Review

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Review

A Mechanistic Discussion on Oxidative Stress and Ferroptosis

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Abstract: Ferroptosis is a recent form of non-apoptotic cell death, which occurs due to imbalance of iron homeostasis (iron overload). Oxidative stress due to the impairment of the antioxidant system is a major pathophysiology during ferroptosis, which eventually results in lipid peroxidation. The cellular and physiological biomarker of ferroptotic cell death includes major alteration in the glutathione peroxidase 4 (GPX4) antioxidant system and lipid peroxidation induced loss of plasma membrane integrity. This review elaborates the mechanism of oxidative stress which triggers ferroptosis.

Keywords: oxidative stress; ferroptosis; iron; cell death; lipid peroxidation

Introduction

Programmed Cell Death (PCD) can be described as sequential events that lead to the controlled and organized destruction of the cell (Lockshin and Zakeri, 2004). PCD is required for maintaining the homoeostasis and normal development which broadly includes autophagy, apoptosis, necrosis, necroptosis (Liu *et al*., 2013; Dixon *et al*., 2012; Fuchs and Steller, 2011; Degterev*et al*., 2005; Brennan *et al*., 2000). Ferroptosis is established as a non-apoptotic pathway, which is triggered by imbalance in iron homeostasis and eventually by iron overload in the cells (Dixon *et al*., 2012). The oxidative stress is pathophysiologically controlled by molecules, which are the products of oxidative metabolism and provoke oxidative injury, are collectively known as Reactive Oxygen Species (ROS). Ferroptotic cell death is induced by oxidative stress and triggered by the accumulation of toxic Lipid-Reactive Oxygen Species (L-ROS) by inhibiting or inactivating glutathione biosynthesis or the glutathione-dependent antioxidant enzyme, glutathione peroxidase 4 (GPX4) (Dixon *et al*. 2012; Skouta *et al*., 2014; Cao and Dixon, 2016).

Cysteine uptake, along with the export of glutamate, is required for the biosynthesis of GPX4 which occurs by the antiport named System X_c^-. Biochemically, it shows increase in iron- dependent ROS accumulation and oxidation of Poly Unsaturated Fatty Acids (PUFAs) in the plasma membrane and hence it alters iron homoeostasis and lipid peroxidation metabolism in cells (Chen *et al*., 2020). Accumulation of L-ROS causing oxidative stress eventually depletes the plasma membrane integrity. Regulatory genes of lipid and amino acid metabolism play crucial role in ferroptosis (Cao and Dixon, 2016). Ferroptosis causes neurological issues, ischemic-reperfusion, acute renal failure among others (Munshi and Bhattacharya 2022; Chen *et al*. 2020; Gao *et al*., 2015; Linkermann *et al*., 2014; Angeli *et al*., 2014; Skouta *et al*., 2014; Dixon *et al*., 2012).

Approximately 65% of the iron is bound to hemoglobin and 10% is the constituent of myoglobin, cytochrome and iron containing enzymes and at about 25% of iron is bound to ferritin and haemosiderin. The storage capacity of ferritin is 4500 atoms of iron / molecule. Transferrin is the other molecule that helps the iron to remain in bound state in plasma (2 atoms of iron/ molecule). Only little amount iron is remained in free state. This inner pool of iron should be maintained to prevent the potential health disturbances produced due to both iron deficiency and iron overload. Normal iron homeostasis is maintained by the balance between absorbed dietary iron and required iron of body (Valko, 2005). Iron changes its ionic states between +3 (ferric ion, oxidized state) and +2 (ferrous

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state, reduced state) (Chatterjee *et al*., 2014b). Iron plays an important role in maintaining physiological function. It is present in heme which is essential for haemoglobin formation and serves as coenzyme for several catalytic reactions. There are two states of iron in human body: a. Bound Fe^{2+}; remains in form of ferritin such as haemoglobin and iron sulfur nanoclusters and b. Free Fe^{2+}; remains in heme or non-iron sulfur nanoclusters. Free irons remain in both ferrous and ferric form. Excessive free ferrous iron causes harm to cell by generating hydroxyl radicals (OH°) via Fenton reaction (Chen *et al*., 2020) and then induces oxidative stress which eventually cause lipid peroxidation.

Fe^{2+} mediated toxicity may be aggravated by increased oxidative stress and DNA damage. Interestingly, Fe^{2+} mediated cell death does not always occur via apoptotic pathway (Chew *et al*., 2011). It also has the capacity to induce hemolytic cleavage of hydrogen peroxide (H_2O_2), iron overload may cause the generation of aggressive hydroxyl radical (OH°) or other iron centered radicals and in such case Fe^{2+} becomes hazardous metal (Gutteridge, 1982; Eaton *et al*., 2002). As most of the enzymes that cause site specific oxidation of the lipid molecules are either iron containing or iron dependent (lipoxygenase), iron pool catalyze the formation of alcoxy radicals (L-O°) that cause PUFA fragmentation (Cheng and Li, 2007). These enzymes also inhibit lipophilic iron chelators that can chelate intracellular free iron molecules and induce ferroptosis (Kuhn *et al*., 2015; Abeysinghe *et al*., 1996; Barradas *et al*., 1989).

Stress mechanism of ferroptosis

Imbalance in the antioxidant system results in excessive ROS production and can trigger lipid peroxidation to damage plasma membrane and induce ferroptosis (Hassannia *et al*., 2019). Certain concentration of ROS can increase the repair of damages DNA, strand breaks and promote cell survival, but excessive ROS severely damages biofilm, protein and nucleic acid and finally leads to cell death (Chen *et al*., 2020). The generative mechanism of ferroptosis involves iron overloading, lipid peroxidation and downstream execution whereas the regulatory mechanism includes Glu/GPX4 pathway as well as mevalonate pathway and trans-sulfuration pathway (Chen *et al*., 2020).

Role of system Xc^-

Amino acids, vitamins that are required for the growth and proliferation of mammalian cells in culture and found that cystine (Cys2) which is the oxidized thiol-containing form of cysteine (Cys) one of the most essential amino acids. In absence of Cys2, cells fail to grow as there will be rapid depletion of Cys-containing antioxidant tripeptide GSH (c-L-glutamyl-L-cysteinglycine) and subsequently the cells die due to the accumulation of L-ROS (Banni *et al*., 1977; Eagle *et al*., 1961; Eagle, 1959; Eagle, 1955). System X_C^- is a Cystine/Glutamate (Cys/Glu) antiporter which play an important role in ferroptosis. It causes uptake of Cys along with the export of Glu (Dixon *et al*., 2014; Dixon *et al*. 2012). It is a heterodimeric, amino acid antiporter which is present at the cell surface and composed of twelve pass transmembrane transporter protein named SLC7A11 (Sato *et al*., 1999). This system plays important role in Glutathione synthesis. Glutathione is a tripeptide made of glutamine, cysteine and glysine. Glu is linked to Cys by GCL to form glutamylcysteine. This dipeptide, Glutamylcysteine is linked to Gly by the GSS to form the final GSH molecule (Griffith, 1999). Free GSH has antioxidant property (Griffith, 1999; Staal, 1998). GPX4 is the GSH dependent enzyme that helps in reduction of hydroperoxidase (LOOH) to lipid alcohols (L-OH) (Seiler *et al*., 2008; Ursini *et al*., 1985). Inactivation of GPX4 is required for induction of ferroptosis as it involves lipid peroxidation of PUFA chains of Membrane lipids and causes iron dependent accumulation of L-ROS (Yang *et al*., 2014; Skouta *et al*., 2014; Angeli*et al*., 2014, Dixon *et al*., 2012; Cheng and Li, 2007). Oxygen and Fe^{2+} also help in lipid peroxidation and cause L-ROS accumulation (Cao and Dixon, 2016). This accumulation can be prevented by continuous uptake of Cys2 and GSH synthesis. It was found that ferroptosis can only happen when there is direct or indirect inactivation of GPX4 and when highly oxidizable PUFA arachchidonic acid is present in the membrane (Cao and Dixon, 2016). Subsequent studies demonstrated that both lipophilic antioxidants and iron chelators can block this cell death (Yonezawa *et al*., 1996; Murphy *et al*., 1990; Murphy *et al*., 1989; De Brabander*et al*., 1979).

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Iron metabolism pathway

The iron present in ferric state (Fe^{3+}) forms a complex with transferrin and then the complex bind to Transferrin Receptor 1 (TFR1) on the cell membrane and then it is transplanted into the cell via endocytosis (Hao *et al*., 2018). By the action of Six-Transmembrane Protein of prostate 3 (STEAP3) Fe^{3+} is degraded into highly reactive Fe^{2+} and this ferrous form then translocate from endosome to the cytoplasm via Divalent Metal Transporter 1 (DMT1) (Chen *et al*., 2020). Thus, an unstable pool of iron is generated inside the cytoplasm. Under normal condition, part of this Fe^{2+} pool is stirred in ferritin to protect cells and tissues from iron mediated damage, while another part of the Fe^{2+} are pumped out through ferroportin on the cell membrane. Thus, the intracellular iron concentration remains stable (Chen *et al*., 2020). When the iron overload occurs, this balance is disrupted. As a result, Fe^{3+} and hydroxyl radicals can be directly catalyzed by Fenton chemical reaction. As hydroxyl radicals are most unstable oxygen free radicals and are active lethal ROS, they can easily get electrons to form other molecules (like O^{2-}) which can cause lipid peroxidation and ferroptosis. Fe^{3+} can be reduced to Fe^{2+} by the superoxide radical (O^{2-}) reaction (Hassannia *et al*., 2019). Under stress condition Ferritin is self-degraded to Fe^{2+} by the process called iron autophagy and then induces ferroptosis (Chen *et al*., 2020). Increase of iron uptake by TFR1 or reduction of stable iron by self-degradation of ferritin, leads to overload of iron inside the cell which causes oxidative damage and cause ferroptosis (Yang *et al*., 2008). This iron overload also induces noncanonical pathway of ferroptosis (Hassannia *et al*., 2018; Li *et al*., 2017). Iron export protein CDGSH iron sulfur domain 1 (CISD1) in the mitochondrial membrane reduces the accumulation of iron and production of L-ROS in the mitochondria thus prevents ferroptosis.

Lipid metabolism pathway and accumulation of lipid peroxides

Newly synthesized fatty acids must transform the long chain fatty acids into coenzyme A (CoA) for getting into the phospholipids (PLs) and this is mediated by Acetyl-CoA synthetase (Hassannia *et al*., 2019). Acetyl-CoA Synthetase Long Chain Family Member 4 (ACSL4) catalyzes the acylation reaction of Arachidonic Acid (AA) and Adrenic Acid (AdA) and this enzyme is important to execute ferroptosis (Doll *et al*., 2017). Now this acylation products combine with Phosphatidylethanolamines (PE) into membrane phospholipid by the action of Lypophosphatidylcholine Acetyltransferase 3 (LPCTA3) and produce PE-AA and PE-AdA (Kagan *et al*., 2017). ACSL4 and LPCTA3 make the cell membrane rich in sensitive PUFAs and lipoxygenase (LOX) especially 15-LOX (Yang *et al*., 2016) and then oxidize PUFAs into lipid hydroperoxides (PE-AA-O-OH or PE-AdA-O-OH) (Wenzel *et al*., 2017). When there is accumulation of Fe^{2+} in the cytoplasm the lipid hydroperoxide forms toxic L-ROS and cause cell damage. These radicals capture electrons from adjacent PUFA and launch a new round of lipid oxidation reaction and cause more serious damage (Kagan *et al*., 2017). ACSL (Kagan *et al*., 2017) and vitamin E (Wenzel *et al*., 2017) can block ferroptosis. Phosphatidylethanolamine-Binding Protein (PEBP1) increases the binding of LOX15 and increases the PUFAs in the cell membrane and promotes ferroptosis (Wenzel *et al*., 2017). The integration of PUFAs into the PLs after acylation and the production of lipid free radicals determine the advancement of ferroptosis (Dixon *et al*., 2019). PUFA can be incorporated into the PLs in different ways (phosphatidylcholine, phosphatidylinositol or phosphatidylethanolamine, PE) and it is dependent on the length of the carbon chain and the degree of unsaturation (Magtanong *et al*., 2016). Acylated chains of AA and AdA should be integrated with PE to induce ferroptosis (Kagan *et al*., 2017). L-ROS attack the mammalian phospholipid bilayer membrane and functions through 2 different pathways (Hassannia *et al*., 2019).

Non-enzymatic lipid peroxidation pathway

In such case free radicals (OH°) captures the hydrogen ions of PUFAs which are present in the plasma membrane and form a phospholipid free radical which acts with the oxygen molecule (O_2) to produce the Phospholipid hydrogen peroxide radical (PLOO°) which can also capture hydrogen atoms from PUFAs and produces Phospholipid hydroperoxide (PLOOH) and a new PL°. This newly synthesized phospholipid radical can again start a new oxidation reaction (Hassannia *et al*., 2019).

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Lipid peroxidation pathway

Lipoxigenase (LOX) plays major role in this pathway, and it causes dehydrogenation of PUFAs to form PLOOH. PLOOH is decomposed into alcoxy radical (PLO°) and in the presence of Fe^{2+} it can attack other PUFAs to trigger a chain reaction of lipid peroxidation. PLOOH through decomposition can produce 4-Hydroxynonenal (4-HNE) and Malondialdehyde (MDA). They along with PUFA-PLs reduce the stability of the cell membrane and increase permeability and thus resulting into cell death via ferroptosis (Hassannia *et al*., 2019).

GPX4 pathway

GPX4 is a selenoprotein, synthesized in the presence of selenium (Se) and it can eliminate PLOOH of PUFAs by transforming it into inactivated phosphatidylcholine (PLOH) because of this the lipid peroxidation process be prevented. Thus, ferroptosis will be inhibited (Hassannia *et al*., 2019). On the other hand, Cys uptake inside the cell is required to synthesize GSH and this GSH is an essential cofactor of GPX4 and maintains its function. GPX4 and GSH are antioxidants and eliminate ROS. GPX4 eliminates PLOOH and converts them into alcohol (PL-OH) under the assistance of GSH. GPX4 has antagonistic function of active Fe^{2+} and converts H_2O_2 into H_2O (Imai *et al*., 2003). When, there is high concentration of extracellular glutamate, the synthesis of GSH gets affected (Chen *et al*., 2020). Latest research indicates that, Interferon γ (IFNγ), which is released by CD8+ T-cells, can downregulate the expression of Glutathione antiporter and reduce the uptake of cystine by tumour cell which affects the production and activity of GPX4 and enhance the occurrence of Ferroptosis to have an anti-tumour effect (Wang *et al*., 2019).

Mevalonate (MVA) pathway

It regulates the cholesterol synthesis and isoprene modification of the small G-protein after translation. In this pathway at first, Acetyl-CoA forms 3-Hydroxyl-3-Methylglutaryl Coenzyme A (HMGCoA) by the action of 3-Hydroxyl-3-Methulreductase (HMGCR). HMGCoA reduces to MVA then produces Isoprenyl Pyrophosphate (IPP) under the action of a series of enzymes. This IPP produces Famesyl Pyrophosphate (FPP) under the action of famesyl phosphate synthetase. This FPP generates squalene by the action of Squalene Synthease (SQS). Then cholesterol is formed under the action of squalene cyclase (Kim *et al*., 2014). This plays important role in GPX4 maturation. During the synthesis of GPX4, selenocysteine must be inserted into its catalytic center to function as antioxidant. IPP plays important role in this step (Warner, 2000). It promotes the formation of isoprene transferase and thus promotes the integration of selenocysteine on the GPX4 catalytic subunit (Kim *et al*., 2014).

Other pathways

p53 reduces the uptake of cystine by decreasing the expression of SLC7A11 which inhibits the transporter activity, and this decreases GSH synthesis, inhibit GPX4 activity, increase L-ROS and cause ferroptosis (Jiang *et al*., 2015). Activation of MAPK pathway can induce ferroptosis in cancer cells when RAS/RAF/MEK/ERK will be activated (Xie *et al*., 2016).

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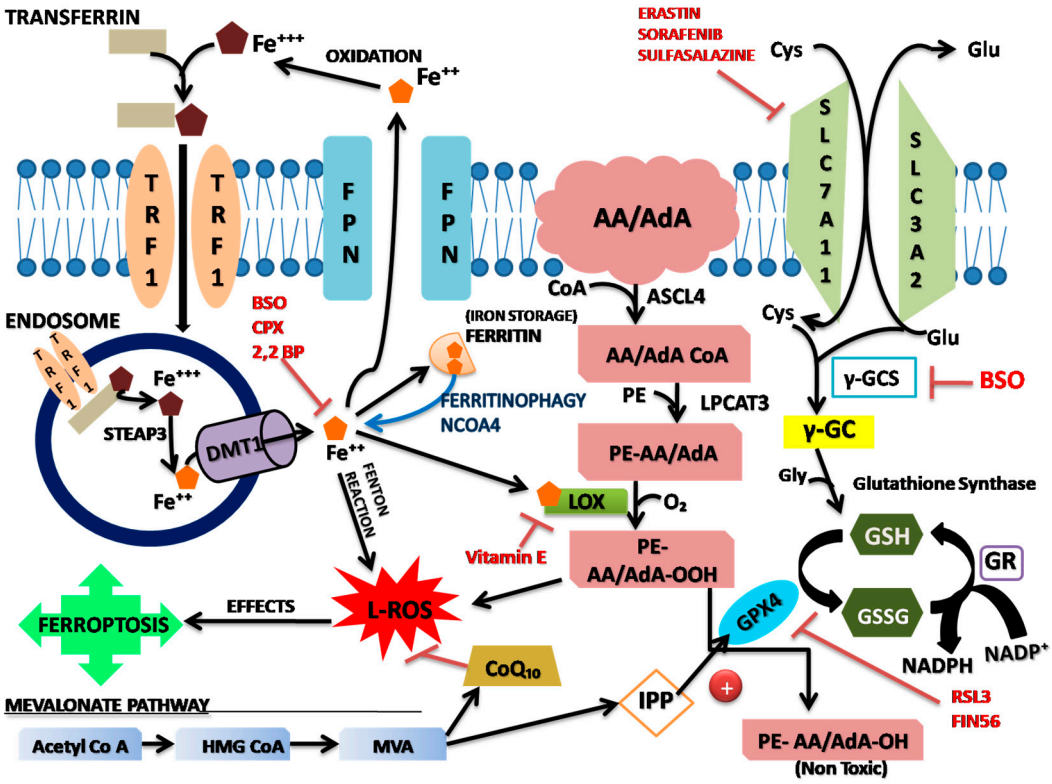

Figure 1. Schematic diagram of oxidative stress during ferroptotic cell death.

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