Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1778 (2008) 143 – 152
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbamem

Different oxidized phospholipid molecules unequally affect bilayer packing

Francesco M. Megli *, Luciana Russo

Dipartimento di Biochimica e Biologia Molecolare "E. Quagliariello", Università di Bari, and Centro di Studio sui Mitocondri e Metabolismo Energetico, CNR, Via E. Orabona, 4-70126 Bari, Italy

Received 9 May 2007; received in revised form 13 September 2007; accepted 18 September 2007
Available online 17 October 2007

Abstract

The aim of this study was to gain more detailed knowledge about the effect of the presence of defined oxidized phospholipid molecules in phospholipid bilayers. After chromatographic and mass spectrometry analysis, the previously used product of the Fenton reaction with unsaturated lecithins proved to consist of a plethora of oxidatively modified lecithins, unuseful either for the detailed study of the effects brought about in the bilayer or as the source of defined oxidized phospholipid molecules. The latter, particularly 2-(ω-carboxyacyl)- and 2-(*n*-hydroperoxyacyl)-lecithins, can be more conveniently prepared by chemical or enzymatic synthesis rather than by chemical or physical oxidation. The effect of those molecules and of commercially available 12-hydroxy-stearic and dodecanedioic acid was studied in planar supported phospholipid bilayers (SPBs) by use of EPR spectrometry. The SPBs also contained 2-(5-doxylstearoyl)-lecithin as the spin probe, and the EPR spectral anisotropy loss, indicative of bilayer disordering, was measured as a function of the molar percentage of oxidized lipid. Most oxidized lipid molecules examined in this study were able to induce bilayer disordering, while hydroperoxyl group-bearing acyl chains appeared to be much less effective. It is concluded that the effects of different oxidized phospholipids on phospholipid bilayer structure cannot be generalized, as happens with batch-oxidized phospholipids, and that the use of defined oxidized phospholipid molecular species for membrane oxidative stress guarantees a more reliable and detailed response.

Keywords: Phospholipid bilayer; Lipoperoxidation; EPR; Spin labeling; Bilayer disordering; EPR spectral anisotropy

1. Introduction

Lipid peroxidation occurring in biomembranes after oxidative stress-formed ROS and RNS attack is receiving much attention, and despite its occurrence in many pathological states, as routinely shown based on the presence of MDA and conjugated dienes, lipoperoxidation products are currently studied in models prepared by chemical or physical oxidation of lecithins, under different aggregation forms [1–7]. Invariably, these protocols produce a complex ensemble of oxidized phospholipid molecules, the composition of which can be usually resolved only by HPLC/mass spectrometry analysis. Inspection of the literature data leads to the conclusion that the oxidation mixture composition strongly depends upon the particular protocol and the reaction conditions in such a critical way that, in our opinion, formation of defined oxidation products is not always guaranteed.

In our previous research [8–11] we adopted the Fenton reaction as the oxidation model putatively closest to the one occurring in the living cell [12] even if, given the large number of oxidation models and the wide composition variety of outcoming oxidized mixtures, it seems difficult to decide which one will best mimic that naturally occurring in living cells under given oxidative stress conditions. In those studies we were able to attribute the observed bilayer disordering to oxidized phospholipids in general, based on results from model membranes including Fenton-oxidized lecithins. Nonetheless, increasingly detailed knowledge of lipoperoxidized products accumulating from HPLC/mass spectrometry analysis has made attribution of the observed effects to the whole oxidized phospholipid mixture appear more and more a rough approximation. At the same time, the convenience of studies aimed at defining membrane altering properties of specific oxidized phospholipid molecules [13] has progressively emerged. Unfortunately, mass spectrometry

* Corresponding author. Tel./fax: +39 080 5443367/17.
E-mail address: f.m.megli@biologia.uniba.it (F.M. Megli).

0005-2736/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2007.09.014

analysis showed that various attempts to further purify defined single molecules from the Fenton oxidation melange [4] in quantitative yield by reversed-phase flash chromatography were unsuccessful.

As a preliminary test of a different strategy, we checked the effects exerted on the bilayer by free fatty acids simulating those present in oxidized phospholipids, that is dodecanedioic (DDA) and azelaic acid (mimicking a carboxyl terminated acyl chain) and 12-hydroxystearic acid (12-(OH)-SA) (mimicking hydroxyl-bearing fatty acids). Hence, as an alternative route to oxidized phospholipid molecules, we adopted chemical and enzymatic synthesis through already established methods, yielding some of the major phospholipid oxides commonly present in the oxidation mixture [1–7], such as 2-azelaoyl-lecithin (AzEPC), 2-glutaroyl-lecithin (GlEPC), 1-palmitoyl-2-(13-hydroperoxy-10,12-octadecanedienoyl)-lecithin (HPPLPC, 2-(13-HPODE)-lecithin), 1-stearoyl-2-(15-hydroperoxy-5,8,11,13-eicosatetraenoyl)-lecithin (HPSAPC, 2-(13-HPETE)-lecithin, in pure form and in reasonably high yield (in our experimental mass scale).

It will be shown that disturbance to the host phospholipid bilayer by these molecules strongly varies with their chemical nature, most of them confirming our previous results obtained by use of the Fenton mix. Nonetheless, the observed differences between defined lipid oxides, undetectable when using the oxidation mixture, confirm our lack of detailed knowledge of their impact on bilayer structure and suggest the usefulness of studies aimed at characterizing specific oxidized phospholipid molecules under the aspect of the peculiar disturbing effect exerted on membranes.

2. Materials and methods

2.1. Materials

5-doxylstearic acid (5DSA), glutaric acid, azelaic acid, dodecanedioic acid, 12-hydroxystearic acid, lecithin and 2-*lyso*-lecithin from egg yolk, 1-palmitoyl-2-linoleoyl-glycero-3-phosphocholine (PLPC), 1-stearoyl-2-arachidonoyl-glycero-3-phosphocholine (SAPC) and lipoxidase (type V from soybean, EC 1.13.11.12) were purchased from Sigma; 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.

2.2. Phospholipid modification

Oxidation of PLPC and SAPC was performed by the Fenton reaction, as already described [8]. Oxidized lipids were freed from non-oxidized material by reverse-phase column chromatography [10]. Further separation of different oxidized species was attempted by the same method, as described below, followed by preparative TLC developed with $CHCl_3/CH_3OH/H_2O$ 65:25:4 (vol). 2-(5-doxylstearoyl)-lecithin (5DSPC) was obtained by coupling 5DSA to egg yolk lysolecithin according to Boss et al. [14], and purified by flash-chromatography. AzEPC and GlEPC were obtained by the method of Schlame [15] starting from egg yolk lysolecithin. These products were purified by preparative TLC developed with $CHCl_3/CH_3OH/NH_4OH_{conc}$ 65:25:8 (vol). HPPLPC and HPSAPC were obtained by action of lipoxidase on PLPC and SAPC, respectively [16,17], and purified by reverse-phase preparative column chromatography using $CH_3OH/(C_2H_5)_2/H_2O$ 95:5:2 (vol) (see Preparative flash chromatography). The identity and purity of all synthesised molecular species was confirmed by analytical TLC and by mass spectrometry [3–5]. Quantitative phosphorus analysis was performed according to Nakamura [18].

2.3. Preparative flash chromatography

Two 12-cm long (1-cm i.d.) RP18 Silicagel columns were packed with the Büchi Cartridger and coupled in series. The crude final oxidized phospholipid mixture was extracted by the method of Bligh and Dyer [19], dissolved in a small volume of $CH_3OH/(C_2H_5)_2O/H_2O$ 95:5:2 and eluted with the same solvent with 12 ml/min flow-rate. The eluent was simultaneously monitored at both 206 nm (λ_{max} of normal phospholipid) and 235 nm (λ_{max} of oxidized phospholipid conjugated dienes) with a Cary 50 UV-VIS spectrophotometer equipped with a Hellma continuous-flow cuvette. Based on OD_{235}, fractions containing oxidized phospholipids eluting between 2.5-min and 5.5-min retention time were pooled, concentrated, and re-eluted through the same columns with $CH_3OH/(C_2H_5)_2O/H_2O$ 95:5:6, under the same conditions as those for the first run.

2.4. Mass spectrometry

Analysis of oxidized PLPC or SAPC was performed by electrospray ionization mass spectrometry (ESI-MS), using an LCQ Thermo ionic trap mass spectrometer, equipped with an electrospray ionization interface. Samples (5 μl of a water/methanol 1:1 solution containing 5 to 10 nmol phospholipid phosphorus) were introduced into the spectrometer by a syringe pump using a flow rate of 10 μl/min. Data acquisition was performed by the Xcalibur software in the *m/z* range comprised between 50 and 800 Thompson in MS-full scan and positive polarity mode. ESI interface settings were: 6 kV source voltage, 15 V capillary voltage, 190 °C, capillary temperature. Based on other authors' tandem MS analysis of the products of the Fenton reaction [4], oxidized molecular species were recognized by use of the Mass Frontier software yielding the calculated molecular weight of selected structures and related isotopic distribution. Attribution of a structure to a chosen spectral peak was based on the following criteria: matching of the observed to the calculated isotopic distribution, identity of the observed and calculated molecular weight, the structure's chemical coherence with the reaction producing it. These criteria were preliminarily validated by use of known standard phospholipid samples, both synthetic and commercial (not shown).

2.5. Analysis of EPR spectral anisotropy

A nitroxide *g*-value (which determines the centre of the spectrum) and the hyperfine splitting constant A (which determines the distance between hyperfine lines) are orientation dependent and axially symmetric, and in a spin labelled phospholipid the symmetry axis (the *z*-axis) is parallel to the fatty acid long axis. In hydrated oriented planar phospholipid bilayers on a solid support, phospholipid molecules orient perpendicularly to the supporting glass slide so that the fatty acid chain long axis is normal to its plane as is the *z*-axis of the nitroxide ring bound to the fatty residue [20]. Molecular tumbling of a nitroxide ring placed at a rigid chain position such as C-5 is strongly reduced resulting in its molecular frame being rigidly oriented with the spin label's *z*-axis aligned along the sample's *z*-axis orientation [21]. Under these conditions the sample behaves as an ordered crystal and, when the labels are oriented relative to the laboratory frame, the spectrum will change when the sample is rotated relative to the static magnetic field, thus yielding two well-separated EPR spectra. One is dominated by $g_{\|}$ and $A_{\|}$ (the parallel components of tensors *g* and *A*, respectively), only visible when the nitroxide *z*-axis (coaxial with both the fatty chain long axis and the glass slide normal) is parallel to the applied magnetic field. The other is dominated by $g_{\perp}$ and $A_{\perp}$ (the perpendicular components of tensors *g* and *A*, respectively), only appearing when the glass slide normal is perpendicular to the applied magnetic field [22,23]. The resulting angular dependence of EPR spectra of planar phospholipid bilayers reveals an ordered ensemble of phospholipid molecules. On the contrary, if the label's orientation is isotropic within the sample, then no dependence of the spectrum on the sample orientation will appear. In general, an intermediate degree of orientation will be revealed by a superimposition of the parallel and the perpendicular EPR spectra, indicating the existence of a fraction of either disordered fatty acid chains or an isotropic phospholipid phase.

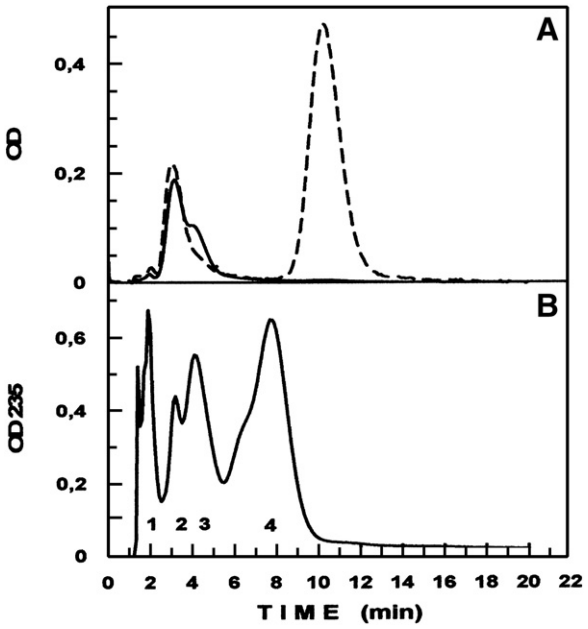

Fig. 1. (A) Elution profile of oxidized PLPC with $CH_3OH/(C_2H_5)_2O/H_2O$ 95:5:2, followed at (----) 206 nm and at (—) 235 nm. (B) Elution profile of oxidized phospholipids contained in the leading peak in panel A with $CH_3OH/(C_2H_5)_2O/H_2O$ 95:5:6.

The extent of disorder can be estimated from the height of the parallel spectrum bands appearing in the perpendicular EPR spectrum. The best-resolved parallel spectrum low-field band appearing in the perpendicular spectrum is the most convenient marker of disorder. Even more conveniently, EPR bands are best resolved in the second derivative presentation of spectra. From this presentation, the numerical value of an empirical disorder index R'' can be calculated as the ratio between the heights of the parallel spectrum low field band second derivative to the second derivative of the perpendicular spectrum low field band [8].

2.6. Measurement of EPR spectral anisotropy

200 nmol phospholipid in 50-μl ethanol, containing 2 mol% lipid spin label was deposited on a thin narrow glass slide and taken to dryness under vacuum (1–2 mBar, 37 °C). 5–10 μl distilled water was layered onto the bottom of a flat quartz tissue cell well (Wilmad WG-806-A, well dimensions: 7 × 23 × 0.5 mm) and the slide was placed into the well with its rear side adhering to the water layer. After covering the well, the phospholipid layer was allowed to hydrate as judged by the transparency of the sample [8].

The cell was inserted vertically into the cavity of a 9-GHz Varian E-9 Century Line EPR spectrometer at room temperature and oriented by means of a small goniometer mounted on top of it so that the normal to its plane (coincident with both the fatty acid chain direction and the sample's *z*-axis) was either perpendicular (⊥) or parallel (||) to the magnetic field (H) direction. Instrumental settings were: 338/335 mT (⊥/||) 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 (⊥/||). The degree of spectral anisotropy loss, indicative of membrane disordering, was gauged from the R'' parameter value, calculated from the perpendicular EPR spectra

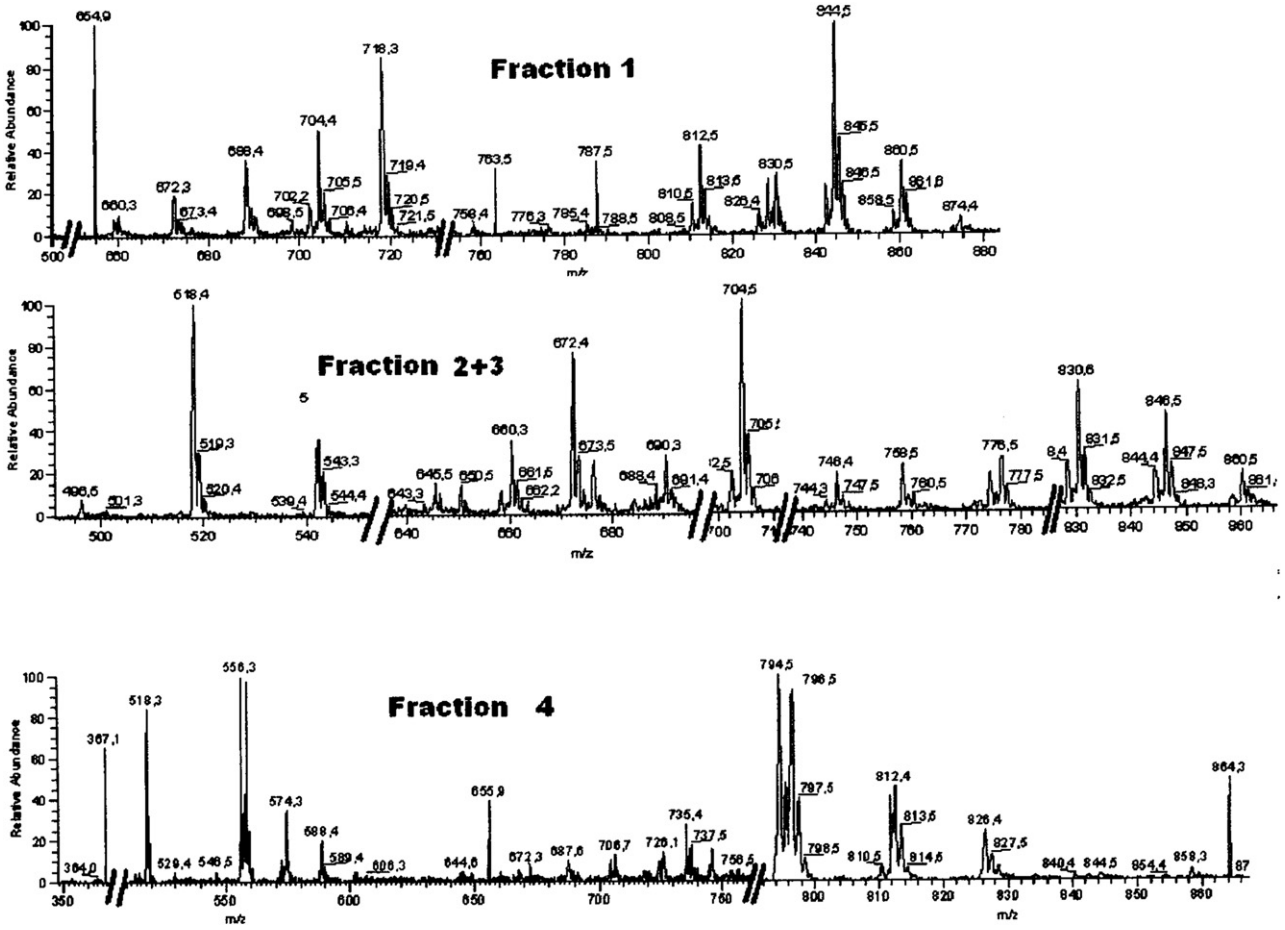

Fig. 2. Mass (ESI/MS) spectra of Fenton-oxidized PLPC oxides found in fractions 1–4 (see Fig. 1) after chromatographic separation.

Table 1
Mass spectrometry analysis of PLPC fractions after the Fenton oxidation

Oxidized product in fraction no	1 [a]	2+3	4	*m/z* (H^+)	*m/z* (Na^+)
1-palmitoyl-2-lyso-GPC	•	•	•	496.5	518.5
1-lyso-2-linoleoyl-GPC			•	520.4	544.4
1-lyso-2-(9,13-dioxo-11-hydroxy-10-octadecenoyl)-GPC			•	566.4	588.4
1-lyso-2-(9-oxo-10,12-octadecanedienoyl)-GPC			•	534.3	556.3
1-lyso-2-(9-oxo-12-octadecenoyl)-GPC			•	536.5	558.5
1-palmitoyl-2-(8-oxo-octanoyl)-GPC		•		636.0	658.0
1-palmitoyl-2-(9-oxo-nonanoyl)-GPC	•	•	•	650.4	672.4
1-palmitoyl-2-(9-oxo-8-hydroperoxy-nonanoyl)-GPC			•	682.5	704.5
1-palmitoyl-2-(9,12-dioxo-10-dodecenoyl)-GPC			•	704.1	726.1
1-palmitoyl-2-nonanedioyl-GPC	•	•	•	666.4	688.4
1-palmitoyl-2-octanedioyl-GPC		•		652.5	674.5
1-palmitoyl-2-(8-oxo-nonanedioyl)-GPC	•	•		680.2	702.2
1-palmitoyl-2-(9-hydroxy-10-dodecenedioyl)-GPC		•		722.3	744.3
1-palmitoyl-2-(9-hydroxydodecanedioyl)-GPC		•		724.4	746.4
1-palmitoyl-2-(9-hydroperoxy-10,12-octadecanedienoyl)-GPC			•	790.4	812.4
1-palmitoyl-2-(9-hydroxy-10,12-octadecanedienoyl)-GPC			•	774.5	796.5
1-palmitoyl-2-(9-oxo-10,12-octadecanedienoyl)-GPC			•	772.5	794.4
1-palmitoyl-2-(12-epoxy-9-idroxy-10-octadecenoyl)-GPC	•			790.5	812.5
1-palmitoyl-2-(9,12,13-trihydroxy-10-octadecenoyl)-GPC	•	•		808.5	830.5
1-palmitoyl-2-(8,9,14-trihydroxy-10,12- octadecanedienoyl)-GPC	•	•		806.5	828.5
1-palmitoyl-2-(8-oxo-9,14-dihydroxy-10,12- octadecanedienoyl)-GPC	•		•	804.4	826.4
1-palmitoyl-2-(9,13-dioxo-12-hydroperoxy-10- octadecenoyl)-GPC		•		820.5	842.5
1-palmitoyl-2-(9,13-dioxo-12-hydroperoxy-octadecanoyl)-GPC	•		•	822.5	844.5
1-palmitoyl-2-(13-oxo-12-hydroperoxy-9-hydroxy-octadecanoyl)-GPC		•		824.5	846.5
1-palmitoyl-2-(13-oxo-12-hydroperoxy-8,9-dihydroxy-10-octadecenoyl)-GPC	•	•		838.5	860.5
1-palmitoyl-2-(8,13-dioxo-12-hydroperoxy-9-hydroxy-10-octadecenoyl)-GPC	•	•	•	836.5	858.5

[a] Numbers refer to those in Fig. 1, panel B.

derivative as previously mentioned. According to its definition, R''-value increases with the degree of bilayer disorder.

3. Results

The separation of Fenton-oxidized PLPC from the unreacted phospholipid is shown in Fig. 1, panel A. As predictable from the expected higher polarity, the oxidized phospholipid broad peak eluted earlier (panel A, 2–5 min) than PLPC (panel A, 9–13 min, dashed line, 206 nm), as revealed by the absorption at 235 nm (solid line). Further fractionation of this peak, aimed at isolating pure molecular species of oxidized PLPC, is shown in panel B of the same figure. To this purpose, a more polar solvent composed of $CH_3OH/(C_2H_5)_2O/H_2O$ 95:5:6 was used to resolve fractions 1–4, as monitored by the absorption at 235 nm. These fractions (of which fractions 2 and 3 were pooled) were further purified by preparative TLC in order to get rid of overlapping peak ends, and then analysed by mass spectrometry in order to establish their molecular identity. Representative parts of the mass spectra of fractions 1, 2+3 and 4 are reported in Fig. 2. The results of this analysis, reported in Table 1, represent the best fit of structures hypothesized by use of the Mass Frontier software to the observed mass peaks, also taking into account results published by other authors [4]. Each $[MH]^+$ peak was overwhelmed by its $[MNa]^+$ complex, arising from the use of 10 mM NaCl during the phospholipid extraction procedure. Even if full validation of these data requires tandem MS analysis, we consider them an adequate criterion for evaluating the results of RP-18 column chromatography and preparative TLC application to the separation of the Fenton mixture. In fact MS data show that no fraction contained a pure compound and that all fractions shared many oxidation products, revealing that the oxidized phospholipid peak in panel A consisted of a complicated and unresolvable ensemble of oxidized species. Very similar results (not shown) were also obtained from the oxidation of SAPC by

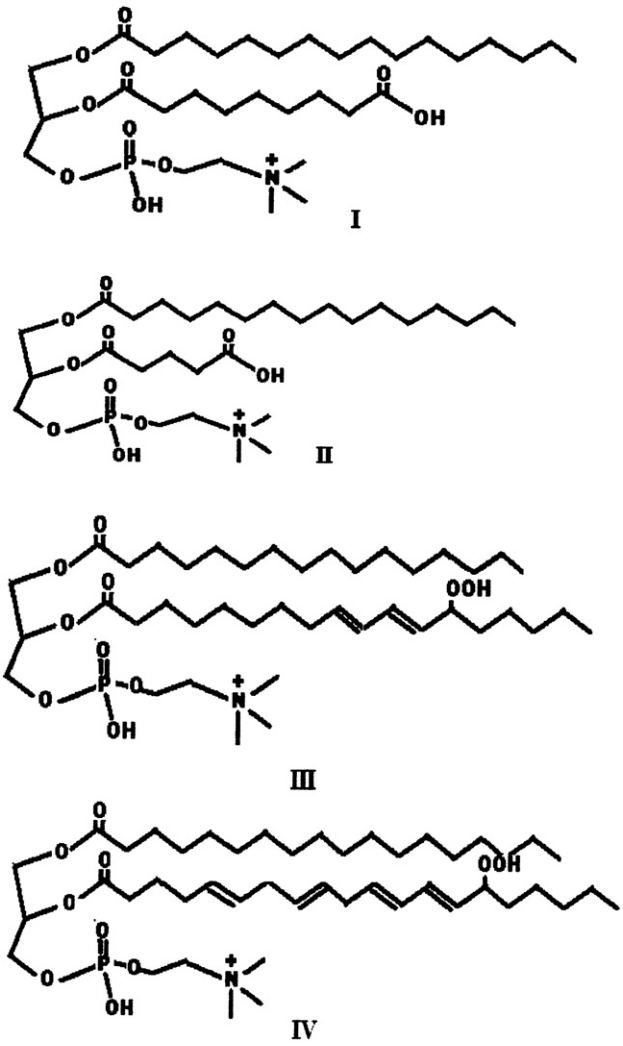

Fig. 3. Structure of I, 1-palmitoyl-2-azelaoyl-lecithin (AzEPC); II 1-palmitoyl-2-glutaroyl-lecithin (GlEPC); III, 1-palmitoyl-2-(13-hydroperoxy-10,12-octadecanedienoyl)-lecithin (HPPLPC), and IV, 1-stearoyl-2-(15-hydroperoxy-5,8,11,13-eicosatetraenoyl)-lecithin (HPSAPC).

the Fenton reagent, in keeping with results of other authors [4], rendering further investigation of MS analysis superfluous and definitely discouraging the use of chemical oxidation methods for preparative purposes.

On the contrary, the oxidized lecithin species shown in Fig. 3 were obtained in high amounts by already described chemical or enzymatic synthesis [3,5,15–17] and in the pure form after preparative column chromatography, as confirmed by chromatographic and mass spectrometry analysis (data not shown). In addition to these molecular species, free fatty acid chains bearing carboxyl or hydroxyl groups the same as those present in oxidized lecithins [1–7] (Table 1) are commercially available, and were considered useful to mimic the presence of oxidized phospholipid in the bilayer.

The loss of EPR spectral anisotropy of 5-DSPC in supported planar bilayers, visible as the loss of angular dependence of EPR spectra has already been successfully exploited as a valid tool, useful to assess the degree of geometrical order of the fatty acid chain packing [8–11,20–24]. Accordingly, fatty acid chains disordering in spin labelled SPBs is readily seen as the increasing mutual superimposing of parallel and perpendicular EPR spectra, irrespectively of the sample orientation with respect to the magnetic field (see Materials and methods).

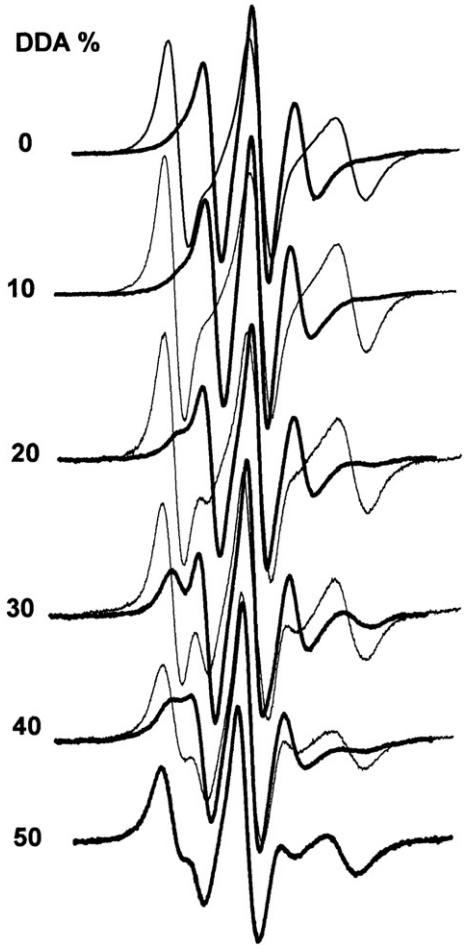

Fig. 4. EPR spectra of 5DSA-spin labelled SPBs made of egg yolk lecithin containing the indicated percentages of dodecanedioic acid. Orientation of sample normal: bold, ⊥ H; hairline, || H.

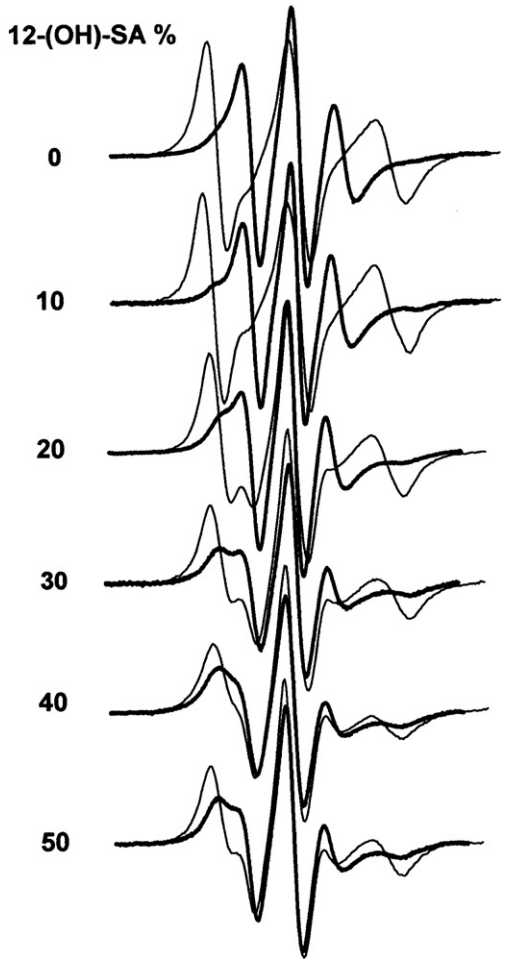

Fig. 5. EPR spectra of 5DSA-spin labelled SPBs made of egg yolk lecithin containing the indicated percentages of 12-hydroxystearic acid. Orientation of sample normal: bold, ⊥ H; hairline, || H.

With this criterion in mind, the phospholipid bilayer disordering arising in egg yolk lecithin SPBs after inclusion of increasing molar percentages of dodecanedioic acid (DDA) is clearly evident from the loss of anisotropy of EPR spectra of 5-DSA enclosed therein, reported in Fig. 4, starting from 10% per mol. Progressive addition of dicarboxylic acid simulates the growth of an ω-carboxyl-truncated form of oxidized PUFA-containing phospholipids such as those from PLPC after membrane lipoperoxidation. Since our study concerns the phospholipid bilayer structure, the maximum DDA molar percentage was kept below 50% in order to enable lecithin to maintain the bilayer framework. This percentage was enough to completely abolish spectral angular dependency (anisotropy), so that the parallel and perpendicular EPR spectra appear indistinguishable (Fig. 4, bottom), indicating complete bilayer disordering. These observations were confirmed by similar spectra obtained after inclusion of azelaic acid in the SPBs (results not shown).

Similar results are reported in Fig. 5, in which growth of hydroxy-groups after oxidative attack on the membrane is simulated by addition of increasing amounts of 12-hydroxystearic acid (12-(OH)-SA).

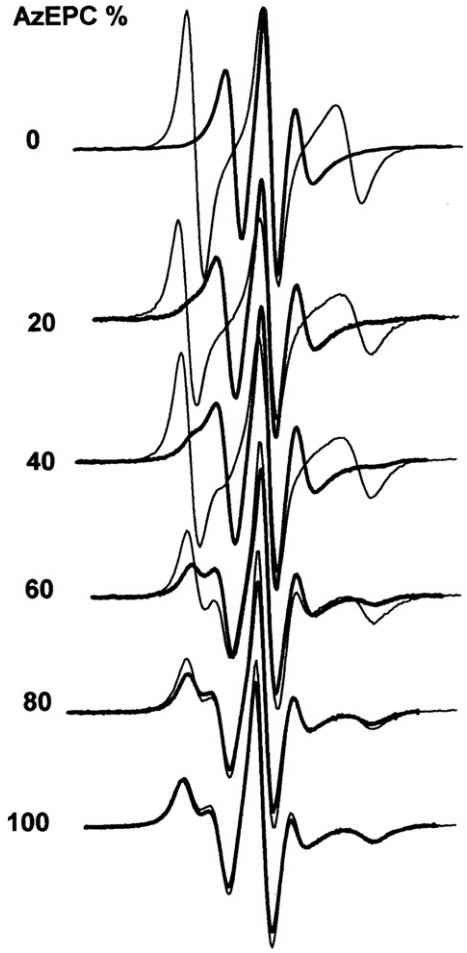

Fig. 6. EPR spectra of 5DSPC-spin labelled SPBs made of egg yolk lecithin containing the indicated percentages of 2-azelaoyl-lecithin. Orientation of sample normal: bold, ⊥ H; hairline, || H.

As expected from these preliminary experiments, oxidized lecithins containing carboxyl groups (namely 2-azelaoyl-lecithin, 2-glutaroyl-lecithin, (Fig. 3) [3–5] usually found in oxidation mixtures of PLPC and of SAPC respectively) were also able to disorder the fatty acid packing. In fact, inclusion of AzEPC in 5-DSPC spin labelled SPBs, leads to the EPR spectra shown in Fig. 6. As can be seen, and in agreement with the spectra in Fig. 4, increasing amounts of the carboxyl-terminated fatty chain-containing phospholipid were able to disturb and completely destroy the acyl residue order in egg yolk lecithin supported planar bilayers. The disordering effect of the carboxyl group in the hydrophobic core of the bilayer was definitely confirmed by inclusion of 2-glutaroyl-lecithin in SPBs, as evident from the spectra in Fig. 7.

Unexpectedly, the planar bilayers made of pure hydroperoxy-acyl species, 1-palmitoyl-2-(13-hydroperoxy-10,12-octadecanedienoyl)-lecithin (HPPLPC) and 1-stearoyl-2-(15-hydroperoxy-5,8,11,13-eicosatetraenoyl)-lecithin (HPASPC) showed either moderate or no disordering at all, respectively, as evident from the spectra reported in Fig. 8 compared with those of pure PLPC and pure SAPC SPBs in the same figure. EPR spectra of SPBs made of mixed oxidized/non-oxidized species in intermediate molar percentages were almost always similar to the blanks and have not been included in this figure.

An overview of the present data is portrayed in Fig. 9 as the trend of the R'' parameter, formerly introduced as the disorder parameter, for some of the presented EPR spectral series. For comparison, the R''-trend is reported as a function of the oxidized fatty acid chain equivalent percentage, so that 100% single chain-oxidized lecithin corresponds to 50% oxidized fatty acid. From this plot, the similarity of the effect of a carboxyl-terminated fatty chain, either bound or free, readily emerges (the closely matching trends of R'' calculated from free azelaic acid and azelaoyl-PC EPR spectra are not reported). In contrast with the carboxylic species, the ineffectiveness of the hydroperoxyl moiety in disordering the bilayer is readily apparent.

4. Discussion

The importance of studies dedicated to unveiling the properties of oxidized phospholipids and their related impact on bilayer structure and characteristics is better understood by considering that key indicators of oxidative stress found in many pathologies [25–27], such as malondialdehyde and

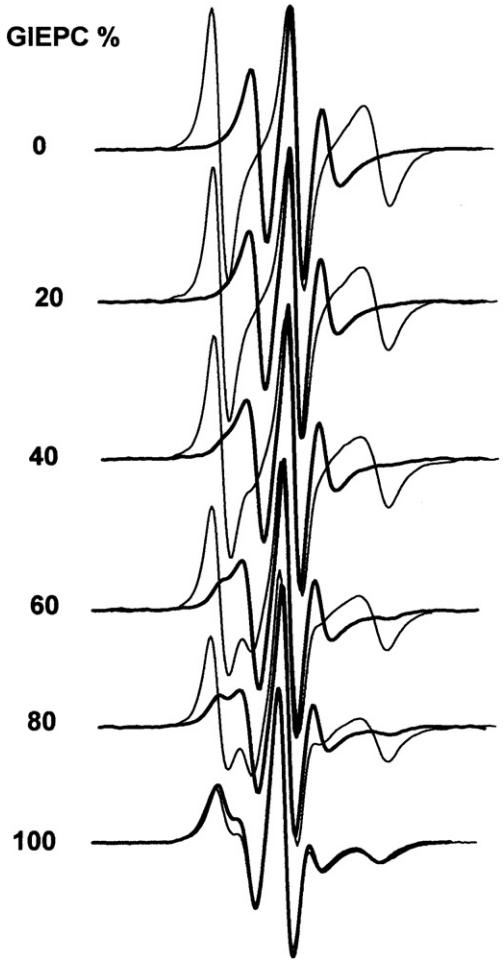

Fig. 7. EPR spectra of 5DSPC-spin labelled SPBs made of egg yolk lecithin containing the indicated percentages of 2-glutaroyl-lecithin. Orientation of sample normal: bold, ⊥ H; hairline, || H.

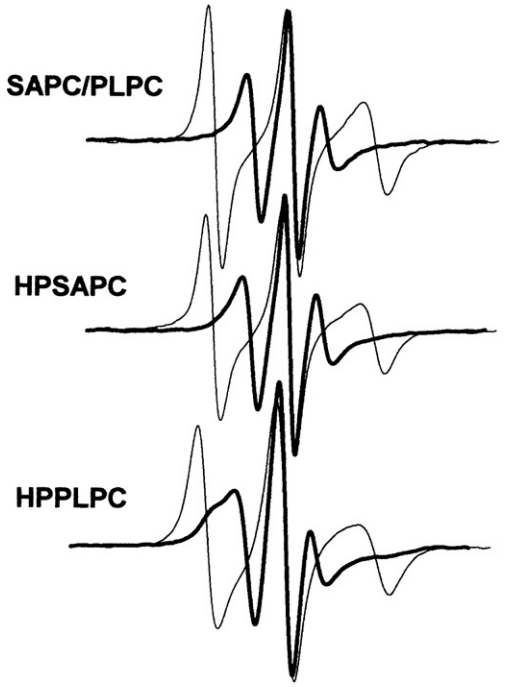

Fig. 8. EPR spectra of 5DSPC spin label in SPBs made of: pure SAPC or PLPC (top), 100% 2-(13-HPETE)-lecithin (HPSAPC) (center), 100% 2-(9-HPODE)-lecithin (HPPLPC) (bottom) as indicated. Orientation of sample normal: bold line, ⊥ H; hairline, || H.

conjugated dienes, originate from phospholipid oxidation [13]. Even though the search for deleterious effects of the so-detected ROS at the level of enzymes and nucleic acids is important for understanding the role of oxidative stress in pathology, the same importance should be given to the consideration that oxidized phospholipid molecules (the remains of those delivering MDA and conjugated dienes, signalling ROS attack) stay in the cell membrane and accumulate. Moreover, lipid peroxidation is a chain reaction leading to rapid propagation and spreading of free radicals in the attacked tissue cells, causing even greater accumulation of oxidized lipids under pathological conditions [25,26]. By considering that the radical chain reaction propagates through the bisallyl systems pathway, necessarily stopping at the (mono-un) saturated phospholipid domain boundaries, it can be argued that lipoperoxide accumulation might reach unpredictably high local levels in PUFA-rich membrane micro-domains. Owing to these molecules different chemical-physical properties, the so-formed locally high concentration of lipid oxides, might give rise to phase separation, as already demonstrated in aqueous lipid suspensions [11]. Therefore, artificial membranes containing varying percentages of oxidized phospholipids better model oxidized membrane micro-domains than whole membranes. From another point of view, given the existence of lipid micro-domains in natural membranes, no closely matching model can ever be built, even using natural phospholipid extracts. In fact, once phospholipids have been extracted from natural membranes, any information about their topology will be lost, and the resulting model membrane will take up the average composition of the original membrane, not necessarily similar to any natural micro-domain. Hence, membrane models must be legitimately considered as realistic test-benches useful to help understanding of which local membrane alterations can be elicited by lipoperoxides, and not as strictly natural membrane models.

Oxidative damage to phospholipid acyl chains causes rearrangement of double bonds, chain shortening, and the introduction of a variety of polar groups such as hydroperoxyl, hydroxyl, carbonyl and carboxyl moieties [13,28] in the hydrophobic core of the bilayer. As a consequence, structural characteristics and dynamics of lipid bilayers can be affected, as already proposed [29–31], possibly visible as functional impairment of integral enzymes. Malfunctioning of some ion carrier proteins in the presence of lipid oxides has been advanced as the molecular basis for some diseases [32]. Yet in spite of the obvious relevance to the understanding of the molecular basis of oxidative stress-related pathologies, the impact of oxidized lipid species on the phospholipid bilayer has received scarce attention. In this context, the recent application of powerful analytical methods of lipidomics, such as mass spectrometry (tandem ESI/MS) coupled to HPLC to identify lipid oxide molecules in the affected membranes of pathological tissues [33–37], underlines the importance of and need for intensive research dealing with these molecules, at least from the analytical point of view. In our opinion, it is equally important that these studies be complemented by the study of the impact of the recognized oxidized species on the bilayer structure and properties. To this aim, in order to build model membranes apt for these studies, the mass range should be up-scaled from the analytical to the preparative scale.

Batch oxidation of phospholipids is easily performed by several different methods [1–7], and we were able to demonstrate membrane alteration by Fenton-oxidized lecithins [8]. In our hands, a decrease in phospholipid acyl chain order was detected by the loss of anisotropy in EPR spectra of spin labelled oriented planar layers [8,22,23], and the presence of lipoperoxides has been confirmed in the oxidation mixture [4]. Similar membrane defects were also demonstrated for phospholipids from isolated rat liver mitochondria maintained in respiration state IV in vitro [9], as well as for liver mitochondrial phospholipids from rats treated with carbon tetrachloride [10].

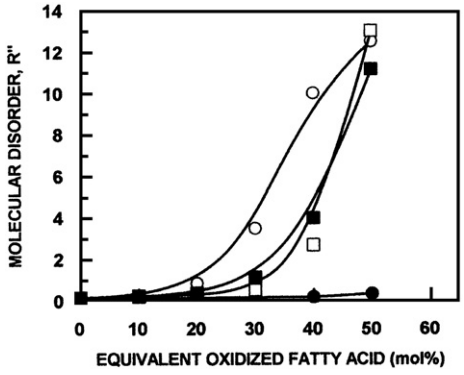

Fig. 9. Profile of EPR spectral anisotropy loss in egg yolk lecithin SPBs containing variable amounts of various oxidized lipids, expressed as the R'' value measuring the phospholipid bilayer disordering. ○, 12-(OH)-SA; ●, HPPLPC; □, DDA; ■, GlEPC. R''-values were calculated from the second derivative of EPR spectra (not shown) as described in [8].

Nevertheless, taking into account the accumulating knowledge of the variety of oxidized phospholipid species from tandem ESI/MS analyses, it seemed more appropriate for us to update our previous results by considering the various oxidized molecules separately, and to study in greater detail how each one impinges upon the membrane, as we recently did in the study of lipoxidized monolayers [13]. As a first attempt at the preparation of single oxidized phospholipid molecules in useful amounts, chromatographic fractionation of the previously used Fenton oxidation mixture proved useless. In fact, the separation of well-resolved peaks appearing in the chromatogram in Fig. 1 did not correspond to separated molecular species, as shown by mass spectrometry analysis (Fig. 2 and Table 1), indicating that even if batch phospholipid oxidation might represent an attractively rich source of lipoperoxides, yet it can turn out to be of limited use for preparative purposes. Better definition of the oxidized molecular species formed during the Fenton reaction, as already given by other authors [4], is not required at this stage of our study. In fact, our use of ESI-MS is limited to the qualitative evaluation of chromatographic separation of those species. To this end, our data are sufficient to suggest discarding the oxidation/chromatography preparative strategy, and do not require further analysis, such as tandem MS, for our present purpose.

Before adopting chemical synthesis as the alternative route to the desired amount of phospholipid oxides, the effects of the presence of carboxylic and hydroxyl groups in the bilayer were tested by using commercially available simpler molecules such as 12-hydroxystearic acid, dodecanedioic acid and azelaic acid (results not shown). The rationale for using modified fatty acids to bring polar groups into the bilayer was the same as that for the use of fatty acid spin labels to bring the nitroxide group therein. This spin probe, specifically 5-DSA, was used as the spin label in experiments with modified fatty acids for the sake of homogeneity. Top EPR spectra in Fig. 4 and in Fig. 5 show perfect angular dependence, signalling ordered fatty acid packing. Thereafter, by increasing amounts of modified fatty acids the spin label readily reveals progressive and complete loss of packing order.

Since its first observation, anisotropy loss in oriented planar samples was attributed to fatty acid chain disordering [24] even though, in general, it would also signal transition of the membrane to an isotropic phase (un-aligned samples such as micelles or vesicles). In fact, even if acyl chain disordering has been clearly indicated in lipid monolayers containing oxidised phospholipids [13], completely isotropic spectra from unaligned planar samples (e.g., those in Fig. 4 at 50% DDA and Fig. 6, at 100% AzEPC) strongly resemble EPR spectra from 5-DSPC in multi-lamellar vesicles (MLVs). Even if the preparation protocol of oriented samples rules out formation of these structures in the presence of high percentages of oxidized phospholipids, the possibility of phase separation and formation of an isotropic phase in our planar samples cannot be excluded, due to the strong chemical–physical differences between oxidised phospholipids and normal ones. As a matter of fact, transition to micelles had already been considered [8] and experimentally observed in aqueous vesicle suspensions [11] containing more than 40% by mole Fenton-oxidized PLPC. In this case, the observed anisotropy loss could consequently represent the signal for membrane breakdown due to extreme oxidative stress.

The striking inefficacy of hydroperoxyl-lecithins in disordering compared to the strong effect of carboxyacyl-lecithins can be speculatively attributed to the presence of the long hydrophobic tail remaining in the former. It can be argued that the longer the hydrophobic residue following the oxidized polar group, the stronger it anchors to the hydrophobic core of the bilayer, so that chain disorienting could turn out to be the result of competing forces between the oxidatively formed polar group and the remaining (if any) hydrophobic fatty acid tail. The case for 2-(ω-carboxyl) moiety might represent an example of complete imbalance towards the most polar end, as indicated by its dramatic effects reported in Figs. 6 and 7 (see also Fig. 9), while complete inefficacy of HPSAPC could represent complete imbalance towards the most non-polar end. Further support for this idea might be found in the relatively stronger disordering by shorter HPPLPC in comparison to HPSAPC, as apparent in Fig. 8. Nonetheless, this argument does not apply to the disorienting efficacy of 12-(OH)-SA, even if at this stage this result must be taken as a mere suggestion that a more precise comparison should be made with better suited 2-(12-hydroxystearoyl)-lecithin and 2-(13-hydroxy-10,12-octadecanedienoyl)-lecithin. The issue requires more chemical synthesis work, currently underway in our laboratory, and is also being investigated by use of a different spin probe, 3-doxyl-cholestane, as the different orientation of the coordinate system of this probe provides another perspective on planar samples [22].

5. Concluding remarks

The present results strongly support the idea that different structural characteristics of oxidized phospholipids result in differential influence on the phospholipid bilayer structure, and represent a further step in the knowledge of how lipoperoxidation impinges upon the membrane structure, with respect to our previous studies and other authors' work. In fact, the use of products of batch oxidation of phospholipids (or whole membranes) cannot reveal how an evoked membrane structure/property alteration is related to the presence of a particular oxidized group. As we have shown, the carboxyl group seems to create much more disturbance than the hydroperoxyl, while the latter in turn exhibits differences from the hydroxyl group. More differences seem to arise from the length of the remaining hydrophobic tail, and maybe also from the bisallyl system. In this context, the present data suggest more research into other synthetic oxidized lecithin molecules bearing hydroxyl instead of hydroperoxyl groups in both conjugated dienes and saturated chain forms. Studies of the impact of lipoperoxides on other membrane properties, such as the fluidity and polarity gradients, phase transition temperature and lateral phase separation, are also in progress.

Relating the outcome of these studies to the natural membrane is a difficult task, as is modelling an oxidatively-stressed living membrane. In fact, owing to the presence of membrane lipid micro-domains and their peculiar composition-dependent sensitivity to ROS/RNS attack, phospholipid oxides can

qualitatively and quantitatively vary from one micro-environment to the next. Further variability in the type of lipid oxides is expected from one membrane type to another, based on the variety of anti-oxidative defence systems of living cells that can steer the oxidation reaction chain to different products and stop it at different levels. On the other hand, ESI-MS lipidomic analysis reveals in great detail the existence of a large number of lipid peroxide species both in models and in oxidatively stressed natural membranes, also in pathological tissues [1–7]. The task of recomposing an artificial membrane putting together the right lipids, both normal and oxidized, in the right places and in the right amount in order to model a pathologically oxidized natural membrane does not seem a realistic proposition. As an alternative strategy, in our study simple model membranes have been more conveniently used as test-benches, useful to characterize the behaviour of an oxidized species in detail and from many aspects. Given the availability of quantitative preparation protocols, different amounts of oxidized species could be tested, regardless of the natural percentage in order to focus on their effects rather than on an unpredictable natural membrane model. Even if this study is just at the beginning and this approach requires novel chemical synthetic routes to the many oxidized phospholipid molecules, the expected outcome is a panel of features to be attached to each lipid oxide structure, useful to predict the deleterious effects it may produce in a membrane once it has been found therein.

Acknowledgements

The authors would like to thank Dr. Ilario Losito for mass spectrometry analysis, Mr. Domenico Munno for laboratory assistance, and Ms. Elena Conte for skillful experimental collaboration.

References

[1] A. Reis, M.R. Domingues, F.M. Amado, A.J. Ferrer-Correia, P. Domingues, Separation of peroxidation products of diacyl-phosphatidylcholines by reversed-phase liquid chromatography-mass spectrometry, Biomed. Chromatogr. 19 (2005) 129–137.

[2] J. Adachi, N. Yoshoka, M. Sato, K. Nakagawa, Y. Yamamoto, Y. Ueno, Detection of phosphatidylcholine oxidation products in rat heart using quadrupole time-of-flight mass spectrometry, J. Chromatogr., B. 823 (2005) 37–43.

[3] M.S. Göpfert, F. Siedler, W. Siess, A. Sellmeyer, Structural identification of oxidized acyl-phosphatidylcholines that induce platelet activation, J. Vasc. Res. 42 (2004) 120–132.

[4] A. Reis, P. Domingues, A.J.V. Ferrer-Correira, M.R.M. Domingues, Fragmentation study of short-chain products derived from oxidation of diacylphosphatidylcholines by electrospray tandem mass spectrometry: identification of novel short-chain products, Rapid Commun. Mass Spectrom. 18 (2004) 2849–2858.

[5] A.D. Watson, N. Leitinger, M. Navab, K.F. Faull, J.L. Hörkö, W. Witztum, D. Palinski, R.G. Schwenke, W. Salomon, G. Sha, A.M. Subbanagounder, J.A. Fogelman, Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interaction and evidence for their presence in vivo, J. Biol. Chem. 272 (1997) 13597–13607.

[6] J. Terao, S.S. Shibata, S. Matsushita, Selective quantification of arachidonic acid hydroperoxides and their hydroxy derivatives in reverse-phase High Performance Liquid Chromatography, Anal. Biochem. 169 (1988) 415–423.

[7] J. Terao, I. Asano, S. Matsushita, Preparation of hydroperoxy and hydroxy derivatives of rat liver phosphatidylcholine and phosphatidylethanolamine, Lipids 20 (1985) 312–317.

[8] F.M. Megli, K. Sabatini, EPR studies of phospholipid bilayers after lipoperoxidation. 1. Inner molecular order and fluidity gradient, Chem. Phys. Lipids 125 (2003) 161–172.

[9] F.M. Megli, K. Sabatini, Respiration state-IV-generated ROS destroy the mitochondrial bilayer packing order in vitro. An EPR study, FEBS Lett. 550 (2003) 185–189.

[10] F.M. Megli, K. Sabatini, Mitochondrial phospholipid bilayer structure is ruined after liver oxidative injury in vivo, FEBS Lett. 573 (2004) 68–72.

[11] F.M. Megli, L. Russo, K. Sabatini, Oxidized phospholipids induce phase separation in lipid vesicles, FEBS Lett. 579 (2005) 4577–4584.

[12] B. Halliwell, J.M.C. Gutteridge, Role of free radicals and catalytic metal ions in human disease: an overview, Methods Enzymol. 186 (1990) 1–85.

[13] K. Sabatini, J.-P. Mattila, F.M. Megli, P.K.J. Kinnunen, Characterization of two oxidatively modified phospholipids in mixed monolayers with DPPC, Biophys. J. 90 (2006) 4488–4499.

[14] W.F. Boss, C.J. Kelley, F.R. Landsberger, A novel synthesis of spin label derivatives of phosphatidylcholine, Anal. Biochem. 64 (1974) 289–292.

[15] M. Schlame, R. Haupt, I. Wiswedel, W.J. Kox, B Rüstow, Identification of short chain oxidized phosphatidylcholine in human plasma, J. Lipid Res. 37 (1996) 2608–2615.

[16] J. Eskola, S. Laakso, Bile salt-dependent oxygenation of polyunsaturated phosphatidylcholines by soybean lipoxigenase-1, Biochim. Biophys. Acta 751 (1983) 305–311.

[17] M. Perez-Gilabert, G.A. Veldink, J.F.G. Vliegenthart, Oxidation of dilineoyl phosphatidylcholine by lipoxigenase 1 from soybean, Arch. Biochem. Biophys. 354 (1998) 18–23.

[18] G.R. Nakamura, Microdetermination of phosphorus, Anal. Chem. 24 (1952) 1372.

[19] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.

[20] P. Jost, L.J. Libertini, V.C. Hebert, O.H. Griffith, Lipid spin labels in lecithin multilayers. A study of motion along fatty acid chain, J. Biol. Mol. 59 (1971) 77–98.

[21] O.H. Griffith, P. Jost, Lipid spin labels in biological membranes, in: L.J. Berliner (Ed.), Spin Labeling, Academic Press, New York, 1976, pp. 453–523.

[22] I.C.P. Smith, K.W. Butler, Oriented lipid systems as model membranes, in: L.J. Berliner (Ed.), Spin Labeling, Academic Press, New York, 1976, pp. 411–451.

[23] J. Seelig, Anisotropic motion in liquid crystalline structures, in: L.J. Berliner (Ed.), Spin Labeling, Academic Press, New York, 1976, pp. 373–409.

[24] P. Jost, A.S. Waggoner, O.H. Griffith, Spin labelling and membrane structure, in: L. Rothfield (Ed.), Structure and function of biological membranes, Academic Press, New York, 1971, pp. 83–144.

[25] M. Gago-Dominguez, J.E. Castelao, M.C. Pike, A. Sevanian, R.W. Haile, Role of lipid peroxidation in the epidemiology and prevention of breast cancer, Cancer Epidemiol. Biomark. Prev. 14 (2005) 2829–2839.

[26] L.G. Wood, P.G. Gibson, M.L. Garg, Biomarkers of lipid peroxidation, airway inflammation and asthma, Eur. Respir. J. 21 (2003) 177–186.

[27] P. Cejas, E. Casado, C. Belda-Iniesta, J. De Castro, E. Espinosa, A. Redondo, M. Sereno, M.A. Garcia-Cabezas, J.A.F. Vara, A. Domìnguez-Càceres, R. Perona, M. Gonzales-Baron, Implication of oxidative stress and cell membrane lipid peroxidation in human cancer (Spain), Cancer Causes Control 15 (2004) 707–719.

[28] W.A. Pryor, in: W.A. Pryor (Ed.), Free Radicals in Biology, Academic Press, New York, 1976, pp. 15–16.

[29] S.P. Gabbita, D.A. Butterfield, K. Hensley, W. Shaw, J.M. Carney, Aging and caloric restriction affect mitochondrial respiration and lipid membrane status: an electron paramagnetic resonance investigation, Free Radic. Biol. Med. 23 (1997) 191–201.

[30] S.P. Gabbita, R. Subramaniam, F. Allouch, J.M. Carney, D.A. Butterfield,

Effects of mitochondrial respiratory stimulation on membrane lipids and proteins: an electron paramagnetic resonance investigation, Biochim. Biophys. Acta 1372 (1998) 163–173.
[31] M.F. Nepomuceno, A. Alonso, L. Pereira-Da-Silva, M. Tabak, Inhibitory effect of dipyridamole and its derivatives on lipid peroxidation in mitochondria, Free Radic. Biol. Med. 23 (1997) 1046–1054.
[32] J.I. Kourie, Interaction of reactive oxygen species with ion transport mechanism, Am. J. Physiol. 275 (1998) C1–C24 (Cell Physiol. 44).
[33] H. Yin, N.A. Porter, New insights regarding the autoxidation of polyunsaturated fatty acids, Antioxid. Redox Signal. 7 (2005) 170–184.
[34] P.W. Connelly, D. Draganov, G.F. Maguire, Paraoxonase-1 does not reduce or modify oxidation of phospholipids by peroxynitrite, Free Radic. Biol. Med. 38 (2005) 164–174.
[35] G. Spiteller, Lipid peroxidation in aging and age-dependent diseases, Exp. Gerontol. 36 (2001) 1425–1457.
[36] X. Han, R.W. Gross, Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples, Mass Spectrom. Rev. 24 (2005) 367–412.
[37] X. Han, R.W. Gross, Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics, J. Lipid Res. 44 (2003) 1071–1079.