Reactive oxygen species/oxidative stress contributes to progression of kidney fibrosis following transient ischemic injury in mice

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Kim J, Seok YM, Jung K-J, Park KM. Reactive oxygen species/oxidative stress contributes to progression of kidney fibrosis following transient ischemic injury in mice. Am J Physiol Renal Physiol 297: F461–F470, 2009. First published May 20, 2009; doi:10.1152/ajprenal.90735.2008.—Recently, kidney fibrosis following transplantation has become recognized as a main contributor of chronic allograft nephropathy. In transplantation, transient ischemia is an inescapable event. Reactive oxygen species (ROS) play a critical role in ischemia and reperfusion (I/R)-induced acute kidney injury, as well as progression of fibrosis in various diseases such as hypertension, diabetes, and ureteral obstruction. However, a role of ROS/oxidative stress in chronic kidney fibrosis following I/R injury remains to be defined. In this study, we investigated the involvement of ROS/oxidative stress in kidney fibrosis following kidney I/R in mice. Mice were subjected to 30 min of bilateral kidney ischemia followed by reperfusion on day 0 and then administered with either manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin (MnTMPyP, 5 mg/kg body wt ip), a cell permeable superoxide dismutase mimetic, or 0.9% saline (vehicle) beginning at 48 h after I/R for 14 days. I/R significantly increased interstitial extension, collagen deposition, apoptosis of tubular epithelial cells, nitrotyrosine expression, hydrogen peroxide production, and lipid peroxidation and decreased copper-zinc SOD, manganese SOD, and glucose 6-phosphate dehydrogenase activities in the kidneys 16 days after the procedure. MnTMPyP administration minimized these postischemic changes. In addition, MnTMPyP administration significantly attenuated the increases of α-smooth muscle actin, PCNA, S100A4, CD68, and heat shock protein 47 expression following I/R. We concluded that kidney fibrosis develops chronically following I/R injury, and this process is associated with the increase of ROS/oxidative stress.

interstitial fibrosis; ischemia-reperfusion; superoxide; α-smooth muscle actin; manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin

ISCHEMIA AND REPERFUSION (I/R) occurs in various clinical settings, including transplantation and vascular surgery. In kidney transplantation, I/R causes acute kidney injury (AKI) and chronic fibrotic changes in kidneys, such as extension of the interstitial area, infiltration of leukocytes, and collagen deposition (1, 7). These unwanted fibrotic consequences following transplantation lead to chronic allograft nephropathy and are recognized as one of main contributors of graft loss (2, 27). I/R excessively produces reactive oxygen species (ROS) beyond the scavenging capacity of this organ for ROS, simultaneously impairs antioxidant enzymes, and causes cell damage by lipid peroxidation, DNA breakdown, and protein damage. This leads to AKI (9, 10, 12). Antioxidant treatment has been used to prevent AKI in various clinical settings and experimental models (5, 17). Recently, we found that increased ROS after I/R in the kidneys of mice sustained for longer than 2 wk and an herbal extract (which may effect an antioxidant) reduced the ROS stress and kidney fibrosis following I/R (34). This indicates that oxidative stress following I/R may be a contributor to renal fibrosis following I/R injury. Furthermore, ROS has been suggested as an important fibrogenic factor in chronic allograft nephropathy following transplantation (4). However, the role of ROS/antioxidant enzymes in chronic alteration of kidneys after transient I/R injury remains to be defined.

Therefore, we investigated the association of ROS/antioxidant systems with kidney fibrosis following I/R injury via administration of manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin (MnTMPyP), a cell permeable superoxide dismutase mimetic. Recently it has been demonstrated that the pretreatment of MnTMPyP effectively reduced I/R-induced oxidative stress in mice (12, 21). In the present study, we report that kidney fibrosis develops chronically following I/R injury, that this process is associated with the increase of ROS/oxidative stress, and that MnTMPyP administration reduces the progress of kidney fibrosis after I/R.

MATERIALS AND METHODS

Animal preparation. Experiments were performed in 8-wk-old male BALB/c mice. Mice were allowed free access to water and standard mouse chow. In all cases, studies were conducted according to the animal experimental procedures approved by the Kyungpook National University Institution Animal Care and Use Committee. To induce kidney fibrosis after I/R, animals were anesthetized with pentobarbital sodium (60 mg/kg body wt ip; Sigma, St. Louis, MO) before surgery and subjected to occlusion of renal pedicles using nontraumatic microaneurism clamps (Roboz, Rockville, MD) for 30 min as described previously (12, 14, 29). Some mice were subjected to sham operation. To minimize the variation of post-I/R fibrosis, mice whose plasma creatinine (Pcr) concentration increased between 2.0 and 3.0 mg/dl 24 h after ischemia were used for further studies. The mortality 16 days after I/R was 89.2 ± 2.0 and 3.0 mg/dl 24 h after ischemia were used for further studies. The mortality 16 days after I/R was 89.2 ± 3.1% (n = 56). Some mice were administered intraperitoneally with MnTMPyP (5 mg/kg body wt; Calbiochem, San Diego, CA), 0.9% saline (vehicle-MnTMPyP), α-tocopherol (vitamin E; 50 mg/kg body wt; Sigma), or olive oil (vehicle-vitamin E; Sigma) beginning at either 48 h or 8 days after ischemia until euthanasia daily.

Histology. Kidneys were perfused via the left ventricle with 30 ml of PBS for 2 min and then paraformaldehyde (2%, 75 mM L-lysine, 10 mM sodium periodate; Sigma) solution. After that, kidneys were excised and placed in paraformaldehyde overnight at 4°C. Tissue samples were then cut into 2-μm paraffin-embedded sections using a microtome (Leica, Bensheim, Germany). Paraffin sections were stained with periodic acid Schiff (PAS) and Masson’s trichrome to determine histological changes and collagen deposition, respectively. PAS and trichrome stainings were carried out according to standard protocols. Images were collected using a digital camera (Carl Zeiss). Each experimental animal group consisted of more than four mice. The area of interstitium, collagen deposition, and superoxide formation were analyzed in 10 fields (0.1 mm²/field) per the outer

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medulla using the LabWorks 4.5 software (Ultra-Violet Products, Cambridge, UK).

**Morphological score.** Morphological damage levels were scored as described previously (15). Briefly, 50 tubules in the outer medullar of kidney were analyzed by using a score of 0, no damage; 1, mild damage with rounding of epithelial cells and dilated tubular lumen; 2, severe damage with flattened epithelial cells, loss of nuclear staining, dilated lumen, and congestion of lumen; and 3, destroyed tubules with flat epithelial cells lacking nuclear staining and congestion of lumen. Four kidneys from each experimental condition were used \( (n = 4) \). Ten fields per slide were used for the counting.

**Renal functional parameters.** To evaluate concentrations of PCr and blood urea nitrogen (BUN), 70 μl of blood was taken from the orbital sinus 24 and 48 h and 3, 5, 8, 12, and 16 days after I/R \( (n = 6 \text{ per time point}) \). PCr concentration was measured using the Beckman Creatinine Analyzer II (Beckman, Brea, CA), and BUN concentration was measured using the BUN kit (Asan Pharm, Seoul, Korea).

**TUNEL assay.** TUNEL assay was carried out with the in situ cell death detection kit, Fluorescein (Roche, Mannheim, Germany) following the manufacturer’s protocol. Briefly, 2 μm of kidney sections were deparaffinized, rehydrated, and treated with proteinase K \( (20 \mu g/ml; \text{Invitrogen}) \) in TE buffer \( (50 \text{ mM Tris base, } 1 \text{ mM EDTA, } 0.5\% \text{ Triton X-100, pH 8.0; Sigma}) \). After that, the sections were incubated with TUNEL reagent mixture for 30 min at room temperature (RT) and then washed with PBS three times for 5 min. Finally, the sections were mounted with Prolong Gold anti-fade reagent (Invitrogen) and observed under a confocal microscope (Carl Zeiss). Images were collected and merged using Zeiss LSM Image Examiner software. TUNEL-positive cells were counted in 10 fields \( (0.1 \text{ mm}^2/\text{field}) \) per kidney. Each experimental animal group consisted of four mice.

![Fig. 1. Concentrations of plasma creatinine (PCr) (A) and blood urea nitrogen (BUN) (B) and chronic increases of interstitial cell number (C and D) and collagen deposition (E and F) after ischemia/reperfusion (I/R). Mice were subjected to 30 min of bilateral renal ischemia followed by reperfusion on day 0, and kidneys were harvested 24 h and 8 and 16 days after reperfusion. Day 0 indicates before ischemia. Kidney sections were stained with periodic acid Schiff (PAS) (C and D) and Masson trichrome (E and F). C: arrows indicate interstitial cells. D: numbers of interstitial cells were counted in a 0.1-mm² field of the outer medulla of the kidney (10 fields per kidney). F: collagen depositions were quantified as the number of pixels of blue color in a 0.1-mm² field of the outer medulla of the kidney using LabWorks analysis software (10 fields per kidney). Results are expressed as means ± SE \( (n = 4–6) \). *\( P < 0.05 \) vs. day 0. Scale bars = 50 μm.](http://ajprenal.physiology.org/processed/doi/10.220.32.247/131820-F462-FIG1)}

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**Fig. 1.** Concentrations of plasma creatinine (PCr) \( (A) \) and blood urea nitrogen (BUN) \( (B) \) and chronic increases of interstitial cell number \( (C \text{ and } D) \) and collagen deposition \( (E \text{ and } F) \) after ischemia/reperfusion \( (I/R) \). Mice were subjected to 30 min of bilateral renal ischemia followed by reperfusion on day 0, and kidneys were harvested 24 h and 8 and 16 days after reperfusion. Day 0 indicates before ischemia. Kidney sections were stained with periodic acid Schiff (PAS) \( (C \text{ and } D) \) and Masson trichrome \( (E \text{ and } F) \). C: arrows indicate interstitial cells. D: numbers of interstitial cells were counted in a 0.1-mm² field of the outer medulla of the kidney \( (10 \text{ fields per kidney}) \). F: collagen depositions were quantified as the number of pixels of blue color in a 0.1-mm² field of the outer medulla of the kidney using LabWorks analysis software \( (10 \text{ fields per kidney}) \). Results are expressed as means ± SE \( (n = 4–6) \). *\( P < 0.05 \) vs. day 0. Scale bars = 50 μm.
Immunofluorescence. Immunofluorescent staining was performed as described previously (14, 30). Tissue sections were deparaffinized with xylene, rehydrated with 100, 95, and 80% ethanol, and then washed with PBS for 10 min each. The sections were incubated in PBS containing 0.1% SDS (Sigma) for 5 min and washed in PBS for 10 min. To reveal antigen epitope, sections were boiled in 10 mM sodium citrate buffer (pH 6.0) for 20 min using an autoclave, cooled at RT for 20 min, and then washed with PBS three times for 5 min. Sections were blocked with PBS containing 1% bovine serum albumin (blocking buffer) for 30 min at RT and then incubated with monoclonal anti-α-smooth muscle actin (α-SMA; 1:500 dilution; Sigma), anti-CD68 (1:50 dilution; Serotec), polyclonal anti-S100A4 (1:100 dilution; Thermo Labsystems, Vantaa, Finland), or anti-heat shock protein 47 (HSP47; 1:20 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies diluted in blocking buffer overnight at 4°C. Following this, they were then washed with PBS three times for 5 min. Sections were incubated with FITC-conjugated horse anti-mouse IgG (1:100 dilution; Vector Laboratories, Burlingame, CA), rabbit anti-rat IgG (1:100 dilution; Vector Laboratories), or goat anti-rabbit IgG (1:100 dilution; Vector Laboratories) for 60 min at RT and then washed with PBS three times for 5 min. To detect cell nuclei, DAPI (Sigma) was placed on sections for 1 min. Finally, the sections were mounted with the Prolong Gold anti-fade reagent (Invitrogen) and observed under a confocal microscope (Carl Zeiss). S100A4-, CD68-, or HSP47-positive cells were counted in 10 fields (0.1 mm²/field) per the outer medulla of kidneys. Each experimental animal group consisted of four mice.

Measurement of lipid peroxidation and H₂O₂. To determine the levels of lipid peroxidation in kidney tissues, malondialdehyde concentration was measured as described previously (12, 14). Briefly, kidney lysates were mixed with 1 ml of thiobarbituric acid-trichloroacetic acid-HCl solution (0.375% thiobarbituric acid, trichloroacetic acid in 0.25 N HCl, pH 2.0) and boiled at 100°C for 15 min. Absorbance was measured at 535 nm wavelength. H₂O₂ levels in kidney tissues were determined using the ferric oxide-sensitive dye, xylenol orange, as described previously (12). H₂O₂ oxidizes iron (II) to iron (III) in the presence of sorbitol, which acts as a catalyst. Iron (III) forms a purple complex with xylenol orange. Absorbance was measured at 560 nm wavelength.

Western blot analysis. Western blot analyses were performed as described previously (14, 15, 29). Briefly, renal tissue proteins were separated on 10% SDS-PAGE gels and then transferred to an Immobilon P463ROS AND FIBROSIS FOLLOWING TRANSIENT ISCHEMIA AJP-Renal Physiol • VOL 297 • AUGUST 2009 • www.ajprenal.org
membrane (Millipore, Bedford, MA). The membranes were blocked and incubated with monoclonal anti-nitrotyrosine (1:1,000; Cayman, Ann Arbor, MI), anti-α-SMA (1:4,000; Sigma), anti-PCNA (1:4,000; Santa Cruz), and anti-β-actin (1:4,000; Sigma) antibodies overnight at 4°C. After being washed, the membranes were incubated with horseradish peroxidase-conjugated horse anti-mouse (Vector Laboratories) antibody diluted 1:4,000 for 1 h at RT. Finally, the membranes were exposed to a Western Lighting Chemiluminescence Reagent (PerkinElmer, Boston, MA). The density of the immunoblot was analyzed using the LabWorks 4.5 software (Ultra-Violet Products).

Enzyme activity assay. Tissues in sucrose buffer (0.32 M sucrose, 10 mM Tris•HCl, pH 7.4; Sigma) were homogenized on ice with a Dounce homogenizer. The homogenates were centrifuged at 1,000 g for 5 min, and then the supernatants were centrifuged at 15,000 g for 30 min. The supernatants were the cytosolic fraction. The precipitates were washed twice with sucrose buffer to collect mitochondria-containing pellets. The pellets were resuspended in PBS containing 0.1% Triton X-100, disrupted four times using a sonicator (4710 Series; Cole-Palmer, Chicago, IL) at 40% of maximum setting for 10 s and centrifuged at 15,000 g for 30 min (12, 14). Catalase activity in the cytosolic fraction was measured by the decomposition of H2O2, determined by a decrease in absorbance at 240 nm (12, 14). Manganese SOD (MnSOD) and copper-zinc SOD (CuZnSOD) activities were measured in the mitochondrial and cytosolic fractions using spectrophotometrical assay by pyrogallol at 340 nm, respectively (12, 14). One unit of SOD activity was defined as the quantity of enzyme that reduces the superoxide-dependent color change by 50%. Glucose 6-phosphate dehydrogenase (G6PD) activity in the cytosolic fraction was measured by following the rate of NADP+ reduction at 340 nm (14).

Fig. 4. Inhibition of increases of nitrotyrosine expression (A), tissue H2O2 (B), and lipid peroxidation (C) following I/R by manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin (MnTMPyP) administration. Mice were subjected to 30 min of bilateral renal ischemia followed by reperfusion on day 0 and then administrated with either MnTMPyP (a cell permeable SOD mimetic; 5 mg/kg body wt) or vehicle (0.9% saline) intraperitoneally beginning at 48 h after I/R for 14 days daily. Sixteen days after reperfusion, kidneys were harvested. A: nitrotyrosine expressions were determined by Western blot analysis. The β-actin was used as equivalent protein loading marker. Productions of tissue H2O2 (B) and lipid peroxidation (C) were determined as described in MATERIALS AND METHODS. Results are expressed as means ± SE (n = 4–6). *P < 0.05 vs. respective sham; #P < 0.05 vs. respective vehicle.

Fig. 5. Inhibition of the decreases of MnSOD (A), CuZnSOD (B), G6PD (C), and catalase (D) following I/R by MnTMPyP administration. Mice were subjected to 30 min of bilateral renal ischemia followed by reperfusion on day 0 and then administrated with either MnTMPyP or vehicle intraperitoneally for 14 days beginning at 48 h after the onset of the procedures. Sixteen days after reperfusion, the activities of MnSOD (A), CuZnSOD (B), G6PD (C), and catalase (D) were measured as described in MATERIALS AND METHODS. Results are expressed as means ± SE (n = 6). *P < 0.05 vs. respective sham; #P < 0.05 vs. respective vehicle.
**RESULTS**

I/R induces kidney fibrosis 16 days after the procedure. I/R resulted in dramatic increases of PCr (Fig. 1A) and BUN (Fig. 1B). Morphological damage attributable to I/R was reflected by dilation, flattening, and congestion of tubules in the outer medulla of the test kidneys. Interstitial cell number also increased 24 h after reperfusion (Fig. 1, A and B). Eight and sixteen days after I/R, PCr and BUN returned to near normal levels (Fig. 1, C and D). However, histological restoration of test kidneys remained incomplete compared with normal kidneys (Fig. 1C). Eight and sixteen days after I/R, the number of interstitial cells was significantly greater when compared with normal kidneys (Fig. 1, C and D), and many atrophic tubules were observed (Fig. 1C). We also determined collagen deposition, a typical index of fibrosis, by Masson trichrome staining. Eight and sixteen days after I/R, collagen was noted predominantly in the interstitium of the outer medulla and gradually increased over time (Fig. 1, E and F). These results suggest that kidney tissue acutely damaged by I/R may recover in part. Also, when kidneys morphologically undergo chronic progression of fibrosis, they may be exposed continuously to fibrogenic factors.

Increases of ROS and oxidative stress following I/R are sustained for 16 days. To investigate whether ROS/oxidative stress continue beyond the functional recovery of kidneys following I/R, we examined levels of H2O2 and lipid peroxidation up to 16 days after I/R. H2O2 levels in kidney tissues peaked at 24 h, and this level was sustained for up to 16 days after I/R (Fig. 2A). The pattern of lipid peroxidation was similar to H2O2 formation (Fig. 2B). These results indicate that, following I/R, kidneys are continually exposed to ROS stress via incomplete removal of increased ROS or continuous production of ROS.

To determine whether these increases in H2O2 and lipid peroxidation were due to impairments of kidney antioxidant scavenging systems, we determined the activities of MnSOD, CuZnSOD, catalase, and G6PD in the kidney tissues. I/R dramatically decreased the activities of MnSOD, CuZnSOD, catalase, and G6PD 24 h after I/R (Fig. 3, A–D). These decreased activities were not completely recovered until 8 and 16 days after I/R (Fig. 3, A–D). These results indicate that an abnormal redox condition is sustained for 16 days after I/R and may be associated with the progression of kidney fibrosis.

Administration of MnTMPyP reduces the changes of redox condition after I/R. To investigate whether the increased ROS/oxidative stress after I/R is involved in kidney fibrosis, we administrated MnTMPyP into the mice subjected to I/R beginning at 48 h after I/R for 14 days daily. MnTMPyP administration significantly reduced nitrotyrosine expression (Fig. 4A), H2O2 (Fig. 4B), and lipid peroxidation (Fig. 4C) 16 days after I/R when compared with vehicle administration. In addition, MnTMPyP administration attenuated the decreases of MnSOD, CuZnSOD, and G6PD activities following I/R (Fig. 5). In the present study, although we cannot clarify whether these increased enzyme activities are regulated directly or indirectly by MnTMPyP, the increased enzyme activities by MnTMPyP may be associated with its antioxidant functions and increased cell survival by its administration.

Administration of MnTMPyP reduces the cell damage after I/R. To investigate that MnTMPyP affects tubular cell death and survival and following accumulation of interstitial cells, we determined kidney epithelial cell damage by scorings of tubular damage and TUNEL and interstitial cell accumulation by cell counting. MnTMPyP administration attenuated the...
post-I/R increase of morphological damage (Fig. 6A) and tubular epithelial cell apoptosis (Fig. 6, B and C) in the outer medulla of kidneys when compared with vehicle administration.

Interstitial cell accumulation and S100A4, CD68, and HSP47 expressions implicate in kidney interstitial fibrosis after I/R. I/R dramatically increased the area of interstitium and interstitial cell numbers, consistent with our previous study (13). The increases of extension of interstitium and accumulation of interstitial cells were significantly attenuated by MnTMPyP (Fig. 7, A and B), suggesting that the accumulation of interstitial cells and extension of interstitium is associated with oxidative stress. It has been demonstrated that fibroblasts derived from marrow stromal cells (26, 31) and epithelial-to-mesenchymal cell transition (6, 32, 38). To characterize the cells observed in posts ischemic fibrotic kidneys, we carried out immunofluorescent staining using anti-S100A4 as a fibroblast or macrophage marker (20, 22), anti-CD68 antibodies as a macrophage-specific marker (9), or anti-HSP47 as a chaperone protein for mature collagen molecules in cells actively engaged in collagen synthesis (11). Sixteen days after I/R, S100A4-positive cells were predominately expressed in the interstitium around atrophic tubules (Fig. 7C). The post-I/R increases in S100A4-positive cell number were significantly lower in MnTMPyP-administered mice than in vehicle-administered mice (Fig. 7, C and D). CD68-positive cells were significantly lower in MnTMPyP-administered mice than vehicle-administered mice (Fig. 7, C and E). These results indicate that ROS/oxidative stress stimulates increases of fibroblasts/macroages. In the kidneys exposed to I/R, HSP47-positive cells were predominately localized in the interstitium (Fig. 7C). Interestingly, HSP47-positive cells were detected in some of the tubular cells of kidneys exposed to I/R but not in the tubular cells of sham-operated kidneys (Fig. 7C). This suggests a phenotypic conversion of tubular epithelial cells to collagen-synthetic cells, such as fibroblasts. The increases of the number of HSP47-positive cells following I/R were

Fig. 7. Inhibition of increases of interstitial cell (A and B), S100A4 (C and D), CD68 (C and E), and heat shock protein 47 (HSP47) (C and F) expressions following I/R by MnTMPyP administration. Mice were subjected to 30 min of bilateral renal ischemia followed by reperfusion on day 0 and then administered with either MnTMPyP or vehicle intraperitoneally beginning from 48 h after I/R for 14 days daily. A: kidneys were perfusion fixed for PAS staining. Arrows indicate interstitial cells. B: numbers of interstitial cells were counted in a 0.1-mm² field of the outer medulla of the kidney (10 fields per kidney). C: S100A4, CD68, and HSP47 expressions were detected by immunofluorescent staining using anti-S100A4, anti-CD68, and anti-HSP47 antibodies, respectively. Arrows indicate S100A4-, CD68-, and HSP47-positive cells in the tubulointerstitial cells, respectively. Arrowheads indicate HSP47-positive cells in tubular epithelial cells. D–F: S100A4-, CD68-, and HSP47-positive cells were quantified in a 0.1-mm² field of the outer medulla of the kidney (10 fields per kidney). Visible blue color indicates nucleus stained by DAPI. Visible red color is a 650–710-nm-emitted color to detect general morphology without treatment with antibodies. Results are expressed as means ± SE (n = 4). *P < 0.05 vs. respective sham; #P < 0.05 vs. respective vehicle. Scale bars = 30 μm.
significantly inhibited by MnTMPyP administration (Fig. 7, C and F).

To determine the effect of MnTMPyP on kidney fibrosis after I/R, we determined the collagen deposition and the expressions of α-SMA, a specific marker of myofibroblast, and PCNA, a marker of cell proliferation. MnTMPyP administration significantly lowered the increases in the collagen deposition (Fig. 8, A and B) and expressions of α-SMA (Fig. 8, C–E) and PCNA (Fig. 8F). The expression of α-SMA was highly localized in the interstitium of the outer medulla of MnTMPyP-administered kidneys (Fig. 8, C and D). These results indicate that increased ROS/oxidative stress following I/R may stimulate macrophage accumulation and, in part, a conversion of tubular epithelial cells and/or accumulated macrophages to collagen synthetic cells.

MnTMPyP administration after recovery of kidney function reduces fibrosis. To investigate whether the antifibrotic effect of MnTMPyP was involved in effect on repair after I/R injury, we determined PCr concentrations 3, 5, 8, 12, and 16 days after I/R. PCr concentrations in mice that were administered MnTMPyP beginning at 48 h after I/R were no significant difference when compared with PCr in vehicle-administered mice (Fig. 9A). Furthermore, we examined whether MnTMPyP administration beginning at 8 days after I/R, when PCr levels return to normal, inhibits fibrosis after I/R (Fig. 9B). MnTMPyP administrations both beginning at 48 h and 8 days after I/R significantly reduced α-SMA expressions when compared with vehicle administration (Fig. 9C). The reduction of α-SMA ex-

Fig. 8. Inhibition of increases of collagen deposition (A and B), α-smooth muscle actin (α-SMA) (C–E), and PCNA (F) expressions following I/R by MnTMPyP administration. Mice were subjected to 30 min of bilateral renal ischemia followed by reperfusion on day 0 and then administered with either MnTMPyP or vehicle intraperitoneally beginning from 48 h after I/R for 14 days daily. A: kidneys were perfusion fixed for Masson trichrome staining. B: collagen deposition was quantified as the number of pixels of blue color in a 0.1-mm² field of the outer medulla of the kidney using LabWorks analysis software (10 fields per kidney). C: α-SMA localization was detected by immunofluorescent staining using anti-α-SMA antibody. Double arrows indicate vessels expressing α-SMA. Arrows indicate α-SMA-positive interstitial cells. D: α-SMA-positive areas were quantified as the number of pixels of FITC-color in a 0.1-mm² field of the outer medulla of the kidney (10 fields per kidney). Visible blue color indicates nucleus stained by DAPI. Visible red color is a 650–710-nm-emitted color to detect general morphology without treatment with antibodies. E and F: expressions of α-SMA and PCNA were determined by Western blot analysis using anti-α-SMA and anti-PCNA antibodies, respectively. The densities of blots were quantified using Lab Works analysis software. Results are expressed as means ± SE (n = 4). *P < 0.05 vs. respective sham; #P < 0.05 vs. respective vehicle. Scale bars in A and C = 50 and 30 μm, respectively.
pression was significantly greater in mice MnTMPyP administered beginning at 48 h after I/R than mice MnTMPyP administered beginning at 8 days after I/R (Fig. 9C). These data suggest that MnTMPyP reduces the postischemic fibrosis by attenuating the constantly generated ROS stress.

To compare the antifibrotic effect of MnTMPyP with other ROS scavenger, mice were administered vitamin E, a ROS scavenger, beginning at either 48 h or 8 days after I/R daily until euthanasia (Fig. 9A–C). PCr concentrations in vitamin E-administered mice were not significantly different when compared with those in vehicle-administered mice, indicating that vitamin E did not affect postischemic recovery (Fig. 9A and B). Vitamin E administration beginning at 48 h after I/R slightly attenuated α-SMA expression, whereas vitamin E administration beginning at 8 days after I/R did not (Fig. 9C). The inhibition of α-SMA expression by MnTMPyP administration was significantly greater than that by vitamin E (Fig. 9C). Probably the strong antifibrotic effect of MnTMPyP may be due to its various functions, such as inhibitor of the production of peroxynitrite and H2O2 (39) and the oxidation of mitochondrial and nuclear proteins (40), NADPH/GSH:superoxide oxidoreductase as well as peroxynitrite reductase (12, 15, 18).

DISCUSSION

Recently, the progression of fibrosis following transplantation has become widely recognized as a contributor of chronic allograft loss (2, 35). It is also associated with changes in hemodynamics, increases in oxidative stress and angiotensin II and its receptors, as well as inflammatory responses (3, 19, 33). Thus it has been suggested that inhibition of these mediators, especially ROS/oxidative stress, may protect against subsequent interstitial fibrosis after tissue transplantation. However, previous attempts to limit fibrotic change by targeting ROS with antioxidants have reported only minor improvements (6–8). In this study, we show that the overproduction of ROS/oxidative stress promotes progression of subsequent kidney fibrosis following I/R and that a superoxide-targeted antioxidant, MnTMPyP, significantly prevents this progression.

The process of renal fibrosis is characterized by numerous phenotypic changes, including the loss of epithelial cell membrane transporters and their functions, the acquisition of a more fibroblastic phenotype leading to production of extracellular matrix elements, such as collagen and fibronectin, and expression of α-SMA commonly found in myofibroblasts (25, 28, 36). In addition, PCNA overexpression in fibrotic kidneys has been detected in atrophic tubular cells and interstitial fibrotic cells and showed no correlation with mitosis (41). Kim et al. (16) reported that N-acetylcysteine, a free radical scavenger, attenuated α-SMA expression and induced cell cycle arrest in fibrosis-associated hepatic stellate cells. In our present study, the deposition of collagen and the expressions of α-SMA and PCNA were shown to increase 16 days after I/R, an effect that was significantly inhibited by MnTMPyP. This suggests that
ROS/oxidative stress may play a role in inducing synthesis of collagen, change of cell phenotypes, increase of interstitial cells, and cell proliferation. Although recent studies have reported that oxygen radical scavengers to treat progressive kidney diseases are ineffective (23, 24, 37), our animal experimental results show that antioxidant strategies to treat progressive renal disease are effective. It may be associated with the property of antioxidant scavengers and timing of treatment. We found in present study that earlier and longer treatment of both MnTMPyP and vitamin E present more potent antifibrotic function compared with later and shorter treatment. In addition MnTMPyP presented more strong antifibrotic effect than vitamin E. It may be due to the various functions of MnTMPyP. MnTMPyP is a cell-permeable SOD mimetic and functions as an inhibitor of the production of peroxynitrite and hydrogen peroxide (39) and the oxidation of mitochondrial and nuclear proteins (40) and as a NADPH/GSH:superoxide oxidoreductase and a peroxynitrite reductase (12, 15, 18). In conclusion, this study demonstrates NADPH/GSH:superoxide oxidoreductase and a peroxynitrite permeable SOD mimetic and functions as an inhibitor of the later and shorter treatment. In addition MnTMPyP presented that earlier and longer treatment of both MnTMPyP and vitamin E present more potent antifibrotic function compared with recipients of cadaveric renal transplants. Transplantation 57: 211–217, 1994.


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