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Pure MnTBAP selectively scavenges peroxynitrite over superoxide: Comparison of pure and commercial MnTBAP samples to MnTE-2-PyP in two different models of oxidative stress injuries, SOD-specific *E. coli* model and carrageenan-induced pleurisy

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Abstract

MnTBAP is often referred to as an SOD mimic in numerous models of oxidative stress. We have recently reported that pure MnTBAP does not dismute superoxide, but commercial/ill-purified samples are able to perform $O_2^{\bullet-}$ dismutation with low-to-moderate efficacy via non-innocent Mn-containing impurities. Herein, we show that neither commercial nor pure MnTBAP could substitute for SOD enzyme in the SOD-deficient *E. coli* model, while MnTE-2-PyP-treated SOD-deficient *E. coli* grew as well as wild-type strain. This SOD-specific system indicates that MnTBAP does not act as an SOD mimic in vivo. In another model, carrageenan-induced pleurisy in mice, inflammation was evidenced by increased pleural fluid exudate, and neutrophil infiltration and activation: these events were blocked by 0.3 mg/kg of MnTE-2-PyP and to a slightly lesser extent with 10 mg/kg of MnTBAP. Also, 3-nitrotyrosine formation, an indication of the peroxynitrite existence *in vivo*, was blocked by both compounds; again MnTE-2-PyP was 33-fold more effective. Pleurisy model data

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indicate that MnTBAP exert some protective actions in common with MnTE-2-PyP, which are not $O_2^{\bullet-}$-related, and can be fully rationalized if one considers that the common biological role shared by MnTBAP and MnTE-2-PyP is related to their reduction of peroxynitrite and carbonate radical, the latter arising from $ONOO^-$ adduct with CO_2. The log k_{cat} ($O_2^{\bullet-}$) value for MnTBAP is estimated to be about 3.16, which is ~5 and ~7 orders of magnitude smaller than the SOD activity of the potent SOD mimic MnTE-2-PyP and Cu, Zn-SOD, respectively. This very low value indicates that MnTBAP is very inefficient in dismuting superoxide to be of any biological impact, which was confirmed in the SOD-deficient *E. coli* model. Peroxynitrite scavenging ability of MnTBAP, however, is only ~2.5 orders of magnitude smaller than that of MnTE-2-PyP and is not significantly affected by the presence of the SOD-active impurities in commercial MnTBAP sample (log $k_{red}(ONOO^-)$ = 5.06 for pure and 4.97 for commercial sample). The reduction of carbonate radical is equally fast with MnTBAP and MnTE-2-PyP. The dose of MnTBAP required to yield oxidative stress protection and block nitrotyrosine formation in the pleurisy model is >1.5 orders of magnitude higher than that of MnTE-2-PyP, which could be related to the smaller ability of MnTBAP to scavenge peroxynitrite. The slightly better protection observed with the commercial MnTBAP sample (relative to the pure MnTBAP one) could arise from its impurities, which, by scavenging $O_2^{\bullet-}$, reduce consequently the overall peroxynitrite, and secondary ROS/RNS levels. These observations have profound biological repercussions as they may suggest that the effect of MnTBAP observed in numerous studies may conceivably relate to peroxynitrite scavenging. Moreover, provided that *pure* MnTBAP is unable to dismute superoxide at any significant extent, but is able to partially scavenge peroxynitrite and carbonate radical, this compound may prove valuable to distinguish $ONOO^-/CO_3^{\bullet-}$ from $O_2^{\bullet-}$ pathways.

Keywords

SOD mimic; peroxynitrite scavenger; carbonate radical scavenger; MnTBAP; MnTE-2-PyP; SOD-deficient *E. coli*; carrageenan-induced pleurisy in mouse

Introduction

There has been a great deal of interest in developing superoxide dismutase (SOD) mimics and peroxynitrite scavengers as both mechanistic probes and therapeutic drugs for treating oxidative stress injuries [1–13]. The most potent porphyrin-based compounds developed based on structure-activity relationships (SAR), such as Mn (III) *meso*-tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (MnTE-2-PyP; Fig. 1), its hexyl analogue MnTnHex-2-PyP and Mn (III) *meso*-tetrakis(*N*,*N*′-diethylimidazolium-2-yl)porphyrin, bear positively charged *ortho* pyridyl or di-*ortho* imidazolyl moieties close to the metal site, which thus offer the thermodynamic and electrostatic facilitation [1–4,14–16] for the reaction with negatively charged superoxide and peroxynitrite, and are the most efficient in helping SOD-deficient *E. coli* to grow aerobically [4,14,17]. Along with SAR, a stringent SOD model based on the aerobic growth of SOD-deficient *E. coli* in restricted 5 amino acid medium has proven extremely useful to assess the potentialities of Mn porphyrins as candidate therapeutics for ameliorating diseases/conditions that have oxidative stress in common [4,14]. Negatively charged porphyrins, which lack thermodynamic and electrostatic facilitation for $O_2^{\bullet-}$ dismutation, are either poor SOD mimics (*e.g.*, MnTSPP) or not at all able to dismute $O_2^{\bullet-}$ (*e.g.*, MnTBAP; Fig. 1) [4,18,19], and in turn can poorly or not at all substitute for the SOD in SOD-deficient *E. coli* [19]. Although commercial preparations of MnTBAP have been widely used to test mechanistic concepts and/or for therapeutic purposes, the preparation of a pure MnTBAP sample showed unambiguously that MnTBAP *per se* has no SOD-like activity in aqueous systems [18], and that all common commercially available MnTBAP preparations contain varying amounts of highly SOD-active Mn oxo/hydroxo/acetato species as contaminants; such impurities are responsible for the observed *in vitro* SOD-activity. Moreover, all of these commercial preparations exhibit modest

to high ability to inhibit xanthine oxidase [18], which is a common source of ROS *in vivo* [20].

Due to a high number of studies indicating that MnTBAP is effective in ameliorating *in vivo* oxidative stress injuries, we kept trying to understand the origin of such effects. Our *in vitro* and *in vivo* experiments suggest that while MnTBAP is not an SOD mimic, the benefits often observed with MnTBAP in cell/animal models may conceivably be related, at least in part, to its capacity to scavenge peroxynitrite or other reactive species derived from it. Thus we analyzed herein the ability of pure and impure MnTBAP to reduce $ONOO^-$.

Finally we devised *in vivo* experiments to investigate whether pure MnTBAP and commercial MnTBAP differ with respect to their *in vivo* behavior in mediating oxidative stress in two well-established models: an SOD-deficient *E. coli* model [21–23] and an inflammatory, mouse carrageenan-induced pleurisy model [24–26]. We compared the effects of MnTBAP to MnTE-2-PyP, which is both a potent SOD mimic and peroxynitrite scavenger [4,27].

Experimental

Materials

Pure MnTBAP was synthesized and characterized as previously reported [18]. A commercial sample of MnTBAP was bought from Alexis (catalogue # 430-069-M010, lot# L06883); analyses of this commercial sample showed 3% of Mn-free ligand. Another batch of commercial MnTBAP (lot# 19498) was found unsuitable for any cell/animal testing as it contained ~24% of the Mn-free ligand [28]. MnTE-2-PyP was prepared and thoroughly characterized as described in [4,28]. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Milan, Italy). The compounds for animal work were obtained from Sigma-Aldrich Company Ltd. (Milan, Italy) and all other chemicals were of the highest commercial grade available.

Kinetics of peroxynitrite scavenging

Stopped-flow kinetic measurements were carried out as described before [27] using an SX-17MV Stopped-Flow from Applied Photophysics coupled with a 1-cm-long mixing cell. Briefly, MnTBAP samples (6 μM) in 100 mM phosphate buffer pH 7.0 were mixed in a 1:1 v/v ratio with 10- to 40-fold molar excess of $ONOO^-$ in 15 mM NaOH; the final pH was 7.3. The reaction was monitored by the change in the absorbance of the Soret band of MnTBAP (468 nm [18]), and the plots were fitted to a single exponential function. All experiments were carried out at 37 °C. The pH was measured at the outlet of the stopped-flow.

E. coli growth experiments

Escherichia coli strains used in this study were AB1157, wild type (*F-thr-1; leuB6; proA2; his-4; thi-1; argE2; lacY1; galK2; rpsL; supE44; ara-14; xyl-15; mtl-1; tsx-33*), and JI132, SOD-deficient, $sodA^-sodB^-$ (same as AB1157 plus (*sodA::mudPR13*)25 (*sodB-kan*)1-Δ2). Both strains were obtained from J. A. Imlay [21]. The experiments were carried out as described in detail in [14]. Briefly, cultures were grown aerobically in either casamino acid (M9CA) medium or in a 5 amino acid restricted medium (L-leucine, L-threonine, L-proline, L-arginine, L-histidine) in flasks on a water bath shaker at 37°C and 200 rpm. The effect of Mn porphyrins on the growth of the SOD-deficient strains was followed at 10 (M9CA medium) and 20 hours (5 amino acids medium), turbidimetrically at 700 nm (to minimize the interference of compounds studied) and compared to the growth curves of both strains in the absence of Mn porphyrin (controls). Deionized water was used throughout the study.

Animals

Six to eight week old CD1 male mice (Charles River, Calco, Italy) were used for the study. The animals were housed in a controlled environment and provided with standard rodent chow and water. The study was approved by the University of Messina Review Board for the care of animals. All animal experiments complied with regulations in Italy (D.M. 116192), Europe (O.J. of E.C. L 358/1 12/18/1986) and USA (Animal Welfare Assurance No A5594-01, Department of Health and Human Services, USA).

Carrageenan-induced pleurisy

Pleurisy was induced by carrageenan as previously described [29]. We anesthetized the mice with isoflurane and made a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected and saline (0.2 ml) or saline containing 1% (w/v) λ-carrageenan (Sigma-Aldrich Ltd, 0.2 ml) was injected into the pleural cavity. The skin incision was closed with a suture and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were killed. The chest was carefully opened and the pleural cavity rinsed with 2 ml of saline solution containing heparin (5 U/ml) and indomethacin (10 μg/ml). The exudate and washing solution were removed by aspiration and the total volume measured. Any exudate, which was contaminated with blood, was discarded. The amount of exudate was calculated by subtracting the volume injected (2 ml) from the total volume recovered. The leukocytes in the exudate were suspended in phosphate-buffer saline (PBS, 0.01M, pH7.4) and counted with an optical microscope in a Burker's chamber after vital Trypan Blue staining.

Experimental groups

Mice were allocated into one of the following groups: (1) administration of carrageenan only (**CAR group**, *N*=10), (2a) commercial MnTBAP (10.0 mg/kg) given as an intraperitoneal (i.p.) bolus 30 minutes before carrageenan (**CAR + commercial MnTBAP group**, *N*=10), (2b) same as 2a except that pure MnTBAP (10.0 mg/kg) was used instead of commercial MnTBAP (**CAR + pure MnTBAP group**, *N*=10), (2c) same as 2a except that MnTE-2-PyP (0.3 mg/kg) was used instead of commercial MnTBAP (**CAR + MnTE-2-PyP group**, *N*=10), (3) a sham-operated group in which identical surgical procedures to the CAR group was performed, except that the saline (vehicle) was administered instead of carrageenan (**Sham group**, *N*=10). (4a) **Sham + commercial MnTBAP group:** Same as Sham group except for the administration of commercial MnTBAP (10.0 mg/kg) which was given 30 minutes before the saline injection (*N*=10), (4b) same as 4a except that pure MnTBAP (10.0 mg/kg) was used instead of commercial MnTBAP (**Sham + pure MnTBAP group**, *N*=10), (4c) same as 4a except that MnTE-2-PyP (0.3 mg/kg) was used instead of commercial MnTBAP (**Sham + MnTE-2-PyP group**, *N*=10).

Histological examination

Lungs were taken 4 h after carrageenan or vehicle injection. Tissues were fixed for 1 week in 10 % (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Lung sections were then deparaffinized with xylene, stained with hematoxylin and eosin. All sections were studied using light microscopy (Dialux 22 Leitz).

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined in lung tissues after carrageenan-injection as previously described [30]. Subplantar and lung tissues obtained from ten animals per group were homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) using a Polytron homogenizer (3 cycles

of 10 sec at maximum speed) and centrifuged for 30 min at 20,000 × g at 4° C. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM hydrogen peroxide. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μM of peroxide/min at 37 °C and was expressed in units per gram of wet tissue.

Immunohistochemical localisation of 3-nitrotyrosine

At the end of the experiment, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with 1) with anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v). Sections were washed with PBS, and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). In order to confirm that the immunoreaction for the nitrotyrosine was specific some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity.

Statistical evaluation

All values in the figures and text are expressed as mean ± standard error of the mean (s.e.m.) from 10 mice for each group. For the in vivo studies n represents the number of animals studied. In the experiments involving histology, the figures shown are representative of at least three experiments (histological coloration) performed on different experimental days on the tissues section collected from all the animals in each group. The results were analyzed by one-way ANOVA followed by a Bonferroni's post-hoc test for multiple comparisons and the effect of on was assessed with 2-way ANOVA for repeated measures and followed by Student's t test. A p-value of less than 0.05 was considered statistically significant.

Results

$ONOO^-$ scavenging and $O_2^{\bullet-}$ dismuting properties of the Mn porphyrins

The ability of the pure and the commercial MnTBAP samples to scavenge peroxynitrite was determined by stopped-flow measurements and is summarized in Table 1, which includes also the data on MnTE-2-PyP and Cu, Zn-SOD for comparison. Although the SOD activity of the pure and the commercial (impure) MnTBAP samples differ by 2 orders of magnitude [18], their ability to scavenge peroxynitrite is essentially the same (Table 1). This indicates that while the redox-active impurities in the commercial samples (Mn oxo/hydroxo/acetate clusters) possess SOD activity in their own right and alter the properties of the MnTBAP sample, these impurities do not play any significant role in peroxynitrite scavenging.

We have previously observed that the ability of Mn porphyrins to scavenge peroxynitrite and dismute superoxide is governed by the closely related SAR [27] as both reactions are predominantly affected by the electron-deficiency and redox-cycling of the Mn site. It follows, thus, that $\log k_{cat}(O_2^{\bullet-})$ and $\log k_{red}(ONOO^-)$ are related by a linear relationship (Fig. 2), from which the SOD activity of pure MnTBAP may be estimated. Because of the low SOD activity of the pure MnTBAP ($\log k_{cat} < 3.50$), only an upper limit for the rate constant was accessible by the direct experimental measurement [18]. Conversely, the determination of the peroxynitrite scavenging ability of MnTBAP could be achieved accurately (Table 1). Given

the linear relationship between the SOD and peroxynitrite scavenging properties of Mn porphyrins (Fig. 2), the log k_{cat} ($O_2^{\bullet -}$) value for MnTBAP is estimated to be about 3.16.

Protection of SOD-deficient *E. coli*

SOD-deficient *E. coli* can only grow under aerobic conditions in restricted five amino acid-medium if a compound is added to substitute for superoxide dismutase enzyme. In the presence of such a functional SOD mimic, *E. coli* can, then, synthesize other essential amino acids by utilizing enzymes that are superoxide sensitive [22,23]. Neither pure nor commercial 20 or 200 μM MnTBAP protects SOD-deficient *E. coli* against superoxide toxicity when grown aerobically in restricted medium (Fig. 3), while 20 μM MnTE-2-PyP was fully protective. In M9CA, which is a richer medium, only MnTE-2-PyP was fully protective at 20 μM, whereas only a slight growth of SOD-deficient *E. coli* was observed with the commercial MnTBAP preparation at a 10-fold higher concentration (200μM) (Fig. 3). Therefore, pure MnTBAP does not substitute for SOD in either rich (M9CA) or restricted (5 amino acid) medium (Figs. 3A and 3B). The growth of *E. coli* in M9CA and 5 amino acid restricted medium in the presence and absence of compounds is shown here at 10 and 20 hours, respectively. In both media wild type was in stationary phase, while SOD-deficient *E. coli* still did not grow. We however followed the growth slightly longer and into the stationary phase to able to notice the effect of MnTBAP, if any. We have previously reported the growth curves of SOD-deficient *E. coli* [4,17], which clearly showed that MnTE-2-PyP protected and accelerated the growth of SOD-deficient mutants to a rate that is comparable to that of the wild type cells.

Effect of MnTBAP and MnTE-2-PyP on carrageenan-induced pleurisy, lung MPO activity, and histological examination

When compared to the sham-treated group, intrapleural injection of carrageenan led to the development of acute pleurisy producing turbid exudate containing a large amount of PMNs within 4 hours (Fig. 4A, B). The presence of pleural exudate and the number of inflammatory cells in the pleural cavity at 4 hours after carrageenan administration was significantly reduced by the treatment with commercial or pure MnTBAP (both at 10 mg/kg) or MnTE-2-PyP (0.3 mg/kg) (Fig. 4A, B). When tested at 0.3 mg/kg commercial or pure MnTBAP had no protective effect (n=6/group, not shown). The presence of inflammatory cells in the pleural cavity appeared to be related to the influx of leukocytes into the lung tissue. Indeed, myeloperoxidase activity was significantly elevated at 4 h after carrageenan injection in vehicle-treated mice (Fig. 4C, $p < 0.01$), and this was significantly inhibited by commercial or pure MnTBAP (both at 10 mg/kg) or MnTE-2-PyP (0.3 mg/kg) (Fig. 4C, $p < 0.01$).

No histological alterations were observed in lung tissues collected from vehicle-treated mice (Fig. 5). On the contrary, histological examination of lung sections collected at 4 h from all of carrageenan-treated mice showed tissue injury as well as inflammatory cells infiltration. Commercial or pure MnTBAP or MnTE-2-PyP significantly reduced lung injury at 4 h after carrageenan injection as evidenced by histological examination.

Effects of MnTE-2-PyP and MnTBAP administration on carrageenan-induced immunohistochemical localization of 3-nitrotyrosine in the lung tissues after pleurisy

No positive staining for nitrotyrosine (Fig. 6a) was found in lung sections from vehicle-treated mice. Enhanced staining for nitrotyrosine was evident in lung sections obtained from mice at 4h after carrageenan administration (Fig. 6b) and this was reduced by MnTE-2-PyP (Fig. 6c). The same has been observed with 10 mg/kg of commercial MnTBAP (Figure 7, [26]).

Discussion

Superoxide and its progeny peroxynitrite are among the major reactive oxygen and nitrogen species implicated in a series of physiological processes and pathological conditions [31,32]. The ability of Mn porphyrins to dismute superoxide or scavenge peroxynitrite is an intrinsic property that is quantified, by physicochemical means, via measurement of the rate constants associated with the processes.

MnTBAP has been widely used in *in vivo* animal models of oxidative stress injuries as an SOD mimic often showing protective effects. Based on kinetic and thermodynamic grounds there is no basis for such an SOD activity. Indeed we have prepared and characterized a pure MnTBAP [18] and showed that it has no SOD-like activity in aqueous system (log $k_{cat} < 3.5$, [18]; of note Cu, Zn-SOD has log k_{cat} ~9 [33–35]). We compared this pure sample to several common commercial preparations, among which the apparent log k_{cat} varied by >350-fold [18]. Furthermore, we have shown that the SOD-like activity in the commercial samples arise from impurities (Mn oxo/hydroxo/acetato complexes), whose levels differed from one batch to another even within the same supplier [18]. In addition to scavenging $O_2^{\bullet -}$, the commercial preparations also have significant ability to inhibit xanthine oxidase, which may affect superoxide levels *in vivo* not by scavenging it, but by decreasing its production [20]. Some researchers have also proposed other alternative role/s for MnTBAP, such as induction of hemeoxygenase-1 [36] or modulation of Ca^{2+} levels [37,38], which do not invoke a MnTBAP superoxide dismuting ability *per se.* The possibility of MnTBAP action *via* superoxide reductase (in aqueous media) or superoxide oxidase (in lipophilic environments, such as biological membranes) mechanisms was fully contemplated [18] and found unlikely on thermodynamic grounds [18].

Herein, as a continuation of our efforts to understand the *in vivo* effects reported on commercial MnTBAPs, we decided to determine the peroxynitrite scavenging ability of MnTBAP and to test a pure MnTBAP *in vivo*. We used two different samples to study the possible effects of MnTBAP: a pure preparation [18] and a commercial (impure) one. We utilized an *SOD-specific* model, which is based on the inability of a SOD-deficient *E. coli* strain (JI132) to grow under aerobic conditions in the absence of a suitable SOD mimic [21–23], and a mouse carrageenan-induced pleurisy model, where inflammation has been previously ascribed at least in part to the involvement of superoxide and peroxynitrite [26,39].

Because of the nature of its formation and decomposition, $ONOO^-$ cannot be directly measured *in vivo* [40] and investigators have been relying on markers of its production. To this end, detection of the formation of 3-nitrotyrosine has been widely used for over a decade as a "footprint marker" to verify its presence [40]. However, several other *in vivo* sources of nitrating oxidants exist (for example, hydrogen peroxide, nitrite and myeloperoxidase [40–42]. Therefore, detection of 3-nitrotyrosine *in vitro* or *in vivo*, can be reliably used as a surrogate "footoprint marker" for $ONOO^-$, only, if it is blocked pharmacologically with agents that remove superoxide (i.e., superoxide dismutase mimetics), nitric oxide (i.e., nitric oxide synthase NOS inhibitors) and specifically by catalytic scavengers of $ONOO^-$ such as metalloporphyrins [40,43,44]. We have previously demonstrated that the expression of spinal 3-nitrotyrosine during the development of morphine antinociceptive tolerance is derived from *in situ* formation of $ONOO^-$ since it was blocked by each class of the above-mentioned pharmacological agents targeting $ONOO^-$ indirectly or directly [45].

SOD-deficient *E. coli* strain (JI132) exhibits several auxotrophies and grows slowly aerobically as it has little protection against superoxide; it grows, however, as well as wild type (AB1157) under anaerobic conditions or in the presence of a compound that is able to behave as an SOD mimic and, thus, substitute for the SOD enzyme [22,23]. Such a behavior has

provided us with a very useful model to quickly screen the *in vivo* activity of SOD mimics [3,4,14,16,17,19]. *E. coli* experiment undoubtedly shows that neither commercial nor pure MnTBAP preparations at 20 or 200 μM levels can substitute for the SOD enzyme in both nutrient-restricted and rich medium (Fig. 3). The potent SOD mimic, MnTE-2-PyP (log k_{cat} = 7.76 [4]), allows full growth of SOD-deficient *E. coli* at 20 μM (Fig. 3). The very low SOD-like activity of the commercial (Alexis) MnTBAP (k_{cat} = 5.16, Table 1 [18]), which arises from its impurities (SOD-active Mn oxo/hydroxo/acetato complexes [18]), is clearly insufficient to protect SOD-deficient *E. coli* (Fig. 3). The same inefficacy of MnTBAP has been found with two other SOD-specific systems, MnSOD [46], and Cu, ZnSOD [47] knock-out yeast where Calbiochem MnTBAP was used.

Pleurisy model

Carrageenan-induced local inflammation is commonly used to evaluate non-steroidal anti-inflammatory drugs (NSAID). Therefore, carrageenan-induced local inflammation (pleurisy) is a useful model to asses the contribution of mediators involved in vascular changes associated with acute inflammation. In particular, the initial phase of acute inflammation (0–1h) which is not inhibited by NSAID such us indomethacin or aspirin, has been attributed to the release of histamine, 5-hydroxytryptamine and bradykinin, followed by a late phase (1–6h) mainly sustained by prostaglandin release and more recently has been attributed to the induction of inducible cyclooxygenase (COX-2) in the tissue [25]. The development of inflammation in response to carrageenan has also been associated with increased pleural exudate production, myeloperoxidase activity, accumulation of polymorphonuclear cells in pleural cavity and the production of several reactive species including superoxide, nitric oxide and peroxynitrite resulting in increased nitrotyrosine formation [13]. Indeed, carrageenan-induced inflammation is markedly reduced in neutropenic animals and in animals treated with either superoxide dismutase mimics such as M40403 or M40401, inhibitors of nitric oxide synthase or peroxynitrite scavengers [24,25,48–52]. The effect of such compounds on the decreased neutrophil infiltration may occur through various pathways including inhibition of the expression of adhesion molecules, activation of redox-sensitive transcription factors and generation of cytokines [13]. Such pathways would likely involve the positive loop of the oxidative injury: the decreased levels of ROS/RNS by porphyrins would modulate cellular transcription activity resulting in decreased inflammatory cell and cytokine recruitment which would in turn decrease levels of secondary ROS/RNS. The data obtained herein show that an efficient SOD mimic and peroxynitrite scavenger, MnTE-2-PyP (at dose of 0.3 mg/kg) decreased exudate volume, MPO activity, infiltration of PMNs and nitrotyrosine levels (Figures 4,5, and 6). At the same dose MnTBAP was ineffective. Similar anti-inflammatory effects were observed with MnTBAP but at doses at least 33-fold higher (10 mg/kg) than those used in the MnTE-2-PyP group (Figures 4,5,6 and 7) [26]. The anti-inflammatory effects of the impure (commercial) MnTBAP sample were just slightly more pronounced than those obtained with pure MnTBAP (Figures 4,5 and 6). Our results confirm the general notion [40] that whatever the origin of the beneficial effect of MnTBAP is, it is significantly lower when compared to MnTE-2-PyP. Most importantly, they show that while pure MnTBAP has no SOD-like activity in aqueous systems [18] and is not efficient in $O_2^{\bullet-}$-specific systems (*i.e.*, the SOD-deficient *E. coli* model), it is able to diminish oxidative stress to some extent. Such data point to other possible roles of MnTBAP in oxidative stress injuries. Furthermore, the data also indicate that MnTBAP exert some protective actions in common with MnTE-2-PyP, which are not $O_2^{\bullet-}$-related.

Due to the complexity of the mouse pleurisy model when compared to a more straightforward SOD-deficient *E. coli* system, the actual roles of MnTBAP in modulating oxidative stress is less clear-cut. In aqueous systems, the commercial MnTBAP sample has a moderate SOD activity (associated with its impurities) and is able to inhibit considerably xanthine oxidase

[18], which may reduce, therefore, *in vivo* levels of $O_2^{\bullet -}$ and H_2O_2 and indirectly the levels of peroxynitrite. Such inhibition of xanthine oxidase, however, is not observed in the case of MnTE-2-PyP [4] and is considerably smaller with pure MnTBAP [18]. The effect of pure MnTBAP on other enzymatic systems or the modulation of Ca^{2+} levels [37,38] have not been explored. We also suggested [18] the possibility that MnTBAP may localize (upon protonation) in lipid membranes, *where* due to the different oxidation potential of the $O_2^{\bullet -}/O_2$ couple [53], it may act [18,54] as a superoxide scavenger and protect against lipid peroxidation [18]. This, however, can occur only if MnTBAP localizes in relatively acidic cellular compartments *and* if such compartments are major sources/targets of ROS/RNS.

As an additional difference between MnTBAP and MnTE-2-PyP is that the latter is readily reduced *in vivo* by a number of flavoenzymes [55,56]. Such a facile reduction plays a major role in the antioxidant activity of this and similar Mn(III) *ortho N*-alkylpyridylporphyrins [56]; thus MnTE-2-PyP may remove O_2^- *in vivo* through flavoenzyme-superoxide oxido-reductase activity rather than superoxide dismutase activity. Further, such a reduction, allows Mn(II) porphyrins to reduce peroxynitrite by two electrons thus avoiding the formation of nitrogen dioxide, and to reduce carbonate radical 5 to 10 times faster than the Mn(III) compounds. So far we have not found any biological reductant capable of reducing Mn(III) TBAP to Mn(II)TBAP and this limits the redox activity of MnTBAP to being oxidized by peroxynitrite or carbonate radical. In Figure 8 we illustrate the differential reactivity between the two porphyrins discussed herein.

At present, the *in vivo* data presented here can be fully rationalized if one considers that the common biological role shared by MnTBAP and MnTE-2-PyP is related to their peroxynitrite scavenging activities and the reduction of carbonate radical. The log k_{cat} ($O_2^{\bullet -}$) value for MnTBAP is estimated to be about 3.16 (based on the relationship given in Fig. 2), which is ~5 and ~7 orders of magnitude smaller than the SOD activity of the potent SOD mimic MnTE-2-PyP and Cu, Zn-SOD, respectively. This very low value indicates that MnTBAP is very inefficient at dismuting superoxide to be of any biological impact, which was confirmed in the SOD-deficient *E. coli* model. Worthwhile noting, however, is that peroxynitrite scavenging ability of MnTBAP is only ~2.5 orders of magnitude smaller than that of MnTE-2-PyP and is not significantly affected by the presence of the SOD-active impurities in commercial MnTBAP sample. Additionally, the reduction of carbonate radical by MnTBAP (log k_{red} $CO_3^{\bullet -}$ = 9.1) and MnTE-2-PyP (log k_{red} $CO_3^{\bullet -}$ = 8.5 for $Mn^{III}P$ and 9.5 for $Mn^{II}P$) is comparably fast [27]. These observations have profound biological repercussions as they may suggest that the effect of MnTBAP may conceivably relate to peroxynitrite and carbonate radical scavenging. The previous report on carrageenan induced pleurisy with same dose of commercial MnTBAP used in this study showed a significant decrease in levels of nitrotyrosine (Figure 7, [26]). The effect on nitrotyrosine levels was also reported in another model, carrageenan-induced-paw edema. MnTBAP further decreased nitrotyrosine levels in cultured H9C2 cardiomyocytes following a brief exposure to peroxynitrite and decreased nitrotyrosine formation in *in vivo* myocardial ischemia-reperfusion [57]. The dose of MnTBAP required to yield oxidative stress protection, and decrease damage from peroxynitrite, in the pleurisy model is >1.5 orders of magnitude higher than that of MnTE-2-PyP, which could be related to the smaller ability of MnTBAP to scavenge peroxynitrite and the lesser impact of carbonate radical which is formed only after peroxynitrite reacts with CO_2. The slightly better protection observed with the commercial MnTBAP sample (relative to the pure MnTBAP one) could arise from its impurities, which by scavenging $O_2^{\bullet -}$ [18], reduce consequently the overall peroxynitrite, and secondary ROS/RNS levels. Of note is that a direct translation of the relative *in vitro* properties (rate constants) of MnTBAP and MnTE-2-PyP to their relative *in vivo* effects, particularly dosing requirements is most likely unattainable, as these porphyrins are by their own chemical nature, which governs uptake and biodistribution (e.g., overall charge, unrelated functional groups, size), considerably different. A direct comparison between the

levels of these porphyrins in tissue was not easily achievable, as there is no reliable method for the determination of MnTBAP levels in biological samples and adaptation of the recently reported method for MnTE-2-PyP *via* reductive Mn to Zn transmetallation [1] to the MnTBAP case is far from straightforward (e.g., the ascorbate-reduction of MnTBAP to its Mn(II) analogue is precluded by its the very low Mn^{III}/Mn^{II} reduction potential [18]). Because the development of *anionic* Mn porphyrin-based compounds (such as MnTBAP) as SOD mimic is severely hampered by unfavorable electrostatics [19], the positively-charged MnTE-2-PyP and related analogues [3,14] are still the most reliable positive controls as potent SOD mimics and $ONOO_2^-/CO_3^{\bullet-}$ scavengers.

In very practical terms, the parallel study of MnTE-2-PyP (as an indicator of both ROS and RNS) and pure MnTBAP (as an indicator of RNS) in an oxidative stress model may conceptually be useful in distinguishing the major contributions of ROS vs RNS pathways, as summarized in Table 2. ((Table 2))

Concluding remarks

In summary, physicochemical, *in vitro*, and *in vivo* data suggest that, whereas MnTBAP is not an SOD mimic, its overall *in vivo* efficacy may arise from its role as peroxynitrite scavenger. This may shed some light on the controversial effects reported for MnTBAP and justify further studies, particularly mechanistic ones, given that pure compounds are utilized or the levels and properties of the impurities in the samples are well established and characterized. Provided that *pure* MnTBAP is unable to dismute superoxide at any significant extent [18] but is able to partially scavenge peroxynitrite and carbonate radical, this compound may prove a valuable tool to distinguish the major contributions of $ONOO^-$-dependent from $O_2^{\bullet-}$-dependent pathways.

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Abbreviations Charges are omitted for clarity

$MnTBAP^{3-}$
Mn(III) *meso*-tetrakis(*p*-carboxylatophenyl)porphyrin

$MnTE\text{-}2\text{-}PyP^{5+}$
Mn(III) *meso*-tetrakis(*N*-ethylpyridinium-2-yl)porphyrins (AEOL10113)

SOD-deficient
E. coli JI132

SOD-proficient
wild type *E. coli* AB1157

$MnTSPP^{3-}$
Mn(III) *meso*-tetrakis(*p*-sulfonatophenyl)porphyrin

$MnBr_8TSPP^{3-}$
Mn(III) β-octabromo-*meso*-tetrakis(*p*-sulfonatophenyl)porphyrin

MnP
: manganese porphyrin

MPO
: myeloperoxidase

PMN
: polymorphonuclear leukocyte

CAR
: carrageenan

SOD
: superoxide dismutase

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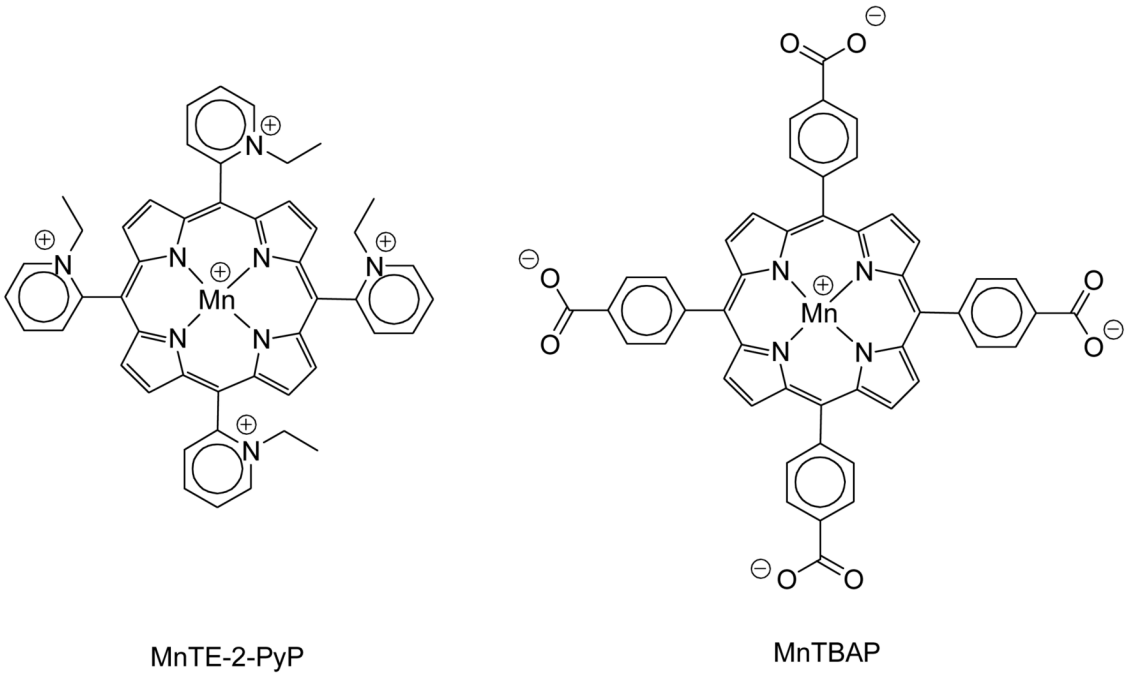

Figure 1.
Structural diagram of MnTE-2-PyP and MnTBAP.

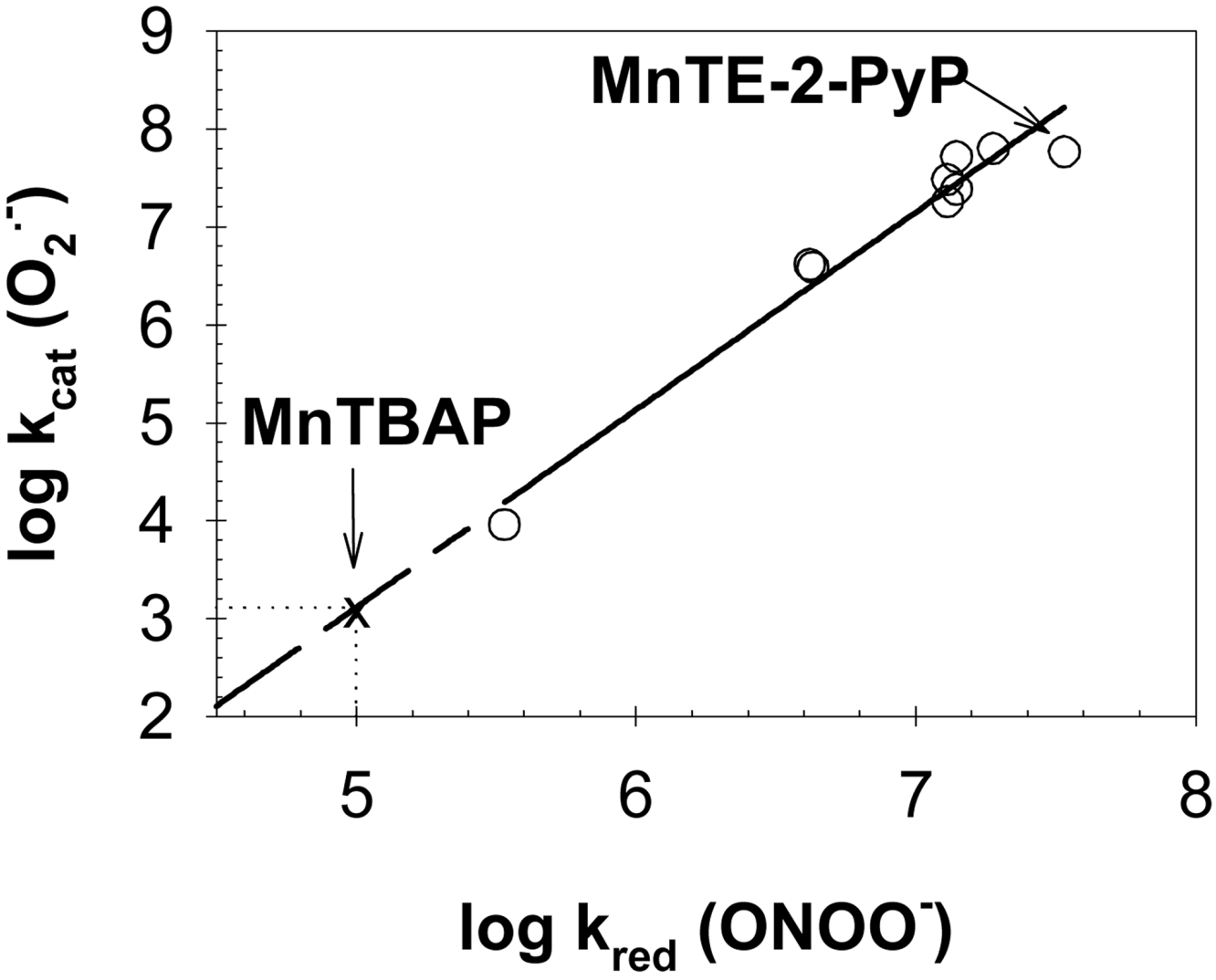

Figure 2.
Linear relationship between the SOD-like and peroxynitrite scavenging activities of Mn porphyrins ($y = -6.98 + 2.02\ x$; $R^2 = 0.96$). Data points from [27]. The MnTBAP point was not included in the linear regression as log k_{cat} ($O_2^{\bullet -}$) was too low to be measured accurately (< 3.50); this value was estimated to be ~3.16 based on log k_{red} ($ONOO^-$) of 5.02 determined for pure MnTBAP (see Table 1).

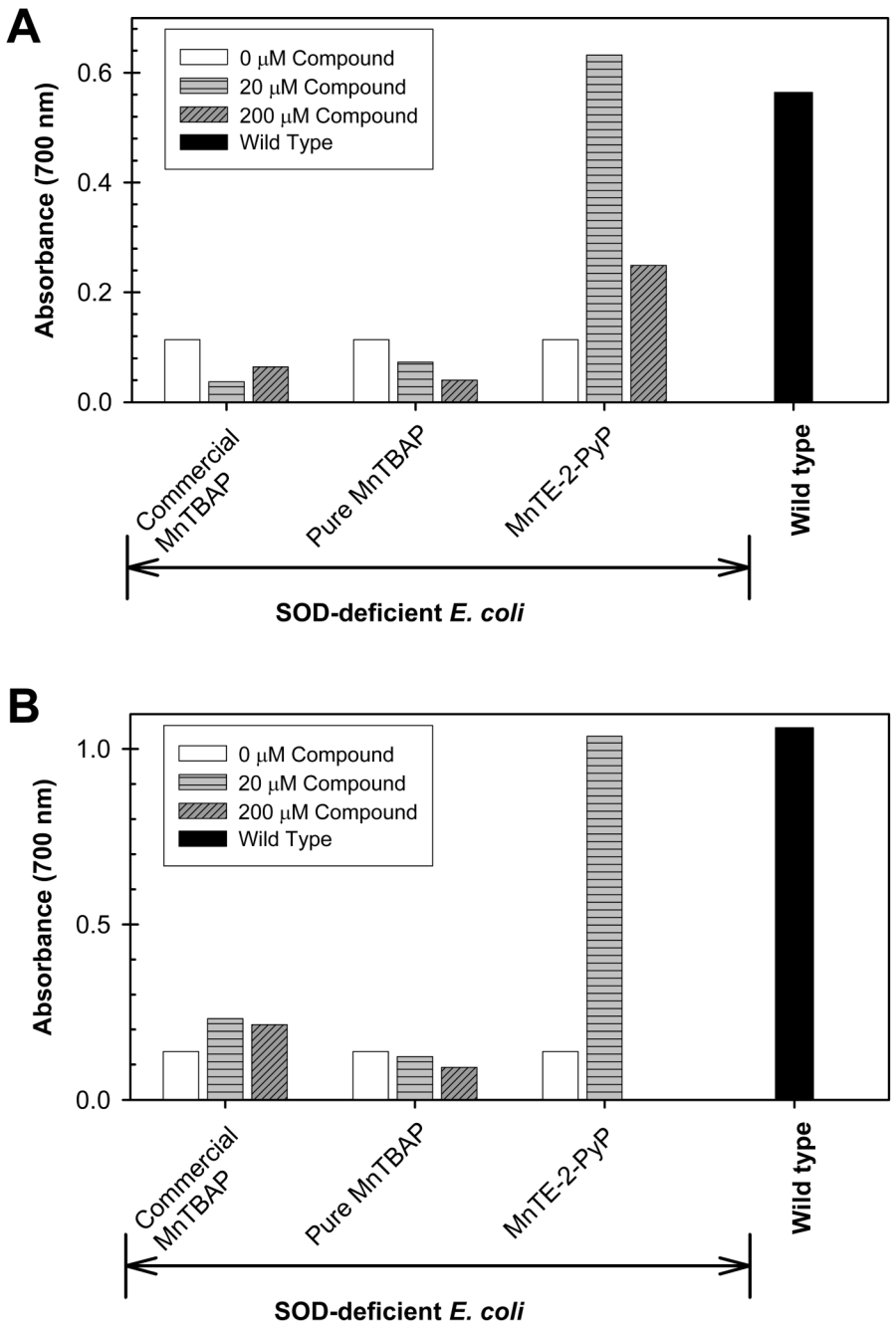

Figure 3.

Aerobic growth of SOD-deficient (JI132) and wild type (SOD-proficient, AB1157) *E. coli* strains in the presence and absence of 20 or 200 μM of commercial MnTBAP, pure MnTBAP, and MnTE-2-PyP in (**A**) 5 amino acid-medium after 20 hours, and (**B**) in nutrient rich M9CA medium at 10 hours. Figures are representative of at least 3 experiments performed on different days.

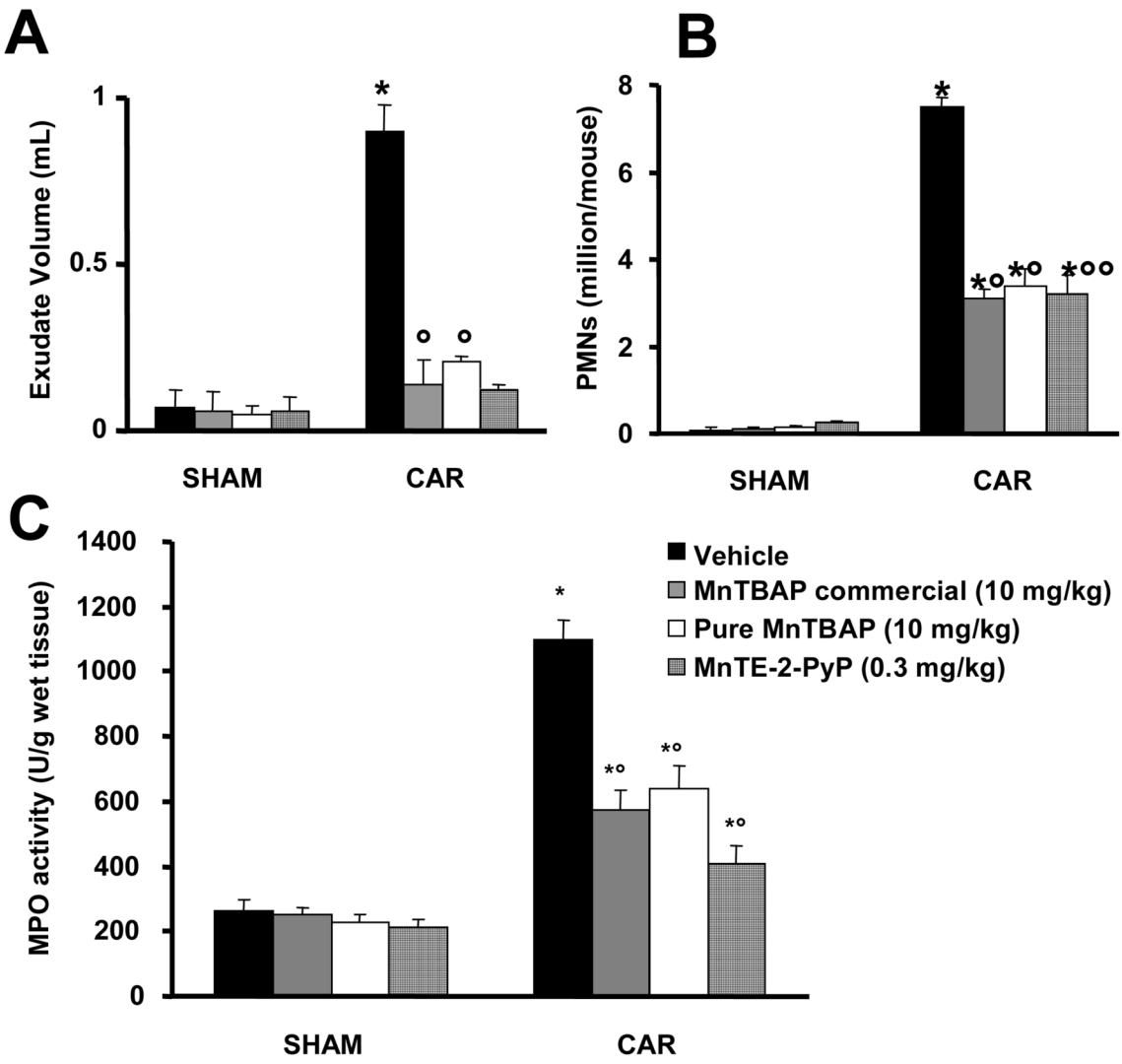

Figure 4.

Effects of commercial MnTBAP, pure MnTBAP, and MnTE-2-PyP on carrageenan-induced pleural exudate production, accumulation of polymorphonuclear cells in pleural cavity and myeloperoxidase activity (MPO). A significant production in pleural exudate (**A**), polymorphonuclear and (**B**) cells infiltration was observed in pleural cavity from vehicle-treated mice at 4h after carrageenan administration. Furthermore, MPO activity in lung tissues was significantly elevated at 4 h after carrageenan administration in vehicle-treated mice (**C**). The treatment with commercial or pure MnTBAP, and MnTE-2-PyP significantly reduced the presence of pleural exudate (**A**) and the number of inflammatory cells (**B**) as well as lung tissue levels of MPO (**C**). Data are means ± SEM of 10 mice for each group. *$p < 0.01$ *vs.* SHAM; °$p < 0.01$ *vs.* carrageenan group.

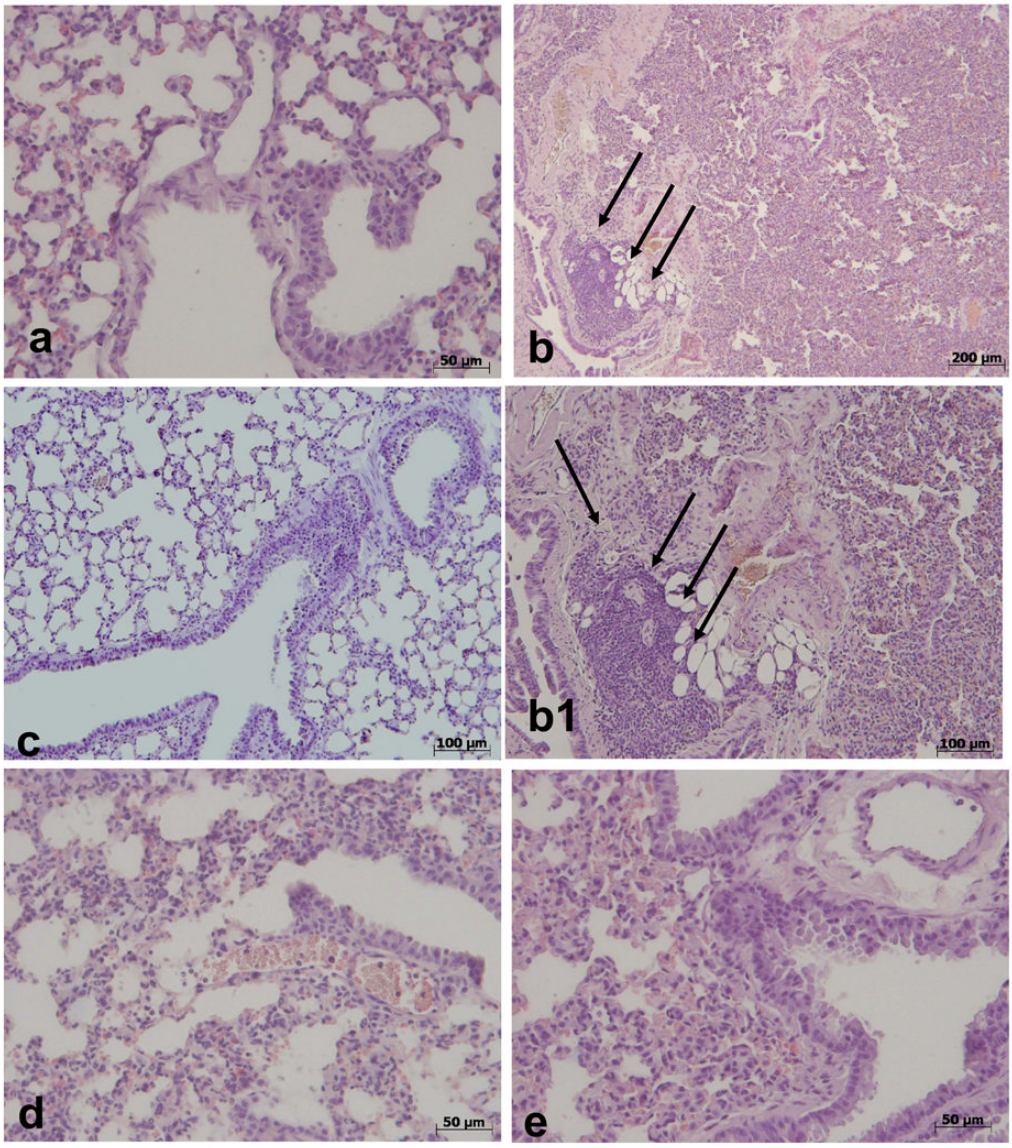

Figure 5.

Effects of commercial MnTBAP, pure MnTBAP and MnTE-2-PyP on lung injury. Lung tissues collected at 4 h were stained with hematoxylin and eosin. No injury was observed in the lung tissues collected from sham (vehicle-treated) mice (**a**). Histological examination of lung sections collected at 4 h from all carrageenan-injected mice showed tissue injury as well as inflammatory cells infiltration (**see arrows, b and b1**). The treatment of mice with commercial MnTBAP (**c**), pure MnTBAP (**d**), and MnTE-2-PyP (**e**) significantly reduced the lung injury at 4 h after carrageenan injection. Figure is representative of at least 3 experiments performed on different days.

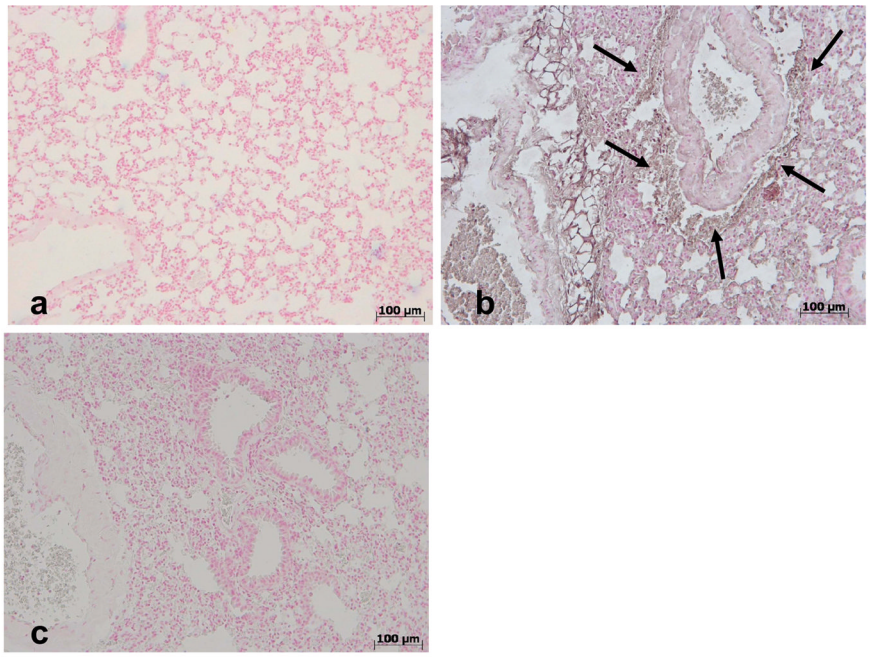

Figure 6.
Effects of MnTE-2-PyP on nitrotyrosine formation in the lung. No positive staining for nitrotyrosine was observed in lung tissues obtained from sham mice (**a**). In contrast, tissue sections obtained from carrageenan-treated mice at 4h after carrageenan administration demonstrate positive staining for nitrotyrosine (see arrows **b**) and this was blocked by MnTE-2-PyP (**c**). Figure is representative of at least 3 experiments performed on different experimental days.

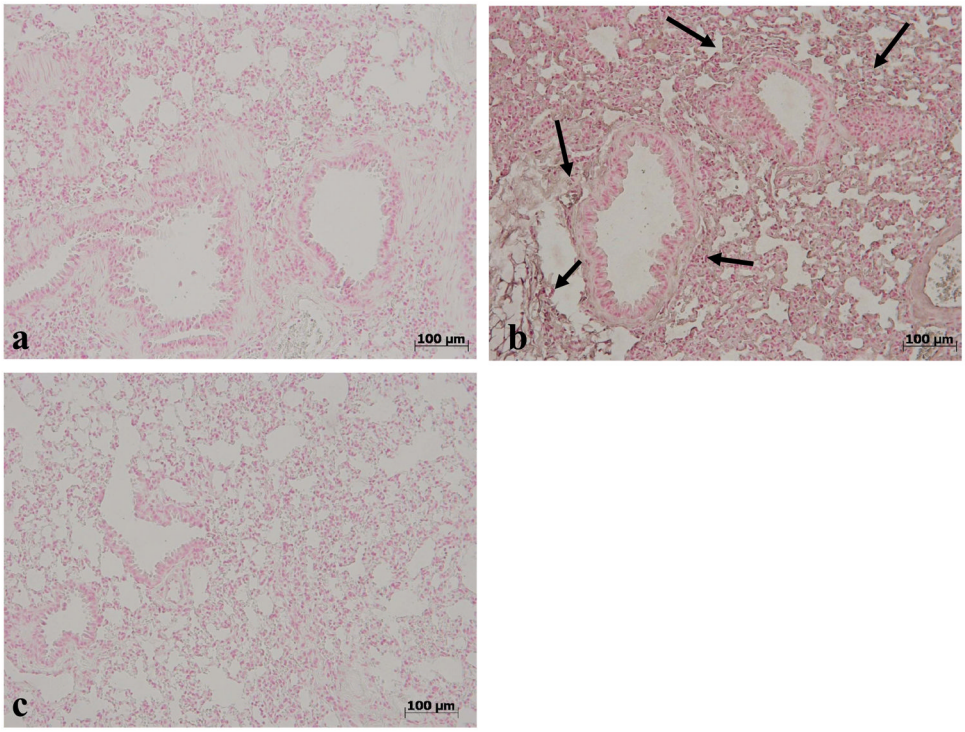

Figure 7.
Effects of commercial MnTBAP on nitrotyrosine formation in the lung. No positive staining for nitrotyrosine was observed in lung tissues obtained from sham mice (**a**). On the contrary, tissue sections obtained 4h after the administration of carrageenan demonstrate positive staining for nitrotyrosine (see arrows, **b**) and this was blocked by MnTBAP (10 mg/kg, **c**). Figure is representative of at least 3 experiments performed on different experimental days.

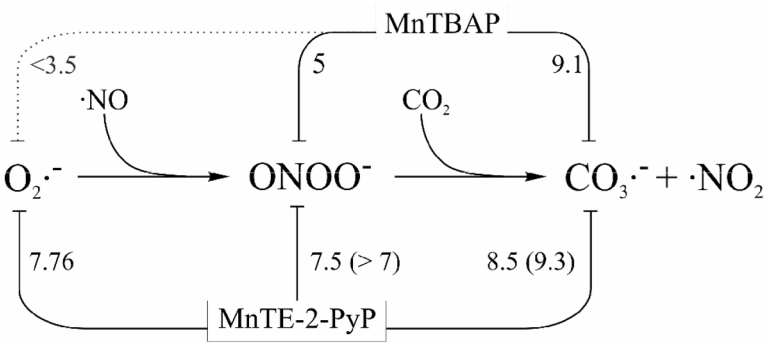

Figure 8.
Comparison of the reactivity of MnTBAP and MnTE-2-PyP. The numbers are the log k for the Mn(III) porphyrins and the reactants linked with each line (numbers in parenthesis correspond to the reactions with the corresponding Mn(II) species). Data are from [4,18,27, 55] and this work.

Table 1

The SOD-like (k_{cat} for $O_2^{\bullet-}$ dismutation),, peroxynitrite and carbonate radical scavenging (k_{red} for the reduction of $ONOO^-$ and $CO_3^{\bullet-}$) properties of Mn(III) porphyrins and Cu, Zn-SOD.

Compound	log k_{cat} ($O_2^{\bullet-}$)	log k_{red} ($ONOO^-$)	log k_{red} ($CO_3^{\bullet-}$)
MnTE-2-PyP	7.76[a]	7.53[f] (> 7)[g,h]	8.5[f] (9.3)[f,g]
Pure MnTBAP	<3.50[b] (3.16)[c,d]	5.02[d]	~9.1[j]
Commercial MnTBAP	5.16[b]	4.96[d]	9.1[f]
Cu, Zn-SOD	*ca* 9[e]	3.97[i]	–

[a] Ref. [4].

[b] Ref. [18].

[c] This work.

[d] Calculated using the log k_{cat} ($O_2^{\bullet-}$) *vs*. log k_{red} ($ONOO^-$) relationship given in Fig. 2.

[e] Ref. [33–35].

[f] Ref. [27].

[g] Value for the corresponding Mn(II) species.

[h] Ref. [55].

[i] Ref. [58].

[j] The estimated value is based on the similar reactivity of pure and commercial MnTBAP with $ONOO^-$.

Table 2

Possible use of MnTE-2-PyP and pure MnTBAP as tools in oxidative stress mechanistic studies.

RNS scavenger		**Potent ROS *and* RNS scavenger (*e.g.*, MnTE-2-PyP and congeners)**	
		Effect Observed	**Effect Not Observed**[a]
Pure MnTBAP	Effect Observed	$ONOO^-$ may be a major player	MnTBAP probably regulates ROS/RNS *via* alternative pathways (*e.g.*, enzyme inactivation; see text)
	Effect Not Observed[a]	$O_2^{\bullet -}$ may be a major player	Likely not an oxidative stress event

[a] given that cell uptake/biodistribution is granted.