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Redox Phospholipidomics of Enzymatically Generated Oxygenated Phospholipids as Specific Signals of Programmed Cell Death.

VE Kagan[1,2,4,5,6,10,#], **YY Tyurina**[1,2], **WY Sun**[1,2,12], **II Vlasova**[10], **H Dar**[1,2], **VA Tyurin**[1,2], **AA Amoscato**[1,2], **R. Mallampalli**[7], **PCA van der Wel**[11], **RR He**[12], **AA Shvedova**[8], **D. Gabrilovich**[9], **H. Bayir**[1,3,#]

[1]Center for Free Radical and Antioxidant Heath, University of Pittsburgh, USA [2]Department of Environmental and Occupational Health, University of Pittsburgh, USA [3]Critical Care Medicine, University of Pittsburgh, USA [4]Chemistry, University of Pittsburgh, USA [5]Pharmacology and Chemical Biology, University of Pittsburgh, USA [6]Radiation Oncology, University of Pittsburgh, USA [7]The Ohio State University, Columbus, Ohio, USA [8]Exposure Assessment Branch, NIOSH/CDC, Morgantown, WV, USA [9]The Wistar Institute, Philadelphia, Pennsylvania, USA [10]Laboratory of Navigational Redox Lipidomics, IM Sechenov Moscow State Medical University, Moscow, Russian Federation [11]Zernike Institute for Advanced Materials, University of Groningen, Groningen, The Netherlands [12]International Cooperative Laboratory of Traditional Chinese Medicine Modernization and Innovative Drug Development of Chinese Ministry of Education (MOE), College of Pharmacy, Jinan University, Guangzhou, Guangdong, China

Abstract

High fidelity and effective adaptive changes of the cell and tissue metabolism to changing environments requires strict coordination of numerous biological processes. Multicellular organisms developed sophisticated signaling systems of monitoring and responding to these different contexts. Among these systems, oxygenated lipids play a significant role realized via a variety of re-programming mechanisms. Some of them are enacted as a part of pro-survival pathways that eliminate harmful or unnecessary molecules or organelles by a variety of degradation/hydrolytic reactions or specialized autophageal processes. When these “partial” intracellular measures are insufficient, the programs of cells death are triggered with the aim to remove irreparably damaged members of the multicellular community. These regulated cell death mechanisms are believed to heavily rely on signaling by a highly diversified group of molecules, oxygenated phospholipids (PLox). Out of thousands of detectable individual LPox species, redox phospholipidomics deciphered several specific molecules that seem to be diagnostic of specialized death programs. Oxygenated cardiolipins (CLs) and phosphatidylethanolamines (PEs) have been

[#]To whom correspondence should be addressed: kagan@pitt.edu or bayihx@ccm.upmc.edu.

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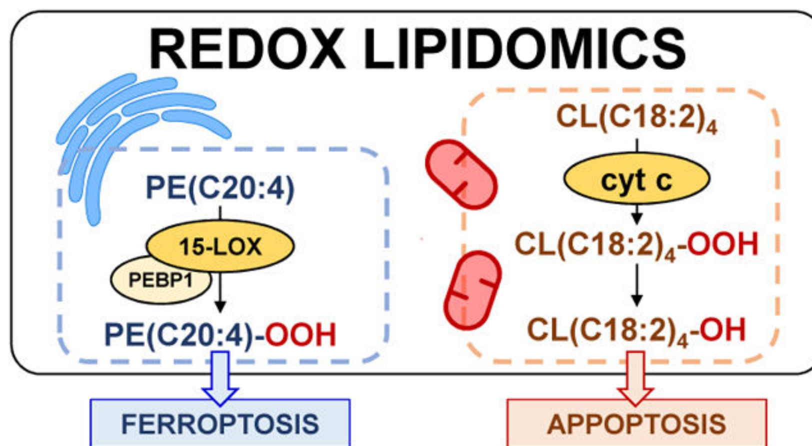
identified as predictive biomarkers of apoptosis and ferroptosis, respectively. This has led to decoding of the enzymatic mechanisms of their formation involving mitochondrial oxidation of CLs by cytochrome c and endoplasmic reticulum-associated oxidation of PE by lipoxygenases. Understanding of the specific biochemical radical-mediated mechanisms of these oxidative reactions opens new avenues for the design and search of highly specific regulators of cell death programs. This review emphasizes the usefulness of such selective lipid peroxidation mechanisms in contrast to the concept of random poorly controlled free radical reactions as instruments of non-specific damage of cells and their membranes. Detailed analysis of two specific examples of phospholipid oxidative signaling in apoptosis and ferroptosis along with their molecular mechanisms and roles in reprogramming has been presented.

Graphical Abstract



> The weariest and most loathed worldly life That age, ache, penury and imprisonment Can lay on nature, is a paradise To what we fear of death.”
>
> William Shakespeare,
>
> Measure for Measure (1603).

Programs of Regulated Cell Death or Re-programming Cell Populations?

Fidelity and quality control of biological systems depend, to a large extent, on the reprogramming or elimination of unnecessary or harmful cells and their components [1, 2]. Reprogramming, including trans-generational reprogramming [3, 4], is commonly engaged when the responses to changing environments are still reversible, while elimination is triggered usually when the effects of damaging or toxic materials exceed the repair capacities of cells. For a long time, cell death has been viewed as a catastrophic and mostly chaotic chain of events. Over the last 3-4 decades, this point of view has changed as experimental biology has discovered several organized and highly regulated cascades of cell death, thus “erasing” the differences between reprogramming and death [5, 6].

The emotional characterizations of the meaning of death have been transferred into the field of biology, particularly cell biology, where rationally designed sophisticated programs of

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elimination of unnecessary or harmful cells have earned the anthropocentric term "death." One of the greatest Masters in describing the tragedy of human death and loss of the beloved people, W. Shakespeare, emphasized the "sweetness" of death that liberates from suffering, pain and loathed life. His words may sound like an appreciation of useful consequences of elimination or reprogramming mechanisms helping to rid of harmful attributes of life, yet are associated with the tragic symbolism of death. Interestingly, the emotional and philosophical categories of death are commonly applied to biological processes starting from cells.

The programs taking care of excessive or harmful cells and described as mechanisms of "regulated cell death" are important for the maintenance of healthy cell populations. The genetically-controlled mechanisms of individual death of organisms - phenoptosis - are widely spread at higher levels and have been described in prokaryotes, unicellular eukaryotes, and all kingdoms of multicellular eukaryotes (animals, plants, and fungi) [7]. Phenoptosis, although tragic at the individual level, serves an important evolutionary function. Two types of phenoptosis have been considered: i) acute phenoptosis - rapid deterioration of an organism induced by an essential biological function (eg, breeding), and ii) age-induced slow phenoptosis - slow deterioration and death of an organism due to accumulated stresses over long periods of time [8].

Independently of the "emotional flavors" of all these biochemical and biological processes and responses [9], understanding the molecular mechanisms of reprograming and death programs and pathways is essential for the very pragmatic goals of deciphering the etiology and pathogenesis of injury and disease conditions. Indeed, insufficient levels of elimination of harmful materials lead to the accumulation of antigenic/genotoxic materials - typical of cancer or auto-immune diseases; vice versa, exceedingly massive elimination leads to tissue and organ injury - eg, brain injury, sepsis, radiation injury, chronic degenerative diseases, etc (Fig. 1). In addition to the general biological, philosophical and even social aspects of the interpretation of death, this dualism of the elimination processes is remarkably important in biomedicine and will be discussed in the current review. Of particular importance to understanding the etiology and pathogenesis of disease is not only the philosophy behind, but also the effectiveness of controlling the fine balance between preserving and eliminating death signals. The balance that ultimately controls the transition of biological units from live thermodynamically open systems into dead material is fundamental to our understanding of the etiology and pathogenesis of disease.

Oxidative Stress/Injury, Free Radicals and Antioxidants.

Paradoxically, among the instruments of elimination or cell death, one the most important is the molecule of life, oxygen, that is involved in many oxidative reactions. Over the last six decades, the concept of free radicals and antioxidants has been developed, propagated and subjected to experimental testing and clinical trials [10-13]. The simplicity of the concept - uncontrolled free radical chemical reactions that cause injury, disease and even death are counteracted by a network of antioxidant mechanisms maintaining health - has attracted remarkable attention of experimental researchers as well as clinicians [14, 15]. As a subclass of these processes, the term oxidative stress has been created to encompass multiple, in most

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cases unknown or poorly understood, events setting the stage for the disbalance between pro-oxidant free radical reactions and their antioxidant regulators [16, 17]. Essentially, a huge variety of redox signals with their effects on biological functions has been trivialized into a concept of free radicals <-> antioxidant balance or dysbalance as if only these two states were meaningful (Fig. 2). This over-simplified concept ignored the molecular mechanisms of action of oxygenated molecules and their functions. Instead, the war on toxic and injurious free radicals and triumph of beneficial antioxidants have been declared. The major endogenous antioxidant molecules - water-soluble and lipid-soluble and their cascades have been identified and the interactions thoroughly investigated [18]. Along with the endogenous radical scavenging molecules in mammalian cells, the myriads of natural molecules of plant, fungal and bacterial origin have been isolated and their antioxidant characteristics evaluated [19-21]. Synthetic efforts of chemists yielded a multitude of novel classes of radical scavenging molecules as well as an arsenal of chemically modified and perfected natural antioxidants - such as modified homologues of vitamins E and C [22-24]. The multitude of effective chain breaking radical scavenging tools with phenolic and aromatic amino-groups as well as sulfhydryls - have been well characterized in chemical systems and simple physical-chemical model systems [25, 26]. Unfortunately, specific regulatory functions of antioxidant molecules as a part of regulatory cascades controlling the production and degradation of specific signaling molecules has been neglected. Instead, a huge amount of work on perfection and optimization of antioxidants and their mixtures has been performed and raised the “plank” of expectations very high - to the level of a new revolution in health improvements, disease prevention and the stimulation of extraordinary new therapies. This has been followed by a series of multiple sobering clinical trials. Subsequent decades of these trials and analysis of their results were disappointing. A journey through the PubMed database clearly quantitates the results (Table 1): not a single one of the conducted trials has been was successful in producing a positive effect. The conclusion was clear: the concept of chemical free radical chain reactions and their correction by chain-breaking radical scavengers/antioxidants failed as a biomedical preventive or therapeutic endeavor [10-13]. New ideas and a better understanding of redox regulation and its enzymatic mechanisms had to be developed. While the general schemas of random chemical lipid peroxidation and enzymatic lipid peroxidation seem to be quite similar (Fig. 3), detailed analysis reveals dramatic differences in the selectivity and specificity of catalytic mechanisms and products. This is best illustrated in the case of lipids containing polyunsaturated fatty acids (PUFA)

Enzymatically regulated vs. free radical-mediated lipid peroxidation.

The susceptibility of polyunsaturated fatty acid (PUFA)-containing lipids to radical oxidation in chemical systems could be characterized by the rate constant of H-atom abstraction, which increases with the number of bis-allylic -CH_2-centers in the molecule [27]. Based on this simple chemical rule, one can expect that highly polyunsaturated lipid substrates with multiple double bonds with several bis-allylic sites will be the preferred oxidation substrates. In contrast, the enzymatic process is directed towards oxidation substrates specifically positioned within the catalytic site of the enzyme, such that specificity of the reaction products may deviate significantly from this chemical rule. In other words,

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oxidation programs realized via enzymatic mechanisms may yield products inconsistent with the dominance of bis-allylic sites in the substrates. With this in mind, we performed this type of analysis using two types of genetic models with genetically manipulated levels of PUFA phospholipids. In particular, based on previously published results we compared the oxidizability of phospholipids, i.e. the total amount of major oxidizable PUFA-phosphatidylethanolamines (PEs) and their oxidation products in WT and ACSL4 (acyl-CoA synthase 4) KO mouse kidney and embryonic fibroblast (Pfa1) cells (Fig. 4 a-c) and WT and FATP2 (fatty acid transport protein 2) KO polymorphonuclear myeloid-derived suppressor (PMN-MDSCs) cells (Fig 4. d-f) in the presence and absence of RSL3 – a pro-ferroptotic agent. Both ACSL4 and FATP2 control delivery and activation of PUFA, particularly AA, into phospholipids [28-32]. We focused our analysis on AA-PEs with three bis-allylic centers in the *sn*-2 position of the phospholipid - accounting for the majority of oxidizable PUFA-PEs (44-56%, Fig. 4 a, d) and oxidation products (66-76%, Fig. 4 b, e). DHA-PEs, another major group of oxidizable PUFA-PEs with five bis-allylic centers in the *sn*-2 position, contribute a significant amount of oxidation substrates (10-17%, Fig. 4 a, d) but displayed a relatively lower oxidizability (lower levels of oxidation products (3-12%, Fig. 4 b, e). Notably, no significant correlation was observed between oxidation levels (mole%) of PUFA-Pes and the rate constants of hydrogen abstraction for their corresponding PUFAs in the *sn*-2 position predicted by chemical reactivity (Fig. 4 c, f). Based on these calculations other assessments of this kind may be performed using the published data on PUFA content in specific classes of phospholipids and their oxidation preferences in cells and tissues where enzymatic machineries may be involved in the catalytic process. These data indicate that cellular lipid peroxidation is not a simple chemically-driven free radical-mediated oxidation process but it rather represents a complex of biochemical reactions each of which dictates its selectivity with toward the substrates and specificity with regards to the oxidation products generated [27, 33]. Another support for the strictly selective and controlled lipid peroxidation can be provided by the results showing that the oxidation levels of PUFA-PEs (mole%) are significantly decreased in accordance with the lack of one of the oxidation substrates, AA-containing species of PE, in ACSL4-deficient Pfa1 cells compared to WT cells during execution of ferroptotic program in these cells. (Fig. 4 c).

Analytical Techniques for Detection of Lipid Peroxidation Products.

Over the last three decades, several distinctive genetically pre-determined cell death programs have been discovered and their molecular mechanisms described. Notably, redox disbalance and lipid peroxidation have been claimed as inherent features of essentially each of them [33-36]. These claims, however, have been made on the basis of experimentally indiscriminative and mostly non-specific analytical protocols that were not able to provide information on the molecular identity of the oxidation reactions and their targets. Among the most common characterizations were assessments of protein carbonyls [37, 38], 4-hydroxynonenal [39], malondialdehyde [40, 41], antioxidant depletion [42, 43] and similar features incapable of identifying of the specific oxidations products with the predictive characteristics of biomarkers [44]. Deficiencies of these molecular protocols have been revealed even in *in vitro* experiments with cells but became particularly obvious when *in vivo* assessments of cell death in tissues of animals or available samples from human

subjects have been attempted [41, 43, 44]. This ambiguity of analytical protocols has been overcome with the advent of new LC-MS based approaches with a variety of different specific protocols [45-49]. Given that polyunsaturated lipids represent the most vulnerable substrates in pro-oxidant environments, many of the current LC-MS based techniques were designed to detect lipid peroxidation products. Among the first findings in the new field of research - redox lipidomics or oxidative lipidomics [48, 50-58] - was the documentation of thousands of individual molecular species of oxidatively modified phospholipids [48, 50-58]. These included several major categories of products: i) the primary molecular oxidation products - hydroperoxy-containing fatty acid residues with the same length of the hydrocarbon chain, ii) the secondary products with a variety of oxygen-containing functionalities (epoxy-, hydroxy, oxo-, etc) retaining the same chain length, iii) oxidatively-truncated electrophilic products with oxygen-containing functionalities, and iv) conjugates of oxidatively-truncated electrophilic lipid products with target proteins or “oxidatively lipidated proteins” (Fig. 5). The analytical power of redox lipidomics - high sensitivity and resolution - has resulted in the identification of numerous oxidatively modified lipid species and set the stage for several fields of research seeking to find specific features of lipid peroxidation products causatively related to particular types of physiological re-programming mechanisms or conditions associated with acute injuries or chronic diseases [48, 50, 51, 53, 55, 56, 59, 60].

While conventional high mass resolution LC-MS/MS protocols can fully structurally characterize diversified lipids and their oxidation products, they provide no information on the distribution of lipid molecules of interest in cells and tissues. Given the obvious importance of this type of knowledge, much effort has been concentrated on the development of mass-spectrometric imaging (MSI) of lipids. Matrix-assisted laser desorption-ionization (MALDI) protocols have become the most commonly employed techniques which allowed to get substantial data on the localization of different types of lipid molecules in tissues, particularly brain, and their changes associated with injury or disease conditions [61-63]. Direct detection of oxidized lipids has not been yet achieved; however, much useful information on the redox reactions in tissues lipid have been obtained through the MSI analysis of the major substrates of lipid peroxidation, PUFA-phospholipids [64]. In spite of substantial progress in MALDI-technologies in the topographical identification of different lipids, including low abundance and higher mass classes of them, such as CLs [65], he major deficiency remained a relatively low spatial resolution incompatible with the subcellular mapping of their major lipid components of individual cells and their organelles. Revolutionizing breakthrough in this area has been associated with the introduction of gas-cluster ion beam time-of-flight secondary-ion MS which, with its spatial resolution ~1 micron, permits subcellular analysis of essential lipids in individual cells and their organelles (Fig. 6) [66-68]. Further improvements in this technology promise the opportunity to directly visualize the peroxidized lipids and their changes produced by physoiological and/or pathological conditions at the subcellular level.

Reprogramming via Apoptosis.

Among the first applications of redox lipidomics were the studies of cell death programs. It seemed tempting to examine the specific meaning of the common notion that execution of

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cell death programs is associated with lipid peroxidation (Fig. 7). While changes of lipid mediators have been related to cell death, none of them were found directly involved in the execution of death signaling [52]. The new opportunities offered by redox *phospholipidomics* turned out to be successful and revealed a highly selective engagement of two phospholipids in the course of intrinsic mitochondria-dependent apoptosis in a number of cell models as well as in tissues of animals (eg, via exposure to acute brain injury, lung challenge by inhalation of nanoparticles [49-51, 53, 69, 70]). Detailed studies revealed the mechanism of mitochondria-confined peroxidation of CL in a complex process of its trans-migration from the inner mitochondrial membrane through the intermembrane space to the outer mitochondrial membrane [71-74]. During this journey - initiated by the decreased membrane potential across the IMM - the physical encounter and binding of CL to a hemoprotein cytochrome c (cyt c) occurs. The significance of this interaction was quite unexpected: within the complex with CL, cyt c changes its catalytic competence in the process of conversion from a mobile electron carrier between respiratory complexes III and IV into a potent peroxidase capable of oxidizing many organic compounds, including PUFA-CLs (Fig. 6) [75]. In this process a hexa-coordinate Fe transitions into a penta-coordinate state as a result of weakening and rupture of the Fe-Met(80) bond (Fig. 8) [76]. This dramatic change is accompanied by a sharp drop of the cyt c/CL redox potential (by ~400mV) such that, in contrast to native cyt c, the complex cannot act as an acceptor of electrons from respiratory complex III [75]. As a result, the normal flow of electrons through the disarrayed electron transport chain is no longer possible, thus causing a massive production of superoxide-anion-radicals [77, 78]. As the latter can be converted - spontaneously or in the Mn-superoxide dismutase-catalyzed reaction - into H_2O_2 [79], pro-apoptotic mitochondria become a source of oxidizing equivalents feeding the peroxidase cycle of cyt c/CL complexes (Fig. 9) [80]. Typical of the peroxidase cycle is the sequential production of reactive intermediates [81] - cation-radicals of the porphyrins, compounds I and II with protein immobilized radicals [82] capable of effective H-abstraction from the bis-allylic positions and leading to the formation of the alkyl and, in the presence of oxygen, peroxyl radicals (Fig. 9). The exact nature of the changes in cyt c structure upon its interaction with CL and leading to the “weakening” and breach of the Fe-(Met80) bond and hexa-coordinate state of Fe is still a matter of active on-going studies. The results may be interpreted as supporting the significant unfolding of the protein [83, 84] or relatively small changes of the protein structure but highly increased dynamics of the specific portions of the protein (eg, a highly dynamic loop formed by the residues 70-85) [85-87]. In either structural rearrangement, the formation of the penta-coordinate state of Fe is essential for its peroxidase function.

While CL is the major intramitochondrial substrate of attack on phospholipids, redox lipidomics studies revealed that another anionic phospholipid, phosphatidylserine (PS) was also involved in the oxidation process during apoptosis [88, 89]. As PS is an extramitochondrial phospholipid, the role of cyt c in its oxidation is less clear although complexes of cyt c/PS reveal significant, albeit lower than cyt c/CL complexes, peroxidase activity (Fig. 6) [90]. It is likely, although not unequivocally proven, that released from mitochondria into the cytosol cyt c may form complexes with abundant extra-mitochondrial PS. In apoptosis the significance of this event has been interpreted in terms of facilitated

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trans-migration of PS from the inner to the outer surface of the plasma membrane and the appearance of PSox on the cell surface [57, 91]. The biological significance of this has been associated with a much higher phagocytosing activity of macrophages and microglia [91-93]. Given the well-established anti-inflammatory (pro-resolving) effects of phagocytosing macrophages with predominantly expressing the M2 phenotype, the role of enhanced phagocytosis of apoptotic cells bearing PSox by macrophages may be quite significant, yet insufficiently studied [93].

The function and identity of CLox products in apoptosis have been the subject of detailed studies [48-51, 53-56, 58, 69, 94-96]. Selectivity of CL oxidation is caused by the high affinity of cyt c towards CL binding. It has been estimated that dissociation constants for cyt c/CL complexes are on the order of $2.0\text{-}4.2 \times 10^{-5}$ M [97]. Thus, it is likely that tightly bound PUFA-CL species represent the oxidation substrates for the cyt c/CL complexes. It has been established that oxidized CL species (CLox) bind less avidly with cyt c [98]. This suggests that after oxidation, CLox is liberated from the complex. One can speculate that during apoptosis cyt c is released into the cytosol in complexes with CL and/or CLox. The latter, however, are not stable and their dissociation leaves cytosolic cyt c available for interactions with alternative substrates. Given that the extra-mitochondrial concentration of CL is very low, PS may get involved in the interactions with cyt c. The cyt c/PS complex can also act as a peroxidase resulting in oxidation of PUFA-PS species [90].

In terms of the mechanisms of oxidation, cyt c/CL complexes are engaged in typical peroxidase cycle [82, 99]. The peroxide bond -O-O- in hydroperoxy-phospholipids is weak and can be readily cleaved to yield secondary CL oxidation products, frequently with oxidatively truncated, shortened chains [100, 101]. Common among those oxidiatively-truncated products, are electrophilic moieties that can react with the nucleophilic sites in proteins and produce covalent-adducts. If their formation is non-random and follows a specific pattern, these protein aggregates may form oligomeric structures in plasma membranes thus affecting its integrity and contribute to the execution of death. In lieu of this, it is noteworthy that apoptosis-associated CLox products are enriched with electrophilic epoxy- and oxo-derivatives [94, 102]. However, their role in plasma membrane disturbances and formation of apoptotic membrane “pores” and “blebs” has not been directly established.

Translocations of CL and CLox to the surface of mitochondria and its release into the cytosol may be followed by the appearance of CL/CLox on cell surface. These signals have been associated with two major effects on professional phagocytes - activation of phagocytosis realized via CD36 and, independently of this, on bonding with the TLR4/Md2 complex leading to the strong suppression of LPS-induced cytokine production, ie, immune-paralysis [103]. The suggested mechanisms for this may be similar to the effects of immature lipid A that prevents the hetero-oligomerizaton of TLR4 with Md2, which is necessary for the stimulation of the pro-inflammatory cytokine response [104-106]. In spite of the obvious importance, the significance of these effects as regulators of the inflammatory responses has not been sufficiently studied *in vivo.*

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Reprogramming via Ferroptosis: mechanisms, significance and applications.

Ferroptosis is the type of cellular response to the changing redox environments associated with disturbed iron homeostasis, the accumulation of lipid peroxidation products, and deficiency of the thiols system, particularly of GPX4 (a seleno-enzyme catalyzing the reduction of phospholipid hydroperoxides to their respective alcohols) (Fig. 6) [107-110]. Lipid peroxides are believed to be primarily responsible for the cell demise but the direct evidence supporting this point of view is scarce because the majority of the available experimental material is based on the employment of surrogate measurements potentially correlating with lipid peroxidation [111, 112]. The most commonly utilized protocol is based on the fluorescent assessments of BODIPY 581/591 C11 that may be co-oxidized congruently with the development of the lipid peroxidation response [112, 113]. Direct LC-MS based redox lipidomics assessments showed remarkable specificity of the ferroptosis-associated changes in the levels of lipid oxidation products determined by the effects of pro-ferroptotic stimulation (eg, treatment with a GPX4 inhibitor, RSL3) and elimination of these signals by an anti-ferroptotic agent, Ferrostatin-1 [52]. In spite of hundreds of oxidatively-modified individual phospholipids detected in cells undergoing ferroptosis, only four of them “survived” the scrutiny of intensive filtering and sieving through several criteria: i) significantly increased content (3-fold) in ferroptotic *vs* control cells; ii) correlation with cell death; iii) reduced contents of non-oxygenated oxidizable precursors in Acsl4 KO cells; iv) elevated levels in Gpx4 KO cells *in vitro* and Gpx4 KO mice *in vivo* [52]. These predictive ferroptotic signals were identified as hydroperoxy-derivatives of arachidonoyl- (C20:4) or adrenoyl- (C22:4, the product of AA elongation) phosphatidylethanolamines [52]. This high selectivity within the oxidation profile was also supported by independent findings demonstrating a strong suppressive effects of genetic or pharmacological (rosiglitazone) depletion of ACSL4 - an enzyme responsible for Co-A-activation of arachidonoyl residues [31, 52] as well as lyso-phospholipid acyltransferase (LPCAT3) facilitating arachidonoyl re-acylation of lysophospholipids (Fig. 4) [52]. In other words, the availability of sufficient amounts of arachidonoyl-PE was necessary for the successful completion of the ferroptotic program.

Establishment of the nature of this exclusive selectivity of PE oxidation in ferroptosis has uncovered the role of 15-lipoxygenase (15-LOX) [52], a typical dioxygenase capable of oxidizing not only free fatty acids but also membrane phospholipids [114]. Mammalian LOXes oxidize PUFA localized in their a U-shaped FA binding channel where distinct amino acids control FA orientation positioning the selected pentadiene structure opposite the non-heme iron in the catalytic site (Fig. 10, [115]) [116, 117]. Dependent on the depth of the channel relative to the site of the catalytic LOX-iron, arachidonic acid oxidation can occur at the 5th, 8th, 12th, or 15th carbon of the AA. These different LOXs are designated as 5-, 8-, 12-, and 15-LOX, respectively. The iron (III) at the LOX catalytic site has 5 coordination bonds with the protein’s amino acids with the hydroxy-group occupying the sixth coordination position [118]. Due to the very tight alignment of PUFA, Fe(III)-OH abstracts a hydrogen from substrates at the bis-allylic carbon in a highly site- and stereo-specific fashion. The intermediates of this reaction are the carbon-centered radical and Fe(II)-OH_2.

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An oxygen molecule delivered through the special oxygen channel attaches to the rearranged PUFA radical causing the formation of an oxygen-centered peroxy-radical that, upon hydrogen transfer from Fe(II)-H_2O, is converted into the molecular hydroperoxy-product [119]. As an oxidized PUFA is produced, the catalytic iron is converted back into Fe(III)-OH (Fig. 11). One can see that this mechanism is markedly different from the peroxidase mechanisms operated in CL oxidation by cyt c/CL complexes (see above).

It has been established that the catalytic competence of the 15-LOX towards AA-PE is strongly facilitated by the formation of its complex with a scaffold-protein, phosphatidylethanolamine binding protein 1 (PEBP1), that allosterically adapts the enzyme towards accommodating a bulkier substrate and also limits access to the catalytic site by other phospholipids with larger polar heads (eg, phosphatidylcholine) [59]. In terms of oxidation specificity, the 15th carbon in AA-PE and the 17th carbon in adrenoyl-PE (AdA-PE) have been determined as preferred sites of oxygenation [120]. The essential role of 15LOX/PEBP1 complexes in ferroptosis has been established *in vivo* in models of acute traumatic brain injury and kidney failure by folic acid and sepsis as well as in airway epithelium in asthma [59]. Importantly, in the majority cases when ferroptosis has been detected, GPX4 degradation was a hallmark of the response [109, 121, 122]. Thus, it has become obvious that GPX4 represents the major check-point in regulation of ferroptosis. A more detailed study of the mechanisms of this degradation indicated the involvement of specific proteolytic mechanisms, chaperone-mediate degradation [123].

Central to understanding the intricacies of the ferroptotic program is the identification of the proximate death executing mechanism. While HOO-AA-PE is a predictive ferroptosis biomarker, the role of this oxygenated phospholipid as an immediate instrument of cell death has to be further explored. Because the hydroperoxy-group confers instability leading to oxidative truncation of HOO-AA-PE, the resulting electrophilic products may operate as modifiers of sensitive nucleophilic sites in one or more proteins that will ultimately form “pores” leading to the permeability of plasma membranes and cellular demise. Identification of HOO-AA-PE as a precursor of these electrophilic truncated products makes the search for the immediate executioners of cell death feasible via their isolation and combined redox lipidomics/proteomics analysis.

Concluding remarks:

Programmed cell death mechanisms are important adaptive factors of cell populations in response to changed environments caused by chemical and physical factors, pathogens etc. By eliminating the unnecessary or harmful (damaged beyond repair) individual cells, these programs are beneficial and they enhance the overall vitality and survival of the organism. When the scale of the demise exceeds the allowable limits they may become pathogenic and correcting/limiting the execution of the programs becomes a necessity. Understanding the molecular mechanisms of the programs is therefore critical to maintenance of optimal health. Given the central role of lipid peroxidation with regulated cell death pathways, redox lipidomics is one of the most powerful tools in achieving this goal. Here we presented two cases - apoptosis and ferroptosis - when understanding/deciphering the mechanisms has led to important and useful interventions. Clearly, these first experimental steps are only the

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beginning of the required conceptual developments and transforming new ideas and approaches into more effective therapies.

Lipid mediators have been known as signaling molecules for more than five decades. Their diversity defines their important roles in the regulation of many physiological functions [54, 124-126]. The production of these signals follows a typical temporal chain of events that begins with the phospholipase A_2-catalyzed hydrolysis of phospholipids followed by the oxygenation step catalyzed by one of the isoforms of cyclooxygenases or LOXs [127]. Lately, it has become apparent that oxygenated lipid mediators esterified into phospholipids represent a very rich source of signaling molecules whose diversity may be remarkably high and reach hundreds of thousands of individual molecular species. The significance of this signaling pathway and deciphering of the meaning of the individual "words" in this language has become possible due to the development of redox phospholipidomics and this new research field started bringing its first significant results. Identification of specific oxygenated species of phospholipids as signals of apoptotic and ferroptotic death as predictive biomarkers of these programs and the re-programming of cell populations as a response to acute injury or chronic disease condition offers an entirely unique opportunity to monitor these responses *in vivo* [48, 53, 55, 56, 59, 69, 128, 129]. Given the important role that these types of cell death programs play in the pathogenesis of several types of injury and diseases, redox phospholpidomics can be utilized for the detection of cell death mechanisms in the overall disease process thus guiding the temporally optimized treatments representing new mechanism-based therapeutic approaches. It is becoming clear that a search for a single effective "silver bullet" for the treatment of diseases related to redox dis-homeostasis is based on the elusive simplistic concept of counterbalancing the exceedingly high production of free radicals by antioxidants. Understanding the specific enzymatic phospholipid oxidation mechanisms triggering cell death is a new approach that has to be tested as an alternative to antioxidant-based therapeutic strategies. Quantitative redox phospholipidomics assessments of biomarkers of cell death programs triggered and executed at specific stages of disease will dictate and guide the rational combinations of different specific inhibitors as well as the temporally harmonized regimens of their application.

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Abbreviations:

PLox	oxygenated phospholipids
CL	cardiolipin
CLox	oxidized cardiolipin species
PS	phosphatidylserine
PSox	oxidized phosaphatidylserine species
PE	phosphatidylethanolamine

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AdA-PE	adrenoyl- phosphatidylethanolamine
AA-PE	arachidonoyl-phosphotidylethanolamine
Cyt c	cytochrome c
APLT	aminophospholipid translocase
FATP2	fatty acid transport protein 2
ACSL4	acyl-CoA synthase 4
LPCAT	lysophosphatidylcholine acyltransferase
15-LOX	15-lpoxygenase
GPX4	glutathione peroxidase 4
PEBP1	phosphatidylethanolamine binding protein
NDPK-D	nucleoside diphosphate kinase D
RSL3	(1S,3R)-methyl 2-(2-chloroacetyl)-1-(4-(methoxycarbonyl)phenyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate
PUFA	polyunsaturated fatty acid
LA	linoleic acid
AA	arachidonic acid
AdA	adrenic acid

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Highlights:

Enzymatic phospholipid peroxidation generates death signals

Redox lipidomics establishes cardiolipin peroxidation in apoptosis

Cytochrome c/cardiolipin acts as a peroxidase complex

Hydroperoxy-phosphatidylethanolamines as biomarkers of ferroptosis

Lipoxygenases peroxidize phosphatidylethanolamines in ferroptosis

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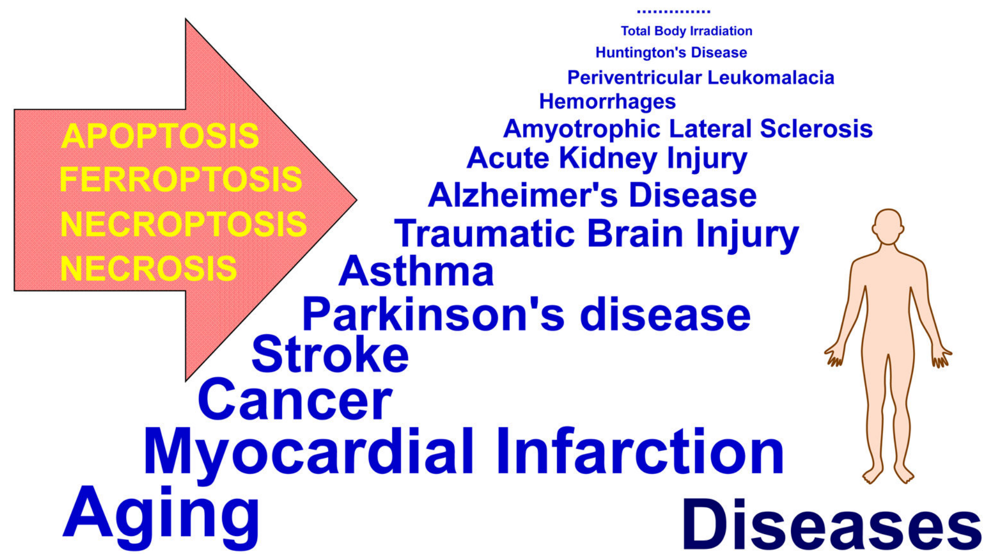

Fig. 1.
Acute injuries and chronic diseases associated with programmed cell death pathways.

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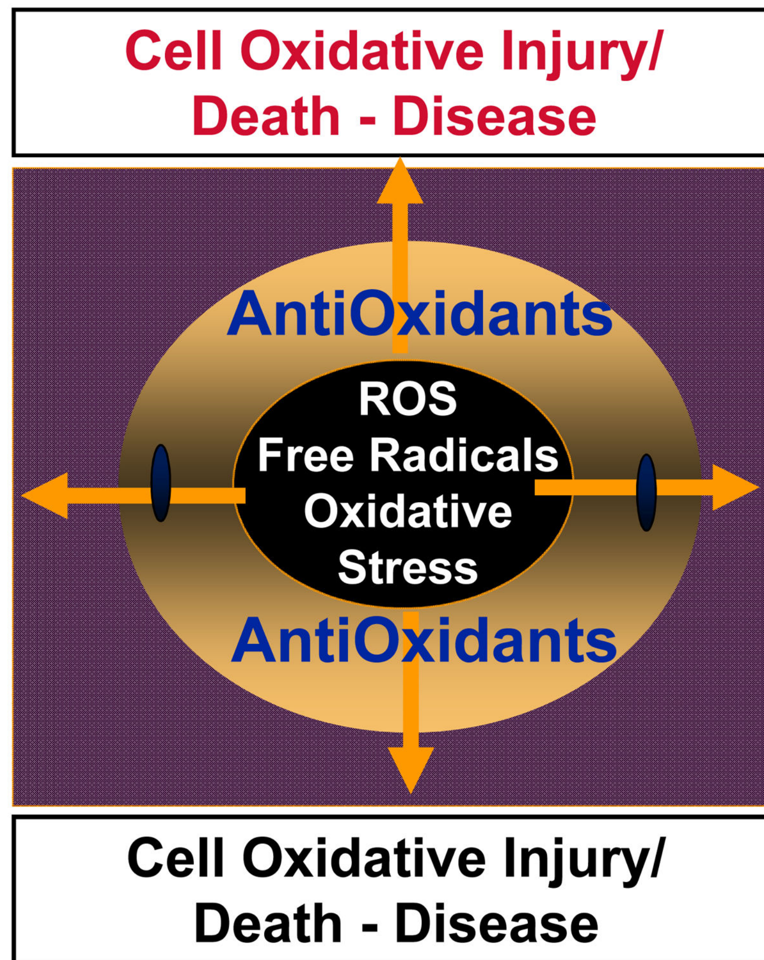

Fig. 2.
The concept of free radical/antioxidant balance or predominance of uncontrolled free radical reactions leading to oxidative injury, disease and death

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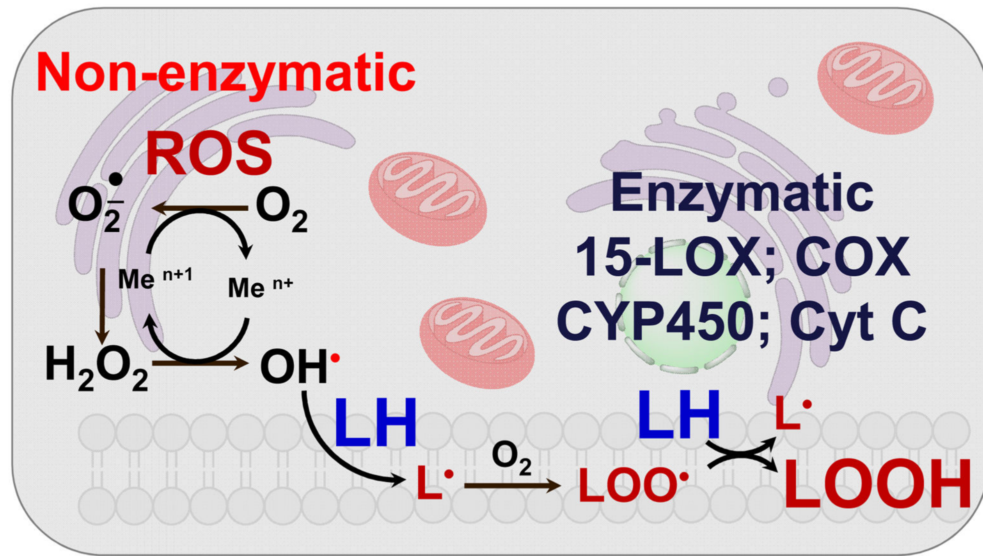

Fig. 3.

"Apparent similarities" in the general schemas of enzymatic and non-enzymatic lipid peroxidation.

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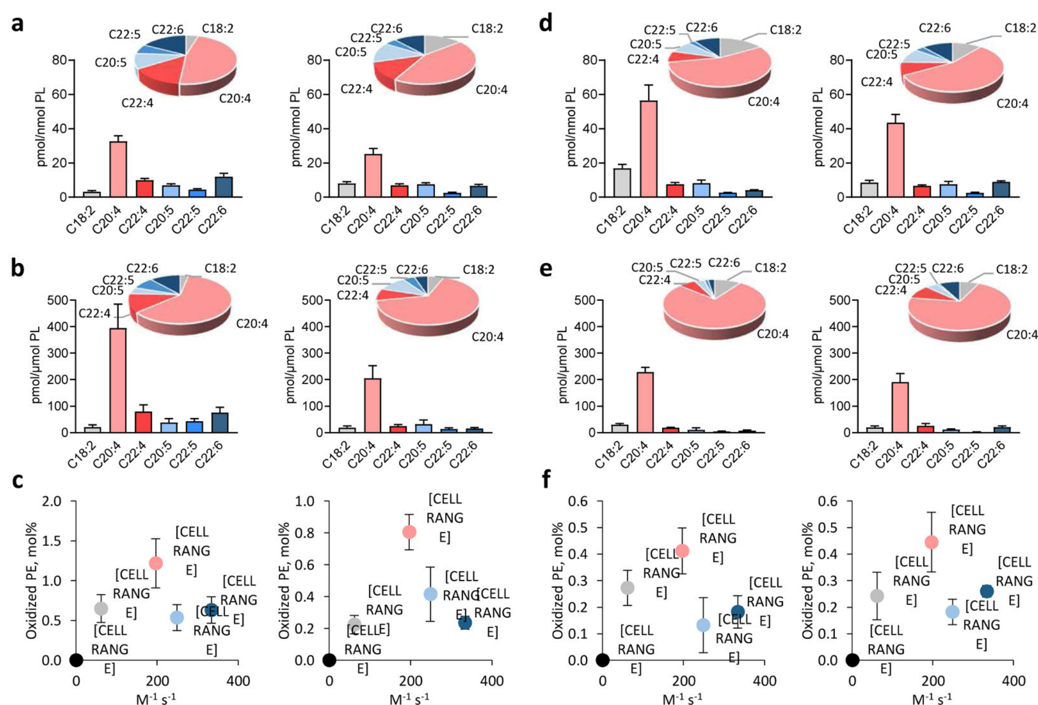

Fig. 4. Relationship between contents of phosphatidylethanolamine (PE) oxidation products in cells and rate constants of H-atom abstraction for polyunsaturated fatty acids (PUFAs).

a) Contents of PUFA-PEs in Pfa1 WT (*left*) and ACSL4 KO (*right*) cells treated with RSL3 (100 nM, 6 h). Data are means ± SD, $n = 4$. **b**) Contents of PUFA-PEs oxidation products in Pfa1 WT (*left*) and ACSL4 KO (*right*) cells treated with RSL3 (100 nM, 6 h). Data are means ± SD, $n = 4$. **c**) Relationship between mole percentages of PUFA-PEs oxidation products in Pfa1 WT (*left*) and ACSL4 KO (*right*) cells treated with RSL3 (100 nM, 6 h) and rate constants of H-atom abstraction for their corresponding PUFAs. Data are means ± SD, $n = 4$. **d**) Contents of PUFA-PEs in PMN WT (*left*) and FATP2 KO (*right*) cells. Data are means ± SD, $n = 4$. **e**) Contents of PUFA-PEs oxidation products in PMN WT (*left*) and FATP2 KO (*right*) cells. Data are means ± SD, $n = 4$. **f**) Relationship between mole percentages of PUFA-PEs oxidation products in PMN WT (*left*) and FATP2 KO (*right*) cells and rate constants of H-atom abstraction for PUFAs. Data are means ± SD, $n = 4$. PEs and their oxidation products are classified by polyunsaturated fatty acyl chain on *sn*-2. Rate constants of H-atom abstraction for PUFAs were assayed in liposomes [21].

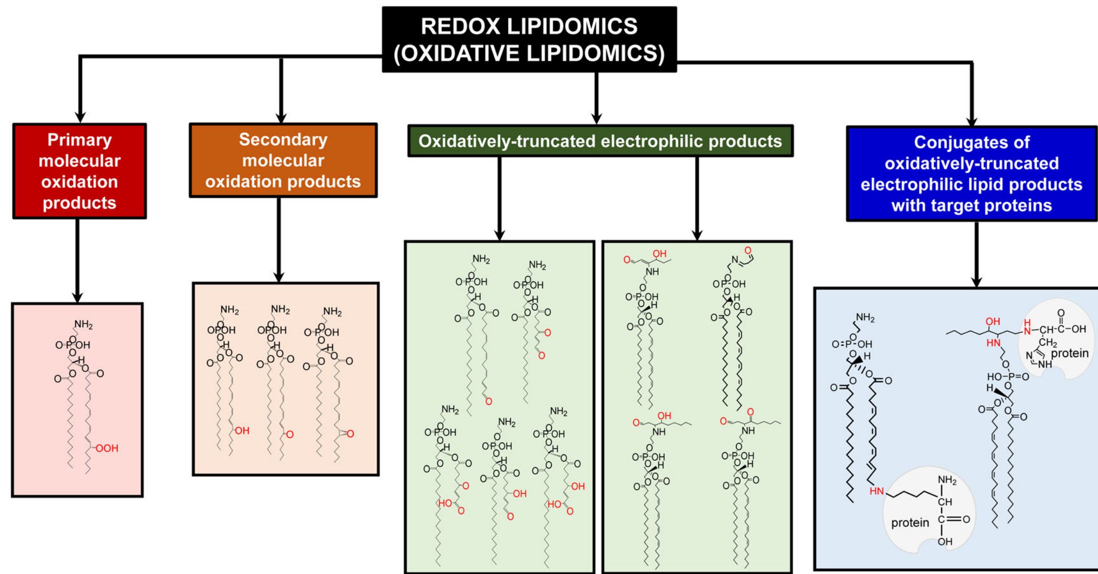

Fig. 5.

Major types of lipid oxidation products detectable in cells and tissues revealed by LC-MS based redox lipidomics. These products include: hydroperoxy-, hydroxy-, oxo-, epoxy- and oxidatively truncated phospholipid molecular species as well as conjugates of electrophilic lipid products with proteins.

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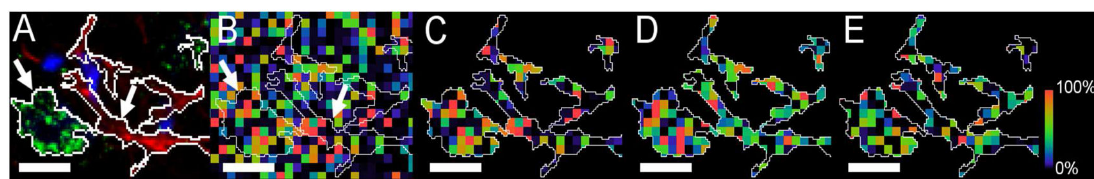

Fig. 6.

(**A**) **NeuN** (green, neurons) and **GFAP** (red, astroglia) signals marked with arrows and their thresholded overlays used to define cell body limits (bar=10 μm). (**B**) The PE(36:4p) SIMS signal (m/z 722.5) from the same region rendered as a pseudocolored heatmapped panel, intensity-scaled relative to the field of view. The thresholded NeuN and GFAP signals have been overlaid on the SIMS image. (**C**) PE(36:4p) cropped to the NeuN and GFAP thresholds. (**D**) PE(38:4) m/z 766.5, and, (**E**) PE(40:6) m/z790.5 also thresholded and cropped.

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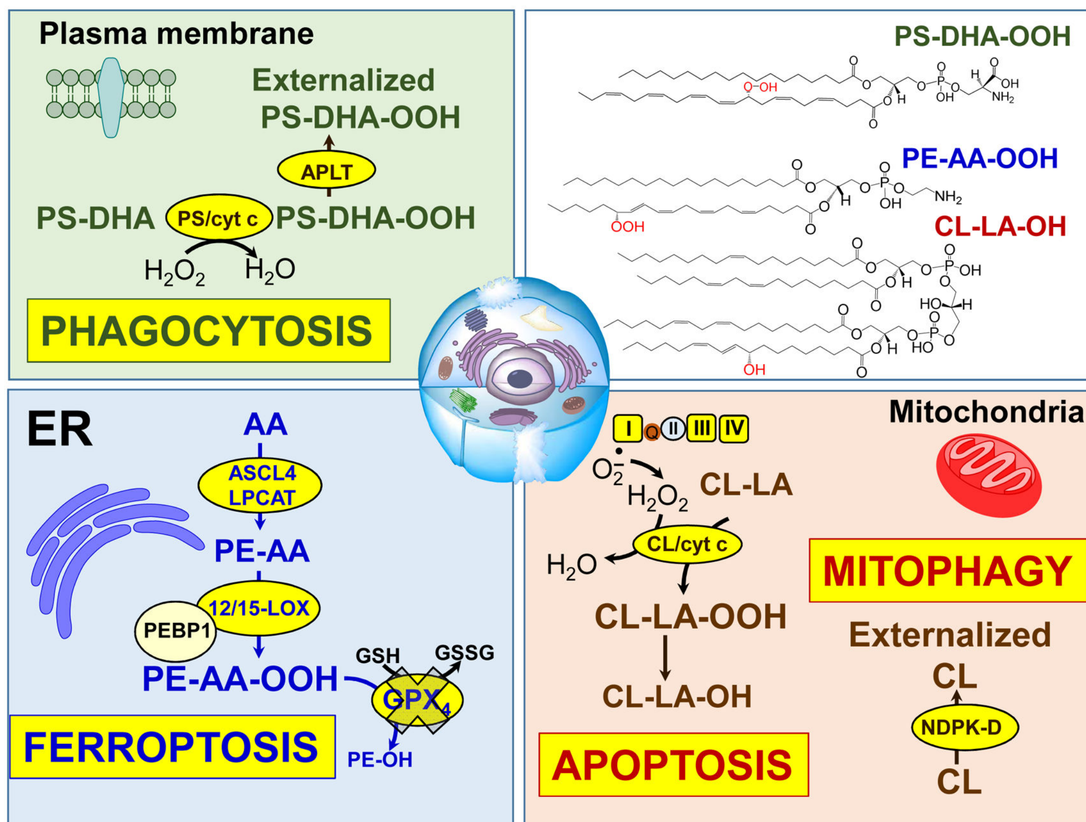

Fig. 7.

Oxygenated phospholipids associated with execution of programmed cell death processes and elimination of damaged mitochondria and cells. Formation of CL/cyt c complex with peroxidase activity in mitochondria results in the oxidation of CL, release of cyt c from mitochondria and triggering of the apoptotic cell death pathway. Released cyt c interacts with PS to form cyt c/PS complexes that cause PS oxidation and externalization on the cell surface. Externalized PS/PSox serve as an "Eat-me" signal for phagocytes. Damaged mitochondria externalize CL to be eliminated via the mitophagy pathway. Interaction of 15LOX with PEBP1 during ferroptosis results in the generation of the ferroptotic cell death signal, hydroperoxy-arachidonoyl-PE.

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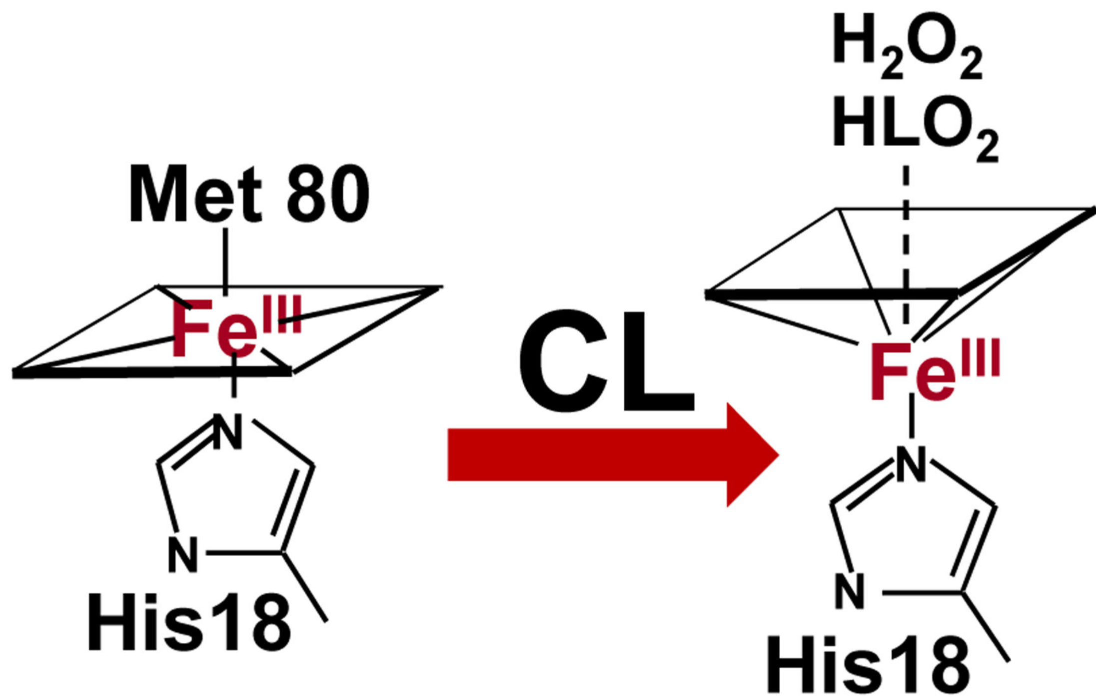

Fig. 8.

Cardiolipin induces restructuring and unfolding of cyt c accompanied by the loss or exchange of the distal heme iron ligand Met80 and facilitates heme interaction with small molecules.

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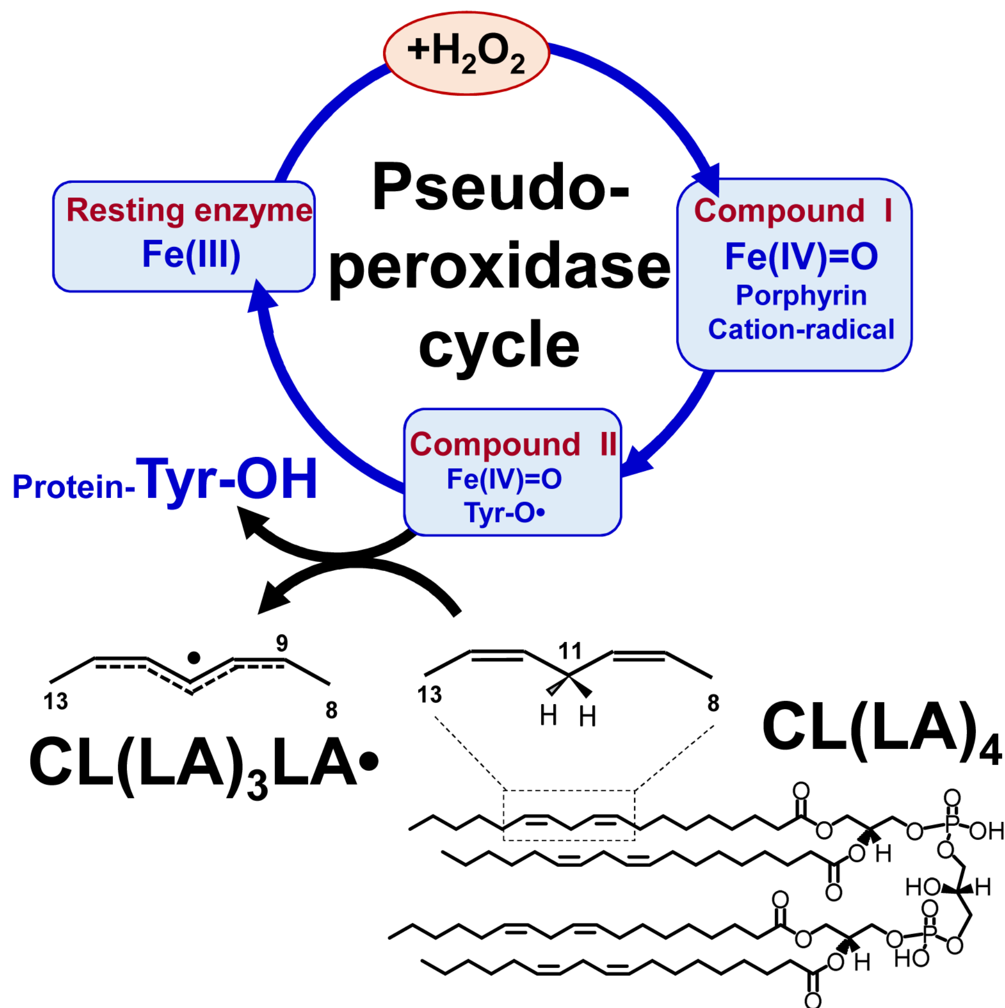

Fig. 9.

Catalytic cycle of pseudo-peroxidases [74, 75]. Interaction between hydrogen peroxide and native ferric pseudo-peroxidase heme leads to the formation of Compound I which is most likely oxoferryl porphyrin-π-cationic radical. Compound I oxidizes amino acid residues (Tyr, Trp, His) located near the heme with the formation of protein based radicals (most likely, Tyr67 of cyt c) and oxoferryl heme (Compound II) [76]. Oxoferryl heme iron can oxidize protein amino acids and peroxidase substrates. The protein-based tyrosyl radicals are the alternative reactive intermediates of pseudo-peroxidases, which oxidize CLH in the mitochondrial membrane as exemplified by TLCL oxidation.

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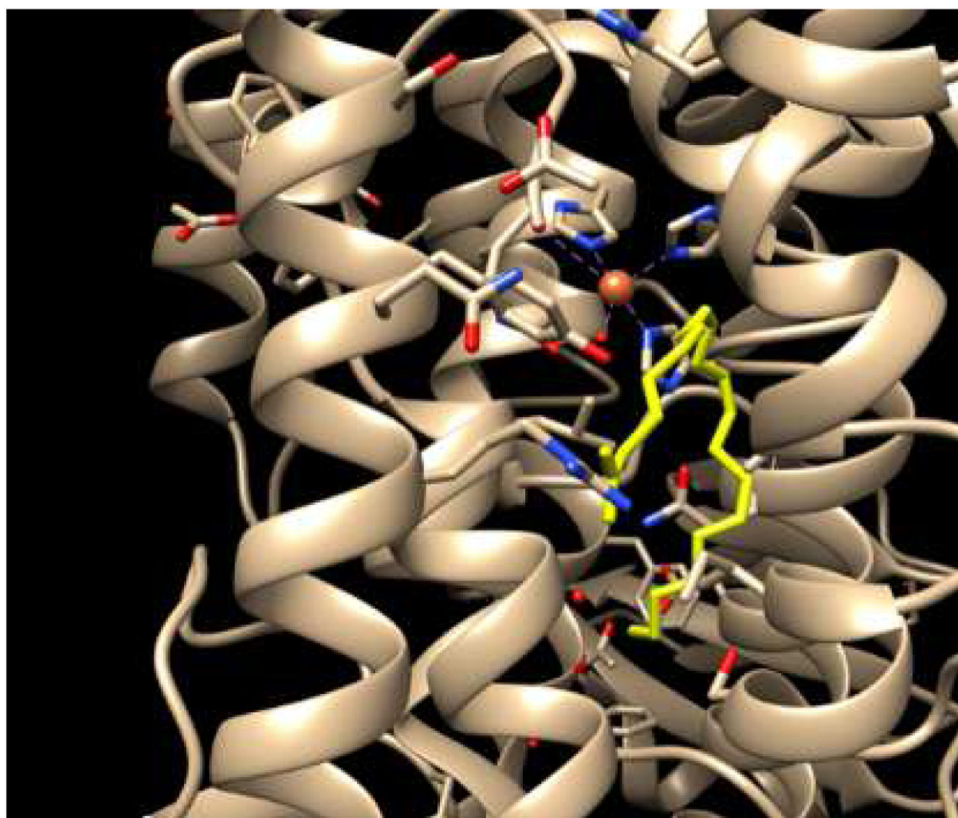

Fig. 10.

X-ray structure showing the constrained substrate placement within the active site of 8R-lipoxygenase (PDB 4QWT) [101]. The arachidonic acid (yellow sticks) is bound in the active site in a specific orientation, such the protein directs the oxidation to occur in a site-specific fashion.

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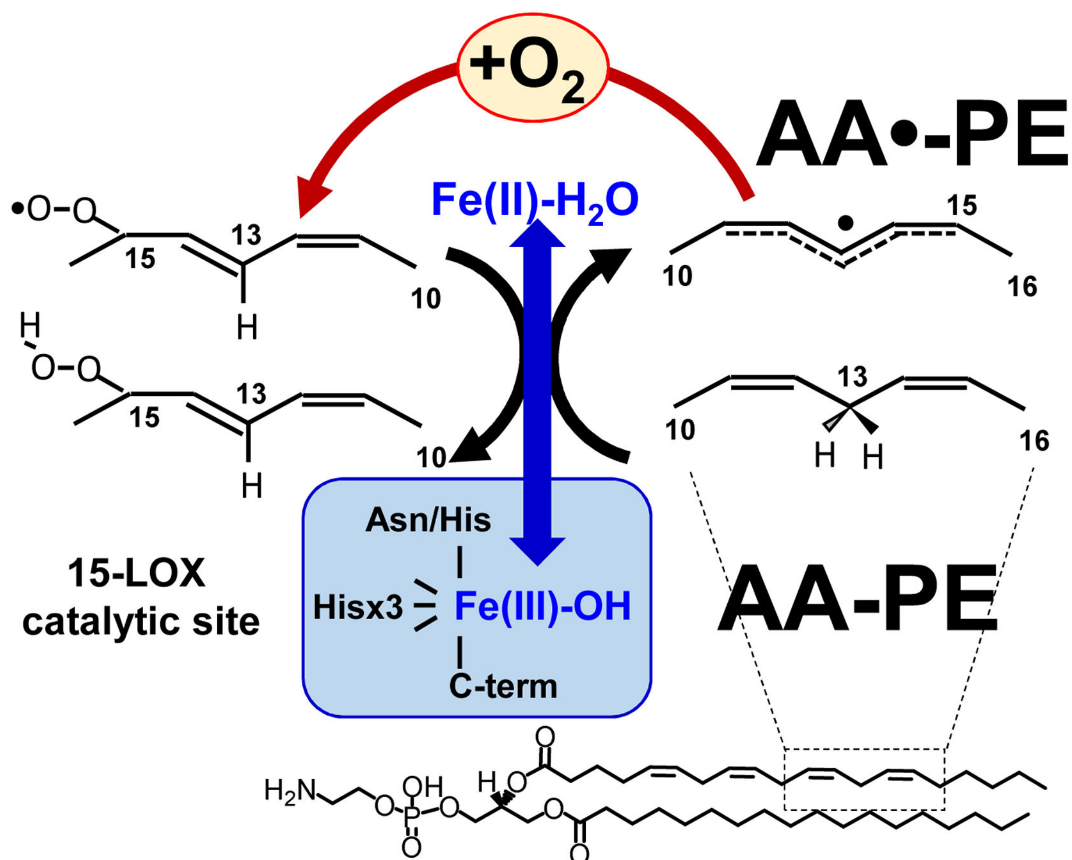

Fig. 11.

Mechanism of LOX catalyzed reaction of PUFA oxidation. The blue arrow shows changes in Fe-containing active site of LOX during FA oxidation. Black arrows follow the reactions catalyzed by redox-active Fe in the LOX catalytic site. Red arrows show the Fe-independent radical rearrangements with simultaneous reaction of oxygen insertion into oxidized FA. Details are in the text.

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Table 1.

Diseases associated with generation of free radicals and antioxidant clinical trials

	The number of entries in Pubmed
Disease	6,526,878
Disease + Free radicals	45,972
Antioxidant Clinical Trials	28,983
Antioxidant Clinical Trials + NIH Funded	18
Antioxidant Clinical Trials + NIH Funded + Successful	0
Antioxidant Clinical Trials + Successful + Positive results	0