Respiration state IV-generated ROS destroy the mitochondrial bilayer packing order in vitro. An EPR study

Francesco M. Megli*, Karen Sabatini

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

Received 17 June 2003; revised 17 July 2003; accepted 23 July 2003

First published online 5 August 2003

Edited by Vladimir Skulachev

Abstract The aim of the present study was to detect defective structural properties in bilayers of mitochondrial phospholipids after oxidative stress of isolated mitochondria in vitro, reportedly during respiration state IV. The structural behaviour of extracted phospholipids was studied by electron paramagnetic resonance (EPR) spectrometry in oriented phospholipid bilayers spin-labelled with 5-doxyl-lecithin, by detecting of the degree of EPR spectral anisotropy loss, indicative of the phospholipid bilayer packing order. Bilayers of phospholipids from untreated mitochondria showed the highest spectral anisotropy, hence highly ordered structure, while chemically oxidised phospholipid yielded almost completely disordered supported phospholipid bilayers. Samples from mitochondria after respiration state IV showed bilayer disorder increasing with oxidation time, while inclusion of the antioxidant resveratrol in the respiration medium almost completely prevented bilayer disordering. On the other hand, β-*n*-doxylstearoyl-lecithin spin-labelled mitochondria showed unchanged order parameter *S* at C positions 5, 12 and 16 after respiration state IV, confirming the insensitivity of this parameter to phospholipid oxidative stress. It is concluded that reactive oxygen species attack to the membrane affects lipid packing order more than fluidity, and that EPR anisotropy loss reveals oxidative damage to the bilayer better than the order parameter.

Key words: Lipoperoxidation; Phospholipid bilayer; Mitochondrion; Electron paramagnetic resonance; Spectral anisotropy; Membrane fluidity

1. Introduction

Phospholipid damage after oxidative stress is widely maintained to be implicated in the onset and/or development of many diseases [1], probably by impairing integral enzyme functioning, such as some ion transporters [2]. In these cases it was proposed that oxidative stress brought about an indirect effect on the functionality of the membrane by altering the phospholipid environment rather than the enzyme itself.

In studies dealing with lipoxidation, phospholipid damage is usually assessed by measuring malondialdehyde (MDA) formation by the thiobarbituric acid-reactive substances (TBARS) assay and/or detecting conjugate dienes spectrophotometrically. Both methods only yield a rough indication of lipid oxidation, without any information about the real amount of oxidised phospholipids formed and related damage to the membrane. The latter aspect in turn is of great concern in relation to the influence exerted by phospholipids on the functioning of many integral enzymes [3,4], and the possibility that they may be affected by the surrounding phospholipid altered status rather than by direct reactive oxygen species (ROS) attack. Nevertheless, apart from some studies concerning changes of the membrane fluidity gradient [5–7], little attention has been given to detection of structural damage to the phospholipid bilayer after lipoperoxidation. Those studies were performed by electron paramagnetic resonance (EPR) measurement of the order parameter *S* in membranes probed with phospholipid or fatty acid spin labels. Although a statistically highly significant variation in *S* was reported, its value shifts by tiny amounts and reveals so little fluidity change as to judge it physiologically insignificant.

Recently, we investigated how lipoxidation influenced a different (and largely dismissed) EPR spectral feature useful for evaluation of the phospholipid bilayer structural integrity, namely spectral anisotropy, also termed spectral angular dependence, in spin-labelled oriented phospholipid bilayer on a solid support (SPB) [8]. This feature is able to reveal disordering of the bilayer phospholipid chains [9–11] and, possibly, transition from bilayer to non-bilayer structures. By this method we discovered that SPBs containing chemically oxidised phospholipids dramatically lose spectral anisotropy and that this loss strictly follows the oxidation state of phospholipids. In addition, detection of anisotropy loss proved much more apt at revealing bilayer structural alterations after lipoxidation than the order parameter *S*, which in our hands proved rather insensitive to oxidative stress of phospholipids in vesicles.

In this paper we present a similar study of mitochondrial phospholipids after oxidative stress of isolated mitochondrial membranes provoked by respiration state IV in vitro. It will be shown that SPBs made with phospholipids from mitochondria subjected to ROS-generating incubation conditions display a strong loss of EPR spectral anisotropy, while those from untreated mitochondria display unaltered full angular dependence of EPR spectra. Furthermore, inclusion of antioxidants in the incubation medium such as resveratrol almost

*Corresponding author. Fax: (39)-080-5443317.
E-mail address: f.m.megli@biologia.uniba.it (F.M. Megli).

Abbreviations: n-DSPC, β-*n*-doxylstearoyl-lecithin; EPR, electron paramagnetic resonance; SPB, supported phospholipid bilayer; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00861-5

completely prevented anisotropy loss of mitochondrial phospholipids, while oxidation of mitochondria by external reagents, such as Fenton reagent, had limited effect. In contrast, measurements of the order parameter S in mitochondria after the same treatment in vitro showed that the membrane fluidity gradient was unchanged, confirming the insensitivity of S to lipoxidation.

2. Materials and methods

2.1. Materials

Spin-labelled lecithin was synthesised by coupling either 5- or 12- or 16-doxylstearic acid (Sigma) to egg yolk *lyso*-lecithin (Sigma) by the method of Boss et al. [12].

2.2. Preparation of mitochondria

Mitochondria were isolated from rat liver as described [13] and resuspended in 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.35. Mitochondrial respiratory control index, measured immediately after the preparation by the Clark electrode in 145 mM KCl, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA 0.1% bovine serum albumin, 30 mM HEPES, pH 7.4 as described in [13], usually ranged between 2.8 and 3.2. Mitochondrial protein was assayed by the method of Bradford [14]. Mitochondrial lipids were extracted according to Bligh and Dyer [15] and phospholipid phosphorus was assayed by the Nakamura [16] method.

2.3. Oxidation procedures

Extracted mitochondrial lipids were dispersed in Tris 0.5 mM pH 7.4 buffer/methanol 9:10 at 1 mM final concentration and treated with 0.5 mM $FeCl_2$ and 0.25 mM H_2O_2 (Fenton reagent) at 37°C for different time intervals. Mitochondria were resuspended in KCl 125 mM Tris 20 mM pH 7.4 (1 mM phospholipid phosphorus final concentration) and treated with Fenton reagent as concentrated as above, in the same conditions. At the end, extraction followed according to Bligh and Dyer [15] and the lipids were redissolved in absolute ethanol at 1 mM concentration along with 4% by mol β-5-doxylstearoyl-lecithin (5-DSPC) for the preparation of SPBs.

In both cases, lipoxidation was checked by the formation of MDA by the TBARS assay [17].

2.4. Spin labelling of mitochondria

Spin-labelled small unilamellar vesicles (SUV) were prepared by sonication (Branson sonifier W-250 D operated at 40 W output for 15 min with intervals, in an ice bath) of an EYPC/*n*-DSPC 2:1 by mol mixture in 125 mM KCl, 25 mM HEPES, 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 ultrafilter. 5 mg freshly prepared mitochondrial membranes were 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 0.43 M sucrose was layered onto its bottom. Spin-labelled mitochondria were pelleted by centrifugation at 10 000 rpm for 10 min [18] and resuspended immediately in respiration medium (1 mg mitochondrial protein per ml final concentration).

2.5. Mitochondrial ROS production

Five milligram of either unlabelled or spin-labelled mitochondria were suspended in respiration medium at a concentration of 1 mg/ml and incubated with the indicated succinate concentration without ADP (respiration state IV) for different time intervals at 37°C. For time intervals longer than 0.5 h, the proper amount of succinate, calculated on the basis of oxygen consumption rate, was re-added every 30 min in order to keep its concentration constant. Atmospheric air was gently bubbled in under hydrostatic pressure throughout the whole incubation step to ensure oxygen supply in all parts of the bulky sample. Spin-labelled mitochondria were incubated for 30 min maximum in order to avoid EPR signal disappearance, while control mitochondria were kept in parallel at 37°C without substrate and air. At this stage, occurrence of lipoperoxidation was checked by assaying MDA with the TBARS test.

2.6. EPR spectral anisotropy measurement

A 50 μl ethanol solution of spin-labelled phospholipids was deposited on a thin narrow glass slide and taken to dryness under vacuum (1–2 mbar). 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 [8].

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 are accordingly labelled 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.

2.7. Membrane fluidity measurement

After the respiration step, spin-labelled mitochondria were pelleted at 10 000 rpm for 10 min and after resuspension in 100 μl respiration medium, a 25 μl sample was introduced into a glass capillary. EPR spectra were scanned as above, at room temperature with field set and radiating field shifted to 339 mT and 9.35 GHz, respectively.

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

2.8. Spectral analysis

Nitroxide spin labels are characterised by three-line EPR spectra, defined by the central g value and hyperfine splitting constant A. The former establishes the EPR spectrum position on the magnetic field scale and the latter measures the separation of two consecutive bands, usually in mT. Either parameter has cylindrical symmetry, taking on one value relative to the xy plane (more appropriately, the mean of the two close values relative to x- and y-axis, $1/2(g_{xx}+g_{yy})$ and $1/2(A_{xx}+A_{yy})$) and a different one along the z-axis, g_{zz} and A_{zz} (crystal values). In an experimental spectrum, these values are called $g_\perp$ and $A_\perp$, $g_\parallel$ and $A_\parallel$. $A_\perp$ and $A_\parallel$ represent the shortest and the largest splitting, respectively [11].

Given the doxylstearic acid geometry, the nitroxide z-axis is rigidly bound and parallel to the fatty acid long axis so that the chain's dynamics is strictly reflected onto the nitroxide dynamics [9]. In these conditions, the motional freedom of the doxyl fatty acid chain is expressed by the spin label order parameter S, defined as follows [11,19]:

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

and the highest and lowest motional freedom states are described by $S=0$ and $S=1$, respectively. It must be stressed that the order measured by S is dynamic in nature in the sense that high S values reveal motional order, that is stiffness, and not geometrical order.

In oriented planar phospholipid bilayers, phospholipid molecules bind perpendicularly to the solid glass support so that the fatty acid chains, along with the rigidly bound nitroxide z-axis, are also normal to it and form a rigid ordered ensemble. For a nitroxide ring placed at a rigid chain position, such as C position 5 or less, molecular tumbling is strongly reduced (high S values) and the EPR spectrum determined by $g_\parallel$ and $A_\parallel$ is only visible when the sample z-axis (coaxial with both the fatty chain long axis and the glass slide normal) is parallel to the applied magnetic field. Conversely, the perpendicular spectrum determined by $g_\perp$ and $A_\perp$ will appear only when the glass slide normal is perpendicular to the applied magnetic field. Therefore the two spectra are well separated and not simultaneously visible, the perpendicular switching to the parallel one only upon rotating the sample by 90°, showing angular dependence. In this situation, loss of spectral anisotropy is readily revealed by the appearance of the parallel spectrum components superimposed onto the perpendicular spectrum (and vice versa), signalling bilayer disordering [9,10] as we observe after lipoxidation. Its most prominent signs are displayed as a more or less pronounced downfield shoulder of the low field perpendicular band and an upfield downward smaller band, with spectral separation close to $A_\parallel$ (ranging between 5 and 6 mT for 5-DSPC). Superimposition of

the perpendicular and parallel spectra helps detection of anisotropy loss after different treatments.

3. Results and discussion

Non-oxidised phospholipids extracted from untreated fresh mitochondria yielded oriented phospholipid bilayer showing highest EPR spectral anisotropy, as is seen in Fig. 1A. This pair of spectra clearly reveals the presence of highly ordered phospholipid bilayers. In contrast, mitochondrial phospholipids exhaustively oxidised with Fenton reagent were unable to build up ordered bilayers, as revealed by their SPB EPR spectra reported in Fig. 1B, in which spectral cross-contamination of perpendicular and parallel components indicates a considerable loss of angular dependence. Oxidation of whole mitochondria with Fenton reagent for 3 h had no influence on phospholipid structural behaviour, as resulting SPBs appeared to be still highly ordered (Fig. 1C), and oxidation had to be prolonged to 16 h to show an effect (result not shown). Spectra A and B in Fig. 1 are useful as standard spectra for normal and oxidised mitochondrial phospholipids, respectively.

ROS production in mitochondria and related consequences are well-established phenomena [20–22] and generation of ROS in mitochondria under respiration state IV has been used to produce oxidative stress in those membranes [5–7,13]. Under these conditions, lipoxidation was confirmed by detection of MDA throughout the whole experiment. Oxidation of mitochondrial phospholipids by physiologically generated ROS during respiration state IV, obtained as described in [13], strongly impairs their capability of building up ordered bilayers. As can be seen from Fig. 2, SPBs of phospholipids from control mitochondria (panel A), incubated for up to 180 min without succinate/air, show unaltered bilayer packing order in comparison with those made of phospholipids from mitochondria subjected for increasing time to respiration state IV (panel B, 30 min with 2 mM succinate and air), which in contrast show increasing disorder, until anisotropy is completely lost after 3 h incubation, revealing that the bilayers are completely disordered (panel C). In the latter case, exhaustive late-stage lipoxidation should be attributed more to the propagation step [1] of the radical chain reaction than to primary ROS production by now exhausted mitochondria.

It is important to note that oxidation by endogenously generated ROS under physiological conditions appears to be much stronger and more devastating than that brought about by either externally added chemicals (Fig. 1C) or chemical reaction (Fig. 1B). This observation suggests that physiolog-

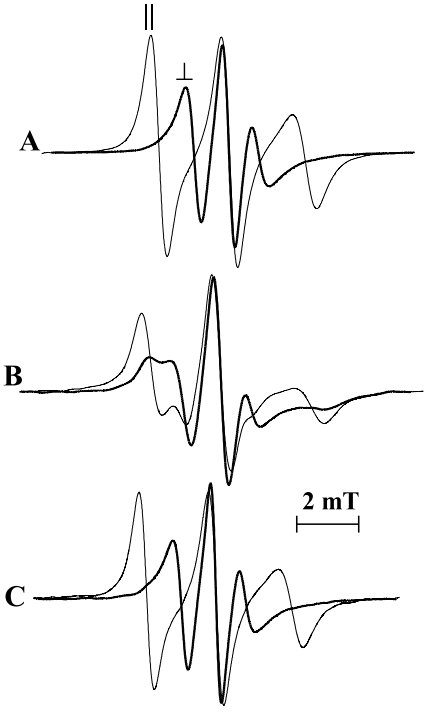

Fig. 1. EPR spectra of SPBs made with (A) phospholipid extract from untreated control mitochondria, (B) phospholipid extract after oxidation with Fenton reagent in aqueous buffer/methanol 9:10 and (C) phospholipid extract from mitochondria treated with Fenton reagent for up to 3 h. Bold line, perpendicular spectrum as indicated in A.

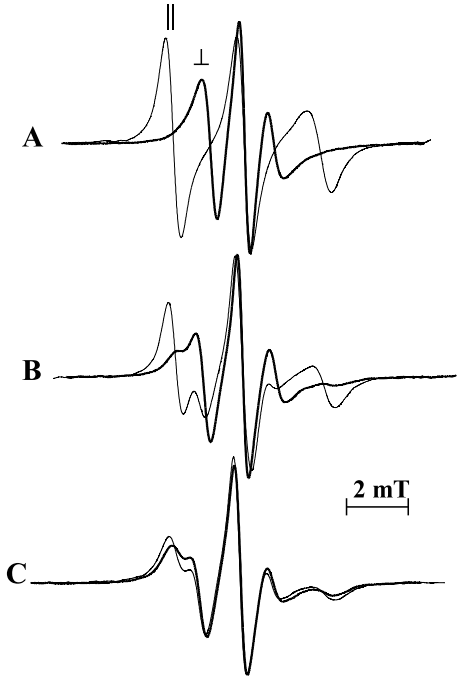

Fig. 2. EPR spectra of 5-DSPC spin-labelled SPBs made with phospholipid extracts from (A) control mitochondria after 180 min incubation without substrate/air, (B) mitochondria after 30 min and (C) after 180 min respiration in the presence of 2 mM succinate and air at 37°C (state IV). Bold line, perpendicular spectrum as indicated in A.

Table 1
Order parameter S^a of *n*-DSPC in variously treated isolated mitochondria

C(*n*)	5	12	16
Mt[b]	0.649	0.319	0.115
Mtox[bc]	0.643	0.319	0.131

[a]Typical standard deviation ranges between 0.006 and 0.01.
[b]Mitochondria were spin-labelled as described in Section 2.
[c]Mitochondria were incubated for 30 min at 37°C in the presence of 5.4 mM succinate under an air stream (see Section 2).

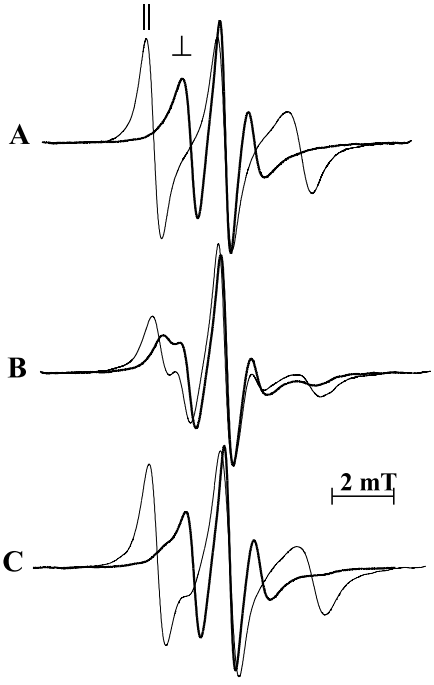

Fig. 3. EPR spectra of 5-DSPC spin-labelled SPBs made with phospholipid extracts from mitochondria after 90 min incubation without substrate/air (control) (A), with 5.4 mM succinate (B) in the absence and (C) in the presence of 1 mM resveratrol. Bold line, perpendicular spectrum as indicated in A.

ical oxidation might develop along completely different and more effective pathways than in vitro models.

The effect of the presence of a renowned antioxidant, resveratrol, in the incubation medium is reported in Fig. 3. Panel B of this figure reports on the spectral anisotropy of SPBs of phospholipids from mitochondria after 90 min incubation with 5.4 mM succinate under air supply. From this figure, an intermediate spectral anisotropy loss with respect to that appearing in Fig. 2B,C can be seen. In striking contrast, the spectra in Fig. 3C resemble more the control spectra in panel A (90 min incubation without succinate/air), revealing the negligible anisotropy loss of SPBs of phospholipids from mitochondria incubated in the same conditions and 1 mM resveratrol. Nonetheless, the radical-scavenging action attributed to it [23–26] might not reach radicals deeply buried in some membrane recess, explaining the observed less than 100% disorder reversal. In any case, large prevention of disordering by the antioxidant fully confirms the dependence of anisotropy loss and bilayer disordering on ROS attack. It is reasonable to retain that oxidative stress is also responsible for similar structural damage of the phospholipid bilayer in intact mitochondrial membranes and even in vivo, and suggests for the first time the possible nature of membrane damage at the level of phospholipids after lipoxidation by ROS attack.

At the same time, it is to be noted that while spectral anisotropy reveals these effects quite well, the order parameter fails. As already noted in the case of phospholipid vesicles [8], results in Table 1 show that phospholipid spin labels in mitochondria subjected to 30 min respiration state IV present the same membrane fluidity as those in control mitochondria do along the whole acyl chain. The limit of 30 min oxidation is imposed by the observed weakening and disappearance of the EPR signal upon longer incubation times, similarly to what has been reported by other authors [5,6] (however, since the reaction environment is oxidising in nature, we disagree with the concept of 'reduction' of the nitroxyl moiety in favour of overoxidation [20]). Comparison between the fluidity gradients reported in Table 1 reveals that either the order parameter is unable to detect tiny fluidity variations or oxidative damage results more in scrambling acyl chains than in fluidising the bilayer. In any case, our results confirm that fluidisation, if any, ranges within the experimental error limits and reveal it has no physiological influence.

As an alternative interpretation of our data, since angular dependence is also lost in ordered systems with a curved surface, (e.g. micelles and vesicles: note that the spectra in Fig. 2C look remarkably similar to those of vesicles), we cannot exclude transition to non-bilayer structures after oxidation, such as the hexagonal phase, with notable implications in the case of mitochondrial membranes.

In both cases, by comparing the information gained from the set of spectra in Fig. 2 with that obtained from Table 1, our results show that the study of EPR spectral anisotropy of phospholipid bilayers is able to monitor the occurrence of these phenomena better than membrane fluidity measurements.

Acknowledgements: K.S. was supported by a fellowship from ESF (P.O.P. '94–'99). The authors thank Mr D. Munno for skilful assistance in laboratory work.

References

[1] Halliwell, B. and Gutteridge, J.M.C. (1990) Methods Enzymol. 186, 1–85.
[2] Kourie, J.I. (1988) Am. J. Physiol. 275, C1–C24.
[3] Kinnunen, P.K. (1991) Chem. Phys. Lipids 57, 375–399.
[4] Kinnunen, P.K. (2000) Cell Physiol. Biochem. 10, 243–250.
[5] Gabbita, P.S., Butterfield, D.A., Hensley, K., Shaw, W. and Carney, J.M. (1997) Free Radic. Biol. Med. 23, 191–201.
[6] Gabbita, P.S., Subramaniam, R., Allouch, F., Carney, J.M. and Butterfield, D.A. (1998) Biochim. Biophys. Acta 1372, 163–173.
[7] Nepomuceno, M.F., Alonso, A., Pereira-Da-Silva, L. and Tabak, M. (1997) Free Radic. Biol. Med. 23, 1046–1054.
[8] Megli, F.M. and Sabatini, K. (2003) Chem. Phys. Lipids (in press).
[9] Smith, I.C.P. and Butler, K.W. (1976) in: Spin Labeling (Berliner, L.J., Ed.), pp. 411–451, Academic Press, New York.
[10] Jost, P., Libertini, L.J., Hebert, V.C. and Griffith, O.H. (1971) J. Mol. Biol. 59, 77–98.
[11] Griffith, O.H. and Jost, P.C. (1976) in: Spin Labeling (Berliner, L.J., Ed.), pp. 453–523, Academic Press, New York.
[12] Boss, W.F., Kelley, C.J. and Landsberger, F.R. (1974) Anal. Biochem. 64, 289–292.
[13] López-Torres, M., Gredilla, R., Sanz, A. and Barja, G. (2002) Free Radic. Biol. Med. 32, 882–889.
[14] Bradford, M.M. (1976) Biochemistry 72, 248–254.
[15] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[16] Nakamura, G.R. (1952) Anal. Chem. 24, 1372.
[17] Ramanathan, L., Das, N.P. and Li, Q.-T. (1994) Biol. Trace Elem. Res. 40, 59–70.
[18] Megli, F.M., Mattiazzi, M., Di Tullio, T. and Quagliariello, E. (2000) Biochemistry 39, 5534–5542.
[19] Gaffney, B.J. (1976) in: Spin Labeling (Berliner, L.J., Ed.), pp. 567–571, Academic Press, New York.
[20] Korshunov, S.S., Skulachev, V.P. and Starkov, A.A. (1997) FEBS Lett. 416, 15–18.

[21] Gredilla, R., Barja, G. and Lopez-Torres, M. (2001) J. Bioenerg. Biomembr. 33, 279–287.
[22] Han, D., Antunes, F., Daneri, F. and Cadenas, E. (2002) Methods Enzymol. 349, 271–280.
[23] Tadolini, B., Juliano, C., Piu, L., Franconi, F. and Cabrini, L. (2000) Free Radic. Res. 33, 105–114.
[24] Stojanovic, S., Sprinz, H. and Brede, O. (2001) Arch. Biochem. Biophys. 391, 79–89.
[25] Zini, R., Morin, C., Bertelli, A., Bertelli, A.A. and Tillement, J.P. (1999) Drugs Exp. Clin. Res. 25, 87–97.
[26] Keana, J.F.W. (1979) In: Spin Labeling II (Berliner, L.J., Ed.), pp. 115–172, Academic Press, New York.