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Spin traps inhibit formation of hydrogen peroxide via the dismutation of superoxide: implications for spin trapping the hydroxyl free radical

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To enhance the sensitivity of EPR spin trapping for radicals of limited reactivity, high concentrations (10–100 mM) of spin traps are routinely used. We noted that in contrast to results with other hydroxyl radical detection systems, superoxide dismutase (SOD) often increased the amount of hydroxyl radical-derived spin adducts of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) produced by the reaction of hypoxanthine, xanthine oxidase and iron. One possible explanation for these results is that high DMPO concentrations (~ 100 mM) inhibit dismutation of superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2). Therefore, we examined the effect of DMPO on $O_2^{\cdot-}$ dismutation to H_2O_2. Lumazine ± 100 mM DMPO was placed in a Clark oxygen electrode following which xanthine oxidase was added. The amount of H_2O_2 formed in this reaction was determined by introducing catalase and measuring the amount of dioxygen regenerated. Lumazine was used as the xanthine oxidase substrate to increase the percentage of H_2O_2 generated via $O_2^{\cdot-}$ dismutation as compared to direct divalent O_2 reduction. In the presence of 100 mM DMPO, H_2O_2 generation decreased 43%. DMPO did not scavenge H_2O_2 nor alter the rate of $O_2^{\cdot-}$ production. The effect of DMPO was concentration-dependent with inhibition of H_2O_2 production observed at [DMPO] > 10 mM. Inhibition of H_2O_2 production by DMPO was not observed if SOD was present or if the rate of $O_2^{\cdot-}$ formation increased. The spin trap 2-methyl-2-nitroso-propane (MNP, 10 mM) also inhibited H_2O_2 formation (81%). However, α-phenyl-*N*-*tert*-butylnitrone (PBN, 10 mM), 3,3,5,5 tetramethyl-1-pyrroline *N*-oxide (M_4PO, 100 mM), α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN, 100 mM) had no effect. These data suggest that in experimental systems in which the rate of $O_2^{\cdot-}$ generation is low, formation of H_2O_2 and thus other H_2O_2-derived species (e.g., $^{\cdot}OH$) may be inhibited by commonly used concentrations of some spin traps. Thus, under some experimental conditions spin traps may potentially prevent production of the very free radical species they are being used to detect.

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMPO/$^{\cdot}CH_3$, 2,2,5-trimethyl-1-pyrrolidinyloxyl; DMPO/$^{\cdot}OH$, 2,2-dimethyl-5-hydroxypyrrolidinyloxyl; DMPO/$^{\cdot}OOH$, 2,2-dimethyl-5-hydroperoxy-1-pyrrolidinyloxyl; DTPA, diethylenetriaminepentaacetic acid; EPR, electron paramagnetic resonance; $HO_2^{\cdot}$, perhydroxyl radical; H_2O_2, hydrogen peroxide, HBSS, Hanks' balanced salt solution; lumazine, 2,4-pteridinediol; Me_2SO, dimethyl sulfoxide; M_4PO, 3,3,5,5-tetramethyl-1-pyrroline *N*-oxide; MNP, 2-methyl-2-nitroso-propane; $^{\cdot}OH$, hydroxyl radical; $O_2^{\cdot-}$, superoxide anion, in this paper, we use $O_2^{\cdot-}$ to represent the equilibrium mixture of $O_2^{\cdot-}$ and $HO_2^{\cdot}$; PBN, α-phenyl-*N*-*tert*-butylnitrone; 4-POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; PBS, phosphate-buffered saline; SOD, superoxide dismutase; TBA, thiobarituric acid; TCA, trichloroacetic acid.

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Introduction

Increasingly, reactive oxygen species are being linked to the pathogenesis of human diseases [1]. The univalent reduction of dioxygen results in the generation of the superoxide radical. In solution, $O_2^{\cdot-}$ exists in equilibrium, $pK_a = 4.8$, with its protonated form, $HO_2^{\cdot}$ [2]. At pH 7.5, $O_2^{\cdot-}/HO_2^{\cdot}$ reacts with itself (dismutes) to form hydrogen peroxide and O_2, $k_{obs} = 2.0 \cdot 10^5$ M^{-1} s^{-1} [2]. Superoxide dismutases accelerate this reaction by 10^4, $k = 2 \cdot 10^9$ M^{-1} s^{-1} [3].

Since both $O_2^{\cdot-}$ and H_2O_2 are only mild to moderate oxidizing agents, it has been suggested that the primary mechanism whereby they cause cell injury is by acting as precursors for more toxic oxidants [4]. In vitro

$O_2^{\cdot-}$ and H_2O_2 react in the presence of a transition metal catalyst such as iron to generate the extremely reactive hydroxyl radical by a reaction scheme referred to a the iron-catalyzed Haber-Weiss reaction or the superoxide-driven Fenton reaction, as outlined below [5,6]:

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (1)$$

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+} \quad (2)$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + O_2 \quad (3)$$

The application of spin trapping techniques in conjunction with electron paramagnetic resonance spectroscopy (EPR) to experimental biologic systems has provided a powerful tool for studying the role of oxygen-centered free radicals in human disease [7,8]. With this technique, a nitrone or nitroso compound, termed a spin trap, is included in the experimental system [7,8]. Under optimal conditions the free radical of interest will react with the spin trap to generate a long-lived nitroxide free radical, which yields an EPR spectrum whose hyperfine splittings provide information about the original free radical present.

Most commonly used free radical detection systems measure reaction products formed through the interaction of the radical of interest and a particular substrate. These substrates are chosen in part because they do not interact with other oxidants, thereby increasing the specificity of the detection system. Spin trapping is therefore unique in that most spin traps have the capacity to react with numerous free radical species thereby allowing simultaneous detection of multiple free radicals species. Specificity is preserved by the unique EPR pattern of each of the resulting spin adducts.

Because of the rapid reaction ($k = \sim 10^9\ M^{-1}\ s^{-1}$) between hydroxyl radical many spin traps, as well as the relative stability of the resulting spin adduct ($t_{1/2}$ up to 2.6 h in the case of DMPO/$^{\cdot}$OH spin adduct) [7,8], spin trapping is well suited for detection of this free radical species. In contrast, the rate of reaction of $O_2^{\cdot-}$ with commonly used spin traps is quite slow. For example, rate constants reported for the reaction of $O_2^{\cdot-}$ with the most commonly used spin trap, DMPO, have ranged from 1.2 to 15.7 $M^{-1}\ s^{-1}$ [9–11]. This rate constant increases to $6.6 \cdot 10^3\ M^{-1}\ s^{-1}$ under conditions of low pH where $HO_2^{\cdot}$ rather than $O_2^{\cdot-}$ predominates [10]. Spin trapping of $O_2^{\cdot-}$ with DMPO is further compromised by the relative instability of the resultant spin adduct, DMPO/$^{\cdot}$OOH ($t_{1/2} = 27$ s and 91 s at pH 9 and 5, respectively) [12]. DMPO/$^{\cdot}$OOH decomposes to DMPO/$^{\cdot}$OH as well as several other species [9], the relative yield of which remains unclear but is quite small. In order to enhance the sensitivity of the system for superoxide detection, high concentrations of spin traps (10–100 mM) are routinely used to offset the poor reaction kinetics.

Although, the ability of spin traps to react with multiple free radical species enhances their potential usefulness, under some conditions this could pose problems. In cases in which spin traps are being used to detect a free radical whose formation is dependent on the presence of another free radical species (e.g., hydroxyl radical generation from $O_2^{\cdot-}$ by the Haber Weiss reaction), the spin trap could alter formation of the terminal free radical product by irreversibly 'trapping' and thereby limiting the availability of the precursor free radical. Consistent with this possibility, we had previously noted [13,14] that the presence of SOD often increased the concentration of hydroxyl radical-derived spin adducts of DMPO produced by the oxidation of (hypo)xanthine by xanthine oxidase in the presence of iron. In most superoxide-generating systems SOD inhibits hydroxyl radical generation, because the continuous formation of hydroxyl radical depends on $O_2^{\cdot-}$ mediated reduction of Fe^{3+} [15–17]. One possible explanation for our data was that the high DMPO concentration present (100 mM) decreased the spontaneous dismutation of $O_2^{\cdot-}$ to H_2O_2 (reaction 4) by serving as a competitive substrate for $O_2^{\cdot-}$ (reaction 5).

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$

$$O_2^{\cdot-} + DMPO + H^+ \rightarrow DMPO/{}^{\cdot}OOH \quad (5)$$

The potential importance of such a phenomenon is considerable for the design and interpretation of experiments in which spin trapping techniques are applied to complex free radical generating systems. The possible impact of the presence of spin traps on biochemical reactions has been addressed by some [18,19] but not other [20–24] reviews dealing with spin trapping techniques. Therefore, in order to provide further insight into the likelihood of such events, the effect of commonly used spin traps on the generation of H_2O_2 from $O_2^{\cdot-}$ was investigated under experimental conditions which would be likely encountered in biological systems.

Materials and Methods

Reagents

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), 3,3,5,5 tetramethyl-1-pyrroline *N*-oxide (M_4PO), α-phenyl-*N*-*tert*-butylnitrone (PBN), α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN), 2-methyl-2-nitrosopropane (MNP), 2,4-pteridinediol (lumazine), oxypurinol, hypoxanthine, xanthine, catalase, 2-deoxyribose, and diethylenetriaminepentaacetic acid (DTPA) were from Sigma Chemicals, St. Louis, MO. Xanthine oxidase was

from Boehringer-Mannheim Biochemicals, Indianapolis, IN.

Formation of hydrogen peroxide

Formation of H_2O_2 during the oxidation of xanthine, lumazine, or hypoxanthine by xanthine oxidase was quantitated by measuring evolution of O_2 with a Clark oxygen electrode (YSI Model 53, Yellow Springs Instruments, Yellow Springs, OH) following addition of catalase to the reaction mixture, using a modification of previously described methods [25]. A 3 ml solution of (hypo)xanthine (2 mM) or lumazine (0.1 mM) in 0.1 M sodium pyrophosphate buffer (pH 8.3) was placed into the chamber of the Clark electrode and allowed to equilibrate to 25 °C. Then the desired amount of xanthine oxidase (13.3–53.2 munits/ml) was added and the resulting decrease in O_2 content of the buffer was then recorded. At the desired time point, usually after 20% of the O_2 had been consumed, catalase (167 units/ml) was injected into the solution through the chamber side port using a Hamilton syringe and regeneration of previously consumed O_2 recorded. Since catalase converts two molecules of H_2O_2 to one molecule of O_2 and two molecules of H_2O there is a two to one relationship between H_2O_2 present and catalase-induced O_2 regeneration. The effect of the various spin traps on the amount of H_2O_2 generated was measured by including desired concentrations of each agent in the initial reaction mixture prior to the introduction of xanthine oxidase. In some cases SOD (30 units/ml) was also included. In experiments using hypoxanthine as the xanthine oxidase substrate where the rate of O_2 consumption was high, oxypurinol (10 μM) was added just prior to the addition of catalase to prevent any difficulty in data interpretation due to the continued consumption of O_2 by the primary reaction. To confirm that none of the spin traps scavenged H_2O_2, a solution of 40 μM H_2O_2 ± the spin trap of interest was placed in the oxygen electrode chamber and the percentage of O_2 saturation adjusted to read 50%. After 10 min of incubation, catalase (167 units/ml) was added and the amount of O_2 generation recorded.

Spin trapping

Detection of spin adducts of DMPO was performed using a Varian E104A EPR spectrometer (Varian Associates, Palo Alto, CA) according to previously described techniques [13,14]. Briefly, a 0.5 ml solution was prepared in a glass tube by adding reagents in the following order – HBSS (pH 7.5 containing 0.2 mM hypoxanthine), 0.1 mM DTPA, 0.1 mM ferrous ammonium sulfate, 140 mM Me_2SO, and 100 mM DMPO. Xanthine oxidase was added to initiate $O_2^{\cdot-}$ production. The reaction mixture was then transferred to a quartz EPR flat cell, which was in turn placed into the cavity of the EPR spectrometer. Sequential EPR spectra were then obtained at 25 °C. Although iron was added for convenience as ferrous iron to the reaction mixture, we have found that it undergoes oxidation to ferric iron (monitored as O_2 consumption) within 60 s of its addition to the phosphate containing buffers employed in our studies. Any H_2O_2 generated as a consequence disappears rapidly due to its subsequent interaction with the iron chelate. Thus, at the time of xanthine oxidase addition, ferric iron is the primary form of this transition metal in the system [a].

Unless otherwise noted, EPR spectrometer settings were: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; response time, 1 s; sweep rate, 12.5 G/min; and receiver gain, $5.0 \cdot 10^4$.

Formation of thiobarbituric acid-reactive 2-deoxyribose oxidation products

As a complimentary method of measuring hydroxyl radical generation the formation of thiobarbituric-acid (TBA)-reactive oxidation products of 2-deoxyribose was quantitated as previously described [26]. The reaction of 2-deoxyribose with hydroxyl radical generates malonaldehyde which when boiled in the presence of TBA yields a poorly characterized chromophore that absorbs at 532 nm [27]. Briefly, the desired reaction constituents were added to a solution of 5 mM 2-deoxyribose (pH 7.5); the reaction was then initiated by the addition of xanthine oxidase. After 40 min of incubation at 25 °C the reaction was terminated by the addition of 1.0 ml TCA (6.0%) and 0.5 ml TBA (1% w/v in 0.5 M NaOH). The solution was transferred to glass tubes and heated at 100 °C for 10 min, following which A_{532} was determined using a Beckman DU-30 spectrophotometer (Beckman Instruments, Palo Alto, CA).

Superoxide formation

The rate of $O_2^{\cdot-}$ formation by the various reaction mixtures was measured as the SOD-inhibitable reduction of ferricytochrome *c* as previously described [28].

[a] The use of 0.1 mM iron may seem to be an inappropriately large amount of iron to investigate the role of catalytic iron in the chemistry initiated by the superoxide-driven Fenton reaction. However, in these experiments we have chosen DTPA as the iron chelating agent rather than EDTA, because Fe-DTPA produces significantly less artifactual EPR signals than Fe-EDTA in our spin trapping experiments. The rate constant for the reaction of $O_2^{\cdot-}/HO_2^{\cdot}$ (pH 7.0) with Fe(III) EDTA is $1.9 \cdot 10^6$ $M^{-1} s^{-1}$, whereas with Fe(III) DTPA it is $< 10^4$ $M^{-1} s^{-1}$ [42]. Thus, to achieve the same rate of formation of Fe(II) chelate with the same flux of $O_2^{\cdot-}/HO_2^{\cdot}$ it is necessary to have $> \sim 100$-times more Fe(III) DTPA than Fe(III) EDTA in the incubation. Both Fe(II) DTPA and Fe(II) EDTA readily react with H_2O_2 to form hydroxyl radical.

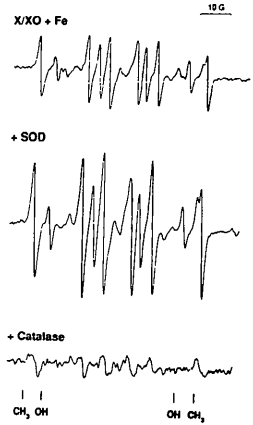

Fig. 1. EPR spectra representative of the results of eight separate experiments which were obtained following the addition of xanthine oxidase (26.6 mU/ml) to a solution (in HBSS, pH 7.5) of hypoxanthine (2 mM), DMPO (100 mM), Me_2SO (140 mM), DTPA (0.1 mM), and ferrous ammonium sulfate (0.1 mM), designated HX/XO + Fe. Under these conditions essentially all of the iron was present initially in the ferric form due to the rapid oxidation of ferrous iron in the phosphate buffer employed (see text). Also shown are results obtained under the same conditions except that 30 units/ml SOD (+SOD) or 500 units/ml catalase (+catalase) was included in the reaction mixture. High and low field peaks corresponding to the spin adducts DMPO/$^{\cdot}CH_3$ and DMPO/$^{\cdot}OH$ are designated CH_3 and OH, respectively.

Statistical analysis

Data were analyzed statistically using the paired Student's *t*-test. Although results are sometimes expressed as the percentage of control for enhanced clarity, raw data was always used for statistical analysis.

Results

Effect of SOD and catalase on formation of hydroxyl radical as detected by spin trapping and the deoxyribose oxidation assay

Similar to results in earlier studies [13,14], we found (Fig. 1) that addition of SOD (30 units/ml) to a reaction mixture of (hypo)xanthine (2 mM), xanthine oxidase (26.6 munits/ml), ferric iron (0.1 mM), DTPA (0.1 mM), Me_2SO (140 mM), and DMPO (100 mM) either increased or at best had no effect on the magnitude of EPR peak amplitudes of the resulting hydroxyl radical-derived DMPO spin adducts (DMPO/$^{\cdot}CH_3$ and DMPO/$^{\cdot}OH$). Catalase, however, markedly inhibited apparent hydroxyl radical formation (Fig. 1).

TABLE I

Effects of SOD and catalase on hydroxyl radical generation as detected by the deoxyribose assay

Shown is the effect of SOD (30 units/ml) or catalase (500 units/ml) on the amount of hydroxyl radical formed by the reaction of xanthine oxidase (26.6 mU/ml) and hypoxanthine (2 mM) in the presence of either 0.1 mM Fe^{3+}-DTPA or Fe^{3+}-NTA in PBS (pH 7.5), as quantitated by the formation of TBA-reactive 2-deoxyribose oxidation products. Results are expressed as the mean percentage of hydroxyl radical formed in the presence of SOD or catalase with each iron chelate relative to a control system, which did not contain either antioxidant enzyme. Results are the mean of three separate experiments.

	% Control	
	Fe-DTPA	Fe-NTA
Catalase	15	45
SOD	48	61

The above spin trapping results were in contrast to those obtained under similar experimental conditions where formation of TBA-reactive 2-deoxyribose oxidation products was used as the experimental method for quantitating hydroxyl radical formation (Table I). In the deoxyribose system both SOD and catalase inhibited formation of hydroxyl radical-induced products (Table I).

Previous work has shown [14] that the effect of SOD on the magnitude of apparent hydroxyl radical formation, as detected by DMPO spin trapping, could not be explained by an effect of SOD on the stability of the resulting DMPO spin adducts. In contrast to 2-deoxyribose and other substances used for hydroxyl radical detection, DMPO also has the capacity to react with $O_2^{\cdot-}/HO_2^{\cdot}$. This suggested the possibility that the presence of the high concentration of DMPO employed in the above experiments inhibited the dismutation of $O_2^{\cdot-}$ to H_2O_2, thus limiting the availability of H_2O_2 to react with Fe^{2+} to generate hydroxyl radical. SOD, by increasing the rate of the dismutation reaction, would therefore reverse this phenomenon. This possibility was subsequently examined.

Effect of DMPO on the dismutation of superoxide to hydrogen peroxide

In order to examine the effect of DMPO on dismutation of $O_2^{\cdot-}$ to H_2O_2 a solution of (hypo)xanthine was placed into the chamber of a Clark oxygen electrode. Xanthine oxidase was then introduced and the rate of O_2 consumption monitored. At a defined time point, oxypurinol was added to terminate the xanthine/xanthine oxidase reaction and H_2O_2 was quantitated by measuring the amount of O_2 regenerated after the injection of catalase into the reaction mixture. Inclusion of 100 mM DMPO in the reaction mixture

decreased the amount of H_2O_2 generated. However, these results were highly variable and difficult to reproduce from day to day. In the presence of DMPO the amount of H_2O_2 detected ranged from 34 to 100% of control with a mean of 78% ($n = 11$).

One difficulty with the above experimental design is that formation of H_2O_2 during the oxidation of xanthine by xanthine oxidase occurs via two mechanisms [29]. In the first mechanism the oxidation of xanthine is coupled to the univalent reduction of O_2 to O_2^- with subsequent dismutation of O_2^- to form H_2O_2. Secondly, the enzyme can oxidize xanthine so as to divalently reduce O_2 directly to H_2O_2, bypassing the O_2^- intermediate. Multiple experimental factors including pH, temperature, and relative substrate/enzyme ratio influence the percentage of H_2O_2 generated by the univalent or divalent reduction mechanism [29–31]. Since DMPO was postulated to only affect the dismutation reaction, it seemed possible that the variability of our results using the xanthine/xanthine oxidase system was due to our inability to adequately control divalent reduction of O_2 directly to H_2O_2.

In contrast to the oxidation of xanthine by xanthine oxidase, Nagano and Fridovich have shown [31] that oxidation of lumazine by the enzyme results in a much greater percentage of univalent O_2 reduction, up to 91% under their experimental conditions. Consequently, the affect of 100 mM DMPO on the amount of H_2O_2 resulting from the oxidation of lumazine by xanthine oxidase was examined. As seen in Fig. 2, the presence of 100 mM DMPO decreased H_2O_2 generation to 56.8 ± 4.0% of control (mean ± S.E., $n = 10$, $P < 0.00002$). The magnitude of inhibition varied directly with the concentration of DMPO (Fig. 3). The similar rate of SOD-inhibitable ferricytochrome *c* reduction in the presence and absence of 100 mM DMPO (Table II) indicated that inhibition of xanthine oxidase/lumazine induced O_2^- generation by DMPO was not an explanation for these data and is consistent with data previously reported [32]. To eliminate the possibility that DMPO was directly svavenging H_2O_2, 40 μM H_2O_2 was placed in the oxygen electrode chamber in the presence and absence of DMPO for 5–10 min, following which remaining H_2O_2 was quantitated by injection of catalase. No difference was noted between the control and DMPO incubated samples. Similarly, addition of DMPO after the completion of the xanthine oxidase/lumazine reaction had no affect on the magnitude of H_2O_2 detected with the addition of catalase.

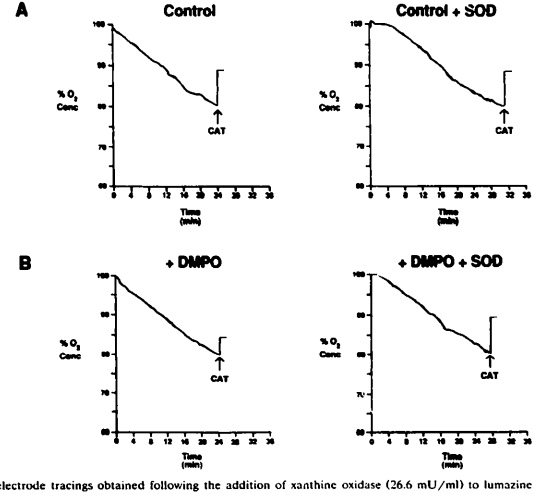

Fig. 2. (A) Oxygen electrode tracings obtained following the addition of xanthine oxidase (26.6 mU/ml) to lumazine (0.1 mM) in 0.1 sodium pyrophosphate buffer (pH 8.2) ± SOD (30 units/ml) and the regeneration of O_2 following the subsequent addition of catalase (167 units/ml) to the system. Oxygen regeneration results from the catalase-mediated conversion of H_2O_2 to H_2O and 1/2 O_2. (B) Oxygen electrode tracing obtained under the same conditions as in tracing A except that 100 mM DMPO was also present in the reaction mixture. Results are representative of ten separate experiments.

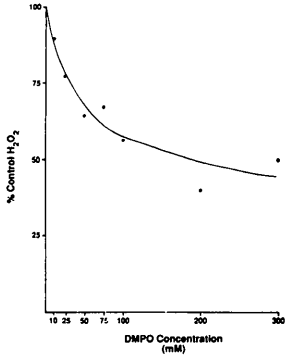

Fig. 3. Effect of the presence of increasing concentrations of DMPO on the generation of H_2O_2 formed by the reaction of xanthine oxidase (26.6 mU/ml) and lumazine (0.1 mM) in 0.1 M sodium pyrophosphate buffer (pH 8.2). Results are expressed as the mean percentage of H_2O_2 detected by the O_2 electrode assay in five separate experiments relative to paired reactions occurring in the absence of DMPO.

Effect of SOD and variations in the rate of superoxide generation on DMPO-mediated inhibition of H_2O_2 formation

The above data were consistent with a direct effect of DMPO on the dismutation of $O_2^{\cdot-}$ to H_2O_2. Further evidence in support of this hypothesis was sought. Since SOD increases the rate constant of the $O_2^{\cdot-}$ dismutation reaction by a factor of $\sim 10^4$, we reasoned that SOD would prevent DMPO-mediated inhibition of H_2O_2 generation. Accordingly, the generation of H_2O_2 by xanthine oxidase/lumazine was measured in the presence of 100 mM DMPO ± SOD. The addition of SOD to the DMPO-containing reaction mixture increased the amount of H_2O_2 recovered to that of the non-DMPO treated control (Fig. 2).

Because the dismutation of $O_2^{\cdot-}/HO_2^{\cdot}$ is a second order in $[O_2^{\cdot-}/HO_2^{\cdot}]$, the ability of a fixed concentration of DMPO to act as a competitive substrate of the $O_2^{\cdot-}$ dismutation reaction should decrease as the steady state concentration of $O_2^{\cdot-}$ increases. Consequently, the effect of increasing the rate of $O_2^{\cdot-}$ generation, and therefore its steady-state concentration, on the ability of DMPO to decrease generation of H_2O_2 during the reaction was assessed. As shown in Table II, as the rate of $O_2^{\cdot-}$ formation increased there was a corresponding decrease in the inhibition of H_2O_2 production observed in the presence of 100 mM DMPO. No effect of DMPO on the detection of the rate of $O_2^{\cdot-}$ formation was observed (Table II). This likely reflects the ability of 80 μM ferricytochrome c to outcompete 100 mM DMPO for $O_2^{\cdot-}$ as would be predicted from the reaction rates of $O_2^{\cdot-}$ with these two compounds [9–11,33]. On the other hand, the rate of O_2 consumption resulting from the oxidation of lumazine by 26.6 and 53.2 units/ml xanthine oxidase appeared greater in the presence of DMPO (Table II). this difference was statistically significant at the highest xanthine oxidase concentration. This result would be predicted if DMPO prevented $O_2^{\cdot-}$ dismutation with its resultant generation of O_2 (reaction 4), thereby resulting in an 'apparent' increase in O_2 consumed as previously observed by Bannister et al. [34].

Effect of the concentration of DMPO on the spin trapping of hydroxyl radical

As noted earlier, the rate of reaction between hydroxyl radical and DMPO is very rapid indicating a high efficiency of hydroxyl radical trapping by DMPO. This, in conjunction with the data presented above, suggested that when spin trapping hydroxyl radical being produced by an iron-supplemented $O_2^{\cdot-}$ generating system, the inhibition of H_2O_2 generation by high concentrations of DMPO may offset the enhanced

TABLE II

Effect of the rate of superoxide production on the ability of DMPO to inhibit hydrogen peroxide formation

Shown is the rate of $O_2^{\cdot-}$ formation (μM/min), O_2 consumption (μM/min), and H_2O_2 generation (expressed as the percentage of previously consumed O_2 recovered following the addition of catalase) resulting from the oxidation of lumazine (0.1 mM) by the three different concentrations of xanthine oxidase (munits/ml) in 0.1 M sodium pyrophosphate buffer (pH 8.2). Although the rate of $O_2^{\cdot-}$ generation was not significantly affected by the presence of 100 mM DMPO, the amount of apparent H_2O_2 generation was inhibited when lower concentrations of xanthine oxidase were employed as demonstrated by the differences in catalase-induced O_2 recovery in the presence (+DMPO) or absence (−DMPO) of the spin trap. In addition the presence of DMPO appeared to enhance the rate of O_2 consumption observed with higher concentrations of xanthine oxidase.

[XO]	$O_2^{\cdot-}$		O_2		% O_2 Recovery	
	−DMPO	+DMPO	−DMPO	+DMPO	−DMPO	+DMPO
13.3	0.8	0.9	1.5	1.1	46	15 *
26.6	1.4	2.2	2.3	2.9	41	20 *
53.2	3.8	3.9	3.2	4.9 *	46	33

* Statistically different from control value, $P < 0.05$.

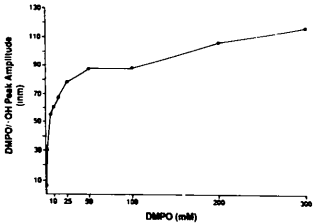

Fig. 4. Effect of DMPO concentration on the amplitude of the second DMPO/·OH peak resulting from the spin trapping of hydroxyl radical generated by the reaction of xanthine oxidase (26.6 munits/ml) and lumazine (0.1 mM) in the presence of 0.1 mM ferrous ammonium sulfate and 0.1 mM DTPA in 0.1 M sodium pyrophosphate buffer (pH 8.2). Results are representative five separate experiments.

efficiency of hydroxyl radical spin trapping. In order to assess that possibility, EPR spectra were obtained during the generation of hydroxyl radical by the reaction of xanthine oxidase, lumazine, and iron in the presence of increasing concentrations of DMPO. As shown in Fig. 4, increasing the concentration of DMPO 30-fold (10 mM to 300 mM) resulted in only about a 2-fold increase in DMPO/·OH peak amplitudes.

Effect of other spin traps on the dismutation of superoxide

DMPO is only one of several commonly used spin traps. Little information exists regarding the rate constants for the reactions of other spin traps with $O_2^{\cdot-}$ [9,10,35]. Consequently, we examined whether the effect noted above with DMPO would also be observed with other spin traps. A concentration related inhibition of H_2O_2 generation by the reaction of xanthine oxidase with lumazine was also detected with MNP. For example, 10 mM MNP decreased H_2O_2 generation to 19.8% of control ($n = 11$, $P < 0.0000001$). This inhibition was reversed by the presence of SOD and decreased as the rate of $O_2^{\cdot-}$ generation increased as observed with DMPO. No evidence of direct scavenging of H_2O_2 by MNP was detected. As measured by its ability to inhibit xanthine oxidase/lumazine-mediated reduction of ferricytochrome *c*, the rate constant for the interaction of $O_2^{\cdot-}$ and MNP was estimated to be $1.6 \cdot 10^4$ M^{-1} s^{-1} [b]. In contrast to the results with MNP and DMPO, H_2O_2 formation was 83, 89, and 84% of control in the presence of 100 mM 4-POBN, 100 mM M_4PO, and 10 mM PBN, respectively ($n = 4$-7, $P > 0.05$). The PBN concentration could not be increased further because of its insolubility in the buffer system employed.

Discussion

Due in part to its high degree of sensitivity and specificity, spin trapping has become an increasingly popular technique for the study of free radical formation in biologic and other experimental systems [7,8,18–24]. In order to maximize detection of free radical species of relatively low reactivity, such as $O_2^{\cdot-}$, spin traps are commonly employed in high concentrations. Using the reaction of xanthine oxidase with lumazine as a continuous source of $O_2^{\cdot-}$ generation, we demonstrated a concentration-dependent inhibition of H_2O_2 production by DMPO. The fact that we never observed 100% inhibition of H_2O_2 formation likely reflects the portion of H_2O_2 generated in this reaction by the direct divalent reduction of O_2 to H_2O_2. At pH 7.5, SOD increases the rate constant for the dismutation of $O_2^{\cdot-}$ from $2.0 \cdot 10^5$ M^{-1} s^{-1} to $1.9 \cdot 10^9$ M^{-1} s^{-1} and in turn it prevented DMPO inhibition of H_2O_2 generation [c]. In addition it was found that increasing the rate of $O_2^{\cdot-}$ generation, and thus its steady state concentration, also decreased DMPO-mediated inhibition. Finally, the presence of DMPO appeared to enhance the rate of O_2 consumption resulting from the oxidation of lumazine by xanthine oxidase as would be expected if the regeneration of O_2, which occurs as a consequence of the dismutation reaction (reaction 4), was inhibited. These data strongly suggest that DMPO interferes with $O_2^{\cdot-}$ dismutation to H_2O_2 (reaction 4) by acting as a competitive substrate for $O_2^{\cdot-}$ (reaction 5).

The potential for such an event may be easily overlooked in the design of spin trapping systems since the rate constant for the dismutation reaction at pH 7.5 is at least 10^4-fold greater than the fastest reported [9–11]

[b] This rate constant was determined using that the rate constant of $1.1 \cdot 10^6$ M^{-1} s^{-1} for the reaction of $O_2^{\cdot-}/HO_2^{\cdot}$ with ferricytochrome *c* in the pH 8.2 pyrophosphate buffer used for these competition experiments [33]. The MNP solution was freshly prepared; this rate constant represents an observed rate constant for the MNP, without regard to equilibrium between MNP dimer and blue monomer.

[c] In the experiments described here we used 30 U/ml of SOD, as measured by the standard cytochrome *c* assay [43]. In this assay 1 unit is equivalent to 0.1 μg/ml of bovine CuZn SOD [43]; thus, 30 U/ml of SOD = $9 \cdot 10^{-8}$ M. Comparing the rates of the reaction of $O_2^{\cdot-}/HO_2^{\cdot}$ with DMPO and SOD at pH 7.4 we have:

$$\frac{\text{rate with SOD}}{\text{rate with DMPO}} = \frac{2 \cdot 10^9\ M^{-1}\ s^{-1} \cdot 9 \cdot 10^{-8}\ M \cdot [O_2^{\cdot-}/HO_2^{\cdot}]}{30\ M^{-1}\ s^{-1} \cdot 0.1\ M \cdot [O_2^{\cdot-}/HO_2^{\cdot}]}$$

$$\frac{\text{rate with SOD}}{\text{rate with DMPO}} = 60/1$$

i.e., SOD outcompetes DMPO for $O_2^{\cdot-}/HO_2^{\cdot}$ by a factor of 60:1 in our experiments.

rate constant for the reaction of $O_2^{\cdot}$ with DMPO. However, our calculations based on a steady state $O_2^{\cdot-}$ concentration of 1 μM show that the high DMPO concentration (100 mM) present in the spin trapping system would negate this difference [d]. Consistent with our data these calculations predict that H_2O_2 generation should be inhibited by DMPO concentrations above ~ 10 mM [d], supporting the suggestion of Finkelstein et al. [9] that at high spin trap concentrations the spontaneous dismutation reaction of $O_2^{\cdot-}$ could be ignored in calculating the constants of the spin traps DMPO and TMPO with $O_2^{\cdot-}$. Our data also add further support to the observations of Bannister and colleagues who hypothesized that the enhanced rate of O_2 consumption they observed during the generation of $O_2^{\cdot-}$ by the neutrophil NADPH oxidase system in the presence of DMPO was the result of the prevention of $O_2^{\cdot-}$ dismutation [34].

Although the simplest explanation for our data involves DMPO acting as a competitive inhibitor of the dismutation reaction, other reactions could also contribute. One of us (GRB) has recently demonstrated [36] that the $O_2^{\cdot-}$ spin adduct of DMPO, DMPO/$^{\cdot}$OOH, also reacts rapidly with $O_2^{\cdot-}$ (apparent rate constant of $6.6 \cdot 10^6$ M^{-1} s^{-1} at pH 7.4). In addition, $O_2^{\cdot-}$ has been shown to react with DMPO/$^{\cdot}$OH, a minor decomposition product of DMPO/$^{\cdot}$OOH [14,37–39], although the rate constant of this reaction has not been reported. Furthermore, it has been shown that other nitroxides will react with $O_2^{\cdot-}$ at reaction rates between $1.1 \cdot 10^3$ and $1.3 \cdot 10^6$ M^{-1} s^{-1} [40,41]. Therefore, it is possible that some of the inhibition of H_2O_2 generation observed with DMPO results from the interaction of $O_2^{\cdot-}$ with DMPO/$^{\cdot}$OOH (reaction 6) or DMPO/$^{\cdot}$OH (reaction 7) rather than DMPO itself (reaction 5).

$$\text{DMPO/}^{\cdot}\text{OOH} + O_2^{\cdot-} \rightarrow \text{degradation products} + O_2 \quad (6)$$

$$\text{DMPO/}^{\cdot}\text{OH} + O_2^{\cdot-} \rightarrow \text{degradation products} + O_2 \quad (7)$$

These possibilities seem less likely however, since the reaction between $O_2^{\cdot-}$ and various nitroxides has been reported to be 'SOD-like' [40,41], that is accelerating the rate of $O_2^{\cdot-}$ dismutation to H_2O_2 rather than preventing H_2O_2 production.

Since DMPO is only one of several commonly used spin trapping agents, the possibility that other such compounds could influence $O_2^{\cdot-}$ dismutation to H_2O_2 was also examined. Only one of the four spin traps examined, MNP, demonstrated an ability to decrease the production of H_2O_2 by the xanthine oxidase/lumazine reaction in a concentration-dependent manner. In contrast to the results with MNP, neither PBN, 4-POBN, nor M_4PO appeared to affect the dismutation reaction. There should be a direct relationship between the inhibitory capacity of each spin trap and its ability to react with $O_2^{\cdot-}$. However, little information is available as to the rate constants for the reaction of each of these spin traps with $O_2^{\cdot-}$. The second-order rate constant for the reaction of $O_2^{\cdot-}$ with M_4PO has been reported to be 1 M^{-1} s^{-1} [35]. Finkelstein and colleagues reported that 4-POBN was approximately thirty times slower than DMPO in trapping $O_2^{\cdot-}$ although no specific rate constant was given [9]. To our knowledge the rate constant for the reaction of $O_2^{\cdot-}$ with PBN or MNP has not been reported. Our data [b] suggest an 'effective' rate constant of approximately $1.6 \cdot 10^4$ M^{-1} s^{-1}.

The results of our work have important implications for the application of spin trapping techniques for the detection of hydroxyl radical and other oxidants formed as secondary products of $O_2^{\cdot-}$. Consistent with our previous experience [13,14] and in contrast to results with other hydroxyl radical detection systems (see Refs. 15–17, and Table I), the addition of SOD to the reaction of (hypo)xanthine and xanthine oxidase in the presence of a catalytic iron chelate enhanced or had no effect on the magnitude of the EPR spectral peaks of the hydroxyl radical generated spin adducts of DMPO. It seems likely that this phenomenon was due to SOD-reversible inhibition of H_2O_2 formation by DMPO, resulting in decreased hydroxyl radical production.

The rate of $O_2^{\cdot-}$ generation during the above experiments is likely similar to that produced in many biologic systems where spin trapping has been utilized for the investigation of potential hydroxyl radical production. Since hydroxyl radical generation is directly dependent on the presence of H_2O_2, in experimental

[d] At pH 7.4 k_{obs} (for DMPO + $O_2^{\cdot-}$/$HO_2^{\cdot}$) = 30 M^{-1} s^{-1} [10], and k_{obs} (for $O_2^{\cdot-}$/$HO_2^{\cdot}$ dismutation) = $2 \cdot 10^5$ M^{-1} s^{-1} [2]. If $[O_2^{\cdot-}/HO_2^{\cdot}]_{ss} = 1$ μM, then the rate of disappearance of $O_2^{\cdot-}$/$HO_2^{\cdot}$ by the spin trapping reaction (or the rate of DMPO/$^{\cdot}$OOH formation) will be:

$$d[O_2^{\cdot-}/HO_2^{\cdot}]/dt = -30\ M^{-1}\ s^{-1}\ [DMPO][1 \cdot 10^{-6}\ M]$$

For [DMPO] = 0.1 M this rate is $-3 \cdot 10^{-6}$ Ms^{-1}. The rate of disappearance of $O_2^{\cdot-}$/$HO_2^{\cdot}$ by dismutation (without SOD) will be:

$$d[O_2^{\cdot-}/HO_2^{\cdot}]/dt = -2k_{obs}[O_2^{\cdot-}/HO_2^{\cdot}]^2$$

At pH 7.4 and $[O_2^{\cdot-}/HO_2^{\cdot}]_{ss} = 1$ μM this rate will be $-4 \cdot 10^{-7}$ Ms^{-1}. Thus, ~ 90% of the disappearance of $O_2^{\cdot-}$/$HO_2^{\cdot}$ will be via the spin trapping reaction. However, $[O_2^{\cdot-}/HO_2^{\cdot}]_{ss} = 1$ μM is quite high. The actual steady state concentration of $O_2^{\cdot-}$/$HO_2^{\cdot}$ in a spin trapping system such as described here is on the order of 10 nM or less [36]. At this concentration the rate of disappearance of $O_2^{\cdot-}$/$HO_2^{\cdot}$ via dismutation (pH 7.4) is $-4 \cdot 10^{-11}$ Ms^{-1}. Thus, even at 10 mM DMPO, the spin trapping reaction will capture more than 99% of the $O_2^{\cdot-}$/$HO_2^{\cdot}$ produced and negligible H_2O_2 would be formed from the dismutation reaction of $O_2^{\cdot-}$/$HO_2^{\cdot}$.

systems in which the rate of $O_2^{\cdot-}$ generation is relatively low and particularly if $O_2^{\cdot-}$ dismutation is the only means of H_2O_2 production, the use of high concentrations of spin traps such as DMPO to 'enhance sensitivity' could conceivably prevent the generation of the very free radical species which the system is trying to detect. We found that a 30-fold increase (10–300 mM) in DMPO concentration did not markedly increase the concentration of DMPO/$^{\cdot}$OH generated by the reaction of xanthine oxidase and lumazine in the presence of iron-DTPA. This likely reflects the combined effects of DMPO being a highly efficient hydroxyl radical trap ($k \geq 10^9$ M^{-1} s^{-1} [7,8]) and the inhibition of H_2O_2 generation by increasing DMPO concentrations. Thus, in designing spin trapping experiments investigators should consider using lower spin trap concentrations that will only modestly decrease the efficiency of hydroxyl radical spin trapping, but which will provide less interference with the dismutation of $O_2^{\cdot-}$.

In summary, we have shown that when used at concentrations routinely employed in spin trapping systems two commonly used spin traps, DMPO and MNP, have the potential for decreasing the formation of H_2O_2 via the dismutation of $O_2^{\cdot-}/HO_2^{\cdot}$. Investigators using spin trapping techniques to assess formation of hydroxyl radical or alternatively, lipid radicals, whose generation is dependent on the formation of H_2O_2 from $O_2^{\cdot-}$ need to consider the effect of these spin trapping agents on the chemistry of the systems under study.

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