Evaluation of various spin traps for the *in vivo in situ* detection of hydroxyl radical

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The ideal spin trap for the *in vivo in situ* detection of $HO^{\bullet}$ is one that reacts specifically with this free radical at near diffusion controlled rates. Further, the corresponding spin trapped adducts must be sufficiently long-lived to allow the acquisition of EPR spectra for prolonged periods of time. Herein, we report on our studies in which we explored the ability of various imidazole *N*-oxides, an isoquinoline *N*-oxide and pyrroline *N*-oxides to specifically react with $HO^{\bullet}$ at the expense of $O_2^{\bullet -}$. Further, we estimated the rate constant for spin trapping $HO^{\bullet}$. Finally, we measured the stability of the corresponding spin trapped adducts. Our data suggest that imidazole *N*-oxides, in particular 2,2-dimethyl-4-methoxycarbonyl-2*H*-imidazole 1-oxide (**3**), appear to be the best spin traps for the *in vivo in situ* detection of $HO^{\bullet}$ in real time.

Introduction

It is widely believed that the initiating toxic event generated by radiation for both normal tissue and tumor cells is the production of hydroxyl radical ($HO^{\bullet}$). Ionizing radiation generates $HO^{\bullet}$ from water through a process involving at least two steps. The highest yield products are commonly characterized as follows:[1]

$$H_2O + h\nu \longrightarrow H_2O^{+\bullet} + e^- (\longrightarrow e_{aq})$$

$$H_2O^{+\bullet} + H_2O \longrightarrow HO^{\bullet} + H_3O^+$$

In *in vitro* models, $HO^{\bullet}$ scavenging experiments have been the primary method used to demonstrate that this free radical is responsible for the observed cytotoxicity.[2] The use of such methods *in vivo* is far more problematic, due to a number of kinetic, dynamic and metabolic factors that impact the ability of the scavenger to report reliable outcomes. Although similar radiolytic events are believed to occur in animals exposed to ionizing radiation, tissue-associated homeostatic mechanisms may neutralize free radicals before they can exert their effects. Thus, tissue measurements of $HO^{\bullet}$ evolution are necessary to bridge the gap between cellular and animal models in assessing the biological effects of ionizing radiation and the physiological responses to this oxidant stress. Thus, a rational approach to the beneficial modulation of these mechanisms requires the detection and quantification of $HO^{\bullet}$ and other free radicals in animal models where and when they are produced.

For the past decade, we have refined spin trapping/EPR spectroscopy to allow the identification of free radicals in mice in real time and at the site of their evolution.[3] Several years ago, we reported our initial *in vivo in situ* experiments detecting free radicals derived from $HO^{\bullet}$, produced in an irradiated leg tumor of a mouse.[4] This required a low frequency EPR spectrometer, which can make *in vivo* measurements of free radicals in living tissue deep in animals and probes capable of spin trapping these reactive species.[3b] Further, the corresponding spin trapped adduct must have a sufficiently long lifetime to allow its characterization.[3b]

Despite the success we have had in our initial efforts at the *in vivo in situ* detection of $HO^{\bullet}$, there are a number of limitations that prevented us from advancing further. One only needs to examine the spin trapping system used, namely α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (4-POBN) in the presence of EtOH, to appreciate the depth of the problem.[5] Ethanol and 4-POBN must be localized in the same tissue compartment so that the α-hydroxyethyl radical, formed as the result of the reaction of $HO^{\bullet}$ with EtOH, can be spin trapped by 4-POBN. Even though EtOH is diffusible into many compartments within a tissue, 4-POBN, which has a small *n*-octanol–water partition coefficient (0.09), may not easily diffuse into cells.[5b] Thus, the $HO^{\bullet}$ detected by 4-POBN–EtOH is undoubtedly limited to interstitial and vascular spaces. In addition, α-hydroxyethyl radical is a secondary free radical, in our case a "marker" of $HO^{\bullet}$ formation. Therefore, additional experiments are required to verify that $HO^{\bullet}$ was responsible for the EPR spectrum corresponding to 4-POBN–$CH(OH)CH_3$. Although this has been accomplished in aqueous buffers and in isolated cell models,[5a] it is considerably more difficult to prove, *in vivo*.

The ideal spin trap for the *in vivo in situ* detection of $HO^{\bullet}$ is one that reacts specifically with this free radical at near diffusion controlled rates. Further, the corresponding spin trapped adducts must be sufficiently long-lived to allow the acquisition of EPR spectra for prolonged periods of time. In this report, we synthesized a number of spin traps, surveyed their specificity for $HO^{\bullet}$, measured the rate of spin trapping $HO^{\bullet}$ and estimated the stability of the corresponding spin trapped adducts.

Results and discussion

Spin trapping hydroxyl radical

As early as the mid-1970s, it was clear that nitrones could spin trap oxygen-centered free radicals[6] like $HO^{\bullet}$ and $O_2^{\bullet -}$, yet the efficiencies of these reactions were rarely defined. Rather, it was assumed that the reaction of nitrones with free radicals was limited by the rate of free radical formation.[7] In the intervening years, this hypothesis has been found to be confined to a selected group of nitrones and a few free radicals.[8] Of particular interest to us have been nitrones with specificity towards $HO^{\bullet}$.[8f,9] Our immediate goal is to arrive at unique compounds,

Table 1 Spin trapping of $HO^{\bullet}$

Spin trap	Splitting constant/G	$t_{1/2}$/min	Rate constant/ 10^9 M^{-1} s^{-1}
1	$A_N = 14.0$, $A_H = 16.0$	11.3 ± 1.4	1.38 ± 0.29 (1.39 ± 0.60)[a]
2	$A_N = 14.2$, $A_D = 2.5$	14.9 ± 1.6	0.98 ± 0.18
3	$A_N = 14.0$, $A_H = 15.2$	12.6 ± 0.4	(1.04 ± 0.19)
4	$A_N = 14.1$, $A_H = 15.5$	17.4 ± 2.3	0.99 ± 0.11 (1.41 ± 0.72)
5	$A_N = 15.2$, $A_H = 8.2$	8.7 ± 0.8	(2.74 ± 0.61)
6	$A_N = A_H = 14.9$	116.0 ± 7.0	0.55 ± 0.22
7	$A_N = A_H = 14.7$	61.2 ± 5.9	2.1 (lit.)[b]

[a] Rate constants in parentheses indicate that $HO^{\bullet}$ was generated from H_2O_2/Fe^{2+}, otherwise, the $HO^{\bullet}$ was generated from photolysis of H_2O_2. [b] From ref. 8*a*.

which, when introduced into animals, can *in vivo in situ* spin trap $HO^{\bullet}$ in real time. Herein, we examine several different classes of nitrones and compare their ability to spin trap $HO^{\bullet}$ at the expense of $O_2^{\bullet -}$.

For these initial investigations, we selected representative examples of imidazole *N*-oxides, isoquinoline *N*-oxides and pyrroline *N*-oxides and contrasted their capability to spin trap $HO^{\bullet}$ (Table 1). Apparent rate constants were obtained, as a measure of their effectiveness, by estimating the relative rate of spin trapping $HO^{\bullet}$ as compared to that for 4-POBN. This acyclic nitrone was chosen as a competitive inhibitor, since the EPR spectrum for 4-POBN–OH does not interfere with the spectral lines of each of the spin trapped adducts evaluated. The reactions of the spin trap and 4-POBN with $HO^{\bullet}$ can be expressed as eqns. (1) and (2). From these equations, the rate of

$$HO^{\bullet} + \text{Spin Trap} \longrightarrow \text{Spin Trap–OH} \quad (1)$$

$$HO^{\bullet} + \text{4-POBN} \longrightarrow \text{4-POBN–OH} \quad (2)$$

$HO^{\bullet}$ elimination can be represented as eqn. (3). In the absence

$$V = -\mathrm{d}[HO^{\bullet}]/\mathrm{d}t = k_{app}[\text{Spin Trap}][HO^{\bullet}] + k_{\text{4-POBN–OH}}[\text{4-POBN}][HO^{\bullet}] \quad (3)$$

of 4-POBN, eqn. (3) can be described as eqn. (4). By dividing

$$v = -\mathrm{d}[HO^{\bullet}]/\mathrm{d}t = k_{app}[\text{Spin Trap}][HO^{\bullet}] \quad (4)$$

eqn. (4) into eqn. (3) and rearranging the terms, the competing reactions can be represented as eqn. (5). Determining the rates

$$V/v = 1 + k_{\text{4-POBN–OH}}[\text{4-POBN}]/k_{app}[\text{Spin Trap}] \quad (5)$$

V and v is cumbersome, resulting too often in estimations that are prone to error. However, the relative concentration of the spin trapped adduct, as determined by peak height, is directly related to the k_{app} and $k_{\text{4-POBN–OH}}$ at a given concentration of a spin trap. As such, we can assign the term A_o to be the peak height of the spin trapped adduct of the hydroxyl radical in the absence of 4-POBN and term A to be the peak height of the spin trapped adduct of hydroxyl radical in the presence of 4-POBN at various concentrations.[8d] If the concentration of the spin trap is fixed, then a plot of A_o/A *versus* [4-POBN] becomes a straight line, the slope is $k_{\text{4-POBN–OH}}/k_{app}$[Spin Trap].[8d] By using the known rate constant[8a] for the formation of $k_{\text{4-POBN–OH}}$, 1.9×10^9 M^{-1} s^{-1}, the k_{app} for the various nitrones studied can be calculated (Fig. 1, Table 1). As can be seen in Table 1, the apparent rate constants are comparable, ranging from 0.55 to 2.7×10^9 M^{-1} s^{-1}.

As important as rate constants are in predicting spin trapping efficiency, the stability of the spin trapped adduct can be considered a more important variable in determining the feasibility of observing an EPR spectrum, *in vivo*, especially where metabolic factors greatly contribute to the elimination of the spin trapped adduct. Consider, for example, the reaction of $HO^{\bullet}$ with 5,5-dimethyl-3,4-dihydropyrrole *N*-oxide (DMPO, **7**) and 4-POBN. Even though the rate constants[8a] are similar, in the range of $2–5 \times 10^9$ M^{-1} s^{-1}, the first-order half-lives of the corresponding spin trapped adducts in aqueous solutions are remarkably different, from under a min[8a,10] for 4-POBN–OH to as long as 1 h[8a,b] for DMPO–OH, depending on experimental conditions (see Table 1).

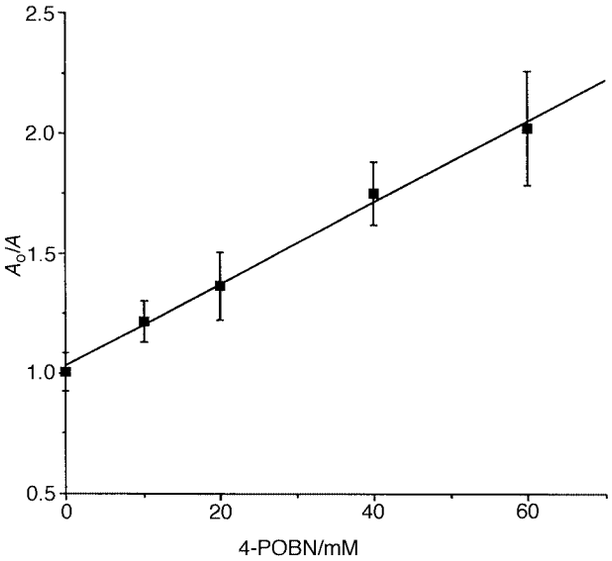


Fig. 1 A representative plot of the inhibition by 4-POBN of the spin trapping of $HO^{\bullet}$ by nitrone **4**. From these data, the apparent rate constant was found to be 1.12×10^9 M^{-1} s^{-1}. Methods are described in the Experimental section. Rate constants for the spin trapping of $HO^{\bullet}$ by this and other spin traps are presented in Table 1.

The decay of the spin trapped adducts seems to follow two different reaction pathways.[8a,b,f,h,10] Early on, when the concentration of the spin trapped adduct is high, the rate of disappearance is second order, consistent with a disproportionation reaction.[10b–d] Over time, as the concentration of the spin trapped adduct decreases, the dismutation pathway no longer dominates, shifting instead to a first-order decay—a simple solvolysis reaction.[8b,10b] Evidence in support of this scheme comes from the finding of a pH-dependent rate of hydrolysis[10a–e] and time-delayed production of *tert*-butylhydroaminoxyl from PBN–OH.[10b] *In vivo*, the first-order decay is the more important of the two mechanisms for elimination of the spin trapped adducts under investigation. For this reason, half-lives of the various hydroxy-spin trapped adducts shown in Table 1 were based on first-order kinetics (Fig. 2). Of interest is the finding that spin trapped adducts derived from the reaction of $HO^{\bullet}$ with pyrroline *N*-oxides exhibited considerably longer half-lives than the corresponding reaction with imidazole *N*-oxides, with the half-life of hydroxy spin trapped adduct derived from pyrroline *N*-oxide **6** approaching nearly 2 h (Table 1). As exciting as these results have been, the half-life of the hydroxyl spin trapped adduct of isoquinoline *N*-oxide **5** was surprisingly poor (Table 1).

Spin trapping of superoxide

Next, we determined the specificity of the spin traps shown in Scheme 1. One of the problems with pyrroline *N*-oxide **7** is that DMPO–OH can be derived from the spin trapping of $HO^{\bullet}$, the decomposition of DMPO–OOH or metal ion-catalyzed aerobic oxidation.[8a,11] Thus, with pyrroline *N*-oxide **7**, it is frequently difficult to determine whether $HO^{\bullet}$ or $O_2^{\bullet -}$ was actually spin trapped, especially intracellularly. While synthetic

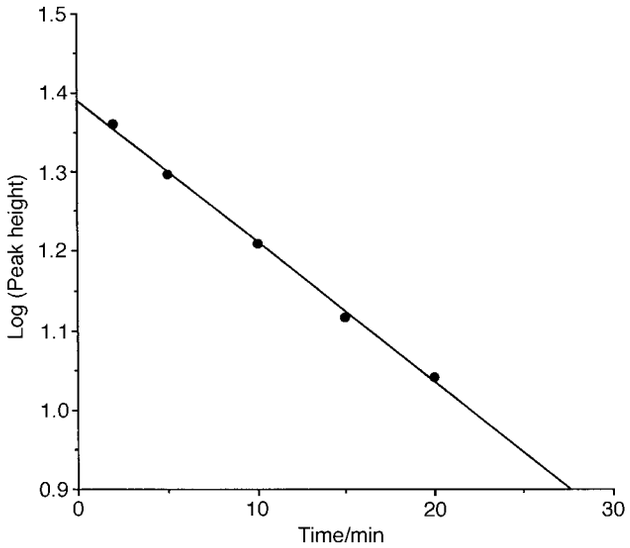
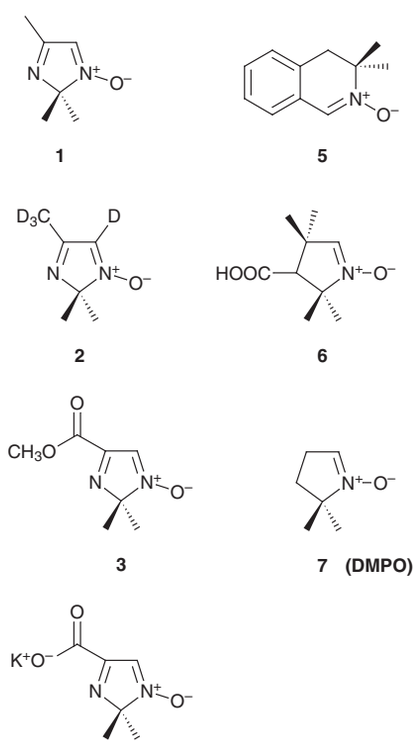


Fig. 2 A representative plot of the log of the signal height *versus* time of the decay of the spin trapped adduct derived from the reaction of $HO^{\bullet}$ with nitrone **4**. The calculated half-life was 17 min. Methods are described in the Experimental section. The half-life of this and other spin trapped adducts of $HO^{\bullet}$ are presented in Table 1.



Scheme 1

modifications to pyrroline *N*-oxide **7** have yielded nitrones with enhanced specificity toward $HO^{\bullet}$,[8f,9] we are far from the most practical nitrone for the *in vivo in situ* detection of $HO^{\bullet}$. Therefore, we investigated which of the nitrones in Scheme 1 might exhibit the requisite specificity we seek. For these experiments, we used two sources of $O_2^{\bullet -}$, a hypoxanthine–xanthine oxidase system and PMA-stimulated neutrophils. In each case, the rate of $O_2^{\bullet -}$ production was 5–10 μM min^{-1}. The results, independent of the source of $O_2^{\bullet -}$, are the same and are shown in Table 2. Several observations are readily apparent. First, isoquinoline *N*-oxide **5** not only reacted with $HO^{\bullet}$, but this nitrone was effective at spin trapping $O_2^{\bullet -}$.[12] Second, 2,2-dimethyl-4-methoxycarbonyl-2*H*-imidazole 1-oxide **3** does not spin trap $O_2^{\bullet -}$, which is in agreement with literature findings. Third,

Table 2 Spin trapping of $O_2^{\bullet -}$

Spin trap	Splitting constant/G
1	$A_N = 14.0$, $A_H = 16.0$
2	$A_H = 14.2$, $A_D = 2.5$
3	—
4	$A_N = 13.3$, $A_H = 15.6$
5	$A_N = 14.2$, $A_H = 4.3$
6	—
7	$A_N = 14.3$, $A_H^{\beta} = 11.4$, $A_H^{\gamma} = 1.3$

Hyperfine splitting constants for the spin trapped adducts of $O_2^{\bullet -}$ were found to be identical whether hypoxanthine–xanthine oxidase or PMA-stimulated human neutrophils were used as the source of this free radical.

imidazole *N*-oxide **1**, its deuterium-containing analog **2**, and **4**, like pyrroline *N*-oxide **7**, appear to have spin trapped $O_2^{\bullet -}$, even though the hyperfine splitting constants for the spin trapped adducts of **1** and **2**, from their reaction with $HO^{\bullet}$ and $O_2^{\bullet -}$, are the same (compare data in Table 1 and Table 2). This conclusion is based on the finding that superoxide dismutase (SOD) (30 U mL^{-1}), but not catalase (300 U mL^{-1}), completely inhibited the EPR spectrum. It is, of course, conceivable that initially imidazole *N*-oxides **1** and **2** spin trapped $O_2^{\bullet -}$. Rapid decomposition of the corresponding hydroperoxy-spin trapped adduct resulted in the recording of an EPR spectrum corresponding to that of the hydroxyl radical adduct of imidazole *N*-oxides **1** and **2**. In any event, these data are surprising, considering previous reports have suggested that imidazole *N*-oxides **1** and **4** do not react with $O_2^{\bullet -}$.[13] Finally, it is worth noting that with pyrroline *N*-oxide **6**, we did record an EPR spectrum from its reaction with $O_2^{\bullet -}$. However, this EPR spectrum was so small that it was insignificant. Therefore, for all practical purposes pyrroline *N*-oxide **6** appears to be specific for $HO^{\bullet}$.

The specificity of each of the above nitrones toward $HO^{\bullet}$ at the expense of $O_2^{\bullet -}$ is based upon whether an EPR spectrum was recorded. The absence of an EPR signal may be attributed to: (a) the rate of reaction is slow, (b) the half-life of the spin trapped adduct is too short-lived to be observed by EPR spectroscopy at ambient temperature, or (c) the reaction yields non-radical products that would be undetectable by EPR spectroscopy. Of these three possibilities, we favor the short-lived spin trapped adduct theory. First, $O_2^{\bullet -}$ reacts with the pyrroline *N*-oxide **7** at only 12 M^{-1} s^{-1} at pH 7.4[8a] and at an even slower rate of 1 M^{-1} s^{-1} with 3,3,5,5-tetramethyl-1-pyrroline *N*-oxide at this same pH.[9] Despite these slow rate constants, recordable EPR spectra have been obtained.[8a,9,14] Second, the half-lives of the spin trapped adducts of acyclic nitrones such as 4-POBN and PBN are less than 1 min, so short that under experimental conditions such as ambient temperatures in buffered aqueous solutions at pH 7.4, an EPR spectrum is rarely recorded.[8a] Finally, the primary reaction of $O_2^{\bullet -}$ in aqueous solutions is disproportionation,[15] its addition to a double bond is considerably slower and results in formation of a secondary free radical which in this case should lead to an aminoxyl.

Conclusion

In this study, three classes of spin traps: 2*H*-imidazole 1-oxides, isoquinoline *N*-oxides, and pyrroline *N*-oxides were investigated as to their specificity and efficiency at spin trapping $HO^{\bullet}$ and $O_2^{\bullet -}$, as well as the stability of the corresponding spin trapped adducts. While 4-carboxy-3,3,5,5-tetramethyl-1-pyrroline *N*-oxide **6** exhibited a high degree of specificity toward $HO^{\bullet}$, the laborious synthetic scheme suggests its limited utility for *in vivo in situ* detection of $HO^{\bullet}$. This is particularly so, when one considers the enormous cost of preparing ^{15}N and deuterium-containing spin traps of pyrroline *N*-oxide **7**

and analogs of 4-ethoxycarbonyl-3,3,5,5-tetramethyl-1-pyrroline *N*-oxide.[8f,16] In contrast, 2,2-dimethyl-4-methoxycarbonyl-2*H*-imidazole 1-oxide **3** has been found to be the most selective of the spin traps investigated, specifically reacting with $HO^{\bullet}$ at the expense of $O_2^{\bullet -}$. In contrast to pyrroline *N*-oxides, isotope labeled 2*H*-imidazole 1-oxides can readily be prepared with reasonable yields.[13b] Although the half-life of the hydroxyl radical adduct of the imidazole *N*-oxide **3** is shorter than that of the corresponding spin trapped adduct of pyrroline *N*-oxide **7**, the specificity of **3** and the ability to prepare readily isotope-labeled imidazole *N*-oxides make this class of nitrones viable candidates for the *in vivo in situ* detection of $HO^{\bullet}$.

Experimental

Reagents

Hypoxanthine, xanthine oxidase, catalase, diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma Chemical Company (St. Louis, MO). α-(4-Pyridyl 1-oxide)-*N*-*tert*-butylnitrone (4-POBN) was purchased from Aldrich Chemical Company (Milwaukee, WI). Superoxide dismutase (SOD) was obtained from Boehringer Mannheim (Indianapolis, IN). Phorbol 12-myristate 13-acetate (PMA) was obtained from Midland Chemical Co. (Brewster, NY). 5,5-Dimethyl-3,4-dihydropyrrole *N*-oxide (DMPO) was synthesized as presented in the literature[17] and purified by Kugelrohr distillation. 2,2,4-Trimethyl-2*H*-imidazole 1-oxide (**1**), 2,2-dimethyl-4-methoxycarbonyl-2*H*-imidazole 1-oxide (**3**), and 4-carboxy-2,2-dimethyl-2*H*-imidazole 1-oxide (**4**) were prepared according to literature procedures.[13,18] 3,3-Dimethyl-3,4-dihydroisoquinoline *N*-oxide (**5**) was generously provided by Marion Merrell Dow (Cincinnati, OH).

4-Carboxy-3,3,5,5-tetramethyl-1-pyrroline *N*-oxide (6). To a solution of 4-ethoxycarbonyl-3,3,5,5-tetramethyl-1-pyrroline[19] (1.4 g, 7.1 mmol) in absolute ethanol (15 mL) was added sodium borohydride (530 mg, 14.2 mmol). This reaction was stirred overnight at room temperature, at which point, the mixture was evaporated *in vacuo*. The remaining residue was taken up into water (10 mL) and extracted with chloroform. The organic solution was dried over anhydrous Na_2SO_4. After filtration, the remaining solution is rotary evaporated, giving 4-ethoxycarbonyl-3,3,5,5-tetramethyl-1-pyrrolidine as an oil (1.25 g, 89%). The absence of an imine peak in the IR spectrum confirms the success of the reduction.[18] IR (Neat liquid) 1750 (C=O) cm^{-1}.

This crude pyrrolidine was oxidized without further purification using the method of Murahashi and co-workers.[20] To a mixture of crude 4-ethoxycarbonyl-3,3,5,5-tetramethyl-1-pyrrolidine (1.25 g, 6.3 mmol) in methanol (10 mL) at 0 °C under argon was added sodium tungstate dihydrate (83 mg, 250 μmol) and H_2O_2 (30%, 1.6 mL, 13.86 mmol). The reaction was continually stirred anaerobically at room temperature for 3 h at 0 °C. Methanol was then removed *in vacuo*. To the remaining residue, saturated NaCl was added. After extraction with chloroform, the organic solution was dried over anhydrous Na_2SO_4, filtered, and rotary evaporated to dryness, giving 4-ethoxycarbonyl-3,3,5,5-tetramethyl-1-pyrroline *N*-oxide as an oil. IR (Neat liquid) 1750 (C=O), 1590 (C=N–O) cm^{-1}. Crystallization with hot pentane yielded the desired product (900 mg, 69%) as a white solid, mp 44–45 °C.[19] NMR ($CDCl_3$) δ: 1.1–1.6 (m, 15H, CH_3), 2.9 (s, 1H, $HCCO_2$), 4.2 (q, 2H, OCH_2), 6.61 (s, 1H, HC=N–O).

4-Carboxy-3,3,5,5-tetramethyl-1-pyrroline *N*-oxide (**6**) was prepared by heating a solution of 4-ethoxycarbonyl-3,3,5,5-tetramethyl-1-pyrroline *N*-oxide (1 g, 4.7 mmol) dissolved in ethanol (95%, 20 mL) to reflux with dilute sodium hydroxide (1 M, 5 mL) for several hours. Upon cooling in an ice bath, the reaction was concentrated *in vacuo* and passed through a Dowex 50 (H^+ form) column, until the presence of the product was no longer visible. Fractions were collected and rotary evaporated, giving 4-carboxy-3,3,5,5-tetramethyl-1-pyrroline *N*-oxide (**7**) (720 mg, 83%) as a white solid from chloroform–hexane, mp > 240 °C with decomposition. NMR (D_2O) δ: 1.2–1.6 (m, 12H, CH_3), 2.9 (s, 1H, $HCCO_2$), 7.1 (s, 1H, HC=N–O).

EPR Spectral measurements

The spectra of spin adducts, derived from the reaction of $HO^{\bullet}$ and $O_2^{\bullet -}$ with the nitrones in Scheme 1, were recorded using an EPR spectrometer (Varian Associates E-9) at 25 °C. Reaction mixtures were transferred to a flat quartz cell, fitted into the cavity of the EPR spectrometer and spectra were recorded at room temperature. Instrumentation settings were microwave power, 20 mM; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; response time, 1 s; and sweep, 12.5 G min^{-1}.

Generation of superoxide

Superoxide was generated using two different enzymatic sources hypoxanthine–xanthine oxidase at pH 7.4 and PMA-stimulated human neutrophils.

Neutrophil isolation and preparation. Neutrophils were isolated from heparinized venous blood of normal human volunteers as previously described,[21] with minor modification. Briefly, neutrophils and red blood cells were separated from other cellular components by centrifugation with Lymphoprep at 327 × g for 20 min at 4 °C. The resulting leukocyte suspension was sedimented at 37 °C over a 6% dextran in 0.85% saline solution followed by osmotic lysis of contaminating erythrocytes. Neutrophils were then suspended in HBSS containing 0.1 mM DTPA and stored on ice until use. The concentration of the cells was adjusted accordingly and the viability was more than 95% as determined by exclusion of Trypan blue dye.

Superoxide-generation from stimulated neutrophils. To stimulate $O_2^{\bullet -}$ production by neutrophils, PMA (100 ng mL^{-1}) was added to a mixture of cells ($1–10 \times 10^6$ mL^{-1}) and ferricytochrome c. The assay was performed at 25 °C for PMA. The rate of $O_2^{\bullet -}$ production[22] was 5–10 μM min^{-1} as determined by SOD-inhibitable reduction of ferricytochrome c (80 μM), monitored at 550 nm with a spectrophotometer using an extinction coefficient of 21 mM^{-1} cm^{-1}.

Superoxide-generation from hypoxanthine–xanthine oxidase. Production of $O_2^{\bullet -}$ was determined by mixing hypoxanthine (400 μM), ferricytochrome c (80 μM) and sufficient xanthine oxidase in sodium phosphate buffer (30 mM) containing DTPA (0.1 mM), at pH 7.4. The rate of $O_2^{\bullet -}$ generation[22] was estimated by measuring the SOD-inhibitable reduction of ferricytochrome c (80 μM) at 550 nm using an extinction coefficient of 21 mM^{-1} cm^{-1}.

Spin trapping of hydroxyl radical

Photolysis of H_2O_2 was used as the source of $HO^{\bullet}$, when a solution of the spin trap (100 mM), and H_2O_2 (88 mM) was irradiated for 1 min in sodium phosphate buffer (30 mM) at pH 7.4 containing DTPA (0.1 mM). The reaction mixture was transferred to a flat quartz cell and fitted into the cavity of the EPR spectrometer. Spectra were recorded 1 min after termination of photolysis. The Fenton reaction was used as an alternative source of $HO^{\bullet}$ by mixing H_2O_2 (88 mM) and Fe^{2+} (0.1 mM) in phosphate buffer at pH 7.4.

Spin trapping of superoxide

Spin trapping experiments were performed by mixing the spin trap (100 mM), hypoxanthine (400 μM), and sufficient xanthine oxidase in sodium phosphate buffer (30 mM) containing DTPA

(0.1 mM), at pH 7.4 to reach the desired flux of $O_2^{\bullet -}$. Control experiments contained SOD (30 U mL^{-1}) and/or catalase (300 U mL^{-1}). The reaction mixtures were transferred to a flat quartz cell and fitted into the cavity of the spectrometer, and the spectra were recorded 2 min after mixing. Hyperfine splitting constants are presented in Table 2.

Spin trapping of $O_2^{\bullet -}$ by stimulated neutrophils was undertaken by mixing cells (5–10 × 10^6 mL^{-1}), spin trap (100 mM), PMA (100 ng mL^{-1} in DMSO) and sufficient buffer to reach a final volume of 0.25 mL. Reaction mixtures were transferred to a quartz EPR flat cell open to air, fitted into the cavity of an EPR spectrometer and spectra were recorded at 25 °C. Hyperfine splitting constants are presented in Table 2.

Stability of the spin trapped adducts of HO$^{\bullet}$

The stability of the spin trapped adducts of HO$^{\bullet}$ was estimated and presented in Table 1. In a typical experiment, the spin trap (60 mM) with H_2O_2 (88 mM) was photolyzed for 1 min and transferred to the EPR flat quartz cell and introduced to the cavity of the spectrometer. EPR spectra were recorded at room temperature at various time intervals until the peak height decreased to several half-lives.

Rate of the spin trapping of HO$^{\bullet}$

To determine the apparent rate constant for the spin trapping of HO$^{\bullet}$ by the spin traps used in this study, the mixture of spin trap (60 mM), H_2O_2 (88 mM) and 4-POBN (0–60 mM) was photolyzed for 1 min. The reaction mixtures were immediately transferred to an EPR flat quartz cell and introduced into the cavity of the EPR spectrometer. EPR spectra were recorded 1 min after the termination of the photolysis. Apparent rate constants for the spin trapping of HO$^{\bullet}$ are presented in Table 1.

Acknowledgements

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Paper 9/01566C