Susceptibility for Hydroperoxide Formation of Phosphatidylcholine and Phosphatidylethanolamine in Liposomes

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(Received October 28, 1994)

Summary To compare the peroxidative susceptibilities of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in liposomes, multilamellar vesicles (MLVs) were prepared with equimolar L-α-dilinoleoyl PC (DLPC) and L-α-dilinoleoyl PE (DLPE), and with soya PC and soya PE having a uniform constituent fatty acids. The hydroperoxide formation at 37°C in the presence of a water-soluble radical initiator was examined by chemiluminescence-high-performance liquid chromatography (CL-HPLC), and the effect of heterogeneous distribution of PC and PE on peroxidation was investigated. No difference was found between the hydroperoxidation of PC and PE in MLVs systems, except that soya PC was more susceptible to peroxidation than soya PE in the L-α-dipalmityl PC (DPPC)-based liposomes. No correlation was found between the amount of phospholipids distributed in the external leaflet of MLVs and hydroperoxide formation. This result suggested that the unsaturation of constituent fatty acids in phospholipids is more important than the difference in the polar head group of phospholipids regarding their peroxidizabilities in liposomes.

Key Words liposome, phospholipid, peroxidation, hydroperoxide, external leaflet, CL-HPLC

Lipid peroxidation has been noted as one of the causative agents of various diseases (1, 2). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major constituent lipids of biomembranes. In biological membranes such as erythrocytes and in mixed liposomal models, PC and PE are known to distribute asymmetrically between the outer and inner layers (3, 4). To investigate the differences in peroxidative susceptibilities, the quantitation of hydroperoxide formation regarding the difference in their polar head group is needed. For such

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investigations, the rate of chain initiation must be controlled and the breakdown of hydroperoxide formed should be minimized. The fatty acid composition of PC and PE should be uniform because the oxidizability is affected by the content of doubly allylic hydrogens (5). In this study, liposomes containing PC and PE with the same constituent fatty acids were prepared and subjected to water-soluble radical initiator-dependent peroxidation (6, 7), and the peroxidative susceptibilities of PC and PE were investigated by means of chemiluminescence-high performance liquid chromatography (CL-HPLC).

EXPERIMENTAL

Chemicals. l-α-Dilinoleoyl PC (DLPC) and l-α-dilinoleoyl PE (DLPE) were purchased from Avanti Polar Lipids (Birmingham, AL); soya PC (purity 98.6%) was from Nippon Oil & Fats Co. (Tokyo); soya PE with the same fatty acid composition as that of soya PC was synthesized from soya PC via transphosphatidylolation by cabbage phospholipase D (8). The fatty acid compositions of soya PC and (soya PE) were analyzed by gas-liquid chromatography as their methyl esters: 16:0, 38.1% (38.3%); 18:0, 11.4% (10.6%); 18:1, 32.5% (32.2%); 18:2, 15.1% (16.0%); and others, 2.9% (3.0%). l-α-Dipalmitoyl PC (DPPC) and cytochrome c (from horse heart, type VI) were products of Sigma (St. Louis, MO). Luminol (3-aminophthaloyl hydrazine) and 2,2'-azobis(2-amidinopropane) di-hydrochloride (AAPH) were from Wako Pure Chemical Co. (Osaka). 2,4,6-Tri-nitrobenzenesulfonic acid (TNBS) was obtained from Tokyo Kasei Kogyo Co. (Tokyo).

Methods. As unsaturated PE alone cannot form a bilayer upon hydration under physiological conditions (9), DPPC was added to PE to stabilize the bilayer. Phospholipid mixtures, with equimolar of DLPE/DLPC, DLPE/DPPC, DLPC/DPPC, soya PE/soya PC, soya PE/DPPC and soya PC/DPPC, were dissolved in 1 ml of chloroform and dried by evaporation to form a thin film on the glass vial wall. The film was suspended in 4 ml of Tris-HCl buffer (10 mM, pH 7.4) and vortexed for 30 sec to get multilamellar vesicles (MLVs) (10). Samples for quantitative analysis of the free amino group of PE in liposomes were prepared with 0.15 M saline instead of Tris-HCl buffer. The final phospholipid concentration was determined by phosphorus analysis (11). A 1-ml aliquot of MLVs solutions (1 μmol of total phospholipid) was incubated with AAPH (final concentration 10 mM) at 37°C. Another liposome series composed of equimolar PC with increasing amount of PE (DLPE/DLPC, 0/1; 0.5/1; 1/1 in molar ratios) was also prepared. A 1-ml aliquot of those MLVs solutions contained 0.25 μmol of PC. Vesicles contained over 50 mol% of PE were easily aggregated when incubated with AAPH. At different times during the incubation, the phospholipids were extracted from the incubation mixture with 1 ml of chloroform/methanol (2:1, v/v) containing 0.002% butylated hydroxytoluene as an antioxidant. Twenty microliters of the phospholipid fraction in chloroform layer was injected directly into a chemilumi-
nescence-high-performance liquid chromatograph for the phospholipid hydroperoxide determination (12, 13). The free amino group in the polar head of PE was determined by the method of Kumar and Gupta (14) using TNBS as a probe. The absorbance of TNBS derivative at 410 nm was measured with a Shimadzu UV-350A Recording Spectrophotometer. In MLVs, it has been reported that TNBS preferentially reacts with the free amino group of PE located in the external leaflet (4).

RESULTS AND DISCUSSION

In DLPC/DPPC, DLPE/DPPC, soya PC/DPPC and soya PE/DPPC MLVs, equimolar amounts of oxidizable PC and PE were included in different vesicles, whereas in DLPE/DLPC and in soya PE/soya PC MLVs, they were included in the same vesicles. Such two kinds of MLVs systems (DPPC-based liposomes and DLPC- or soya PC-based liposomes) were used to compare the peroxidizability of PC and PE. Phospholipid hydroperoxide formation in AAPH-induced peroxidation was confirmed by CL-HPLC assay (Fig. 1). When DLPC and DLPE, soya PC and soya PE were included in the same vesicles, no difference was observed between

![Fig. 1. CL-HPLC chromatograms of phospholipid hydroperoxides formed in equimolar systems of soya PC/DPPC, soya PE/DPPC and soya PE/soya PC liposomes after incubation with a water-soluble radical-inducer for 20 min at 37°C. PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide.](image-url)
Fig. 2. Hydroperoxidation of phosphatidylcholine and phosphatidylethanolamine in (a) DLPC/DPPC and DLPE/DPPC systems and (b) a DLPE/DLPC system. Values are expressed as nmol PCOOH (PEOOH) per umol PC (PE). Points shown are the M±SD of four analyses from two independent experiments.

Fig. 3. Hydroperoxidation of phosphatidylcholine and phosphatidylethanolamine in (a) soya PC/DPPC and soya PE/DPPC systems and (b) a soya PE/soya PC system. Values are expressed as nmol PCOOH (PEOOH) per umol PC (PE). Points shown are the M±SD of four analyses from two independent experiments.

the amount of PEOOH and PCOOH formed (Figs. 2b and 3b). The results indicated the same susceptibilities of PC and PE to AAPH-induced peroxidation. However, in DPPC-based vesicles, a higher amount of hydroperoxide was produced from DLPC than from DLPE at 20 min and 40 min of incubation, but the difference was not significant (Fig. 2a). High accumulation of PCOOH rather than PEOOH was observed in soya liposomes, in which the system consisted of complexed constituent fatty acids (Fig. 3a).

The peroxidative difference between the two kinds of MLVs systems (DPPC-based liposomes and DLPC- or soya PC-based liposomes) observed in the present study might be attributed to the heterogeneous distribution of phospholipids in the liposomes. One cause might be owing to the effect of asymmetric distribution of PC and PE, and the other might be the effect of phase separation. Although the
Table 1. Distribution of phosphatidylethanolamine in the external leaflet of MLVs.

<table>
<thead>
<tr>
<th>PE/PC</th>
<th>$A_{\text{total}}$</th>
<th>$A_{\text{external}}$</th>
<th>external/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLPE/DLPC</td>
<td>0.263±0.015</td>
<td>0.088±0.006</td>
<td>33.5</td>
</tr>
<tr>
<td>DLPE/DPPC</td>
<td>0.253±0.026</td>
<td>0.214±0.042</td>
<td>84.6</td>
</tr>
<tr>
<td>soya PE/soya PC</td>
<td>0.222±0.017</td>
<td>0.103±0.010</td>
<td>46.4</td>
</tr>
<tr>
<td>soya PE/DPPC</td>
<td>0.199±0.013</td>
<td>0.170±0.006</td>
<td>85.4</td>
</tr>
</tbody>
</table>

The total absorbance represents the amount of PE-2,4,6-trinitrobenzenesulfonic acid chromophore measured ($A_{\text{total}}$), which corresponds to about 75 nmol of PE. Whereas the PE content in the external leaflet of MLVs is represented by $A_{\text{external}}$. The percent of PE in the external leaflet is presented in the column of external/total. Values are M±SD of six analyses from two independent experiments.

distributions of PC and PE in the outer layer were not confirmed in the present study, the amount of free amino groups of PE located in the external leaflet of DLPE/DLPC and soya PE/soya PC liposomes was suggested to be much lower than in that of DLPE/DPPC and soya PE/DPPC liposomes, respectively (Table 1). As the amount of TNBS derivative formed is considered to be equivalent to the PE distribution in liposomes, one-third of the PE was expected to exist in the external leaflet of DLPE/DLPC liposomes, and five-sixths of the PE in the external leaflet of DLPE/DPPC liposomes. Similarly, one-half of the PE should be distributed in the external leaflet of soya PE/soya PC liposomes, and five-sixths of the PE in the external leaflet of soya PE/DPPC liposomes. However, Figs. 2 and 3 gave no evidence that the PEOOH content in DPPC-based liposomes is higher than that of either DLPE/DLPC or soya PE/soya PC liposomes. Thus, no correlation was suggested between the distribution of PE in the external leaflet of liposomes and the formation of PEOOH. On the other hand, it is possible that there was a phase separation in unsaturated PC/DPPC and unsaturated PE/DPPC liposomal systems at 37°C (15). This phase separation might promote aggregation of unsaturated hydrocarbon chains and contribute to the difference in the formation of PCOOH and PEOOH in DPPC-based soya liposomes. These results imply that the peroxidation in liposomes is dependant on the nature of the constituent hydrocarbon chains and less on the polar head groups. The low oxidizability observed on soya PC and soya PE compared with DLPC and DLPE could be interpreted in the same way, because the molar ratio of double bonds in the acyl group to phosphorus in soya PC and soya PE (<0.8) was much lower than that of DLPC and DLPE (=4.0). In the MLVs systems consisting of equimolar PC with increasing amounts of PE (DLPE/DLPC, 0/1; 0.5/1; 1/1 in molar ratios), the amount of PCOOH formed was about twice that of PEOOH in PE/PC (0.5/1 in molar ratio) liposomes, and the amount of PCOOH formed was almost the same as PEOOH formation in PE/PC (1/1 in molar ratio) liposomes (Table 2). These results showed that the difference in the polar head group is not so much significant for their peroxidative susceptibilities as the constituent fatty acids or the concentration of phospholipids.
Table 2. Phospholipid hydroperoxide contents of MLVs containing a constant amount of DLPC and an increasing amount of DLPE. The distribution of PE is also indicated.

<table>
<thead>
<tr>
<th>DLPE/DLPC in molar ratio</th>
<th>PCOOH (nmol)</th>
<th>PEOOH (nmol)</th>
<th>total OOH (nmol)</th>
<th>$A_{\text{total}}$</th>
<th>$A_{\text{external}}$</th>
<th>external/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/1</td>
<td>38.8 (5.8)</td>
<td></td>
<td>38.8 (5.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5/1</td>
<td>121.6 (16.9)</td>
<td>119.9 (15.1)</td>
<td>241.6 (16.9)</td>
<td>0.226</td>
<td>0.099</td>
<td>43.8</td>
</tr>
<tr>
<td>1/1</td>
<td>92.4 (8.5)</td>
<td>47.9 (4.7)</td>
<td>140.3 (8.5)</td>
<td>0.098</td>
<td>0.031</td>
<td>31.6</td>
</tr>
</tbody>
</table>

The contents of PCOOH and PEOOH are expressed as nmol OOH per μmol of DLPC. Values are the average of four measurements in two individual experiments after 30 min of peroxidation. The distribution of PE is expressed as the same in Table 1, but the data are the mean of two experiments.

Several researchers have observed that PE undergoes oxidation more rapidly than PC in metal ion-mediated peroxidation of emulsion systems (16). On the other hand, it has been reported that PE accumulates only a small amount of peroxide in normal saline, which may be attributed to more rapid decomposition of PEOOH than PCOOH, presumably due to the metal chelating ability of PE (17). Kawakatsu et al. (18) synthesized DLPC and DLPE and compared their oxidation kinetics in the presence of ferrous ion and ascorbic acid in emulsion systems, DLPE was found to be more susceptible to ferrous ion-induced peroxidation than DLPC at pH 5.8–7.0. This has been suggested to be due to chelation of ferrous ion by PE. The externally distributed phospholipids labelled with a fluorescent probe in neural membranes has been reported to be more sensitive to the iron-induced peroxidation in the early period than the inner layer (19). On the other hand, the present study showed different results from these previous reports. The contribution of metal ion-induced decomposition of phospholipid hydroperoxide was small in the present system, because of the monophasic increase in the oxygen consumption curve in the AAPH-induced reaction, whereas the ferrous ion-induced reaction showed the biphasic kinetics (20). Our results showed that there was no significant difference in hydroperoxide formation between PC and PE in the MLVs systems, except that the soya PC was more susceptible to peroxidation than soya PE in DPPC-based liposomes.

The close packing of phospholipids and the hydrophilicity of AAPH did not allow the initiator to diffuse into the interior of the membrane. The attack on phospholipid of radicals generated from AAPH firstly occurred at the surface of the membrane and produced phospholipid radical. This initiation step depended only on the concentration of AAPH. The lipid radical reacted rapidly with oxygen to give a lipid peroxy radical, which attacked another phospholipid molecule to yield peroxide and a new lipid radical. The propagation proceeded in the interior of the membrane and was independent of the phospholipid distribution on the surface of the membrane. Under the present experimental conditions, unsaturation of the constituent fatty acids in phospholipids was more important than the difference in

the polar head group of phospholipids regarding their peroxidizabilities in liposomes.

REFERENCES


