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DIFFERENTIAL EFFECT OF LIPID PEROXIDATION ON MEMBRANE FLUIDITY AS DETERMINED BY ELECTRON SPIN RESONANCE PROBES

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The effect of lipid peroxidation on membrane fluidity was examined in sonicated soybean phospholipid vesicles. Following iron/ascorbate dependent peroxidation, the vesicles were labeled with a series of doxyl stearate spin probes which differed in the site of attachment of the nitroxide free radical to the fatty acid. Comparison of motional and partitioning parameters derived from electron spin resonance spectra of the probes indicated that the membranes were less fluid following peroxidation. However, the magnitude of the fluidity decrease was markedly dependent on the intramembrane location, as well as on the extent of lipid peroxidation. The effect of lipid peroxidation on fluidity was maximal in the membrane microenvironment sampled by 12-doxyl stearate, whereas other regions of the bilayer were less affected. These findings indicate that lipid peroxidation leads to an alteration of the transbilayer fluidity gradient.

Introduction

Peroxidation of the unsaturated fatty acids of phospholipids is accompanied by alteration of the structural and functional characteristics of membranes (reviewed in Ref. 1). In addition to alteration of biochemical functions, such as enzyme inactivation, lipid peroxidation also affects physical properties of membranes, including fluidity, ion permeability and surface potential [1]. Vladimirov and co-workers have reported increased membrane rigidity (decreased fluidity) following peroxidation of model and biological membranes based on experiments with the fluorescence probes pyrene, perylene and 4-dimethylchalcone. [1,2]. Although these probes were considered to differ with respect to intramembrane location, the magnitude of the fluidity decrease was similar for each probe [2]. Decreased fluidity has also been found following peroxidation of phospholipid vesicles, erythrocytes and microsomes [3–5] in studies employing the fluorescent probe diphenylhexatriene, a probe of membrane microviscosity [6]. By contrast, results obtained with fatty acid spin probes suggested that fluidity increased following peroxidation of erythrocytes [7]. The contradictory experimental results obtained with fluorescence [2–5] and spin [7] probes suggest that the observed effect of lipid peroxidation on fluidity may be dependent on the particular probe used to label the membrane.

In this paper we have examined the effect of lipid peroxidation on membrane fluidity in sonicated phospholipid vesicles (liposomes) by using a series of doxyl stearate spin probes which differ in the site of attachment of the free radical label to the fatty acid. Since these probes are oriented in membranes such that the long axis of the probe is parallel to the phospholipid fatty acids [8], different locations within the membrane can be examined by varying the position of the doxyl substituent on the fatty acid. In this paper, we show that motional and partitioning parameters derived from electron spin resonance spectra of such probes

indicate that membrane fluidity decreases following peroxidation. However, the magnitude of the fluidity decrease is not uniform throughout the membrane, but is dependent on the specific location within the bilayer.

Materials and Methods

Materials

A mixture of soybean lipids (asolectin) was obtained from Sigma Chemical Co. Lipid composition was determined by fractionation of the mixture on a silicic acid column using sequential elution with chloroform for neutral lipids, acetone for glycolipids, and methanol for phospholipids [9]. The weight percentage of each lipid type eluted from the column was determined gravimetrically following removal of the solvent. The composition of the lipids used in these experiments was approx. 90% phospholipid, 5% glycolipid and 5% neutral lipid. The composition of the phospholipid fraction was determined by thin-layer chromatography on silica gel H plates using chloroform/methanol/acetic acid/water (65 : 35 : 2 : 1, v/v) [10]. Identification of the phospholipids, visualized by iodine vapor, was made by comparison to standards. Amounts of individual phospholipids were determined by lipid phosphorus assay [11]. The phospholipid fraction consisted almost entirely of an equimolar amount of phosphatidylcholine and phosphatidylethanolamine, with trace amounts of cardiolipin and lysophosphatidylethanolamine.

Doxyl stearic acid spin probes, *N*-oxyl-4',4'-dimethyloxazolidine derivatives of ketostearic acid, labeled at carbon number 5, 7, 10, 12 or 16 were obtained from Molecular Probes, Inc. (Junction City, OR). Stock solutions (25 mg/ml) of the spin probes were prepared in ethanol.

Liposome preparation

The lipids were dissolved in chloroform and dried under N_2 to form a thin film. Residual solvent was removed under vacuum at room temperature for at least 1 h. The lipids were hydrated at room temperature in 130 mM KCl/20 mM potassium phosphate (pH 7.5). This buffer was used in all experiments. The opaque lipid suspension (20 mg/ml) was sonicated (6 × 30 s) with a microprobe at output setting 4 with a Branson model 185 sonifier under N_2 in an ice/water bath with intermittent 30 s cooling periods. The resulting translucent suspension showed no lipid peroxidation as a result of the liposome preparation procedure when assayed by the thiobarbituric acid method [12].

Lipid peroxidation procedure

Lipid peroxidation was initiated by incubating liposomes (2 mg/ml) in the presence of 0.2 mM $FeSO_4$ and 0.4 mM ascorbate. Stock solutions of $FeSO_4$ were prepared in ice-cold deoxygenated water and used immediately. Reaction mixtures of 10 ml total volume, in flasks open to the atmosphere, were agitated at 60 oscillations/min throughout the incubation period at 37°C in a shaking water-bath. Aliquots equivalent to 1 mg of lipid were removed at various times during the incubation and placed in tubes containing 0.02 ml butylated hydroxytoluene (0.5% in ethanol). Lipid peroxidation was assayed by the thiobarbituric acid method [12]. Absorbance values measured at 532 nm were converted to malondialdehyde equivalents using a molar absorptivity of $1.56 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ [12]. Authentic malondialdehyde was prepared hy hydrolysis of 1,1,3,3-tetramethoxypropane (Aldrich) in 2 M HCl [13].

Electron spin resonance spectroscopy

Following lipid peroxidation, the reaction mixtures were centrifuged at 105 000 × *g* for 45 min at 4°C. The vesicles were resuspended in fresh buffer at a final concentration of 20 mg/ml. Phospholipid analysis [14] of the final suspension indicated complete recovery of lipid after centrifugation. Spin probes were mixed with liposome suspensions such that the probe-to-lipid weight ratio was 1 : 240 and the final concentration of ethanol (added as a vehicle for the probes) was 0.3% (v/v). Spectra of the spin-labeled membranes in sealed glass capillary tubes were recorded at 24°C at 9.1 GHz with a Varian E-109 spectrometer. Signal-averaged spectra were obtained from four scans over a 100G range at 5 mW microwave power.

For 5- and 7-doxyl stearates, the empirical order parameter (S) was derived from the inner and outer hyperfine spectral splittings and calculated as described by Gaffney [15]. For the remaining

probes, the outer hyperfine splittings were not resolved and the order parameter was calculated from the inner splittings only [16]. Empirical rotational correlation times (τ) were derived from the spectral line heights and midfield linewidth as described in [17]. The partitioning of 10- and 12-doxyl stearate probes between aqueous and lipid phase was evaluated from the split highfield lines in these spectra. The partitioning parameter (f), representing the fraction of the total amount of probe located in the lipid phase, was calculated as described by Marsh [18].

Results

Time-course of lipid peroxidation

Lipid peroxidation was initiated by the addition of $FeSO_4$ and ascorbate to vesicle suspensions [19,20]. The rate and extent of lipid peroxidation induced by the combined iron/ascorbate system were markedly dependent on the presence of both components in the reaction mixture (Fig. 1). In the presence of the combined initiation system, lipid peroxidation, as reflected by the increase in thiobarbituric acid-reactive material (expressed as nmol malondialdehyde/mg lipid), reached nearly maximal level after 3 h of incubation. Peroxidation of lipid due to autoxidation (neither iron nor ascorbate present) was negligible throughout the incubation period (Fig.1) Addition of the antioxidant butylated hydroxytoluene inhibited the iron/ascorbate dependent lipid peroxidation by 98%.

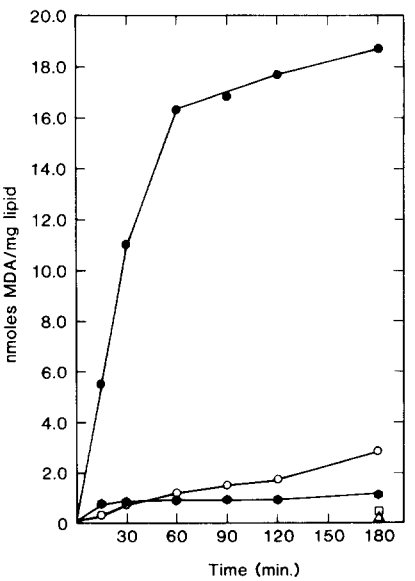

Fig. 1. Representative time-course of lipid peroxidation. Liposomes were suspended in 130 mM KCl/20 mM potassium phosphate (pH 7.5) and incubated aerobically at 37°C. Aliquots were removed at the indicated times and assayed for malondialdehyde (MDA) formation. Additions to the reaction mixture were 0.2 mM $FeSO_4$ and 0.4 mM ascorbate (●); 0.4 mM ascorbate (○); 0.2 mM $FeSO_4$ (hexagons); none (△); and 0.2 mM $FeSO_4$, 0.4 mM ascorbate and 90 μM butylated hydroxytoluene (□).

Effect of lipid peroxidation on membrane fluidity

When incorporated into membranes, fatty acid spin probes undergo anisotropic motion characterized by lateral (side-to-side) motion within the plane of the bilayer as well as rapid rotation about the long axis of the probe [8]. An empirical order parameter (S), a measure of membrane fluidity, can be derived directly from the spectral line splittings reflecting the amplitude of the motional anisotropy [15,16]. In an isotropic environment (e.g., spin probe dispersed in homogeneous solution) the probe motion is no longer anisotropic and $S = 0$, whereas in a rigid environment (e.g., spin probe incorporated into a host crystal) the motional anisotropy is maximal and $S = 1$. In membranes, the amplitude of the motional anisotropy, and hence the order parameter, is dependent on the particular intramembrane location sampled by the probe [15,16].

An order parameter profile for soybean liposomes was determined by using a series of doxyl stearate spin probes in which the free radical label was attached to different carbon number of the fatty acid. The order parameter profile demonstrated the existence of a transbilayer fluidity gradient, as previously reported [21]. The effect of lipid peroxidation on the order parameter profiles of sonicated liposomes is shown in Fig. 2A. At low levels of lipid peroxidation (i.e., not more than 2 nmol malondialdehyde/mg lipid), the order parameter profile was shifted slightly to higher values, but was within experimental error of that obtained prior to peroxidation (i.e., unperoxidized liposomes). At higher levels of peroxidation (i.e., 10 nmol malondialdehyde/mg lipid), a significant increase in order parameter was obtained for 7-, 10- and 12-doxyl stearates, whereas order parameters for 5- and 16-doxyl stearates were essentially

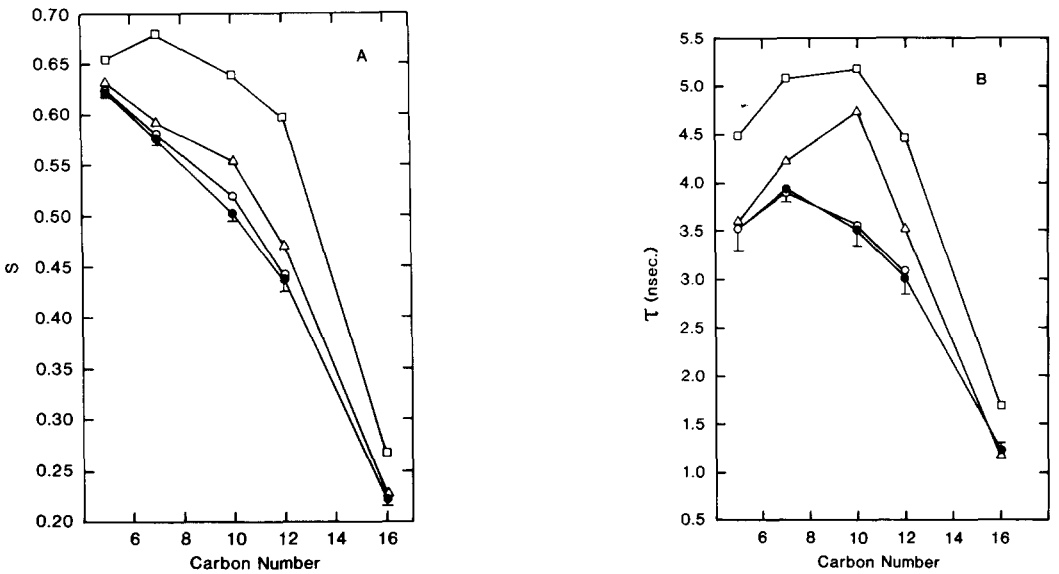

Fig. 2. Effect of lipid peroxidation on order parameter and rotational correlation time profiles. Order parameter (A) and rotational correlation time (B) profiles were determined prior to lipid peroxidation (●) and after peroxidation to an extent equivalent to 2 (○), 10 (△), and 20 (□) nmol malondialdehyde/mg lipid. Error bars represent standard deviations based on 5–8 measurements for each probe.

unaffected. The order parameters for the latter two probes were increased significantly only at maximal levels of lipid peroxidation (i.e., 15–20 nmol malondialdehyde/mg lipid). These data indicate that fluidity, as reflected by increased order parameters, decreased in a differential manner that was dependent on the intramembrane location as well as on the extent of lipid peroxidation. Comparison of the order parameters for the various probes in vesicles peroxidized to an extent equivalent to 20 nmol malondialdehyde/mg lipid (Table I) indicated that the magnitude of the increase in order parameter ranged from 6% for 5-doxyl stearate to 36% for 12-doxyl stearate.

A second empirical parameter, the rotational correlation time (τ), was also derived from the spectra using intensity and linewidth measurements [17]. This parameter describes the rate of motion of the probe as it rotates about its long axis [8]. The increased molecular ordering as a result of lipid peroxidation, based on order parameter calculations, suggested that the rate of probe rotational motion would decrease and therefore τ would increase. As shown in Fig. 2B, the correlation time profiles before and after lipid peroxidation confirmed this expectation. Comparison of the rotational correlation time profiles (Fig. 2B) with the order parameter profiles (Fig. 2A) showed that both parameters followed similar trends that were dependent on intramembrane location and extent of lipid peroxidation. Comparison of the τ values for the various probes in vesicles peroxidized to an extent equivalent to 20 nmol malondialdehyde/mg lipid (Table I) indicated that the magnitude of the change in motional rate ranged from 27% for 5-doxyl stearate to a maximum of 48% for 10- and 12-doxyl stearates.

The relationship between the extent of lipid peroxidation and the alteration of membrane fluidity as reflected by the order parameter is shown in Fig. 3 for 5- and 12-doxyl stearates. When the ratio of the order parameter after peroxidation to that obtained before peroxidation was plotted as a function of the amount of malondialdehyde formed, sigmoidal-shaped plots qualitatively similar to those in Fig. 3 were obtained for all the probes. Similar results were also obtained when the ratio of the rotational correlation times (after and before peroxidation) was plotted in this manner. The major difference ob-

TABLE I

ORDER PARAMETERS AND ROTATIONAL CORRELATION TIMES OF SPIN PROBES BEFORE AND AFTER LIPID PEROXIDATION

Probes are designated by the carbon number at which the doxyl label is attached to stearic acid. S_0 and τ_0 are the initial average values of the order parameter and rotational correlation time, respectively, before lipid peroxidation. S and τ are the maximal values of the order parameter and rotational correlation time, respectively, after lipid peroxidation to an extent equivalent to 20 nmol malondialdehyde/mg lipid. Numbers in parentheses are standard deviations, $n = 5–8$.

Probe	S_0	S/S_0	τ_0 (ns)	τ/τ_0
5-DS	0.622 (±0.002)	1.063	3.52 (±0.24)	1.27
7-DS	0.576 (±0.004)	1.180	3.94 (±0.15)	1.29
10-DS	0.503 (±0.007)	1.296	3.51 (±0.15)	1.48
12-DS	0.437 (±0.010)	1.364	3.01 (±0.16)	1.49
16-DS	0.220 (±0.004)	1.214	1.23 (±0.02)	1.37

served between the various probes was the magnitude of the change in these parameters obtained at maximal levels of lipid peroxidation (Table I). At maximal levels of peroxidation, comparison of the magnitude of the changes in both order parameter and rotational correlation times indicated that the greatest alteration of fluidity was at the level of 12-doxyl stearate.

During the course of these experiments, we also observed a differential effect of lipid peroxidation on the partitioning of the various spin probes into liposomes. The partitioning parameter (f), evaluated from the intensities and linewidths of the two high-field lines corresponding to spin probe in lipid and aqueous phases, represents the fraction of spin probe located in the lipid phase [18]. Prior to lipid peroxidation, more than 99% of the total amount of each probe was located in the membrane ($f = 1.0$). Following peroxidation, the partitioning of 10- and 12-doxyl stearates into the membrane decreased slightly, whereas the partitioning of the other probes was not affected. The partitioning parameters for 10- and 12-doxyl stearates were decreased to similar extents at all levels of peroxidation. The minimum value of f (0.93 ± 0.02) for these probes was obtained at the same level of lipid peroxidation (15–20 nmol malondialdehyde/mg lipid) at which maximal increases were observed for order parameters and rotational correlation times (Fig. 3 and Table I).

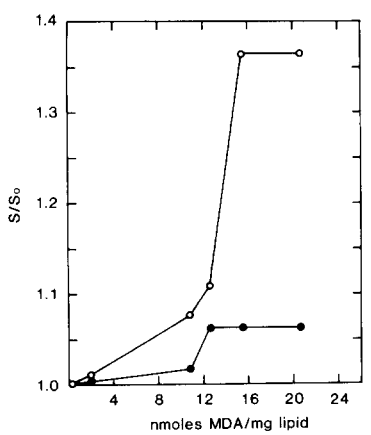

Fig. 3. Relationship between membrane fluidity and extent of lipid peroxidation. The ratio of the order parameters measured after peroxidation (S) to that before peroxidation (S_0) is shown for 5-doxyl stearate (●) and for 12-doxyl stearate (○). MDA, malondialdehyde.

Discussion

The data reported in this paper indicate that membrane fluidity, as reflected by electron spin resonance spectra of fatty acid nitroxide probes, decreases following lipid peroxidation. This result agrees with those obtained by others using fluorescence probes [2–5]. However, we have found that the magnitude of the fluidity decrease following peroxidation is dependent on the intramembrane location sampled by the various probes. In particular, the magnitude of the fluidity change, evaluated from motional and partitioning parameters of spin probes, is greatest in that part of the bilayer sampled by 10- and 12-doxyl stearates. This region of

the bilayer is expected to contain a large proportion of fatty acid double bonds susceptible to lipid peroxidation. Therefore, the loss of unsaturation in this region of the bilayer as a result of lipid peroxidation [5,22] may account for the decreased fluidity. However, cross-linking, either between lipid radicals [5] or by malondialdehyde [23], may also contribute to the observed effect.

In their study of fluidity in peroxidized membranes labeled with fluorescent probes which were presumably located in different regions of the bilayer, Dobretsov et al. [2] did not observe a differential effect like that which we have described. The uniform decrease in fluidity found with various fluorescence probes [2] suggests that the environments, and hence the intramembrane locations, sampled by these probes were not sufficiently different to allow observation of a differential effect. In addition, the results obtained with fluorescence probes may be influenced by interaction of the probes with lipid peroxidation products. For example, Barrow and Lentz [3] have shown that diphenylhexatriene is converted to a nonfluorescent product in the presence of oxidized lipid, thereby altering considerably the value of the microviscosity parameter.

A previous spin label study of fluidity in erythrocyte membranes peroxidized by gamma-irradiation has been reported by Grzelinska et al. [7]. In that study, the methyl esters of 5-doxyl palmitate and 12-doxyl stearate were used to monitor membrane fluidity. By contrast with our results, no change in fluidity was observed at the level of 5-doxyl palmitate, whereas fluidity increased slightly at the level of 12-doxyl stearate [7]. The latter is opposite to our findings as well as those of others using fluorescence probes [2–5]. However, the data of Grzelinska et al. [7] were obtained using a high ratio of spin probe to erythrocyte lipid (approx. 1 : 20) in the presence of 0.5% ethanol. In addition to the disordering effect of the fatty acid spin probes at such high concentrations [8], 0.5% ethanol (about 0.1 M) is sufficient to fluidize a variety of membranes to a significant extent [24]. Thus, the results observed by Grzelinska et al. [7] may be attributable to the combined fluidizing effects of high concentrations of spin probe and ethanol, rather than to lipid peroxidation.

The results of this study demonstrate that lipid peroxidation produces a differential alteration in transbilayer membrane fluidity. Although the model membrane system used in this study is representative in a general way of biological membranes, the quantitative aspects of this effect would be expected to depend upon the specific lipid and acyl chain composition of a particular membrane. However, consideration of the characteristic chemistry of lipid peroxidation, which leads to selective modification of unsaturated groups within acyl chains, suggests that differential alteration of transbilayer fluidity may be a general consequence of membrane lipid peroxidation. Such an alteration in the natural fluidity gradient of a biological membrane may play a role in alterations of membrane functions caused by lipid peroxidation.

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