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Impact of SOD-Mimetic Manganoporphyrins on Spin Trapping of Superoxide and Related Artifacts

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Abstract

The superoxide dismutase (SOD)-mimetic effectiveness of [meso-tetrakis (R)porphyrinato]manganese with R = 1,3-di-*N*-ethylimidazolium-2-yl (Mn-TDEIP), 1,3-di-*N*-methylimidazolium-2-yl (Mn-TDMIP), 1,3-di-*N*-propylimidazolium-2-yl (Mn-TDPIP), *N*-ethyl-2-pyridyl (Mn-T2EPyP), 4-sulphonatophenyl (Mn-TSP), 1-methyl-4-pyridyl (Mn-T4PyP), 4-carboxyphenyl (Mn-TBAP), and β -octabromomeso-tetrakis(4-carboxyphenyl porphyrinato)manganese (MnBr₈TBAP) was compared with Cu, Zn SOD. Superoxide generated by reaction of xanthine oxidase with hypoxanthine was trapped with 5-tert-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BMPO), forming BMPO–OOH, which was monitored by electron paramagnetic resonance. Manganoporphyrins with redox potentials ranging from –0.190 to 0.346 V relative to the standard hydrogen electrode were selected for this study. With 0.1 μ M manganoporphyrins and 20 mM BMPO, the effectiveness of the manganoporphyrins in inhibiting formation of BMPO–OOH increases in the order Mn-TSP < Mn-TBAP < MnBr₈TBAP < Mn-T4PyP < Mn-T2EPyP < Mn-TDEIP ~ Mn-TDMIP ~ Mn-TDPIP ~ Cu, Zn SOD. However, at higher concentrations of manganoporphyrin and BMPO, a BMPO–OH signal was observed. The formation of BMPO–OH was not inhibited by catalase or dimethylsulfoxide, which demonstrated that it was not produced from hydroxyl radical. The artifactual formation of BMPO–OH is attributed to oxidation of the water adduct of BMPO by the manganoporphyrins or decomposition

of BMPO–OOH. Although spin trapping is an effective method for evaluating SOD-mimetic efficacy, caution must be exercised to ensure that artifact signals are not interpreted improperly.

1 Introduction

There currently is substantial interest in identifying and analyzing pharmaceuticals that mimic the properties of antioxidant enzymes. One such example is Mn-TDEIP [manganese(III) meso-tetrakis(1,3-di-*N*-ethylimidazolium-2-yl)porphyrin], which has been shown to have activity similar to that of metalloprotein superoxide dismutases (SOD) [1]. Low-molecular-weight mimetics are of particular interest because of their ability to cross cellular membranes, unlike SOD enzymes, which cannot cross because of their large size [2]. Because of these properties, low-molecular-weight catalytic antioxidants may ultimately be used for treatment of disorders in which high-level oxidative stress is initiated in cells. Earlier studies of rats have shown that Mn-TDEIP extends the life span of animals with amyotrophic lateral sclerosis (ALS) [3], prevents the toxicity of the sulfur mustard gas analogue 2-chloroethyl ethyl sulfide in cultured pulmonary epithelial cells and in vivo [4, 5], and decreases pulmonary fibrosis due to radiation poisoning [6–8]. Manganoporphyrins with other substituents also have been shown to possess SOD activity [1].

The effectiveness of manganoporphyrins as SOD-mimetics has been measured by inhibition of cytochrome *c* reduction [9, 10]. Spin-trapping studies of reactions involving radicals have the advantage that characteristic signals can be monitored for individual radical species and therefore radical chemistry can be elucidated directly. The scavenging of superoxide radical by the antioxidant manganoporphyrin, Mn-TBAP, has been demonstrated by spin trapping [11], but spin trapping has not been used to determine the SOD-mimetic effectiveness of other manganoporphyrins. Manganoporphyrins (Table 1) with redox potentials ranging from –0.190 to 0.346 V relative to the standard hydrogen electrode were examined in this study by spin trapping. Superoxide was generated by reaction of xanthine oxidase with hypoxanthine [12–15]. The effectiveness of each complex at 0.1 μ M in decreasing the concentration of spin-trapped superoxide was monitored by electron paramagnetic resonance (EPR) spectroscopy. At higher concentrations of the manganoporphyrins, artifactual production of the hydroxyl radical adduct of the spin trap was observed.

2 Materials and Methods

The spin trap 5-tert-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BMPO) was synthesized and purified in the lab of Dr. Gerald Rosen, University of Maryland [19]. Cu, Zn SOD was provided by DDI Pharmaceuticals (Mountain View, CA, USA). Manganoporphyrins were gifts from Aeolus Pharmaceuticals (Mission Viejo, CA) and obtained from the following sources: Mn-4PyP (AEOL 10110) and Mn-TBAP (AEOL 10201) (Porphyrin Products, Logan, Utah); Mn-T2EPyP (AEOL 10113), Mn-TDMIP (AEOL 10123), Mn-TDEIP (AEOL 10150) and Mn-TSP (AEOL 10203) (Albany Molecular Research); and Mn-TDPIP (AEOL 10158) (Johnson Matthey Pharma Services, Devens, MA, USA). MnBr₈TBAP (AEOL 10216) was synthesized by R. Kachadourian, National Jewish Health [20]. All other reagents were purchased from Sigma-Aldrich (Milwaukee, WI, USA). The activity of xanthine oxidase with xanthine was measured spectrophotometrically using cytochrome *c* reduction at 550 nm [21].

2.1 EPR Spectroscopy

X-band continuous-wave EPR spectra were recorded with a Bruker EMX Plus spectrometer (Bruker BioSpin, Billerica, MA, USA) at room temperature using an ER4119 HS resonator. Samples were contained in Pyrex capillaries with outer diameter (OD) of 1 mm supported in

4 mm OD quartz tubes. Instrument parameters were: sweep width, 80 G; center field, 3362.6 G; power, 20.0 mW; frequency, 9.4 GHz; gain, 5.02×10^4 ; modulation frequency, 100.0 kHz; time constant, 41 ms; sweep time, 42 s. A modulation amplitude of 0.50 G was used to optimize resolution of small hyperfine splittings and 1.00 G was used for quantitating signals. Peak heights were calculated based on simulations through the noise. Replicate experiments indicate that uncertainties are about 10% of the values measured.

2.2 Spin Trapping of Superoxide

BMPO was selected for the superoxide trapping experiments because of the longer half-life of BMPO–OOH than of other superoxide spin adducts [19, 22]. Superoxide was generated by reaction of xanthine oxidase with hypoxanthine [12, 13] at pH 7.4. The hypoxanthine concentration was held constant at 300 μ M. To select conditions for the SOD-mimetic studies the concentration of xanthine oxidase was varied between 0.0125 and 0.075 units/mL. For concentrations between 0.0125 and 0.050, superoxide radical measured by spin trapping with BMPO increased linearly ($n = 4$). To ensure that the spin-trapping experiments were performed in a linear response regime, a xanthine oxidase concentration of 0.025 units/mL was selected. Reactions took place in 100 mM KPO₄ buffer (pH 7.4) with 1 mM diethylene-triaminepentaacetic acid (DTPA). To check the kinetics for formation and decay of BMPO–OOH, the magnetic field was held constant, and the amplitude of the BMPO–OOH signal was monitored as a function of time; the results agreed with those previously reported by Zhao et al. [22]. The maximum signal intensity was observed 8 min after mixing.

For the SOD-mimetic experiments, the manganoporphyrin concentrations were 0.1 μ M. This low value was selected to be in the range where differences in effectiveness could be distinguished. At higher concentrations of manganoporphyrins, the concentrations of BMPO–OOH were low and difficult to distinguish for most of the mimetics. The BMPO concentration was held constant at 20 mM, which gave adequate signal-to-noise ratio. The amplitudes of the EPR signals were time-dependent. For reproducibility, the scans were initiated at 90 s after mixing, and three scans (42 s/scan) were signal-averaged.

2.3 Artifact Analysis

While searching for optimum concentrations of BMPO and manganoporphyrin for the SOD-mimetic experiments, it was observed that BMPO–OH was formed at higher concentrations of BMPO and manganoporphyrin. To examine the possibility of artifact signals, manganoporphyrin concentrations were varied between 0.5 and 10 μ M, and BMPO concentrations were varied between 20 and 100 mM.

3 Results and Discussion

Reactive free radicals, such as superoxide, persist in aqueous solution for very short times, making direct detection difficult. Reaction with a spin trap produces a longer-lived radical that can be detected by EPR [23, 24]. BMPO is particularly effective for trapping superoxide, and produces a distinctive EPR signal [22, 25]. Superoxide was produced by the reaction of xanthine oxidase with hypoxanthine and trapped with BMPO. The resulting BMPO–OOH EPR signal (Fig. 1A) was in good agreement with that reported by Zhao et al. [22]. Figure 1 shows the effects of both authentic and mimetic SODs on the concentration of BMPO–OOH. Addition of Cu, Zn SOD (Fig. 1J) eliminated all traces of BMPO–OOH. The relative effectiveness of inhibition (Fig. 1) increases in the order Mn-TSP (90%) < Mn-TBAP (86%) < MnBr₈TBAP (74%) < Mn-T4PyP (63%) < Mn-T2EPyP (27%) < Mn-TDEIP ~ Mn-TDMIP ~ Mn-TDPIP ~ Cu, Zn SOD, which correlates with increasing redox potential (Table 1). The numbers in parentheses are the percentages of BMPO–OOH signal

remaining, relative to control (Fig. 1A). The correlation between effectiveness of the manganoporphyrins as SOD-mimetics and redox potential is consistent with prior studies [10].

These in vitro spin-trapping experiments demonstrated that at 0.1 μM concentrations, three manganoporphyrins, Mn-TDEIP, Mn-TDMIP, and Mn-TDPIP, which have redox potentials close to that of Cu, Zn SOD, greatly decreased the concentration of BMPO-OOH. The manganoporphyrins with redox potentials further from that of Cu, Zn SOD were less effective. The relative efficiencies in cells may be significantly different from in vitro due to the importance of lipophilicity and transport across membranes as evaluated in other studies [7, 26].

Spin trapping with BMPO has an advantage over the cytochrome *c* spectrophotometric reduction assay (550 nm) that is commonly used to detect superoxide [9, 27] because an EPR signal that is characteristic of trapped superoxide is monitored.

3.1 Artfactual Formation of BMPO-OH

An artifactual signal of BMPO-OH dominated the EPR spectra at higher concentrations of either BMPO or Mn-TDEIP. The characteristic signal for BMPO-OH [22] has different hyperfine splitting and persists longer than BMPO-OOH. The intensity of the artifact increased in a nearly linear manner when the Mn-TDEIP concentration was increased between 2.5 and 10 μM and BMPO was held constant at 20 mM. Spectra obtained with 2.5 and 5 μM Mn-TDEIP with 20 mM BMPO are shown in Fig. 2A and B, respectively. Signal intensity also grew in a linear manner when the Mn-TDEIP concentration was held constant and BMPO was increased to 100 mM (Fig. 2C). The detection of BMPO-OH raised the question whether hydroxyl radical was formed and trapped. Addition of catalase (1,000 units/mL) did not prevent the formation of BMPO-OH (Fig. 2D), which indicated that the artifact was not formed from H_2O_2 . Additionally, a system containing only BMPO and Mn-TDEIP (Fig. 2E) also produced a BMPO-OH signal. The signal resulting from only BMPO and Mn-TDEIP was further examined (Fig. 3A). Again, the addition of catalase (1,000 units/mL) had no effect on the signal. If the BMPO-OH was the result of hydroxyl radical trapping, dimethylsulfoxide (DMSO) should be a competitive inhibitor [24]. However, the addition of DMSO had no impact on the amplitude of the BMPO-OH signal (Fig. 3C). The addition of 500 units/mL Cu, Zn SOD (Fig. 3D) inhibited the BMPO-OH signal, suggesting that superoxide plays a role in the artifact formation. The addition of catalase with Cu, Zn SOD (Fig. 3E) also inhibited the signal formation. No signal resulted from the addition of 500 units/mL Cu, Zn or Mn SOD and BMPO (not shown), which indicated that manganoporphyrins are required for the artifact formation.

The other manganoporphyrins tested showed similar artifact signals but to differing degrees relative to Mn-TDEIP. For example, MnBr₈TBAP (Fig. 2F) produced a diminished BMPO-OH signal relative to that of Mn-TDEIP.

Earlier work has shown that careful control experiments must be performed when using nitron spin traps, such as BMPO or 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), in solutions that contain redox-active compounds. DMPO can undergo nucleophilic attack by water and then react with mild oxidants, such as Fe^{3+} or quinones, to form DMPO-OH [28, 29]. Based on DMPO spin trapping, it was originally proposed that hydroxyl radical was produced by the reaction of H_2O_2 and Cu, Zn SOD [30]. It was subsequently shown that much of the DMPO-OH signal in that system was produced by nucleophilic attack and subsequent oxidation [31]. One source of the observed BMPO-OH signal is attributed to nucleophilic attack on BMPO by water with subsequent oxidation by the manganoporphyrin complex. The redox process forms Mn(II) porphyrins, which are then reoxidized to the

resting Mn(III) form, producing superoxide. Support for this mechanism is provided by the presence of the artifact signal with only Mn-TDEIP and BMPO in the reaction mixture; the addition of xanthine oxidase and hypoxanthine caused the artifact to grow. The approximately linear dependence of the BMPO–OH concentration on both manganoporphyrin and BMPO is consistent with a bimolecular reaction that is first order in each reagent. BMPO–OH can also be formed by decomposition of DMPO–OOH [32], and by analogy decomposition of BMPO–OOH. These alternate pathways to the formation of BMPO–OH must be considered when dealing with redox-active compounds, such as manganoporphyrins, to ensure that the results are correctly interpreted.

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Fig. 1.

Impact of SOD and SOD-mimetics on O_2^- concentrations monitored by spin trapping. All reaction mixtures contained 20 mM BMPO, 300 μ M hypoxanthine, 0.025 units/mL xanthine oxidase, and KPO_4 buffer (pH 7.4). Plus *A* control, *B* 0.1 μ M Mn-TDEIP, *C* 0.1 μ M Mn-TDMIP, *D* 0.1 μ M Mn-TDPIP, *E* 0.1 μ M Mn-T2EPyP, *F* 0.1 μ M MnBr₈TBAP, *G* 0.1 μ M Mn-4PyP, *H* 0.1 μ M Mn-TBAP, *I* 0.1 μ M Mn-TSP, *J* 0.1 μ M Cu, Zn SOD

Fig. 2.

Impact of higher concentrations of manganoporphyrins on the artifactual signal. *A* 20 mM BMPO, 300 μ M hypoxanthine, 0.025 units/mL xanthine oxidase, 2.5 μ M Mn-TDEIP in KPO_4 /DTPA buffer, *B* same components as in *A* but with 5 μ M Mn-TDEIP, *C* same components as in *A* but with 100 mM BMPO and 5 μ M Mn-TDEIP, *D* same components as in *A* but with addition of 1,000 units/mL of catalase, *E* same components as in *A* but without xanthine oxidase and hypoxanthine, and *F* same components as in *A* but substituting 2.5 μ M MnBr₈TBAP

Fig. 3.

Impact of Cu, Zn SOD, catalase, and DMSO on the artifactual signal. *A* 50 mM BMPO, 2.5 μ M Mn-TDEIP in KPO₄ buffer, *B* same components as in *A* but with addition of 1,000 units/mL catalase, *C* same components as in *A* but with addition of 5 mM DMSO, *D* same components as in *A* but with addition of 500 units/mL Cu, Zn SOD, *E* same components as in *D* but with addition of 1,000 units/mL catalase

Table 1

SOD-mimetic manganoporphyrins

Abbreviation	R ₁	R ₂	Redox potential (V) ^b
Mn-TDEIP		H	+0.346 [16]
Mn-TDMIP		H	+0.320 [16]
Mn-TDPIP		H	+0.320 [16]
Mn-T2EPyP		H	+0.228 [9, 16]
MnBr ₈ TBAP		Br	+0.128 [17]
Mn-4PyP		H	+0.060 [18]
Mn-TSP		H	-0.160 [10]
Mn-TBAP		H	-0.190 [18]

The porphyrins have the structure shown below with the R groups listed in the table

^aE_{1/2} relative to the standard hydrogen electrode. The value of E_{1/2} for MnSOD is +0.35 V