Reactive oxygen species differently regulate renal tubular epithelial and interstitial cell proliferation after ischemia and reperfusion injury

Kim J, Jung KJ, Park KM. Reactive oxygen species differently regulate renal tubular epithelial and interstitial cell proliferation after ischemia and reperfusion injury. Am J Physiol Renal Physiol 298: F1118–F1129, 2010. First published February 17, 2010; doi:10.1152/ajprenal.00701.2009.—Reactive oxygen species (ROS) function as an inducer of cell death and survival or proliferative factor, in a cell-type-specific and concentration-dependent manner. All of these roles are critical to ischemia-induced renal functional impairment and progressive fibrotic changes in the kidney. In an effort to define the role of ROS in the proliferation of tubular epithelial cells and of interstitial cells in kidneys recovering after ischemia and reperfusion (I/R) injury, experimental mice were subjected to 30 min of bilateral kidney ischemia and administered with manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin (MnTMPyP), a superoxide dismutase mimetic, from 2 to 14 days after I/R for 14 days daily (earlier and longer) and from 8 to 15 days after I/R for 8 days daily (later and shorter). Cell proliferation was assessed via 5′-bromo-2′-deoxyuridine (BrdU) incorporation assays for 20 h before the harvest of kidneys. After I/R, the numbers of BrdU-incorporating cells increased both in the tubules and interstitium. MnTMPyP administration was shown to accelerate the proliferation of tubular epithelial cells, presenting tubule-specific marker proteins along tubular segments, whereas it attenuated the proliferation of interstitial cells, exhibiting α-smooth muscle actin, fibroblast-specific protein-1, F4/80, and NADPH oxidase-2 proteins; these results indicated that ROS attenuates tubular cell regeneration, but accelerates interstitial cell proliferation. Earlier and longer MnTMPyP treatment more effectively inhibited tissue superoxide formation, the increment of interstitial cells, and the decrement of epithelial cells compared with later and shorter treatment. After I/R, apoptotic cells appeared principally in the tubular epithelial cells, but not in the interstitial cells, thereby indicating that ROS is harmful in tubule cells, but is not in interstitial cells. In conclusion, ROS generated in the I/R-injured tissues induces apoptotic and necrotic cell death via lipid peroxidation, DNA breakdown, protein dysfunction, and the regulation of intracellular signaling pathways (16, 23, 24, 26–28). A number of studies have shown that, although high concentrations of ROS induce apoptotic and/or necrotic cell death, ROS at low concentrations stimulates cell survival and proliferation via the mitogenic pathways (17, 29). Recently, we determined that the removal of ROS-generating tissue macrophages affects the regeneration of tubular epithelial cells, a process required for the restoration of kidney function (19). Hence, our study, along with those of other researchers, demonstrates that ROS may perform differently in the restoration of damaged tubular epithelial cells and interstitial cells depending on the cell type and its concentration. However, the function of ROS in kidney epithelial cell regeneration and fibrosis after I/R remains to be clearly elucidated.

In the present study, we evaluated the hypothesis that ROS performs differently on the proliferation of tubular epithelial cells and interstitial fibroblasts in the kidneys after I/R injury. In this study, we determined that the removal of ROS increased after I/R injury accelerated tubular epithelial cell proliferation, whereas it attenuated interstitial cell proliferation in kidneys recovering from I/R insult, thereby indicating that ROS is crucial to the restoration of damaged tubular epithelial cells or fibrotic changes.

MATERIALS AND METHODS

Animal preparation. Experiments were conducted with 8-wk-old BALB/c male mice. Mice were permitted free access to water and standard mouse chow. In all cases, studies were conducted in accordance with the animal experimental procedures approved by the Kyungpook National University Institution Animal Care and Use Committee. The animals were anesthetized with pentobarbital sodium (60 mg/kg body wt ip; Sigma, St. Louis, MO) before I/R or sham surgery. Kidney ischemia was subjected to the occlusion of renal pedicles using nontraumatic microaneurysm clamps (Roboz, Rockville, MD) for 30 min as previously described (24, 37). Some mice were administered intraperitoneally with manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin [MnTMPyP, a cell permeable superoxide dismutase mimetic (SOD) mimetic; 5 mg/kg body wt; Calbiochem, San Diego, CA] and 0.9% saline (vehicle) beginning at either 48 h or 8 days after ischemia until 24 h before harvest each day. In an effort to minimize variation, only the mice whose PCr concentrations reached levels between 2.0 and 3.0 mg/dl at 24 h after reperfusion were used for further studies. Some mice were administered intraperitoneal injec-
tions of 5'-bromo-2'-deoxyuridine (BrdU; 50 mg/kg body wt; Sigma) 20 h before kidney harvest.

**Histology.** Kidneys were perfused via the left ventricle with 30 ml of PBS for 2 min and then PLP (2% paraformaldehyde, 75 mM L-lysine, and 10 mM sodium metaperiodate; Sigma) solution. Afterward, the kidneys were excised and placed in PLP for 4 h at 4°C. Paraffin-embedded tissue samples were then sliced into 2-μm sections using a microtome (Leica, Bensheim, Germany). Paraffin sections were stained with periodic acid-Schiff (PAS) to determine the number of tubular epithelial or interstitial cells. PAS staining was conducted in accordance with the manufacturer’s protocols. Images were collected with a digital camera (Carl Zeiss, Oberkochen, Germany). Each experimental animal group consisted of more than four mice. The numbers of epithelial or interstitial cells were counted in 10 fields (0.1 mm²/field) of the outer medulla using LabWorks 4.5 software (Ultra-Violet Products, Cambridge, UK).

**Renal functional parameter.** To evaluate the concentration of PCr, 70 μl of blood were obtained from the orbital sinus 1, 2, 3, 4, 5, and 16 days after I/R (n = 6/time point). The concentration was measured using a Beckman Creatinine Analyzer II (Beckman, Brea, CA).

**Immunofluorescence.** Paraffin-embedded 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 for 10 min in PBS. To reveal the antigen epitope, the sections were boiled in 10 mM sodium citrate buffer (pH 6.0) for 10 min using an autoclave, cooled for 20 min at room temperature, and then washed three times with PBS for 5 min each. The sections were blocked with PBS containing 1% BSA (blocking buffer) for 30 min at room temperature and then incubated with monoclonal anti-α-smooth muscle actin (α-SMA; 1:500 dilution; Sigma), -Na-K-ATPase (1:20 dilution; Santa Cruz, Santa Cruz, CA), -F4/80 (1:50 dilution; Santa Cruz), -BrdU (1:50 dilution; Vector Laboratories) for 60 min at room temperature, and then washed three times in PBS for 5 min each. The sections were stained with periodic acid-Schiff (PAS) to determine the number of tubular epithelial or interstitial cells. The terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay was conducted using the In Situ Cell Death Detection Kit and Fluorescein (Roche, Mannheim, Germany) in accordance with the manufacturer’s recommendations. In brief, 2 μm of kidney sections were deparaffinized, rehydrated, and treated with proteinase K (20 μg/ml; Invitrogen) in TE buffer (50 mM Tris Base, 1 mM EDTA, and 0.5% Triton X-100, pH 8.0, Sigma). Afterward, the sections were incubated for 30 min with TUNEL reagent mixture at room temperature and then washed three times with PBS for 5 min each. Finally, the sections were mounted with Prolong Gold anti-fade reagent (Invitrogen) and visualized under a confocal microscope (Carl Zeiss).

**Statistics.** The results were expressed as the means ± SE. Statistical differences among groups were calculated via ANOVA followed by a least-significant difference post hoc comparison using the SPSS 12.0 program. Differences between groups were considered statistically significant at a P value of <0.05.

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**Fig. 1.** Concentration of plasma creatinine (PCr) after ischemia and reperfusion (I/R). Mice were subjected to 30 min of bilateral renal ischemia or sham operation on day 0. PCr concentration was determined at the indicated times (n = 6/time point). Results are expressed as means ± SE. P < 0.05 vs. respective 0 day (before surgery) (*), vs. respective sham (#), and vs. respective 1 day ($).
RESULTS

Periodic changes of tubular epithelial and interstitial cell proliferation in recovering kidney from I/R injury. Consistent with the results of our previous studies (28, 36, 37), PCr levels in mice subjected to 30 min of bilateral renal ischemia were shown to increase dramatically 1 day after reperfusion, and then returned to almost normal 8 days later (Fig. 1), thereby indicating that the kidney tubules were gradually restored over time. To evaluate periodic changes in tubular epithelial and interstitial cell proliferation after I/R insult, mice subjected to ischemia were treated with BrdU 20 h before harvest, and the kidney sections were immunostained with BrdU antibody. BrdU incorporation experiments are commonly utilized in the detection of proliferating cells in vivo, as it is incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle), instead of thymidine (22). Significant increases in BrdU-positive tubular epithelial cells are initially observed in the deep cortex, just above the outer medulla (which is most susceptible to I/R insult), from 3 days after I/R, and then within the outer medulla and inner medulla beginning at 4 days (Fig. 2, A and B). BrdU incorporation peaked 4 days after reperfusion in every region of the kidney (Fig. 2, A and B). After this peak, the number of BrdU-incorporated cells was reduced gradually in all kidney regions (Fig. 2, A and B), along
with a reduction in the concentration of PCR. The incorporation of BrdU in the tubular epithelial cells in the outer medulla, which is the most severely damaged region, was greatest and remained for the longest time, when compared with the inner medulla and cortex (Fig. 2, A and B).

Significant increases in the numbers of BrdU-incorporated interstitial cells were noted on the outer medulla and cortex (Fig. 2, A and B).

Beginning 4 and 8 days after reperfusion, respectively (Fig. 2, A and C). However, any significant increase in BrdU-positive interstitial cells was not noted in the inner medulla (Fig. 2, A and C). Unlike the BrdU incorporation noted in the tubular epithelial cells, BrdU incorporation in the interstitial cells increased continuously over time; nevertheless, the PCR levels returned to normal (Fig. 2, A and C), thereby suggesting that the progressive expansion of the interstitial area is associated with interstitial cell proliferation; additionally, these two cell types have different fates in the recovering kidney.

MnTMPyP administration to mice recovering from I/R injuries accelerates the proliferation of tubular epithelial cells, whereas it attenuates the proliferation of interstitial cells in the kidneys. We have recently shown that kidneys recovering from I/R injury tend to progress to fibrosis (28, 36); although PCR and BUN levels return almost to normal levels ~1 wk later, the ROS generated by I/R persists for 16 days, at least, after reperfusion, and the removal of ROS using antioxidant drugs attenuates the fibrotic progression of kidneys, evidencing less profound expansions of the interstitial area compared with nontreated kidneys (28). This suggests that ROS is importantly involved in the repair process of kidneys damaged by I/R. Therefore, to determine whether ROS is involved in the increases of interstitial and tubular epithelial cell proliferation, mice were treated with MnTMPyP, a cell permeable SOD mimetic, for a total of 14 days from 2 to 15 days after reperfusion (later and shorter administration). We initially determined whether MnTMPyP administration reduced superoxide generation in kidney tissues by measuring ethidium oxidized from DHE by the superoxide anion. As noted in Fig. 3, the administration of MnTMPyP...
effectively reduced superoxide generation. This rate of reduction was greater in the animals treated with MnTMPyP earlier and for longer periods compared with those treated later and for shorter periods (Fig. 3).

MnTMPyP administration accelerated post-I/R BrdU incorporation in the tubular epithelial cells (Fig. 4, A and B; arrows indicate BrdU-incorporated tubular epithelial cells). This acceleration was more pronounced in mice treated with MnTMPyP earlier and for a longer period compared with those treated later and for a shorter time (Fig. 4, A and B). Because we injected BrdU in mice 20 h before kidney harvest, the BrdU incorporation data did not include the regenerated cells before BrdU injection, implying only the 20-h periods of cell proliferation; therefore, we evaluated the total number of tubular epithelial cells in the PAS-stained kidney sections (Fig. 5, A and B). The number of tubular epithelial cells in the MnTMPyP-treated mice was significantly higher in the animals treated earlier and for a longer period compared with the vehicle-treated mice, and the mice treated later and for a shorter time (Fig. 5, A and B), thereby indicating that ROS levels increased after I/R result in a slowing of the restoration of tubular epithelia after I/R injury.

In contrast with the observed tubular epithelial cell proliferation, the administration of MnTMPyP significantly inhibited the proliferation of interstitial cells (Fig. 4, A and C; arrowheads indicate BrdU-incorporated interstitial cells). We noted no differences in the numbers of BrdU-incorporated interstitial cells between the earlier and longer MnTMPyP administration group and the later and shorter group (Fig. 4, A and C); however, the increase in interstitial cell numbers in the PAS-stained sections was significantly lower in the earlier and longer MnTMPyP administration group compared with the later and shorter group (Fig. 5, A and C). The difference between the BrdU- and PAS-stained sections may be attributable to the fact that BrdU incorporation reflected only cell proliferation occurring during the 20 h before kidney harvest, but the PAS data reflected all periods of cell proliferation until 16 days after I/R. When cell proliferation was evaluated by immunofluorescent staining using anti-PCNA, an index protein of cell proliferation, antibody, in consistence with the BrdU incorporation result (Fig. 4, A–C), the administration of MnTMPyP significantly inhibited the increase of PCNA-positive interstitial cells (Fig. 6, A and C). The PCNA-positive tubular epithelial cells were greater in the earlier and longer MnTMPyP administration group than in the later and shorter group (Fig. 6, A and B). The higher number of PCNA-positive cells (Fig. 6, A–C) compared with BrdU-incorporated cells (Fig. 4, A–C) may be involved in the differences of two experiments. PCNA, as a cofactor for DNA polymerase, is expressed in nonproliferating cells that are arrested in the G1 phase of the cell cycle and

Fig. 5. Effect of MnTMPyP administration on changes in the tubular epithelial (A and B) and interstitial (A and C) cell numbers after I/R. Mice were subjected either to 30 min of bilateral renal ischemia or sham operation on day 0 and then treated with either MnTMPyP or vehicle intraperitoneally for a total of 14 days from day 2 to 15 after reperfusion (earlier and longer) and for a total of 8 days from day 8 to 15 after reperfusion (later and shorter). The kidney section harvested on day 16 was stained with periodic acid-Schiff (PAS) (A). Numbers of tubular epithelial (B) and interstitial (C) cells were counted in a 0.1-mm² field of the outer medulla of kidney sections (10 fields/kidney). Results are expressed as the means ± SE (n = 4). P < 0.05 vs. respective sham (*), vs. respective vehicle (#), and vs. respective MnTMPyP (earlier and longer) (†). Scale bar indicates 50 µm.
repaired the damaged DNA, as well as in proliferating cells. BrdU, as a thymidine analog, incorporation occurs only in the DNA synthetic period (11, 12, 34). These data clearly demonstrate that increased ROS levels after I/R treatment inhibit the restoration of damaged kidney tubular epithelial cells, whereas they accelerate interstitial cell proliferation, thereby indicating that ROS regulates cell proliferation, in a cell-type-specific and concentration-dependent manner.

Characteristics of proliferating cells in tubules and interstitium in recovering mouse kidneys from I/R injury. To characterize the proliferating cells, the kidney sections were double-stained using anti-BrdU and –Na-K-ATPase (a marker of tubular epithelial cells), -AQP1 (a marker of cells of a proximal tubule and thin limb of Henle’s loop), or -AQP2 (a marker of principal cells of a collecting duct) antibodies. BrdU-positive cells observed in the outer medulla evidenced tubule-specific proteins, along with tubule segments (Fig. 7A). The postischemic proliferations of the proximal tubular cells evidencing AQP1 and weak Na-K-ATPase expression and the distal tubular epithelial cells exhibiting strong Na-K-ATPase expression were significantly greater in the earlier and longer MnTMPyP-administered mice compared with the vehicle-administered and later and shorter MnTMPyP-administered groups (Fig. 7, A–C). However, the numbers of BrdU- and AQP2-positive epithelial cells in the collecting ducts did not differ between the vehicle-treated vs. both MnTMPyP-treated groups (Fig. 7, A and D). The data show that the effects of ROS on the regulation of tubular epithelial cell proliferation may occur in a cell-type-specific manner, even within the same urineiferous tubule, and also in a concentration-dependent manner.

When the kidney section was double-stained using anti-BrdU and -α-SMA (a marker of myofibroblasts), -FSP1 (a marker of fibroblasts), or -F4/80 (a marker of macrophages) antibodies, the majority of the BrdU-positive cells expressed α-SMA protein 16 days after I/R (Fig. 8, A and B). Some interstitial cells with BrdU incorporation expressed FSP1 protein 16 days after I/R (Fig. 8, A and C). The α-SMA gene is activated during the conversion of fibroblasts into myofibroblasts (40). The data indicate that the proliferation of fibroblasts in the interstitium progresses largely after the differentiation of fibroblasts to myofibroblasts. F4/80-positive interstitial cells with BrdU incorporation were very rare 16 days after I/R, thereby indicating that the increased number of macrophages in kidneys after I/R may be attributable to the infiltration of circulating monocytes/macrophages. MnTMPyP treatment resulted in a significant reduction in the number of BrdU- and α-SMA-, FSP1-, or F4/80-positive cells in the interstitium 16 days after I/R when compared with the vehicle-treated mice.
(Fig. 8, A–D), thereby indicating that I/R-generated ROS accelerates the proliferation of interstitial cells, including myofibroblasts, fibroblasts, and macrophages.

A number of previous studies have demonstrated that NOX2 promotes cell proliferation in a variety of cells, including fibroblasts (4, 18, 33, 39, 44). Therefore, we evaluated NOX2 expression, which has been detected in phagocytes, fibroblasts, and myofibroblasts (5). The level of NOX2 expression was increased significantly 16 days after I/R (Fig. 9, A and B). This increase was attenuated by MnTMPyP treatment; earlier and longer treatment resulted in more dramatic effects than later and shorter treatment (Fig. 9, A and B). The majority of the NOX2-positive cells appeared in the interstitial cells, and these cells were only rarely detected in the tubular epithelial cells (Fig. 9C). Consistent with the results of Western blotting (Fig. 9A), the number of NOX2- and BrdU-positive interstitial cells
increased significantly 16 days after I/R, and these increases were attenuated by MnTMPyP treatment (Fig. 9, C and D), thereby suggesting that NOX2-generated ROS is associated with the proliferation of interstitial cells. Taken together, the data indicate that the levels of ROS in the interstitial cells are higher than in the tubular epithelial cells, and that the interstitial cells evidence higher tolerance to high concentrations of ROS, even though high ROS levels are required for their functions, including cell proliferation; however, tubular epithelial cells are susceptible to high ROS concentrations. To test this possibility, we detected superoxide-producing cells via DHE staining and apoptotic cells by TUNEL assay in the interstitial and tubular epithelial cells 24 h after I/R, when the most severe tubular cell damage and the greatest increase in

Fig. 8. Characteristics of BrdU-incorporating cells on interstitium in kidneys subjected to I/R injury. Mice were subjected to 30 min of either bilateral renal ischemia or sham operation on day 0 and then treated with either MnTMPyP or vehicle intraperitoneally for a total of 14 days from day 2 to day 15 after reperfusion (earlier and longer) and for a total of 8 days from day 8 to day 15 after reperfusion (later and shorter). BrdU was injected in mice 20 h before kidney harvest. A: kidney sections were double-stained using anti-BrdU and α-smooth muscle actin (α-SMA, a marker of myofibroblast), fibroblast-specific protein-1 (FSP1, a marker of fibroblast), or F4/80 (a marker of macrophage) antibodies. V indicates a vessel expressing α-SMA. Arrows, double arrows, and arrowhead indicate proliferating myofibroblasts (BrdU- and α-SMA-positive), fibroblast (BrdU- and FSP1-positive), and macrophages (BrdU- and F4/80-positive), respectively. Visible blue color indicates the DAPI-stained nucleus. B–D: BrdU-positive myofibroblasts (B), fibroblasts (C), or macrophages (D) were counted in a 0.1-mm² field of the outer medulla of kidneys (10 fields/kidney). The results are expressed as the means ± SE (n = 4). P < 0.05 vs. respective sham (*) and vs. respective vehicle (#). Scale bar indicates 20 μm.
infiltrated cell accumulation were observed. Apoptotic cells appeared mostly in the tubules, whereas they were rarely noted in the interstitium (Fig. 10). Both the interstitial and tubular epithelial cells evidenced higher superoxide levels compared with the cells of the sham-operated kidney (Fig. 10), thereby indicating that the high ROS levels generated by I/R induce apoptotic cell death in the tubular epithelial cells, but not in the interstitial cells.

DISCUSSION

The results of this study demonstrated that ROS induces accelerated interstitial cell proliferation, which subsequently causes renal insufficiency via the expansion of the interstitial area and extracellular matrix deposition, whereas it inhibits the tubular epithelial cell proliferation required for the restoration of renal function in the kidneys recovering from I/R injury. This suggests that ROS is a critical determining factor in desirable or nondesirable consequences and acts in a cell-type-specific manner, even within the same tissue type. Additionally, our data demonstrate that ROS scavenging begins at an earlier stage after I/R injury and is sustained for a longer time, more effectively preventing fibrotic changes and accelerating tubular cell restoration than when administered later and for a shorter time. This implies that the level of superoxide is an important factor to the regulation of cell-type-specific proliferation. Although superoxide production was normalized completely after earlier and longer treatment of MnTMPyP in the ischemic mice, the changes in cellular proliferation (both tubular and interstitial) persisted to a significant level. It suggests that, although the superoxide plays a critical role in the post-I/R cell proliferation, it is not the only factor. Thus our results demonstrate that an appropriate antioxidant therapy must consider this cell-type specificity, as well as the timing and duration of treatment. Furthermore, we determined that kidney tubular epi-

![Fig. 9. Effect of MnTMPyP treatment on NADPH oxidase-2 (NOX2) expression in kidneys subjected to I/R injury. Mice were subjected to either 30 min of bilateral renal ischemia or sham operation on day 0 and then treated with either MnTMPyP or vehicle intraperitoneally for a total of 14 days from day 2 to day 15 after reperfusion (earlier and longer) and for 8 days from day 8 to day 15 after reperfusion (later and shorter). BrdU was injected in mice 20 h before kidney harvest. A: NOX2 expression was determined by Western blot analysis using anti-NOX2 antibody. B: the densities of the blots were quantified using Lab Works analysis software. C: kidney sections were double-stained using anti-BrdU (red color) and -NOX2 (green color) antibodies. Arrows indicate BrdU- and NOX2-positive interstitial cells in the outer medulla. Visible blue color indicates DAPI-stained nucleus. D: BrdU- and NOX2-positive cells were counted in a 0.1-mm² field of the outer medulla of kidneys (10 fields/kidney). The results are expressed as the means ± SE (n = 4). P < 0.05 vs. respective sham (*), vs. respective vehicle (#), and vs. respective MnTMPyP (earlier and longer) (†). Scale bar indicates 50 μm.](http://www.ajprenal.org/)

**A**

NOX2

β-Actin

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>MnTMPyP (earlier and longer)</th>
<th>MnTMPyP (later and shorter)</th>
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<tbody>
<tr>
<td>Sham</td>
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<td>Ischemia</td>
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**B**

|  | Vehicle | MnTMPyP (earlier and longer) | MnTMPyP (later and shorter) |
|  |         |                             |  |
|  | 1       | *                            | * # †                        |
|  | 2       | #                            |                             |
|  | 3       |                              |                             |
|  | 4       |                              |                             |
|  | 5       |                              |                             |
|  | 6       |                              |                             |
|  | 7       |                              |                             |

**C**

Sham + vehicle

Ischemia + vehicle

Sham + MnTMPyP (earlier and longer)

Ischemia + MnTMPyP (earlier and longer)

**D**

|  | Vehicle | MnTMPyP (earlier and longer) | MnTMPyP (later and shorter) |
|  |         |                             |  |
|  | 0       | *                            | * # †                        |
|  | 5       | #                            |                             |
|  | 10      |                              |                             |
|  | 15      |                              |                             |
|  | 20      |                              |                             |
|  | 25      |                              |                             |

**Fig. 9. Effect of MnTMPyP treatment on NADPH oxidase-2 (NOX2) expression in kidneys subjected to I/R injury. Mice were subjected to either 30 min of bilateral renal ischemia or sham operation on day 0 and then treated with either MnTMPyP or vehicle intraperitoneally for a total of 14 days from day 2 to day 15 after reperfusion (earlier and longer) and for 8 days from day 8 to day 15 after reperfusion (later and shorter). BrdU was injected in mice 20 h before kidney harvest. A: NOX2 expression was determined by Western blot analysis using anti-NOX2 antibody. B: the densities of the blots were quantified using Lab Works analysis software. C: kidney sections were double-stained using anti-BrdU (red color) and -NOX2 (green color) antibodies. Arrows indicate BrdU- and NOX2-positive interstitial cells in the outer medulla. Visible blue color indicates DAPI-stained nucleus. D: BrdU- and NOX2-positive cells were counted in a 0.1-mm² field of the outer medulla of kidneys (10 fields/kidney). The results are expressed as the means ± SE (n = 4). P < 0.05 vs. respective sham (*), vs. respective vehicle (#), and vs. respective MnTMPyP (earlier and longer) (†). Scale bar indicates 50 μm.**
Kidney tubules possess a remarkable regenerative capacity after injury and have shown to be restored within only a few days after the insult (25, 46). In present study, we found that the recovery of renal function occurred before the complete restoration of cell numbers. The earlier functional recovery may be associated with the recovery of cell function in reversibly damaged cells; the kidney cells after I/R injury undergo 1) irreversible cell death including necrosis, 2) reversible cell damage, or 3) no damage. If the recovery process is not appropriate, the ischchemically injured kidney is predisposed to renal fibrosis (2, 28). Thus the later cell proliferation of tubular epithelial and interstitial cells may be highly associated with the fate of the recovering kidneys.

At present, our results indicate that ROS may be an important determining factor of the fate of the recovering kidney. Excessively generated ROS under pathological conditions not only leads to the destruction of cellular components, including DNA, protein, and lipid peroxidation, followed by apoptotic and/or necrotic cell death (21, 23), but also triggers unscheduled mitogenic pathways via the upregulation of growth factors (e.g., epidermal growth factor and vascular endothelial growth factor) (32). Clement and Pervaiz (10) have reported that high, moderate, and low concentrations of hydrogen peroxide can induce cell death, growth arrest, and proliferation, respectively. In the present study, earlier and longer treatment with MnTMPyP, which more profoundly reduced superoxide generation compared with later and shorter treatments, also more dramatically attenuated the proliferation of interstitial myofibroblasts, thereby indicating that I/R-generated ROS is a critical factor in the induction of fibrotic changes in kidneys injured by I/R in a concentration-dependent manner. Our previous study showed that MnTMPyP and α-tocopherol, a ROS scavenger, reduced I/R-induced kidney fibrosis, depending on the period of treatment and the starting time points; the results of this study supported this notion (28). Chade et al. (9) previously suggested that chronic antioxidant intervention more effectively improved renal fibrosis by renovascular disease than acute intervention.

In the present study, the removal of ROS was shown to accelerate the proliferation of tubular epithelial cells, whereas it attenuates the proliferation of interstitial cells. Thus the different roles of ROS in the tubule cells and interstitial cells may be caused by the different intracellular ROS levels and redox status in the tubule cells and fibroblasts. There have been several reports thus far asserting that the role of ROS in cell proliferation occurs in a cell-type-specific manner, even within the same tissue (1, 8, 13). Anilkumar et al. (1) reported that the levels of NOX2, a major ROS generator, differ in a cell-specific manner, resulting in different levels of intracellular ROS and different cell responses. In this study, we determined that earlier and longer MnTMPyP treatment, which reduced tissue ROS levels more greatly than was noted with later and shorter-duration treatment, also accelerates tubular epithelial cell proliferation more dramatically than later and shorter-duration treatment. This indicates that the different responses between tubular epithelial and interstitial cells may be attributable to different levels of intracellular ROS. In fact, we determined that interstitial cells that harbored higher quantities of superoxide did not die, whereas the tubular epithelial cells that harbored relatively lower amounts of superoxide were severely injured, as shown in the TUNEL staining results depicted in Fig. 10. These data support the notion that cell susceptibility against ROS occurs in a cell-type-specific manner.

Acute kidney injury (AKI) is a common complication of critical illness and is characterized by high patient morbidity and mortality (7, 43). Although antioxidant therapies in experimental settings have proven useful for the treatment of progressive kidney diseases and AKI, antioxidant intervention was not utilized widely in clinical settings because of its ineffectiveness (31, 35, 42). Our present results show that it is necessary to treat early and continuously to prevent a progression of secondary renal diseases following AKI and that an effective strategy of anti-oxidative intervention for the treatment of progressive renal disease and AKI must take into...
consideration the timing of treatment and duration, as well as the specificity of cell types, even within the same tissues.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


