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Towards the mechanisms involved in the antioxidant action of Mn^{III} [*meso*-tetrakis(4-*N*-methyl pyridinium) porphyrin] in mitochondria

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Abstract Aerobic organisms are afforded with an antioxidant enzymatic apparatus that more recently has been recognized to include cytochrome *c,* as it is able to prevent hydrogen peroxide generation by returning electrons from the superoxide ion back to the respiratory chain. The present study investigated the glutathione peroxidase (GPx), superoxide dismutase (SOD) and cytochrome *c*-like antioxidant activities of *para* Mn(III)TMPyP in isolated rat liver mitochondria (RLM) and mitoplasts. In RLM, Mn^{III}TMPyP decreased the lipid-peroxide content associated with glutathione (GSH) depletion consistent with the use of GSH as a reducing agent for high valence states of Mn^{III}TMPyP. SOD and cytochrome *c* antioxidant activities were also investigated. Mn^{II}TMPyP was able to reduce ferric cytochrome *c,* indicating the potential to remove a superoxide ion by returning electrons back to the respiratory chain. In antimicyn A-poisoned mitoplasts, Mn^{III}TMPyP efficiently decreased the EPR signal of DMPO-OH adduct concomitant with GSH depletion. The present results are consistent with SOD and GPx activities for Mn^{III}TMPyP and do not exclude cytochrome *c*-like activity. However, considering that *para* Mn^{III}TMPyP more efficiently reduces, rather than oxidizes, superoxide ion; electron transfer from the Mn^{II}TMPyP to the respiratory chain might not significantly contribute to the superoxide ion removal, since most of Mn^{II}TMPyP is expected to be produced at the expense of NADPH/GSH oxidation. The present results suggest GPx-like activity to be the principal antioxidant mechanism of Mn^{III}TMPyP, whose efficiency is dependent on the NADPH/GSH content in cells.

Keywords Manganese *meso*-tetrakis porphyrins · SOD · Rat liver mitochondria · Cytochrome *c* · Superoxide ion

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Introduction

The manganese porphyrins have been used in studies contributing to advances in the enzymology of the antioxidant enzymes, superoxide dismutase, and catalase (Meunier 1992; Batinic-Haberle 2002; Spasojevic et al. 2003; Day et al. 1997). In fact, literature data report the antioxidant effects of manganese porphyrins such as scavenging $O_2^{-\bullet}$, H_2O_2, and $ONOO^-$, as well as reaction with lipid peroxides (Patel and Day 1999; Simonson et al. 1997; Shaffer et al. 1987; Dolphin et al. 1971; Ferrer-Sueta et al. 1999, 2003, 2006). Different factors influence the catalytic mechanism of the metalloporphyrins and consequently its biological effects: capacity of membrane binding,

meso ligands, substituents, and metal center (Ferrer-Sueta et al. 2003; Inada et al. 2007; Pessoto et al. 2009).

Manipulation of the *meso* substituent has been a strategy to attain desirable antioxidant activity of manganese porphyrins. The introduction and modifications of the *meso* substituents interfere with the redox potential and electrostatic facilitation of water soluble porphyrins (Richards et al. 1996; Tabata et al. 1996; Batinić-Haberle et al. 1997).

Previous studies with Mn^{III}TMPyP (Mn^{III}5,10,15,20-[*meso*-tetrakis(*N*-methylpyridyl)porphyrin]) have demonstrated the impact of *ortho*, *meta,* and *para N*-methylpyridyl substituents on the SOD activity of these compounds. The *ortho* isomer exhibited the most efficient SOD-like activity assigned to an appropriated combination of adequate redox potential and electrostatic facilitation. (Takeuchi et al. 1994; Sen and Krishnan 1997; Giraudeau et al. 1979; Autret et al. 1996; Tagliatesta et al. 1996; Binstead et al. 1991; Gali et al. 1996). However, other studies consider the rate constant for the reduction of Mn^{III}TMPyP by superoxide ions to be ~4× 10^7 $M^{-1}.s^{-1}$, while that for the oxidation of Mn^{II}TMPyP by the same reactive species is 100-fold higher (Faraggi 1984) than the proposed Therefore, manganese porphyrins probably act in cells as a NADPH/GSH:$O_2^{-\bullet}$ oxidoreductase rather than as a SOD mimic (Faulkner et al. 1994).

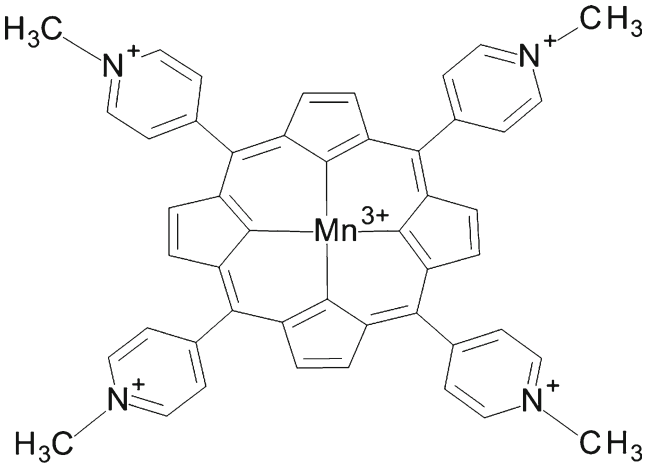
Despite the capacity of manganese porphyrins to promote superoxide ion dismutation or reduction, hydrogen peroxide is the final product either way. More recent literature has demonstrated a paradox for SOD activity. While promoting superoxide ion depletion, SOD also generates hydrogen peroxide and the effectiveness of the antioxidant protection is dependent on efficient associated activity of catalase and the GSH/GPx (gluthatione/gluthatione peroxidase) system. In this scenario, the scavenger of superoxide ions by the respiratory cytochrome *c* rises as an efficient antioxidant system because, in contrast to SOD, it is able to replace the electron back into the respiratory chain leading to complete reduction of molecular oxygen to water and not to hydrogen peroxide, a prooxidant species (Mano et al. 2009; Pereverzev et al. 2003; Goldsteins et al. 2008). In fact, catalase activity has also been considered for Mn^{III}TMPyP, but at physiological pH this mechanism is not favored (Pessoto et al. 2009). Furthermore, the capacity of GSH to reduce both resting and high valence states of the cationic *meso*-tetrakis porphyrins, and the reactivity of these porphyrins with nitrosative species and flavins (Ferrer-Sueta et al. 1999, 2003, 2006), implies that the antioxidant activity of these porphyrins is related to a multiplicity of catalytic properties. Thus it is important to also consider GPx-like activity, and in particular pay attention to a possible cytochrome *c* mimetic activity for Mn^{III}TMPyP; this latter has not yet been considered in the research. In particular, regarding the capacity of the manganese porphyrins to oxidize superoxide ions for electron transfer back to the respiratory chain, the low potential values exhibited by *meta* and *para* isomers of Mn^{III}TMPyP ($E_{1/2}$=+0.052 and +0.06 V, respectively), although unfavorable for SOD activity, is favorable to promote cytochrome *c* reduction ($E_{1/2}$=+0.25 V) (Batinić-Haberle et al. 1998).

The present study investigated GPx-, SOD-, and cytochrome *c*-like activities of *para* Mn^{III}TMPyP in isolated rat liver mitochondria (RLM) and mitoplasts.

Material and methods

Chemicals

Meso tetrakis porphyrin was purchased from Mid Century Chemicals (Posen, IL, USA). All other chemicals were purchased from Sigma Chemical Co.



Mn^{III} [*Meso*-Tetrakis(4-*N*-Methyl Pyridinium) Porphyrin

Electronic absorption spectra measurements

The electronic absorption spectra of porphyrins were measured in the course of the reaction with peroxides by using a Shimadzu Model 1501 MultiSpec (Tokyo, Japan), employing the photodiode array scan mode. The spectral resolution was 0.5 nm, and the spectra were obtained with a time interval of 1 s. The optical path length was 1 cm for all measurements.

Isolation of rat liver mitochondria

Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar rats that had fasted overnight. The homogenate was prepared in 250 mM sucrose, 1.0 mM EGTA, and 5.0 mM HEPES buffer, pH 7.2. The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA, and the final pellet was diluted in 250 mM sucrose to a protein concentration of 80 to 100 mg/ml. The protein concentration was determined by the biuret method, and modified by the addition of cholate.

Standard incubation conditions

The experiments were performed in a standard medium containing 250 mM sucrose, 10 mM HEPES, pH 7.2 and an oxidizing cocktail of NAD-linked substrates (malate, pyruvate, α-ketoglutarate, 5 mM). For lipid peroxidation experiments, osmotic support was provided by 120 mM KCl, 2 mM phosphate and 10 mM HEPES, pH 7.2, to avoid sucrose interference in the method in order to ensure an accurate estimation of lipid peroxidation. Oxygen consumption experiments were performed in a standard medium containing 250 mM sucrose, 10 mM HEPES, pH 7.2, 2 mM phosphate, 1 mM $MgCl_2$ and 0.5 mM EGTA. Other additions are indicated in the figure legends.

Mitochondrial swelling

Swelling was estimated from the decrease in apparent absorbance at 540 nm using a model U-2001 spectrophotometer Hitachi (Tokyo, Japan).

Preparation of mitoplasts

Mitoplasts were prepared from isolated mitochondria by digitonin treatment as described previously (Tagliatesta et al. 1996). Briefly, mitochondria (40 mg/ml) were mixed with an equal volume of H medium containing digitonin and stirred for 15 min on ice. The sample was diluted 6-fold with the medium and centrifuged at 10000*g* for 10 min. The pellet was washed once with H medium and the suspension centrifuged a final time (10,000*g* for 10 min).

EPR

For EPR analysis, mitoplasts (1 mg) were placed in 150 μL of the buffer (230 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl; pH adjusted to 7.4 with Mops) in the absence or presence of mitochondrial respiratory substrates or inhibitors. DMPO (160 mM) was added and the EPR spectra were recorded on a Bruker ECS106 spectrometer. Instrument settings were as follows: receiver gain, 5-10%; microwave power, 20 mW; microwave frequency, 9.81 GHz; modulation amplitude, 0.505 G; time constant, 1.3 s; scan time, 167.7 s; scan width, 100 G. The EPR traces shown in the figures are summations of five scans.

SOD activity

Xanthine oxidase was used to produce $O_2^{\bullet-}$, and the reduction of ferricytochrome *c* was its standard indicating activity (McCord and Fridovich 1969). Reduction of cytochrome *c* was followed at 550 nm. Assays were conducted in the presence and absence of 0.1 mM EDTA in 0.05 M phosphate buffer, pH 7.8. All measurements were done at 25 °C.

Lipid oxidation assays

TBARS (thiobarbituric reactive substances) production The optical density of the organic layer was determined at 535 nm. Under these conditions, the levels of TBARS were calculated as concentration of malondialdehyde (TBARS) using its molar extinction coefficient 1.56×10^5 $M^{-1}cm^{-1}$.

Lipid hydroperoxide (LOOH) measurement The oxidation of Fe^{2+} by LOOH generates Fe^{3+} that reacts with xylenol orange, forming the colored compound that absorbs at 560 nm. LOOH concentration was calculated from $\varepsilon_{560nm}=6.4\times10^4$ $M^{-1}cm^{-1}$.

Results

In highly pro-oxidant conditions, Mn^{III}TMPyP caused the exacerbation of the oxidative damages in isolated RLM

Mn^{III}TMPyP was incubated with isolated RLM for the analysis of peroxide consumption in reducing and prooxidant conditions (Fig. 1). Figure 1 shows that Mn^{III}TMPyP significantly decreased the basal peroxide content of isolated RLM. The concentration of LOOH obtained in the presence of Mn^{III}TMPyP plus GSH was

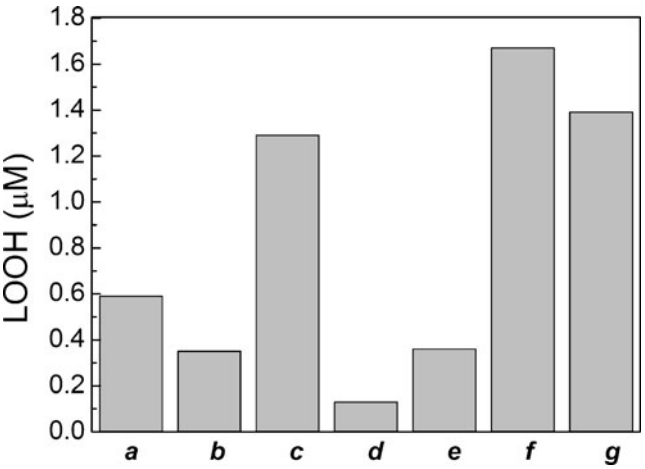
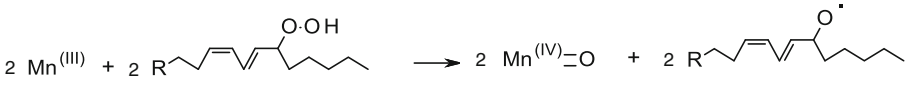


Fig. 1 LOOH content in isolated RLM incubated at different conditions. Column *a* RLM (negative control), *b* + GSH, *c* + Fe^{II} (positive control), *d* + Mn^{III}TMPyP, *e* + Mn^{III}TMPyP and GSH, *f* + Mn^{III}TMPyP and Fe^{II}, *g* + Mn^{III}TMPyP and GSH and Fe^{II}. The experimental conditions were: 1 mg/mL RLM in the presence and in the absence of 50 μM GSH; 500 μM Fe^{II} (incubated in the presence of 2 mM citrate) and 0.5 μM Mn^{III}TMPyP. LOOH concentration was calculated from $\varepsilon_{560nm}=6.45\times10^4$ $M^{-1}cm^{-1}$

higher than that obtained in the absence of the reducing tripeptide. These results suggest that, at a relatively low peroxide concentration, reduction of Mn^{III}TMPyP to Mn^{II}TMPyP at expense of GSH depletion prevents peroxide consumption by the high valence states of the porphyrin. In fact, the Mn^{II} EPR signal was detected when GSH was added to Mn^{III}TMPyP-containing mitoplasts (not shown). In RLM, LOOH and GSH compete with each other for the reaction with Mn^{III}TMPyP. In the absence of exogenous added GSH, the relatively low concentration of the endogenous tripeptide and the affinity of the porphyrin to the inner mitochondrial membrane, favor its conversion to the high valence states, as resulting from the reaction with LOOH. The high valence states of Mn^{III}TMPyP are then recycled by GSH in a GPx-like mechanism. Consistent with GSH depletion by Mn^{III}TMPyP, Fig. 1 shows also that the manganese porphyrin exacerbated the LOOH production by lipid peroxidation promoted by Fe^{II}-citrate.



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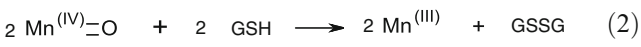

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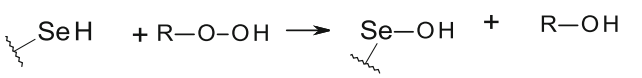

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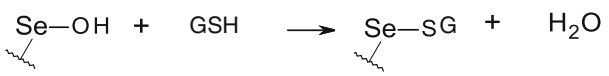

(5)

(6)

The reduction of peroxides promoted by GPx does not involve peroxide and GSH free radical intermediates. Particularly due to the association with the negatively charged inner

However, an important question was raised: could the decrease of LOOH content promoted by Mn^{III}TMPyP be considered an antioxidant effect for RLM?

To answer this question, the occurrence of mitochondrial swelling, formation of TBARs and both GSH and mitochondrial membrane SH depletion were checked in the presence of Mn^{III}TMPyP added to RLM and challenged by *t*-BuOOH and Fe^{II}-citrate (Fig. 2A–C).

Figure 2A–c shows that the LOOH consumption by Mn^{III}TMPyP showed in Fig. 1, column *d* is accompanied by a discrete mitochondrial swelling, a discrete increase in TBARS content and depletion of GSH and SH contents. The consumption of LOOH concomitant with GSH depletion is consistent with a GPx-like mechanism exhibited by Mn^{III}TMPyP. However, the deleterious effect of Mn^{III}TMPyP on RLM, evidenced by the occurrence of mitochondrial swelling, results from the different mechanisms for peroxide reduction exhibited by Mn^{III}TMPyP (Eqs. 1–3) and GPx (Eqs. 4–6).

mitochondrial membrane, Mn^{III}TMPyP can generate free radicals close to the membrane proteins, leading to the crosslink of proteins that characterizes the occurrence of mitochondrial permeability transition. Consistently, Mn^{III}TMPyP exacerbated the mitochondrial swelling as well as SH and GSH depletion caused by *t*-BuOOH (Fig. 2A and B). This result shows that the GPx-like mechanism exhibited by Mn^{III}TMPyP is not efficient in protecting mitochondria from an excess of peroxides. However, the significant consumption of the mitochondrial basal peroxide content by Mn^{III}TMPyP, decreases the extensive formation of TBARS by Fe^{II}-citrate (Fig. 2C). The low yield of TBARs content in the presence of Mn^{III}TMPyP is not a consequence of the reaction of the porphyrin with aldehydes because the porphyrin was unable to react with malonaldialdehyde and diphenylacetaldehyde (not shown). In this condition, the significant decrease of TBARs generated by Fe^{II}-citrate in the presence of Mn^{III}TMPyP suggests that HOOH was also consumed by

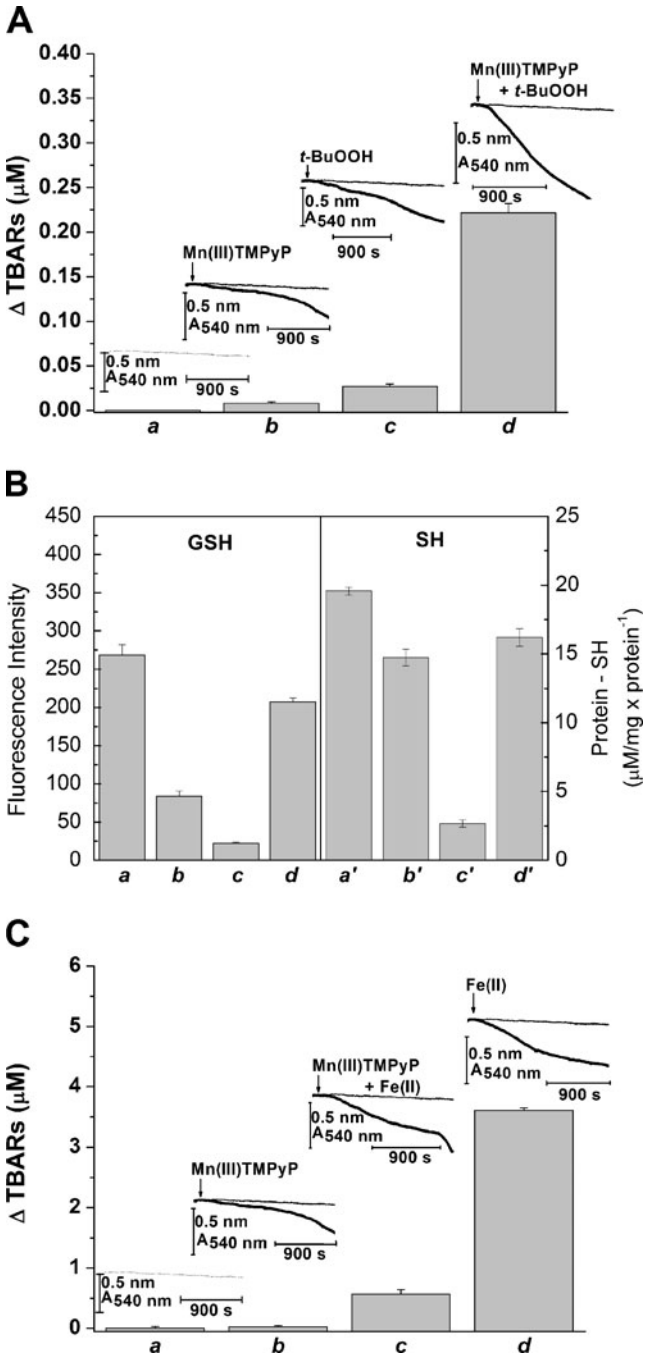

Fig. 2 Effect of $Mn^{III}TMPyP$ on isolated RLM challenged by pro-oxidant conditions. **A** Mitochondrial membrane TBARS induced by *t*-BuOOH. **B** GSH and SH content of RLM challenged by *t*-BuOOH at different conditions. *a* and *a′* RLM, *b* and *b′* + *t*-BuOOH, *c* and *c′* + *t*-BuOOH and $Mn^{III}TMPyP$, *d* and *d′* + $Mn^{III}TMPyP$. The experimental conditions are 0.4 mg/mL RLM; 500 μM*t*-BuOOH and 20 μM $Mn^{III}TMPyP$. **C** Mitochondrial membrane TBARs induced by Fe^{II}-citrate. The experimental conditions were: 1 mg/mL RLM, 2 μM $Mn^{III}TMPyP$, 500 μM Fe^{II}, incubated in the presence of 2 mM citrate. The insets of panels A and C show mitochondrial swelling at the corresponding conditions but with 0.4 mg/mL RLM, 800 μM*t*-BuOOH, 20 μM $Mn^{III}TMPyP$, 50 μM Fe^{II}. Levels of TBARs were calculated as concentration of TBARs using the molar extinction coefficient ε_{535nm} 1.56×10^5 $M^{-1}cm^{-1}$

$Mn^{III}TMPyP$, decreasing the generation of $OH^{\bullet}$. In RLM, due to the availability of axial ligands for manganese, it is possible that the porphyrin can promote heterolytic scission of the *O-O* bond, leading to a less deleterious peroxide consumption. However, the protective effect of $Mn^{III}TMPyP$ against lipid damage promoted by Fe^{II}-citrate could also be associated to the scavenger of superoxide ion.

The inefficient SOD activity has been assigned to *para* $Mn^{III}TMPyP$, because this compound has a low potential value ($E_{1/2}$=+0.06 V) in comparison with the *ortho* isomer ($E_{1/2}$=+0.22 V), a potential value close to that exhibited by SOD (Batinić-Haberle et al. 1998). However, the low redox potential of *para* $Mn^{III}TMPyP$ implicates the manganese porphyrin has the capacity to transfer electrons to cytochrome *c* and to cytochrome *c* oxidase.

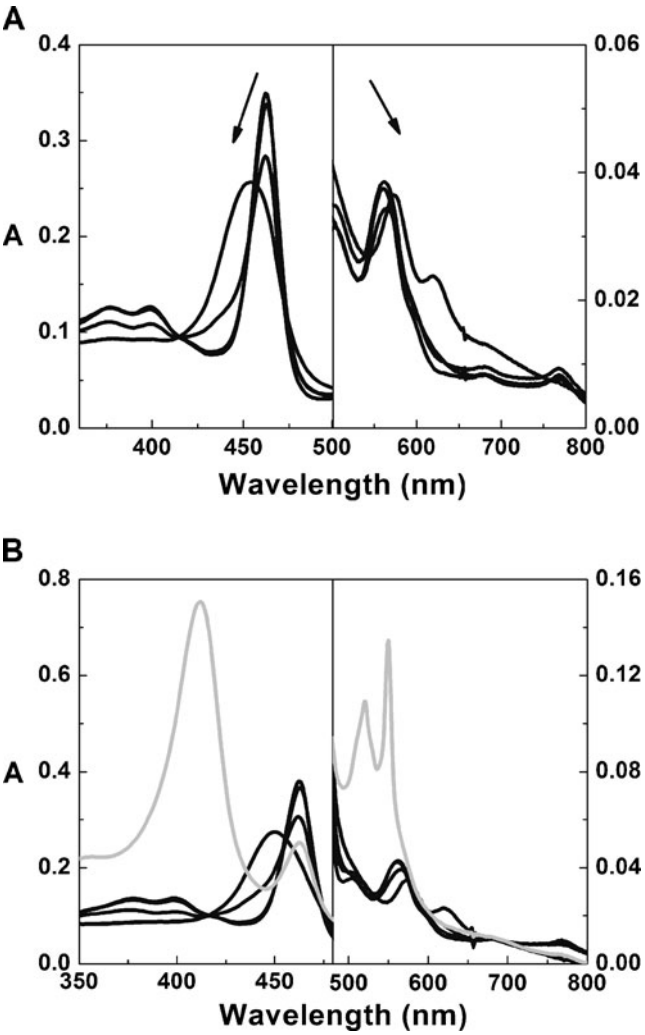

Fig. 3 Changes in the electronic absorption spectra of $Mn^{III}TMPyP$ and cytochrome *c* in the presence of xanthine and xanthine oxidase. **A** Spectra of 16 μM $Mn^{III}TMPyP$ with 800 μM xanthine, immediately after the addition of 0.6 U/mL xanthine oxidase and 600 s after the addition of reagents as indicated by the *arrows*. **B** The same conditions as in A, except 16 μM cytochrome *c* was added after 600 s incubation

Therefore, in sequence, the protective effect of Mn^{III}TMPyP against superoxide ions was also investigated when considering both SOD and cytochrome *c* mechanisms.

Mn^{III}TMPyP is reduced by superoxide ions and reoxidized by ferric cytochrome c

Figure 3A shows that Mn^{III}TMPyP is one electron-reduced by the superoxide ion generated by the xanthine/xanthine oxidase system (X/XO). The porphyrin was incubated with X/XO, and after xanthine depletion, Fe^{III}cytochrome *c* was added to the medium. The addition of Fe^{III}cytochrome *c* to the Mn^{II}TMPyP solution led to the reoxidation of the porphyrin concomitant with cytochrome *c* reduction (Fig. 3B).

In sequence, the effect of Mn^{III}TMPyP on mitoplasts challenged by superoxide production was investigated.

Mn^{III}TMPyP abolishes ion superoxide EPR signal at expenses of GSH depletion

The SOD activity of Mn^{III}TMPyP was investigated by using superoxide ion detection by EPR-DMPO spin trapping measurements of the antimycin A (AA)-poisoned mitoplasts, a model reported by Derick et al. 2001 for accessing the vectorial release of the superoxide ion. The model is based on data from previous literature that reported the production of superoxide ions by intact mitochondria and submitochondrial particles in the presence of AA (Boveris et al. 1976), which has been assigned to an increase in the steady-state levels of the ubisemiquinone pool caused by the inhibition of electron transfer at the UQ_I site in the Q cycle. In the present study, these measurements were associated with concomitant GSH and SH dosage.

Figure 4A, line *a*, shows the basal composite EPR signal exhibited by mitoplasts respiring on the endogenous substrate and resulting from the basal oxidative processes normally occurring in the organelle. The basal EPR signal was not increased by the addition of Mn^{III}TMPyP (Fig. 4A, line *b*). As expected, the spectral component corresponding to the DMPO-OH adduct signal (a^H=14.91; a^N=14.92 EPR, line width = 1.13 Gauss, inset Fig. 4A) increased in the presence of AA associated or not to succinate addition (Fig. 4, lines *c* and *d*, respectively). One more EPR signal with parameter a^N=14.90; a^H=1.05 and Gaussian line width = 1.05 was also detected in all conditions and corresponds to an unknown species. Superoxide dismutase drastically decreased the EPR signal of the DMPO-OH adduct in the absence and in the presence of succinate (Fig. 4, lines *e* and *f*, respectively), consistent with a DMPO-OH adduct formed by the spontaneous decay of a DMPO-superoxide adduct (DMPO-OOH) (Finkelstein et al. 1980). In subsequent experiments, SOD was replaced by 2 μM Mn^{III}TMPyP in mitoplast samples containing AA

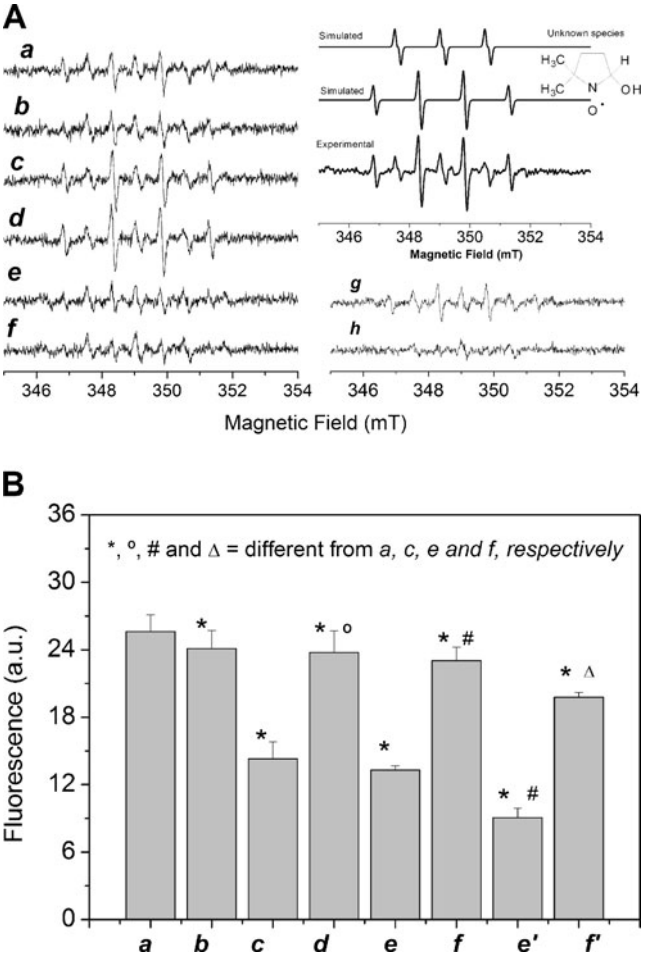

Fig. 4 The comparative effects of SOD and Mn^{III}TMPyP on the superoxide ion generation and GSH depletion in AA-poisoned mitoplasts. **A** The effect of SOD and Mn^{III}TMPyP on DMPO-OH adduct : *a* mitoplasts, *b* + Mn^{III}TMPyP, *c* + AA, *d* + AA and succinate, *e* + AA and SOD, *f* + AA and succinate and SOD, *g* + AA and Mn^{III}TMPyP, *h* + AA and succinate and Mn^{III}TMPyP. The inset shows the experimental EPR composite spectra from mitoplasts incubated with AA and succinate and the indicated simulated components. **B** Effect of SOD and Mn^{III}TMPyP on the corresponding GSH content: *a* mitoplasts, *b* + Mn^{III}TMPyP, *c* + AA, *d* + AA and succinate, *e* + AA and SOD, *f* + AA and succinate and SOD, *e′* + AA and Mn^{III}TMPyP, *f′* + AA and succinate and Mn^{III}TMPyP. The experimental conditions were: 1 mg/mL mitoplasts; 2 mM succinate; 10 μM EGTA; 20 μM Mn(III)TMPyP; 1 μM AA and 2 μM catalase

and AA plus succinate and the results are presented in Fig. 4A, lines *g* and *h*, respectively. Interestingly, in the presence of AA (Fig. 4A, line *g*), Mn^{III}TMPyP was distinctly less efficient than SOD but totally abolished the DMPO-OH signal in the presence of AA plus succinate (Fig. 4A, line *h*). This result is consistent with a GSH-dependent efficiency of Mn^{III}TMPyP.

The GSH (Fig. 4B) and SH (not shown) contents were determined for mitoplasts samples submitted to the same experimental conditions used for EPR measurements, except the presence of DMPO. Mn^{III}TMPyP led to a discrete but

significant decrease in GSH content probably by using NADPH/GSH to be converted to the reduced form. In mitoplasts treated with AA and AA plus succinate, the presence of SOD was associated with significant GSH depletion. However, in the presence of succinate the GSH content was 40% higher. A partial protective effect of succinate against lipid peroxidation of RLM had been reported (Tretter et al. 1987); however this paradoxical result was probably a consequence of GSH production by the deglutathionylation of SQR-SSG (glutathionylated succinate coenzyme Q reductase) that has been associated with superoxide ion production in mitochondria (Chen et al. 2007). The Mn^{III}TMPyP-promoted decreasing of the DMPO-OH signal was also associated with GSH depletion. The GSH depletion detected in the presence of Mn^{III}TMPyP was 30 and 15% higher than that promoted by SOD in the presence of AA and AA plus succinate, respectively. In the present study, the generation of superoxide ion in AA-poisoned mitoplasts in the absence and presence of the additives was not associated with a significant decrease of mitochondrial membrane SH content (not shown). This result suggests that the consumption of GSH as a reducing agent for peroxides and free radicals was efficient in preventing significant oxidation of the protein SH content. However, it is important to note that, although not associated with a decrease in protein SH content, the conditions that unequivocally led to the depletion of GSH make RLM more vulnerable to subsequent oxidative stressing events.

Discussion

Data from the literature show protective effects of metalloporphyrins associated with distinct antioxidant properties such as the scavenging of $O_2^{-\bullet}$, H_2O_2, $ONOO^-$ and reaction with lipid peroxides (Inada et al. 2007; Batinić-Haberle et al. 1998; Fariss et al. 2005). The properties of metalloporphyrins are strongly dependent on the central metal, axial ligands, structure and position of *meso* ligands and topology in biological membranes mediated by the hydrophobicity and net charge (Inada et al. 2007; Pessoto et al. 2009; Araujo et al. 2010). The SOD mimicking of Mn^{III}*N*-alkylpyridylporphyrins have been credited to the presence of positive charges in a close proximity to the metal center, providing thermodynamic and kinetic facilitation for the reaction with negatively charged superoxides (Faulkner et al. 1994).

Based on a consensus of SOD activity, most of the literature concerned with the biological effects of MnTMPyP has reported protective effects in cultured cells and SOD null E.coli and assigned them to the catalysis of superoxide ion dismutation (Faulkner et al. 1994; Wang et al. 2004; Liang et al. 2009; Batinić-Haberle et al. 2010; Strathmann et al. 2010; Sharma and Gupta 2007). However, from comparative studies with the negative partner, we have observed that the structural characteristics facilitating the reduction of Mn^{III}TMPyP by ion superoxides also favored the reduction by GSH. Consistently, previous studies by Faulkner et al. (Faulkner et al. 1994) credited the ability of Mn^{III}TMPyP and other manganese porphyrin to protect SOD null E coli upon manganese complexes, acting like ion superoxide oxidoreductases rather than as superoxide dismutases. According to this mechanism, NADPH and GSH reduce Mn^{III}TMPyP, which, in the reduced form, catalyzes the reduction of the ion superoxide to hydrogen peroxide.

The results presented here using the model of vetorial ion superoxide production (Derick et al. 2001), associated with GSH (Fig. 4C) and SH (not shown) dosages, are consistent with SOD, GPx-like and superoxide ion oxidoreductase as proposed by Faulkner et al. 1994, because all of them are expected to deplete GSH. However, the less efficient decrease of the superoxide ion signal, associated with a more intense GSH depletion promoted by Mn^{III}TMPyP in comparison with SOD, strongly suggests that the porphyrin acts as GPx, oxidoreductase or both. In fact, at high GSH/NADPH levels, the reduction of Mn^{III}TMPyP leads the porphyrin to act as a superoxide oxidoreductase. The oxidoreductase activity of Mn^{II}TMPyP on superoxide ions increases the hydrogen peroxide concentration. At decreasing GSH concentrations, Mn^{III}TMPyP can act as a peroxidase leading to more intense GSH depletion. Also, the present results do not support an efficient catalase or cytochrome c antioxidant mechanism because all of them are expected to preserve GSH content. In Fig. 3A and B, it was demonstrated that, in vitro, ion superoxide can reduce Mn^{III}TMPyP, and that the reduced manganese porphyrin can transfer one electron to cytochrome c. However, the low potential of para Mn^{III}TMPyP, relative to SOD and cytochrome c, is thought to make the manganese porphyrin poorly competitive with these enzymes regarding superoxide ion oxidation. However, whatever the alternative mechanism is leading to the formation of Mn^{II}TMPyP, the reduced manganese porphyrin can use one reducing equivalent to reduce the superoxide ion to hydrogen peroxide and ferric cytochrome c to the ferrous form. The latter reaction has a partially protective role since the reduction of cytochrome c prevents the reaction of the hemeprotein with peroxides and consequent free radical production. Therefore, the ion superoxide oxidoreductase mechanism and the reduction of ferric cytochrome c are dependent on a reducing microenvironment with high availability of NADPH and GSH as provided by cytosol. Consistently, protective effects of Mn^{III}TMPyP have been observed for cells (Liang et al. 2009; Batinić-Haberle et al. 2010; Strathmann et al. 2010; Sharma and Gupta 2007; Lee and Park 2004; Lee et al. 2005) and for isolated RLM. Protective effects have been inferred from punctual meas-

urements such as TBARS (Inada et al. 2007) or have not been found (Pessoto et al. 2009). In the present study, a decrease of Fe^{II}-induced TBARS content by Mn^{III}TMPyP was also found, but the concomitant slight increase in the LOOH content, associated with the innocuous effect against mitochondrial swelling, suggests a delay in the lipid peroxidative process rather than effective antioxidant activity. Previously, we had studied the biological effects of anionic Fe^{III} and Mn^{III}TPPS4 [(meso-tetrakis (para-sulfonatophenyl) porphyrins], in RLM modulated by the metal center; efficient antioxidant activity for the manganese anionic partner was not also found (Pessoto et al. 2009). Differently of Mn^{III}TMPyP that exacerbated the swelling caused by t-BuOOH (Fig. 2A), Mn^{III}TPPS4 delayed this event. These different effects could be related to the reactivity of the cationic and anionic porphyrins with GSH. When challenged by peroxides, the more reactive cationic porphyrin probably leads to a faster GSH depletion than the anionic partner. Therefore, the biological effects of Mn^{III}TMPyP can be realized in the following scenario. In conditions in which there is increased production of superoxide ions, such as ischemia and reperfusion, para Mn^{III}TMPyP that was previously reduced by NADPH and/or GSH could act as a superoxide reductase by reducing superoxide ions to hydrogen peroxide. It is important to note that this is a less efficient antioxidant mechanism than that exhibited by SOD, since the elimination of one mol of superoxide ion is accompanied by stoichiometric consumption of reducing agents such as NADPH and GSH. In addition, the elimination of the hydrogen peroxide produced by superoxide ion reduction via the GPx mechanism will consume additional reducing equivalents. The reoxidation of the Mn^{II}TMPyP to reduce superoxide ion to HOOH is associated with a decrease of cell reducing agents NADPH and GSH in a condition in which the peroxide level was enhanced by SOD and superoxide ion reductase (Mn^{III}TMPyP) activities. This condition can make a large contribution to the oxidative damage of cells. The peroxidase activity of Mn^{III}TMPyP in a condition of GSH depletion is expected to generate free radicals even if the heterolytic cleavage of peroxides is prevalent. The generation of free radicals occurs because the recycling of high valence states of the manganese porphyrin back to the resting form can occur at expenses of LOOH and LOH oxidation. Furthermore, although Mn^{II}TMPyP can efficiently transfer one electron to Fe^{III}cytochrome c and cytochrome c oxidase, it is important to consider that the majority of the reduced porphyrin was formed by NADPH and GSH oxidation, rather than by superoxide ion oxidation. In this context, protective reducing equivalents present in GSH and NADPH are replaced to water molecules, a poor reducing agent. Therefore, the protective effects of Mn^{III}TMPyP observed in the cells result from the access of porphyrin to the reducing environment provided by the cytosol.

Taken together, the literature data and the present study indicated that a complete SOD activity is not necessarily desirable to provide antioxidant properties for manganese porphyrins against superoxide ions. The dismutation of superoxide ions by manganese porphyrins suggests two steps: oxidation of superoxide ions generating the reduced form of the porphyrin and subsequent reduction of another superoxide ion to hydrogen peroxide, with recycling of the porphyrin to its resting state. However, for manganese porphyrins with low redox potential, the first step is not favored and the oxidation of the superoxide ion to molecular O_2 is preferentially performed by SOD and cytochrome *c*. The reduced forms of these porphyrins are formed at the expense of GSH and NADPH, and their recycling to the resting form by the reduction of superoxide ions favors the oxidative stress, spends cell-reducing equivalents and produces hydrogen peroxide. In this regard, in a comparative study of the protective effects of *ortho meta* and *para,* Mn^{III}TMPyP Batinić-Haberle et al. 1998, reported protective effects of the *ortho* isomer for the SOD null E coli and toxic effects for *para* Mn^{III}TMPyP. It is important to consider that due to the high redox potential of *ortho* Mn^{III}TMPyP, the protective effects could result not only from a complete SOD activity, but also from cytochrome *c*-like activity. After being reduced by a superoxide ion, one part of the reduced porphyrin content could be reoxidized by the respiratory chain in a cytochrome *c*-like mechanism. In fact, the toxic effects of *para* Mn^{III}TMPyP cannot be explained exclusively by low SOD activity, but they are expected in a superoxide ion reductase activity at the expenses of reducing equivalents, as the prevalent mechanism.

The present study points to desirable characteristics to be searched for in a drug design targeting the superoxide ion. Rather than a SOD-like activity, a cytochrome *c*-like activity is desirable.

Conclusion

Inside cells and in mitochondria, Mn^{III}TMPyP is expected to be converted to the reduced form at the expense of the NADPH/GSH content. Mn^{II}TMPyP acts as a superoxide ion reductase generating hydrogen peroxide. The consumption of hydrogen peroxide by GPx and Mn^{III}TMPyP leads to additional GSH depletion. In highly prooxidant conditions in which a significant depletion of NADPH/GSH was installed in association with the increase of LOOH content, Mn^{III}TMPyP can also act as a peroxidase and contributes to the oxidative stress. Therefore, the protective action of Mn^{III}TMPyP against superoxide ion and peroxides is limited by GSH levels.

The capacity of Mn^{II}TMPyP to promote one-electron transfer to cytochrome *c,* and the consequent potential to return electrons to the respiratory chain, does not characterize an efficient antioxidant mechanism since, in mitochondria, the reduced form of the porphyrin is generated predominantly by NADPH/GSH, rather than by the superoxide ion.

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