Critical Review

Iron and Ferroptosis: A Still Ill-Defined Liaison

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Abstract

Ferroptosis is a recently described form of regulated necrotic cell death, which appears to contribute to a number of diseases, such as tissue ischemia/reperfusion injury, acute renal failure, and neurodegeneration. A hallmark of ferroptosis is iron-dependent lipid peroxidation, which can be inhibited by the key ferroptosis regulator glutathione peroxidase 4(Gpx4), radical trapping antioxidants and ferroptosis-specific inhibitors, such as ferrostatins and liproxstatins, as well as iron chelation. Although great strides have been made towards a better understanding of the proximate signals of distinctive lipid peroxides in ferroptosis, still little is known about the mechanistic implication of iron in the ferroptotic process. Hence, this review aims at summarizing recent advances in our understanding to what is known about enzymatic and non-enzymatic routes of lipid peroxidation, the involvement of iron in this process and the identification of novel players in ferroptotic cell death. Additionally, we review early works carried out long time before the term "ferroptosis" was actually introduced but which were instrumental in a better understanding of the role of ferroptosis in physiological and pathophysiological contexts. © 2017 IUBMB Life, 69(6):423–434, 2017

Keywords: *ferritinophagy; glutathione peroxidase 4; Gpx4; labile iron pool; lipid peroxidation; regulated necrosis; electrophile signaling*

Introduction

The term cell death, first mentioned in 1842 (1), describes the ultimate ending of all essential cellular processes in living cells. As such, cell death antagonizes all cellular functions, thus representing an equally important mechanism for proper development and tissue homeostasis of multicellular organisms. Yet under certain conditions, including physical and chemical injuries of the plasma membrane and other cell constituents, cells are unable to control the cell death process, which is therefore commonly referred to as "accidental/explosive" or necrotic cell death. In contrast to this unregulated form of cell death, nature has evolved a variety of highly controlled cell death modalities

Abbreviations: *Gpx4, glutathione peroxidase; RCD, regulated cell death; HD, Huntington's disease; GSH, glutathione; System x_c^-, Na^+-independent cystine-glutamate antiporter; Nrf2, nuclear factor E2-related factor; keap1, kelch-like ECH-associated protein 1; α-toc, α-tocopherol; Alox12, arachidonate 12-lipoxygenase; ; Alox5, arachidonate 5-lipoxygenase; Alox15, arachidonate 15-lipoxygenase; AA, arachidonic acid; ; AdA, adrenic acid; PE, phosphatidylethanolamine; BSO, L-buthionine sulfoximine; RSL3, (1S, 3R)-RAS synthetic lethal 3; FIN, ferroptosis inducing agents; DFO, deferoxamine; Fer-1, ferrostatin-1; Lip-1, liproxstatin-1; PUFAs, polyunsaturated fatty acids; FAs, fatty acids; CoA, coenzyme A; Acsl4, acyl-CoA synthetase long chain family member 4; Lpcat3, lysophosphatidylcholine acyltransferase 3; HT-1080, human fibrosarcoma cells; PL, phospholipid; p53, tumor protein p53; xCT, solute carrier family 7 member 11; CoQ_{10}, coenzyme Q_{10}; LOX, lipoxygenase; NADH, reduced form of nicotinamide adenine dinucleotide; NADPH, NADH phosphate; LIP, labile iron pool; $•O_2^-$, superoxide radical; H_2O_2, hydrogen peroxide; •OH, hydroxyl radical; PL-OOH, phospholipid hydroperoxide; PL-OO•, phospholipid peroxyl radical; PL-O•, phospholipid alkoxyl radicals; PLA_2, phospholipase A2; MEF, mouse embryonic fibroblast; ROS, reactive oxygen species; MAM, mitochondria-associated membranes*

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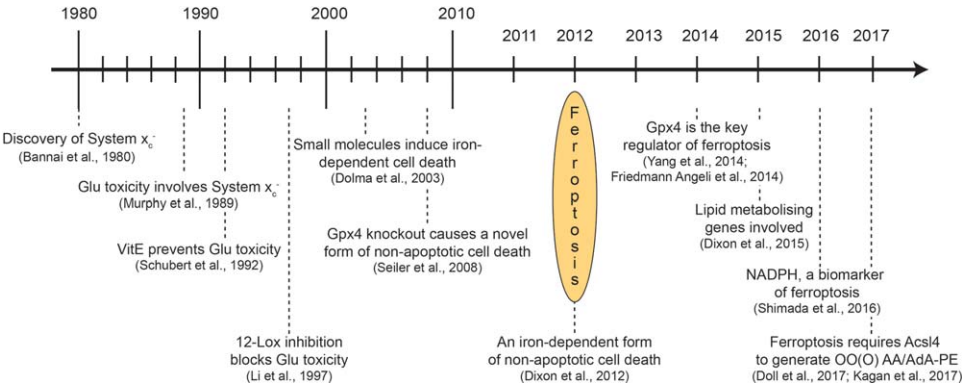

FIG 1

Schematic timeline depicting important discoveries in the field of ferroptosis research.

(regulated cell death, RCD), which ensure that during normal development and tissue homeostasis the final decision of life versus death is properly orchestrated. Starting with the recognition of apoptosis, the prime example of "programmed cell death" (2), extensive research performed in recent years has led to identification of a number of regulated necrotic cell death routines, such as necroptosis, netosis, entosis, and cyclophilin D-mediated cell death (3). Among these forms of nonapoptotic cell death, ferroptosis (derived from the Greek word ptosis, meaning "a fall," and ferrum, the Latin word for iron) has been first described in 2012 as an iron-dependent form of RCD, which is characterized by the requirement of redox active iron (4). At the heart of ferroptosis is the selenoenzyme glutathione peroxidase 4 (Gpx4), which for its unique activity to prevent uncontrolled peroxidation of phospholipids (PLs) has been proposed to be the most central downstream ferroptosis regulator (5,6). While a certain amount of iron is essential for cell proliferation and survival (7), iron accumulation along with an increase in lipid-associated radicals and lipid peroxides, both hallmarks of ferroptosis (4), may trigger cell death. In fact, these proferroptotic conditions have been linked with many pathologies including acute traumatic injuries and neurodegenerative diseases including Alzheimer's, Parkinson's, and Huntington's disease (HD; *8*). Even though a role of iron in the ferroptotic process has been postulated, its involvement in ferroptosis remains poorly understood. More specifically, the generation of toxic lipid-derived peroxide species may result from enzymatic or non-enzymatic processes that both involve different forms of catalytically active iron. Therefore, a critical evaluation of the exact role of iron during ferroptosis is of great interest not only for a better understanding of the molecular signature of ferroptosis, but also for the development of potent antiferroptotic strategies based on iron chelation.

Early Days in "Ferroptosis Research"

Ferroptotic cell death is characterized by iron-dependent generation of lipid peroxidation products that leads to a sudden cell death with necrotic phenotype (4). Hence, mechanisms that act upstream of this chain of events including cystine (*i.e.*, the oxidized form of cysteine) uptake, synthesis of the antioxidant tripeptide glutathione (GSH) and proper functioning of Gpx4 must be regarded as key cellular processes suppressing ferroptosis (8). Yet perturbations of any of these events have been linked with cell death long before the term ferroptosis was actually coined (Fig. 1). Perhaps one of the earlier discoveries in this respect includes data that came from works on the metabolic interaction between glutamate and cystine. Cystine, the predominant form of cysteine found in the extracellular space, is taken up by cells via the concentration-driven Na^+-independent cystine-glutamate antiporter (system x_c^-), which releases one molecule of glutamate per molecule cystine taken up (9). System x_c^- is a heterodimeric amino acid transporter consisting of the cell surface antigen heavy chain (4F2) and the solute carrier family 7 member 11 (xCT) light chain that are linked by an intermolecular disulfide bond (10). Expression of *xCT* is among others regulated by the nrf2-keap1 pathway (nrf2, nuclear factor E2-related factor- kelch-like ECH-associated protein 1; (11,12)), which enhances transcription under oxidative stress; treatment with electrophilic agents or during high oxygen tensions (13–15). Under atmospheric oxygen conditions cysteine hardly exists in its reduced form, so cells or organoids cultured *ex vivo* are highly dependent on system x_c^- to maintain the intracellular cysteine levels necessary for GSH biosynthesis (16). Consequently, the removal of cystine from cell culture media leads to intracellular GSH deprivation followed by cell death (17). Interestingly, another amino acid transporter namely cystinuria-related type II membrane glycoprotein (related to $b^{0,+}$ amino acid transporter) facilitates the Na^+-independent cystine transport when expressed in COS-7 cells (18). Expression of this transporter *in vivo* is, however, limited to brush border membranes of kidney and small intestine, thus ruling out a general effect in cystine uptake. In stark contrast to the *in vitro/ex vivo* situation, *in vivo* cysteine concentrations in plasma and extracellular fluids are by far not as low as in cell culture, and thus cysteine is available in its reduced form by a different set of amino acid transporters, such as the alanine, serine, cysteine-preferring amino acid transport system, large amino acid transporter 2, and excitatory amino acid carrier1 (in neuronal cells). Hence, all these systems are present *in vivo* to cope

with the cells' demand for cysteine to prevent cysteine starvation and GSH depletion (18–21). Thus, the function of system x_c^- is complemented by other systems to provide intracellular cysteine *in vivo*. Nevertheless, some cell types, particularly of the central nervous system (area postrema, subfornical organ, habenular nucleus, hypothalamic area, and ependymal cells of the lateral wall of the third ventricle in the adult mouse brain) maintain high expression of system x_c^- (22). In this regard, cystine uptake, which is inevitably coupled to the release of glutamate, must be tightly controlled as high extracellular glutamate concentrations can cause oxidative glutamate toxicity to cell types that depend on system x_c^- like immature cortical neurons (by inhibition of cystine uptake; (23,24)). Of note, glutamate also acts as a neurotransmitter and elevated levels can lead to excitotoxicity and neuronal cell death via glutamate receptor overstimulation (25).

Early reports by Murphy et al. (1989) and Schubert et al. (1992) indicated that antioxidant supplementation (e.g., α-tocopherol, α-toc) prevents glutamate-induced oxidative stress and cell death in neuronal cell lines lacking *N*-methyl-D-aspartate receptor (23,26). Soon thereafter, the same laboratory discovered that inhibition of arachidonate 12-lipoxygenase (Alox12), an iron-containing lipid dioxygenases, effectively inhibited oxidative glutamate toxicity, and cell death in the hippocampal cell line HT22 and in primary cortical neurons (27). Conversely, treatment of cells with exogenous arachidonic acid (AA), a Alox12 substrate, further potentiated this kind of cell death (27). As this form of cell death induced by oxidative stress in neuronal cells was distinct from apoptosis it was later dubbed "oxytosis" (28). Some years later, by using conditional knockout mouse embryonic fibroblast (MEF) cell line and mouse models for Gpx4, Seiler and colleagues provided first evidence that knockout of Gpx4 causes a novel form of non-apoptotic cell death (which we now know is ferroptosis) which entails the activation of 12/15-lipoxygenase (LOX) and associated lipid peroxidation (29). This form of cell death phenocopied that of cells treated with the GSH depleting agent L-buthionine sulfoximine (BSO), indicating that Gpx4 is the limiting GSH using enzyme in cells.

Yang and Stockwell published the finding of synthetic lethal compounds including Erastin and RAS synthetic lethal 3 (RSL3; later referred to as "ferroptosis inducing agents," FIN's) that facilitate an iron-dependent subroutine of nonapoptotic cell death in cancer cells expressing an oncogenic isoform of RAS (30,31). In their synthetic lethal screen, specificity towards oncogenic RAS^{V12} transformed tumor cells was ensured as these cells exhibit elevated iron levels through increased expression of transferrin receptor 1 and downregulation of the iron storage protein ferritin (31).

Ferroptosis: Morphology, Mechanisms, Modulators, and Transgenic Mouse Studies

Morphological Features of Ferroptosis

The term ferroptosis was coined in 2012 by Stockwell's group (4). Ferroptotic cells show very distinct bioenergetic and morphological features including depletion of the intracellular pool of nicotinamide adenine dinucleotide (NADH) [NADH phosphate (NADPH)] but not that of ATP, as well as loss of membrane integrity accompanied by oncosis with morphologically normal nuclei and shrinking mitochondria that show increased membrane density and outer mitochondrial membrane rupture (4,6,32–34). Based on these features, ferroptosis can be readily distinguished from other cell death paradigms including apoptosis (caspase-dependent, chromatin condensation, membrane "budding") and autophagy (formation of autophagosomes) (4). Even though phenotypically similar to H_2O_2-induced necrosis, ferroptosis differs from this form of death in bioenergetic terms (ATP pool depleted in H_2O_2-induced necrosis while remaining unchanged during ferroptosis) (4). The hallmark of ferroptosis is an iron-dependent PL peroxidation that is counteracted by the GSH and Gpx4 system (4–6). Accordingly, the chelation of intracellular iron using deferoxamine (DFO) is sufficient to protect cells from FIN- and Gpx4 knockout-induced cell death (4,6), again reinforcing the importance of iron in ferroptosis. Whether the phenotypical appearance of ferroptosis is a direct consequence of the breakdown of plasma membrane integrity by massive lipid peroxidation (35), or the consequence of further downstream signaling events, however, remains to be explored.

Modulators of Ferroptosis

First described to target voltage-dependent anion channel 2 (36), the FIN Erastin primarily acts through efficient inhibition of system x_c^- (4), whereas RSL3 is an alkylating agent able to directly inactivate Gpx4 via alkylation of the catalytic selenocysteine (5,37). The discovery of FIN's was closely followed by the development of ferroptosis-specific inhibitors [*i.e.*, Ferrostatin-1 (Fer-1) and Liproxstatin-1 (Lip-1)] (4,6,8,38). Fer-1 was shown to ameliorate cell death in rat corticostriatal brain slices expressing mutant *huntingtin*, an *ex vivo* model for HD, in a cellular model of periventricular leukomalacia, as well as in a proximal tubule (mouse) damage assay (Fe^{2+} hydroxyquinoline) (38). Lip-1 was able to suppress ferroptosis in immortalized fibroblasts in the absence of Gpx4 as well as in an inducible mouse model of Gpx4 depletion and a pre-clinical model of hepatic ischemia/reperfusion (6). Mechanistically, both compounds were able to prevent the accumulation of PL peroxides. Given a common structural element, which consists of at least two secondary amines in close proximity to each other, it is hypothesized that Fer-1 and Lip-1 may have redox cycling activity and thus possibly operate as lipophilic reducing agents and/or radical scavengers (38–40).

The generation of PL peroxides is directly dependent on the starting material: polyunsaturated fatty acids (PUFAs) are easily attacked by reactive oxygen species (ROS), while monounsaturated and saturated fatty acids (FAs) are much less reactive. Consequently, genome-wide genetic screens performed independently in different cellular contexts identified enzymes involved in PUFA metabolism, that is, acyl-CoA synthetase long chain family member 4 (Acsl4) and lysophosphatidylcholine acyltransferase 3 (Lpcat3), as critical determinants of ferroptosis sensitivity

((34,41); as further detailed below). In particular, Acsl4, an enzyme that ligates long PUFAs to Coenzyme A (CoA), appears to be critically involved in ferroptosis execution not only in fibroblasts but also in a subset of triple negative breast cancer cell lines (34). In line with this, an extensive redox lipidomics analysis of cells undergoing ferroptosis revealed that doubly and triply oxygenated species of AA and adrenic acid (AdA) containing phosphatidylethanolamines (OO(O)AA/AdA-PE) are decisive to direct cells to undergo ferroptosis (34,42).

Yet the cellular lipid composition is not the only factor that determines the cells' responsiveness to ferroptosis downstream of Gpx4 as a recent report indicated that the cellular levels of the reduced forms of NADH and NADPH may also be used as a predictor for the outcome of a ferroptotic event (32). Additionally, the amount of accessible intracellular iron and the corresponding production of PL peroxides are ultimately considered as the driving forces of ferroptosis downstream of Gpx4 (31,43). In this context, both transferrin (the major source of iron in cell culture) and glutamine uptake pathways were shown to be required for Erastin-induced ferroptosis (44). While excessive iron can be stored intracellularly in the ferritin complex (45), autophagic/lysosomal processes, known as "ferritinophagy," are able to release iron from these intracellular stores providing supply during times of iron shortage (45,46). Ferritin break down seems to be particularly interesting in terms of ferroptosis as lysosome inhibitors are able to ameliorate the initial ferroptotic oxygen radical burst in human fibrosarcoma cells (HT-1080) triggered by Erastin/RSL3 (47). Knockdown of the ferritinophagy-specific nuclear receptor coactivator 4 significantly decreases the ferroptotic response to Erastin in human pancreas carcinoma cells (PANC1) and HT-1080 (46), thus corroborating that ferritinophagy is an essential event to disturb the cellular iron equilibrium and drive the production of oxygen radicals in perinuclear compartments during ferroptosis (47).

Mechanisms of Ferroptosis

When speaking of canonical signaling cascades one usually assumes the propagation of a stimulus via various forms of post-translational protein modifications (*e.g.*, phosphorylation, methylation/acetylation, ubiquitination etc.) that are propagated from an upstream to a downstream effector molecule. This concept, however, does not strictly apply to ferroptosis, where the core events rather recapitulate a series of metabolic processes constituting the major antioxidant system of the cell. Inhibition of system x_c^- mediated either by high concentrations of extracellular glutamate or small molecule inhibitors, such as Erastin, Sulfasalazine, Sorafenib (9,48,49), represents so far the most "upstream" point of ferroptosis induction that ultimately leads to deprivation of intracellular GSH and thus reduced substrate availability of Gpx4 (4), which is particularly relevant in cells and organoid systems cultured *ex vivo* (Fig. 2). System x_c^- transports cystine/cystathionine into the cell in exchange for glutamate, which is secreted into the extracellular space (50,51). Under oxidative conditions, system x_c^- is the only transporter that can provide cellular cysteine in its oxidized form cystine. Alternatively, cells may sustain intracellular cysteine levels by the reverse transsulfurylation pathway converting methionine to homocysteine, cystathionine, and in a final step to cysteine (52). Interestingly, decreased uptake of cystine has been recently linked to the tumor protein p53 (p53) via suppression of *xCT* expression, indicating that the modulation of ferroptosis may present another function of p53's oncosuppressive repertoire (53), although further studies are required to support this assumption. Once inside the cell, cystine is reduced to cysteine by GSH or thioredoxin reductase 1 (54). Under physiological conditions, cysteine is available through various amino acid transporter systems as described earlier (18–21). Apart from protein synthesis cysteine is mainly used for the synthesis of GSH, which occurs stepwise by the enzymes γ-glutamylcysteine synthetase (γ-GCS; the direct target of BSO) (55) and glutathione synthetase (GSS; (56,57)). GSH is the major cellular antioxidant in mammals and the substrate of many different redox enzymes, of which Gpx4 is most critical in terms of ferroptosis. Gpx4 is the sole isoform of the glutathione peroxidase family that is able to efficiently reduce PL peroxides and cholesterol peroxides to their corresponding alcohols (58). Pharmacologically, Gpx4 can be efficiently inhibited by RSL3 (5) or altretamine (59), thereby PL peroxides accumulate which direct cells to undergo ferroptosis. Specifically, OO(O)AA/AdA-PEs have been proposed as markers of ferroptosis and are considered a prerequisite for the generation of a yet to be further characterized lethal lipid signal (42). Accordingly, lowering the abundance of AA/AdA-PE in cell membranes by genetic ablation of *Acsl4* (and Lpcat3 to some extent), pharmacological inhibition of Acsl4 by thiazolidinediones (and triascin C) or by enriching membranes with monounsaturated FAs (unpublished data) all prevent the generation and propagation of lipid peroxidation and consequently ferroptosis in MEFs (34,42). Alternatively, antioxidants [*e.g.*, α-toc or idebenone, a hydrophilic analog of coenzyme Q_{10}(CoQ_{10})], antiferroptotic compounds (Fer-1 or Lip-1) or iron chelators (DFO or ciclopirox olamine) are able to halt ferroptosis (immortalized fibroblasts, HT-1080, and in a model of glutamate-induced neurotoxicity organotypic hippocampal slides; (4,6,38,60,61)). In line with this, inhibition of the mevalonate pathway and subsequent depletion of CoQ_{10} increases lipid peroxidation and cell death upon FIN treatment (FIN56) in HT-1080 cells (61).

In Vivo Relevance of Ferroptosis Unmasked by Gpx4 Knockout Studies

As a direct link between a certain human disease and ferroptosis is still missing, knockout studies performed in mice have helped to greatly improve our understanding of the *in vivo* relevance of ferroptosis. Early studies using "classical" knockout approaches showed that loss of Gpx4 causes an early embryonic lethal phenotype at the gastrulation stage (62), which suggested that Gpx4 is a vitally important enzyme among the glutathione peroxidase family of proteins (63). Subsequent studies using conditional knockout mouse model(s) for Gpx4

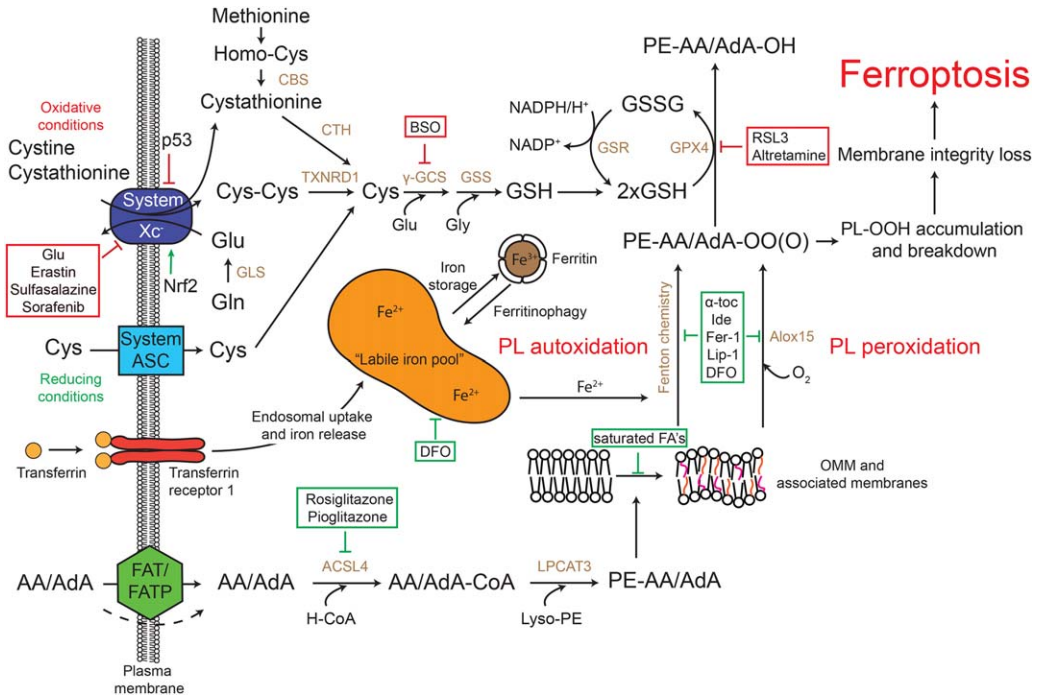

FIG 2

Ferroptosis mechanisms including known modulators. The uptake of cystine (Cys-Cys)/cystathionine in exchange for glutamate by system x_c^- represents the most upstream event of the ferroptosis cascade under oxidative extracellular conditions, while under reducing conditions cysteine is directly taken up via system ASC (alanine, serine, cysteine-preferring). Once inside the cell, cystine is reduced to cysteine by either GSH or thioredoxin reductase (TXNRD1). Tumor protein p53 (p53) suppresses the expression of system x_c^- component xCT, while the nrf2/keap1 (nuclear factor E2-related factor/kelch-like ECH-associated protein 1) pathway promotes xCT expression. Cystathionine taken up by system x_c^- or derived from reverse transulfurylation of methionine can be used as another cysteine source. Subsequently, cysteine is used for the biosynthesis of GSH in two enzymatically catalyzed reactions γ-GCS and GSS to generate GSH. GPX4 uses two GSH molecules as electron donors to reduce phospholipid hydroperoxides (PL-OOH) to the corresponding alcohols leaving GSSG (oxidized GSH) as a byproduct. GSSG is reduced to GSH by glutathione reductase using NADPH. The accumulation of PL-OOH is a hallmark of ferroptosis, which critically depends on the amount of AA/AdA esterified into PLs. Therefore, the molecular pathway responsible for the uptake (fatty acid translocase, fatty acid transport protein, or free diffusion of AA/Ada), activation (ACSL4) and esterification (LPCAT3) of AA/AdA into PLs influences the sensitivity of cellular membranes to PL-peroxidation. The amount of redox-active Fe^{2+} (LIP) represents another critical factor for the formation of PL-OOH via Fenton chemistry. LIP is regulated by the cellular iron homeostasis. Transferrin-mediated iron uptake (via transferrin receptor 1) and ferritinophagic release of iron increase the LIP, while storage into ferritin decreases it. Double/triple oxygenated phosphatidylethanolamines (OO(O)-AA/AdA-PE) have emerged as early markers of ferroptosis. Iron-catalyzed enzymatic (Alox15) as well as non-enzymatic (possibly Fenton chemistry) processes are involved in the generation of OO(O)-AA/AdA-PE. If not detoxified via GPX4 OO(O)-AA/AdA-PE accumulate and propagate lipid peroxidation to other PLs which ultimately leads to membrane integrity loss and ferroptosis. A variety of pharmacologic inhibitors have been shown to induce ferroptosis (red boxes). Compounds that inhibit ferroptosis (green boxes) include α-toc, idebenone, Fer-1, Lip-1, and DFO. High concentrations of saturated FAs prevent the enrichment of PUFAs at the sn-2 position of PLs. Abbreviations: CBS, cystathionine-β-synthase; CTH, cystathionine gamma-lyase; BSO, L-buthionine sulfoximine; RSL3, (1S, 3R)-RAS synthetic lethal; GLS, glutaminase; OMM, outer mitochondrial membrane.

(29) demonstrated that Gpx4 is essential for neuroprotection in hippocampus, cortex, cerebellum and motor neurons (29,64–66), as well as for photoreceptor cells (67), kidney tubular cells (6), and CD8-positive T cells (68). Importantly, in some cells, such as endothelial and hepatic cells (69,70), tissue injury induced by the genetic loss of Gpx4 can be masked by α-toc when present either in high concentrations in the mouse chow or by α-toc fortification in the diet, suggesting that α-toc concentrations need be considered in future ferroptosis studies due to α-toc's well-documented antiferroptotic effects. This fact might be particularly relevant not only for studies performed in animal models of disease evaluating the potential contribution of ferroptosis to certain diseases, but, more importantly, also in future clinical trials assessing the efficacy of potentially antiferroptotic drugs aiming to ameliorate cell and tissue demise in diseases linked with ferroptosis, such as neurodegeneration and ischemia/reperfusion scenarios (8).

Iron-Catalyzed Oxygen Radical Formation in Cells

As a result of respiration and some dedicated enzymatic reactions, aerobic organisms constantly expose themselves to a

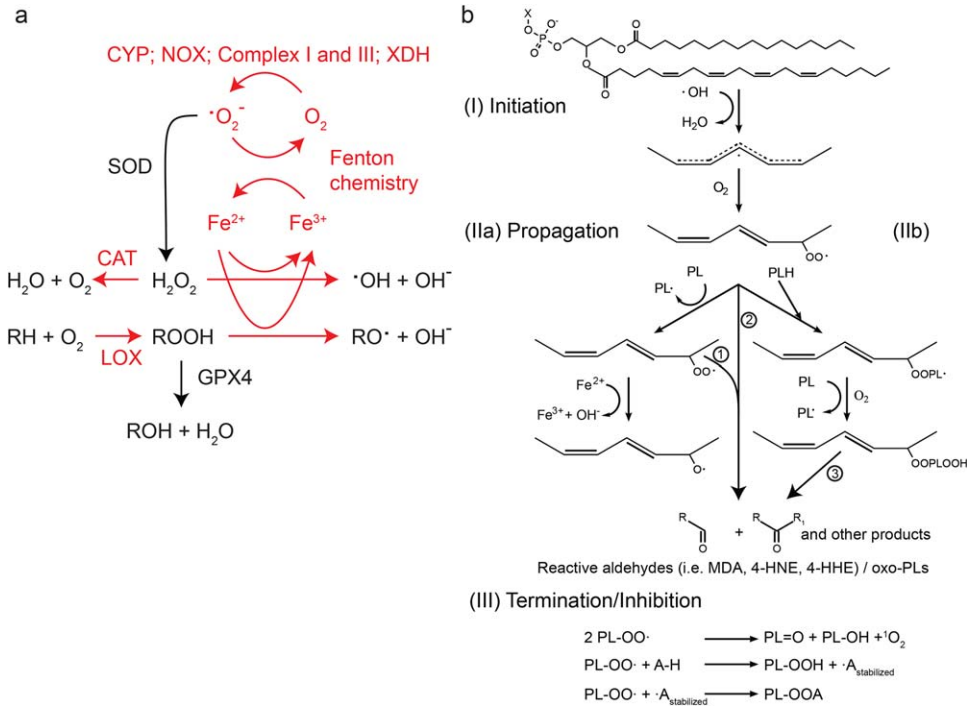

FIG 3

*The role of iron in the generation of cellular oxygen radicals and lipid peroxidation. (a) Scheme depicting a selection of reactions that contribute to the formation of soluble and lipid-derived ROS in cells. Fenton chemistry describes the redox reaction of iron (Fe^{2+}) with different peroxide species to generate hydroxyl (·OH) or alkoxyl (RO•) radicals. Ferric iron (Fe^{3+}) can be recycled to Fe^{2+} by superoxide ($•O_2^-$) generating molecular oxygen (O_2). Enzymes and reactions involving iron as catalytically active element are colored in red. Abbreviations: CYP, cytochrome P450; NOX, NADPH oxidase; CAT, catalase; LOX, lipoxygenase; GPX4, glutathione peroxidase 4; SOD, superoxide dismutase; OH^-, hydroxide; H_2O_2, hydrogen peroxide; RH, lipid molecule; ROOH, lipid hydroperoxide. (b) Autoxidation radical chain reactions of PUFA containing PLs can be divided into three stages: (I) Initiation, a free radical (*i.e.*, •OH) abstract hydrogen from bis-allylic positions of PLs to generate carbon-centered radicals. This carbon centered radical can react with molecular oxygen to form a PL-peroxyl radical (PL-OO•). Propagation reactions of PL-OO• include (IIa) hydrogen abstraction from a PL molecule to form a PL-hydroperoxide (PL-OOH) or (IIb) addition of PL-OO• to the bis-allylic position of another PL to form PL-OO-PL• dimers. In the presence of Fe^{2+}, PL-OOH can undergo reductive cleavage to generate a PL-alkoxyl radical. Autoxidation reactions of PUFA containing PLs form a variety of electrophilic species (MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; 4-HHE, 4-hydroxyhexenal; oxygenated PLs and other products) following different routes: Hock rearrangement and cleavage of PL-OOH (1); Dioxetane cleavage of PL-OO• (2); ß-scission of dihydroxy peroxides in the presence of Fe^{2+} or when labile dimers (*i.e.*, PL-OO-PL-OOH) break at the crosslinking peroxide bond (3). The radical reaction is terminated/inhibited every time two radicals react with each other or when antioxidants "consume" radical species (III).*

variety of reactive molecules derived from the partial reduction of molecular oxygen. These species include the superoxide radical ($•O_2^-$), hydrogen peroxide (H_2O_2), the hydroperoxyl, and hydroxyl radical (•OH) as well as lipid-associated ROS represented by hydroperoxides, peroxyl radicals and alkoxyl radicals (71). Loosely bound iron or iron-complexes (*i.e.*, heme or [Fe-S] clusters) are the essential reactive elements of the many enzymes that participate in the formation (LOX, cytochrome P450, xanthine oxidase, NADPH oxidases, mitochondrial complex I and III) but also decomposition (catalase, peroxidases) of these reactive forms of oxygen ((72,73); Fig. 3a). Additionally, eukaryotic cells contain small amounts of uncoordinated and redox-active Fe^{2+}, the so-called "labile iron pool" (LIP) (74). Lysosomes contain a particularly large LIP, which arises from their function in the uptake of exogenous iron as well as the recycling of endogenous iron sources like ferritin and mitochondria (75,76). Interestingly, the presence of functional lysosomes has recently been described to be required for the execution of ferroptosis in cancer cells (47). Mechanistically, free Fe^{2+} catalyzes the formation of •OH and hydroxide from H_2O_2 in a reaction named after the 19th century chemist Henry J. H. Fenton ("Fenton reaction"; Fig. 3a). In the cellular compartment, the catalytic cycle of the Fenton reaction can be complemented by the regeneration of Fe^{2+} via oxidation of $•O_2^-$ to oxygen. Accordingly, both Fenton chemistry and iron-dependent enzymes may generate reactive forms of oxygen that can trigger oxidative damage to nearby biomolecules, such as proteins, DNA, and lipids. Consequently, the depletion of cellular iron by chelation thus affects not one but probably most iron-dependent sources of oxygen radicals, making it difficult to decipher the damaging contribution of an individual species. For an overview of already identified molecular targets of H_2O_2 and $•O_2^-$ with implications for

cellular proliferation and cell death the reader is redirected to a recent review by Dixon and Stockwell (77).

PL Peroxidation Drives Ferroptosis

Ferroptosis execution specifically requires the accumulation of peroxides in membranes, a process that can be visualized by various redox-sensitive fluorescent probes including BODIPY 581/591 C11, Liperfluo and Spy-LHP (4,6,29,34,42). Due to their lipophilic character, these molecules reside in the PL bilayer and are thus shielded to some extent from non-lipid derived ROS (H_2O_2, $\bullet O_2^-$, •OH, etc.) (78,79). Early studies have established the radical-based nature of autoxidation events (80,81), which ultimately generate PL hydroperoxides and their electrophilic breakdown products (82). PLs [*e.g.*, PE and phosphatidylcholine (PC)] contain two esterified acyl chains of which mostly the polyunsaturated acyl chain in the sn-2 position undergoes autoxidation. The prevailing kinetic concept of autoxidation reactions dictates that the number of bis-allylic positions [H atoms of bis-allylic positions can be easier abstracted than from their allylic counterpart (83)] in acyl chains must correlate with their oxidizability (60), although in the context of ferroptosis the situation seems to be more complex. While mouse Acsl4 knockout fibroblasts are generally resistant to ferroptosis, supplementation of certain FAs can revert their resistance. Supplementation of MEFs with a panel of FAs revealed that the position of the last double bond position rather than the degree of unsaturation impacted susceptibility to ferroptosis: Eicosatetraenoic acid (C20:4n-6) supplementation sensitized fibroblasts to undergo ferroptosis, while eicosapentaenoic acid (C20:5n-3) failed to do so (34). This observation argues against a sole non-enzymatic (*i.e.*, Fenton reaction) mechanism, and implies the participation of enzymatic (substrate-specific) processes to initiate ferroptotic PL-peroxidation. It is still under intense investigation whether iron-mediated autoxidation or enzyme-steered peroxidation of PLs by LOXs is responsible for the accumulation of toxic PL peroxides during ferroptosis it can be assumed that not just one but a mixture of both processes are involved (37,42,60).

Nonenzymatic PL Autoxidation

Polyunsaturated acyl chains, esterified into the sn-2 position of PLs are the prime targets of autoxidation, whereas acyl chains (saturated and monounsaturated) of the sn-1 position hardly participate in autoxidation reactions (84). The oxygen-dependent radical chain reaction can be schematically described in three stages, known as initiation (I), propagation (II) and termination (III) of the radical chain-reaction (Fig. 3b). Step (I) involves a free radical (*i.e.*, •OH), which abstracts hydrogen from a polyunsaturated acyl chain of a PL. The resulting carbon-centered radical readily reacts with molecular oxygen to form a PL peroxyl radical (PL-OO•) (85) (II), which in turn can propagate the reaction in multiple ways [the following reactions are chemically well-documented for methyl linolate, but hypothetically similar reactions occur with PLs containing polyunsaturated acyl chains (84)]. PL-OO• abstracts hydrogen from another PL molecule (IIa) and forms PL-OOH and a PL radical which propagates the chain reaction. In the presence of Fe^{2+}, PL-OOH can be converted to PL alkoxyl radicals (PL-O•) which also contributes to chain propagation (86). Alternatively, PL-OO• reacts via addition (IIb) to the polyunsaturated acyl chain of another PL, which effectively forms PL dimers that are linked via a peroxide bond (87,88). These dimers along with other intermediate products (PL-OO• and PL-OOH) are instable molecules that suffer decomposition reactions (reviewed in detail in *88*) producing the electrophilic end products of PL autoxidation (reactive aldehydes and oxygenated PLs; (82,89–91)). Termination or inhibition of the chain reaction generally occurs when two radicals of the chain reaction react with each other to form stable molecules or when chain-breaking antioxidants (*i.e.*, α-toc) effectively "consume" radicals necessary for chain propagation (60) (III).

Enzymatic PL Peroxidation

Enzymatic peroxidation of PLs (LOX-catalyzed) can also participate in the formation of PL-OOH. However, the role of the LOX family proteins in ferroptotic PL peroxidation is still debated as direct genetic proof is still lacking that knockout of one or several LOX isoforms indeed confers strong protection against ferroptosis in a manner similar to that conferred by Acsl4 (34). Arachidonic (C20:4), linoleic (C18:2) and linolenic (C18:3) acids are the most prominent substrates for different LOX isoforms that use molecular oxygen to form hydroperoxyl groups at carbon position 5, 12, or 15 (Alox5, Alox12, or Alox15) of acyl chains (92). Strikingly, Alox15 has a unique substrate requirement among the LOX family as it is able to directly oxygenate AA containing PLs without prior release of esterified AA by phospholipase A_2 (PLA_2; (93–95)). Even though Alox5 and Alox12 cannot oxygenate PLs directly, they have been shown to provide oxygenated acyl precursors that can get incorporated into lyso-PE/PC of platelets and neutrophils thereby similarly generating oxygenated PLs (96,97). The responsible lyso-PL acyl-CoA transferase has not been identified yet. Interestingly, during RSL3-trigged ferroptosis oxygenated AA/AdA-CoA was not detected, which led to the assumption that the oxygenation event that drives ferroptosis occurs after and not before AA/AdA incorporation into PL (42). Accordingly, only LOX isoforms that can use PUFA-containing PLs as substrates (*i.e.*, Alox15) would be relevant in a ferroptotic scenario. Considering the very short-lived nature of oxygenated acyl-CoAs, which makes their detection technically challenging we cannot formally exclude a contribution of these precursors, and consequently of any LOX enzyme, to produce oxygenated PLs (96). In fact, the knockout or pharmacologic inhibition of Alox15 confers resistance to BSO-induced cell death in MEF (29); however no rescuing effect is observed in (inducible) Alox15/Gpx4 double knockout mice and derived MEFs (6,98). Additional knockdown of Alox5 (siRNA-mediated) in the inducible Alox15/Gpx4 double knockout MEF conferred a modest delay of ferroptosis, which indicated that not a single, but multiple LOX enzymes are collectively fueling

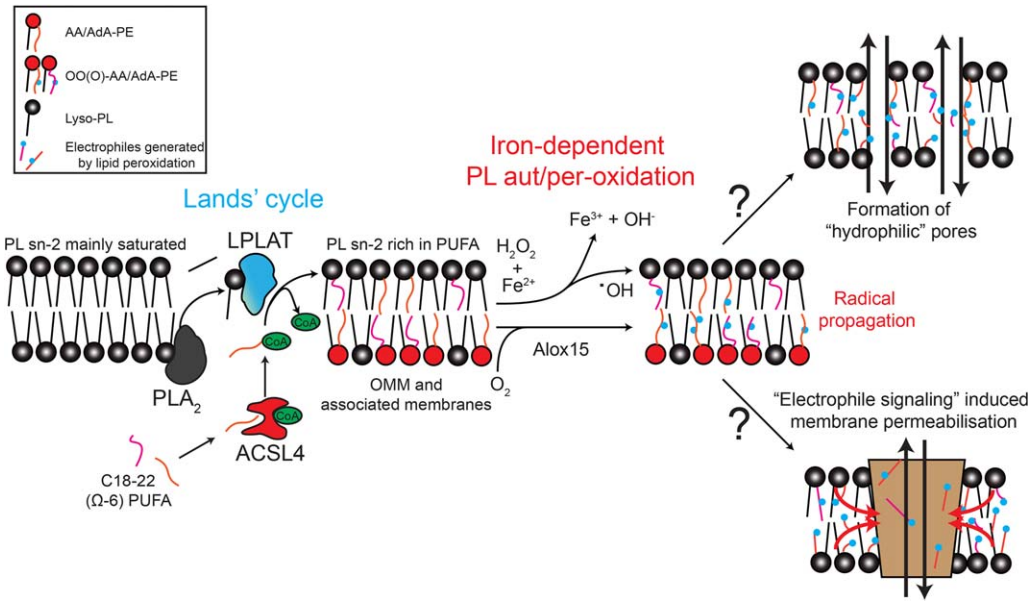

FIG 4

PL acyl chain remodeling (Lands cycle) is crucial for ferroptosis execution. The PL acyl chain remodeling pathway (Lands cycle) is responsible for the enrichment of membranes with PUFA. De-novo PL synthesis generates mostly PLs with saturated or monounsaturated acyl chains at the sn-2 position. PLA_2 removes the sn-2 standing acyl chain setting the stage for LPLAT to re-esterify the position using PUFA-CoAs. In terms of ferroptosis, the critical PUFA-CoA substrates, representing Ω-6 PUFAs (carbon chain length 18–22), are generated by acyl-CoA long-chain family member 4 (ACSL4). Membranes (outer mitochondrial membranes (OMM) and associated membranes) enriched with Ω-6 PUFAs are prone to undergo iron–dependent lipid peroxidation possibly via Fenton chemistry or enzymatic oxygenation (e.g., *12/15 LOX, 15-Alox). AA/AdA-PEs are primary targets of lipid peroxidation during ferroptosis (PE is marked in red, mainly localized to cytosol facing membrane leaflets). Radical propagation, if not halted by the cellular antioxidant systems, spreads lipid peroxidation to other PUFA-containing PLs in a randomized fashion. The exact mechanism that leads to loss of membrane integrity and ultimately cell death is not understood. Hypothetically, PL hydrogen peroxides and breakdown products (electrophiles) accumulate in the membrane. Two mechanisms are proposed that could support the phenotypic features of ferroptotic cell death ("oncosis") observed during* in vitro *experiments. First, altered membrane properties could allow the formation of hydrophilic pores and consequently lead to osmotic catastrophe. Second, lipophilic electrophiles formed during the lipid peroxidation event could act as specific signaling molecules by modifying so far unknown downstream players* (i.e., *membrane proteins) inducing membrane permeabilization. Abbreviations: OO(O)-AA/AdA-PE, double/triple oxygenated phosphatidylethanolamines.*

ferroptotic PL peroxidation (6). In fact, an "all LOX knockdown" approach shows protection against erastin-induced ferroptosis (HT-1080 and G-401 cells), but is ineffective in RSL3-induced ferroptosis in G-401 cells (37). These results demonstrate a clear difference between ferroptosis induction by GSH deprivation and direct inhibition of the ferroptosis regulator Gpx4 (when GSH levels remain normal). LOX-mediated PL oxygenation seems to be only critical for ferroptosis when GSH is depleted while posing an insignificant contribution otherwise. A careful consideration of individual expression profiles as well as the determination of potential functional overlaps among the LOX family might help deciphering the role of specific LOX isoforms in future ferroptosis studies.

Oxygenated AA-/AdA-Containing PE Species Spark Lipid Peroxidation during Ferroptosis

The amount of PUFAs that are esterified into PLs determines the oxidizability of cellular membranes (99,100). To incorporate PUFAs into newly synthesized PLs, the sn-2 positioned, mostly saturated or mono-unsaturated acyl residue of the PL has to be liberated by PLA_2 and subsequently replaced by a polyunsaturated acyl group (catalyzed by the family of lysophospholipid acyl transferases, LPLAT; Lands cycle; Fig. 4). Specifically, AA-/AdA-containing PE species are emerging as important proferroptotic precursors, that can—if oxygenated—direct cells to undergo ferroptosis (42). PE is after PC the second most abundant PL in eukaryotic cells (101). Its conical shape (small hydrophobic head with large hydrophilic tails) prevents PEs from producing functional lipid bilayers on its own, but instead it incorporates into preexisting bilayers to enhance membrane curvature and support fusion/fission events (102,103). Importantly, PEs are actively translocated into the cytosol-facing membrane leaflet by ATP-driven PL flippases (P4 subfamily of P-type ATPases; (104,105)), thereby statistically (and physically) representing the first line of PLs that can be modified by iron-catalyzed or LOX-mediated oxygenation in the cytosol. This explains why the generation of OO(O)AA/AdA-PE is strongly associated with ferroptosis, while other oxygenated PLs, are less prominent during ferroptosis (42). In addition to this bias towards PE, 1-steaoryl-2-

arachidonoyl-3-PE has been shown to be a much better substrate for human recombinant ALOX15-mediated oxygenation than for instance 1-steaoryl-2-arachidonoyl-3-PC (42). As nonenzymatic radical propagation reactions do probably not discriminate between the headgroups of PUFA-containing PLs, the accumulation of certain PL peroxides may reflect the local PL distributions. In this regard, the localization of the ferroptosis-derived PL peroxidation may critically influence the generated products. Considering PUFA-PE rich membranes (*i.e.*, the outer mitochondrial membrane; (106)) as initiation sites of ferroptotic lipid peroxidation, one could infer that the generation of OO(O)AA/AdA-PEs is the consequence of an early ferroptotic event that ignites the production of a yet to be identified lethal lipid signal. The final execution mechanism of ferroptosis, which ultimately leads to membrane permeabilization and "osmotic catastrophe" (Donnan effect) of cells, still remains mysterious and is therefore subject to speculations (Fig. 4). One possibility is the accumulation of PL-peroxides and their breakdown products in membranes that may give rise to "hydrophilic pores" (35,107). Consequently, water and other solutes diffuse across membranes, which would lead to a loss of compartmentalization and cell death. Alternatively, electrophiles (*i.e.*, reactive aldehydes, oxygenated PLs) generated as a consequence of PL-peroxidation could act as downstream signaling molecules that might modify/activate a set of jet unrecognized effector proteins (108–110). In this scenario, the specificity of the signal could be achieved either by close proximity of the target protein and site of PL-peroxidation or by high affinity of certain electrophiles to distinct targets (protein, DNA; (108,111)). In this respect, hundreds of oxidized PL species have been already identified to regulate mitochondrial metabolism during acute activation of human platelets (112). Corroborating with the proposed "electrophile signaling" mechanism, upregulation of several reactive aldehyde detoxifying enzymes (aldo-keto reductases; AKR1C1, AKR1C2, and AKR1C3) protect cells from system x_c^- inhibition-induced ferroptosis (49). Notably, metabolite analysis of erastin-treated HT-1080 cells revealed that both GSH and its oxidized form are depleted in ferroptotic cells, either as a consequence of electrophile-dependent GSH conjugation or controlled GSH turnover to ameliorate cellular cysteine deprivation (5,113,114).

Concluding Remarks

We are just beginning to understand the complexity of cellular mechanisms and signaling events that contribute to ferroptosis. Intriguing questions concern the series of iron-dependent processes that are necessary to produce pro-ferroptotic lipid signals: Is an activity of LOX enzymes required or indispensable? Putative functional redundancies as well as highly variable expression profiles of LOX make this question a hard nut shell to crack. Is there one (or several) specific iron metabolism-related gene(s) whose targeted knockout confers a robust and solid protection against ferroptosis as we have seen it for Acsl4? So far, no iron metabolism-related gene has been found in genetic screens arguing either for complex redundancies among these genes or for a less specific function of iron in the ferroptotic process. Other questions revolve around the final ferroptotic executioner mechanisms based on PL-peroxidation breakdown products. For instance, why do Ω6 but not Ω3 fatty acids (considering similar chain length and degree of saturation) sensitize *Acsl4* knockout fibroblasts to ferroptosis? This question might be closely related to the potential role of 4-hydroxynonenal (4-HNE) as the main lipid-derived electrophile signal downstream of PL peroxidation. The type of reactive aldehyde produced from the decomposition of lipid peroxides highly depends on the position of the last double bond as oxygenation of Ω6 FAs is required for the formation of 4-HNE, while the same process will produce mainly 4-hydroxy-2-hexenal (HHE) using Ω3 fatty acids (89,115–118). Hence, the application of robust technological advances in chemoproteomic and (oxi)lipidomic tools, electrophile signaling (119), and genome-wide CRISPR/Cas9-mediated screens will yield a dramatically improved understanding of still unsolved questions in the ferroptosis chain of events.

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