HYDROXYL RADICAL PRODUCTION INVOLVED IN LIPID PEROXIDATION OF RAT LIVER MICROSOMES*

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Summary: The spin-traps, 5,5'-Dimethyl-1-pyrroline-1-oxide and phenyltertiarybutylnitrone have been used to investigate the primary free radical involved in lipid peroxidation. In this report, we wish to present evidence that a NADPH-dependent hydroxyl radical (OH·), which may be the primary free radical that initiates lipid peroxidation, is generated in liver microsomes. A mechanism for the generation of the hydroxyl radical in liver microsomes is also postulated.

Introduction

Hochestein and Ernster (1) have demonstrated NADPH-dependent enzymic lipid peroxidation in liver microsomes. This raises the question of whether or not a free radical is involved in lipid peroxidation. Pederson and his co-workers (2) showed that the superoxide radical ($O_2^{\cdot-}$), which may be involved in lipid peroxidation, was generated by a purified NADPH-cytochrome c reductase from rat liver microsomes. Several investigators (3-6) have suggested that singlet oxygen production is associated with the NADPH-dependent lipid peroxidation in liver microsomes. By using several scavengers with specificity for the OH radical, Fong et. al. (7) suggested that the OH radical initiates lipid peroxidation in liver microsomes. To date, however, no direct evidence identifying the OH radical as being involved is available.

We previously reported (8) that by using a spin-trapping technique that we were able to detect free radical formation in the NADPH-dependent lipid peroxidation of liver microsomes. These free radicals, however, were associated with added substrates such as tris buffer, alcohol, dimethyl sulfoxide, etc. In this previous report (8), it was also suggested that cytochrome P-450 may be involoved in these free radical events.

The study presented here is an attempt to shed more light on the mechanism of free radical production, particularly the primary free radical which initiates

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Abbreviations: ESR——Electron Spin Resonance, DMPO——5,5'-Dimethyl-1-pyrroline-1-oxide, PBN——Phenyltertiarybutylnitrone, Metyrapone——2-Methyl-1,2-di-3-pyridyl-1-propanone, pCMB——p-Chloromercuribenzoate, EDTA——Ethylenediaminetetraacetic acid, NADPH——Nicotinamide adenine dinucleotide phosphate-reduced.

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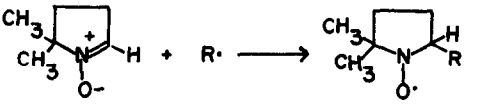
lipid peroxidation in liver microsomes. This was accomplished by designing a detection method which requires shorter time between the induction of lipid peroxidation and the observation by ESR in the absence of any aubstrate.

In addition to PBN(phenyltertiarybutylnitrone), the spin-trap, DMPO (5,5'-Dimethyl-1-pyrroline-1-oxide) was chosen for this investigation. If a short-lived free radical reacts with DMPO, as well as with PBN, a long-lived nitroxide radical is formed which can be easily identified by ESR. The mechanism being:



It has been shown that the β-hydrogen hyperfine splitting of the DMPO spin adduct is extremely sensitive to the nature of R· attached (9,10).

By using these spin-traps, we can demonstrate that a NADPH-dependent hydroxyl radical that may initiate lipid peroxidation, is in fact generated in liver microsomes.

Materials and Methods

Liver microsomes were prepared from 3-4 months old male Wistar rats. Animals were sacrificed, livers were quickly removed and washed with a 0.05 M phosphate buffer containing 0.15 M KCl and 0.25 M sucrose, PH 7.4 (Buffer A). Livers then were minced and homogenization was carried out in buffer A with a Teflon-glass homogenizer. The homogenate was centrifuged at 11,000 rpm for 25 minutes. This step was repeated once. The pellets were suspended in Buffer A with gentle homogenization and centrifuged at 25,000 rpm for 90 minutes. The pellets then were resuspended in a 0.05 M phosphate buffer, 0.15 M KCl and 1.0 mM EDTA, PH 7.4 (Buffer B) with gentle homogenization. The microsome suspension was centrifuged at 16,000 rpm for 30 minutes. The pellets were resuspended in a 1.0 mM phosphate buffer containing 0.15 M KCl, PH 7.4 (Buffer C) and were stored at -80°C.

Protein was determined by the Lowry et. al. method (11). ESR spectra were recorded with a Varian Model E-4 spectrometer equipped with an on-line V-72 16K mini-computer. Samples were prepared for ESR measurement by mixing the reaction mixture containing 1.8 mg/ml microsomes, 2.2×10^{-5} M Fe^{+2}, 4.4×10^{-5} M EDTA, 7.0 mM DMPO in 0.15 M KCl solution or 0.12 M PBN and 0.15 M KCl in 0.05 M phosphate buffer, PH 7.4 with 0.74 mM NADPH. Final volume of each sample was 0.54 ml. The samples were placed immediately in the ESR after mixing with no incubation. Unless otherwise specified, all ESR spectra were recorded under these conditions. The determinations of hyperfine splitting parameters and g values were made by direct comparison with a known standard, tempol (2,2,6,6,-tetramethyl-4-hydroxy-piperidine-1-oxy) or by direct fieldial measurements. The ultraviolet irradiation source used was a 100 watt high pressure Hg lamp.

5,5'-Dimethyl-1-pyrroline-1-oxide (DMPO), phenyltertiarybutylnitrone (PBN) and 2-methyl-1,2-di-3-pyridyl-1-propanone (Metyrapone) were obtained from Aldrich Chemical Company. Xanthine and p-chloromercuribenzoate,sodium salt were purchased from Calbiochem. Linoleic acid, xanthine oxidase, NADPH (type III enzymically reduced) and sucrose were obtained from Sigma Chemical Company. Other chemicals and solvents used were reagent grade.

Results and Discussion

Several investigators have shown that OH and OH_2 radicals are the major

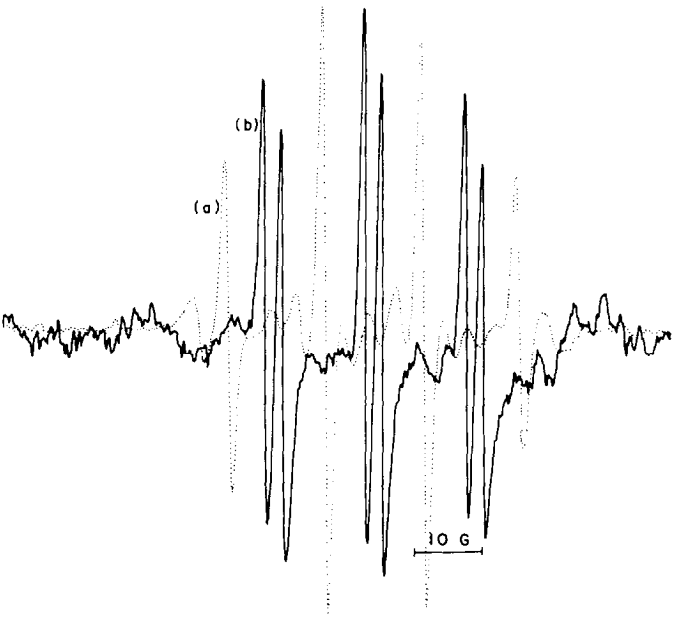


Fig. 1 (a) The ESR spectrum of the DMPO-OH radical adduct obtained upon a 15-second pulse u.v. irradiation of 1% H_2O_2 in the presence of 2.5 x 10^{-2} M DMPO. $a^N = a^H_\beta$ = 15.0 G and g = 2.0062 (the dotted line). (b) The ESR spectrum of the PBN-OH radical adduct obtained upon a 15-second pulse u.v. irradiation of 1% H_2O_2 in the presence of 0.12 M PBN. a^N = 15.5 G, a^H_β = 2.75 G and g = 2.0061 (the solid line).

intermediates generated in the ultraviolet photolysis of aqueous hydrogen peroxide (12-14).

In studies of the ultraviolet photochemical dissociation of hydrogen peroxide with DMPO in aqueous solution, Harbour et. al. (10) were able to obtain an ESR spectrum consisting of a 1:2:2:1 quartet with hyperfine splittings of $a^N = a^H_\beta$ = 15.3 G and g = 2.0060. This signal was identified as the DMPO-OH radical adduct. When PBN was used instead of DMPO, an ESR spectrum consisting of a triplet of doublets with hyperfine splittings of a^N = 15.3 G, a^H_β = 2.75 G and g = 2.0057 was obtained. We have repeated the work of Harbour et. al. and observed the same spectra with only slightly different splittings (see Fig. 1).

Liver microsomes were known to catalyze NADPH-dependent lipid peroxidation (1). When either DMPO or PBN was added to a reaction mixture containing microsomes, Fe^{+2}, EDTA and NADPH without any incubation time, an ESR signal, identical to the DMPO-OH radical adduct (Fig. 2a) or the PBN-OH radical adduct (Fig. 3a) as shown in Fig. 1, was obtained. The signal is NADPH-dependent. No signal can

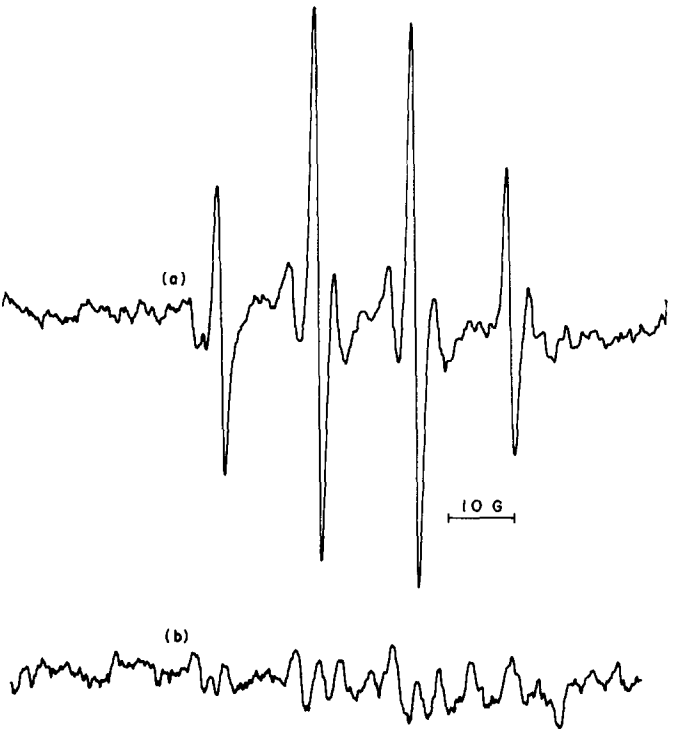


Fig. 2 The ESR spectrum of the DMPO-OH radical adduct obtained when the reaction mixture containing 1.8 mg/ml microsomes, 2.2 x 10^{-5} M Fe^{+2}, 4.4 x 10^{-5} M EDTA, 7.0 mM DMPO at PH 7.4 in 0.15 M KCl solution was mixed (a) with 0.74 mM NADPH, (b) without NADPH. $a^N = a^H_\beta$ = 15.0 G and g = 2.0062

be seen in tha absence of NADPH (Fig. 2b or Fig. 3b). In the conventional assay method the reaction mixture is usually incubated at 37°C for 20 minutes. When the reaction mixture containing either DMPO or PBN was incubated at 37°C for 20 minutes, no DMPO-OH or PBN-OH radical adduct was detected. This finding suggests that the DMPO-OH and the PBN-OH radical adducts cannot be detected using the technique described in (8). Dissolving DMPO into a 0.05 M phosphate buffer, PH 7.4, alone results in autoxidation and a complicated ESR spectrum. This observation indicates that DMPO is labile in 0.05 M phosphate buffer, PH 7.4. In the previous report (8), we have shown that in the presence of small concentrations of ethanol,a PBN-ethanol radical adduct was formed in liver microsomes. When DMPO was added to a reaction mixture containing microsomes, Fe^{+2}, EDTA, NADPH and 0.9% ethanol, PH 7.4, an ESR spectrum consisting of doublets of triplets shown in Fig.4, which is possibly a DMPO-ethoxy radical adduct, was obtained instead of the quartet typical of the DMPO-OH radical adduct. The

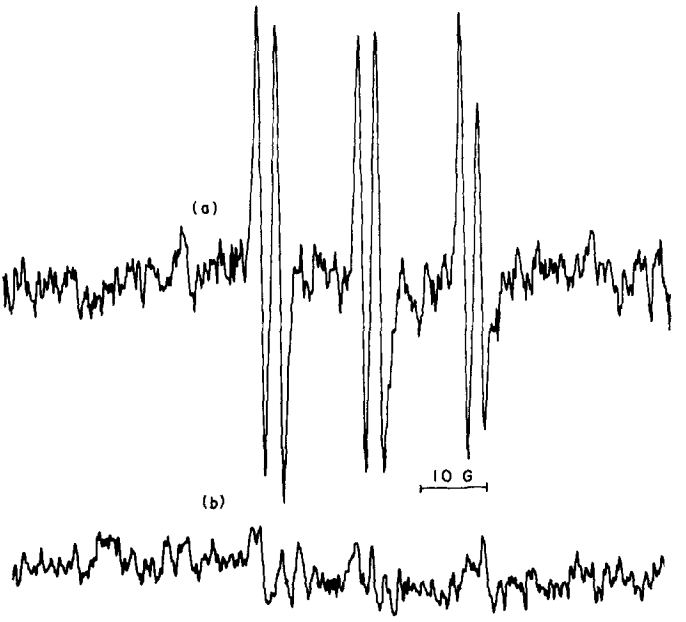


Fig. 3 The ESR spectrum of the PBN-OH radical adduct obtained when the reaction mixture containing 1.8 mg/ml microsomes, 2.2 x 10^{-5} M Fe^{+2}, 4.4 x 10^{-5} M EDTA, 0.12 M PBN, 0.15 M KCl in 0.05 M phosphate buffer, PH 7.4 was mixed (a) with 0.74 mM NADPH, (b) without NADPH. a^N = 15.5 G, a^H_β = 2.75 G and g = 2.0061

NADPH-dependent DMPO-OH radical adduct, therefore, cannot be observed in the presence of ethanol which has been shown to act as a scavenger for OH radical (27)

Metyrapone (15,16), a specific inhibitor of cytochrome P-450 was used in this system and found to have no effect on the DMPO-OH radical adduct in liver microsomes (Table I). This suggests that cytochrome P-450 is not involved in the formation of the hydroxyl radical. p-Chloromercuribenzoate (pCMB) (6), a potent inhibitor of cytochrome P-450 reductase, however, at very low concentrations (8.0 x 10^{-5}M) completely inhibited the formation of the DMPO-OH radical adduct (Table I). This finding suggests that cytochrome P-450 reductase must play an essential role in the formation of the DMPO-OH radical adduct. It has been well documented that in the presence of oxygen and NADPH, cytochrome P-450 reductase can generate superoxide radicals (17). Since only neutral free radicals are trapped by the nitrone spin-traps such as PBN or DMPO (18), $O_2^{\cdot-}$ cannot contribute directly to the ESR signal detected. The hyperfine splittings of the DMPO-OOH radical adduct are very much different from that of the DMPO-OH radical adduct (10). The protonated form of $O_2^{\cdot-}$, namely, $HO_2\cdot$ therefore does not contribute to the ESR signal detected. This suggests that $O_2^{\cdot-}$ must act to

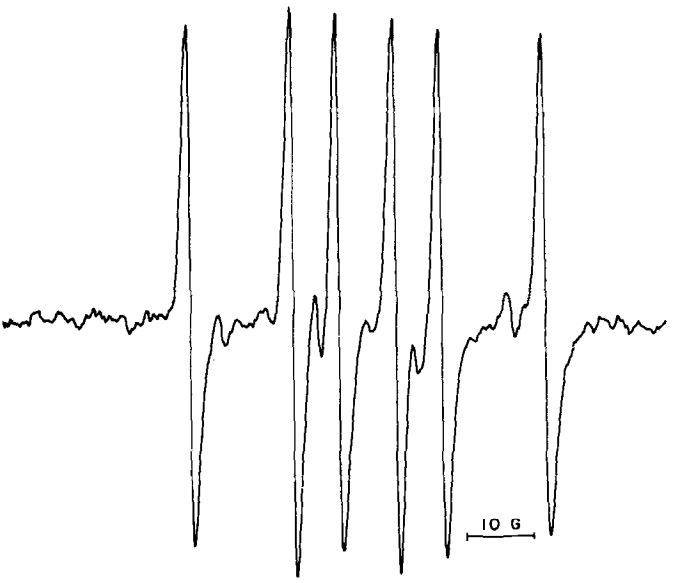


Fig. 4 The ESR spectrum of the DMPO-ethoxy radical adduct obtained when the reaction mixture containing 1.8 mg/ml microsomes, 2.2 x 10^{-5} M Fe^{+2}, 4.4 x 10^{-5} M EDTA, 7.0 mM DMPO, 0.9% ethanol and 0.74 mM NADPH at PH 7.4 in 0.15 M KCl solution. a^N = 15.8 G, a^H_β = 22.8 G and g = 2.0068

generate OH· which may in turn abstract a hydrogen from a methylene carbon atom of an unsaturated fatty acid of phosphatidylcholine and thus initiate lipid peroxidation.

Any reaction mixture which generates $O_2^{\cdot-}$ will also be producing H_2O_2, by the dismutation of $O_2^{\cdot-}$ (19) following the scheme:

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (1)$$

If the amount of H_2O_2 can accumulate, $O_2^{\cdot-}$ and H_2O_2 will react with each other, by the Haber-Weiss reaction (20) to generate OH·.

$$H_2O_2 + O_2^{\cdot-} \longrightarrow O_2 + OH^- + OH\cdot \qquad (2)$$

This scheme is supported by the fact that linoleic acid strongly inhibited the formation of the DMPO-OH radical adduct (Table I), thus suggesting that OH· is the primary free radical that initiates lipid peroxidation in liver microsomes.

Studies in several laboratories (21-23) have shown that the EDTA-iron complex is required for the maximum activity of the NADPH-dependent lipid peroxidation in liver microsomes. The function of EDTA may appear to elevate the redox potential of iron (22). Studies in our laboratory indicate that ferric iron was only 40-50% as active as ferrous iron for the formation of the DMPO-OH or the PBN-OH radical adduct in NADPH-dependent lipid peroxidation in liver microsomes

Table I. Effects of Metyrapone, pCMB and linoleic acid on the formation of the PBN-OH or the DMPO-OH radical adduct. The reaction mixture contained 1.8 mg/ml microsomes, 2.2 x 10^{-5} M Fe^{+2}, 4.4 x 10^{-5} M EDTA, 7.0 mM DMPO in 0.15 M KCl, PH 7.4. Various amounts of inhibitors were then added to the reaction mixtures. The reaction was initiated by adding 0.74 mM NADPH.

Inhibitors	conc.(mM)	%control*
none	---	100
Metyrapone	0.47	93
	0.93	94
pCMB	0.04	40
	0.08	0
linoleic acid	29.0	38
	54.0	25

*note: % control was calculated by comparing the ESR signal intensities.

The results presented above led us to postulate a mechanism, modified from Fong et. al.'s (7), for the conversion of $O_2^{\cdot-}$ into OH·. The mechanism being:

Oxidized P-450 reductase + NADPH $\longrightarrow$ Reduced P-450 reductase + $NADP^+$ (a)

Reduced P-450 reductase + O_2 $\longrightarrow$ Oxidized P-450 reductase + $O_2^{\cdot-}$ (b)

$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$ (c)

$O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + OH^- + OH\cdot$ (d)

$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH^- + OH\cdot$ (e)

Either reaction (a) or (b) or both is inhibited by pCMB. Reaction (c) and reaction (d) are dismutation and Haber-Weiss reactions, respectively. Reaction (e) is the well known Fenton reaction (24).

Two results, described in the following, will serve to further confirm this postulated mechanism.

First, the mixture of an iron salt and H_2O_2 referred to as Fenton's reagent (24) has been employed as OH· generating system. By introducing DMPO into a reaction mixture containing Fe^{+2}, EDTA and H_2O_2, we observed a DMPO-OH radical adduct (Fig. 5a). This supports the postulated mechanism that ferrous iron is necessary for the maximal formation of the hydroxyl radical.

Second, in the presence of oxygen and xanthine, xanthine oxidase has been shown to produce substantial amounts of $O_2^{\cdot-}$ (25,26). By using ethanol and benzoate as scavengers, Beauchamp et. al. (27) demonstrated that hydroxyl radicals are generated in the reaction mixtures containing xanthine, xanthine oxidase, O_2 and EDTA. When xanthine and xanthine oxidase were mixed under aerobic condition

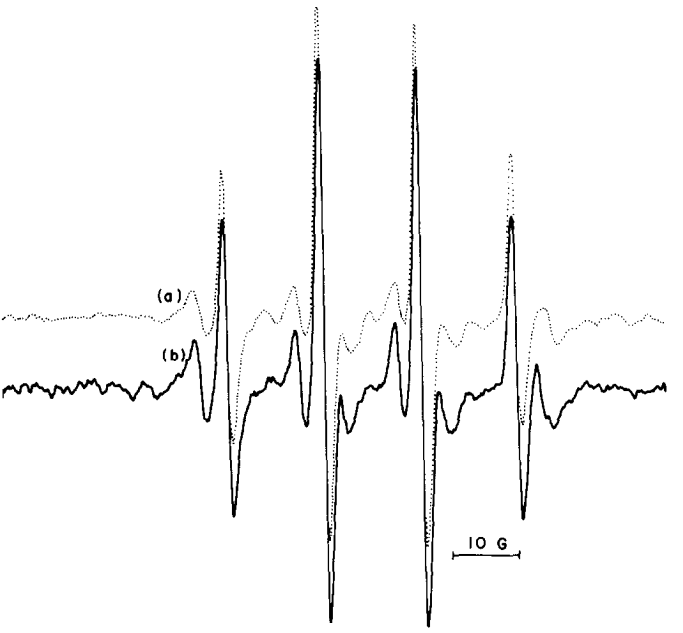


Fig. 5 (a) The ESR spectrum of the DMPO-OH radical adduct obtained when 6.2 mM DMPO was added to the reaction mixture containing 2.0×10^{-4} M Fe^{+2}, 4.0×10^{-4} M EDTA, 0.5% H_2O_2 in 0.15 M KCl solution. $a^N = a^H_\beta = 15.0$ G, g = 2.0062 (the dotted line). (b) The ESR spectrum of the DMPO-OH radical adduct obtained when 6.2 mM DMPO was mixed into the reaction mixture containing 2.2×10^{-5} M Fe^{+2}, 4.4×10^{-5} M EDTA, 0.08 mg/ml xanthine oxidase, 5.0×10^{-5} M xanthine in 0.15 M KCl solution, PH 7.4. $a^N = a^H_\beta = 15.0$ G, g = 2.0062 (the solid line).

to which was added Fe^{+2}, EDTA and DMPO, again, an ESR spectrum, identical to the DMPO-OH radical adduct, was obtained (Fig. 5b). This also confirms that $O_2^{\cdot-}$ can serve to generate a more reactive oxidant, OH·.

Thus, the evidence presented strongly suggests that a hydroxyl radical (OH·), which is generated indirectly from cytochrome P-450 reductase, may be the primary free radical that initiates lipid peroxidation in liver microsomes.

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