Note

Oxygen-centered Radical Formation in Liver Homogenates and Microsomes upon the Addition of Lipid Hydroperoxides

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Peroxidation of membrane polyunsaturated lipids is a naturally occurring free radical chain reaction that has been suggested to be a mechanism of tissue damage.^{1,2)} We have reported that tissue lipid peroxidation accompanies a very weak chemiluminescence.^{3~5)} Previously, we observed an intense chemiluminescence in rat liver homogenates to which lipid hydroperoxides had been added.⁶⁾ A system has been devised as a model for studying the light emitting species producing the chemiluminescence in tissue lipid peroxidation. Spectroscopic analysis revealed that the major light emitters are singlet molecular oxygen and excited carbonyl compounds. Almost the same spectrum was observed for liver chemiluminescence in rats orally given oxidized lipids.^{4,5)} These chemiluminescences were inhibited by free radical scavengers, *e.g.* α -tocopherol and butyl hydroxytoluene.^{4,6)} So, the occurrence of some short-lived free radicals is postulated to be essential for the chemiluminescence mechanism.^{4~6)} However, lipid free radicals produced in biological systems in the presence of lipid hydroperoxides have not been yet confirmed. In this study, we demonstrated by ESR the formation of oxygen-centered free radicals in rat liver homogenates and microsomes upon the addition of lipid hydroperoxides. In order to catch the short-lived radicals, the spin trap method was applied.

Male Wistar rats, purchased from Nihon Rat Co., were bred to a body weight of 200 g and used in this study. The animals were fed on a commercial laboratory chow (type F-2, Funabashi Farm Co.) which contains all required minerals and vitamins. Rats were anesthetized with ether, and their liver were perfused with ice-cold saline, excised and blotted. A liver homogenate was prepared in 0.15 M potassium phosphate buffer (pH 7.4) with a teflon-glass homogenizer. Liver microsomes were prepared as described by May and McCay.⁷⁾ Methyl linoleate hydroperoxides were obtained as described previously.⁵⁾ As spin traps, PBN (phenyl *N*-tert-butyl nitron, Eastman Kodak

Co.) and 4-POBN (α -(4-pyridyl)-1-oxide)-*N*-tert-butyl nitron, Aldrich Chemical Co.) were used. When 4-POBN was used as a spin trap, DETAPAC (1 mM, *N,N*-bis(2-[bis(carboxymethyl)amino]ethyl)glycine, Sigma) was included in the buffer to prevent the formation of adducts with 4-POBN and hydroxy radical.⁸⁾ Iron-DETAPAC is unable to catalyze OH $\cdot$ formation from hydrogen peroxide plus superoxide.^{9,10)} ESR spectra were recorded in quartz flat cells with a Varian E-4 spectrometer with 100 kHz magnetic field modulation at ambient temperature under atmospheric conditions. A liver homogenate or microsomal suspension in phosphate buffer was mixed with an equal volume of a suspension of methyl linoleate hydroperoxides in water plus 0.5% sodium deoxycholate, and then the spin trap reagent was added before the ESR recording. Incubation of a liver homogenate with methyl linoleate hydroperoxides and PBN resulted in the formation of a radical adduct with characteristic hyperfine splitting constants of $a_N = 13.8$ G and $a_H = 2.2$ G (Fig. 1C). In the absence of lipid hydroperoxides, no significant ESR signal was observed (Fig. 1A). A characteristic ESR doublet, a non-adduct signal, was recorded for a liver homogenate upon the addition of lipid hydroperoxides without PBN (Fig. 1B). This non-adduct signal (1.83 G) was assigned as an ascorbate radical. It has been established that the radical from ascorbic acid has the proton hyperfine structure of a 1.76~1.84 G doublet.^{11,12)} The ESR constants of this non-adduct doublet in Fig. 1B agreed well with those of the ascorbate radical reported by

FIG. 1. ESR Spectra of PBN Adducts in Liver Homogenate Systems.

The systems contained; (A) liver homogenate (10% w/v) and 20 mM PBN, (B) liver homogenate and 11.2 mM methyl linoleate hydroperoxides, and (C) liver homogenate, methyl linoleate hydroperoxides and PBN.

FIG. 2. ESR Spectrum of 4-POBN Adducts in a Liver Homogenate System.

The system consisted of 10% (w/v) liver homogenate (in 0.15 M phosphate buffer, pH 7.4, containing 1 mM DETAPAC), 22 mM methyl linoleate hydroperoxides and 40 mM 4-POBN.

Laroff *et al.*¹¹⁾ and Sasaki *et al.*¹²⁾ The signal of the spin adduct of PBN in Fig. 1C was assigned to lipid oxy radicals since the hyperfine splitting constants were strictly consistent with those of alkoxy radical adducts of PBN ($a_N = 13.8\text{G}$, $a_H = 2.2\text{G}$) observed during CCl_4 metabolism in rat liver microsomes.^{13,15)} With regard to the possible trapping of lipid peroxy radicals, it has been shown that most peroxy spin adducts of PBN, if they occur, decompose at room temperature.¹⁴⁾ Thus, it is quite possible that $\text{LOO}^\bullet$ radicals are trapped with PBN but do not survive under the experimental conditions. The signal of $\text{LO}^\bullet$ -PBN adducts predominated and they were stable in the present system for at least several hours. When a liver microsomal suspension was incubated with methyl linoleate hydroperoxides and PBN, unknown radical adducts of PBN (triplet of doublets, $a_N = 16.1\text{G}$, $a_H = 3.0\text{G}$) were detected, but the $\text{LO}^\bullet$ -PBN adducts in Fig. 1C were not observed. To obtain more information about the lipid free radicals, another spin trap, 4-POBN, was used. The ESR spectrum obtained on incubating a liver homogenate with methyl linoleate hydroperoxides and 4-POBN is shown in Fig. 2. The spin adduct signal showed a strong triplet of doublets with hyperfine splitting constants of $a_N = 15.8\text{G}$ and $a_H = 2.6\text{G}$, overlapping with non-adduct doublets of the ascorbate radical. The hyperfine features of the triplet of doublets shown in Fig. 2 were consistent with those of $\text{LOO}^\bullet$ -4-POBN adducts ($a_N = 15.8\text{G}$, $a_H = 2.6\text{G}$) obtained through the action of lipoxygenase on linoleic acid and the NADPH-dependent microsomal peroxidation system in the presence of 4-POBN.⁸⁾ Thus, the radical that was spin trapped by 4-POBN during incubation of a liver homogenate with methyl linoleate hydroperoxides was recognized to be a lipid peroxy radical, $\text{LOO}^\bullet$. The ESR spectrum obtained on incubating liver microsomes with methyl linoleate hydroperoxides and 4-POBN is shown in Fig. 3. The spin adduct signal showed a clear triplet of doublets, without any ascorbate radical signal, with hyperfine splitting constants of $a_N = 15.8\text{G}$ and $a_H = 2.6\text{G}$, indicating the formation of $\text{LOO}^\bullet$ -4-POBN adducts.

The results demonstrate that oxygen-centered radicals such as lipid alkoxy and peroxy radicals are generated in liver homogenates and microsomes on incubation with lipid hydroperoxides. The results support the idea that tissue chemiluminescence involves the generation of singlet oxygen and excited carbonyls mainly due to the dimolecular decomposition of lipid peroxy radicals *via* the tetroxide structure.⁶⁾

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FIG. 3. ESR Spectrum of 4-POBN Adducts in a Liver Microsome System.

The system contained liver microsomal suspension (0.88 mg of protein/ml in 0.15 M phosphate buffer, pH 7.4, with 1 mM DETAPAC), 22 mM methyl linoleate hydroperoxides and 40 mM 4-POBN.

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