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Detection of oxygen consumption during very early stages of lipid peroxidation by ESR nitroxide spin probe method

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Oxygen consumption during the very early stages of the spontaneous peroxidation of egg yolk phosphatidylcholine membranes was studied by monitoring the oxygen concentration in the aqueous phase of the sample using a spin-probe closed-chamber method. The method depends on the broadening by oxygen of the proton superhyperfine lines of the electron spin resonance spectra of the nitroxide radical spin probe 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxyl. It is concluded that this method is useful in monitoring lipid peroxidation and that it monitors the onset of the peroxidation process before the commonly used thiobarbituric acid assay detects the peroxidation products.

Introduction

Lipid peroxidation and the biological defense mechanisms against it have been studied intensively (for reviews see Refs. 1 and 2). The degree of lipid peroxidation is usually measured by the detection of one of three products: malondialdehyde, lipid hydroperoxides, or lipids containing conjugated diens [3]. Although the process of peroxidation involves the reaction of oxygen, few studies have been performed on the processes of lipid peroxidation from the side of oxygen consumption [4–7]. The merits of the oxygen uptake method as a measure of peroxidation have been described in Refs. 8 and 9.

In this work, we investigated oxygen uptake during peroxidation of egg yolk phosphatidylcholine (PC) in multilamellar liposomes without a special initiation procedure. Results were compared with the level of lipid peroxidation as monitored by standard methods.

Oxygen uptake was investigated by an electron spin resonance (ESR) closed-chamber method using nitroxide radical spin probes [10–13]. This method has a number of advantages over the prevalent polarographic method (Clark electrode) for investigation of oxygen consumption during lipid peroxidation. Spin probes do not consume oxygen, as occurs with the Clark electrode, thereby allowing measurement of very slow processes of oxygen consumption. The ESR method requires no stirring that might damage samples and is not susceptible to interference from various small molecules, including lipids and H_2O_2, which seriously disturb the Clark electrode [14,15].

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Abbreviations: BHT, butylated hydroxytoluene; CTPO, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxyl; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; PC, phosphatidylcholine; ESR, electron spin resonance.

The ESR method is as sensitive as the polarographic method in terms of oxygen concentration (1 μM) but requires less sample volume (0.5 μl–0.2 ml) compared with that needed for the polarographic method (2 ml). It is thus much more sensitsive on the basis of numbers of lipid and oxygen molecules required for the experiment. Sensitivity of the pressure-transducer method is best expressed in terms of the least amount of oxygen molecules (not concentration) needed for detection and is about 1 μmol at best [8,9] compared with 10 pmol (1–100 pmol) of the ESR method. Using this ESR closed-chamber method, we have observed oxygen consumption before lipid peroxidation is detected by a prevalent method (thiobarbituric acid assay).

Experimental section

Egg yolk phosphatidylcholine (PC), L-α-dimyristoylphosphatidylcholine, BHT, EDTA and DTPA were obtained from Sigma (St. Louis, MO). CTPO was purchsaed from Aldrich (Milwaukee, WI).

The membranes used in this work are multilamellar dispersions of lipids prepared in the following way. The lipids dissolved in chloroform were dried with a stream of nitrogen and further dried under reduced pressure (≈ 0.1 mmHg). In some samples, suitable amounts of BHT were added to the chloroform solution. Deionized distilled water (Milli-Q water purification system, Millipore) containing $1.1 \cdot 10^{-4}$ M CTPO and 3 mM NaN_3 was added to dried lipid at 25°C and vortexed vigorously. In some samples, suitable amounts of $FeCl_2$, EDTA or DTPA were added to the distilled water. In some cases, lipid peroxidation was induced by adding to the liposome suspension $FeCl_2$ ($5 \cdot 10^{-5}$ M final concentration), EDTA ($5 \cdot 10^{-5}$ M) and H_2O_2 ($1.6 \cdot 10^{-4}$ M) (Fenton reaction). For ESR measurements, 0.2 ml of the sample containing 15 mg of lipids was placed in a sealed Pasteur pipet.

Fig. 1 shows the principle of oxygen measurements using the spin-probe closed-chamber method. ESR spectra of nitroxide radicals exhibit three lines because of the hyperfine interaction of the unpaired electron with the nitrogen nucleus ($I_N = 1$) (Fig. 1A). Close examination reveals that

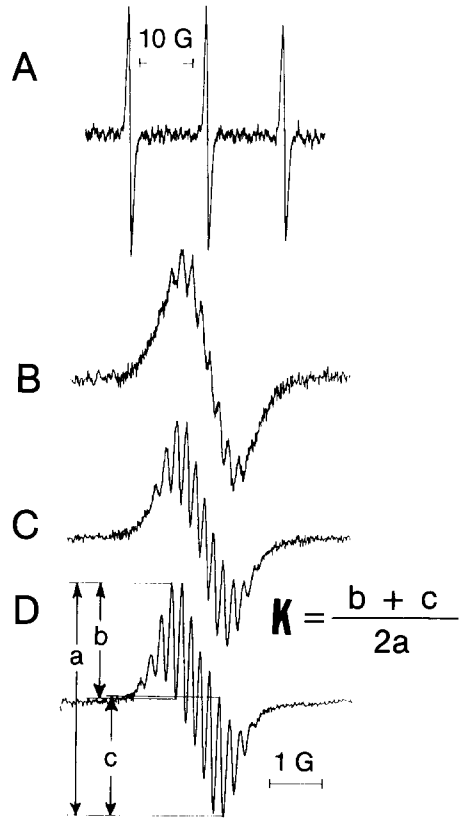

Fig. 1. The full ESR spectrum of the spin probe CTPO ($1.1 \cdot 10^{-4}$ M) in water saturated with air at 37°C is shown in A. In B, C and D, the central peak of CTPO is shown with expanded abscissa (magnetic field scan range, by a factor of 10) in water saturated at 37°C with 100% air, 50% air + 50% N_2, and 100% N_2 respectively. The definition of the K parameter is also shown. Spectra were recorded at 10°C with an incident microwave power of 1 mW, field modulation amplitude of 0.04 G, and a field scan rate of 0.33 G/min.

each line consists of another group of lines because of proton superhyperfine interactions ($I_H = 1/2$) as seen in Fig. 1B,C,D. The resolution of the proton superhyperfine lines greatly depends on the oxygen concentration because bimolecular collisions of the spin probe with molecular oxygen, a paramagnetic molecule, broaden each line via Heisenberg exchange interactions. Compare Fig. 1B, C and D. The spectral change has been parameterized (K parameter in Fig. 1D) against oxygen concentration [12,13].

ESR spectra were obtained at 10°C on a Varian E-109 X-band spectrometer with Varian temperature-control accessories, and E-231 Varian multi-

purpose cavity (rectangular TE_{102} mode).

After each ESR measurement, the whole sample was used for evaluation of lipid peroxidation. The level of lipid peroxidation was assessed by the thiobarbituric acid method according to Asakawa and Matsushita [16], which is a modification of the method described by Buege and Aust [3].

Results and Discussion

The concentration of molecular oxygen in aqueous phase was measured by an ESR spin-probe method using CTPO as a spin probe [11–13,17].

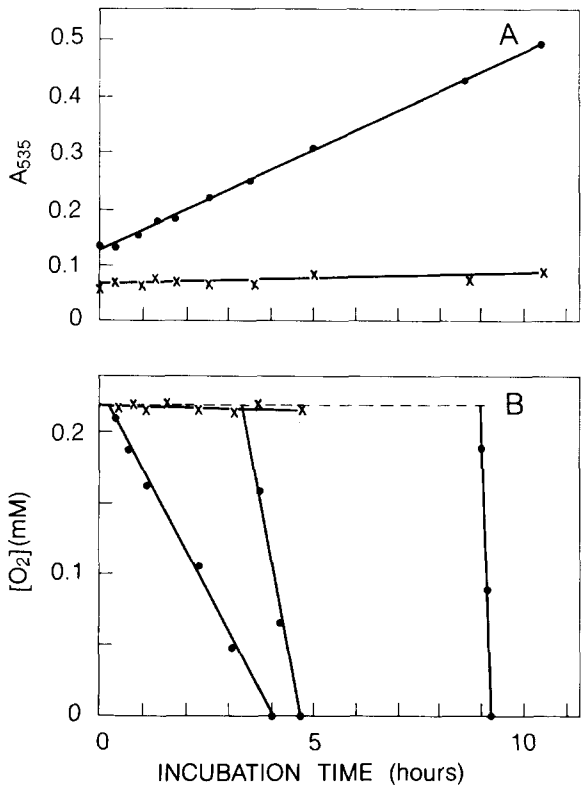

Fig. 2. (A) The time course of absorption change at 535 nm (A_{535}) in thiobarbuturic acid assay of egg yolk PC membrane suspension. Peroxidation was induced by the Fenton reaction, and the sample was constantly aerated. ●, no addition; ×, with BHT, (BHT:PC = 1:500 mol/mol). 0.1 absorption unit is equivalent to $1.2 \cdot 10^{-5}$ M malondialdehyde in the sample. (B) Oxygen consumption rate is measured by ESR spin-probe closed-chamber method. The figure shows the time course of oxygen consumption in 75 mg/ml egg yolk PC membrane suspension at 37°C. Aliquots were taken from the same aerated stock sample used in A at times 0, 3 and 9 h after initiation. Symbols as in A.

The concentration of CTPO used in this work was 10^{-4} M. There is no dependence of the measured oxygen-consumption rate on CTPO concentration when the concentration is below 10^{-3} M. A 2-fold decrease in the oxygen-consumption rate was observed when the CTPO concentration was increased to 10^{-2} M. At a very high concentration, partition of CTPO into the membrane starts to become significant, resulting in reactions with radicals produced during lipid peroxidations, as has been observed with lipid-soluble spin probes [18]. We did not observe reduction of CTPO during ESR measurements as was the case with lipid-soluble probes [18]. These results indicate that CTPO does not interfere with the measurement of the true rate of oxidation in the membrane. In studies of melanin [12] and illuminated chloroplasts [17], both spin probe and polarographic measurements of oxygen consumption give the same results.

In Fig. 2, we show the parallel progress of lipid peroxidation measured by a thiobarbituric acid method (A) and oxygen consumption measured by the ESR closed-chamber method (B) in egg yolk PC membrane suspension. To accelerate the peroxidation process, initiation was made by the Fenton reaction (induction of hydroxyl radicals). The sample was incubated at 37°C with constant aeration. A portion of the sample was transferred to a

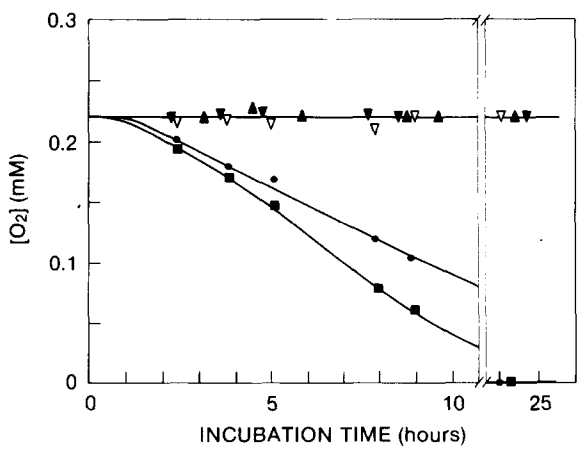

Fig. 3. The time course of the oxygen concentration in a closed chamber with the sample of deionized distilled water (▲), 0.1 mM $FeCl_2$ (▼), 75 mg/ml egg yolk PC membranes (●), 75 mg/ml egg yolk PC membranes + 0.1 mM $FeCl_2$ (■) and 75 mg/ml DMPC membranes (▽) at 37°C.

sealed Pasteur pipet at times 0, 3 and 9 h to measure the oxygen-consumption rate by the sample with the ESR method (Fig. 2B). The amounts of the sample (3 mg of lipids in 0.2 ml aqueous solution) used for ESR measurements and thiobarbituric assays are the same and are comparable to the lipid concentrations employed for the thiobarbituric assay in literature [3,16]. In the presence of BHT, we detected neither lipid peroxidation nor oxygen consumption. These results indicate that the spin-probe closed-chamber technique is a valid method to evaluate oxygen consumption that is coupled with peroxidation of lipids in membranes.

In the following part of this communication, we concentrate our attention on the early stages of lipid peroxidation without initiation procedures.

Fig. 3 (closed circles) shows a rather dramatic decrease of oxygen concentration in a suspension of egg yolk PC membranes. This is accelerated in the presence of 0.1 mM $FeCl_2$. No oxygen consumption is observed in dimyristoylphosphatidylcholine membrane suspensions. No oxygen consumption by the Haber-Weiss pathway was observed (0.1 mM $FeCl_2$ in distilled water) [19].

Chelating reagents such as EDTA and DTPA

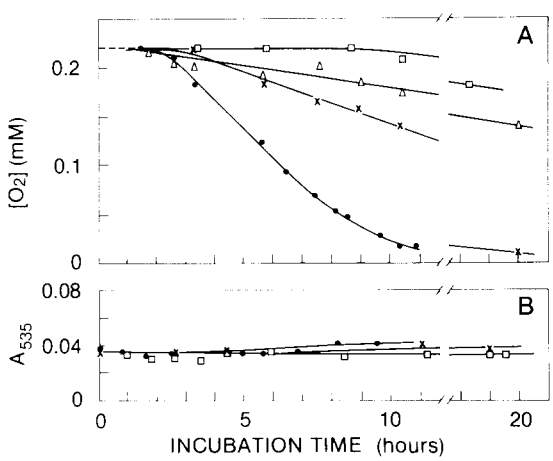

Fig. 4. (A) The time course of oxygen concentration in 75 mg/ml egg yolk PC membrane suspension in a closed chamber (without aeration) with: water only (●), EDTA (△, EDTA : PC = 1 : 20, mol/mol), DTPA (□, DTPA : PC = 1 : 20, mol/mol), and BHT (×, BHT : PC = 1 : 500, mol/mol). Samples were incubated at 37°C. (B) The time course of absorption change at 535 nm (A_{535}) in thiobarbituric acid assay of egg yolk PC membrane suspension. Symbols are the same as in A. 0.1 absorption unit is equivalent to $1.2 \cdot 10^{-5}$ M malondialdehyde in the sample.

(chelator : PC = 1 : 20, mol/mol) inhibit oxygen consumption in egg yolk PC membranes (Fig. 4A), which is consistent with the growing evidence of the involvement of transition metal ions in the initiation of the peroxidation process [20,21]. It has been shown that EDTA inhibits the catalysis by metal ions of the oxidation of unsaturated fatty acids [22] and the decomposition of lipid hydroperoxides [23]. In the present work, protection by DTPA has been found to be more effective than that by EDTA. The addition of the antioxidant BHT (BHT : PC = 1 : 500 mol/mol) in the membrane decreases the rate of oxygen consumption. It is concluded from the results shown in Fig. 3 and 4A that the loss of molecular oxygen is coupled with peroxidation of egg yolk PC.

At observation times employed in these oxygen-consumption studies, only slight lipid peroxidation can be detected by the thiobarbituric acid assay, which is the most common method to evaluate lipid peroxidation [16] (Fig. 4B). These results indicate that measurement of oxygen consumption is a better method to observe lipid peroxidation at its early stages than evaluating the peroxidation products.

In summary, we showed that (1) observation of oxygen concentration by a spin-probe closed-chamber technqiue is a useful method to observe lipid peroxidation (Fig. 2), and that (2) observation of oxygen consumption is a very sensitive monitor of the onset of lipid peroxidation, especially in its very early stages (Figs. 3 and 4). Removal of oxygen is important in membrane studies because autooxidation may proceed, as shown by the oxygen consumption by egg yolk PC membranes, during the time of the experiment.

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