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**Brief Communication**

CHOLESTEROL PROTECTS THE PHOSPHOLIPID BILAYER FROM OXIDATIVE DAMAGE

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Abstract—The measurement of fluorescence lifetime distribution of 1,6-diphenyl-1,3,5-hexatriene is used for the detection of oxidative damage produced in phospholipid membranes by ionizing radiation. The recently developed method is based on the linear relationship between the width of the probe lifetime distribution and the logarithm of the dose. The molecular origin of the damage resides in the production of hydroperoxide residues at the level of acyl chains double bonds. A chemiluminescence assay was used to quantitate the amount of produced hydroperoxides. Consequences of the produced damages include an increased disorder in the upper portion of the bilayer, accompanied by the penetration of water molecules. In the presence of the physiological concentration of cholesterol in phospholipid bilayers, the amount of hydroperoxides produced by ionizing radiation is dramatically reduced. The packing effect of cholesterol in phospholipid bilayers is well recognized, as well as its influence on the reduction of water concentration in the bilayer. The dramatic reduction of hydroperoxides concentration observed when irradiation is performed in the presence of cholesterol probably originates from a steric hindrance to the radical chain reaction through the unsaturated lipids due to the presence of cholesterol.

Keywords—Free radicals, Cholesterol, Fluorescence, Ionizing radiation, Membrane, Oxidative damage, Oxygen, Phospholipids, Quenching

INTRODUCTION

Ionizing radiation has been demonstrated to produce oxidative damage on phospholipid membranes.¹ In erythrocyte ghosts and in phospholipid bilayers, damage induced by ionizing radiation in the presence of oxygen has been observed after irradiation with doses as low as 50 cGy, well within the dose range used for cell survival studies.^{2,3} The sensitivity of membrane lipids to low radiation dose represents a new finding based on the peculiar properties of the fluorescence decay of 1,6-diphenyl-1,3,5-hexatriene (DPH). DPH shows a complex decay in membranes, described by two lifetime components, continuously distributed following a lorentzian function.⁴ The DPH lifetime is inversely related to the dielectric constant of the environment. The reason for its complex decay in membranes resides in the heterogeneity of the value of di-

electric constant along the bilayer depth, originated by a gradient of water concentration.⁵ Due to the possibility of an accurate determination of DPH lifetime distribution by the use of the harmonic response technique,⁶ the width of DPH lifetime distribution has been found to be a sensitive monitor of lipid membrane damage.^{2,3}

The molecular mechanism for the production of damage on unsaturated phospholipids by radiation has been described as a radical chain reaction, leading to the formation of hydroperoxides.¹ A model to explain the molecular origin of the observed modifications of DPH fluorescence decay has been proposed. When hydrophilic hydroperoxide residues are produced in the bilayer core, hydrophobic interaction between adjacent phospholipids are looser, and the upper portion of the membrane, from the aqueous surface to the level of the hydroperoxide residues is disordered,^{2,3} allowing the penetration of water molecules.⁷ DPH is not fluorescent in aqueous environments. After radiation damage has been produced and the membranes are labeled with DPH, the fluorescence can only originate from the deeper portion of the hydrophobic core of the bi-

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layer, which has not been disturbed by the formation of oxidation products and where water molecules did not penetrate. This deep portion of the bilayer has more homogeneous values of dielectric constant,⁵ so that DPH lifetime distribution is narrower.^{2,3} This effect is only observable if irradiation of lipids is performed in the presence of oxygen. The method for detecting the damage produced by ionizing radiation is thus the measurement of the width of DPH lifetime distribution. A linear relationship has been established between the width decrease and the logarithm of the radiation dose.^{2,3} Moreover, we previously showed that the amount of the disordering effect of the damage is directly related to the number and position of double bonds in the acyl residues, being higher in phospholipids with multiple unsaturations and when the unsaturations are located deeper in the bilayer.³

Hydrophobic interactions between adjacent phospholipids (i.e., the packing of phospholipid bilayer), can also be studied by the property of oxygen to quench the fluorescence. The fluorescence intensity arising from labeled bilayers decreases with the increase of oxygen pressure.⁸ Being a very small molecule with a quenching efficiency of one per collision, the kinetics of oxygen diffusion through the bilayer is a sensitive monitor of the packing of the system. A large difference, of about a factor of 50, between oxygen diffusion in the gel and in the liquid-crystalline phospholipid phases has been found.⁸ The tight packing of phospholipids in the gel phase can be perturbed easily due to the presence of molecules different from phospholipids. For example, the addition of 10 mol% cholesterol to the gel phase renders the oxygen diffusion quite similar to that determined in a 50% mixture of the gel and liquid-crystalline phases.⁸

In the present communication, we report results obtained using the above techniques, both the determination of the fluorescence decay with the harmonic response technique and the measurement of oxygen quenching of the fluorescence intensity, for the study of the damage produced by ionizing radiation on phospholipid membranes. The bilayers are composed of unsaturated phospholipids and cholesterol. Cholesterol has been added at the average concentration of most biological membranes^{9,10}; that is, 30 mol%. Its presence has been found to have a profound effect on the DPH lifetime distribution in vesicles exposed to ionizing radiation. The formation of oxidative damage, as hydroperoxides concentration, has been measured using a recently developed chemiluminescence method.¹¹ In samples composed of unsaturated phospholipids and in the absence of cholesterol, a linear relationship has been found between the radiation dose

and the concentration of hydroperoxides. Instead, when 30 mol% cholesterol is added to the phospholipids, no appreciable production of hydroperoxides has been detected for radiation doses up to 11 Gy.

MATERIALS AND METHODS

Multilamellar phospholipid vesicles composed either of dioleoyl- or dipalmitoleoyl-phosphatidylcholine (DOPC and DPOPC, respectively) (Avanti Polar Lipids Inc., Pelham, AL, USA) were prepared by evaporating the chloroform solution of phospholipids and cholesterol (Sigma Chemical Co., St. Louis, MO, USA) under nitrogen stream, resuspending the dried film in phosphate buffered saline solution (PBS) (Sigma Chemical Co.), warming the samples at 37°C and vortexing. Final phospholipids concentration was 0.3 mM. When added, cholesterol concentration was 30 mol% with respect to phospholipids, keeping the final total lipids concentration of 0.3 mM constant. Vesicles were prepared in the dark and used immediately.

Samples (2.5 ml) were irradiated with a cobalt-60 source, Gammacell 220 (Atomic Energy Canada Limited), at the rate of 11 Gy/min. Irradiation were performed at 0°C.

Labeling of the vesicles with 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes Inc., Eugene, OR, USA) was performed immediately after irradiation, by adding to each of the 2.5 ml samples, 0.5 μ l of a 2.5 mM solution of DPH in dimethylsulfoxide (DMSO) (Sigma Chemical Co.) under mild stirring, at room temperature and under red light. Samples were incubated for 30 min, then transferred into the cuvette and allowed to equilibrate at 20°C in the cell holder compartment of the fluorimeter for 10 min prior measurement. Labeling of the vesicles with 2-dimethylamino-6-lauroylnaphthalene (Laurdan) (Molecular Probes Inc.) was performed using the same procedure, by adding 0.5 μ l of a 2.5 mM solution of Laurdan in DMSO. During incubation with Laurdan, samples were also deoxygenated by nitrogen bubbling. The final molar ratio between the probes and the phospholipids was 1:600.

DPH lifetime measurements were performed using a K2 phase fluorometer (ISS Inc., Champaign, IL, USA) equipped with a xenon arc lamp. Excitation was at 360 nm and emission was observed through a Janos 418 cutoff filter. An additional polarizer was inserted in the excitation light path, with an angle of 30°. A solution of 2,2'-*p*-phenylene-bis-(5-phenyl)oxazole (POPOP) in ethanol was used as the reference (lifetime = 1.35 ns). During measurement, samples were continuously

stirred. Sample compartment was kept at $20 \pm 1^\circ\text{C}$ by a water circulating bath. Phase and modulation data were collected for 11 modulation frequencies, logarithmically spaced in the range from 2 to 150 MHz. Data were analyzed using the Globals Unlimited software¹² (Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champaign). The fitting function was the sum of two continuously distributed lorentzian components.

Oxygen quenching experiments were performed using Laurdan as the fluorescent probe,⁸ using a procedure similar to that reported. Laurdan emission spectra were acquired using a photon counting fluorimeter, model PC1 (ISS Inc.), equipped with a xenon arc lamp and an oxygen pressure cell,¹³ thermostated to $20 \pm 1^\circ\text{C}$ by a water circulating bath. The measurements in the absence of oxygen were performed on deoxygenated samples and under nitrogen atmosphere. During measurements, samples were continuously stirred. Oxygen pressure was applied by means of a steel needle inserted in the cuvette, connected to the oxygen tank, and slowly bubbling oxygen at the bottom of the cuvette. After the desired oxygen pressure was obtained, samples were allowed to equilibrate for 20 min before spectra acquisition. Fluorescence emission spectra were acquired from 400 nm to 550 nm, using excitation at 360 nm and excitation and emission monochromator bandwidths of 8 nm. The intensity for the I_0/I ratio reported in the Stern-Volmer plots was calculated integrating the intensity of the overall Laurdan emission spectra collected under nitrogen atmosphere and at different oxygen pressures.

Hydroperoxide concentration was measured on 3 ml samples composed of DOPC multilamellar vesicles, with or without 30 mol% cholesterol, using a luminol-based chemiluminescence technique¹¹ recently devel-

oped in the laboratory of Prof. F. Ursini, that will be briefly described in the following. The reaction mixture (1 ml) contained 30 μM luminol, 0.1% (w/v) Triton X-100, 0.1 ml of methanol in 0.1 M 3-[cyclohexylamino]-1-propanesulfonic acid at pH 10, and the samples or the hydroperoxides standards. Reaction was started by adding 4 μM hemin. Chemiluminescence measurements were performed using a luminometer equipped with photon counting electronics (SEAS, Milano, Italy). The insertion of the sample, the stirring, and the addition of the starting substrate (hemin) are computer controlled. The blank subtraction and the luminescence emission was performed using the instrument accompanying software.

RESULTS AND DISCUSSION

The fluorescence decay of DPH in multilamellar vesicles composed either of DOPC or of DPOPC is described by two components, continuously distributed following a lorentzian function² (Fig. 1). The main component, with relative intensity of about 95%, is centered at about 8 ns, while the less intense component is centered at about 0.5 ns. The full width at half maximum (width, in the following) of the main component is about 1.1 ns, while the short component shows a quite narrow distribution, with a width of about 0.03 ns. This description of DPH decay accounts for the following:

1. Regarding the main distributed component, as due to the gradient of polarity across the bilayer,³ DPH is located at various depths along the bilayer normal, and its fluorescence lifetime is inversely related to the value of the dielectric constant. The origin of the gradient of polarity across the mem-

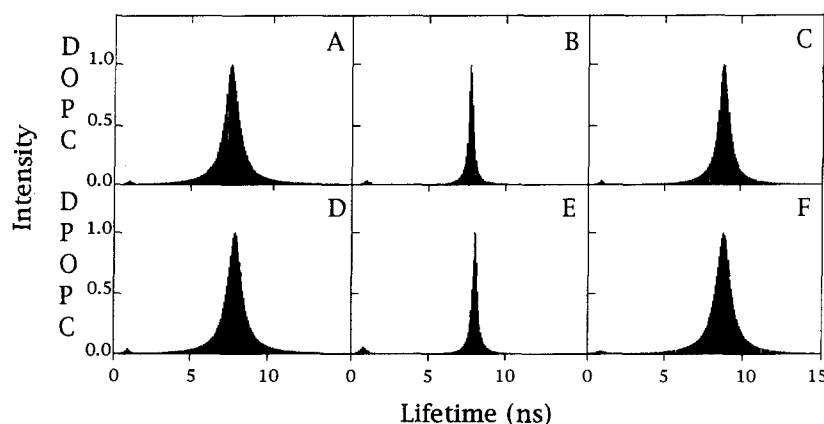


Fig. 1. DPH lifetime distributions in multilamellar vesicles composed of DOPC (upper row) and of DPOPC (lower row) not irradiated (A and D), after a dose of 44 Gy in the absence of cholesterol (B and E) and after a dose of 44 Gy in the presence of 30 mol% cholesterol (C and F).

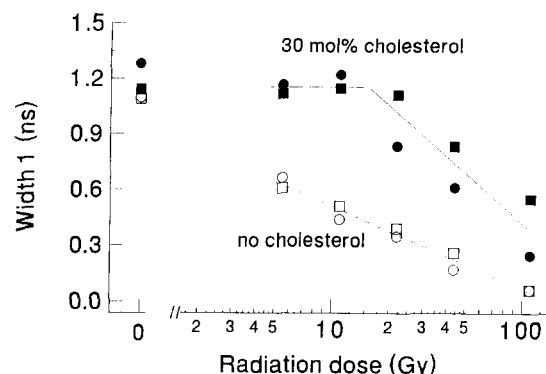


Fig. 2. Values of the full width at half maximum of the lifetime distribution of the main component of DPH fluorescence decay (width 1) in ns, as a function of the logarithm of the radiation dose, in multilamellar vesicles composed of DOPC ($\circ$ and $\bullet$) and of DPOPC ($\square$ and $\blacksquare$), in the absence (open symbols) and in the presence (filled symbols) of 30 mol% cholesterol. Lines have been drawn to highlight the results and do not represent curve fittings.

brane is due to different water concentration across the bilayer.⁵

- Regarding the short lifetime component, as due to the photophysical properties of DPH.³ The presence of products of molecular degradation of the phospholipids can contribute to the broadening and to the increased intensity of this short component.^{3,14}

When the phospholipid vesicles are irradiated in the presence of oxygen, both components of DPH decay are affected.^{2,3} The main component displays a distribution that is progressively sharper with the increase of the radiation dose (Figs. 1 and 2). The decrease of the width of this component shows a linear inverse relationship with the logarithm of the dose (Fig. 2). The short component of DPH decay is not affected by radiation dose lower than about 50 Gy. Above this dose, its relative amplitude and its width increase with the dose (Fig. 3). These results have been observed in both the phospholipids used (Figs. 2 and 3) and are in agreement with previously reported data.^{2,3} As summarized in the introduction, the molecular origin of the observed modification of DPH decay in phospholipid vesicles after ionizing radiation has been attributed to a series of events: (a) a radical chain reaction leading to the production of hydroperoxides at the level of the unsaturation of the acyl residues¹; (b) breaks in van der Waals interactions between adjacent acyl chains, with the consequent disordering of the upper portion of the bilayer, from the lipid-water interface to the level of the formed hydroperoxide^{3,7}; and (c) the increase of water concentration in the upper portion of the bilayer.^{2,3} Because DPH is not fluorescent in the presence of water, its fluorescence can only originate from probe

molecules deep in the bilayer interior, where more homogeneous dielectric constant values have been determined.⁵ Thus, DPH experiences a more homogeneous environment and its lifetime distribution is sharper.³

If the phospholipid vesicles contain 30 mol% cholesterol, the effect of ionizing radiation on the width of DPH lifetime distribution is dramatically reduced (Figs. 1 and 2), while the presence of cholesterol in unirradiated control samples has no effect on the width of DPH distribution. Using both phospholipids, in the presence of cholesterol no variation of the width of DPH distribution can be observed after doses up to 11 Gy. At higher doses, a sharper distribution is observed, but always broader than the distribution obtained in the absence of cholesterol (Fig. 2). In the presence of cholesterol, the short-lived component of DPH decay shows a lower increase of both its fraction and width with the dose than that observed in the absence of cholesterol (Fig. 3).

The quantitative determination of hydroperoxides in DOPC samples after exposure to ionizing radiation has been performed using a novel, extremely sensitive chemiluminescence technique.¹¹ The results of two separate set of experiments are reported in Figure 4. In the absence of cholesterol, a linear increase of the concentration of hydroperoxides is observed as a function of the radiation dose, from about 10 nM in the blank sample to about 300 nM after a dose of 11 Gy. In the presence of cholesterol, the concentration of hydroperoxides shows a negligible variation.

A major difference can be observed between the dose-response curve reported in Figure 2 and the plot of hydroperoxides concentration as a function of the radiation dose (Fig. 4). The width of DPH lifetime varies linearly with the logarithm of the dose, while the hydroperoxide concentration varies linearly with the dose. This result can be explained by considering

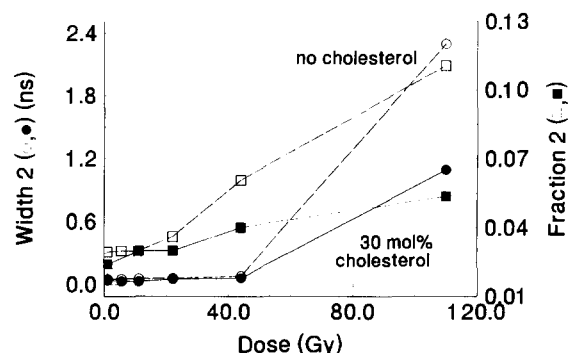


Fig. 3. Values of the full width at half maximum (width 2) in ns, and of the relative intensity (fraction 2) of the lifetime distribution of the short short-lived component of DPH fluorescence decay as a function of the radiation dose, in multilamellar vesicles composed of DPOPC, in the absence (open symbols) and in the presence (filled symbols) of 30 mol% cholesterol.

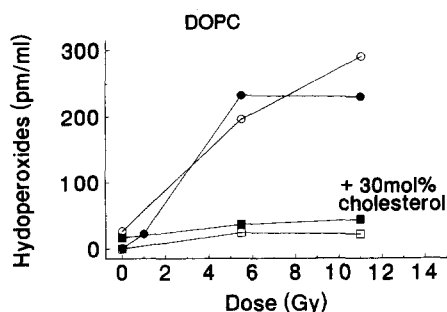


Fig. 4. Variation of hydroperoxide concentration as a function of the radiation dose in DOPC vesicles, in the absence (●, ○) and in the presence of 30 mol% cholesterol (■, □). Determinations were performed using a luminol-based chemiluminescence technique.¹² Filled and open symbols represent two set of separate experiments.

that the distribution of DPH lifetimes monitors the change of heterogeneity in the bilayer depth, that can be also considered a variation of the membrane order. The presence of each hydroperoxide residue can modify the bilayer packing in a relatively long range, so that most of the disordering effect due to oxidation products occurs for a relatively low concentration of these same products. This model can also explain the extreme sensitivity of the fluorescence technique, showing a 50% decrease of the width of DPH lifetime distribution after a dose of 5.5 Gy, corresponding to the presence of $0.2 \mu\text{M}$ hydroperoxides (i.e., 0.07 mol% hydroperoxides with respect to phospholipids). Of course, this explanation needs further investigation. Actually, oxidative damage on membrane lipids has already been extensively reported, but after doses of ionizing radiation higher for about two orders of magnitude (see, for a comparison, refs. 2 and 15). In agreement with our results, using radiation dose >50 Gy, lipid decomposition products have been detected.¹⁵

Quenching of Laurdan fluorescence with oxygen was previously used to study the packing of phospholipid bilayers in the two gel and liquid-crystalline phases, and the alteration of this packing introduced by cholesterol.⁸ The reason for using Laurdan resides in its extreme spectral sensitivity to polarity changes; that is, to the penetration of water in the bilayer after damage is produced by ionizing radiation.⁷ Large differences, of a factor of about 50, were reported for the oxygen diffusion between the two phospholipid phases, to be attributed to a very tight packing of the bilayer in the gel phase, when compared to the liquid-crystalline phase.⁸ Oxygen quenching experiments are now reported, performed on DOPC vesicles, irradiated with a dose of 20 Gy. Stern-Volmer plots have been calculated, by the ratio between the fluorescence intensity in the absence of oxygen (I_0) and that at a given oxygen pressure (I) vs. the oxygen pressure (Fig. 5).

The results show a significant difference between the slope obtained using irradiated and unirradiated DOPC vesicles (Fig. 5). If 30 mol% cholesterol is added to DOPC, the difference in slope between irradiated and unirradiated vesicles is reduced (Fig. 5).

From the above results, we can conclude that in the presence of the average physiological cholesterol concentration, hydroperoxides are produced by ionizing radiation on unsaturated phospholipids at lower concentrations than in the absence of cholesterol. As for the quantitative measurement of the produced hydroperoxides, their decreased production when cholesterol is present can be explained by a real protective effect, due to a steric hindrance to the diffusion of the radical chain propagation reaction,¹ because of its presence between two adjacent unsaturated acyl chains. As for the fluorescence measurements, the protective effect of cholesterol can be explained by its property of increasing the order of bilayers in the liquid-crystalline phase.¹⁶ After oxidative damage has been produced by ionizing radiation, the presence of cholesterol can maintain the order of the system by promoting a tighter packing of the bilayer, by locating itself in the discontinuities between the hydrophobic interactions, created by the presence of oxidation products. The lack of production of local disorder can also decrease the penetration of both water and hydroxyl radicals, reducing the quenching of DPH fluorescence and the production of oxidation, respectively. Although cholesterol itself can be oxidized, a higher radiation dose is required to oxidize the cholesterol molecule than that required for the oxidation of phospholipid double bonds.^{3,17} At higher radiation doses, also in the presence of cholesterol, the damage can be observed, both for the sharpening of the lifetime distribution of the main component of DPH decay (Fig. 2) and

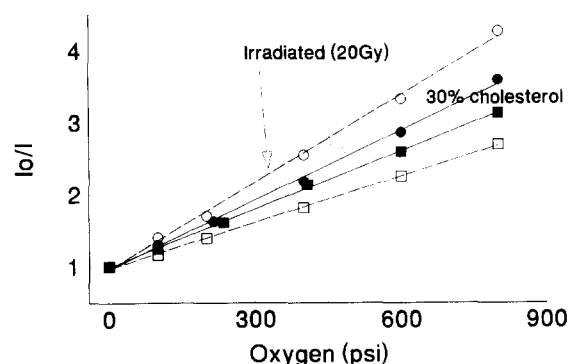


Fig. 5. Stern-Volmer plots obtained from DOPC multilamellar vesicles labeled with Laurdan. Unirradiated vesicles (□ and ■) and vesicles irradiated with a dose of 20 Gy (○ and ●) in the absence (open symbols) and in the presence (filled symbols) of 30 mol% cholesterol. Lines represent linear regression fit.

for the increase in intensity and width of the short component (Fig. 3), but of a lower amount than that observed in the absence of cholesterol. A protective effect of cholesterol against erythrocyte oxidative stress has been reported,^{18,19} as well as a negative correlation between membrane cholesterol concentration and the degree of lipid peroxidation in human red cells.²⁰ Cholesterol has been also proposed to exhibit its protective effect by intercepting blood and tissue oxidants, giving rise to the various oxisterols.²¹

As a final consideration, the measurement of the kinetics of oxygen quenching appears to constitute a promising tool for investigations on membrane defects produced by oxidation (i.e., could constitute an early monitor of oxidative damage). Compared to the measurement of DPH lifetime distribution, the oxygen quenching technique requires less sophisticated instrumentation and the obtained data are straightforward, with no need of complex analysis.

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ABBREVIATIONS

DOPC—dioleoyl-phosphatidylcholine
 DPOPC—dipalmitoleoyl-phosphatidylcholine
 DPH—1,6-diphenyl-1,3,5-hexatriene
 Laurdan—2-dimethylamino-6-lauroyl-naphthalene