Published in final edited form as:

Free Radic Biol Med. 2008 June 1; 44(11): 1935–1944. doi:10.1016/j.freeradbiomed.2008.02.016.

Cardiolipin deficiency leads to decreased cardiolipin peroxidation and increased resistance of cells to apoptosis

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Abstract

Cardiolipin (CL), a unique mitochondrial phospholipid synthesized by CL synthase (CLS), plays important, yet not fully understood, roles in mitochondria-dependent apoptosis. We manipulated CL levels in HeLa cells by knocking down CLS using RNA interference and selected a clone of CL-deficient cells with ~45% of its normal content. ESI-MS analysis showed that CL molecular species were the same in deficient and sufficient cells. CL deficiency did not change mitochondrial functions (membrane potential, reactive oxygen species generation, cellular ATP levels) but conferred resistance to apoptosis induced by actinomycin D (ActD), rotenone or gamma-irradiation. During ActD induced apoptosis, decreased CL peroxidation along with suppressed cytochrome (cyt) *c* release were observed in CL-deficient cells, while Bax translocation to mitochondria remained similar to that in CL-sufficient HeLa cells. The amounts of loosely-bound cyt *c* (releasable in high ionic strength conditions) were the same in CL-deficient and CL-sufficient cells. Given that CL peroxidation during apoptosis is catalyzed by CL/cyt *c* complexes and CL oxidation products are essential for cyt *c* release from mitochondria, our results suggest that CL deficiency prevents adequate assembly of productive CL/cyt *c* complexes and CL peroxidation resulting in increased resistance to apoptosis.

Keywords

Cardiolipin; cardiolipin synthase; cytochrome *c*; reactive oxygen species

Introduction

Elimination of unwanted or irreparably damaged cells by programmed death (or apoptosis) heavily relies on the involvement of mitochondrial mechanisms [1]. It has been established that enhanced production of reactive oxygen species (ROS) and oxidation of a mitochondria specific phospholipid, cardiolipin (CL), along with the activation of pro-apoptotic proteins of Bcl-2 family, Bax/Bak, are essential components of the apoptotic response, which culminates in mitochondrial permeabilization and release of pro-apoptotic factors into the cytosol [2]. We have recently demonstrated a unique catalytic role of cytochrome (cyt) *c* in CL oxidation [3].

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The mechanism includes high affinity interactions of cyt *c* with CL yielding complex with a strong peroxidase activity highly specific towards CL. ROS, particularly H₂O₂, are required to feed the peroxidase activity and CL oxidation. Because oxidized CL does not retain cyt *c* [4], CL oxidation seems to be essential for cyt *c* release from mitochondria [3,5,6]. There is general agreement that CL oxidation plays an essential role in mitochondrial permeabilization and release of cyt *c*, however the molecular mechanism underlying this role still needs clarification [7].

A promising approach to understand CL function in apoptosis is the manipulation of its content and oxidizability in mitochondria. In yeast, ablation of CL synthase (CLS), that catalyzes the key and final step of multi-stage CL synthesis *de novo* [8], did not significantly perturb the level and activity of cyt *c* oxidase and overall mitochondrial functions reportedly due to accumulation of the precursor, phosphatidylglycerol (PG), that was capable to functionally substitute the requirements in CL [9]. Recently, attempts have been made to use the RNA interference (RNAi) protocol to knock-down CLS and decrease CL content in mammalian cells [10]. While the real scale of CL depletion was not accurately assessed, based on this approach, the authors concluded that inhibition of CLS was associated with an increased sensitivity to apoptosis - likely due to a weakened binding of cyt *c* with CL. In contrast, changes of CL induced by mutations of tafazzin gene - whose product is proposed to act as an acyl-transferase affecting the CL remodeling pathway - did not change sensitivity to apoptotic challenges in cells from patients with Barth syndrome [11]. Thus, the influence of CL content and/or CL acyl composition on the sensitivity of cells to apoptosis is a subject of current scientific debate. Characterization of CL's apoptotic role is important not only for targeted drug discovery but also for understanding of cyt *c*/CL interactions in normally functioning mitochondria.

Here, we used RNAi approach to knock down CLS in HeLa cells and employed a thorough screening of clones to select those with significantly lowered CL content. We further utilized ESI-MS analysis to characterize molecular species of CL in the clones, particularly those with highly oxidizable polyunsaturated acyls; we also used two-dimensional high performance thin layer chromatography (2D-HPTLC) with fluorescence HPLC to quantitatively assess CL oxidation. We report that CL deficiency is associated with decreased CL oxidation and increased resistance of cells to apoptosis, contrasting the results described by Choi *et al* [10].

Materials and methods

Materials

Rotenone, actinomycin D (ActD), antimycin A, alamethicin and ATP bioluminescent somatic cell assay kit were purchased from Sigma (St. Louis, MO). N-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red), dihydroethidium (DHE) and JC-1 were purchased from Invitrogen (Carlsbad, CA). Antibodies against cyt *c*, Bax and Smac/Diablo were from Pharmingen (San Diego, CA). Anti COX-IV antibody was from Abcam (Cambridge, MA) and anti-actin antibody was from Calbiochem (San Diego, CA). HPTLC plates (5 × 5 cm) were purchased from Whatman (Florham Park, NJ). All other reagents were purchased from Sigma-Aldrich unless indicated.

Generation of stable CLS knockdown HeLa cells with siRNA expression vector

Two 19 nucleotide sequences (A: 5'-CCCATGGACAATCCCGAAT-3'; B: 5'-GTGCTCTTGATCCACTTGC-3') matching human CLS gene (Gene Bank, NM_019095) were chosen to construct pSEC hygro vector (Ambion, Austin, TX). In addition, a vector containing a siRNA sequence (Ambion) with limited homology to any known sequences in the human genome was used as control. The resulting plasmids were transfected into HeLa cells using Optifect™ Reagent (Invitrogen) according to the manufacturer's instruction.

Transfected cells were cultured in the conditioned DMEM medium supplemented with 15% FBS, 25 mM HEPES, 50 mg/L uridine, 110 mg/L pyruvate, 2 mM glutamine, 1 × nonessential amino acids, 2'-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin. The stably transfected clones were selected with 300 µg/ml of hygromycin B.

Lipid extraction and 2D-HPTLC analysis

Total cellular lipids were extracted as described by Folch *et al* [12] with minor modifications [13], which allowed highly reproducible quantitative analysis of all lipid fractions, including acidic phospholipids. Briefly, NaCl was replaced by KCl; the upper phase was re-extracted once with Folch lower phase containing 0.1N HCl and once with lower phase alone; non-lipid contaminants were removed from the extract by Sephadex G-25 instead of traditional aqueous washing. Lipid extracts were separated and analyzed by 2D-HPTLC on silica G plates. The plates were first developed with a solvent system consisting of chloroform:methanol:28% ammonium hydroxide (65:25:5 v/v). After the plate was dried with a forced N₂ blower to remove the solvent, it was developed in the second dimension with a solvent system consisting of chloroform:acetone:methanol:glacial acetic acid:water (50:20:10:10:5 v/v). For separation of PG, 1D-HPTLC with a solvent system consisting of chloroform:methanol:glacial acetic acid (32.5:14:4 v/v) was used. Phospholipids were visualized by exposure to iodine vapors and identified by comparison with authentic standards. Lipid phosphorus was determined by a micro-method [14]. For ESI-MS and fluorescence HPLC analysis, the phospholipid spots on the silica plates were visualized by spraying the plates with deionized water. After this, the spots were scraped and phospholipids were extracted by chloroform:methanol:water (20:10:2 v/v). Extracted phospholipids were divided into aliquots for determinations of phosphorus, ESI-MS and fluorescence HPLC analysis.

Mass spectra of phospholipids

Mass spectra of phospholipids were obtained after direct infusion into a linear ion trap mass spectrometer LXQ (Thermo Electron, San Jose, CA). After 2D-HPTLC separation, phospholipids were collected, evaporated under N₂, re-suspended in chloroform:methanol 1:2 v/v (20 pmol/µl) and directly used for acquisition of negative-ion ESI mass spectra using a flow rate of 5 µl/min. The electrospray probe was operated at a voltage differential of -3.5-5.0 kV. Source temperature was maintained at 150°C. Spectra were acquired using full range zoom (500-2000 m/z) scans. Tandem mass spectrometry (MS/MS analysis) of individual molecular CL species was used to determine the fatty acid composition. MSⁿ analysis was carried out with relative collision energy ranged from 20-40% and with activation q value at 0.25 for collision-induced dissociation and q value at 0.7 for pulsed-Q dissociation technique. PG molecular species were quantified by comparing the peak intensities with that of an internal standard PG (C12:0/C12:0) which was added in the sample prior to MS-analysis. The chemical structures of CL and PG were confirmed by using Lipid Map Data Base and ChemDraw format (www.lipidmaps.org).

Mitochondrial membrane potential and cellular ATP content

Mitochondrial membrane potential was determined using the 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) staining. Briefly, cells were incubated with 10 µg/ml of JC-1 at 37 °C for 20 min and then washed twice with PBS. The harvested cells were resuspended in PBS and then analyzed immediately by FACScan flow cytometry (BD Biosciences, Rutherford, NJ) equipped with CellQuest software. The ratio of red (aggregates) and green (monomer) fluorescence was used as a relative measure of mitochondrial membrane potential.

Cellular ATP content was measured by using an adenosine 5'-triphosphate bioluminescent somatic cell assay kit (Sigma) according to the manufacturer's instruction.

Subcellular fractionation

At the end of incubation, spontaneously detached cells were combined with those removed by trypsinization and resuspended in lysis buffer A (250 mM sucrose, 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin) containing 0.05% digitonin for 3 min on ice, and then centrifuged at $8,500 \times g$ for 5 min. The resulting supernatants were collected as cytosolic fractions. For whole cell lysates, harvested cells were resuspended in lysis buffer A with 1% Triton X-100 on ice for 1 hr, and then centrifuged at $10,000 \times g$ for 5 min to get the supernatants.

Western blotting

The samples were separated on 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with antibodies against cyt c (clone 7H8.2C12, Pharmingen), Bax (clone 6A7, Pharmingen), Smac/Diablo (clone 7, Pharmingen), COX-IV (Abcam) or actin (Calbiochem) (loading control) followed by horseradish peroxidase-coupled detection. The protein band profile was analyzed by densitometry using Labworks Image Acquisition and Analysis Software (UVP, Upland, CA).

Biomarkers of Apoptosis

At the end of incubation, still adherent cells were trypsinized and pooled with the cells already detached. Samples were then analyzed according to the instruction of Caspase-GloTM 3/7 assay kit (Promega, Madison, WI) for caspase 3/7 activity and annexin V-FITC apoptosis detection kit (Biovision, Mountain View, CA) for PS externalization.

Immunofluorescence assay

Briefly, cells were fixed with 4% paraformaldehyde/PBS, permeabilized with 0.2% Triton X-100/PBS and blocked in 2% BSA/PBS. Anti-Bax antibody was used at 1:100 in 2% BSA/PBS, followed by FITC-conjugated goat anti-mouse IgG antibody (1:200) (Upstate, Lake Placid, NY). Cells were mounted in Fluoromount-G (Southern Biotech) and then examined under a fluorescent microscope (Nikon eclipse TE200).

Superoxide generation

Production of superoxide radicals was examined using a superoxide-mediated oxidation-sensitive fluorogenic dye, DHE. Briefly, cells were incubated with 5 µM of DHE for 30 min at the indicated time point, and then collected by trypsinization and resuspended in PBS. The fluorescence of ethidium was measured using a FACScan flow cytometer (BD Biosciences) supplied with the CellQuest software. Mean fluorescence intensity from 10,000 cells was acquired using a 585/42-nm bandpass filter.

Evaluation of phospholipid hydroperoxides

CL hydroperoxides were determined by fluorescence HPLC of products formed in microperoxidase (MP) 11-catalyzed reaction with a fluorogenic substrate, Amplex Red. Oxidized phospholipids were hydrolyzed by pancreatic phospholipase A2 (2 U/ml) in 25 mM phosphate buffer containing 1.0 mM CaCl₂, 0.5 mM EDTA and 0.5 mM SDS (pH 8.0 at RT for 30 min). After that, Amplex Red and MP-11 were added and samples were incubated for 40 min at 4°C. A Shimadzu LC-100AT vp HPLC system equipped with fluorescence detector (RF-10Ax1, Ex/Em=560/590 nm) and an autosampler (SIL-10AD vp) was used for the analysis of products separated by HPLC (Eclipse XDB-, C18 column, 5 µm, 150 × 4.6 mm). The mobile phase was composed of NaH₂PO₄ (25 mM, pH 7.0)/methanol (60:40 v/v).

Alamethicin induced Cytochrome C release in isolated mitochondria

Mitochondria were isolated from HC and B23 cells using differential centrifugation. Briefly, harvested cells were resuspended in MIB buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, cocktail protease inhibitor, pH 7.4). Cells were transferred to a Dounce homogenizer and lysed with 30 gentle strokes with B-type pestle at 0 °C. The cell lysate was centrifuged at $700 \times g$ for 10 min at 4 °C to remove unbroken cells and cell debris. The resulting supernatant was centrifuged at $5,000 \times g$ for 10 min, and the pellets were collected as crude mitochondria. Mitochondria were resuspended in either low ionic buffer (250 mM sucrose, 1 mM potassium P_i , 0.5 mM EGTA and 1 mM $MgCl_2$) or high ionic buffer (250 mM sucrose, 25 mM potassium P_i , 300 mM KCl, 0.5 mM EGTA and 1 mM $MgCl_2$), and incubated on ice for 15 min in the presence or absence of 100 $\mu g/ml$ alamethicin. The samples were then centrifuged at $10,000 \times g$ for 10 min at 4 °C. The amounts of cyt *c* in the resulting supernatant and pellets were determined using Western blot.

Statistics

Data are expressed as mean $\pm$ S.D. as indicated in figure legends. Changes in variables for different assays were analyzed by a two-tailed Student's *t* test for comparisons. Differences were considered significant at $P < 0.05$.

Results

Cardiolipin synthase RNAi knock down decrease CL level in HeLa cells

To assess the role of CL, its interactions with cyt *c* and peroxidation in apoptosis we used an RNAi protocol to manipulate the activity of CLS and the level of CL in HeLa cells. Two 19-nucleotides sequences (A and B, see 'Materials and Methods') matching human CLS mRNA (Gene Bank, NM_019095) were chosen as RNAi targets and were constructed into pSEC hygro vector (Ambion, TX). Clones stably transfected with RNAi vectors were selected by hygromycin B (300 $\mu g/ml$). The contents of CL in the resulting clones were evaluated by 2D-HPTLC (Fig. 1A). Out of 48 clones examined, significantly decreased levels of CL were observed in clones HeLa/A23 and HeLa/B23: 15.9 ± 1.8 and 9.2 ± 1.5 pmol CL/nmol of total lipid phosphorus, which corresponded to ~75% and ~45% of the CL content in HeLa control (HC) cells that were stably transfected with a pSEC hygro vector harboring a small interfering RNA (siRNA) with limited sequence homology to the human genome and contained 20.8 ± 1.4 pmol CL/nmol of total lipid phosphorus. The content of other major phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (Sph) and phosphatidylinositol (PI) remained unchanged (Table 1) with a notable exception of PG (see below).

We chose to use the clone B23 with the lowest content of CL in most of further experiments (thereafter called CL-deficient cells vs CL-sufficient cells/HC cells). Some results for the clone A23 with ~25% decrease of CL content are also presented. Detailed ESI-MS analysis showed that molecular speciation of CL in CL-deficient and CL-sufficient cells were similar (Fig. 1B). Based on MS fragmentation experiments, we established that the major molecular species of CL were represented by $C_{(16:1)2}/C_{(18:1)2}$, $C_{(16:0)1}/C_{(18:1)1}/C_{(18:2)2}$, $C_{(18:1)2}/C_{(18:2)2}$, $C_{(18:1)1}/C_{(18:2)2}/C_{(20:4)1}$ and $C_{(16:0)1}/C_{(18:1)1}/C_{(20:4)1}/C_{(22:5)1}$ (Table 2). Depletion of CL was accompanied by a significant (about 1.6-fold) increase in the content of its precursor, PG (Table 1). Direct phospholipids analysis (Table 1) revealed that a 44% decrease of CL in absolute scale was equal to 11.6 pmol/nmol CL between HC cells (20.8 pmol/nmol CL) and B23 cells (9.2 pmol/nmol CL). Correspondingly, a significant increase of PG (57%) in B23 vs HC cells constituted only 1.9 pmol/nmol PG (5.2 pmol/nmol and 3.3 pmol/nmol PG in B23 cells and HC cells, respectively). This comparison indicates that the reduction of CL (11.6 pmol/nmol) could be hardly compensated by the increase of PG (1.9 pmol/nmol).

Characterization of CL-deficient cells

CL-deficient cells had similar growth rate as CL-sufficient cells with the doubling time of 29.0 ± 1.4 and 25.0 ± 1.3 hrs, respectively. Because CL is believed to be essential for maintaining mitochondrial functions, we evaluated several relevant parameters. CL-deficient cells maintained their mitochondrial membrane potential as assessed by JC-1 fluorescence (Fig. 1C). The ratio of red (595 nm) fluorescence characteristic of functional mitochondria to green fluorescence (525 nm) characteristic of impaired mitochondria was the same in CL-sufficient and CL-deficient cells. Western blotting with antibody against subunit IV of cytochrome c oxidase (COX-IV) and cytochrome c showed that CL-deficient cells and CL-sufficient cells had similar amounts of these proteins, i.e. similar amounts of mitochondria (Figs. 1D and 1E). In line with this, measurements of ATP revealed no significant difference between the levels in CL-sufficient cells and CL-deficient cells (Fig. 1F). Thus significantly reduced content of CL had no major impact on viability and bioenergetics of cells under optimal conditions.

In the previous study, Jiang et al. [15] have demonstrated that CL deficiency in yeast cells resulted in decreased activity of adenine nucleotide translocase (ANT), a central component of mitochondrial permeability transition pore (mtPTP). However, the ANT content remained unchanged when compared to the control strain as revealed by ^3H carboxyatractylate binding. Consistent with the data of Jiang *et al*, we found that CL deficient HeLa cells had the same level of ANT as the control cells (Figs. 1D and 1E).

CL deficiency increases resistance to apoptosis

It is possible that important roles of CL in apoptotic signaling are dissociated from its participation in normal bioenergetics processes in mitochondria [5]. Therefore, we examined sensitivity of the cells to stimulation by several pro-apoptotic stimuli. CL-sufficient and CL-deficient cells were challenged with 100 ng/ml of ActD for 24 and 32 hrs. CL-deficient cells displayed markedly increased resistance to apoptosis. This was evidenced by a significantly decreased release of cytochrome c from mitochondria and its accumulation in the cytosolic fraction (~4% vs ~30% in CL-sufficient cells) (Figs. 2A and 2B). In clone A23 cells the resistance was less pronounced than in the clone B23 after ActD treatment, about ~12% of cytochrome c was found in cytosolic fraction. CL can bind cytochrome c, resulting in its association with mitochondrial membranes. Therefore, we tested whether release of another pro-apoptotic factor, Smac/Diablo, which does not directly interact with CL, was affected by CL deficiency. Similar to cytochrome c, ActD induced release of Smac/Diablo was diminished in CL-deficient cells compared to CL-sufficient cells (Figs. 2A and 2B). It has been suggested that release of Smac/Diablo is associated with mtPTP under certain circumstances [16]. To experimentally address possible involvement of mtPTP in decreased release of Smac/Diablo in CL-deficient B23 cells, we studied the effect of cyclosporine A (CyA, an mtPTP inhibitor) on ActD induced apoptosis. As shown in figure 2C, CyA exerted no effect on ActD (100 ng/ml) induced PS externalization at low concentrations (0.5, 1 and 2 μM). At a higher concentration (5 μM), CyA synergistically enhanced ActD induced cell death (Fig. 2C). Therefore, the effect of CyA on ActD induced Smac/Diablo release was analyzed at a lower dose (2 μM). We found that CyA did not affect ActD induced cytosolic accumulation of Smac/Diablo (Figs. 2D and 2E), indicating that mtPTP was not likely involved in the release of Smac/Diablo under the experimental settings used. Further, caspase-3/7 activation was stronger in CL-sufficient cells than in CL-deficient cells. In CL-sufficient cells, a 9.2- and 16.5-fold increase (vs untreated cells) was detected after 24 and 32 hrs incubation with ActD, respectively. Under the same conditions, only a 2.6- and 4.9-fold activation of caspase-3/7 was found in CL-deficient cells (Fig. 2F). Analysis of PS externalization further revealed that CL-deficient cells were less sensitive to ActD treatment. Around 40% and 70% (vs ~4% in untreated cells) of CL-sufficient cells were Annexin V-FITC positive after 24 and 32 hrs incubation with ActD, respectively. In contrast, only about 13% and 27% of CL-deficient cells exposed PS on the surface under the same conditions (Fig. 2G).

Clone A23 also displayed an increased resistance to ActD induced PS externalization, albeit less pronounced than clone B23. Around 22% and 44% of the clone A23 cells showed PS externalization under the same treatments.

We further employed rotenone, an inhibitor of mitochondrial respiratory complex I, as an alternative pro-apoptotic agent, to determine whether the observed suppression of apoptotic responses in CL-deficient cells is specific to ActD. In this case too, we found that PS externalization was significantly less manifested in CL-deficient cells than in CL-sufficient cells (Fig. 2H). Finally, we used γ -irradiation (20Gy) as an apoptotic trigger, and again detected increased resistance of CL-deficient cells compared to CL-sufficient cells as documented by PS externalization (Fig. 2I). Combined, these results indicate that CL deficiency is associated with increased resistance to apoptosis induced by different agents. To identify possible molecular signaling pathways involved in de-sensitization of CL-deficient cells to pro-apoptotic stimulation we tested several mechanisms.

ActD induced Bax translocation occurs in CL-deficient cell

Release of cyt *c* from mitochondria into the cytosol is regulated by proteins of Bcl-2 family. Translocation of Bax from the cytosol to the outer mitochondrial membrane (OMM) is a critical triggering step in mitochondrial apoptotic signaling. It has been reported that Bax-dependent mitochondrial permeabilization requires the presence of CL [17]. Therefore, we determined whether deficiency of CL would lead to inhibition of Bax redistribution. Cytosolic Bax was decreased to a comparable extent in both CL-sufficient and CL-deficient cells following ActD treatment (Figs. 3A and 3B). Immuno-fluorescence staining (Fig. 3C) revealed punctuate distribution profiles of Bax in both cell lines treated with ActD, indicating its mitochondrial localization. These results are consistent with several reports that CL is dispensable for the redistribution of Bax during apoptosis [18,19]

ROS generation in CL-sufficient and CL-deficient cells

Generation of ROS is one of the prerequisites for the execution of apoptotic program which is controlled by Bax signaling [20]. Disrupted mitochondrial electron transport – primarily at Complexes I and III - is believed to be the major source of ROS during apoptosis [2]. We next examined whether deficiency of CL in mitochondria resulted in an impaired ROS generation. CL-sufficient and CL-deficient cells were exposed to rotenone (1 μ M) or antimycin A (2 μ M) (complex I and III inhibitors, respectively) for 4 hrs. Rotenone and antimycin A induced massive generation of superoxide that was equally effective in CL-sufficient and CL-deficient cells (Figs. 4A and 4B). In addition, similar levels of superoxide generation were detected in ActD treated CL-sufficient and CL-deficient cells (1.12 and 1.10 fold over untreated cells, respectively) (Fig. 4C). Thus, CL deficiency is not associated with changes in ROS production during apoptosis.

CL deficiency leads to decreased CL oxidation during apoptosis

Previously, we demonstrated that oxidation of CL is essential for mitochondria permeabilization and release of pro-apoptotic factors [3]. Therefore, we next examined CL oxidation in CL-sufficient and CL-deficient cells following ActD treatment. The content of CL hydroperoxides (CL-OOH) in ActD treated CL-sufficient cells (100 ng/ml, 18 hrs) was found to increase almost 20-fold to 153.3 ± 19.2 pmol CL-OOH/nmol CL vs 6.6 ± 4.3 pmol CL-OOH/nmol CL in untreated cells. In contrast, a significantly lower level of CL-OOH accumulation was detected in CL-deficient cells under the same conditions (72.9 ± 2.1 pmol CL-OOH/nmol CL vs 20.8 ± 16.8 pmol CL-OOH/nmol CL in untreated cells, a 3.5-fold increase) (Fig. 5). Notably, the peroxidation was CL specific, as more abundant phospholipids – PC, PE – did not undergo significant oxidation (Fig. 5). Earlier studies established that CL oxidation during ActD induced apoptosis is driven by the peroxidase activity of cyt *c*/CL

complexes whereby ROS (H_2O_2 formed by dismutation of superoxide radicals) are utilized as a source of oxidizing equivalents [3]. Because ROS production was essentially identical in the two cell lines, the reduced CL oxidation in CL-deficient cells is likely due to a less effective catalysis of oxidation e.g. less effective formation of peroxidase cyt *c*/CL complexes.

Releasable pool of cyt *c* is not changed by CL deficiency

Most of cyt *c* (up to 80-85%) in the inter-membrane space is either solubilized or electrostatically bound to the membrane and can be readily released in high ionic strength conditions by membrane permeabilization [21]. As a strong complexer of cyt *c*, available CL in the outer leaflet of the inner mitochondrial membrane (IMM) is likely responsible for tight membrane association of the remaining cyt *c* [5]. It is possible that CL deficiency may cause changes of binding of cyt *c* within mitochondria and hence alter the release of cyt *c* during apoptosis [22]. To address experimentally this possibility we compared the release of cyt *c* from mitochondria obtained from CL-sufficient and CL-deficient cells using alamethicin, a channel-forming peptide antibiotic [23]. There were no detectable differences in fractions of cyt *c* that could be washed away by either low or high ionic strength buffer in CL-sufficient and CL-deficient cells. In both types of cells treated with alamethicin, the high ionic strength buffer (300 mM KCl) facilitated the release of approximately 80% of total cyt *c*, while the low ionic strength buffer (1 mM potassium Pi) removed only a small fraction of cyt *c* (approximately ~10% of total) from mitochondria (Fig. 6). Thus CL deficiency did not substantially affect the pool of releasable cyt *c* in mitochondria.

Discussion

CL is a structurally unique dimeric phospholipid combining high hydrophobicity (due to the presence of four acyl chains) with two negative charges on the phosphate groups. As a result CL readily and strongly interacts with membranes and binds with a broad spectrum of proteins in mitochondria [8]. CL participates in the formation of the cristae structure of IMM and is involved in the organization of electron transporters into supermolecular assemblies and maintenance of the activity of several other IMM proteins such as adenine nucleotide translocator (ANT), phosphate, pyruvate and carnitine carriers as well as Complex V [24,25]. While the affinity of CL binding to these proteins is poorly characterized, studies of stoichiometry indicate the presence of multiple binding sites in CL/protein complexes in mitochondria.

Synthesis of CL utilizes PG and CDP-diacylglycerol as substrates in a reaction catalyzed by CLS [26]. Disruption of CLS gene in yeast results in complete CL deficiency and PG accumulation [9]. Mitochondria of CL null strain showed only moderate dysfunction reportedly due to partial substitution of CL by its negatively charged precursor, PG [9,27] In line with the previous reports, our results show that CL deficiency did not cause obvious changes in mitochondrial function: mitochondrial membrane potential, ATP production, ROS generation remained unchanged compared to CL-sufficient cells.

Cyt *c* has a high affinity for CL (K_d is on the order of 2 nM at low ionic strength). However CL and cyt *c* are spatially separated in normal mitochondria such that most of CL is confined to the IMM (with more than 50% of it localized in the inner leaflet of IMM), while cyt *c* is located in the intermembrane space. Although CL is present in large excess compared to cyt *c*, their different compartmentalization and interactions of CL with other proteins result only in a small fraction of cyt *c*/CL complexes – about 10-12% of the total cyt *c* - in mitochondria [21,28]. This CL-associated cyt *c* is tightly (electrostatically plus hydrophobically) bound to the membrane and represents a ready to act peroxidase complex [3]. The other two pools of cyt *c* include loosely (electrostatically) bound or solubilized forms of cyt *c* and can be easily removed from mitochondria by high ionic strength buffers in the presence of channel-forming

antibiotics such as alamethicin. Our results show that these interactions between CL and cyt *c* was not changed by CL deficiency. Indeed, the majority of cyt *c* could be released by treatment of mitochondria from both CL-sufficient and CL-deficient cells by alamethicin in high ionic strength buffer. In low ionic strength conditions, only a minor fraction of cyt *c* was releasable by alamethicin from either CL-sufficient or CL-deficient mitochondria. This clearly demonstrates that deficiency of CL does not affect “looseness” of cyt *c* association with mitochondrial membranes in normal conditions.

CL is reportedly involved in several mechanisms participating in the execution of the mitochondrial stage of apoptosis [7]. One of them is CL oxidation leading to detachment and release of cyt *c* from mitochondria [5,29]. This is preceded by trans-membrane migration of CL early in apoptosis resulting in its appearance in the OMM (up to 50% of total) at the expense of its disappearance from the IMM [3,30]. In CL-sufficient mitochondria, this creates conditions for effective and quantitatively adequate formation of cyt *c*/CL complexes, activation of their peroxidase activity, and CL oxidation during apoptosis [3,33]. The oxidized CL participates further in mitochondrial permeabilization and the release of cyt *c*. Current results demonstrate, for the first time, that this essential chain of events cannot be fully realized in CL-deficient cells. We showed that CL deficiency is associated with decreased CL oxidation upon pro-apoptotic stimulation. Reduced CL oxidation is not due to down-regulation of ROS production. We speculate that the effect is likely due to a less effective formation of productive cyt *c*/CL peroxidase complexes. As a result, the release of cyt *c* from mitochondria is hampered and the execution of apoptotic program is inhibited. A minimal stoichiometry of cyt *c*/CL electrostatic interactions is 1:4, which corresponds to the total formal charges of cyt *c* and CL of +8e and -2e at neutral pH, respectively. There is likely a significant excess of CL over cyt *c* at the sites of its location, particularly during apoptosis, and thus even CL-deficient cells should seemingly have enough CL to bind all cyt *c* molecules [29,31]. However, CL is not free but rather interacts with mitochondrial electron-transporting complexes [32] and other proteins resulting in a competition for binding to CL between cyt *c* and other proteins. Some CL molecules are bound to proteins (e.g., cyt *c* oxidase), very strongly and never detach in normal conditions while other CL molecules bind less tightly and are available for exchange. One can expect that reduction of total CL content reduces the number of free and weakly bound CL molecules and thus increases the free energy penalty of releasing of CL molecules from proteins to become available for interaction with cyt *c*. We previously showed that peroxidase activity of cyt *c* rapidly increases as a function of CL/cyt *c* ratio [33]. CL deficiency (and increased free energy cost of availability of CL molecules) should predominantly reduce the probability of formation of cyt *c*/CL complexes with a large number of lipid molecules thus greatly decreasing the average peroxidase activity of cyt *c* molecules and the rate of CL peroxidation, as cyt *c* is the major catalyst of CL peroxidation in cells [3].

The above considerations are consistent with decreased CL peroxidation in CL-deficient cells and its role in the release of cyt *c* from mitochondria. Priault *et al* have shown that mitochondrial lipid oxidation is required for Bax-induced cell death [34]. Brain mitochondria can only release minimal amounts of cyt *c* (~18%) when incubated with recombinant, oligomeric Bax protein, however, up to 65% of the mitochondrial cyt *c* was released when mitochondria were incubated with both recombinant Bax and Complex I inhibitors to induce ROS generation and oxidize CL [35]. Accordingly, antioxidants supplementation and overexpression of mitochondrial antioxidant enzymes, particularly peroxiredoxin III, glutaredoxin 2 and glutathione peroxidase 4, protect cells against apoptosis [36-39].

A two-step model of cyt *c* release from mitochondria in the process of apoptosis [5] explains the pro-apoptotic effect of CL oxidation by an increased detachment of cyt *c* from the membrane as a result of reduced content of non-oxidized CL. Our data do not contradict directly this model, the oxidative detachment of cyt *c* from the membrane can be important. The

observed antiapoptotic effect of CL-deficiency supports the likelihood that CL oxidation products serve as triggers of apoptosis. It is likely that oxidized CL participates in mitochondrial membrane permeabilization. The specific mechanism(s) of this participation are not quite clear. Whether CL oxidation products can transiently destabilize the mitochondrial outer membrane, act as components and facilitators of protein machinery by interacting with Bax, ANT, function via both pathways or through additional mechanisms – remains to be elucidated.

ANT has been suggested to be part of mtPTP, a multi-protein complex associated with the release of pro-apoptotic proteins from mitochondria under certain experiment settings [16]. Thus ANT may be involved in apoptosis resistance in the CL deficient cells [40]. However, CL deficiency had no effect on the ANT content as demonstrated by other investigators [15] and us. Furthermore, blocker of mtPTP, CyA, exerted no protection on ActD induced HeLa cell apoptosis. Finally, Kokoszka *et al* [40] reported that ANT is a non-essential component of the mtPTP, and there was no difference in TNF- α induced apoptosis between ANT knockout and control hepatocytes. Therefore, we conclude that ANT is not likely to be involved in the resistance of CL deficient HeLa cells to apoptosis.

Recently, Choi *et al* employed the same RNAi approach to knock down CLS and deplete CL in cells [10]. These workers arrived at the conclusion that CL deficiency leads to increased sensitivity of HeLa cells to apoptosis [10] – an obvious disagreement with our data. We believe there are several reasons for this discrepancy. First, we performed a thorough screening of clones produced and utilized assessments of CL content to select those with ~75% and ~45% of CL compared to non-transfected controls or cells transfected with siRNA negative control. When this robust selection was omitted from our protocol, the mixed cell population did not elicit any significant difference in their CL content *vs* CL-sufficient or wild-type HeLa cells. Importantly, we directly assessed the content of CL *vs* total content of all phospholipids in the selected clones as well as in CL-sufficient cells. In contrast, Choi *et al* chose a protocol based on the integration of [3 H] palmitate into CL. It has been reported that palmitate is a poor substrate for CLS and does not significantly integrate into CL [42]. Thus only a negligibly small and hardly representative fraction of total CL was employed for the assessment of the efficiency of RNAi protocol used in these studies. Consequently, it is difficult to conclude whether CL deficiency has indeed been induced in experiments reported by Choi *et al*.

Our ESI-MS assessments indicate that no significant changes of molecular speciation of CL – particularly with regards to most oxidizable molecular species such as $C_{(18:2)4}$, $C_{(18:1)2}/C_{(18:2)2}$, $C_{(18:1)1}/C_{(18:2)2}/C_{(20:4)1}$ and $C_{(16:0)1}/C_{(18:1)1}/C_{(20:4)1}/C_{(22:5)1}$ – were associated with its deficiency in HeLa B23 and A23 clones. This may explain the difference between our results demonstrating increased resistance to apoptosis in CL-deficient clones and those by Valianpour *et al* [11]. These authors examined cells with unchanged total amount of CL and its hydrolysis products, i.e., CL-sufficient cells, but with changed molecular speciation of CL. Interestingly, this did not result in significantly changed sensitivity to apoptosis.

It is likely that CL deficiency affects sensitivity to apoptosis during the initial stage of CL/cyt *c* complex formation, after which availability of cyt *c* and H_2O_2 play an important role. Apparently, ~45% of CL is insufficient for effective CL oxidation and cyt *c* release. What drives redistribution of CL in mitochondria – tBid, other pro-apoptotic proteins - Bax/Bak possibly via activation of PLA_2 - is still unclear. Migration and redistribution of CL during apoptosis depends on the translocation of Bax from the cytosol to mitochondria [43]. Consequently, translocation of Bax to mitochondria is coupled to the increase of the peroxidase activity of mitochondria [43]. This effect of Bax should be diminished in CL-deficient cells. Dependence on H_2O_2 is likely less critical for at least two reasons. First, cyt *c*/CL complexes have a very negative redox potential (approximately by -400 mV *vs* cyt *c* alone) and cannot act as electron acceptors from complex III. As a result disrupted e-transport generates sufficient

amounts of superoxide and H₂O₂ to feed the peroxidase reaction. Second, CL-OOH is also effective as a source of oxidizing equivalents for the peroxidase reactions of cyt *c*/CL complexes [44]. Thus the dependence of CL oxidation on H₂O₂ is transient.

In conclusion, our results identify CL and its oxidation products as determinants of cell's sensitivity to apoptosis such that CL deficiency and decreased CL peroxidation are associated with the increased resistance to pro-apoptotic stimulation. This opens new avenues for using CL biosynthesis and remodeling pathways, particularly inhibitors of CLS and CL-acyl transferase as interesting new targets for antiapoptotic drug discovery.

Acknowledgments

This work is supported by NIH NIAID U19 AI068021 and The Human Frontier Science Program.

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List of Abbreviations

ActD	actinomycin D
ANT	adenine nucleotide translocase
CL	cardiolipin
CL-OOH	CL hydroperoxides
CLS	cardiolipin synthase
CyA	cyclosporine A
cyt	cytochrome
COX-IV	subunit IV of cytochrome <i>c</i> oxidase

IMM	inner mitochondrial membrane
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
RNAi	RNA interference
siRNA	small interfering RNA
ROS	reactive oxygen species
Sph	sphingomyelin
2D-HPTLC	two-dimensional high performance thin layer chromatography

Fig. 1.

Comparison of phospholipids (cardiolipins) and mitochondrial characteristics in CL-sufficient HeLa cells and CL-deficient HeLa/B23 cells generated by RNAi knock down of CLS. (A) Representative 2D-HPTLC phospholipid profile of HeLa control (HC) cells. (B) Mass spectrometry analysis of molecular species of CL in HC and B23 cells. Extracted phospholipids were analyzed by electrospray ionization mass spectrometry in negative mode. (C) Flow cytometry evaluation of mitochondrial membrane potential using JC-1. (D, E) Western blot and densitometric analysis of COX-IV, cyt *c* and ANT levels in HC and B23 cells. Whole cell lysates (50 µg) from HC and B23 cells were analyzed by Western blot with antibodies against COX-IV, cyt *c*, ANT or actin (loading control). The levels of COX-IV, cyt *c* or ANT were

expressed as mean densitometric ratios over actin. (F) Assessment of ATP level in HC and B23 cells using ATP bioluminescent somatic cell assay kit. Data presented are means $\pm$ S.D. (n=3).

Fig. 2.

Sensitivity of HC and B23 cells to apoptosis induced by different stimuli. (A, B) Western blot and densitometry analysis of cyt c and Smac/Diablo in cytosolic fractions of HC and B23 cells following ActD treatment (100 ng/ml, 18 hrs). At the end of incubation, still adherent cells were trypsinized and pooled with the cells already detached. Harvested samples were resuspended in lysis buffer A (250 mM sucrose, 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin) containing 0.05% digitonin on ice for 3 min. Samples were then centrifuged at $8,500 \times g$ for 5 min. The amounts of cyt c and Smac/Diablo in the resulting supernatant were analyzed by Western blot using antibodies against cyt c and

Smac/Diablo, respectively. Actin was used as a loading control. The levels of cyt *c* and Smac/Diablo were expressed as the mean densitometric ratios of cyt *c* or Smac/Diablo over actin, respectively. (C) Effect of CyA on ActD induced apoptosis in HC and B23 cells. Cells were treated with 100 ng/ml ActD in the presence or absence of CyA (0.5, 1 and 2 μ M) for 24 h. (D, E) Western blot and densitometric analysis of Smac/Diablo accumulation in cytosolic fractions of HC and B23 cells. (F) Assessment of ActD induced caspase-3/7 activation in HC and B23 cells using a caspase-3/7 assay kit (Promega). (C, G–I) Evaluation of PS externalization in HC and B23 cells treated with: (C) CyA, (G) ActD (100 ng/ml) for 24 or 32 hrs, (H) rotenone (1 or 2 μ M) for 48 hrs, or (I) γ -irradiation at a dose of 20 Gy (24, 48 or 72 hrs). At the end of incubation, still adherent cells were trypsinized and pooled with the cells already detached. Harvested samples were then analyzed for phosphatidylserine externalization by flow cytometry using an annexin V/PI kit. Data presented are means $\pm$ S.D. (n = 3). [#]*P*<0.05, **P*<0.01 vs HC under the same condition.

Fig. 3.

Subcellular distribution of Bax in HC and B23 cells following ActD treatment. Cells were treated with 100 ng/ml of ActD for 18 hrs. (A, B) Western blot and densitometric analysis of cytosolic Bax. At the end of incubation, still adherent cells were trypsinized and pooled with the cells already detached. Harvested samples were resuspended in lysis buffer A containing 0.05% digitonin on ice for 3 min. Samples were then centrifuged at $8,500 \times g$ for 5 min. The resulting supernatant (cytosolic fraction) was analyzed by Western blot with antibodies against Bax and actin (loading control). The levels of Bax were expressed as the mean densitometry ratio of Bax over actin. Data presented are means $\pm$ S.D. (n=3). $*P < 0.01$ vs same cells without ActD treatment. (C) Immunofluorescent staining of Bax. Cells were fixed with 4% of

paraformaldehyde, permeabilized with 0.2% of Triton X-100 and probed with antibody against Bax (1:100) followed by FITC-conjugated goat anti-mouse IgG antibody (1:200). Cells were examined under a fluorescent microscope (Nikon eclipse TE200) with a 40 × objective len.

Fig. 4.

Superoxide generation in HC and B23 cells triggered by rotenone, antimycin A, and ActD. Cells were treated with (A) rotenone (1 μ M) for 4 hrs, (B) antimycin A (2 μ M) for 4 hrs, (C) ActD (100 ng/ml) for 12 hrs. Cells were then incubated with 5 μ M of DHE for 30 min at 37°C. Collected cells were resuspended in PBS and fluorescence of ethidium was analyzed using FACScan supplied with CellQuest software. Mean fluorescence intensity from 10,000 cells was acquired using a 585-nm bandpass filter. Data presented are means $\pm$ S.D. (n=3). # P <0.05, * P <0.01 vs sample from same cells without treatment.

Fig. 5. Estimation of phospholipid hydroperoxides in HC and B23 cells following ActD treatment. Cells were treated with 100 ng/ml of ActD for 18 hrs. At the end of incubation, lipids were extracted and resolved by 2D-HPTLC. Spots of phospholipids were scraped from the HPTLC plates and amounts of phospholipid hydroperoxides were determined by a fluorescent HPLC based Amplex-Red assay. Data presented are means $\pm$ S.D. (n=3). * P <0.01 vs HC cells under the same condition.

Fig. 6.

Western blot and densitometric analysis of alamethicin induced cyt *c* release in mitochondria isolated from HC and B23 cells. Mitochondria were suspended in low ionic strength buffer (250 mM sucrose, 1 mM potassium P_i, 0.5 mM EGTA and 1 mM MgCl₂) or high ionic buffer (250 mM sucrose, 25 mM potassium P_i, 300 mM KCl, 0.5 mM EGTA and 1 mM MgCl₂) in the presence or absence of 100 µg/ml of alamethicin for 15 min on ice. Samples were then centrifuged at 10,000 × g for 10 min at 4 °C. The cyt *c* contents in the resulting supernatants and pellets were analyzed by Western blot with anti-cyt *c* antibody. The cyt *c* amounts in samples were normalized to the amounts of cyt *c* in mitochondria pellets from HC and B23 cells without alamethicin treatment, respectively. Data presented are means ± S.D. (n=3).

**P*<0.01 vs mitochondria from same cells without alamethicin treatment.

Table 1

Quantification of phospholipids levels in HC and B23 cells.

Phospholipid	pmol/nmol total phospholipid phosphorus	
	HC	B23
CL	20.8 ± 1.4	9.2 ± 1.5 [*]
PG	3.3 ± 0.5	5.2 ± 0.1 [*]
PE	241.4 ± 21.6	253.8 ± 18.9
PC	535.5 ± 31.9	547.7 ± 42.7
PS	63.7 ± 5.4	65.4 ± 3.9
PI	79.2 ± 11.0	74.1 ± 7.1
Sph	58.0 ± 6.3	49.8 ± 15.0

Data presented are means ± S.D. (n = 3).

* $P < 0.01$ vs HC cells

Table 2

Major cardiolipin molecular species in HC and B23 cells

Phospholipids are designated as follows: tetra-acyl 74:8 CL, where 74 indicates the summed number of carbon atoms at sn-1, sn-2 and sn-1', sn-2' positions and 8 designates the summed number of double bonds at sn-1, sn-2 and sn-1', sn-2' positions. These individual CL molecular species were detected by ESI as deprotonated species of CL in the negative ionization mode at m/z ratios from 737.5 to 1500.0. These m/z values indicate ratios of mass to charge for singly charged $[M-H]^-$ ions and doubly charged $[M-2H]^{2-}$ ions, respectively.

Molecular Species	$[M-2H]^{2-}$ m/z	$[M-H]^-$ m/z	Possible fatty acid composition	Content, pmol/nmol CL	
				HC	B23
66:1	688.5	1378.0	$(C_{16:1})_1/(C_{16:0})_2/(C_{18:0})_1$	5.8 ± 0.3	6.2 ± 0.3
68:4	699.5	1399.9	$(C_{16:1})_2/(C_{18:1})_2$ $(C_{16:0})_2/(C_{18:2})_2$ Minor	55.7 ± 2.8	61.8 ± 3.1
70:6	711.5	1424.0	$(C_{16:1})_1/(C_{18:1})_1/(C_{18:2})_2$	68.9 ± 3.4	70.2 ± 3.5
70:5	712.5	1425.9	$(C_{16:0})_1/(C_{18:1})_1/(C_{18:2})_2$ $(C_{16:1})_1/(C_{18:1})_2/(C_{18:2})_1$	108.2 ± 5.4	110.6 ± 5.5
72:9	722.5	1446.0	$(C_{16:0})_1/(C_{18:2})_1/(C_{18:3})_1/(C_{20:4})_1$ $(C_{16:1})_1/(C_{18:2})_2/(C_{20:4})_1$	22.2 ± 1.1	23.7 ± 1.2
72:8	723.5	1448.0	$(C_{18:2})_4$ $(C_{16:1})_1/(C_{18:1})_1/(C_{18:2})_1/(C_{20:4})_1$ Minor	45.5 ± 2.3	46.0 ± 2.3
72:6	725.5	1452.0	$(C_{18:1})_2/(C_{18:2})_2$	52.9 ± 2.7	54.0 ± 2.7
72:5	726.5	1454.0	$(C_{18:1})_3/(C_{18:2})_1$	32.3 ± 1.6	36.5 ± 1.8
72:4	727.5	1456.0	$(C_{18:1})_4$ $(C_{18:0})_1/(C_{18:1})_2/(C_{18:2})_1$	44.1 ± 2.2	46.3 ± 2.3
72:4	728.5	1458.0	$(C_{16:1})_1/(C_{18:1})_2/(C_{20:1})_1$	14.3 ± 0.7	16.3 ± 0.8
74:9	736.5	1474.0	$(C_{18:1})_1/(C_{18:2})_2/(C_{20:4})_1$	33.4 ± 1.7	34.0 ± 1.7
74:8	737.5	1476.0	$(C_{18:1})_1/(C_{18:2})_2/(C_{20:3})_1$ $(C_{18:1})_2/(C_{18:2})_1/(C_{20:4})_1$	21.7 ± 1.1	20.5 ± 1.0
74:7	738.5	1478.0	$(C_{16:0})_1/(C_{18:0})_1/(C_{20:4})_1/(C_{20:3})_1$	5.8 ± 0.3	2.9 ± 0.8
76:12	747.5	1495.9	$(C_{16:0})_1/(C_{18:1})_1/(C_{20:5})_1/(C_{22:6})_1$	5.5 ± 0.3	5.5 ± 0.3
76:10	749.5	1500.0	$(C_{16:0})_1/(C_{18:1})_1/(C_{20:4})_1/(C_{22:5})_1$	4.7 ± 0.2	4.6 ± 0.2