatory role of MYDGF has also been reported. For example, MYDGF can inhibit NF-κB signaling and significantly reduce the inflammation of endothelial cells and macrophages.[34] Moreover, Houseright et al[8] found that MYDGF can act as a neutrophil inhibitor, limits neutrophil inflammation in response to tissue damage through the HIF-1α pathway, and this provides further support for the effect of MYDGF and its mechanisms. However, the function of MYDGF and its action mechanism has not been investigated in renal I/R model. In this

study, we found that SCr, BUN in serum, MDA, TNF-α, IL-6, IL-1β, and Bax levels of kidney in rhMYDGF group were decreased, and the expression levels of SOD, GSH-Px, and Bcl-2 were increased compared with I/R group. These results indicated that rhMYDGF can play anti-inflammatory and antiapoptosis protective roles in the process of renal I/R injury. Our in vivo and in vitro data showed that a high concentration of rhMYDGF may have an inhibitory effect. However, Korf-Klingebiel et al[5] revealed that injection of recombinant Mydgf (10 μg) into the LV cavity of FVB/N mice can significantly alleviate the myocardial injury after MI. The differences between our study and previously published works may have several possible explanations but are most likely due to differences in the models, target organ, and cell types. And the exact mechanism needs further investigation. In addition, pharmacokinetics, metabolic, and toxicological studies of rhMYDGF will be carried out in our future study to determine the optimal concentration in improving renal I/R injury.

In recent years, literatures have confirmed that the process of renal I/R injury involves the change of many molecular pathways, especially the protein therapy targeting I/R, which mainly plays a protective role in the kidney through various classical pathways. Recent studies have shown that many molecules can induce phosphorylation of Akt, and then regulate oxidative stress. Our results showed that rhMYDGF exerts its anti-inflammatory, antiapoptotic, and antioxidant effects independently through activation of the Akt pathway.[35] Numerous studies have shown that MYDGF functions through the Akt pathway. An article from He et al[36] reported that MYDGF protected renal podocytes from injury and prevented DKD progression through activation of the Akt/Bcl-2 associated death promoter pathway. Data also confirmed that MYDGF promoted the viability, proliferation, migration, and invasion of human bladder cancer cells[37] and renal clear cell carcinoma (KIRC)[38] by regulating the PI3K/Akt pathway. Here, the expression level of p-Akt in the I/R group, H/R group, and H_2O_2-treated group were significantly increased after rhMYDGF pretreatment, thus suggesting that rhMYDGF can reduce oxidative stress, inflammatory response, and apoptosis in the course of renal I/R injury through activating Akt signaling pathway. Furthermore, our data in vivo and in vitro showed that the anti-inflammatory, antiapoptotic, and antioxidant effects of rhMYDGF could be abolished by LY.

The present study has some limitations. First, MYDGF is a recognized secretory protein, but the receptor mediating its action has not been clearly identified. Second, the study of Zhao et al[39] showed that rhMYDGF could mediate the proliferation of human coronary artery endothelial cells, and it is possible to further study whether rhMYDGF can improve angiogenesis after renal I/R injury. Also, since renal I/R injury triggers multiple cell death modalities, and our data indicated that rhMYDGF affects oxidative stress, future studies should seek to elucidate whether rhMYDGF is extensively involved in other types of programmed cell death, such as necroptosis, pyroptosis, and ferroptosis. Moreover, we have found that rhMYDGF can activate the Akt pathway in the present study, and the precise

mechanisms between rhMYDGF and the Akt pathway need further studies. Finally, our current study was limited to I/R injury, however, kidney transplantation model is more in line with clinical practice. And subsequent studies will attempt to observe the role of MYDGF in renal transplantation models.

In conclusion, we provide here the first evidence that MYDGF plays a crucial role in the prevention of renal I/R injury by reducing the levels of oxidative stress, inflammation, and apoptosis in renal tubular epithelial cells by activating the Akt pathway, providing a new therapeutic target for the study of the pathophysiological mechanisms of I/R injury and subsequent drug development.

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