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Mn porphyrin-based SOD mimic, MnTnHex-2-PyP⁵⁺, and non-SOD mimic, MnTBAP³⁻, suppressed rat spinal cord ischemia/reperfusion injury via NF-κB pathways

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Abstract

Herein we have demonstrated that both superoxide dismutase (SOD) mimic, cationic Mn(III) *meso*-tetrakis(*N*-n-hexylpyridinium-2-yl) porphyrin (MnTnHex-2-PyP⁵⁺), and non-SOD mimic, anionic Mn(III) *meso*-tetrakis(4-carboxylatophenyl)porphyrin (MnTBAP³⁻), protect against oxidative stress caused by spinal cord ischemia/reperfusion via suppression of nuclear factor kappa B (NF-κB) pro-inflammatory pathways. Earlier reports showed that Mn(III) *N*-alkylpyridylporphyrins were able to prevent the DNA binding of NF-κB in an aqueous system, whereas MnTBAP³⁻ was not. Here, for the first time, in a complex *in vivo* system—animal model of spinal cord injury—a similar impact of MnTBAP³⁻, at a dose identical to that of MnTnHex-2-PyP⁵⁺, was demonstrated in NF-κB downregulation. Rats were treated subcutaneously at 1.5 mg/kg starting at 30 min before ischemia/reperfusion, and then every 12 h afterward for either 48 h or 7 days. The anti-inflammatory effects of both Mn porphyrins (MnPs) were demonstrated in the spinal cord tissue at both 48 h and 7 days. The down-regulation of NF-κB, a major pro-inflammatory signaling protein regulating astrocyte activation, was detected and found to correlate well with the suppression of astrogliosis (as glial fibrillary acidic protein) by both MnPs. The markers of oxidative stress, lipid peroxidation and protein carbonyl formation, were significantly reduced by MnPs. The favorable impact of both MnPs on motor neurons (Tarlov score and inclined plane test) was assessed. No major changes in glutathione peroxidase- and SOD-like

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Declaration of interest

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activities were demonstrated, which implies that none of the MnPs acted as SOD mimic. Increasing amount of data on the reactivity of MnTBAP³⁻ with reactive nitrogen species (RNS) (NO/HNO/ONOO⁻) suggests that RNS/MnTBAP³⁻-driven modification of NF-κB protein cysteines may be involved in its therapeutic effects. This differs from the therapeutic efficacy of MnTnHex-2-PyP⁵⁺ which presumably occurs *via* reactive oxygen species and relates to NF-κB thiol oxidation; the role of RNS cannot be excluded.

Keywords

superoxide dismutase mimetics; ischemia reperfusion; MnTnHex-2-PyP⁵⁺; MnTBAP³⁻; oxidative stress; NF-kappa B

Introduction

Spinal cord injury

The spinal cord is extremely vulnerable to ischemic injury. The descending thoracic or thoracoabdominal aortic aneurysm surgery may cause spinal cord ischemia because of aortic cross-clamping, and can lead to severe neurologic complications including paraplegia and paraparesis [1,2]. The incidence of paraplegia and paraparesis still ranges from 5 to 35% [2]. Several strategies have been used clinically and experimentally to prevent paraplegia due to the spinal cord ischemia, and have included the use of shunts, systemic hypothermia, spinal fluid drainage, and infusion of scavengers of reactive species [3,4]. However, none of these have thus far been successful in preventing this unpredictable complication.

Cross-clamping of abdominal aorta for extended intervals of 30–60 min leads to low flow state and results, therefore, in irreversible spinal cord neuronal damage and loss of neurologic function. Such scenario is consistent with the anatomy of the spinal vasculature. The spinal cord receives a major portion of nutritive perfusion from the segmental radicular arteries arising from the descending aorta and from the largest segmental medullary artery—known as “artery of Adamkiewicz.” This artery provides a significant flow into the anterior spinal artery at the lumbosacral enlargement of the spinal cord. The nerves which supply the lower limbs originate from the lumbar enlargement. The magnitude of the insult to spinal function will be affected by the factors that impact the perfusion through the collateral circulation. The period of ischemia is followed by reperfusion when the major oxidative damage occurs. The reactive oxygen and nitrogen species produced during reperfusion induce lipid peroxidation and neuronal cell death in the spinal cord. These species also induce upregulation of pro-inflammatory pathways, including nuclear factor kappa B (NF-κB), which amplify primary oxidative stress and thus contribute to the ischemic injury. Prevention of oxidative stress-induced damage has thus the potential to reduce the spinal cord ischemia/reperfusion (I/R) injury [5,6].

Therapeutic effects of Mn(III) porphyrins in central nervous system injuries

Mn porphyrin (MnP)-based superoxide dismutase (SOD) mimics exhibit remarkable therapeutic effects in diseases which have oxidative stress in common, including injuries of central nervous system (CNS). Several recent reviews have summarized those effects [7–

14]. While the favorable effects of cationic *N*-substituted pyridylporphyrins, such as Mn(III) *meso*-tetrakis(*N*-n-hexylpyridinium-2-yl)porphyrin (MnTnHex-2-PyP⁵⁺), are expected, those of anionic Mn(III) *meso*-tetrakis(4-carboxylatophenyl) porphyrin (MnTBAP³⁻) are far from understood. MnTBAP³⁻, anionic Mn porphyrin, has no SOD-like activity; its therapeutic effects have often been erroneously assigned to its SOD-like activity, and in particular mitochondrial MnSOD-like activity [15–36]. Even a redox-inert Zn analog, ZnTBAP⁴⁻, was claimed to have SOD-like activity [34]. In addition, MnTBAP³⁻ has no significant catalase-like activity (Table I) [37]. Cationic MnTnHex-2-PyP⁵⁺, though, is a potent SOD mimic (Table I) [12,38–55]. Here we used a rat spinal cord injury model to test these two MnPs of vastly different thermodynamics and kinetics with regards to reactive oxygen and nitrogen species, with the goal to further our understanding of the origin of their therapeutic efficacy.

Some of the therapeutic effects of MnPs are listed below and in Figure 1. Melov et al. demonstrated the impact of MnTBAP³⁻ on prolonged survival and lower systemic pathology of MnSOD knockout *SOD^{tm1Cje}(–/–)* mice. A prolonged survival was assigned to mimicking mitochondrial MnSOD [56]. Yet mice developed the pathology, including pronounced movement disorder progressing to total debilitation by 3 weeks of age, which was ascribed to the inability of MnTBAP³⁻ to cross blood–brain barrier. Its anionic charge would most likely diminish its ability to cross any other anionic phospholipid-based membranes, including mitochondrial membranes. The Mn aqua/hydroxo/acetato complexes, common impurities in MnTBAP³⁻ preparations [57,58], might have contributed to the brain toxicity related to manganism, due to prolonged treatment of young animals (starting at day 3 of age) at a dose of 5 mg/kg/day [59–61]. Danxia Liu's group has used MnTBAP³⁻ for years in a rodent spinal cord contusion injury model and has assigned its therapeutic efficacy to the scavenging of either ONOO⁻ or broad spectrum of reactive species, including superoxide, H₂O₂, and ·OH radical [62,63]. Aladag et al. discussed the efficacy of MnTBAP³⁻ in rat subarachnoid hemorrhage model with regards to its SOD-like activity [64]. Rarely, the lack of therapeutic effect of MnTBAP³⁻ was reported [31,65,66]. Mn(III) *N,N'*-diethylimidazolylporphyrin, MnTDE-2-ImP⁵⁺ (AEOL10150), was efficacious in a spinal cord compression injury when administered intrathecally as a single injection at 5-min post-reperfusion [67]. The intravenous (*iv*) administration was inefficacious [67]. MnTDE-2-ImP⁵⁺ has thermodynamics and kinetics similar to MnTE-2-PyP⁵⁺, but is bulkier and in turn has different bioavailability. In the same model, MnTE-2-PyP⁵⁺ was protective even when given *iv* as a single dose of 8 mg/kg at 15 min after spinal cord compression. Neurologic assessment at day 21 post-injury (rotarod with fixed and accelerated speed of rotation, coarse and fine screen upside down) demonstrated ~30% reduction in neurologic injury. Histological assessment, performed as reported [67], indicated that vehicle (non-injured) *versus* MnP-treated group scored 28 *versus* 21 (Batinic-Haberle et al., unpublished). In a traumatic spinal cord injury, a Fe(III) porphyrin with pyridylbenzoates in porphyrin *meso* positions (WW-85) suppressed inflammation and recovered limb function; the effects were ascribed to ONOO⁻-related chemistry/biology [68].

MnTnHex-2-PyP⁵⁺ and MnTBAP³⁻ were directly compared only with respect to the aerobic growth of single-cell SOD-deficient organisms, *Escherichia coli* and *Saccharomyces*

cerevisiae: while former was fully efficacious at as low as 0.3–10 μM , MnTBAP^{3-} was not protective at up to 200 μM [51]. No single animal study explored those two compounds. When the data from two separate studies are compared, $\text{MnTnHex-2-PyP}^{5+}$ appears to be 100-fold more efficacious than MnTBAP^{3-} in blocking the development of rat morphine antinociceptive tolerance [36,69]. In another carrageenan-induced rat paw inflammation study, a similar analog, $\text{Mn(III) } N\text{-ethylpyridylporphyrin}$ (MnTE-2-PyP^{5+}), was ~ 1.5 log units more efficacious than MnTBAP^{3-} . The lower efficacy of MnTBAP^{3-} relative to MnTE-2-PyP^{5+} was attributed to ~ 2.5 log units lower ONOO^- reducing ability [69].

Several Mn(III) -substituted *N*-pyridyl- or *N,N'*-diimidazolylporphyrins suppress inflammation caused by I/R insult *via* suppression of $\text{NF-}\kappa\text{B}$ pro-inflammatory pathways [50,93,94]. The master transcription factor, $\text{NF-}\kappa\text{B}$, controls antiapoptotic and anti-inflammatory pathways involved in CNS injuries [95–98]. The data on the involvement of SOD mimics in the oxidation of $\text{NF-}\kappa\text{B}$ thiol *via* $\text{H}_2\text{O}_2/\text{GSH}$ [99,100] and/or $\text{HNO/NO/H}_2\text{S}$ reactions [101–103] made us decide to explore whether $\text{NF-}\kappa\text{B}$ is a common pathway in suppression of rat spinal cord I/R injury by MnTBAP^{3-} and $\text{MnTnHex-2-PyP}^{5+}$.

Materials and methods

A total of 52 female Sprague-Dawley rats weighing between 200 and 250 g were obtained from Rijeka University's Laboratory Animals. Two rats died during this study because of surgical complications. None of them had any neurological disorder prior to surgery. All work described in this study was performed according to the protocols approved by the Ethical Committee of the Faculty of Medicine, University of Rijeka.

Spinal cord injury procedure and study design

Rats were housed two per cage, exposed to a 12-h light/dark cycle, and had a free access to food and water. Animals were anesthetized with intraperitoneal (*ip*) injections of ketamine hydrochloride (0.1 mg/g body weight) and xylazine hydrochloride (0.02 mg/g body weight). The *ip* injection of cephalosporin (10 mg/kg) was administered prior to skin incision. Postoperative analgesia was provided with tramadol for 12 h; 150 U/kg of heparin was administered subcutaneously to all animals immediately before the procedure.

After the surface cleaning of the surgical area, a mid-line incision was made. The abdominal aorta was explored through transperitoneal approach. The intestines were exteriorized and wrapped in wet gauze. I/R injury of the spinal cord was induced by the occlusion of abdominal aorta. Abdominal aorta was clamped for 45 min with a proximal (just below the left renal artery) and a distal (just above the aortic bifurcation) clip as illustrated in Figure 2A. At the end of the surgery, the anterior abdominal wall was sutured using 5/0 polypropylene suture.

The animals were randomly divided into four groups: (1) I/R + $\text{MnTnHex-2-PyP}^{5+}$ group, in which subcutaneous (*sc*) injection of 1.5 mg/kg $\text{MnTnHex-2-PyP}^{5+}$ was given 30 min before I/R, followed by ischemia and reperfusion ($n = 16$); (2) I/R + MnTBAP^{3-} group, in which *sc* injection of 1.5 mg/kg MnTBAP^{3-} was given 30 min before I/R, followed by ischemia and reperfusion ($n = 16$); (3) I/R + vehicle group, in which the aorta was occluded for 45 min

followed by 48 h or 7 days of reperfusion ($n = 16$); (4) control—the sham group, which included animals subjected to anesthesia and laparotomy without ischemia ($n = 4$) and was analyzed at 48 h after surgical procedure. The study design schedule is illustrated in Figure 2B. Drugs were administered 30 min before surgical procedure, timing of which is relevant to thoracic surgeries. After closing the abdominal wall, drug administration continued every 12 h either for duration of 48 h or 7 days.

The first three groups consisted of two subgroups based on time post-reperfusion. One subgroup was sacrificed after 48 h and another subgroup was sacrificed after 7 days post-reperfusion. At either 48-h or 7-day post-reperfusion, half of the animals per subgroup were anesthetized with xylazine hydrochloride and ketamine hydrochloride, perfused transcardially with 0.9% saline and 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4), and sacrificed. The L₃–S₁ segments of the spinal cord were extracted. The tissue was fixed in 4% PFA for 24 h and embedded in paraffin wax for immunofluorescence investigation. Another half of each subgroup was not perfused with PFA. Their spinal cord was immediately immersed in liquid nitrogen and stored at -80°C for spectrophotometry (thiobarbituric acid reactive substances (TBARS), protein carbonyls, and enzymes) and Western blot analyses (protein carbonyls).

Motor assessment of spinal cord injury

Post-ischemic motor dysfunction was assessed every 24 h after reperfusion. A trained observer who was blinded to the group of animals evaluated the hindlimb motor function using Tarlov score and inclined plane test. Tarlov scale evaluated the animals as follows: grade 0, paraplegic with no evident lower extremity motor function; grade 1, poor lower extremity motor function, weak antigravity movement only; grade 2, moderate lower extremity function with good antigravity strength but inability to pull legs under the body; grade 3, able to stand but unable to walk normally; grade 4, minimal hopping; and grade 5 complete recovery and normal gait/hopping. In addition, animals were tested for their ability to stand on an inclined plane while facing upward at angles of 35° , 45° , and 50° angle; scores (0–3) were assigned for each inclination relative to pre-injury performance and then averaged for inclusion into the composite score. A composite neuromotor score (0–8) was generated by combining the scores for each of these tests, with a score of 8 indicating maximal motor function.

Sample preparation

Spinal cord tissue was weighted and homogenized in ice-cold phosphate-buffered saline (pH 7.4). The homogenates were aliquoted (100 μL) and used for lipid peroxidation and protein carbonyl content assessment. Remaining homogenate was centrifuged at 13,000 rpm for 30 min and the obtained supernatant was used as a source for enzyme assay. Supernatant was aliquoted and stored at -20°C to measure SOD and glutathione peroxidase (GPx) activities. All procedures were performed at 4°C .

Lipid peroxidation assay

Lipid peroxidation was determined spectrophotometrically by measuring the levels of TBARS, using a modification of the method described by Ohkawa et al. [104]. Malondialdehyde (MDA) was used as an external standard.

Protein oxidation assay

Protein oxidation was determined using the OxyBlot™ kit, according to the manufacturer's instructions. The method is based on immunochemical detection of protein carbonyl groups derivatized using 2,4-dinitrophenylhydrazine (DNPH). Briefly, spinal cord samples were reacted with DNPH and the samples were electrophoresed and electrotransferred to nitrocellulose membranes. Subsequently, the primary rabbit anti-DNP antibody (1:150) and horse-radish peroxidase-conjugated goat anti-rabbit antibody (1:300) were applied to detect protein carbonyl groups. Membranes were visualized by chemiluminescence using the Pierce SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). The bands were recorded using the Kodak Image Station CF 440 and the densitometric analyses of the proteins of all molecular weights were done using the Kodak 1D 3.5 imaging software (Eastman Kodak, Inc., Rochester, NY, USA).

Enzyme assays

SOD activity was determined using the method that employs xanthine and xanthine oxidase to generate $O_2^{\cdot-}$. The $O_2^{\cdot-}$ was in turn reacted with 2-iodophenyl-3-(4-nitrophenyl)-5-phenyltetrazolium chloride, to form a red formazan dye whose absorbance at 505 nm was detected. One unit of enzyme activity was defined as the quantity of SOD required to cause a 50% inhibition of the absorbance change per minute of the blank reaction. GPx activity was measured according to the Paglia and Valentine method [105]. GPx catalyzes the reduction of cumene hydroperoxide using glutathione as a reducing agent. Oxidized glutathione was reduced with NADPH (β -nicotin-amide adenine dinucleotide phosphate, reduced) in the presence of glutathione reductase, whereby NADPH was oxidized to $NADP^+$. Oxidation of NADPH is monitored as decrease in the absorbance at 340 nm, whose rate was directly proportional to the sample's GPx activity.

Immunofluorescence microscopy

Immunofluorescence staining of glial fibrillary acidic protein (GFAP) and NF- κ B was done on 3- μ m-thick formalin-fixed, paraffin-embedded spinal cord tissue sections. Following deparaffinization, the antigen retrieval was performed using citric acid (pH 6.6) for 15 min in water bath at 95°C. The sections were then rinsed under fluent water for next 15 min. Non-specific binding sites were blocked using 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). In the subsequent step, the sections were incubated overnight at 4°C with primary antibodies: anti-NF- κ B p65 antibody—ChIP Grade (1:100, ab7970, Abcam) and purified mouse anti-GFAP monoclonal antibody (1:100, cat. no. 556328, BD Pharmingen). Incubation with secondary antibodies, Alexa fluor 488 goat anti-rabbit IgG (1:1000, Invitrogen) for NF- κ B and Alexa fluor 488 goat anti-mouse IgG (1:1000, Invitrogen) for GFAP, were applied for 1 h at RT. The sections were washed using PBS 3 $\times$ 5 min after each step. After being coverslipped in fluorescence mounting media, the

signal was detected by fluorescence microscopy using an Olympus microscope BX51/BX52 (Tokyo, Japan). Images of the spinal cord ventral gray matter were analyzed by ImageJ software (NIH, USA). Positive cells were counted per mm². The spinal cord tissues from four animals of the same study subgroup were analyzed. From each sample, three serial cross sections were obtained and on each four fields of 280 × 280 μm area were analyzed.

MnPs

A pure MnTBAP³⁻, synthesized and characterized as reported [57], was used in this study. It is fairly difficult to prepare the MnTBAP³⁻ of high purity, especially to eliminate all low molecular weight Mn(II)-related impurities [57]. The compound of same purity was used in reported animal model study [58]. It was also used in all studies related to *E. coli* and *S. cerevisiae* [51]. MnTnHex-2-PyP⁵⁺ of highest purity was synthesized and characterized by us as reported [106], and used herein and in all our earlier studies. This compound is presently in pre-clinical development and has been tested in non-human primate model of pulmonary radioprotection [41]. The doses selected here were in the range of doses used in reported studies. Aqueous solutions of 5.26 mM MnTnHex-2-PyP⁵⁺ and 5.88 mM MnTBAP³⁻ were used for rat *sc* injections.

Statistics

All statistical analyses were performed using Statistica software version 8.0 (StatSoft Inc., Tulsa, OK, USA). TBARS levels were expressed as nmol MDA/mg protein. SOD and GPx activities were expressed in U/mg protein. In densitometric analyses of protein carbonyl levels, the values were expressed as relative optical densities of the related bands. Area of astrocytes' perikarya and the intensity of GFAP and NF-κB staining were expressed as number of positive cells/mm². Statistical significance for the biochemical analyses data was calculated according to the one-way analysis of variance (ANOVA), followed by Duncan's multiple range post-hoc test. For the GFAP and NF-κB staining, the adjacent coronal sections were analyzed, their means calculated, and used for the Kruskal–Wallis analysis. Post-hoc group comparisons were accomplished utilizing the Mann–Whitney *U* test. Results are expressed as means ± SEM. In all comparisons, *P* < 0.05 was considered statistically significant.

Results

Neurologic status

The neurologic status was assessed daily through 48-h or 7-day post-reperfusion using composite neuroscore comprised from Tarlov score and inclined plane test. The assessments at 48 h and 7 day are given in Figure 3A, while daily assessments are shown in Figure 3B. All the animals in the control (sham) group were neurologically normal (grade 8). Motor scores in I/R groups at 48-h and 7-day post-reperfusion were significantly lower (*P* < 0.05) than those in the control group. The scores at 48-h post-reperfusion in the groups treated with I/R + MnTnHex-2-PyP⁵⁺ or I/R + MnTBAP³⁻ were significantly higher versus group treated with vehicle. In I/R + MnTnHex-2-PyP⁵⁺ group, there is a significant difference between motor scores assessed at 48 h and 7 day. The motor scores in the I/R + MnTnHex-2-PyP⁵⁺ group were significantly higher (*P* < 0.05) compared with those in I/R +

MnTBAP³⁻ group and also in group treated with vehicle at 7 day post-reperfusion. Also, the motor scores in I/R + MnTBAP³⁻ group were significantly higher ($P < 0.05$) compared with those in I/R + vehicle group. For most of 7 days, MnTnHex-2-PyP⁵⁺ was more efficacious in suppressing motor neuron injury than MnTBAP³⁻ (Figure 3B).

Analysis of fibrous astrocytes, GFAP, and NF- κ B expression

Fibrous astrocytes in the white matter are characterized in the fluorescent microscope by GFAP (Figure 4A). The presence of GFAP in the gray matter of the ventral horn of the spinal cord indicates the glial scar formation, an inflammatory process. The absence of GFAP in the white matter of the spinal cord indicates the astrocytes death [107,108]. Rats which were treated with MnTnHex-2-PyP⁵⁺ or MnTBAP³⁻ or received a vehicle showed a high number of GFAP-positive cells ($P < 0.01$) in comparison with the control group. The highest number of GFAP-positive cells in I/R + vehicle was statistically significant compared with MnTnHex-2-PyP⁵⁺-treated and MnTBAP³⁻-treated groups either at 48-h or 7-days post-reperfusion ($P < 0.01$). There was no statistical difference between the number of GFAP-positive cells in the groups treated with MnTnHex-2-PyP⁵⁺ and MnTBAP³⁻ (Figure 4B).

Once activated, NF- κ B translocates from cytosol to nucleus to trigger the transcription of genes involved in cytokine production and cell survival. NF- κ B has also been implicated in processes of synaptic plasticity. We used immunofluorescence to detect the expression of NF- κ B in the gray matter of ventral horn. Positive cells were those with nuclear accumulation of NF- κ B (Figure 5A). The number of NF- κ B-positive cells in the ventral horn of the spinal cord was significantly increased ($P < 0.01$) in all experimental groups *versus* control group. The NF- κ B expression in the I/R + vehicle rats was significantly higher relative to I/R + MnTnHex-2-PyP⁵⁺ or I/R + MnTBAP³⁻ group either at 48-h or 7-day post-reperfusion. NF- κ B expression in I/R + MnTnHex-2-PyP⁵⁺ or I/R + MnTBAP³⁻ group at 48-h post-reperfusion was similar to that in control group, and significantly lower relative to I/R + vehicle group ($P < 0.01$). At 7 days post-reperfusion, the NF- κ B expression in I/R + MnTnHex-2-PyP⁵⁺ and I/R + MnTBAP³⁻ groups increased compared to 48-h post-reperfusion, but the difference was not significant (Figure 5B).

Evaluation of oxidative stress

In order to verify the presence of spinal cord lipid and protein oxidative damage induced by I/R, the levels of lipid peroxidation products, TBARS, as well as the protein carbonyl formation were measured. Figure 6A shows spinal cord TBARS levels in control rats, rats treated with vehicle, and rats treated with either MnTnHex-2-PyP⁵⁺ or MnTBAP³⁻. An overall ANOVA revealed a statistically significant effect of MnTnHex-2-PyP⁵⁺ and MnTBAP³⁻ on the TBARS levels. Duncan's multiple range test indicated that spinal cord TBARS levels in I/R injured rats treated with MnTnHex-2-PyP⁵⁺ or MnTBAP³⁻ were significantly higher in comparison with control group. Further, the TBARS levels in I/R + MnTnHex-2-PyP⁵⁺ or I/R + MnTBAP³⁻ group were significantly decreased in comparison with those in I/R + vehicle group (Figure 6A). There were no differences demonstrated with respect to the time post-reperfusion.

Figure 6B shows the immunoblots of oxidized proteins in the spinal cord and Figure 6C shows the quantification of blots in the rats from all experimental groups. A statistically significant effect of MnTnHex-2-PyP⁵⁺ and MnTBAP³⁻ treatment on the levels of oxidized proteins was demonstrated ($P = 0.01$). In the I/R + vehicle rats, the levels of oxidized protein were significantly increased to $146.0\% \pm 8.8\%$ at 48-h and to $163\% \pm 7.4\%$ at 7-day post-reperfusion relative to the control group (100%). The MnTnHex-2-PyP⁵⁺ and MnTBAP³⁻ treatment significantly reduced the I/R-induced increase in the level of oxidized proteins both at 48-h and 7-day post-reperfusion *versus* I/R + vehicle group ($123.2\% \pm 8.3\%$, $132\% \pm 5.4\%$, $125.8\% \pm 5.9\%$, and $137.5\% \pm 4.7\%$).

Antioxidant enzyme activities

Figure 7A and B shows the activities of SOD and GPx enzymes in the rat spinal cords at 48-h and 7-day post-I/R. At 48-h post-reperfusion, the significantly increased GPx activity was found in I/R + MnTnHex-2-PyP⁵⁺ group compared to that in control, but not relative to I/R + vehicle group. GPx activity of the spinal cord decreased to a control level at 7 days post-reperfusion in all groups.

A modest decrease in SOD activity in I/R + MnTnHex-2-PyP⁵⁺ group, and less so in I/R + MnTBAP³⁻ group, was demonstrated relative to a control group. The effect was more pronounced at 48 h than at 7 days (Figure 8). The data clearly demonstrate that neither MnTnHex-2-PyP⁵⁺ nor MnTBAP³⁻ acted as SOD mimic.

Discussion

Suppression of I/R spinal cord injury by MnPs

Impact of MnPs on motor neuron function—Both MnPs improved motor neuron function as evaluated by Tarlov score and inclined plane test (Figure 2). When assessed on day-to-day basis, there is a trend suggesting that MnTnHex-2-PyP⁵⁺ is superior over MnTBAP³⁻ (Figure 2B). Such observation may be explained by higher ability of more lipophilic MnTnHex-2-PyP⁵⁺ to cross blood–brain barrier [109]. As this is the only set of data from this study indicating the advantage of MnTnHex-2-PyP⁵⁺ over MnTBAP³⁻, we have decided to stay away from strong conclusions. A combined dose-dependent/mechanistic study is in progress and aims to differentiate further between the therapeutic effects of these two MnPs.

Impact of MnPs on astrocyte fibrosis and NF- κ B expression—The activation of astrocytes, known as reactive astrogliosis, is a hallmark of astrocytes' response to all forms of CNS injuries [110]. Its molecular triggers in ischemia/reperfusion are still largely unknown, but seem to involve NF- κ B, p38 MAPK (mitogen-activated protein kinase), and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathways [95,107,108,110,111]. The expression of GFAP has become a prototypical marker for immunohistochemical identification of fibrous astrocytes. The astrocytes are the most abundant glial cells of the spinal cord and are involved in the scarring process of the spinal cord following ischemic injury. The increase of GFAP expression is essential for the process of reactive astrogliosis and glial scar formation [112]. Herein, we have found that astrocytes exhibited upregulation

of GFAP post-I/R (Figure 4). The number of GFAP-positive cells was significantly reduced in injured spinal cord treated with either MnTnHex-2-PyP⁵⁺ or MnTBAP³⁻ (Figure 4).

The trend in astrogliosis, affected by MnPs, is paralleled by NF- κ B expression (Figures 4 and 5). The NF- κ B proteins comprise a family of structurally related eukaryotic transcription factors that are involved in the control of a large number of physiological cellular processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis [107]. NF- κ B is present as a latent, inactive complex in the cytoplasm. When a cell receives extracellular signal, NF- κ B rapidly enters the nucleus and activates gene expression. Upon ischemic injury, the excessive activation of NF- κ B leads to a destructive inflammatory response, phenomenon referred to as I/R injury [96]. I/R caused large increase in the NF- κ B-positive cells, which was greatly reduced by both MnPs almost to the level in control rats. The suppression of NF- κ B with MnPs (Figure 5) reduced secondary injury, as demonstrated by reduced astrogliosis (Figure 4), lipid peroxidation, and protein oxidation (Figure 6), which in turn improved the motor neuron activity (Figure 3).

Impact of MnPs on oxidative stress—Reactive species were upregulated by I/R oxidative insult. The primary oxidative stress was further maintained/enhanced by the upregulation of NF- κ B-driven pro-inflammatory pathways [97,98]. This resulted in oxidation of biomolecules as evidenced here with lipids and proteins. Both MnPs suppressed lipid peroxidation and protein oxidation to a similar extent (Figure 6). MnPs presumably scavenged reactive species formed during a short period of initial I/R injury. Yet, the impact of initial oxidative insult is further amplified over a long course of time by excessive upregulation of NF- κ B [93,94]. We have clearly shown in our rat middle cerebral artery occlusion (MCAO) I/R studies [93,94] that MnPs were protective when administered as a single injection given either very early at 5 min after reperfusion, or as late as 6 h after post-reperfusion. Such data show that the effect does not occur at the moment of reperfusion only (when most of the reactive species are produced), but at cellular transcription also. The effect of MnP, however, fades away eventually unless its administration continues over a longer period of time in order to suppress cycling secondary inflammatory processes. Several MnPs (MnTE-2-PyP⁵⁺, MnTDE-2-ImP⁵⁺, and MnTnHex-2-PyP⁵⁺) suppress NF- κ B activation in stroke and subarachnoid hemorrhage [50,94]. Based on reported data and evidence provided herein, we may safely conclude that both MnPs suppress I/R spinal cord injury by impacting secondary oxidative stress, and that such effect is in great part controlled by NF- κ B downregulation.

Impact of MnPs on endogenous antioxidative defenses—GPx activity: At 48-h post-reperfusion, due to ischemic injury, the GPx activity was increased in all groups relative to control non-injured, non-treated group of rats (Figure 7). At 7 days it was restored to control levels. SOD-like activity: The increased activity of SOD by MnPs has not been observed herein, which suggests that none of MnPs acted as SOD mimic (Figure 7). If any, the data indicate that SOD activity was somewhat reduced at 48 h. It is still possible that SOD enzyme had been upregulated, but was inactivated by reactive oxygen species (ROS)/reactive nitrogen species (RNS). The nitration of MnSOD enzyme with ONOO⁻ has been reported [113,114]. MnTBAP³⁻ is able to nitrate aromatic compounds with ONOO⁻

[114,115]. The upregulation *versus* inactivation of SOD enzymes will be addressed in future studies.

Our data imply that pathways other than SOD-related pathways are implicated in the therapeutic actions of these two MnPs. The data agree with recent discussion of Liochev on (perhaps) irrelevance of the *in vivo* SOD-like actions of MnPs in their therapeutic potential [116]. These data further agree with a study on rat kidney I/R, where MnTnHex-2-PyP⁵⁺, being a part of a mixture of drugs, upregulated numerous endogenous antioxidative defenses (including SOD and GPx). The upregulation of SOD strongly implied that MnP had not acted as SOD mimic in that model (For further discussion, read the section “*Reactivity toward signaling proteins*”).

Are the therapeutic effects of vastly different MnTnHex-2-PyP⁵⁺ and MnTBAP³⁻ controversial or simply not yet understood?

SOD-like activity—While on the first sight the issue may be viewed as controversial, it may, however, simply result from the insufficient knowledge of the chemistry and biology of MnPs. We have thus far gained knowledge on at least some of the pathways which may be operative. Yet, we are constantly reminded that (i) still we know little about the chemistry and biology of metal complexes and (ii) therefore other pathways may be operative. This is the first *in vivo* animal study providing a direct comparison of MnTnHex-2-PyP⁵⁺ and MnTBAP³⁻ on their therapeutic efficacy in rat spinal cord I/R injury (Figure 1). The strong therapeutic effects obtained herein, under identical experimental conditions, have justified further dose-related and mechanistic studies.

MnTnHex-2-PyP⁵⁺ is an efficacious SOD mimic with rate constant for the catalysis of O₂^{•-} dismutation of log k_{cat}(O₂^{•-}) = 7.34 (Table I) [7,8,10,12,14]. For the family of SOD enzymes, log k_{cat}(O₂^{•-}) is reported to be in the range of 8.64–9.3 (Table I). Its high SOD-like activity is a result of the favorable thermodynamics (E_{1/2} = + 314 mV vs NHE) and kinetics, which are based on five positive charges in close proximity to the metal site. This allows for the superior catalysis of O₂^{•-} dismutation (Eqs. 1 and 2). Additionally, its lipophilic character, based on four hydrophobic n-hexylpyridyl substituents, greatly contributes to its transport across membranes mostly across blood–brain barrier and mitochondrial membranes [109]. This in turn results in its high therapeutic efficacy. However, anionic compound, MnTBAP³⁻, lacks proper thermodynamics for O₂^{•-} dismutation; the relatively high electron richness of Mn site as indicated by its negative metal-centered reduction potential, E_{1/2} = –196 mV vs NHE precludes its reduction with O₂^{•-} in the 1st step of the dismutation process (Eq. 1, charges on periphery of MnP are omitted) [10,12,57], thus preventing a catalytic cycle.

Further, with four negatively charged carboxylates on its periphery, there is a repulsion of anionic $O_2^{\cdot -}$ from the anionic carboxylates of $MnTBAP^{3-}$. Consequently, $MnTBAP^{3-}$ is not an SOD mimic. Thus, $MnTnHex-2-PyP^{5+}$ does, but $MnTBAP^{3-}$ does not, allow SOD-deficient organisms—prokaryotic *E. coli* and eukaryotic yeast *S. cerevisiae*—to grow aerobically as well as wild-type strains [51]. These assays are $O_2^{\cdot -}$ -specific and are routinely used to evaluate the SOD-like potency of MnPs and other types of SOD mimics. The reader is directed for further details to references [7,8,10,12,14,117].

Catalase-like activity—None of MnPs, including $MnTnHex-2-PyP^{5+}$ and $MnTBAP^{3-}$, have any significant catalase-like activity ([37] Maia et al., SFRBM Meeting 2014, submitted) (Table I). The k_{cat} (H_2O_2) is 5.84 and 28.3 $M^{-1}s^{-1}$, while it is $1.5 \times 10^6 M^{-1}s^{-1}$ for the catalase enzyme itself, thus representing 0.0004 and 0.0018% of enzyme activity.

Reactivity toward cellular reductants— $MnTnHex-2-PyP^{5+}$ cycles with cellular reductants, such as GSH and ascorbate, with Mn in + 3 and + 4 oxidation states (see details [12,133,136]). Yet, $Mn^{III}TBAP^{3-}$ is very electron-rich (as indicated by its very negative $E_{1/2}$) and, therefore, is very stable in its Mn + 3 oxidation state. It, therefore, does not favor reaction (reduction) with electron-donating species, such as ascorbate and glutathione. Only strong and not biologically relevant reductant, such as sodium dithionite— $Na_2S_2O_4$, is able to reduce it to $Mn^{II}TBAP^{4-}$. Alternatively, ligand binding to its Mn site may change its reduction potential, thus facilitating reduction of Mn site from + 3 to + 2 (see below).

Reactivity toward RNS—We have thus far also explored the chemistry and biology of MnPs with RNS, $\cdot NO$, HNO , and $ONOO^-$ [10,12,117,124]. $MnTBAP^{3-}$ reacts with $ONOO^-$ at more than 2 log units slower than $MnTnHex-2-PyP^{5+}$, $k_{red} = 1.05 \times 10^5 M^{-1}s^{-1}$ (Table I) [69]. The lower $k_{red}(ONOO^-)$ is due to the reluctance of a fairly electron-rich Mn site to bind the anionic $ONOO^-$. The additional unfavorable electrostatics, that is, the repulsion of anionic $ONOO^-$ from anionic $MnTBAP^{3-}$ is, however, absent in its reactions with $\cdot NO$ and HNO . $MnTBAP^{3-}$ reacts with $\cdot NO$ only after binding with an appropriate ligand modifies its reduction potential; the nitrosylated complex $(NO)Mn^{II}TBAP^{4-}$ was formed in the presence of $\cdot NO$ and endogenous antioxidant, uric acid [137]. Quite recently, Doctorovich's group has reported that $MnTBAP^{3-}$ rapidly reacts with HNO , a species which has recently gained increased interest due to its biological relevance [126] (Table I). The product is identical to the one formed in reaction with $\cdot NO$ after urate binding: $(NO)Mn^{II}TBAP^{4-}$. The reactivity of $MnTBAP^{3-}$ toward HNO is supported by thermodynamics. The pH-dependent reduction potential of the $\cdot NO/HNO$ couple at pH 7 is $E' = + 550$ mV vs NHE: $\cdot NO + 1e^- + H^+ \rightarrow HNO$ [138]. If oxidation of nitroxyl, given by $HNO \rightarrow \cdot NO + 1e^- + H^+ (E' = + 550$ mV vs NHE), couples with reduction of MnP, $Mn(III)P^+ + 1e^- \rightarrow Mn(II)P$ where $E_{1/2} = -194$ mV vs NHE, then $E = + 356$ mV. This indicates a thermodynamically favored process. The spontaneity of the reaction would depend on pH; at a very low gastric pH, it would not be favored, but it would occur at neutral pH. Further, Filipovic et al. have recently demonstrated that HNO and a metal center of an Fe porphyrin can eventually S-nitrosate proteins via H_2 S-assisted mechanism [102]. The same has been shown with Mn(II) cyclic polyamine-based SOD mimic [139]. This suggests that similar mechanism may be operative

for MnTBAP³⁻-driven inhibition of NF-κB and in turn suppression of pro-inflammatory pathways.

Reactivity toward signaling proteins—The reactivity of MnTBAP³⁻ toward RNS, and the impact on NF-κB, lead us to hypothesize that MnTBAP³⁻ may be involved in the modification of NF-κB protein cysteine, followed by the inactivation of its transcriptional activity. Such scenario may explain the beneficial effects of MnTBAP³⁻. Our hypothesis is based on several recent studies where modification of cysteines of signaling proteins, with their subsequent inactivation, has been implicated in the modes of actions of two different classes of SOD mimics. The modification of cysteines was shown to occur *via* either ROS [99,100,140] or RNS [103]. It has recently been shown that Mn(II) cyclic polyamine-based SOD mimic could react with ·NO, whereby oxidizing and reducing it [101]. Such compound presumably *oxidatively modifies* cysteine of Keap1 *via* ·NO/HNO/H₂S biology, whereby activating transcription factor Nrf2. Nrf2, in turn, controls levels of endogenous antioxidant defenses. The Nrf2 upregulation is a likely scenario in the pro-oxidant action of a natural compound, curcumin, commonly regarded as antioxidant [141]. Sheng et al. published extensively on a stroke I/R MCAO model, where MnTnHex-2-PyP⁵⁺ and MnTDE-2-ImP⁵⁺ inhibit DNA binding of NF-κB due to the modification of the cysteines in their p50 and p65 subunits [50,94]. This agrees well with substantial evidence provided by Tome's group that few Mn(III) *N*-substituted pyridylporphyrins, rather than acting as antioxidants, act indeed as pro-oxidants and oxidize cysteines of NF-κB whereby suppressing its transcriptional activities [100,140]. That is further in agreement with the data from Piganelli's group which suggest that oxidation of cysteine of p50 subunit of NF-κB, upon nuclear translocation, suppresses immune response during beta islet cell transplant [11,142]. Our work on rat kidney I/R injury demonstrated that MnTnHex-2-PyP⁵⁺ (as a part of mixture containing growth factors and amino acids of Krebs cycle) upregulates a number of endogenous antioxidant enzymes — including mitochondrial and extracellular SOD, GPx, lactoperoxidase, inducible nitric oxide synthase (iNOS), and thioredoxin reductase 1—and a set of peroxiredoxins—heat shock protein 70 (HSP-70) and phospho-heat shock factor 1 (pHSF-1) [42]. Importantly, this study clearly showed that MnP had not acted as an SOD mimic, but perhaps as a pro-oxidant inducing adaptive responses such as activating Nrf2 *via* oxidation of Keap1, in a manner similar to Mn(II) cyclic polyamine [102,103]. Such mode of action is further substantiated with data from Tome's group indicating that MnTE-2-PyP⁵⁺ can *S*-glutathionylate other thiol-bearing proteins, such as complexes I, III, and IV of mitochondrial electron transport chain [99,100].

It is important to note that in all cases listed here, we do observe antioxidant effects; thus it is no wonder that researchers were frequently (and incorrectly) assigned them to antioxidative actions. We have already stressed that it is crucial to differentiate the effects we observe and actions that produce such effects [12,117].

Concluding remarks

We have summarized our discussion in Figure 8. It indicates the possible involvement of RNS-related chemistry in the therapeutic efficacy of MnTBAP³⁻ and ROS-based effects of MnTnHex-2-PyP⁵⁺, both of which exhibit suppression of NF-κB-controlled pro-

inflammatory pathways in common. The mechanism of MnTBAP^{3-} action on NF- κ B was hypothesized to be related to RNS-based modification of cysteine which would inhibit its activation. Such conclusions are based on the (i) MnTBAP^{3-} /RNS-related chemistry; (ii) Mn(III) *N*-substituted pyridylporphyrins-driven and *N,N'*-disubstituted imidazolylporphyrins-driven inactivation of NF- κ B in a stroke I/R rat injury [50,93,94]; (iii) curcumin-driven suppression of astrogliosis/scar formation *via* NF- κ B pathways [111]; (iv) inhibition of NF- κ B DNA binding due to the *S*-glutathionylation of p65 subunit in a cancer study [100,143]; (v) islet cell transplant study [144], where two analogous MnPs suppressed immune responses *via* oxidation of p50 subunit followed by subsequent inhibition of NF- κ B DNA binding [11,142]; (vi) oxidation of Keap1 (cysteine) of Nrf2 by cyclic polyamine-based SOD mimic [102,103]; (vii) parthenolide oxidation of Keap1 and activation of Nrf2 in a normal tissue with subsequent upregulation of endogenous antioxidant defenses [145], which is similar to the effect of MnTnHex-2-PyP $^{5+}$ in suppression of rat kidney I/R injury [42,45]; (viii) role of Nrf2 in the inhibition of NF- κ B and p38 MAPK phosphorylation in the hyper-activation of microglia cells/astrogliosis [95]; and (ix) role of NF- κ B in astrogliosis and CNS injuries in general [95,107,108,110,111].

Taken together, data reported elsewhere and our data strongly suggest that NF- κ B may be the common pathway for the involvement of two MnPs having entirely different chemistry and biology in suppression of I/R-driven inflammation. MnTnHex-2-PyP $^{5+}$ has SOD-like properties, while MnTBAP^{3-} is not an SOD mimic. Further, neither of the two acted in this model as SOD mimic. The chemistry and biology of MnPs is complex and difficult to comprehend even to the scientists with chemical background. It is thus of no surprise that biologists rely on the manuscripts, most of which are written by biologists also [116,146–148]; they would have to put in additional efforts in order to fully understand the coordination chemistry of metalloporphyrins that accounts for their therapeutic effects.

Understanding the mechanism of action of redox-active drugs may be irrelevant from clinical perspective as long as they are promising therapeutically [146]. Yet, their mechanism of action is critical for drug development, and especially when such drugs are used as tools to gain insight into the cellular metabolism and pathways involved in pathological disorders. Incorrect assignments of the pathways do not benefit drug development, understanding of the cellular redoxome, and identifying their interventions when perturbed. Increased understanding of their actions will result in increased understanding of cellular redox biology under physiological and pathological conditions. It is important to understand that what we observe may be the consequence of a complex set of interactions. Multiple approaches, both pharmacological and genetic, along with direct measurements of cellular and subcellular localization of SOD mimics are essential to safely identify the species or pathways involved [7,8,10,12,14,117]. Only such experimental strategies could eventually allow researchers to safely propose that mimicking either CuZnSOD or MnSOD accounts at least in part for the observed therapeutic effects of those compounds [149–151].

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Abbreviations

I/R	ischemia/reperfusion
IF	immunofluorescence
iNOS	inducible nitric oxide synthase
GPx	glutathione peroxidase
HSP-70	heat shock protein 70
MnTnHex-2-PyP⁵⁺	Mn(III) <i>meso</i> -tetrakis(<i>N</i> -n-hexylpyridinium-2-yl)porphyrin
MnTBAP³⁻	Mn(III) <i>meso</i> -tetrakis(4-carboxylatophenyl)porphyrin
MnP	Mn(III) porphyrin
Mn^{III}P	MnP with Mn in + 3 oxidation state
Mn^{II}P	MnP with Mn in + 2 oxidation state
MnTDE-2-ImP⁵⁺	Mn(III) <i>meso</i> -tetrakis(<i>N,N'</i> -diethylimidazolium-2-yl)porphyrin—AEOL10150
MnTE-2-PyP⁵⁺	Mn(III) <i>meso</i> -tetrakis(<i>N</i> -ethylpyridinium-2-yl)porphyrin—AEOL10113
$E_{1/2}$	metal-centered reduction potential for Mn ^{III} P/Mn ^{II} P redox couple
$\log k_{\text{cat}}(O_2^{\cdot -})$	rate constant for the catalysis of $O_2^{\cdot -}$ dismutation to O_2 and H_2O_2 by MnP
$\log k_{\text{red}}(ONOO^-)$	rate constant for the reduction of peroxynitrite to $\cdot NO_2$ with MnP
$\log k_{\text{cat}}(H_2O_2)$	rate constant for the catalysis of H_2O_2 dismutation to O_2 and H_2O by MnP
sc	subcutaneous
ip	intraperitoneal
iv	intravenous
GFAP	glial fibrillary acidic protein
NF-κB	nuclear factor kappa B
Nrf2	nuclear factor (erythroid-derived 2)-like 2
DAPI	4',6-diamidino-2-phenylindole
TBARS	thiobarbituric acid-reactive substances

MCAO

middle cerebral artery occlusion

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Highlights

- An SOD mimic, MnTnHex-2-PyP⁵⁺, and non-SOD mimic, MnTBAP³⁻, exert anti-inflammatory effects on rat spinal cord I/R injury.
- Neither MnTnHex-2-PyP⁵⁺ nor MnTBAP³⁻ acted as SOD mimic in this model.
- Downregulation of NF-κB pathways appears to be a common pathway for both MnPs, and results in suppression of lipid peroxidation, protein carbonyl formation, astrogliosis and NF-κB expression, and restoration of motor neuron function.

Figure 1.

Therapeutic effects of MnTnHex-2-PyP⁵⁺ [12,38–55] and MnTBAP³⁻ [20,35,63,64,70–90]. Structure of MnTnHex-2-PyP⁵⁺ and references related to reported studies are shown in blue in left circle. The structure of MnTBAP³⁻ and its references are shown in red color in right circle. The central oval lists the same type of animal and cellular studies where both compounds are studied independently. The smallest circle lists the same type of studies where MnTnHex-2-PyP⁵⁺ was efficacious (blue references), but MnTBAP³⁻ (red reference) was not. There is no single animal study with direct comparison of these two compounds. Both MnPs were only tested side-by-side in cellular studies: aerobic growth of SOD-deficient eukaryotic *S. cerevisiae* and prokaryotic *E. coli* [51,91,92].

Figure 2.

The illustration of the aorta clamping positions (A) and study design schedule (B). (A) I/R injury of the spinal cord was induced by the occlusion of abdominal aorta. Abdominal aorta was clamped for 45 min with a proximal (just below the left renal artery) and a distal (just above the aortic bifurcation) clip; (B) rats in I/R + MnTnHex-2-PyP⁵⁺, I/R + MnTBAP³⁻, and I/R + vehicle groups underwent 45 min of spinal cord ischemia. The treatment started 30 min prior to ischemia and continued every 12 h until 48 h or 7 days. Rats from control (sham) group did not have I/R injury. They were sacrificed at 48-h post-sham procedure.

Figure 3. Neurologic status of rats after I/R injury as affected by the treatment with MnTnHex-2-PyP⁵⁺ and MnTBAP³⁻. (A) The neurologic status noted at 48 h (2D) and on 7 day (7D); (B) Neurologic score was taken daily. The status was assessed by Tarlov score and inclined plane test every day after ischemic insult, and is expressed as mean $\pm$ S.E.M. $P < 0.05$.

Figure 4.

Impact of MnPs on the expression of GFAP in fibrotic astrocytes at 48-h and 7-day post-I/R injury. (A) Immunofluorescence of GFAP. Immunofluorescence microscopy of paraffin sections of rat spinal cord stained with GFAP, a marker of astrocyte activation (reactive astrogliosis). The highest number of GFAP positive astrocytes was detected in the I/R + Vehicle group in comparison with the control group, or MnTnHex-2-PyP⁵⁺-treated or MnTBAP³⁻-treated group. The treatment with MnTnHex-2-PyP⁵⁺ or MnTBAP³⁻ significantly decreased the number of GFAP-positive cells/mm² either 48-h or 7-day post-reperfusion relative to I/R + Vehicle. (B) Quantification of GFAP-positive cells (fibrotic astrocytes) per mm² in the gray matter of ventral horn of the spinal cord. Each value is mean $\pm$ S.E.M. **P* < 0.01 I/R + vehicle vs control, and I/R + vehicle vs I/R + MnTnHex-2-PyP⁵⁺ or I/R + MnTBAP³⁻.

Figure 5.

Impact of MnPs on the expression of NF-κB at 48-h and 7-day post-I/R injury. (A) Immunofluorescence of NF-κB. Immunofluorescence microscopy of paraffin sections of rat spinal cord stained with NF-κB. I/R injury of the spinal cord causes NF-κB translocation to the nucleus. The highest number of nuclear NF-κB-positive astrocytes is in the I/R + Vehicle group. The treatment with MnTnHex-2-PyP⁵⁺ or MnTBAP³⁻ decreases the number of nuclear NF-κB-positive cells, either 48-h or 7-day post-reperfusion. (B) Quantification of the impact of MnPs on NF-κB expression. The number of NF-κB-positive cells/mm² in the ventral horns of spinal cord is significantly increased ($*P < 0.01$) in all experimental groups *versus* control group analyzed at 48 h. Both MnPs greatly inhibit upregulation of NF-κB relative to I/R + Vehicle. Each value is the mean $\pm$ S.E.M.; $*P < 0.01$ I/R + vehicle *vs* control and I/R + vehicle *vs* I/R + MnTnHex-2-PyP⁵⁺ or I/R + MnTBAP³⁻.

Figure 6.

Evaluation of the impact of MnPs on the oxidative stress in rat spinal cord 48 h and 7 days after I/R injury. A) TBARS levels (nmol MDA/mg protein) represent means $\pm$ SEM; B) representative immunoblots of protein carbonyls as a measure of oxidized proteins; and C) Quantified protein carbonyls (% of the control). The following are presented: control group; rats with I/R injury treated with MnTnHex-2-PyP⁵⁺, MnTBAP³⁻, or vehicle. I/R + vehicle is significantly different from control and I/R + MnTnHex-2-PyP⁵⁺ or I/R + MnTBAP³⁻ (* P < 0.01). There were no differences between 48-h and 7-day treatment groups.

Figure 7.

Impact of MnPs on the activities of GPx (A) and SOD enzymes (B). A) GPx activity; * $P < 0.05$ significant difference control vs I/R + MnTnHex-2-PyP⁵⁺ at 48 h; * $P < 0.05$ significant difference between 48 h and 7 days of reperfusion in the same group; B) SOD activity; * $P < 0.05$ significant difference of I/R + MnTnHex-2-PyP⁵⁺ at 48-h post-I/R vs control and I/R + MnTBAP³⁻ at 48-h and 7-day post-I/R vs control; * $P < 0.05$ significant difference between 48 h and 7 day time points in the same group of MnTnHex-2-PyP⁵⁺-treated rats and $P < 0.05$ significant difference between I/R + MnTnHex-2-PyP⁵⁺ and I/R + MnTBAP³⁻ groups at 7-day post-I/R injury. The bars represent mean values $\pm$ S.E.M.

Figure 8. RNS-related chemistry of MnTBAP³⁻ and ROS-based pathways of MnTnHex-2-PyP⁵⁺ are presumably involved in suppression of NF-κB-controlled pro-inflammatory pathways and, in turn, I/R injury. MnPs were injected *sc* starting at 30 min before 45-min-ischemia and then every 12 h at 1.5 mg/kg for either 48-h or 7-day post-I/R. The suppression of astrogliosis, NF-κB expression, and oxidative stress (lipid peroxidation and protein carbonyl formation) was demonstrated by both MnPs. Further, motor neuron activity was significantly restored by both MnPs. While I/R itself increases GPx activity, no impact of MnPs on SOD and GPx activities was observed, suggesting that none of MnPs acted as SOD mimic in this model of oxidative stress.

Table I

Rate constants for the reaction of MnPs with different reactive species.

	MnTnHex-2-PyP ⁵⁺	MnTBAP ³⁻	SOD
$E_{1/2}$ (mV vs NHE)	+314 ^a	– 194 ^b	~+300 ^c
$\log k_{\text{cat}}(\text{O}_2^{\cdot-})$	7.48 ^a	3.16 ^b	8.64–9.20 ^c
$\log k_{\text{cat}}(\text{H}_2\text{O}_2\text{-CAT})$	~1.6 ^d	1.1 ^d	1.1 ^e
$\log k(\text{NO})$	~6 ^f	NR ^g	<i>h</i>
$\log k_{\text{red}}(\text{O}_2)$	4 ⁱ	NR	– 0.36 ^j
$\log v_0(\text{HA}^-)$	1.7 ^k	NR	ND
$\log k_{\text{cat}}(\text{H}_2\text{O}_2\text{-GPx})$	~4 ^d	NR	<i>l</i>
$\log k_{\text{red}}(\text{ONOO}^-)$	7.11 ^m	5.02 ^m	3.97 ^c
$\log k_{\text{red}}(\text{CO}_3^{\cdot-})$	9.38 ^m	9.54 ^m	<i>n</i>
$\log k(\text{HNO})$	~4 ^o	~5 ^p	~6 ^q

For comparison, the values for SOD enzymes are also listed. Mn(III) *N*-substituted pyridylporphyrins have thermodynamics and kinetics of the Mn site similar to those of SOD enzymes. In turn, both undergo same reactions. Yet, sterical factors are entirely different precluding high reactivity of SOD metal site with species other than $\text{O}_2^{\cdot-}$. The reactions which occur with high rate constants and may, therefore, be in common with the actions of those MnPs are grouped in red.

NR, not reactive; N/A, not applicable; ND, not determined

^aRef [91];

^bRef [57];

^cRefs [118–122];

^dRef [37] and Maia et al. SFRBM Meeting 2014, submitted;

^eRef [123];

^fthe rate constant of the reactivity of Mn^{II}TnHex-2-PyP⁴⁺ with $\cdot\text{NO}$ is estimated based on the reactivity of Mn^{II}TE-2-PyP⁴⁺ with $\cdot\text{NO}$ [124]. This reactivity is biologically relevant as cationic Mn(III) *N*-alkylpyridylporphyrins get readily reduced with cellular reductants, such as ascorbate due to its abundance within cell at millimolar levels. The rate constant for the reaction of Mn^{III}P with $\cdot\text{NO}$ is low and estimated based on the reactivity of Mn^{III}TE-2-PyP⁵⁺ to be $\log k(\text{NO}) = -3.70$ [124];

^gin its Mn + 3 oxidation state, MnTBAP³⁻ is not reactive to $\cdot\text{NO}$ unless ligand binding, such as urate, occurs prior to reduction and modifies its reduction potential;

^hrelates to CuZnSOD and only when the metal, that is, Cu is in + 1 oxidation state [125];

ⁱrelates to the reaction of reduced Mn^{II}P with oxygen, O_2 , which leads to the production of superoxide and subsequently peroxide and is of critical importance in the chemistry/biology of Mn(III) *N*-alkylpyridylporphyrins. Once in cell, MnTnHex-2-PyP⁵⁺ is reduced to Mn + 2 oxidation state with cellular reductants. MnTBAP³⁻ cannot be reduced to Mn + 2 oxidation state with cellular reductants to cycle with oxygen; yet once reduced with stronger reductant (such as sodium dithionite), it would favor reoxidation more than electron-deficient MnPs. Based on the data from Doctorovich's group [126], it can undergo reduction to Mn + 2 oxidation state while binding HNO;

^jrelates to Cu^IZnSOD reaction with O_2 [127,128];

^k the high reactivity of MnPs with cellular reductant, ascorbate, plays an important role in the biology of Mn(III) *N*-substituted pyridylporphyrins [12,129,130]. ^v₀ relates to initial rate, MnTBAP³⁻ is non-reactive toward ascorbate. HA⁻ is a monodeprotonated ascorbate which is a main species at physiological pH.

^l At low H₂O₂ levels, Cu in CuZnSOD gets reduced and produces 'OH bound to metal site, acting as peroxidase; at high H₂O₂ levels, SOD undergoes oxidative degradation [131,132].

^m Ref [133];

ⁿ Ref [134];

^o Estimated based on the data on MnTE-2-PyP⁵⁺ and the similar redox properties and reactivities of these two MnPs with O₂^{•-} and ONOO⁻—it relates to the reaction of MnP with either Angeli's salt or toluene sulfohydroxamic acid;

^p [126] relates to the reaction of MnP with HNO;

^q relates to CuZnSOD [135].