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Review article

# Iron catalysis of lipid peroxidation in ferroptosis: Regulated enzymatic or random free radical reaction?

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ABSTRACT

Duality of iron as an essential cofactor of many enzymatic metabolic processes and as a catalyst of poorly controlled redox-cycling reactions defines its possible biological beneficial and hazardous role in the body. In this review, we discuss these two "faces" of iron in a newly conceptualized program of regulated cell death, ferroptosis. Ferroptosis is a genetically programmed iron-dependent form of regulated cell death driven by enhanced lipid peroxidation and insufficient capacity of thiol-dependent mechanisms (glutathione peroxidase 4, GPX4) to eliminate hydroperoxy-lipids. We present arguments favoring the enzymatic mechanisms of ferroptotically engaged non-heme iron of 15-lipoxygenases (15-LOX) in complexes with phosphatidylethanolamine binding protein 1 (PEBP1) as a catalyst of highly selective and specific oxidation reactions of arachidonoyl- (AA) and adrenoyl-phosphatidylethanolamines (PE). We discuss possible role of iron chaperons as control mechanisms for guided iron delivery directly to their "protein clients" thus limiting non-enzymatic redox-cycling reactions. We also consider opportunities of loosely-bound iron to contribute to the production of pro-ferroptotic lipid oxidation products. Finally, we propose a two-stage iron-dependent mechanism for iron in ferroptosis by combining its catalytic role in the 15-LOX-driven production of 15-hydroperoxy-AA-PE (HOO-AA-PE) as well as possible involvement of loosely-bound iron in oxidative cleavage of HOO-AA-PE to oxidatively truncated electrophiles capable of attacking nucleophilic targets in yet to be identified proteins leading to cell demise.

*"At once he became an enigma. One side or the other of his nature was perfectly comprehensible; but both sides together were bewildering."*
Jack London, The Sea Wolf

## 1. Introduction

Cooling of the Earth's crust during the Archean eon created conditions compatible with the utilization of iron for life-sustaining processes - electron transfer, biochemical catalysis and, later, binding and transport of oxygen [1,2]. The role of reduced iron as the electron donor in many reactions, including photosynthesis, and oxidized iron as the universal terminal electron acceptor in respiratory chains, as well as the catalytic propensities of iron as a key cofactor for a variety of enzymatic reactions determined its irreplaceable functions in all living organisms [3–7]. The newly emerging field of radical enzymology points to a possibly important role of iron in primordial hydrogen abstraction reactions occurring anaerobically in primitive organism through tightly coordinated alkyl radical driven mechanisms [8]. The essential high chemical reactivity of iron also imposed the creation of

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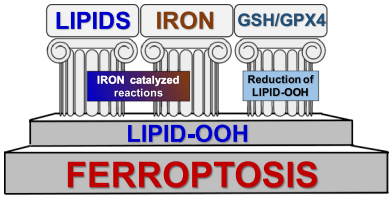



**Fig. 1.** Three pillars of ferroptosis.

highly specialized, complex mechanisms of competition for iron between host cells and invading bacterial pathogens [1], along with those for transport and control of extra- and intra-cellular iron, based on tight binding of this metal by specialized proteins. Paradoxically, the same high catalytic activity of iron may represent the threat to cell's existence through catalysis of poorly controlled Fe-dependent reactions or, as has been recently established, via regulated death programs (eg, apoptosis, ferroptosis) (Fig. 1; [9,10]). This duality of the iron-driven mechanisms, at times representing bewildering conceptual and experimental challenges, is the main subject of the review.

### 1.1. Mishandling of iron, pathogenic mechanisms

In mammalian cells, both deficiency and excess of iron perturb the homeostasis, and physiological conditions can be restored by iron supplementation or chelation, respectively [3,11,12]. Release of iron from transport and storage proteins and formation of low molecular mass iron complexes with cellular ligands (Fe-$L_b$) is viewed as a toxicologically significant event [13–15]. Acute poisoning in humans with iron has been associated with liver, kidney and lung toxicity [11]. In models of chronic iron overload in rodents, it has been observed that the content of iron markedly increases in liver, spleen and kidney with concomitant accumulation of aliphatic aldehydes, which are markers of lipid peroxidation [16–18]. Recent studies indicate that an iron storage protein, ferritin, can release its payload in acidic environment of lysosomes hence create conditions potentially leading to enhanced prooxidant effects. This mishandling of iron has been associated with directly or indirectly with the pathogenesis of worsening of a number of disease conditions including neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases [19]. It is tempting to speculate this dysregulation of iron handling may cause ferroptotic cell death as a pathogenic factor [20,21].

### 1.2. Enzymatic and non-enzymatic iron-catalyzed (bio)chemical reactions

The wealth of biochemical reactions that are mediated by iron reflects its facile redox interconversion between oxidation states and its ability to form complexes with various cellular ligands. Although the oxidation state of iron can vary from -2 to + 7, in biological systems this metal predominantly exists in the relatively stable 2 + (ferrous; $Fe^{(II)}$) and 3 + (ferric; $Fe^{(III)}$) oxidation states. In addition, reactive $Fe^{(IV)}$-oxo species can be formed as short-lived intermediates in a number of biological reactions [22]. Many iron-containing enzymes have been structurally and functionally characterized; however, reliable and accurate protocols for analyzing the cellular distribution of $Fe^{(II)}$ and $Fe^{(III)}$ in Fe-$L_b$ have not been established thus far. Hence, the mechanistic and derivative chemistry of Fe-$L_b$ remains poorly understood and their biological role is often deduced from analyses of the chemical properties of low molecular mass iron complexes with non-physiological ligands.

Since the late 1950s [23,24], a considerable research effort has been directed towards understanding the mechanisms of iron-induced lipid peroxidation in biological membranes. It is commonly believed that non-enzymatic lipid peroxidation is the consequence of the attack on polyunsaturated lipids by hydroxyl radical ($HO^{\bullet}$) generated in so-called Fenton reaction ($Fe^{(II)} + H_2O_2 \rightarrow Fe^{(III)} + HO^{\bullet} + HO^-$; [25,26]); a search in the PubMed database with keywords “iron”, “hydroxyl radical”, “lipid peroxidation”, and “Fenton reaction” renders 198,911, 18,942, 67,330, and 3795 entries, respectively. Detailed studies of lipid peroxidation in isolated mitochondria and membranes of the endoplasmic reticulum (microsomal fraction) catalyzed by exogenously added “free” or "loose" iron have been published [27–31]. However, the relevance of these data to physiological iron-driven mechanisms of lipid peroxidation with a strict control of available iron remains elusive.

Cells consistently produce $H_2O_2$ [32], in many reactions of redox metabolism, particularly those yielding superoxide anion radicals that readily dismutate to $H_2O_2$ [33]. Due to its high reactivity, $HO^{\bullet}$ is one of the most toxic species that can be formed in biological systems. $HO^{\bullet}$ reacts at diffusion-controlled rates with the majority of biomolecules [34], including lipids [35]. Thus, $HO^{\bullet}$ cannot “travel” far from the site of its generation, no further than the nearest molecule. This remarkably high reactivity of $HO^{\bullet}$ imposes kinetic restrictions on the ability of potential exogenous protectors from oxidation of essential biomolecules by $HO^{\bullet}$. Assuming that a radical scavenger reacts with $HO^{\bullet}$ at a diffusion-controlled rate, the concentration of such an effective protector should be at least one order of magnitude higher than that of the intracellular targets. This is well illustrated by cellular thiols, which are present at mM concentrations [36]. As many synthetic candidate radical scavengers exert significant cytotoxicity at such high concentrations, this sets the limit for their applications as protectors against $HO^{\bullet}$. Mother Nature has devised several alternative mechanisms to counteract the high reactivity of $HO^{\bullet}$, mostly based on the prevention of the Fenton chemistry via the elimination of its major precursor, $H_2O_2$ [37,38].

### 1.3. Extra- and intra-cellular mechanisms of iron delivery control its redox activity

Given the high and likely detrimental redox activity of loose iron in cells and extracellular compartments, transportation and delivery of this “high risk” catalyst to its final destinations remains under constant control of specialized proteins (Fig. 2; [3–5,7,12,39–42]). Extracellular iron in the plasma is almost exclusively in the ferric form and bound to circulating transferrin (reviewed in [43]). Transferrin bound iron (TBI) initially binds to transferrin receptor 1 (Tfr1) on the cell surface, which triggers endocytosis of the TBI-Tfr1 complex. In the acidic environment of the endosome, iron is released from transferrin and reduced by ferric reductases of the Steap family [44,45]. Endosomal iron can then be transported across the membrane to the cytosol by a dedicated ferrous-iron transmembrane transporter, Divalent Metal Transporter 1 (DMT1) [46] or by a multispecific metal transporter, ZRT/IRT-like Protein (ZIP) 14 or 8 [47,48]. In some settings, iron presented to the cell surface is not bound to transferrin and can be directly transported into the cell without endocytosis by transporters on the cell surface. Uptake of dietary iron at the apical surface of the intestinal epithelial cell is the most notable example of this type of uptake. In some physiological conditions, e.g. after a large dietary bolus of iron, or pathophysiological settings, e.g. when transferrin is saturated in hereditary iron overload, non-transferrin-bound iron may be present in the portal or systemic circulation and be taken up by transporters on the cell surface. Again, ferric reductase activities encoded by Steaps or the intestinal ferric reductase, duodenal cytochrome b (dCytB) [49] are required prior to transport by DMT1 or ZIP8/14 [50,51].

Upon entering the cytosol, $Fe^{(II)}$ is likely coordinated by a pool of small and macro-molecules that escort the iron to different sites within the cell. These molecules serve to limit the redox activity of iron and promote its interaction with appropriate targets within the cell. The major small molecule proposed to coordinate iron is reduced glutathione (GSH) [52]. GSH can coordinate $Fe^{(II)}$ through a single thiol ligand contributed by its reduced cysteine residue and therefore binds iron with relatively low affinity. Nevertheless, it is present in the cytosol at very high concentrations (2–10 mM), and speciation plots of Fe

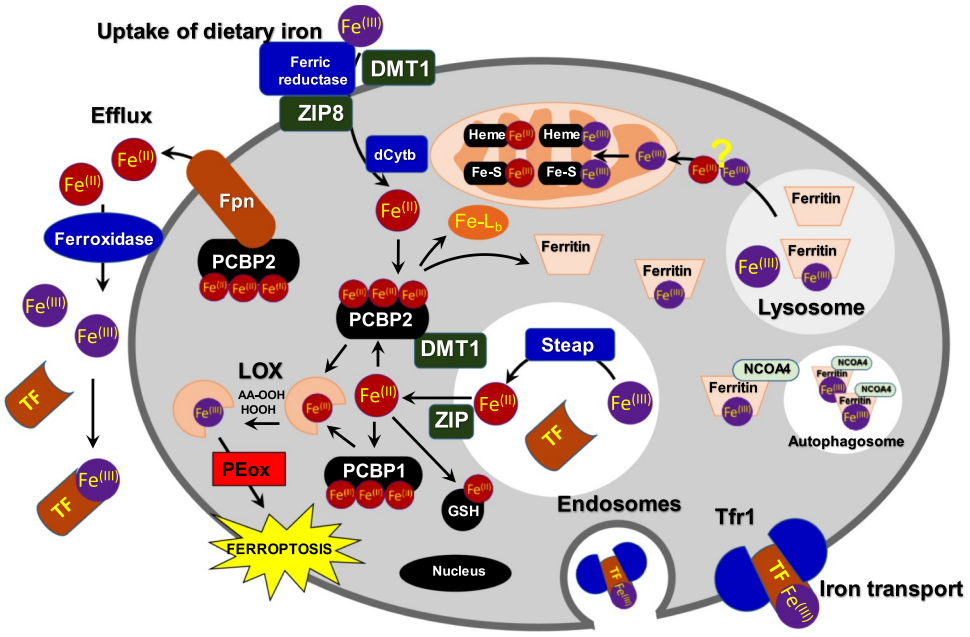



**Fig. 2.** Schema of cellular iron homeostasis. The schema illustrates the various metalloproteins and metallochaperons involved in the influx of dietary iron into the cell, its import/transport into different organelles via different proteins, and its efflux from the cell. dCytB: Duodeneal cytochrome B, DMT1 (SLC11A2): Natural resistance-associated macrophage protein 2, Fe-$L_b$: Low molecular mass iron complexes with cellular ligands, FE-S: Iron-Sulphur clusters, Ferritin: Protein that stores Iron, Fpn (SLC40A1): Ferroportein-1 iron transporter, Ferric reductase: catalyses reduction of Fe(III) to Fe(II), Ferroxidase: catalyses oxidation of $Fe^{(II)}$ to $Fe^{(III)}$, GSH: Reduced Glutathione, HEME: Heme, NCOA4: Nuclear receptor coactivator 4, Autophagic cargo receptor for ferritin., LOX: Lipoxygenase, AA-OOH; Hydroperoxy-arachidonic acid, HOOH; hydrogen peroxide; PCBP1/2: Poly(rC)-binding protein-1, Iron chaperone, PE: Phosphatidylethanolamine lipid, Steap: Metalloreductase, TF: Transferrin, Tfr1: Transferrin Receptor protein 1, ZIP14/8 (SCL39A14): Zinc and iron permease.

(II) and GSH suggest that this is the major small molecule ligand for iron in the cytosol.

Macromolecular ligands for cytosolic iron include the iron chaperones of the Poly rC Binding-Protein (PCBP) family [53]. These are multifunctional adaptor proteins that bind single-stranded nucleic acids, other proteins, and iron. The most abundant PCBPs are PCBP1 and PCBP2, both of which can bind $Fe^{(II)}$ in a 3:1 stoichiometry with low micromolar affinity [54,55]. PCBP2 can directly interact with DMT1 to bind iron and facilitate its transfer to the cytosol [56]. A similar interaction with heme oxygenase, a membrane associated enzyme that liberates iron from heme, allows PCBP2 to capture iron released from heme [57]. PCBP2 can also interact with PCBP1, which binds cytosolic iron and delivers it to client proteins, such as ferritin, the iron storage protein [55], and to non-heme iron enzymes, such as 2-oxoglutarate-dependent dioxygenases (which have mononuclear iron centers) [58] and monooxygenases of the fatty acid hydroxylase/desaturase type (which have dinuclear iron centers) [59]. Cytosolic lipoxygenases implicated in ferroptosis contain mononuclear iron centers and, as such, are likely to receive iron from PCBP chaperones, although this has not been directly demonstrated. Under conditions of iron deficiency, iron stored in cytosolic ferritin can undergo recycling by autophagic turnover of ferritin in the lysosome [60–62]. The autophagic cargo receptor for ferritin is Nuclear Co-Activator 4 (NCOA4), another multifunctional protein that can modulate transcription of nuclear genes or bind to ferritin to direct it into the autophagosome [63–65]. Lysosomal $Fe^{(III)}$ released from degraded ferritin can be returned to the cytosol or directed to the mitochondria for incorporation into mitochondrial iron cofactors (Heme, Fe-S clusters, non-heme iron centers). Mechanisms of lysosomal iron transfer in mammalian cells are not completely understood as yet.

Finally, iron can exit cells through the sole iron transmembrane exporter, ferroportin (Fpn) [66–68]. PCBP2 may direct cytosolic iron to Fpn for export, as PCBP2 has been found to bind directly to Fpn and, in some cell types, PCBP2 depletion can impair Fpn-mediated iron efflux [69]. $Fe^{(II)}$ that exits the cell is rapidly converted to $Fe^{(III)}$ for loading onto transferrin, a reaction that is catalyzed by multicopper ferroxidases, such as the circulating enzyme ceruloplasmin and the membrane-bound hephaestin [70,71]. The efflux of $Fe^{(II)}$ through Fpn appears to be coupled to the extracellular oxidation and loading of iron onto transferrin, as rates of $Fe^{(II)}$ efflux are augmented by the presence of extracellular ferroxidases and transferrin.

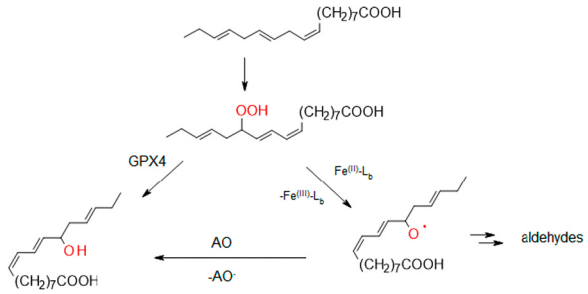
### 1.4. Iron is central to the execution of the ferroptotic death program

A new wave of attention to specific mechanisms of iron has been induced by its central role in a recently identified type of genetically programmed cell death, ferroptosis [72]. Three major pillars of ferroptosis – *iron catalysis* of *lipid peroxidation* yielding the hydroperoxy-products and their (also iron-catalyzed) *cleavage to reactive, oxidatively-truncated electrophiles* (instead of the effective reduction to alcohols by glutathione peroxidase type 4 (GPX4)) – are the most important stages of the ferroptotic program (Fig. 1). Two major concepts on the role of iron-catalyzed reactions - stochastic free radical catalysis by Fe-$L_b$ vs tightly regulated enzymatic process controlled by Fe-protein(s) – have been considered as the mechanisms ultimately leading to the ferroptotic cell demise.

While these alternative mechanisms may seem a relatively narrow and even technical issue, in fact they reflect the general approach and philosophy of a very broad area of research in contemporary redox biology. Indeed, the success in understanding of free radical mechanisms of organic oxidation reactions in liquid phase inspired the transfer of these powerful concepts to the field of biology and encouraged the emergence of free radical biomedicine. Among the most popular concepts was iron-catalyzed free radical lipid peroxidation and membrane injury as one of the leading pathogenic mechanisms in a multitude of diseases, including major cardiovascular, neurodegenerative and neoplastic conditions [29]. The idea of uncontrolled free radical chain reactions as the major mechanism of injury and the opportunity of "fixing" it by antioxidants was so obvious and attractive that numerous experimental, pre-clinical and very expensive and time-consuming clinical trials have been funded and conducted. The results were disappointingly uniform - these studies did not reveal clinically significant positive results [73]. While meta-analysis indicated that a serious rethinking of the underlying concepts and technological methodologies may be necessary [74], the tendency to directly describe and interpret biological phenomena in terms of random non-enzymatically controlled chemical reactions remains persistent.

### 1.5. Lipid peroxidation is a hallmark of ferroptosis

The new concept of ferroptosis may represent a unique opportunity and testing ground for understanding the role of iron and radical-driven reactions in cell metabolism and fate. In cells, GPX4 catalyses the




**Scheme 1.** Inhibition of GPX4 leads to the formation of alkoxyl radicals.

reduction of fatty acid and phospholipid hydroperoxides to alcohols [75–77]. It is hypothesized that inhibition of GPX4 leads to reductive cleavage of hydroperoxides by $Fe^{(II)}$-$L_b$ to alkoxyl radicals (Scheme 1; [78]), which sets the stage for the formation of aliphatic aldehydes [78] that readily react with thiol and amino groups in proteins [79]. Random induction of lipid peroxidation can be envisioned as a process where Fenton-like reactions lead to a stochastic formation of lipid hydroperoxides. The paramount difference between random and enzymatically-induced lipid peroxidation in ferroptotic cells lays in the initial substrate specificity of the reactions, which would define the nature of the end-reaction products.

### *1.6. 15-LOX-driven enzymatic generation of ferroptotic signals*

The role of the enzymatically regulated process catalyzed by lipoxygenases [LOXes] (most likely 15-LOX) is supported by several lines of evidence, including selectivity towards peroxidation substrates and specificity of the oxidation products formed. Indeed, among thousands of molecular species of oxidizable phospholipids, two molecular species of arachidonoyl-PE and two of adrenoyl-PE were identified as substrates of 15LOX-catalyzed oxygenation reactions [80]. Moreover, only four hydroperoxy-PE - 15-HpETE- (and 17-HOO-AdA-PE) were identified as specific oxidation products catalyzed by 15-LOX in the presence of a scaffold protein, PEBP1 [81]. The latter forms a complex with both isoforms of 15-LOX – 15LO1 and 15-LO2 – in which the catalytic competence of the enzyme is changed from free AA to AA-PE. This is achieved via allosteric changes in the organization of the catalytic site of 15-LOX induced by PEBP1 as well as by the ability of the latter to bind up to nine free AA thus depleting free AA in the microenvironment of 15LOX. These features of AA-PE oxidation are compatible with the enzymatic nature and genetically conserved mechanisms of the overall ferroptotic process. Noteworthy, not only eukaryotic (mammalian) 15-LOXes but also prokaryotic (bacterial) 15-LOX can be involved in the selective and specific oxidation of AA-PE to 15-HpETE-PE and ferroptosis of the host organism [82]. For example, 15-LOX of a Gram-negative pathogen, *P. aeruginosa*, can oxidize AA-PE in the host bronchioepithelial cells via a “theft-ferroptosis” pathway thus facilitating and propagating its effective colonization, particularly in immune-compromised organisms (eg, in cystic fibrosis and antiobiotic-resistant pneumonias) [82].

Further support to the notion for controlled lipid peroxidation is the strong dependence of ferroptosis on several lipid-metabolizing proteins specific for arachidonic and adrenic acids, such as ASCL4 required for the biosynthesis of oxidation substrates AA-PE and AdA-PE [80,83]. In addition, a trans-acylase, LPCAT3, participating in the maintenance of sufficient levels of these oxidation substrates is also a significant required constituent of the ferroptotic program [80] (Fig. 3).

## 2. Non-enzymatic iron-catalyzed free radical lipid peroxidation as a pro-ferroptotic mechanism

The anti-ferroptotic activity of a variety of free radical scavengers (Table 1) has provided a foundation for the hypothesis that ferroptosis is triggered by random, Fe-$L_b$-catalyzed free radical reactions. Zilka et al. favored the random free radical pathway vs. LOX-catalyzed oxidation because, in liposomal suspensions, Ferrostatin-1 (Fer-1) and Liproxstatin-1 (Lip-1) proved better scavengers of $ROO^{\bullet}$ than inhibitors of LOXs [84] as anti-ferroptotic agents [80,85,86].

The authors argued that the observations made in their model system can be extrapolated to intact cells, as they are consistent with the greater potency of Fer-1 and Lip-1 relative to α-tocopherol and specific inhibitors of LOXs to impede ferroptosis. However, validation of this hypothesis would require assessment of the incorporation of α-tocopherol in liposomes as reported in ref. [84], as well as analysis of both the intracellular concentrations and the compartmentalization of Fer-1 and Lip-1 in experiments with intact cells.

Fer-1 contains an esterified carboxylic group that is most likely hydrolyzed by intracellular esterases. The latter reaction would maintain a continuous uptake of Fer-1 from the incubation medium and can lead to relatively high intracellular concentrations of the carboxylate anion of this amine. Furthermore, Fer-1 and Lip-1 are lipophilic amines/imines that may accumulate in acidic intracellular compartments, such as lysosomes, through a mechanism referred to as “ion trapping” [90,91]. This mechanism includes the protonation of basic groups in lipophilic organic molecules and is exemplified by the compartmentalization of a number of drugs containing amino groups, whose lysosomal concentrations are orders of magnitude higher than those in the extracellular milieu [90,91]. Hence, it would be interesting to assess (i) the cytosolic and lysosomal concentrations of both Fer-1 and Lip-1, with the notion that lysosomes are viewed as a major source of redox active Fe-$L_b$ [92–95], including in cells undergoing ferroptosis [96], and (ii) whether at the concentrations found in cells Lip-1 and Fer-1 inhibit LOXs. Of note, different homologues of tocopherols and tocotrienols may act as inhibitors of LOX by occupying the substrate binding pocket [80]. It may be particularly important to assess the effects of tocopherols as inhibitors of AA-PE oxidation by 15-LOX/PEBP1 complexes involved in the execution of ferroptotic program [80]. It is also tempting to speculate that Fer-1 and Lip-1 can bind iron. Although iron complexes of Fer-1 and Lip-1 have not been characterized thus far, organic amines and imines, including benzene-1,2-diamine derivatives are known to chelate iron [97–101]. Some of these complexes have been found to exhibit catalase-like activities [102,103].

Over the past 80 years, considerable progress has been made in the development of free radical chemistry [104]. However, the assessment of preponderant free radical reactions in complex biological matrices remains challenging. This is due, at least in part, to the limited methods and/or lack of specific molecular probes for analysis of short-lived species. Indeed, the discrimination of iron-, oxygen-centered radicals-, and LOXs-induced ferroptosis has proven difficult because of the overlapping reactivity of the probes used to analyze this death pathway (Table 1): (i) the iron chelators and inhibitors of ferroptosis, Deferoxamine, Ciclopirox, and Deferiprone are good scavengers of free radicals [105–107] that also disrupt cellular processes such as DNA repair, cell division signals, and protein synthesis by mechanisms that are not fully understood [108]; (ii) in addition to reactions with oxygen-centered radicals, the antioxidants used to impede ferroptosis have the potential to reduce metal ions [100,109,110] and to act as substrates of peroxidases [111,112]; (iii) most inhibitors of LOXs are efficient scavengers of free radicals [113]; and (iv) $Fe^{(IV)}$-$L_b$-oxo complexes may account for the deleterious effects of Fe-$L_b$ [114–116]. Recent studies with synthetic low molecular mass iron complexes have shown that they can utilize oxygen and $H_2O_2$ for inner-sphere hydroxylation of aromatic ligands [117,118], C-H hydroxylation of alkanes [119–121], and epoxidation of alkenes [122–124]. However, the question of whether Fe-$L_b$-oxo complexes are formed in cells and contribute to the toxicity of iron remains to be resolved. It should also be mentioned that triggering of lipid peroxidation in ferroptotic cells may be preceded or paralleled by Fe-$L_n$- or $Fe^{(iv)}$-$L_b$-oxo-induced modification of the enzymatic activities of redox-sensitive proteins [125–127].

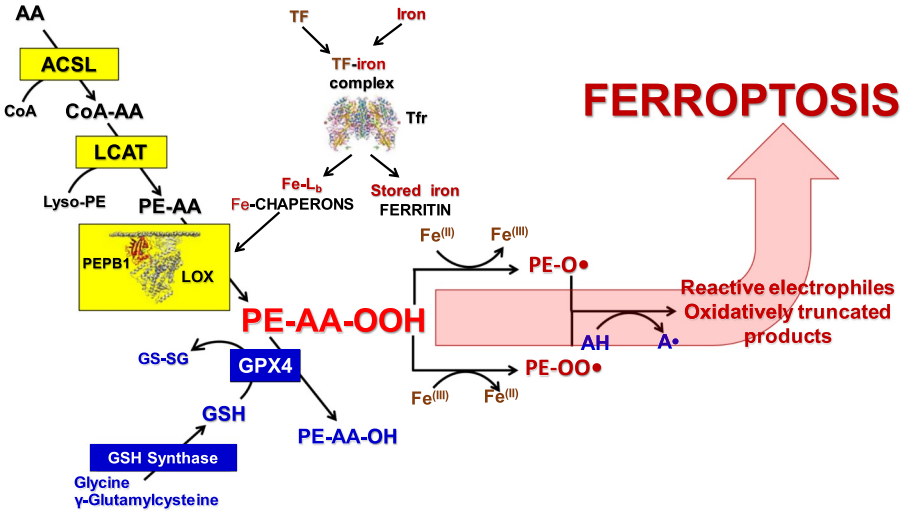



**Fig. 3.** Schema illustrating three pillars of ferroptotic program: Lipid peroxidation, Iron handling pathways and GPX4-dependent reduction of hydroperoxy-phospholipids. ACSL, Acyl-CoA synthase; LCAT, Lyso-phospholipid acyl transferase; PEBP1, Phosphatidylethanolamine binding protein 1; LOX, Lipoxygenase; GSH, Glutathione; GS-SG, Oxidized glutathione; AA, arachidonic acid; PE-AA-OOH, Hydroperoxy-arachidonoyl phosphatidylethanolamine; PE-AA-OH, Hydroxy-arachidonoyl phosphatidylethanolamine;GPX4, Glutathione peroxidase 4; AH, Antioxidants (phenols, aromatic amines); Fe-$L_b$, Low molecular mass iron complexes with cellular ligands; TF, Transferrin; Tfr, Transferrin receptor.

### *2.1. Combination of enzymatic generation of HOO-AA-PE and their oxidative cleavage in ferroptosis*

The nature of the immediate lipid peroxidation products acting as proximate pro-ferroptotic signals still remains to be elucidated.

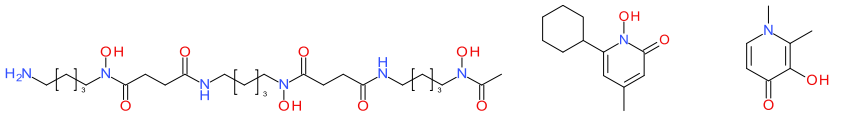
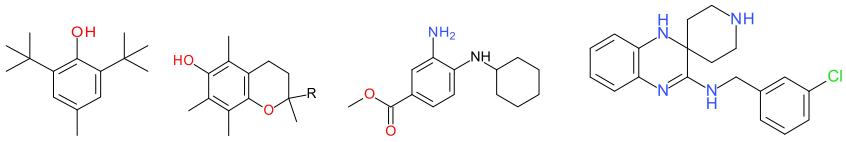
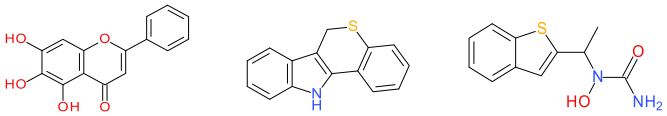
It is likely that the overall process includes two stages: (i) selective and specific enzymatic production of 15-HOO-AA-PE by 15-LOX; and ii) oxidative cleavage of these initial HOO-derivatives to proximate electrophiles capable of interacting with protein targets to cause the formation of pores in plasma membranes, or to rupture them. One can envision that two types of oxidatively-truncated products can be formed from HOO-AA-PE – with the carbonyl function either on the shortened AA-residue esterified into PE or on the leaving aldehyde (Scheme 2).

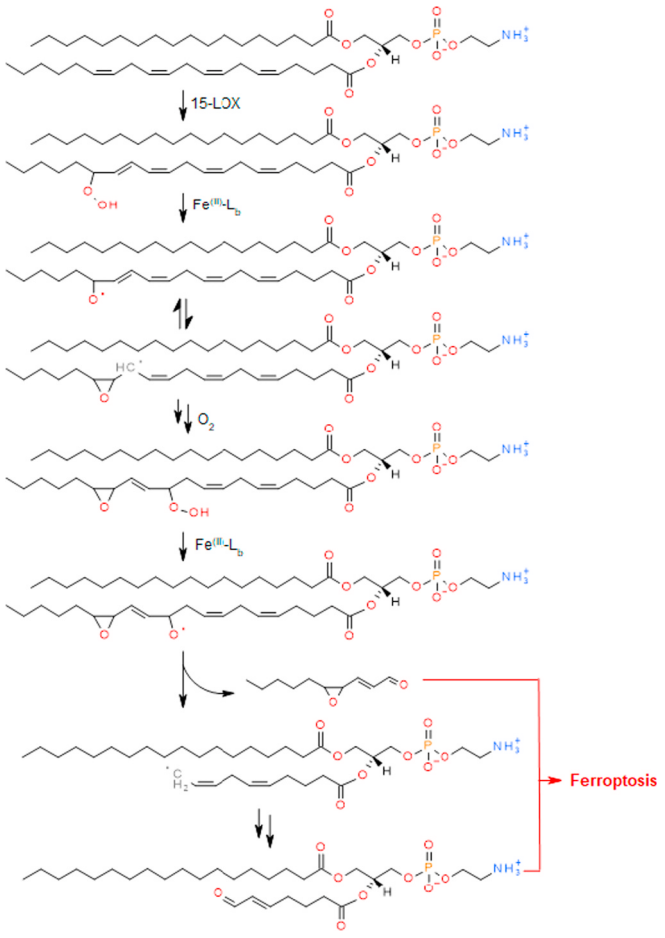
In the first scenario, the specificity of PE-derived electrophiles will be retained – in contrast to the free aldehydes that can be formed during oxidative truncation of any (phospho)lipid. Much more work is necessary to identify the chemical nature of these proximal PE oxidation products, the mechanisms of their formation, as well as their protein targets and adducts. The specificity of the process of oxidative truncation of HOO-AA-PE catalyzed by Fe-protein complexes or by Fe-$L_b$ needs to be thoroughly investigated. Furthermore, the role of coordination of iron by different intracellular chaperons and their contributory role in the strict control of iron redox-cycling activity via its tight "caging" within redox "silent" complexes needs to be established.

The pleiotropic mechanisms of action of ferroptosis inhibitors, the uncertainty of characteristic morphological features as well as biochemical biomarkers of ferroptosis makes it difficult to specifically ascertain the engagement of this cell death mechanism, particularly in vivo as illustrated by the following example. While iron is predominantly coordinated within proteins, it can also be present in metabolically available pools of kinetically labile iron, often referred to as the labile iron pool. The levels of the labile iron pool are regulated by iron-responsive factors that balance iron uptake and utilization with deposition into ferritin or efflux [128]. Increased levels of labile iron may create pro-oxidant conditions and cause cell injury and death, possibly ferroptosis. Much of the labile iron pool is coordinated by iron chaperones of the PCBP family. In the absence of these, the redox activity of the labile iron pool might be significantly altered.

We performed LC-MS-based lipidomics analysis on liver tissue from C57Bl6 mice specifically lacking PCBP1 in hepatocytes (C.C. Philpott, in preparation). These mice exhibit marked disturbances in lipid metabolism. We were interested in determining whether accumulation of pro-ferroptotic biomarkers of PE oxidation could be associated with

**Table 1**
The major types of small molecule inhibitors of ferroptosis.

| | | Ref. |
|---|---|---|
| Iron chelators |  Deferoxamine Ciclopirox Deferiprone | [10,84,87] |
| Antioxidants |  BHT α-tocopherol Ferrostatin-1 Liproxstatin-1 | [10,84] |
| LOX inhibitors |  Baicalein PD 146176 Zileuton | [72,85,88,89] |




**Scheme 2.** Proposed reaction sequence leading to ferroptosis. Reactions with two arrows denote multiple steps that are omitted from the reaction scheme.

PCBP1-deficiency. Redox phospholipidomics analysis of liver homogenates revealed 56 molecular species of oxygenated PE. However, the levels of HOO-AA-PE previously identified as biomarkers of ferroptosis [80], were not significantly elevated in the livers of PCBP1-deficient compared to WT mice (Fig. 4).

We detected 53 oxidized species of the most abundant phospholipid, PC. Among those, the levels of nine species were different ($p < 0.05$) between WT and PCBP1-deficient samples, but only two species exhibited a > 2-fold increase and these specifically accumulated in the PCBP1-deficient livers. However, neither of these PC oxidation products have been associated with the execution of the ferroptotic program. Because PCBP1 functions as an iron chaperone for other mononuclear iron enzymes, lipoxygenases that specifically modify AA-PE may have reduced activity in PCBP1-deficient tissues. Furthermore, 15-LOX is expressed at very low levels in murine liver [129]. Thus, the GPX4 activity may be sufficient to reduce HOO-AA-PE to alcohols (HO-AA-PE), and prevent the triggering of the ferroptotic program. These observations suggest that perturbations in the reactivity of the labile iron pool may not be sufficient to trigger ferroptosis. This also illustrates the complexity of the task of revealing the involvement of ferroptosis in the aberrant reactions of lipid metabolism and lipid peroxidation in vivo.

## 3. Concluding remarks

Discovery and characterization of several programmed cell death mechanisms has already facilitated the identification of their regulators with potential therapeutic effects. Further work on their improvement requires exact knowledge of specific features and metabolic networks engaged in the execution of death programs. Interestingly, all three major pathways of regulated cell death – apoptosis, necroptosis and ferroptosis - are believed to include lipid peroxidation as an essential part of the program. Indeed, oxygenation of cardiolipins has been shown to be essential to apoptosis, whereas oxygenation of phosphatidylethanolamines is characteristic of ferroptosis. Lipid peroxidation has been postulated as an important part of necroptotic cell death program but specific lipid substrates have not been identified so far. While the name ferroptosis emphasizes the central role of iron-driven reactions in this type of regulated cell death, the nature of the mechanisms involved remains controversial. A wealth of experimental data support the contribution of strictly controlled “caged” iron-dependent processes catalyzed by 15-LOX/PEBP1 complexes with their high selectivity and specificity towards the substrates and products of the reactions. There are also results pointing to the role of loosely-bound iron in ferroptosis. These two seemingly opposing concepts may be resolved within the framework of a two-stage process: catalysis by 15-LOX/PEBP1 complex yielding 15-OOH-AA-PEs and oxidative cleavage/truncation of hydroperoxy-products. The latter may include less specific participation of small molecule iron ligands or other yet to be identified Fe-peptides/proteins. The resolution of this conundrum strongly depends on the identification of the immediate ferroptosis-inducing proteins responsible for the disruption of the cell membrane leading to cell demise. This task requires refined and detailed redox proteomics-lipidomics analyses aimed at detecting of protein conjugates with oxidatively truncated PEox products.

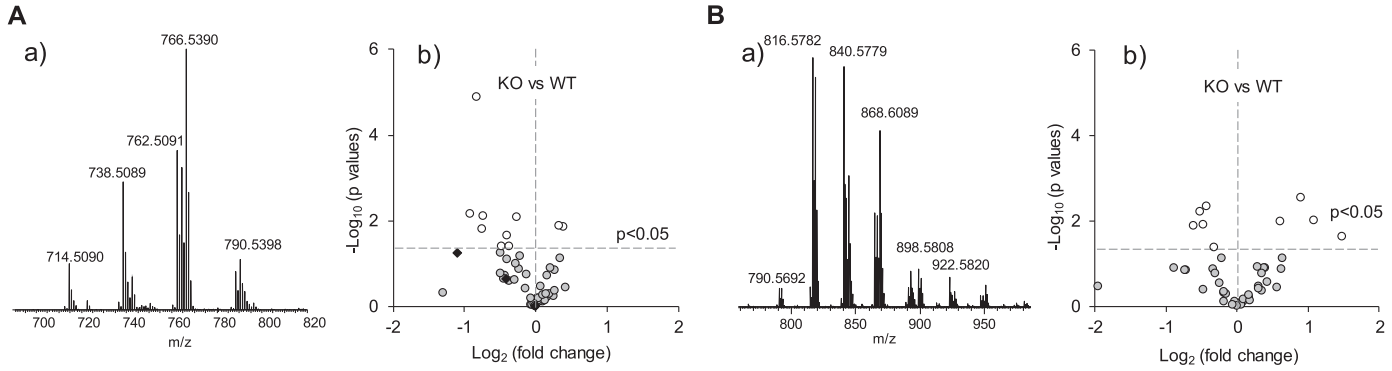



**Fig. 4.** Assessment of oxygenated phospholipid species in mouse liver. Typical MS spectra of phosphatidylethanolamine (PE) (A-a) and phosphatidylcholine (PC) (B-a) obtained from WT mouse liver. Analysis of oxygenated PE species (A-b) and oxygenated PC (B-b) in liver of PCBP1-deleted (KO) vs. wild type (WT) mice. Male mice were 5–6 weeks old and maintained on a synthetic, defined diet containing adequate, but not elevated, amounts of iron (50 ppm). Ferroptotic cell death signals (hydroperoxy-PE species) are shown as closed diamonds. N = 6.

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