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NITROXIDE SOD-MIMICS: MODES OF ACTION

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Low molecular weight superoxide dismutase mimics have been shown to afford protection from oxidative damage. Such SOD-mimics can readily permeate cell membrane achieving sufficiently high levels both inside and outside the cell to effectively detoxify intracellular $O_2^{\cdot-}$. Preliminary findings also indicated that metal-based and metal-free SOD-mimics can protect hypoxic cells from H_2O_2-induced damage. The present study explored the possibility that SOD-mimics such as desferrioxamine-Mn(III) chelate [DF-Mn] or cyclic nitroxide stable free radicals could protect from $O_2^{\cdot-}$-independent damage. Killing of monolayered V79 Chinese hamster cells was induced by H_2O_2 or by t-butyl hydroperoxide (t-BHP) and assayed clonogenically. Neither catalase nor native SOD protected the cells from t-BHP. In contrast, both DF-Mn and cyclic nitroxides protected suggesting cytotoxic processes independent of $O_2^{\cdot-}$ or of $O_2^{\cdot-}$-derived active species. The inhibition of the damage by both metal-free and metal-based SOD mimics is attributable to either SOD-mimic reacting with reduced transition metal to block the Fenton reaction and/or intercepting and detoxifying intracellular organic free radicals.

KEY WORDS: Superoxide, superoxide dismutase mimic, ESR, spin-labels, nitroxides, t-butyl hydroperoxide, cytotoxicity.

ABBREVIATIONS: ESR, electron spin resonance; t-BHP, t-butyl hydroperoxide; TP, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; DTPA, diethylenetriamine pentaacetic acid; DF, desferrioxamine; XO, xanthine oxidase; HX, hypoxanthine; Cyt-c^{III}, ferricytochrome c; DF-Mn, desferrioxamine-Mn(III).

INTRODUCTION

The deleterious effects of superoxide-derived reactive species and $O_2^{\cdot-}$ itself along with the physiological role of superoxide dismutase (SOD) are well documented.[1] In particular, attention has focused on SOD and its protective effects against various pathogenic conditions such as ischemia/reperfusion or inflammation.[2] Since SOD might be antigenic and because its delivery into the cells is limited, attempts were concentrated on synthesis of non-immunogenic, stable, low molecular weight SOD mimics that readily enter cells.[3–5] Certain chelates of transition metals were found to possess SOD-like activity and to protect various biological systems against oxidative damage.[6–8] Such chelates, however, might dissociate and lose their SOD-like activity, and even have the potential to become cytotoxic by participating in Fenton reactions.[3,4] To preempt such problems metal-independent SOD-mimics would be desirable. Cyclic nitroxide stable free radicals which are commonly used in electron spin resonance (ESR) spectroscopy to probe molecular motion in membranes and whole cells, transmembrane potential, and intracellular oxygen and pH,[9,10] were recently found to react with $O_2^{\cdot-}$ radical.[11] Both 5- and 6-membered ring nitroxides oxidize $O_2^{\cdot-}$ yielding oxygen and the respective ESR-silent cyclic hydroxylamines which are then reoxidizable to the parent nitroxides.[11,12]

Recent studies showed that nitroxide stable free radicals protect against oxidative damage.[13,14] Since both the nitroxide and its respective hydroxylamine react with $O_2^{\cdot-}$, the protective effect is attributable to the removal of $O_2^{\cdot-}$. Preliminary experiments indicated, however, that both metal-based and metal-free SOD-mimics also protect cells from H_2O_2 in hypoxic conditions where $O_2^{\cdot-}$ radical is most likely absent. To study other possible modes of protection from $O_2^{\cdot-}$-independent damage, cell killing by H_2O_2 under hypoxia or by t-BHP was determined. Both oxic and hypoxic protective effects of nitroxide were investigated and compared with those manifested by desferrioxamine-Mn(III) 1:1 chelate (DF-Mn). The present results show that nitroxides as well as DF-Mn protect cells from $O_2^{\cdot-}$-independent damage. The possible mechanisms responsible for cell protection are discussed.

MATERIALS AND METHODS

Chemicals

Desferrioxamine (DF) was a gift from Ciba Geigy; hypoxanthine (HX) was purchased from Calbiochem; MnO_2 and 4-hydroxy-2,2,6,6-tetramehtylpiperidine-1-oxyl (TP) were purchased from Aldrich Chemical Co.; xanthine oxidase (EC 1.2.3.2 xanthine: oxygen oxidoreductase) (XO) grade III from buttermilk, superoxide dismutase (SOD), t-butyl hydroperoxide (t-BHP), and ferricytochrome c (Cyt-c^{III}) were obtained from Sigma; H_2O_2 was bought from Fisher. Xanthine oxidase was further purified on a G25 sephadex column. All chemicals were prepared and used without further purification. The 1:1 green complex of DF-Mn(III) was prepared as previously reported[4,5] and assayed spectrophotometrically using $\varepsilon^{320\,nm} = 1570\,M^{-1}\,cm^{-1}$. The pink DF-Mn complex[5] was also prepared but could not be used because it turned green upon isolation. Distilled-deionized water was used throughout all experiments.

Hydrogen Peroxide Assay

H_2O_2 was assayed using a YSI Model 27 Industrial Analyzer (Yellow Springs Instruments) equipped with a selective electrode for H_2O_2.

Electron Spin Resonance

ESR spectra were recorded using gas-permeable teflon capillary as earlier described[11] on a Varian E4 (or E9) X-band spectrometer, with field set at 3357 G, modulation frequency of 100 KHz, modulation amplitude of 1 G and non-saturating microwave power.

Cell Survival Analysis

Survival of Chinese hamster V79 cells in tissue culture was assessed by clonogenic assay.[14] Inoculated cells were incubated 16–24 hours prior to experimental procedures, in complete medium at 37°C and then were exposed to H_2O_2 or t-BHP. Following treatment, cells were trypsinized, rinsed, counted, and plated in triplicate for macroscopic colony formation. Following appropriate incubation periods, colonies were fixed, stained, and lastly counted with the aid of a dissecting microscope.

For hypoxic experiments the cells were plated into specially designed glass flasks sealed with soft rubber stoppers. 19-gauge needles were pushed through to act as entrance and exit ports for a humidified gas mixture of 95% N_2/5% CO_2 (Matheson Gas Products). Each flask was also equipped with a ground glass side arm vessel which when rotated and inverted could deliver 0.2 ml of medium containing H_2O_2. Stoppered flasks were connected in series and mounted on a reciprocating platform and gassed at 37°C for 45 min prior to and throughout the experiment. After 45 min the effluent gas phase was < 10 ppm O_2 as measured by Thermox probe.

Ferricytochrome c Reduction Assay

The SOD-inhibitable Cyt-c^{III} reduction assay,[26] was used to determine rate constants of reaction with $O_2^{\cdot-}$. Superoxide radicals were generated at 25 ± 0.2°C in aerated phosphate buffer containing 50 μM DTPA, 5 mM HX, and 10–18 μM Cyt-c^{III}. In experiments conducted with DF-Mn, DTPA was replaced with 50 μM DF to avoid dissociation of the SOD-mimic. The reaction was started by adding 0.01 U/ml XO and the rate of Cyt-c^{III} reduction, in the absence and in the presence of the competing substrate, was spectrophotometrically followed at 550 nm.

RESULTS

Superoxide Reaction with Nitroxides and DF-Mn SOD-mimics

The reactivities at pH7 of nitroxide, and DF-Mn towards $O_2^{\cdot-}$ were compared using the SOD-inhibitable Cyt-c^{III} reduction assay.[15] Formation rates of Cyt-c^{II} were monitored, in the absence (V) and in the presence (v) of varying substrate concentrations. Data were analyzed by plotting V/v as a function of [substrate] and the rate constant k_1 was calculated, knowing $k_{CytC+superoxide}$, according to:

$$V/v = 1 + k_1 \times [\text{substrate}]/k_{CytC+superoxide} \times [\text{Cyt-}c^{III}] \quad (1)$$

For DF-Mn k_1 may represent k_{cat} but for TP which is present in a large excess k_1 actually represents the second order reaction rate constant of $O_2^{\cdot-}$ with the nitroxide. The rate constants calculated for DF-Mn and TP at pH7 are $1.5 \times 10^7\ M^{-1}s^{-1}$ and $5 \times 10^5\ M^{-1}s^{-1}$ respectively.

Oxic Effect of DF-Mn and TP

To compare the protective effects of the cyclic nitroxide and DF-Mn against oxidative damage, monolayered Chinese hamster V79 cells were exposed to 1.2 mM H_2O_2 for 1 h, in the absence and in the presence of either TP, DF-Mn, catalase, or SOD. DF, when used, was preincubated with the cells for 2 h. The cellular damage was assessed by clonogenically monitoring cell viability (Figure 1A). In control experiments performed with TP and DF-Mn in the absence of H_2O_2, no cytotoxic effect was observed. Catalase, though not SOD, protected the cells from H_2O_2 cytotoxicity. DF (0.1 mM) added 2 h prior to H_2O_2 afforded partial protection, 1 mM DF fully protected, but 1 mM DTPA had no protective effect (data not shown). To check if the nitroxide destroys H_2O_2, 1 mM TP was added to 5 mM HX/0.1 U/ml XO reaction mixture, aliquots were sampled and the [H_2O_2] determined using a YSI specific selective

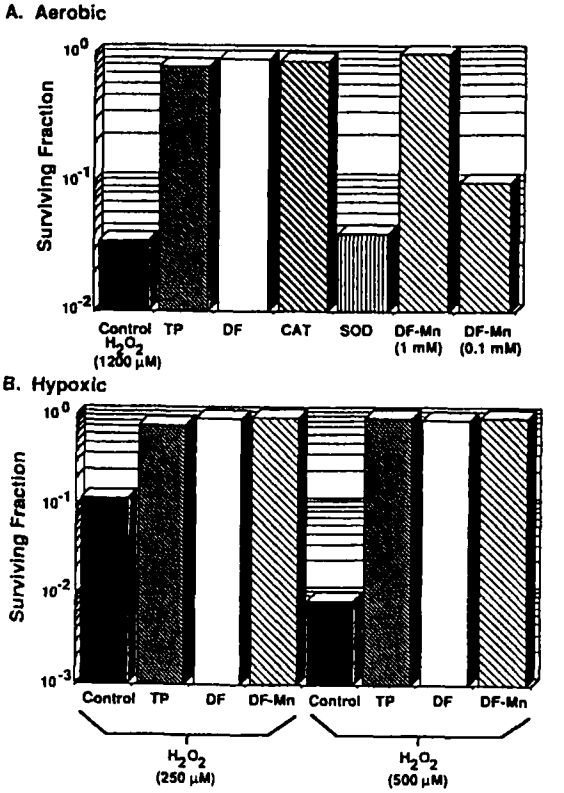


FIGURE 1 Protection from H_2O_2-induced cytotoxicity. Monolayered Chinese hamster V79 cells in full growth medium at 37°C were exposed for 1 h to: A) 1.2 mM H_2O_2 under 95% air/5% CO_2; in the presence of a) control, no additives; b) 5 mM TP; c) 1 mM DF; d) 100 U/ml catalase (CAT); e) 350 U/ml SOD; f) 1 mM DF-Mn; g) 0.1 mM DF-Mn. B) 250 or 500 μM H_2O_2 under 95% N_2/5% CO_2 in the presence of a) control, no additives; b) 5 mM TP; c) 0.5 mM DF; d) 1 mM DF-Mn.

electrode. The [H_2O_2] was not lessened in the presence of TP, thus ruling out any catalase-like activity of the nitroxide. The time-dependence of [TP] was determined using ESR spectroscopy but no change in TP ESR signal was apparent in the absence or the presence of H_2O_2.

Anoxic Effect of DF-Mn and TP

To study the effects of DF-Mn and TP on $O_2^{\cdot}$-independent damage, the cell survival experiments were repeated under hypoxic conditions. H_2O_2 cytotoxicity under hypoxia was greater then in air and was fully inhibitable by either catalase or 0.5 mM DF (Figure 1B). Both TP (5 mM) and DF-Mn (1 mM) protected the cells from the hypoxic cytotoxicity of H_2O_2 (Figure 1B), suggesting that both types of SOD-mimics can protect from $O_2^{\cdot}$-independent damage. To further examine such a possibility the effect of organic peroxide was studied.

Protection from t-Butyl Hydroperoxide (t-BHP)

Cytotoxicity induced in various biological test systems by organic radicals generated

from peroxides such as t-BHP has been previously reported.[16,17] Exposure of monolayered V79 cells in full growth medium to 3 mM t-BHP for several hours in air resulted in extensive cell killing. Both types of SOD-mimics protected the cells from t-BHP toxicity. In contrast, neither 350 U/ml SOD nor 100 U/ml catalase afforded any protection (Figure 2).

DISCUSSION

Role of Metals

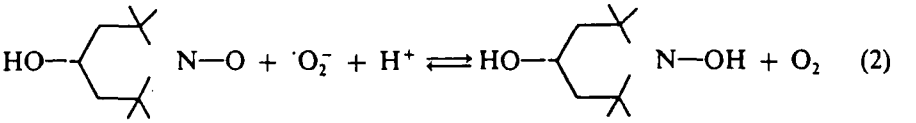
The metal chelator DF protected the cells both under oxic and hypoxic conditions indicating that transition metal ions, most likely iron, play a key role in the damage induced by either H_2O_2 or t-BHP. Unlike DTPA, DF can enter the cell, although quite slugglishly. Therefore, the requirement for a prolonged preincubation of DF with the cells and the failure of DTPA to protect suggest that the critical damage site is intracellular. The DNA scission observed for t-BHP treated V79 cells further corroborates this conclusion.[17]

Mechanisms of Damage

Oxidative biological damage mediated by transition metals and H_2O_2 is frequently attributed to the Haber-Weiss reaction (or superoxide-driven Fenton reaction). Accordingly any reagent that can remove H_2O_2 (i.e. catalase), dismutase $O_2^{\cdot-}$ (i.e. SOD), bind the metals (i.e. DF), or sucessfully compete for $^{\cdot}OH$ radicals is anticipated to diminish the biological damage. Therefore when attempting to discern by which path cytoprotection is provided from oxidative damage, several potential mechanisms must be addressed.

Superoxide Dismutase-like Activity

Cyclic nitroxides as well as DF-Mn have been previously found to afford protection from oxidative damage induced under air.[6,11,13,14] Nitroxides and their respective hydroxylamines react with $O_2^{\cdot-}$ and may protect by intercepting cellular superoxide.



(2)

In view of the relatively high rates of reaction with $O_2^{\cdot-}$ achievable with TP or DF-Mn concentrations used, such protection is anticipated and may be attributed to their SOD-mimetic activity facilitating the removal of $O_2^{\cdot-}$. The present results, however, show that a) both H_2O_2 and t-BHP exert cytotoxicity that does not require $O_2^{\cdot-}$ participation; and b) both types of SOD-mimics can protect cells from $O_2^{\cdot-}$-independent damage. The hypoxic protection afforded by TP or DF-Mn might be also ascribed to the unlikely but feasible case of catalase-facilitated intracellular dismutation of H_2O_2 to generate sufficient oxygen to produce $O_2^{\cdot-}$. Such an assumption, however, is untenable in the case of t-BHP cytotoxicity since cell killing induced by t-BHP does not involve $O_2^{\cdot-}$ or H_2O_2 and was not inhibitable by SOD or catalase[18] (Figure 2).

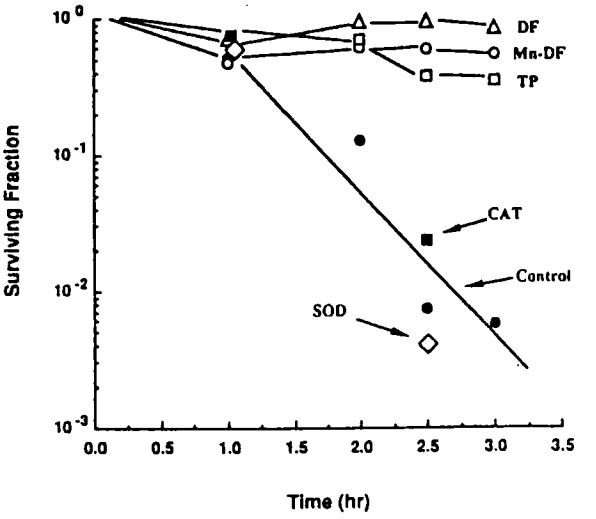


FIGURE 2 Protection from t-BHP-induced cytotoxicity. Monolayered Chinese hamster V79 cells in full growth medium at 37°C were exposed under 95% air/5% CO_2 to 2 mM t-BHP for various periods of time in the presence of various additives. a) control, no additives (●); b) 5 mM TP (△); c) 0.5 mM DF (▲); d) 1 mM DF-Mn (○); e) 100 U/ml CAT ■; f) 360 U/ml SOD (◇).

Metal Binding

Anoxic protection can result from binding of adventitious labile cellular metals, such as iron, by DF originating from DF-Mn. Metal-based SOD-mimics have been shown to dissociate in the presence of DTPA, EDTA, or proteins[4,5] and are prone to dissociate in a biological environment. The failure of 0.1 mM DF-Mn, despite its high reaction rate with $O_2^{\cdot-}$, to fully protect the cells (see Figure 1A) supports this conclusion. In the case of cyclic nitroxide no evidence was found for nitroxide-metal complex formation as no metal effect on the nitroxide ESR signal was detected.

Reoxidation of Reduced Metals

Alternatively, it is plausible that nitroxides reoxidize bound, reduced metal ions. A commonly proposed mechanism accounting for the metal-catalyzed H_2O_2-induced cell damage involves the redox reactions of unbound or chelated metal ions.[19] In particular, redox reactions of metals coordinated to biological macromolecules can act as catalysts for the Haber-Weiss reaction and lead to site-specific damage inflicted by deleterious species generated in the vicinity of the critical targets. According to that mechanism, metal binding to critical cellular component is essential for site-specific DNA damage:

$$L + M^{n+} \cdot \rightleftarrows L - M^{n+} \tag{3}$$

in which M^{n+} denotes oxidized transition metal such as Cu^{2+} or Fe^{3+}, whereas L represents coordinating ligand irrespective of the number of bound ligand molecules or coordination number. The bound metal is reducible either metabolically or by reagents such as $O_2^{\cdot-}$ or ascorbate:

$$L - M^{n+} + O_2^{\cdot-} \text{ (or other reductant)} \rightleftarrows L - M^{(n-1)+} + O_2 \quad (4)$$

which in turn can react with H_2O_2 to form a peroxo complex:[20]

$$L - M^{(n-1)+} + H_2O_2 \rightarrow L - M^{(n-1)+} - H_2O_2 \quad (5)$$

that can generate higher oxidation state of the metal and/or $^{\cdot}OH$ radical

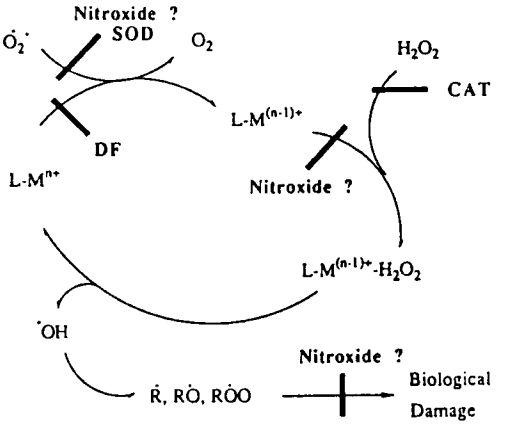
$$L - M^{(n-1)+} - H_2O_2 \rightarrow L - M^{n+} + OH^- + {}^{\cdot}OH \quad (6a)$$

$$L - M^{(n-1)+} - H_2O_2 \rightarrow L - M^{(n+1)+} + 2OH^- \quad (6b)$$

Such reactions have previously been proposed to operate in various systems, including DNA cleavage induced by $(OP)_2Cu(II)$ and H_2O_2.[19] Preliminary experiments showed that 5-membered cyclic nitroxides oxidize iron(II) bound to DNA thus corroborating the assumption that cyclic nitroxides can inhibit reactions 5 and 6, thus, diminishing oxidative damage.

Detoxifying Organic Radicals

Because t-BHP cytotoxicity requires neither $O_2^{\cdot-}$ nor H_2O_2 it is possible that cyclic nitroxides can protect also by intercepting organic radicals, such as $R^{\cdot}$, $RO^{\cdot}$, or $ROO^{\cdot}$, and also detoxify radical sites in cellular macromolecules. Cyclic nitroxide free radicals are quite stable in cells but were found to undergo radical–radical reactions with various carbon-centered and oxygen-centered radicals.[21] It is very likely therefore, that detoxifying deleterious primary and secondary radicals contribute in a major fashion to the protective effect of those SOD-mimics. There is also the possibility that metal ions are instrumental in the t-BHP-induced cellular damage. If that were the case, the stable nitroxide radicals as well as the dissociated metal-dependent SOD-mimics may be operative through competition for either reduction (nitroxides) or actual binding of the metal (DF).



SCHEME I

The present results are not sufficient to discern among the contributions of above mentioned mechanisms (see scheme I) and further research is needed. It is likely, however, that the low-molecular, cell-permeable SOD-mimics operate in more than a single mechanism.

In conclusion, the present findings show that although both metal-based and

metal-free SOD mimics react with and remove $O_2^{\cdot}$ *in vitro* the cytoprotection afforded by these agents against oxidative damage might be independent of their SOD-activity.

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