Cholesterol attenuates and prevents bilayer damage and breakdown in lipoperoxidized model membranes. A spin labeling EPR study

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ABSTRACT

The stabilizing effect of cholesterol on oxidized membranes has been studied in planar phospholipid bilayers and multilamellar 1-palmitoyl-2-linoleoyl-phosphatidylcholine vesicles also containing either 1-palmitoyl-2-glutaroyl-phosphatidylcholine or 1-palmitoyl-2-(13-hydroxy-9,11-octadecanediényl)-phosphatidylcholine oxidized phosphatidylcholine in variable ratio. Lipid peroxidation-dependent membrane alterations in the absence and in the presence of cholesterol were analyzed using Electron Paramagnetic Resonance spectroscopy of the model membranes spin labelled with either cholestane spin label (3-DC) or phosphatidylcholine spin label (5-DSPC). Cholesterol, added to lipid mixtures up to 40% final molar ratio, decreased the inner bilayer disorder as compared to cholesterol-free membranes and strongly reduced bilayer alterations brought about by the two oxidized phosphatidylcholine species. Furthermore, Sepharose 4B gel-chromatography and cryo electron microscopy of aqueous suspensions of the lipid mixtures clearly showed that cholesterol is able to counteract the micelle forming tendency of pure 1-palmitoyl-2-glutaroyl-phosphatidylcholine and to sustain multilamellar vesicle formation. It is concluded that membrane cholesterol may exert a beneficial and protective role against bilayer damage caused by oxidized phospholipid formation following reactive oxygen species attack to biomembranes.

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1. Introduction

Cholesterol associates with phospholipids in building up the lipid bilayer of cellular membranes and contributes to determine properties of the lipid bilayer such as fluidity and hydrophobicity, water permeation and lateral phase organisation (lipid rafts) [1–6]. Cholesterol is also critical in stabilizing the phospholipid bilayer as shown by its capacity to counteract the membranolytic action of lyso-PC and to form bilayers when associated with this micelle-forming lipid [7–9]. Recently, molecular dynamics simulation studies of thermodynamic effect of cholesterol on dipalmitoylphosphatidylcholine bilayers showed its ability to restrict bilayer deformations and to prevent pore formation, also indicating its possible capacity to modulate the functional properties of membranes [10,11]. Protective effect of cholesterol against membrane peroxidation is also suggested by its ability to slow down oxidized lipids lateral mobility [12] and to prevent membrane leakage after lipoperoxidation-dependent lyso-phosphatidylcholine formation [13]. Many of these effects have been recently reviewed and measured more precisely by improved EPR methods [14].

In contrast to the membrane stabilizing effect of cholesterol, oxidized phosphatidylcholines arising from membrane oxidative stress bring about a variety of membrane alterations [15–19], including disordering (fatty acid disorienting) and destabilization (micelle formation) of the phospholipid bilayer [20–22]. These studies showed the capacity of conjugated dienes phosphatidylcholines to disorder the bilayer hydrophobic core, and pointed out the membranolytic properties of cleaved chain PC in general, and particularly of carboxyacyl-PC (PGPC and palmitoyl-azelaoyl-PC), similar to lyso-PC. This similarity prompted the present study, aimed at establishing whether cholesterol was also able to exert a stabilizing effect on oxidized PC-rich bilayers similar to that observed on lyso-PC-containing model membranes.

This paper reports on the effects of cholesterol, added in 40% molar ratio to lipid mixtures, on the ordering of PLPC supported planar lipid bilayers (SPB) containing variable percentage of either conjugated diene phosphatidylcholines (HOP LPC, 1-palmitoyl-2-(13-hydroxy-9,11-octadecanediényl)-3-phosphoglycercholine) or carboxyacyl phosphatidylcholine (PGPC, 1-palmitoyl-2-glutaroyl-3-phosphoglycercholine). Furthermore, the vesicle formation
ability of these PGPC/PLPC mixtures after addition of 40% cholesterol was studied, and their behavior was compared to that of similarly composed bilayers containing lyso-PC in place of oxidized PC.

Bilayer disordering was detected and measured by EPR spectral anisotropy loss of either 5-doxylstearoyl-phosphatidylcholine or 3-doxylcholestane spin label in SPBs, while vesicle formation was assessed by Sepharose 4B gel-chromatography and by Cryo Electron Microscopy of the lipids aqueous suspension. It will be shown that SPB disordering provoked by the considered oxidized PC species is strongly reduced by the presence of cholesterol, and that micelle formation of PGPC is completely reversed to vesicle formation, in striking analogy with the known effect exerted by cholesterol on lyso-PC.

Our results allow attribution of a new protective role of cholesterol against membrane oxidative stress, and enrich the already known membrane stabilizing effect of this lipid with a new aspect, possibly related to ROS-linked pathologies.

2. Materials and methods

2.1. Materials

5-Doxylstearic acid (5-DSA), 3-doxyl-cholestane (3-DC, Fig. 1), glutaric anhydride, lyso-PC (from egg yolk and 1-palmitoyl-2-lyso-phosphatidylcholine), and lipoxidase (type V from soybean, EC 1.13.11.12) were purchased from Sigma; 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC) was from Avanti Polar Lipids. Solvents were Baker HPLC grade. Lichroprep RP18 (40–63 μm) silicagel and TLC chromatoplates (0.2-mm or 0.5-mm thick) were from Merck. Sepharose 4B was purchased from Amersham Biosciences.

2.2. Phospholipid modification

1-acyl-2-(5-doxylstearoyl)-phosphatidylcholine (Fig. 1, 5-DSPC) was obtained by coupling 5-doxylstearic acid to lyso-phosphatidylcholine according to Boss et al. [23], and purified by reverse-phase (RP18 silicagel) flash-chromatography [20]. 2-Glutaryl-phosphatidylcholine (PGPC, Fig. 1) was obtained by the method of Watson et al. [24] starting from lyso phosphatidylcholine and purified by preparative TLC developed with CHCl₃/CH₃OH/NH₄OHconc 65:25:8 (by volume). 1-Palmitoyl-2-(13-hydroxy-9,11-octadecanediynoyl)-phosphatidylcholine (HOP LPC, Fig. 1) was prepared by NaBH₄ reduction [15] of 1-palmitoyl-2-(13-hydroperoxy-9,11-octadecanediynoyl)-phosphatidylcholine obtained by action of lipoxidase on PLPC [25,26]. HOP LPC was extracted from the reaction mixture by the method of Bligh and Dyer [27] and purified by RP18 Silicagel reverse-phase preparative column flash-chromatography, using CH₃OH/(C₂H₅)₂O/H₂O 95:5:2 (vol) [20]. Molecular structure and purity of oxidized phosphatidylcholine species were confirmed by mass spectrometry and by TLC [20], respectively. Quantitative phosphorus analysis was performed according to Nakamura [28].

2.3. Analysis of EPR spectral anisotropy

A nitroxide g-value (establishing the EPR spectrum center) and hyperfine splitting constant A (determining the distance between hyperfine lines) are orientation-dependent [29,30], and the orientation of the nitroxide ring reference frame with respect to that of a spin labelled lipid molecule is dependent on the geometry of the lipid-nitroxide bond(s) [31]. In a lipid bilayer, non-axial g and A components are time-averaged to axial symmetry by fast rotation of the spin labelled molecule around the long molecular axis coincident with the z-axis for 5-DSPC spin label and with the y-axis for 3-DC. In the case of a spin label located on a fatty acyl tail of phosphatidylcholine, the motion of the nitroxide z-axis, parallel to the acyl chain long axis, is described as a wobbling motion into a cone having its axis oriented perpendicular to the bilayer’s local plane. A similar description applies to the motion of the nitroxide ring y-axis of 3-DC spin label, lying parallel to the cholestane long axis instead of the z-axis. The smaller the cone width, the closer the nitroxide z- or (y-) axis to the bilayer normal (as it happens at position C-5 of the stearic acid), so that the average direction of these axes can be fairly approximated to be locally perpendicular to the bilayer.

Fig. 1. Molecular structure of HOP LPC, 1-palmitoyl-2-(13-hydroxy-9,11-octadecanediynoyl)-phosphatidylcholine; PGPC, 1-palmitoyl-2-glutaroyl-phosphatidylcholine; 5-DSPC, 2-(5-doxylstearoyl)-phosphatidylcholine; 3-DC, 3-doxylcholestane.
In hydrated oriented planar phospholipid bilayers on a solid support (SPB), the fatty acid chain (or cholestane) long axis tends to align (within the limits of the ideal cone containing the wobbling motion) normally to the sample plane, as it does either the z-axis of the nitroxide ring bound to the fatty residue [32], or the y-axis of the probe bound to the cholestane molecule [31], giving rise to a defined orientation of spin labels, the more the lower the C-position, also reflecting the orientation of the surrounding lipid matrix.

As a consequence of the anisotropy of nitroxide magnetic tensors \( \mathbf{g} \) and \( \mathbf{A} \), the EPR spectrum changes as the orientation of the planar sample normal is changed relative to the static magnetic field, thus yielding two distinct EPR spectra, as described in ref. [34] (p. 381), by shifting the value of the angle \( \phi \) formed by the sample normal and the external field direction from 0° to 90°. One (the parallel spectrum) is determined by \( g_z \) and \( A_z \) (the axial components of tensors \( g \) and \( A \), respectively, relative to either 5-DSPC z-axis or 3-DC y-axis), and is only visible when the external magnetic field direction is parallel to the sample normal, coaxial with the nitroxide z-axis (5-DSPC) or y-axis (3-DC). The other (the perpendicular spectrum) is defined by \( g_x \) and \( A_x \) (the time-averaged transverse components of tensors \( g \) and \( A \), respectively, corresponding to the average value of 5-DSPC x- and y-components, and of 3-DC x- and z-components), only appearing when the magnetic field is applied perpendicular to the planar sample normal [33,34]. The resulting angular dependence of EPR spectra of the probes in planar phospholipid bilayers reveals an ordered ensemble of spin labels, reflecting ordered alignment of the host phospholipid fatty acids ensemble. On the contrary, if the label’s orientation distribution is isotropic (probe disordering) within the sample, then no dependence of the spectrum on the sample orientation will hold, and EPR spectra similar to powder spectra will appear independently of the sample orientation. In general, the onset of either misaligned fatty acid chains or an isotropic phospholipid phase is revealed by appearance of the parallel EPR spectrum superimposed to the perpendicular one to a variable extent.

It is relevant to note that the meaning of “order” we refer to is different from the one expressed by the commonly used order parameter \( S \). In more detail (see ref. [31], p. 474), the latter is named \( S_{\text{motion}} \) and implies the concept of fluidity. According to the wobble model its value depends on the half-width of the ideal cone in which the wobbling motion of the spin label axis occurs, and is experimentally calculated by the familiar expression (ref [31], p. 481):

\[
S_{\text{motion}} = \left( A_{\text{max}} - A_{\text{min}} \right) / \left( A_{zz} - \frac{1}{2} (A_{xx} + A_{yy}) \right)
\]

in which \( A_{\text{max}} \) and \( A_{\text{min}} \) are measured from powder like spectra.

In planar oriented bilayers, a different \( S \) parameter, namely \( S_{\text{space}} \) (see ref. [31] p. 493), is introduced to describe the order of the spin label long axis, that is its orientation with respect to the planar sample normal. Put together, these parameters build up an overall order parameter, named \( S_{\text{aniso}} \), according to the following relationship (ref. [31], p. 494):

\[
S_{\text{aniso}} = S_{\text{motion}} \cdot S_{\text{space}}
\]

in which \( S_{\text{aniso}} \) is described by the following expression (ref [31], p.492-493):

\[
S_{\text{aniso}} = \left( A_{||} - A_1 \right) / \left( A_{zz} - \frac{1}{2} (A_{xx} + A_{yy}) \right)
\]

where \( A_{||} \) and \( A_1 \) are measured from the parallel and the perpendicular EPR spectra of a planar sample (ref. [31], p.493). All parameters range between 0 and 1, so that \( S_{\text{motion}} \rightarrow 1 \) implies restricted dynamics, as it happens at position C-5 of 5-DSPC (and in the polar head region in the case of 3-DC). Under this condition, \( S_{\text{aniso}} \) is now dominated by \( S_{\text{space}} \), that is by the spin label orientation, hence by spatial order.

According to the above expression of \( S_{\text{aniso}} \), when also \( S_{\text{space}} \) approaches 1 (that is, in an ordered sample) then \( (A_1 - A_{||}) \) approaches to \( (A_x - \frac{1}{2}(A_{xx} + A_{yy})) \), that is the single crystal value, showing more distinct separation of \( A_x \) from \( A_{||} \) in a planar sample (e.g., EPR spectra in ref. [31], p. 477). This relationship, in case of motionally restricted spin labels, renders the measure of \( A_x \) and \( A_{||} \) in a planar sample as a direct measurement of the static spatial order of the spin label and of its surrounding matrix. On this basis, orienting of fatty acids along the planar sample normal in a motion restricted matrix (at least in C-5) is indicated by the separation of the powder-like spectrum components into a couple of distinct parallel and perpendicular spectra. These spectra are observed separately upon changing the sample normal orientation with respect to the external magnetic field by varying the angle \( \phi \) value, as mentioned above. For motionally restricted planar samples, in order to distinguish the spatial order we deal with from the more commonly used motion-dependent order, generally expressed by \( S \) (without any subscript), we often use the terms “alignment” or “orientation” better than “order” and “disorder”.

The extent of alignment disordering was semi-quantitatively estimated from the height of the parallel spectrum bands appearing in the perpendicular EPR spectrum [35]. In the case of 5-DSPC, the better-resolved parallel spectrum low-field band appearing in the perpendicular spectrum was conveniently taken as the most indicative of chain misalignment. Even more conveniently, EPR bands are best resolved in the second derivative presentation of perpendicular spectra. From this presentation, the numerical value of an empirical misalignment index \( R' \) could be calculated from EPR spectra second derivative as the ratio of the height of the low-field band of the parallel spectral component to that of the perpendicular component [35].

In the case of the 3-DC spin label, a similar parameter has previously been exploited [33] as the \( B/C \) ratio of the height of the parallel spectrum low field band (named B), scanned with the external magnetic field parallel to the sample normal (hence to the nitroxide y-axis as well), to the height of the central band (named C) of the same spectrum. This ratio has been reported to be indicative of 3-DC nitroxide y-axis tilt towards the xz-plane, that is the probe misalignment shown to follow the alignment loss of the hydrophobic core of the bilayer [2,3].

2.4. Measurement of SPB EPR spectral anisotropy

Phospholipid (200 nmol, also including 2 mol% lipid spin label) in 50-μl ethanol solution was deposited on a thin 6×18-mm glass slide and taken to dryness under vacuum (1–2 mBar, 37 °C). A narrow strip of filter paper, soaked in distilled water, was placed at the bottom of a flat quartz tissue cell well (Wilmark WG-806-A, well dimensions: 7×23×0.5 mm) and the slide rear side was made to adhere to it. After covering the well, the phospholipid layer was allowed to hydrate as judged by the transparency of the sample [35].

The cell was vertically inserted into the cavity of a 9 GHz Varian E-9 Century Line EPR spectrometer and oriented by means of a small goniometer mounted on top of it, so that the normal to the well (coincident with both the fatty acid chain direction—or the cholestane long axis—and the sample’s normal) was either perpendicular (\( \perp \)) or parallel (\( \parallel \)) to the magnetic field (\( H \)) direction. EPR spectra were scanned at room temperature, and instrumental settings were 338/335 mT (\( \perp/\parallel \)), respectively) field set with 12 mT scan width in all presented spectra, 100 kHz and 0.2 mT modulation frequency and amplitude. Radiating field power and frequency were 20 mW and 9.5/9.4 GHz (\( \perp/\parallel \)), respectively. The degree of spectral anisotropy loss, indicative of membrane disordering, was estimated from the \( R' \) parameter value, calculated from the derivative of 5-DSPC EPR perpendicular spectra, or from the \( B/C \) value, deduced from 3-DC parallel EPR spectra band-heights, as previously defined. According to its definition, \( R' \) value increases with the degree of bilayer disorder, while the \( B/C \) ratio value decreases with it.
2.5. Vesicle preparation

The desired phospholipid mixture (1–4 μmol lipid P) was dissolved in 0.5 ml CHCl₃ and dried to a thin film under an N₂ stream in a test tube at 37 °C. After further drying for 20 to 60 min under vacuum (oil pump), the film was hydrated with 0.5 ml of a 100 mM KCl, 10 mM Tris–HCl, pH 8 buffer for 20 to 60 min at 37 °C [36].

SUVs (small unilamellar vesicles) were obtained by ultrasonic irradiation (Branson sonifier) of egg yolk phosphatidylcholine MLV preparations under an N₂ stream at 40 W output for 15 min with 60-s/20-s on/off intervals in an ice bath.

MLV to be observed by cryo Electron Microscopy were prepared as above and extruded through a polycarbonate filter (with 100 nm pore diameter) by means of an Avestin Miniextruder, and the size distribution of these preparations was checked by gel-chromatography to be compatible with that of spontaneously swollen MLV.

2.6. Sepharose 4B gel-chromatography

Samples (0.5-ml, corresponding to 4% of the bed volume) were loaded on a Sepharose 4B column (15-cm × 1-cm ID), previously equilibrated with the vesicle buffer [37–39]. The column was eluted with MLV buffer at 10 ml/h flow rate, and the effluent was monitored at 300-nm wavelength [37] using a Hellma continuous-flow cuvette in a Cary 50 spectrophotometer coupled to a desktop computer via a Varian interface. Optically dense lipid particles (MLV, SUV, micelles) were visualized as optical density (OD) peaks, and the elution pattern was plotted as a function of elution volume, Vs relative to the void volume, V0. V0 was calibrated with Dextran Blue, and MLVs always eluted at V0/V0 = 1. SUVs, characterized by a diameter of about 20 nm [40] and eluting later, were also used as an additional standard particle. Reported fractionation range of Sepharose 4B is 60 kDa–2×10⁵ kDa for globular proteins, approximately corresponding to 5–35 nm particle diameter, respectively.

2.7. Cryo electron microscopy

For this observation, extruded MLW were always used in order to render the vesicle dimension compatible with the size of the holes in the holey carbon grids for microscopy. In principle, cryo electron microscopy was carried out as described elsewhere [41,42]. A portion (3.5 μl) of the sample was mounted on a holey carbon grid (Quantifoil, Germany), blotted and plunged into liquid ethane for quick freezing under vacuum (oil pump), the liquid was hydrated with 0.5 ml of a 100 mM KCl, 10 mM Tris–HCl, pH 8 buffer for 20 to 60 min at 37 °C [36].

MLV to be observed by cryo Electron Microscopy were prepared as above and extruded through a polycarbonate filter (with 100 nm pore diameter) by means of an Avestin Miniextruder, and the size distribution of these preparations was checked by gel-chromatography to be compatible with that of spontaneously swollen MLV.

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3. Results and discussion

The effect of cholesterol on fatty acid misalignment was analyzed in SPB by use of 3-DC spin label (Fig. 1). EPR spectra of this spin probe in planar preparations of HOPLPC, PGPC (Fig. 1) and lyso-PC scanned with the external magnetic field parallel to the sample normal are reported in Fig. 2. The presented parallel EPR spectrum of pure PLPC SPB without cholesterol (−C column) only shows a tiny trace of the perpendicular component and is typical of an ordered matrix with fatty acid chains aligned parallel to the membrane normal. In contrast, planar preparations of pure HOPLPC and pure PGPC yield powder pattern spectra, indicative of a motionally restricted disordered matrix with misaligned fatty acid chains, in principle attributable to either disordered bilayers or micelles. Fatty acid misalignment is evident from the EPR spectrum of 3-DC in lyso-PC planar preparation without cholesterol as well, as also expected from micellar structures or disordered bilayers. B/C parameter [33] values calculated from these spectra are 0.75 for PLPC, 0.08 for HOPLPC, and very close to zero for PGPC and lyso-PC. Parallel EPR spectra of SPB made of the same lipids to which 40% by mol cholesterol was added are reported in the right column (+C) of this figure. While the ordered spectrum of PLPC SPB remains unchanged as expected, the spectra of all modified PC samples show a considerable increase of the parallel component (arrowed), indicating that addition of cholesterol consistently reorients all phospholipids' bilayer core. B/C values calculated from these spectra are seen to increase up to 0.55 for HOPLPC, and to mid-scale values for lyso-PC and PGPC (0.45 and 0.32, respectively), showing the highest orientation recovery in HOPLPC SPB and the lowest in PGPC bilayers. The former is attributed to the already observed HOPLPC better compatibility with the bilayer structure than cleaved chain PC [21,22].

In consideration of the possibility of cholesterol-rich domains formation after addition of cholesterol, and of the possible preferential localization of 3-DC therein, the reorienting effect of cholesterol was also studied with 5-DSPC spin label (Fig. 1), better suited to probe phospholipid-rich domains, if any. EPR spectra of 5-DSPC in planar preparations of PLPC, PGPC and lyso-PC, in the absence and in the presence of 40% cholesterol are reported in Fig. 3 (−C and +C column, respectively). HOPLPC SPB were not included in these measurement due to this lipid proven inability to misalign 5-DSPC in any case [20,22]. Spectra were scanned from SPB oriented with their normal perpendicular to the external magnetic field, yielding the spectral presentation used for derivation and calculation of the order index R′, as described [35]. In the absence of cholesterol, 5-DSPC enclosed in SPB made of PGPC (either pure or in 2:1 mixture with PLPC), or of lyso-PC, showed powder pattern spectra, indicative of a motionally restricted disordered matrix in sharp contrast with pure PLPC, as already reported [20,22]. Also in the case of this spin label, powder-like spectra are compatible with the presence of either micelles or disordered bilayers. The values of the disorder index R′ calculated from these spectra are: 0.28 (PLPC), 15.6 (PGPC), 8 (lyso-PC). The EPR spectrum of lyso-PC SPB after addition of 40% cholesterol (−C column) shows an increase of the perpendicular component (arrowed) revealing a recovery of fatty acid orientation (R' = 2.7) to a extent similar to that estimated by the change of B/C parameter value in 3-DC spectra. No change of EPR spectral anisotropy is observed upon addition of 40% cholesterol to pure PGPC planar preparation (R' = 13.8), in keeping with the lowest order recovery observed with 3-DC. Upon addition of 40% cholesterol to PGPC/PLPC 2:1 bilayers, 5-DSPC also displays successful reorienting of the fatty acid core as demonstrated by the increase of the EPR perpendicular component (arrowed), yielding a decrease of R′ disorder index from a value as high as 15 (−C, very close to that of pure PGPC) to 1.3 (+C).
A more complete picture of cholesterol effect on fatty acids misalignment upon gradual increase of OXPL and lyso-PC molar percentage is presented in Fig. 4, reporting B/C and R'' values calculated from more EPR spectra similar to those shown in Figs. 2, 3. In the upper panel, B/C values of EPR spectra of 3-DC in SPB of various mixtures of OXPC/PLPC and lyso-PC/PLPC in the presence (full symbols) and in the absence (empty symbols) of 40% by mol cholesterol are reported. In general, higher B/C values of all plots in the presence of cholesterol show that the bilayers are considerably more ordered than in the absence of cholesterol, at all oxidized PC molar ratios. Furthermore, the trend of the plots indicates that cholesterol helps fatty acid chains to keep their orientation almost completely parallel to the membrane normal up to about 70% oxidized PC (full diamonds and triangles) or lyso-PC (full squares). Thereafter, misalignment sets up, though never reaching completeness even with 100% modified PC. The lower B/C profiles obtained in the absence of cholesterol (upper panel, void symbols), reaching full disordering with 100% modified PC, show that all the tested bilayers are much more prone to disordering at all oxidized PC molar percentages (empty diamonds and triangles). This trend is also common to lyso-PC containing SPB (empty squares), indicating in general that membranes without cholesterol are quite “undefended” against the disordering effect of oxidized PC.

A similar trend is revealed by R'' index calculated from perpendicular EPR spectra of 5-DSPC enclosed in SPB containing the same lipid mixtures (HOPLC omitted), reported in the lower panel of Fig. 4. As it can be seen from these plots closely resembling 3–DC disordering trend, the presence of cholesterol shifts the percentage of PGPC triggering the onset of bilayer disordering (revealed by the sharp R'' increase) to higher values, that is from 40% (void diamonds: cholesterol absent) to about 70% (full diamonds). SPBs containing lyso-PC show a similar behavior, though less dramatic (empty vs. full squares). The similarity of B/C trend to that of R'' in all cases, and the close match between the values of oxidized PC (and lyso-PC) molar percentages setting on bilayer disordering deduced from both parameters also reveals that the nitroxide response is independent of its lipid support as expected from a homogeneous distribution of both spin labels. This consideration suggests that the observed effect involves the membrane as a whole.

The reorienting effect of cholesterol addition is more evident in 100% HOPLC (Fig. 4, upper panel, full vs. empty triangles, and Fig. 2) and 100% lyso-PC bilayers (Fig. 4, both panels, full vs. void square Figs. 2, 3), than in 100% PGPC SPB, with 5-DSPC showing no restructuring effect of cholesterol on pure PGPC bilayers (Fig. 4, lower panel, full vs. open diamond, and Figs. 2, 3). However, this is the conclusion drawn by the EPR analysis, within the method’s limits. Indeed, when considering the disorder origin one must keep in mind that the EPR method is unable to distinguish between planar disordered bilayers and non-planar ordered bilayers, since the parallelism of fatty acids to the sample normal is lost in both cases. In fact, identical powder-like spectra of 5-DSPC can be indifferently obtained from either internally ordered multi-lamellar vesicles [31] or internally disordered planar bilayers [32], and in general from round shaped structures (including micelles) in which the fatty acid long axis (and the nitroxide z-axis) aligns along all possible spatial directions.

Consolidated knowledge of lyso-PC structural behavior helps in attributing the observed spectral presentation to the correct structure. Indeed, the renowned micelle-forming ability of this lipid [43,44] confirms correct attribution of 5-DSPC and 3–DC EPR spectra in pure lyso-PC planar preparations shown in Figs. 2 and 3 (bottom left) to the presence of micelles, also in keeping with the previously observed full EPR spectral anisotropy loss in lyso-PC planar preparations [22]. The anisotropy recovery of EPR spectra visible in Figs. 2 and 3, bottom right, upon cholesterol addition to lyso-PC SPB is consistent with the transition of micelles to ordered planar structures, also in agreement with the formation of lamellar structure reported in many previous studies of lyso-PC/cholesterol mixture [7–11]. Therefore, the plots of B/C and R'' of lyso-PC planar preparations in the absence of cholesterol can be interpreted in terms of growing formation of micelles (starting around 50% lyso-PC), while those obtained in the presence of cholesterol are taken as an indication of moderately growing fatty acids disordering in planar bilayers (starting at about 70–80% lyso-PC).

Lyso-PC data analysis also helps interpreting the behavior of PGPC-containing bilayers. In PGPC/PLPC SPB, both 3–DC and 5-DSPC reveal a sharp transition from order to disorder just above 50% (by mol) PGPC in PLPC. As it is seen in both panels of Fig. 4 (solid lines, open diamonds), in PGPC/PLPC 2:1 (PGPC = 67% of total phospholipid) R'' value increases to 15, and B/C value decreases to 0.07. Sepharose 4B gel-chromatography of the aqueous suspension of this mixture, reported in Fig. 5 (dotted line), displays an elution profile with principal elution peak at V_e/V_0 = 1.6 typical of micelles, similar to that of the aqueous suspension of
lyso-PC [21,22]. As we previously observed MLV formation from 1:1 PGPC/PLPC aqueous suspension [21], we attribute the order–disorder transition observed upon increasing PGPC/PLPC molar ratio from 1:1 to 2:1 in planar preparations to bilayer breakdown and micelles formation. Addition of cholesterol (40% final mole ratio) to PGPC/PLPC 2:1 brings about a drastic structural change, as shown by the orientation recovery of 5-DSPC visible in Fig. 3: PLPC/PGPC 1:2, +C EPR spectrum vs. –C spectrum. At the same time, in Fig. 4 $R''$ value of 67% PGPC mixture decreases from 15 to 1.3 (lower panel, solid line, full diamonds), while B/C increases to 0.664 (upper panel, solid line, full diamonds). This structural change is also visible in the chromatographic elution profile of PGPC/PLPC/C 2:1:2 (Fig. 5) in which the main elution peak shifts to $V_e/V_0 = 1$ (solid line), typical of multi-lamellar vesicles [21,22]. It is concluded that abolition of the order–disorder transition in PGPC/PLPC 2:1 EPR spectra after cholesterol inclusion indicates prevention of bilayer breakdown and of micelle formation. Though the onset of SPB disordering is apparently shifted by cholesterol to higher PGPC percentage (70–80%, Fig. 4 both panels, solid lines, full diamonds), this disordering may no longer be linked to micelle presence.

Indeed, no apparent reorienting effect by cholesterol is evident in 100% PGPC SPB probed with 5-DSPC, as apparent in Fig. 4: $R''$ barely decreases from 15.6 to 13.8, while B/C is seen to increase from 0.02 to 0.32, indicating only a modest 3-DC orientation recovery (see also Figs. 2, 3). Also in this case, the EPR method alone is unable to provide more detail about the structure, while Sepharose 4B gel-chromatography suggests correct attribution of pure PGPC SPB disordering to the presence of micelles. In fact, the elution profile of pure PGPC aqueous suspension (Fig. 5, dashed line) confirms abundant micelle formation, with the main peak eluting at $V_e/V_0 = 1.6$. Yet, also in this case, addition of cholesterol brings about a structural change as shown by Sepharose 4B gel chromatography of the aqueous suspension of PGPC/C 60:40. The elution profile of this mixture (Fig. 5, solid line) is superimposed to PGPC/PLPC/C 40:20:40 elution profile, and is typical of MLV as well. This pattern reveals bilayer formation, thus indicating the presence of bilayers also in the planar samples, though highly disordered. Therefore, the observed EPR anisotropy loss of PGPC/C SPB (Fig. 3) is attributed to disordered bilayers rather than to micelles. It is concluded that cholesterol is able to reshape the bilayer structure of PGPC planar preparations, as it happens with lyso-PC and with PGPC/PLPC 2:1, yet not to successfully reorient the inner bilayer core to a large extent. In this case, EPR spectral analysis by alone is unable to yield structural information. Nonetheless, in case of anisotropic EPR spectra, this ambiguity is removed, since the bilayers must be both planar and ordered to display orientation dependence of the enclosed spin label EPR spectrum.

![Fig. 5. Elution profile of Sepharose 4B gel-chromatography of aqueous suspensions of: 100% PGPC (dashed line); 2:1 PGPC/PLPC (dotted line); PGPC/C 3:2 and PGPC 2:1:2 (solid line).](image)

![Fig. 6. Cryo Electron Micrograph of aqueous suspensions of: A, either pure PGPC or pure lyso-PC; B, PGPC/C 3:2; C, lyso-PC/C 3:2; D, pure PLPC. Experimental conditions are described under Materials and methods.](image)
Correct attribution of Sepharose 4B elution profile to either MLV or micelles (V_0/V = 1 or V_0/V = 1.6, respectively) was checked by cryo Electron Microscopy, in order to exclude any possibility of artifactual structures possibly behaving like MLV under gel-chromatography. Cryo electron micrographs of extruded aqueous suspensions of PGPC 100% and of PGPC/C 60:40 by mole (‘yielding elution profiles identical to those reported in Fig. 5, not shown) are presented in Fig. 6. In agreement with the gel-chromatography size analysis, vesicular structures with about 100-nm diameter similar to those formed by pure PLPC (shown in panel D) are clearly visible in 60:40 PGPC/C (panel B) and lyso-PC/C (panel C) mixtures. The blank micrograph of pure PGPC aqueous suspension (panel A, common also to lyso-PC, not shown) is considered confirmative of micelles presence since particles eluting at V_0/V = 1.6 must be tiny structures made of transparent organic matter lacking the right optical contrast useful to make them appear in microphotographs. From this figure, the newly described transition of PGPC micelles (panel A) to PGPC/C vesicles (panel B) is readily apparent, as is the already described transition of lyso-PC micelles to lyso-PC/C vesicles, definitely confirming the fundamental restructurung role of cholesterol on oxidized phosphatidylcholine-containing membranes.

A possible explanation of this effect on PGPC membrane formation may be based on the very different behavior of the glucorolyl residue with respect to the parent arachidonic acid, due to both its reduced length and ionisation. Escape of this residue to the outer water medium has been long since proposed [45–47] and recently confirmed [17,18,48]. We argue that the single fatty acyl tail remaining in the hydrophobic core gives rise to a hydrophobic region geometry of PGPC very similar to the one of lyso-PC, yielding an explanation of the observed micelle-forming ability on the basis of molecular shape [49,50]. Moreover, this similarity allows application of geometrical criteria adopted to explain the transition of lyso-PC micelles to lyso-PC/cholesterol bilayers [51,52], to the case of PGPC/cholesterol mixtures, thus explaining the gel-chromatographic behavior of PGPC aqueous suspensions in the absence and in the presence of cholesterol. In addition, this explanation also applies to the anisotropy recovery of EPR spectra of PGPC/PLPC planar samples upon addition of cholesterol, in analogy to lyso-PC planar preparations.

In conclusion, the present results show that membrane cholesterol is able to consistently keep fatty acids in the normal orientation (in the case of HOPNPC SPB) and to transform PGPC-containing micelles into fairly internally aligned bilayers, though not at the maximum extent as in the case of lyso-PC. A protective role of cholesterol on membranes against ROS attack to unsaturated lipids has already been advanced [53–55]. Our results show that a new structurally important protection role is also exerted by cholesterol on lipoperoxidized membranes based on this lipid ability to contrast fatty acid disordering and to maintain the bilayer structure in spite of conspicuous oxidation of the constituent phospholipids. On this basis, similar protection is also predictable against bilayer disordering by other cleaved-chain oxidized PCs (palmitoyl-olaeoyl-phosphatidylcholine, palmitoyl-oxonanoyl-phosphatidylcholine and palmitoyl-oxyvaleroyl-phosphatidylcholine) of which micelle-forming tendency has been previously observed [22].

The bilayer-stabilizing role of cholesterol is thereby enlarged to encompass peroxidized membranes.

**Errata corrig**

The structure of the molecule referred to as HOPNPC (1-palmitoyl-2-(3-hydroxy-9,11-octadecanediyl)-phosphatidylcholine) correctly drawn in Fig. 1 does not correspond to the one reported in refs. [21,22]. The authors want to make clear that the latter was erroneously drawn, and that the error is limited to the mere drawing. The correct structure of HOPNPC is the one reported in Ref. [20], as checked by MS of lipoxigenase-1 reaction product, in agreement with Refs [25,26], and is the same as that in Fig. 1 of the present paper.

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