J Chin Med Assoc

ORIGINAL ARTICLE

Magnolol reduces myocardial injury induced by renal ischemia and reperfusion

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Abstract

Background: Magnolol is a component of the bark of *Magnolia officinalis*, which is a traditional herbal remedy used in China. In this study, we investigated whether magnolol can reduce myocardial injury induced by renal ischemia and reperfusion (I/R).

Methods: Renal I/R was elicited by a 60-minute occlusion of the bilateral renal arteries and a 24-hour reperfusion in Sprague-Dawley rats. Magnolol was administered intravenously 10 minutes before renal I/R to evaluate its effects on myocardial injury induced by renal I/R.

Results: Renal I/R significantly increased the serum levels of creatine phosphokinase (CPK), lactate dehydrogenase (LDH), and cardiac troponin I and caused myocardial damage. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling–positive nuclei and caspase-3 activation was significantly increased in the myocardium, indicating increase of apoptosis. Echocardiography revealed left ventricular dysfunction, as evidenced by reduction of left ventricular ejection fraction and left ventricular fractional shortening. Furthermore, serum levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 were significantly elevated, while the IL-10 level was suppressed. However, intravenously, pretreatment with magnolol at doses of 0.003 and 0.006 mg/kg 10 minutes before renal I/R significantly prevented the increases of CPK, LDH, and cardiac troponin I levels, as well as the histological damage and the apoptosis in the myocardium. Echocardiography showed significant improvement of left ventricular function. Furthermore, the increases in TNF-α, IL-1β, and IL-6 and the decrease in IL-10 were significantly limited, while Bcl-2 was increased and Bax was decreased in the myocardium. Phosphorylation of Akt and extracellular signal-regulated kinases 1 and 2 was increased, while phosphorylation of p38 and c-Jun N-terminal kinase was reduced.

Conclusion: Magnolol reduces myocardial injury induced by renal I/R. The underlying mechanisms for this effect might be related to modulation of the production of pro- and anti-inflammatory cytokines and the limiting of apoptosis.

Keywords: Apoptosis; Magnolia; Plant bark; Renal artery; Reperfusion

1. INTRODUCTION

Renal ischemia and reperfusion (I/R) occur in kidney surgery, kidney transplantation, and cardiac surgery[1] and may result in acute kidney injury (AKI), a common and serious complication of kidney surgery, kidney transplantation, cardiac surgery, and vascular surgery.[2–4] AKI may in turn lead to acute cardiac dysfunction, the so-called type 3 cardiorenal syndrome.[5,6] There are several pathways through which AKI can affect the heart, including fluid overload, hyperkalemia, acidemia, pericarditis, and the accumulation of myocardial depressant factors.[6–9] Additionally, renal I/R increases the production of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 and decreases the level of anti-inflammatory cytokines, such as IL-10.[10–13] This increase of proinflammatory cytokines and decrease of anti-inflammatory cytokines may enhance inflammatory responses and induce apoptosis, both of which contribute to the development of myocardial injury.[14–18] Cardiovascular death is one of the major causes of mortality in patients with AKI.[5,19]

Although cardiovascular death is one of the major causes of mortality in patients with AKI, there is no specific and effective treatment for myocardial dysfunction resulting from AKI.[20] As such, an effective therapy to prevent or treat myocardial

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Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or material discussed in this article.

Journal of Chinese Medical Association. (2022) 85: 584-596.

Received December 25, 2020; accepted November 5, 2021.

doi: 10.1097/JCMA.0000000000000727.

dysfunction resulting from AKI would significantly improve clinical outcomes. Magnolol (Fig. 1A) is a component of the bark of *Magnolia officinalis*, which has traditionally been used to treat allergy, cough, and gastroenterological disease in China.[21,22] Magnolol possesses strong anti-inflammatory and antioxidant effects.[23,24] Magnolol has been reported to attenuate myocardial stunning and reduce myocardial infarct size,[25,26] and we recently reported that magnolol attenuates renal I/R injury.[10] However, the protective effects of magnolol on myocardial injury induced by renal I/R are not known and have never been reported. Therefore, we conducted this study to determine whether magnolol can reduce myocardial injury induced by renal I/R. The roles of cytokines and apoptosis were also investigated. The results of our study reveal the protective effects and mechanisms of magnolol against myocardial injury induced by renal I/R and may provide a rationale for the use of magnolol to decrease myocardial injury in kidney surgery, kidney transplantation, cardiac surgery, and vascular surgery.

2. METHODS

2.1. Chemicals and reagents

Magnolol (with purity ≥95%) was bought from Sigma-Aldrich (St. Louis, MO, USA). Magnolol was dissolved in 40% (v/v) propylene glycol and then dissolved in normal saline to the desired concentration. The final concentration of propylene glycol in the administered magnolol solution was 4×10^{-3}% (v/v). The serum levels of TNF-α, IL-1β, IL-6, and IL-10 were determined by using a commercially available ELISA set (R&D Systems, Minneapolis, MN, USA). The diaminobenzidine chromogen used for the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was purchased from Boehringer (Mannheim, Germany).

2.2. Animals

Fifty-seven male Sprague-Dawley rats (body weight, 250–280 g) were used in this study. All the rats were cared for humanely in accordance with the "Guide for the Care and Use of Laboratory

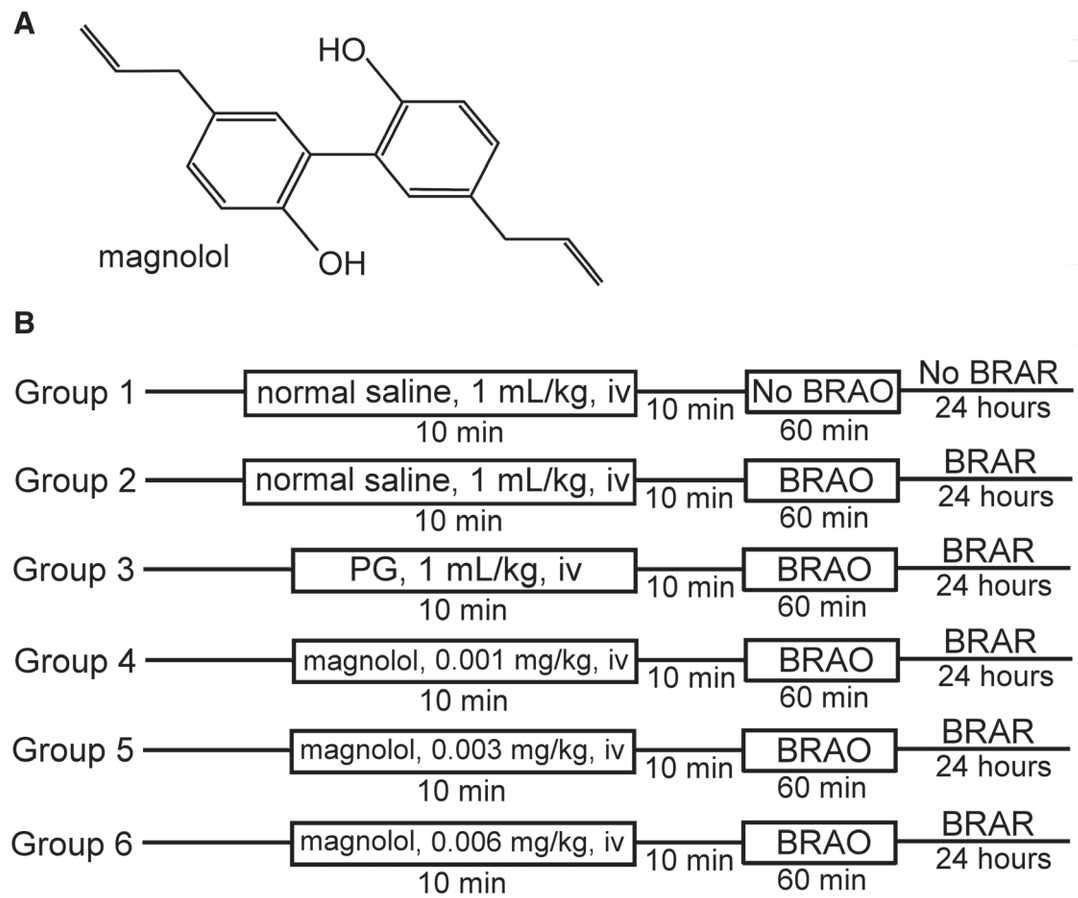

Fig. 1 The chemical structure of magnolol and the experimental protocol. A, The chemical structure of magnolol. B, The experimental protocol. The rats in group 1 underwent the same surgical procedures as the rats in the other five groups, and an intravenous infusion of normal saline (1 mL/kg) for 10 min, without bilateral renal artery occlusion (BRAO) or reperfusion (BRAR). The rats in group 2 received an intravenous infusion of normal saline (1 mL/kg) for 10 min. The rats in group 3 received an intravenous administration of 1 mL/kg of 4×10^{-3}% (v/v) propylene glycol (PG) for 10 min. The rats in group 4 received an intravenous administration of 0.001 mg/kg of magnolol for 10 min. The rats in group 5 received an intravenous administration of 0.003 mg/kg of magnolol for 10 min. The rats in group 6 received an intravenous administration of 0.006 mg/kg of magnolol for 10 min. Ten minutes after the above pretreatments, all rats except those in group 1 underwent a 60-min BRAO and a 24-h BRAR. Because there have not been any previous reports like our current study, there is no reference substance or treatment that has been previously established as having any effect when tested by the assay used in our current study. Therefore, no positive controls were included in the current study.

Animals" (National Academic Press, USA, 1996). The current study was approved by the Animal Experiment Committee of the Taipei Veterans General Hospital (approval number: IACUC 2017–082; approval date: July 10, 2017).

2.3. Animal preparation

The animal preparation techniques used in this study have been previously reported.[10] Briefly, the rats were anesthetized with pentobarbiturate (30 mg/kg, intraperitoneal) and were placed on a heating pad. The body temperature of each rat was monitored and maintained at 37°C throughout the experiments. A small midline incision was performed at the abdomen, through which the bilateral renal pedicles were occluded for 60 minutes with nontraumatic microvascular clamps to induce ischemia. Successful occlusion of the bilateral renal arteries was verified by the cyanotic change of the kidneys. After the 60-minute warm ischemia of the bilateral kidneys, the microvascular clamps were gently removed for reperfusion. Reperfusion was confirmed by the restoration of blood flow. The abdominal incision was then closed in layers with 4-0 silk suture. All the procedures were performed under aseptic conditions. The animals were then allowed to recover with free access to food and water. Sham animals underwent the same surgical procedure without the placement of the microvascular clamps. The rats were sacrificed 24 hours after the reperfusion or sham operation. The blood of the rats was then collected, and their kidneys and hearts were harvested for examination.

2.4. Grouping and experimental protocol

A total of 57 rats were randomly allocated into six groups (Fig. 1B). The rats in group 1 were subjected to the surgical procedures described above and intravenous infusion of normal saline (1 mL/kg) for 10 minutes but without renal artery occlusion or reperfusion (sham group, 8 rats). The rats in group 2 were administered an infusion of normal saline (1 mL/kg) for 10 minutes (renal I/R group, 10 rats). The rats in group 3 received an infusion of 4×10^{-3}% (v/v) propylene glycol (1 mL/kg) for 10 minutes (PG group, 10 rats). The rats in group 4 received an infusion of 0.001 mg/kg of magnolol for 10 minutes (Mag–0.001 group, 10 rats). The rats in group 5 received an infusion of 0.003 mg/kg of magnolol for 10 minutes (Mag–0.003 group, 9 rats). The rats in group 6 received an infusion of 0.006 mg/kg of magnolol for 10 minutes (Mag–0.006 group, 10 rats). Ten minutes after the aforementioned pretreatments, the rats in all the groups except those in group 1 were subjected to a 60-minute bilateral renal artery occlusion and 24-hour renal reperfusion. The blood pressure and heart rate of each rat were recorded before the various pretreatments (baseline 1), after the pretreatments (baseline 2), 60 minutes after the bilateral renal artery occlusion, and at 3 and 24 hours after the bilateral renal artery reperfusion. During the experiment and 24 hours after reperfusion, the tail-cuff blood pressure and heart rate was measured using an indirect blood pressure meter (Softron Biotechnology, Beijing, China). During the measurement of the blood pressure, we tried to minimize stress for the animals.[10,27]

In this study, the dose of 0.001, 0.003, and 0.006 mg/kg of magnolol was used based on the methods of our previous report.[10] We used a 60-minute bilateral renal artery occlusion and 24-hour renal reperfusion also based on the methods of our previous report.[10]

2.5. Echocardiography evaluation of left ventricular function

Twenty-four hours after the reperfusion or sham operation, transthoracic echocardiography was performed under light anesthesia induced by inhalation of a 1% isoflurane and oxygen mixture through a nose cone. The left ventricular end-diastolic diameter, left ventricular end-systolic diameter, left ventricular posterior wall thickness at diastole, left ventricular posterior wall thickness at systole, interventricular septal wall thickness at diastole, interventricular septal wall thickness at systole, left ventricular end-diastolic volume, and left ventricular end-systolic volume were measured by M-mode and two-dimensional parasternal short-axis scans at the level of the papillary muscles using a 8- to 15-MHz linear transducer in a SEQUOIA 512 machine (ACUSON, Mountain View, CA, USA). All measurements were performed in a blinded manner and represented the mean of at least 5 consecutive cardiac cycles. Five rats from each group received the echocardiography examination. Cardiac contractile functions as represented by left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were calculated by computer algorithms.[28]

2.6. Biochemical analysis of kidney and myocardial injury

At the end of the experiments, we collected blood from six rats in each group. Blood urea nitrogen (BUN), creatinine, creatine phosphokinase (CPK), lactate dehydrogenase (LDH), and cardiac troponin I levels in the serum were measured by colorimetric methods adapted to an auto-analyzer. The serum levels of BUN and creatinine were expressed as mg/dL, while the serum levels of CPK and LDH were expressed as U/L. The serum level of cardiac troponin I was expressed as ng/mL.

2.7. Histological examination of kidney and myocardial injury

At the end of the experiments, the kidneys and the left ventricles of four rats from group 1 and five rats from the remaining groups were fixed in 10% buffered formaldehyde for the histological examination of myocardial injury. The left ventricles were dehydrated, embedded in paraffin, sectioned into 5-μm-thick slices, and mounted on glass slides. The sections were then deparaffinized with xylene and counterstained with hematoxylin and eosin.

Histological injury to the kidneys was evaluated in a blinded manner by a pathologist through determinations of focal glomerular necrosis, dilatation of Bowman's capsule, degeneration of tubular epithelium, necrosis in tubular epithelium, tubular dilatation, and interstitial inflammatory infiltration. Histological changes were scored on a 4-point scale. The score was 0 if there were no changes. The score was 1 if the changes were mild and focal. The score was 2 if the changes were intermediate and multifocal. The score was 3 if the changes were prominent and extensive.[10,18]

Histological injury to the myocardium was examined in a blinded manner by a pathologist. The following morphological criteria were used to grade the severity of myocardial injury: score 0, no damage; score 1 (mild), interstitial edema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling and necrosis; score 3 (severe), necrosis with the presence of contraction bands and neutrophil infiltrate; and score 4 (highly severe), widespread necrosis with the presence of contraction bands, neutrophil infiltrate, and hemorrhage.[29,30]

2.8. TUNEL staining analysis of the myocardium

The TUNEL staining analysis methods used in this study have been previously reported.[10,18] Briefly, at the end of the experiments, the left ventricles of four rats from group 1 and five rats from the remaining groups were sectioned into 3- to 4-μm-thick slices and fixed in acetone. Endogenous peroxide activity was quenched by incubation in 3% hydrogen peroxide in methanol. Slides were rinsed with PBS blocking solution and incubated with a permeabilization solution. Samples were incubated with terminal deoxynucleotidyl transferase and detection

buffer conjugated with horseradish peroxidase. A diaminobenzidine chromogen was used. Counterstaining with hematoxylin was performed. Six to eight randomly selected areas per section and two to three sections per left ventricle were examined in a blinded manner by a pathologist. The number of TUNEL-positive nuclei was counted and expressed as the percentage of the total number of cellular nuclei at a magnification of ×400.

2.9. Determination of serum levels of TNF-α, IL-1β, IL-6, and IL-10

At the end of the experiments, we collected blood from six rats in each group to determine the serum levels of TNF-α, IL-1β, IL-6, and IL-10. The serum levels of TNF-α, IL-1β, IL-6, and IL-10 were determined through ELISA by using a commercially available ELISA kit. The ELISA was performed according to the manufacturer's instructions. All samples and standards were measured in duplicate.[10,18]

2.10. Western blot analysis for activated caspase-3, TNF-α, Bcl-2, Bax, Akt, phospho-Akt, ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, and phospho-JNK in the myocardium

The Western blot analysis methods used in this study have been previously reported.[10,18] Briefly, at the end of the experiments, the left ventricles of four rats in each group were homogenized in buffer at 4°C. After centrifugation, protein concentrations were determined using a modified Bradford assay. Equivalent amounts of protein samples were loaded and separated on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), the membranes were incubated with various appropriate antibodies at 1:1000 (v/v) dilution in 5% nonfat dry milk. The membranes were then incubated in 5% nonfat dry milk in TBST containing secondary antibody conjugated to horseradish peroxidase. The peroxidase activity of the membranes was visualized with an enhanced chemiluminescence substrate system, after which the membranes were exposed to hyperfilms. β-actin (1:2000 dilution) was detected as a loading control for protein quantity. The optical density for each band was determined using NIH Image 1.6 and normalized against the background density for each gel.

2.11. Statistical analysis

All values were expressed as mean ± SD. A computer program (SPSS 18.0; SPSS Inc., Chicago, IL, USA) was utilized for statistical analysis. Hemodynamic variables were analyzed using two-way analysis of variance with repeated measures. Multiple comparisons of repeated measures were performed using within-subject contrasts. The data were analyzed using one-way analysis of variance and the Bonferroni post hoc multiple comparison test. The ordinal values of the kidney and myocardial injury scores were analyzed using the Kruskal-Wallis and the Mann-Whitney nonparametric tests. The Pearson correlation coefficient was used to assess the correlation between TNF-α levels in the serum and the myocardium. Differences were considered significant at p values of <0.05.

3. RESULTS

3.1. Mortality and hemodynamic changes

Fifty-seven rats were included in this study (Fig. 1B). The mortality rates of the six groups were not significantly different (p = 0.872; Table 1). The baseline systolic blood pressures and heart rates were not significantly different for the six groups (Table 2). The renal I/R significantly decreased the systolic blood pressures and heart rates of the rats in group 2 through group 6.

Table 1

Mortality

Group	Treatment protocol	Number	Mortality	Number included
1	Sham	8	0	8
2	Renal I/R	10	1	9
3	PG	10	1	9
4	Mag–0.001	10	1	9
5	Mag–0.003	9	0	9
6	Mag–0.006	10	1	9

Groups 2, 3, 4, and 6 each had one rat that gradually became weak and hypotensive after the surgery. These four rats died during the reperfusion period. No significant differences in the mortality rates were observed among the groups. The Pearson's chi-squared p value was 0.872.
I/R = ischemia and reperfusion; Mag–0.001 = magnolol, 0.001 mg/kg; Mag–0.003 = magnolol, 0.003 mg/kg; Mag–0.006 = magnolol, 0.006 mg/kg; PG = propylene glycol.

Although the systolic blood pressures and heart rates remained significantly lower than the baseline values in group 2 through group 6 after reperfusion for 24 hours, the administration of magnolol at the dose of 0.006 mg/kg significantly limited the decrease of the heart rate (416 ± 21 vs 357 ± 38 beats/min in group 2; $p < 0.01$). Because there have not been any previous reports like our current study, ie, reports determining the protective effects of any substance or medicine against myocardial injury induced by renal I/R, there is no reference substance or treatment that has previously been established as having any protective effects when tested by the assay used in our current

Table 2

Hemodynamic changes during the experiments

Group	Treatment protocol	Baseline 1	Baseline 2	RAO (1 h)	RAR 3 h	RAR 24 h
SBP, mmHg						
1	Sham	103 ± 4	100 ± 4	102 ± 3	101 ± 5	102 ± 5
2	Renal I/R	104 ± 5	102 ± 6	71 ± 4[a,d,g]	78 ± 8[a,d,g]	71 ± 7[a,d,g]
3	PG	103 ± 9	104 ± 8	73 ± 7[a,d,g]	79 ± 7[a,d,g]	69 ± 15[a,d,g]
4	Mag–0.001	102 ± 7	103 ± 5	72 ± 12[a,d,g]	82 ± 7[a,d,g]	73 ± 9[a,d,g]
5	Mag–0.003	104 ± 7	103 ± 4	71 ± 5[a,d,g]	79 ± 5[a,d,g]	76 ± 6[a,d,g]
6	Mag–0.006	105 ± 7	104 ± 6	72 ± 5[a,d,g]	80 ± 6[a,d,g]	79 ± 6[a,d,g]
HR, beats/min						
1	Sham	463 ± 21	456 ± 26	447 ± 28	457 ± 24	450 ± 22
2	Renal I/R	459 ± 20	461 ± 21	383 ± 32[a,d,h]	396 ± 30[a,d,h]	357 ± 38[a,d,g]
3	PG	457 ± 28	450 ± 27	387 ± 36[a,e,h]	394 ± 27[b,e,g]	361 ± 32[a,d,g]
4	Mag–0.001	453 ± 27	443 ± 29	389 ± 33[b,e,h]	402 ± 37[c,h]	378 ± 32[a,e,g]
5	Mag–0.003	454 ± 22	449 ± 19	382 ± 25[a,d,h]	386 ± 22[a,d,g]	395 ± 22[a,d,h]
6	Mag–0.006	459 ± 23	446 ± 22	406 ± 22[a,e]	413 ± 20[b,f,i]	416 ± 21[b,f,j,k]

Values are mean ± SD. Rats in group 1 received the same surgical procedure without administration of magnolol, renal artery occlusion, or reperfusion. The hemodynamic data shown for group 1 were observed at the same time points as those for the other five groups. Eight rats were included in group 1. Nine rats were used in the other five groups.
baseline 1 = baseline before treatment; baseline 2 = baseline after treatment; HR = heart rate; I/R = ischemia and reperfusion; Mag–0.001 = magnolol, 0.001 mg/kg; Mag–0.003 = magnolol, 0.003 mg/kg; Mag–0.006 = magnolol, 0.006 mg/kg; PG = propylene glycol; RAO = renal artery occlusion; RAR = renal artery reperfusion; SBP = systolic blood pressure.
[a] $p < 0.001$ vs baseline 1;
[b] $p < 0.01$ vs baseline 1;
[c] $p < 0.05$ vs baseline 1;
[d] $p < 0.001$ vs baseline 2;
[e] $p < 0.01$ vs baseline 2;
[f] $p < 0.05$ vs baseline 2;
[g] $p < 0.001$ vs group 1;
[h] $p < 0.01$ vs group 1;
[i] $p < 0.05$ vs group 1;
[j] $p < 0.01$ vs group 2;
[k] $p < 0.01$ vs group 3.

study. Therefore, no positive controls were included in the current study.

3.2. Biochemical analysis and histological examination of kidney injury

To evaluate the AKI induced by renal I/R, blood was collected from six rats in each group for biochemical analysis of kidney function, and the kidneys of four rats from group 1 and five rats from group 2 through group 6 were harvested for histological examination of tissue damage at the end of the experiments. Renal I/R significantly increased the BUN level (153 ± 23 vs 17 ± 6 mg/dL in group 1; $p < 0.001$; Fig. 2A) and creatinine level (3.23 ± 0.78 vs 0.31 ± 0.13 mg/dL in group 1; $p < 0.001$; Fig. 2B). However, magnolol at doses of 0.003 and 0.006 mg/kg significantly limited the increases in the BUN level (54 ± 18 and 31 ± 13 mg/dL; $p < 0.001$ vs group 2) and creatinine level (0.94 ± 0.66 and 0.73 ± 0.46 mg/dL; $p < 0.001$) induced by renal I/R. Magnolol at the dose of 0.001 mg/kg and propylene glycol did not significantly change the increases in the BUN level (136 ± 19 and 142 ± 21 mg/dL; $p > 0.99$ vs group 2) and

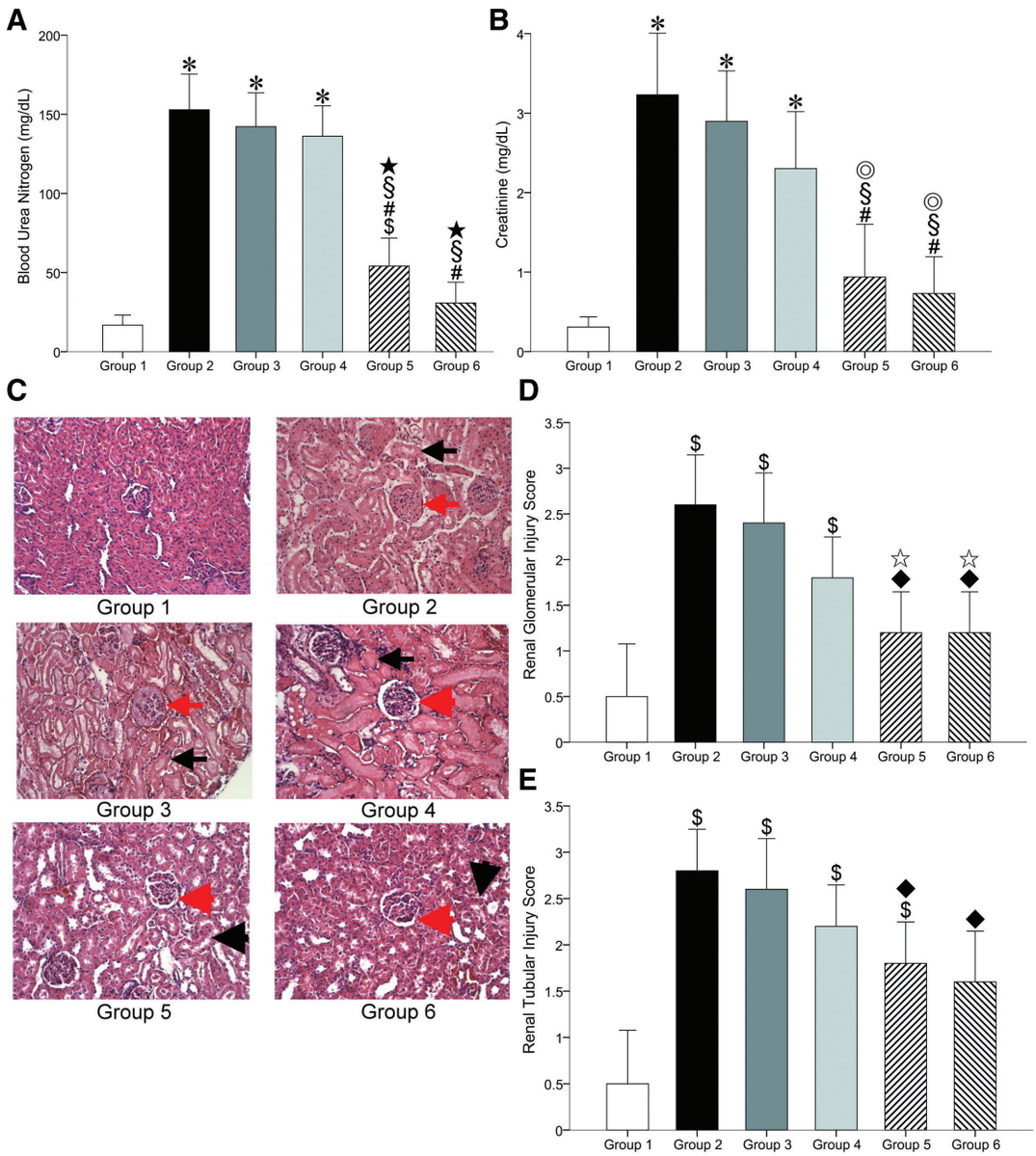

Fig. 2 Acute kidney injury induced by renal ischemia and reperfusion. A, The blood urea nitrogen level. B, The creatinine level. C, Representative photomicrographs of hematoxylin and eosin staining of kidney sections (×200 magnification). The renal glomeruli and tubules of the kidneys of the rats in group 1 appear normal. Necrosis of renal glomeruli (red arrows) and tubules (black arrows) was found in the kidneys of the rats in groups 2 and 3. Dilatation of Bowman's capsule (red arrowheads) and necrosis of renal tubules were present in the kidneys of the rats in group 4. Dilatation of Bowman's capsule and renal tubules (black arrowheads) were found in the kidneys of the rats in groups 5 and 6. D, Histological injury scoring of the renal glomeruli. E, Histological injury scoring of the renal tubules; *$p < 0.001$ vs group 1; \$$p < 0.05$ vs group 1; #$p < 0.001$ vs group 2; ◆$p < 0.05$ vs group 2; §$p < 0.001$ vs group 3; ☆$p < 0.05$ vs group 3; ★$p < 0.001$ vs group 4; and ◎$p < 0.01$ vs group 4. Blood from six rats from each group was collected for analysis of blood urea nitrogen and creatinine levels at the end of the experiments. The kidneys of four rats from group 1 and 5 rats each from group 2 through group 6 were used for histological examination of kidney injury at the end of the experiments.

creatinine level (2.30 ± 0.72 and 2.90 ± 0.63 mg/dL; $p = 0.187$ and $p > 0.99$, respectively).

The results of the histological examination of the renal glomeruli and tubules in the kidneys of the rats in group 1 appeared normal (Fig. 2C). However, renal I/R induced significant degeneration and necrosis in the renal glomeruli and tubules in the kidneys of the rats in group 2. The injury scores of the renal glomeruli and tubules of the rats in group 2 were significantly higher than those of the rats in group 1 ($p < 0.05$ vs group 1; Fig. 2D, E). However, magnolol at doses of 0.003 and 0.006 mg/kg significantly reduced these increases to the injury scores of the renal glomeruli and tubules ($p < 0.05$ vs group 2). Magnolol at the dose of 0.001 mg/kg and propylene glycol did not significantly affect the changes to the injury scores of the renal glomeruli ($p = 0.095$ and $p = 0.690$) and tubules ($p = 0.151$ and $p = 0.690$).

3.3. Biochemical analysis of myocardial injury

At the end of the experiments, blood was collected from six rats in each group for biochemical analysis of myocardial injury. Renal I/R significantly increased the serum levels of CPK (1243 ± 645 vs 287 ± 114 U/L in group 1; $p < 0.001$; Fig. 3A), cardiac troponin I (0.67 ± 0.30 vs 0.01 ± 0.01 ng/mL in group 1; $p < 0.001$; Fig. 3B), and LDH (3812 ± 1631 vs 189 ± 89 U/L in group 1; $p < 0.001$; Fig. 3C). However, magnolol at doses of 0.003 and 0.006 mg/kg significantly reduced these increases in the serum levels of CPK (475 ± 117 and 337 ± 76 U/L; $p < 0.01$ and $p < 0.001$ vs group 2, respectively), cardiac troponin I (0.03 ± 0.04 and 0.02 ± 0.01 ng/mL; $p < 0.01$ and $p < 0.001$), and LDH (610 ± 367 and 361 ± 131 U/L; $p < 0.001$) induced by renal I/R. Magnolol at the dose of 0.001 mg/kg and propylene glycol did not significantly change the increases in the serum levels of CPK (669 ± 300 and 854 ± 285 U/L; $p = 0.064$ and $p = 0.670$ vs group 2, respectively), cardiac troponin I (0.50 ± 0.33 and 0.60 ± 0.35 ng/mL; $p > 0.99$), and LDH (2629 ± 1257 and 3089 ± 1188 U/L; $p = 0.689$ and $p > 0.99$).

3.4. Echocardiography examination of the left ventricular function

To evaluate the effects of renal I/R on the left ventricular function, five rats from each group received a transthoracic echocardiography examination after a 24-hour bilateral renal artery reperfusion (Fig. 4A). Renal I/R significantly impaired the left ventricular function, as evidenced by decreases of LVEF (83 ± 2% vs 95 ± 3% in group 1; $p < 0.001$; Fig. 4B) and LVFS (46 ± 2% vs 71 ± 3% in group 1; $p < 0.001$; Fig. 4C). However, magnolol at doses of 0.003 and 0.006 mg/kg significantly limited the reductions of LVEF (89 ± 2% and 91 ± 2%; $p < 0.01$ and $p < 0.001$ vs group 2, respectively) and LVFS (54 ± 2% and 57 ± 2%; $p < 0.001$) induced by renal I/R. Magnolol at the dose of 0.001 mg/kg and propylene glycol did not significantly change the decreases of LVEF (85 ± 2% and 81 ± 2%, $p > 0.99$ vs group 2) and LVFS (49 ± 2% and 45 ± 2%; $p = 0.793$ and $p > 0.99$).

3.5. Histological examination of myocardial injury

At the end of the experiments, the left ventricles of four rats from group 1 and five rats from group 2 through group 6 were used for histological examinations of myocardial tissue damage. The myocardium in the left ventricle of the rats in group 1 appeared normal (Fig. 5A). However, renal I/R resulted in interstitial edema, myocardial cell swelling, and the disruption of myocardial fibers. The myocardial injury scores of the rats in group 2 were significantly higher than those of the rats in group 1 ($p < 0.05$ vs group 1; Fig. 5B). However, the administration of magnolol at doses of 0.003 and 0.006 mg/kg significantly reduced the histological damage. The myocardial injury scores of the rats in group 5 and group 6 were significantly decreased ($p < 0.05$ vs group 2). Magnolol at the dose of 0.001 mg/kg and propylene glycol did not significantly change the myocardial injury induced by renal I/R ($p = 0.222$ and $p = 0.690$).

3.6. TUNEL staining analysis of the myocardium

At the end of the experiments, the left ventricles of four rats from group 1 and five rats from group 2 through group 6 were used for TUNEL staining. Few positive TUNEL cells were identified in the myocardium of the rats in group 1 (Fig. 6A). In contrast, renal I/R induced numerous positive TUNEL cells in the myocardium of the rats in group 2. Positive TUNEL cells, expressed as a percentage of total cells, were significantly higher in the myocardium of the rats in group 2 (22.2 ± 4.3% vs 0.7 ± 0.2% in group 1; $p < 0.001$; Fig. 6B). However, magnolol at doses of 0.003 and 0.006 mg/kg significantly inhibited this increase in positive TUNEL cells induced by renal I/R (11.4 ± 2.6% and 8.2 ± 3.0%; $p < 0.001$ vs group 2). Magnolol at the dose of 0.001 mg and propylene glycol did not, in contrast, significantly change the increase in positive TUNEL cells (16.4 ± 2.4% and 20.3 ± 3.2%, $p = 0.082$ and $p > 0.99$, respectively).

3.7. Western blot analysis of activated caspase-3 in the myocardium

At the end of the experiments, the left ventricles of four rats from each group were used for Western blot analysis of the activated caspase-3 level. Western blot analysis demonstrated that renal I/R significantly increased the activated caspase-3 in the myocardium of the rats in group 2 (lane 2, $p < 0.001$ vs group 1; Fig. 6C). However, magnolol at doses of 0.003 and 0.006 mg/kg significantly prevented this increase in activated

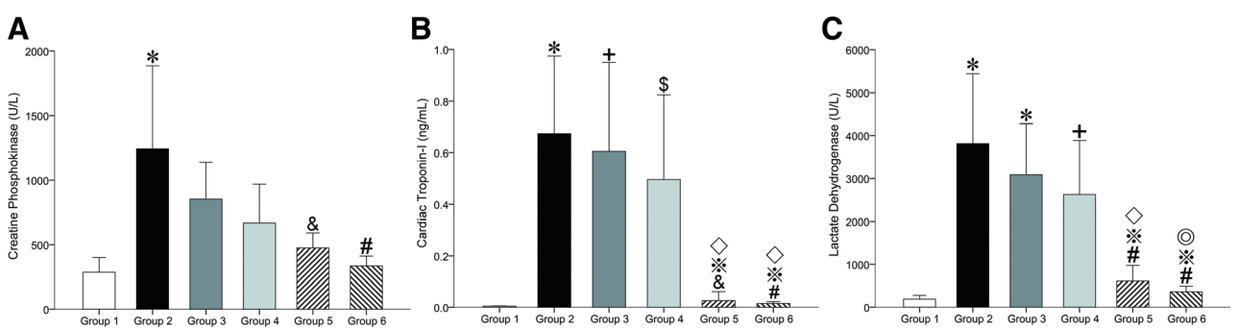

Fig. 3 Biochemical analysis of myocardial injury. A, The creatine phosphokinase level. B, The cardiac troponin I level. C, The lactate dehydrogenase level. *$p < 0.001$ vs group 1; +$p < 0.01$ vs group 1; $$p < 0.05$ vs group 1; #$p < 0.001$ vs group 2; &$p < 0.01$ vs group 2; ※$p < 0.01$ vs group 3; ◎$p < 0.01$ vs group 4; and ◇$p < 0.05$ vs group 4. Blood from six rats from each group was collected for analysis at the end of the experiments.

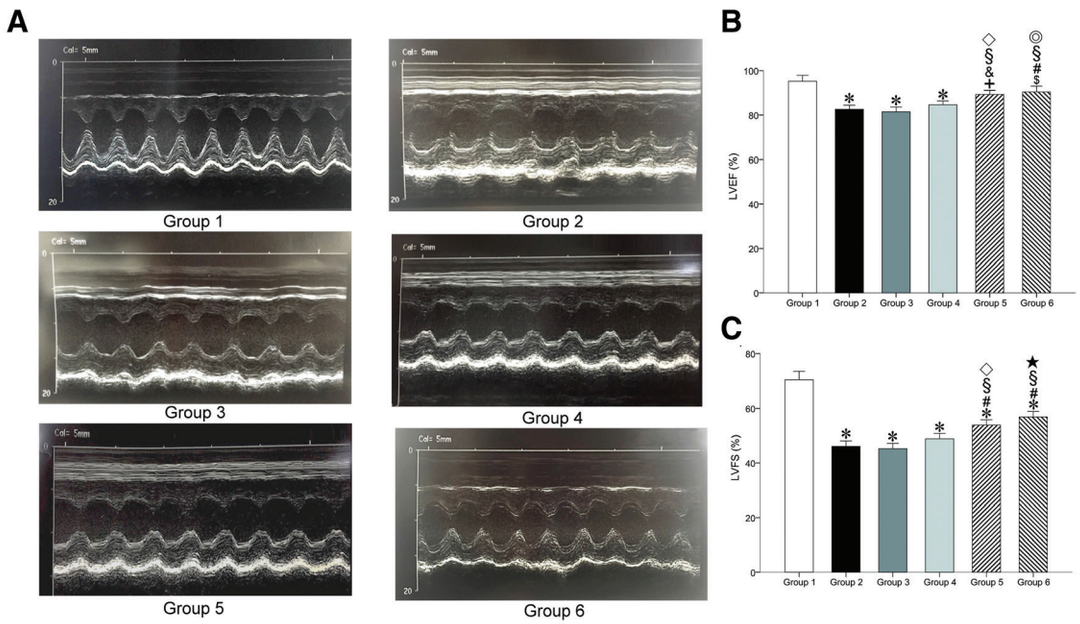

Fig. 4 Echocardiographic evaluation of the left ventricular function. A, Representative M-mode echocardiograms. B, The left ventricular ejection fraction (LVEF). C, The left ventricular fractional shortening (LVFS). *$p < 0.001$ vs group 1; +$p < 0.01$ vs group 1; \$$p < 0.05$ vs group 1; #$p < 0.001$ vs group 2; &$p < 0.01$ vs group 2; §$p < 0.001$ vs group 3; ★$p < 0.001$ vs group 4; ◎$p < 0.01$ vs group 4; and ◇$p < 0.05$ vs group 4. Five rats from each group received the transthoracic echocardiography examination after 24-h bilateral renal artery reperfusion.

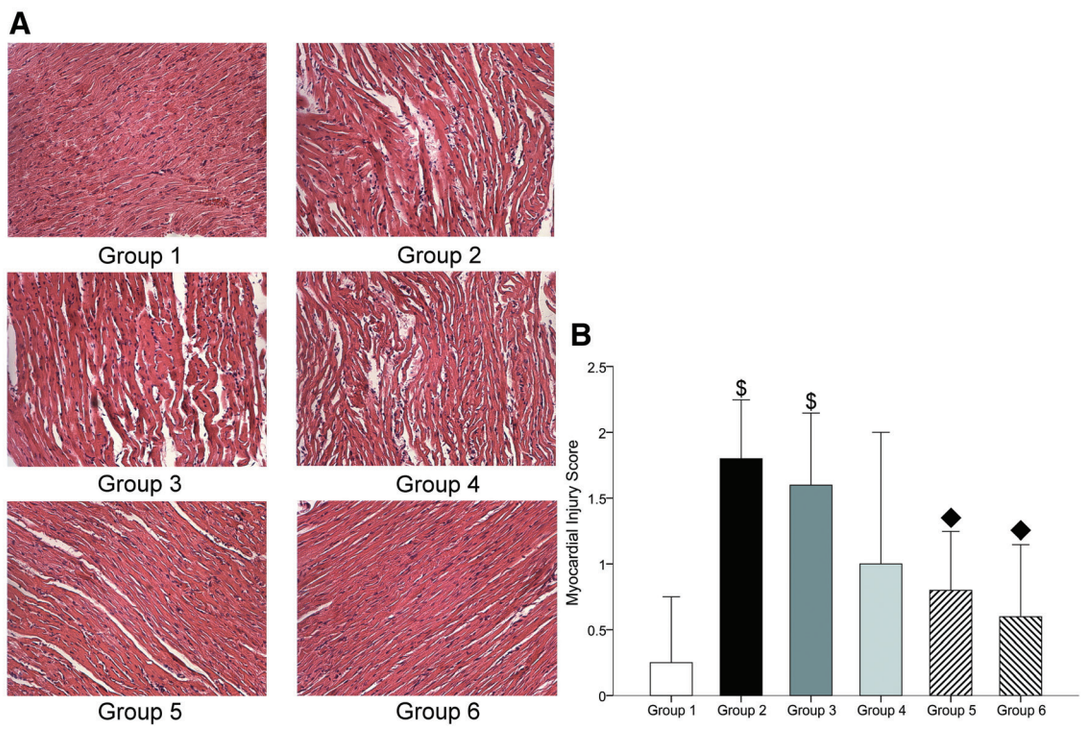

Fig. 5 Histological examination of myocardial injury. A, Representative photomicrographs of hematoxylin and eosin staining of myocardial sections (×200 magnification). The myocardium in the left ventricles of the rats in group 1 appeared normal. Renal I/R resulted in interstitial edema, myocardial cell swelling, and disruption of myocardial fibers in group 2. Administration of magnolol at doses of 0.003 and 0.006 mg/kg ameliorated the histological changes in the myocardium induced by renal I/R (groups 5 and 6). B, Histological injury scoring of the myocardium. \$$p < 0.05$ vs group 1; ◆$p < 0.05$ vs group 2. The left ventricles of four rats from group 1 and 5 rats each from group 2 through group 6 were used for histological examination of myocardial injury at the end of the experiments.

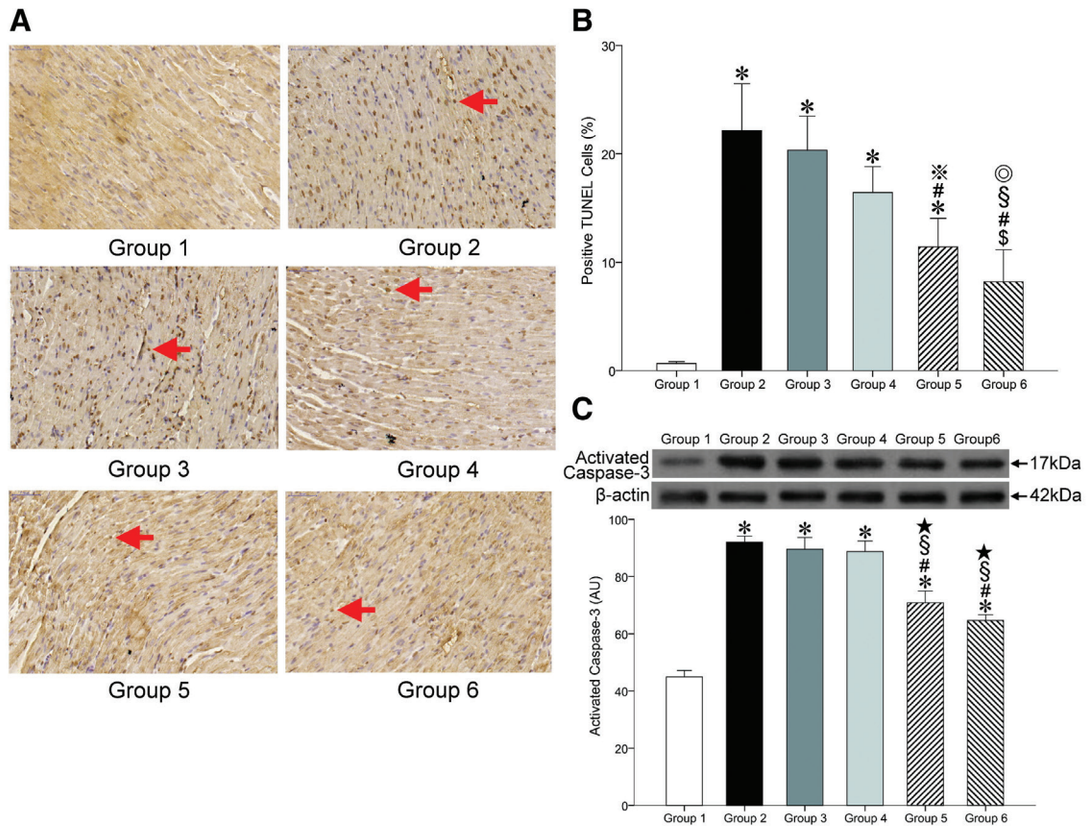

Fig. 6 Analysis of apoptosis in the myocardium. A, Representative photomicrographs of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining of the myocardium (×400 magnification). Dark brown staining (red arrows) indicates TUNEL-positive nuclei. B, The percentage of TUNEL-positive nuclei. C, Assay of activated caspase-3 in the myocardium. A representative Western blot of activated caspase-3 is shown (top). The density of activated caspase-3 was analyzed using arbitrary units (AU; bottom). *$p < 0.001$ vs group 1; $$p < 0.05$ vs group 1; #$p < 0.001$ vs group 2; §$p < 0.001$ vs group 3; ※$p < 0.01$ vs group 3; ★$p < 0.001$ vs group 4; and ◎$p < 0.01$ vs group 4. The left ventricles of four rats from group 1 and five rats each from group 2 through group 6 were used for TUNEL staining. The left ventricles of four rats from each group were used for the Western blot analysis of activated caspase-3.

caspase-3 in the myocardium induced by renal I/R (lanes 5 and 6, $p < 0.001$ vs group 2). In contrast, magnolol at the dose of 0.001 mg/kg and propylene glycol did not significantly affect the increase in activated caspase-3 in the myocardium (lanes 4 and 3, $p > 0.99$).

3.8. Determination of serum levels of TNF-α, IL-1β, IL-6, and IL-10

At the end of the experiments, blood was collected from six rats in each group to determine the serum levels of TNF-α, IL-1β, IL-6, and IL-10. Renal I/R significantly increased the serum levels of the proinflammatory cytokines, including TNF-α (26 ± 6 vs 2 ± 1 pg/mL in group 1; $p < 0.001$; Fig. 7A), IL-1β (237 ± 10 vs 21 ± 4 pg/mL; $p < 0.001$; Fig. 7B), and IL-6 (254 ± 35 vs 44 ± 7 pg/mL; $p < 0.001$; Fig. 7C). However, magnolol at doses of 0.003 and 0.006 mg/kg significantly reduced these increases in the serum levels of TNF-α (6 ± 2 and 4 ± 1 pg/mL; $p < 0.001$ vs group 2), IL-1β (115 ± 10 and 102 ± 7 pg/mL; $p < 0.001$), and IL-6 (186 ± 42 and 165 ± 18 pg/mL; $p < 0.05$ and $p < 0.01$). Magnolol at a dose of 0.001 mg/kg also significantly limited the increases in the serum levels of TNF-α (14 ± 5 pg/mL; $p < 0.001$) and IL-1β (210 ± 11 pg/mL; $p < 0.001$). However, magnolol at a dose of 0.001 mg/kg did not significantly change the increase in the serum level of IL-6 (236 ± 40 pg/mL; $p > 0.99$). Furthermore, propylene glycol did not significantly affect the increases in the serum levels of TNF-α (23 ± 3 pg/mL; $p > 0.99$), IL-1β (233 ± 13 pg/mL; $p > 0.99$), and IL-6 (245 ± 47 pg/mL; $p > 0.99$).

The serum level of IL-10—an anti-inflammatory cytokine—was significantly decreased by renal I/R in the rats of group 2 (72 ± 16 vs 193 ± 11 pg/mL in group 1; $p < 0.001$; Fig. 7D). However, magnolol at doses of 0.001, 0.003, and 0.006 mg/kg significantly inhibited the decrease in the serum level of IL-10 (124 ± 15, 165 ± 20, and 199 ± 11 pg/mL vs group 2; $p < 0.001$), while propylene glycol did not significantly affect the decrease in the serum level of IL-10 (76 ± 12 pg/mL; $p > 0.99$).

3.9. Western blot analysis of TNF-α in the myocardium

At the end of the experiments, the left ventricles of four rats in each group were used for Western blot analysis of the TNF-α level. Western blot analysis revealed that renal I/R significantly increased the TNF-α level in the myocardium of rats in group 2 (lane 2, $p < 0.001$ vs group 1; Fig. 7E). However, magnolol at doses of 0.003 and 0.006 mg/kg significantly reduced the increase of the TNF-α level in the myocardium induced by renal I/R (lanes 5 and 6, $p < 0.001$ vs group 2), whereas magnolol at the dose of 0.001 mg/kg and propylene glycol did not significantly change the increase of the TNF-α level (lanes 4 and 3, $p > 0.99$).

In the analysis of the TNF-α levels in the serum and in the myocardium, we found a significant correlation between the TNF-α levels in the serum and the myocardium ($r = 0.900$, $p < 0.01$; Fig. 7F).

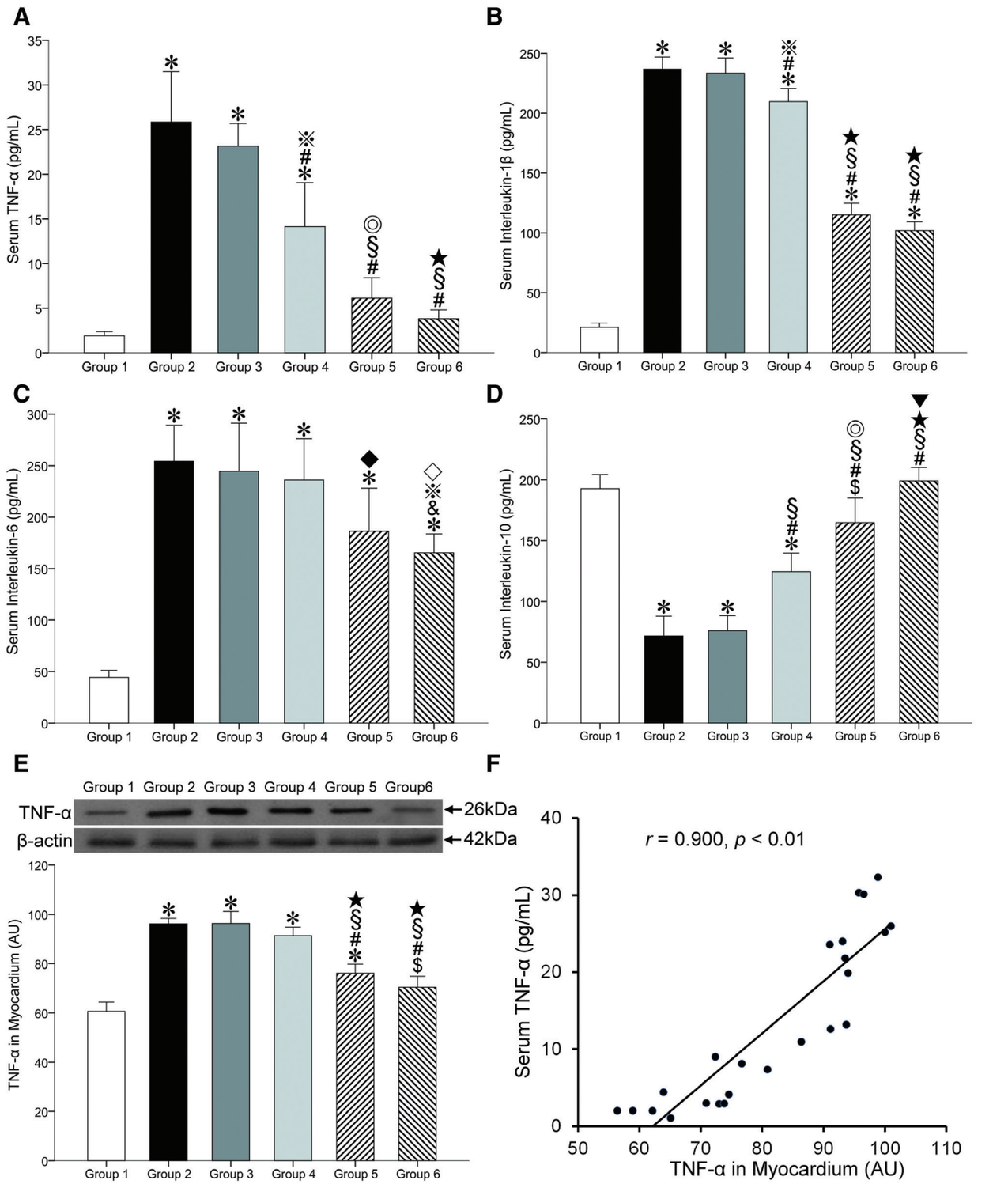

Fig. 7 Analysis of tumor necrosis factor-α, interleukin-1β, interleukin-6, and interleukin-10. A, Serum level of tumor necrosis factor-α (TNF-α). B, Serum level of interleukin-1β. C, Serum level of interleukin-6. D, Serum level of interleukin-10. E, Assay of TNF-α in the myocardium. A representative Western blot of TNF-α is shown (top). The density of TNF-α was analyzed using arbitrary units (AU; bottom). F, Correlation between the TNF-α level in the serum and the myocardium (r = 0.900, $p < 0.01$). *$p < 0.001$ vs group 1; \$$p < 0.05$ vs group 1; #$p < 0.001$ vs group 2; &$p < 0.01$ vs group 2; ◆$p < 0.05$ vs group 2; §$p < 0.001$ vs group 3; ※$p < 0.01$ vs group 3; ★$p < 0.001$ vs group 4; ◎$p < 0.01$ vs group 4; ◇$p < 0.05$ vs group 4; and ▼$p < 0.01$ vs group 5. Blood was collected from six rats in each group to determine the serum levels of TNF-α, interleukin-1β, interleukin-6, and interleukin-10. The left ventricles of four rats from each group were used for the Western blot analysis of TNF-α in the myocardium.

3.10. Western blot analysis for Bcl-2, Bax, Akt, phospho-Akt, ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, and phospho-JNK in the myocardium

At the end of the experiments, the left ventricles of four rats from each group were used for Western blot analysis of the Bcl-2, Bax, phospho-Akt, phospho-ERK1/2, phospho-p38, and phospho-c-JNK levels. Renal I/R significantly decreased the Bcl-2 levels in the myocardium of the rats in group 2 (lane 2, $p < 0.001$ vs group 1; Fig. 8A). However, magnolol at doses of 0.003 and 0.006 mg/kg significantly prevented this decrease in the Bcl-2 levels in the myocardium induced by renal I/R (lanes 5 and 6, $p < 0.001$ vs group 2).

Renal I/R significantly increased the levels of Bax in the myocardium of the rats in group 2 (lane 2, $p < 0.001$ vs group 1; Fig. 8B), while magnolol at doses of 0.003 and 0.006 mg/kg significantly prevented this increase in the Bax levels (lanes 5 and 6, $p < 0.001$ vs group 2).

Renal I/R significantly reduced the phospho-Akt levels in the myocardium of the rats in group 2 (lane 2, $p < 0.001$ vs group 1; Fig. 8C). However, magnolol at doses of 0.003 and 0.006 mg/kg significantly limited this decrease in the phospho-Akt levels in the myocardium of the rats in groups 5 and 6 (lanes 5 and 6, $p < 0.01$ and $p < 0.001$ vs group 2).

Renal I/R significantly decreased the levels of phospho-ERK1/2 in the myocardium of the rats in group 2 (lane 2, $p < 0.001$ vs group 1; Fig. 8D), while magnolol at doses of 0.003 and 0.006 mg/kg significantly inhibited this decrease in the levels of phospho-ERK1/2 (lanes 5 and 6, $p < 0.001$ vs group 2).

Renal I/R significantly increased the levels of phospho-p38 and phospho-JNK in the myocardium of the rats in Group 2 (lane 2, $p < 0.001$ vs group 1; Fig. 8E, F), while magnolol at doses of 0.003 and 0.006 mg/kg significantly limited these increases in the levels of phospho-p38 and phospho-JNK (lanes 5 and 6, $p < 0.001$ vs group 2).

Magnolol at the dose of 0.001 mg/kg and propylene glycol did not significantly affect the changes in the levels of Bcl-2, Bax, phospho-Akt, phospho-ERK1/2, phospho-p38, and phospho-JNK in the myocardium induced by renal I/R.

4. DISCUSSION

In this study, we demonstrated for the first time that magnolol reduces myocardial injury induced by renal I/R. In addition to AKI (shown in the supporting information), renal I/R induced myocardial injury, as evidenced by elevated serum levels of CPK, cardiac troponin I, and LDH, reduced LVEF and LVFS shown by echocardiographic examinations, histological tissue damage, and increased apoptosis in the myocardium. However, pretreatment with magnolol at doses of 0.003 and 0.006 mg/kg significantly attenuated the myocardial injury induced by renal I/R. Renal I/R is one of the major causes of AKI,[1] and AKI is associated with increased mortality.[2–4] An abrupt primary worsening of kidney function may lead to acute cardiac dysfunction, known as type 3 cardiovascular syndrome.[5,6] Unlike type 1 cardiorenal syndrome, type 3 cardiorenal syndrome has not been systematically studied, and the incidence and prevalence of type 3 cardiorenal syndrome are not known. The pathophysiology of type 3 cardiorenal syndrome is also not well understood, and a specific and effective treatment for it is lacking.[5,6,20] The current study better clarifies the mechanisms of myocardial injury induced by renal I/R and may provide a rationale for the use of magnolol to prevent myocardial injury in kidney surgery, kidney transplantation, cardiac surgery, and vascular surgery.

We previously reported that myocardial I/R results in AKI, demonstrating that a local I/R insult may induce injury to a distant organ. However, ischemic preconditioning and the administration of baicalein—a component of the root of *Scutellaria baicalensis*—attenuate the AKI induced by myocardial I/R.[16,18] In the current study, we demonstrated that renal I/R leads to myocardial injury and left ventricular dysfunction. There is a high degree of cross talk between the kidney and the cardiovascular system. Chuasuwan and Kellum[5] reported that AKI and left ventricular dysfunction seem to be risk factors for each other. AKI leads to left ventricular dysfunction, and this in turn leads to the progression of kidney failure, triggering a vicious cycle. They suggested that measures be taken to stop this cycle in order to protect against secondary organ damage and prevent further injury to both organs. In this study and our previous report,[10] we demonstrated that magnolol protects against AKI and myocardial injury induced by renal I/R, such that magnolol might be used to prevent AKI and myocardial injury in kidney surgery, kidney transplantation, cardiac surgery, and vascular surgery.

Magnolol possesses protective effects against I/R injury in many organs. As noted above, we recently demonstrated that magnolol protects the kidneys against AKI induced by renal I/R.[10] We also previously reported that magnolol protects the myocardium against stunning induced by brief myocardial I/R.[26] Subsequently, Jin et al[25] reported that magnolol decreases myocardial infarct size and prevents myocardial dysfunction induced by myocardial I/R. Furthermore, magnolol has been reported to limit muscle damage in the hind limbs of rats induced by I/R.[31] The present study, however, is the first report demonstrating that magnolol reduces myocardial injury induced by renal I/R injury.

The results of this study also demonstrated that magnolol inhibits myocardial apoptosis induced by renal I/R, and relatedly, our recent study found that magnolol reduced apoptosis in the kidneys induced by renal I/R.[10] Magnolol has likewise been demonstrated to inhibit the apoptosis of HT22 neuroblastoma cells induced by trimethyltin. The activation of Jun N-terminal kinase (JNK) and p38 induced by trimethyltin was inhibited by magnolol in that study.[32] Moreover, pretreatment with magnolol at a dose of 10^{-7} g/kg 15 minutes before a 2-hour warm hepatic ischemia has been reported to decrease the number of apoptotic cells in the rat liver.[33] However, the same research group later reported that a more intense nuclear apoptotic signal was observed in liver grafts treated with high concentrations of magnolol under serum-reduced conditions. Increased mitochondrial cytochrome c release and subsequent caspase-3 activation were also observed.[34] Magnolol has also been shown to induce apoptosis and inhibit tumor growth.[35] The effects of magnolol on apoptosis might be different under different doses and different experimental conditions.[22]

We recently demonstrated that magnolol decreases the production of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, and increases the production of anti-inflammatory cytokines, such as IL-10, in renal I/R.[10] Magnolol has also been shown to reduce the levels of TNF-α, IL-1β, and IL-6 in mice with lipopolysaccharide-induced lung injury.[36] Furthermore, Yang et al[37] reported that magnolol decreases TNF-α mRNA expression and increases IL-10 mRNA expression in the small intestine in lipopolysaccharide-induced septic rats.

Kelly[38] reported that renal ischemia induced by occlusion of the bilateral renal arteries for 30 minutes followed by various periods of reperfusion (6-48 hours) increases TNF-α in the serum and in the myocardium. Elevated levels of IL-1β and intercellular adhesion molecule-1 mRNA were also found in the myocardium. Transthoracic echocardiography 48 hours after reperfusion revealed decreased LVFS. Interestingly, the study further demonstrated that increased myocardial apoptosis could be found in the myocardium 48 hours after a 15-minute occlusion of the bilateral renal arteries—a brief period of renal ischemia insufficient to induce azotemia. However, increased myocardial apoptosis was not noted in rats with significant

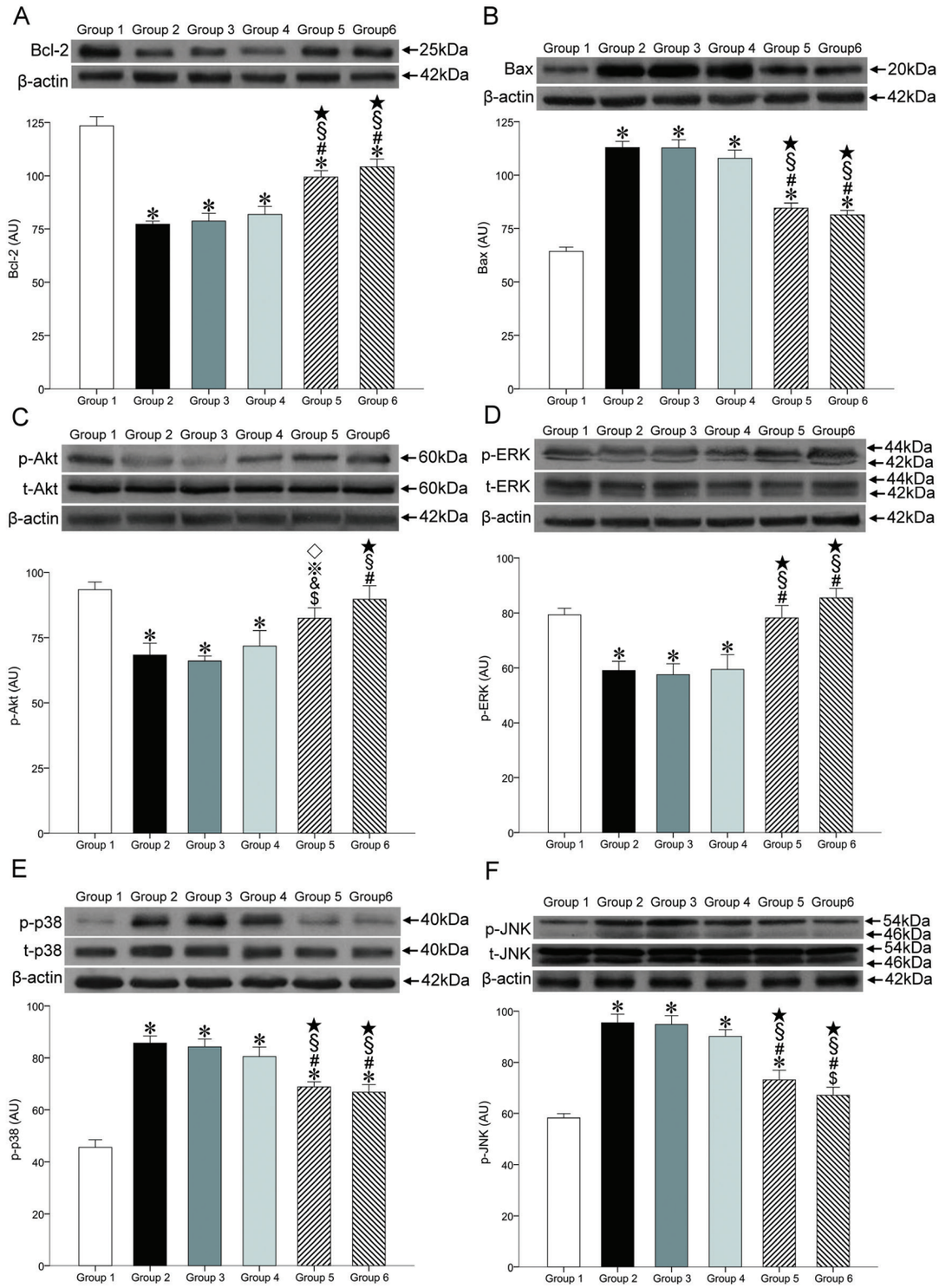

Fig. 8 Western blot analysis of Bcl-2, Bax, Akt, ERK, p38, and JNK. A, Bcl-2 assay. B, Bax assay. C, Phospho-Akt (p-Akt) and total Akt (t-Akt) assay. D, Phospho-extracellular signal-regulated kinases 1 and 2 (p-ERK) and total ERK (t-ERK) assay. E, Phospho-p38 (p-p38) and total p38 (t-p38) assay. F, Phospho-c-Jun N-terminal kinase (p-JNK) and total JNK (t-JNK) assay. Representative Western blots of Bcl-2, Bax, p-Akt, t-Akt, p-ERK, t-ERK, p-p38, t-p38, p-JNK, and t-JNK are shown (top). The densities of Bcl-2, Bax, p-Akt, p-ERK, p-p38, and p-JNK bands were analyzed using arbitrary units (AU; bottom). *$p < 0.001$ vs group 1; \$$p < 0.05$ vs group 1; #$p < 0.001$ vs group 2; &$p < 0.01$ vs group 2; §$p < 0.001$ vs group 3; ※$p < 0.01$ vs group 3; ★$p < 0.001$ vs group 4; and ◇$p < 0.05$ vs group 4. The left ventricles of 4 rats from each group were used for the Western blot analysis of Bcl-2, Bax, p-Akt, t-Akt, p-ERK, t-ERK, p-p38, t-p38, p-JNK, and t-JNK in the myocardium.

azotemia induced by bilateral nephrectomy. Kelly thus suggested that renal I/R rather than azotemia is necessary for the increase of myocardial apoptosis observed.

Youssef et al[39] reported that renal I/R induced by 30-minute occlusion of the bilateral renal arteries followed by 24-hour reperfusion results in both AKI and myocardial injury, in association with elevations of the TNF-α and caspase-3 levels in the kidneys and the myocardium. They also revealed increases in the content of malondialdehyde, nitric oxide, and inducible nitric oxide synthase and decreases of glutathione and antioxidative enzyme activity in both the kidneys and the myocardium. They further demonstrated that the administration of sitagliptin—a dipeptidyl peptidase-4 inhibitor—and furosemide attenuates both the AKI and myocardial injury induced by renal I/R.

The present study has several limitations. First, the definite blood flow of the kidneys was not determined. However, the model of renal I/R used in this study is comparable to that widely used in experiments on renal I/R.[10–13,38,39] Second, with respect to the parameters of left ventricular function, only LVEF and LVFS were demonstrated in this study, while the other parameters were not presented. However, the serum levels of CPK, cardiac troponin I, and LDH and the histological damage to the myocardium were analyzed in this study. Third, TUNEL staining was used to evaluate apoptosis of the myocardium in this study, and while TUNEL staining is a sensitive method commonly used for detecting apoptosis,[10,12,18,34] it is not specific.[40] However, we also investigated caspase-3 activation, which provides a specific and quantitative analysis of apoptosis.[40] Fourth, magnolol has both antioxidant and anti-inflammatory effects.[24,41,42] Its antioxidant effects might contribute the myocardial protection induced by renal I/R. There might be a cross talk between the antioxidant and anti-inflammatory effects. However, we did not investigate the role of antioxidant effects of magnolol in this study. Fifth, although it is possible that renal sympathetic nerve might be clamped during the 60-minute occlusion of bilateral renal pedicles, we did not investigate whether the renal sympathetic nerve was clamped or not in this study.

In conclusion, we demonstrated for the first time in this study that magnolol reduces myocardial injury induced by renal I/R. The underlying mechanisms for this protective effect might be related to limiting apoptosis, possibly through the inhibition of both extrinsic and intrinsic apoptotic pathways, including the decrease of TNF-α production and the modulation of pro- and antiapoptotic signaling elements. Our findings better clarify the mechanisms of myocardial injury induced by renal I/R and the protective effects of magnolol and may provide a rationale for the use of magnolol to prevent myocardial injury in kidney surgery, kidney transplantation, cardiac surgery, and vascular surgery. However, further research on other animal species and investigations of the roles of other signaling elements are necessary.

ACKNOWLEDGMENTS

This study was supported by grants from the Taipei Veterans General Hospital (V106C-192, V107C-187, and V109C-194), the Taipei Veterans General Hospital—National Taiwan University Hospital Joint Research Program (VN106-08 and VN107-08), the Taipei Veterans General Hospital—National Defense Medical Center Joint Research Program (DV104-4), and the Ministry of Science and Technique (MOST 108-2314-B-075-065 and MOST 110-2314-B-075-060), Taiwan. This work was assisted, in part, by the Division of Experimental Surgery, Department of Surgery, Taipei Veterans General Hospital. We thank Shiang-Rong Jeang for her excellent technical support in the experiments.

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