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# AN ESR STUDY ON LIPID PEROXIDATION PROCESS. FORMATION OF HYDROGEN ATOMS AND HYDROXYL RADICALS

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SUMMARY: Lipid peroxidation process was studied by the combination of ESR spectroscopy and spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide. Autoxidation of egg lecithin phosphatidylcholine dispersed in water was found to produce hydrogen atoms and hydroxyl radicals. It was also found that for producing such reactive species, unsaturated fatty acid moieties are needed. The ESR spectrum obtained from this model system was identical with that from body fluid such as serum, indicating that hydrogen atoms and hydroxyl radicals could be generated in living bodies during the course of lipid peroxidation. 

Detailed exploration of lipid peroxidation process is of extreme importance because in cellular systems, peroxidation is known to cause fatal damage of the membrane.[1,2] For instance, lipid hydroperoxides, produced in this process, have been reported to initiate further oxidation[3] and to react with other compounds of biological importance.[4] Involvement of free radicals in such undesirable effect has been considered to be responsible. The evidence, however, for the participation of radicals in lipid peroxidation has been based on indirect observations such as consumption of oxygen,[5] formation of peroxides and hydroperoxides,[6] or the formation of malondialdehyde.[7] Direct detection of such radicals has failed.

ESR spectroscopy can offer a technique that may provide the evidence necessary to confirm the involvement of such labile

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species. In the present study, therefore, we combined ESR spectroscopy with spin-trapping.[8] The spin-trapping method consists of using a spin trap, a diamagnetic compound, which forms relatively stable nitroxide radicals by reacting covalently with unstable free radicals. The "trapped" species, spin adducts, can be observed by conventional ESR spectroscopy. The hyperfine splitting constants (hfsc) of the adduct provide the information that can aid in the identification and quantification of the original radical.

## MATERIALS AND METHODS

5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Aldrich Chemical Co. (Wisconsin, USA). Egg lecithin phosphatidylcholine (LPC) and other reagents were obtained from Nakarai Chemicals (kyoto, Japan). Serum was obtained from a New Zealand white rabbit weighting 2.5 kg. Autoxidation of LPC was carried out as follows. To LPC (18 mg) taken into a pyrex test tube, 60 μL of potassium phosphate buffer (60 mM, PH 7.8) containing DMPO (15 μL) and N,N-bis(2-[bis(carboxymethyl)amino]ethyl)glycine (DETAPAC)[9] was added and the system was allowed to stir vigorously for 2 min. This sample was allowed to stand at 37 °C under the atmospheric pressure of oxygen for 10 min and measured in an aqueous round cell (0.8 mm i.d. and 160 mm long) on a JEOL Model ME3X ESR spectrometer (X-band, 100 kHz field modulation, JEOL, Tokyo, Japan). The hfsc values from the spectra were measured by $Mn^{2+}$ in MgO as a reference. Also, computer simulation was used for the confirmation of the values. All the experiments were carried out in the dark.

## RESULTS AND DISCUSSION

From rabbit serum (200 μL) added DMPO (20 μL) and incubated at 37 °C for 10 min, the ESR spectrum composed of several different lines was obtained, as represented in Fig.1a. In order to characterize each ESR line appearing in the spectrum, a model system consisting of LPC was used. The ESR spectrum obtained by the procedure in MATERIAL AND METHOD is shown in Fig.1b. This spectrum is produced by the same lines as those in Fig.1a although the hfsc values differed because of the difference in the concentration of DMPO included. When as a reference, 60 μL potassium phosphate buffer (60 mM, pH 7.8) containing DMPO (15 μL) and DETAPAC (1 mM) was incubated under the same conditions, no signal was observed. Both the ESR specta in Fig.1a and b was analyzed to consist of the

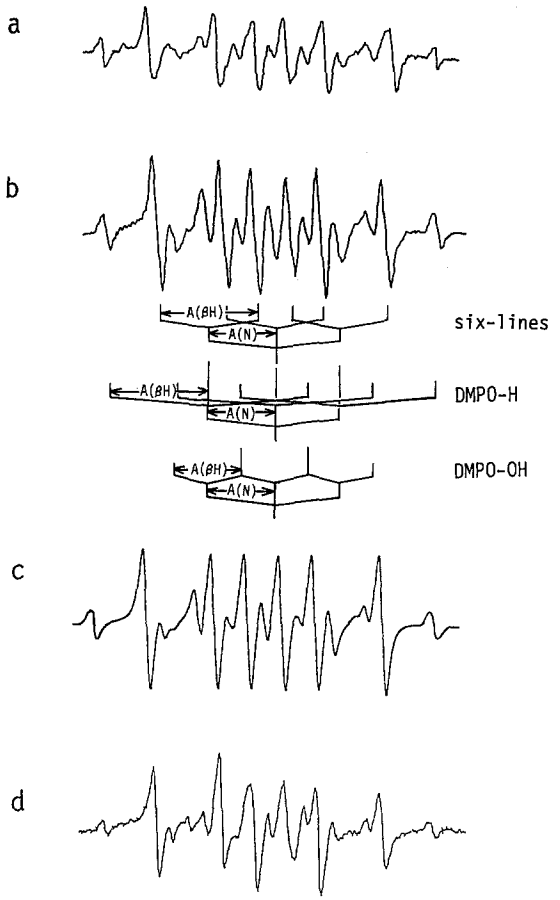


Fig.1. (a). An ESR spectrum of radicals produced in rabbit serum (200 μL) and trapped by DMPO (20 μL). Conditions: temperature, 37 °C; incubation time, 10 min. (b). An ESR spectrum of radicals, produced in the LPC-dispersed system and trapped by DMPO, and stick diagrams for the lines. Conditions: temperature, 37 °C; incubation time, 10min. (c). A computer-simulated spectrum for Fig.1b. The spectrum is composed of DMPO-H (a(N)=1.50 mT and a(βH)=2.32 mT), DMPO-OH (a(N)=1.47 mT and a(βH)=1.47 mT), and the six-lines (a(N)=1.57 mT and a(βH)= 2.28 mT). The intensity ratio taken for DMPO-H, DMPO-OH and the six-lines is 1:0.4:3.8. (d). An ESR spectrum of spin-trapped radicals obtained by incubating LA (50 μL) and DMPO (15 μL).

lines due to the adducts of hydrogen atoms (DMPO-H) and hydroxyl radicals (DMPO-OH) and unidentified six-lines with equal intensities. This assignment is illustrated by the stick diagrams in Fig.1b. Thus measured hfsc values are: a(N)=1.55 mT and a(βH)=2.34 mT for DMPO-H, a(N)=1.43 mT and a(βH)=1.43 mT for DMPO-OH, and a(N)=1.61 mT and a(βH)=2.45 mT for the six-lines in Fig.1a and a(N)=1.50 mT and a(βH)=2.32 mT for DMPO-H, a(N)=1.47 mT and a(βH)=1.47 mT for DMPO-OH, and a(N)=1.57 mT and a(βH)=2.28 mT for

the six-lines in Fig.1b. Using these values, computer simulation for the spectrum shown in Fig.1b was carried out and the resulting spectrum was in good agreement with that in Fig.1b, as represented in Fig.1c. Since the values for DMPO-H and DMPO-OH differed from those reported previously in an aqueous solution,[10] the effect of LPC and DMPO present, on the hfsc values, was investigated as follows. Hydrogen atoms and hydroxyl radicals were produced by ultrasound in an aqueous DMPO solution (25 mM)[11,12] and the hfsc's of the lines produced were obtained as follows: a(N)=1.66 mT and a($\beta$H)=2.25 mT for DMPO-H and a(N)=1.49 mT and a($\beta$H)=1.49 mT for DMPO-OH. Subsequently, DMPO and LPC were added to this solution and the system was allowed to stir. The hfsc values decreased by the addition of these compounds and those determined for DMPO-OH and DMPO-H, on the addition of 3 M DMPO and 18 mg of LPC, were in good agreement with those obtained in the LPC-dispersed system, suggesting the formation of hydrogen atoms and hydroxyl radicals in the lipid peroxidation process.

In order to see if these species are generated from the fatty acid moieties of LPC, linolenic acid (LA), having three double bonds, was used instead of LPC. When LA (50 $\mu$L) was added to DMPO (15 $\mu$L) and incubated under the same conditions, the spectrum composed of the lines due to DMPO-H (a(N)=1.50 mT and a($\beta$H)=2.37 mT), DMPO-OH (a(N)=1.45 mT and a($\beta$H)=1.45 mT) and the six-lines (a(N)=1.53 mT and a($\beta$H)=2.24 mT) was also obtained, as depicted in Fig.1c. On the other hand, upon utilizing saturated fatty acid such as palmitic acid, no signal was produced. These results suggest that for the generation of such labile species, unsaturated fatty acid moiety should be involved.

To confirm the evolution of hydrogen atoms and hydroxyl radicals during the course of lipid peroxidation, scavengers which can compete for these two species with DMPO were used. The rate constant for the reaction of·OH with DMPO has been reported to be

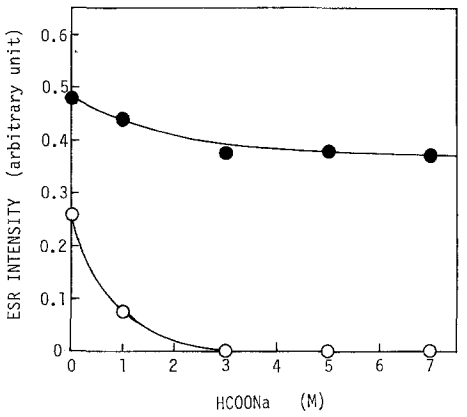


Fig.2. Effect of the change in the concentration of HCOONa on the formation of DMPO-H(●) and DMPO-OH(○).

4.3 x $10^9$ $M^{-1}s^{-1}$.[13] An experiment was performed by incubating a dispersion of LPC (18 mg) in a sodium formate solution (50 μL) containing DMPO (15 μL). Sodium formate ($k_{HCOO^- + \cdot H}$ = 3 x $10^8$ $M^{-1}s^{-1}$ and $k_{HCOO^- + \cdot OH}$ = 3 x$10^9$ $M^{-1}s^{-1}$) is a strong scavenger for both the species.[14,15] In the ESR spectrum obtained, the lines due to DMPO-H and DMPO-OH decreased with increase in the concentration of the scavenger, as depicted in Fig.2. Also the elimination of such species was observed in methanol ($k_{CH_3OH + \cdot OH}$ = 9 x $10^8$ $M^{-1}s^{-1}$) and allyl alcohol ($k_{CH_2=CHCH_2OH + \cdot H}$ = 3.6 x $10^9$ $M^{-1}$ $s^{-1}$) which are good scavengers for •H and •OH, respectively.[14,15] These results obtained with the scavengers imply the formation of the spin adducts, DMPO-H and DMPO-OH, by the direct attack of •H and •OH to DMPO. This means that lipid peroxidation process involves hydrogen atom and hydroxyl radical generating systems.

Further support for the generation of hydrogen atoms from fatty acid moiety of LPC was obtained by the following experiments. DMPO-H could be generated in the different series of reactions by the attack of hydrated electron to DMPO, followed by the protonation. If DMPO-H is formed by this process, the ESR intensity of DMPO-H must be lowered at higher pH. However, no change in the ESR intensity of the DMPO-H adduct was observed in the

pH range from 4 to 12. Consequently, it is inferred that •H is generated in lipid peroxidation process. Also, an experiment was carried out in $D_2O$ (60 μL) dispersed with LPC (18 mg). The spectrum obtained was consistent with that shown in Fig.1b. This result supports the above consideration that hydrogen atoms are generated by the peroxidation of unsaturated fatty acid portion of LPC.

The assignment for the six-lines remains unsolved.

From all the results shown above, it is strongly indicated that toxic species, hydrogen atoms and hydroxyl radicals, are generated from the unsaturated fatty acid moieties of LPC in the peroxidation process and that these two reactive species are also formed in rabbit serum. These species may be responsible for the complex lipid peroxidation system occurring in living bodies.

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