

Original Contribution

Manganese porphyrin reduces renal injury and mitochondrial damage during ischemia/reperfusion

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Abstract

Renal ischemia/reperfusion (I/R) injury often occurs as a result of vascular surgery, organ procurement, or transplantation. We previously showed that renal I/R results in ATP depletion, oxidant production, and manganese superoxide dismutase (MnSOD) inactivation. There have been several reports that overexpression of MnSOD protects tissues/organs from I/R-related damage, thus a loss of MnSOD activity during I/R likely contributes to tissue injury. The present study examined the therapeutic benefit of a catalytic antioxidant, Mn(III) *meso*-tetrakis(*N*-hexylpyridinium-2-yl)porphyrin (MnTnHex-2-PyP⁵⁺), using the rat renal I/R model. This was the first study to examine the effects of MnTnHex-2-PyP⁵⁺ in an animal model of oxidative stress injury. Our results showed that porphyrin pretreatment of rats for 24 h protected against ATP depletion, MnSOD inactivation, nitrotyrosine formation, and renal dysfunction. The dose (50 μg/kg) used in this study is lower than doses of various types of antioxidants commonly used in animal models of oxidative stress injuries. In addition, using novel proteomic techniques, we identified the ATP synthase-β subunit as a key protein induced by MnTnHex-2-PyP⁵⁺ treatment alone and complex V (ATP synthase) as a target of injury during renal I/R. These results showed that MnTnHex-2-PyP⁵⁺ protected against renal I/R injury via induction of key mitochondrial proteins that may be capable of blunting oxidative injury.

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Kidneys were the first successfully transplanted organs and are the most commonly performed transplants today. Approximately 65,000 patients are currently on waiting lists for renal transplantation; however, only 10,000 donor kidneys were available in 2005 [9]. Renal transplantation is one of the most successful solid-organ transplant procedures as measured by 1-year survival rates. However, significant graft loss occurs due to delayed (>1 year) graft function and processes of chronic

allograft nephropathy (CAN). The precise cause of CAN after renal allografts remains unclear, but seems to be related to pathological changes due to organ preservation techniques and ischemia/reperfusion (I/R) injury before and after surgical implantation [6,7,15,17,20]. It is well established that excessive oxidant production occurs during renal I/R [11,25,31,33]. The increase in oxidants overwhelms endogenous antioxidants leading to increased cellular injury. Several studies using models of I/R have clearly demonstrated free radical or reactive oxygen species damage as evidenced by increased lipid peroxidation and loss of function of cellular antioxidant proteins, including catalase, glutathione peroxidase, and superoxide dismutases Cu,ZnSOD (SOD1) and MnSOD (SOD2) [11,15]. Finally, I/R has been shown to result in mitochondrial

Abbreviations: BN–PAGE, blue native polyacrylamide gel electrophoresis; 2D-DIGE, two-dimensional fluorescence differential in-gel electrophoresis.

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dysfunction. Mitochondria are extremely sensitive to I/R injury, with subsequent changes in oxidative phosphorylation, ATP depletion, rise in intracellular calcium, mitochondrial swelling, and loss of respiratory complex activity [18,19,34,38].

Previously, using a rat model of renal I/R [11] and transplantation [26,27], we reported that MnSOD, the major mitochondrial antioxidant, was inactivated before the onset of renal dysfunction, suggesting that loss of mitochondrial antioxidant protection might play an important role in I/R-related renal injury. MnSOD is the major antioxidant in the mitochondria. Its main function is to catalyze the dismutation of $O_2^{\bullet -}$, which is continually generated via misfires (~1% of total oxygen consumption) in the electron transport chain and from other sources. MnSOD is a nuclear-encoded protein that is transported into the mitochondria via an amino-terminal targeting sequence and is subsequently cleaved to form its native homotetrameric structure of 96 kDa. Studies with MnSOD knockout mice provide unequivocal evidence that MnSOD is essential for life [22,24]. This is not the case for the other two superoxide dismutase family members, cytosolic SOD1 and extracellular SOD (SOD3), for which no lethality is observed after gene deletion [5,36].

MnSOD heterozygous knockout mice (expressing 50% of the normal complement of MnSOD) seem to develop normally and, in the absence of oxidative stress, fail to show any overt phenotypic change compared to wild-type animals [22,24]. However, recent studies have demonstrated that mitochondria from MnSOD heterozygous knockout mice do show oxidative damage and alterations in mitochondrial function [39]. Thus, it seems apparent that any condition that results in a loss of MnSOD activity (even only a 50% reduction) in mitochondria would have serious, potentially lethal consequences. Importantly, our laboratory demonstrated a 50% reduction in MnSOD activity in kidneys exposed to warm I/R [11]. In addition, there have been several reports that overexpression of MnSOD protects tissues/organs from I/R-related damage [1,7,8,20]; thus a loss of MnSOD activity during I/R likely contributes to tissue injury.

Experiments were designed to evaluate the therapeutic potential of a newly designed metalloporphyrin-based antioxidant, MnTnHex-2-PyP⁵⁺, in the rat renal I/R model. This porphyrin has drawn attention because it is significantly more lipophilic than the widely used MnTE-2-PyP⁵⁺, while possessing the same ability to eliminate $O_2^{\bullet -}$ [2] and ONOO⁻ [16]. Due to its lipophilicity MnTnHex-2-PyP⁵⁺ was around 10-fold more efficient in allowing SOD-deficient *Escherichia coli* to grow aerobically than MnTE-2-PyP⁵⁺ [32]. The data provided thus far indicate that the most potent Mn porphyrins studied *in vivo* are very effective in decreasing levels of oxidant species [30,35]. But there is also growing evidence that porphyrins may do more than quench oxidant production [10,37]. For example, porphyrins have been shown to inactivate transcription factors AP-1, NF- κ B, and HIF-1, either through eliminating reactive species or through directly oxidizing them [37], therefore affecting expression of corresponding genes. The goal of the current study was to determine whether MnTnHex-2-PyP⁵⁺ could blunt MnSOD inactivation and renal injury in our rat renal I/R model. Our data show that MnTnHex-2-PyP⁵⁺ treatment for a short period

(30 min) was ineffective, whereas longer treatment (24 h) was renal protective and seemed to involve upregulation of important mitochondrial proteins.

Materials and methods

Rat ischemia/reperfusion model

Animals were treated according to UAMS IACUC guidelines. Male inbred Fisher 344 rats weighing 250–300 g were used in this study. Ethrane was used to anesthetize the rats, followed by shaving and prepping with betadine. A 2-ml bolus of 0.9% (w/v) NaCl was administered intravenously and an incision made 1 cm superior to the symphysis pubis to the tip of the xyphoid process. The abdominal organs were retracted to the left to allow access to the retroperitoneum. A right nephrectomy was performed so that renal function would reflect the function of the left kidney alone. The left renal artery and vein were clamped for 40 min, resulting in a color change of the left kidney, during which time moist gauze was placed in the abdominal cavity. After 40 min, the clamp was released and the kidney turned red (~3 min), indicating proper reperfusion. Post-operatively, the animals were given 2 ml of 0.9% (w/v) NaCl in the abdominal cavity and placed under a heating lamp to recover from the anesthesia. Immediately after the onset of reperfusion and 18 h before sacrifice, rats were placed in metabolic cages to collect and measure urine. Urine was analyzed for creatinine concentration (uCr) using a Roche Mira Classic analyzer. At the end of the reperfusion (18 h), the animals were anesthetized (Ethrane), the left kidney was removed, and blood was collected via intracardiac puncture.

Experimental groups

MnTnHex-2-PyP⁵⁺ was synthesized as previously described [2]. Rats were divided into the following groups:

- I/R group. Rats were subjected to a right nephrectomy and renal artery occlusion (40 min) followed by 18 h reperfusion ($n=5$).
- I/R + MnTnHex-2-PyP⁵⁺ group (24 h). Rats received an iv (penile vein) bolus of MnTnHex-2-PyP⁵⁺ (50 μ g/kg in 0.5 ml saline) 24 h before right nephrectomy and renal I/R ($n=5$).
- I/R + MnTnHex-2-PyP⁵⁺ group (30 min). Rats received an iv (penile vein) bolus of MnTnHex-2-PyP⁵⁺ (50 μ g/kg in 0.5 ml saline) 30 min before right nephrectomy and renal I/R ($n=5$).
- Sham-operated group. Rats underwent identical surgery (nephrectomy), but without the I/R episode ($n=5$).
- Sham + MnTnHex-2-PyP⁵⁺ group. Rats received an iv bolus (penile vein) of MnTnHex-2-PyP⁵⁺ (50 μ g/kg in 0.5 ml saline) 24 h before right nephrectomy ($n=5$).

Assessment of renal function

Creatinine clearance

At the time of kidney harvesting, rats were prepared for determination of creatinine clearance (CrCl) [40]. Maximum blood volumes (via intracardiac puncture) were recovered

followed by determination of serum creatinine (sCr) using a Roche Mira Classic serum analyzer calibrated for rat samples: $CrCl = (uCr \times uVolume) / (sCr \times 1080 \text{ min})$.

Histological assessment

Renal tissue was formalin fixed, paraffin embedded, and processed as described [21]. Nitrotyrosine staining was performed using the polyclonal anti-nitrotyrosine antibody (1:700) as described [26]. Renal tissue injury was assessed in tissue sections stained using the periodic acid–Schiff (PAS) reaction, which permits assessment of tubular integrity.

Renal extract preparation

Renal extracts were made from frozen tissue by homogenization (0.1 g/ml), using a Polytron homogenizer, in buffer containing 50 mM potassium phosphate, pH 7.4, and 1 mM phenylmethylsulfonyl fluoride. Solubilized extracts were sonicated and centrifuged at 10,000 rpm (5 min, 4°C) to remove tissue debris. Protein concentrations were determined using the Coomassie Plus protein assay reagent (Pierce).

Western blot analysis

MnSOD Western analysis was performed using the polyclonal anti-MnSOD antibody (Upstate Biotechnology; 1:1000) or the polyclonal anti-ATP synthase- β subunit antibody (Invitrogen; 1:10,000). Probed membranes were washed three times and immunoreactive proteins were detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

MnSOD activity assay

Enzymatic activity of MnSOD was determined in renal extracts by the cytochrome *c* reduction method using 1 mM KCN as described by Fridovich et al. [28].

Blue native polyacrylamide gel electrophoresis (BN–PAGE)

Renal mitochondria were isolated by differential centrifugation in a sucrose-containing buffer. BN–PAGE was performed with minor modifications as described by Brookes et al. [4]. Briefly, mitochondrial pellets (200 μ g protein) were resuspended in 50 μ l of extraction buffer containing aminocaproic acid (0.75 M) and BisTris (50 mM, pH 7). *L n*-dodecyl-D-maltoside (lauryl-maltoside, 6.25 μ l, 10% w/v) was added to this suspension and incubated on ice (20 min). Samples were centrifuged (14,000g for 10 min), and 6.3 μ l of Coomassie Brilliant Blue G (5%, in aminocaproic acid, 0.5 M) was added to 50 μ l of the supernatant. Subsequently, samples (80 μ g) were quickly loaded into a native polyacrylamide gel (6%). Electrophoresis (40 V) used an anode buffer comprising BisTris (50 mM, pH 7) and cathode buffer comprising Tricine (50 mM), BisTris (15 mM, pH 7), and Coomassie Brilliant Blue G (0.02% w/v). After 1 h, the cathode buffer was replaced with one containing less Coomassie blue (0.002%), and electrophoresis

was resumed at 110 V, until the blue dye front reached the bottom. The gel was then stained (1 h) with a Coomassie blue solution (Coomassie Brilliant Blue G (0.05% w/v), Coomassie Brilliant Blue R-250 (0.05% w/v) in 25% isopropanol, 10% acetic acid) followed by destaining (20% isopropanol, 5% acetic acid).

Two-dimensional fluorescence differential in-gel electrophoresis

As a complementary approach to BN–PAGE, samples were also subjected to a highly quantitative, state-of-the-art two-dimensional “differential in-gel electrophoresis” (2D-DIGE) proteomics analysis. Renal homogenates were sent to Applied Biomics (Hayward, CA, USA) and were pre-labeled with one of three CyDyes, which are matched in terms of size and charge and, therefore, do not alter protein migration in either dimension (IEF or SDS–PAGE) (see also www.appliedbiomics.com Web site). The excitation and emission wavelengths of the CyDyes are resolvable with filters such that each protein spot can be quantified. Equal protein amounts of all three fluorescently labeled renal samples were mixed and loaded onto the same electrophoresis gel. Proteins were resolved using 2D electrophoresis (isoelectric focusing followed by SDS–PAGE). The gel was then scanned with a sensitive imager and DeCyder software was used to locate and analyze the protein spots based on their specific fluorescence.

Finally, protein spots of interest were excised from the gel using a ProPic (Genomic Solutions) imaging and spot-picking robot. Protein digestion was performed with a ProGest (Genomic Solutions) in-gel enzymatic digestion robot using sequencing-grade modified trypsin (Promega). The resulting peptide mixture was loaded using an autosampler onto a trapping column (Symmetry 300 C18 5 μ m NanoEase; Waters) using a CapLC XE (Waters) system, a switching valve, and a flow rate of 20 μ l/min. Peptides were separated by nanoflow capillary HPLC using a CapLC XE pump (Waters) operating at 12 μ l/min; flow rate was controlled with a splitter in front of the switching valve. Peptides were eluted at 300 nl/min onto a self-packed PicoFrit (New Objective) 75 μ m \times 20 cm column (Jupiter 4u Proteo 90A; Phenomenex). The eluant was analyzed in-line by ESI–MS/MS using a Micromass Q-ToF Micro (Waters) mass spectrometer operating in the positive ion mode. Data acquisition was performed in a data-dependent fashion, and the resulting data were processed to generate a peak list file using MassLynx (Waters) and searched against the NCBI nonredundant database using the Mascot search engine (www.matrixscience.com).

Cell culture

A normal rat kidney proximal tubular cell line (NRK-52E; ATCC No. CRL-1571) was used as recently described [12]. Cells were maintained in a humidified incubator gassed with 5% CO₂–95% air at 37°C in DMEM containing 5% fetal calf serum. After treatment with MnTnHex-2-PyP⁵⁺ (2 μ M; 24 h), cells were trypsinized in 0.25% trypsin/EDTA and lysed in PBS plus 1% Triton at 4°C for 30 min. After lysis,

cells were centrifuged at 10,000g for 5 min and the supernatant was assayed for protein.

Statistical analysis

All data were expressed as means±SEM. Comparisons between groups were performed using one-way ANOVA. We used the Tukey test as the post hoc ANOVA; differences were considered statistically significant if the *p* value was less than 0.05 with a power of 0.80.

Results and discussion

Experiments were designed to determine whether MnTnHex-2-PyP⁵⁺ could reduce renal injury using the rat renal I/R model. Male Fisher rats were pretreated (30 min) with MnTnHex-2-PyP⁵⁺ (50 µg/kg; iv) before renal I/R. Surprisingly, renal function, as measured by creatinine clearance, was not protected in the rats given the porphyrin (MnP) 30 min before ischemia compared to rats undergoing I/R without drug (I/R) (Fig. 1). Due to the intense brown color of the porphyrin, we could see that the compound was quickly delivered to the kidney during this 30-min incubation. Because the 30-min treatment group was not protective we extended the time of porphyrin treatment to 24 h. Rats receiving MnTnHex-2-PyP⁵⁺ 24 h before ischemia (I/R+MnP, 24 h), showed improved renal function compared to rats undergoing I/R without drug (I/R) (Fig. 1). Rats not exposed to I/R (sham-operated animals) but treated with the same concentration of MnTnHex-2-PyP⁵⁺ (SH+MnP) showed no change in renal function, suggesting the compound had little renal toxicity at the dose given. The lack of toxicity was not surprising given the low dose (50 µg/kg) and route of administration (iv) used in these studies; other reports showing protective effects in vivo have used higher and often multiple doses of Mn-porphyrins or other antioxidants (from ~1 to 10 mg/kg, with porphyrins, and cyclic polyamines to as high as

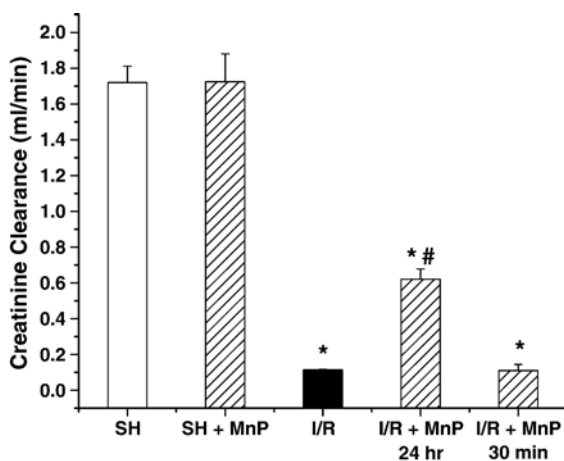


Fig. 1. Effects of MnTnHex-2-PyP⁵⁺ on creatinine clearance after I/R (40 min ischemia/18 h reperfusion). Creatinine clearance was determined in rats receiving MnTnHex-2-PyP⁵⁺ (50 µg/kg; 30 min or 24 h before ischemia) (MnP). Values are expressed as means±SEM (*n*=5). **p*<0.05 compared with sham (SH); #*p*<0.05 compared with I/R.

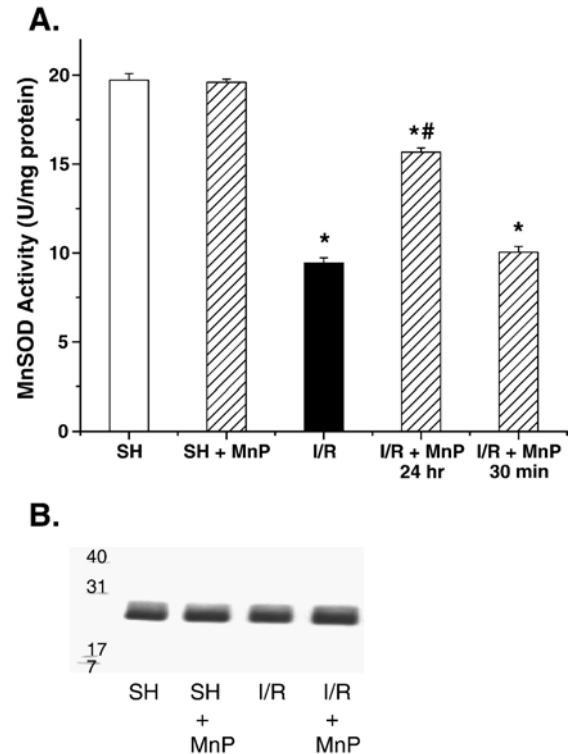


Fig. 2. Effects of MnTnHex-2-PyP⁵⁺ on MnSOD inactivation after I/R (40 min ischemia/18 h reperfusion). (A) MnSOD activity of renal extracts was determined using the cytochrome *c* assay in rats receiving MnTnHex-2-PyP⁵⁺ (50 µg/kg; 24 h or 30 min before ischemia) (MnP). Values are expressed as means±SEM (*n*=5). **p*<0.05 compared with sham (SH); #*p*<0.05 compared with I/R. (B) MnSOD Western blot showing equal protein levels in the four rat groups (MnP was 24-h treatment).

600 mg/kg with nitroxides) and a different route of administration (intraperitoneal) [3,10,13,14,23,29,30,35,41]. It is also important to note that previous studies using higher doses of MnTnHex-2-PyP⁵⁺ did show cellular toxicity [32]. These data suggest that at the dose used in these studies, the longer incubation time (24 h) was necessary for porphyrin-mediated protection against renal I/R injury.

Additional studies examined the ability of MnTnHex-2-PyP⁵⁺ to blunt MnSOD inactivation in our rat model of renal I/R. MnTnHex-2-PyP⁵⁺ failed to increase MnSOD activity in sham-treated rats (SH+MnP), suggesting that the dose and duration (30 min or 24 h) of treatment did not increase basal antioxidant capacity (Fig. 2A). This indicated that MnTnHex-2-PyP⁵⁺ was not acting as a classic “SOD mimetic” in our system. However, rats receiving MnTnHex-2-PyP⁵⁺ 24 h before I/R (I/R+MnP, 24 h) showed improved MnSOD activity compared to rats exposed to I/R without drug. Consistent with the previous findings, no protection was observed in the 30-min pretreatment group (I/R+MnP, 30 min). No alterations in MnSOD protein levels were noted in any of the rat groups (Fig. 2B). These data suggest that 24 h pretreatment with MnTnHex-2-PyP⁵⁺ protects MnSOD from inactivation in rats exposed to renal I/R. Thus, this compound may represent one therapeutic approach to preserving mitochondrial function and improving renal function during ischemia and reperfusion.

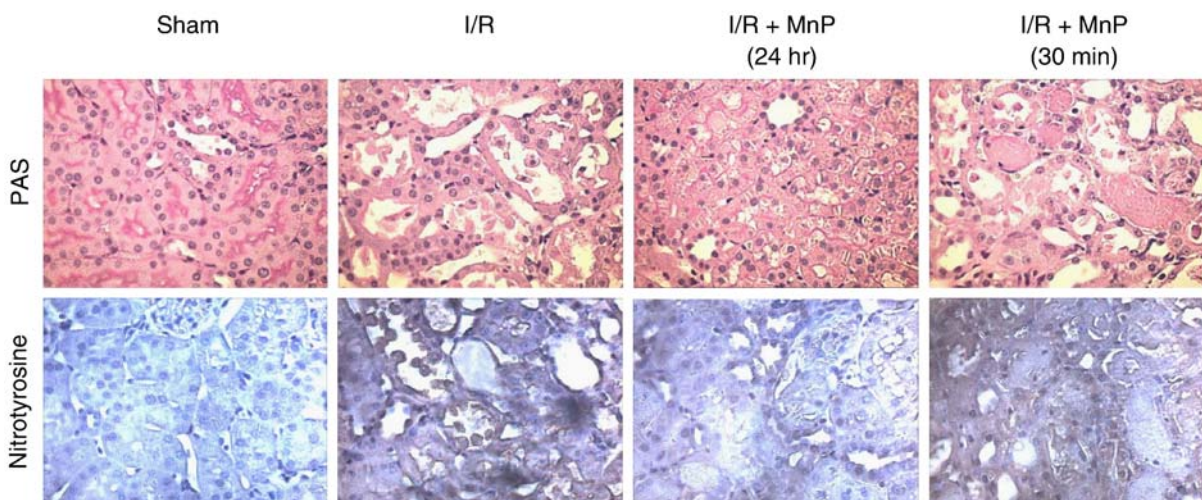


Fig. 3. PAS and nitrotyrosine (1:500) staining of renal tissue after I/R with and without pretreatment of MnTnHex-2-PyP⁵⁺ (MnP); original magnification 400 \times . These sections are representative of the rats used in each group ($n=5$).

PAS staining was used to examine tubular histopathological changes associated with loss of membrane integrity and also revealed improved renal histology in rats receiving MnTnHex-2-PyP⁵⁺ 24 h before I/R. Specifically, rats undergoing I/R without drug showed extensive tubular damage (dilation, loss of brush border, extensive tubular casts, and debris), whereas rats receiving pretreatment with MnTnHex-2-PyP⁵⁺ (I/R + MnP, 24 h) showed less severe tubular damage (Fig. 3). Likewise, MnTnHex-2-PyP⁵⁺ (24 h) also decreased the levels of nitrotyrosine within the kidney during I/R. Again, the 30-min pretreatment group (I/R + MnP, 30 min) did not protect against tubular damage or nitrotyrosine staining.

We used a relatively new proteomic approach called two-dimensional fluorescence differential in-gel electrophoresis to

begin to dissect the mechanism by which the porphyrin protects against tyrosine nitration and I/R-mediated renal injury. This is a highly quantitative, state-of-the-art proteomics analysis. In 2D-DIGE, three separate protein samples (rat kidney homogenates) were pre-labeled with three distinct size and charge-matched, spectrally resolvable CyDye fluorophores. Thus, three samples can be compared to one another simultaneously, i.e., A compared to B, A to C, and B to C. This quantitative “crosschecking” provides an internal control for the accuracy and precision of each protein spot, making 2D-DIGE far superior to conventional 2D gel overlay methods.

Kidneys from rats receiving MnTnHex-2-PyP⁵⁺ alone (50 $\mu\text{g}/\text{kg}$; 24 h or 30 min before kidney harvest) were compared to control rats without drug to screen for alterations in

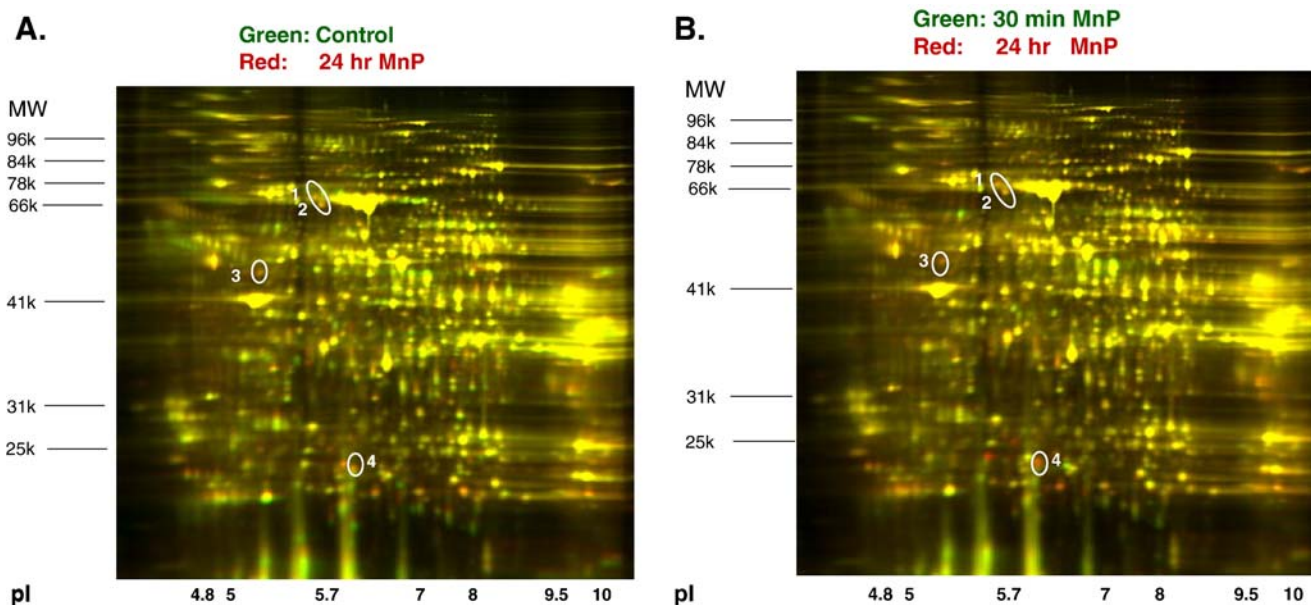


Fig. 4. Two-dimensional differential in-gel electrophoresis of renal proteins after treatment with porphyrin (MnP). Rats were injected with MnTnHex-2-PyP⁵⁺ (50 $\mu\text{g}/\text{kg}$ 24 hr before kidney harvest). Renal proteins (200 μg) were subjected to 2D-DIGE (Applied Biomics). Proteins were separated by isoelectric point (pI), from pH \sim 3 to 10 along the horizontal axis, and by molecular mass, from \sim 100 to 20 kDa, vertically. The circles represent the spots which were increased by porphyrin with respect to (A) control and (B) the 30-min pretreatment group.

renal proteins by drug alone. The scale and scope of the 2D-DIGE approach is very broad. For example, this analysis revealed 1840 distinct protein spots that could be rigorously quantified. Of these spots, 6.1% were increased by ≥ 1.5 -fold and 5.8% were decreased by ≥ 1.5 -fold. Only 15 spots increased or decreased by ≥ 2 -fold. Because only the 24-h drug treatment proved to be protective in vivo, the comparison of 24-h drug to control is the most relevant; spots showing ≥ 1.5 -fold increases/decreases in 24-h drug (relative to control) were chosen for identification. Fig. 4 shows the results when the control kidney is compared to 24 h MnTnHex-2-PyP⁵⁺ (Fig. 4A), as well as the 24-h versus the 30-min comparison (Fig. 4B). Based on the labeling done with these samples, protein spots that are increased (relative to control in Fig. 4A and 30-min drug treatment in Fig. 4B) show up as red, and spots that are decreased show up green; equal alignment and intensity of red and green yield yellow, meaning that these proteins are unchanged.

Using the naked eye, numerous red and green spots are readily evident; however, the DeCyder software makes quantitative comparisons via calculations of 3D “volumes” (spot area \times intensity). The circled numbers represent four spots that were increased ≥ 1.5 -fold in the kidneys of rats treated for 24 h with MnP compared either to control animals (Fig. 4A) or to rats treated for 30 min with MnP (Fig. 4B). Some additional red spots in the 24-h vs 30-min comparison were evident, but were not due to an induction of protein at 24 h. Rather, these spots were due to the fact that 30 min of MnP treatment decreased protein expression. In this regard, the advantage of correlating functional and proteomic data is necessary and apparent. For example, we know that MnP treatment for 30 min did not confer protection from I/R; hence proteins that changed in control versus the 30-min treatment group would not be candidate targets to microsequence, even if these same proteins changed in the control versus 24-h treatment group. We chose to identify the protein depicted in spot 3. A robotic system (ProPic from Genomic Solutions) was used to excise the spot. After in-gel trypsin digestion and mass spectroscopy, spot 3 was identified as the ATP synthase- β subunit (Table 1). The enzyme appeared to be significantly upregulated in rats treated with MnTnHex-2-PyP⁵⁺ (24 h) compared to control rats alone. Additional evidence that MnTnHex-2-PyP⁵⁺ induces the ATP synthase- β subunit was seen in our in vitro model using normal rat renal proximal tubule cells (NRK cells) [12]. Treatment of these cells with MnTnHex-2-PyP⁵⁺ (2 μ M; 24 h) resulted in increased protein expression of ATP synthase- β subunit (Fig. 5).

Finally, studies using BN-PAGE, a proteomic technique that separates and detects membrane-bound mitochondrial com-

Table 1
Summary of mass spectroscopy findings on excised gel spot 3

| Spot | Protein name | Mass | Total peptides | Unique peptides | Mascot score | Coverage |
|------|------------------------------|--------|----------------|-----------------|--------------|----------|
| 3 | ATP synthase β subunit | 51,171 | 10 | 10 | 456 | 28% |

Anti-ATP Synthase-beta

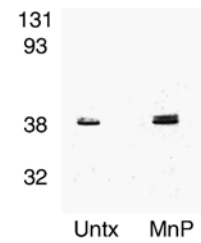
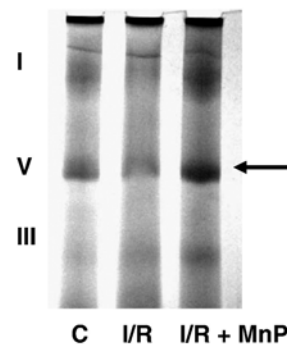


Fig. 5. ATP synthase- β subunit Western blot showing porphyrin (MnP)-mediated induction of ATP synthase- β subunit in normal rat kidney proximal tubule cells treated with MnTnHex-2-PyP⁵⁺ (2 μ M; 24 h). Cellular proteins (25 μ g) were separated by 12% SDS-PAGE and blotted with an ATP synthase- β subunit polyclonal antibody (Molecular Probes; 1:10,000). Untx indicates untreated renal cells. Gel is representative of three separate experiments.

A. BN-PAGE



B.

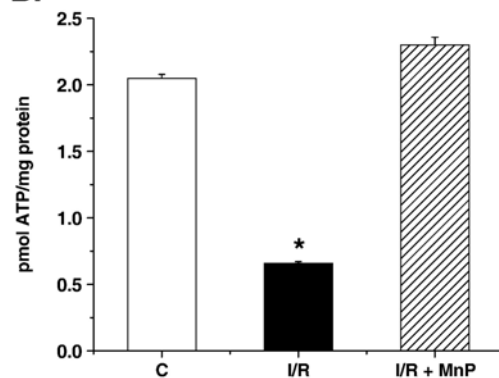


Fig. 6. (A) BN-PAGE showing alterations in mitochondrial complex proteins during I/R and restoration after porphyrin pretreatment. Renal mitochondria (80 μ g) isolated from rat kidney tissue after I/R were electrophoresed (6% native), stained, and destained as described under Materials and methods. The arrow indicates complex V (ATP synthase), which differs in mitochondria of controls (C), I/R rats, and rats pretreated with MnTnHex-2-PyP⁵⁺ (50 μ g/kg; 24 h before ischemia) (MnP). The Roman numerals represent the migration of mitochondrial electron transport complexes (I, NADH dehydrogenase; V, ATP synthase; III, ubiquinol/cytochrome *c* oxidoreductase). The gel is representative of three separate experiments. (B) ATP levels of renal extracts after I/R or with pretreatment with MnTnHex-2-PyP⁵⁺ (MnP). Values are expressed as means \pm SEM ($n=5$). * $p < 0.05$ compared with control (C).

plexes, were used to pinpoint which respiratory complexes revealed the most intense changes in patterns or function during I/R. To our knowledge, this technique has not been applied to evaluate critical changes in mitochondrial proteins in renal I/R. Mitochondria were isolated from control and ischemic kidneys without or with pretreatment with MnTnHex-2-PyP⁵⁺ (24 h) followed by I/R and analyzed using BN–PAGE, during which the complexes remain intact (shown by Roman numerals). Interestingly, the mitochondria from the ischemic kidney appeared to have an altered level of complex V (ATP synthase), which may indicate complex instability or inactivation (Fig. 6A). In addition, not only were mitochondria from rats pretreated with MnTnHex-2-PyP⁵⁺ protected from renal I/R injury; but also the porphyrin seemed to restore the levels of complex V (ATP synthase) comparable to control levels. It is important to remember that complex V comprises 14 subunits; thus future studies could use the modified protocol of 2D-BN–PAGE [4] to determine precisely which subunits of ATP synthase are altered during renal I/R. The precise mechanism by which upregulation of ATP synthase might protect the kidney from I/R injury has not been evaluated previously, but increased ATP production before ischemia would likely serve to protect mitochondria from I/R-mediated injury. Further studies were completed to determine ATP levels in the renal homogenates of control rats versus rats undergoing I/R and I/R+MnTnHex-2-PyP⁵⁺ pretreatment (Fig. 6B). These data support our prior report showing that I/R reduces renal ATP levels [11], but importantly that pretreatment with MnTnHex-2-PyP⁵⁺ (24 h) significantly restored ATP levels. Thus, these new studies suggest that the induction of complex V using MnP also correlates with increased ATP levels.

To our knowledge, this is the first report showing induction of a key protein involved with mitochondrial respiration after treatment with a manganese porphyrin. Interestingly, the finding of altered ATP synthase complements the results of 2D-DIGE (Fig. 5), which revealed an induction of ATP synthase- β subunit in response to MnTnHex-2-PyP⁵⁺ pretreatment. Importantly, these findings also suggest that MnTnHex-2-PyP⁵⁺ may prevent renal injury via induction of protective factors. Although we have not identified the full complement of proteins that are altered in response to MnTnHex-2-PyP⁵⁺ treatment, our data demonstrate the feasibility of using proteomic techniques to detect specific proteins altered by drug treatment in this model of renal I/R. It is important to note that only 15 of 1840 proteins were significantly altered by MnTnHex-2-PyP⁵⁺ and that this induction occurred independent of oxidant stress (i.e., in control rats). Future studies will be performed to better understand the molecular mechanism by which 24-h pretreatment of MnTnHex-2-PyP⁵⁺ leads to protein induction, as well as identification of these proteins.

Interestingly, our studies showed that MnTnHex-2-PyP⁵⁺ (at the dose and time points studied) did not upregulate MnSOD protein or activity in the cell or rat model but did significantly blunt nitration of renal proteins during renal I/R (Fig. 3). These data suggest that MnTnHex-2-PyP⁵⁺ is not acting as a classic SOD mimetic, but rather seem to block oxidant production indirectly during renal I/R. The precise mechanism of this

protection remains unknown, but may relate to its ability to induce ATP synthase- β subunit and prevent ATP depletion (Fig. 6), which would decrease mitochondrial superoxide production (and protein nitration), leading to less MnSOD inactivation. This, in turn, would preserve mitochondrial and renal function during renal I/R.

In conclusion, a proteomic approach was used to identify abnormalities of specific mitochondrial proteins in renal I/R and the reversal of these abnormalities by MnTnHex-2-PyP⁵⁺ pretreatment. Importantly, the porphyrin used here was protective at a single dose of 50 μ g/kg, which is significantly lower than doses of various types of antioxidants commonly used in animal models of oxidative stress injuries. Prevention or rapid reversal of these mitochondrial modifications may preserve mitochondrial function and improve long-term success of renal transplants. Notably, renal damage clearly occurs during I/R and is an unavoidable complication of cross-clamping the donor kidney for removal during transplant surgery. Therefore, strategies to limit the extent of renal damage during ischemic periods will increase the number of viable donor organs and improve graft function after renal transplantation.

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References

- [1] Abunasa, H. J.; Smolenski, R. T.; Morrison, K.; Yap, J.; Sheppard, M. N.; O'Brien, T.; Suzuki, K.; Jayakumar, J.; Yacoub, M. H. Efficacy of adenoviral gene transfer with manganese superoxide dismutase and endothelial nitric oxide synthase in reducing ischemia and reperfusion injury. *Eur. J. Cardiothorac. Surg.* **20**:153–158; 2001.
- [2] Batinic-Haberle, I.; Spasojevic, I.; Stevens, R. D.; Hambright, P.; Fridovich, I. Manganese(III) meso-tetrakis(ortho-N-alkylpyridyl)porphyrins: synthesis, characterization, and catalysis of O₂⁻ dismutation. *J. Chem. Soc. Dalton Trans.* 2689–2696; 2002.
- [3] Benov, L.; Batinic-Haberle, I. A manganese porphyrin suppresses oxidative stress and extends the life span of streptozotocin-diabetic rats. *Free Radic. Res.* **39**:81–88; 2005.
- [4] Brookes, P. S.; Pinner, A.; Ramachandran, A.; Coward, L.; Barnes, S.; Kim, H.; Darley-Usmar, V. M. High throughput two-dimensional blue-native electrophoresis: a tool for functional proteomics of mitochondria and signaling complexes. *Proteomics* **2**:969–977; 2002.
- [5] Carlsson, L. M.; Jonsson, J.; Edlund, T.; Marklund, S. L. Mice lacking extracellular superoxide dismutase are more sensitive to hyperoxia. *Proc. Natl. Acad. Sci. USA.* **92**:6264–6268; 1995.
- [6] Chan, P. H. Role of oxidants in ischemic brain damage. *Stroke* **27**:1124–1129; 1996.
- [7] Chen, Z.; Siu, B.; Ho, Y. S.; Vincent, R.; Chua, C. C.; Hamdy, R. C.; Chua, B. H. Overexpression of MnSOD protects against myocardial ischemia/reperfusion injury in transgenic mice. *J. Mol. Cell. Cardiol.* **30**:2281–2289; 1998.
- [8] Chen, Z.; Siu, B.; Ho, Y. S.; Vincent, R.; Chua, C. C.; Hamdy, R. C.; Chua,

- B. H. Overexpression of MnSOD protects against myocardial ischemia/reperfusion injury in transgenic mice. *J. Mol. Cell. Cardiol.* **30**:2281–2289; 1998.
- [9] Cooper, J. T.; Chin, L. T.; Krieger, N. R.; Fernandez, L. A.; Foley, D. P.; Becker, Y. T.; Odorico, J. S.; Knechtle, S. J.; Kalayoglu, M.; Sollinger, H. W.; D'Alessandro, A. M. Donation after cardiac death: the University of Wisconsin experience with renal transplantation. *Am. J. Transplant.* **4**:1490–1494; 2004.
- [10] Crow, J. P.; Calingasan, N. Y.; Chen, J.; Hill, J. L.; Beal, M. F. Manganese porphyrin given at symptom onset markedly extends survival of ALS mice. *Ann. Neurol.* **58**:258–265; 2005.
- [11] Cruthirds, D. L.; Novak, L.; Akhi, K. M.; Sanders, P. W.; Thompson, J. A.; MacMillan-Crow, L. A. Mitochondrial targets of oxidative stress during renal ischemia/reperfusion. *Arch. Biochem. Biophys.* **412**:27–33; 2003.
- [12] Cruthirds, D. L.; Saba, H.; MacMillan-Crow, L. A. Overexpression of manganese superoxide dismutase protects against ATP depletion-mediated cell death of proximal tubule cells. *Arch. Biochem. Biophys.* **437**:96–105; 2005.
- [13] Cuzzocrea, S.; Mazzon, E.; Dugo, L.; Caputi, A. P.; Riley, D. P.; Salvemini, D. Protective effects of M40403, a superoxide dismutase mimetic, in a rodent model of colitis. *Eur. J. Pharmacol.* **432**:79–89; 2001.
- [14] Cuzzocrea, S.; Mazzon, E.; Dugo, L.; Di Paola, R.; Caputi, A. P.; Salvemini, D. Superoxide: a key player in hypertension. *FASEB J.* **18**:94–101; 2004.
- [15] Dobashi, K.; Ghosh, B.; Orak, J. K.; Singh, I.; Singh, A. K. Kidney ischemia–reperfusion: modulation of antioxidant defenses. *Mol. Cell. Biochem.* **205**:1–11; 2000.
- [16] Ferrer-Sueta, G.; Vitturi, D.; Batinic-Haberle, I.; Fridovich, I.; Goldstein, S.; Czapski, G.; Radi, R. Reactions of manganese porphyrins with peroxy-nitrite and carbonate radical anion. *J. Biol. Chem.* **278**:27432–27438; 2003.
- [17] Fujimura, M.; Morita-Fujimura, Y.; Kawase, M.; Copin, J. C.; Calagui, B.; Epstein, C. J.; Chan, P. H. Manganese superoxide dismutase mediates the early release of mitochondrial cytochrome c and subsequent DNA fragmentation after permanent focal cerebral ischemia in mice. *J. Neurosci.* **19**:3414–3422; 1999.
- [18] Gupta, M.; Dobashi, K.; Greene, E. L.; Orak, J. K.; Singh, I. Studies on hepatic injury and antioxidant enzyme activities in rat subcellular organelles following in vivo ischemia and reperfusion. *Mol. Cell. Biochem.* **176**:337–347; 1997.
- [19] Henke, W.; Jung, K.; Polster, F. Effects of preservation solutions on cortical and medullary mitochondria of rat kidney. *Cell. Mol. Biol.* **41**:319–326; 1995.
- [20] Keller, J. N.; Kindy, M. S.; Holsberg, F. W.; St Clair, D. K.; Yen, H. C.; Germeyer, A.; Steiner, S. M.; Bruce-Keller, A. J.; Hutchins, J. B.; Mattson, M. P. Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxy-nitrite production, lipid peroxidation, and mitochondrial dysfunction. *J. Neurosci.* **18**:687–697; 1998.
- [21] Kerby, J. D.; Verran, D. J.; Luo, K. L.; Ding, Q.; Tagouri, Y.; Herrera, G. A.; Diethelm, A. G.; Thompson, J. A. Immunolocalization of FGF-1 and receptors in glomerular lesions associated with chronic human renal allograft rejection. *Transplantation* **62**:190–200; 1996.
- [22] Lebovitz, R. M.; Zhang, H.; Vogel, H.; Cartwright, J.; Dionne, L.; Lu, N.; Huang, S.; Matzuk, M. M. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc. Natl. Acad. Sci. USA* **93**:9782–9787; 1996.
- [23] Leinenweber, S. B.; Sheng, H.; Lynch, J. R.; Wang, H.; Batinic-Haberle, I.; Laskowitz, D. T.; Crapo, J. D.; Pearlstein, R. D.; Warner, D. S. Effects of a manganese (III) porphyrin catalytic antioxidant in a mouse closed head injury model. *Eur. J. Pharmacol.* **531**:126–132; 2006.
- [24] Li, Y.; Huang, T. T.; Carlson, E. J.; Melov, S.; Ursell, P. C.; Olson, J. L.; Noble, L. J.; Yoshimura, M. P.; Berger, C.; Chan, P. H., et al. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat. Genet.* **11**:376–381; 1995.
- [25] Linas, S. L.; Whittenburg, D.; Parsons, P. E.; Repine, J. E. Ischemia increases neutrophil retention and worsens acute renal failure: role of oxygen metabolites and ICAM 1. *Kidney Int.* **48**:1584–1591; 1995.
- [26] MacMillan-Crow, L. A.; Crow, J. P.; Kerby, J. D.; Beckman, J. S.; Thompson, J. A. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc. Natl. Acad. Sci. USA* **93**:11853–11858; 1996.
- [27] MacMillan-Crow, L. A.; Cruthirds, D. L.; Akhi, K. M.; Sanders, P. W.; Thompson, J. A. Mitochondrial tyrosine nitration precedes chronic allograft nephropathy. *Free Radic. Biol. Med.* **31**:1603–1608; 2001.
- [28] McCord, J. M.; Fridovich, I. Superoxide dismutase: an enzymic function for erythrocyte hemocuprein (hemocuprein). *J. Biol. Chem.* **244**:6049–6055; 1969.
- [29] Nilakantan, V.; Zhou, X.; Hilton, G.; Shi, Y.; Baker, J. E.; Khanna, A. K.; Pieper, G. M. Antagonizing reactive oxygen by treatment with a manganese(III) metalloporphyrin-based superoxide dismutase mimetic in cardiac transplants. *J. Thorac. Cardiovasc. Surg.* **131**:898–906; 2006.
- [30] Nin, N.; Cassina, A.; Boggia, J.; Alfonso, E.; Botti, H.; Peluffo, G.; Trostchansky, A.; Bathiany, C.; Radi, R.; Rubbo, H.; Hurtado, F. J. Septic diaphragmatic dysfunction is prevented by Mn(III) porphyrin therapy and inducible nitric oxide synthase inhibition. *Intensive Care Med.* **30**:2271–2278; 2004.
- [31] Noiri, E.; Nakao, A.; Uchida, K.; Tsukahara, H.; Ohno, M.; Fujita, T.; Brodsky, S.; Goligorsky, M. S. Oxidative and nitrosative stress in acute renal ischemia. *Am. J. Physiol. Renal Physiol.* **281**:F948–F957; 2001.
- [32] Okado-Matsumoto, A.; Batinic-Haberle, I.; Fridovich, I. Complementation of SOD-deficient *Escherichia coli* by manganese porphyrin mimics of superoxide dismutase activity. *Free Radic. Biol. Med.* **37**:401–410; 2004.
- [33] Paller, M. S.; Hoidal, J. R.; Ferris, T. F. Oxygen free radicals in ischemic acute renal failure in the rat. *J. Clin. Invest.* **74**:1156–1164; 1984.
- [34] Piantadosi, C. A.; Zhang, J. Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. *Stroke* **27**:327–331; 1996.
- [35] Piganelli, J. D.; Flores, S. C.; Cruz, C.; Koepp, J.; Batinic-Haberle, I.; Crapo, J.; Day, B.; Kachadourian, R.; Young, R.; Bradley, B.; Haskins, K. A metalloporphyrin-based superoxide dismutase mimic inhibits adoptive transfer of autoimmune diabetes by a diabetogenic T-cell clone. *Diabetes* **51**:347–355; 2002.
- [36] Reaume, A. G.; Elliott, J. L.; Hoffman, E. K.; Kowall, N. W.; Ferrante, R. J.; Siwek, D. F.; Wilcox, H. M.; Flood, D. G.; Beal, M. F.; Brown Jr., R. H.; Scott, R. W.; Snider, W. D. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat. Genet.* **13**:43–47; 1996.
- [37] Tse, H. M.; Milton, M. J.; Piganelli, J. D. Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: implication for their use in targeting oxidation–reduction reactions in innate immunity. *Free Radic. Biol. Med.* **36**:233–247; 2004.
- [38] Weinberg, J. M.; Venkatachalam, M. A.; Roeser, N. F.; Nissim, I. Mitochondrial dysfunction during hypoxia/reoxygenation and its correction by anaerobic metabolism of citric acid cycle intermediates. *Proc. Natl. Acad. Sci. USA* **97**:2826–2831; 2000.
- [39] Williams, M. D.; Van Remmen, H.; Conrad, C. C.; Huang, T. T.; Epstein, C. J.; Richardson, A. Increased oxidative damage is correlated to altered mitochondrial function in heterozygous manganese superoxide dismutase knockout mice. *J. Biol. Chem.* **273**:28510–28515; 1998.
- [40] Wood, P. A.; Hamm, D. A.; Chen, P. Y.; Sanders, P. W. Studies of arginine metabolism and salt sensitivity in the Dahl/Rapp rat models of hypertension. *Mol. Genet. Metab.* **64**:80–83; 1998.
- [41] Zahmatkesh, M.; Kadkhodae, M.; Moosavi, S. M.; Jorjani, M.; Kajbafzadeh, A.; Golestani, A.; Ghaznavi, R. Beneficial effects of MnTBAP, a broad-spectrum reactive species scavenger, in rat renal ischemia/reperfusion injury. *Clin. Exp. Nephrol.* **9**:212–218; 2005.