

Invited Mini Review

Mass spectrometry-based approaches to explore metabolism regulating ferroptosis

Chi Thi Ngoc Nguyen[#], Seon Min Kim[#] & Yun Pyo Kang^{*}

College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 08826, Korea

Ferroptosis is a type of programmed cell death distinct from apoptosis or necroptosis. Ferroptosis is well characterized by an iron-dependent accumulation of lipid peroxides and disruption of cellular membrane integrity. Many metabolic alterations can prevent or accelerate ferroptosis induction. Recent advances in analytical techniques of mass spectrometry have allowed high-throughput analysis of metabolites known to be critical for understanding ferroptosis regulatory metabolism. In this review, we introduce mass spectrometry-based analytical methods contributing to recent discovery of various metabolic pathways regulating ferroptosis, focusing on cysteine metabolism, antioxidant metabolism, and poly-unsaturated fatty acid metabolism. [BMB Reports 2022; 55(9): 413-416]

INTRODUCTION

Ferroptosis is a type of programmed cell death that is morphologically, biochemically, and genetically distinct from other forms of regulated cell death. It is characterized by iron-dependent accumulation of lipid peroxides and disruption of cellular membrane integrity. Since the discovery of ferroptosis (1), many ferroptosis-related metabolic alterations have been studied, suggesting possible intertwinement of diverse metabolic pathways in ferroptosis induction.

Much evidence has shown that Cyst(e)ine metabolism plays a crucial role in ferroptosis prevention. Inhibition of cysteine uptake and cysteine depletion can induce ferroptosis by depleting intracellular glutathione (GSH) and accumulating glutamic acid (1, 2). While GSH works as a co-factor of glutathione peroxidase 4 (GPX4) to detoxify lipid peroxidation (3), glutamate accelerates ferroptosis by accumulating reactive oxygen species (2). In the same context, glutamate-cysteine ligase catalytic sub-

unit (GCLC), a rate-limiting GSH synthetic enzyme, also protects against ferroptosis by converting glutamate into non-toxic gamma-glutamyl peptides (2). On the other hand, there are other parallel ferroptosis protective enzymes. FSP1 in the plasma membrane and mitochondrial DHODH can reduce coenzyme Q₁₀ (CoQ₁₀) producing dihydro-CoQ₁₀ (CoQ₁₀H₂), a solid radical-trapping antioxidant (RTA) (4-6). GTP cyclohydrolase 1 has also been shown to suppress ferroptosis, producing another RTA of tetrahydrobiopterin and contributing to CoQ₁₀ synthesis (7). Enzymes involved in the mevalonate pathway can also protect against ferroptosis by generating CoQ₁₀ and GPX4 (8). In contrast, many ferroptosis-inducible metabolic enzymes including LPCAT3, ASCL4, FADS1, and ELOVL5 can accumulate poly-unsaturated fatty acid (PUFA)-containing phospholipids (9-11) as well as LOXs and POR oxidizing PUFA containing lipids (9, 12, 13). Since all these factors can regulate ferroptosis, precise metabolic alterations need to be evaluated to find the primary source of ferroptosis under various biological conditions.

Modern mass spectrometry based analytical techniques play a crucial role in the discovery of metabolic alteration regulating ferroptosis by extensive analysis of metabolites such as the sulfhydryl residue containing cysteine metabolites, RTAs, and membrane phospholipids. This review discusses the journey of elucidating central ferroptosis mechanisms with present cutting-edge analytical methods.

CONTENTS

Current understanding of ferroptosis-regulating metabolism with mass spectrometry technique

Cyst(e)ine metabolism: Since the discovery of ferroptosis, the role of cyst(e)ine metabolism in ferroptosis regulation has been emphasized. Dixon *et al.* have found that erastin, a potent inhibitor of cystine-glutamate antiporter xCT, can induce non-apoptotic iron-dependent cell death and named it ferroptosis (1). In their study, they examined the uptake of [¹⁴C]-cystine into cells using a scintillation counter to evaluate xCT inhibition by erastin treatment (1). In addition to extracellular cystine uptake, another route of cellular cysteine supply is through trans-sulfuration (TSS) pathway. Therefore, measuring TSS pathway activity is crucial to determine the major source of cysteine. Indeed, a stable isotope-labeled tracing approach has been introduced with ¹³C labeled serine, a major carbon source of the TSS

*Corresponding author. Tel: +82-2-880-7857; E-mail: Yunpyo.Kang@snu.ac.kr

[#]These authors contributed equally to this work.

<https://doi.org/10.5483/BMBRep.2022.55.9.109>

Received 23 June 2022, Revised 27 July 2022,
Accepted 8 August 2022

Keywords: Analytical method, Ferroptosis, Mass spectrometry

pathway (Fig. 1A). As no ^{13}C labeled cysteine fractions were found in non-small cell lung cancer cell (NSCLC) lines, it was concluded that these cells highly relied on extracellular cysteine uptake for cysteine supply, not the TSS pathway. This result further explains why NSCLC cells are susceptible to cysteine deprivation mediated ferroptosis, emphasizing the development of cysteine deprivation as an effective therapeutic approach to treat lung cancer (2).

Cysteine is a rate-limiting building block for the synthesis of GSH, a potent antioxidant and the cofactor of GPX4 which prevents ferroptosis. Therefore, measuring intracellular cysteine and GSH would be important to understand the precise cause of ferroptosis. Notably, as both cysteine and GSH are easily oxidized outside cells due to their high reduction potential, their thiol group needs to be protected during sample preparation and instrumental analysis. Hence, several agents have been used to derivatize the sulfhydryl residue of cysteine and GSH. These reagents could be alkylating agents conjugated to a chromophore or fluorophore include monobromobimane, 4-

fluoro-7-sulfamoylbenzofurazan, 7-fluoro-benzo-2-oxa-1,3-diazole-4-sulphonate, 5-iodoacetamidofluorescein, 2-chloro-1-methylpyridinium iodide and 2-chloro-1-methylquinolinium tetrafluoroborate (14, 15); or maleimide-thiol conjugation such as N-ethyl maleimide (NEM), propionoyl, hexanoyl, and 4-methylcyclohexylcarbonyl maleimide (16). Thiol derivatization assures the accuracy of the quantitative method by preventing thiol oxidation and increases the method's sensitivity by enhancing MS ionization efficiency (17). Recently, NEM has been used to alkylate the sulfhydryl residue of cysteine and GSH followed by LC-MS analysis to quantify the depletion of intracellular cysteine and GSH under a cysteine deprived condition (2, 18).

System xCT regulates not only cysteine uptake, but also glutamate exportation. Therefore, xCT inhibition can result in the accumulation of glutamate that accelerates ferroptosis by ROS accumulation (2). Importantly, the mass spectrometry-based metabolomics approach plays a pivotal role not only in the quantification of glutamate, but also in the discovery of unexpected glutamate metabolism detoxifying glutamate stress under a cysteine-

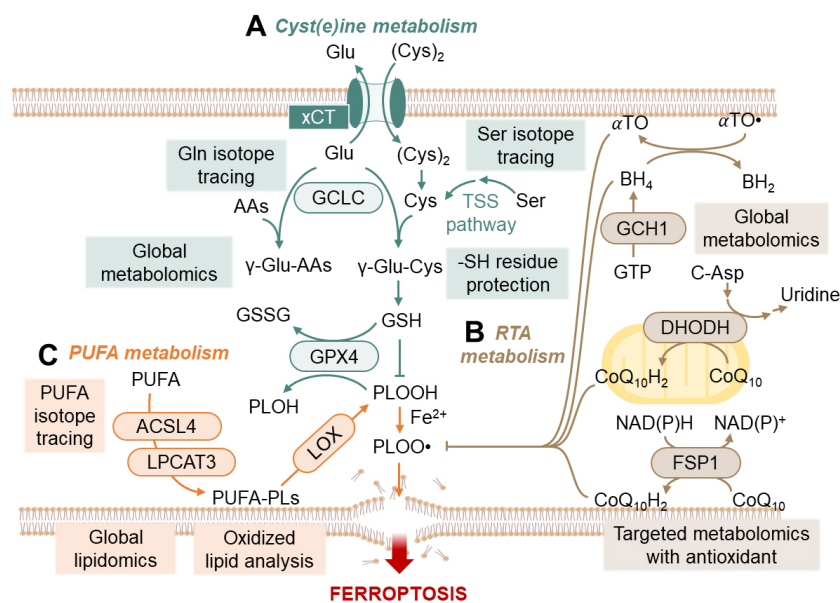


Fig. 1. Mass-spectrometry based analytical methods to study the role of metabolism in regulating ferroptosis. (A) The role of cysteine metabolism. Stable isotope-labeled serine and glutamine tracing approach are used to evaluate trans-sulfuration pathway and GCLC activity, respectively. The global metabolomics approach is used to analysis of γ -Glu-AAs. The cysteine and GSH is quantified after alkylating thiol group due to its high reactivity. (B) The role of radical-trapping antioxidant metabolism. $\text{CoQ}_{10}\text{H}_2$ and CoQ_{10} is quantified with an antioxidant to protect the auto-oxidation of $\text{CoQ}_{10}\text{H}_2$ during sample preparation and instrumental analysis. Also, the global metabolomics approaches are used to determine metabolic consequence of DHODH and GCH1 alteration, respectively. (C) The role of poly-unsaturated fatty acid metabolism. LC-MS/MS-based analysis including global lipidomics and targeted oxidized lipid analysis are applied to study role of poly-unsaturated lipid regulatory enzymes. RTA: radical-trapping antioxidant, $(\text{Cys})_2$: cystine, Cys: cysteine, Ser: serine, TSS: trans-sulfuration, Gln: Glutamine, GCLC: glutamate-cysteine ligase catalytic subunit, γ -Glu-Cys: γ -glutamyl-cysteine, GSH: glutathione, GSSG: oxidized glutathione, AAs: amino acids, γ -Glu-AAs: γ -glutamyl-amino acids, GPX4: glutathione peroxidase 4, PLOOH: phospholipid hydroperoxide, PLOH: phospholipid alcohol, PLOO \cdot : phospholipid peroxy radical, CoQ_{10} : coenzyme Q_{10} , $\text{CoQ}_{10}\text{H}_2$: dihydrocoenzyme Q_{10} , FSP1: ferroptosis suppressor protein 1, DHODH: dihydroorotate dehydrogenase, BH_4 : tetrahydrobiopterin, BH_2 : dihydrobiopterin, GCH1: guanosine triphosphate cyclohydrolase 1, PUFA: poly-unsaturated fatty acid, PUFA-PLs: phospholipids containing polyunsaturated fatty acid, ACSL4: acyl-CoA synthetase long-chain family member 4, LPCAT3: lysophosphatidylcholine acyltransferase 3, LOX: lipoxygenase.

depleted condition. With a non-targeted metabolite profiling approach, dramatic accumulation of γ -glutamyl-peptides in cystine-starved cells was observed (Fig. 1A). By applying a stable isotope-labeled glutamine tracing approach, it was further confirmed that γ -glutamyl-peptides could be directly synthesized from glutamate and other amino acids through GCLC, not from the canonical GSH degradation pathway (Fig. 1A) (2). These results further emphasize that simultaneous targeting of cystine uptake and the production of γ -glutamyl-peptides provide a synergistic therapeutic potential for anti-cancer therapy.

Radical-trapping antioxidant metabolism: Recent studies have discovered the function of coenzyme Q₁₀ (CoQ₁₀) metabolism to prevent ferroptosis independent of GPX4, a primary enzyme that can prevent ferroptosis. Both cytosolic FSP1 (4, 5) and DHODH in mitochondria (6) can reduce CoQ₁₀ to di-hydro CoQ₁₀ (CoQ₁₀H₂), an element suppressing ferroptosis by quenching radicals.

Interestingly, two independent research groups have discovered that FSP1 protein is a novel ferroptosis preventive protein by applying different assays of genetic suppressor screening (4) or CRISPR screening (5) approach to GPX KO or GPX inhibitor-treated cells, respectively. On the other hand, chromatography coupled to mass spectrometry-based global metabolomics approach has shown that GPX4 inhibitor-treated cells consistently accumulate uridine while depleting its precursor of carbamoyl aspartate, suggesting the potential role of DHODH, an enzyme localized on the mitochondrial inner membrane, in preventing ferroptosis (Fig. 1B) (6). These findings imply that in addition to the genetic screening approach, metabolomics analysis might be helpful for finding novel ferroptosis regulatory factors.

To validate the function of ferroptosis preventive enzymes, the ratio of CoQ₁₀ to CoQ₁₀H₂ must be measured. Like the sulfhydryl residue containing metabolite analysis, oxidation of CoQ₁₀H₂ to CoQ₁₀ during sample preparation and instrumental analysis can change the ultimate CoQ₁₀/CoQ₁₀H₂ ratio and express an unreliable result. Therefore, antioxidants such as butylhydroxytoluene (BHT) or tert-butylhydroquinone were added to extraction solvent (5). Due to electroactive groups of CoQ₁₀, the electrochemical detector showed advantage in detecting these metabolites (5). Alternatively, a mass spectrometer detector can be used to measure CoQ₁₀ and CoQ₁₀H₂ quantity with stable isotope-labeled internal standard (Fig. 1B) (6).

CRISPR/dCas9 overexpression screening approach has shown that GCH1 is another GPX4-independent ferroptosis suppressive protein (7). Applying FT-ICR-MS-based global metabolite profiling followed by a UPLC-QTOF-MS-based targeted metabolomics approach has further confirmed that GCH1 overexpressed cells can protect against ferroptosis by producing tetrahydrobiopterin (BH₄) which works as a radical-trapping antioxidant and a cofactor for CoQ₁₀ synthesis (Fig. 1B) (7). Consistently, CRISPR Screening combined with global metabolomics approach revealed that the BH₄ synthesis metabolic pathway could protect against ferroptosis by working as an RTA and by synergizing with Vitamin E (19). Moreover, They further

showed that methotrexate could synergize ferroptosis induction by inhibiting DHFR, which catalyzes the regeneration of BH₄ (19), emphasizing the therapeutic potential by inducing synergistic ferroptosis of cancer cells.

Poly-unsaturated fatty acid metabolism: Oxidation of polyunsaturated fatty acid (PUFA)-containing lipids is a prominent phenotype of ferroptosis. It is known that many PUFA-containing lipid regulatory enzymes are ferroptosis inducible (9-11, 20). Combining the gene expression analysis in the GPX4 deleted cells resistant against ferroptosis and the genome-wide CRISPR-based genetic screening assay, Doll *et al.* revealed that acyl-CoA synthetase long-chain family member 4 (ACSL4) might induce ferroptosis (10). To explore the mechanism of ACSL4-dependent ferroptosis induction, they have applied both global lipidomics and targeted oxidized lipid analysis (Fig. 1C). By observing the depletion of PUFA-containing phospholipids and their oxidized phosphatidylethanolamine (PE) species in ACSL4 KO cells, they validated the contribution of ACSL4 to accumulation of PUFA esterified phospholipids and their peroxidation products (10). The role of PUFA esterified phospholipids in ferroptosis is well demonstrated by Kagan *et al.* (9). By combining the LC-MS/MS based quantitative oxidized fatty acid analysis and the platelet-activating factor acetylhydrolase treatment, they were able to obtain the distribution of free- and esterified- oxidized PUFA residues. By doing so, they showed that treatment of RSL3, a potent GPX4 inhibitor, resulted in accumulation of oxidized esterified PUFA in wild-type cells while the free form of oxidized PUFAs was accumulated in ACSL4 KO cells (9). Furthermore, by applying stable isotope labeled PUFA tracing, they validated the incorporation of PUFA in phospholipids and the function of 15-lipoxygenase in producing pro-ferroptotic oxygenated PE species (9). The deuterated arachidonic acid (AA-d8) tracing shows that the arachidonic acid can be elongated into the adrenic acid (AdA), which are predominantly oxidized by lipoxygenases (9). Further, another stable isotope labeled tracer of [U-¹³C₁₈] linoleic acid is used to show that ELOVL5 and FADS1 can induce ferroptosis in cancer cells by synthesizing AA and AdA (11).

In addition to studying lipid metabolism, oxidized lipids have been analyzed to validate metabolic pathways that regulate ferroptosis. For instance, Yang *et al.* have evaluated the consequence of GPX4 inhibition by applying LC-MS/MS-based phosphatidylcholine hydroperoxide analysis (3). Similar approaches have validated GPX4-independent ferroptosis protective function of FSP1 (6). The LC-MS/MS based oxidized lipid analysis method also has been applied to show the accumulation of oxidized lipids in tumor cells via IFN- γ generated from CD8⁺ T-cell (21) and to validate the functional role of oxidized lipid of 1-stearyl-2-15-HpETE-3-phosphatidylethanolamine (SAPE-OOH) activating the phagocytic clearance of ferroptotic cells by interacting with TLR2 receptors on macrophages (22). The predominant presence of SAPE-OOH in arachidonoyl-enriched ferroptotic cells compared to naive cells, while targeted oxidized lipid analysis was used to evaluate how the SAPE-

OOH concentration changed the rate of phagocytosis toward ferroptotic cells (22).

Overall, MS-based lipid analytical platform is a crucial tool to precisely evaluate ferroptosis phenotype in various biological and pathological conditions.

CONCLUSIONS

This mini-review described a mass spectrometry-based analysis to understand ferroptosis regulatory metabolism. In addition to this, there might be other ferroptosis-regulatory metabolic pathways that need to be evaluated. For instance, coenzyme A (CoA) is known to contribute to ferroptosis regulation by being involved in HMG-CoA in the mevalonate pathway, which is essential for CoQ₁₀ synthesis (23). However, the precise role of CoA derivatives has not been well understood. Furthermore, although lipid oxidation is a hallmark of ferroptosis, the function of lipid oxidative fragments still needs to be evaluated. Therefore, developing methods for quantitative and comprehensive analysis of CoA derivatives and oxidized lipids is needed to understand the new functional metabolism that plays a crucial role in ferroptosis. Another essential technique would be the matrix-assisted laser desorption/ionization (MALDI) based mass imaging and isotope tracing, which can be used in tissue-specific metabolism analysis (24). This technique will let us know spatial metabolic alterations that regulate ferroptosis in multiple organs. Overall, the development of mass spectrometry approach will provide a better chance to understand the novel mechanism of ferroptosis.

ACKNOWLEDGEMENTS

This research was supported by the New Faculty Startup Fund from Seoul National University and the National Research Foundation of Korea (NRF-2022M3A9I2017587, NRF-2022R1C1C1003619).

CONFLICTS OF INTEREST

The authors have no conflicting interests.

REFERENCES

1. Dixon SJ, Lemberg KM, Lamprecht MR et al (2012) Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* 149, 1060-1072
2. Kang YP, Mockabee-Macias A, Jiang C et al (2021) Non-canonical glutamate-cysteine ligase activity protects against ferroptosis. *Cell Metab* 33, 174-189. e7
3. Yang WS, SriRamaratnam R, Welsch ME et al (2014) Regulation of ferroptotic cancer cell death by GPX4. *Cell* 156, 317-331
4. Doll S, Freitas FP, Shah R et al (2019) FSP1 is a glutathione-independent ferroptosis suppressor. *Nature* 575, 693-698
5. Bersuker K, Hendricks JM, Li Z et al (2019) The CoQ oxidoreductase FSP1 acts parallel to GPX4 to inhibit ferroptosis. *Nature* 575, 688-692
6. Mao C, Liu X, Zhang Y et al (2021) DHODH-mediated ferroptosis defence is a targetable vulnerability in cancer. *Nature* 593, 586-590
7. Kraft VA, Bezjian CT, Pfeiffer S et al (2019) GTP cyclohydrolase 1/tetrahydrobiopterin counteract ferroptosis through lipid remodeling. *ACS Cent Sci* 6, 41-53
8. Zheng J and Conrad M (2020) The metabolic underpinnings of ferroptosis. *Cell Metab* 32, 920-937
9. Kagan VE, Mao G, Qu F et al (2017) Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis. *Nat Chem Biol* 13, 81-90
10. Doll S, Proneth B, Tyurina YY et al (2017) ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. *Nat Chem Biol* 13, 91-98
11. Lee JY, Nam M, Son HY et al (2020) Polyunsaturated fatty acid biosynthesis pathway determines ferroptosis sensitivity in gastric cancer. *Proc Natl Acad Sci U S A* 117, 32433-32442
12. Shah R, Shchepinov MS and Pratt DA (2018) Resolving the role of lipoxygenases in the initiation and execution of ferroptosis. *ACS Cent Sci* 4, 387-396
13. Zou Y, Li H, Graham ET et al (2020) Cytochrome P450 oxidoreductase contributes to phospholipid peroxidation in ferroptosis. *Nat Chem Biol* 16, 302-309
14. Banjac A, Perisic T, Sato H et al (2008) The cystine/cysteine cycle: a redox cycle regulating susceptibility versus resistance to cell death. *Oncogene* 27, 1618-1628
15. Peng H, Chen W, Cheng Y, Hakuna L, Strongin R and Wang B (2012) Thiol reactive probes and chemosensors. *Sensors* 12, 15907-15946
16. Fontaine SD, Reid R, Robinson L, Ashley GW and Santi DV (2015) Long-term stabilization of maleimide-thiol conjugates. *Bioconjugate Chem* 26, 145-152
17. Zhao S and Li L (2020) Chemical derivatization in LC-MS-based metabolomics study. *Trends Analyt Chem* 131, 115988
18. Kang YP, Torrente L, Falzone A et al (2019) Cysteine dioxygenase 1 is a metabolic liability for non-small cell lung cancer. *Elife* 8, e45572
19. Soula M, Weber RA, Zilka O et al (2020) Metabolic determinants of cancer cell sensitivity to canonical ferroptosis inducers. *Nat Chem Biol* 16, 1351-1360
20. Dixon SJ, Winter GE, Musavi LS et al (2015) Human haploid cell genetics reveals roles for lipid metabolism genes in nonapoptotic cell death. *ACS Chem Biol* 10, 1604-1609
21. Wang W, Green M, Choi JE et al (2019) CD8+ T cells regulate tumour ferroptosis during cancer immunotherapy. *Nature* 569, 270-274
22. Luo X, Gong HB, Gao HY et al (2021) Oxygenated phosphatidylethanolamine navigates phagocytosis of ferroptotic cells by interacting with TLR2. *Cell Death Differ* 28, 1971-1989
23. Shimada K, Skouta R, Kaplan A et al (2016) Global survey of cell death mechanisms reveals metabolic regulation of ferroptosis. *Nat Chem Biol* 12, 497-503
24. Wang L, Xing X, Zeng X et al (2022) Spatially resolved isotope tracing reveals tissue metabolic activity. *Nat Methods* 19, 223-230