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Cytochrome c/cardiolipin relations in mitochondria: a kiss of death

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Abstract

Recently, phospholipid peroxidation products gained a reputation as key regulatory molecules and participants in oxidative signaling pathways. During apoptosis, a mitochondria-specific phospholipid, cardiolipin (CL), interacts with cytochrome *c* (cyt *c*) to form a peroxidase complex that catalyzes CL oxidation; this process plays a pivotal role in the mitochondrial stage of the execution of the cell death program. This review is focused on redox mechanisms and essential structural features of cyt *c*'s conversion into a CL-specific peroxidase that represent an interesting and maybe still unique example of a functionally significant ligand change in hemoproteins. Furthermore, specific characteristics of CL in mitochondria – its asymmetric trans-membrane distribution and mechanisms of collapse, regulation of its synthesis, remodeling and fatty acid composition – are given significant consideration. Finally, new concepts in drug discovery based on the design of mitochondria-targeted inhibitors of cyt *c*/CL peroxidase and CL peroxidation with anti-apoptotic effects are presented.

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Keywords

cytochrome *c*; cardiolipin; peroxidase; lipidomics; oxidative stress; apoptosis; autophagy; mitochondrial targeting

Introduction

“Life is pleasant. Death is peaceful. It's the transition that's troublesome.”

Isaac Asimov (1920 - 1992)

As a triplet biradical with two parallel spins, molecular oxygen readily interacts with other radicals – e.g., lipid alkyl radicals, thiyl radicals – but it has a very poor reactivity towards molecules with fully paired electrons (non-radicals). As a kid, everyone was amazed by a famous experiment in chemistry class when the teacher burned a small strip of iron in an atmosphere of oxygen. Radicals generated by the high temperature of the flame and combustion facilitated the oxidation of iron. Remarkably, iron is vital to functions of diverse enzymes where it catalyzes reactions with oxygen; however, the chemistry of life does not burn our body. On the contrary, aerobically living cells have developed a safe and sophisticated machinery to *activate* oxygen and catalyze slow and well controlled oxidation (but not combustion) processes. Yet, oxygen radicals are continuously produced in our body via a univalent reduction of molecular oxygen. While all one-electron products of oxygen reduction are called “reactive oxygen species”, only one of them – the hydroxyl radical HO[•] (a three electron reduction intermediate of oxygen) – is notorious for its remarkably high and calamitously indiscriminate reactivity towards most biomolecules.

It is a common belief that strict control and elimination of superoxide and hydrogen peroxide (H₂O₂) are protective mechanisms preventing cell damage and death [1]. Recently, however, superoxide radicals and hydrogen peroxide gained a reputation as regulatory molecules and participants in oxidative signaling pathways. Superoxide dismutases (SODs) – in the mitochondrial matrix and intermembrane space, in the cytosol, and in extracellular compartments – convert superoxide radicals into H₂O₂. Thus, SODs may be acting as important regulators and sources of H₂O₂. An important process through which cells utilize H₂O₂ for signaling purposes is the peroxidase catalytic cycle of hemoproteins¹. While activation of H₂O₂ by peroxidases is usually effectively controlled by the participating protein moieties, it is still a high risk endeavor; changes in the redox environment, protein structure, or genotoxic events may lead to unregulated activation of H₂O₂ and the production of hydroxyl radicals. In this review, we will focus on cyt *c* – a well known hemoprotein electron transporter in mitochondria – to illustrate possible mechanisms and consequences stemming from peroxidase activation of this protein by physiologically relevant anionic phospholipids.

¹Peroxidases are diverse and widespread enzymes capable of two-electron reduction of peroxides at the expense of various oxidizable substrates [4]. There are non-heme peroxidases (thiol peroxidase, NADH peroxidase, etc.) and heme-containing peroxidases. Among the latter are plant enzymes – ascorbate peroxidase, cytochrome *c* peroxidase etc. – and animal peroxidases – cyclooxygenase superfamily, which includes prostaglandin H synthase (cyclooxygenase), myeloperoxidase, lactoperoxidase and others. Most of heme peroxidases can subtract electrons from specific and nonspecific substrates and generate corresponding free radicals and protein-centered radicals (A[•] → A[•]). In addition, some peroxidases (i.e., cyclooxygenase-2) may catalyze only one-electron reduction of peroxides, thus producing O-centered radicals. A similar catalytic mechanism is utilized by various heme proteins including hemoglobin, myoglobin and cytochrome *c*. Lipids represent one class of physiologically important reducing substrates for peroxidases. Prostaglandin H synthase is capable of specific oxidation of arachidonic acid. Myeloperoxidase is less discriminative towards lipid substrates and catalyzes peroxidation of different lipids [5,6]. Catalytic properties of cyt *c*/CL complexes and their specificity towards peroxidation of anionic phospholipids – cardiolipin, phosphatidylserine, phosphatidylinositol – are considered in this review.

Multiple functions of cytochrome c in cells

Over last two decades, we witnessed the collapse of an old dogma of biochemistry: one gene->one protein->one function. Discoveries of new functions of cytochrome *c* are one of stunning hallmarks of this paradigm shift. In addition to its well established role as an electron shuttle between respiratory complexes III and IV in mitochondria, the antioxidant role of cytochrome *c* has been linked to its propensity to catalyze the oxidation of superoxide radicals to molecular oxygen. Thus, cytochrome *c* can act as a superoxide scavenger [2]. In addition to mechanisms associated with cytochrome *c*'s electron-transporting capacities, it has been identified as a critical cell death factor capable of initiating the caspase cascade via its binding to apoptosis protease activating factor, Apaf-1, and the formation of apoptosomes [3]. Mueller et al. have demonstrated that cytochrome *c* catalyzes the amidation of fatty acids and the formation of important physiological regulators – long chain fatty acyl glycines – through yet to be identified pathways [7,8]. All these important biological activities of cytochrome *c* are realized in its native structure. Studies from several laboratories documented that unfolding of the cytochrome *c*'s globule reveals a new function as a peroxidase [9]. The structural destabilization of the protein can be induced by chemical modification (i.e. oxidation, nitration) [10-12] or by its association with hydrophobic anions, including anionic phospholipids [13-19]. Our studies uncovered the mechanisms through which cytochrome *c* peroxidase activity propagates the oxidation of a mitochondria-specific phospholipid, cardiolipin, and the pivotal role of this reaction in the execution of apoptosis (via mitochondrial membrane permeabilization and release of pro-apoptotic factors from mitochondria) [20,21]. The mechanisms and essential features of cytochrome *c*'s conversion into a peroxidase represent an interesting and maybe still unique example for a functionally significant ligand change in hemoproteins. These properties of cytochrome *c* may represent an interesting case of the recently developed concept of “intrinsically disordered” or “ill-structured” proteins, whereby anionic membrane phospholipids induce the “controlled chaos” [22,23] required for the emergence of a new peroxidase function. These structural rearrangements of cytochrome *c* – induced by its binding with CL, the appearance of the peroxidase function, CL peroxidation and the subsequent “dreadful” consequences for both mitochondria and cells – are the major focus of this review.

Interactions of cytochrome c with anionic phospholipids lead to peroxidase activation

Binding modes of cytochrome c to anionic phospholipids

During the last three decades, studies of cytochrome *c* revealed several protein binding sites for anionic lipids; at least 30% of the protein surface constitutes so-called A-, C- and L- candidate binding domains believed to participate in interactions with anionic lipids (see schema 1) [24-26]. Both the penetration of phospholipid acyl chains into the protein globule as well as the protein integration into the phospholipid bilayer of the membrane were suggested as possible binding modes. Interactions of cytochrome *c* with anionic phospholipids are complex, and multiple factors can contribute to the unfolding capacity of the lipids.

Electrostatic forces are one of the major factors that govern cytochrome *c*-lipid interactions. Positively charged cytochrome *c* molecules (isoelectric point is near pH 10, net charge is +8e at neutral pH) are strongly attracted to the negatively charged headgroups of anionic lipids [20,24,27,28]. There are no signs of unfolding of cytochrome *c* and activation of its peroxidase activity in the absence of electrostatic interactions - with non-charged (zwitterionic) lipids such as phosphatidylcholine (PC) - or with anionic phospholipids in high-ionic strength buffers, as evidenced by electrophoretic measurements of the cytochrome *c*/lipid complex [17,19,29]. Site A has been designated as the anion-binding center that likely includes the basic residues Lys72 and Lys73 [24,25]. By studying interactions of the spin-labeled protein with spin-labeled phospholipids, Kostrzewa et al. found that the membrane interface of the protein includes Lys72, Lys86 and Lys87 [30]. Similarly, recent mutation studies of yeast cytochrome *c* revealed an involvement of Lys72 and Lys73 in cytochrome *c*-CL binding [31]. The Nantes' group reported the existence of an additional

electrostatic binding site on cyt *c*, named L site, which includes Lys-22, Lys-25, His-26, Lys-27, and His-33, and participates in protein-membrane interactions at pH<7.0. Through simultaneous interactions of sites L and A with CL-containing membranes, cyt *c* can promote vehicle fusion at low pH [26].

Electrostatic interactions between cyt *c* and lipid membranes, however, are not the only factors that affect unfolding of the protein. Hydrogen bonding between the C site represented by Asn52 and protonated acidic phospholipids was proposed to stabilize the high affinity binding of the protein [24,25]. By detecting tertiary rearrangements, it was demonstrated that cyt *c*-CL binding is a two step process involving high and low affinity sites, which are believed to be A- and C-sites, respectively [19]. Studies of cyt *c* binding with fluorescence resonance energy transfer (FRET) from labeled lipid to heme have also confirmed the presence of two interaction modes – an electrostatic low affinity binding to deprotonated CL molecules and a high affinity binding stabilized by electrostatic and H-bonding to partially protonated CL [32]. The partial involvement of these two binding sites depended on pH, ionic strength and the mole fraction of CL; yet both binding modes were operational at physiologically relevant conditions.

Hydrophobic interactions between nonpolar acyl residues of the lipid molecules and nonpolar regions of cyt *c* (normally buried inside the protein) also effect the formation of the cyt *c*/lipid complex [17,28,33]. Tuominen et al. suggested that the C-site-mediated interaction of cyt *c* includes a “lipid anchorage” and provided a direct demonstration of phospholipid acyl chain interaction with the hydrophobic interior of the protein [28]. It has been proposed that a single CL molecule could be involved in both the electrostatic interaction of a head group with Lys72 and in the hydrophobic anchoring of the protein by acyl chain insertion either in the channel surrounded by Asn52, Lys72 and Lys73 or between the non-polar polypeptide strands 67–71 and 82–85 [31,32]. Our recent finding that the reaction rate of both membrane bound and free cyt *c* with fatty acid hydroperoxides is about three orders of magnitude higher than the rate of H₂O₂-dependent peroxidase activity strongly argues in favor of the importance of hydrophobic interactions (Belikova et al., unpublished observations).

It has been postulated that the formation of the complex commences with electrostatic interactions that promote the penetration of cyt *c* into the lipid bilayer, where it interacts hydrophobically with interior lipids [17,19]. This model suggests that the presence of unsaturated lipids is a requirement for effective membrane interfacing of cyt *c* and formation of the complex. Indeed, alterations in saturation of the lipid acyl chains have a dramatic effect on cyt *c* binding and unfolding. Mono-unsaturated tetraoleoyl cardiolipin (TOCL) was found to be a much stronger inducer of cyt *c* conformational changes and activator of its peroxidase activity than saturated tetramyristoyl cardiolipin (TMCL); the structural rearrangements triggered by polyunsaturated tetralinoleoyl cardiolipin (TLCL) appeared to be even stronger than those initiated by TOCL [17]. In support of this hypothesis, interaction of cyt *c* with CL evaluated by competitive binding with acridine 10-nonyl bromide (nonyl acridine orange, NAO) increased in the same order TMCL << TOCL < TLCL < BHCL (bovine heart cardiolipin), i.e. interactions progressed proportional to the number of double bonds in the CL acyl chains.

An interesting mode for cyt *c* interactions with anionic phosphatidylglycerol (PG) has been proposed by Oellerich et al. on the basis of viscosity and turbidity of lipid/protein mixtures. The binding mechanism depends on the lipid to protein ratio. At low coverage of the membrane surface, the binding is peripheral; at a ratio from 18:1 to 12:1 partial protein penetration into the membrane occurs. At lower ratios, peripheral binding dominates again [16]. In line with this observation, the analysis of FRET from anthrolylvinyl-labeled PC to cyt *c* heme indicated that the high affinity binding involves a partial penetration of the protein into the lipid bilayer to the depth of several proximal carbons of the acyl chains [32]. Approximate estimates of

heme location relative to the membrane/aqueous interface by FRET from NBD-labeled CL (1,1',2-trioleoyl-2'-[12;-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-cardiolipin) are in agreement with these findings [34].

In summary, the molecular description of cyt *c* interactions with CL (and other anionic phospholipids) is not complete. There are at least two sites on the cyt *c* protein surface that can contribute to CL binding. The binding may be realized via different modes whose relative contributions depend on the experimental conditions, such as pH, ionic strength, lipid composition and lipid/protein ratios. However, detailed information on the structure of “foldons” in cyt *c*/CL complexes and the molecular dynamics of CL-driven protein unfolding is lacking. Further studies to better our understanding of cyt *c*-membrane interactions are warranted.

Structural rearrangements and peroxidase activity of cyt *c* upon its interactions with anionic lipids

Interaction of cyt *c* with negatively charged lipid membranes induces considerable disruption of the native compact structure of the protein and induces intermediate conformations between the native and fully unfolded states, called a “molten globule”. This state, an “alternative folding”, is defined as a compact conformation with a secondary structure comparable to that of the native state and fluctuating tertiary conformation due to a high enhancement of intramolecular motion [13,27,35,36]. In solution, stability and unfolding of cyt *c* were extensively studied using deuterium exchange [37] and other experimental techniques [38-41]. Stabilities of different regions of the protein were found to be very dissimilar: five distinct domains of cyt *c* (foldons) with non-equivalent stabilities were identified, which participate in cooperative folding–unfolding of the protein in a stepwise sequential way [37]. These structural domains are folded around the heme of cyt *c* which is covalently attached to the polypeptide chain by residues Cys14 and Cys17.

Binding of cyt *c* to membranes is accompanied by changes in the tertiary protein conformation and opening of a heme crevice [13-15]. An early finding of Fe-S(Met80) bond disruption upon binding was further advanced by analysis of the heme configuration using resonance Raman spectroscopy, which revealed the coexistence of a mixture of hexa-coordinated low spin states (His-Fe-Me and His-Fe-His) or hexa-coordinated low spin (His-Fe-His) and high spin states (His-Fe-H₂O and His-Fe-) in DOPG bound protein, depending on the lipid to protein ratio [16]. These coordination states of heme are similar to those present in microperoxidases, heme-containing peptides produced by proteolytic digestion of cyt *c* [42,43].

Changes in the heme environment also affected CD spectra, causing shifts in the 375-425 nm region (heme moiety) as well as changes in the UV-region (250-280 nm) that were observable even at relatively low CL/cyt *c* ratios (2:1 and 4:1). By detecting changes in 416 nm dichroic signal upon cyt *c* binding to CL, Sinibaldi et al. described two-step alterations in the heme pocket associated with Fe-S(Met80) bond disruption and rearrangements of the tertiary structure [19]. They found that the peroxidase activation of cyt *c* also followed a two-step transition profile, thus demonstrating a strong link between conformational and functional properties of the protein. In concordance with this observation, an acidic environment further favored the high affinity binding of the protein to CL and the destabilization of its tertiary structure [32,44] as well as simultaneously enhanced peroxidase activity of CL-bound cyt *c* (Borisenko, unpublished observations). The appearance of Trp59 fluorescence (quenched by its proximity to heme in native cyt *c*) is also characteristic of CL binding to cyt *c*; this effect indicates a substantial conformational shift in the protein, leading to a proportional increase in the peroxidase activity [18].

Because high affinity binding of CL with cyt *c* – accompanied by unfolding of the protein – is realized largely through an electrostatic interface between negatively charged phosphates on CL and positively charged lysines on cyt *c*, as well as through hydrophobic interactions of CL's acyl groups with a hydrophobic domain of the protein, other negatively charged phospholipids may also bind and unfold cyt *c* via analogous mechanisms. Indeed, several anionic phospholipids tested for their ability to change the structure of cyt *c* – phosphatidic acid (PA), dioleoyl-glycero-3-phosphoinositol 4,5-bisphosphate (PIP2) and dioleoyl-glycero-3-phosphoinositol 3,4,5-trisphosphate (PIP3) – revealed significant binding with cyt *c* accompanied by structural rearrangements of the protein. CL and PA were most effective as inducers of cyt *c* unfolding assessed by several criteria such as the levels of Trp59 fluorescence, a full disruption of the Fe-S(Met80) bond and a nitrosylation of the cyt *c* heme. Similarly, CL and PA were stronger inducers of cyt *c*'s peroxidase activity than other anionic phospholipids [18].

The activation energy of the peroxidase reaction, ΔG_{perox} , was found to be ~ 1 kcal/mol less than the free energy needed to unfold the cyt *c* domain containing Trp59 (ΔG_{fluor}) and lower by 0.3-0.6 kcal/mol than the ΔG for disruption of Fe-S(Met80). This interesting kinetic effect is consistent with the assessment of a 5 to 12 kcal/mol range of stability energies for the five domains of cyt *c* [45-47] compared to an activation energy for the peroxidase catalytic function of ~ 3.8 kcal/mol [48]. ΔG_{perox} appeared to be very close to ΔG of heme nitrosylation, probably because it is very similar to the formation of the complex between heme and hydrogen peroxide – a prerequisite for the catalysis of the hydrogen peroxide reduction [18]. These comparisons of structural parameters and peroxidase activity indicate that the peroxidase function of cyt *c* is strongly activated under conditions that do not markedly change the protein tertiary structure (assessed by Trp59 fluorescence). In other words, unfolding can favor peroxidase activity, yet this activity does not require *complete* protein unfolding. Peroxidase activity is upregulated when the dissociation energy of the Fe-S(Met80) bond is lowered due to protein-lipid interaction, yet the bond is still retained, thus suggesting that a relatively small perturbation of the protein structure by electrostatic interactions with membrane components is sufficient for the substitution of H₂O₂ (or nitric oxide, NO) for Met80 and for peroxidase activation. This alternative protein (un)folding and disruption of the Fe-S(Met80) bond inevitably leads to an enhancement of peroxidase activity, similar to that caused by chemical modifications such as carboxymethylation [49] and interactions with peroxynitrite [50] or hypochlorite [51], or complete unfolding in the presence of guanidine hydrochloride. The augmented availability of heme iron in cyt *c*/CL complex for small molecules is reminiscent of microperoxidases in which one of hexa-coordinated ligands, H₂O, can be readily displaced by a variety of exogenous ligands resulting in high peroxidase activity [52].

In summary, high affinity interactions of cyt *c* with anionic phospholipids may involve binding at two different sites to the membrane, partial unfolding of the protein globule and partial insertion of the protein into the membrane. Structural transitions of cyt *c* include: (i) opening of the heme crevice (detected by CD and NMR spectroscopy); (ii) reduction of the volume of hydrophobic core (detected by NMR spectroscopy); (iii) disruption of the 6th ligation of heme Fe with the Met80 residue (detected by UV-VIS and Raman spectroscopy); (iv) emergence of penta-coordinated high spin heme and hexa-coordinated Fe with a new ligand, presumably His33 (shown by Raman and EPR spectroscopy); (v) rearrangement of the protein's tertiary structure with the preservation of its secondary structure (observed by CD spectroscopy); and (vi) emergence of Trp59 fluorescence that is completely quenched by heme in the native protein.

To satisfy these experimental observations, the conformation of the protein in the complex with the membrane has to include electrostatic and hydrophobic protein-lipid interactions, relocation of His33 from the proximal to the distal side of the heme and transition of Trp59

from the plane of the heme to the plane perpendicular to the heme (i.e., a relocation whereby a minimal shift in distance will produce the highest gain in fluorescence quantum yield). We propose the following model for the protein-lipid interactions (see Scheme 2): cyt *c* binds to an anionic phospholipid via Lys72 (A-site) and then to a second anionic lipid via Lys27 (L-site). As a result, the heme will be located perpendicular to the plane of the membrane. Subsequent concerted rearrangements of the tertiary structure include: (i) a slight shift of a low energy foldon (residues 72-86 according to [37]) along the membrane surface and out of the heme plane, (ii) a substantial movement of a low energy foldon (residues 37-59) out of the heme plane and rearrangement of a high energy foldon (residues 20-36) around the heme, leading to the occupation of a distal heme pocket by His33. According to this structure, the heme edge has to be inserted into the membrane and the hydrophobic residues Ile81 or Phe82 anchor the protein in the membrane. The heme and the heme pocket become readily available for interaction with phospholipid acyl chains. Three other high energy foldons (residues 1-19, 60-71 and 88-104) do not undergo perturbations. Residues 1-19 and 88-104 are located above the heme, while residues 60-71 interact with the protein coils of residues 72-86. This model of cyt *c* structural rearrangements by CL (anionic lipids) is associated with minimal energy cost.

Structure of cyt *c* catalytic site

Highly specialized peroxidases, like horseradish peroxidase (HRP) or myeloperoxidase (MPO), have particular heme environments, allowing the catalytic sites to effectively bind, and cleave H₂O₂. The exceptionally high reaction rates – 10^6 - 10^7 M⁻¹s⁻¹ of peroxidases [53] are dependent on the presence of His as a 5th iron ligand in the proximal heme pocket as well as His and Arg residues in the distal heme pocket. The latter two residues participate in the so-called “push-pull” catalysis of H₂O₂ cleavage [54-56] and the formation of the first reactive intermediate, compound I (schema 3). Analysis of HRP mutants revealed that substitution of distal Arg and His was associated with 10³- and 10⁵-fold decreases in the rate of Compound I formation, respectively [55,57]. Mutation of the proximal His slowed this reaction 10⁶ fold [58], thus clearly indicating the hierarchy of these amino acids in catalysis.

Although cyt *c* possesses some of the pre-requisites of a peroxidase – a heme moiety and a proximal His – it has a very weak peroxidase activity in its native state [59,60]. The catalytic site of cyt *c* lacks Arg or His in the distal pocket of the heme. Moreover, the distal ligand – Met80 – is located 2.5Å away from the Fe, thus precluding access of the heme to H₂O₂ and other peroxides in the native protein [20]. The above considerations based on crystallography or NMR studies of *native* cyt *c* are hardly applicable to its markedly changed organization in complexes with anionic lipid membranes [17,27,28,35,61]. Unfortunately, neither crystallographic data nor detailed NMR analysis of these complexes with anionic phospholipids are currently available. However, several studies employing less direct techniques are indicative of electronic and structural changes in the protein/CL complexes favoring its peroxidase function. Upon binding and unfolding of cyt *c* by anionic lipids or surfactants as well as after its chemical modification (for example, by carboxymethylation, oxidation or nitration [49-51]), Met80 moves away from the heme site and thus releases the sixth iron coordination bond. Cyt *c* bound to CL displays a shift of the Soret band to a shorter-wavelength region suggesting a high-spin state of heme in the new cyt *c* conformation (Belikova unpublished observations). Raman spectroscopy studies confirmed that a penta-coordinated high spin state is one of the major forms of heme in cyt *c* both bound to DOPG vesicles and unfolded by detergents [16,62]. This heme coordination state is characteristic of peroxidases and is believed to be important for peroxidase function [63].

Weakening and disruption of the Met80-Fe bond, the distal movement of Trp59 from the heme and changes of the protein tertiary structure facilitate engagement of several essential ligands – His26, His33, Arg38 and Arg91 – in the catalytic peroxidase process in cyt *c*/CL complexes.

Raman spectroscopy analysis of ferrous and ferri cyt *c*/PG complexes revealed the presence of heme with hexa-coordinated ligands represented by His33 or His26 [16,62], thus suggesting a close location of His to the heme in the distal pocket.

The structural rearrangements in cyt *c*/CL complexes dramatically affect its redox properties. In complexes with CL, the redox potential for the Fe(II)/Fe(III) couple is ~400 mV more negative than in intact cyt *c* [64]. As a result, two important functions of free cyt *c* – electron shuttling between complexes III and IV of the mitochondrial respiratory chain and scavenging of superoxide radicals – become unfeasible. In peroxidases, the Fe(II)/Fe(III) redox potential is linked to the stability of highly oxidized heme intermediates via electron donation from anionic axial ligands to the heme [65-66]. The stability of these intermediates is essential for peroxidase catalysis. Structural factors contributing to the regulation of the redox potential in hemoproteins include the hydrophobicity of the heme pocket, the nature of the axial ligands on iron and electrostatic interactions at the active site. In cyt *c*, His-Met ligation of iron and a hydrophobic protein core are two important factors that determine its positive redox potential (+260 mV) [67,68]. Loss of these features upon binding of cyt *c* with CL is likely accountable for a substantially more negative redox potential, which falls into the range characteristic of peroxidases (f. ex., the redox potentials of cyclooxygenase (COX) and HRP are -160 mV and -250 mV, respectively). The CL-induced shift in the redox potential may be a substantial contributor to the higher peroxidase activity of cyt *c*/CL complexes.

Heterolytic and homolytic pathways of cyt *c* peroxidase catalysis

Heme-containing peroxidases can oxidize reducing substrates by utilizing different peroxides (e.g., H₂O₂, and organic peroxides including lipid hydroperoxides) as a source of oxidizing equivalents. As a heme-protein with a proximal His18, cyt *c* can function as a peroxidase, albeit at a very low rate of about 1 M⁻¹s⁻¹ [59,60]. Partially unfolded cyt *c* in its complexes with CL exerts almost a 100-fold higher catalytic activity in the presence of H₂O₂ than the native protein [20], yet it is still a relatively weak peroxidase [17]. H₂O₂ has been traditionally recognized as the preferred source of oxidizing equivalents for many peroxidases such as horseradish peroxidase, lactoperoxidase, and myeloperoxidase, which do not display significant peroxidase activity with fatty acid hydroperoxides [53]. In contrast, cyt *c*/CL peroxidase activity can be supported by other hydroperoxides, particularly those of lipids. In fact, oxidized CL (hydroperoxy-CL, CL-OOH) is evidently a very good source of oxidizing equivalents for the peroxidase activity of cyt *c*. Low concentrations of CL-OOH – accumulated during its storage or by its initial oxidation in the cyt *c* catalytic peroxidase cycle – effectively propagated the peroxidation of CL without any additional supplementation with H₂O₂. Our recent studies demonstrated that peroxidase activity of cyt *c*/CL complexes can be enhanced up to 1,000-fold when fatty acid hydroperoxides were utilized as a source of oxidizing equivalents instead of H₂O₂ (Belikova et al., unpublished results). In a way, this is similar to the requirement in arachidonic acid hydroperoxides (Ar-OOH) for the full expression of the peroxidase function of cyclooxygenases (COX-1 and COX-2), which catalyze the reduction of lipid hydroperoxides (i.e. PGG₂ derivatives) 2-3 orders of magnitude more effectively compared to H₂O₂ [69]. Interestingly, both cyclooxygenases lack Arg in the distal pocket, while Gln adjacent to His is not involved in the peroxidase reaction. Instead, COXs have enlarged hydrophobic domains that are likely involved in the binding of bulky fatty acid moieties and the cleavage of the O-O bond by the distal His and heme iron [70-72]. Similarly to COX, cyt *c*/CL complexes accommodate an esterified fatty acid residue of CL (and probably free fatty acid) in their hydrophobic heme pockets upon interaction with the anionic membrane [28].

Two major catalytic mechanisms potentially involved in the cleavage of hydroperoxides by peroxidases are the homolytic and heterolytic pathways [53]. Homolytic splitting of H₂O₂ leads to the formation of highly and indiscriminately reactive HO[•]. As a consequence, the homolytic

mechanism is likely associated with a non-specific oxidation of reducing substrates as well as the oxidative modification of the enzyme itself. Homolytic splitting of hydroperoxies by hemin and several hemoproteins such as HRP mutants, cytochrome *c* peroxidase (CcP) mutants, myoglobin, and cytochrome P450 has been reported [54,55,73-76]. Based on earlier studies of the Mason group with cyt P450 and lipoxygenase [77,78], Iwahashi et al. [79] proposed a mechanism for a one-electron reductive homolytic decomposition of fatty acid hydroperoxide (FA-OOH) by cyt *c*, leading to the formation of alkoxyl radicals:

where *R* is a protein chain, *Por* is heme, *R-Por-Fe(III)* is ferri cyt *c*, *R-Por-Fe(IV)=O* is the oxyferryl state of heme in cyt *c*, and *LO*[•] is an alkoxyl radical of a fatty acid.

In contrast, heterolytic cleavage of the O-O bond – leading to the formation of Compound I – requires significantly higher activation energy than homolysis (370 versus 40 kcal/mol [71]). Several peroxidases, such as HRP, CcP, MPO, and COX utilize this pathway [53]. The employment of either of these alternative peroxidase mechanisms depends on several factors, such as the spin-state of the heme (high or low), the organization of catalytic pocket, and the nature and properties of the oxidant. A “Push-pull” catalysis relies on positively charged Arg and His residues to shift the electron density sufficiently for H₂O₂ heterolytic splitting. Accordingly, the His42Leu mutant of HRP is characterized by the preference for homolytic cleavage of H₂O₂ and a significantly lower rate of peroxidase reaction [55,57]. In concurrence with these considerations, the catalytic site of native cyt *c* lacking Arg or His in close vicinity of the heme in the distal pocket should favor the homolytic peroxidase mechanism.

The heterolytic mechanism of peroxidase action utilizes reactive intermediates leading to oxidation products specific for a particular enzyme (i.e., PGH₂ produced by COX, and hypochlorous acid by MPO). In contrast to classic peroxidases, no spectroscopic evidence has been obtained so far for the formation of compounds I and II for chemically modified cyt *c* or cyt *c*/CL complexes. Combined with readily detectable protein-centered radicals [80,81], this is indicative of the generation of highly unstable heme intermediates. Stereospecific oxidation by cyt *c*/CL complexes can be achieved via interactions with relatively stable protein-centered radical intermediates generated during the cleavage of the O-O bond. The specificity of cyt *c* as a peroxidase may be due to the proper orientation of strongly-bound reducing substrates such as polyunsaturated cardiolipins.

Mass spectrometric studies identified several species of CL-OOH and CL-OH as major oxidation products formed by complexes of cyt *c* with polyunsaturated CL both *in vitro* and *in vivo* [20,21,82,83]. This indicates that not only H₂O₂ but also CL-OOHs are utilized as sources of oxidizing equivalents in this reaction. It is possible that both mechanisms – homolytic and heterolytic – may be involved at different stages of cyt *c* peroxidase reactions. Initiation of the reaction by H₂O₂ and subsequent switching to CL-OOH or FA-OOH (see below) may be also associated with different contributions of each of the two mechanisms. Interestingly, employment of both catalytic mechanisms has been demonstrated for mammalian cyclooxygenases [84]. A highly specific COX-1 isoform catalyzes primarily a two-electron reduction of FFA-OOH via its heterolytic cleavage. In contrast, COX-2 catalyzes both one- and two-electron reductions [84].

Protein-derived radicals and oligomerization of cyt c by the peroxidase activity of its complexes with anionic phospholipids

Characteristic reactive intermediates of peroxidase-catalyzed reactions are protein-immobilized radicals [85-88]. The formation of these radicals is markedly enhanced in enzymes that have relatively unstable Compounds I and II. Particularly, the highly reactive Compound I in COX-2 (half-life ~100 ms) produces a tyrosyl radical that is presumably involved in the oxidation of other protein groups, and enzyme inactivation occurs within seconds [89]. Cyt *c* contains several potentially oxidizable aminoacid residues: four tyrosines, some of which (Tyr67, Tyr48) are within 5.0 Å of the heme porphyrin ring, and one tryptophan residue [90]. The radical intermediates from these residues can be detected by low temperature EPR spectroscopy [85] as well as by an immuno-spin trapping technique in which the immunoreactive protein-immobilized spin adducts formed during interaction of radicals with a spin trap, DMPO, can be detected by an anti-DMPO antibody [86,88,91]. EPR spectroscopy experiments demonstrated that anionic phospholipids facilitate the H₂O₂-dependent production of protein immobilized radicals on cyt *c*. The effectiveness of dioleoyl (DO) phospholipids in inducing protein-derived radicals increased in the order DOPA > TOCL > DOPS > DOPC (DOPS, dioleoylphosphatidylserine). In the absence of reducing substrates or spin-traps, recombination of protein-derived tyrosyl radicals results in oligomerization of the protein via dityrosine cross-links (which are non-dissociable by S-S reducing reagents) and the disappearance of its monomeric form [51,92]. The strengths of phospholipids in inducing peroxidase dependent oligomerization of cyt *c* ranked similarly to their effects on peroxidase activity and the formation of protein-derived radicals: TOCL ~ DOPA > PIP3 > PIP2 > DOPS. The importance of cyt *c*/CL oligomerization is that it may facilitate not only the accumulation of homo-oligomers but also cause a hetero-oligomerization by co-oxidation of other proteins. This may be particularly relevant to proteins that have anionic lipid binding sites, such as alpha-synuclein, resulting in an accumulation of poorly digestible cross-linked aggregates characteristic of neurodegenerative diseases. Interestingly, both cyt *c* and alpha-synuclein are abundant components of Lewy bodies which accumulate in the brain of patients with Parkinson's disease.

Peroxidase function of cyt c/CL complexes in apoptosis

Multiple functions of cyt *c* – in mitochondrial electron transport, peroxidase oxidation of CL, interactions with Apaf-1 in the cytosol – raise a question about regulation and switching mechanisms involved in its diverse pathways. One of these mechanisms is a marked negative shift of cyt *c*'s redox potential upon its interaction with CL, thus precluding its operation as an electron acceptor from mitochondrial complex III or from superoxide radicals (see above). Another important regulatory mechanism is availability of oxidizing equivalents - H₂O₂ or lipid hydroperoxides - feeding the peroxidase cycle of cyt *c*/CL complexes. Disrupted electron transport, particularly at Complexes I and III, are considered the major sources of superoxide production. Enzymatic mechanisms – by MnSOD in the matrix or Cu,Zn-SD in the intermembrane space – and spontaneous (non-enzymatic) pathway convert superoxide radicals into H₂O₂ [93,94]. A mitochondrial outer membrane enzyme, monoamine oxidase, catalyzes deamination of biogenic amines and directly generates H₂O₂. In addition, 12/15-lipoxygenase is a potent cytosolic source of lipid hydroperoxides. Both monoamine oxidase and 12/15-lipoxygenase have been implicated in the development of cell death [95,96]. Finally, strict compartmentalization of CL, that prevents its random non-specific binding with cyt *c*, likely represents one of the most effective regulators of cyt *c*'s peroxidase activity – as detailed below.

Collapse of CL asymmetry in mitochondria during apoptosis

Peroxidase function of cyt *c* requires its direct physical interaction with CL. Normally, however, CL is confined almost exclusively (>80%) to the inner mitochondrial membrane (IMM) [20,97-101] whereby it is distributed between the inner and the outer leaflets at a ratio of 60:40 [102,103]. Thus, binding of cyt *c* to CL depends on the availability of the latter in the outer leaflet of the IMM. Moreover, significant demand for high-affinity CL binding by other mitochondrial proteins such as mitochondrial respiratory Complexes I, III and IV as well as other mitochondrial proteins also limits access of cyt *c* to CL [104,105]. However, within mitochondrial contact sites – zones of close apposition of the inner and outer membranes – the content of CL may be high and comparable to that of PC and PE – up to 24% of total lipids [101].

During apoptosis, the asymmetric distribution of CL collapses and the level of CL in the outer mitochondrial membrane increases to 40% of its total content, while 60% of it still resides in the inner membrane [20]. About 30-40% of the CL present in the IMM of apoptotic cells, is confined to its inner leaflet [20]. The decrease of the CL level in the inner membrane is accompanied by changes in the inter-membrane distribution of CL. Only 30-40% of CL is found in the inner leaflet of the inner membrane in mitochondria of apoptotic cells [20].

tBid and Scramblase-3: involvement in CL trans-membrane redistribution in apoptosis?

The mechanisms of CL mitochondrial translocation are not well understood. It has been shown that CL inter- and intra-membrane changes in mitochondria occur at early stages of apoptosis [20], before dissipation of membrane potential and prior to PS externalization on the cell surface [106]. Activities of two proteins have been associated with apoptotic trans-migration of CL: tBid and phospholipid scramblase-3 (PLS-3) [103,107,108]. Early work has established that tBid has a CL binding domain [109,110]. While tBid is bound to the outer mitochondrial membrane at both contact and non-contact sites [111], it preferentially inserts into the negative lipids of the mitochondrial contact sites between the inner and outer membranes [112], possibly in proximity to the polyunsaturated CL species [101]. A significant trans-membrane distribution of CL from the inner to the outer leaflets of the inner mitochondrial membrane and subsequent appearance of CL in the outer mitochondrial membrane was observed in tBid-treated mitochondria [100,103].

PLS-3 is another mitochondrial protein believed to play a role in CL translocation during apoptosis [107]. PLS-3 is a member of the PLS family responsible for the bi-directional movement of phospholipids [113]. PLS-3 contains 295 amino acids and its gene is located in chromosome 17 in humans [114]. PLS-3 is activated by calcium and disruption of its calcium binding motif results in its inactivation [107]. Cells transfected with this inactive PLS3 mutant contained fewer mitochondria that were larger than those in control cells [107]. These cells were also less sensitive to UV- and tBid-induced apoptosis. In contrast, cells overexpressing PLS-3 displayed increased sensitivity to UV-induced apoptosis and enhanced CL translocation [107]. PLS-3 undergoes post-translational modification by phosphorylation of a specific Thr residue (Thr21) by protein kinase C delta (PKC δ) [115]. Using pyrene-PC labeled liposomes, He et al., [116] assessed the lipid flip-flop activity of PLS-3 and showed that the phosphomimetic mutant of PLS-3 (T21D) was more effective than wild-type protein or phosphoinhibitory mutant (T21A) [116]. However, the exact mechanisms of how phosphorylation of PLS-3 leads to CL translocation are not known. It has been reported that phosphomimetic form of PLS-3 (T21D) facilitates the mitochondrial targeting of tBid [116]. Thus, apoptosis-associated phosphorylation and activation of PLS-3 may be involved – possibly via tBid-dependent pathways – in trans-membrane re-distribution of CL that is critical to the execution of the mitochondrial stage of the apoptotic program.

Biosynthesis and remodeling of CL

Compartmentalization/topography and sufficiency of CL via its binding to cyt *c* is an important regulatory mechanism of apoptosis. Therefore, the supply of CL via its *de novo* biosynthesis and remodeling may affect the sensitivity of cells to apoptosis. As a unique mitochondrial phospholipid, CL may be represented predominantly by one or only few molecular species in some tissues (e.g., heart, skeletal muscle, liver, kidney or intestines) [83,117-119] or by hundreds of individual species in other tissues (e.g., brain) [120-122]. There are two major metabolic pathways for CL biosynthesis and turnover. *De novo* synthesis of CL takes place in mitochondria. Several enzymes catalyzing the multi-stage CL synthesis – those responsible for the formation of phosphatidic acid from acyl-CoA fatty acid and glycerol-3-phosphate, production of activated phosphatidyl (phosphatidyl-CMP), its transfer to another glycerol-3-phosphate and consequent hydrolysis of the latter yielding phosphatidylglycerol – are localized in mitochondria [123]. The final and rate-limiting synthetic step whereby PG is combined with CDP-diacylglycerol to yield CL, is catalyzed by CL synthase, the active site of which is exposed to the mitochondrial matrix [124-126]. Knocking down CL synthase by using RNA interference results in a significant decrease of CL content [127,128]; however, the CL molecular species in CL-deficient cells remain unchanged [128]. Importantly, CL-deficiency was associated with the increased resistance to apoptosis induced by actinomycin D, X-ray irradiation and rotenone in HeLa cells, likely due to decreased amounts of productive cyt *c*/CL complexes participating in the peroxidation of CL [128]. Recently, Gonzalves et al. demonstrated that in the type II apoptotic response, CL is important for the anchoring, translocation and embedding of caspase 8 in the mitochondrial membrane. This event is vital for caspase 8 oligomerization and further release of apoptotic factors from mitochondria to cytosol [129].

The second mechanism of CL transformation includes re-acylation or remodeling of CL acyl chains [130]. Remodeling has been recognized as an important step of CL post-synthetic maturation. It is believed that the *de novo* synthesis predominantly contributes to the diversity of CL molecular species, while acyl-specific remodeling yields a limited number of CL molecular species [131]. Neuronal CL remodeling occurs shortly after birth in mammalian species and causes alterations in the physical properties of the mitochondrial membrane [120]. CL remodeling can occur both in mitochondria [131-133] as well as in the endoplasmic reticulum [134]. The re-acylation process requires a hydrolysis of CL by phospholipase A₂ [131] to monolyso-CL (MLCL) or dilyso-CL (DLCL) and its coenzyme A dependent [134] or independent re-acylation [135]. Coenzyme A dependent re-acylation of MLCL is orchestrated by acyl-CoA:lysocardiolipin acyltransferase 1 (ALCAT1). ALCAT1 is localized in the endoplasmic reticulum and recognizes both MLCL and DLCL as substrates with a preference for linoleoyl-CoA and oleoyl-CoA as acyl donors [134]. CoA-independent phospholipid transacylase (or so-called “tafazzin”) can catalyze the acylation of both lyso-phosphatidylcholine with CL-derived acyl groups and the acylation of MLCL with phosphatidylcholine-derived acyl groups [135]. It has been reported that defects in CL remodeling associated with Barth syndrome lead to accumulation of CL derivatives with abnormal fatty acid composition (reviewed in [136]). Remodeling of CL species may be important as a mechanism controlling the level of CL oxidation by cyt *c*. For example, substitution of abundant and highly polyunsaturated C22:6, C22:5, and C20:4 species of CL in the brain for less oxidizable C18:2 or non-oxidizable mono-unsaturated C18:1 should inevitably result in lower susceptibility to pro-apoptotic agents. Conversely, targeted manipulation of CL to more oxidizable molecular species may lead to a desirable decreased resistance to apoptosis in tumor cells.

Peroxidation of cardiolipins by cyt *c*

The distinction of CLs as substrates of peroxidase activity of cyt *c* is mostly associated with their participation in the execution of the mitochondrial stage of apoptosis. Polyunsaturated

phospholipids are known precursors of many important signaling molecules through their hydrolytic or oxidative metabolism; this is particularly relevant to eicosanoid and docosanoid pathways [137-144]. Phospholipids containing polyunsaturated acyl groups are the major substrates of non-enzymatic free radical oxidation that can be initiated and propagated as a chain reaction. Interestingly, *cyt c* catalyzed peroxidation of CLs also utilizes polyunsaturated molecular species, whereas saturated and monounsaturated CL molecules do not undergo peroxidation [92]. However, *cyt c* catalyzed peroxidation reactions display significant specificity: anionic phospholipids, particularly CL, PS and PI – are the preferred oxidation substrates. This is most likely due to the proximity of these *cyt c*-bound phospholipids to the sites where reactive peroxidase intermediates are generated [83,145,146]. In different types of cultured cells triggered to apoptosis (e.g., by gamma-irradiation, or staurosporine or actinomycin D exposure) as well as in animal tissues with a significant number of apoptotic cells (e.g. induced by traumatic brain injury or gamma-irradiation), accumulation of phospholipid hydroperoxides decreases in the order CL>>PS>>PI>>PE>PC [21,82,83,120, 128,147,148]. A notable example is the selective and robust oxidation of two anionic phospholipids – CL in mitochondria and PS outside of mitochondria – in the small intestine of gamma-irradiated mice (Schema 4) [83]. Two molecular species of CL containing C18:2 as potentially oxidizable species indeed underwent oxidation after exposure of mice to total body irradiation. In line with this argument, several hydroperoxy-derivatives – (C_{18:2})₃/ (C_{18:2}-OOH)₁; (C_{18:2})₂/ (C_{18:2}-OOH)₂; (C_{18:2})₁/ (C_{18:2}-OOH)₃; (C_{18:2}-OOH)₄ – were detectable in the MS of CLs from irradiated animals. Notably, more abundant phospholipids with higher contents of more polyunsaturated acyl chains – PC, PE, and PI – remained non-oxidized [83]. This underscores the role of *cyt c*/CL interactions as major factors in determining the substrate specificity of CL oxidation.

Selective oxidation of CL was also found in the lungs of mice exposed to hyperoxia. A 7.5-fold increase of pulmonary CL-hydroperoxide content (to 33.8 ± 8.0 pmol/nmol CL) was detected after hyperoxia (72 h, 99.9% of oxygen) compared to the normal lungs of C57BL/6 mice. The MS analysis of CL oxidation products identified CL molecular species containing hydroperoxy-linoleic acid (C_{18:2}-OOH) along with palmitic C_{16:0}, linoleic C_{18:2}, and stearic C_{18:0} fatty acids [149]. Accumulation of CL hydroperoxides was also characteristic of the lung of C57BL/6 mice exposed through inhalation (for 4 consecutive days, 5 h/day) to single walled carbon nanotubes. Up to 87.1 ± 9.7 pmol of CL hydroperoxides per nmol of CL were detected in lungs at 1 and 7 days after inhalation associated with a robust inflammatory response.

The major products of *cyt c*-catalyzed peroxidation include different hydroperoxy- and hydroxy-derivatives [17,18,20,82,83]. The latter are formed by the peroxidase activity of *cyt c* whereby phospholipid-hydroperoxides (PL-OOH) are utilized as a source of oxidizing equivalents and reduce PL-OOH to PL-OH at the expense of oxidation of new CL molecules [82,83]. Our recent results indicate that alternatively to the reductive metabolism, CL-OOH may be involved in hydrolytic reactions catalyzed by *cyt c* [150]. This phospholipase A2-like activity of *cyt c* yields two major products – non-oxidized monolyso-CL and hydroperoxy-free fatty acids [150]. It is possible that this pathway is involved in CL remodeling as well as the production of oxygenated fatty acids with potentially important physiological functions.

Possible role of CL in interactions between autophagy and apoptosis

In addition to apoptosis, autophagy represents an alternative pathway of programmed cell death (the so called autophagic (type II) cell death). It is well accepted that these two types of cell death mechanisms are interconnected, while the link between autophagy and apoptosis is highly ambiguous [151]. Autophagy is an evolutionary conserved mechanism that provides cells with a mechanism for the continuous turnover of damaged and obsolete macromolecules and organelles (e.g. mitophagy and reticulophagy) [152], and may serve as a mechanism of

adaptation to stress (and hence suppress apoptosis), whereas in many other circumstances, it constitutes an alternative cell-death pathway (type II) [153]. For instance, Bax/Bak double knockout mouse embryonic fibroblast cells failed to undergo apoptosis when challenged by DNA damaging reagents such as etoposide. Instead, massive autophagy and delayed cell death was observed [154]. Amaravadi *et al.* found that autophagy inhibition with either chloroquine or ATG5 shRNA enhanced the ability of either p53 activation or alkylating drug therapy-induced apoptosis in a Myc-induced model of lymphoma [155]. The cytoprotection via autophagy was mainly attributed to its ability of removing protein aggregates and injured organelles, and regulating the cell cycle. In a different experimental setting, perturbations of the apoptotic machinery in lipopolysaccharide treated U937 monocytoid cells and macrophages by the pan-caspase inhibitor Z-VAD-FMK resulted in autophagic cell death, which could be attenuated by RNAi-mediated knockdown of beclin [156]. The cytotoxicity of autophagy could be explained by the destructive potential of uncontrolled massive autophagy. Autophagic removal of mitochondria has been shown to be triggered following a process of induction/blockade of apoptosis. While the detailed molecular mechanisms of autophagy cargo recognition remain unclear, the existence of a selective autophagy of mitochondria (mitophagy) [157,158] indicates that specific mitochondrial signal(s) are involved in triggering the autophagy signaling pathway and “tag” the damaged mitochondria. Indeed, UTH1, which encodes a mitochondrial protein in yeast, has been demonstrated to be required for effective targeting of mitochondria for autophagic degradation [159]. We hypothesized that – in analogy to phosphatidylserine externalization on plasma membrane during apoptosis, and subsequent uptake and digestion of apoptotic cells by professional phagocytes - externalized CL might serve as a mitochondrial version of an “eat-me” signal in an autophagy signaling pathway. Moreover, accumulation of peroxidized CL may act as a molecular switch that initiates the development of pro-apoptotic events when autophageal mechanisms fail to effectively eliminate damaged mitochondria as depicted on Schema 5. Thus CL-mediated signaling may be a key-point in regulation of both autophagy and apoptosis. As a matter of fact, Kissova *et al.* previously reported that the inhibition of the oxidation of mitochondrial lipids slowed down mitochondria autophagy [160]. Dadakhujaev *et al.* showed that TrkA overexpression causes ROS accumulation via reduced catalase expression, ultimately leading to autophagic cell death [161]. Further experimental testing of this hypothesis is warranted to define the possible roles of CL externalization/oxidation in these fundamental mechanisms of programmed cell death.

Inhibition of CL peroxidation as a new approach to anti-apoptotic drug discovery

The discovery of the specific oxygenase activity of cyt *c* towards CL peroxidation and its essential role in the execution of the apoptotic program indicates possible directions for an effective regulation of apoptosis. Prevention of CL peroxidation may be important because it can be accomplished in mitochondria before the release of pro-apoptotic factors into the cytosol, i.e. prior to “the point-of-no-return” associated with the activation of the caspase cascades [20]. To achieve a substantial effectiveness of the anti-apoptotic action, our recent efforts have been focused on designing and developing several mitochondria-targeted inhibitors of CL peroxidation (Schema 6).

The peroxidase reaction of cyt *c*/CL complexes requires a source of oxidizing equivalents – such as H₂O₂ – to feed and maintain the peroxidation cycle. It is likely that apoptotic disruption of electron transport and diversion of electron flow to molecular oxygen acts as the major supplier of superoxide radicals and its dismutation product, H₂O₂. This implies that effective electron scavengers might be potent inhibitors of CL, provided their levels in mitochondria can be increased sufficiently. We took advantage of the well known high effectiveness of stable nitroxide radicals as acceptors of electrons from respiratory carriers [162] and conjugated the electron-scavenging cargo to mitochondria-targeted vehicles. Two different types of vehicles

have been employed: (i) fragments of the known antibiotic gramicidin S with high affinity to the mitochondrial inner membrane and (ii) a positively-charged organic cation, triphenylphosphonium, that is readily “electrophorized” into mitochondria due to their membrane potential [163]. In both cases, we found that targeted delivery of nitroxides achieved its goal – re-routing of electron flow from oxygen and preventing superoxide production [164]. As a result, CL peroxidation was blocked in a number of different cell types triggered to apoptosis by different pro-apoptotic stimuli – actinomycin D, staurosporine, or gamma-irradiation [164]. This strategy proved to be successful and resulted in effective prevention of cyt *c* release into the cytosol, hence protection against apoptosis. Most importantly, *in vivo* utilization of nitroxide conjugates showed significant protection against hemorrhagic shock induced in rats [165,166] as well as against irradiation of mice (Greenberger et al. unpublished observations). Optimization of this strategy may turn out to bear promise in a number of disease conditions where massive apoptosis represents the major contributor to the mechanisms of pathogenesis.

An alternative strategy to block CL peroxidation may be based on inhibition of the peroxidase function of cyt *c*/CL complexes. We tested two different pathways with the intent to achieve selectivity of the inhibitory effect. First, we chose to utilize precursors of NO donors which could be activated by the peroxidase function emerging in cyt *c* during apoptosis upon its interaction with CL. We designed and synthesized several oximes, whose oxidation converts them into NONOates; the latter are known to readily release NO[•] [167]. Indeed, we were able to demonstrate that the peroxidase function of cyt *c*/CL complexes caused the production of NO[•] from (2-hydroxyamino-vinyl)-triphenylphosphonium (HVTP) [167]. As expected, NO[•] acted as a potent reductant for the reactive peroxidase intermediates and prevented CL peroxidation in model systems and in cells. Experiments with mouse embryonic cells demonstrated that HVTP displayed significant protective potency against apoptosis induced in cells by either actinomycin D or irradiation [167].

Another attractive opportunity to prevent accumulation of CL peroxidation products is to utilize an alternative substrate capable of competing with endogenous CL substrates. If successful, this may re-direct the oxidizing power of cyt *c* as a peroxidase to non-essential CL-like substrates that will be ineffective in inducing the mitochondrial permeability transition and the release of pro-apoptotic factors from mitochondria. To experimentally explore this concept, we chose to use chemically modified CL, NBD-CL, and demonstrated that this conjugate formed high affinity complexes with cyt *c* and blocked cyt *c*-catalyzed oxidation of peroxidase substrates, oxidation of polyunsaturated TLCL and accumulation of TLCL hydroperoxides [34]. Upon incorporation in mitochondria, NBD-CL inhibited peroxidase activity in these organelles and, hence, may act as a promising regulator of apoptosis.

The above examples are only a few of many feasible approaches that may control and regulate peroxidase activity of cyt *c* towards CL peroxidation. Currently, experiments are underway to utilize effective ligands of cyt *c* heme to occupy its sixth coordination bond (Met80-Fe) to strongly and irreversibly “knock-out” the peroxidase potential of cyt *c*/CL complexes.

Creating CL deficiency is another good strategy to achieve increased resistance of cells to apoptosis. As mentioned above, we were successful in creating clones of CL-deficient HeLa cells in which CL content was decreased to 40mol% of its levels in parental cells. This success was associated with a markedly increased resistance to apoptosis induced by gamma-irradiation, rotenone and actinomycin D [128].

Not only inhibition but also stimulation of cyt *c*/CL peroxidase activity and CL peroxidation may be important for elaborating new therapeutic strategies. As an example, it may be very significant to enhance CL peroxidation in tumor cells, hence trigger their apoptotic machinery

towards initiation of programmed cell death. One of potentially important approaches may be based on changing the molecular speciation of cardiolipins to enrich them with highly oxidizable individual chains readily interacting with cyt *c*. We examined this hypothesis by enriching cells with docosahexaenoic acid and noted that this led to increased sensitivity to proapoptotic stimulation [20]. Our further attempts are directed towards a selective delivery of the desired precursors of fatty acids into mitochondria using different targeting vehicles.

Future experiments will also elucidate a possible direction of work focused on studies of the CL remodeling pathway – the tafazzin gene – as a potentially important way to regulate CL speciations in targeted tissues, hence manipulating their sensitivity to apoptosis.

Concluding Remarks

The organization of native cyt *c* favors its most common function as an electron shuttle between complexes III and IV of mitochondria. Hexa-coordination of heme-iron, utilization of not readily oxidizable Met(80) as the distal ligand, lack of Arg and His residues in close proximity to heme, remote location of electron-accepting Trp or Tyr residues – all of these features decrease the occurrence of peroxidase functions in native cyt *c*. However, the binding of cyt *c* to anionic phospholipids unfolds the protein and converts it from an electron shuttle into a potent peroxidase. A removal of a relatively weak ligand, Met(80), changes of spin-state and structural rearrangements pave the way for opening of the heme catalytic site to small molecules (including H₂O₂, FA-OOH) to bolster its catalytic activity to levels comparable to those of genuine peroxidases. In mitochondria, this peroxidase activity displays remarkable specificity towards cardiolipin, causing oxidation as well as hydrolysis of CL-OOH but not other more abundant phospholipids. In cells, this specificity is utilized during the execution of the apoptotic program realized via accumulation of CL-OOH. Normally, the regulation of cyt *c* as a peroxidase is achieved through the very low availability of CL that prevents the formation of productive cyt *c*/CL complexes. Upon pro-apoptotic stimulation, phosphorylation and activation of scramblase-3 likely triggers the trans-membrane redistribution of CL facilitating its interactions with cyt *c*. Phosphorylation of cyt *c* is another regulatory factor that can fine-tune its interactions with CL. The newly discovered role of cyt *c* in apoptosis allows the exploitation of this knowledge for drug discovery purposes. Indeed several types of new, mitochondria-targeted compounds have been designed and successfully tested as anti- and/or pro-apoptotic agents based on their ability to manipulate CL peroxidation in cyt *c*/CL complexes.

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List of Abbreviations

cyt c	Cytochrome c
CL	cardiolipin
CL-OOH	hydroperoxy-CL
MLCL	monolyso-CL
DLCL	dilyso-CL
TOCL	tetraoleoyl cardiolipin
TMCL	tetramyristoyl cardiolipin
TLCL	tetralinoleoyl CL
BHCL	bovine heart cardiolipin
NBD-CL	1,1',2-trioleoyl-2'-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-cardiolipin
PC	phosphatidylcholine
PA	phosphatidic acid
PIP2	dioleoyl-glycerol-3-phosphoinositol 4,5-bisphosphate
PIP3	dioleoyl-glycerol-3- phosphoinositol 3,4,5-trisphosphate

PS	phosphatidylserine
PG	phosphatidylglycerol
FRET	fluorescence resonance energy transfer
SOD	superoxide dismutase
NO	nitric oxide
H₂O₂	hydrogen peroxide
HRP	horseradish peroxidase
MPO	myeloperoxidase
COX	cyclooxygenase
CcP	cytochrome <i>c</i> peroxidase
FA-OOH	fatty acid hydroperoxide
ALCAT1	acyl-CoA:lysocardiolipin acyltransferase 1
PLS-3	phospholipid scramblase-3
PKCδ	protein kinase C delta
IMM	inner mitochondrial membrane
HVTP	(2-hydroxyamino-vinyl)-triphenyl-phosphonium

Schema 1.

Possible cardiolipin (CL) binding sites on cytochrome *c*.

Structure of native cyt *c* (1HRC). Several domains that are likely involved in interactions with CL and acting as heme-iron ligands include the following amino acid residues: Lys72, Lys73, Lys86 and Lys87 (A-site), Asn52 (C-site) and Lys22, Lys25, His26, Lys27 and His33 (L-site). Met80 and His18 form coordination bonds with heme. Intrinsic fluorescence of Trp59 is quenched by the proximity to the heme moiety.

Schema 2.

Proposed structural model of alternative (un)folding of cyt *c* bound to anionic membrane surface. The model explains reported physico-chemical properties of cyt *c* including its peroxidase activity.

A – side view, membrane interface is at the bottom of the protein; **B** – view from the membrane side. Protein backbone is encoded by color in accord with unfolding energy (in the order from low to high energy: white, red, yellow, green, and blue [37]). Amino acid residues important for binding with the membrane and peroxidase activation are highlighted.

Schema 3.

Catalytic mechanism of horseradish peroxidase.

A – ferric enzyme with H₂O₂ bound as a ligand in the 6th coordination position of iron; **B** – oxoferryl iron and porphyrin centered radical (Compound I).

Schema 4.

Oxidative lipidomics "Hit-map" of small intestine from mice subjected to total body irradiation.

A non-random, cyt c-driven mechanism is involved in the catalysis of γ -irradiation induced peroxidation of intestinal phospholipids. Selective oxidation of CL followed by oxidation of PS takes place after γ -irradiation and is a part of the intestinal apoptosis *in vivo*.

Cross-roads of Mitophagy and Apoptosis

Normal:

- H_2O_2 is not available
- SCR-3 is not phosphorylated therefore it is inactive
- Cyt-c is Tyr phosphorylated

Mitophagy:

- H_2O_2 is not available
- SCR-3 is phosphorylated therefore it is active
- SCR-3 enhances translocation of CL to OMM
- CL transmembranes asymmetry collapses
- Cyt-c is Tyr phosphorylated so it does not interact with CL

Apoptosis:

- Cyt-c is dephosphorylated
- CL/cyt c complexes are formed
- ETC is disrupted and more O_2^- is formed; H_2O_2 is formed
- H_2O_2 becomes available
- CL is oxidized
- Cyt c has lower affinity for CL-OOH than for CL, cyt c gets detached from membranes
- CL-OOH contributes to OMM permeabilization
- Cyt c is released

Schema 5.

Cross-roads of mitophagy and apoptosis.

Autophagy and apoptosis are two processes that may be mutually inhibitory; autophagy usually precedes apoptosis while triggering of apoptosis is associated with blocked autophagy. It is likely that these two pathways are intrinsically interconnected via molecular switches that turn on the autophagy process (with still inhibited apoptosis) followed by activation of apoptosis (with turned off autophagy). Both processes function as parts of an essential combined mechanism of elimination of irreparably damaged cells. Phospholipid signaling, particularly deregulation of characteristic for normal cells asymmetry of phospholipids, has been discovered as one of important factors in both autophagy and apoptosis. Collapse of cardiolipin asymmetry in mitochondria and covalent association of phosphatidylethanolamine (or phosphatidylserine) with LC3_1 are the two major events in signaling, culminating in apoptotic cell death and mitophagy, respectively. It is possible that changes of cardiolipin asymmetry in mitochondria are at the center of the chain of events leading to cell death. This chain includes several consecutive levels of regulation:

1. Synthesis of cardiolipin (CL) and its molecular speciation with a balance of poly- and mono-unsaturated molecular forms as well as saturated CLs).
2. Scramblase-3 (SCR-3) is inactive; maintenance of asymmetry of CL between the inner and outer mitochondrial membranes; CL and cyt c are spatially separated.

3. Regulation of cyt *c*/CL interactions via cyt *c* phosphorylation hindering binding of negatively charged CL to cyt *c*.
4. Low levels of H₂O₂ production leading to insufficiency of oxidizing equivalents.
5. Cyt *c* can undergo phosphorylation of its Tyr 97 (in the heart) [168] Tyr 48 (in the liver) [169] likely via a cAMP dependent pathway. Phosphorylation of Y97 is associated with changes of the absorbance at 695 nm which suggests subtle structural changes in the heme environment [168]. Peroxidase activity of cyt *c*/CL complex involves formation of Tyr radicals [20]. It is possible that phosphorylation of Y97 and Y48 affects binding of cyt *c* with CL as well as its peroxidase activity.
6. During initiation of autophagy, SCR phosphorylation (resulting in its activation) moves CL to the outer mitochondrial membrane and stimulates (turns on) mitophagy. There is no CL oxidation at this time, because cyt *c* may be phosphorylated and H₂O₂ is still unavailable. As damage develops, cyt *c* can be dephosphorylated and more avidly binds with CL. This disrupts electron transport and stimulates H₂O₂ production. As a result, CL gets oxidized, thus initiating apoptosis and the end of autophagy (mitophagy).

Scheme 6.

Inhibition of CL-activated peroxidase activity of cyt *c* and prevention of CL oxidation in mitochondria leading to suppression of apoptosis.

Peroxidase activity of cyt *c*/CL complexes leads to CL oxidation and accumulation of products required for the release of pro-apoptotic factors from mitochondria. Consequently, agents and factors that inhibit the peroxidase activity and prevent CL oxidation may act as anti-apoptotic agents. A new approach to regulate the cyt *c* peroxidase activity is based on the use of modified CL with an oxidizable and fluorescent 7-nitro-2,1,3-benzoxadiazole (NBD) moiety (NBD-CL). NBD-CL forms high-affinity complexes with cyt *c* and blocks cyt *c*-catalyzed oxidation of several peroxidase substrates, cyt *c* self-oxidation, and, most importantly, inhibits cyt *c*-

dependent oxidation of polyunsaturated CL and accumulation of CL hydroperoxides. Mitochondrial targeting of such agents may lead to discovery of new potent drugs. Several options shown on the schema include mitochondria-targeted conjugates of nitroxide radicals (TEMPO) with hemi-gramicidin S (GS) or triphenyl-phosphonium. Specifically, GS-TEMPO is selectively accumulated in mitochondria where it acts as an electron scavenger capable of preventing superoxide formation and its dismutation into H_2O_2 that is necessary for CL oxidation. GS-TEMPO is also an effective anti-apoptotic agent. Mitochondria-targeted donors of nitric oxide ($NO^\cdot$) – such as 2-(hydroxyamino-vinyl)-triphenyl-phosphonium (HVTP) - activatable by peroxidase activity of cyt c owe their anti-apoptotic potency to the $NO^\cdot$ -dependent reduction of reactive intermediates of the peroxidase cycle.