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Review

Apoptotic interactions of cytochrome *c*: Redox flirting with anionic phospholipids within and outside of mitochondria

H. Bayir [a,b,c,*], B. Fadeel [j], M.J. Palladino [e,i], E. Witasp [j], I.V. Kurnikov [a,d], Y.Y. Tyurina [a,d], V.A. Tyurin [a,d], A.A. Amoscato [f], J. Jiang [a,d], P.M. Kochanek [b,c], S.T. DeKosky [g], J.S. Greenberger [h], A.A. Shvedova [k], V.E. Kagan [a,d,e,*]

[a] *Center for Free Radical and Antioxidant Health, Pittsburgh, PA 15219, USA*
[b] *Safar Center for Resuscitation Research, Pittsburgh, PA 15260, USA*
[c] *Department of Critical Care Medicine, Pittsburgh, PA 15260, USA*
[d] *Department of Environmental and Occupational Health, Pittsburgh, PA 15219, USA*
[e] *Department of Pharmacology, Pittsburgh, PA 15260, USA*
[f] *Department of Pathology, Pittsburgh, PA 15260, USA*
[g] *Department of Neurology, Pittsburgh, PA 15260, USA*
[h] *Department of Radiation Oncology, University of Pittsburgh, Pittsburgh, PA 15260, USA*
[i] *Pittsburgh Institute for Neurodegenerative Diseases, Pittsburgh, PA 15260, USA*
[j] *Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden*
[k] *Physiology/Pathology Research Branch, Health Effects Laboratory Division, NIOSH, Morgantown, WV, Morgantown, WV, USA*

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Abstract

Since the (re)discovery of cytochrome *c* (cyt *c*) in the early 1920s and subsequent detailed characterization of its structure and function in mitochondrial electron transport, it took over 70 years to realize that cyt *c* plays a different, not less universal role in programmed cell death, apoptosis, by interacting with several proteins and forming apoptosomes. Recently, two additional essential functions of cyt *c* in apoptosis have been discovered that are carried out via its interactions with anionic phospholipids: a mitochondria specific phospholipid, cardiolipin (CL), and plasma membrane phosphatidylserine (PS). Execution of apoptotic program in cells is accompanied by substantial and early mitochondrial production of reactive oxygen species (ROS). Because antioxidant enhancements protect cells against apoptosis, ROS production was viewed not as a meaningless side effect of mitochondrial disintegration but rather playing some – as yet unidentified – role in apoptosis. This conundrum has been resolved by establishing that mitochondria contain a pool of cyt *c*, which interacts with CL and acts as a CL oxygenase. The oxygenase is activated during apoptosis, utilizes generated ROS and causes selective oxidation of CL. The oxidized CL is required for the release of pro-apoptotic factors from mitochondria into the cytosol. This redox mechanism of cyt *c* is realized earlier than its other well-recognized functions in the formation of apoptosomes and caspase activation. In the cytosol, released cyt *c* interacts with another anionic phospholipid, PS, and catalyzes its oxidation in a similar oxygenase reaction. Peroxidized PS facilitates its externalization essential for the recognition and clearance of apoptotic cells by macrophages. Redox catalysis of plasma membrane PS oxidation constitutes an important redox-dependent function of cyt *c* in apoptosis and phagocytosis. Thus, cyt *c* acts as an anionic phospholipid specific oxygenase activated and required for the execution of essential stages of apoptosis. This review is focused on newly discovered redox mechanisms of complexes of cyt *c* with anionic phospholipids and their role in apoptotic pathways in health and disease.
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Keywords: Cytochrome *c*; Phospholipid; Mitochondrion

* Corresponding authors. Bayir is to be contacted at Safar Center for Resuscitation Research, 3434 Fifth Avenue Pittsburgh, PA 15260. Tel.: +1 412 383 1900; fax: +1 412 624 0943. Kagan, Center for Free Radical and Antioxidant Health, Department of EOH Bridgeside Point, 100 Technology Drive, Suite 350, Pittsburgh, PA 15219, USA. Tel.: +1 412 624 9479; fax: +1 412 624 9361.
E-mail addresses: bayihx@ccm.upmc.edu (H. Bayir), vkagan@eoh.pitt.edu (V.E. Kagan).

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"Happy families are all alike; Every unhappy family is unhappy in its own way." Lev Tolstoy, "Anna Karenina"

As discerning as the above principle postulated by Lev Tolstoy is in describing societal interactions, it is entirely inapplicable on a cellular level. In fact, quite the opposite can be said about cells: all happy cells are happy in their own well differentiated ways, while unhappy, injured cells are all alike in the ways they end their life through two major death pathways — necrosis or apoptosis. Decoding these pathways, particularly apoptosis has become one of the important foci of cell biology research and new important details are incessantly emerging. This review concentrates on a new role that cytochrome *c* (cyt *c*) complexes with anionic phospholipids – a mitochondria-specific phospholipid, cardiolipin (CL), and plasma membrane phosphatidylserine (PS) – play in apoptotic signaling. We will consider newly discovered pathways through which complexes of cyt *c* with CL and PS, respectively act as a mitochondrial "sensor" during the execution phase of apoptosis and/or as an externalized plasma membrane "eat-me" signal during apoptotic corpse removal (programmed cell clearance). Some of the implications for these lipid-dependent signaling events in human disease will also be discussed.

1. Electron donor/acceptor functions of cytochrome *c* in mitochondria

Cyt *c* is an abundant hemoprotein whose concentration in the intermembrane space of mitochondria may be as high as 0.5–1.0 mM [1]. This renders cyt *c* an effective shuttle of electrons between respiratory complexes III and IV, a function essential for uninterrupted energy metabolism. The quantitatively significant presence of cyt *c* at the mitochondrial membrane crossroads also makes it a potentially important participant of other redox reactions in which not only its electron acceptor/donor features but its catalytic properties are essential. Both participation in electron transport and action as an oxidant of superoxide radicals (a newly ascribed antioxidant function of cyt *c* [2]) are based on tunneling of electrons to its heme-iron and do not require immediate interactions of the heme with the electron donors, complex III or superoxide anion radicals, respectively.

Hexa-coordinate arrangement of cyt *c* heme iron whereby tetra-coordinate association with porphyrin and two additional coordinate bonds with Met_{80} and His_{18}, respectively, ideally fit these electron donor/acceptor functions. In fact, the hexa-coordinate structure precludes involvement of cyt *c* in other duties, such as redox-catalysis of peroxidase reactions, typical of many hemoproteins [3,4]. In line with this, peroxidase activity of solubilized cyt *c* is very low [5]. Interestingly, Met_{80} can undergo oxidative modifications resulting in loss of cyt *c*'s hexa-coordinate state [6,7]. Not surprisingly, oxidants – H_2O_2, organic hydroperoxides – can convert cyt *c* into a peroxidase via oxidation of its Met_{80} [8–10]. However, the physiological relevance of these harsh oxidative conditions resulting in modified forms of cyt *c* with pronounced peroxidase activity remained uncertain. It has been known for a long time that different negatively charged membrane-active molecules such as detergents and some phospholipids can bind to cyt *c* in model systems and stimulate its peroxidase activity [11,12]. The importance of cyt *c* conversion into a peroxidase in vivo is not fully understood.

2. Cardiolipin binding confers peroxidase activity on cytochrome *c*

Recently, we have reported that CL, which is essential for normal functions of several mitochondrial protein complexes [13–16], avidly binds to cyt *c* resulting in an extraordinary enhancement of its catalytic peroxidase activity [17]. The binding includes initial electrostatic attractions of positively charged Lys residues (likely 72 and 73) with negatively charged phosphate groups on CL followed by hydrophobic interactions of one of the polyunsaturated fatty acid residues of CL with a hydrophobic pocket of cyt *c* [18]. Tight interaction between cyt *c* and CL likely involves formation of the hydrogen bond between CL and Asn_{52} in cyt *c* [18]. The binding constants of cyt *c* with different polyunsaturated molecular species of CL are very high (on the order of 10^9 M^{-1}); thus the complexes produced cannot be easily dissociated by disruptors of electrostatic interactions such as high ionic strength conditions [147].

Cyt *c* has a highly conserved primary structure across different species. As shown in Fig. 1, a particular segment of the cyt *c* molecule that includes Met_{80} as well Lys_{72}, Lys_{73} and Tyr_{48}, Tyr_{67}, Tyr_{74} remains invariant in different species. Because these particular amino acid residues are essential for either binding of cyt *c* with CL (Lys_{72}, Lys_{73}) or likely participate in realization of its catalytic peroxidase activity (Met_{80}, Tyr_{48}, Tyr_{67}, Tyr_{74}), it is tempting to speculate that this peroxidase function and structural organization of cyt *c* once emerged, remained evolutionary conserved.

In normal mitochondria, CL is confined to the inner (about 65% of total CL) and outer (about 35% of total CL) leaflets of the inner mitochondrial membrane (IMM) whereas its presence in the outer mitochondrial membrane (OMM) is negligible [17,19,20]. Thus, physical contact between cyt *c* and CL can only take place on the intermembrane surface of the outer IMM leaflet. Furthermore, a significant fraction of CL is localized within the contact sites between the IMM and OMM [21–23] and is also engaged in interactions with other mitochondrial proteins [13–16,24]. As a result, only a limited amount of CL is available for binding with cyt *c*. Consequently, cyt *c*/CL complexes normally represent only a small portion of both cyt *c* (about 10–15%) and CL (2–3%) [17,25].

Recent focus on the involvement of CL in apoptosis revealed that the triggering mechanisms engaging Bcl-2 family members require CL [22,23,26,27] and cause massive trans-membrane migration of CL as well as its hydrolysis [28,29]. Both peroxidation and hydrolysis of CL occur during apoptosis and are important in its execution, particular in release of pro-apoptotic factors [17,30]. One of the members of the Bcl-2 family of proteins, Bid (more specifically, truncated Bid or tBid), appears early in apoptotic

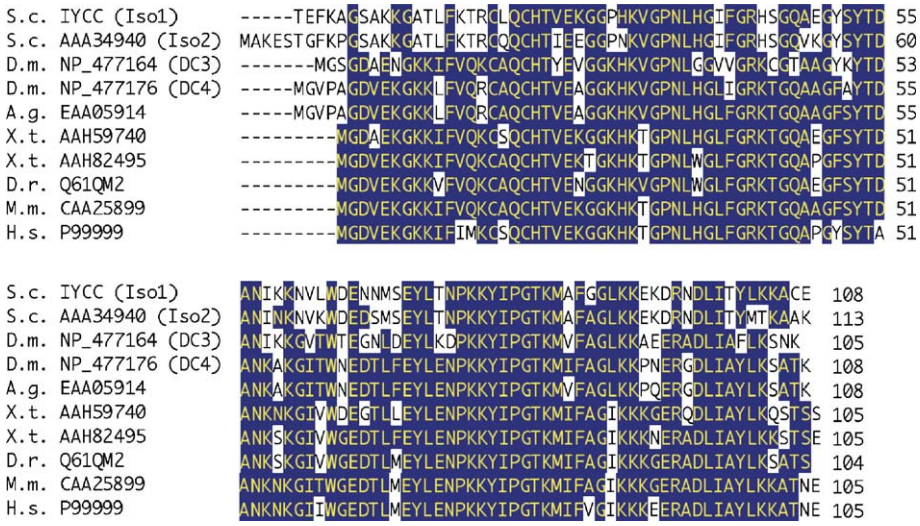

Fig. 1. Alignment of eukaryotic cytochrome C protein sequences. Species abbreviation and NCBI protein accession designations are indicated. S.c. (*Saccharomyces cerevisiae*, yeast); D.m. (*Drosophila melanogaster*, fruitfly); A.g. (*Anopheles gambiae*, mosquito); X.t. (*Xenopus tropicalis*, frog); D.r. (*Danio rerio*, zebra fish); M.m. (*Mus musculus*, mouse); and H.s. (*Homo sapien*, human). ClustalW alignment performed by MegAlign 5.0 (DNASTAR).

mitochondria and likely participates in trans-membrane migration of CL and its hydrolysis to mono-lyso-CL [28,29]. During apoptosis, a significant enrichment of the outer leaflet of IMM as well as of OMM with CL sets the stage for its binding to cyt *c* and production of the CL/cyt *c* complex with peroxidase activity.

3. Cytochrome *c* is a cardiolipin oxygenase

The peroxidase activity of cyt *c*/CL complexes reveals a unique ability to selectively oxidize CL during apoptosis [17,31] thus generating CL hydroperoxides (CL-OOH) required for the release of pro-apoptotic factors from mitochondria into the cytosol [17]. This critical role of CL oxidation in apoptosis implies that its susceptibility to oxidation may control progression of the apoptotic program. We established that, unlike its polyunsaturated molecular species, mono-unsaturated CLs are not readily oxidized by cyt *c* [17]. Thus changes in the polyunsaturation pattern of CL molecular species may be responsible for differential sensitivity of cells to pro-apoptotic stimuli. In fact, different tissues display very specific patterns of CL molecular species. Our novel lipidomics analysis data indicate that mass spectra of liver CL are dominated by one major peak (with *m/z* ratio of 724 for doubly charged and 1448 for singly charged ions) corresponding to tetralinoleoyl CL (TLCL) ($C_{18:4}$) (Fig. 2). In mass spectra of the brain CLs, multiple peaks (with *m/z* ratios of 723.4, 738.5, 750.4, 762.1 and 774.4, for doubly-charged ions) are detectable (Fig. 3). The MS–MS structural analysis identified several major molecular species of CL in brain mitochondria as TLCL [$(C_{18:2})_4$ CL]; $C_{(18:1)_3}C_{(20:4)_1}$; $(C_{16:0})_1(C_{18:1})_1(C_{20:4})_1(C_{22:4})_1$; $(C_{18:1})_2(C_{20:4})_1(C_{22:6})_1$; $C_{(18:0)_1}C_{(18:1)_1}C_{(22:6)_2}$. This indicates that brain mitochondria contain molecular species of CL with long-chain polyunsaturated fatty acid residues ($C_{20:4}$, $C_{22:4}$, $C_{22:6}$) highly susceptible to oxidation (Fig. 3). It is tempting to speculate that these specific features of brain CLs may determine, at least in part, their sensitivity to pro-apoptotic stimulation by excitatory amino acids, catechols, etc. Interestingly, several molecular species of polyunsaturated CL are also found in *Drosophila* (with *m/z* 671.9, 685.4, 697.9, 711.8, 723.8 corresponding to $(C_{16:1})_4$; $(C_{16:1})_3(C_{18:2})$; $(C_{16:1})_2(C_{18:2})_2$;

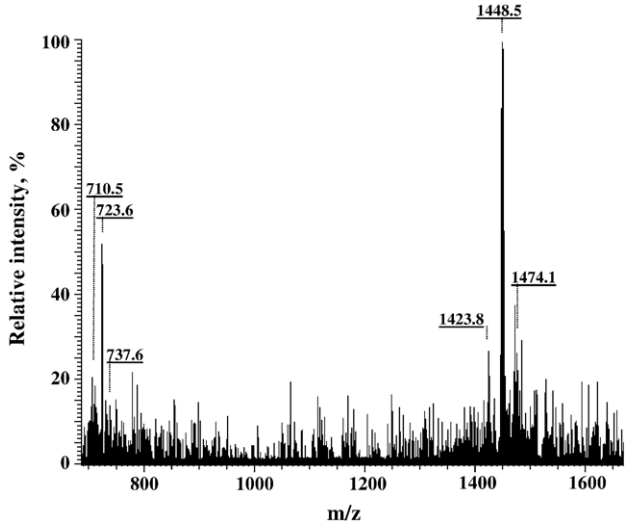

Fig. 2. ESI mass spectrometry of CL isolated from C57BL/6J mouse liver mitochondria. CL was separated by 2D-HPTLC, extracted from the HPTLC plate and subjected to electrospray ionization mass spectrometry by direct infusion into a triple quadrupole mass spectrometer (TSQ70, Finnigan). Sheath flow was 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 keV in the negative ion mode. Mass spectra were obtained by scanning in the range of 200–1600 M/z. Source temperature was maintained at 70 °C. The major species in mitochondria consisted of TLCL [$(C_{18:2})_4$ CL], *m/z* 723.6 and *m/z* 1448.5 for the double and single charge species, respectively. Other minor species of CL [$(C_{18:2})_3$, $(C_{16:1})_1$], *m/z* 710.5 double and *m/z* 1423.8 single charge species, respectively and [$(C_{18:2})_3$, $(C_{20:2})_1$], *m/z* 737.6 and *m/z* 1474.1 for double and single charge species, respectively.

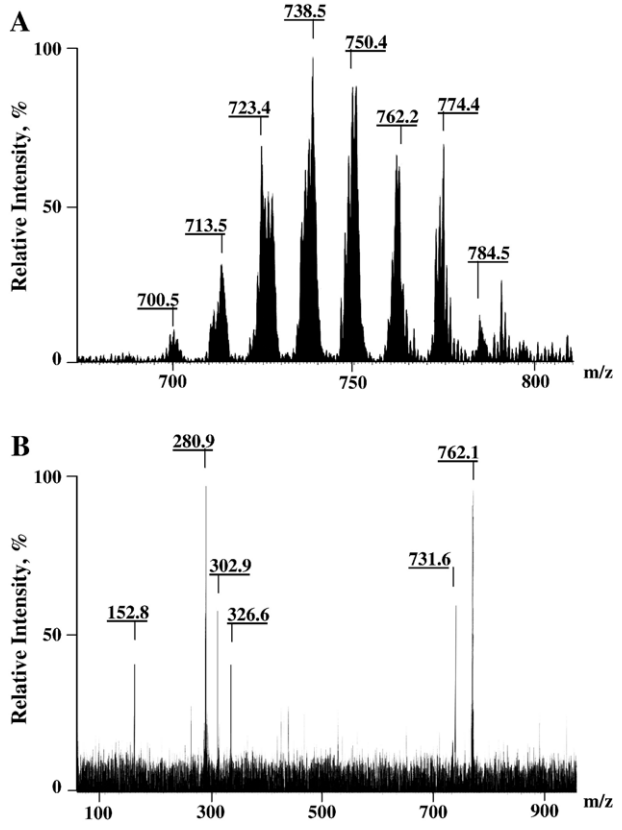

Fig. 3. ESI tandem mass spectrometry of CL isolated from mouse brain mitochondria. CL was separated by 2D-HPTLC, extracted from the HPTLC plate and subjected to electrospray ionization mass spectrometry by direct infusion into a triple quadrupole mass spectrometer (Micromass, Inc., Manchester, England). 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 keV in the negative ion mode. Mass spectra for doubly charged CL species were obtained by scanning in the range of 400–950 M/z. Source temperature was maintained at 70 °C. (A) Typical negative ion ESI mass spectrum of different molecular species of mouse brain mitochondria cardiolipins. The major species in mouse brain consisted of TLCL [$(C_{18:2})_4$ CL], *m/z* 723.4; $C_{(18:1)_3}C_{(20:4)_1}$, *m/z* 738.5; $(C_{16:0})_1(C_{18:1})_1(C_{20:4})_1(C_{22:4})_1$, *m/z* 750.4; $(C_{18:1})_2(C_{20:4})_1$ $(C_{22:6})_1$, *m/z* 762.2; $C_{(18:0)_1}C_{(18:1)_1}C_{(22:6)_2}$, *m/z* 774.4, all of which represent the $(M\text{-}2H)^{2-}$ species. Mass spectra prototypical of 3 independent experiments are presented. (B) ESI tandem mass spectrometry of CL, *m/z* 762.1. Daughter ions of linoleic acid (*m/z* 280.9), arachidonic acid (*m/z* 302.9) and docosahexaenoic acid (*m/z* 326.6) fragments are shown.

$(C_{16:1})(C_{18:2})_3$; $(C_{18:2})_4$, respectively) (Fig. 4) suggesting that CL oxidation may be involved in its apoptotic pathways as well.

On the other hand, cancer cells that contain more saturated CL species may use this as a strategy to develop resistance to pro-apoptotic stimuli and escape from apoptotic action of antitumor drugs (Tyurin et al., unpublished data). In this context, a recent study of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-triggered apoptosis of cancer cell lines has demonstrated a vital link between death receptor signaling at the plasma membrane and changes in mitochondrial membrane lipids (including CL) occurring before or in parallel with the activation of apical caspases [29]. The latter findings thus point towards new potential strategies for anti-cancer drugs that, by targeting cellular lipid metabolism, could selectively kill cancer cells.

4. Role of cytochrome *c* peroxidase activity in apoptosis

The role of cyt *c* changes from mostly an electron-carrier in normal mitochondria to mostly a CL-specific peroxidase during apoptosis. The switch between these two functions is due to cyt *c*'s association with CL, whose availability at the sites of location of cyt *c* sets the limit for peroxidase activity. It is likely, that transmembrane migration of CL early in apoptosis and its interaction with cyt *c* regulates peroxidase activity of cyt *c*. Not surprisingly, siRNA-driven depletion of cyt *c* results in proportionally enhanced resistance of cells to apoptosis [17]. In line with this, our preliminary experiments indicate that cyt $c^{-/-}$ mouse embryonic fibroblasts (MEF) cells transfected with mutated cyt *c* ($Met_{(80)}Ala$) exerted a higher sensitivity to pro-apoptotic stimulation (Jiang et al, unpublished results). Indeed, substitution of Met for Ala – which is a weaker heme ligand than Met – facilitates access of H_2O_2 to the heme catalytic site of cyt *c* and enhances its peroxidase activity. Interestingly, the transgenic cyt *c* "knock-in" mouse expressing cyt *c* that is defective (mutant) for interactions with Apaf-1 and apoptosome activation, effectively supports electron transport functions in mitochondria [32]. However, the novel peroxidase function of cyt *c* described herein, and its importance for apoptosis signaling in vivo, was not evaluated in these studies.

Peroxidase activity of cyt *c*/CL complex requires H_2O_2 or organic (lipid) hydroperoxides for catalytic oxidation of CL and

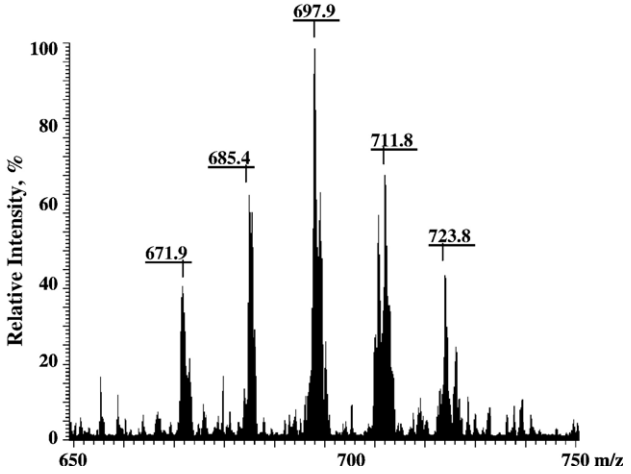

Fig. 4. Typical negative ion ESI mass spectrum of different molecular species of *Drosophila* cardiolipins. Lipids were analyzed by electrospray ionization mass spectrometry by direct infusion into a triple quadrupole mass spectrometer (TSQ70, Finnigan). Sheath flow was adjusted to 5 μl/min and the solvent consisted of chloroform:methanol (1:2, v/v). The electrospray probe was operated at a voltage differential of −3.5 keV in the negative ion mode. Mass spectra for doubly charged CL species were obtained by scanning in the range of 400–950 M/z. Source temperature was maintained at 70 °C. Different CL molecular species present in *Drosophila* were observed $(C_{16:1})_4$, *m/z* 671.9; $(C_{16:1})_3(C_{18:2})$, *m/z* 685.4; $(C_{16:1})_2(C_{18:2})_2$, *m/z* 697.9; $(C_{16:1})(C_{18:2})_3$, *m/z* 711.8; $(C_{18:2})_4$, *m/z* 723.8. Mass spectra prototypical of 3 independent experiments are presented.

other substrates. The amounts of available H_2O_2 are among the factors that normally limit CL oxidation. Apoptosis is usually accompanied by pronounced oxidative stress causing massive production of ROS-superoxide radicals [33–35] whose dismutation (spontaneous or catalyzed by mitochondrial Mn-SOD) yield H_2O_2 [36–38]. Interestingly, a recent report described a specific apoptosis-activated H_2O_2-generating role of p66 [39], known to be an adaptor-protein in a tyrosine-kinase regulated cascade in normal mitochondria [40].

GSH peroxidase, thioredoxin, glutaredoxin 2, and peroxyredoxin systems in mitochondria as well as catalase in peroxisomes are important H_2O_2 regulators [41–44]. Genetic manipulations of these H_2O_2-regulating systems have been shown to affect sensitivity of cells to apoptosis [45–48]. Overexpression of mitochondrial antioxidant enzymes regulating phospholipid oxidation such as phospholipid hydroperoxide glutathione peroxidase inhibits release of cyt *c* from mitochondria by suppressing the peroxidation of CL thereby preventing apoptosis [49,50]. Similarly, Enoksson et al. [51] showed that mitochondrial glutaredoxin 2 can prevent CL oxidation and thereby mitigates cyt *c* release. H_2O_2-regulating antioxidants, particularly GSH and its precursors, are effective at inhibiting apoptosis. Interestingly, another mitochondrial protein that has been implicated in cell death, apoptosis-inducing factor (AIF) [52,53] may realize its potential via control of GSH. The oxidoreductase function of AIF is required for the maintenance of GSH levels under conditions of cellular stress [54]. In light of the dual roles of cyt *c* in the life and death of the cell, it is instructive to also consider AIF as a potentially important redox regulator of H_2O_2 and associated cyt *c*-dependent apoptotic pathways.

5. Antioxidant regulation of cyt *c*/CL peroxidase activity

The heme-site in partially-unfolded cyt *c*/CL peroxidase complex is accessible to small molecules, suggesting a mechanism to regulate cyt *c* catalytic activity. Mitochondrial reductants (e.g., ascorbate) may interact with the peroxidase reactive intermediates (compounds I and II as well as with protein-derived radicals) and, hence, inhibit CL peroxidation. While this feat of ascorbate has been established for a number of peroxidases, its role in the regulation of cyt *c*/CL-catalyzed oxidations is not known. Another small-molecule regulator of peroxidase activity, NO, can indeed effectively inhibit peroxidase activity of cyt *c*/CL complexes. We demonstrated that typical penta- and hexa-coordinate heme-nitrosyl complexes of cyt *c* are readily formed in the presence of CL and prevent H_2O_2-induced oxidation of polyunsaturated CL and other substrates in simple biochemical model systems [148]. Provided these reactions are realized in mitochondria during apoptosis, mitochondrial NOS (mtNOS) and this NO-dependent mechanism may serve a novel protective utility whose role and functions remain conjectural [55]. Peroxynitrite, stemming from interactions of NOS-generated NO with superoxide radicals inadvertently formed by electron transport in mitochondria [56] is a well-characterized inhibitor of mitochondrial functions [57]. Moreover, peroxynitrite causes nitration of MnSOD resulting in loss of its catalytic competence, so this adds another detrimental aspect of dysregulated mtNOS [58]. Thus functions of NOS cannot be considered protective in mitochondria. However, combined with the role of p66 in specific mechanisms for H_2O_2 generation without intermediate production of superoxide [39], effects of NO as scavengers of reactive intermediates of cyt *c*/CL peroxidation may be essential in preventing accidental CL oxidation and release of pro-apoptotic factors.

Because H_2O_2 is very important as a co-factor for the cyt *c*/CL peroxidase reaction, prevention of its formation may be an effective antiapoptotic strategy. Nitroxide radicals have ideal chemical propensities for this function because they can act as effective electron acceptors from components of mitochondrial respiratory complexes [59]. As a result, nitroxides would prevent formation of superoxide radicals and their dismutation to H_2O_2. In addition, the product of nitroxide reduction, nitroxide hydroxylamine, is a potent free radical scavenger whose reaction with oxidizing radicals yields nitroxides. Thus recycling of nitroxides, which occur during their radical scavenging, may be important in their protective action. Not surprisingly nitroxides have demonstrated significant protective potency in different types of redox-driven disease conditions in animal models [60–66]. Unfortunately the therapeutic potential of nitroxides is difficult to exploit because it requires their very high (mM) concentrations. One may assume that mitochondrial targeting of nitroxides should lead to a significant decrease of required protective concentrations. Recently, three successful attempts have been reported in which different conjugates of nitroxides directing them to mitochondria or cells resulted in a several-fold decrease in nitroxide concentrations required to exert antiapoptotic effects [17,67–69].

6. Oxidized CL binds to cyt *c* poorly resulting in release of cyt *c* from mitochondria

CL oxidation designates an important stage of the execution of apoptotic program because oxidized CL (in contrast to non-oxidized CL) does not effectively bind cyt *c* [70,71]. As a result of CL oxidation, cyt *c* is no longer retained by mitochondrial membranes and can be released into the cytosol. Several reports indicate that CL oxidation parallels and is required for the release of cyt *c* and other proapoptotic factors from mitochondria into the cytosol [72,73]. Further, the 2-step model of cyt *c* release postulates that disruption of cyt *c* binding to CL within mitochondria is followed by its release through pores in the outer mitochondrial membrane likely involving interactions with pro-apoptotic Bcl-2 family members such as Bax [25,30].

7. CL oxidation, cyt *c* release: implications for CNS diseases

Many CNS diseases are associated with oxidative stress and lipid peroxidation [74–78]. For example, in the central nervous system, programmed cell death contributes to neuronal death after ischemia [79], epilepsy [80], and traumatic brain injury [81]. Programmed cell death pathways have also been identified in adults after stroke [82] and in pediatric patients dying of sudden infant death syndrome [83]. Accumulation of

isoprostanes, end-products of lipid peroxidation, has been demonstrated in early stages of several neurodegenerative diseases as well as in brain trauma [84–86]. This identification of isoprostanes – formed after de-esterification of peroxidized phospholipids – in the disease process, however, does not identify the origin of phospholipids from which they were derived. Several studies indicate that CL is one of the important oxidizable substrates, particularly in conditions associated with enhanced apoptosis. For example, during nerve growth factor (NGF) withdrawal-mediated neuronal apoptosis in vitro, Kirkland et al. [87] showed that CL concentration decreased to a greater extent than did the concentrations of other more abundant phospholipids (phosphatidylcholine (PC) or phosphatidylethanolamine (PE)). The decrease in CL content was temporally associated with increased ROS production; augmentation of cellular GSH concentration by NAC blocked loss of CL, and the decrease in mitochondrial mass in NGF-deprived neurons [87,88]. Similarly, in SOD1 mutant amyotrophic lateral sclerosis (ALS) mice, cyt *c* binding was disrupted in mitochondria, and mitochondrial lipids, including CL, were found to be significantly more oxidized in mutant mice than in wild-type animals [89]. The findings in ALS transgenic mice are compatible with the novel oxygenase function of cyt *c*, as proposed in the present review.

As important as these reported studies may be, they suffer from a serious technical deficiency that quantitations of CL concentrations and oxidation were made indirectly based on its interactions with a fluorescent reagent, *N*-nonyl-acridine orange (NAO) [87]. Recent assessments indicate that specificity of NAO is not sufficient to accurately interpret its changes with CL content and/or peroxidation [90,91]. Therefore, direct estimates of CL oxidation are necessary to prove its participation in neuronal apoptosis. In our preliminary experiments, we demonstrated that CL oxidation occurs early in neuronal apoptosis, and precedes cyt *c* release, PS externalization and GSH depletion [92]. Moreover, direct MS and chromatographic measurements of CL oxidation indicate that it may be used as an important biomarker of apoptosis in vivo. In particular, in brain trauma, selective CL oxidation occurs as one of the early apoptotic biomarkers after traumatic brain injury [92].

8. Flirting of cytochrome *c* with PS results in PS oxidation and externalization

Release of cyt *c* from mitochondria into the cytosol is associated with the well-known central event in intrinsic apoptosis signaling: the apoptotic protease-activating factor-1 (Apaf-1)-driven formation of apoptosomes and downstream caspase activation designating a point-of-no-return in apoptosis [93,94]. However, only a small fraction of cyt *c* is commonly utilized in this process. Moreover, redox characteristics of cyt *c* are not essential for this process [95]. Recent findings show that the redox catalytic potency of cyt *c* is utilized in extra-mitochondrial reactions of cyt *c*. Indeed, results obtained in in vitro model systems have demonstrated that the peroxidase catalytic competence of cyt *c* can also be realized with another anionic phospholipid, PS [96,97]. In addition to CL, preferential oxidation of PS, predominantly in the plasma membrane, is also observed in cells undergoing apoptosis [98–101]. Notably, CL oxidation and PS oxidation are temporally separated. PS oxidation occurs before its externalization but after cyt *c* release (Jiang et al., unpublished data). Finally, PS peroxidation is blocked in cells overexpressing anti-apoptotic genes such as Bcl-2 and is sensitive to pan-caspase inhibitors [98,99,102]. A series of experiments in both intact cells and in vitro model systems indicate that cyt *c*/PS complexes act as major catalysts of PS oxidation during apoptosis [31]. Overall, these findings support the notion that the execution of apoptosis involves oxidative stress that also targets PS in the plasma membrane.

PS externalization is an essential "eat-me" signal for macrophage engulfment of mammalian apoptotic cells [99,103]. Moreover, our studies have shown that the concomitant and selective oxidation of PS also functions to generate crucial recognition signals during programmed cell clearance [98–100]. Importantly, recent studies have confirmed the presence of oxidized PS on the surface of apoptotic cells (in contrast, PE on the cell surface was found not to be oxidized during apoptosis) [104]. Furthermore, the mechanism of PS-dependent apoptotic corpse clearance appears to have been conserved through evolution [105].

Currently, externalization of PS is the only general recognition ligand for phagocytes on apoptotic cell surface [97,106]. However, recent studies have suggested that certain proteins may also be exposed on the cell surface during programmed cell clearance, including the PS-binding protein, annexin I [107] and calreticulin, a protein that binds to LDL-receptor related protein (LPR) on the engulfing cell and was suggested to cooperate with PS as a recognition signal [108]. In other words, engulfing cells evidently prefer a "high protein, high fat" diet. Indeed, given that PS exists either as oxidized or non-oxidized species on the surface of apoptotic cells [99,104], phagocytes may prefer not simply "high fat" but rather a "high rancid fat" diet. This adds a further degree of complexity to the process of cell clearance, and may allow for a more selective removal of cell corpses, without inadvertent engulfment of non-apoptotic cells.

In addition to oxidized phospholipids, lysophospholipids (LPC and LPS) act as chemotactic factors secreted from apoptotic cells in a caspase-dependent manner and serve to attract macrophages to sites of cell attrition [109]. Lysophospholipids have also been identified as putative "eat-me" signals on the apoptotic cell surface, which are recognized by naturally occurring IgM antibodies [110]. Of note, oxidation of phospholipids could represent a step on the way to their hydrolysis and formation of lysophospholipids [111].

9. Regulation of inflammatory responses by PS and oxidized PS

Inflammation is a beneficial host response to foreign challenge or tissue damage that ultimately leads to recovery from infection and to restoration of tissue structure and function [112,113]. However, the inflammatory reaction sometimes proceeds to a chronic state and can thus, paradoxically,

constitute a detrimental process. The accumulation and persistence of leukocytes, including polymorphonuclear granulocytes or neutrophils, is a hallmark of chronic inflammation. Indeed, the release of proteolytic enzymes, ROS, and other harmful contents from activated neutrophils is believed to be responsible for the tissue destruction evidenced in chronic inflammatory conditions [114]. Neutrophil apoptosis at sites of inflammation and the subsequent macrophage engulfment of these cells is thought to constitute a mechanism of safe clearance that may limit tissue injury. Of note, the process of programmed cell clearance is not a passive event, but plays an active role in the resolution of inflammation, through production of anti-inflammatory cytokines such as TGF-β by macrophages and downregulation of pro-inflammatory mediators such as TNF-α [115–117].

Chronic granulomatous disease (CGD) is a rare, inherited condition characterized by severe recurrent bacterial and fungal infections and an inability of neutrophils and other phagocytes to generate ROS, which are needed for intracellular killing of microorganisms; the underlying genetic defect is a mutation in the NADPH oxidase [118]. Our previous in vitro studies have shown that phorbol ester-triggered externalization of the "eat-me" signal PS is defective in neutrophils obtained from CGD patients [119], and one may speculate that the absence of this crucial recognition signal on the surface of activated neutrophils may disrupt the clearance of cells in vivo, thus contributing to the formation of inflammatory granulomas and tissue destruction evidenced in these patients. Indeed, an increased accumulation of neutrophils was observed in peritoneal exudates of NADPH oxidase (phox)-defective mice injected with heat-inactivated bacteria, indicative of a clearance defect in this model of CGD [120]. Our ongoing studies have confirmed that NADPH oxidase-defective, PS-negative neutrophils from CGD patients are poorly recognized by normal macrophages, upon in vitro co-cultivation (Fadeel et al., unpublished observations). In addition, macrophages from CGD patients were shown to be severely compromised in their ability to produce anti-inflammatory mediators such as TGF-β [121], which may contribute to the persistence of inflammation in this disease.

Recent technological developments in the field of nanomaterials and carbon nanotubes have enabled the study of their toxicity, particularly to the lung [122,123]. Exposure of mice to single walled carbon nanotubes induced an unusually robust inflammatory response with a very early onset of pulmonary fibrosis [122]. This has been associated with poor recognition of non-functionalized carbon nanotubes by macrophages. Notably, our studies showed that coating of nanotubes with PS (but not with PC) resulted in a remarkable improvement in their recognition by macrophages suggesting that PS acts as an "eat-me" signal not only on the surface of apoptotic cells but also on nanotubes [124]. PS-dependent signaling changes cytokine responses of macrophages from a pro-inflammatory to an anti-inflammatory pattern [117,125]. Therefore, PS-coated nanotubes may be utilized as a novel tool for the regulation of inflammatory responses [126].

Circulating anti-phospholipid antibodies in patients with systemic lupus erythematous and alcoholic liver disease recognize and bind selectively to apoptotic, but not viable cells [127,128]. Detailed analysis showed that the antibodies were mainly directed towards oxidized phospholipids (CL and PS) and that oxidation of phospholipids was essential to generate epitopes for many anti-phospholipid antibodies [129]. Of note, the recognition of apoptotic cells coated by anti-phospholipid antibodies or other auto-antibodies through Fc receptors was suggested to lead to pro-inflammatory macrophage responses [130]. Thus, it is possible that anti-phospholipid antibodies targeting externalized PS on apoptotic cells might favor inflammatory processes, under certain pathological conditions.

10. Oxidized phospholipids, natural antibodies: implications for atherogenesis

Atherosclerosis is widely recognized as a chronic inflammatory disease that encompasses both innate and adaptive immune responses [131]. Although numerous risk factors have been identified, atherosclerosis is clearly initiated and sustained by hypercholesterolemia and associated elevations of serum low-density lipoproteins (LDL). Once trapped in the vessel wall, LDL may undergo modifications, including oxidation (thus forming oxidized LDL, oxLDL), and the resulting products can then activate inflammatory responses that further promote atherogenesis [132,133]. Macrophages are central in atherosclerosis; upon activation, these cells initiate the oxidation of LDL and rapidly take up oxLDL through specific scavenger receptors, leading to foam cell formation. Macrophages also secrete a variety of pro-inflammatory factors that affect lesion progression.

Membranes of apoptotic cells are in many aspects similar to oxLDL insofar as they contain increased levels of oxidized phospholipids, including PS (discussed above). Chang and colleagues [134] have shown that monoclonal antibodies against oxLDL bind to the surface of apoptotic cells and inhibit their uptake by macrophages, thus providing further evidence of oxidation-specific epitopes on apoptotic cells. Moreover, C-reactive protein (CRP) was recently found to bind both oxLDL and "late" apoptotic cells (i.e., apoptotic cells that have undergone secondary necrosis due to prolonged in vitro culture) through recognition of a common ligand, oxidized phosphatidylcholine (PC) [135]. These oxidation-specific epitopes can be considered as pathogen-associated molecular patterns (PAMPs), and are recognized by natural antibodies and other innate immune receptors, including scavenger receptors [131]. Natural antibodies are thought to play a role in the recognition and removal of senescent cells, cell debris, and other self-antigen, and it has been hypothesized that this "house-keeping" role against oxidation-dependent neoepitopes (including oxLDL) [136] has contributed in large part to their evolutionary selection. Natural antibodies were shown to block the uptake of oxLDL by macrophages and could thus prevent foam cell formation [137]. Similarly, these antibodies could perhaps play a role in the programmed clearance of apoptotic and senescent cells displaying similar oxidation-specific epitopes or PAMPs.

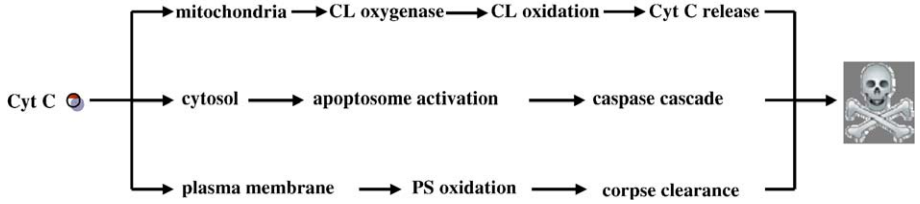

Fig. 5. War and peace: the manifold functions of cytochrome *c*. Cyt *c* is essential for oxidative phosphorylation and the production of ATP under normal conditions. Moreover, as depicted in this diagram, cyt *c* plays several important roles during the execution and resolution stages of cell death. Hence, cyt *c* acts as a cofactor in the activation of apoptosomes in the cytosol, resulting in the activation of downstream caspases. Cyt *c* has also recently been ascribed a novel function as a CL oxygenase within mitochondria, and is thus implicated in the early steps of the apoptotic program. Finally, cyt *c* may catalyze the peroxidation of plasma membrane phospholipids including PS, leading to the exposition of recognition signals for macrophages and the engulfment of the cell corpse.

An interesting recent study showed that immunization of mice with syngeneic apoptotic cells led to high titers of antisera that reacted not only with apoptotic immunogens but also to a panel of different oxidation-specific epitopes, including oxidized PC, that are also found on oxLDL [138]. Moreover, oxidized phospholipids on apoptotic cells were shown to activate endothelial cells to induce monocyte adhesion, a pro-inflammatory response (and a rate-limiting step in atherosclerosis) that was abrogated by antibodies specific for oxidized PC. Normally, apoptotic cell corpses are swiftly removed by phagocytes without inciting an inflammatory response (as discussed above). However, under certain conditions, such as the pathological accumulation of excessive numbers of dying cells, or an accumulation of dying cells that exceeds the clearance capacity of the tissue, apoptosis could also trigger inflammation [139]. One may speculate that the oxidative modification of phospholipids that occurs during apoptosis is a mechanism through which apoptotic cells could promote pro-inflammatory responses [128,138]. Alternatively, such responses could ultimately be protective by enhancing the clearance of apoptotic cells through the recruitment (for instance via LPC-dependent chemotaxis) of sufficient numbers of other inflammatory (phagocytic) cells to the apoptotic lesion. Finally, although oxidized phospholipids may promote chronic inflammation in atherosclerosis, other data suggest that they can also inhibit inflammation and protect mice from lethal endotoxin shock [140]. Clearly, phospholipids (and more specifically, their oxidatively modified counterparts) are involved in a range of biological processes that are now gradually being unraveled [141,142].

11. Concluding remarks

The multitude of molecular players in apoptosis and the intricate interactions are reminiscent of the numerous characters and the convoluted plots of many classic Russian novels. However, cyt *c* has emerged as one of the key protagonists in the drama of cellular suicide (apoptosis). In the present review, we have described some of the novel roles of cyt *c* within and outside of mitochondria that are associated with its redox catalytic competence as a peroxidase specific for the anionic phospholipids, CL and PS (Fig. 5).

The core death machinery has been conserved through evolution, from worms to mammals [143]. However, cyt *c* appears to be important as a co-factor for caspase activation downstream of mitochondria only in mammalian cells, but not in the nematode (indeed, the *C. elegans* homolog of Apaf-1, CED-4, lacks a cyt *c*-binding domain) [94]. Nevertheless, it remains to be determined whether cyt *c* exerts other, conserved roles in apoptosis also in nematodes and other model organisms, for instance, in the facilitation of PS peroxidation during programmed cell clearance. Similarly, the importance of cyt *c* as a mitochondrial CL oxygenase has not been studied yet in non-mammalian cells. Given the highly conserved domains of the cyt *c* molecule important for its redox peroxidase function, it could perhaps serve as a conserved mechanism for the release from these organelles of pro-apoptotic factors, such as cyt *c* or AIF (designated WAH-1 in *C. elegans*) [144].

Dysregulated apoptosis is common in human disease [145]: excessive apoptosis is typical of degenerative diseases including neurodegenerative diseases and immune deficiency states, whereas insufficient apoptosis contributes to cancer and autoimmune diseases. This emphasizes the urgency in developing new therapeutic approaches to control apoptosis. Indeed, multiple attempts to use different antiapoptotic agents have been included in drug discovery programs [145,146]. Thus, an increasing number of compounds targeting a diverse range of apoptosis-related molecules are being explored at the preclinical and clinical levels. However, essentially all of these attempts are based on the development of disruptors of protein–protein interactions. The recent discovery, reported herein, of CL/cyt *c* complexes involved in the execution of early stages of apoptosis makes this a novel and attractive target for drug discovery. New approaches could thus be envisioned based on the use of disruptors of protein–lipid interactions, rather than of protein–protein interactions, to yield effective antiapoptotic agents for the treatment of human pathologies.

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