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MEMBRANE LIPID FLUIDITY AFFECTS THE NITROXIDE RADICAL DECAY OF 5-DOXYL STEARIC ACIDS IN ISOLATED RAT HEPATOCYTES

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We investigated the effect of membrane fluidity on the nitroxide radical decay rate of 5-doxyl stearic acid in spin-labeled rat hepatocytes. The half-time ($t_{1/2}$) for the EPR signal decay of 5-doxyl stearic acids incorporated into the membranes of isolated rat hepatocytes was 12 min (mean value). When spin-labeled hepatocytes were separated into membrane and cytosol fractions, the $t_{1/2}$ of the membrane fraction was prolonged by more than 2 hrs. However, when the cytosolic fraction was added to the membrane fraction, the radical decay reaction recovered ($t_{1/2}$ was 27 min). Incubation of hepatocytes with a stream of 95% O_2 at 37°C for 2 hrs prolonged $t_{1/2}$ by 106% and was associated with a 18% decrease in water-soluble antioxidant content. When the measurement temperature was changed from 24°C to 37°C, $t_{1/2}$ was shortened with a decrease in the order parameter (S). The $t_{1/2}$ and S in hepatocytes treated with phosphatidylcholine (PC) were reduced by 14% and 0.008, respectively. Conversely, after treatment with phosphatidylethanolamine (PE), PC + cholesterol and PE + cholesterol, $t_{1/2}$ and S increased by 14% and 0.014, 20% and 0.018 and 29% and 0.040, respectively. These findings suggest that the nitroxide radical decay of 5-doxyl stearic acids incorporated into hepatocyte membranes is mediated by the antioxidants in the cytosol fraction, and that the nitroxide radical decay rate is affected not only by water-soluble antioxidant content but also by the membrane lipid fluidity of the hepatocytes.

KEY WORDS: Nitroxide radical decay, 5-Doxyl stearic acid, Hepatocytes, Antioxidant content, Membrane fluidity, EPR, Spin label

INTRODUCTION

The electron paramagnetic resonance (EPR) spin-label method is a technique which permits investigation of the microenvironment of biological membranes by monitoring the EPR spectrum of a spin-label probe. This method, therefore, has been widely used to study not only membrane fluidity[1] but also transmembrane flip-flop[2] or lateral diffusion[3] movements in membranes. Since the spin-label probe, 5-doxyl stearic acid has a free radical moiety (nitroxyl function), the decay of the spin-label radical in membranes can be also observed by means of the EPR spin-label technique.

In this study, we applied the EPR spin-label method to isolated rat hepatocytes in order to examine the nitroxide radical decay rate in hepatocyte membranes.

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We further investigated the influences of intracellular antioxidant content and membrane lipid fluidity on the decay rate of nitroxide radicals.

MATERIALS AND METHODS

Chemicals

Collagenase (from Clostridium histolyticum) was obtained from Wako Chemical Co., Tokyo, Japan. 1-Diphenyl-2-picrylhydrazyl (DPPH), L-cysteine and cholesterol were purchased from Nacalai Tesque Co., Kyoto, Japan. Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and 5-doxyl stearic acid (5-DSA) were also obtained from Sigma Chemical Co., St. Louis, MO, USA.

Preparation of Isolated Hepatocytes

Hepatocytes were isolated from male Wistar rats weighing 180 ~ 220 g by the collagenase perfusion method[4]. Isolated hepatocytes whose viability was greater than 85% according to the results of the trypan blue exclusion test were suspended in Krebs-Henseleit buffer (pH 7.4) solution at a concentration of 1×10^6 cells/ml.

Treatment of Hepatocytes with Lipids or O_2

Five ml of cell suspension was incubated with PC (10 mg), PE (10 mg) or cholesterol (5 mg) at 37°C for 20 min. The treated cells were washed once with Krebs-Henseleit buffer solution and subjected to EPR spectroscopic examination or measurement of water-soluble antioxidant content. Hepatocytes which were incubated at 37°C for 1 or 2 hours in Krebs-Henseleit buffer gassed with a stream of O_2 (95%): CO_2 (5%) were employed to assess the effect of intracellular antioxidant content on radical decay.

Spin Labeling

An ethanol solution of a spin-label probe (5-DSA) was prepared at a concentration of 500 μg/ml and stored at −20°C. Sixty μl aliquots were taken from the stock solution of the spin-label probe and transferred to incubation tubes. After removal of the solvent with nitrogen gas, 5 ml of cell suspension (1×10^6 cells/ml) were added and incubated at 37°C for 10 min with gentle shaking. Spin-labeled cells were pelleted by low speed centrifugation and transferred to a 50 μl capillary tube for use in an EPR spectrometer. To stabilize spin-labeling conditions and measurement temperature, EPR measurement was usually started 10 min after spin-labeling.

EPR Spectroscopy

EPR measurements were carried out on a FE2XG spectrometer (JEOL, Japan). EPR spectra were obtained at room temperature (24°C) with the following settings: microwave power, 4 mW; sweep width, 50 G; sweep time, 2 min; modulation amplitude, 2.5 G; and time constant, 0.03 sec. In the experiments in which the measurement temperature was changed, the EPR spectrometer was equipped with the digital variable temperature system (ES-DVT1, JEOL, Japan).

The values of outer and inner hyperfine splitting of the 5-DSA EPR spectra were observed to calculate the order parameter (S) according to Gaffney's equation[5]. An increase in the order parameter reflects a decrease in membrane lipid fluidity, which means an increase in lipid order. A decrease in the order parameter, on the other hand, reflects an increase in membrane lipid fluidity.

Radical decay was further evaluated by measuring the height of the mid-field peak of the EPR spectrum; the height of the EPR spectrum was measured at the beginning ($h(0)$) of measurement and t minutes later ($h(t)$), and the radical decay rate was assessed by sequential changes in the ratio $h(t)/h(0)$.

Subcellular Fractionation

Hepatocytes spin-labeled with 5-DSA were fragmented by sonicating for 1 min and were further separated into membrane (pellet) and cytosol (supernatant) fractions by centrifugation at 100,000X g for 20 min.

Measurement of Antioxidant Content

The water-soluble antioxidant content of hepatocytes was measured by the Glavind's method[6]. Cell homogenates were added to methanol solutions of DPPH, and antioxidant content was assessed by changes in absorbance reading at 517 nm. L-cysteine was used as the standard.

RESULTS

5-DSA, a spin-label probe, yielded a broad EPR spectrum after its incorporation into hepatocytes (Figure 1). The order parameter of 5-DSA incorporated into isolated hepatocytes was 0.669 ± 0.004 ($n = 13$, Mean ± SE). The EPR spectra of spin-label probes decayed in a time-dependent manner. To represent the radical decay rate, we used a $t_{1/2}$ value, defined as the time necessary for 50% decay of the mid-field line height of the initial EPR spectrum. The $t_{1/2}$ for hepatocytes was 12 ± 1 min ($n = 13$, Mean ± SE).

When hepatocytes spin-labeled with 5-DSA were homogenized and fractionated into the membrane and cytosol fractions by centrifugation, only the membrane fraction yielded an EPR signal; the signal intensity hardly decayed at all ($t_{1/2} > 2$ hr). However, recombination of the membrane fraction and the cytosol fraction resulted in an EPR signal decay recovery ($t_{1/2} = 27$ min) (Figure 2).

Incubation of hepatocytes at 37°C for 1 hour prolonged $t_{1/2}$ by 55% and associated with a 9% decrease in water-soluble antioxidant content. Incubation for 2 hours prolonged $t_{1/2}$ by 106% with a 18% decrease in water-soluble antioxidant content. However, the lipid fluidity (order parameter) of the membrane remained unchanged.

When the EPR measurement temperature was raised from room temperature (24°C) to 30°C or 37°C, membrane lipid fluidity increased; the order parameter of 5-DSA decreased, and the radical decay rate became faster; $t_{1/2}$ became shorter (Figure 3). The radical decay rate ($t_{1/2}$) correlated with the membrane fluidity (S), significantly ($r = 0.957$, $p < 0.001$).

The $t_{1/2}$ and S in hepatocytes treated with phosphatidylcholine (PC) were reduced by 14% and 0.008, respectively. Conversely, after treatment with

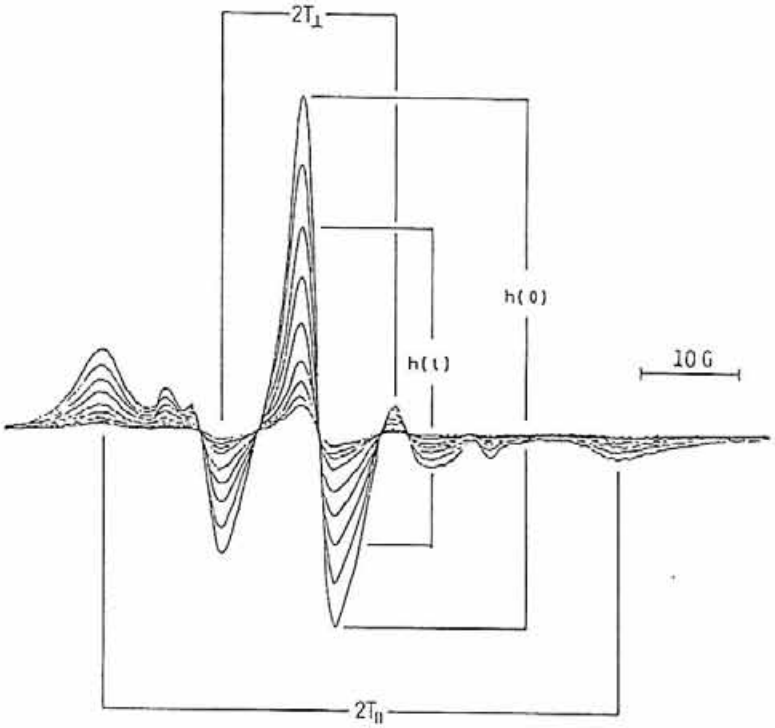

FIGURE 1 EPR spectra of 5-doxyl stearic acids incorporated into isolated rat hepatocyte membranes. The EPR spectra, which were recorded at 5-minute intervals, decayed in a time-dependent manner.

phosphatidylethanolamine (PE), PC+cholesterol and PE+cholesterol, $t_{1/2}$ and S increased by 14% and 0.014, 20% and 0.018 and 29% and 0.040, respectively (Figure 4). The membrane fluidity and the radical decay rate of PE+cholesterol treated hepatocytes were significantly lower than those of PC treated ones. At the end of these treatments, the water-soluble antioxidant content of the hepatocytes was 97 ± 4% (PC treated), 101 ± 5% (PE treated) and 100 ± 5% (PE+cholesterol treated), compared with the control (lipid untreated) value ($n = 5$, Mean ± SE).

DISCUSSION

Applying a lipophilic spin-label probe to isolated hepatocytes, we have examined the time-dependent nitroxide radical decay in hepatocyte membranes. Incubating hepatocytes with 5-DSA yielded a broad EPR spectrum, indicating that most spin-label probes were localized in hepatocyte membranes. The subcellular fractionation experiment showed that the EPR signal of the membrane fraction hardly decayed at all when the membrane fraction was separated from the cytosol fraction, and that recombination of the membrane and cytosol fractions resulted in recovery of membrane radical signal decay. Additionally, incubation of hepatocytes at 37°C for 1–2 hrs prolonged radical's $t_{1/2}$ and decreased water-soluble antioxidant

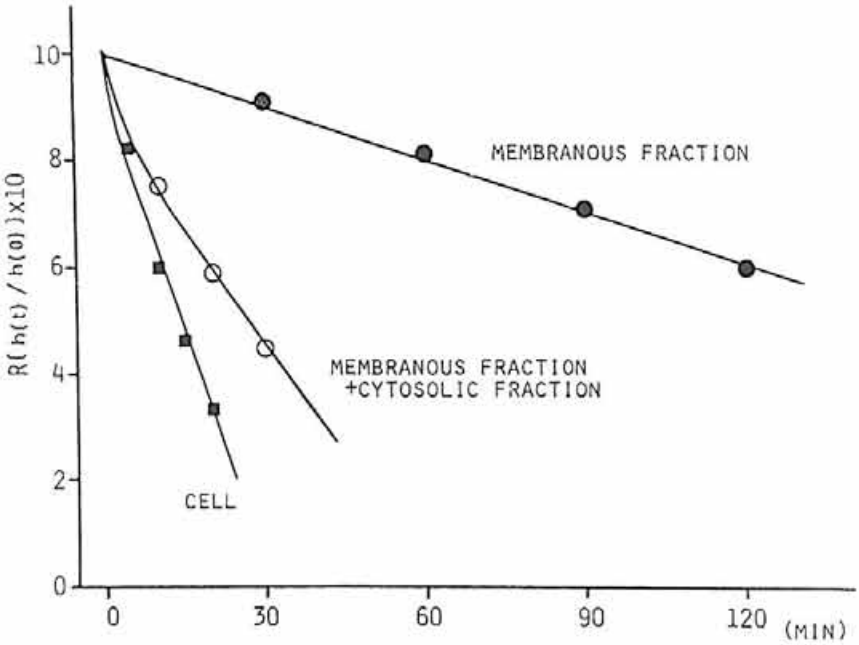

FIGURE 2 Time-dependent decay curve of EPR signal intensity of 5-doxyl stearic acid incorporated into isolated rat hepatocytes. Each point represents the mean value of separate experiments (cell; $n = 13$, membranous fraction; $n = 4$, membranous + cytosolic fraction; $n = 4$).

content. These findings suggest that nitroxide radicals in membranes decayed by cytosolic antioxidants rather than by membrane antioxidants, and that the radical decay rate is affected by the water-soluble antioxidant content.

The biological membrane has been conceptualized as a fluid lipid bilayer and membrane lipid fluidity influences various cellular functions[7,8]. In the present study, the effect of lipid fluidity on nitroxide radical decay in hepatocyte membranes was also examined. Since membrane lipid fluidity is affected by temperature or membrane lipid composition[7,8], an experimental design to change temperatures or to treat hepatocytes with lipids has been employed. We found a clear correlation between the membrane fluidity and the membrane radical decay rate in the temperature changing experiment. The same tendency was also observed in the lipid treating experiment, but a significant correlation between the fluidity and the radical decay rate could not be observed because of difficulty in steady treatment of hepatocytes with lipids.

Some previous reports have shown that sulfhydryl compounds, such as glutathione and cysteine, and ascorbate play an important role in reducing radicals of spin-label probes in biological systems[9,10]. In a liposome study, nitoxide radicals of 5-doxyl stearic acids in liposomal membranes were reduced by ascorbate addition[11]. Some investigators reported that membrane fluidity influenced the membrane permeability of water-soluble substances, such as ascorbate[11,12]. Moreover, the membrane fluidity has been reported to modulate membranous enzyme activities[7,13]. Iannone *et al.* reported that the nitroxide reduction was mediated by

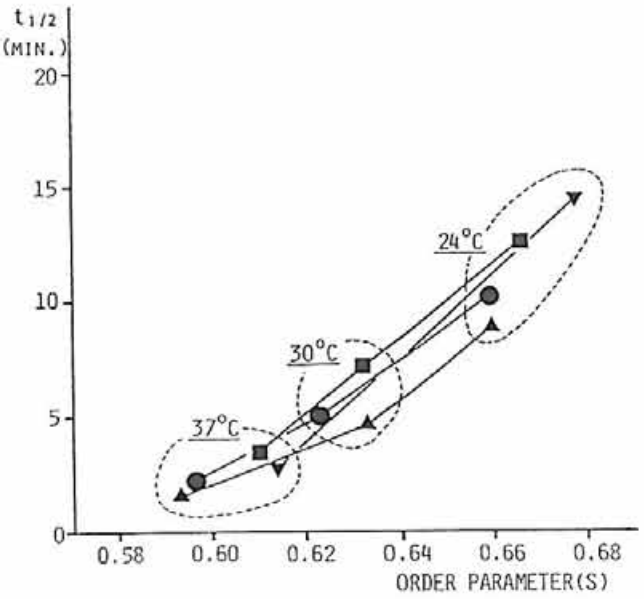

FIGURE 3 Effects of measurement temperature on membrane lipid fluidity and the radical decay rate of 5-doxyl stearic acid in isolated rat hepatocytes. The experimental data obtained from the same cell suspension were connected with a line.

NADPH-dependent cytochrome *c* reductase in microsomal membranes[14]. Chen *et al.* reported that the nitroxide reduction was enzyme-mediated and occurred at the level of the ubiquinone in the respiratory chain in the mitochondria[15,16]. Considering these previous findings, we suggested that membrane fluidity might influence the nitroxide radical decay through the passive permeability change of cytosomal antioxidants and further through the alteration of the membranous redox-enzyme activity which mediates the reaction between nitroxide radicals and cytosomal antioxidants.

Since the membrane lipid compositon of hepatocytes is altered by starvation[7], chronic ethanol ingestion[17] and obstructive jaundice[18], it is important to notice membrane fluidity in liver disease. Most of previous studies on radical decay have emphasized the amount of radicals and antioxidants[9,10,19,20] and have paid little attention to the environment of the membranes where the radical decay reaction occurred. The biological radical decay system is complex; however, our results indicate that membrane fluidity is an important factor influencing the radical decay in the membrane.

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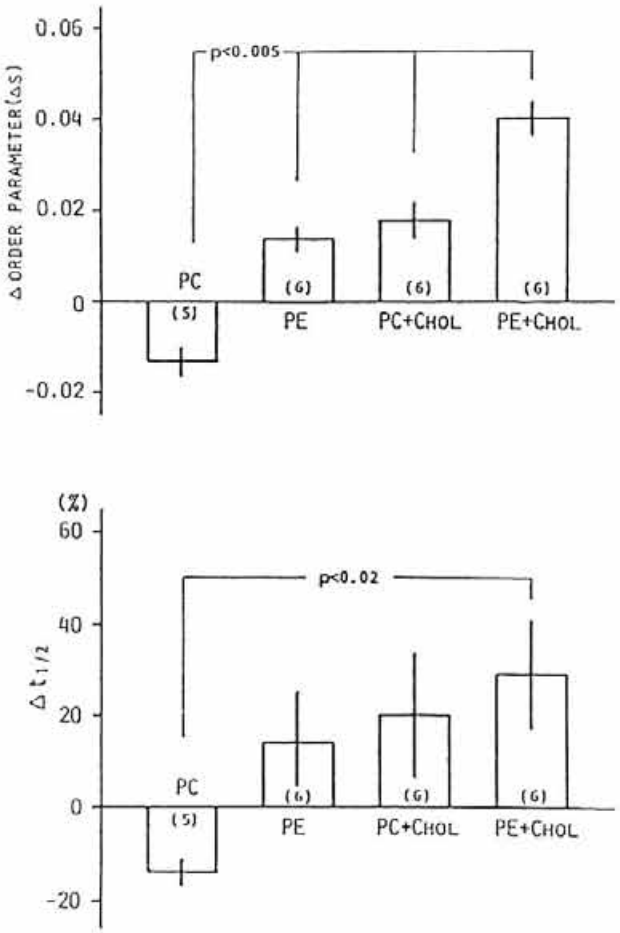

FIGURE 4 Effects of lipid treatment on membrane lipid fluidity and the radical decay rate of 5-doxyl stearic acid in isolated rat hepatocytes. Each value represents the mean ± SE ($n = 5 \sim 6$). Abbreviations are as follows: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PC + Chol, PC mixed with cholesterol; PE + Chol, PE mixed with cholesterol.

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