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# Time-dependant protective effects of mangenese(III) tetrakis (1-methyl-4-pyridyl) porphyrin on mitochondrial function following renal ischemia-reperfusion injury

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**Abstract**

This study examined the time-dependent effects of a cell permeable SOD mimetic, MnTMPyP, on mitochondrial function in renal ischemia-reperfusion injury (IRI). Male SD rats were subject to either sham operation or bilateral renal ischemia for 45 min followed by reperfusion for 1, 4 or 24 h. A sub-set of animals was treated with either saline vehicle or 5 mg/Kg of MnTMPyP (i.p.). EPR measurements showed that at 1-h reperfusion MnTMPyP prevented a decrease in aconitase activity ($p < 0.05$) and attenuated the increase in the high spin heme at $g=6$ and oxidation of 4Fe4S to 3Fe4S signal at $g=2.015$ ($p < 0.01$). MnTMPyP was effective in preventing loss of mitochondrial complexes and prevented the loss of cytochrome *c* and Smac/Diablo from mitochondria early in reperfusion. Following 24 h of reperfusion MnTMPyP was effective in attenuating caspase-3 and blocking apoptosis ($p < 0.05$). In conclusion, MnTMPyP has biphasic effects in renal IRI, inhibiting mitochondrial dysfunction at the early phases of reperfusion and prevention of apoptosis following longer durations of reperfusion.

**Keywords:** *Renal ischemia-reperfusion, superoxide, mitochondria, EPR, MnTMPyP, apoptosis*

## Introduction

Acute kidney injury is a significant clinical problem, with mortality rates as high as 50%. Renal ischemia reperfusion (IR) is one of leading causes of acute kidney injury. There are multiple pathways that are causative in renal IRI, with reactive oxygen species (ROS) being a major contributor. ROS generated in a single ischemic event or by irradiation may also be involved in pre-conditioning and prevention of kidney injury during subsequent ischemic attacks [1]. Because of these reasons, there is substantial interest in understanding the underlying mechanisms of ROS-mediated cell death and in developing therapies that target ROS in renal IRI.

Several SOD mimetics have been successfully used to prevent ischemia-reperfusion injury in heart, brain and kidney. For example, free radical scavengers such as Tempol and EUK-134 attenuate nitrative/oxidative stress and improve renal function following both *in vivo* and *in vitro* renal IRI [2,3]. We have also previously shown that the cell permeable SOD/catalase mimetic, Mangenese(III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP), prevents apoptosis in an *in vitro* model of ATP depletion-recovery [4] and in a rat model of renal IRI [5]. Recently, it has been shown that MnTMPyP is also effective in attenuating the increase in alpha smooth muscle actin and kidney fibrosis following chronic renal IRI in mice [6].

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However, the protective effects of MnTMPyP at the early stages of reperfusion and the mechanisms involved have not been clearly elucidated.

There are a few studies that have examined the role of mitochondria and mitochondrial function in IRI and other disease pathologies. For example, in a model of hepatic IR, there is oxidative inactivation of key mitochondrial proteins and subsequent injury which MnTMPyP is able to attenuate [7]. Another SOD mimetic, MnTM-2-PyP(5+), prevented NADP+ isocitrate dehydrogenase inactivation in diabetic rats [8]. It was also shown recently that pulsatile shear stress of human aortic endothelial cells increased mitochondrial membrane potential (DeltaPsi(m) partly by MnSOD up-regulation [9]. In substrate deprived glial cells, MnTMPyP blocks the depolarization of mitochondrial membrane potential and in attenuating increased cell death [10]. Despite these studies, however, it is not clear whether MnTMPyP can prevent mitochondrial dysfunction following renal IRI. The current study was thus undertaken to determine the time-dependant effects of MnTMPyP on mitochondrial function and apoptosis following *in vivo* renal IRI. EPR is a specific and direct technique to investigate the active centres of mitochondrial proteins viz., hemes and Fe-S clusters. In our earlier study, we have demonstrated that *ex vivo* EPR spectroscopy could be used to study the changes in the metal centres of mitochondrial proteins in cardiac tissues during doxorubicin (DOX)-induced cardiomyopathy and the mechanism of inhibition of cytochrome *c* oxidase inactivation by mito-Q [11]. Here, EPR is used to characterize the metal active centres viz., hemes and Fe-S clusters in renal tissues, their changes due to IRI, and treatment with MnTMPyP at different time points to understand the mechanism of IRI.



## Methods

### *Animal model of renal IR injury*

Male Sprague Dawley (SD) rats (175–220 g) were purchased from Harlan (Madison, WI, USA). All experiments were approved by the Animal Care and Use committee at the Medical College of Wisconsin. The animals were anaesthetized with sodium pentobarbital (50 mg/Kg i.p.). Renal ischemia-reperfusion was induced by bilateral clamping of the renal arteries for 45 min followed by revascularization for 1, 4 or 24 h. A sub-set of animals was treated with TMPyP (Sigma, St. Louis, MO, USA) (5 mg/kg, i.p. 30 min prior to surgery), MnTMPyP (Alexis Corporation, San Diego, CA, USA) (5 mg/kg, i.p, 30 min prior to surgery) or saline vehicle. For the 24-h time point animals, an additional dose of TMPyP or MnTMPyP was given after 6 h of reperfusion in order to account for metabolic loss of the compounds. We used i.p. injection to administer MnTMPyP to the rats, so as to be able to compare this study with our previous report in which we had shown that MnTMPyP protects against acute tubular necrosis and apoptosis following renal IRI [5]. Sham controls underwent surgery without renal artery clamping. Animals were sacrificed at the end of 1, 4 or 24 h, kidneys isolated and quick frozen in liquid nitrogen and stored at –80°C until further analysis.

### *Electrochemical measurements*

Measurements were performed on a CH Instruments Model 2000 Voltametric analyser. A three-electrode system in a small volume cell (0.5–2 mL) was utilized with a Platinum (Pt) wire (0.5 mm) working electrode, an Ag/AgCl reference and a Pt wire as auxiliary electrode.

### *Electrochemical deposition of MnTMPyP*

Mn(III)TMPyP entrapped polypyrrole (PPy) films on Pt electrode were prepared by a two-step procedure. First PPy films were polymerized on Pt electrode according to the published report [12]. The irreversible oxidation of 0.4 M pyrrole in 0.1 M KCl occurred at ~ +700 mV, giving a polymer structure in the potential range of 0–900 mV vs Ag/AgCl electrode under argon atmosphere. Then, Mn(III)TMPyP (0.5 mM) were electrochemically deposited on PPy-Pt electrode by applying the potential from 0 mV and +1200 mV vs Ag/AgCl electrode in the presence of 0.1 M KCl as supporting electrolyte. The morphologies of the MnTMPyP in PPy matrix was characterized by scanning electron microscopy (data not shown).

### *EPR measurements*

The X-band EPR of kidney tissues were recorded at liquid helium temperatures on a Bruker E500 ELEXYS spectrometer with 100 kHz field modulation, equipped with an Oxford Instrument ESR-9 helium flow cryostat and a DM-0101 cavity. For EPR measurements, kidney tissues were excised and immersed in liquid nitrogen, minced to appropriate sizes under liquid nitrogen and then transferred to a 4 mm quartz EPR tube (Wilmad-Lab Glass, Buena, NJ). The kidney tissue weight in each EPR experiment was ~ 0.25 g. The volume of the tissue sample in the EPR tube was always greater than the working zone of the cavity in each EPR experiment. The only variable was the packing factor (i.e. filling of EPR tubes with tissues). However, this tissue packing factor was not perceived to be a major problem, as reloading of tissues in the same EPR tube (with a different packing) resulted in EPR spectra with a 5% variation in signal intensity. Spectrometer conditions were as follows: microwave frequency, 9.635 GHz; modulation frequency, 100 kHz; modulation amplitude, 10 G; receiver gain, 85 dB;

time constant, 0.01 s; conversion time, 0.08 s; sweep time, 83.9 s. EPR spectra were obtained over the temperature range 4–50 K using an incident microwave power of 5 mW and a modulation amplitude of 10 G. The spectrometer was calibrated with the radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) exhibiting an EPR signal centred at $g=2.0036$.

### *Mitochondrial isolation*

Kidney tissues from 1, 4 and 24 h IRI animals were gently homogenized in ice-cold mitochondrial isolation buffer (250 mM sucrose, 20 mM HEPES, pH 7.9, 10 mM KCl, 1.0 mM EDTA, 1.5 mM $MgCl_2$) with protease inhibitors (Roche, mini cocktail tablets consisting of broad spectrum serine, cysteine and metalloprotease inhibitors such as aprotinin, bestatin, calpain inhibitor, leupeptin, pepstatin, PMSF and trypsin inhibitor) until the liquid was uniform and then centrifuged for 40 min at 750 g at 4°C. The supernatant was centrifuged at 10 000 g for 15 min at 4°C to separate cytosolic and mitochondrial fractions. The cytosol was collected in a separate tube while the mitochondrial pellet was washed three-times with mitochondrial isolation buffer. The mitochondrial pellet was resuspended in RIPA buffer (Cell Signaling, Danvers, MA, USA) consisting of the following : 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% NP-40;1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM $Na_3VO_4$ and 1 μg/ml leupeptin and stored at -80°C until assay. Protein concentrations in the cytosolic and mitochondrial fractions were measured by the Bio-Rad protein assay.

### *Western blotting*

Thirty micrograms of mitochondrial and cytosol fraction from sham and experimental tissue were electrophoresed on 10%, 15% or 4–20% criterion gels (dependent the protein size) and transferred to polyvinylidene fluoride (PVDF) membrane. After blocking for 1 h, the blots were incubated with one of the following primary antibodies: cytochrome *c* (BD biosciences, San Jose, CA, 1:1000); Smac/Diablo (AbCam, Cambridge, MA, 1:1000); OxPhos cocktail (MitoSciences Eugene, OR, 1:1000) overnight at 4°C. After washing, the membranes were probed with secondary conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) antibodies at 1:10 000 for 1 h at room temperature. The blots were visualized with ECL-Plus and exposed to film.

### *SOD activity*

The amount of SOD activity from 1, 4 or 24 h kidney homogenates was measured with a commercially available SOD assay kit (Cayman Chemical, Ann Arbor, MI). This kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Absorbance was read at 450 nm according to manufacturer's instructions and expressed as units per milligram of protein.

### *Aconitase activity*

Aconitase activity from 1, 4 or 24 h kidney homogenates was measured with a commercially available aconitase assay kit (Cayman Chemical, Ann Arbor, MI). This assay utilizes the coupled enzymatic reactions converting citrate to isocitrate and isocitrate to alpha-ketoglutarate as the basis for quantifying the enzyme activity. Absorbance was monitored once every minute at 340 nm for 15 min at 37°C according to manufacturer's instructions and expressed as nmol/min/mg of protein.

### *Caspase-3 activity*

Caspase-3 activities in 1, 4 or 24 h kidney tissue homogenates were determined by using commercially available colourimetric kits (Chemicon International, Temecula, CA). This kit utilizes the detection of the chromophore p-nitroaniline (pNA), which was measured spectrophotometrically at 405 nm, after cleavage from labelled substrate N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA). Released pNA was quantified by generating a pNA standard curve, with caspase activity expressed as micromolar per minute per milligram protein. Caspase-3 activity was expressed as micromolar per minute per milligram of protein.

### *TUNEL Assay for apoptosis*

Apoptosis was measured by the TUNEL assay using ApopTag® technology (Chemicon International, Temecula CA) as per manufacturer's instructions. A minimum of five sections per slide and four slides per group were counted by this method.

## Results

### *Superoxide dismutase and catalase-like activities of MnTMPyP*

Figure 1 shows the cyclic voltammogram (CV) of MnTMPyP deposited in PPy-Pt electrode in 0.1 M phosphate buffer solution (pH 7.4). Redox peaks characteristic of MnTMPyP was observed at –0.12 V and –0.38 V, respectively, indicating the MnTMPyP electrodeposited in PPy matrix. Upon $KO_2$ addition, generating superoxide anion radicals ($O_2^{\bullet-}$) the anodic oxidation peaks at nearly 0.0 V and cathodic reduction peaks at –0.43 V increased significantly, exhibiting

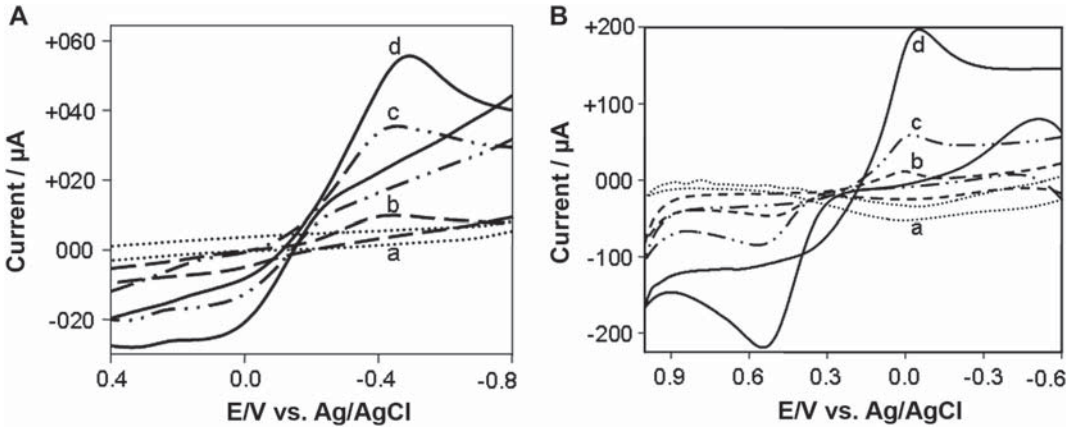


Figure 1. (A) Typical CVs of MnTMPyP-PPy-Pt electrode in (a) PBS buffer plus (b) 25 µM $KO_2$, (c) 250 µM $KO_2$ and (d) 1000 µM $KO_2$ containing 100 µM DTPA at a scan rate of 50 mV/s. (B) CVs obtained at PPy-Pt electrode in (a) PBS buffer plus (b) 25 µM $H_2O_2$, (c) 100 µM $H_2O_2$ and (d) 500 µM $H_2O_2$ containing 0.5 mM MnTMPyP, 25 mM $HCO_3^-$ and 100 µM DTPA at a scan rate of 50 mV/s.



superoxide dismutase activity. The bifunctional electrocatalytic activity of MnTMPyP-modified electrode is attributable to the dismutation of $O_2^{\bullet-}$ to $O_2$ and $H_2O_2$ via a cyclic redox reaction of its Mn(II/III) complex moiety. The following mechanism may explain the enhanced anodic oxidation current observed in Figure 1A.

$$\text{MnTMPyP (II)} - e^- \rightarrow \text{MnTMPyP (III)} \quad (1)$$

$$\text{MnTMPyP (III)} + O_2^{\bullet-} \rightarrow \text{MnTMPyP (II)} + O_2 \quad (2)$$

Similarly, the reduction current was enhanced in the cathodic scan according to the reactions

$$\text{MnTMPyP (III)} + e^- \rightarrow \text{MnTMPyP (II)} \quad (3)$$

$$\text{MnTMPyP (II)} + 2H^+ + O_2^{\bullet-} \rightarrow \text{MnTMPyP (III)} + H_2O_2 \quad (4)$$

The electrochemical response of the MnTMPyP on PPy-Pt in 0.1 M phosphate buffer solution containing 25 mM $HCO_3^-$ is shown in Figure 1B. Increase in $H_2O_2$ concentrations showed remarkable enhancement in the anodic current at +0.55 V and cathodic current at –0.05 V. These data suggest that the MnTMPyP catalyses the disproportionation of $H_2O_2$ at physiological pH with catalase-like activity.

### *Effect of renal IR injury on the EPR spectra of kidney tissues*

The *ex vivo* EPR spectrum of normal kidney tissue is shown in Figure 2 (upper panel shows the EPR trace and lower panel is the bar graph for heme proteins and 3Fe-4S clusters). EPR is used here to monitor the oxidative stress from the changes in the paramagnetic metal active centres of the mitochondrial proteins during the renal ischemia-reperfusion. The EPR of mitochondria rich kidney tissues was analysed by computer simulation [11] to exhibit signals due to the high ($g=6.0$) and low ($g=3.0$) spin hemes and a trinuclear 3Fe-4S ($g=2.015$), tetranuclear, 4Fe4S ($g=1.98$) and binuclear, 2Fe2S ($g=1.94$) clusters. Further, the iron-sulphur cluster signals were characterized by the temperature dependence EPR. For instance, the signal at $g=2.015$ is typical of a 3FeS4 cluster exhibiting high EPR signal intensity at 10 K and diminished to base level at ~ 35 K. The EPR spectra of ischemia followed by reperfusion for 1 h or 4 h showed a time-dependent increase in the high spin heme $g=6$ signal and also oxidation of 4Fe4S to 3Fe4S signal at $g=2.015$ (Figure 2). MnTMPyP attenuated the changes in these signals at the earlier time points. Following the 24-h reperfusion time point, there was a partial recovery of the 3Fe-4S signal in ischemic kidneys, which was attenuated by MnTMPyP. The high spin heme protein signal was still increased following 24 h of reperfusion and MnTMPyP was unable to attenuate this.

### *IR-induced loss of aconitase activity is restored by MnTMPyP at the early phase of reperfusion*

Since MnTMPyP decreased the oxidation of 4Fe4S signal to 3Fe4S signals, we next determined whether the activity of aconitase, an important Fe-S protein in the mitochondria, was decreased following renal IR injury. As shown in Figure 3, there was a significant loss of aconitase activity at the very early phases of reperfusion (1 h) and this was restored by 24 h of reperfusion. Treatment with MnTMPyP prevented the loss of aconitase activity at 1 h of reperfusion, although it did not have a profound effect on aconitase activity following 4 h of reperfusion (Figure 3). At the 24-h time point, aconitase activity was restored in all groups.

### *Effect of MnTMPyP on SOD activity following renal IRI*

To further determine whether mitochondrial antioxidant proteins were also inactivated by renal IRI, we

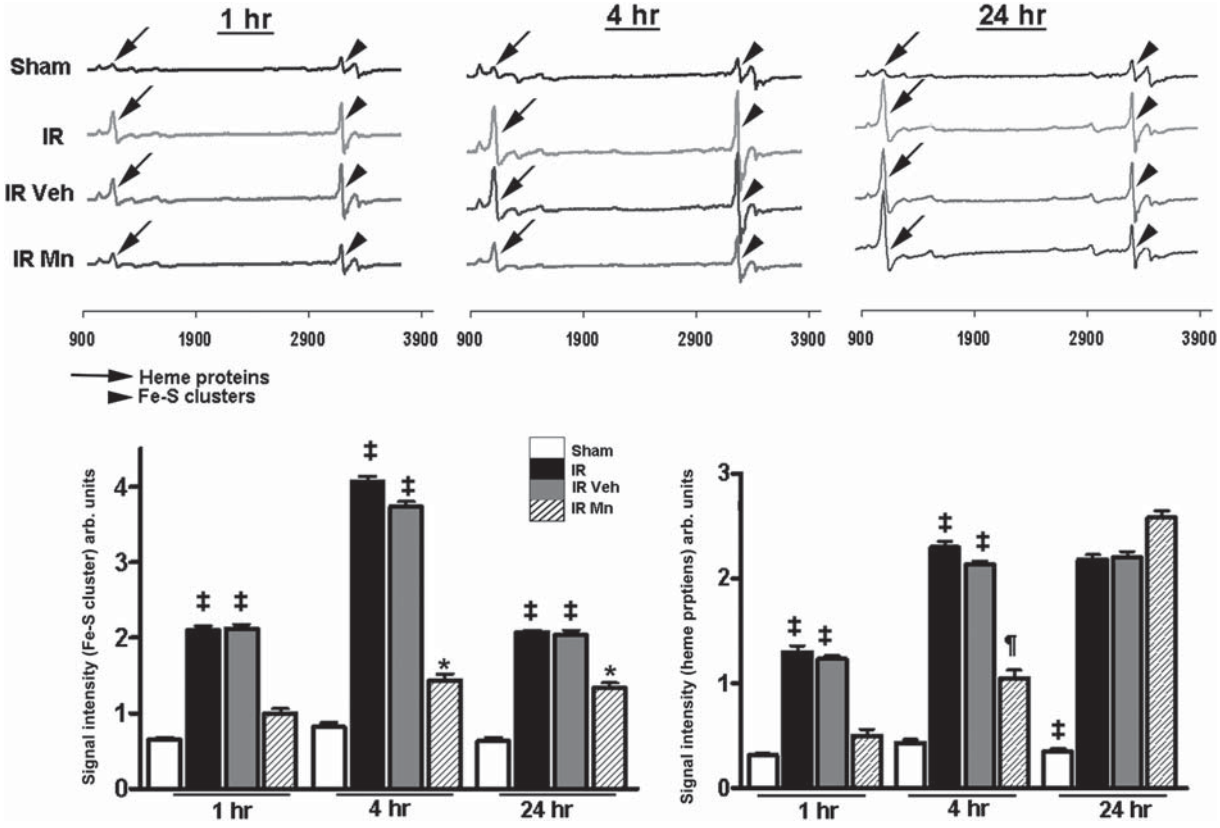


Figure 2. (Top) Time course X-band EPR spectra of renal tissues isolated from sham, IR, IR Veh and IR Mn treated rats at 1 h, 4 h and 24 h of reperfusion. EPR were performed at 10 K using a 5 mW microwave power. (Bottom) EPR signal intensities of heme signal at $g=6.0$ and 3Fe-4S cluster signal at $g=2.015$ for the above. Values are given as mean ± SE. $^{*}p < 0.05$ vs controls. $^{‡}p<0.001$, $^{¶}p<0.01$.

evaluated SOD activity in sham, IR, IR vehicle and IR Mn treated groups. Although there was no effect of renal IRI on SOD activity at either 1 h or 4 h of reperfusion, SOD activity was significantly decreased following 24 h of reperfusion (Figure 4. MnTMPyP did not significantly alter SOD activity at any time point (Figure 4).

### *MnTMPyP prevents loss of mitochondrial complex (I–IV) at the early time points of renal IRI*

We performed Western Blot analysis of mitochondrial complex proteins (I–IV) using an ox-phos antibody cocktail in all groups. There was a significant loss of complex II and IV and a more moderate decrease in complex III protein levels after 1 h of reperfusion (Figure 5). By 4 h of reperfusion, there was restoration of complexes I, II and IV, but levels of complex III were still lower in rats that underwent renal IRI. Interestingly, the protein levels of complex IV displayed a biphasic pattern, with initial decrease at 1 h of reperfusion and a secondary loss at 24 h of reperfusion (Figure 5). MnTMPyP prevented the early loss of all of the mitochondrial complex proteins at 1 h of reperfusion with the most profound effect on complex II (Figure 5). However, MnTMPyP was unable to prevent the loss of complex IV following 24 h of reperfusion.

### *Effect of MnTMPyP on cytochrome c and Smac/Diablo levels in mitochondria following renal IRI*

Since release of pro-apoptotic proteins such as cytochrome *c* and Smac/Diablo from the mitochondria to the cytosol is critical to triggering apoptosis, we next determined if IRI decreased cytochrome *c* and Smac/Diablo levels in mitochondria and if MnTMPyP had any effect. There was no change in mitochondrial cytochrome *c* levels at 1 h of reperfusion, but by 4 h of reperfusion there was a decrease in mitochondrial

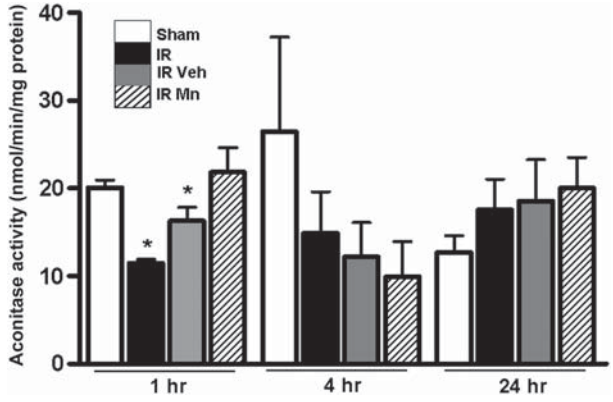


Figure 3. Aconitase activity was measured in kidney homogenates from sham, IR, IR Veh and IR Mn treated rats at 1 h, 4 h and 24 h of reperfusion. Results are means ± SEM. $n=3$–4 per group. $^{*}p < 0.05$ IR and IR Veh vs sham and IR Mn.

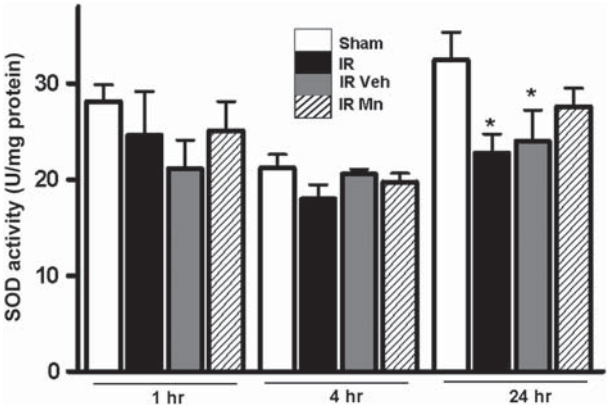


Figure 4. SOD activity in sham, IR, IR Veh and IR Mn rats following 1 h, 4 h and 24 h reperfusion. Results are means ± SEM. $n = 3$–5 per group. $^{*}p < 0.05$ IR and IR Veh vs sham and IR Mn.

cytochrome *c* protein levels (Figure 6A) and MnTMPyP prevented the loss of cytochrome *c* (Figure 6A). Interestingly, by 24 h of reperfusion, the mitochondrial decrease of cytochrome *c* was partially restored in the IR and IR Mn treated animals (Figure 6A). Smac/Diablo protein levels in the mitochondria displayed a pattern similar to cytochrome *c*. At the very early stages of reperfusion (1 h) there was a tendency for Smac/Diablo levels to be decreased (Figure 6B, top panel) and MnTMPyP partially restored this. Following 4 h of reperfusion, there was a more profound loss of Smac/Diablo from the mitochondria, with MnTMPyP almost completely restoring the protein levels back to levels seen in sham animals (Figure 6B, bottom panel).

### *MnTMPyP partially attenuates apoptosis at the late phase of renal IRI*

Caspase-3 activation was evaluated in sham, IR, IR Vehicle and IR MnTMPyP treated rats. Caspase-3 was activated beginning at 4 h of reperfusion and remained high until the 24 h time point (Figure 7A). MnTMPyP decreased caspase-3 activation only following 24 h of reperfusion (Figure 7A).

We next determined the effect of MnTMPyP on apoptosis following the different time points of renal IRI. There was a tendency for apoptotic nuclei to be increased at the very early phase of reperfusion, however this increase was not statistically significant (Figure 7B). Similar to our earlier report [5], by the late reperfusion time point (24 h), there was a dramatic increase in number of apoptotic nuclei in both IR and IR vehicle treated animals compared to sham operated rats (Figure 7B), which was attenuated by MnTMPyP.

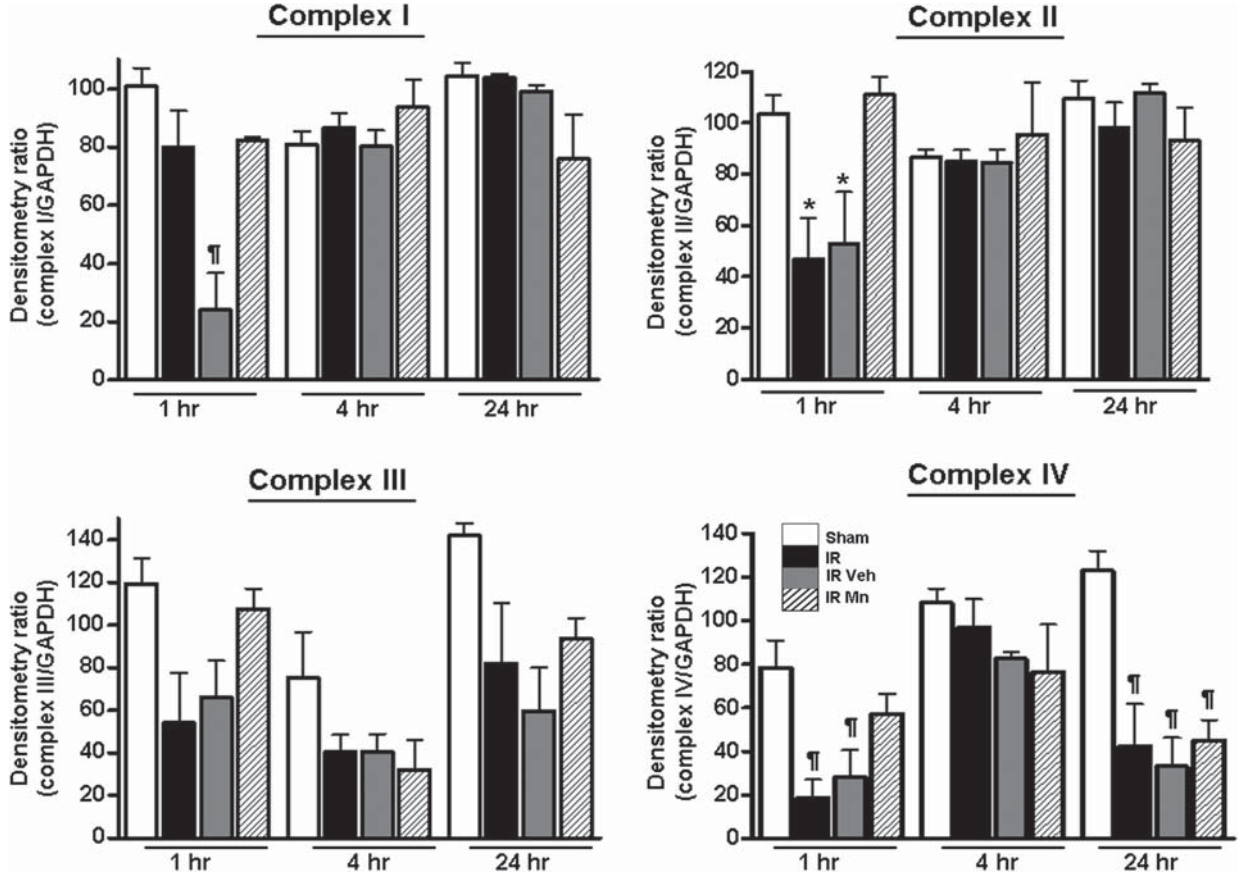


Figure 5. Bar graphs showing densitometry ratio of complex/GAPDH in kidney homogenates from sham, IR, IR Veh and IR Mn treated rats at 1 h, 4 h and 24 h of reperfusion. $n = 3$ per group. $^{*}p < 0.05$ IR and IR Veh vs sham and IR Mn, $^{¶}p < 0.01$ IR Veh vs sham, IR and IR Mn (complex I), $^{¶}p < 0.01$ IR and IR Veh vs sham (complex IV).

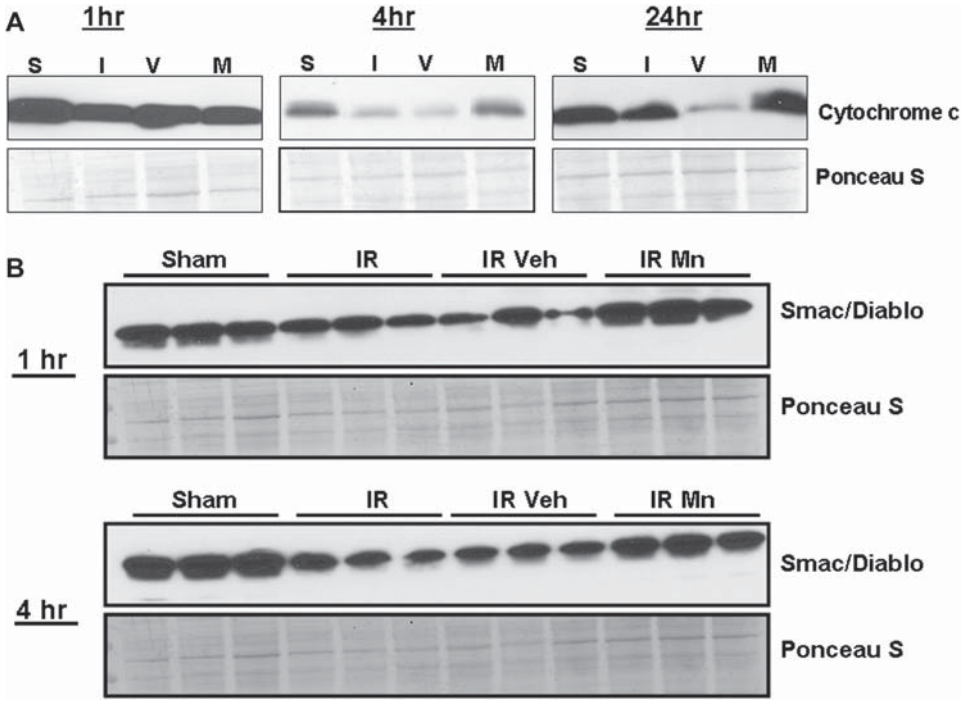


Figure 6. (A) Western Blot showing levels of cytochrome *c* in mitochondrial fractions isolated from kidneys from sham, IR, IR Veh and IR Mn animals at all time points. The blot is normalized to Ponceau S. Cytochrome *c* was decreased in mitochondrial fractions only at the 4-h time point and this was prevented by MnTMPyP. (B) Western Blot showing decreased levels of Smac/Diablo in mitochondrial fractions IR and IR Veh treated animals following the early stages of reperfusion (1 h and 4 h). MnTMPyP partially prevented the loss of Smac/Diablo at these time points of reperfusion.



To determine whether the protective effects of MnTMPyP were due to Mn or it's ligand, we treated a sub-set of animals with TMPyP and subjected them to renal IR injury. There was an expected increase in serum creatinine in both IR and IR vehicle treated animals at the 24-h reperfusion time point compared to sham (sham: $1.07 \pm 0.055$ mg/dL; IR: $3.45 \pm 0.56$ mg/dL; IR Veh: $3.93 \pm 0.17$ mg/dL, $n = 5$–6; $p < 0.01$ IR and IR Veh vs sham). Treatment with MnTMPyP significantly attenuated the IR-mediated increase in serum creatinine (IR Mn $1.88 \pm 0.23$ mg/dL; $n = 6$; $p < 0.01$ vs IR and IR Veh). TmPyP treatment had only a very slight tendency to decrease serum creatinine levels compared to IR and IR Veh, but this was not statistically significant ($n = 3$; $2.72 \pm 0.24$ mg/dL). Further, TMPyP was unable to dismutate superoxide as measured by the electrochemical method and *in vivo* as measured by DHE fluorescence (data not shown), indicating that this compound acts by a different mechanism than MnTMPyP.

## Discussion

In our study, we have shown that the cell permeable SOD/catalase mimetic, MnTMPyP, is effective in attenuating mitochondrial dysfunction and mitochondrial release of pro-apoptotic proteins in the early phase of reperfusion and prevents apoptosis in the late phases of reperfusion in renal IRI. Although there is evidence that MnTMPyP prevents the oxidative modification of mitochondrial proteins and depolarization of mitochondrial membrane potential in hepatic and brain IRI [7,10], there have been relatively few studies examining the role of MnTMPyP in renal IRI [5] and this is the first report to show the mitochondrial protective effects of MnTMPyP in the ischemic kidney.

Electrochemical measurements using MnTMPyP indicated that this compound exhibited both superoxide dismutation and catalase-like properties, indicating that the protective effects of MnTMPyP are due to scavenging of both superoxide and $H_2O_2$. This result is in agreement with other reports showing that MnTMPyP is protective against $H_2O_2$ mediated injury in rat astroglial cells [13] and can restore catalase levels following global cerebral ischemia [14]. *Ex vivo* EPR spectra of kidney tissues from the various time points of reperfusion (1 h, 4 h and 24 h) showed an increase in both the cytochrome *c*/heme $a_3$ signal of complex IV and 3Fe-3S clusters in IR and IR vehicle treated animals compared to sham and IR MnTMPyP treated animals. The heme $a_3$/cytochrome *c* signal perhaps is due to its oxidation and cytochrome *c* release into membrane and the disassembly of 4Fe4S to 3Fe4S cluster during IR is indicative of oxidative stress. MnTMPyP was effective in attenuating the early increases in cytochrome *c*/heme $a_3$, but was ineffective in decreasing the signal intensity at the 24 h reperfusion time point. This result was not entirely surprising to us because cytochrome *c* and Smac/Diablo release from

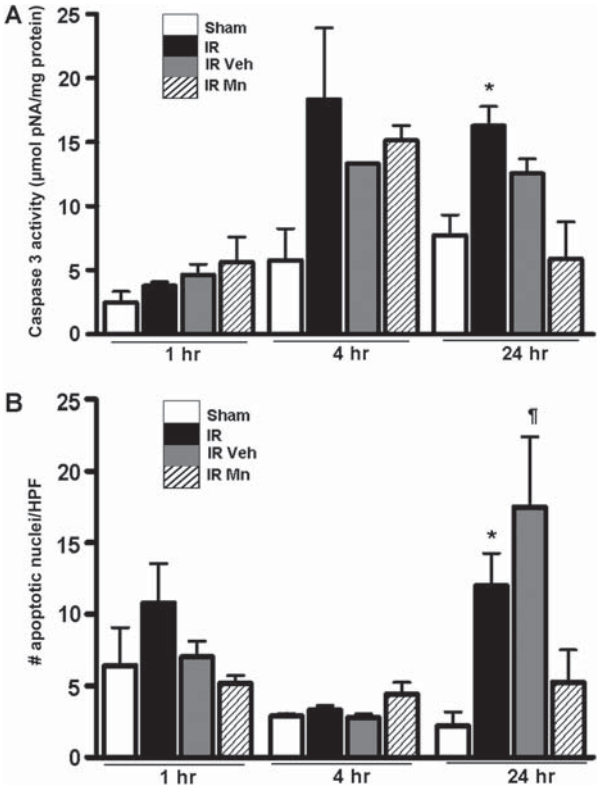


Figure 7. (A) Caspase-3 activity in sham, IR, IR Veh and IR Mn kidney homogenates at 1 h, 4 h and 24 h of reperfusion. Results are means ± SEM. $n = 3$–6 per group. $^{*}p < 0.05$ vs sham and IR Mn. (B) Number of apoptotic nuclei/high power field (HPF) as evaluated by TUNEL staining. Results are ± SEM. $n = 3$–5 per group. $^{*}p < 0.01$ vs sham and IR Mn, $^{¶}p < 0.01$ vs sham and IR Mn.

the mitochondria was also maximal at the early stage of reperfusion (4 h) and was significantly attenuated by MnTMPyP at this time point. Cytochrome *c* has been reported to be nitrated in a model of chronic allograft nephropathy [15] and it is likely that it is a target of nitration in our model of injury also.

Corresponding with the increase in oxidation of 4Fe-4S clusters by EPR, we found that aconitase activity (a mitochondrial Fe-S protein) was decreased in the early time points of IRI. Other investigators have shown a similar loss of aconitase in cardiac models of ischemia-reperfusion [16,17], presumably by oxidative inactivation. In the kidney, although there are no studies examining the effect of IRI on aconitase activity, there are some reports showing inactivation of aconitase following cisplatin administration in LLC-$Pk_1$ cells [18] and the administration of the renal toxicant, S-(1,1,2,2,-tetrafluoroethyl)-L-cysteine in kidney homogenates [19]. Additionally, aconitase is the main target in the kidneys of ageing mice [20]. The aconitase activity was somewhat restored at the later stages of reperfusion in our studies, suggesting that repair mechanisms may be activated by this phase. Our study is the first to show the temporal patterns of aconitase inactivation and the effects of SOD mimetics in a model of renal IRI. The restoration of aconitase activity by MnTMPyP following 1 h of reperfusion correlates with other investigations showing that other SOD mimetics such as MnTE2PyP and the hydroxyl scavenger, DMTU, can prevent oxidation and inactivation of aconitase in brain IRI and in cisplatin-induced nephrotoxicity [21,22].

Based on the data we got, the exact reason for higher aconitase activity at 24 h of reperfusion is not clear, but may be explained by the compensatory elevation in mitochondrial aconitase or a repair mechanism that restores aconitase activity. A similar compensatory elevation in m-aconitase was reported in MPTP-treated mice [23].

Another reason for the non-correlation between EPR analysis and the aconitase activity is that EPR looks at the Fe-S active centres but not exactly the activity. It is also important to note that the increase in 3Fe4S cluster EPR signal is also contributed by several other Fe-S cluster proteins (other than aconitase) such as Complex I and Complex II which may be still oxidized at 24 h of reperfusion. Ultimately, the low-temperature EPR may be the best method to monitor disassembly of iron-sulphur clusters, although evaluation of aconitase activity may give information about whether mitochondrial dysfunction in the early stages of reperfusion is repaired by the later phases.

Investigators have previously shown that brief periods of cardiac ischemia can increase superoxide levels and surprisingly increase complex I activity, whereas longer durations of ischemia results in loss of complex I and IV activities [24]. Racay et al. [25] recently have also shown that complex IV activity was inhibited by 80% within 1 h of reperfusion, with a corresponding increase in mitochondrial calcium levels. In our study, surprisingly, there was a loss of complex I in IR vehicle treated rats but not in the IR animals after 1 h reperfusion. The exact reasons for this discrepancy are not clear, but it could be related to increased susceptibility to mitochondrial injury in the vehicle-treated animals compared to IR alone. It should also be noted that in the kidney, the effect of hypoxia reoxygenation on mitochondrial complex I activity is controversial, with one study showing a preservation of complex I following hypoxia-reoxygenation of proximal tubular epithelial cells [26] and another study indicating energy deficits in hypoxia-reoxygenation due to complex I inactivation prior to mitochondrial membrane permeability and loss of cytochrome *c*. Nevertheless, even the loss of complex I in IR-treated animals was transient as the complex I protein levels were completely restored by 4 h of reperfusion. In an *in vivo* model, Sammut et al. [27] have shown that complexes I and IV are inactivated after 24 or 48 h of cold ischemia. These studies agree well with our results, showing a loss of complexes II, III and IV in early reperfusion stages. Interestingly, there was a secondary loss of complex IV at the 24 h time point. Although it is not yet clear what the biphasic pattern

of complex IV levels is due to, it is possible that post-translational modifications such as oxidation or nitration could be responsible for this secondary loss of the proteins. In fact, the 24 and 30 kDa sub-units of complex I, complex III and the alpha sub-unit of ATP synthase have all been shown to be nitrated following cardiac ischemia-reperfusion injury [28].

Apoptosis is mediated by two signalling pathways, extrinsic or Fas/Fas mediated cell death that is triggered by cell surface death receptors and the intrinsic or mitochondrial pathway which involves the release of pro-apoptotic proteins such as cytochrome *c* and Smac/Diablo. In this study, we found that levels of both cytochrome *c* and Smac/Diablo were decreased in the mitochondrial fractions following 4 h of reperfusion and MnTMPyP prevented this loss at both time points. This concurs well with our previous report that MnTMPyP is also able to prevent mitochondrial release of cytochrome *c* in the early phase of ATP depletion-recovery in a cell culture model of renal IRI [4]. It is likely that cytochrome *c* is also targeted for nitration [15] and possible proteasomal degradation. Interestingly, caspase-3 was activated beginning at 4 h of reperfusion, although MnTMPyP had a preventive effect on caspase-3 activation only at the late phase of reperfusion (24 h). Previously, we had shown that oxidative stress and apoptosis was increased at the 24-h time point of reperfusion and MnTMPyP was effective in attenuating both of these indices [5]. Not surprisingly, there was no activation of apoptosis at the early stages of reperfusion (1 h or 4 h), indicating that IRI has biphasic effects in renal IRI.

In conclusion, these data show that renal IRI has dramatic effects on mitochondrial function and apoptosis at different time points of reperfusion and that MnTMPyP protects against renal IR injury by different mechanisms depending on reperfusion time. At the early phases of reperfusion, MnTMPyP has a profound effect on inhibiting mitochondrial dysfunction and following longer durations of reperfusion MnTMPyP prevents caspase-3 activation and apoptosis. Future studies using mitochondria targeted antioxidants or over-expression of mitochondrial antioxidant proteins (e.g. MnSOD) to prevent mitochondrial dysfunction in renal IRI are warranted.

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