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Degradation of DMPO Adducts from Hydroxyl and 1-Hydroxyethyl Radicals by Rat Liver Microsomes

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Hydroxyl and 1-hydroxyethyl radical adducts of 5,5-dimethylpyrroline N-oxide (DMPO) were prepared by photolysis, and mechanisms for loss of their EPR signals in rat liver microsomal suspensions were evaluated. Rates of NADPH-dependent EPR signal loss were more rapid in phosphate buffer than in Tris buffer. Addition of superoxide dismutase (SOD) partially protected the adducts when Tris was used as a buffer, but was relatively ineffective in the presence of phosphate. The ferrous iron chelator bathophenanthrolene partially protected the spin adducts in the presence and absence of phosphate, but complete protection was observed when SOD was also added. The spin adducts were unstable in the presence of Fe^{+2} and $K_3Fe(CN)_6$, but Fe^{+3} alone had little effect on the EPR signals. The data are consistent with two mechanisms for microsomal degradation of DMPO spin adducts under these conditions. Microsomes form superoxide in the presence of oxygen and NADPH, which attacks these DMPO spin adducts directly. The spin adducts are also degraded in the presence of Fe^{+2}, and phosphate stimulates this iron-dependent destruction of DMPO spin adducts.

Keywords: EPR spectroscopy, nitroxide decay, nitroxide metabolism, spin adduct decay, spin trapping

INTRODUCTION

Spin trapping and EPR spectroscopy are useful methods to study free radical reactions in chemical and biological systems. In spin trapping, a reactive free radical is allowed to react with a spin trapping agent to form a secondary radical called a spin adduct, which produces a characteristic EPR spectrum. One limitation of these methods is that the spin adducts must be sufficiently stable under the assay conditions for subsequent EPR analyses. Although several laboratories have studied conversion of stable nitroxides to EPR-silent products in liver microsomes[1–3] or more complex biological systems[4–6], much less is known about enzymatic reactions which degrade spin adducts.

Our laboratories have developed methods using DMPO (5,5-dimethylpyrroline N-oxide) as a spin trapping agent to measure hydroxyl radical (·OH) formation by rat liver microsomes[7,8]. When ethanol is present, EPR signals from 1-hydroxyethyl radical adducts of DMPO can also

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be observed. However, addition of superoxide dismutase (SOD) increased the EPR signal intensities of both adducts[9], suggesting that superoxide formed by the microsomes may degrade these compounds. These DMPO spin adducts are also unstable in the presence of ferrous iron[10], which may be present in microsomal incubations. The experiments in this report were designed to study mechanisms leading to loss of EPR signals of DMPO-OH and DMPO-HER in rat liver microsomes in more detail.

MATERIALS AND METHODS

Liver microsomes were prepared from male, Sprague-Dawley rats (Sasco, Omaha), and were washed twice to minimize contamination with catalase and non-heme iron[8]. The final microsomal pellets were overlayed with isotonic KCl, and were frozen at –70°C until just prior to use. Microsomal protein concentrations were determined with the Lowry method[11] and are indicated in figure legends. DMPO, iron chelating agents, and biochemicals used in these experiments were purchased from the Sigma Chemical Company. All other chemicals were of reagent grade. Phosphate, Tris and KCl solutions were thoroughly treated with Chelex-100 resin (Bio-Rad) to decrease concentrations of contaminating trace metals.

Spin adducts of DMPO were prepared immediately prior to experiments by exposing aqueous solutions of DMPO and hydrogen peroxide to 30 sec of UV light from an Oriel Medium Pressure illumination system, equipped with a 75 watt mercury arc lamp. When hydroxyl radical adducts (DMPO-OH) were desired, DMPO (200 mM) and H_2O_2 (25 mM) were mixed and irradiated. When 1-hydroxyethyl radical adducts (DMPO-HER) were desired as the predominant spin adducts, DMPO (400 mM), H_2O_2 (25 mM) and ethanol (7.5 M) were mixed prior to irradiation. Non-reacted H_2O_2 was removed by addition of catalase (120 units/ml), and the solutions were bubbled 15 min with N_2 to remove excess oxygen. Aliquots of the spin adduct solutions were then added to microsomes or other chemical mixtures as indicated in the figure legends. Although these aliquots contained non-reacted DMPO and ethanol which might produce spin adducts, this is unlikely to occur in the presence of excess catalase[7]. Furthermore, the spectrometer conditions used to monitor signal loss of pre-formed spin adducts could not detect newly-generated spin adducts when aliquots of non-irradiated DMPO and ethanol were added to microsomes under comparable conditions.

Aliquots of photolytically prepared DMPO adducts were added to a suspension of microsomes, and this mixture was siphoned into a quartz flat cell in a pre-tuned cavity of an EPR spectrometer, with the magnetic field locked on one of the major peaks of the spin adduct. After briefly recording a baseline for EPR signal stability, the suspension was returned to a test tube, quickly mixed with an NADPH-generating system consisting of $NADP^+$ (0.3 mM), glucose-6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (0.5 units/ml)[7,8]. The suspension was quickly siphoned back into the quartz cell, essentially as described by Iannone *et al.*[2], and only about 20 sec passed before NADPH-dependent loss of EPR signals could be monitored.

Rates of DMPO spin adduct loss were found to be dependent on the initial concentration of the spin adduct in the incubation mixture. Because the concentrations of spin adducts recovered varied among experiments, and because some signal loss occurred continually by natural decay processes during the time that the EPR experiments were conducted, calculation of rate constants for signal loss became impractical. Instead, paired observations which differed only by the presence or absence of a particular additive were routinely performed. Characteristic results from at least three separate paired experiments are shown in the figures.

Tris buffer (25 mM, pH 7.4) was added to all solutions to control the pH, and no EPR signals which could be assigned to Tris radicals were

observed. In order to maintain similar osmotic conditions in all microsomal incubations, sufficient KCl was added so that the sum of KCl and phosphate concentrations (40 mM, pH 7.4) equalled 160 mM. In experiments designed to test for hydroxyl radical generation, microsomes were incubated with DMPO (40 mM), azide (0.1 mM), and the NADPH-generating system above[7,8]. Final concentrations of all other additions are indicated in the text and figure legends.

All experiments were conducted with a Bruker EPR 300E spectrometer. Typical operating conditions were: center field, 3480 G; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; gain, 10^5; microwave frequency, 9.75 Ghz;, and microwave power, 19.9 mW. Other conditions are given in the figure legends. Concentrations of the spin adducts tested were estimated by computer integration of peak area of the spectra, and comparison with the spectrum of a known concentration of the stable nitroxide TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy free radical).

RESULTS

When liver microsomes were incubated with DMPO, azide, and an NADPH-generating system, but in the absence of phosphate buffer, EPR signals of the type shown in Figure 1A could be

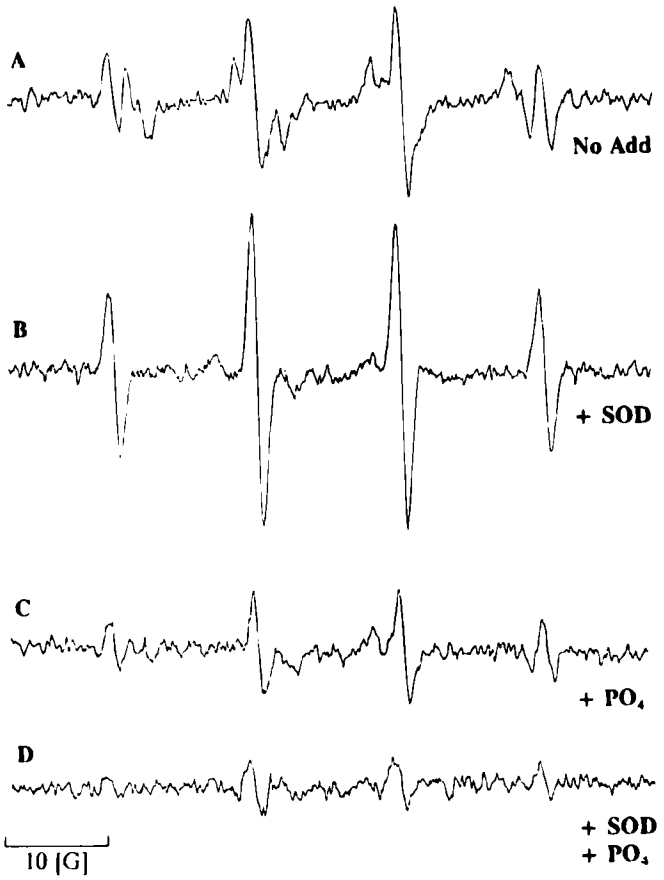

FIGURE 1 Effects of superoxide dismutase (SOD) and phosphate on spin trapping of oxygen radicals with DMPO in rat liver microsomes. Microsomes (2 mg/ml) were incubated with DMPO, azide, Tris buffer, and an NADPH-generating system as described in Materials and Methods. Other additions consisted of: 160 mM KCl (A,B); 100 units/ml SOD (B,D); and 40 mM phosphate, pH 7.4, plus 120 mM KCl (C,D). The EPR spectrometer gain was 1×10^6, and 5 spectral scans of 84 sec each were accumulated.

observed. The spectrum consists of signals from hydroxyl and superoxide radical adducts of DMPO, as indicated previously[8]. Addition of SOD (Fig. 1B) resulted in loss of superoxide radical adduct signals, and increased the intensity of the DMPO-OH signal. When the same experiment was conducted in the presence of phosphate buffer (Fig. 1C), the EPR signals were considerably less intense, and addition of SOD had little effect (Fig. 1D). The apparent effect of phosphate to decrease DMPO spin adduct signal intensity was a perplexing observation, and has not been previously reported. Because protective properties of SOD were eliminated in the presence of phosphate, it seemed important to investigate these effects further.

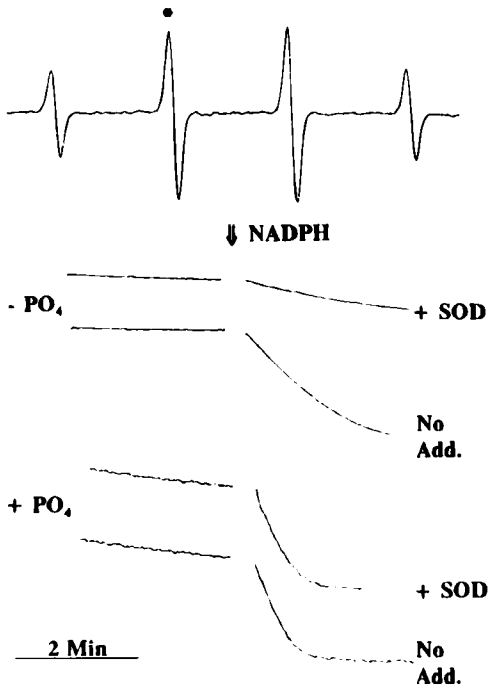

FIGURE 2 Effects of SOD and phosphate on NADPH-dependent decay of DMPO-OH signals in microsomes. Microsomes (1.5 mg/ml) were mixed with DMPO-OH (approximately 1.2 μM) and the EPR spectrometer field was locked on the peak indicated by the asterisk (*). After recording a basal rate of signal decay, the scan was briefly interrupted for addition of an NADPH generating system. SOD (100 units/ml) and phosphate (40 mM, pH 7.4) were added where indicated. KCl concentrations were 120 or 160 mM in the presence or absence of phosphate, respectively.

Effects of SOD and phosphate buffer on the signal stability of the photolytically prepared DMPO-OH adduct in microsomes are shown in Figure 2. The DMPO-OH spin adduct has a half-life of about 2.6 hours in aqueous solutions at pH 7.4[12], and a slow rate of signal loss was observed in the absence of NADPH. After addition of NADPH, rates of DMPO-OH signal loss increased noticeably, particularly when phosphate was also present. NADPH addition did not influence rates of DMPO-OH signal loss in the absence of microsomes, or when boiled microsomes were tested (not shown), indicating that this signal loss was an enzymatic process. Addition of SOD provided substantial protection of DMPO-OH when phosphate was absent, but had little effect when phosphate was present (Fig. 2).

Iron chelators partially protected DMPO-OH spin adducts in microsomal suspensions. For example, deferoxamine (DFO), a chelator of Fe^{+3}, and bathophenanthrolene disulfonic acid (BP), a chelator of Fe^{+2}, both decreased rates of signal loss under these conditions (Fig. 3). Although SOD alone did not protect DMPO-OH in the presence of phosphate buffer (Fig. 2), the combination of SOD with DFO (Fig. 3) or BP (not shown) completely prevented NADPH-dependent loss of the DMPO-OH signals. Similar effects of these chelators were observed in the absence of phosphate buffer (not shown).

The DMPO-HER spin adduct is more intrinsically stable than the DMPO-OH adduct. For example, very little EPR signal loss was observed when DMPO-HER was mixed with microsomes in the absence of NADPH (Fig. 4). As observed with DMPO-OH, NADPH-dependent signal loss was partially prevented by SOD in the absence (not shown), but not in the presence of phosphate buffer (Fig. 4). Addition of BP alone slowed rates of signal loss somewhat, but the DMPO-HER signal was maintained in the presence of both BP and SOD (Fig. 4). Surprisingly, when DFO was evaluated in similar experiments, loss of the DMPO-HER signal was often observed even in

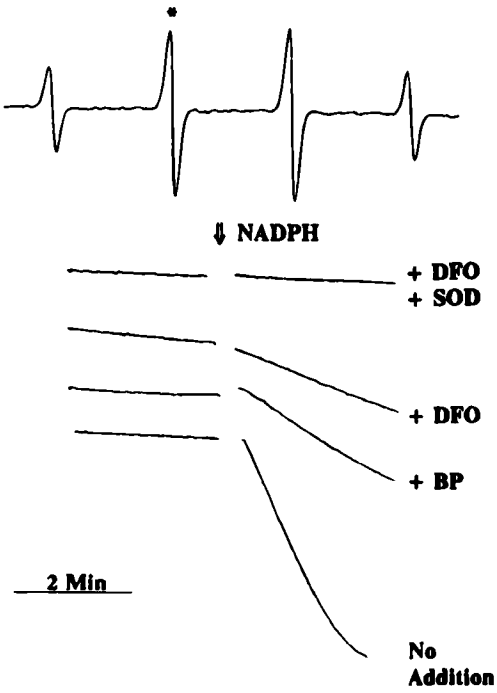

FIGURE 3 Effects of iron chelators and SOD on DMPO-OH signal decay in phosphate-buffered microsomes. Microsomes (0.5 mg/ml) were mixed with DMPO-OH (approximately 1.5 μM) in the presence of 40 mM phosphate buffer, pH 7.4. Deferoxamine (DFO, 1 mM), bathophenanthrolene (BP, 1 mM) and SOD (100 units/ml) were added where indicated. Other conditions are as in Fig. 2.

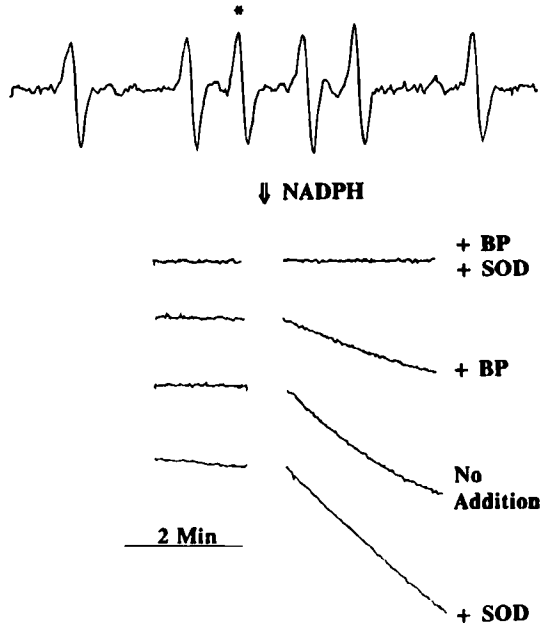

FIGURE 4 Effects of iron chelators and SOD on DMPO-HER signal loss in phosphate-buffered microsomes. Microsomes (1 mg/ml) were mixed with photolytically prepared DMPO-HER (approximately 0.5 μM), and the field was locked on the peak indicated by the asterisk. Other conditions are as in Fig. 3.

the absence of NADPH (not shown). The explanation for this unanticipated effect of DFO on the DMPO-HER signal, which was not observed with DMPO-OH (Fig. 3), has not been determined.

Some preliminary experiments were conducted with the HER spin adduct of POBN [(α-pyridyl-l-oxide)-N-t-butylnitrone]. However, the POBN-HER spin adduct appeared to be stable in microsomal incubations, in the presence and absence of phosphate, indicating that problems with DMPO spin adduct stability are not necessarily observed with other spin trapping agents.

Because iron chelators usually protected DMPO spin adducts (Figs. 3,4), effects of iron salts were studied further in chemical reaction systems which did not contain microsomes. DMPO-OH and DMPO-HER adducts were prepared separately, and were then mixed to produce a spectrum with signals from both adducts. The resulting spin adduct mixture was diluted 1:1 with water, phosphate buffer, or solutions of iron salts. When the spin adduct stock solution was diluted with water (Fig. 5A) or phosphate buffer (Fig. 5B), the resulting spectra were identical, indicating that the adducts are stable in phosphate buffer. When aqueous or phosphate-buffered solutions of $FeSO_4$ (0.1 mM) were tested (Fig. 5C,D), the DMPO-OH signal was lost, and the intensity of the DMPO-HER adduct was markedly reduced. $FeCl_3$ (0.1 mM) had little effect on the EPR signals from these spin adducts (Fig. 5E). However, dilution of the spin adduct mixture into a solution of potassium ferricyanide (1 mM, Fig. 5F) resulted in rapid loss of both signals. Additional experiments indicated substantial signal loss within 3 min when only 0.05 mM $K_3Fe(CN)_6$ was present.

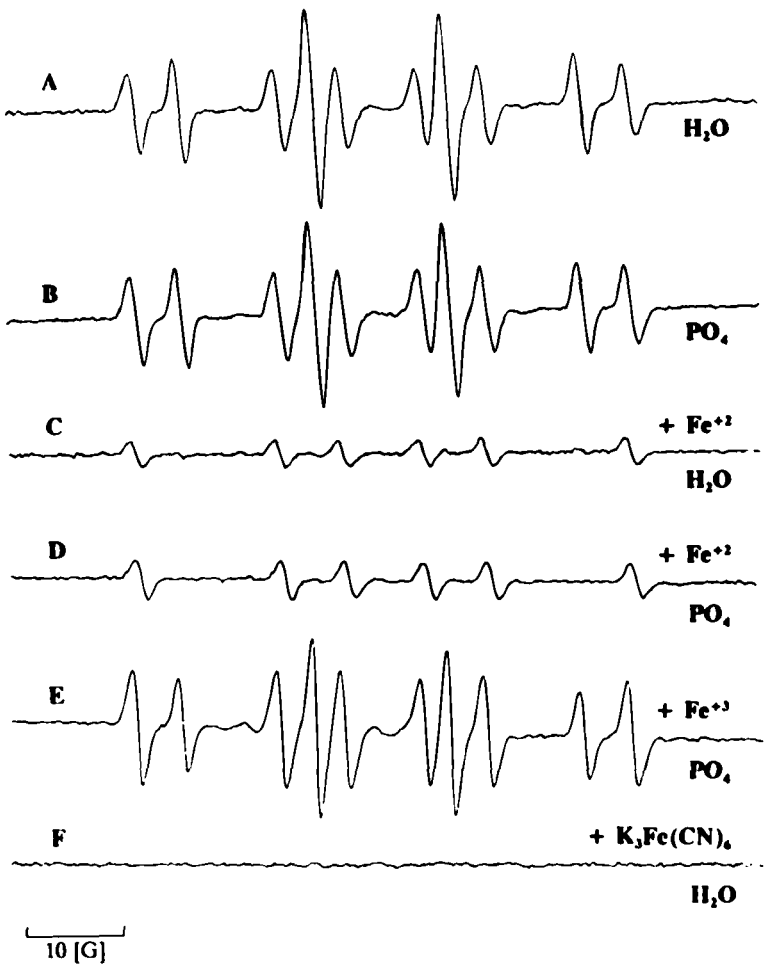

FIGURE 5 Effects of $FeSO_4$, $FeCl_3$, and $K_3Fe(CN)_6$ on DMPO spin adducts. Mixtures of DMPO-OH and DMPO-HER were prepared by photolysis, and were diluted 1:1 into water or phosphate buffer (40 mM, pH 7.4). Immediately after mixing, the solutions were subjected to EPR spectroscopy, using a gain of 2.5×10^6, and one scan of 84 sec was obtained. The iron salts were freshly dissolved in water, and were added just prior to starting the scans to minimize redox changes or precipitation in the presence of phosphate. The final iron concentrations were 0.1 mM for $FeSO_4$ and $FeCl_3$, and 1.0 mM for $K_3Fe(CN)_6$.

DISCUSSION

A number of laboratories have evaluated mechanisms for loss of EPR signals of stable nitroxides in microsomal incubations[1–3], and reduction to EPR-silent hydroxylamines is widely accepted. This hypothesis is supported by partial restoration of the signals after addition of $K_3Fe(CN)_6$ and other oxidants[2,3,5]. Superoxide formed by microsomal enzymes can also oxidize some hydroxylamines[13]. However, the data of this report indicate that NADPH-dependent loss of EPR signals of DMPO-OH and DMPO-HER in microsomes can be completely explained through formation of superoxide and changes in the redox state of iron which is present in the suspensions. Furthermore, the contribution of these two pathways to spin adduct decay were modified by the choice of buffer used for the experiments.

In the absence of phosphate buffer, SOD alone provided partial protection of DMPO-OH (Fig. 2)

and DMPO-HER (Results). These observations suggest that superoxide-dependent spin adduct destruction is responsible for much of the signal loss, as previously suggested in studies using non-microsomal incubation systems[14–16].

In the presence of phosphate buffer, SOD alone did not protect either DMPO spin adduct (Figs. 2,4). However, iron chelators such as DFO and BP provided some protection of DMPO-OH, and completely prevented signal loss when SOD was also present (Fig. 3). Similarly, BP also protected DMPO-HER (Fig. 4). Both spin adducts were non-enzymatically destroyed by Fe^{+2} in the absence or presence of phosphate, whereas Fe^{+3} had little effect on EPR signals when added as $FeCl_3$ (Fig. 5). Taken together, these data suggest that the high rates of DMPO spin adduct destruction in the presence of phosphate is at least partially dependent on formation of Fe^{+2}. These observations can be explained from previously reported effects of phosphate on iron chemistry, as discussed below.

It is well known that phosphate chelates ferric ions in preference to ferrous ions[17]. We have recently shown that ferric-phosphate chelates are rapidly reduced by microsomes in the presence of NADPH, and that microsomal non-heme iron is mobilized in the presence of phosphate and NADPH[18]. Thus, iron which contaminates reagents or iron bound to microsomes can form a complex with phosphate and enter into a redox cycling mechanism in which ferric-phosphate is enzymatically reduced by NADPH.[18] The data in this report are consistent with DMPO spin adduct destruction under these conditions. For example, iron chelators should inhibit this process: DFO by binding Fe^{+3} and preventing its reduction, and BP by binding Fe^{+2} and preventing its subsequent reactions.

A plausible hypothesis for Fe^{+2}-dependent signal loss is that the metal ion binds to the nitroxide moiety and reduces it to a hydroxylamine or other EPR-silent products. If this were the case, phosphate might also accelerate this process by speeding the rate of Fe^{+2} autoxidation[17,19].

Both NAD(P)H-cytochrome c reductase[2,3] and cytochrome P-450[1] have been suggested to catalyze loss of EPR signals from stable nitroxides. Because loss of DMPO spin adduct signals can be explained by effects of superoxide and iron, there is no reason to invoke direct roles for these enzymes in DMPO spin adduct catabolism. However, both enzymes are potential sources of superoxide[20], and the reductase reduces certain Fe^{+3} chelates[21]. Because superoxide is capable of reducing Fe^{+3}, some of the protective effects of SOD might be explained by decreased rates of Fe^{+2} formation[17].

The fate of the DMPO spin adducts in these microsomal experiments has not been determined. Both superoxide and ferrous iron can donate electrons to reduce nitroxides to the corresponding hydroxylamines, but addition of the mild oxidant $K_3Fe(CN)_6$ not only failed to prevent signal loss in microsomes, but directly caused the signals to be lost (Fig. 5). It is possible that the hydroxylamines of these spin adducts are unstable and further degrade to products which cannot produce EPR signals. Alternatively, oxidation by $K_3Fe(CN)_6$, and possibly by superoxide and ferrous-oxygen complexes could generate EPR-silent products which are not hydroxylamines. Similarly, Samuni *et al.*[14] have previously shown that DMPO-OH signals lost in a superoxide-generating system could not be restored by addition of ferricyanide or a combination of H_2O_2 and Cu^{+2}.

Phosphate is widely used to buffer reaction mixtures for free radical studies, even though iron chelation by phosphate is recognized as a possible complication[17], and phosphate accelerates autoxidation of ferrous iron[17,19]. However, it now appears that phosphate not only stimulates certain free radical reactions in liver microsomes[18], but it may also contribute to accelerated degradation of some spin adducts. For example, because phosphate does not cause substantial changes in most microsomal oxidations or production of superoxide[18], the apparent poor yield of DMPO-OH observed in

microsomal spin trapping experiments in the presence of phosphate buffer (Fig. 1) is probably best explained by iron-dependent destruction of DMPO-OH spin adducts, rather than by decreased rates of ·OH formation.

Acknowledgements

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References

[1] G. M. Rosen and E. J. Rauckman (1977) Formation and reduction of a nitroxide radical by liver microsomes, *Biochemical Pharmacology*, **26**, 675–678.

[2] A. Iannone, A. Bini, H. M. Swartz, A. Tomasi and V. Vannini (1989) Metabolism in rat liver microsomes of the nitroxide spin probe Tempol, *Biochemical Pharmacology*, **38**, 2581–2586.

[3] A. Iannone, A. Tomasi, V. Vannini and H. M. Swartz (1990) Metabolism of nitroxide spin labels in subcellular fraction of rat liver. I Reduction by microsomes, *Biochimica et Biophysica Acta*, **1034**, 285–289.

[4] K. Chen, P. D. Morse II and H. M. Swartz (1988) Kinetics of enzyme-mediated reduction of lipid soluble nitroxide spin labels by living cells, *Biochimica et Biophysica Acta*, **943**, 477–484.

[5] A. Iannone, H. Hu, A. Tomasi, V. Vannini and H. M. Swartz (1989) Metabolism of aqueous soluble nitroxides in hepatocytes: effects of cell integrity, oxygen, and structure of nitroxides, *Biochimica et Biophysica Acta*, **991**, 90–96.

[6] A. Samuni, A. Samuni and H. M. Swartz (1989) The cellular-induced decay of DMPO spin adducts of ·OH and $\cdot O_2^-$, *Free Radical Biology & Medicine*, **6**, 179–183.

[7] P. B. McCay, L. A. Reinke and J. M. Rau (1992) Hydroxyl radicals are generated by hepatic microsomes during NADPH oxidation: relationship to ethanol metabolism, *Free Radical Research Communications*, **6**, 335–346.

[8] L. A. Reinke, S. M. Bailey, J. M. Rau and P. B. McCay (1994) Oxygen radical formation in well-washed rat liver microsomes: spin trapping studies, *Free Radical Research*, **20**, 51–60.

[9] L. A. Reinke, D. R. Moore, C. M. Hague and P. B. McCay (1994) Metabolism of ethanol to 1-hydroxyethyl radicals in rat liver microsomes: comparative studies with three spin trapping agents, *Free Radical Research*, **21**, 213–222.

[10] L. A. Reinke, J. M. Rau and P. B. McCay (1994) Characteristics of an oxidant formed during iron (II) autoxidation, *Free Radical Biology & Medicine*, **16**, 485–492.

[11] O. H. Lowry, M. J. Rosebrough, A. L. Farr and R. J. Randall (1951) Protein measurement with the Folin phenol reagent, *Journal of Biological Chemistry*, **193**, 265–275.

[12] E. Finkelstein, G. M. Rosen and E. J. Rauckman (1980) Spin trapping of superoxide and hydroxyl radical: practical aspects, *Archives of Biochemistry and Biophysics*, **200**, 1–16.

[13] E. J. Rauckman, G. M. Rosen and B. B. Kitchell (1979) Superoxide radical as an intermediate in the oxidation of hydroxylamines by mixed function amine oxidase, *Molecular Pharmacology*, **15**, 131–137.

[14] A. Samuni, C. Murali Krishna, P. Riesz, E. Finkelstein and A. Russo (1989) Superoxide reaction with nitroxide spin-adducts, *Free Radical Biology & Medicine*, **6**, 141–148.

[15] H. Kosaka, Y. Katsuki and T. Shiga (1992) Spin trapping study on the kinetics of Fe^{2+} autoxidation: Formation of spin adducts and their destruction by superoxide, *Archives of Biochemistry and Biophysics*, **293**, 401–408.

[16] B. E. Britigan, T. J. Coffman and G. R. Buettner (1990) Spin trapping evidence for the lack of significant hydroxyl radical production during the respiration burst of human phagocytes using a spin adduct resistant to superoxide-mediated destruction, *Journal of Biological Chemistry*, **265**, 2650–2656.

[17] D. M. Miller, G. M. Buettner and S. D. Aust (1990) Transition metals as catalysts of "autoxidation" reactions, *Free Radical Biology & Medicine*, **8**, 95–108.

[18] L. A. Reinke, D. R. Moore, J. M. Rau and P. B. McCay (1995) Inorganic phosphate promotes redox cycling of iron in liver microsomes: effects on free radical reactions, *Archives of Biochemistry and Biophysics*, **316**, 758–764.

[19] B. Tadolini (1987) Iron autoxidation in MOPS and HEPES buffers, *Free Radical Research Communications*, **4**, 149–160.

[20] H. Kuthan and V. Ullrich (1982) Oxidase and oxygenase function of the microsomal cytochrome P450 monooxygenase system, *European Journal of Biochemistry*, **126**, 583–588.

[21] L. A. Morehouse, C. E. Thomas and S. D. Aust (1984) Superoxide generation by NADPH-cytochrome P-450 reductase: the effect of iron chelators and the role of superoxide in microsomal lipid peroxidation, *Archives of Biochemistry and Biophysics*, **232**, 366–377.