

## Pretreatment of sildenafil attenuates ischemia-reperfusion renal injury in rats

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<sup>1</sup>Internal Medicine, Chungnam National University Hospital, Daejeon; <sup>2</sup>Pathology, Gangnam Severance Hospital, Seoul; and <sup>3</sup>Internal Medicine, Daejeon Saint Mary Hospital, Daejeon, Republic of Korea

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**Choi DE, Jeong JY, Lim BJ, Chung S, Chang YK, Lee SJ, Na KR, Kim SY, Shin YT, Lee KW.** Pretreatment of sildenafil attenuates ischemia-reperfusion renal injury in rats. *Am J Physiol Renal Physiol* 297: F362–F370, 2009. First published May 27, 2009; doi:10.1152/ajprenal.90609.2008.—Sildenafil was the first selective inhibitor of phosphodiesterase-5 (PDE5) to be widely used for treating erectile dysfunction. Many recent studies have investigated the cardioprotective role of sildenafil in animal models. We evaluated the protective effects of sildenafil in experimental renal ischemia-reperfusion (IR) injury in two studies. In *study 1*, male Sprague-Dawley rats were divided into four groups: sham, sildenafil-treated sham, vehicle-treated IR, and sildenafil-treated IR groups. In *study 2*, we divided the rats into two groups: sildenafil-treated IR rats and PD98059 (ERK inhibitor)+sildenafil-treated IR rats. Functional parameters of the kidney were evaluated at the molecular and structural levels. Blood urea nitrogen (BUN) and serum creatinine levels were lower in sildenafil-treated IR rats than in vehicle-treated IR rats. The expression of inducible (iNOS) and endothelial nitric oxide synthase (eNOS) proteins in sildenafil-treated IR rats was significantly higher than in vehicle-treated IR rats. Pretreatment with sildenafil in IR rats increased ERK phosphorylation and reduced the renal Bax/Bcl-2 ratio, renal caspase-3 activity, and terminal dUTP nick end-labeling-positive apoptotic cells. In contrast, PD98059 treatment increased BUN and serum creatinine levels and attenuated the sildenafil-induced expression of pERK, iNOS, eNOS, and Bcl-2. PD98059 also increased caspase-3 activity but did not decrease the sildenafil-induced accumulation of cGMP. In conclusion, this study suggests that sildenafil has antiapoptotic effects in experimental IR renal injury via ERK phosphorylation, induction of iNOS and eNOS production, and a decrease in the Bax/Bcl-2 ratio.

apoptosis; ERK

RENAL ISCHEMIA, WHETHER CAUSED by shock or during surgery or transplantation, is a major cause of acute kidney injury. Although reperfusion is essential for the survival of ischemic tissue, there is good evidence that reperfusion itself causes additional cellular injury. Renal ischemia-reperfusion (IR) injury is caused by multiple insults involving tubular cell apoptosis, oxygen free radical formation, mitochondrial dysfunction, inflammatory cytokine generation, and neutrophil sequestration (22, 25, 26). It is important to improve the ability of organs to tolerate ischemic injury, and the effects of IR injury in kidney transplantation have been studied (7, 15). IR injury is closely associated with increased acute rejection and late allograft failure (39). Nitrogen oxide (NO) is an important mediator of the physiological and pathological processes in renal IR injury (26, 27, 46). Endogenous NO is synthesized by different nitric oxide synthase (NOS) isoforms, which have been cloned

and characterized: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Renal reperfusion following ischemia activates NOS, and increases the expression of NOS proteins (5, 29, 36). Despite these findings, the role of NO in IR injury of the kidney remains controversial. Some have reported that NO induces cellular cytotoxicity and tissue injury via lipid peroxidation, DNA damage, and proapoptotic effects, which are part of IR injury (4, 16, 17). However, many studies have demonstrated that increased NOS activity is associated with reduced IR injury and increased blood flow in the ischemic region (37). Nonselective NOS inhibitors such as *N*<sup>G</sup>-nitro-L-arginine methyl ester worsen the postischemic renal function, whereas L-arginine (a NO precursor) can reverse the NOS inhibitor-induced deterioration of renal function (6).

Sildenafil citrate inhibits phosphodiesterase-5 (PDE5), which catalyzes the breakdown of cGMP, one of the primary factors involved in smooth muscle relaxation. Sildenafil induces the upregulation of eNOS and iNOS, which generate NO (35). NO activates guanylate cyclase, resulting in enhanced formation of cGMP, which activates PKG. Subsequently, PKG opens the mitochondrial KATP channels conferring cardioprotective effects against IR injury (24).

Sildenafil is used to treat erectile dysfunction in men (2). Several recent studies have shown that sildenafil also offers benefits in pulmonary arterial hypertension and congestive heart failure (1, 20, 28).

In addition, sildenafil can improve the neurological outcome and reduce neurological injury after transient spinal cord IR following clamping of the thoracic aorta (23). Moreover, sildenafil provided cardioprotection against ischemia when infused at the onset of reperfusion in rabbits (8). Sildenafil also attenuated cardiomyocyte apoptosis in a chronic model of doxorubicin cardiotoxicity and reduced the hypertrophic response to isoprenaline in the mouse heart (14). Apoptosis is also induced by renal IR injury associated with MAPK activation, Bax, Bcl-2, and caspase (33, 34), and sildenafil increased ERK phosphorylation and the Bcl-2/Bax ratio in the heart (10, 11, 24).

Studies have shown that the inhibition of phosphodiesterase decreases the renal injury. Guan et al. (19) showed that treatment with zaprinast, a cGMP-specific phosphodiesterase inhibitor, 24 h after 60 min of bilateral renal artery clamping in the rat, accelerated renal recovery by improving regional renal blood flow. Early treatment with sildenafil ameliorated the progression of renal damage in a 5/6 nephrectomy model (32).

Based on the tissue-protective effects of sildenafil, we hypothesized that sildenafil would attenuate renal IR injury. Thus we studied the protective mechanism involved in sildenafil pretreatment.

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## MATERIALS AND METHODS

### Animals and Drug Treatment

All experiments were performed using 8-wk-old male Sprague-Dawley (SD) rats (Samtako, Kyoung Gi-Do, Korea), weighing 250~260 g. The rats were fed a standard laboratory diet and cared for using a protocol approved by the Institutional Animal Care and Use Committee of Chungnam National University Medical School. We performed two studies. In *study 1*, we divided the rats into four groups: sham-operated rats (S group), sildenafil-treated sham-operated rats (S-Sild group), vehicle-treated IR rats (IR group), and sildenafil-treated IR rats (IR-Sild group). IR rats were killed at 1, 2, 4, or 24 h after reperfusion of the left ischemic kidney. Sham and sildenafil-treated sham-operated rats were killed at 1.58, 2.58, 4.58, or 24.58 h after the abdominal incision for the sham surgery. Kidneys were prepared for Western blotting, real-time RT PCR, and histology. Blood urea nitrogen (BUN) and serum creatinine levels were determined at 0, 4, 24, 48, 72, 120, and 168 h after reperfusion of the left ischemic kidney in IR rats, and at 0.58, 1.58, 2.58, 4.58, and 24.58 h after surgery in the sham and sildenafil-treated sham-operated rats (Fig. 1). In *study 2*, we divided the rats into two groups: sildenafil-treated IR rats and PD98059+sildenafil-treated IR rats. The animals were killed at 24 h after reperfusion of the left ischemic kidney. BUN and serum creatinine levels were determined at 24 h after reperfusion of the left ischemic kidney (Fig. 1). Kidneys were prepared for Western blotting, real-time RT PCR, and histology.

The IR injury involved clamping of the left renal artery and vein for 35 min after nephrectomy of the right kidney. Sildenafil pretreatment consisted of 0.5 mg/kg sildenafil (Viagra; Pfizer Australia, West Ryde, NSW, Australia), administered once intraperitoneally 1 h before ischemia of the left kidney. The vehicle (0.9% NaCl) was administered in the same manner and volume. PD98059 (1 mg/kg; InSolution PD98059; Calbiochem) pretreatment was administered once intravenously at 0.5 h before ischemia of the left kidney. The rats were anesthetized with a mixture of ketamine (50 mg/ml, 10 mg/kg; Ketalar; Bayer, Leverkusen, Germany) and xylazine (2%, 0.1 ml/kg; Rompun, Bayer) in solution (2 ml/kg) given intraperitoneally.

### Tissue Preparation

After the rats were anesthetized, the left kidneys were excised immediately and cut into three coronal sections. Two pieces of kidney were snap-frozen in liquid nitrogen and kept at  $-70^{\circ}\text{C}$  for subsequent RNA extraction and protein analysis; the other piece was fixed in 10% buffered formaldehyde at room temperature, and then embedded in Paraplast (Sherwood Medical, St. Louis, MO) for light microscopy and terminal dUTP nick end-labeling (TUNEL).

### Light Microscopic Examination

Pieces of kidney embedded in Paraplast were cut into 4- $\mu\text{m}$  sections and mounted on glass slides. Then, the sections were deparaffinized with xylene, counterstained with periodic acid-Schiff (PAS), and examined under a light microscope ( $\times 200$  magnification, Dialux 22; Leitz, Milan, Italy). We evaluated the loss of the brush-border membrane, vacuolation, and desquamation of epithelial cells in renal tubules beginning 24 h after cisplatin treatment. A renal pathologist blinded to the study groups examined five different fields of the outer medullary area in every slide. The magnitude of the damage to the tubular epithelial cells was determined in PAS-stained sections by counting the numbers of tubules injured in the outer medulla.

### RNA Extraction, Semiquantitative RT-PCR, and Real-Time PCR

Total RNA was extracted from kidney sections stored at  $-70^{\circ}\text{C}$  using an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1  $\mu\text{g}$  of total RNA using an oligo dT primer (Amersham Pharmacia, Piscataway, NJ), dNTPs (Amersham Pharmacia), Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Grand Island, NY), 0.1 M DTT, and buffer in a volume of 20  $\mu\text{l}$ . Then, the 20  $\mu\text{l}$  of cDNA were diluted to a total volume of 100  $\mu\text{l}$ . Using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), PCR was used to amplify cDNA for iNOS [primers 5'-TCC CAG ACC CCA TAA CAA CAG-3' (sense) and 5'-TGA GGG TGC AGC GAA CTT TA-3' (antisense)], eNOS [5'-TCC CAG ACC CCA TAA CAA CAG-3' (sense) and 5'-TGA GGG TGC AGC

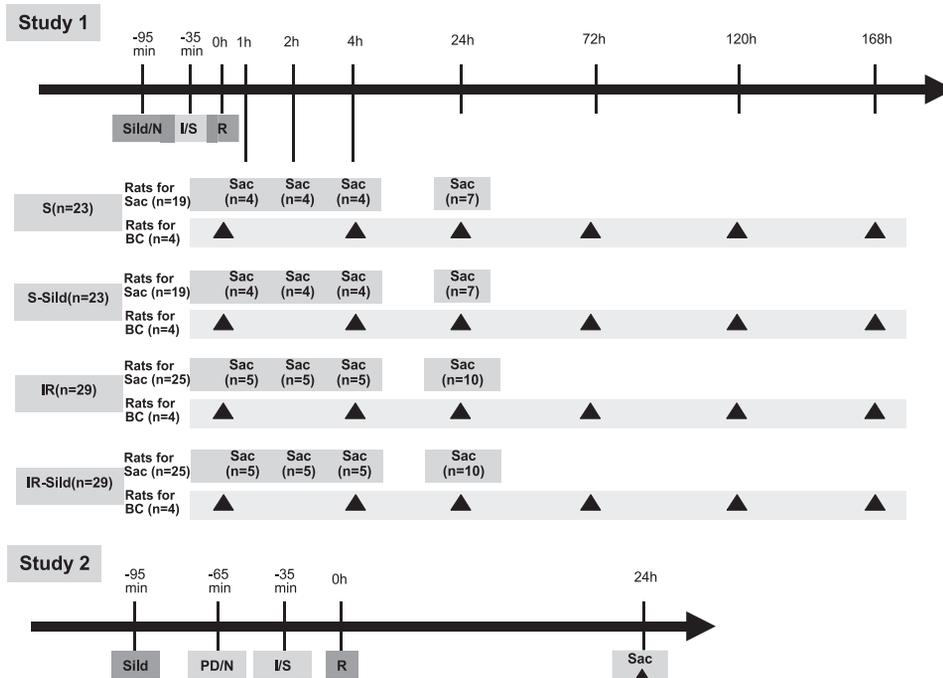


Fig. 1. Diagram of the study design. In *study 1*, the rats were divided into sham-operated rats (S), sildenafil-treated sham-operated rats (S-Sild), vehicle-treated ischemia-reperfusion rats (IR), and sildenafil-treated ischemia-reperfusion rats (IR-Sild). The rats were subjected to a right nephrectomy followed by 35 min of ischemia and reperfusion of the left kidney. After reperfusion of the ischemic left kidney, blood was sampled at several time points and the rats were killed. Age- and time-matched sham-operated control rats were observed in parallel. In *study 2*, the rats were divided into sildenafil-treated ischemia-reperfusion (IR-Sild) and sildenafil+PD98059-treated ischemia-reperfusion (IR-Sild-PD) groups. Blood was sampled, and rats were killed 24 h after reperfusion of the ischemic left kidney.

Sild=sildenafil injection, N=normal saline injection, PD=PD98059 injection, I=ischemia operation, S=sham operation, R=reperfusion of ischemic kidney, Sac=sacrifice, BC=blood sampling for chemistry, ▲ = blood sampling time

GAA CTT TA-3' (antisense)], and GAPDH [5'-TCC CAG ACC CCA TAA CAA CAG-3' (sense) and 5'-TGA GGG TGC AGC GAA CTT TA-3' (antisense)]. The amplification reaction volume was 20  $\mu$ l, which consisted of 10  $\mu$ l iQ SYBR Green PCR Master Mix, 1  $\mu$ l primers, 1  $\mu$ l cDNA, and 8  $\mu$ l H<sub>2</sub>O. Amplification and detection were performed using a thermal cycler (Rotor-Gene 6000; Corbett Research, Mortlake, NSW, Australia). The PCR conditions consisted of denaturation at 95°C for 10 min, followed by 40 cycles consisting of 10 s at 95°C, 15 s at 60°C, and 20 s at 72°C. The fluorescence of SYBR green was measured at the end of each cycle using the comparative threshold cycle (Ct) method. The cDNA was quantified using the following formula

$$2^{-\Delta\Delta Ct} = 2^{-[(Ct \text{ of target gene} - Ct \text{ of GAPDH in treated rats}) - (Ct \text{ of target gene} - Ct \text{ of GAPDH in sham rats})]}$$

#### Western Blotting

Kidney sections stored at -70°C were homogenized in protein extraction solution (PRO-PREP; iNtRON, Sungnam-si, Korea). The homogenates were centrifuged (12,000 g, 15 min, 4°C), and the protein concentrations in the supernatant were determined using the Bradford method (Bio-Rad, Hercules, CA). The centrifuged proteins were mixed with the same volume of 2 $\times$  SDS sample buffer [62.5 mM Tris·HCl (pH 6.8), 6% (wt/vol) SDS, 30% glycerol, 125 mM DTT, and 0.3% (wt/vol) bromophenol blue], so that the ultimate concentration of the mixture was 1 $\times$  (500  $\mu$ g/100  $\mu$ l). The homogenates were heated at 94°C for 5 min in Laemmli sample buffer. Then, 40  $\mu$ g of total protein were loaded in a stacking polyacrylamide gel and resolved on 8 and 15% polyacrylamide gels with a standard biotinylated molecular weight marker. The samples were wet-transferred to a 0.2- $\mu$ m nitrocellulose membrane (Amersham Pharmacia), which was stained with Coomassie blue to assess equal loading of protein. Subsequently, the blots were blocked for 1 h with 5% nonfat dry milk in TBST buffer (20 mM Tris·HCl, pH 7.6, 0.8% NaCl, and 0.05% Tween 20) and incubated overnight at 4°C with 1:1,000 cleaved/total caspase-3 antibody, ERK/pERK, and Bcl-2 (Cell Signaling Technology, Beverly, MA), and 1:1,000 iNOS, eNOS, Bax, and  $\beta$ -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After washing in TBST, the blots were incubated with 1:1,000 secondary anti-rabbit or anti-goat IgG-HRP-linked antibody (Cell Signaling Technology) for 1 h. The blots were washed again in TBST, and the bands were detected using enhanced chemiluminescence (Millipore, Billerica, MA) and exposed to film. The optical density for quantification was obtained with Gel-Pro Analyzer version 3.1 (Media Cybernetics, Silver Spring, MD).

#### cGMP Measurement

Outer medullary area tissue lysate (50 mg) was used. The tissues were chopped and incubated in cell lysis buffer (R&D Systems, Minneapolis, MN). Then, the cells were frozen at -20°C and thawed with gentle mixing. The freeze/thaw cycle was repeated twice. The mixture was centrifuged (600 g, 10 min, 4°C) to remove cellular debris. The supernatant was aliquoted and stored at -20°C for cGMP determination. cGMP levels were assayed in duplicate using an ELISA kit (R&D Systems, Minneapolis, MN). A set of standards (2.1–500 pmol/ml) was assayed in duplicate with the samples. Non-specific binding and background signals were subtracted from each reading, and the average optical density was calculated. Values are presented as picomoles of cGMP per milligram protein.

#### Caspase-3 Activity Assay

Caspase-3 activities in the lysates were determined using a Chemicon caspase colorimetric activity assay kit (Chemicon, Temecula, CA). The tissues were chopped and incubated in cell lysis buffer for 10 min. After centrifugation (5 min, 10,000 g), the supernatant was

aliquoted and analyzed immediately, according to the manufacturer's protocol. The assay is based on the spectrophotometric detection of the chromophore *p*-nitroaniline (pNA) after cleavage from the labeled substrate LEHD-pNA. Free pNA can be quantified using a microtiter plate reader at 405 nm. The fold-increase in caspase-3 activity was determined by comparing optical density readings from the S-Sild, IR, and IR-Sild groups to those of the S group in *study 1*, and by comparing optical density readings from the IR-Sild-PD group to those of the IR-Sild group in *study 2*.

#### TUNEL

TUNEL was performed *in situ* using an Apoptosis Detection Kit (S7100-KIT; Chemicon). The procedure was modified from the manufacturer-recommended protocol. First, the 4- $\mu$ m-thick paraffin-embedded sections were dewaxed in three changes of xylene for 10 min each. These were rehydrated in a graded series of alcohol from absolute, to 95, 80, and 70%, to water. After rinsing in PBS for 5 min, the sections were incubated in 0.3% H<sub>2</sub>O<sub>2</sub> at room temperature to eliminate endogenous peroxidase activity. Then, the sections were washed in two changes of distilled water for 5 min each, followed by PBS for another 5 min. Proteinase K (10  $\mu$ g/ml in 0.1 M Tris, 50 mM EDTA, pH 8) was applied on the sections, which were incubated for 15 min at room temperature. After the sections were washed in four changes of distilled water for 2 min each, and one change of PBS for 5 min, two drops of equilibration buffer were applied to the sections for 0.5 to 1 min to facilitate the penetration of terminal dUTP transferase, the TdT enzyme. A reaction mixture was prepared by mixing the reaction buffer and TdT enzyme in a ratio of 5:1. Then, this reaction mixture was applied to the sections, which were incubated in a humidified chamber for 1 h at 37°C to allow extension of the nick ends of the DNA fragments with digoxigenin-dUTP. During the incubation process, coverslips were put on the sections to prevent them from drying out. After incubation, the coverslips were removed carefully, without disturbing the tissues, and the sections were washed in a stop buffer (1 ml of stock in the kit and 34 ml of distilled water) for 30 min at 37°C. After the sections were washed in PBS for 5 min, two drops of peroxidase-conjugated anti-DIG antibody were applied for 30 min at room temperature. After rinsing of the sections in three changes of PBS followed by one change in Tris buffer, color was developed using 0.05% diaminobenzidine (DAB) with 0.006% H<sub>2</sub>O<sub>2</sub> as a substrate. Finally, the sections were washed in water, counterstained with methyl green, dehydrated through a graded alcohol series, cleared in xylene, and mounted with Permount for light microscopic observation. For negative controls, distilled water was used instead of TdT enzyme.

#### Statistical Analysis

The data are reported as the means  $\pm$  SD. Multiple comparisons among groups were performed using the *t*-test and ANOVA with the post hoc Bonferroni test correction (SPSS 11.0 for Windows; SPSS, Chicago, IL). The difference among groups was considered statistically significant at *P* < 0.05.

## RESULTS

### Effects of Sildenafil on Renal Function and Histology

BUN levels were significantly elevated in the vehicle-treated IR group at 24, 48, and 72 h after reperfusion, compared with time-matched sham-operated rats. In contrast, intraperitoneal injection of sildenafil reduced BUN levels compared with vehicle-treated IR rats at 24, 48, and 72 h after reperfusion. Serum creatinine levels were also significantly elevated in the vehicle-treated IR group at 24 and 48 h after reperfusion, compared with time-matched sham-operated rats, whereas sildenafil treatment reduced serum creatinine levels, compared

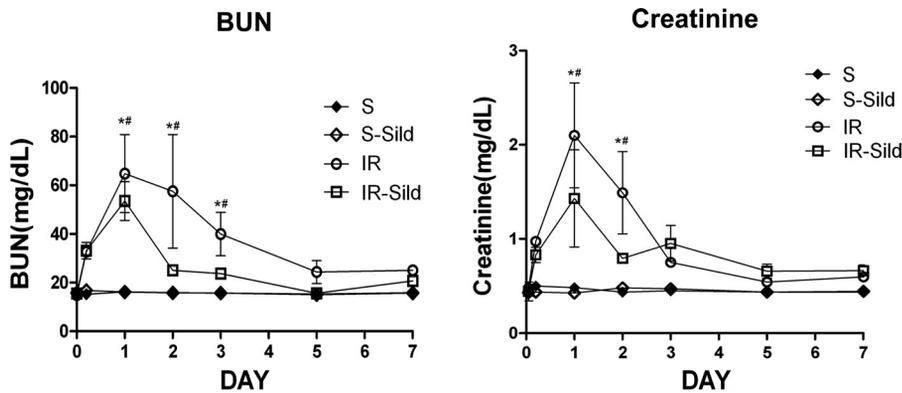


Fig. 2. Effects of sildenafil on renal function showing the temporal changes in serum blood urea nitrogen (BUN) and creatinine. Group definitions are the same as for Fig. 1. \* $P < 0.01$  vs. sham group. # $P < 0.05$  vs. vehicle-treated IR group.

with vehicle-treated IR rats, at 24 and 48 h after reperfusion ( $P < 0.05$ , Fig. 2).

Histological examination revealed loss of the brush border, vacuolation, and desquamation of epithelial cells in renal tubular epithelium in rats 24 h after vehicle-treated IR, whereas pretreatment with sildenafil attenuated the renal tubular injuries ( $P < 0.05$ , Fig. 3).

#### Antiapoptotic Effects of Sildenafil

**Expression of Bcl-2/Bax.** Bcl-2 and Bax expression were measured in kidney samples 24 h after reperfusion in vehicle- and sildenafil-treated IR rats. Protein was extracted, and Bcl-2, Bax, and  $\beta$ -actin were identified using Western blotting. The expression of Bcl-2 and Bax was normalized against  $\beta$ -actin expression. The level of Bax was elevated in the kidneys of IR rats compared with the kidneys of the sham rats and rats treated only with sildenafil ( $P < 0.05$ ). Pretreatment with sildenafil prevented the elevation of Bax in the kidneys of IR rats. No difference was observed in Bcl-2 levels between sildenafil- and vehicle-treated IR rats. However, the Bax/Bcl-2 ratio was elevated in the kidneys of IR rats compared with the kidneys of the sham and sildenafil-only rats ( $P < 0.05$ ). The ratio of Bax to Bcl-2 was calculated as an index of apoptotic signaling. Pretreatment with sildenafil prevented the elevation of the Bax/Bcl-2 ratio in the kidneys of IR rats ( $P < 0.05$ , Fig. 4).

**Caspase-3 activity assay.** Caspase-3 activity increased significantly in the kidneys of IR rats compared with those of the sham and sildenafil-only rats ( $P < 0.05$ ). Sildenafil administration reduced caspase-3 activity in IR rats ( $P < 0.05$ , Fig. 4).

**TUNEL.** The number of TUNEL-positive cells increased in the kidneys of IR rats compared with those of the sham and sildenafil-only rats ( $P < 0.01$ ). Sildenafil administration prevented the increase in the number of TUNEL-positive cells ( $P < 0.05$ , Fig. 5).

#### Expression of cGMP and eNOS/iNOS

cGMP accumulation was measured in kidney samples at 4.58 and 24.58 h in sham-operated and sildenafil-pretreated sham-operated rats, and at 4 and 24 h after reperfusion in IR rats. cGMP accumulation increased significantly in the kidney in the sildenafil-treated IR group, compared with the vehicle-treated IR group at 4 and 24 h after reperfusion.

eNOS and iNOS expression was measured in kidney samples at 1, 2, 4, and 24 h after reperfusion in vehicle- and sildenafil-treated IR rats. iNOS mRNA expression peaked at 2 h in both the vehicle- and sildenafil-treated IR groups, and no difference was found between these two groups. eNOS mRNA expression peaked at 4 h in both groups, but the increase was greater in the sildenafil-treated IR group than in the vehicle-treated IR group ( $P < 0.05$ ). Regarding the levels of proteins, eNOS was

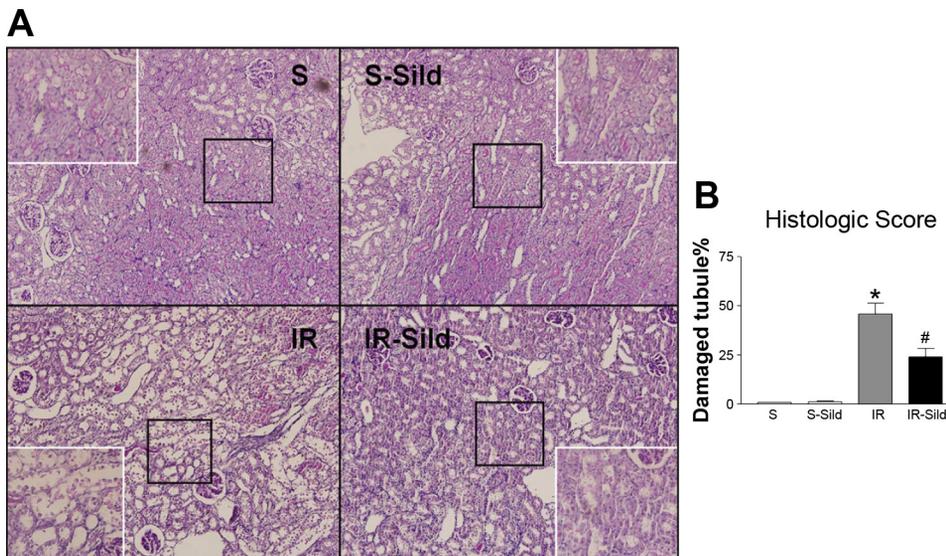
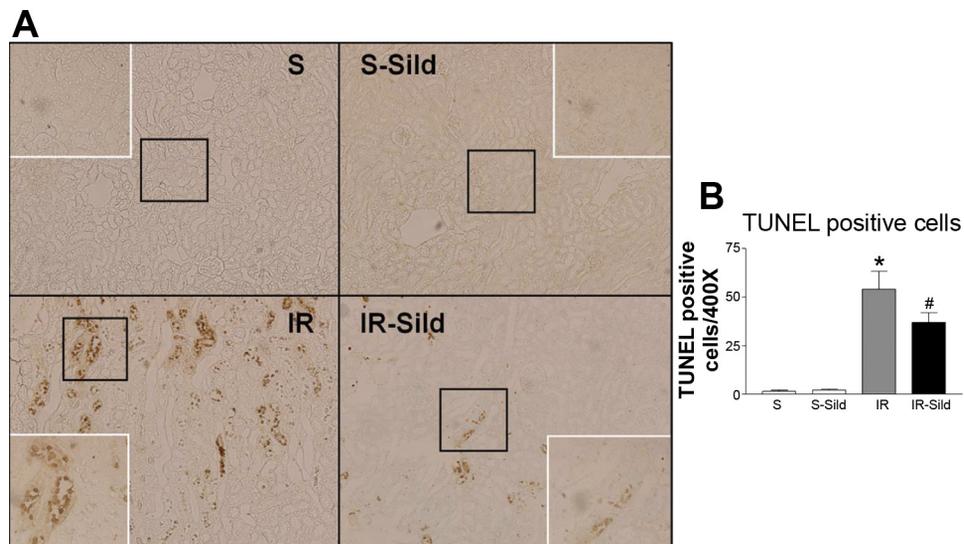


Fig. 3. Effects of sildenafil on renal histology (original magnification  $\times 200$ ). A: changes in renal histology 24 h after renal IR injury. The vehicle-treated IR kidneys showed marked injury, with sloughing of tubular epithelial cells and loss of the brush border. These changes were reduced by sildenafil treatment. B: percentage of damaged tubules. \* $P < 0.01$  vs. sham group. # $P < 0.05$  vs. vehicle-treated IR group.

Fig. 4. A: densitometric analysis of Bax and Bcl-2 immunoblots. Sildenafil pretreatment decreased the Bax/Bcl-2 ratio in IR. B: representative photomicrographs of Bax and Bcl-2 immunostaining in vehicle-treated IR group. C: colorimetric analysis of caspase-3 activity. Sildenafil pretreatment suppressed caspase-3 activity at 4 and 24 h after reperfusion. \* $P < 0.05$  vs. sham group. # $P < 0.05$  vs. vehicle-treated IR group.

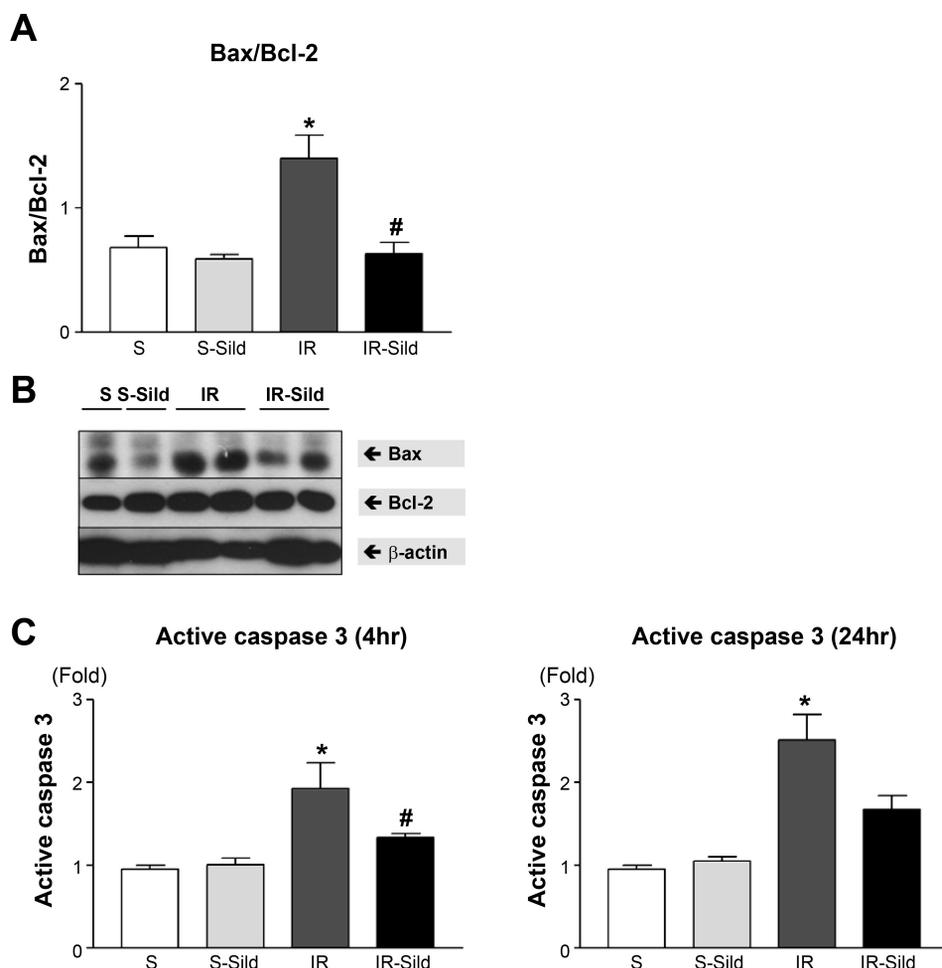


elevated in the kidneys of sildenafil-treated IR rats compared with those of the vehicle-treated IR group 4 and 24 h after reperfusion. iNOS was elevated 24 h after reperfusion in the sildenafil-treated IR group compared with the vehicle-treated IR group ( $P < 0.05$ , Fig. 6).

*ERK Phosphorylation and Kidney Injury*

ERK expression was measured in kidney samples at 2, 4, and 24 h after reperfusion in vehicle- and sildenafil-treated IR rats. The increased ERK phosphorylation was prolonged in the

Fig. 5. A: representative photomicrographs of terminal dUTP nick end-labeling (TUNEL; original magnification  $\times 400$ ). Vehicle-treated IR kidneys contained more TUNEL-positive cells. Sildenafil pretreatment reduced the number of TUNEL-positive cells. B: quantitative analysis of TUNEL-positive cells. \* $P < 0.01$  vs. sham group. # $P < 0.05$  vs. vehicle-treated IR group.



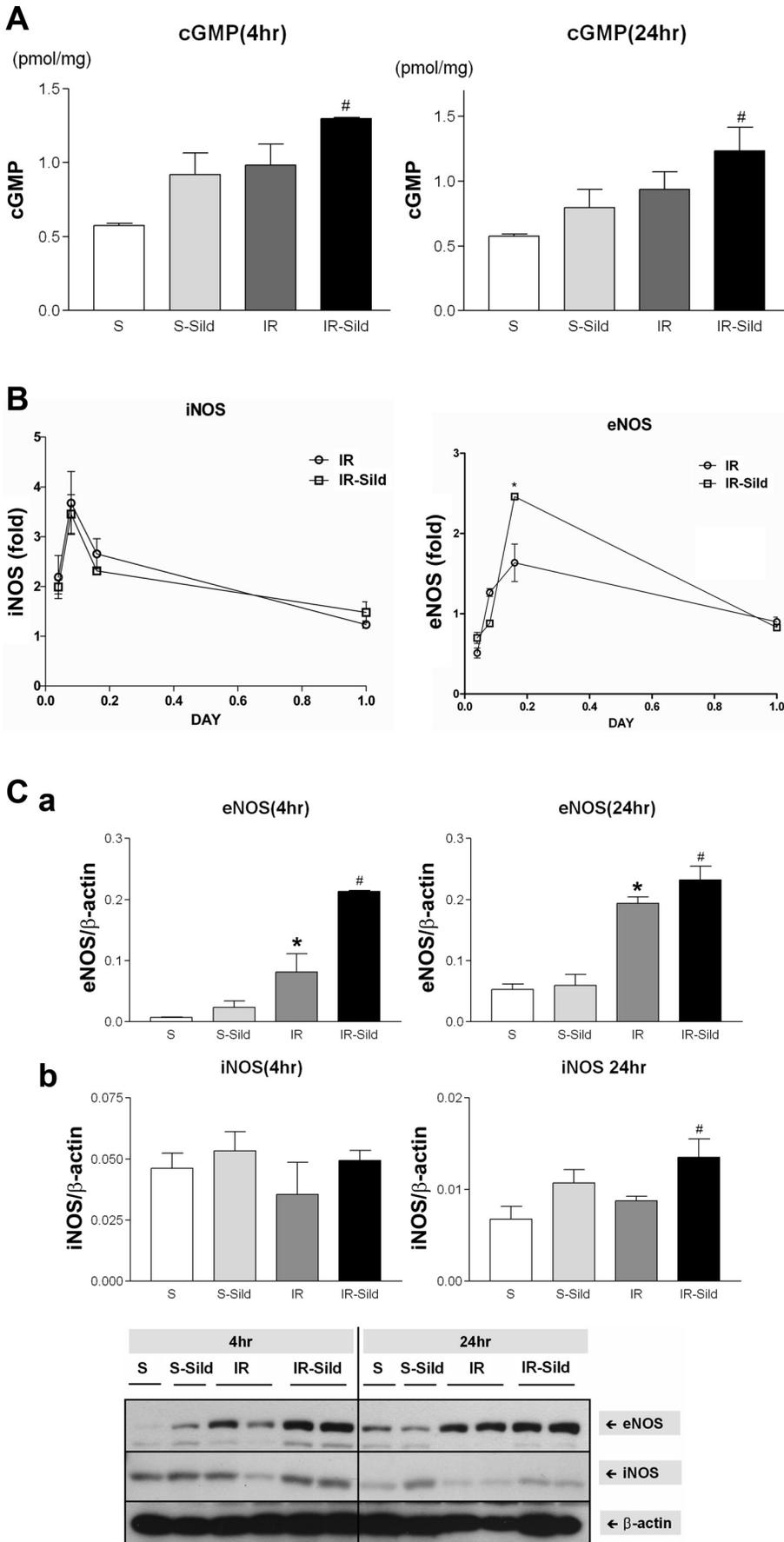


Fig. 6. Renal expression of cGMP, inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS). *A*: cGMP accumulation in the outer medullary area tissue isolated from rats. Sildenafil pretreatment resulted in increased cGMP levels in IR rats. *B*: temporal analysis of iNOS and eNOS using semiquantitative RT-PCR and real-time PCR. *C-a*: densitometric analysis of iNOS and eNOS immunoblots. Sildenafil pretreatment resulted in increased iNOS and eNOS. *C-b*: representative photomicrographs of immunostaining for iNOS and eNOS. \**P* < 0.05 vs. sham group. #*P* < 0.05 vs. vehicle-treated IR group.

sildenafil-treated IR group compared with the vehicle-treated IR group. PD98059 pretreatment of sildenafil-treated IR rats significantly reduced the phosphorylation of ERK, increased BUN and serum creatinine, decreased eNOS and iNOS production, increased Bax/Bcl-2 ratio, and increased caspase activity. By contrast, the accumulation of cGMP was not affected by PD98059 pretreatment of sildenafil-treated IR rats. ( $P < 0.05$ , Fig. 7).

## DISCUSSION

In this study, we demonstrated that sildenafil has renoprotective effects against renal IR injury, attenuating the renal tubular damage, decreasing apoptosis, and suppressing the increases in the BUN and serum creatinine. This protection is due

primarily to the inhibition of apoptosis and necrosis, as clearly revealed by our finding that pretreatment with sildenafil increased iNOS/eNOS and decreased the activation of caspase-3, TUNEL-positive cells, and the Bax/Bcl-2 ratio.

Apoptosis is one of the important mechanisms of cell death in cultured renal tubular cells and isolated kidneys following renal IR injury. Apoptosis is associated with the upregulation of proapoptotic protein (Bax), downregulation of antiapoptotic protein (Bcl-2), and increased caspase-3 activity (33, 34, 42). We observed a significant increase in Bcl-2 and decreases in Bax and cleaved caspase-3 in the sildenafil-treated IR rats compared with the vehicle-treated IR rats, demonstrating the antiapoptotic capacity of sildenafil in renal IR injury. Furthermore, the number of TUNEL-positive cells was significantly

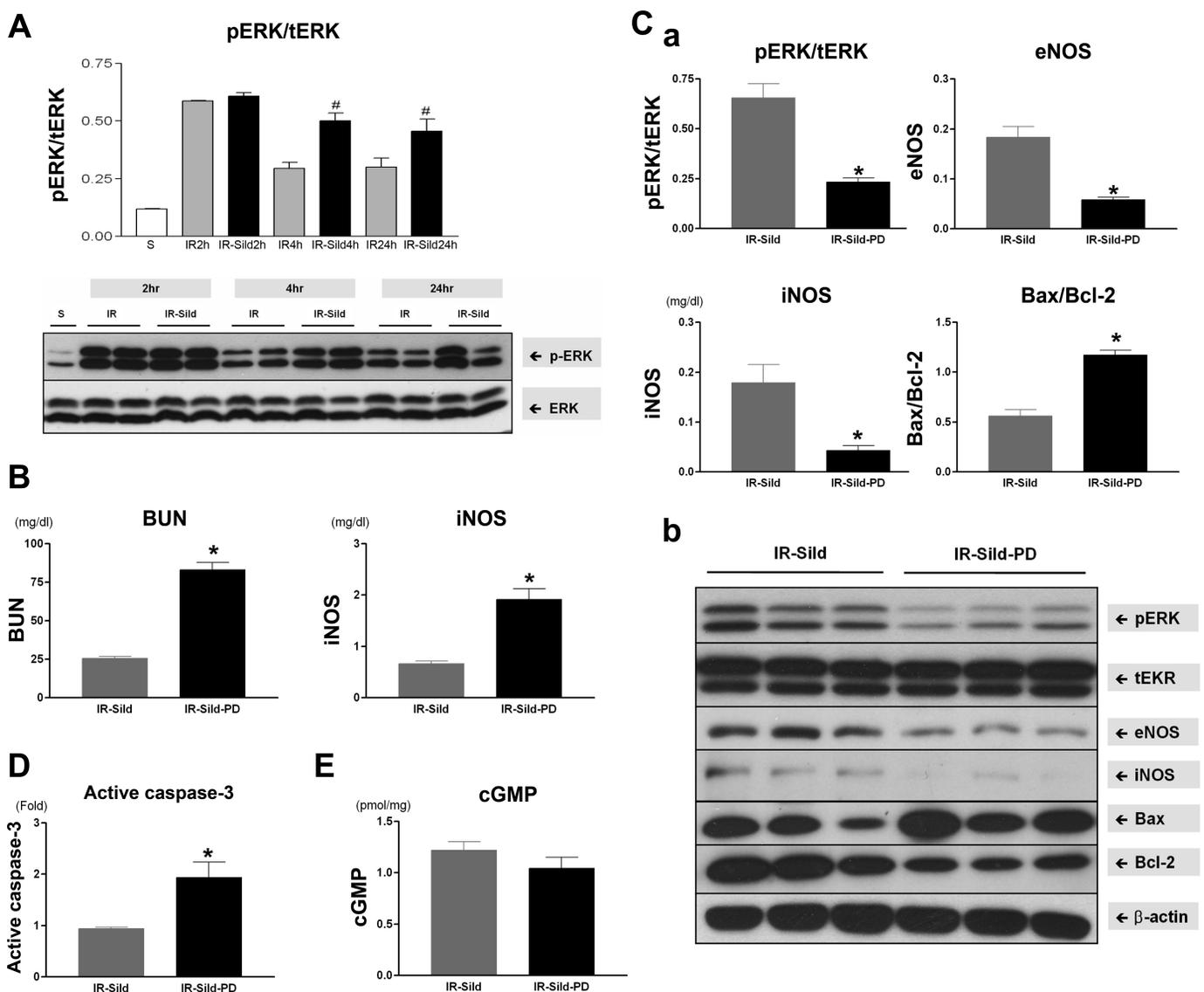


Fig. 7. A: representative photomicrographs of immunostaining for ERK in kidney samples at 2, 4, and 24 h after reperfusion in vehicle- and sildenafil-treated IR rats. Sildenafil pretreatment prolonged the increase in ERK phosphorylation at 4 and 24 h after reperfusion in IR rats. B: BUN and serum creatinine levels. C-a: densitometric analysis of ERK, eNOS, iNOS, Bax, and Bcl-2 immunoblots. PD98059 pretreatment increased the Bax/Bcl-2 ratio and decreased eNOS and iNOS levels after sildenafil-treated IR. C-b: representative photomicrographs of ERK, eNOS, iNOS, Bax, and Bcl-2 immunostaining. D: colorimetric analysis of caspase-3 activity. PD98059 pretreatment increased caspase-3 activity 24 h after reperfusion in sildenafil-treated IR rats. E: cGMP accumulation in the outer medullary area tissue isolated from rats. PD98059 pretreatment did not decrease cGMP accumulation in sildenafil-treated IR rats. \* $P < 0.05$  vs. sildenafil-treated IR group. <sup>#</sup> $P < 0.05$  vs. vehicle-treated IR group.

lower in the kidneys from sildenafil-treated IR rats compared with the vehicle-treated IR rats.

The ERK cascade mediates cell growth and survival signals in many cell types, and ERK activation protects renal epithelial cells from oxidative injury (21). Sildenafil induces ERK activation (24), although the exact mechanism of the sildenafil-mediated enhancement of ERK activity is not known. Our results also demonstrated that sildenafil significantly enhanced ERK activation, compared with the vehicle-treated IR rats. PD98059 attenuated the sildenafil-induced increase in ERK phosphorylation and also diminished the sildenafil-induced renoprotective effect in IR injury.

Many studies have documented an interaction between NO and ERK. However, the detailed mechanism explaining the interaction between these molecules in either direction has not been fully elucidated. It has been reported that the activation of ERK by sildenafil leads to increased NOS activity and subsequent rapid NO release (47). In cardiac tissue, NO enhances the formation of cGMP, which activates PKG (30). It has also been reported that NO release is mediated by NOS-induced phosphorylation and activation of ERK1/2 in isolated rat cardiomyocytes and the intact heart, suggesting that ERK activation is downstream of NOS (43). Our data showed that PD98059 did not diminish the sildenafil-induced accumulation of cGMP, although it did diminish the expression of cytoprotective proteins, including iNOS, eNOS, and Bcl-2. To our knowledge, there is no report on the time course of sildenafil-induced ERK phosphorylation. Das et al. (9, 11) reported that ERK plays an important role in both the early and delayed phases of sildenafil-induced cardioprotection against IR injury in mice. However, our data showed no difference in ERK phosphorylation between the IR and IR-Sild groups at 2 h after reperfusion, but there was a difference at 4 and 24 h after reperfusion. Our results show that ERK phosphorylation occurs downstream of cGMP production within a sildenafil-induced signaling cascade.

Sildenafil, a PDE-5 inhibitor, prevents the breakdown of NO-driven cGMP, primarily in vascular smooth muscle cells, and is therefore a potent vasodilator (2). In renal IR injury, endogenous NO is synthesized by eNOS and iNOS (40). Many studies have confirmed that eNOS-mediated NO production plays a pivotal protective role in IR-induced acute renal failure (18, 44, 45). However, the role of iNOS in renal IR remains controversial. Some studies have demonstrated that renal IR injury was efficiently attenuated by a genetic deficiency or the pharmacological blockade of iNOS (3, 12, 25, 41), while other recent studies clearly demonstrated that iNOS-generated NO inhibited IR-induced renal damage (31, 38). This difference may be due to the different levels of NO production associated with the degree or method of IR injury. We demonstrated that sildenafil significantly enhanced iNOS and eNOS in the sildenafil-treated IR rats compared with the vehicle-treated IR rats. However, Elrod et al. (13) reported that sildenafil has a protective effect, independent of the NO/cGMP pathway (i.e., independent of iNOS, eNOS, and cGMP levels) in a model using 30 min of ischemia and 24 h of reperfusion. This means that other protective pathways involving sildenafil exist, so that further studies are needed.

In conclusion, this study revealed that sildenafil improved the recovery of renal injury during IR by enhancing the activation of ERK, induction of iNOS and eNOS production,

and decreasing the Bax/Bcl-2 ratio. The ability of sildenafil to induce ischemic tolerance suggests that there are advantages in its application in kidney transplantation. Clinical studies are necessary to evaluate the therapeutic properties of sildenafil in preventing IR injury.

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