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Lipid radicals: Properties and detection by spin trapping★✪

Klaus Stolze✉, Natascha Udilova and Hans Nohl

Institute of Pharmacology and Toxicology, Veterinary University of Vienna, A-1210 Vienna, Austria

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Unsaturated lipids are rapidly oxidized to toxic products such as lipid hydroperoxides, especially when transition metals such as iron or copper are present. In a Fenton-type reaction Fe^{2+} converts lipid hydroperoxides to the very short-lived lipid alkoxyl radicals. The reaction was started upon the addition of Fe^{2+} to an aqueous linoleic acid hydroperoxide (LOOH) emulsion and the spin trap in the absence of oxygen. Even when high concentrations of spin traps were added to the incubation mixture, only secondary radical adducts were detected, probably due to the rapid rearrangement of the primary alkoxyl radicals. With the commercially available nitroso spin trap MNP we observed a slightly immobilized ESR spectrum with only one hydrogen splitting, indicating the trapping of a methinyl fragment of a lipid radical. With DMPO or 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) adducts were detected with carbon-centered lipid radical, with acyl radical, and with the hydroxyl radical. We also synthesized lipophilic derivatives of the spin trap DEPMPO in order to detect lipid radical species generated in the lipid phase. With all spin traps studied a lipid-derived carbon-centered radical was obtained in the anaerobic incubation system Fe^{2+}/LOOH indicating the trapping of a lipid radical, possibly generated as a secondary reaction product of the primary lipid alkoxyl radical formed. Under aerobic conditions an SOD-insensitive oxygen-centered radical adduct was formed with DEPMPO and its lipophilic derivatives. The observed ESR parameters were simi-

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✉Corresponding author: Prof. Dr. Hans Nohl, Institute of Pharmacology & Toxicology, Veterinary University of Vienna, Veterinärplatz 1, A-1210 Vienna, Austria, tel. (43 1) 25077 4400; fax: (43 1) 25077 4490; e-mail: Hans.Nohl@vu-wien.ac.at

Abbreviations: DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; DBPMPO, 5-(dibutoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; DPPMPO, 5-(dipropoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ESR, electron spin resonance; $L^{\bullet}$, lipid radical; $LO^{\bullet}$, lipoxyl radical; $LOO^{\bullet}$, lipidperoxyl radical; LOOH, lipid hydroperoxide; Me_2SO, dimethylsulphoxide; MNP, 2-methyl-2-nitroso-propane; PBN, α-phenyl-*N*-t-butyl nitrone; SOD, superoxide dismutase.

lar to those of alkoxyl radical adducts, which were independently synthesized in model experiments using Fe^{3+}-catalyzed nucleophilic addition of methanol or t-butanol to the respective spin trap.

Oxidative degradation of polyunsaturated lipids leads to a variety of toxic products such as lipid hydroperoxides, hydroxyalkenals and other products [1]. In the case of linoleic acid, oxidative attack starts with H-abstraction from position 11 thereby forming a lipid free radical delocalized between positions 9 and 13 [2]. Molecular oxygen rapidly adds to this primary lipid alkyl free radical (preferentially at positions 9 and 13) which after one-electron reduction results in a mixture of 9- and 13-hydroperoxylinoleic acid. The necessary hydrogen atom is provided either by a second lipid molecule (leading to chain propagation) or by an antioxidant present in the system (leading to chain termination).

Spin trapping of lipid-derived radicals using the lipophilic spin trap α-phenyl-*N*-t-butyl nitrone (PBN) has already been extensively studied [3]. Unfortunately, the short half-life of PBN adducts with oxygen-centered radical spin adducts ($t_{1/2}$ of PBN/$^{\bullet}$OH: 38 s [4]) restricts its use to the detection of carbon-centered radical adducts only. Furthermore, the similarity of the ESR spectra of different PBN spin adducts renders spectral identification of the trapped species rather difficult. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), on the other hand, shows ESR spectra with distinct, characteristic hyperfine splitting parameters and can be used for the detection of carbon-centered as well as hydroxyl radicals. Its superoxide adduct is, however, very unstable ($t_{1/2}$ of DMPO/$^{\bullet}$OOH: 45 s [5]) and its decay leads to artifactual "DMPO/$^{\bullet}$OH" adducts [5].

The phosphonic acid compound, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO), with an increased stability of the superoxide spin adduct has recently been described by Fréjaville *et al.* [6]. The half-life of DEPMPO/ $^{\bullet}$OOH is about 10 times higher compared to the respective DMPO adduct [5, 6]. Whereas various studies have shown that DEPMPO is one of the most suitable spin traps for the detection of superoxide radicals, its usefulness under conditions of lipid peroxidation, where in addition to hydrophilic superoxide and hydroxyl radicals also lipophilic radicals such as L$^{\bullet}$, L–O$^{\bullet}$, and L–O–O$^{\bullet}$, are expected to be formed [7, 8], has not been studied in detail until now. Furthermore, variation of the phosphonic acid residue with respect to its lipophilic properties (n-octanol/water partition coefficient) should give additional information about the site of free radical formation [9].

In the present study different spin traps were incubated with a Fenton type system consisting of Fe^{2+} (0.1 mM) and linoleic acid hydroperoxide (mixture of 9- and 13-hydroperoxyoctadecadienoic acid, 1.0 mM) in aerobic (oxygen bubbled) as well as anaerobic aqueous solution (nitrogen bubbled). The different spin adducts obtained reflect the differences in the stabilities of the respective spin adducts as well as the effect of oxygen on the secondary reactions which occur after the Fenton-like reaction between lipid hydroperoxide and Fe^{2+}.

MATERIALS AND METHODS

Chemicals. 2-Methylpyrroline, bis-(n-butyl)phosphite, bis-(ethyl)phosphite, bis-(2-ethylhexyl)phosphite, bis-(n-propyl)phosphite, and *m*-chloroperbenzoic acid were purchased from Aldrich-Sigma chemical company. Petroleum ether (high boiling, 50–70°C) was from Fluka (Buchs, Switzerland). All other chemicals were purchased from Merck (Darmstadt, Germany).

Synthesis of the compounds. Synthesis and characterization of the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO) and its lipophilic derivatives 5-(dipropoxyphosphoryl)-5-methyl-1-pyr-

roline *N*-oxide (DPPMPO) and 5-(dibutoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DBPMPO) was performed in two steps from 2-methylpyrroline and the respective dialkylphosphite according to Fréjaville *et al.* [6] and Barbati *et al.* [10] with minor adaptations published elsewhere [9]. Briefly, 2-methylpyrroline was added at room temperature under nitrogen to a small excess of dialkylphosphite and stirred for several days giving the respective dialkyl-(2-methyl-2-pyrrolidinyl) phosphonate in almost quantitative yield. Separation of the crude product from excess of phosphite was done by extraction with 2 M HCl (DEPMPO, DPPMPO) or by column chromatography using silica gel (DBPMPO). Oxidation to the *N*-oxide was performed by slow addition of *m*-chloroperbenzoic acid (dissolved in chloroform), to a solution of the respective amine in chloroform at –10°C. The crude product was purified by column chromatography on silica gel using a petroleum ether/ethanol gradient. The final purification step was done immediately before the ESR experiments on a 1 mL solid phase extraction column Chromabond C-18 (100 mg) obtained from Macherey-Nagel (Düren, Germany).

Preparation of lipid hydroperoxides. Linoleic acid hydroperoxide was synthesized according to O'Brien [11]. Briefly, linoleic acid was air-oxidized for 72 h at room temperature in the dark. The oxidation mixture was dissolved in petroleum ether (boiling range 40–60°C) and extracted four times with water /methanol (1:3, v/v). The obtained aqueous methanol was then extracted four times with light petroleum. The methanolic phase was then evaporated under reduced pressure and the obtained hydroperoxide was dissolved in ethanol and stored in liquid nitrogen. The concentration of hydroperoxide was calculated using an absorption coefficient of ε_{233nm} = 25250 $M^{-1} \cdot cm^{-1}$ in ethanol [11].

Instruments. For ESR experiments the Bruker spectrometers ER 200 D-SRC 9/2.7 with the data system ESP1600 or the Bruker spectrometer ESP300E were used, operating at 9.6 GHz with 100 kHz modulation frequency and equipped with a rectangular TE_{102} or a TM_{110} microwave cavity.

RESULTS

Figure 1a shows the Fe^{2+}-initiated (0.1 mM) formation of lipid-derived free radical adducts from an anaerobic mixture of 9- and 13-hydroperoxy-linoleic acid (1.0 mM) in phosphate buffer (20 mM, pH 7.4) in the presence of the spin trap DMPO (49 mM).

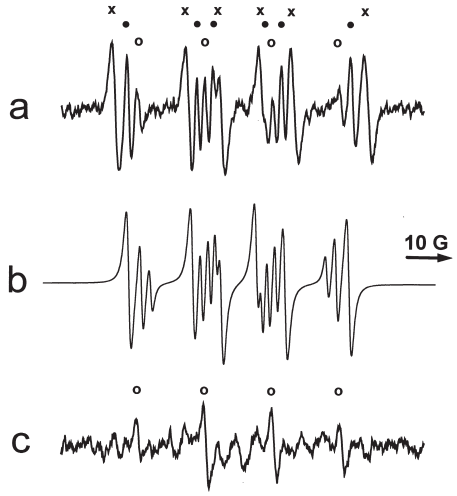


Figure 1. Formation of lipid-derived free radical adducts from linoleic acid hydroperoxide (1.0 mM) and Fe^{2+} (0.1 mM) in the presence of the spin trap DMPO (49 mM).

a) Experimental spectrum and b) computer simulation of an anaerobic system (N_2), showing the DMPO adducts of 1.) a carbon-centered radical (marked "x", a_N = 16.25 G, a_H = 23.50 G), 2.) an acyl radical (marked "•", a_N = 15.30 G, a_H = 18.95 G) and 3.) a hydroxyl radical (marked "o", a_N = 14.95 G, a_H = 14.95 G). c) Aerobic system (O_2), showing only traces of the DMPO/ $^{\cdot}OH$ species. Spectrometer settings: sweep width, 80 G, modulation amplitude, 1.02 G, microwave power, 20 mW, time constant, 0.66 s receiver gain 4×10^5, scan rate, 28.70 G/min. In c) 11 scans were accumulated.

The composite ESR spectrum was subjected to computer simulation for spectral interpretation (Fig. 1b). Three different species can be

distinguished, which were identified by comparison of its ESR parameters with data previously published in the literature [12]: 1.) a carbon-centered adduct, (a_N = 16.25 G; a_H = 23.50 G; approx. 50%), 2.) an acyl radical adduct (a_N = 15.30 G; a_H = 18.95 G, approx. 40%) and 3.) the hydroxyl radical adduct (a_N = 14.95 G; a_H = 14.95 G, approx. 10%), which can always be detected in solutions containing iron ions due to nucleophilic addition of water to the spin trap, even if hydroxyl radicals are not produced.

Figure 1c shows the accumulation of 11 ESR spectra obtained in an aerobic, oxygen-bubbled incubation system under otherwise identical conditions as in Fig. 1a. Except for traces of the DMPO/$^{\cdot}$OH adduct, no significant amounts of other radical adducts were detected.

In Fig. 2a the spin trap DPPMPO (50 mM) was used instead of DMPO under otherwise identical conditions as in Fig. 1a (anaerobic system). Except for the additional phosphorous coupling, the ESR parameters were quite similar to those of the DMPO system and the assignment of the ESR lines to the respective spin adducts was done in analogy to the DMPO system: 1.) a carbon-centered radical adduct (a_P = 48.48 G; a_N = 15.12 G; a_H = 22.18 G, approx. 80%), 2.) an acyl radical adduct (a_P = 49.20 G; a_N = 14.11 G; a_H = 17.05 G, approx. 10%) and 3.) the hydroxyl radical adduct (a_P = 46.95 G; a_N = 14.00 G; a_H = 13.20 G, approx. 20%). All hyperfine splitting constants were calculated by computer simulation of the spectrum (not shown) and comparison with the experimental spectrum, as was demonstrated in Fig. 1b for the three respective DMPO adducts.

In contrast to the aerobic DMPO system shown in Fig. 1c, the respective aerobic incubation using DPPMPO (50 mM) clearly showed an second species in addition to the DPPMPO/$^{\cdot}$OH adduct (ESR data see above in Fig. 2a). The additional lines (a_P = 50.30 G; a_N = 13.20 G; a_H = 11.30 G) are tentatively assigned to an alkoxyl radical adduct, most probably a secondary DPPMPO/$^{\cdot}$OL species formed by addition of oxygen to the carbon-centered radical detected in Fig. 2a, followed by rearrangement or reduction to the detected alkoxyl species. The trapping of a superoxide radical can be excluded since the formation of this species was SOD-insensitive (data not shown). However, the differentiation between alkoxyl- and alkylperoxyl radical

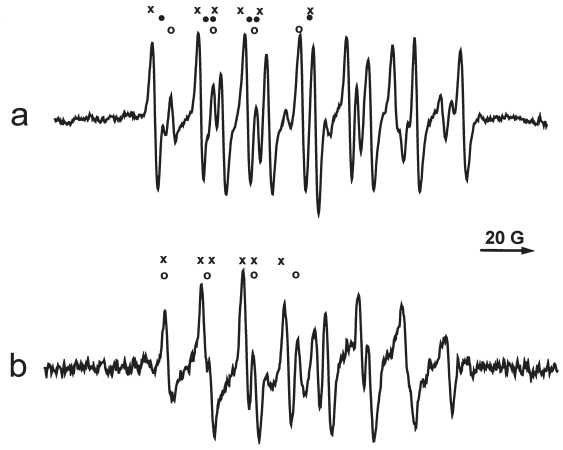


Figure 2. Formation of lipid-derived free radical adducts from linoleic acid hydroperoxide (1.0 mM) and Fe^{2+} (0.1 mM) in the presence of the spin trap DPPMPO (50 mM).

a) Anaerobic system (N_2) showing the DPPMPO adducts of 1.) a carbon-centered radical (marked "x", a_P = 48.48 G, a_N = 15.12 G, a_H = 22.18 G), 2.) an acyl radical (marked "•", a_P = 49.20 G, a_N = 14.11 G, a_H = 17.05 G) and 3.) a hydroxyl radical (marked "o", a_P = 46.95 G, a_N = 14.00 G, a_H = 13.20 G). Spectrometer settings: sweep width, 160 G, modulation amplitude, 1.44 G, microwave power, 20 mW, time constant, 0.16 s, receiver gain 4 × 10^5, scan rate, 115 G/min, 3 scans accumulated. b) Aerobic system (O_2), showing the DPPMPO/$^{\cdot}$OL species (marked "x", a_P = 50.30 G, a_N = 13.20 G, a_H = 11.30 G, approx. 70%) and the DPPMPO/$^{\cdot}$OH adduct (approx. 30%). Spectrometer settings: same as in a) except for the time constant (0.08 s) and the scan rate (230 G/min).

adducts of DEPMPO and its lipophilic derivatives is difficult, since alkylperoxyl radical adducts of DEPMPO (such as DEPMPO/$^{\cdot}$OOCH$_3$) have been reported to be stable for several minutes [6], in contrast to the DMPO system which does not form stable adducts with alkylperoxyl radicals [13].

Similar results were obtained when the spin trap DBPMPO was used. Figure 3a shows the composite ESR spectrum obtained in an aerobic incubation of linoleic acid hydroperoxide (1.0 mM) and Fe^{2+} (0.1 mM) in phosphate buffer (20 mM, pH 7.4) containing DBPMPO (50 mM). Computer simulation of the experimental spectrum shown in Fig. 3a revealed the formation of two species: the DBPMPO/·OL species (marked "x", simulated in Fig. 3b using the following set of parameters: a_P = 50.30 G; a_N = 13.25 G; a_H = 11.30 G, ΔH = 1.45 G, approx. 45%) and the DBPMPO/·OH adduct (marked "o", simulated in Fig. 3c with the following parameters: a_P = 46.90 G; a_N = 14.05 G; a_H = 13.50 G, ΔH = 1.60 G, approx. 55%).

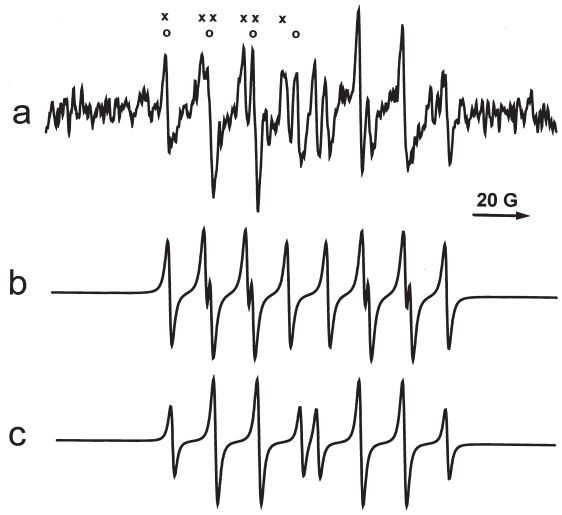


Figure 3. Formation of lipid-derived free radical adducts from linoleic acid hydroperoxide (1.0 mM) and Fe^{2+} (0.1 mM): aerobic system in the presence of the spin trap DBPMPO (50 mM).

a) Experimental spectrum, spectrometer settings: sweep width, 160 G, modulation amplitude, 1.44 G, microwave power, 20 mW, time constant, 0.33 s, receiver gain 4×10^5, scan rate, 57.4 G/min. b) Computer simulation of the DBPMPO/·OL species (x): a_P = 50.30 G, a_N = 13.25 G, a_H = 11.30 G, ΔH = 1.45 G. c) Computer simulation of the DBPMPO/·OH adduct (o): a_P = 46.90 G, a_N = 14.05 G, a_H = 13.50 G, ΔH = 1.60 G.

The ESR spectrum obtained using the spin trap MNP (20 mM) is shown in Fig. 4a. This spectrum was recorded in an anaerobic, phosphate-buffered (20 mM, pH 7.4) incubation

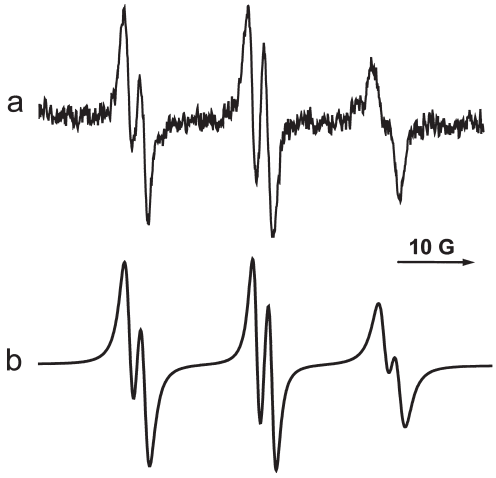


Figure 4. Formation of lipid-derived free radical adducts from linoleic acid hydroperoxide (1.0 mM) and Fe^{2+} (0.1 mM): anaerobic system in the presence of the spin trap MNP (20 mM) in phosphate buffer (20 mM, pH 7.4).

a) Experimental spectrum (N_2), spectrometer settings: sweep width, 60 G, modulation amplitude, 0.90 G, microwave power, 20 mW, time constant, 0.16 s, receiver gain 1×10^5, scan rate, 21.5 G/min, 10 scans accumulated. b) Computer simulation with the following set of parameters: a_N = 16.33 G, a_H = 1.95 G, line width ΔH = 1.3 G, 1.05 G and 1.6 G, respectively.

mixture containing linoleic acid hydroperoxide (1.0 mM) and Fe^{2+} (0.1 mM). The best fit obtained by computer simulation is shown in Fig. 4b, with the following set of parameters: a_N = 16.33 G; a_H = 1.95 G, line width ΔH = 1.3 G, 1.05 G and 1.6 G, respectively. The spectrum shows a partially immobilized carbon-centered radical species attached to MNP with a CH-fragment [14]. Oxygen-centered radical adducts with MNP are, however, not stable enough for ESR detection.

When the same anaerobic incubation system was used in the presence of the spin trap PBN (10 mM), the experimental spectrum shown in Fig. 5a was obtained: two different PBN radical adducts (carbon-centered radicals) were obtained, whose ESR parameters were determined by computer simulation (shown in Fig. 5b).

The greater line width of the first species (a_N = 16.40 G; a_H = 3.20 G, ΔH = 0.95 G) most probably indicates motional restriction due to

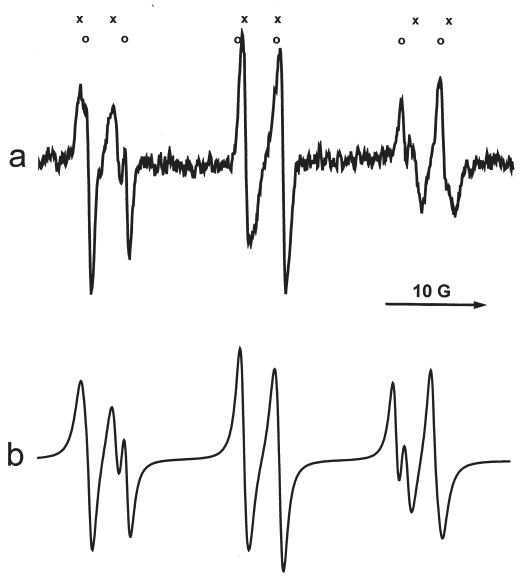
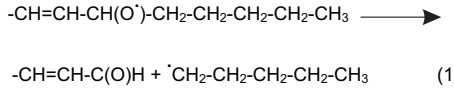


Figure 5. Formation of lipid-derived free radical adducts from linoleic acid hydroperoxide (1.00 mM) and Fe^{2+} (0.1 mM): anaerobic system in the presence of the spin trap PBN (10 mM):

a) Experimental spectrum (N_2). Spectrometer settings: sweep width, 50 G, modulation amplitude, 0.51 G, microwave power, 20 mW, time constant, 0.33 s, receiver gain, 4×10^5, scan rate, 17.9 G/min. b) Computer simulation with the following set of parameters: species 1: a_N = 16.40 G, a_H = 3.20 G, ΔH = 0.95 G (marked "x"), species 2: a_N = 15.55 G, a_H = 3.79 G, ΔH = 0.72 G (marked "o").

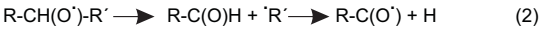
a higher molecular mass of the trapped radical. The second species (a_N = 15.55 G; a_H = 3.79 G, ΔH = 0.72 G) with its lower line width is probably the spin adduct of a secondary radical formed by fragmentation of the initially formed primary alkoxyl radical, which does not form a stable adduct with PBN and is subject to rapid rearrangement and fragmentation (such as β-scission [15], eqn. (1)).



(1)

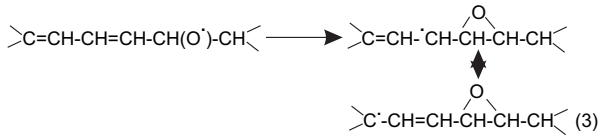
DISCUSSION

The formation of lipid-derived radicals from linoleic acid hydroperoxide in the presence of Fe^{2+} was detected in anaerobic solution with both DMPO and DEPMPO and its lipophilic derivatives. The primary lipid alkoxyl radical was not trapped by any of the spin traps, due to its rapid rearrangement to secondary products, two of which were detected. The first one is an acyl radical adduct, which is formed by β-scission and subsequent H-abstraction [15] (eqn. (2)),



(2)

and the second is a carbon-centered radical. Carbon-centered radicals can be formed in different ways from the initial lipid alkoxyl radical. First, by the epoxidation reaction [12,16] (eqn. (3)),



(3)

and second, by the β-scission reaction mentioned above (eqn. 2).

In addition, the hydroxyl radical adduct was found in minor concentrations, possibly formed by iron-catalyzed nucleophilic addition of water to the spin trap.

Whereas the nitrone spin traps (DMPO, DEPMPO etc.) will give no information regarding the structure of the trapped carbon-centered radical, the additional β-hydrogen splitting observed in the experiment with the nitroso spin trap MNP indicated that a tertiary C-fragment (CH-group) was trapped. This result would be consistent with the trapping of the epoxylinoleic acid radical mentioned above. Trapping of a primary pentyl radical resulting from β-scission, on the other hand, would result in the observation of two additional β-hydrogen splittings in the ESR spectrum (CH_2-fragment).

With PBN two different carbon-centered radical adducts were observed, one of which showed a significantly greater line width. This

observation would be consistent with the trapping of 1.) a linoleic acid derived carbon-centered radical (such as the epoxylinoleic acid radical) and 2.) a fragment with a shorter chain length such as the pentyl radical obtained in the β-scission reaction.

An interesting aspect is the difference between DMPO and the DEPMPO spin traps which was observed in the oxygenated system: with DMPO only traces of the hydroxyl radical adduct were observed, whereas with DEPMPO and its lipophilic derivatives an additional species became visible which was absent in the anaerobic incubation. The ESR spectrum consisted of 12 lines with splitting constants similar to the superoxide adduct. However, the lines did not show the alternating line width typical for the superoxide adduct and their intensity was SOD-insensitive. Most probably these adducts were formed from secondary alkoxyl radicals generated either by transformation of lipid hydroperoxyl radicals into alkoxyl radicals and molecular oxygen [17–22] or by Fe^{2+}-catalyzed decomposition of lipid hydroperoxyl radical adducts (eqn. (4) and (5)).

$$\text{DEPMPO/}^{\cdot}\text{OOL} + Fe^{2+} \longrightarrow \text{DEPMPO/}^{\cdot}\text{OH} + LO^{\cdot} + Fe^{3+} \quad (4)$$

$$\text{DEPMPO} + LO^{\cdot} \longrightarrow \text{DEPMPO/}^{\cdot}\text{OL} \quad (5)$$

On the other hand, Fréjaville *et al.* [6] reported that the DEPMPO/$^{\cdot}$OOCH$_3$ species formed in an UV-irradiated solution of H_2O_2 in the presence of oxygen and Me_2SO is remarkably stable. Therefore, spin adducts of the type DEPMPO/$^{\cdot}$OOL should also be considered as a possible reaction product in the aerobic system.

In summary, DEPMPO and its lipophilic derivatives DPPMPO and DBPMPO can successfully be used when both carbon-centered and oxygen-centered radicals are expected whereas PBN forms the most stable carbon-centered radical adducts. MNP, on the other hand, gives additional information about the number of H-atoms at the radical site of a carbon-centered radical. In general, combination of experiments using different spin traps will give a more complete picture than the use of one particular spin trap alone.

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