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Formation of phosphatidic acid in stressed mitochondria

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ABSTRACT

Mitochondria are an important intracellular source of ROS as well as a sensitive target for oxidative damage under certain pathological conditions such as iron or copper overload. Mitochondrial membranes are rich in the tetraacyl phospholipid cardiolipin. Its integrity is important for efficient oxidative phosphorylation. Mouse liver mitochondria were subjected to oxidative stress by the $Cu^{2+}(Fe^{2+})/H_2O_2$/ascorbate system. Phosphatidic acid was detected in oxidized mitochondria, but not in unperturbed mitochondria. The Cu^{2+}/H_2O_2/and (or not) ascorbate system caused the formation of phosphatidic acid and phosphatidylhydroxyacetone in cardiolipin liposomes. These products proceed via an HO-radical induced fragmentation taking place in the polar moiety of cardiolipin. Mass spectrometry analysis of phosphatidic acid newly formed in mitochondria revealed that it has been derived from fragmentation of cardiolipin. Thus, free-radical fragmentation of cardiolipin in its polar part with the formation of phosphatidic acid is a likely mechanism that damages mitochondria under conditions of oxidative stress.

The generation of reactive oxygen species (ROS)[1] results in mitochondrial dysfunction and is associated with cell disorders and numerous pathological processes [1–4]. Mitochondria are an important intracellular source of ROS as well as a sensitive target for oxidative damage under certain pathological conditions [4–7]. The mitochondrial electron transport chain contains several redox centres that may leak electrons to dioxygen, constituting the primary source of superoxide anion radicals ($O_2^{\cdot-}$), which dismutates to form hydrogen peroxide and dioxygen [3,8,9]. The proportion of dioxygen converted into $O_2^{\cdot-}$ accounts for about 1–2% of the overall dioxygen consumption in mitochondria [8]. Subsequently, interaction of H_2O_2 and $O_2^{\cdot-}$ with proteins bearing metal ions or with free ferrous and copper ions provides conditions to yield highly reactive hydroxyl radicals ($HO^{\cdot}$).

In the living organism, the storage of transitional metals may be disrupted and metal ions can catalyse the formation of free radicals in the Fenton-like reaction [10–14]. Tissue copper levels are increased mainly in the liver due to a genetic disturbance of copper homeostasis, known as Wilson disease, or under conditions associated with oxidative stress, inflammation and infection [15]. Recently several authors reported also excessive hepatic iron storage in addition to copper in patients with Wilson disease [16–18]. It is well known that in conditions of copper and/or iron overload mitochondria are a major target of metal induced toxicity [15,19–21]. However, the underlying molecular mechanisms of oxidative damage to mitochondria remain in many details unknown. Conditions favouring the enhanced formation of hydroxyl radicals can induce the destruction of essential mitochondrial molecules such as lipids, proteins and DNA [22].

Mitochondria contain huge amounts of cardiolipin (CL), a unique phospholipid with dimeric structure, which has four unsaturated fatty acyl chains and two negative charges. CL is required for the optimal activity of several mitochondrial proteins. It is crucial for efficient oxidative phosphorylation and for correct function and structure of the mitochondrial inner membrane [23,24]. In addition, CL becomes associated with cytochrome *c* in damaged mitochondria and participates in the mitochondrial apoptotic pathway. Degradation of CL and transition of CL to the outer mitochondrial membrane are early events in induction of apoptosis [25,26].

We found recently, that γ-irradiated CL or CL subjected to the Fe^{2+}/H_2O_2/ascorbate system is fragmented in its polar part under the formation of phosphatidic acid and phosphatidylhydroxyacetone [27]. This free-radical fragmentation process is typical for lipids bearing a hydroxyl group in β-position to the phosphoester or glycoside bond [27–31].

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[1] *Abbreviations used:* ROS, reactive oxygen species; DOPA, dioleoylphosphatidic acid; DLPA, dilinoleoylphosphatidic acid; SAPA, stearoylarachidonoylphosphatidic acid; TOCL, tetraoleoylcardiolipin, CL, cardiolipin; DOPC, dioleoylphosphatidylcholine.

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It remains unknown, whether a fragmentation of CL with subsequent formation of phosphatidic acid occurs also in stressed mitochondria and under conditions of copper overload. Here, we observed for the first time the formation of phosphatidic acid in stressed mitochondria. This property was absent in control mitochondria. Mass spectrometry analysis revealed that this newly formed phosphatidic acid was derived from cardiolipin.

Materials and methods

Chemicals

Dioleoylphosphatidic acid (DOPA), dilinoleoylphosphatidic acid (DLPA), stearoylarachidonoylphosphatidic acid (SAPA), tetraoleoylcardiolipin (TOCL), cardiolipin (CL) from bovine heart and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Dioleoylphosphatidylhydroxyacetone (DOPHA) was prepared from dioleoylphosphatidylcholine (DOPC) by phospholipase D-catalyzed transphosphatidylation reaction [32] using hydroxyacetone as acceptor. All lipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), sphingomyelin (SM), phosphatidylserine (PS), lyso-phosphatidylcholine, phosphatidylglycerol (PG), ceramide, fatty acids, galactosyl diacylglyceride (GalDAG), diacylglyceride (DAG) and cholesterol used as standards were from Sigma (Deisenhofen, Germany). Trifluoroacetic acid (TFA), 2,5-dihydroxybenzoic acid (DHB), chloroform, methanol and primuline were obtained from Fluka Chemikalien GmbH (Buchs, Switzerland). All chemicals were of the highest purity commercially available.

Isolation of mouse liver mitochondria

Mouse mitochondria were isolated by the standard differential centrifugation method. Twenty-eight-week-old female mice (C57BL × 129S6/SvEv) were euthanized by CO_2 inhalation in accordance with the European directive of protection of vertebrate animals for scientific research. The livers were quickly removed, minced on ice, resuspended in buffer (250 mM sucrose/10 mM Tris–HCl, 5 mM EDTA, pH 7.5) and homogenized with a glass Dounce homogenizer and Teflon pestle. All steps and centrifugations for mitochondrial isolation were performed on ice or at 4 °C. Homogenates were centrifuged at 2500 rpm for 10 min to pellet nuclei and unbroken cells. The supernatant was centrifuged at 7000 rpm for 10 min to form a mitochondrial pellet that was resuspended in the above buffer without EDTA and centrifuged again at 7000 rpm for 10 min. The final mitochondrial pellet was resuspended in 2 ml of 0.01 M phosphate buffer (0.0027 M KCl, 0.137 M NaCl, pH 7.4) and was used within 1 h after isolation.

Protein determination

Protein content was determined by the method of Lowry with bovine serum albumin as standard [33].

Preparation of liposomes

Multilamellar liposomes were prepared using the thin film hydration method [34]. To do this, lipid solutions in chloroform were thoroughly evaporated in a rotary evaporator, and the samples were kept under vacuum for a least 1 h to ensure complete removal of the solvent and formation of a dry lipid film. Then, an appropriate quantity of 0.01 M phosphate buffer (0.0027 M KCl, 0.137 M NaCl, pH 7.4) was added, followed by thorough homogenization using a Vortex mixer at 37 °C. The phospholipid concentration in the liposomes was 0.02 M.

Incubation of samples with metal ions

Liposomes were incubated with Cu^{2+}/H_2O_2 or Cu^{2+}/H_2O_2/ascorbate reagents for 4 h at 37 °C. The final concentrations of Cu^{2+} (as $CuSO_4$) were 0.13, 0.5, 1.0, or 5.0 mM. The final concentrations of H_2O_2 or ascorbate were 1, 2, 5, or 10 mM. The Cu^{2+}:H_2O_2 ratios were varied as follows: 1:2, 1:10. In the Cu^{2+}/H_2O_2/ascorbate system ratios of reagents were as follows: 1:1:1, 1:2:1, 1:2:2, 1:2:5, 1:8:8 and 1:10:10. Mitochondrial samples (0.4 ml with a protein concentration of 10–20 mg/ml) were incubated for 4 h at 37 °C with (a) 1 mM H_2O_2; (b) 1 mM $FeSO_4$ and 1 mM H_2O_2; (c) 1 mM $FeSO_4$, 1 mM H_2O_2 and 1 mM ascorbate; or (d) 1 mM $CuSO_4$ and 2.0 mM H_2O_2. Control samples were incubated in the same way in the absence of any oxidant reagents.

Lipid extraction

Lipids were extracted from the liposomes by a chloroform–methanol mixture (2:1 v/v) by shaking as described in [35]. The lower chloroform layer was concentrated and used for further analysis.

Mitochondrial lipids were extracted using a slightly modified version of a method described earlier [36]. The mitochondrial pellet was resuspended in 1 ml methanol and sonicated for 3 min. Next, chloroform (2 ml) was added and the samples were stirred under nitrogen overnight at 4 °C. Then, 0.4 ml 0.15 M NaCl was added and the mixture was vortexed and centrifuged. The chloroform layer was carefully collected and dried under a stream of nitrogen. Lipids were redissolved in 150 μl chloroform and used for further analysis.

Lipid analysis by high-performance thin-layer chromatography

Lipids were separated on high-performance thin-layer chromatography (HPTLC) plates (silica gel H 60, thickness of the silica gel, 0.3 mm, Merck, Darmstadt, Germany). In case of TOCL and CL from bovine heart, samples were run in a solvent mixture composed of chloroform/methanol/25% aqueous ammonia (13:5:1, v/v/v).

Mitochondrial lipids were separated by two-dimensional high-performance thin-layer chromatography. Samples were first run in methyl acetate/2-propanol/chloroform/methanol/0.25% KCl in water (25:25:25:10:9, v/v/v/v/v). After drying the plates under a stream of nitrogen a solvent mixture composed of chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v)) was used in the second dimension. For each sample, two HPTLC plates were developed.

Lipids spots on HPTLC plates were identified under UV light (λ = 366 nm) after spraying with primuline solution (5 mg of primuline was dissolved in 100 ml of acetone–water (4:1, v/v)) and marked with a pencil. Primuline was used while it is not destructive to lipids. Images of chromatograms were taken with a photographing system (Biostep DH-30/32). The identity of each lipid was established by comparison with the R_f values measured for authentic standards. In any case, lipids were also extracted from Silica gel and analyzed by means of mass spectrometry. Extraction of lipids from TLC silica gel was performed as described previously [27].

Samples and matrix preparation for MALDI-TOF mass spectrometry

The standard technique for the preparation of MALDI-TOF MS samples was used as described in detail previously [27]. Briefly, lipid stock solution of the appropriate concentration was mixed with matrix solution (1:1 v/v) by vortexing. The resulting sample/matrix mixture was directly applied on the sample plate as 1 μl droplets. Samples were rapidly dried under a warm stream of air in order to remove the organic solvent as fast as possible.

For all samples, a 0.5 M DHB solution in methanol containing 0.1% TFA was used as matrix.

In some cases, HPTLC plates were directly scanned in the MALDI-TOF mass spectrometer. TLC plates for MS analysis were prepared according to Ref. [37]. HPTLC plates with lipid samples were cut into stripes and the stripes were attached to a MALDI sample plate with double-page adhesive pads. Several 0.75 μl droplets of matrix solution (100 mg of DHB/ml in acetonitrile/water, 1:1, v/v) were applied around the marked bands. After solvent evaporation and matrix/analyte cocrystallization, the TLC plate was scanned in the mass spectrometer.

Lipid analysis by MALDI-TOF mass spectrometry

All MALDI-TOF mass spectra were acquired on a Bruker Daltonics workstation (Bruker Daltonik GmbH, Germany). The system utilizes a pulsed nitrogen laser emitting at λ = 337 nm. The pressure in the ion chamber was held between 1×10^{-7} and 4×10^{-7} Torr. All measurements were done under delayed extraction conditions improving both mass accuracy and mass resolution. The extraction voltage was 20 kV. All lipid spectra were acquired using a low-mass gate at 400 Da to avoid most of matrix peaks. Samples were analyzed in the positive-ion mode using the reflectron TOF detector. In case of standard MS, 50–128 single laser shots were averaged for each mass spectrum using a laser power of 19–25 arbitrary units. In case of HPTLC-MS, 20–50 single laser pulses were typically applied for acquisition of mass spectra on different positions within the lipids spots using a laser power of 39–45 arbitrary units, frequency 1.0 Hz.

Results

Fragmentation of CL in liposomes treated with copper ions

To investigate the effect of Cu^{2+} on fragmentation of cardiolipin in its polar part, we studied the alteration of this lipid in liposomes containing Cu^{2+}/H_2O_2 or Cu^{2+}/H_2O_2/ascorbate (for 4 h at 37 °C). TOCL and CL from bovine heart (according to the manufacturer's data, this phospholipid contains 95% linoleoyl, 5% oleoyl and some minor fatty acid residues) were used. The organic extract from CL-liposomes was analyzed by means of HPTLC and MALDI-TOF MS.

Untreated TOCL yielded a single spot in HPTLC with a R_f value of 0.6. (Fig. 1a). In contrast to the control (lane 1), two new bands were observed in samples treated with Cu^{2+}, H_2O_2 (lanes 2–5) and absence (lane 2) or presence (lanes 3–5) of ascorbate. These new chromatographic zones with R_f values of 0.1 and 0.85 had the same R_f values as DOPA and DOPHA used as standards. These new products were further analyzed by HPTLC-MALDI-TOF MS. Spectra taken from bands C and D (lane 3, Fig. 1a) were compared with bands A and B taken from the same position of the untreated control (Fig. 1b and c). Spectrum A does not contain any notable peaks. Spectrum C displays two peaks at 723.5 and 745.5 corresponding to DOPA under addition of (2H + Na) or (H + 2Na), respectively. Fig. 1c shows the HPTLC-MALDI spectra obtained directly from zones B (spectrum B) and D (spectrum D). No ion signals were required in spectrum B. Two peaks were found in spectrum D. The *m*/*z* values for these peaks correspond to dioleoylphosphatidylhydroxyacetone (DOPHA) associated with one hydrogen and one sodium cation (779.5) or two sodium ions (801.5). The intensities of newly formed DOPA and DOPHA depend on the concentration of Cu^{2+}, H_2O_2 and ascorbate and their ratio in the samples. The highest yield of these products was found at 1 mM Cu^{2+}, 10 mM H_2O_2 and 10 mM ascorbate (Fig. 1a, lane 3).

A more quantitative evaluation of formation of these new products in copper-induced CL fragmentation was provided by conventional MALDI-TOF mass spectrometry. This approach has the advantage that both educts and products can be seen in the same spectrum. Untreated TOCL yielded three peaks corresponding to mixed protonated and sodiated adducts of TOCL: 1480.1 (M + 2H + Na), 1502.1 (M + H + 2Na) and 1524.1 (M + 3Na) (see Fig. 2a, spectrum A). We observed significant changes at lower masses in samples treated with oxidizing reagents (see Fig. 2a, spectrum B). New peaks appeared at 723.5 and 745.5 Da for DOPA. Further signals were found at 779.5 and 801.5 Da corresponding to DOPHA. Fig. 2b and c shows the changes in the peak intensities of adducts of DOPA and DOPHA detected in two experimental series. The peak intensities of products were highest in samples treated

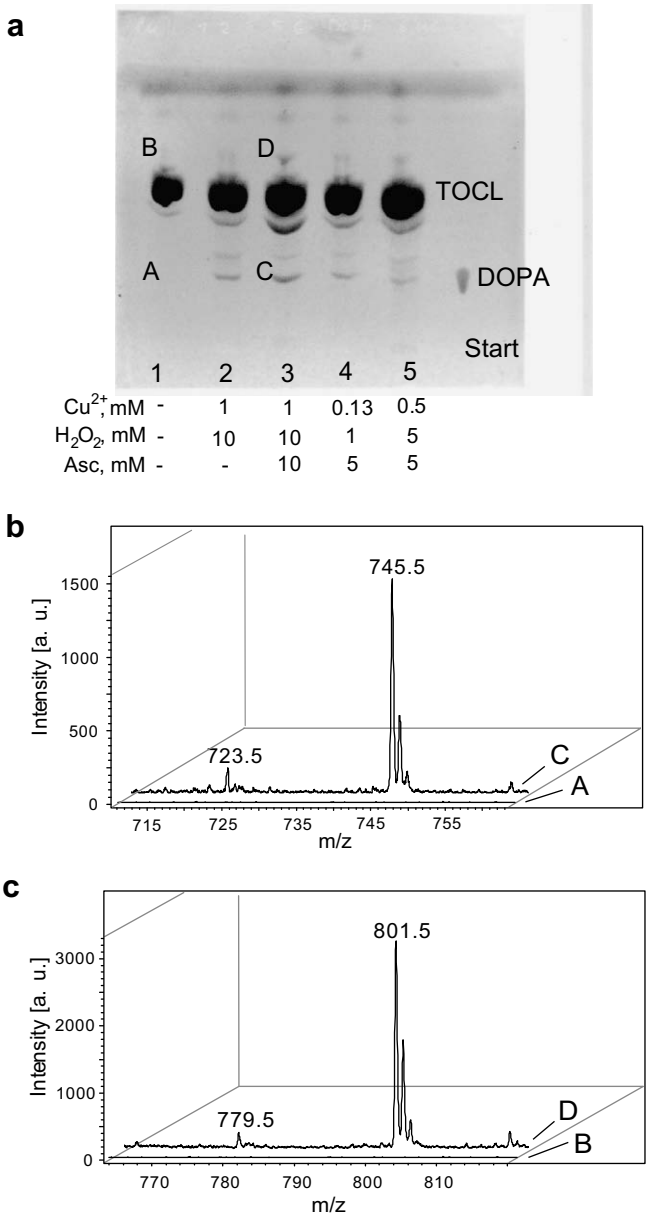

Fig. 1. Copper-induced fragmentation of cardiolipin studied by HPTLC. (a) High-performance thin-layer chromatogram of organic extract from TOCL liposomes incubated for 4 h at 37 °C: lane 1, without reagents; lane 2, with (1:10) Cu^{2+}/H_2O_2 (mM/mM); lanes 3–5 with (1:10:10), (0.13:1:5), or (0.5:5:5) Cu^{2+}/H_2O_2/ascorbate (mM/mM/mM), respectively. Thin-layer chromatogram was stained with primuline. (b) HPTLC-MALDI-TOF mass spectra acquired directly from A (spectrum A) and C (spectrum C) bands on HPTLC lanes 1 and 3, respectively. (c) HPTLC-MALDI-TOF mass spectra acquired directly from B (spectrum B) and D (spectrum D) bands on HPTLC lanes 1 and 3, respectively.

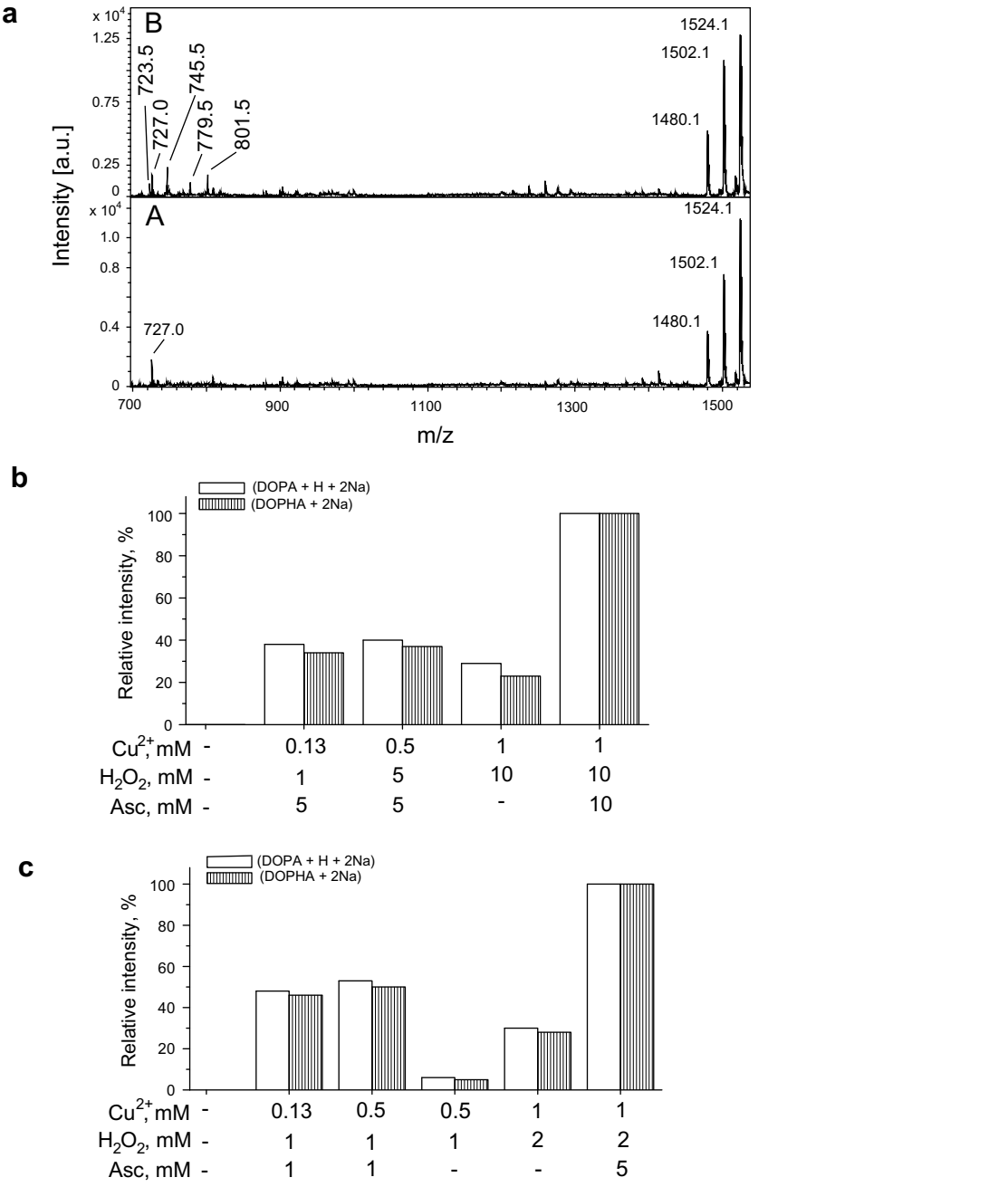

Fig. 2. Copper-induced fragmentation of cardiolipin studied by conventional MALDI-TOF MS. (a) MALDI-TOF mass spectra of the organic extract from TOCL liposomes: A, the control sample; B, the samples incubated for 4 h at 37 °C with 1 mM $CuSO_4$, 10 mM H_2O_2 and 10 mM ascorbate. (a and b) Relative signal intensity of DOPA and DOPHA products after treatment of TOCL with Cu^{2+}/H_2O_2/ascorbate as a function of the concentration of reagents. Intensity of DOPA and DOPHA at 1 mM Cu^{2+}/10 mM H_2O_2/10 mM ascorbate (b); and 1 mM Cu^{2+}/2 mM H_2O_2/5 mM ascorbate (c) was set for 100%. Diagrams show means of three independent experimental series. Standard deviations are always lower than 12%.

with 1 mM Cu^{2+}/10 mM H_2O_2/10 mM ascorbate or 1 mM Cu^{2+}/2 mM H_2O_2/5 mM ascorbate (see Fig. 2b and c, respectively). Omission of ascorbic acid decreased considerably the product yield.

Next, we investigated metal ion-induced fragmentation in bovine heart CL, a naturally occurring CL species that contains mainly linoleoyl fatty acid residues. Tetralinoleoylcardiolipin (TLCL) is a more unsaturated lipid and can be more oxidized in its hydrophobic part upon action of ROS in comparison to TOCL. Mainly peaks from TLCL were observed in mass spectrum obtained for the untreated sample (see Fig. 3a). TLCL showed intense peaks at 1472.0 (M + 2H + Na), 1494.0 (M + H + 2Na) and 1515.9 (M + 3Na). Further less intense peaks were found at 1532.0 and 1548.0. Apparently, these CL species are being oxidized and contain one or two additional oxygen atoms. Full peak description is given in Table 1. Changes in the mass spectra of TLCL upon action of Cu^{2+}/H_2O_2/ascorbate reagents are given in Fig. 3b. In contrast to control, there were several new peaks in the low-molecular region of the spectrum. This finding is an evidence of the presence of new substances formed upon action of the oxidizing reagents on the TLCL-liposomes. The *m*/*z* values for the peaks at 719.5, 741.5 and 763.5 correspond to those for molecular ions of dilinoleoylphosphatidic acid (DLPA) associated with hydrogen and sodium cations (see insert Fig. 3b and Table 1). Those at 775.5 and 797.5 correspond to dilinoleoylphosphatidylhydroxyacetone (DLPHA). All

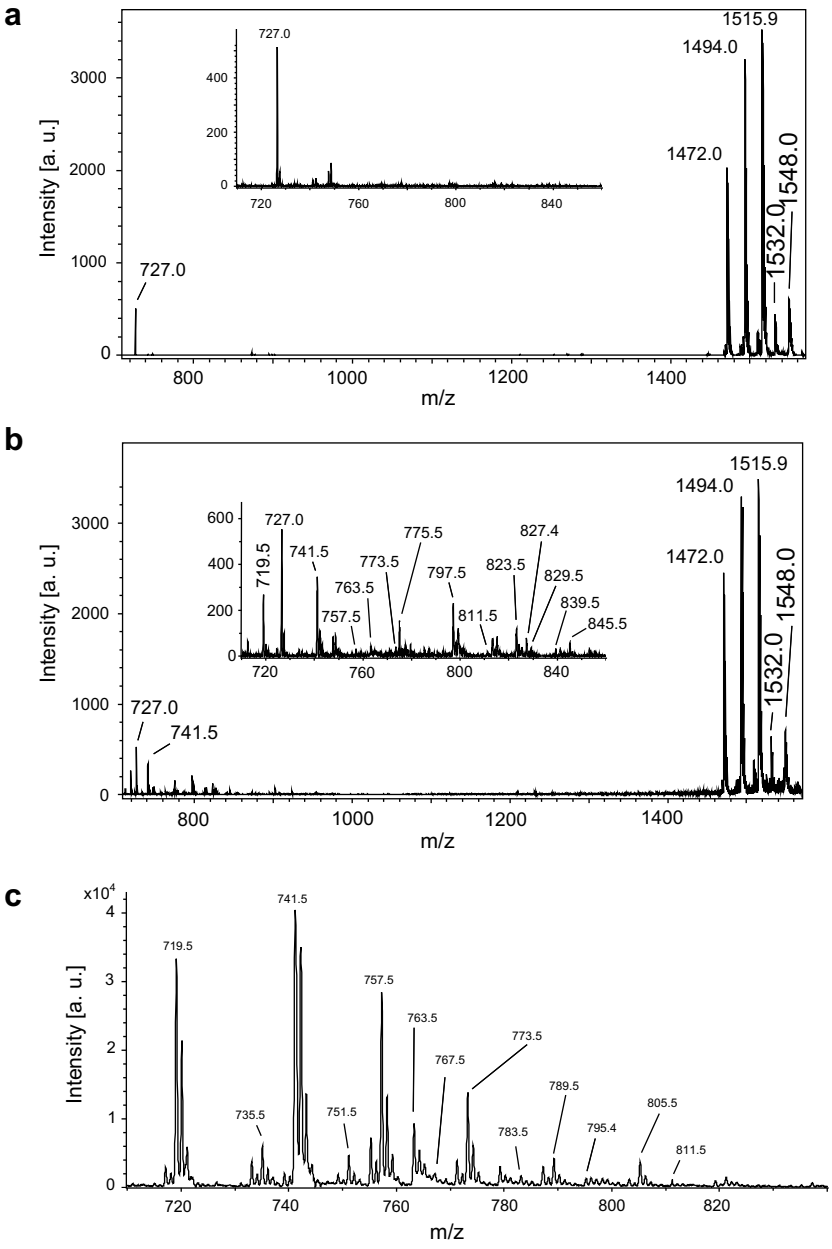

Fig. 3. MALDI-TOF mass spectra of organic extract of CL-liposomes (a and b) and standard DLPA-liposomes (c); (a) the control sample; (b and c) the samples incubated for 4 h at 37 °C with 1 mM $CuSO_4$, 1 mM H_2O_2 and 1 mM ascorbate. Inserts in (a and b) show a part of the mass spectrum within the range of 710–860 Da.

other new peaks in Fig. 3b can be attributed to oxidation products of DLPA and DLPHA. To confirm this conclusion, DLPA was oxidized by Cu^{2+}/H_2O_2/ascorbate system in the same way (see Fig. 3c) as bovine heart CL. Additionally, a series of less intense peaks with a shift of 16, 32, 48 and 64 U towards DLPA was detected in the spectrum of standard DLPA treated with oxidizing reagents. These peaks may result from an addition of molecular oxygen to DLPA (see Table 1). Previously, it has been shown that γ-irradiation of egg PG-liposomes produced the PA consisted of the oxidized and not oxidized molecular species [38]. Formation of some adducts of the DLPA oxidation products (for example at 773.5, 811.5 and 827.5) were also observed in the spectrum of TLCL upon treatment with Cu^{2+}/H_2O_2/ascorbate system (see insert Fig. 3b). The peaks at 823.5, 829.5, 839.5 and 845.5 in Fig. 3b were assigned to oxidation products of DLPHA.

The treatment with Cu^{2+} ions only, either with hydrogen peroxide alone, or ascorbate alone did not cause any changes in the mass spectra or thin-layer chromatograms of both cardiolipin species.

Formation of phosphatidic acid in mitochondria treated with iron or copper ions

Isolated mitochondria were incubated for 4 h at 37 °C without any oxidizing reagents or with Cu^{2+} (or Fe^{2+}), H_2O_2 and ascorbate. Afterwards, mitochondrial lipids were extracted and separated by two-dimensional HPTLC. Fig. 4a shows a thin-layer chromatogram of lipids extracted from the untreated sample. The major mitochondrial phospholipids were phosphatidylcholine, phosphatidylethanolamine, cardiolipin and phosphatidylinositol. Sphingomyelin, phosphatidylserine and lyso-phosphatidylcholine represented the

Table 1
Assignment of the peaks in MALDI-TOF mass spectra of an organic extract of CL or DLPA obtained from the samples incubated for 4 h at 37 °C with 1 mM $CuSO_4$, 1 mM H_2O_2 and 1 mM ascorbate

Peak position (m/z)	Assignment of molecular mass
719.5	DLPA + 2H + Na
727	4DHB − 4H + 5Na
735.5	DLPA + 2H + K
741.5	DLPA + H + 2Na
751.5	DLPA + 2H + Na + 2O
757.5	DLPA + H + Na + K
763.5	DLPA + 3Na
767.5	DLPA + 2H + Na + 3O
773.5	DLPA + H + 2Na + 2O
775.5	DLPHA + H + Na
783.5	DLPA + 2H + Na + 4O
789.5	DLPA + H + 2Na + 3O
795.4	DLPA + 3Na + 2O
797.5	DLPHA + 2Na
805.5	DLPA + H + 2Na + 4O
811.5	DLPA + 3Na + 3O
823.5	DLPHA + H + Na + 3O
827.4	DLPA + 3Na + 4O
829.5	DLPHA + 2Na + 2O
839.5	DLPHA + H + Na + 4O
845.5	DLPHA + 2Na + 3O
1472	TLCL + 2H + Na
1494	TLCL + H + 2Na
1515.9	TLCL + 3Na
1532	TLCL + 3Na + O
1548	TLCL + 3Na + 2O

remainder of phospholipids content. These lipids were identified by comparison with a standard lipid mixture (PC, PE, CL, PI, lyso-phosphatidylcholine, PG, ceramide, fatty acids, GalDAG, DAG and cholesterol) as well as obtaining mass spectra from these spots. Our results obtained by HPTLC and its combination with MALDI-TOF MS are in good agreement with literature data [39]. Untreated mitochondria did not reveal any spots in HPTLC at places where phosphatidic acid and phosphatidylhydroxyacetone were detected, if they were added to the standard lipid mixture or to unperturbed mitochondria samples (data not shown).

Here, we focused our attention on the appearance of phosphatidic acid as fragmentation product of cardiolipin in stressed mitochondria. All main phospholipid classes detected in the control were also found in a sample treated with 1 mM $FeSO_4$, 1 mM H_2O_2 and 1 mM ascorbate (Fig. 4b). In addition, the later sample contained a new less intense spot localized at the place where PA used as standard was found. The position of PA on HPTLC-plate was marked by the sign "P". Fig. 4c shows segments of HPTLC-plates of mitochondria samples treated without reagents (plate A) or with H_2O_2 (plate B), $FeSO_4/H_2O_2$ (plate C), $CuSO_4/H_2O_2$ (plate D) and $FeSO_4/H_2O_2$/ascorbate (plate E). Weak intense spots at position "P" were observed in all mitochondrial samples treated with a mixture of metal ions and hydrogen peroxide (samples C–E), but not in the absence of metal ions (sample B).

MALDI-TOF mass spectra were directly acquired from bands "P" on HPTLC plates (Fig. 5). Spectrum A originating from untreated mitochondria exhibited as expected only background ions, most probably originating from the silica gel. In contrast, spectrum E from mitochondria treated with the $FeSO_4/H_2O_2$/ascorbate system revealed detectable ion signals, whose intensity was clearly distinguished from the background noise. The presence of a large number of peaks evidences that this phospholipid has a varying acyl chain content. Proposed ion structures for the experimental m/z values detectable in spectrum E are given in Table 2. The following PA species can be assigned: PA (38:6), PA (38:5), PA (40:9) and PA (40:8). The spectrum of sample E contains further less intense signals that where not further assigned. Apparently, some of these signals may derive from oxidized CL species (see Table 2). Similar spectra were obtained in mitochondria samples treated with Cu^{2+} in the presence of hydrogen peroxide and ascorbate (data not shown).

Among mitochondrial lipids, both CL and PI are known to yield phosphatidic acid as fragmentation product [27]. Thus, we next analyzed the fatty acid composition of mitochondrial CL and PI and compared it with the fatty acid composition of the newly formed PA. By this approach, we will identify CL as the source of PA. The HPTLC-MALDI mass spectrum obtained directly from the PI band on the HPTLC-plate (see Fig. 4a, band 5) is presented in Fig. 6a. The two more intense peaks at 909.6 and 931.6 are caused by PI (38:4), while the two less intense peaks at 881.6 and 903.6 correspond to PI (36:4). The detailed peak description is given in Table 3. Thus, the acyl region of mitochondrial PI is most likely composed of one C18 and one C20 chain with a total number of four double bonds. A fragmentation of mitochondrial PI would result in formation of PA with the same fatty acid composition. But, we did not find any PA species with 38:4 or 36:4 in stressed mitochondria.

The mass spectrum of mitochondrial CL contained a high number of peaks between 1470 and 1650 Da (Fig. 6b), indicating that CL consists of a large variety of molecular species that are enriched in long chain fatty acids. It is known that cardiolipin acyl chains vary greatly by species and tissue, and can be modified by diet [40,41]. Cardiolipin contains an extraordinarily high percentage of unsaturated fatty acids with linoleic acid (59–90%) being the main one [41]. Liver mitochondrial CL is distinguished from other phospholipids by the presence of linoleoyl in almost all molecular species [40]. Except of linoleic acid (LA) residues, mouse liver mitochondrial CL is known to contain oleic (18:1), linolenoic (18:3), arachidonic (20:4), eicosatrienoic (20:3), eicosapentaenoic (20:5) and docosahexaenoic (DHA) (22:6) acid residues [40]. DHA as well as LA are extensively incorporated into CL (up to 50%) [42]. As mentioned above for TOCL and CL from bovine heart, it is rational to assume that mitochondrial CL gives also three types of adducts (CL + 2H + Na), (CL + H + 2Na) and (CL + 3Na) in mass spectrum displayed in Fig. 6b. The proposed structures of CL molecular ions for the detected m/z values are listed in Table 3. Most intense peaks were found in the regions 1540.0–1544.0, 1564.0–1570.0, 1588.0–1596.0 and 1612.0–1618.0. Cardiolipins with a ratio of 78:14, 80:16, 80:15 and 80:14 for the number of carbon atoms to the number of double bonds in the acyl regions corresponded well to these mass peaks. Further CL species could be assigned with lower intensities by all three mass peaks. These CL species were 72:7, 72:8, 74:10, 76:12, 76:13, 78:13, 82:16, 82:17, 82:18 and 82:19. Thus, cardiolipin isolated from liver mitochondria of mice contained indeed a broad range of fatty acyl chains. A fragmentation of the CL species (78:14, 80:16, 80:15 and 80:14) would predominantly yield PA species bearing 38 or 40 carbon atoms in the acyl chains and a varying number of double bonds. Distribution of PA species in stressed mitochondria corresponds well to the great variety of CL species, but not to acyl chain composition in PI.

Discussion

ROS-induced damage of CL has been intensively studied by many investigators [2,4,23,25,26]. Among the mitochondrial constituents, cardiolipin molecules appear particularly susceptible to ROS attack because of their localization in the inner mitochondrial membrane in close proximity to the site of ROS generation [43,44]. Mitochondria from animals that have been exposed to oxidative stress are specifically depleted of CL [44]. The most extensively investigated ROS-induced destructive process of CL is the peroxidation in the hydrophobic core of CL. In the present work, we focused our attention on the free-radical fragmentation of CL

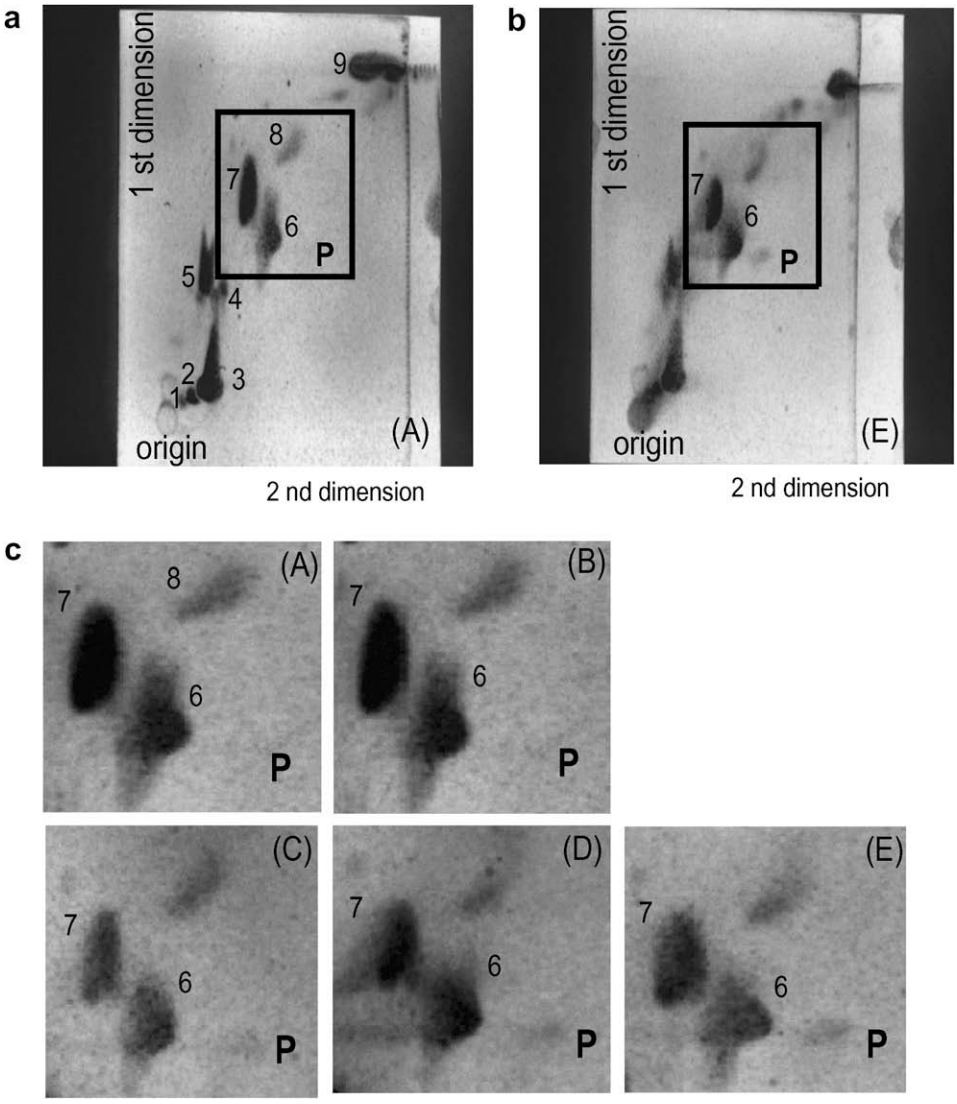

Fig. 4. High-performance thin-layer chromatograms of lipids extracted from mitochondria incubated for 4 h at 37 °C without reagents (a) or with 1 mM $FeSO_4$, 1 mM H_2O_2 and 1 mM ascorbate (b). HPTLC plates (silica gel H 60) were run with methyl acetate/2-propanol/chloroform/methanol/0.25% KCl in water (25:25:25:10:9, v/v/v/v/v) in the first dimension and with chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v)) in the second dimension. Lipids spots stained by primuline were identified as lyso-phosphatidylcholine (1), sphingomyelin (2), phosphatidylcholine (3), phosphatidylserine (4), phosphatidylinositol (5), CL (6), phosphatidylethanolamine (7), unascertained (8), neutral lipids (9). The symbol "P" indicates a silica gel zone on chromatogram corresponding to phosphatidic acid. (c) Segments of high-performance thin-layer chromatograms of lipids extracted from mitochondria incubated for 4 h at 37 °C without reagents (A) or with 1 mM H_2O_2 (B); 1 mM $FeSO_4$ and 1 mM H_2O_2 (C); 1 mM $CuSO_4$ and 2.0 mM H_2O_2 (D); 1 mM $FeSO_4$, 1 mM H_2O_2 and 1 mM ascorbate (E). The zone of the chosen segments is marked by square on the TLC plates showed in the part (a) of this figure.

occurring in the polar part of this lipid. The fragmentation products phosphatidic acid and phosphatidylhydroxyacetone (PHA) are known to be formed in CL-liposomes upon the treatment with the Fe^{2+}/H_2O_2 system [45]. Here we showed for the first time, that the Cu^{2+}/H_2O_2 system is also able to induce CL fragmentation. The presence of ascorbate that efficiently recycles Cu^{+} from Cu^{2+} [46,47] enhanced markedly the product yield. Due to these reactions, conditions for the enhanced formation of hydroxyl radicals are created:

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + HO^{\cdot} + OH^{-}$$
$$Cu^{2+} + Asc^{-} \rightarrow Cu^{+} + Asc^{\cdot}$$

However, the most important result of this study is the evidence for the formation of phosphatidic acid derived from CL in mitochondria subjected to an oxidative stress by metal ions. Thus, CL fragmentation is likely to occur also under *in vivo* conditions. Mitochondria accumulate iron for heme and iron–sulfur cluster formation and copper for the synthesis of enzymes such as copper cytochrome oxidase [48]. The combination of increased iron or copper load and enhanced formation of H_2O_2 in mitochondria might result in the generation $HO^{\cdot}$ radicals via Fenton chemistry. Iron overload to mitochondria increases lipid peroxidation, induces mitochondrial DNA damage and leads to mitochondrial dysfunction [49,50]. Studies with copper-loaded animals have shown that copper is mostly located in the mitochondrial fraction [15]. Copper overload causes mitochondrial swelling, cytochrome inactivation and oxidative stress [15]. There is also clear evidence that iron and copper accumulation is deleterious to mitochondria integrity and function in numerous pathological conditions most important metal storage disorders as hereditary hemochromatosis (iron) [51] and Menkes and Wilson disease (copper) [7,15]. Our findings prove causality of such conditions i.e. for both iron and copper toxicity targeted to mitochondrial lipids and membranes.

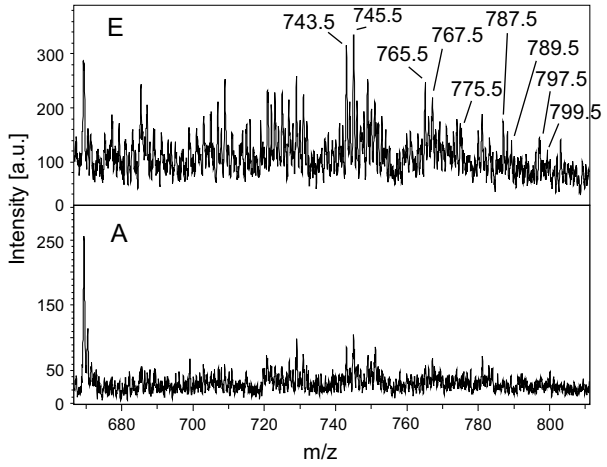

Fig. 5. HPTLC-MALDI-TOF mass spectra acquired directly from bands marked by the symbol "P" on chromatograms shown in Fig. 4(b)-A (spectrum A) and 4(b)-E (spectrum E), respectively. HPTLC bands were sprayed with DHB-solution.

Table 2
Proposed structures of molecular ions detected by HPTLC-MALDI-TOF MS of newly formed phospholipid species in stressed mitochondria

m/z	Phospholipid ion
743.5	PA (38:6) + 2H + Na
745.5	PA (38:5) + 2H + Na
765.5	PA (38:6) + H + 2Na, PA(40:9) + 2H + Na
767.5	PA (40:8) + 2H + Na, PA(38:5) + H + 2Na
775.5	PA (38:6) + 2H + Na + 2O
787.5	PA (38:6) + 3Na, PA(40:9) + H + 2Na
789.5	PA (40:8) + H + 2Na
797.5	PA (38:6) + H + 2Na + 2O
799.5	PA (40:8) + 2H + Na + 2O, PA (38:5) + H + 2Na

Previously, properties of radical intermediates generated from phospholipids were studied using various selective and non-selective methods [27–31]. It has been shown [31] that mainly carbon-centered α-hydroxyl-containing radicals formed from lipids are responsible for the fragmentation process. Oxidative equivalents derived from the $Cu^{2+}(Fe^{2+})/H_2O_2$ system, most likely $HO^{\cdot}$ radicals can induce fragmentation of CL according to Fig. 7.

The fatty acid composition of PA and PHA is determined by the cardiolipin species used. In comparison to TOCL, more complex processes take place in both the hydrophobic and hydrophilic parts of higher unsaturated CL species. Thus, free-radical transformation of TLCL resulted in the production of new lipids containing both oxidized and non-oxidized residues of fatty acids (see Fig. 3 and Table 1). The signal intensity of non-oxidized products exceeded that of the corresponding oxidized products. Formation of peroxidation compounds can occur as a result of fragmentation of already oxidized TLCL or by oxidation of PA and PHA formed in this process.

Cardiolipin represents about 25% of the total phospholipids of mitochondria [39]. Besides CL among mitochondrial phospholipids PI can undergo a free-radical fragmentation reaction with formation of PA [27]. In mitochondrial membranes the PI content is about 10% [39]. In order to identify the source for PA formed in oxidized mitochondria, we separated mitochondrial lipids by two-dimensional HPTLC and analyzed the fatty acid composition of PA by mass spectrometry. According to our results, PA is most likely derived from CL.

We did not detect the formation of PHA in oxidized mitochondria under our experimental conditions. A previous study has shown that γ-irradiation of tetramyristoylcardiolipin yielded high-

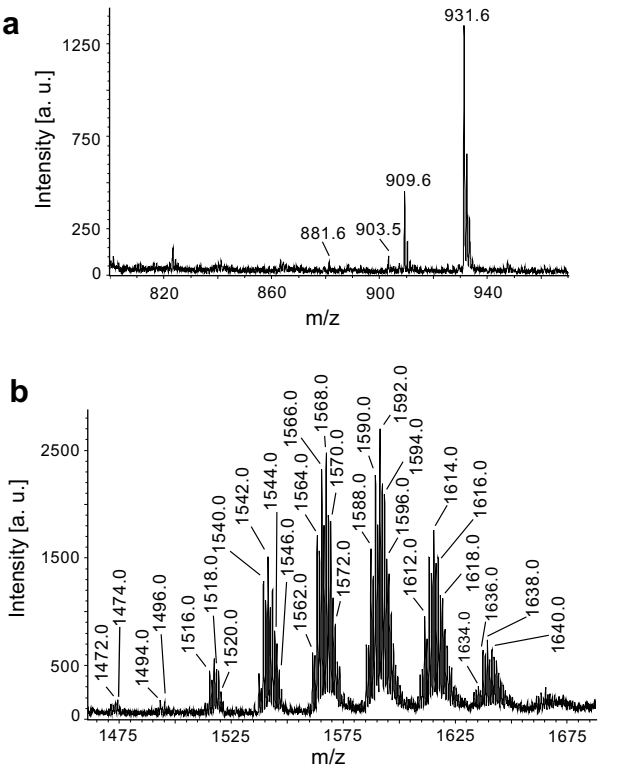

Fig. 6. (a) HPTLC-MALDI-TOF mass spectrum acquired directly from phosphatidylinositol band on HPTLC plate showed in Fig. 4(a)-A; (b) MALDI-TOF mass spectrum of mitochondrial CL.

Table 3
Proposed structures of molecular ions detected by MALDI-TOF MS of mitochondrial PI and CL (in case of CL standard MALDI was used, in case of PI HPTLC-MALDI was used)

m/z	Phospholipid ion
881.6	PI (36:4) + H + Na
903.5	PI (36:4) + H + 2Na
909.6	PI (38:4) + H + 2Na
931.6	PI (38:4) + 2Na
1472	CL (72:8) + 2H + Na
1474	CL (72:7) + 2H + Na
1494	CL (72:8) + H + 2Na
1496	CL (72:7) + H + 2Na, CL (74:10) + 2H + Na
1516	CL (72:8) + 3Na
1518	CL (76:13) + 2H + Na, CL (74:10) + H + 2Na, CL (72:7) + 3Na
1520	CL (76:12) + 2H + Na
1540	CL (76:13) + H + 2Na, CL (74:10) + 3Na
1542	CL (76:12) + H + 2Na
1544	CL (78:14) + 2H + Na
1546	CL (78:13) + 2H + Na
1562	CL (76:13) + 3Na
1564	CL (76:12) + 3Na
1566	CL (78:14) + H + 2Na,
1568	CL (80:16) + 2H + Na, CL (78:13) + H + 2Na
1570	CL (80:15) +2H + Na
1572	CL (80:14) + 2H + Na
1588	CL (78:14) + 3Na
1590	CL (82:19) + 2H + Na, CL (80:16) + H+2Na, CL (78:13) + 3Na
1592	CL (82:18) + 2H + Na, CL (80:15) + H + 2Na
1594	CL (82:17) + 2H + Na, CL (80:14) + H + 2Na
1596	CL (82:16) + 2H + Na
1612	CL (82:19) + H + 2Na, CL (80:16) + 3Na
1614	CL (82:18) + H + 2Na, CL (80:15) + 3Na
1616	CL (82:17) + H + 2Na, CL (80:14) + 3Na
1618	CL (82:16) + H + 2Na
1634	CL (82:19) + 3Na
1636	CL (82:18) + 3Na
1638	CL (82:17) + 3Na
1640	CL (82:16) + 3Na

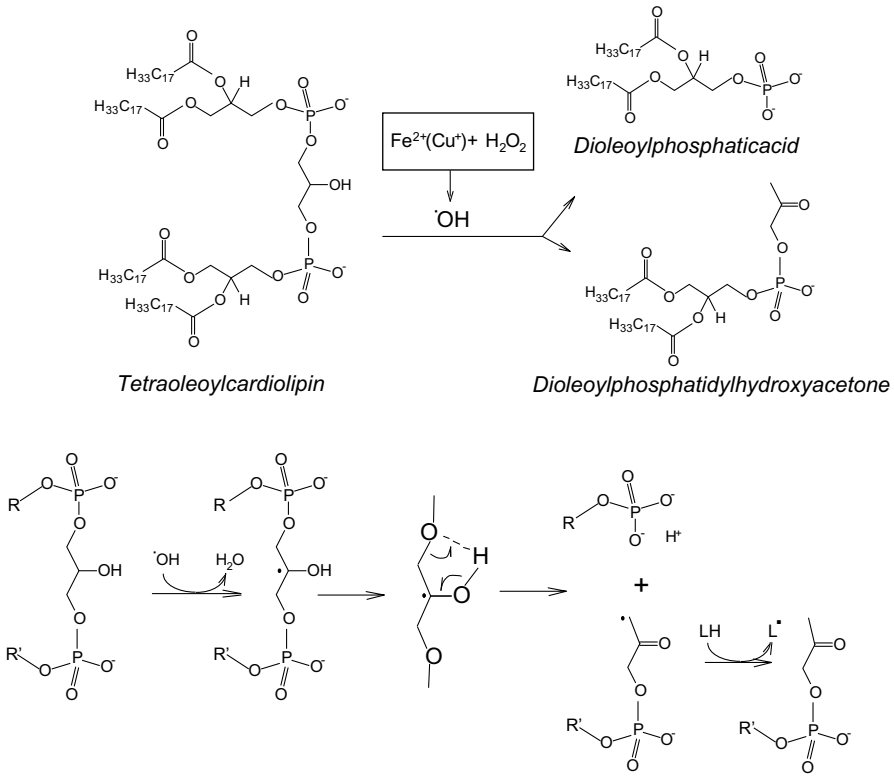

Fig. 7. Scheme for the free-radical fragmentation of tetraoleoylcardiolipin under the influence of metal ions. The lower part shows details of the supposed reaction mechanism, where *R* and *R'* are 1,2-diacylglyceryl residues. LH stands for a species that is involved in the reduction step of phosphatidylhydroxyacetone formation.

er amounts of dimyristoylphosphatidic acid (DMPA) than dimyristoylphosphatidylhydroxyacetone (DMPHA). Furthermore, the yield of DMPHA decreased with increasing saturation of dioxygen [52]. Apparently, PA was formed during CL fragmentation as molecular product, while PHA was formed from the corresponding radical intermediate after interaction with any electron and hydrogen donating group (Fig. 7). Thus, the yield of PHA should depend on other components of the incubation cocktail competing with this radical intermediate. Considering the low level of PA formed in oxidized mitochondria, it is not surprising that we did not detect any PHA species. Maybe more PHA accumulates under conditions of hypoxia. Recent evidence indicates that hypoxia enhances the generation of oxidants in mitochondria [8].

We conclude that under the action of free-radical process initiators i.e. copper and iron, mitochondrial cardiolipin does, along with peroxidation of fatty acid residues, undergo fragmentation by a pathway revealed in this investigation. Disappearance of a part of the membrane cardiolipin pool may be accompanied by changes in the microdomain structure and in the potential of forming a non-bilayer HII phase in the membrane [53], by a decrease in enzyme activity of oxidative phosphorylation system [24,54] and weakening of cytochrome *c* interaction with mitochondrial surface [55], leading to induction of apoptosis. Moreover, the formation of new phospholipids including the bioactive phosphatidic acid may contribute to modulation of physiological functions of mitochondria [56].

Thus, phosphatidic acid formed in mitochondria under the conditions of Fe^{2+}/Cu^{2+}-mediated generation of $HO^{\cdot}$ radicals. This newly formed phosphatidic acid is derived from cardiolipin undergoing a free-radical fragmentation in its hydrophilic part. This damaging mechanism in stressed mitochondria disturbs the integrity of the inner mitochondrial membrane and contributes apparently to induction of apoptosis and a number of disease-associated changes in cell metabolism.

Future studies should focus on cellular and animal models of metal accumulation and toxicity to investigate the biological significance of this newly discovered process. More insight into such molecular mechanisms may pave the way for development of targeted strategies for prevention of mitochondrial and cellular damage in conditions of cellular iron and/or copper overload.

Acknowledgments

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