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Mitochondrial phospholipid bilayer structure is ruined after liver oxidative injury in vivo

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Abstract The purpose of this study was to investigate whether, after oxidative injury in vivo, liver mitochondrial phospholipids suffered from structural defects similar to those we have previously observed after either chemical oxidation or respiration state IV incubation of isolated mitochondria in vitro. Oxidative injury of the liver was simulated by endogastric administration of CCl_4 to rats in variable amounts for different times, under various conditions. Measurements of the phospholipid bilayer packing order were carried out by electron paramagnetic resonance (EPR) spectrometry of oriented planar samples of phospholipids extracted from liver mitochondria, spin labeled with 5-doxylstearoyl-lecithin. Disordering of the bilayer was revealed by the anisotropy loss of EPR spectra and reached a maximum value 4.5 h after CCl_4 administration, vanishing thereafter. The observed disorder also increased with the amount of CCl_4 administered, showing distinct dose-dependence, while administration of resveratrol soon after carbon tetrachloride decreased bilayer disordering by 50%. On the contrary, the order parameter *S* of spin labeled lecithin in isolated mitochondrial membranes from intoxicated rats revealed no change in membrane fluidity after oxidative stress. It is concluded that the phospholipid damage leading to disturbed bilayer geometry after oxidative attack already observed in model membranes and in isolated mitochondria in vitro also occurs in a simulated pathological state in vivo, indicating its possible occurrence also in real oxidative stress-linked pathologies as a contribution to the onset/sustaining of related diseases.

Keywords: Lipoperoxidation; CCl_4-intoxication; Phospholipid bilayer; Mitochondrion; Electron paramagnetic resonance; Spectral anisotropy

1. Introduction

Oxidative attack on membranes by reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) is considered responsible for or a supporter of various pathological states [1–3]. In many cases, oxidation of phospholipid polyunsaturated acyl chains, as revealed by malondialdehyde production and formation of conjugated dienes, is thought to bring about indirect functional damage through alterations of the normal lipid environs of integral proteins [4,5]. Recently, we were able to demonstrate that oxidation of phospholipids leads to alterations of the lipid bilayer consisting mainly in the loss of packing order of phospholipid molecules, visible as the anisotropy loss of electron paramagnetic resonance (EPR) spectra of spin labeled oriented planar layers, possibly as an early stage of a process leading to membrane breakdown. This effect was independent of the oxidizing system, as it was observed both in various lecithin types oxidized by the Fenton reagent [6] and in phospholipids of isolated mitochondria incubated under respiration state IV in vitro [7].

In this paper, we extend our EPR study of the bilayer packing order in oriented layers to liver mitochondrial phospholipids from a living intoxicated animal model, that is rats subjected to endogastric administration of carbon tetrachloride, under various conditions. The CCl_4-treated rat is a widespread animal model for hepato-pathology relying on the renowned intoxicating power of CCl_4 [8], acting as a radical initiator through its prime hepatic metabolite CCl_3 [9,10]. As a consequence, the chemical is capable of raising oxidative stress in the short term and hepatic cirrhosis upon prolonged treatment. We made use of it to find preliminary support for the idea that phospholipid bilayer disorders flank ROS-linked pathologies, and that peroxidized phospholipids at least in part could be considered as the molecular basis of some oxidative stress-induced diseases.

The packing order of oriented planar samples of mitochondrial phospholipids extracted from CCl_4-treated rat liver was monitored by measuring the anisotropy loss of the EPR spectra of spin labeled planar oriented samples as described [6]. We have shown that after administration of CCl_4, phospholipid bilayer disorder increases with treatment time until a maximum value, fading thereafter following a steady state profile, and that the observed disorder is dose-dependent. As observed in the case of isolated mitochondria, the renowned antioxidant resveratrol is able to counteract the effect of CCl_4 treatment, especially when administered simultaneously with carbon tetrachloride. On the contrary, membrane fluidity of intoxicated mitochondria, as measured in spin labeled membranes by the EPR order parameter *S*, proved to be unaffected, as it was in isolated mitochondria in vitro [7].

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Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; *n*-DSPC, β-*n*-doxylstearoyl-lecithin; EPR, electron paramagnetic resonance; SPB, supported phospholipid bilayers

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2. Materials and methods

2.1. Materials

Spin labeled lecithin was obtained by acylation of egg yolk *lyso*-lecithin (Sigma) with either 5- or 12- or 16-doxylstearic acid (Sigma) according to Boss et al. [11]. Resveratrol, 3,4′,5-trihydroxy-*trans*-stilbene, was also from Sigma.

2.2. Treatment of animals

Wistar albino rats weighting 180–220 g after fasting overnight were anaesthetized with diethyl ether. CCl_4 (in mineral oil 1:1 by vol.) was administered in the ratio of 4 g per kg body weight via gavage, and the animals were killed by decapitation after 1–24 h time intervals. Alternatively, CCl_4 was administered in 2–8 g (steps of 2 g) per kg and sacrifice followed after 4.5 h. In another set of experiments, 15 mg resveratrol (about 75 mg per kg body weight) suspended in 0.8 ml maize oil was administered to rats immediately after CCl_4 or after a 2 h time lag. 3–4 animals were used for each experimental datum.

2.3. Phospholipid preparation

Rat liver was taken soon after sacrifice and homogenized in a Potter–Evelhjem homogenizer in 250 mM sucrose and 1 mM Tris–HCl, pH 7.4. Mitochondria were prepared according to standard techniques in the same buffer and, after assaying protein content according to Bradford [12], lipids were extracted by the method of Bligh and Dyer [13]. Phospholipid phosphorus was assayed by the Nakamura method [14] and the presence of conjugated dienes was confirmed by reading phospholipid samples re-dissolved in cyclohexane at 235 nm as described [15].

2.4. Spin labeling of mitochondria

Spin labeled SUVs were prepared by sonication (Branson Sonifier W-250 D operated at 40 W output for 15 min at intervals, in an ice bath) of an EYPC/β-*n*-doxylstearoyl-lecithin (*n*-DSPC) 2:1 by mol mixture in 125 mM KCl, 25 mM HEPES, and 2.5 mM $MgCl_2$. After centrifuging (10 min 10 000 rpm, ALC PK121R centrifuge), the SUV suspension was concentrated to 5 mM in an Amicon ultra-filter. 5 mg of freshly prepared mitochondrial membranes was incubated with 0.1 ml SUV for 20 min at 37 °C. At the end, the incubation vessel was refrigerated in ice and 0.5 ml of 0.43 M sucrose was layered at its bottom. Spin labeled mitochondria were pelleted by centrifugation at 10 000 rpm for 10 min [16] and re-suspended immediately in 50 μl SUV buffer for EPR spectrometry and membrane fluidity measurement.

2.5. Measurement of EPR spectral anisotropy

50 μl ethanol solution of spin labeled phospholipids 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 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 [7].

The cell was inserted into the cavity of a 9-GHz Varian E-9 Century Line EPR spectrometer at room temperature and oriented so that the normal (coincident with the fatty acid chain direction) to its plane was either perpendicular or parallel to the magnetic field direction. Outcoming spectra were accordingly labeled either $\perp$ or $\parallel$, respectively. Instrumental settings were: 338/335 mT (perpendicular/parallel) field set with 12 mT scan width, 100 kHz and 0.2 mT modulation frequency and amplitude. Radiating field power and frequency were 20 mW and 9.4/9.5 GHz (perpendicular/parallel), respectively.

2.6. Membrane fluidity gradient determination

EPR spectra were scanned as above on 25 μl samples in glass capillaries at room temperature with field set and radiating field shifted to 339 mT and 9.35 GHz, respectively.

In both cases, the spectra were digitized by means of an EPR-Data System from STELAR (Mede, Italy) coupled to the spectrometer, using interactive software written by Stan Sykora.

2.7. Spectral analysis

In hydrated oriented planar phospholipid bilayers on solid support, phospholipid molecules orient perpendicularly to the supporting glass slide so that the fatty acid chains' *z*-axis is normal to its plane as is the *z*-axis of a nitroxide ring bound to the fatty acid chain of a spin labeled phospholipid. Molecular tumbling of a nitroxide ring placed at a rigid chain position such as C-5 is strongly reduced, resulting in the molecular frame rigidly oriented along *x*-, *y*- and *z*-axes directions, with the spin label's *z*-axis marking the sample's *z*-axis orientation. Under these conditions, the sample behaves as an ordered crystal and yields two well-separated EPR spectra: one dominated by $g_{\parallel}$ and $A_{\parallel}$ tensors, 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, and the other dominated by $g_{\perp}$ and $A_{\perp}$ tensors, appearing only when the glass slide normal is perpendicular to the applied magnetic field [17,18]. Therefore, the two spectra are not simultaneously visible and the perpendicular switches to the parallel one upon rotating the sample, showing angular dependence. As a consequence, angular dependence of EPR spectra of planar phospholipid bilayers reveals molecular order. Conversely, in this situation, the appearance of parallel EPR spectrum bands in the perpendicular spectrum and vice versa reveals fatty acid chain tilt and is displayed as loss of angular dependence which in turn becomes a telltale sign of packing disorder.

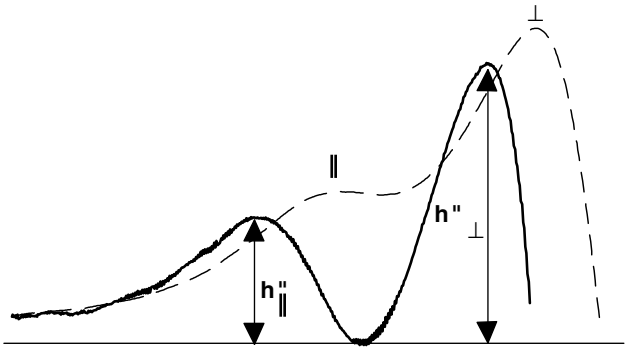

Fig. 1. Low-field band of the perpendicular EPR spectrum of a disordered SPB sample. Dashed line, first derivative; solid line, second derivative. Measurement is shown of the height of the parallel ($h''_{\parallel}$) and perpendicular ($h''_{\perp}$) component second derivative positive lobe, useful for calculation of the R'' parameter.

The extent of disorder can be estimated by the height of the parallel spectrum bands appearing in the perpendicular EPR spectrum. The better-resolved parallel spectrum low-field band of the perpendicular spectrum is the most convenient marker of disorder (Fig. 1, dashed line). EPR bands are best resolved in the second derivative presentation of spectra, as reported in Fig. 1 (solid line) [7]. In order to compare different disorder degrees, we devised an empirical disorder index R'' calculated as the ratio of the parallel spectrum low field band second derivative height to the height of the second derivative of the perpendicular spectrum low field band, as follows:

$$R'' = h''_{\parallel}/h''_{\perp}$$

with the quantities $h''_{\parallel}$ and $h''_{\perp}$ measured as illustrated in Fig. 1.

The mitochondrial membrane fluidity gradient was determined in normal and intoxicated rat liver mitochondria spin labeled with either 5-, 12- or 16-DSPC by measuring the order parameter S from the different spin labels EPR spectra, as follows [19,20]:

$$S = (A_{\parallel} - A_{\perp})/[A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})]$$

3. Results and discussion

Membrane oxidative attack after CCl_4 treatment of rats is a well established phenomenon widely used as a model for oxidative stress in vivo [8–10]. The molecule is quickly metabolized in the liver to the radical species $CCl_3^{\cdot}$ [21], and oxidative attack on membrane components follows, with lipoperoxidation initiated by extraction of a bisallylic hydrogen atom from polyunsaturated fatty acids, as would happen with ROS.

We have previously observed structural damage of the phospholipid bilayer after oxidative attack by ROS on mitochondrial phospholipids in isolated mitochondria in vitro under respiration state IV conditions [7]. By use of chemically oxidized phospholipids, we were also able to establish that lipoperoxidation readily appears as increasingly isotropic EPR spectra of supported planar phospholipid bilayers which in turn become disorderly packed and destabilized to the point of membrane breakdown [8]. We adopted the CCl_4-treated rat model in order to ascertain whether peroxidized phospholipids forming in vivo in a manner similar to lipoperoxidation occurring in some oxidative stress-linked diseases were able to alter the phospholipid bilayer structure in the same way as we previously observed in the above-cited simpler models.

After preliminary confirmation of lipoperoxidation in mitochondria from intoxicated rat liver by detection of conjugated dienes in phospholipid extracts, spin labeled supported phospholipid bilayers (SPB) samples of those phospholipids were scanned by EPR spectrometry. When lipoperoxidation was present, EPR spectra of SPB samples of mitochondrial phospholipids invariantly yielded disordered spectra, as shown by the anisotropy loss apparent in Fig. 2B (4.5 h/0.5 ml CCl_4) and 2 D (4.5 h/1 ml CCl_4). Here, parallel spectrum bands show prominently in the perpendicular spectra and vice versa in comparison with spectra in Fig. 2A (4.5 h/0.5 ml mineral oil, blank), which appear completely free of parallel/perpendicular cross contamination. R'' values of 0.48 and 0.68, respectively, were calculated from the derivative of those spectra, while the blank R'' value ranged around 0.20. Based on our previous studies [6,7], the observed behavior of EPR spectral anisotropy can be attributed to CCl_4 oxidative attack on phospholipids in vivo.

The trend of bilayer disordering with CCl_4 treatment time is reported in Fig. 3 as the variation of the disorder index R'' with time. R'' values were calculated from the derivative of EPR spectra reported in Fig. 2A–C and more, as illustrated in Fig. 1. From Fig. 3 it can be seen that bilayer disordering reaches its maximum extent 4.5 h after CCl_4 treatment of the animals as indicated by an R'' value of 0.47. Thereafter, and as expected in a living model, mechanisms for the removal of intoxication succeeded in abolishing the effect, and phospholipids extracted 24 h after CCl_4 gavage look completely "repaired" judging by the disordering effect vanishing with time (see also Fig. 2C). The solid line was obtained by means of the equations used to describe the steady state approximation [22], and fits data points with $\chi^2 = 0.187$, corresponding to $P < 0.05\%$. This observation might indicate the existence of two competing phenomena: (1) the onset of oxidative stress producing oxidized phospholipids (hence bilayer disordering) and (2) detoxication [10] that is removal of oxidized phospholipids and progressive return to a normal phospholipid pattern with an ordered bilayer.

The dependence of bilayer disordering upon CCl_4 gavage dose was also analyzed and the results are shown in Fig. 4. This figure reports the increase of R'' value as measured from the derivative of spectra similar to that reported in Fig. 2D (1 ml CCl_4, 4.5 h, bold line), with increasing CCl_4 dose after 4.5 h treatment time. Disordering of phospholipid bilayers appears almost linearly linked to the gavage dose, further confirming the dependence of the observed effect on carbon tetrachloride oxidative stress of membranes in vivo.

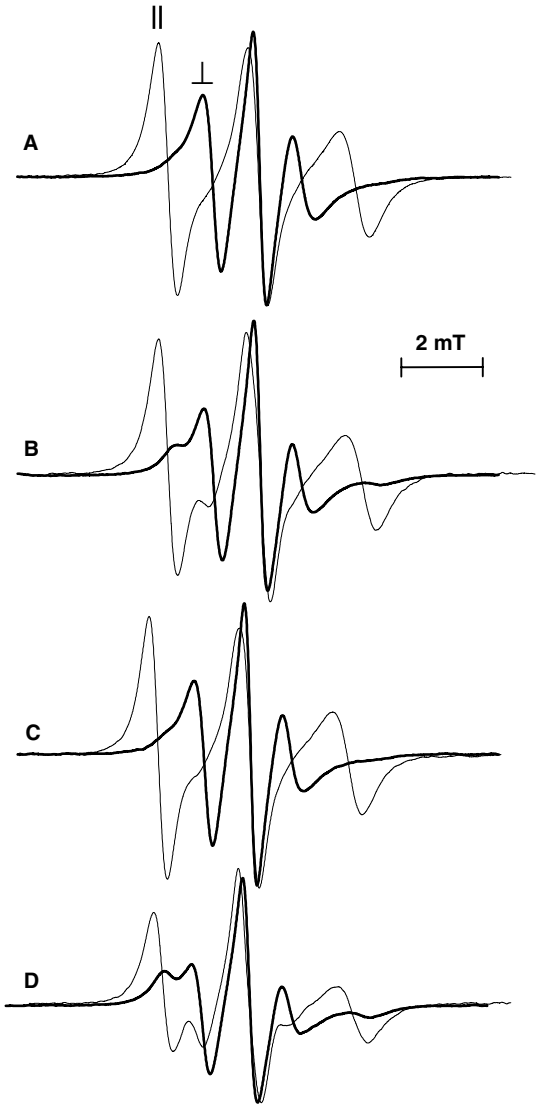

Fig. 2. EPR spectra of SPB samples consisting of liver mitochondrial phospholipids from variously treated rats. Gavage: (A) mineral oil (blank), 4.5 h treatment; (B) 1 ml 1:1 CCl_4/mineral oil, 4.5 h; (C) 1 ml 1:1 CCl_4/mineral oil, 24 h ; and (D) 2 ml 1:1 CCl_4/mineral oil, 4.5 h.

To further prove this dependence and to gain confirmation that oxidative attack in vivo is responsible for the observed effects as we found in model systems, the counteracting effect of antioxidant resveratrol was also studied in vivo via gavage to CCl_4-treated animals. Results are reported in Fig. 5, from which it can be seen that EPR spectra of SPBs made of liver mitochondrial phospholipids from rats treated with CCl_4 and resveratrol (Fig. 5C, $R'' = 0.29$) reveal a much more ordered phospholipid bilayer structure than the one yielding the spectrum in panel B ($R'' = 0.59$), from rats treated with CCl_4 alone, in comparison with the blank (mineral oil/maize oil) spectrum (Fig. 5A, $R'' = 0.19$). The anisotropy recovery of spectra in panel C over spectra in panel B is clearly attributable to the antioxidant properties of resveratrol. Nonetheless, the effect of resveratrol was dependent on the time-lag after CCl_4 treatment. In fact, judging from R'' value, EPR spectra of phospholipid SPBs from rats treated with resveratrol 2 h after CCl_4 gavage showed spectral anisotropy recovery about half as

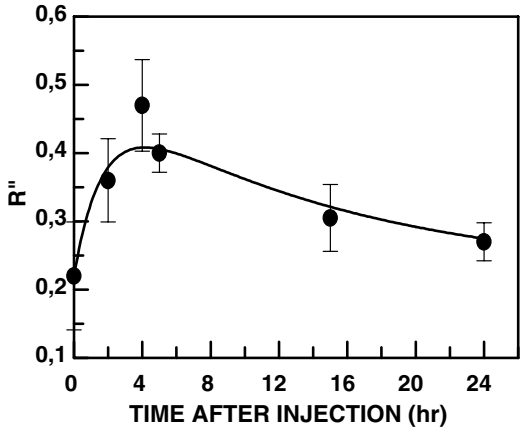

Fig. 3. Profile of anisotropy loss, expressed as R'' value, in spin labeled mitochondrial phospholipid SPBs with CCl_4 treatment time of rats. Each experimental point is the means of three measures of phospholipid extracts from liver mitochondria of three animals. Vertical bars represent S.D.

high as that of simultaneous resveratrol gavage. In fact, in the latter case an R'' value of 0.29 was calculated against $R'' = 0.43$ measured in case of 2 h time lag in resveratrol administration. These values are reported in Fig. 6 as the percentage of the R'' value obtained without resveratrol rating 0.59, taken as 100% disorder.

The data presented so far confirm that oxidative attack on membrane phospholipids in vivo also results in the phospholipid bilayer disordering, and that disorder is readily revealed by the anisotropy loss of EPR spectra of supported planar bilayers of extracted phospholipids. The observed disordering appears to be proportional to the oxidant amount and oxidation time and to respond to antioxidant treatment. The key to this proportionality lies in our previous observations showing that disordering of the bilayers has a molecular basis, since it is proportional to the amount of oxidized phospholipid molecules. This in turn enriches the order index R'' with quantitative valence.

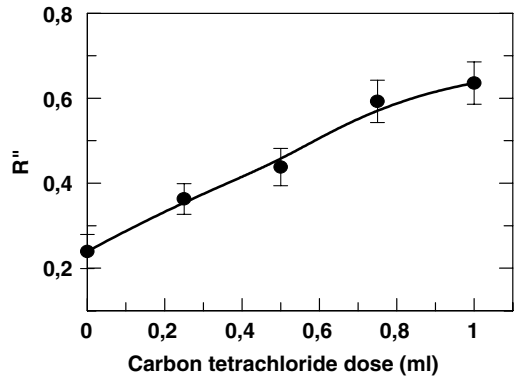

Fig. 4. Trend of anisotropy loss in EPR spectra of liver mitochondrial phospholipid SPBs from rats treated with increasing amounts of CCl_4 for 4.5 h. Anisotropy loss is expressed in terms of the disorder index R'' (see Section 2) calculated from EPR spectra similar to the one in Fig. 2D, bold line. Each data point is the means of four to six measurements on phospholipid extracts from three to four animals. S.D.s are given as vertical bars.

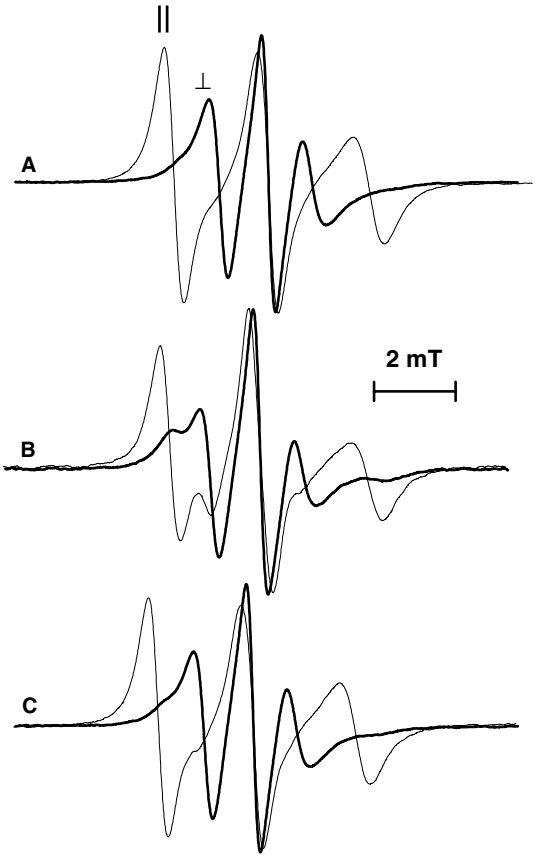

Fig. 5. EPR spectra of liver mitochondrial phospholipid SPBs from rats after (A) placebo, (B) 0.75 ml CCl_4, (C) 0.75 ml CCl_4 plus 15 mg resveratrol gavage. Animals were killed 4.5 h after treatment.

On the contrary, the order parameter S, whose value reflects membrane fluidity, again proved a poor indicator of lipoperoxidation also in the case of in vivo oxidative stress. In fact, mitochondria from rats treated with 0.75 ml CCl_4 and spin labeled with either 5- or 12- or 16-DSPC yielded EPR spectra

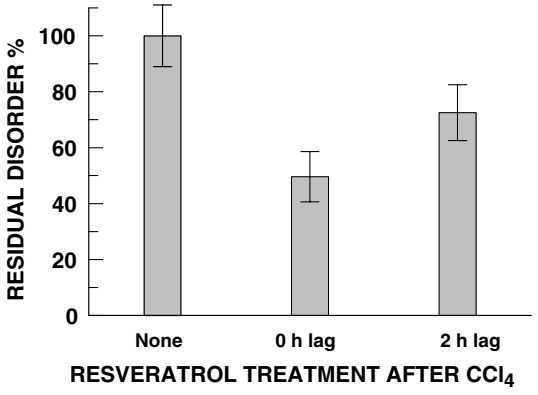

Fig. 6. The residual disorder of EPR spectra of SPBs made with liver mitochondrial phospholipids after different treatments of rats is expressed as the percent ratio of R'' of samples obtained in the presence of resveratrol to R'' value obtained with CCl_4 alone (100% disorder). Rats were treated with 0.75 ml CCl_4 in 1:1 mixture with mineral oil and with 15 mg resveratrol in maize oil after the indicated time lag. Data are the means of 4–6 SPBs measurements of lipid extracts from 3 to 4 animals. Vertical bars represent the S.D. of measurements.

Table 1
Order parameter S of n-DSPC in isolated mitochondria

C(n)	5	12	16
Blank[a]	0.645 ± 0.006	0.313 ± 0.005	0.118 ± 0.008
CCl_4[b]	0.639 ± 0.007	0.309 ± 0.009	0.122 ± 0.006

Liver mitochondria from rats treated with either [a]mineral oil or [b]0.75 ml CCl_4 in 0.75 ml mineral oil were spin labeled as described under Section 2. The S value was calculated from EPR spectra as also described therein. Figures are the means of three experiments and the S.D. is indicated.

from which the calculated S parameter showed minimal or zero difference with respect to the values calculated in the case of placebo-treated rats, as reported in Table 1. Although membrane fluidity variation has been tentatively linked to ROS formation and aging, the present results further confirm our previous observations of scarce sensitivity of membrane fluidity to lipoperoxidation. This link is controversial [23,24] and in some cases membrane fluidity modulation appears to be linked more to cholesterol content variations with aging than to lipoxidation [25,26]. Our present and previous results firmly demonstrate that acyl chain packing order appears much more affected than mobility, and that the order index R'' should be considered a much more reliable reporter of the bilayer status than the order parameter S when lipoxidation is involved.

In the light of our previous results, the present data confirm that oxidative damage to membrane phospholipids by whatever method is reflected in structural defects of the phospholipid bilayer. These structural defects consist mainly in disorder of the phospholipid packing leading to destabilization of the membrane structure and possibly to membrane breakdown. The fact that the same structural defects as those observed in simpler models occur after oxidative stress in a living pathological model suggests that lipoperoxidation, occurring in many other cases such as aging [27,28], hyper-caloric diet [29], inflammation [30,31] and some diseases [1–3] as revealed by the presence of malondialdehyde, can always affect the phospholipid bilayer structure of natural membranes leading also in these cases to membrane disordering. In turn, a ruined phospholipid bilayer no longer provides the proper environment for optimal integral enzymes functioning [5]. From this point of view, oxidized phospholipids and related bilayer disordering can be viewed as the molecular basis for sustaining and spreading some oxidative stress-linked diseases. Furthermore, destabilization of the bilayer might also favor ROS penetration in the inner bilayer and into membrane compartments, promoting overspreading of the oxidative attack to other membrane components and to whole organs.

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