Free Radical Biology & Medicine 46 (2009) 1439–1453

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Review Article

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

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ARTICLE INFO

Article history:
Received 9 January 2009
Revised 2 March 2009
Accepted 4 March 2009
Available online 12 March 2009

Keywords:
Cytochrome *c*
Cardiolipin
Peroxidase
Lipidomics
Oxidative stress
Apoptosis
Autophagy
Mitochondrial targeting
Free radicals

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 transmembrane distribution and mechanisms of collapse, the 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 antiapoptotic effects are presented.

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Abbreviations: cyt *c*, cytochromec; CL, cardiolipin; CL-OOH, hydroperoxy-CL; MLCL, monolyso-CL; DLCL, dilyso-CL; TOCL, tetraoleoyl-CL; TMCL, tetramyristoyl-CL; TLCL, tetralinoleoyl-CL; NBD–CL, 1,1′,2-trioleoyl-2′-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]–CL; PC, phosphatidylcholine; PA, phosphatidic acid; PIP2, dioleoylglycero-3-phosphoinositol 4,5-bisphosphate; PIP3, dioleoylglycero-3-phosphoinositol 3,4,5-trisphosphate; PS, phosphatidylserine; PG, phosphatidylglycerol; FRET, fluorescence resonance energy transfer; SOD, superoxide dismutase; HRP, horseradish peroxidase; MPO, myeloperoxidase; COX, cyclooxygenase; CcP, cytochrome *c* peroxidase; FA-OOH, fatty acid hydroperoxide; ALCAT1, acyl-CoA:lysocardiolipin acyltransferase 1; PLS-3, phospholipid scramblase-3; IMM, inner mitochondrial membrane; HVTP, (2-hydroxyaminovinyl) triphenylphosphonium.

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0891-5849/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.freeradbiomed.2009.03.004

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Life is pleasant. Death is peaceful. It's the transition that's troublesome—Isaac Asimov

Introduction

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 toward molecules with fully paired electrons (nonradicals). As kids, everyone was amazed by a famous experiment in chemistry class in which 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 the iron. Remarkably, iron is vital to the functions of diverse enzymes for which 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. Whereas all one-electron products of oxygen reduction are called "reactive oxygen species," only one of them—the hydroxyl radical $HO^{\cdot}$ (a three-electron reduction intermediate of oxygen)—is notorious for its remarkably high and calamitously indiscriminative reactivity toward most biomolecules.

It is a common belief that strict control and elimination of superoxide and hydrogen peroxide (H_2O_2) 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)[1]—in the mitochondrial matrix and intermembrane space, in the cytosol, and in extracellular compartments—convert superoxide radicals into H_2O_2. Thus, SODs may act as important regulators and sources of H_2O_2. An important process through which cells utilize H_2O_2 for signaling purposes is the peroxidase catalytic cycle of hemoproteins[1]. Whereas activation of H_2O_2 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 H_2O_2 and the production of hydroxyl radicals. In this review, we focus on cytochrome *c* (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.

Multiple functions of cytochrome *c* in cells

Over the past 2 decades, we witnessed the collapse of an old dogma of biochemistry: one gene → one protein → one function. Discoveries of new functions of cyt *c* are one of the 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 cyt *c* has been linked to its propensity to catalyze the oxidation of superoxide radicals to molecular oxygen. Thus, cyt *c* can act as a superoxide scavenger [2]. In addition to mechanisms associated with cyt *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 cyt *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 cyt *c* are realized in its native structure. Studies from several laboratories documented that the unfolding of cyt *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 cyt *c* peroxidase activity propagates the oxidation of a mitochondria-specific phospholipid, cardiolipin (CL), and the pivotal role of this reaction in the execution of apoptosis (via mitochondrial membrane permeabilization and release of proapoptotic factors from mitochondria) [20,21]. The mechanisms and essential features of cyt *c's* conversion into a peroxidase represent an interesting and maybe still unique example of a functionally significant ligand change in hemoproteins. These properties of cyt *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 cyt *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 cyt *c* with anionic phospholipids lead to peroxidase activation

Binding modes of cyt c to anionic phospholipids

During the past 3 decades, studies of cyt *c* revealed several protein binding sites for anionic lipids; at least 30% of the protein surface

[1] Peroxidases are diverse and widespread enzymes capable of two-electron reduction of peroxides at the expense of various oxidizable substrates [4]. There are nonheme 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 heme peroxidases can subtract electrons from specific and nonspecific substrates and generate corresponding free radicals and protein-centered radicals ($A^{-}\rightarrow A^{\cdot}$). 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 toward lipid substrates and catalyzes peroxidation of various lipids [5,6]. Catalytic properties of cyt *c*/CL complexes and their specificity toward peroxidation of anionic phospholipids—cardiolipin, phosphatidylserine, phosphatidylinositol—are considered in this review.

constitutes so-called A-, C-, and L-candidate binding domains believed to participate in interactions with anionic lipids (see Fig. 1) [24–26]. Both the penetration of phospholipid acyl chains into the protein globule and the protein integration into the phospholipid bilayer of the membrane were suggested as possible binding modes. Interactions of cyt *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 cyt *c*–lipid interactions. Positively charged cyt *c* molecules (isoelectric point is near pH 10, net charge is +8*e* 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 cyt *c* and activation of its peroxidase activity in the absence of electrostatic interactions—with uncharged (zwitterionic) lipids such as phosphatidylcholine (PC)—or with anionic phospholipids in high-ionic strength buffers, as evidenced by electrophoretic measurements of the cyt *c*/lipid complex [17,19,29]. Site A has been designated as the anion-binding center that probably 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 cyt *c* revealed an involvement of Lys72 and Lys73 in cyt *c*–CL binding [31]. The Nantes' group reported the existence of an additional electrostatic binding site on cyt *c*, named the L site, which includes Lys22, Lys25, His26, Lys27, and His33 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 the 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.

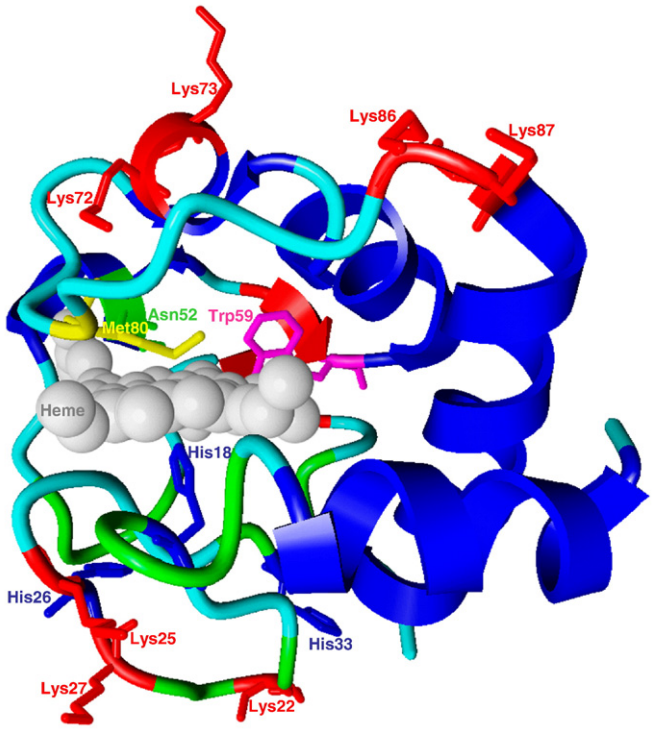


Fig. 1. Possible cardiolipin (CL) binding sites on cytochrome *c*. Structure of native cyt *c*. Several domains that are likely to be involved in interactions with CL and acting as heme–iron ligands include the following amino acid residues: Lys72, Lys73, Lys86, and Lys87 (A site); Asn52 (C site); and 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.

Hydrophobic interactions between nonpolar acyl residues of the lipid molecules and nonpolar regions of cyt *c* (normally buried inside the protein) also affect 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 headgroup with Lys72 and the hydrophobic anchoring of the protein by acyl chain insertion either in the channel surrounded by Asn52, Lys72, and Lys73 or between the nonpolar 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 3 orders of magnitude higher than the rate of H_2O_2-dependent peroxidase activity strongly argues in favor of the importance of hydrophobic interactions (N.A. 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 the saturation of the lipid acyl chains have a dramatic effect on cyt *c* binding and unfolding. Monounsaturated 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) seemed 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-nonenyl bromide (nonenyl acridine orange) increased in the same order, TMCL << TOCL < TLCL < 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 ratios 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 anthroylvinyl-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 ratio. 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 the 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 very dissimilar: five distinct domains of cyt *c* (foldons) with nonequivalent 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–H_2O and His–Fe–) in dioleoyl-phosphatidylglycerol (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 the 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 enhancing the peroxidase activity of CL-bound cyt *c* (G. 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), dioleoylglycero-3-phosphoinositol 4,5-bisphosphate (PIP2), and dioleoylglycero-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 ~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} seemed to be very close to the 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 owing 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 H_2O_2 (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 the cyt *c*/CL complex for small molecules is reminiscent of microperoxidases in which one of the hexa-coordinated ligands, H_2O, 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 the hydrophobic core (detected by NMR spectroscopy); (iii) disruption of the sixth 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 Fig. 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

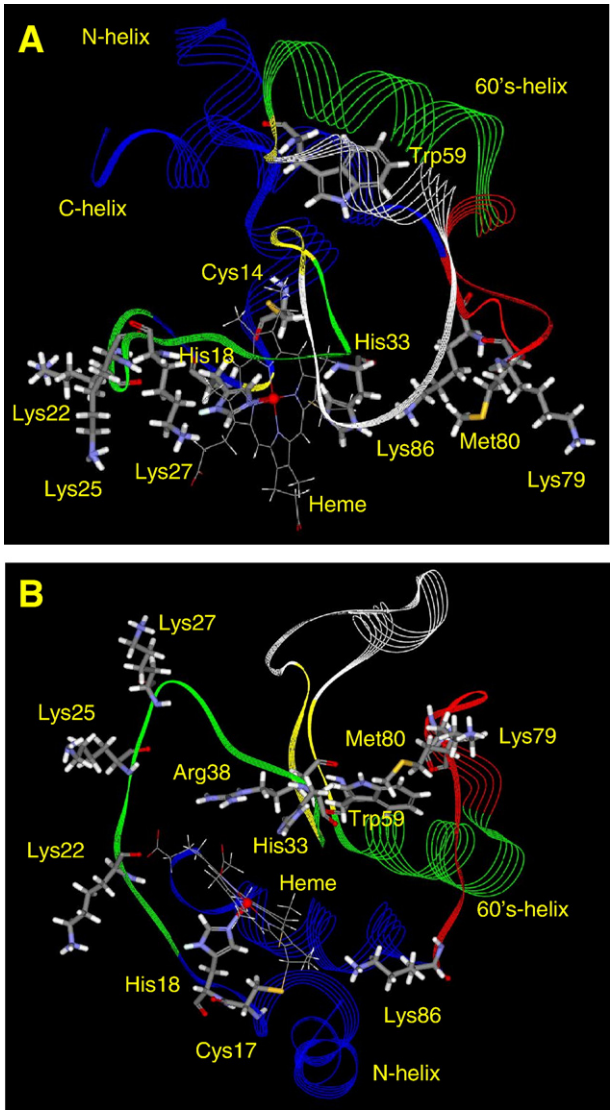


Fig. 2. Proposed structural model of alternative (un)folding of cyt *c* bound to anionic membrane surface. The model explains reported physicochemical properties of cyt *c* including its peroxidase activity. (A) Side view, membrane interface is at the bottom of the protein; (B) view from the membrane side. Protein backbone is encoded by color in accordance 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.

low-energy foldon (residues 72–86 according to [37]) along the membrane surface and out of the heme plane and (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 residue Ile81 or Phe82 anchors 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, whereas 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 H_2O_2. The exceptionally high reaction rates—10^6–10^7 M^{-1} s^{-1}—of peroxidases [53] are dependent on the presence of His as a fifth 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 H_2O_2 cleavage [54–56] and the formation of the first reactive intermediate, compound I (Fig. 3). Analysis of HRP mutants revealed that substitution of distal Arg and His was associated with 10^3- and 10^5-fold decreases in the rate of compound I formation, respectively [55,57]. Mutation of the proximal His slowed this reaction 10^6-fold [58], thus clearly indicating the hierarchy of these amino acids in catalysis.

Although cyt *c* possesses some of the prerequisites 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 2.5 Å away from the Fe, thus precluding access of the heme to H_2O_2 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 analyses 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 the 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 in the Soret band to a shorter-wavelength region, suggesting a high-spin state of heme in the new cyt *c* conformation (N.A. 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 in 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.

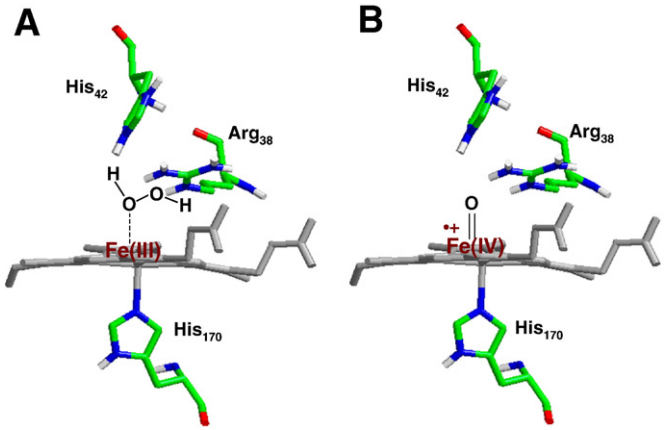


Fig. 3. Catalytic mechanism of horseradish peroxidase. (A) Ferric enzyme with H_2O_2 bound as a ligand in the sixth coordination position of iron. (B) Oxoferryl iron and porphyrin-centered radical (compound I).

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 mW) [67,68]. Loss of these features upon binding of cyt *c* to CL is likely accountable for a substantially more negative redox potential, which falls into the range characteristic of peroxidases (for example, the redox potentials of cyclooxygenase (COX) and HRP are −160 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 various peroxides (e.g., H_2O_2 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^{-1} s^{-1} [59,60]. Partially unfolded cyt *c* in its complexes with CL exerts an almost 100-fold higher catalytic activity in the presence of H_2O_2 than the native protein [20], yet it is still a relatively weak peroxidase [17]. H_2O_2 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 H_2O_2. Our recent studies demonstrated that peroxidase activity of cyt *c*/CL complexes can be enhanced up to 1000-fold when fatty acid hydroperoxides were utilized as a source of oxidizing equivalents instead of H_2O_2 (N.A. Belikova et al., unpublished results). In a way, this is similar to the requirement of arachidonic acid hydroperoxides for the full expression of the peroxidase function of COX-1 and COX-2, which catalyze the reduction of lipid hydroperoxides (i.e., prostaglandin G2 derivatives) 2–3 orders of magnitude more effectively compared to H_2O_2 [69]. Interestingly, both cyclooxygenases lack Arg in the distal pocket, whereas 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]. Similar 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 H_2O_2 leads to the formation of highly and indiscriminately reactive $HO^{\cdot}$. As a consequence, the homolytic mechanism is likely associated with a nonspecific oxidation of reducing substrates as well as the oxidative modification of the enzyme itself. Homolytic splitting of hydroperoxides 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:

$$\mathrm{R{-}Por{-}Fe(III)} + \mathrm{LOOH} \rightarrow \mathrm{R{-}Por{-}Fe(IV)} = \mathrm{O} + \mathrm{LO}^{\cdot} + \mathrm{H}^{+} \qquad (1)$$

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 $LO^{\cdot}$ 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 the 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 H_2O_2 heterolytic splitting. Accordingly, the His42Leu mutant of HRP is characterized by the preference for homolytic cleavage of H_2O_2 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 H_2O_2 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 H_2O_2 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 FA-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 amino acid 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 H_2O_2-dependent production of protein-immobilized radicals on cyt *c*. The effectiveness of dioleoyl 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 nondissociable 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 similar 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 not only facilitate 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 α-synuclein, resulting in an accumulation of poorly digestible cross-linked aggregates characteristic of neurodegenerative diseases. Interestingly, both cyt *c* and α-synuclein are abundant components of Lewy bodies, which accumulate in the brain of patients with Parkinson 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—H_2O_2 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, ZnSOD in the intermembrane space—and spontaneous (nonenzymatic) pathways convert superoxide radicals into H_2O_2 [93,94]. A mitochondrial outer membrane enzyme, monoamine oxidase, catalyzes deamination of biogenic amines and directly generates H_2O_2. 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, which prevents its random nonspecific binding with cyt *c*, probably represents one of the most effective regulators of cyt *c's* peroxidase activity—as detailed below.

Collapse of CL asymmetry in mitochondria during apoptosis

The 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 phosphatidylethanolamine (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, whereas 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 in the CL level in the inner membrane is accompanied by changes in the intermembrane 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 transmembrane redistribution in apoptosis?

The mechanisms of CL mitochondrial translocation are not well understood. It has been shown that CL inter- and intramembrane changes in mitochondria occur at early stages of apoptosis [20], before dissipation of membrane potential and before PS externalization on the cell surface [106]. The activities of two proteins have been associated with apoptotic transmigration 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]. Although tBid is bound to the outer mitochondrial membrane at both contact and noncontact sites [111], it preferentially inserts into the negative lipids of the mitochondrial contact sites between the inner and the outer membranes [112], possibly in proximity to the polyunsaturated CL species [101]. A significant transmembrane 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 were 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 bidirectional movement of phospholipids [113]. PLS-3 contains 295 amino acids and its gene is located on 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 PLS-3 mutant contained fewer mitochondria, which 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 posttranslational modification by phosphorylation of a specific Thr residue (Thr21) by protein kinase Cδ [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 a 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 the 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 transmembrane redistribution 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* are 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 multistage 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 in CL content [127,128]; however, the CL molecular species in CL-deficient cells remain unchanged [128]. Importantly, CL deficiency was associated with increased resistance to apoptosis induced by actinomycin D, X-ray irradiation, and rotenone in HeLa cells, probably 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 reacylation or remodeling of CL acyl chains [130]. Remodeling has been recognized as an important step of CL postsynthetic maturation. It is believed that the de novo synthesis predominantly contributes to the diversity of CL molecular species, whereas 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] and in the endoplasmic reticulum [134]. The reacylation process requires a hydrolysis of CL by phospholipase A_2 [131] to monolyso-CL (MLCL) or dilyso-CL (DLCL) and its coenzyme A-dependent [134] or -independent reacylation [135]. Coenzyme A-dependent reacylation 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 *trans*-acylase (or so-called "tafazzin") can catalyze the acylation of both lysophosphatidylcholine with CL-derived acyl groups and 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 nonoxidizable monounsaturated C18:1 should inevitably result in lower susceptibility to proapoptotic 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 nonenzymatic 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 phosphatidylinositol (dioleoyl-glycero-3-phosphoinositol 4,5-bisphosphate and dioleoyl-glycero-3-phosphoinositol 3,4,5-trisphosphate, 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 γ-irradiation or exposure to staurosporine or actinomycin D) as well as in animal tissues with a significant number of apoptotic cells (e.g., induced by traumatic brain injury or γ-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 γ-irradiated mice (Fig. 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—$(C18:2)_3/(C18:2\text{–}OOH)_1$, $(C18:2)_2/(C18:2\text{–}OOH)_2$, $(C18:2)_1/(C18:2\text{–}OOH)_3$, $(C18:2\text{–}OOH)_4$—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 nonoxidized [83]. This emphasizes 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 in 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 hydroperoxylinoleic acid (C18:2-OOH) along with palmitic C16:0, linoleic C18:2, and stearic C18:0 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 nanomole of CL was detected in lungs at 1 and 7 days after inhalation associated with a robust inflammatory response.

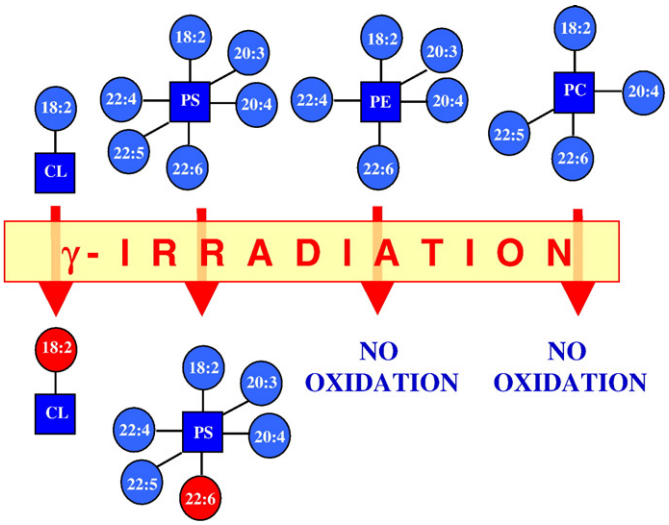


Fig. 4. Oxidative lipidomics "hit map" of small intestine from mice subjected to total body irradiation. A nonrandom, cyt *c*-driven mechanism is involved in the catalysis of γ-irradiation-induced peroxidation of intestinal phospholipids. Selective oxidation of CL followed by oxidation of PS takes place after γ-irradiation and is a part of the intestinal apoptosis in vivo.

The major products of cyt *c*-catalyzed peroxidation include various 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, instead of 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—nonoxidized 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, but the link between autophagy and apoptosis is highly ambiguous [151]. Autophagy is an evolutionarily conserved mechanism used by cells 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 (hence suppressing 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 were 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 to remove protein aggregates and injured organelles and regulate the cell cycle. In a different experimental setting, perturbations of the apoptotic machinery in lipopolysaccharide-treated U937 monocytoid cells and macrophages by the pancaspase 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 after a process of induction/blockade of apoptosis. Whereas 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 signals are involved in triggering the autophagy signaling pathway and "tagging" 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 proapoptotic events when autophageal mechanisms fail to effectively eliminate damaged mitochondria, as depicted in Fig. 5. Thus CL-mediated signaling may be a key point in regulation of both autophagy and apoptosis. As a matter of fact, Kissová 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 antiapoptotic drug discovery

The discovery of the specific oxygenase activity of cyt *c* toward 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 proapoptotic factors into the cytosol, i.e., before the "point of no return" associated with the activation of the caspase cascades [20]. To achieve a substantial effectiveness of the antiapoptotic action, our recent efforts have been focused on designing and developing several mitochondria-targeted inhibitors of CL peroxidation (Fig. 6).

The peroxidase reaction of cyt *c*/CL complexes requires a source of oxidizing equivalents—such as H_2O_2—to feed and maintain the peroxidation cycle. It is likely that apoptotic disruption of electron transport and diversion of electron flow to molecular oxygen act as the major suppliers of superoxide radicals and their dismutation product, H_2O_2. 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 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 owing to their membrane potential [163]. In both cases, we found that targeted delivery of nitroxides achieved its goal—rerouting of the electron flow from oxygen and preventing superoxide production [164]. As a result, CL peroxidation was blocked in a number of cell types triggered to apoptosis by different proapoptotic stimuli–actinomycin D, staurosporine, or γ-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 (J.S. Greenberger et al., unpublished observations). Optimization of this strategy may turn out to bear promise in a number of disease conditions in which 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 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 $NO^{\cdot}$ [167]. Indeed, we were able to demonstrate that the peroxidase function of cyt *c*/CL complexes caused the production of $NO^{\cdot}$ from (2-hydroxyaminovinyl)triphenylphosphonium (HVTP) [167]. As expected, $NO^{\cdot}$ 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 redirect the oxidizing power of cyt *c* as a peroxidase to nonessential CL-like substrates that will be ineffective in inducing the

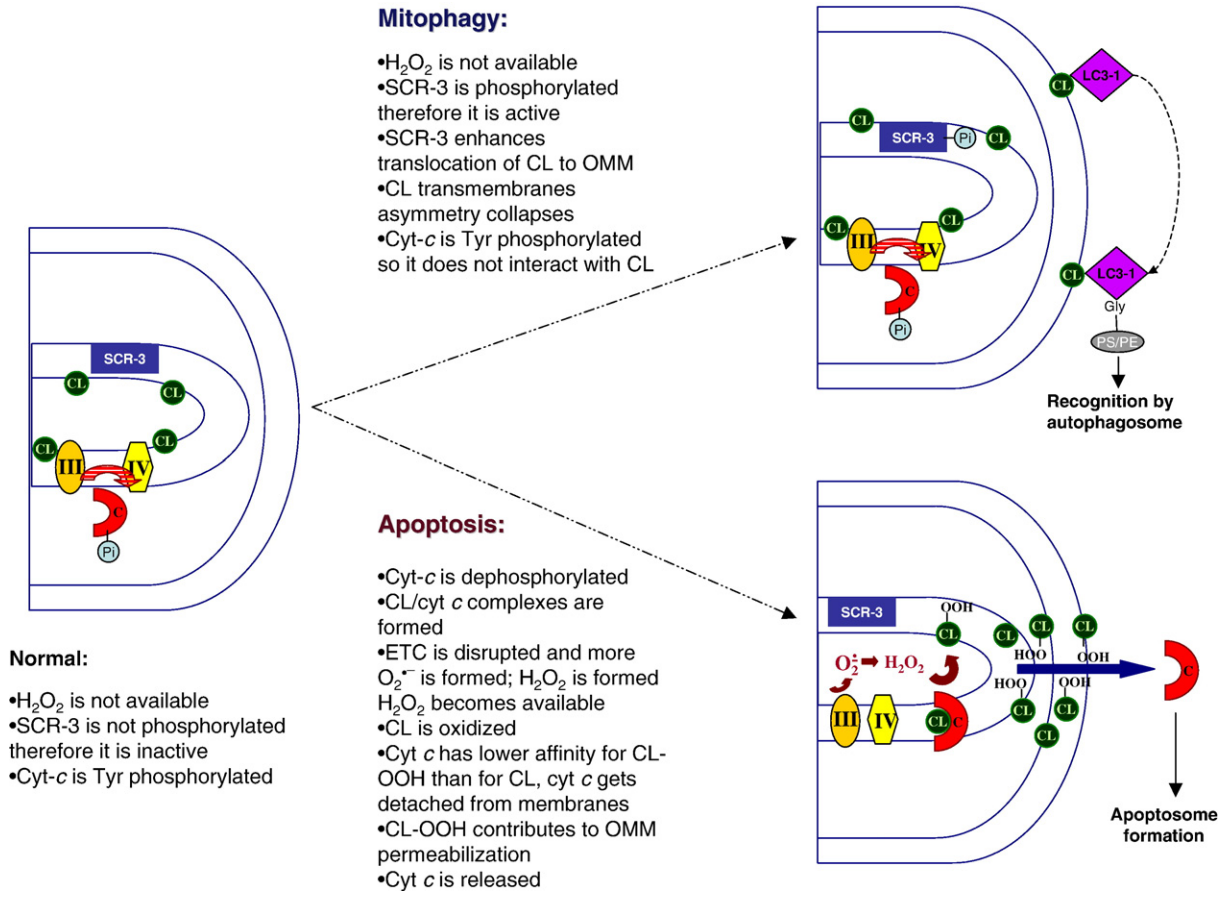


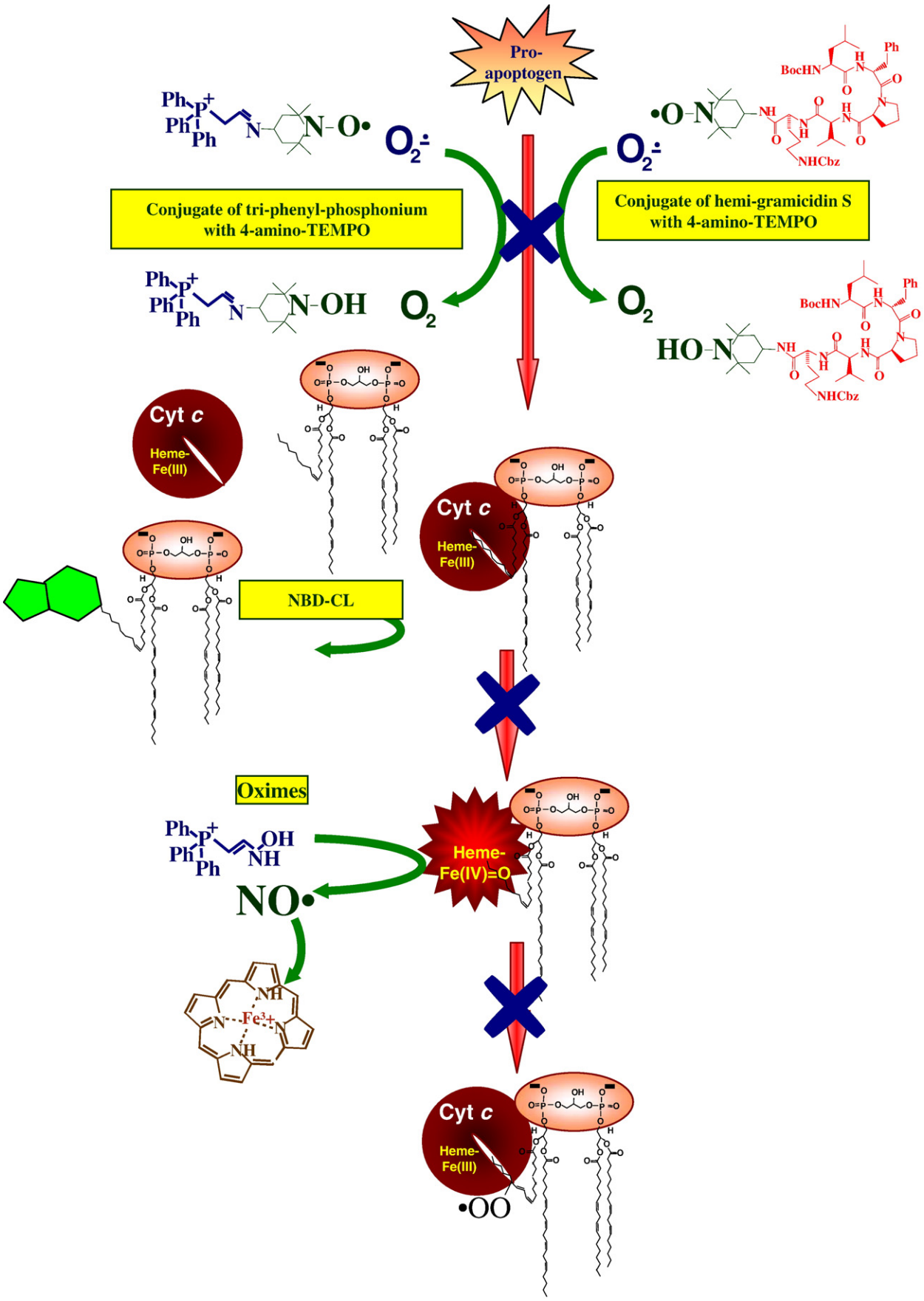
Fig. 5. Crossroads of mitophagy and apoptosis. Autophagy and apoptosis are two processes that may be mutually inhibitory; autophagy usually precedes apoptosis, whereas triggering of apoptosis is associated with blocked autophagy. It is likely that these two pathways are intrinsically interconnected via molecular switches that turn on the autophagy process (with apoptosis still inhibited) followed by activation of apoptosis (with autophagy turned off). Both processes function as parts of an essential combined mechanism of elimination of irreparably damaged cells. Phospholipid signaling, particularly deregulation of asymmetry of phospholipids, characteristic of normal cells, has been discovered as one of the 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 in 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) One level is the synthesis of CL and its molecular speciation with a balance of poly- and monounsaturated molecular forms as well as saturated CLs. (2) Scramblase-3 (SCR-3) is inactive, the asymmetry of CL between the inner and the outer mitochondrial membranes is maintained, CL and cyt *c* are spatially separated. (3) Cyt *c*/CL interactions are regulated via cyt *c* phosphorylation, hindering binding of negatively charged CL to cyt *c*. (4) Low levels of H_2O_2 production lead to insufficiency of oxidizing equivalents. (5) Cyt *c* can undergo phosphorylation of its Tyr97 (in the heart) [168], Tyr48 (in the liver) [169], probably via a cAMP-dependent pathway. Phosphorylation of Y97 is associated with changes in the absorbance at 695 nm, which suggests subtle structural changes in the heme environment [168]. Peroxidase activity of the 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 H_2O_2 is still unavailable. As damage develops, cyt *c* can be dephosphorylated and bind more avidly with CL. This disrupts electron transport and stimulates H_2O_2 production. As a result, CL gets oxidized, thus initiating apoptosis and the end of autophagy (mitophagy).

mitochondrial permeability transition and the release of proapoptotic 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* toward CL peroxidation. Currently, experiments are under way to utilize effective ligands of cyt *c* heme to occupy its sixth coordination bond (Met80–Fe) to strongly and irreversibly knockout 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

Fig. 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 proapoptotic factors from mitochondria. Consequently, agents and factors that inhibit the peroxidase activity and prevent CL oxidation may act as antiapoptotic agents. A new approach to regulating the cyt *c* peroxidase activity is based on the use of modified CL with an oxidizable and fluorescent NBD moiety. NBD–CL forms high-affinity complexes with cyt *c* and blocks cyt *c*-catalyzed oxidation of several peroxidase substrates and 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 the discovery of new potent drugs. Several options shown include mitochondria-targeted conjugates of nitroxide radicals (TEMPO) with hemi-gramicidin S (GS) or triphenylphosphonium. 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 antiapoptotic agent. Mitochondria-targeted donors of $NO^{\cdot}$—such as 2-(hydroxyaminovinyl) triphenylphosphonium—activatable by peroxidase activity of cyt *c* owe their antiapoptotic potency to the $NO^{\cdot}$-dependent reduction of reactive intermediates of the peroxidase cycle.


Pro-apoptogen
Conjugate of tri-phenyl-phosphonium with 4-amino-TEMPO
Conjugate of hemi-gramicidin S with 4-amino-TEMPO
O2÷
O2
Cyt c
Heme-Fe(III)
NBD-CL
Oxines
NO•
Heme-Fe(IV)=O
•OO

CL content was decreased to 40 mol% of its levels in parental cells. This success was associated with a markedly increased resistance to apoptosis induced by γ-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 and hence trigger their apoptotic machinery toward initiation of programmed cell death. One potentially important approach 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 toward a selective delivery of the desired precursors of fatty acids into mitochondria using various 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 Met80 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, Met80, changes in spin state, and structural rearrangements pave the way for opening of the heme catalytic site to small molecules (including H_2O_2, FA-OOH) to bolster its catalytic activity to levels comparable to those of genuine peroxidases. In mitochondria, this peroxidase activity displays remarkable specificity toward 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 proapoptotic stimulation, phosphorylation and activation of scramblase-3 probably triggers the transmembrane 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 proapoptotic agents based on their ability to manipulate CL peroxidation in cyt *c*/CL complexes.

Acknowledgments

This work was supported by NIH Grants NIAID U19AI068021, HL70755, HD057587, NS061817, DAMD 17-01-2-637, and R03TW007320; by the Pennsylvania Department of Health, SAP 4100027294; and by the Human Frontier Science Program.

References

[1] Halliwell, B.; Gutteridge, J. Free Radicals in Biology and Medicine. Clarendon Press, Oxford; 1999.
[2] Skulachev, V. P. Cytochrome c in the apoptotic and antioxidant cascades. *FEBS Lett.* **423:**275–280; 1998.
[3] Liu, X.; Kim, C. N.; Yang, J.; Jemmerson, R.; Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86:** 147–157; 1996.
[4] Passardi, F.; Theiler, G.; Zamocky, M.; Cosio, C.; Rouhier, N.; Teixera, F.; Margis-Pinheiro, M.; Ioannidis, V.; Penel, C.; Falquet, L.; Dunand, C. PeroxiBase: the peroxidase database. *Phytochemistry* **68:**1605–1611; 2007.
[5] Rouzer, C. A.; Marnett, L. J. Mechanism of free radical oxygenation of polyunsaturated fatty acids by cyclooxygenases. *Chem. Rev.* **103:**2239–2304; 2003.
[6] Malle, E.; Marsche, G.; Arnhold, J.; Davies, M. J. Modification of low-density lipoprotein by myeloperoxidase-derived oxidants and reagent hypochlorous acid. *Biochim. Biophys. Acta* **1761:**392–415; 2006.
[7] Mueller, G. P.; Driscoll, W. J. In vitro synthesis of oleoylglycine by cytochrome c points to a novel pathway for the production of lipid signaling molecules. *J. Biol. Chem.* **282:**22364–22369; 2007.
[8] Driscoll, W. J.; Chaturvedi, S.; Mueller, G. P. Oleamide synthesizing activity from rat kidney: identification as cytochrome c. *J. Biol. Chem.* **282:**22353–22363; 2007.
[9] Gebicka, L. Peroxidase-like activity of cytochrome c in the presence of anionic surfactants. *Res. Chem. Intermed.* **27:**717–723; 2001.
[10] Santucci, R.; Brunori, M.; Ascoli, F. Unfolding and flexibility in hemoproteins shown in the case of carboxymethylated cytochrome c. *Biochim. Biophys. Acta* **914:**185–189; 1987.
[11] Chen, Y. R.; Chen, C. L.; Liu, X.; Li, H.; Zweier, J. L.; Mason, R. P. Involvement of protein radical, protein aggregation, and effects on NO metabolism in the hypochlorite-mediated oxidation of mitochondrial cytochrome *c*. *Free Radic. Biol. Med.* **37:**1591–1603; 2004.
[12] Abriata, L. A.; Cassina, A.; Tórtora, V.; Marín, M.; Souza, J. M.; Castro, L.; Vila, A. J.; Radi, R. Nitration of solvent-exposed tyrosine-74 on cytochrome c triggers heme iron-methionine-80 bond disruption: nuclear magnetic resonance and optical spectroscopy studies. *J. Biol. Chem.* **284:**17–26; 2008.
[13] Pinheiro, T. J.; Watts, A. Lipid specificity in the interaction of cytochrome c with anionic phospholipid bilayers revealed by solid-state ^{31}P NMR. *Biochemistry* **33:** 2451–2458; 1994.
[14] de Jongh, H. H.; Ritsema, T.; Killian, J. A. Lipid specificity for membrane mediated partial unfolding of cytochrome c. *FEBS Lett.* **360:**255–260; 1995.
[15] Sanghera, N.; Pinheiro, T. J. Unfolding and refolding of cytochrome c driven by the interaction with lipid micelles. *Protein Sci.* **9:**1194–1202; 2000.
[16] Oellerich, S.; Lecomte, S.; Paternostre, M.; Heimburg, T.; Hildebrandt, P. Peripheral and integral binding of cytochrome *c* to phospholipids vesicles. *J. Phys. Chem. B* **108:**3871–3878; 2004.
[17] Belikova, N. A.; Vladimirov, Y. A.; Osipov, A. N.; Kapralov, A. A.; Tyurin, V. A.; Potapovich, M. V.; Basova, L. V.; Peterson, J.; Kurnikov, I. V.; Kagan, V. E. Peroxidase activity and structural transitions of cytochrome c bound to cardiolipin-containing membranes. *Biochemistry* **45:**4998–5009; 2006.
[18] Kapralov, A. A.; Kurnikov, I. V.; Vlasova, I. I.; Belikova, N. A.; Tyurin, V. A.; Basova, L. V.; Zhao, Q.; Tyurina, Y. Y.; Jiang, J.; Bayir, H.; Vladimirov, Y. A.; Kagan, V. E. The hierarchy of structural transitions induced in cytochrome c by anionic phospholipids determines its peroxidase activation and selective peroxidation during apoptosis in cells. *Biochemistry* **46:**14232–14244; 2007.
[19] Sinibaldi, F.; Fiorucci, L.; Patriarca, A.; Lauceri, R.; Ferri, T.; Coletta, M.; Santucci, R. Insights into cytochrome c–cardiolipin interaction: role played by ionic strength. *Biochemistry* **47:**6928–6935; 2008.
[20] Kagan, V. E.; Tyurin, V. A.; Jiang, J.; Tyurina, Y. Y.; Ritov, V. B.; Amoscato, A. A.; Osipov, A. N.; Belikova, N. A.; Kapralov, A. A.; Kini, V.; Vlasova, I. I.; Zhao, Q.; Zou, M.; Di, P.; Svistunenko, D. A.; Kurnikov, I. V.; Borisenko, G. G. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat. Chem. Biol.* **1:**223–232; 2005.
[21] Belikova, N. A.; Jiang, J.; Tyurina, Y. Y.; Zhao, Q.; Epperly, M. W.; Greenberger, J.; Kagan, V. E. Cardiolipin-specific peroxidase reactions of cytochrome C in mitochondria during irradiation-induced apoptosis. *Int. J. Radiat. Oncol. Biol. Phys.* **69:**176–186; 2007.
[22] Uversky, V. N.; Dunker, A. K. Biochemistry: controlled chaos. *Science* **322:** 1340–1341; 2008.
[23] Gsponer, J.; Futschik, M. E.; Teichmann, S. A.; Babu, M. M. Tight regulation of unstructured proteins: from transcript synthesis to protein degradation. *Science* **322:**1365–1368; 2008.
[24] Rytomaa, M.; Kinnunen, P. K. Evidence for two distinct acidic phospholipid-binding sites in cytochrome c. *J. Biol. Chem.* **269:**1770–1774; 1994.
[25] Rytomaa, M.; Kinnunen, P. K. Reversibility of the binding of cytochrome c to liposomes: implications for lipid–protein interactions. *J. Biol. Chem.* **270:** 3197–3202; 1995.
[26] Kawai, C.; Prado, F. M.; Nunes, G. L.; Di Mascio, P.; Carmona-Ribeiro, A. M.; Nantes, I. L. pH-dependent interaction of cytochrome c with mitochondrial mimetic membranes: the role of an array of positively charged amino acids. *J. Biol. Chem.* **280:**34709–34717; 2005.
[27] Pinheiro, T. J.; Elove, G. A.; Watts, A.; Roder, H. Structural and kinetic description of cytochrome c unfolding induced by the interaction with lipid vesicles. *Biochemistry* **36:**13122–13132; 1997.
[28] Tuominen, E. K.; Wallace, C. J.; Kinnunen, P. K. Phospholipid–cytochrome c interaction: evidence for the extended lipid anchorage. *J. Biol. Chem.* **277:** 8822–8826; 2002.
[29] Nicholls, P. Cytochrome c binding to enzymes and membranes. *Biochim. Biophys. Acta* **346:**261–310; 1974.
[30] Kostrzewa, A.; Páli, T.; Froncisz, W.; Marsh, D. Membrane location of spin-labeled cytochrome c determined by paramagnetic relaxation agents. *Biochemistry* **39:** 6066–6074; 2000.

[31] Kalanxhi, E.; Wallace, C. J. Cytochrome c impaled: investigation of the extended lipid anchorage of a soluble protein to mitochondrial membrane models. *Biochem. J.* **407:**179–187; 2007.

[32] Gorbenko, G. P.; Molotkovsky, J. G.; Kinnunen, P. K. Cytochrome C interaction with cardiolipin/phosphatidylcholine model membranes: effect of cardiolipin protonation. *Biophys. J.* **90:**4093–4103; 2006.

[33] Chattopadhyay, K.; Mazumdar, S. Stabilization of partially folded states of cytochrome c in aqueous surfactant: effects of ionic and hydrophobic interactions. *Biochemistry* **42:**14606–14613; 2003.

[34] Borisenko, G.; Kapralov, A.; Tyurin, V.; Maeda, A.; Stoyanovsky, D.; Kagan, V. Molecular design of new inhibitors of peroxidase activity of cyt c/cardiolipin complexes: fluorescent oxadiazol-derivatized cardiolipin. *Biochemistry* **47:** 13699–13710; 2008.

[35] Muga, A.; Mantsch, H. H.; Surewicz, W. K. Membrane binding induces destabilization of cytochrome c structure. *Biochemistry* **30:**7219–7224; 1991.

[36] Spooner, P. J.; Watts, A. Cytochrome c interactions with cardiolipin in bilayers: a multinuclear magic-angle spinning NMR study. *Biochemistry* **31:**10129–10138; 1992.

[37] Maity, H.; Maity, M.; Englander, S. W. How cytochrome c folds, and why: submolecular foldon units and their stepwise sequential stabilization. *J. Mol. Biol.* **343:**223–233; 2004.

[38] Berners-Price, S. J.; Bertini, I.; Gray, H. B.; Spyroulias, G. A.; Turano, P. The stability of the cytochrome c scaffold as revealed by NMR spectroscopy. *J. Inorg. Biochem.* **98:**814–823; 2004.

[39] Naeem, A.; Khan, R. H. Characterization of molten globule state of cytochrome c at alkaline, native and acidic pH induced by butanol and SDS. *Int. J. Biochem. Cell Biol.* **36:**2281–2292; 2004.

[40] Varhac, R.; Antalik, M.; Bano, M. Effect of temperature and guanidine hydrochloride on ferrocytochrome c at neutral pH. *J. Biol. Inorg. Chem.* **9:** 12–22; 2004.

[41] Wen, X.; Patel, K. M.; Russell, B. S.; Bren, K. L. Effects of heme pocket structure and mobility on cytochrome c stability. *Biochemistry* **46:**2537–2544; 2007.

[42] Tsou, C. L. Cytochrome c modified by digestion with proteolytic enzymes. 1. Digestion. *Biochem. J.* **49:**362–367; 1951.

[43] Marques, H. M.; Perry, C. B. Hemepeptide models for hemoproteins: the behavior of *N*-acetylmicroperoxidase-11 in aqueous solution. *J. Inorg. Biochem.* **75:** 281–291; 1999.

[44] Rytomaa, M.; Mustonen, P.; Kinnunen, P. K. J. Reversible, nonionic, and pH-dependent association of cytochrome c with cardiolipin–phosphatidylcholine liposomes. *J. Biol. Chem.* **267:**22243–22248; 1992.

[45] Maity, H.; Maity, M.; Krishna, M. M.; Mayne, L.; Englander, S. W. Protein folding: the stepwise assembly of foldon units. *Proc. Natl. Acad. Sci. USA* **102:**4741–4746; 2005.

[46] Maity, H.; Rumbley, J. N.; Englander, S. W. Functional role of a protein foldon—an omega-loop foldon controls the alkaline transition in ferricytochrome c. *Proteins* **63:**349–355; 2006.

[47] Krishna, M. M.; Maity, H.; Rumbley, J. N.; Lin, Y.; Englander, S. W. Order of steps in the cytochrome C folding pathway: evidence for a sequential stabilization mechanism. *J. Mol. Biol.* **359:**1410–1419; 2006.

[48] Diederix, R. E.; Ubbink, M.; Canters, G. W. Peroxidase activity as a tool for studying the folding of c-type cytochromes. *Biochemistry* **41:**13067–13077; 2002.

[49] Prasad, S.; Maiti, N. C.; Mazumdar, S.; Mitra, S. Reaction of hydrogen peroxide and peroxidase activity in carboxymethylated cytochrome c: spectroscopic and kinetic studies. *Biochim. Biophys. Acta* **1596:**63–75; 2002.

[50] Cassina, A. M.; Hodara, R.; Souza, J. M.; Thomson, L.; Castro, L.; Ischiropoulos, H.; Freeman, B. A.; Radi, R. Cytochrome c nitration by peroxynitrite. *J. Biol. Chem.* **275:**21409–21415; 2000.

[51] Chen, Y. R.; Deterding, L. J.; Sturgeon, B. E.; Tomer, K. B.; Mason, R. P. Protein oxidation of cytochrome C by reactive halogen species enhances its peroxidase activity. *J. Biol. Chem.* **277:**29781–29791; 2002.

[52] Marques, H. M. Insights into porphyrin chemistry provided by the microperoxidases, the haempeptides derived from cytochrome c. *Dalton Trans.* (39)**:** 4371–4385; 2007.

[53] Everse, J.; Everse, K. E.; Grisham, M. B. Peroxidases in Chemistry and Biology, Vol. II. CRC Press, Boca Raton, FL; 1991.

[54] Poulos, T. L.; Kraut, J. A hypothetical model of the cytochrome c peroxidase: cytochrome c electron transfer complex. *J. Biol. Chem.* **255:**10322–10330; 1980.

[55] Rodriguez-Lopez, J. N.; Smith, A. T.; Thorneley, R. N. Role of arginine 38 in horseradish peroxidase: a critical residue for substrate binding and catalysis. *J. Biol. Chem.* **271:**4023–4030; 1996.

[56] Hiner, A. N.; Raven, E. L.; Thorneley, R. N.; Garcia-Canovas, F.; Rodriguez-Lopez, J. N. Mechanisms of compound I formation in heme peroxidases. *J. Inorg. Biochem.* **91:**27–34; 2002.

[57] Rodriguez-Lopez, J. N.; Lowe, D. J.; Hernandez-Ruiz, J.; Hiner, A. N.; Garcia-Canovas, F.; Thorneley, R. N. Mechanism of reaction of hydrogen peroxide with horseradish peroxidase: identification of intermediates in the catalytic cycle. *J. Am. Chem. Soc.* **123:**11838–11847; 2001.

[58] Newmyer, S. L.; Sun, J.; Loehr, T. M.; Ortiz de Montellano, P. R. Rescue of the horseradish peroxidase His-170→Ala mutant activity by imidazole: importance of proximal ligand tethering. *Biochemistry* **35:**12788–12795; 1996.

[59] Florence, T. M. The degradation of cytochrome c by hydrogen peroxide. *J. Inorg. Biochem.* **23:**131–141; 1985.

[60] Radi, R.; Thomson, L.; Rubbo, H.; Prodanov, E. Cytochrome c-catalyzed oxidation of organic molecules by hydrogen peroxide. *Arch. Biochem. Biophys.* **288:**112–117; 1991.

[61] Bernad, S.; Oellerich, S.; Soulimane, T.; Noinville, S.; Baron, M. H.; Paternostre, M.; Lecomte, S. Interaction of horse heart and Thermus thermophilus type c cytochromes with phospholipid vesicles and hydrophobic surfaces. *Biophys. J.* **86:**3863–3872; 2004.

[62] Droghetti, E.; Oellerich, S.; Hildebrandt, P.; Smulevich, G. Heme coordination states of unfolded ferrous cytochrome C. *Biophys. J.* **91:**3022–3031; 2006.

[63] Dunford, H. B.; Stillman, J. S. On the function and mechanism of action of peroxidases. *Coord. Chem. Rev.* **19:**187–251; 1976.

[64] Basova, L. V.; Kurnikov, I. V.; Wang, L.; Ritov, V. B.; Belikova, N. A.; Vlasova, I. I.; Pacheco, A. A.; Winnica, D. E.; Peterson, J.; Bayir, H.; Waldeck, D. H.; Kagan, V. E. Cardiolipin switch in mitochondria: shutting off the reduction of cytochrome c and turning on the peroxidase activity. *Biochemistry* **46:**3423–3434; 2007.

[65] Gunsalus, I. C.; Meeks, J. R.; Lipscomb, J. D.; Debrunner, P.; Munk, E. Molecular Mechanism of Oxygen Activation. Academic Press, New York; 1974.

[66] Adachi, S.; Nagano, S.; Ishimori, K.; Watanabe, Y.; Morishima, I. Roles of proximal ligand in heme proteins: replacement of proximal histidine of human myoglobin with cysteine and tyrosine by site-directed mutagenesis as models for P-450, chloroperoxidase, and catalase. *Biochemistry* **32:**241–252; 1993.

[67] Di Marino, M.; Marassi, R.; Santucci, R.; Brunori, M.; Ascoli, F. A Spectro-electrochemical study of carboxymethylated cytochrome-c. *J. Electroanal. Chem.* **17:**27–34; 1987.

[68] Barker, P. D.; Nerou, E. P.; Cheesman, M. R.; Thomson, A. J.; de Oliveira, P.; Hill, H. A. Bis-methionine ligation to heme iron in mutants of cytochrome b562. 1. Spectroscopic and electrochemical characterization of the electronic properties. *Biochemistry* **35:**13618–13626; 1996.

[69] Bakovic, M.; Dunford, H. B. Reactions of prostaglandin endoperoxide synthase and its compound I with hydroperoxides. *J. Biol. Chem.* **271:**2048–2056; 1996.

[70] Chubb, A. J.; Fitzgerald, D. J.; Nolan, K. B.; Moman, E. The productive conformation of prostaglandin G2 at the peroxidase site of prostaglandin endoperoxide H synthase: docking, molecular dynamics, and site-directed mutagenesis studies. *Biochemistry* **45:**811–820; 2006.

[71] Liu, J.; Seibold, S. A.; Rieke, C. J.; Song, I.; Cukier, R. I.; Smith, W. L. Prostaglandin endoperoxide H synthases: peroxidase hydroperoxide specificity and cyclo-oxygenase activation. *J. Biol. Chem.* **282:**18233–18244; 2007.

[72] Ichimura, S.; Uchida, T.; Taniguchi, S.; Hira, S.; Tosha, T.; Morishima, I.; Kitagawa, T.; Ishimori, K. Unique peroxidase reaction mechanism in prostaglandin endoperoxide H synthase-2: compound I in prostaglandin endoperoxide H synthase-2 can be formed without assistance by distal glutamine residue. *J. Biol. Chem.* **282:**16681–16690; 2007.

[73] Shimizu, T.; Murakami, Y.; Hatano, M. Glu318 and Thr319 mutations of cytochrome P450 1A2 remarkably enhance homolytic O–O cleavage of alkyl hydroperoxides: an optical absorption spectral study. *J. Biol. Chem.* **269:** 13296–13304; 1994.

[74] Van der Zee, J.; Barr, D. P.; Mason, R. P. ESR spin trapping investigation of radical formation from the reaction between hematin and *tert*-butyl hydroperoxide. *Free Radic. Biol. Med.* **20:**199–206; 1996.

[75] Allentoff, A.; Bolton, J.; Wilks, A.; Thompson, J.; Ortiz de Montellano, P. Heterolytic versus homolytic peroxide bond cleavage by sperm whale myoglobin and myoglobin mutants. *J. Am. Chem. Soc.* **114:**9744–9749; 1992.

[76] Egawa, T.; Yoshioka, S.; Takahashi, S.; Hori, H.; Nagano, S.; Shimada, H.; Ishimori, K.; Morishima, I.; Suematsu, M.; Ishimura, Y. Kinetic and spectroscopic characterization of a hydroperoxy compound in the reaction of native myoglobin with hydrogen peroxide. *J. Biol. Chem.* **278:**41597–41606; 2003.

[77] Chamulitrat, W.; Hughes, M. F.; Eling, T. E.; Mason, R. P. Superoxide and peroxyl radical generation from the reduction of polyunsaturated fatty acid hydroperoxides by soybean lipoxygenase. *Arch. Biochem. Biophys.* **290:**153–159; 1991.

[78] Rota, C.; Barr, D. P.; Martin, M. V.; Guengerich, F. P.; Tomasi, A.; Mason, R. P. Detection of free radicals produced from the reaction of cytochrome P-450 with linoleic acid hydroperoxide. *Biochem. J.* **328** (Pt 2)**:**565–571; 1997.

[79] Iwahashi, H.; Nishizaki, K.; Takagi, I. Cytochrome c catalyses the formation of pentyl radical and octanoic acid radical from linoleic acid hydroperoxide. *Biochem. J.* **361:**57–66; 2002.

[80] Barr, D. P.; Gunther, M. R.; Deterding, L. J.; Tomer, K. B.; Mason, R. P. ESR spin-trapping of a protein-derived tyrosyl radical from the reaction of cytochrome c with hydrogen peroxide. *J. Biol. Chem.* **271:**15498–15503; 1996.

[81] Kagan, V. E.; Borisenko, G. G.; Tyurina, Y. Y.; Tyurin, V. A.; Jiang, J.; Potapovich, A. I.; Kini, V.; Amoscato, A. Oxidative lipidomics of apoptosis: redox catalytic interactions of cytochrome *c* with cardiolipin and phosphatidylserine. *Free Radic. Biol. Med.* **37:**1963–1985; 2004.

[82] Tyurin, V. A.; Tyurina, Y. Y.; Kochanek, P. M.; Hamilton, R.; DeKosky, S. T.; Greenberger, J. S.; Bayir, H.; Kagan, V. E. Oxidative lipidomics of programmed cell death. *Methods Enzymol.* **442:**375–393; 2008.

[83] Tyurina, Y. Y.; Tyurin, V. A.; Epperly, M. W.; Greenberger, J. S.; Kagan, V. E. Oxidative lipidomics of γ-irradiation-induced intestinal injury. *Free Radic. Biol. Med.* **44:**299–314; 2008.

[84] Landino, L. M.; Crews, B. C.; Gierse, J. K.; Hauser, S. D.; Marnett, L. J. Mutational analysis of the role of the distal histidine and glutamine residues of prostaglandin–endoperoxide synthase-2 in peroxidase catalysis, hydroperoxide reduction, and cyclooxygenase activation. *J. Biol. Chem.* **272:**21565–21574; 1997.

[85] Qian, S. Y.; Chen, Y. R.; Deterding, L. J.; Fann, Y. C.; Chignell, C. F.; Tomer, K. B.; Mason, R. P. Identification of protein-derived tyrosyl radical in the reaction of cytochrome c and hydrogen peroxide: characterization by ESR spin-trapping, HPLC and MS. *Biochem. J.* **363:**281–288; 2002.

[86] Detweiler, C. D.; Lardinois, O. M.; Deterding, L. J.; de Montellano, P. R.; Tomer, K. B.; Mason, R. P. Identification of the myoglobin tyrosyl radical by immuno-spin trapping and its dimerization. *Free Radic. Biol. Med.* **38:**969–976; 2005.

[87] Svistunenko, D. A. Reaction of haem containing proteins and enzymes with hydroperoxides: the radical view. *Biochim. Biophys. Acta* **1707:**127–155; 2005.

[88] Ehrenshaft, M.; Mason, R. P. Protein radical formation on thyroid peroxidase during turnover as detected by immuno-spin trapping. *Free Radic. Biol. Med.* **41:** 422–430; 2006.

[89] Smith, W. L.; Song, I. The enzymology of prostaglandin endoperoxide H synthases-1 and -2. *Prostaglandins Other Lipid. Mediat.* **68-69:**115–128; 2002.

[90] Tsong, T. Y. Ferricytochrome c chain folding measured by the energy transfer of tryptophan 59 to the heme group. *Biochemistry* **15:**5467–5473; 1976.

[91] Keszler, A.; Mason, R. P.; Hogg, N. Immuno-spin trapping of hemoglobin and myoglobin radicals derived from nitrite-mediated oxidation. *Free Radic. Biol. Med.* **40:**507–515; 2006.

[92] Tyurina, Y. Y.; Kini, V.; Tyurin, V. A.; Vlasova, I. I.; Jiang, J.; Kapralov, A. A.; Belikova, N. A.; Yalowich, J. C.; Kurnikov, I. V.; Kagan, V. E. Mechanisms of cardiolipin oxidation by cytochrome c: relevance to pro- and antiapoptotic functions of etoposide. *Mol. Pharmacol.* **70:**706–717; 2006.

[93] Barja, G. Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity. *J. Bioenerg. Biomembr.* **31:**347–366; 1999.

[94] Muller, F. L.; Liu, Y.; Van Remmen, H. Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J. Biol. Chem.* **279:**49064–49073; 2004.

[95] Seiler, A.; Schneider, M.; Förster, H.; Roth, S.; Wirth, E. K.; Culmsee, C.; Plesnila, N.; Kremmer, E.; Rådmark, O.; Wurst, W.; Bornkamm, G. W.; Schweizer, U.; Conrad, M. Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell Metab.* **8:** 237–248; 2008.

[96] Toninello, A.; Pietrangeli, P.; De Marchi, U.; Salvi, M.; Mondovì, B. Amine oxidases in apoptosis and cancer. *Biochim. Biophys. Acta* **1765:**1–13; 2006.

[97] Nilsson, O. S.; Dallner, G. Transverse asymmetry of phospholipids in subcellular membranes of rat liver. *Biochim. Biophys. Acta* **464:**453–458; 1977.

[98] Sperka-Gottlieb, C. D.; Hermetter, A.; Paltauf, F.; Daum, G. Lipid topology and physical properties of the outer mitochondrial membrane of the yeast, Saccharomyces cerevisiae. *Biochim. Biophys. Acta* **946:**227–234; 1988.

[99] Gallet, P. F.; Petit, J. M.; Maftah, A.; Zachowski, A.; Julien, R. Asymmetrical distribution of cardiolipin in yeast inner mitochondrial membrane triggered by carbon catabolite repression. *Biochem. J.* **324** (Pt 2)**:**627–634; 1997.

[100] Daum, G. Lipids of mitochondria. *Biochim. Biophys. Acta* **822:**1–42; 1985.

[101] Ardail, D.; Privat, J. P.; Egret-Charlier, M.; Levrat, C.; Lerme, F.; Louisot, P. Mitochondrial contact sites: lipid composition and dynamics. *J. Biol. Chem.* **265:** 18797–18802; 1990.

[102] Hovius, R.; Thijssen, J.; van der Linden, P.; Nicolay, K.; de Kruijff, B. Phospholipid asymmetry of the outer membrane of rat liver mitochondria: evidence for the presence of cardiolipin on the outside of the outer membrane. *FEBS Lett.* **330:** 71–76; 1993.

[103] Tyurin, V. A.; Tyurina, Y. Y.; Osipov, A. N.; Belikova, N. A.; Basova, L. V.; Kapralov, A. A.; Bayir, H.; Kagan, V. E. Interactions of cardiolipin and lyso-cardiolipins with cytochrome c and tBid: conflict or assistance in apoptosis. *Cell Death Differ.* **14:** 872–875; 2007.

[104] Pfeiffer, K.; Gohil, V.; Stuart, R. A.; Hunte, C.; Brandt, U.; Greenberg, M.; Schagger, H. Cardiolipin stabilizes respiratory chain supercomplexes. *J. Biol. Chem.* **278:** 52873–52880; 2003.

[105] Sharpley, M. S.; Shannon, R. J.; Draghi, F.; Hirst, J. Interactions between phospholipids and NADH:ubiquinone oxidoreductase (complex I) from bovine mitochondria. *Biochemistry* **45:**241–248; 2006.

[106] Garcia Fernandez, M.; Troiano, L.; Moretti, L.; Nasi, M.; Pinti, M.; Salvioli, S.; Dobrucki, J.; Cossarizza, A. Early changes in intramitochondrial cardiolipin distribution during apoptosis. *Cell Growth Differ.* **13:**449–455; 2002.

[107] Liu, J.; Dai, Q.; Chen, J.; Durrant, D.; Freeman, A.; Liu, T.; Grossman, D.; Lee, R. M. Phospholipid scramblase 3 controls mitochondrial structure, function, and apoptotic response. *Mol. Cancer Res.* **1:**892–902; 2003.

[108] Gonzalvez, F.; Pariselli, F.; Dupaigne, P.; Budihardjo, I.; Lutter, M.; Antonsson, B.; Diolez, P.; Manon, S.; Martinou, J. C.; Goubern, M.; Wang, X.; Bernard, S.; Petit, P. X. tBid interaction with cardiolipin primarily orchestrates mitochondrial dysfunctions and subsequently activates Bax and Bak. *Cell Death Differ.* **12:** 614–626; 2005.

[109] Lutter, M.; Fang, M.; Luo, X.; Nishijima, M.; Xie, X.; Wang, X. Cardiolipin provides specificity for targeting of tBid to mitochondria. *Nat. Cell Biol.* **2:**754–761; 2000.

[110] Esposti, M. D.; Cristea, I. M.; Gaskell, S. J.; Nakao, Y.; Dive, C. Proapoptotic Bid binds to monolysocardiolipin, a new molecular connection between mitochondrial membranes and cell death. *Cell Death Differ.* **10:**1300–1309; 2003.

[111] Kim, T. H.; Zhao, Y.; Ding, W. X.; Shin, J. N.; He, X.; Seo, Y. W.; Chen, J.; Rabinowich, H.; Amoscato, A. A.; Yin, X. M. Bid–cardiolipin interaction at mitochondrial contact site contributes to mitochondrial cristae reorganization and cytochrome C release. *Mol. Biol. Cell* **15:**3061–3072; 2004.

[112] Van Mau, N.; Kajava, A. V.; Bonfils, C.; Martinou, J. C.; Harricane, M. C. Interactions of Bax and tBid with lipid monolayers. *J. Membr. Biol.* **207:**1–9; 2005.

[113] Van, Q.; Liu, J.; Lu, B.; Feingold, K. R.; Shi, Y.; Lee, R. M.; Hatch, G. M. Phospholipid scramblase-3 regulates cardiolipin de novo biosynthesis and its resynthesis in growing HeLa cells. *Biochem. J.* **401:**103–109; 2007.

[114] Wiedmer, T.; Zhou, Q.; Kwoh, D. Y.; Sims, P. J. Identification of three new members of the phospholipid scramblase gene family. *Biochim. Biophys. Acta* **1467:**244–253; 2000.

[115] Liu, J.; Chen, J.; Dai, Q.; Lee, R. M. Phospholipid scramblase 3 is the mitochondrial target of protein kinase C delta-induced apoptosis. *Cancer Res.* **63:**1153–1156; 2003.

[116] He, Y.; Liu, J.; Grossman, D.; Durrant, D.; Sweatman, T.; Lothstein, L.; Epand, R. F.; Epand, R. M.; Lee, R. M. Phosphorylation of mitochondrial phospholipid scramblase 3 by protein kinase C-delta induces its activation and facilitates mitochondrial targeting of tBid. *J. Cell. Biochem.* **101:**1210–12121; 2007.

[117] Schlame, M.; Ren, M.; Xu, Y.; Greenberg, M. L.; Haller, I. Molecular symmetry in mitochondrial cardiolipins. *Chem. Phys. Lipids* **138:**38–49; 2005.

[118] Han, X.; Yang, K.; Yang, J.; Cheng, H.; Gross, R. W. Shotgun lipidomics of cardiolipin molecular species in lipid extracts of biological samples. *J. Lipid Res.* **47:**864–879; 2006.

[119] Wang, H. Y.; Jackson, S. N.; Woods, A. S. Direct MALDI-MS analysis of cardiolipin from rat organs sections. *J. Am. Soc. Mass Spectrom.* **18:**567–577; 2007.

[120] Bayir, H.; Tyurin, V. A.; Tyurina, Y. Y.; Viner, R.; Ritov, V.; Amoscato, A. A.; Zhao, Q.; Zhang, X. J.; Janesko-Feldman, K. L.; Alexander, H.; Basova, L. V.; Clark, R. S.; Kochanek, P. M.; Kagan, V. E. Selective early cardiolipin peroxidation after traumatic brain injury: an oxidative lipidomics analysis. *Ann. Neurol.* **62:**154–169; 2007.

[121] Cheng, H.; Mancuso, D. J.; Jiang, X.; Guan, S.; Yang, J.; Yang, K.; Sun, G.; Gross, R. W.; Han, X. Shotgun lipidomics reveals the temporally dependent, highly diversified cardiolipin profile in the mammalian brain: temporally coordinated postnatal diversification of cardiolipin molecular species with neuronal remodeling. *Biochemistry* **47:**5869–5880; 2008.

[122] Kiebish, M. A.; Han, X.; Cheng, H.; Lunceford, A.; Clarke, C. F.; Moon, H.; Chuang, J. H.; Seyfried, T. N. Lipidomic analysis and electron transport chain activities in C57BL/6J mouse brain mitochondria. *J. Neurochem.* **106:**299–312; 2008.

[123] Schlame, M.; Rua, D.; Greenberg, M. L. The biosynthesis and functional role of cardiolipin. *Prog. Lipid Res.* **39:**257–288; 2000.

[124] Schlame, M.; Haldar, D. Cardiolipin is synthesized on the matrix side of the inner membrane in rat liver mitochondria. *J. Biol. Chem.* **268:**74–79; 1993.

[125] Zhao, M.; Schlame, M.; Rua, D.; Greenberg, M. L. Cardiolipin synthase is associated with a large complex in yeast mitochondria. *J. Biol. Chem.* **273:** 2402–2408; 1998.

[126] Chen, D.; Zhang, X. Y.; Shi, Y. Identification and functional characterization of hCLS1, a human cardiolipin synthase localized in mitochondria. *Biochem. J.* **398:** 169–176; 2006.

[127] Choi, S. Y.; Gonzalvez, F.; Jenkins, G. M.; Slomianny, C.; Chretien, D.; Arnoult, D.; Petit, P. X.; Frohman, M. A. Cardiolipin deficiency releases cytochrome c from the inner mitochondrial membrane and accelerates stimuli-elicited apoptosis. *Cell Death Differ.* **14:**597–606; 2007.

[128] Huang, Z.; Jiang, J.; Tyurin, V. A.; Zhao, Q.; Mnuskin, A.; Ren, J.; Belikova, N. A.; Feng, W.; Kurnikov, I. V.; Kagan, V. E. Cardiolipin deficiency leads to decreased cardiolipin peroxidation and increased resistance of cells to apoptosis. *Free Radic. Biol. Med.* **44:**1935–1944; 2008.

[129] Gonzalvez, F.; Schug, Z. T.; Houtkooper, R. H.; MacKenzie, E. D.; Brooks, D. G.; Wanders, R. J.; Petit, P. X.; Vaz, F. M.; Gottlieb, E. Cardiolipin provides an essential activating platform for caspase-8 on mitochondria. *J. Cell Biol.* **183:**681–696; 2008.

[130] Ma, B. J.; Taylor, W. A.; Dolinsky, V. W.; Hatch, G. M. Acylation of monolysocardiolipin in rat heart. *J. Lipid Res.* **40:**1837–1845; 1999.

[131] Danos, M.; Taylor, W. A.; Hatch, G. M. Mitochondrial monolysocardiolipin acyltransferase is elevated in the surviving population of H9c2 cardiac myoblast cells exposed to 2-deoxyglucose-induced apoptosis. *Biochem. Cell. Biol.* **86:**11–20; 2008.

[132] Taylor, W. A.; Xu, F. Y.; Ma, B. J.; Mutter, T. C.; Dolinsky, V. W.; Hatch, G. M. Expression of monolysocardiolipin acyltransferase activity is regulated in concert with the level of cardiolipin and cardiolipin biosynthesis in the mammalian heart. *BMC Biochem.* **3:**9; 2002.

[133] Taylor, W. A.; Hatch, G. M. Purification and characterization of monolysocardiolipin acyltransferase from pig liver mitochondria. *J. Biol. Chem.* **278:**12716–12721; 2003.

[134] Cao, J.; Liu, Y.; Lockwood, J.; Burn, P.; Shi, Y. A novel cardiolipin-remodeling pathway revealed by a gene encoding an endoplasmic reticulum-associated acyl-CoA:lysocardiolipin acyltransferase (ALCAT1) in mouse. *J. Biol. Chem.* **279:** 31727–31734; 2004.

[135] Xu, Y.; Malhotra, A.; Ren, M.; Schlame, M. The enzymatic function of tafazzin. *J. Biol. Chem.* **281:**39217–39224; 2006.

[136] Schlame, M.; Ren, M. Barth syndrome, a human disorder of cardiolipin metabolism. *FEBS Lett.* **580:**5450–5455; 2006.

[137] Nigam, S.; Schewe, T. Phospholipase A(2)s and lipid peroxidation. *Biochim. Biophys. Acta* **1488:**167–181; 2000.

[138] McGinley, C. M.; van der Donk, W. A. Enzymatic hydrogen atom abstraction from polyunsaturated fatty acids. *Chem. Commun. (Cambridge)* (23)**:**2843–2846; 2003.

[139] Bazan, N. G. Neuroprotectin D1 (NPD1): a DHA-derived mediator that protects brain and retina against cell injury-induced oxidative stress. *Brain Pathol.* **15:** 159–166; 2005.

[140] Bazan, N. G. Omega-3 fatty acids, pro-inflammatory signaling and neuroprotection. *Curr. Opin. Clin. Nutr. Metab. Care* **10:**136–141; 2007.

[141] Bazan, N. G. Neurotrophins induce neuroprotective signaling in the retinal pigment epithelial cell by activating the synthesis of the anti-inflammatory and anti-apoptotic neuroprotectin D1. *Adv. Exp. Med. Biol.* **613:**39–44; 2008.

[142] Lambert, I. H.; Pedersen, S. F.; Poulsen, K. A. Activation of PLA2 isoforms by cell swelling and ischaemia/hypoxia. *Acta Physiol. (Oxford)* **187:**75–85; 2006.

[143] Schneider, C.; Pratt, D. A.; Porter, N. A.; Brash, A. R. Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. *Chem. Biol.* **14:**473–488; 2007.

[144] Serhan, C. N.; Chiang, N.; Van Dyke, T. E. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat. Rev. Immunol.* **8:**349–361; 2008.

[145] Tyurina, Y. Y.; Kawai, K.; Tyurin, V. A.; Liu, S. X.; Kagan, V. E.; Fabisiak, J. P. The plasma membrane is the site of selective phosphatidylserine oxidation during apoptosis: role of cytochrome c. *Antioxid. Redox Signaling* **6:**209–225; 2004.
[146] Tyurina, Y. Y.; Serinkan, F. B.; Tyurin, V. A.; Kini, V.; Yalowich, J. C.; Schroit, A. J.; Fadeel, B.; Kagan, V. E. Lipid antioxidant, etoposide, inhibits phosphatidylserine externalization and macrophage clearance of apoptotic cells by preventing phosphatidylserine oxidation. *J. Biol. Chem.* **279:**6056–6064; 2004.
[147] Tyurin, V. A.; Tyurina, Y. Y.; Feng, W.; Mnuskin, A.; Jiang, J.; Tang, M.; Zhang, X.; Zhao, Q.; Kochanek, P. M.; Clark, R. S.; Bayır, H.; Kagan, V. E. Mass-spectrometric characterization of phospholipids and their primary peroxidation products in rat cortical neurons during staurosporine-induced apoptosis. *J. Neurochem.* **107:** 1614–1633; 2008.
[148] Jiang, J.; Huang, Z.; Zhao, Q.; Feng, W.; Belikova, N. A.; Kagan, V. E. Interplay between bax, reactive oxygen species production, and cardiolipin oxidation during apoptosis. *Biochem. Biophys. Res. Commun.* **368:**145–150; 2008.
[149] Tyurina, Y. Y.; Wasserloos, K. J.; Stewart, R. C.; Stitt, M. S.; Kaynar, A.; Pitt, B. R.; Kagan, V. E., Oxidative lipidomics of hyperoxic lung injury. In 47th Annual Meeting of the Society of Toxicology, Seattle, WA: *The Toxicologist,* p. 1213; 2008.
[150] Tyurin, V. A.; Jung, M. Y.; Tyurina, Y. Y.; Prieto, T.; Zhao, Q.; Kapralov, A. A.; Belikova, N. A.; Nantes, I. L.; Kagan, V. E., Cardiolipin oxidation, hydrolysis and accumulation of monolysocardiolipins and oxidized free fatty acids during apoptosis: role of cytochrome c. In 48th Annual Meeting of the Society of Toxicology, Baltimore, MD: *The Toxicologist,* 2009.
[151] Maiuri, M. C.; Zalckvar, E.; Kimchi, A.; Kroemer, G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat. Rev. Mol. Cell. Biol.* **8:**741–752; 2007.
[152] Rubinsztein, D. C.; Gestwicki, J. E.; Murphy, L. O.; Klionsky, D. J. Potential therapeutic applications of autophagy. *Nat. Rev. Drug Discovery* **6:**304–312; 2007.
[153] Kim, K. W.; Mutter, R. W.; Cao, C.; Albert, J. M.; Freeman, M.; Hallahan, D. E.; Lu, B. Autophagy for cancer therapy through inhibition of pro-apoptotic proteins and mammalian target of rapamycin signaling. *J. Biol. Chem.* **281:**36883–36890; 2006.
[154] Shimizu, S.; Kanaseki, T.; Mizushima, N.; Mizuta, T.; Arakawa-Kobayashi, S.; Thompson, C. B.; Tsujimoto, Y. Role of Bcl-2 family proteins in a nonapoptotic programmed cell death dependent on autophagy genes. *Nat. Cell Biol.* **6:** 1221–1228; 2004.
[155] Amaravadi, R. K.; Yu, D.; Lum, J. J.; Bui, T.; Christophorou, M. A.; Evan, G. I.; Thomas-Tikhonenko, A.; Thompson, C. B. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J. Clin. Invest.* **117:**326–336; 2007.
[156] Xu, Y.; Kim, S. O.; Li, Y.; Han, J. Autophagy contributes to caspase-independent macrophage cell death. *J. Biol. Chem.* **281:**19179–19187; 2006.
[157] Xue, L.; Fletcher, G. C.; Tolkovsky, A. M. Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. *Mol. Cell. Neurosci.* **14:**180–198; 1999.
[158] Xue, L.; Fletcher, G. C.; Tolkovsky, A. M. Mitochondria are selectively eliminated from eukaryotic cells after blockade of caspases during apoptosis. *Curr. Biol.* **11:** 361–365; 2001.
[159] Kissová, I.; Deffieu, M.; Manon, S.; Camougrand, N. Uth1p is involved in the autophagic degradation of mitochondria. *J. Biol. Chem.* **279:**39068–39074; 2004.
[160] Kissová, I.; Deffieu, M.; Samokhvalov, V.; Velours, G.; Bessoule, J. J.; Manon, S.; Camougrand, N. Lipid oxidation and autophagy in yeast. *Free Radic. Biol. Med.* **41:** 1655–1661; 2006.
[161] Dadakhujaev, S.; Noh, H. S.; Jung, E. J.; Hah, Y. S.; Kim, C. J.; Kim, D. R. The reduced catalase expression in TrkA-induced cells leads to autophagic cell death via ROS accumulation. *Exp. Cell Res.* **314:**3094–3106; 2008.
[162] Chapman, D. A.; Killian, G. J.; Gelerinter, E.; Jarrett, M. T. Reduction of the spin-label TEMPONE by ubiquinol in the electron transport chain of intact rabbit spermatozoa. *Biol. Reprod.* **32:**884–893; 1985.
[163] Murphy, M. P.; Smith, R. A. J. Targeting antioxidants to mitochondria by conjugation to lipophilic cations. *Annu. Rev. Pharmacol. Toxicol.* **47:**629–656; 2007.
[164] Jiang, J.; Belikova, N. A.; Hoye, A. T.; Zhao, Q.; Epperly, M. W.; Greenberger, J. S.; Wipf, P.; Kagan, V. E. A mitochondria-targeted nitroxide/hemigramicidin S conjugate protects mouse embryonic cells against gamma irradiation. *Int. J. Radiat. Oncol. Biol. Phys.* **70:**816–825; 2008.
[165] Fink, M. P.; Macias, C. A.; Xiao, J.; Tyurina, Y. Y.; Delude, R. L.; Greenberger, J. S.; Kagan, V. E.; Wipf, P. Hemigramicidin–TEMPO conjugates: novel mitochondria-targeted antioxidants. *Crit. Care Med.* **35** (9 Suppl.)**:**S461–467; 2007.
[166] Macias, C. A.; Chiao, J. W.; Xiao, J.; Arora, D. S.; Tyurina, Y. Y.; Delude, R. L.; Wipf, P.; Kagan, V. E.; Fink, M. P. Treatment with a novel hemigramicidin–TEMPO conjugate prolongs survival in a rat model of lethal hemorrhagic shock. *Ann. Surg.* **245:**305–314; 2007.
[167] Stoyanovsky, D. A.; Vlasova, I. I.; Belikova, N. A.; Kapralov, A.; Tyurin, V.; Greenberger, J. S.; Kagan, V. E. Activation of NO donors in mitochondria: peroxidase metabolism of (2-hydroxyamino-vinyl)-triphenyl-phosphonium by cytochrome c releases NO and protects cells against apoptosis. *FEBS Lett.* **582:** 725–728; 2008.
[168] Lee, I.; Salomon, A. R.; Yu, K.; Doan, J. W.; Grossman, L. I.; Hüttemann, M. New prospects for an old enzyme: mammalian cytochrome c is tyrosine-phosphorylated in vivo. *Biochemistry* **45:**9121–9128; 2006.
[169] Yu, H.; Lee, I.; Salomon, A. R.; Yu, K.; Hüttemann, M. Mammalian liver cytochrome c is tyrosine-48 phosphorylated in vivo, inhibiting mitochondrial respiration. *Biochim. Biophys. Acta* **1777:**1066–1071; 2008.