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# Lipid Peroxidation in Small and Large Phospholipid Unilamellar Vesicles Induced by Water-Soluble Free Radical Sources

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**The susceptibility of small and large egg yolk phosphatidylcholine unilamellar vesicles to $Fe^{2+}$/histidine-$Fe^{3+}$- and Fenton reagent ($Fe^{2+}$-$H_2O_2$)-induced lipid peroxidation was evaluated by measuring the formation of thiobarbituric acid reactive substances (TBARS). It has been found that surface curvature or phospholipid packing exerts significant effect on the oxidative susceptibility of the unsaturated lipid bilayers and the highly curved and loosely packed small unilamellar vesicles (SUVs) exhibit much less resistance to the oxidative stress induced by the water-soluble free radical sources. The presence of lipid hydroperoxides in sonicated vesicles was excluded as the cause for higher level of lipid peroxidation in the phospholipid SUVs. Instead, the experimental results can be explained by the difference in ability of the water-soluble oxidants to penetrate the two types of lipid membranes. This hypothesis is supported by data obtained from fluorescence lifetime and quenching studies.** 

***Key Words:*** **lipid peroxidation; fluorescence lifetime; fluorescence quenching; SUVs; LUVs; TBARS; transition metal ions.**

Extensive investigations on lipid peroxidation induced by water-soluble free radical inducers such as transition metal ions have been carried out in phospholipid bilayer vesicles (1–4). Generally, two types of phospholipid vesicles, namely, small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs), were employed. Many of such studies were focused on the protective roles of various antioxidants such as $\alpha$-tocopherol (1, 3), with little attention being paid to the possible effect of surface curvature or lipid packing of the membrane bilayers on their susceptibility to oxidation. It is well known that the motional properties of membrane lipids, the surface area available for each lipid polar headgroup and the density of fatty acyl chain packing in SUVs are not the same in their two leaflets and also differ from those in more planar bilayer membranes (e.g., LUVs) (5–7). These should result in different exposures of the acyl chain region, where lipid oxidation occurs, of membrane lipids in SUVs and LUVs to the aqueous medium or the free radical inducer if the latter is water-soluble. Both the initiation and propagation reactions of lipid peroxidation are affected by the accessibility of the free radical inducers to the unsaturated fatty acyl chains. Therefore, the susceptibility of membrane lipids to oxidation mediated by water-soluble reagents should be affected by the choice of lipid vesicles of different size.

In this study, SUVs and LUVs were prepared with identical egg yolk phosphatidylcholine (egg PC) and their susceptibilities to $Fe^{2+}$/histidine-$Fe^{3+}$- and Fenton reagent ($Fe^{2+}$-$H_2O_2$)-induced lipid peroxidation were examined by assays for thiobarbituric acid reactive substances (TBARS) (8). The level of water penetration into the membrane bilayers was assessed by the fluorescence lifetime of 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (DPH-PC) since this parameter is inversely related to the dielectric constant of the environment of the probe (9, 10), which is the fatty acyl chain region of the phospholipid bilayers. The fluorescence emission of DPH-PC was also quenched by water-soluble ionic quenchers in order to estimate the ability of the ions and free radicals in penetrating the lipid bilayer. Our results show that the surface curvature plays an important role on the susceptibility of phospholipid bilayer vesicles to water-soluble reagent-mediated lipid peroxidation. The correlation between the size of the

Abbreviations used: SUVs, small unilamellar vesicles; LUVs, larger unilamellar vesicles; egg PC, egg yolk phosphatidylcholine; TBARS, thiobarbituric acid reactive substances; DPH-PC, 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine.

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vesicles and the level of lipid peroxidation is interpreted in terms of the permeability of the radical inducers in phospholipid membranes.

## MATERIALS AND METHODS

*Materials.* Egg PC, $FeSO_4$, $CuSO_4$, $FeCl_3$, $H_2O_2$, histidine, KI and 2-thiobarbituric acid were purchased from Sigma (St. Louis, MO). DPH-PC was from Molecular Probes (Eugene, OR). All other chemicals were of analytical reagent grade and deionised water was used for all experiments.

*Liposome preparations.* Egg PC SUVs were prepared by sonication according to established procedures (12). In brief, egg PC, with or without DPH-PC, was dispersed above its phase transition temperature in 10 mM Tris-HCl buffer (50 mM NaCl, pH 7.0) at a total lipid concentration of 2.5 mM. The aqueous dispersions were then sonicated under nitrogen for 10 min (30 s on and 30 s off for each cycle) with a Heat Systems Sonicator XL at maximum power output. Temperature was controlled with an ice water bath and no lipid degradation was found as examined by thin-layer chromatography using a solvent system of chloroform:methanol:acetic acid:water (25:15:4:2, v/v). On the other hand, LUVs were prepared from egg PC multilamellar vesicle solutions (2.5 mM) by the method of membrane extrusion with a LiposoFast extruder (AVESTIN, Ottawa) (13). Polycarbonate filters (Nuclepore, Pleasanton, CA) with 100 nm pore size were used and the extrusion was carried out at room temperature. Phospholipid concentrations of the bilayer vesicles were determined by either the method of Bartlett (14) or gravimetry. The vesicles were analysed for their size distribution by elution through a Sepharose CL-4B column (1.0 × 43 cm). More than 90% of the LUVs were recovered from fraction No. 13 (fraction volume: 1 ml), while SUVs were centered in fraction No. 23. The average diameter of the SUVs was about 25 nm and that of the LUVs was 107 nm (12, 15).

*Lipid peroxidation assay.* A $Fe^{2+}$ stock solution was prepared in ice-cold, nitrogen-purged deionised water and kept at 0°C. On the other hand, the histidine (25 mM)-$Fe^{3+}$ (5 mM) stock solution was prepared in 10 mM Tris-HCl buffer containing 50 mM NaCl and the pH adjusted to 6.5. The lipid peroxidation was initiated by addition of the above solutions to the bilayer vesicle (1). The final concentrations of $Fe^{2+}$, $Fe^{3+}$ and histidine were 50 $\mu$M, 50 $\mu$M and 250 $\mu$M, respectively. Similar experiments were repeated with Fenton's reagent (0.1 mM $H_2O_2$ and 0.2 mM $FeCl_2$). In both cases, samples containing 0.5 mM egg PC were incubated in a metabolic shaking incubator for up to 2 h at 37°C. The extent of lipid peroxidation was then assessed in terms of the amount of TBARS formed in the medium (8, 16). The absorbance was read at 532 nm and the results were expressed as malondialdehyde equivalents per mg of lipids by using the molar extinction coefficient of $1.56 \times 10^5$ $M^{-1}$ $cm^{-1}$ (17). Control experiments were conducted in the absence of the "initiators" and their readings were subtracted from those of the samples.

*Fluorescence quenching.* DPH-PC steady-state fluorescence was measured with a Perkin-Elmer LS-50B spectrofluorometer. Excitation and emission wavelengths were 360 nm and 430 nm, respectively. Both $CuSO_4$ and KI solutions were freshly prepared and 0.1 mol% of $Na_2S_2O_3$ was added to the $I^-$ stock solution to inhibit the formation of $I_2$ and $I_3^-$. Quenchers were added to the vesicle suspensions as small aliquots (20 $\mu$l). All measurements were corrected for light scattering and dilution. For dynamic quenching, the decrease in fluorescence intensity is related to the concentration of quencher ([Q]) by the Stern-Volmer equation

$$I_0/I = 1 + K_{SV}[Q], \quad [1]$$

where I and $I_0$ are fluorescence intensities in the presence and absence of a quencher, respectively. $K_{SV}$ is the Stern-Volmer quenching constant.

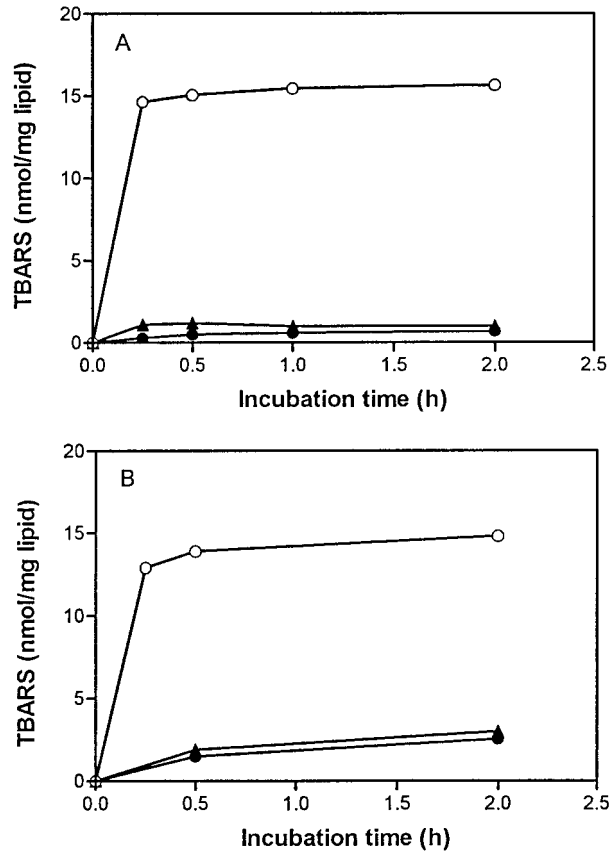


**FIG. 1.** $Fe^{2+}$/histidine-$Fe^{3+}$-induced (A) and Fenton's Reagent-induced (B) TBARS formation in egg PC SUVs (○), egg PC LUVs (●) and LUVs prepared with phospholipids obtained from sonicated egg PC SUVs (▲).

*Fluorescence lifetime measurements.* The fluorescence lifetimes of DPH-PC in egg PC SUVs and LUVs were measured using a K2 multifrequency phase and modulation fluorometer (ISS, Inc., Champaign, IL) as described before (18). The probe to lipid ratio was 1:100. The experimental phase and modulation data were best fitted, in terms of the magnitude of chi-square ($\chi^2$), with the function of unimodal Lorentzian distribution.

## RESULTS AND DISCUSSION

The $Fe^{2+}$/histidine-$Fe^{3+}$- and Fenton's Reagent-induced lipid peroxidation in egg PC SUVs and LUVs was followed by measuring the formation of TBARS in the incubation solution and the results are depicted in Fig. 1. In SUVs, the formation of TBARS was virtually completed within the first 15 min of incubation at 37°C, the amount of TBARS formed being 15.2 ± 0.8 nmol/mg phospholipid, irrespective of the nature of the inducers used. On the other hand, the oxidation reaction was largely inhibited when egg PC LUVs were employed under similar conditions. It is well known that ultrasonic irradiation of lipids containing polyunsaturated fatty acids promotes the production of hydroperoxides and these reactive molecules are capable

**TABLE I**

Continuous Lorentzian Distribution Analysis of DPH-PC Fluorescence Emission Decay[a]

| Vesicles | C (ns) | W (ns) | $\chi^2$ |
|---|---|---|---|
| SUVs | 6.50 ± 0.05 | 0.13 ± 0.06 | 1.78 |
| LUVs | 6.79 ± 0.09 | 0.06 ± 0.05 | 1.38 |

[a] Abbreviations: C, distribution center; W, full width at half-maximum; ns, nanoseconds. Values represent mean and standard deviation obtained from three independent vesicle preparations, while the $\chi^2$ represents the median value obtained from the three fits of the data.

of abstracting hydrogen from lipid to form new lipid alkyl radicals (19–22). It is therefore crucial to examine whether the method used for preparing SUVs was responsible for their higher susceptibility to lipid peroxidation. In doing so, we had prepared LUVs with egg PC obtained from lyophilised SUVs. It was found that the amount of TBARS formed in the post-sonicated LUVs was similar to that in LUVs prepared in the normal way (Fig. 1). Therefore, it appears that the significantly higher concentration of TBARS found in egg PC SUVs was not due to the artefacts introduced during sample preparations.

In compartmentalized systems such as phospholipid vesicles, it is reasonable to assume that free radical inducers in the external medium must first gain access to the unsaturated fatty acyl chains buried inside the membrane bilayer to start the chain reaction of lipid peroxidation. If this is the case, both the transition metal ions and oxygen or the hydroxyl radicals should penetrate into the membrane bilayer and a higher water permeability of the latter would definitely facilitate this process. Numerous physical studies on the acyl chain structure of phospholipid bilayer vesicles suggest that acyl chain packing depends in part on the radius of curvature of the vesicles (23, 24). With the higher surface curvature, the unsaturated fatty acyl chains of SUVs should have a greater exposure to water, resulting in a higher accessibility of these potential targets of peroxidation reactions to the water-soluble oxidants. We have approached this question of membrane permeability in two ways (see below) and by making use of the fluorescence emission of the labelled phospholipid, DPH-PC, whose DPH moiety is expected to be aligned with the membrane lipid acyl chains and located in regions near the bilayer centre (25).

Firstly, the lifetimes of DPH-PC fluorescence emission in egg PC SUVs and LUVs were determined and the results are shown in Table I. Both the lifetime distributional centre and the width at half-maximum of the distribution of DPH-PC fluorescence are different when the measurements were carried out in the two types of vesicles. It is known that the lifetime centre parameter is sensitive to the average environment of the fluorophore and will be decreased by the presence of water due to its higher dielectric constant (9, 10). Therefore, the lower value of lifetime distributional centre of DPH-PC fluorescence in egg PC SUVs (6.50 ± 0.05 ns) indicates a greater degree of interchain hydration, implying a higher level of water penetration as compared with that in egg PC LUVs, where the DPH-PC lifetime distribution centre was 6.79 ± 0.09 ns. The width of distribution depends on the environmental heterogeneity of the fluorophore (26, 27). A slightly wider distribution of DPH-PC fluorescence lifetime might reflect, at least to certain extent, the more heterogeneous nature of the size distribution of SUVs prepared by the method of sonication (12). Since the transition metal ions, oxygen and the hydroxyl radicals are all water-soluble substances, the DPH-PC lifetime data would therefore suggest that these molecules are more accessible to the hydrocarbon interior of SUVs than to that of LUVs.

Secondly, the fluorescence emission of DPH-PC in egg PC SUVs and LUVs was quenched by either $Cu^{2+}$ or $I^-$ and the Stern-Volmer plots are shown in Fig. 2. These plots are apparently linear, implying that dynamic quenching is probably the predominant mechanism involved. Since fluorescence quenching requires direct contact between fluorophore and quencher or at least they should be at only a short distance, both quenchers used in this experiment, $Cu^{2+}$ and $I^-$, must therefore diffuse into the bilayer centre where the DPH

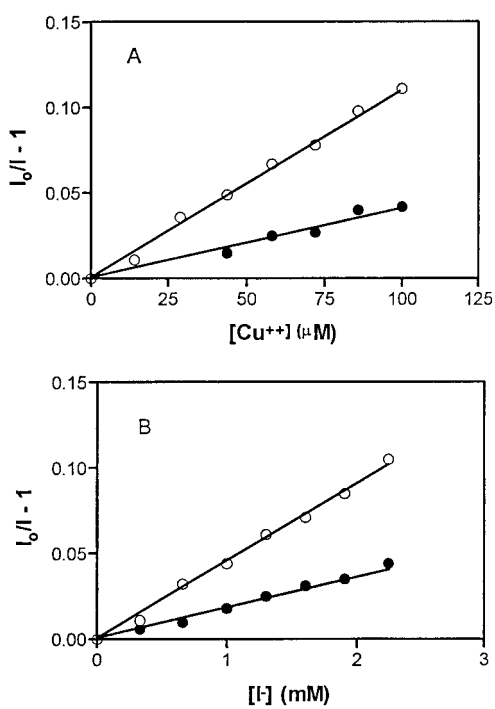


**FIG. 2.** Stern-Volmer plots for the quenching of DPH-PC fluorescence by $Cu^{2+}$ (A) or iodide (B) in egg PC SUVs (○) or egg PC LUVs (●). These experiments were carried out at 25°C.

moiety of DPH-PC molecule resides. This implies that the DPH-PC fluorescence quenching is in fact a diffusion-controlled event. In other words, the level of DPH-PC fluorescence quenching should be directly proportional to the membrane permeabilities of the fluorescence quenchers. Thus, one may conclude from the slopes of the Stern-Volmer plots shown in Fig. 2 that the degree of penetration of the ions in much higher in egg PC SUVs, as compared to that in egg PC LUVs. Because the ratio of the slope of the Stern-Volmer plot of DPH-PC quenching in egg PC SUVs to that in egg PC LUVs appears insensitive to the nature of the quenchers (Fig. 2) and the fluorescence quenching is always more efficient in SUVs, it is likely that the transition metal ions, oxygen and hydroxyl radical may also be able to penetrate into the SUV bilayers more readily than they do to the LUVs.

The difference in susceptibility of phospholipid membranes to lipid peroxidation should also depend on the nature such as size and hydrophobicity of the free radical sources since these properties affect their ability of penetrating into the membrane bilayer. For example, when lipid peroxidation was induced with human haemoglobin, LaBrake and Fung found that both bovine brain phosphatidylserine SUVs and LUVs exhibited very similar level of peroxidation products (28). As a matter of fact, little lipid peroxidation occurred in these SUV and LUV samples after incubation with human haemoglobin for 30 min at 37°C, suggesting that both membranes were quite resistant to the penetration of haemoglobin and/or the transfer of hydrophobic heme groups from haemoglobin into the lipid bilayers. In our case, the free radical reaction inducers are small molecules and equally soluble in water. Therefore, the lipid peroxidation induced by them should be more sensitive to the structural dynamics of the lipid bilayer. In other words, the enhanced lipid peroxidation may be attributed to the looser packing of PC molecules in the highly curved SUVs.

Whether the effect of membrane surface curvature or molecular packing on lipid peroxidation observed in this study has physiological significance is unclear. *In vitro* studies on copper-induced lipid peroxidation in the subfractions of low-density lipoproteins showed that smaller lipoprotein particles displayed diminished resistance to oxidative stress (29, 30). It has been found that the rates of TBARS formation are not related to lipoprotein contents of protein, triacylglycerol, phospholipid and even antioxidant, but are significantly correlated with the lipoprotein content of unesterified cholesterol (29). Therefore, one possible explanation to the above correlation might be that cholesterol has charged the membrane permeability to free radical reaction inducers such as oxygen (31). It appears that our results provide an good explanation that the difference in size or surface packing properties is, at least in part, responsible for the variations in oxidative susceptibility among the lipoprotein subfractions.

In summary, the present study reports the effect of surface curvature or packing of phospholipid bilayer vesicles on their susceptibility to lipid peroxidation induced by transition metal based water-soluble reagents. The results show that SUVs are more prone to lipid peroxidation than LUVs prepared with identical phospholipids. We conclude that higher level of penetration of the water-soluble radical reaction inducers to the highly curved and loosely packed SUV bilayers is responsible for the greater susceptibility of SUVs to lipid peroxidation found in this study.

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