Chapter 2

Spin-Trapping Methods for Detecting Superoxide and Hydroxyl Free Radicals In Vitro and In Vivo

Garry R. Buettner and Ronald P. Mason*

Garry R. Buettner • Free Radical Research Institute, The University of Iowa, Iowa City, IA 52242

Ronald P. Mason • Laboratory of Pharmacology and Chemistry, NIEHS, MD: F0-01, P.O. Box 12233, Research Triangle Park, NC 27709

*Corresponding Author

Tel: (919) 541-3910, Email: mason4@niehs.nih.gov

Critical Reviews of Oxidative Stress and Aging: Advances in Basic Science, Diagnostics and Intervention. (2003) Ed Richard G. Cutler and Henry Rodriguez. World Scientific, New Jersey, London, Singapore, Hong Kong. Volume I. Chapter 2, pp 27-38.

ISBN 981-02-4636-6

1. Summary

Spin trapping has become a valuable tool in the study of transient free radicals as evidenced by the many investigations in which it has been employed.[1] Oxygen-centered radicals are of particular interest because they have been implicated in many adverse reactions *in vivo*. Their short lifetimes and broad line widths make many of these radicals difficult, if not impossible, to detect by direct electron spin resonance (ESR) in room temperature aqueous solutions. Spin trapping provides a means, in principle, to overcome these problems, but it is not without its pitfalls and limitations. We discuss some of these problems in this chapter.

2. Choice of Spin Trap

Two types of spin traps have been developed, nitrone and nitroso compounds. In aqueous solutions, however, oxygen-centered spin adducts in nitroso spin traps such as 2-methyl-2-nitrosopropane are, in general, quite unstable. Thus, the nitrone spin traps are by far the most popular. The most used radical trap for the study of oxygen-centered free radicals is 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), which has been used extensively to study superoxide[1, 2] and hydroxyl radicals[1, 3] in biochemical and biological systems.

3. Superoxide

The spin trapping of superoxide has been of such interest because of the involvement of superoxide in many physiological processes. DMPO/superoxide (the superoxide radical adduct of DMPO) has a distinctive spectrum (a^N = 14.2 G, a_β^H = 11.3 G, and a_γ^H = 1.25 G)[1] that is easily recognizable. However, other oxygen-centered adducts of DMPO such as alkoxyl have a similar appearance.[4] Thus, the real proof that the spectrum observed is indeed due to DMPO/superoxide is gained by using superoxide dismutase (SOD) to inhibit the signal.[5]

Although the DMPO/superoxide spectrum is distinctive, the spin trapping of superoxide is not without its problems. The actual reaction of superoxide with DMPO is very slow (k_{obs} is 60 M^{-1} sec^{-1} at pH 7 and only 30 M^{-1} sec^{-1} at pH 7.4).[6] Thus, in most superoxide-generating systems, the spin-trap concentration must be quite high (-.01 M) in order to outcompete the self-decay, namely, spontaneous dismutation of superoxide. In addition, the DMPO/superoxide adduct is unstable, decaying by a first-order process with a half-life of about 60s at pH 7.[7] Therefore, one must always be prepared to deal with a relatively weak signal; that is, the concentration of DMPO/superoxide will, under most circumstances, be much less than 10μM.

4. Nitric Oxide as an Antioxidant

The DMPO/hydroxyl adducts is the most often reported radical adduct of DMPO ($a^N \cong a_\beta^H = 14.9$ G). Much of the interest in the spin trapping of $^\bullet$OH is due to its formation in the superoxide-dependent Fenton reaction:

$$O_2^{\bullet-} + HO_2^\bullet \xrightarrow{H+} H_2O_2 + O_2 \quad (1)$$

$$O_2^{\bullet-} + Fe(III) \longrightarrow Fe(II) + O_2 \quad (2)$$

$$Fe(II) + H_2O_2 \longrightarrow {}^\bullet OH + OH^- + Fe(II) \quad (3)$$

Thus, SOD will inhibit DMPO/superoxide and/or DMPO/hydroxyl formation if this reaction sequence is operative. However, catalase will always inhibit the formation of hydroxyl in reaction (3) above. A failure of catalase to inhibit the formation of DMPO/hydroxyl when the superoxide-driven Fenton reaction is suspected indicates that something artifactual is occurring or that another mechanism must be sought.

Two additional SOD-inhibitable routes to DMPO/hydroxyl from DMPO/superoxide itself should be considered: the reduction of DMPO/superoxide[5] (a hydroperoxide) to the alcohol DMPO/hydroxyl, for example, by glutathione peroxidase;[8] and the possible hemolytic cleavage of the oxygen-oxygen bond of DMPO/superoxide to produce free $^\bullet$OH,[2] which is subsequently trapped by unreacted DMPO.[9] Finkelstein *et al.* indicate that approximately 3% of DMPO/superoxide decomposes to produce hydroxyl. Unfortunately, no experimental data or details are given to indicate how this estimate was made; thus, it is difficult to assess how this number should be used. Therefore, weak DMPO/hydroxyl signals that are not catalase inhibitable should always be viewed cautiously because they quite often are artifactual rather than the result of the spin trapping of free $^\bullet$OH generated by the system under study. Possible sources of artifactual DMPO/hydroxyl signals are (1) hydrolysis of DMPO to produce DMPO/hydroxyl as an impurity signal;[10] (2) the one-electron oxidation of DMPO followed by hydration of DMPO$^+$;[11] (3) the apparently concerted hydrolysis-oxidation reaction by photochemically excited molecules;[12] and (4) the presence of a strong oxidant such as hypochlorous acid.[13]

To establish the existence of free hydroxyl radical in spin-trapping experiments, it is necessary to perform kinetic competition experiments with hydroxyl radical scavengers.[14] For example, ethanol, formate, and dimethyl sulfoxide can be used in these competition experiments because, upon hydroxyl radical attack, they form carbon-centered radicals that can subsequently be trapped by DMPO:

$$^\bullet OH + DMPO \xrightarrow{k_1} DMPO/hydroxyl \quad (4)$$

$$^\bullet OH + HCO_2^- \xrightarrow{k_2} CO_2^{\bullet-} + H_2O \quad (5)$$

$$CO_2^{\bullet-} + DMPO \xrightarrow{k_3} DMPO/^{\bullet}CO_2^{-} \quad (6)$$

$$^{\bullet}OH + Ch_2CH_2OH \xrightarrow{k_4} {}^{\bullet}CH(OH)CH_3 + H_2O \quad (7)$$

$$^{\bullet}CH(OH)CH_3 + DMPO \xrightarrow{k_5} DMPO/^{\bullet}CH(OH)CH_3. \quad (8)$$

Most artifacts leading to DMPO/hydroxyl radical adduct formation will be excluded by the use of hydroxyl radical scavengers if the scavenger-derived radical adduct is detected, if a corresponding decrease in the DMPO/hydroxyl radical adduct concentration is found, and if quantitative kinetic criteria are used.[14]

Measurement of the initial rates of formation of the DMPO/hydroxyl and DMPO scavenger radical adducts removes the effects of the differential radical adduct decay rates.[14] Using this approach, the relative efficiency of two hydroxyl radical scavengers, if quantitatively predictable from the known rate constants, can be calculated for the reactions of the hydroxyl radical with these scavengers. For example, using formate (k_2) and ethanol (k_4) we can calculate k_2/k_4 from the ratio of the rates of formation of these two radical adducts:

$$\frac{k_2}{k_4} = \frac{d[DMPO/^{\bullet}CO_2^{-}]/dt}{d[DMPO/^{\bullet}CH(OH)CH_3]/dt} \times \frac{[CH_3CH_2OH]}{[HCO_2^{-}]}. \quad (9)$$

In Eq. (9), the ratio k_2/k_4 from spin trapping should agree with the ratio of rate constants for the reaction of the hydroxyl radical with these scavengers as determined from pulse radiolysis. It should be kept in mind that to arrive at this expression, it is assumed that the predominant route of scavenger radical decay is via the trapping reaction. This kinetic approach has been successfully applied to an enzyme-dependent hydroxyl radical-generating system.[15]

A similar approach has been presented by Buettner *et al.*[16] In this approach a $^{\bullet}OH$ scavenger is included in the spin-trapping mixture at a concentration calculated to reduce the intensity of the DMPO/hydroxyl signal by 50%. In other words, the rate of the reaction of $^{\bullet}OH$ with scavenger (Scav) is equal to its rate of reaction with DMPO.

$$k_{Scav}[Scav][^{\bullet}OH] = k_{DMPO}[DMPO][^{\bullet}OH] \quad (10)$$

$$[Scav] = k_{DMPO}[DMPO]/k_{scav}. \quad (11)$$

The spin trapping of hydroxyl radical scavenger-derived radicals is the most reliable method of detecting hydroxyl radical in complex biological systems. Examples of the success of this approach are the detection of spontaneous peroxynitrite hemolysis to form the hydroxyl radical,[17] which was initially very controversial, and the detection of hydroxyl radical from the inactivation of mitochondrial aconitase by superoxide.[18]

Samuni *et al.*[19] have demonstrated that $O_2^{\bullet-}$ reacts very efficiently with DMPO/hydroxyl and DMPO/$^{\bullet}CH_3$ radical adducts, destroying the nitroxide and thus producing an ESR-silent species. If the flux of superoxide is high enough, some DMPO radical adduct may not be observed because of its rapid removal. Thus, a high flux of superoxide would not be desirable if additional free radical reactions are expected in a superoxide spin-trapping system.

5. In Vivo Hydroxyl Radical Detection

The DMPO/hydroxyl adduct has also been detected from cell organelles, intact cells, and organs, and the DEPMPO/hydroxyl adduct has been detected in a living mouse, but the numerous hydroxyl radical-independent pathways to DMPO/hydroxyl make the interpretation of this data problematic.[20] In addition, the determination of free hydroxyl radical using the hydroxyl radical scavenger approach is also somewhat problematic because the presence of classic hydroxyl radical scavengers such as ethanol, dimethyl sulfoxide, or formate can have a severely perturbing influence on the system, especially at the high concentrations that are required to outcompete the reaction of any $^{\bullet}OH$ formed with the numerous biochemicals present at millimolar concentrations. Thus, we believe that the unambiguous determination that free hydroxyl has been spin-trapped requires very careful experimental design and interpretation, especially when the goal is to examine free radical production in cells, organs, or whole animals.

In the study of hydroxyl radical formation *in vivo,* we used the scavenging reaction in which the hydroxyl radical is converted into the methyl radical via its reaction with dimethyl sulfoxide, the most inert of the classical hydroxyl radical scavengers.[21] The methyl radical is then detected as its long-lived phenyl *N-tert*-butylnitrone (PBN) adduct. Alone, DMSO is relatively nontoxic with a 24 h LD_{50} in the rat (*ip*) of 13.7 g/kg and is, therefore, an ideal reagent for the *in vivo* detection of the hydroxyl radical.

We usually assay untreated bile for radical adducts. Experiments are initiated by *ip* injection of DMSO containing PBN, followed by intragastric injection of ferrous sulfate or another hydroxyl radical-generating agent. The resulting PBN/methyl radical adduct (PBN/$^{\bullet}CH_3$) is detected by ESR, and the DMSO- and iron-dependence of *in vitro* adduct formation is demonstrated using collection of bile into dipyridyl, which inhibits *ex vivo* hydroxyl radical generation. Collection directly into dipyridyl is necessary to stop *ex vivo* iron chemistry due to the iron excreted into the bile along with the radical adducts. Having unambiguously demonstrated *in vivo* iron-dependent free radical formation, we next determined the effect of Desferal in this system. Desferal is a ferric iron chelators used to treat iron overload. After the treatment of rats with ferrous sulfate and an *ip* injection of Desferal, the six-line signal from the PBN/$^{\bullet}CH_3$ adduct was almost abolished,

suggesting that Desferal can inhibit hydroxyl radical generation during iron overload, presumably by binding iron in the ferric state.[22]

Since the Fenton reaction requires hydrogen peroxide, we thought that a substance that catalyzes hydrogen peroxide formation would increase the signal. The activity of the herbicide paraquat (PQ^{2+}) is attributed to its ability to catalyze the formation of superoxide and, subsequently, hydrogen peroxide. The herbicide undergoes an enzymatic one-electron reduction to form the paraquat radical-cation, $PQ^{\bullet+}$, which is then oxidized by molecular oxygen to form the superoxide radical, $O_2^{\bullet-}$. Through the participation in repeated cycles of reduction and oxidation, PQ^{2+} catalyzes superoxide radical formation. The formation of superoxide radical and the resulting hydrogen peroxide during the "futile cycling" of PQ^{2+} is thought responsible for its pulmonary toxicity to man. The paraquat radical has been detected using direct ESR in microsomal,[23] heptaocyte,[24] alveolar type II, and Clara cell incubations.[25] Unexpectedly, when we administered paraquat to our iron-poisoned rat model, only a modest increase of radical adduct formation occurred.

In contrast to iron, radical adducts were detected in the bile of copper-poisoned rats only after they had been given paraquat.[26] Apparently hydrogen peroxide was limiting *in vivo* in the copper analog of the Fenton reaction.

$$Cu^{3+} + H_2O_2 \longrightarrow Cu^{2+} + OH^- + {}^{\bullet}OH. \qquad (12)$$

When the experiment was repeated in the absence of copper or PQ^{2+}, no radical adducts were detected, thereby confirming the dependence of radical formation on the co-administration of both copper and PW^{2+}. The fact that copper or PW^{2+} alone caused little detectable radical adduct formation may be attributed to their inability to form hydroxyl radicals at detectable concentrations due to strong defense systems against oxidative stress in living organisms. For instance, GSH binds CU^{1+} as a stable complex that reacts slowly, if at all, with hydrogen peroxide to form the hydroxyl radical.[27]

In an attempt to demonstrate PBN/${}^{\bullet}CH_3$ formation as detected in the bile of animals treated with Cu(II) and PQ^{2+} or with Fe^{2+}, ^{13}C-labeled DMSO was used.[21] The presence of hyperfine splittings in the ESR spectrum from ^{13}C (I=½) allows an unambiguous assignment of the PBN/${}^{\bullet}CH_3$ radical adduct formed *in vivo*. The appearance of ^{13}C-hyperfine splittings is unambiguous proof that the PBN/${}^{\bullet}{}^{13}CH_3$ radical adduct was formed. ESR detection of PBN/${}^{\bullet}{}^{13}CH_3$ from DMSO has also been used to investigate hydroxyl radical generation in rats with chronic dietary iron loading.[28]Desferal completely inhibited *in vivo* hydroxyl radical generation stimulated by high dietary iron intake. No radical adducts were detected in rates which were fed the control diet for the same period of time. This as the first ESR evidence of hydroxyl radical generation in chronic iron-loaded rats.[28]

6. In Vivo and In Vitro Superoxide

Many studies are pursuing the possible production of superoxide of hydroxyl radicals by cell organelles, intact cells, and organs. The detection of superoxide by spin trapping with DMPO has been achieved in all of the above. For success, however, experimental protocols must allow for the relatively short lifetime of DMPO/superoxide[7] and the possible interference by metal catalysts such as iron.[29] For example, in studying free radicals produced in myocardial ischemia/ reperfusion, Arroyo et al.[30] immediately froze the coronary effluents in liquid nitrogen to prevent spin adduct decay. By monitoring the ESR spectra of the effluents immediately after thawing, they were successful in observing DMPO/ superoxide. The DMPO/superoxide adduct has also been detected in perfusate from isolated perfused rat livers subjected to ischemia/reperfusion.[31]

7. DTPA, EDTA, and Desferal

The presence of transition metals (particularly iron) and various chelating agents can significantly alter the results of spin-trapping experiments.[29] Although contaminating catalytic metals can be removed from buffer and biochemical systems,[32] this would be a difficult and uncertain (perhaps impossible) process for cells and organs. Thus, chelating agents are much needed tools. When studying a superoxide-generating system, EDTA will, in general, enhance the catalytic activity of iron in the reaction sequence,[1-3, 29] thereby increasing the yield of DMPO/hydroxyl while decreasing or eliminating the appearance of DMPO/ superoxide. DTPA (diethylenetriaminepentaacetic acid) reduces or eliminates many of the problems generated by catalytic iron in superoxide-generating systems,[29] but under circumstances where a reducing agent stronger than superoxide is responsible for iron reduction, DTPA can increase DMPO/hydroxyl formation.[15] In studying stimulated neutrophils, Britigan *et al.*[33] found DTPA (1-100 μM) to be a very useful tool; it had no effect on neutrophil superoxide production or oxygen consumption, whereas it enhanced the detection of superoxide by DMPO in their cellular experiments.

The iron chelators Desferal (deferrioxamine mesylate) renders iron essentially catalytically inactive in reactions (2)-(3) above. Unfortunately, the hydroxamic acid moieties of Desferal can undergo one-electron oxidation by superoxide (most likely $^{\bullet}$OOH), hydroxyl radical, and horseradish peroxidase.[34-36] The nitroxide radical so formed is stable, for a free radical; nevertheless, it reacts rapidly with cysteine, methionine, glutathione, ascorbate, and a water-soluble form of vitamin E.[35] This radical may also deactivate enzymes, as demonstrated for alcohol dehydrogenase.[35] If Desferal is present at a relatively high concentration (compared to spin trap), it can effectively compete for superoxide and hydroxyl

radical.[36] Since adventitious transition metals are present at only micromolar concentrations and spin traps are used at millimolar concentrations, scavenging by Desferal is perhaps less of a problem than the interference caused by the detection of the Desferal nitroxide radical itself. In any case, the Desferal concentration should be kept as low as possible to minimize scavenging.

Although DMPO is at least 20-fold more sensitive than the reduction of cytochrome *c* for the measurement of superoxide,[37] recent work has focused on the development of spin traps even more sensitive than DMPO. The first of these spin traps is 5-diethyloxy-phosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO). Although the rate of superoxide trapping by DEPMPO is still relatively slow, the DEPMPO/superoxide adduct is 15-fold more persistent than the DMPO/superoxide adduct.[38] In fact, the use of DEPMPO has improved the detection of superoxide during reperfusion of ischemic rat hearts [38, 39] over that which was possible with DMPO,[30] although the experiment is still difficult. The DEPMPO/superoxide radical adduct is detectable from phorbol ester-activated polymorphornuclear leukocytes with as few as 2×10^3 cells.[40] Recently, a nitrone derivative, 5-ethoxycarboxyl-5-methyl-1-pyrroline *N* oxide (EMPO), has been synthesized and found to have a superoxide adduct that is 5-fold more persistent than the DMPO analogue without the considerable spectral complexity of the DEPMPO/superoxide adduct.[41] In fact, the ESR spectrum of EMPO/superoxide adduct is virtually identical to that of the DMPO/superoxide adduct. The ^{15}N-labeled EMPO increases the sensitivity of this radical adduct by 50% as a result of having one-third fewer lines.[42]

Since the introduction of nitrone spin traps as a tool for the detection superoxide and hydroxyl radical, thousands of publications have used this technique. Not a single artifact has been reported for the detection of superoxide with DMPO in all this time. In contrast, many hydroxyl radical-independent pathways to DMPO/hydroxyl have been discovered. Fortunately, the hydroxyl radical scavenger derived trapping approach has exemplified by the trapping of methyl radical from DMSO also appears to be artifact free, although it may not be possible to distinguish the hydroxyl radical from hydroxyl-like species without careful kinetic studies. In retrospect, the nitrone spin-trapping approach to the detection of superoxide and hydroxyl radical has been an outstanding success.

In summary, success in biological spin trapping requires:

(1) the appropriate choice of spin trap for detecting the radicals of interest;
(2) an experimental design that considers the kinetics of the reaction of the radicals of interest with the spin trap and potential competing reactants;
(3) careful attention to possible artifacts;
(4) consideration of the chemistry that adventitious catalytic metals can introduce and
(5) use of appropriate spectrometer parameters to obtain the best spectra possible in the lifetime of the free radical-generating system.

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