Biologically Active Metal-Independent Superoxide Dismutase Mimics

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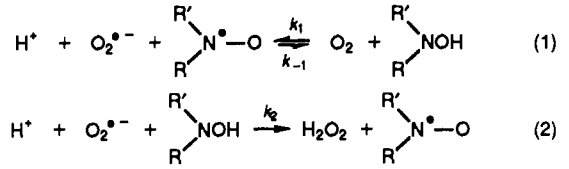
Received August 21, 1989; Revised Manuscript Received October 27, 1989

ABSTRACT: Superoxide dismutase (SOD) is an enzyme that detoxifies superoxide ($O_2^{\bullet -}$), a potentially toxic oxygen-derived species. Attempts to increase intracellular concentrations of SOD by direct application are complicated because SOD, being a relatively large molecule, does not readily cross cell membranes. We have identified a set of stable nitroxides that possess SOD-like activity, have the advantage of being low molecular weight, membrane permeable, and metal independent, and at pH 7.0 have reaction rate constants with $O_2^{\bullet -}$ ranging from 1.1×10^3 to 1.3×10^6 M^{-1} s^{-1}. These SOD mimics protect mammalian cells from damage induced by hypoxanthine/xanthine oxidase and H_2O_2, although they exhibit no catalase-like activity. In addition, the nitroxide SOD mimics rapidly oxidize DNA–Fe^{II} and thus may interrupt the Fenton reaction and prevent formation of deleterious OH radicals and/or higher oxidation states of metal ions. Whether by SOD-like activity and/or interception of an electron from redox-active metal ions they protect cells from oxidative stress and may have use in basic and applied biological studies.

In an oxygen-containing environment, cellular metabolism results in the production of several potentially harmful oxygen-derived species. The first in this cascade of active oxygen metabolites is the one-electron reduction product, superoxide ($O_2^{\bullet -}$). The function of the superoxide-detoxifying metalloenzyme superoxide dismutase (SOD)[1] in protecting against oxygen-mediated biological damage is well documented (McCord & Fridovich, 1969; Fridovich, 1972, 1974, 1975; Gregory & Fridovich, 1973; Hassan & Fridovich, 1977). Increasing intracellular levels of SOD or administering exogenous SOD, among other salutary effects, reportedly lessens inflammation, decreases ischemia-induced reperfusion injury, decreases damage from tumor necrosis factor, and mitigates the damage caused by rheumatoid arthritis (Menander & Huber, 1977; McCord et al., 1979; McCord, 1985; Clark et al., 1988). Such reports have prompted further research to explore the clinical utility of administered SOD. Because $O_2^{\bullet -}$ can potentially be produced both inside and outside cells and because exogenously applied SOD has limited membrane permeabililty, detoxification of intracellularly produced $O_2^{\bullet -}$ by extracellular SOD would be limited. Hence, compounds with SOD-like activity having low molecular weight, biological stability, and membrane permeability have been sought. A number of metal chelates having SOD-like activity have been synthesized (Weinstein & Bielski, 1980; Koppenol et al., 1986; Darr et al., 1987; Nagano et al., 1988). These metal-dependent agents, however, might become ineffective in cells because of metal–ligand dissociation with subsequent random and potentially deleterious binding of the dissociated metal ions to critical cellular constituents (Darr et al., 1987). Thus, metal independence would be a desirable added criterion for a potential biologically useful SOD mimic. We have identified a family of stable cyclic nitroxide free radicals that possess these characteristics. Our initial observation was that a persistent nitroxide spin adduct resulting from the reaction between $^{\bullet}OH$ and DMPO, a commonly used electron spin-trapping agent, reacts with superoxide (Samuni et al., 1989). We extended the initial observation to show that a related stable free radical, 2-ethyl-2,5,5-trimethyl-3-oxazolidine-1-oxyl (OXANO; compound I, Table I), and its hydroxylamine react with $O_2^{\bullet -}$ independent of metal ions (Samuni et al., 1988).



(1)

(2)

Summing reaction 1 and 2 results in

$$2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

and therefore, OXANO act as a low molecular weight, metal-free, SOD mimic. We have synthesized a series of chemically related stable nitroxide radicals with varying pendent groups and physical characteristics and have determined their SOD-like activity. To explore the potential usefulness of these agents, we examined their mechanism of action and effects on mammalian cells exposed to oxidative stress resulting from either HX/XO or direct H_2O_2 exposure.

Experimental Procedures

Chemicals. Desferrioxamine (DF) was a gift from Ciba Geigy; hypoxanthine (HX) was purchased from Calbiochem-Boehringer Co.; 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), 4-hydroxypyrazolo[3,4,-*d*]pyrimidine (allopurinol), *p*-toluenesulfonic acid, 2-amino-2-methyl-1-propanol, 2-butanone, and cyclohexanone were purchased from Aldrich Chemical Co.; tris(oxalato)chromate(III) [$K_3[Cr(C_2O_4)_3]$·

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[1] Abbreviations: EPR, electron paramagnetic resonance; CHD, spiro[cyclohexane-1,2′-doxyl] [spiro[cyclohexane-1,2′-(4′,4′-dimethyloxazolidine-3′-oxyl)]]; DTPA, diethylenetriaminepentaacetate; OXANO, 2-ethyl-2,4,4-trimethyloxazolidine-3-oxyl; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; SOD, superoxide dismutase; DF, desferrioxamine; XO, xanthine oxidase; HX, hypoxanthine.

$3H_2O$] (CrOx) was prepared and recrystallized as previously described (Bailar & Young, 1939); xanthine oxidase (EC 1.2.3.2; xanthine:oxygen oxidoreductase), grade III, from buttermilk, superoxide dismutase (SOD), and grade V ferricytochrome *c* were obtained from Sigma. H_2O_2 was bought from Fisher Scientific Co. XO was further purified on a G-25 Sephadex column. All other chemicals were prepared and used without further purification. Distilled–deionized water was used throughout all experiments.

Synthesis of Oxazolidine Derivatives. Spiro[cyclohexane-1,2′-doxyl] [CHD; spiro[cyclohexane-1,2′-(4′,4′-dimethyl-oxazolidine-3′-oxyl)]] and 2-ethyl-2,4,4-trimethyl-oxazolidine-3-oxyl (OXANO) as well as other nitroxides were synthesized as previously described (Keana et al., 1967). For the general synthesis of the cyclic amines, the appropriate starting ketone was reacted with 2-amino-2-methyl-1-propanol in benzene in the presence of catalytic amounts of *p*-toluenesulfonic acid. As the cyclic structure formed, water was eliminated. The volume of water collected in a Dean–Stark apparatus was monitored and used to gauge the reaction progress. The amines thus produced were purified through fractional distillation under reduced pressure, characterized by 220-MHz ^1H NMR, IR, UV, either EI or CI mass spectroscopy, and subsequently oxidized to the corresponding nitroxides by using *m*-chloroperbenzoic acid. The nitroxides were purified by silica flash chromatography (Still et al., 1978). Water/octanol ratios were determined by placing a quantity of nitroxide in water + octanol within a separatory funnel. The mixture was shaken thoroughly and allowed to separate for 15 min, whereupon aliquots were taken from both fractions and the ratio of nitroxide distribution was determined according to electron paramagnetic resonance (EPR) spectroscopy by comparing the intensities of signal obtained under N_2.

Ferricytochrome c Reduction Assay. The SOD-inhibitable ferricytochrome *c* reduction assay (Fridovich, 1985) was used to determine rate constants of reaction with $O_2^{\bullet -}$. Superoxide radicals were generated at 25 °C in aerated phosphate buffer (50 mM) containing 50 μM DTPA, 5 mM HX, and 10–50 μM ferricytochrome *c* (with or without 65 units/mL catalase). The reaction was started by adding 0.01 unit/mL XO, and the rate of ferricytochrome *c* reduction, in the absence (V) and in the presence (v) of various nitroxides, was spectrophotometrically followed at 550 nm. Both reference and sample cuvettes contained all the agents, with the reference containing 100 units/mL SOD, thereby eliminating spurious reactions from interfering with the determination of rate constants. Data were analyzed by plotting V/v as a function of [nitroxide], and k_1 was calculated, knowing $k_{CytC+superoxide}$, according to $(V/v) - 1 = k_1[\text{nitroxide}]/k_{CytC+superoxide}[\text{Cyt-c}^{III}]$.

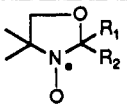
Cell Culture. Chinese hamster V79 cells were grown in F12 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Survival was assessed in all studies by the clonogenic assay. The control plating efficiency ranged between 80 and 90%. Stock cultures of exponentially growing cells were trypsinized, rinsed, plated (5×10^5 cells/dish) into a number of 100-mm Petri dishes, and incubated 16 h at 37 °C prior to experimental protocols. Cells were exposed for 1 h at 37 °C to either 0.5 mM hypoxanthine (HX) + 0.05 unit/mL xanthine oxidase (XO) for varying lengths of time or H_2O_2 at different concentrations. To assess possible modulation in cytotoxicity, catalase, 100 units/mL; SOD, 100 μg/mL; DF, 500 μM; and 5 mM of each of the nitroxides from Tables I and II were added to parallel cultures. CHD was prepared in a stock solution in ethanol and diluted into medium such that the final concentration was 5 mM. This resulted in a final concentration of 1% ethanol in the medium which was not cytotoxic and did not influence the cellular response to HX/XO or H_2O_2. TEMPOL is water soluble and was prepared directly in tissue culture medium. Neither catalase, SOD, DF, CHD, or TEMPOL was cytotoxic alone at the concentrations used. DF was added either 2 h prior to or immediately before and then left on during treatment while the other agents were present only during HX/XO or H_2O_2 treatment. Following treatment, cells were rinsed, trypsinized, counted, and plated for macroscopic colony formation. For each dose determination cells were plated in triplicate and the experiments were repeated a minimum of two times. Plates were incubated 7 days, after which colonies were fixed with methanol/acetic acid, stained with crystal violet, and counted. Colonies containing >50 cells were scored. Error bars represent SD of the mean and are shown when larger than the symbol.

Some studies required exposure to H_2O_2 under hypoxic conditions. For these studies cells dispersed in 1.8 mL of medium were plated into specially designed glass flasks (Russo et al., 1985). The flasks were sealed with soft rubber stoppers, and 19-gauge needles were pushed through to act as entrance and exit ports for a humidified gas mixture of 95% nitrogen/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 at a concentration that when added to the cell monolayer resulted in a final concentration of H_2O_2 of 600 μM. Stoppered flasks were connected in series, mounted on a reciprocating platform, and gassed at 37 °C for 45 min. This gassing procedure results in an equilibrium between the gas and liquid phase (in both the medium over the cell monolayer and the solution in the side arm) and yielded oxygen concentrations in the effluent gas phase of <10 ppm as measured by a Thermox probe (Russo et al., 1985). After 45 min of gassing, the hypoxic H_2O_2 solution was added to the cell monolayer culture. The cells were exposed to H_2O_2 for 1 h under hypoxic conditions. N_2 gas flow was maintained during the H_2O_2 exposure. In parallel flasks, DF and CHD were added as described above and were present during the entire gassing procedure. Following treatment cell survival was assessed as described above.

Electron Paramagnetic Resonance. For EPR experiments samples (0.05–0.1 mL) of either solutions of chemicals or cell suspensions were drawn by a syringe into a gas-permeable teflon capillary of 0.8 mm inner diameter and 0.05 mm wall thickness (Zeus Industrial Products, Inc., Raritan, NJ). Each capillary was folded twice, inserted into a narrow quartz tube which was open at both ends (2.5 mm i.d.), and then placed horizontally into the EPR cavity. During the experiments, gases of desired compositions were blown around the sample without having to disturb the alignment of the tube within the EPR cavity. EPR spectra were recorded 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 nonsaturating microwave power. The EPR spectrometer was interfaced to an IBM-PC through an analog-to-digital converter and a data translation hardware (DT2801), and the spectra were digitized by using commercial acquisition software, enabling subtraction of background signals. To study the kinetics of the spin loss, the spectra were deliberately overmodulated, and the magnetic field was kept constant while the intensity of the EPR signal was followed.

H_2O_2 Assay. Hydrogen peroxide was assayed by using a YSI Model 27 industrial analyzer (Yellow Springs Instru-

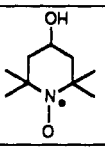
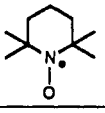
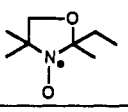
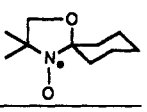
Table I: Five-Membered Oxazolidine-3-oxyl (Doxyl)



nitroxide notation	ring substituents R_1	ring substituents R_2	yield (%)	partition coefficient[a]
I	OXANO	CH_3	C_2H_5	42	10
II		CH_3	C_5H_{11}	52	145
III		CH_3	C_4H_9	49	58
IV	CHD	spirocyclohexyl		77	80
V		CH_3	C_6H_5	22	720

[a] Octanol/water.

Table II: Kinetic Data: SOD-like Activity of Five- and Six-Membered Cyclic Nitroxides

chemical structure:				
nitroxide notation	TEMPOL	TEMPO	OXANO	CHD
steady-state EPR signal (%)[a]	100	100	50	30
$k_{R'RNO+O_2^{\bullet -}}$ (M^{-1} s^{-1})[b]	3.4×10^5	1.3×10^6	1.1×10^3	3.5×10^3

[a] Steady-state EPR signal of nitroxides (% from total R'RNO + R'RNOH) after exposure to 5 mM HX + 0.03 unit/mL XO in air-saturated PBS, pH 7.2. [b] Rate constants were determined at low ionic strength (10 mM HEPES), pH 7.0, and 22 °C.

ments) equipped with a selective electrode for H_2O_2. For analysis of cellular preparations, the cells, except during the brief time required for removal of aliquots for analysis, were kept in T25 culture flasks maintained at 37 °C in complete medium (pH 7.2). Aliquots of 25 μL were sampled from the reaction or cell preparation system at varying time points and injected into the analyzer. [H_2O_2] was determined after calibrating the instrument with known concentrations of H_2O_2. The concentrations of standard H_2O_2 solutions were calibrated according to the iodometric assay (Hochanadel, 1952).

Results

Nitroxide Synthesis and Characterization. To check whether oxazolidineoxyl derivatives other than OXANO manifest SOD-like activity, we synthesized several nitroxides having different ring substituents. Table I shows representative synthesized nitroxides with accompanying physical characteristics. Exposure of these five-membered cyclic nitroxides to $O_2^{\bullet -}$ flux formed by HX/XO resulted in a decrease in their EPR signal, as previously found for OXANO (Samuni et al., 1989). After terminating the HX/XO reaction by allopurinol, the nitroxide spin loss was reversed by adding 0.5 mM ferricyanide, indicating that $O_2^{\bullet -}$ reduces the nitroxide to its respective hydroxylamine (Samuni et al., 1989). On the other hand, no effect of $O_2^{\bullet -}$ on the EPR signal of six-membered ring nitroxides such as TEMPO and TEMPOL was detectable (see Table II). The failure of superoxide to affect TEMPO and TEMPOL apparently suggested that six-membered cyclic nitroxides lack SOD-like activity. As a further check, the reaction of representatives of both five- and six-membered cyclic nitroxides with $O_2^{\bullet -}$ was studied. Superoxide flux was generated by HX/XO reaction system, and the nitroxide effect on the SOD-inhibitable reduction of ferricytochrome *c* was followed spectrophotometrically (Fridovich, 1985). All of the

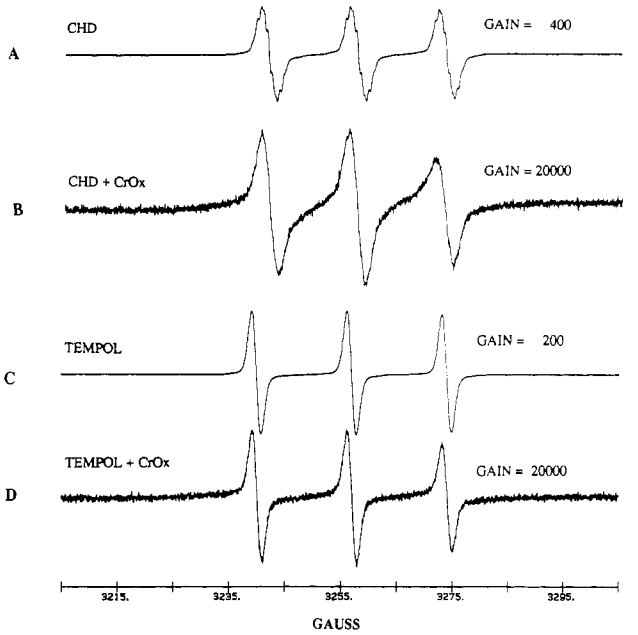


FIGURE 1: EPR spectra of CHD and TEMPOL demonstrating the partitioning of each nitroxide (1 mM) in both the intra- and extracellular space of V79 cells. The EPR signal intensity of the total concentration of CHD or TEMPOL (intra- and extracellular) in 6.4 × 10^7 V79 cells/mL (traces A and C) and in the presence of 110 mM tris(oxalato)chromiate (CrOx) (traces B and D). The gains for individual spectra are as cited in the individual traces.

stable nitroxide radicals studied demonstrated reaction with $O_2^{\bullet -}$. The rate constants of the synthetic nitroxides' reaction with $O_2^{\bullet -}$ at low ionic strength (10 mM HEPES) and pH 7.0 ranged from 1.1×10^3 to 1.3×10^6 M^{-1} s^{-1} (see Table II), as compared with 2.3×10^9 M^{-1} s^{-1} for k_{cat} of native SOD.

None of the nitroxides shown in Tables I and II exhibited cytotoxicity (determined by clonogenic assay) in V79 cells exposed for 1 h at 5 mM. For subsequent studies the most lipophilic nitroxide, CHD, and the most hydrophilic one, TEMPOL, were chosen.

Nitroxide Intracellular Localization. Traces A and C of Figure 1 illustrate the EPR signal from 1 mM CHD and TEMPOL, respectively, suspended with 6.4 × 10^7 V79 cells/mL. This EPR signal represents the total concentration of intra- and extracellular CHD or TEMPOL. Tris(oxalato)chromate is a paramagnetic broadening agent which remains excluded from the intracellular volume space and causes the EPR signal from extracellular species to become nondetectable (Lai, 1988). When cells were added to CHD or TEMPOL in the presence of 110 mM tris(oxalato)chromiate, a much smaller yet observable intracellular signal was detected, as shown in Figure 1B,D. The observable line broadening and loss of the hyperfine structure of the intracellular signal indicate that CHD, though not TEMPOL, has decreased freedom of motion (anisotropy) within the intracellular environment and is located primarily in a membranous compartment as can be anticipated on the basis of the difference between their lipophilicities.

Protection from Oxidative Damage. To expose the cells to oxidative stress, they were incubated with HX/XO. Figure 2 shows a survival curve for cells exposed to HX/XO. Cell survival was not altered when SOD was present during the HX/XO exposure. In contrast, 5 mM CHD or TEMPOL fully protected the cells. The other nitroxides, presented in Tables I and II, afforded similar protection (data not shown). Figure 2 also shows that either catalase or DF provides complete protection from HX/XO-derived damage. Complete

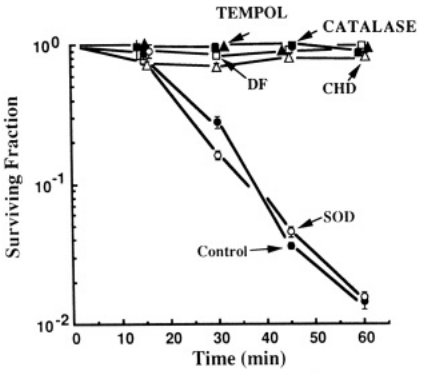


FIGURE 2: Protection from HX/XO-induced cell killing. Chinese hamster V79 cells in full medium at 37 °C were exposed to 0.05 unit/mL XO + 0.5 mM HX for various time periods in the presence of various additives: (●) control, no additives; (■) 100 units/mL catalase; (○) 100 units/mL SOD; (□) 500 μM DF, preincubated for 2 h with the cells prior to addition of XO; (△) 5 mM CHD; (▲) 5 mM TEMPOL.

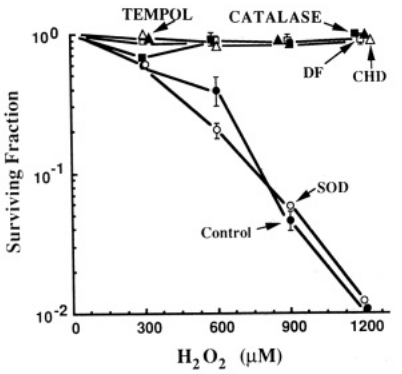


FIGURE 3: Protection from H_2O_2-induced cell killing. The effect of various agents on cell survival was measured by clonogenic assay of Chinese hamster V79 cells exposed in full medium at 37 °C to various concentrations of H_2O_2 for 1 h: (●) control, no additives; (■) 100 units/mL catalase; (○) 100 μg/mL SOD; (□) 500 μM DF, preincubated for 2 h with the cells prior to H_2O_2 addition; (△) 5 mM CHD; (▲) 5 mM TEMPOL.

protection by DF required a 2-h preincubation with DF before cells were exposed to HX/XO, whereas DF addition simultaneously with HX/XO offered only partial protection (data not shown).

One interpretation of the data shown in Figure 2 was that H_2O_2 is the principal cytotoxic species produced by the HX/XO system (Link & Riley, 1988). This assumption is based on the fact that extracellular catalase provided complete protection from HX/XO (Figure 2). To test if cell protection by the SOD mimic resulted by preventing the effect of H_2O_2, cells were exposed to H_2O_2 as shown in Figure 3. The results of these experiments were identical with those shown in Figure 2, in that SOD did not protect, but catalase, DF, TEMPOL, and CHD provided complete protection against H_2O_2 cytotoxicity. At this point it was questioned if CHD might have other features apart from acting as a SOD mimic, namely, whether CHD affects H_2O_2 concentration. Figure 4 shows the concentration of H_2O_2 in tissue culture exposed to HX/XO. With time there was a buildup followed by a slow decline in $[H_2O_2]$. The presence of CHD did not significantly alter the pattern of H_2O_2 accumulation by HX/XO. Thus, the cellular protection afforded by CHD to HX/XO and H_2O_2 could not be attributed to a direct reaction of CHD with H_2O_2.

Even with direct exposure of cells to H_2O_2 there is the possibility that superoxide could be produced intracellularly as a result of the H_2O_2 treatment. If superoxide were produced intracellularly, CHD protection of cells from HX/XO and

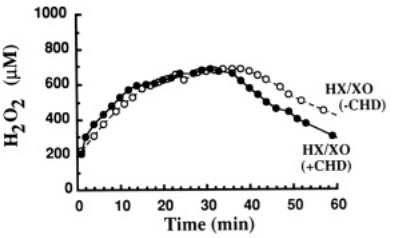


FIGURE 4: Nitroxide effect on accumulation and decay of H_2O_2 upon exposure of cells to HX/XO. Chinese hamster V79 cells were plated in full medium and incubated at 37 °C with 5 mM HX + 0.04 unit/mL XO, sampled at various time points, and assayed for H_2O_2 by using a hydrogen peroxide selective electrode.

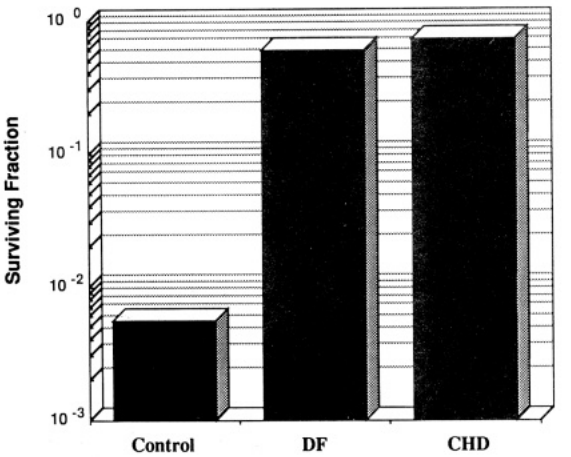


FIGURE 5: Survival of Chinese hamster V79 cells exposed to 600 μM H_2O_2 ± DF or CHD in full medium at 37 °C for 1 h under hypoxic conditions.

H_2O_2 might be expected, given the findings that CHD can penetrate intracellular spaces as shown in Figure 1. To test if the cytoprotection provided by CHD was solely a result of its reaction with superoxide, CHD effectiveness was examined when H_2O_2 was applied to cells incubated in an hypoxic environment, conditions in which the chance for superoxide formation would be significantly limited. As is seen in Figure 5, CHD protects against H_2O_2 cytotoxicity even under hypoxic conditions.

Figure 5 also shows that DF provides complete protection to H_2O_2 cytotoxicity under hypoxic conditions. The pattern of DF protection shown in Figures 2, 3, and 5 suggested that the cytotoxicity of HX/XO and H_2O_2 may directly involve intracellular reduction of H_2O_2 by ferrous ion to produce the highly toxic •OH. It was also questioned whether the aerobic and hypoxic protection by CHD to H_2O_2 exposure was a result of CHD directly accepting electrons from ferrous ions, thereby preventing generation of •OH. Because cellular iron is chelated, the possible reaction of nitroxide with chelated iron(II) was examined by repeating the experiment in the presence of DNA. To study the possibility of nitroxide-induced oxidation of transition metals, CHD was hypoxically mixed with iron(II) in the presence of 0.1 mg/mL salmon DNA. Consequently, DNA–Fe^{III} was formed and the nitroxide EPR signal disappeared. The reaction kinetics were investigated by maintaining either CHD or Fe(II) in excess while the absorbance due to DNA–Fe^{III} and the nitroxide spin loss were monitored respectively (Figure 6). Both the decay of the EPR signal and the appearance of the OD_{353nm} obeyed pseudo-first-order kinetics from which the second-order reaction rate constant was calculated as 44 M^{-1} s^{-1} or 33 M^{-1} s^{-1} by using the data from EPR or optical absorption, respectively. When TEMPOL was hypoxically mixed with DNA–Fe^{II}, a similar reaction took

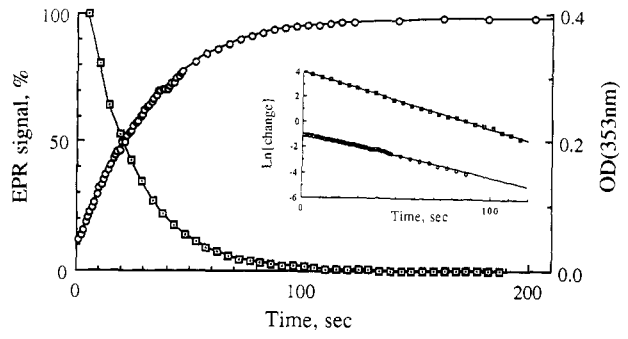


FIGURE 6: Reaction between CHD and DNA-Fe^{II}. CHD in 50 mM MOPS buffer, pH 7.0, was anoxically mixed at 22 °C with DNA-Fe^{II}. All solutions always contained 0.1 mg/mL salmon DNA. The appearance of DNA-Fe^{III} was spectrophotometrically monitored at 353 nm, whereas the spin loss of CHD was monitored by following its EPR signal. To study the time dependence of ΔOD_{353nm} (○), 1 mM CHD was mixed with 0.1 mM Fe(II). To follow the spin loss of CHD (□), 1 mM Fe(II) was mixed with 0.1 mM CHD. Inset: Time dependence of ln (EPR signal) (□) and of ln ($OD_\infty - OD_t$) (○).

place having a second-order reaction rate constant of 40 M^{-1} s^{-1}. The spin loss was completely reversed by adding 2 mM ferricyanide, thus indicating that DNA-Fe^{II} reduced the respective nitroxide to its hydroxylamine.

Discussion

The recent finding that OXANO can function as a metal-independent SOD mimic (Samuni et al., 1988) opened the possibility that nitroxides, which have already found considerable utility in both EPR and NMR spectroscopy (McConnell, 1976; Magin et al., 1986; Swartz et al., 1986), might also exhibit unique biological characteristics. The data presented here evaluating representative five- and six-membered ring nitroxides clearly demonstrate their utility in protecting mammalian cells against oxidative stress. In our experiments using two different means of inducing cytotoxicity, all nitroxides tested afforded protection. Initially, when considering the large difference in catalytic capabilities between native SOD and the nitroxide SOD mimics, it would appear that the mimics would be less than ideal for biological applications. However, since nitroxide SOD mimics can be used at relatively high concentrations without cytotoxicity and are capable of penetrating through membranes into the cellular compartments, the effective detoxification of intracellular superoxide seems plausible.

In both systems used to access cytotoxicity (HX/XO and direct H_2O_2 exposure), extracellular application of SOD did not offer protection. There are several conclusions that can be drawn from this finding. Possibly extracellular superoxide does not have any inherent toxicity, and therefore applying an agent like SOD extracellularly would afford little benefit. Another possibility is that the HX/XO-derived superoxide is only toxic after it crosses the cell membrane. However, the amount of SOD applied (Figure 2) is sufficient to have converted all of the extracellularly generated superoxide to H_2O_2. Therefore, we conclude that exogenously produced superoxide is not in and of itself responsible for cytotoxicity. If exogenous $O_2^{\bullet -}$ is not responsible for the toxicity, what is? Since the HX/XO system produces both H_2O_2 and $O_2^{\bullet -}$ (which yields H_2O_2), the former appears to be the major initial cytotoxic species produced by HX/XO (Link & Riley, 1988). The present results support this conclusion. Both catalase and DF (see Figure 2) protected from both HX/XO and H_2O_2 by inhibiting the Fenton reaction (Rush & Koppenol, 1986):

$$\text{DNA-Fe}^{II} + H_2O_2 \rightarrow \text{DNA-Fe}^{II}\text{-}H_2O_2 \quad (4)$$

The peroxo complex can generate $^{\bullet}OH$ radicals and/or higher oxidation states of the metal (Masarwa et al., 1988):

$$\text{DNA-Fe}^{II}\text{-}H_2O_2 \rightarrow \text{DNA-Fe}^{III} + OH^- + {}^{\bullet}OH \quad (4a)$$

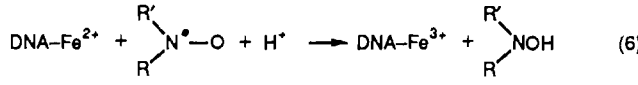
$$\text{DNA-Fe}^{II}\text{-}H_2O_2 \rightarrow \text{DNA-Fe}^{IV} + 2OH^- \quad (4b)$$

By catalyzing H_2O_2 production through reactions 1 and 2, CHD and TEMPOL might be expected to increase cytotoxicity; however, reactions 4, 4a, and 4b constitute only the second half of the Haber–Weiss reaction (Fenton reaction), the first half being a superoxide-driven reduction of ferric ion to the oxidized metal, i.e.

$$Fe^{III} + O_2^{\bullet -} \rightarrow Fe^{II} + O_2 \quad (5)$$

Removal of either the labile intracellular ferric ion (by chelators such as DF) or removal of intracellular superoxide (by SOD mimics such as CHD or TEMPOL) could result in cessation of $^{\bullet}OH$ production and a decrease in toxicity as is demonstrated in Figures 2, 3, and 5.

Further, we have demonstrated that nitroxides are reduced by Fe(II) chelated to DNA. The reaction rate of CHD with DNA-Fe^{II} (see Figure 6) is comparable with that of H_2O_2 plus Fe(II) (68 M^{-1} s^{-1}) (Kozlov et al., 1974). This comparison, however, might be inconsequential because the exact nature, concentrations, and sites of redox-active iron within the cell are not known, nor is the rate of reaction of DNA-Fe^{II} with H_2O_2; hence, direct comparison to the CHD reaction with DNA-Fe^{II} is not possible. Yet, it is possible that where [nitroxide] $\gg$ [H_2O_2], redox-active iron would react with nitroxide in preference to H_2O_2



therefore preempting the intracellular generation of $^{\bullet}OH$ through reactions 4 and 4a.

Nitroxide also protected (Figure 5) when H_2O_2 treatment was conducted under hypoxic conditions, where the availability of oxygen for the formation of intracellular $O_2^{\bullet -}$ generation is greatly if not completely eliminated. It should be noted that, even in the absence of oxygen, intracellular catalase could convert H_2O_2 to oxygen which might then become available to form superoxide. Thus, it cannot be stated categorically that nitroxides protect independent of reactions with intracellular superoxide. Yet, taken together, our data suggest that (a) the major intracellular toxicity of superoxide may be an indirect contribution to $^{\bullet}OH$ production rather than direct reactions with biological molecules like DNA and (b) the beneficial function of intracellular SOD might be in decreasing metal-catalyzed $^{\bullet}OH$ production. Such a function for SOD is consistent with the seemingly incongruous relatively low direct reactivity of superoxide (Sawyer & Valentine, 1981). An alternative explanation for the nitroxide protective effect can be found by assuming that they react with and detoxify secondarily produced organic free radicals. Although nitroxide free radicals are relatively stable, they rapidly couple with carbon-centered and oxygen-centered free radicals and exhibit diffusion-controlled reaction rate constants. Such a detoxification was recently found to decrease lipid peroxidation in cell-free systems (Takahashi et al., 1989; Nilsson et al., 1989).

In addition to ameliorating cytotoxicity as shown in Figures 2, 3, and 5, the penetration of the nitroxides into intracellular spaces could possibly allow for a better understanding of intracellular damage sites of superoxide and/or H_2O_2. The design and synthesis of other nitroxide SOD mimics that can be used to probe specifically the effects of $O_2^{\bullet -}$ within different cellular compartments and organelles may, in part, be based

on simple lipid/water partitioning coefficients. Since the partitioning ratio between membranous and intracellular or extracellular aqueous compartments can be approximated by the octanol/water partition coefficient, then in addition to the design of specific site-directed stable nitroxide radicals one may be able to produce a first generation of mimics with more aqueous or lipid activity (Table I). We are just beginning to explore these possibilities.

The nitroxide based SOD mimics described here are tolerated by mammalian cells and act to protect against oxidative damage, whereas extracellular application of SOD does not offer such protection. It is anticipated that these SOD mimics or similar derivatives may find use as basic biologic probes to study the effects of superoxide acting in specific cellular compartments. Additionally, they might be used to probe the intracellular mechanisms of "redox-active" drugs that ostensibly mediate damage through specific oxygen metabolites. Our finding may ultimately have application in protection from biologic damage caused by postischemic reperfusion injury associated with reopening of arteries after heart attacks or strokes, as well as lessening the life-threatening toxic effects of exposure to elevated oxygen concentration as is sometimes necessary while providing life support during acute care.

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