THE INCREASE OF PHOSPHOLIPID BILAYER RIGIDITY AFTER LIPID PEROXIDATION

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1. Introduction

Many consequences of lipid peroxidation have been described, among them uncoupling of oxidative phosphorylation in mitochondria [1,2], alteration of liver endoplasmic reticulum functions [3], modification of ionic permeability of artificial phospholipid membranes [4] etc. (cf. [5]). While chemical transformations of lipids have been studied [5,6], considerably less information has so far been available about changes of the physical structure of membranes and alterations of the liquid-crystalline state of membrane lipids. However this state is known to influence significantly both biomembrane permeability and membrane-bound enzyme activity [7]. Recent data on the correlation between membrane viscosity and the level of tissue antioxidants in vivo [8] may suggest some relationship between the liquid–crystalline state of membranes and the lipid peroxidation process.

In this communication we have attempted to reveal the effect of lipid peroxidation on viscosity of artificial and biological membranes; in vitro experiments were carried out using the fluorescent probe method.

2. Methods

Pyrene was obtained from Fluka, cholesterol from Sigma, perylene from Aldrich. 3-Methoxybenzanthrone (MBA) was a gift from Dr B. M. Krasovitski (Institute of Monocrystals, Kharkov), and 4-dimethylaminochalcone (DMC) was a gift from Dr T. P. Surikova (2nd Moscow Medical Institute). Thin-layer chromatography of the MBA and DMC preparations did not reveal any coloured impurities. Phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) were isolated from egg phospholipid mixture by chromatography on silica gel as described [9]. Mitochondria and endoplasmic reticulum vesicles were isolated from rat liver as described in [10] and [11], respectively. The phospholipid vesicles (liposomes) were formed by injection of phospholipids dissolved in ethanol into a buffer [12]; the ethanol was subsequently removed by dialysis against the same buffer (0.01 M Tris–HCl, pH 7.5). The protein content of membrane suspensions was determined by the procedure of Lowry et al. [13].

Lipid peroxidation in membrane suspensions (0.4 mg phospholipid/ml) was induced by addition of 5 μM $FeSO_4$ with 0.1 mM ascorbate, developed with stirring at 25°C and stopped by the addition of 1 mM EDTA. Subsequently, fluorescent probes were introduced into the suspension. The amount of lipid peroxidation products was estimated by using 2-thiobarbituric acid and expressed in equivalents of malondialdehyde (MDA) [14].

Fluorescence measurements were made on a Hitachi Perkin-Elmer MPF-2A spectrofluorimeter. Phospholipid concentration was equal to 0.2 mg/ml; DMC, MBA and perylene were 5 μM. Fluorescence was excited at 334 nm (pyrene) or 410 nm (other probes). The fluorescence decay time was measured with an Ortec 9200 nanosecond fluorimeter for pyrene and with a phase fluorimeter [15] for perylene and MBA (since decay time of perylene fluorescence is comparable with the decay of the excited flash in the Ortec 9200 instrument, and the MBA fluorescence decay was not rigorously exponential). The lateral diffusion coefficient of pyrene was calculated according to [16], the rotational diffusion coefficient for perylene and MBA were determined by using the Perrin equation [17]. The fluorescence and absorp-

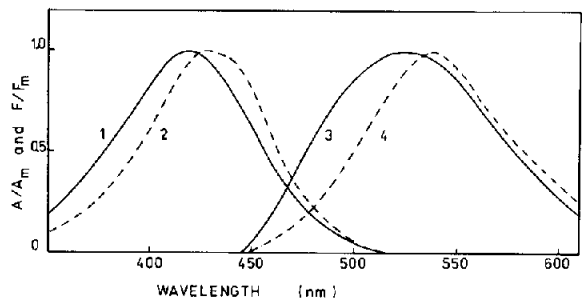

Fig.1. Normalized absorption (1,2) and fluorescence (3,4) spectra of DMC and MBA (5 μM) in phosphatidylcholine liposomes suspension (0.2 mg lipid/ml): DMC, solid lines; MBA, broken lines.

tion spectra of DMC and MBA are shown in fig.1; other characteristics are described [18,19].

3. Results

The addition of ascorbate and Fe^{2+}-ions to phospholipid liposomes brought about a rapid accumulation of lipid peroxidation products (fig.2). This was not so when Fe^{2+}-ions alone or ascorbate alone was introduced into the suspension, or in the complete system in the presence of an antioxidant. The amount of lipoperoxidation products was strongly dependent on the presence of PE: in liposomes prepared from pure PC the concentration of MDA accumulated was several times lower than in those containing 20% PE.

The accumulation of peroxidation products resulted in the alteration of fluorescence parameters of all the used probes, with the rate of pyrene translational diffusion and that of perylene and MBA rotational diffusion decreasing simultaneously (fig.3 and table 1). In addition, an enhancement of DMC fluorescence intensity was observed, which may be interpreted as resulting from restriction of water molecule mobility restriction in the water layers adjoining the membrane surface [19].

It should be noted, however, that the lateral diffusion rate for perylene (fig.3 and table 1) was calculated according to [16] using the experimentally measured ratio of pyrene excimer fluorescence (F_e) to monomer fluorescence intensity (F_m). However, the possibility can not be excluded that the fluorescence quantum yield of the pyrene excimers and/or monomers in a membrane may change. For this reason, a control experiment was carried out in which the shortening of monomer's life time rather than

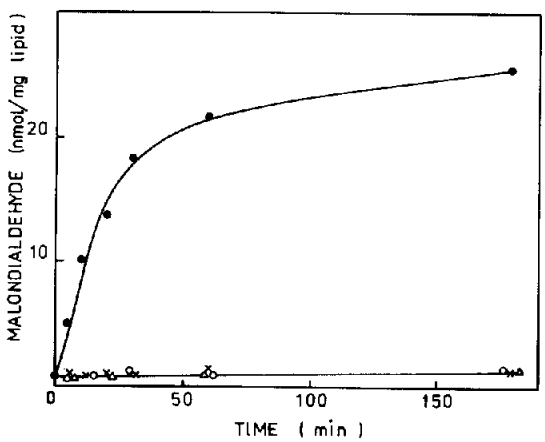

Fig.2. Kinetics of lipid peroxidation products accumulation in PC:PE (4:1) liposomes suspension. At the initial time the following substances were added to the suspension: (○) 5 μM $FeSO_4$; (x) 0.1 mM ascorbate; (●) 5 μM $FeSO_4$ + 0.1 mM ascorbate; (△) 5 μM $FeSO_4$ + 0.1 mM ascorbate + 10 μM ionol (an antioxidant).

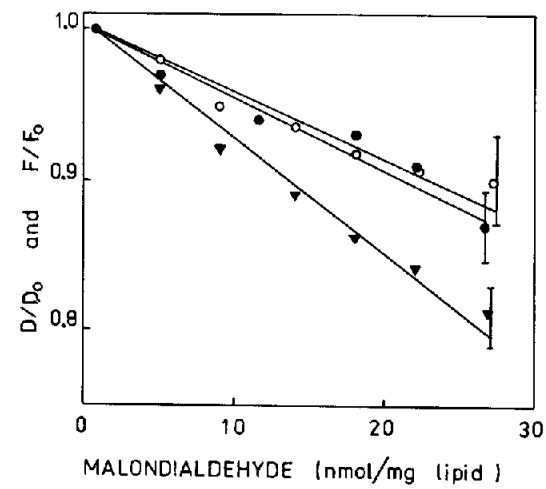

Fig.3. Changes of pyrene lateral diffusion (●), MBA rotational diffusion (▼) and DMC fluorescence intensity (○) in the course of lipid peroxidation in liposomes suspension (PC:PE = 4:1). D, diffusion coefficient; F, fluorescence intensity at 524 nm; D_0 and F_0, the same parameters as [MDA] = 0.6 nmol/mg protein.

Table 1
Changes of pyrene lateral diffusion, of perylene and MBA rotational diffusion and of DMC fluorescence intensity in membranes after 180 min peroxidation

Membranes	D_0/D_{180}			F_{180}/F_0
	Pyrene	Perylene	MBA	DMC
PC + PE (4:1)	1.16 ± 0.05	1.09 ± 0.05	1.22 ± 0.09	1.11 ± 0.03
PC + PE + cholesterol (4:1:2.5)	1.29 ± 0.08	1.20 ± 0.04	1.26 ± 0.07	1.15 ± 0.03
Liver endoplasmic reticulum	1.24 ± 0.09	1.17 ± 0.05	1.15 ± 0.07	1.16 ± 0.04
Liver mitochondria	1.32 ± 0.13[a]	1.14 ± 0.04	1.35 ± 0.12	1.36 ± 0.11

[a] Abnormal excimerization of pyrene [21]. The approximate value is calculated by the ratio F_e/F_m in peroxidized and non-peroxidized membranes

D_0 and D_{180}, diffusion coefficients before and after the peroxidation, respectively; F, fluorescence intensity

F_e/F_m ratio was measured to determine the excimer-monomer ratio. The pyrene excimerization is a process of the dynamic quenching of monomer fluorescence [16] and hence should be described by Stern-Volmer equation [20]:

$$\frac{1}{\tau} - \frac{1}{\tau_0} = k \cdot P$$

where τ and τ_0 are fluorescence decay times with and without excimerization, respectively; P, the pyrene concentration; k, the excimerization rate constant, which is proportional to the lateral diffusion coefficient D [16].

The data obtained are presented in fig.4. It is seen that the slope of the line for liposomes is lower after peroxidation. Hence, the R value and, therefore, the pyrene lateral diffusion coefficient D decrease in phospholipid membranes after lipid peroxidation. A similar effect has also been observed in other membranes studied in our experiments. Thus, the two methods of pyrene diffusion rate determination provide a good qualitative correlation, though some quantitative discrepancies do exist.

It should be pointed out in conclusion that the accumulation of lipid peroxidation products did not produce any chemical destruction of probe molecules in our experiments as estimated by their fluorescence.

4. Discussion

We have used three different fluorescent methods of membrane microviscosity determination based on the investigation of:

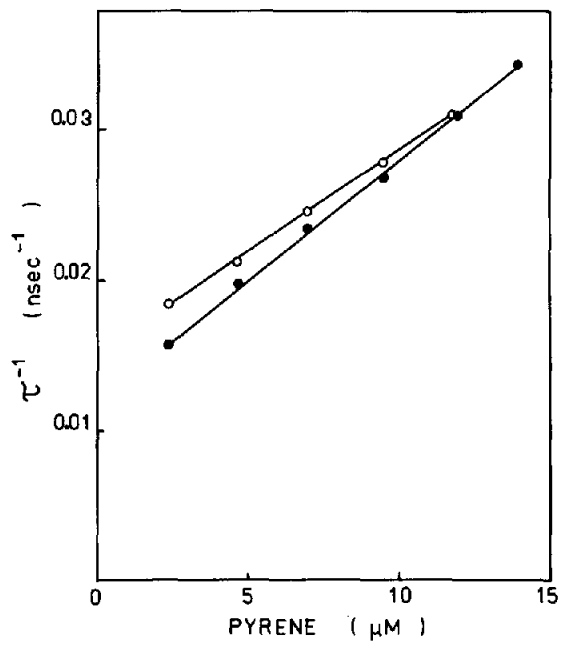

Fig.4. Changes of decay time (τ) of excited pyrene monomers as a result of pyrene excimerization in liposomes (PC:PE=4:1) before (•) and after 180 min peroxidation (○).

(i) Pyrene excimerization kinetics

(ii) Perylene and MBA rotational relaxation rate

(iii) Quenching DMC fluorescence by water molecules.

Although they are basically different, they have demonstrated the same result: the lowering of the membrane 'fluidity' after lipid peroxidation. All the three methods have shown similar changes in membrane viscosity for each sort of membrane investigated.

In spite of the fact that the fluorescence probes used are distributed differently in a membrane, DMC and MBA being closer to the membrane–water interface [18,19] and pyrene [21] and perylene [22] – in the depth of lipid bilayer, they have provided similar values of the increase in membrane viscosity after lipid peroxidation.

Apparently, the latter process brings about an increase in the membrane rigidity throughout its hydrophobic space. It can be supposed that oxidized fatty acid chains are transferred from the membrane depth to the outside [23], in the course of this transfer the portion of unsaturated fatty acid chains will decrease, resulting in the enhancement of the membrane viscosity

It is interesting to compare the degree of lipoperoxidation at which the measurable alteration of membrane fluidity occurs and the peroxidation under which membrane functions become damaged. In mitochondria the respiratory control dropped from 6.7–2.0 after 10 nmol malondialdehyde had been accumulated/mg lipid [2], i.e., at the same degree of peroxidation at which we observed changes in membrane viscosity in the present paper. Hence, the modification of the physical state of the membrane correlates with the loss of its functioning.

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