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The "pro-apoptotic genies" get out of mitochondria: Oxidative lipidomics and redox activity of cytochrome *c*/cardiolipin complexes

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Abstract

One of the prominent consequences of the symbiogenic origin of eukaryotic cells is the unique presence of one particular class of phospholipids, cardiolipin (CL), in mitochondria. As the product originated from the evolution of symbiotic bacteria, CL is predominantly confined to the inner mitochondrial membrane in normally functioning cells. Recent findings identified CL and its oxidation products as important participants and signaling molecules in the apoptotic cell death program. Early in apoptosis, massive membrane translocations of CL take place resulting in its appearance in the outer mitochondrial membrane. Consequently, significant amounts of CL become available for the interactions with cyt *c*, one of the major proteins of the intermembrane space. Binding of CL with cytochrome *c* (cyt *c*) yields the cyt *c*/CL complex that acts as a potent CL-specific peroxidase and generates CL hydroperoxides. In this review, we discuss the catalytic mechanisms of CL oxidation by the peroxidase activity of cyt *c* as well as the role of oxidized CL (CLox) in the release of pro-apoptotic factors from mitochondria into the cytosol. Potential implications of cyt

Abbreviations: 2D-HPTLC, two dimensional high performance thin layer chromatography; CLox, oxidized cardiolipin; DOPC, dioleoyl-L-α-phosphatidylcholine; DTPA, diethylentriaminepentaacetic acid; EPR, electron paramagnetic resonance; ESI, electrospray ionization; HPLC, high performance liquid chromatography; IMM, inner mitochondria membrane; MNP, 2-methyl-2-nitrosopropane; OMM, outer mitochondria membrane; PBS, phosphate buffered saline; Syn, synuclein; TLCL, 1,1′2,2′-tertalinoleoyl cardiolipin; TOCL, 1,1′2,2′-tetraoleoyl cardiolipin

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c/CL peroxidase intracellular complexes in disease conditions (cancer, neurodegeneration) are also considered. The discovery of the new role of cyt *c*/CL complexes in early mitochondrial apoptosis offers interesting opportunities for new targets in drug discovery programs. Finally, exit of cyt *c* from damaged and/or dying (apoptotic) cells into extracellular compartments and its accumulation in biofluids is discussed in lieu of the formation of its peroxidase complexes with negatively charged lipids and their significance in the development of systemic oxidative stress in circulation.

Keywords: Cytochrome *c*; Oxidative lipidomics; Mitochondrial toxicity

1. Introduction

> "Living is no laughing matter: you must live with great seriousness . . ." Nazim Hikmet "On Living", February, 1948.

It is hard to believe that a renowned Turkish poet, Nazim Hikmet, meant cellular aspects of life when he talked about the seriousness of life. Yet, at the molecular level, life may be as serious as our personal and societal interactions. In our body and tissues, cells send signals and communicate with each other; some of these signals are no laughing matter at all because they mean death, apoptotic or necrotic cell death. Among these signaling molecules, lipids are emerging as a new and important class of agents.

It is a common knowledge that lipids are essential for the integrity of cell membranes by constituting their hydrophobic core. For many years, this structural role of lipids as "building blocks" of the membrane bilayer, required for the proper arrangement of membrane proteins, overshadowed their other not less important functions, particularly in signaling mechanisms [1]. A significant variety of different classes of lipids and an enormous number of distinct molecular species of lipids in cells cannot be easily rationalized within a simple concept of their necessity for the maintenance of appropriate membrane fluidity [2].

With the advent of mass-spectrometry-based shotgun and functional lipidomic assessments of global lipid composition and individual species of lipids in normal cells and their changes in disease conditions, it became quite apparent that there is a significant role for lipids, particularly for different modified lipid species, in cell signaling [1,3]. Indeed, membrane lipids are known as sources of precursors for second messengers synthesized most commonly via hydrolytic pathways from parental lipids to yield a variety of signaling molecules as different as inositol-phosphates, diacylglycerols, phosphatidic acid, arachidonic acid and eicosanoids, ceramides, etc. The greatest contribution to the variety of molecular species of lipids is due to numerous combinations of their fatty acid residues with polar head-group structures. In mammals, most lipids contain polyunsaturated fatty acids that are readily oxidizable [4]. As a result, a huge number of oxidatively modified lipid molecules may be formed. Surprisingly, their role in signaling is presently just beginning to emerge.

This review is focused on apoptotic signaling by oxidized phospholipids, more specifically by oxidation products formed early in apoptosis from a mitochondria-specific phospholipid, cardiolipin (CL). It summarizes information on catalytic mechanisms of CL oxidation by the peroxidase activity of cytochrome *c* (cyt *c*) – as it forms complexes with CL – as well as the role of oxidized CL (CLox) in the release of pro-apoptotic factors from mitochondria into the cytosol. Potential implications of cyt *c*/CL peroxidase intracellular complexes in disease conditions (cancer, neurodegeneration) are also considered. Finally, exit of cyt *c* from damaged and/or dying (apoptotic) cells into biofluids is discussed in lieu of the formation of its peroxidase complexes with negatively charged lipids and their significance in the development of systemic oxidative stress in circulation.

2. Cardiolipin in mitochondria: topography changes early in apoptosis

CLs are anionic doubly negatively charged species representing two regular phosphatidyl lipid moieties fused into one molecule via the third glycerol backbone. In mitochondria, about 25 mol% of all lipids is represented by its one unusual mitochondria specific class, CLs [5]. In eukaryotes CLs are confined predominantly to the inner mitochondrial membrane (IMM), whereby over 65 mol% of CLs are located in its inner leaflet thus constituting the phospholipid majority [6].

Each CL molecule contains four fatty acid residues. Therefore, there is a large number of theoretically possible CL molecular species. For example, 10 different fatty acids will give 10^4 possible combinations of CL. Each additional modification of fatty acid residues (e.g., peroxidation of polyunsaturated fatty acids) will increase this number by manifold. Surprisingly, only a few of many possible CL molecular species are present

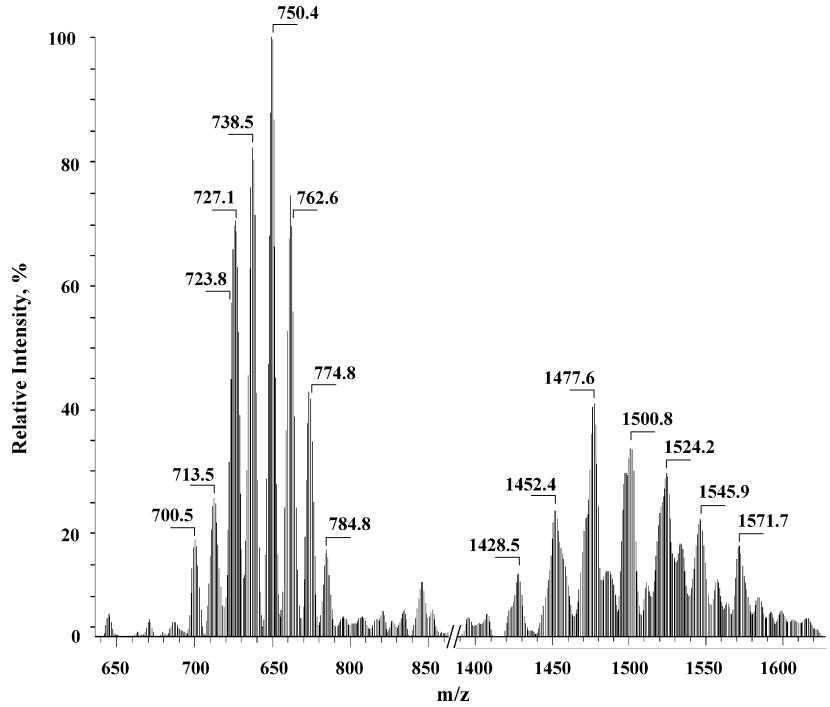


Fig. 1. Typical negative ion ESI mass spectrum of different molecular species of mouse brain mitochondria cardiolipins. Lipids were extracted using Folch procedure [97]. Total lipid phosphorus was determined as described by a micro-method [95]. CL was separated by 2D-HPTLC as described before [96], extracted from the HPTLC plate and subjected to electrospray ionization (ESI) mass spectrometry by direct infusion into a triple quadrupole mass spectrometer (Micromass, Inc., Manchester, England). Sheath flow was adjusted to 5 μl/min and the solvent consisted of chloroform:methanol (1:2, v/v). The electrospray probe was operated at a voltage differential of −3.5 kV in the negative ion mode. Mass spectra for CL species were obtained by scanning in the range of 400–1700 *m/z*. Source temperature was maintained at 70 °C. The major species in mouse brain consisted of TLCL [$(C_{18:2})_4$ CL], *m/z* ratio of 723.8 for doubly charged and 1447.6 for singly charged ions; $(C_{18:1})_3(C_{20:4})_1$, *m/z* ratio of 738.5 for doubly charged and 1477.6 for singly charged ions; $(C_{18:1})_2(C_{20:4})_2$, *m/z* ratio of 750.4 for doubly charged and 1500.8 for singly charged ions; $(C_{18:1})_2(C_{20:4})_1$ $(C_{22:6})_1$, *m/z* ratio of 762.6 for doubly charged and 1524.2 for singly charged ions; $C_{(18:0)_1}C_{(18:1)_1}C_{(22:6)_2}$, (*m/z* ratio of 774.8 for doubly charged and 1545.9 for singly charged ions). Mass spectra prototypical of three independent experiments are presented.

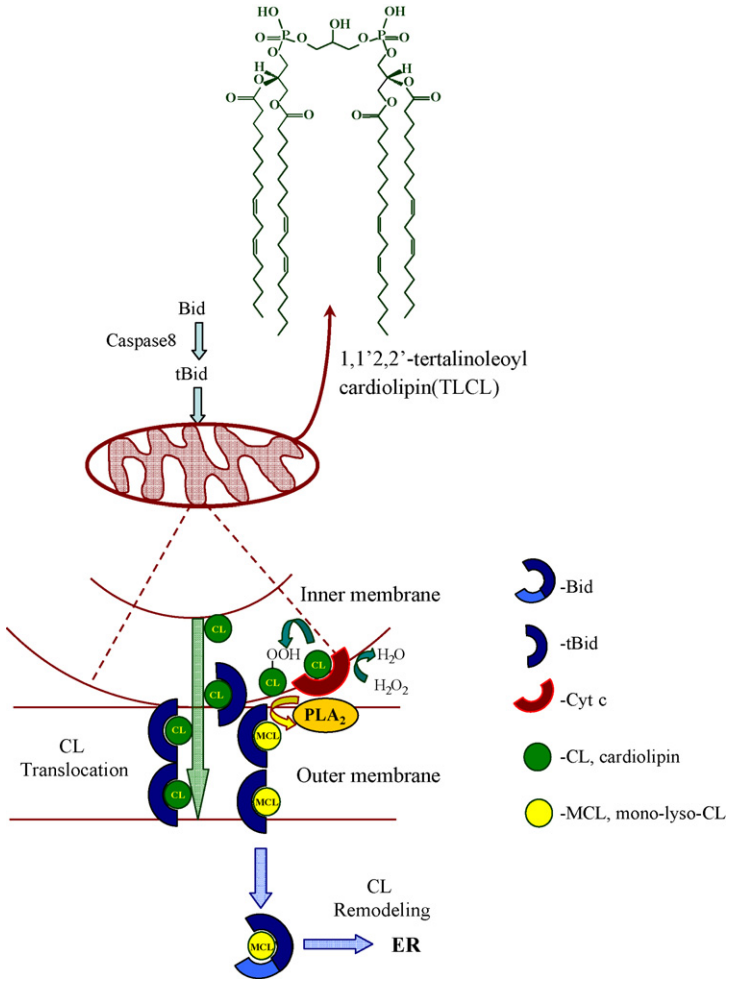
in tissues. Mammalian heart CL contains predominantly linoleic acid ($C_{18:2}$) [7], while CLs in some marine bivalves include docosahexaenoic fatty acid ($C_{22:6}$) [8]. CL extracted from human lymphoblasts has multiple fatty acids including oleic ($C_{18:1}$) and palmitoleic ($C_{16:1}$) [9]. Mouse brain CL is rich in molecular species with long-chain polyunsaturated fatty acid residues such as arachidonic ($C_{20:4}$), docosatetraenoic ($C_{22:4}$), and docosahexaenoic ($C_{22:6}$) (Fig. 1). The reason for this tissue specific changes in the CL fatty acid composition remains unknown [7].

There are two metabolic pathways through which CL is either synthesized *de novo* or undergoes remodeling from its existing species. All necessary enzymes for primary CL synthesis are located in the mitochondria. Initial step in biosynthesis of CL (acylation of glycerol 3-phosphate) takes place in the outer mitochondrial membrane [10], while subsequent reactions occur in the inner membrane [11], finally cardiolipin synthase acts mainly in the matrix to catalyze the last step in the biosynthesis of CL [12]. For the acyl chain remodeling, CL has to be transported into endoplasmic reticulum, where either direct acylation of lyso-CL or by deacylation of CL to monolyso-CL followed by its reacylation takes place [13]. This reaction requires activation of phospholipase A_2 [14] and is coenzyme-A-dependent [13]. It is possible that CL oxidation triggers the remodeling pathway (see below). A defect in CL remodeling might result in abnormalities in the CL fatty acid composition detected in disease states such as Barth syndrome. This is an X-linked human disease associated with a defective phospholipid acyltransferase; patients with Barth syndrome have abnormal mitochondria [15,16]. The defect in CL remodeling is a plausible link between the genotype and phenotype. Two cardinal symptoms of Barth syndrome, namely cardiomyopathy and skeletal myopathy, involve CL-rich tissues [9].

CL is a significant player in the apoptotic cell death program [17] and one of the major factors in tBid-induced destabilization of mitochondrial bioenergetics

[18,19]. Apoptosis-associated proteins such as Bid and tBid [20,21] have CL-binding domains [20,22,23] and reveal dynamic interactions with CL and its metabolites, mono- and di-lyso-CLs [24]. These interactions are likely to be most effective at the contact sites of the inner and outer mitochondrial membranes [25] resulting in changes in CL trans-membrane distribution (see Scheme 1) and its reorganization in micro-domains with a hexagonal H_n configuration favorable for the release of cyt *c* and other pro-apoptotic factors [20,23,26–28]. Therefore, CL is believed to significantly contribute to the outer membrane permeabilization and cyt *c* release during apoptosis.

In normal cells, almost 80% of CL is localized in the inner mitochondrial membrane where it is distributed between the matrix and intermembrane surfaces at a ratio of 60:40 [29–32]. Although there is ~70-fold excess of CL available for 1:1 stoichiometric binding with cyt *c* [5,33], most CL is not free but rather interacts with mitochondrial electron-transport complexes [34,35]. However, in apoptotic cells, the CL content in the outer mitochondrial membrane markedly increases to reach



Scheme 1. Possible involvement of Bid in trans-membrane redistribution of CL in mitochondria during apoptosis. Very early during apoptosis, activated caspase-8 initiates the cleavage of the pro-apoptotic BCL-2 family member, Bid, yielding a truncated fragment (tBid) that translocates to the mitochondria. At the contact sites of the inner and outer mitochondrial membranes, tBid reveals dynamic interactions with CL resulting in changes in CL trans-membrane distribution that likely occur after the production of reactive oxygen species. Thus the CL content in the outer mitochondrial membrane markedly increases. The CL distribution between the two monolayers of the inner membrane also changes in a way that almost 70 mol% of CL is localized in the outer monolayer of the membrane. The CL redistributed to the outer leaflet of the inner mitochondrial membrane becomes available for interactions with cyt *c*. These interactions result in the formation of cyt *c*/CL complex. H_2O_2 gets access to the heme catalytic site of cyt *c* activating it to a peroxidase. The peroxidase activity is specific to CL and generates CL hydroperoxides that induced the release of pro-apoptotic factors from mitochondria into the cytosol. CL oxidation can also trigger the remodeling pathway including hydrolysis of CLox by phospholipase A_2 and subsequent tBid/Bid dependent transport of mono-lyso-CL into endoplasmic reticulum (where its reacylation takes place).

the level of approximately 40 mol%. The CL distribution between the two monolayers of the inner membrane also changes such that almost 70 mol% of CL is found in the outer monolayer while 30 mol% remains confined to the matrix side of the membrane [32]. These membrane translocations of CL occur very early during apoptosis, well before changes in mitochondrial membrane potential or other markers of apoptosis, such as plasma-membrane exposure of PS, but after the production of reactive oxygen species [29,32]. The amounts of CL that may become available for interactions with cyt *c*, hence for tight binding of cyt *c* in the membrane, change dramatically during apoptosis. The changes in CL distribution during apoptosis are likely associated with its interactions with tBid (see Scheme 1). In fact, addition of tBid to mouse liver mitochondria *in vitro* induced significant changes in CL distribution between the inner and outer membranes and accumulation of mono-lyso-CL [36]. In line with this, a marked accumulation of mono-lyso-CL was accompanied by simultaneous mitochondrial trans-membrane migration of tBid and cyt *c* release when low concentrations of exogenous mono-lyso-CL and tBid were added to isolated mitochondria [20].

3. CL binds with cytochrome *c* and changes its catalytic profile

Cyt *c* is a basic protein which under physiological conditions has a net charge of +8 [37]. As a result, it avidly binds with negatively charged membranes, including anionic (phospho)lipid membranes [38–41]. Notably, this interactions confers peroxidase activity on the protein [42]. This is caused by its partial unfolding and weakening of the coordination bond between the heme-iron and Met_{80} [43–45]. As a result, small molecules – like H_2O_2 – get access to the heme site of cyt *c* activating it to a peroxidase. In mitochondria, the peroxidase activity of the cyt *c*/CL complex is specific to CL and acts as CL oxygenase yielding CL hydroperoxides [32].

In resting-state mitochondria, most of CLs and cyt *c* are spatially separated and, therefore, the peroxidase activity of cyt *c* is very low [32]. However, massive transmembrane migration of CL during apoptosis (see above) sets the stage for the elevated content of cyt *c*/CL complexes and markedly enhances the peroxidase activity of cyt *c* [32]. In fact, the amounts of cyt *c* available in the intermembrane space of mitochondria, rather than CL in the outer leaflet of the IMM and the inner leaflet of the OMM, determine the level of peroxidase activity of cyt *c*/CL complexes during apoptosis. Not surprisingly, cells with siRNA manipulated levels of cyt *c* exert lower levels of CL oxygenase activity and sensitivity to apoptosis (see below) that are proportional to the amounts of cyt *c* [32].

Thus during apoptosis, a significant peroxidase activity of cyt *c*/CL complexes can generate CL hydroperoxides, provided sufficient levels of H_2O_2 (a source of oxidizing equivalents required for activation of the peroxidase) are available. One of the important sources of H_2O_2 during apoptosis is dysregulated electron transport capable of generating high concentrations of superoxide whose spontaneous or MnSOD-catalyzed dismutation produces H_2O_2 [46–48]. Moreover, CL-bound cyt *c* has a negative redox potential precluding its participation in electron transport as an electron acceptor from the mitochondrial complex III [49]. Thus electron transport between complexes III and IV cannot be serviced by cyt *c*/CL complexes. Finally, a recently discovered function of p66 (normally, a tyrosine kinase adaptor protein [50]) as a generator of H_2O_2 in apoptosis contributes to the required supplementation with sufficient amounts of oxidizing equivalents to catalyze CL oxygenation [51]. As a consequence, CL oxidation is universally discovered in a number of different cell lines undergoing apoptosis [32]. Ionizing-irradiation is one of many examples of a pro-apoptotic stimulation triggering significant CL oxidation dependent on cyt *c* levels [32]. As shown on Fig. 2, ionizing-irradiation of mouse heart mitochondria results in accumulation of CL oxidation products detectable as increased amounts of total CL hydroperoxides as well as individual molecular species of polyunsaturated CL hydroperoxides in mass spectra. Similarly, in tissues with significant levels of apoptotically dying cells resulting from exposure to damaging factors (e.g., in brain after traumatic injury), accumulation of CL hydroperoxides occurs as a characteristic biomarker [52].

Peroxidase activation of cyt *c*/CL complexes proceeds through the formation of the reactive intermediates—compounds I and II and the production of protein radicals, most commonly tyrosyl radical intermediates [53,54]. Interaction of cyt *c* with CL results in partial unfolding of the protein making possible interactions the heme catalytic site with H_2O_2. As in many other peroxidase reactions, this results in the formation of compounds I and II followed by the generation of protein-derived, most commonly, tyrosyl radicals [55–57]. Cyt *c* has four tyrosine residues some of which are located close to the heme moiety and some are present on the surface of the protein. In the unfolded protein/CL complex, tyrosyl radical abstracts hydrogen from one of the CL polyunsaturated lipid acyl chains yielding a lipid radical (see below). After the addition

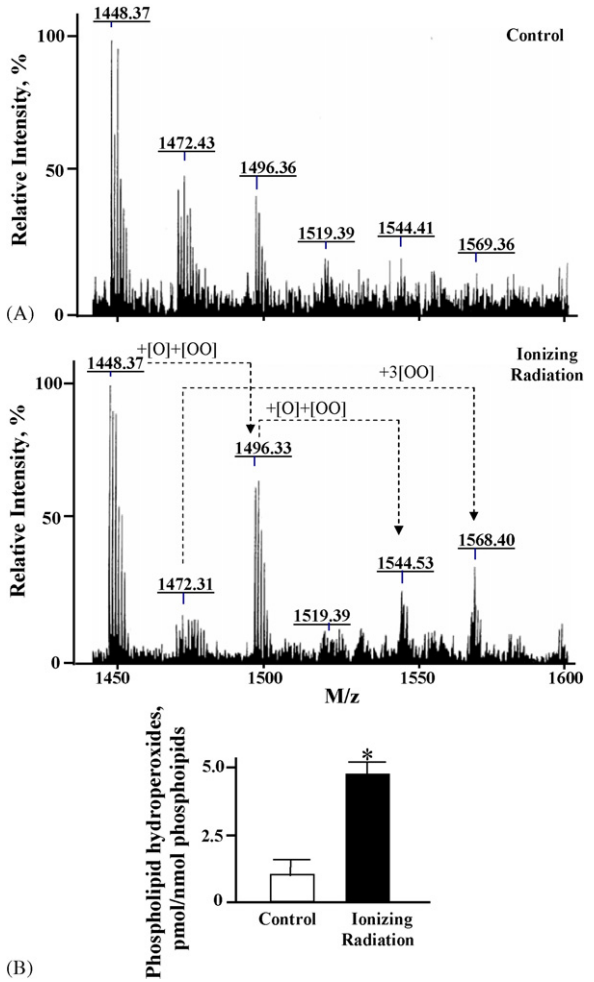


Fig. 2. Oxidation of mouse heart mitochondrial cardiolipin in response to ionizing radiation. Mitochondria were isolated from the hearts of C57BL10 mice. The isolated mitochondria were irradiated to 25 Gy. (A) Typical negative ion ESI mass spectrum of different molecular species of mouse heart mitochondria cardiolipins before and after ionizing radiation. Lipids from mitochondria were extracted using Folch procedure [97]. Total lipid phosphorus was determined as described by a micro-method [95]. Lipids were analyzed by electrospray ionization tandem mass spectrometry by direct infusion into a triple quadrupole mass spectrometer (Micromass, Inc., Manchester, England). Mass spectra for the $(M-H)^-$ CL species were obtained by scanning in the range of 1200–1800 every 1.5 s and summing individual spectra. CID spectra were obtained by selecting the ion of interest and performing daughter ion scanning in Q3 at 400 Da/s using Ar as the collision gas. Mitochondria displayed several species of CL as determined by mass spectrometry (top panel). Tandem mass spectrometry (MS/MS analysis) of individual mitochondrial CL species was used to determine the fatty acyl chain composition. There was a marked decrease in the CL cluster around *m/z* 1472 upon radiation treatment (bottom panel). In addition, there appeared a "new" cluster of mass ions at *m/z* values of 1566, 1568, 1570 and 1572. The new masses correspond to mass ions of 1470 ($(C_{18:3})_2/(C_{18:2})_1/(C_{20:3})_1$ CL), 1472 ($(C_{18:3})_1/(C_{18:2})_2/(C_{20:3})_1$ CL), 1474 ($(C_{18:2})_2/(C_{18:1})_1/(C_{20:4})_1$ CL) and 1476 ($(C_{18:2})_1/(C_{18:1})_2/(C_{20:4})_1$ CL) each with an additional 96 Da. Specifically, peaks at +96 Da relative to peaks in the native lipid spectrum were attributed to tri-peroxy derivatives of CL species. (B) Accumulation of phospholipid hydroperoxides in mouse heart in response to ionizing irradiation. After irradiation of mouse heart, lipids were extracted using Folch procedure [97] and total lipid phosphorus was determined as described by a micro-method [95]. Oxidized phospholipids were hydrolyzed by pancreatic phospholipase A_2 (2 U/μl) in 25 mM phosphate buffer containing 1 mM $CaCl_2$, 0.5 mM EDTA and 0.5 mM SDS (pH 8.0 at room temperature for 30 min). Fatty acid hydroperoxides formed were determined by fluorescence HPLC of resorufin stoichiometrically formed during their microperoxidase 11-catalyzed reduction in the presence of Amplex Red (for 40 min at 4 °C) [32]. Fluorescence HPLC (Eclipse XDB-C18 column, 5 μm, 150 mm × 4.6 mm, mobile phase was composed of 25 mM disodium phosphate buffer (pH 7.0)/methanol (60:40, v/v), Ex = 560 nm, Em = 590 nm), was performed on a Shimadzu LC-100AT HPLC system equipped with fluorescence detector (RF-10Axl) and autosampler (SIL-10AD).

of oxygen, the latter is converted into a peroxyl radical which is further protonated to CL hydroperoxide. As highly potent oxidants, the peroxidase reactive intermediates can attack both endogenous substrates such as CL located in close proximity to the catalytic site, ascorbate, thiols, etc., as well as exogenously added reducing compounds (e.g., different phenolic compounds). Protein radical intermediates can be directly detected in the EPR spectra of cyt *c* complexes with different molecular species of phospholipids [32,56,57]. In addition, spin trapping experiments can be employed to identify specific types of radicals subsequently involved not only in intermolecular protein oxidation but also in the oxidation of other substrates such as lipids. Finally, relatively long-lived radicals of phenolic compounds, e.g., phenoxyl radicals of a hindered phenolic compound, etoposide, are also EPR-detectable. We used a spin-trap, 2-methyl-2-nitrosopropane (MNP), to detect and identify the radicals generated by cyt *c*/CL. Cyt *c* incubated with non-oxidizable TOCL in the presence of H_2O_2 generated EPR signal that was identical to previously reported MNP-adduct of protein-derived (tyrosyl) radical of cyt *c* [55,57] (Fig. 3A). When oxidazable TLCL was used in place of TOCL, cyt *c*/CL complexes produced two types of MNP adducts: one of them was the same protein-derived (tyrosyl) radical–MNP adduct [55,57], while the other one could be identified as a lipid radical (pentadienyl radical) adduct [58,59] (Fig. 3B). No significant radical MNP adduct was detected in the absence of CL, and no adducts were measured in the absence of H_2O_2. Importantly, the same MNP adducts could be distinguished in mitochondria incubated in the presence of H_2O_2 (Fig. 4). Detailed analysis and subtraction of spectra (see figure legend) elicited that superposition of the signals from two radical adducts

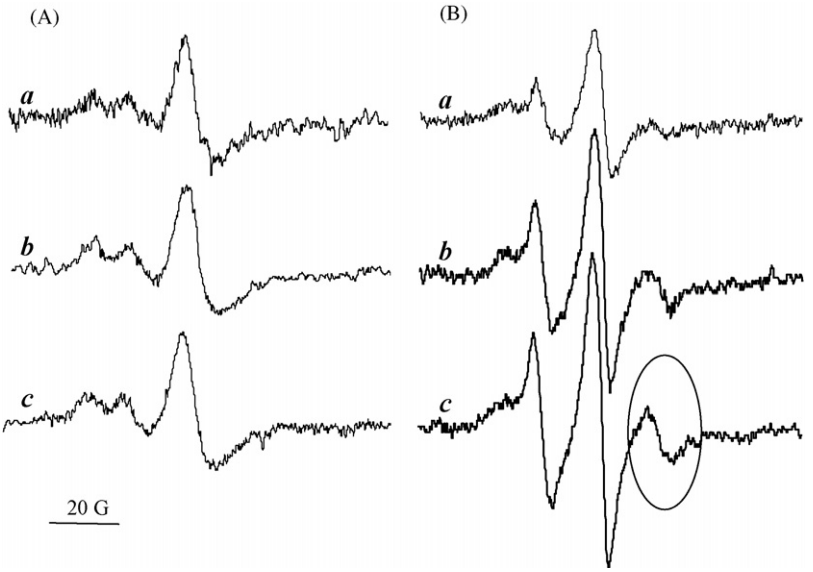


Fig. 3. EPR spectra of spin adducts formed by the peroxidase activity of cyt *c*/CL complexes in the presence of H_2O_2. EPR spectra obtained from the reaction mixture of cytochrome *c* with hydrogen peroxide in the presence of a spin trap, 2-methyl-2-nitrosopropane (MNP) and TOCL/DOPC (A) or TLCL/DOPC (B) liposomes. Cyt *c* (500 μM) was incubated with TOCL/DOPC or TLCL/DOPC liposomes (CL = PC = 5 mM) and MNP (10 mM) for 3 min in the dark at room temperature in 20 mM phosphate buffer pH 7.4, 100 mkM DTPA, then H_2O_2 (2 mM) was added and spectra were recorded: (a) 2.5, (b) 5 and (c) 10 min thereafter. High-field component of MNP adduct of lipid radical is circled. The instrument parameters were as follows: center field 3350 G, scan range 100 G, modulation amplitude, 2 G; time constant, 0.3 s; scan time, 4 min; receiver gain, 5×10^3; microwave power, 20 mW.

– the protein (tyrosyl) radical and the lipid pentadienyl radical – which occurred during activation of the peroxidase activity of cyt *c* in mitochondria. Confirming that, the cyt *c* catalyzed peroxidase reaction readily involves polyunsaturated CLs as its substrate. In line with this, aggregation of cyt *c* and formation of its oligomeric complexes exerted different features in the presence of non-oxidizable mono-unsaturated or readily oxidizable polyunsaturated molecular species of CL. Mitochondria of eukaryotic cells contain significant amounts of readily oxidizable polyunsaturated molecular species of CLs, including TLCL. Mechanisms of aggregations of a poorly oxidizable mono-unsaturated TOCL are substantially different from those for readily oxidizable polyunsaturated TLCL (Fig. 5). As shown on Fig. 5, PAGE gels of cyt *c*/TOCL comlexes incubated in the presence of H_2O_2 reveal formation of dimers, trimers, tetramers of cyt *c*. In contrast, H_2O_2 oxidation of cyt *c*/TLCL complexes resulted predominantly in the formation of very high molecular weight cross-links (note that most of these complexes were too large to enter the running gel). While in the former case, the formation of dityrosine oligomers is the major aggregation pathway, TLCL oxidation products with bifunctional characteristics are mostly involved in its cross-linking. In other words, oxidation of polyunsarturated lipids by cyt *c*/CL complexes was associated with the formation of high molecular weight aggregates that were resistant to reduction. Interestingly, formation of such aggregates is typical of neurodegenerative disease conditions that are commonly associated with oxidative stress (e.g., Parkinson's disease, Alzheimers disease), in which they represent morphological hallmarks of intracellular damage [60–62].

4. Release of pro-apoptotic factors requires CL oxidation

The significance of CL oxidation for the execution of the apoptotic program is determined by the essential role that this oxidized phospholipid plays in the release of pro-apoptotic factors such as Smac/Diablo, cyt *c*, AIF, etc. [32,63,64]. As the action of mediators such as AIF do not depend upon apoptosome formation [65], it would be reasonable to suppose that its actions would be independent of cyt *c* expression. However, in cyt *c*-deficient cells, where CL oxidation does not occur, the other pro-apoptotic factors are not released either [32]. Moreover, addition of exogenous CLox to the mitochondria isolated from cyt *c*-deficient cells initiates the release of pro-apoptotic factors (Smac/Diablo) suggesting a critical role of CLox in general mitochondrial

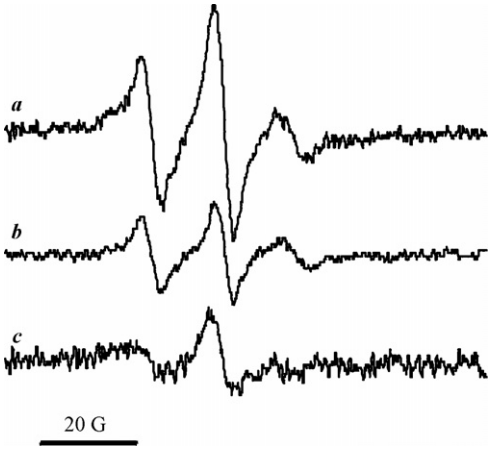


Fig. 4. H_2O_2 induced EPR spectra of spin adducts formed in mitochondria. EPR spectra of MNP adducts from mitochondria. (a) Spectrum of MNP adduct in mitochondria incubated for 10 min with MNP (10 mM) and H_2O_2 (10 mM) in PBS with 200 μM DTPA. (b) Spectrum of lipids extracted from mitochondria after the same incubation, (c) Spectrum of the MNP adduct in mitochondria obtained by subtraction of spectrum (b) from spectrum (a). Spectrum (b) was magnified ×1.6 to equalize the high field component of both spectra that represents the lipid-radical MNP adduct (see Fig. 3B). Note, the spectrum c is similar to the spectrum of MNP adduct of protein-derived radicals (Fig. 3A). H_2O_2 (10 mM) and MNP (10 mM) were added to a suspension of mitochondria (30 mg protein/ml) in PBS with DTPA (200 μM), and incubated for 10 min. After addition of catalase (1 μg/ml), the mitochondria were washed by PBS with 200 μM DTPA (centrifugation 12000 × *g*, 5 min). The final concentration of mitochondria was 30 mg/ml. Relatively high H_2O_2 concentrations (10 mM) were needed to overcome glutathione peroxidase, catalase contamination and also to generate a sufficiently high radical flux for detection of spin adducts by trapping. In the absence of H_2O_2, the magnitude of the spectrum of MNP spin adducts was five times lower. Lipid extraction was performed using Folch procedure [97]. EPR spectra of MNP adducts of lipids were monitored after lipids were resuspended in PBS.

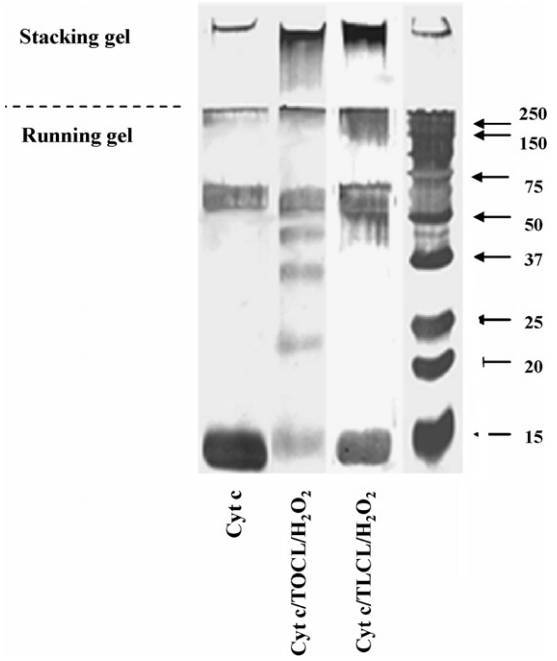


Fig. 5. Typical electrophoresis gel of cyt *c*/CL complexes. Incubation of cyt *c*/TOCL comlexes with H_2O_2 resulted in the formation of dimers, trimers, tetramers of cyt *c*. In contrast, H_2O_2 oxidation of cyt *c*/TLCL complexes induced predominantly the production of very high molecular weight cross-links (note that most of these complexes were too large to enter the running gel). Liposomes (200 μM, DOPC/TOCL or DOPC/TLCL at ratio 1:1) were incubated with cyt *c* (4 μM) in PBS containing 100 μM DTP A at 37 °C for 1 h in the presence of 100 μM H_2O_2 (H_2O_2 was added every 15 min of incubation). Samples (15 μl per well) were loaded onto the gel and electrophoresis was run in 12.5% SDS-PAGE with 5% stacking gel [98]. GelCode SilverSNAP Stain Kit II (Pierce) was used for staining of protein.

pro-apoptotic mechanisms. Currently, it is not known whether CLox acts independently or in conjunction with other components of the pro-apoptotic machinery (Bax, Bak) to trigger the egression of the factors into the cytosol. Thus two pro-apoptotic pathways – trans-membrane migration of CL and the formation of cyt *c*/CL complexes – merge together to generate, upon generation of sufficient sources of oxidizing equivalents (H_2O_2, lipid hydroperoxides), a new signal, oxidized CL, essential for the release of pro-apoptotic factors from mitochondria into the cytosol.

5. Apoptosis in cells with manipulated fatty acid composition of CLs ($C_{22:6}$ or $C_{18:2}$): a possible role in avoiding or stimulating apoptosis

If CL oxidation is an early step of the apoptotic program critical to its propagation and completion, stimulation of CL oxidation is expected to enhance apoptotic responses in cells; vice versa, inhibition of CL oxidation should be associated with a decreased sensitivity to apoptosis. Experimentally, this paradigm can be tested by engineering cells with manipulated levels of CL fatty acids differing in their susceptibility to oxidation. Indeed, we have demonstrated that tetraoleoyl-CL is highly resistant to cyt *c* catalyzed oxidation whereas tetralinoleoyl-CL and more polyunsaturated molecular species of CLs are highly susceptible to pro-oxidant stimuli. With this in mind, we prepared HL-60 cells significantly enriched in CL molecular species containing highly oxidizable $C_{22:6}$ [66]. These cells turned to be highly sensitive to pro-apoptotic stimulation by staurosporine [31,32]. By contrast, cells with high levels of CL species containing oleic acid and saturated fatty acid residues elicited an unusually high resistance to pro-apoptotic stimulation. It is tempting to speculate that

manipulation of CL susceptibility to oxidation may be one of the factors that are utilized by undifferentiated cells – such as stem cells or tumor cells – to obtain resistance to pro-apoptotic signals hence avoid apoptotic cell death. Conversely, drug-discovery programs aimed at eradication of tumor cells may benefit from approaches to stimulate oxidation of CL in their mitochondria by forcing them to integrate polyunsaturated fatty acid residues. One of the approaches can be based on non-toxic nutritional manipulations using sources of polyunsaturated fatty acids (e.g., fish oil).

6. Regulation of CL oxidation in mitochondria—NO, nitroxides, Vitamin E homologues, and etoposide

Given that CL oxidation is one of the very early stages of the apoptotic program, taking place up-stream of the point of no-return (caspase activation and proteolysis), formation of cyt *c*/CL complexes may be an important target for the control of apoptosis. Several approaches may be envisioned to achieve this goal. As CL availability is an important stage for cyt *c* activation to CL oxygenase, regulation of CL trans-migration in mitochondria can be targeted. A role of tBid in stimulation of CL redistribution in apoptotic mitochondria becomes more evident indicating that tools to affect interactions of tBid with mitochondria may be constitute promising strategies [67,68].

Control of redox environment that is conducive of cyt *c*/CL peroxidase activity is another interesting way to affect the CL oxidation stage of apoptosis. High oxidizing potential of the cyt *c*/CL peroxidase intermediates [32,69] offers a number of opportunities for their chemical reduction by both endogenous substrates and exogenous agents. Ascorbate, cysteine, GSH and other low molecular weight thiols, Vitamin E homologues are examples of the former type while different phenolic and SH-containing drugs represent the latter category and may be utilized for the reduction of peroxidase intermediates. These general considerations, however, do not take into account key structural factors that may restrict access of the bulky reducing agents to the catalytic site of cyt *c* accessible only for small molecules. One such small molecule is NO which can act as a reductant for reactive intermediates of cyt *c*/CL peroxidase complexes [69]. Therefore, NO and NO-releasing compounds may participate in regulation of CL oxidation by cyt *c*/CL complexes. Moreover, the role of mitochondrial NOS – whose functions are not well understood – [70] can be rationalized in terms of its participation in the regulation of the peroxidase activity of cyt *c*/CL complexes as an anti-apoptotic mechanism preventing inadvertent CL oxidation.

Because the peroxidase function of cyt *c*/CL complexes requires H_2O_2 or organic (lipid) hydroperoxides [71–73], the removal of these oxidizing equivalents is equivalent to inhibition of the CL oxygenase activity. Obviously, different GSH-dependent mechanisms (GSH peroxidases) [74,75] as well as thioredoxin [76–78], peroxiredoxin [79] may be effective in removing the hydroperoxides through respective enzymatic pathways.

Another protective pathway may be realized through prevention of H_2O_2 formation by eliminating its major source in apoptotic mitochondria, superoxide radicals. Disrupted electron transport is said to play a major role in the production of superoxide radicals on one or more respiratory complexes during apoptosis [80–82]. Dismutation of superoxide radicals that occurs either spontaneously or through catalytic action of MnSOD generates H_2O_2 that feeds the peroxidase reaction of cyt *c*/CL complexes. Therefore, prevention of superoxide production may be one of the most potent approaches to inhibit CL oxidation. Stable nitroxide radicals seem to be almost ideal candidate agents to quench the production of superoxide in mitochondria. This is because nitroxides possess several features that are essential for their ability to control the levels of superoxide radicals and H_2O_2. First, they readily undergo one-electron reduction by electron transport to yield respective hydroxylamines. This prevents one-electron reduction of oxygen to form superoxide radicals. The hydroxylamines formed from nitroxides act as effective radical scavengers to produce nitroxides, ie undergo recycling. Second, nitroxide radicals exert an SOD mimetic activity and dismutate superoxide radicals. The product of this reaction is H_2O_2 which can feed the peroxidase cycle of cyt *c*/CL complexes. However, by acting as an electron acceptor from mitochondrial complexes and by eliminating superoxide radicals, the electron scavenging and SOD activity of nitroxides contribute to prevention of superoxide reaction with NO resulting in peroxynitrite, a potent oxidant known to contribute to dysregulation of electron transport in mitochondria [83,84]. Thus, overall SOD mimetic activity of nitroxides may be an important protective mechanisms, similar to that of MnSOD in mitochondria [85,86]. Third, nitroxides display catalase activity that directly eliminates H_2O_2. These combined propensities of nitroxides make them highly promising mitochondrial anti-apoptotic agents. Unfortunately, these activities of nitroxides require their presence in mitochondria which, in turn, only happens when very high (milomolar) concentrations of nitroxides are utilized *in vivo*. Until recently, this precluded effective use of nitroxides as

anti-apoptotic remedies. Targeting of nitroxides to mitochondria, however, may overcome this problem. In fact, latest developments in the field indicate that an remarkably enhanced anti-apoptotic activity of nitroxides can be achieved by their effective delivery into mitochondria using different approaches [87,88].

Since execution of apoptotic program in mitochondria is associated with CL peroxidation products required for the release of pro-apoptotic factors, lipid antioxidants capable of inhibiting CL peroxidation may act as anti-apoptotic agents. Etoposide, a widely-used antitumor drug, is a prototypical inducer of apoptosis and, at the same time, an effective lipid radical scavenger and lipid antioxidant. We found that CL oxidation during apoptosis is realized not via a random CL peroxidation mechanism but rather proceeds as a result of peroxidase reaction in a tight cyt *c*/CL complex that restrains interactions of etoposide with radical intermediates generated in the course of the reaction [89]. While etoposide can inhibit cyt *c* catalyzed oxidation of CL competing with it as a peroxidase substrate, the inhibition is realized at far higher concentrations than those at which it induces apoptotic cell death. Thus, oxidation of CL by cyt *c*/CL peroxidase complex, which is essential for apoptosis, is not inhibited by pro-apoptotic concentrations of the drug.

7. Peroxidase complexes of cyt *c* can be formed in extramitochondrial compartments: biomedical implications

Hitherto, we discussed double complexes of cyt *c* with CL, however, more complex associations of these two molecules, with other partners are likely to occur, particularly in extramitochondrial compartments. As an example, synaptic terminals contain high levels of α-synuclein (Syn) which has a phospolipid-binding domain. If Syn binds CL, and if the Syn/CL complex thus formed will be able to additionally bind cytosolic cyt *c*, the triple complex Syn/CL/cyt *c* will be generated in which activation of cyt *c* peroxidase activity will still be possible. Moreover, the peroxidase activity, in the presence of H_2O_2 will be inducing oligomerization of their constitutive components to form mixed aggregates similar to Lewy bodies. In both Lewy bodies and in our experimental aggregates, co-localization of Syn and cyt *c* will be overlapping and detectable along with the presence of CL. This has been indeed observed in both model biochemical systems as well as in apoptotic HeLa cells (expressing significant amounts of Syn in the cytosol) upon the *tert*-butyl hydroperoxide induced release of cyt *c*. In line with these observations, co-localization of cyt *c* and Syn was detected in the substantia nigra Lewy bodies in patients with Parkinson's disease [90]. Overall, these combined results are compatible with the hypothesis that during pro-apoptotic damage of mitochondria in synaptic terminals, released cyt *c* and CL are captured by an abundant cytosolic protein, Syn. This mode of action of Syn prevents retrograde spread of pro-apoptotic factors (cyt *c*) to the soma region of the neuronal cells thus protecting them from total cell apoptosis.

Cell damage may be accompanied by a significant release of cyt *c* into extracellular spaces and biofluids. The presence of cyt *c* in plasma and cerebrospinal fluid has been reported in several disease conditions (e.g., brain trauma, sepsis, and systemic inflammatory response syndrome) [91–93]. Because dyslipidemia and elevated contents of free fatty acids are also typical of most common pro-inflammatory diseases, it is possible that complexes of cyt *c* with these negatively charged lipids will be formed resulting in a peroxidase activity. The consequences of their formation can be envisioned in terms of enhanced oxidative stress and antioxidant depletion. Indeed, any H_2O_2 production by inflammatory response would unavoidably trigger its utilization accompanied by consumption of extracellular reductants (ascorbate, thiols). Moreover, oxidation depletes NO leading to vasoconstrictive conditions. Future studies are necessary to better understand the significance of these events and their manifestations in severe pro-inflammatory conditions such as sepsis, preeclampsia, etc.

8. Concluding remarks

The biology of apoptosis in mitochondria, creates conditions for tight interactions of one of the major proteins of the intermembrane space, cyt *c*, with the major membrane phospholipid, CL. The complex thus formed does not function as an electron shuttle in the respiratory chain but rather operates as a CL-specific oxygenase, a qualitatively new catalytic entity with different enzymatic properties. Peroxidase catalytic activity of the cyt *c*/CL complex is essential for the execution of the apoptotic program. Using an oxidative lipidomics approach we established that this is due the production of oxidized molecular species of CL that participate in the release of pro-apoptotic factors from mitochondria into the cytosol. Thus, in contrast to the previously well recognized pro-apoptotic role of cyt *c* in apoptosome formation, this new function of cyt *c* is dependent on its redox properties. Specific features of the redox behavior of cyt *c* and its interactions with different molecular species of CL are important for CL oxidation and its regulation of the sensitivity of cells, including tumor cells, to apop-

tosis. The discovery of the early stage of apoptosis also offers interesting opportunities for new targets in drug discovery programs. One can also envision that employment of new delivery vehicles (e.g., nanoparticles, single walled carbon nanotubes) coated with different molecular species of CL and cyt *c* may be particularly effective in achieving these therapeutic goals.

Reliable fulfillment and coordination of cell functions relies on prodigality of its essential mechanisms. While CL oxidation may be an essential mechanism of apoptotsis, it likely that alternative pathways are turned on when CL oxidation becomes impossible or ineffective as has been demonstrated for yeast cells deficient in CL [94].

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