Detection of Oxygen-derived Radicals in Biological Systems Using Electron Spin Resonance

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Oxygen-centered radicals, particularly the hydroxyl and superoxide radicals, have been postulated in many biochemical reactions and have been implicated in many adverse reactions in vivo. This article begins with a review of spin-trapping detection of oxygen-centered radicals in vitro and concludes with a presentation of our approach to the detection of the hydroxyl radicals in models of acute iron and copper poisoning. — Environ Health Perspect 102(Suppl 10):33–36 (1994)

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Introduction

The high reactivity of most free radicals makes their detection difficult. Table 1 summarizes the most common methods for detecting free radicals in biological systems along with their respective advantages and disadvantages. Electron spin (paramagnetic) resonance (ESR) or EPR is a spectroscopic technique that detects the unpaired electron present in a free radical. As such, it is the only approach (other than superoxide dismutase) that can provide direct evidence for the presence of a free radical. In addition, the analysis of the ESR spectrum generally enables the determination of the identity of the free radical.

Spin trapping is a technique in which a short-lived, reactive free radical combines with a diamagnetic molecule ("spin trap") to form a more stable free radical ("radical adduct") which can be detected by electron spin resonance. Even if the free radical is diatomic such as the superoxide or hydroxyl radical and therefore undetectable in solution (Table 1), its radical adduct will be polyatomic and, in principle, detectable. The most useful radical trap for oxygen-centered free radicals is 5,5-dimethyl-1-pyrroline N-oxide (DMPO).

The DMPO/superoxide and DMPO/hydroxyl radical adducts were first assigned as these nitrooxides by Harbour et al. in 1974 using chemical evidence for their formation (7). We have reviewed the chemical evidence for the structure of these radical adducts and shown them to be oxygen-centered by using [17O₂]oxygen, which explicitly affects the spectrum (2). The first report of spin trapping an oxygen-centered radical in a biological system was the trapping of the light-induced, paraquat-enhanced superoxide radical anion in chloroplasts (3). Since that first report, the use of spin trapping to detect oxygen-centered radicals in biological systems has multiplied. This article centers on the problems involved in proving that the adducts formed are not artifactual rather than on reviewing the vast literature.

If the superoxide radical is being trapped, then the formation of the radical adduct must be inhibited by superoxide dismutase. In fact the rate constant for the reaction of DMPO with superoxide at pH 7.4 is only 30 M⁻¹sec⁻¹ (4), so even at high concentrations of DMPO, superoxide dismutase can easily outcompete the spin trap for superoxide. This is the main reason superoxide has never been detected intracellularly with spin trapping.

Often the formation of the DMPO/OH radical adduct is also inhibited by superoxide dismutase, which implies that a) the hydroxyl radical formation is dependent on superoxide (perhaps by the reduction of ferric iron for the Fenton reaction) or b) superoxide is trapped first and the resulting superoxide radical adduct is reduced to form the hydroxyl radical adduct. This reaction is the reduction of a hydroperoxide to an alcohol that is catalyzed by glutathione peroxidase (5). The high activity of glutathione peroxidase is another reason the DMPO-

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Table 1. Methods for detecting and identifying oxygen-derived free radicals.

| Method            | Advantages                                      | Disadvantages                      |
|-------------------|-------------------------------------------------|------------------------------------|
| Product analysis  | Employs well-established analytical techniques | Does not provide unequivocal evidence |
| Inhibition by free radical scavengers (antioxidants) | Simple technique                   | Little information about structure of radical |
| Inhibition by superoxide dismutase (SOD)         | Simple, highly specific                    | Only applicable to superoxide       |
| Electron spin (paramagnetic) resonance            | Yields structural information, e.g., LOO⁺    | Diatomic radicals cannot be detected in solution |
| Direct                                                      | Wide application                       | May not be long-lived enough to detect in cells or in vivo |
| Indirect (spin trapping)                               |                                                  |                                    |

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superoxide adduct has not been detected intracellularly.

The DMPO/superoxide radical adduct also decomposes slowly to release the hydroxyl radical that may then be trapped (6). A good indication that something artifactual is occurring is the inability of catalase to inhibit hydroxyl radical adduct formation, a most unlikely event. In short, DMPO is not a useful trap for the hydroxyl radical if the hydroxyl radical formation is dependent on superoxide. In addition, the DMPO/hydroxyl radical is a detectable impurity when DMPO is added to buffer due to the nucleophilic addition of water, no matter how the DMPO is purified, so weak signals are suspect (7).

\[ \text{DMPO + Cu}^{2+}, \text{Fe}^{2+} \rightarrow \text{DMPO/OH} \rightarrow \text{DMPO/OH} \]

Recently, we have found that this reaction is catalyzed by copper in the presence of most buffers, which inhibit a similar iron-catalyzed reaction (8).

The best way to prove the existence of the hydroxyl radical in a system is to perform a competition experiment with a hydroxyl radical scavenger. Dimethyl sulfoxide reacts with the hydroxyl radical to form the readily trapped methyl radical (6).

\[ \text{-OH + DMSO} \rightarrow \text{-CH}_3 \]

Formate, which forms the carbon dioxide anion radical upon hydroxyl radical attack, can also be used in these competition experiments (9).

\[ \text{-OH + DMPO} \rightarrow \text{DMPO/OH} \]

\[ \text{-OH + HCO}_2^- \rightarrow \text{CO}_2^- + \text{H}_2\text{O} \]

\[ \text{CO}_2^- + \text{DMPO} \rightarrow \text{DMPO/CO}_2^- \]

Most artifacts leading to DMPO/OH radical adduct formation will be excluded by the use of hydroxyl radical scavengers, if both the scavenger-derived radical adduct is detected and a corresponding decrease in the DMPO/OH radical adduct concentration is found. This approach has been extended with a quantitative kinetic criterion to exclude species that are sufficiently reactive to oxidize the "specific" hydroxyl radical scavengers (10). Measurement of the initial rates of formation of the DMPO/OH and DMPO/scavenger radical adducts removes the effects of the differential radical adduct decay rates (11).

Using this approach, the relative efficiency of hydroxyl radical scavengers such as formate and DMPO is quantitatively predictable from the known rate constants for the reactions of the hydroxyl radical with the compounds \( k_1 \) and \( k_2 \).

\[
k_1 = \frac{d(\text{DMPO/OH})/dt}{k_2} = \frac{2[D\text{MO}]/[\text{DMPO}]/[\text{CO}_2^-]/[\text{DMPO}]^2}{d(\text{DMPO}^-\text{CO}_2^-)/dt} \times [\text{DMPO}]^2
\]

where \( k_1 (4.3 \times 10^8 \text{M}^{-1}\text{sec}^{-1}) \) and \( k_2 (3.2 \times 10^9 \text{M}^{-1}\text{sec}^{-1}) \) are determined from pulse radiolysis experiments (12).

Unfortunately, the -OH adducts of the spin traps currently available appear to be too unstable to use in vivo. Since in vivo Fenton chemistry is nonetheless thought to be an important source of oxidative damage, we have developed a spin-trapping approach to the detection of hydroxyl radical in iron and copper acute poisoning.

Although iron is an essential nutrient, the pathological processes associated with the various forms of iron overload demonstrate that the metal can also be toxic. Iron poisoning may be either acute or chronic. Acute iron poisoning is, in general, restricted to young children following the accidental ingestion of their mothers' iron pills. Acute iron poisoning resulting in death due to the accidental ingestion of oral iron supplements is the single most frequent cause of pediatric ingestion fatalities, being responsible for over 30% of all such fatalities (13). Chronic iron overload has several causes, including excessive dietary intake, genetic hemochromatosis, and multiple blood transfusions.

Findings from several animal model studies into the pathology of iron indicate that oxidative damage to the membranes of cell organelles may be a crucial event in toxicity. In addition, there exists a large body of indirect evidence from in vitro studies involving isolated organelles, cells, and tissue homogenates to suggest that oxidative damage due to hydroxyl radical formation is responsible for the toxic effects of iron (14).

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 methyl radical is then detected as its long-lived phenyl N-t-butyl nitritone (PBN) adduct. Alone, DMSO is relatively nontoxic, with a 24-hr LD50 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. Anesthesia is maintained throughout the experiments, which are initiated by ip injection of DMSO containing PBN, followed by intragastric injection of ferrous sulfate. The resulting PBN/methyl radical adduct (PBN/CH3) is detected by ESR, and the DMSO- and iron-dependence of in vivo adduct formation is demonstrated using collection of bile into dipryridyl (Figure 1). Collection directly into dipryridyl is necessary to stop ex vivo iron chemistry due to the iron excreted into the bile along with the radical adducts. The weak signal detected in the absence of DMSO, which is dependent upon iron, (Figure 1b), indicates the trapping of radicals derived from endogenous molecules. Having demonstrated unambiguously in vivo iron-dependent free radical formation, we next determined the effect of Desferal in this system. Desferal is a ferric iron chelator 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/CH3 adduct was almost abolished, suggesting that Desferal can inhibit hydroxyl radical generation during iron overload, presumably by binding iron in the ferric state (15).

Since the Fenton reaction requires hydrogen peroxide, we thought that a substance which catalyzes hydrogen peroxide formation would increase the signal. The activity of the herbicide paraquat (PQ2+) is attributed to its ability to catalyze the formation of superoxide and, subsequently, hydrogen peroxide. The herbicide undergoes an enzymatic single-electron reduction to form the paraquat radical-cation, PQ+, which is then oxidized by molecular...
The formation of superoxide radical, $O_2^-$, through its 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 (16), hepatocyte (17), alveolar type II, and Clara cell incubations (18). Unexpectedly, when we administered paraquat to our iron-poisoned rat model, only a modest increase of radical adduct formation occurred.

In contrast, radical adducts were detected in the bile of copper-poisoned rats only after they had been given paraquat (Figure 2). Apparently hydrogen peroxide was limiting in vivo in the copper analog of the Fenton reaction.

\[
Cu^{1+} + H_2O_2 \rightarrow Cu^{2+} + OH^- + \cdot OH
\]

When the experiment was repeated in the absence of copper (Figure 2B) or PQ$^{2+}$ (Figure 2C), no radical adducts were detected, thereby confirming the dependence of radical formation on the co-administration of both copper and PQ$^{2+}$. The fact that copper or PQ$^{2+}$ alone causes 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 which reacts slowly, if at all, with hydrogen peroxide to form the hydroxyl radical (19). The doublet in the center of the spectra of Figure 2 is from the ascorbate radical formed from the oxidation of endogenous ascorbate.

In an attempt to identify the radical species detected in bile of animals treated with Cu(II) and PQ$^{2+}$, $^{13}$C-labeled DMSO was used (Figure 3A). The presence of hyperfine splittings in the ESR spectrum from $^{13}$C (I=1/2) allows an unambiguous assignment of the PBN/$^1$CH$_3$ radical adduct formed in vivo. The appearance of $^{13}$C-hyperfine splittings observed in the wings of each of the lines of the nitroxide triplet is proof that the PBN/$^1$CH$_3$ radical adduct was formed during PQ$^{2+}$ and copper intoxication. The use of deuterated PBN enhances spectral resolution. Figure 3B shows the complete spectral simulation. Four radical species were used to simulate this spectrum. Only the assignments of the PBN/$^1$CH$_3$ and ascorbate radical species, however, can be considered unique. The other two PBN adducts, labeled ‘C and ‘Y, remain unidentified.

In conclusion, although the hydroxyl radical is often implicated as being the species responsible for the initiation of oxidative damage in iron- and copper-overload conditions, no ESR evidence for the formation of the hydroxyl radical had been reported until recently (14,15,19,21). We have employed a secondary radical-trapping technique in which the hydroxyl radical reacts with DMSO to form the methyl radical, $^1$CH$_3$, that is then detected as its adduct of the spin trap PBN in the bile of animals given an intragastric dose of ferrous or copper sulfate. The successful in vivo trapping of the DMSO-derived methyl radical in an animal model provides the only ESR evidence of hydroxyl radical formation in vivo due to the administration of iron or copper.

The identity of the methyl radical adduct was proven using $^{13}$C-isotope-substituted DMSO. In the iron model, methyl radical adduct formation was increased only moderately by paraquat, but totally inhibited by Desferal administration. In the copper model, no radical adduct formation could be detected unless paraquat was co-administered, implying hydrogen peroxide was limiting.

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