BMB Reports - Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-22-109

Title: Mass spectrometry-based approaches to explore metabolism regulating ferroptosis

Article Type: Mini Review

Keywords: Analytical method; ferroptosis; mass spectrometry; apoptosis; necroptosis

Corresponding Author: Yun Pyo Kang

Authors: Chi Thi Ngoc Nguyen[1], Seon Min Kim[1], Yun Pyo Kang[1,*]

Institution: [1]College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Republic of Korea,

Manuscript Type: Mini Review

Title: Mass spectrometry-based approaches to explore metabolism regulating ferroptosis

Author's name:
Chi Thi Ngoc Nguyen[1,#], Seon Min Kim[1,#], and Yun Pyo Kang[1,*],

[1]: College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Republic of Korea
[#]: These authors contributed equally to this work.
[*]: Correspondence

Affiliation: College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Republic of Korea, 29-Room #312, Gwanak-ro, Gwanak-gu, Seoul, 08826, South Korea

Keywords: Analytical method, ferroptosis, mass spectrometry.

Corresponding Author's Information: 82-2-880-7857, email: Yunpyo.Kang@snu.ac.kr

ABSTRACT
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.

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 cystine 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 subunit (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 Q_{10} (CoQ_{10}) producing dihydro-CoQ_{10} ($CoQ_{10}H_2$), 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 CoQ_{10} synthesis [7]. Enzymes involved in the mevalonate pathway can also protect against ferroptosis by generating CoQ_{10} 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 [^{14}C]-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 ^{13}C labeled serine, a major carbon source of the TSS pathway (Figure 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 cystine uptake for cysteine supply, not the TSS pathway. This result further explains why NSCLC cells are susceptible to cystine deprivation mediated ferroptosis, emphasizing the development of cystine 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 cystine deprived condition [2, 18].

System xCT regulates not only cystine 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 cystine-depleted condition. With a non-targeted metabolite profiling approach, dramatic accumulation of *γ*-glutamyl-peptides in cystine-starved cells was observed (Figure 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 [2] (Figure 1A). 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 Q_{10} (CoQ_{10}) 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 CoQ_{10} to di-hydro CoQ_{10} ($CoQ_{10}H_2$), 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 [6] (Figure 1B). 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 CoQ_{10} to $CoQ_{10}H_2$ must be measured. Like the sulfhydryl residue containing metabolite analysis, oxidation of $CoQ_{10}H_2$ to CoQ_{10} during sample preparation and instrumental analysis can change the ultimate $CoQ_{10}/CoQ_{10}H_2$ ratio and express an unreliable result. Therefore, antioxidants such as butyl-hydroxytoluene (BHT) or tert-butyl-hydroquinone were added to extraction solvent [5]. Due to electroactive groups of CoQ_{10}, the electrochemical detector showed advantage in detecting these metabolites [5]. Alternatively, a mass spectrometer detector can be used to measure CoQ_{10} and $CoQ_{10}H_2$ quantity with stable isotope-labeled internal standard [6] (Figure 1B).

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 (BH_4) which works as a radical-trapping antioxidant and a cofactor for CoQ_{10} synthesis [7] (Figure 1B). Consistently, CRISPR Screening combined with global metabolomics approach revealed that the BH_4 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 BH_4 [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 (Figure 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 esteried- 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-$^{13}C_{18}$] 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 methods 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-steaoryl-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 CoQ_{10} 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 declare no conflict of interest.

FIGURE LEGEND

Figure 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 ɣ-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. $CoQ_{10}H_2$ and CoQ_{10} is quantified with an antioxidant to protect the auto-oxidation of $CoQ_{10}H_2$ 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.
Abbreviation: RTA: radical-trapping antioxidant, $(Cys)_2$: cystine, Cys: cysteine, Ser: serine, TSS: trans-sulfuration, Gln: Glutamine, GCLC: glutamate-cysteine ligase catalytic subunit, ɣ-Glu-Cys: ɣ-glutamyl-cysteine, GSH: glutathione, GSSG: oxidized glutathione, AAs: amino acids, ɣ-Glu-AAs: ɣ-glutamyl-amino acids, GPX4: glutathione peroxidase 4, PLOOH: phospholipid hydroperoxide, PLOH: phospholipid alcohol, PLOO.: phospholipid peroxyl radical, CoQ_{10}: coenzyme Q_{10}, $CoQ_{10}H_2$: 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, ASCL4: acyl-CoA synthetase long-chain family member 4, LPCAT3: lysophosphatidylcholine acyltransferase 3, LOX: lipoxygenase.

REFERENCES

1. Dixon, S.J., et al., *Ferroptosis: an iron-dependent form of nonapoptotic cell death.* Cell, 2012. **149**(5): p. 1060-1072.
2. Kang, Y.P., et al., *Non-canonical glutamate-cysteine ligase activity protects against ferroptosis.* Cell Metabolism, 2021. **33**(1): p. 174-189. e7.
3. Yang, W.S., et al., *Regulation of ferroptotic cancer cell death by GPX4.* Cell, 2014. **156**(1-2): p. 317-331.
4. Doll, S., et al., *FSP1 is a glutathione-independent ferroptosis suppressor.* Nature, 2019. **575**(7784): p. 693-698.
5. Bersuker, K., et al., *The CoQ oxidoreductase FSP1 acts parallel to GPX4 to inhibit ferroptosis.* Nature, 2019. **575**(7784): p. 688-692.
6. Mao, C., et al., *DHODH-mediated ferroptosis defence is a targetable vulnerability in cancer.* Nature, 2021. **593**(7860): p. 586-590.
7. Kraft, V.A., et al., *GTP cyclohydrolase 1/tetrahydrobiopterin counteract ferroptosis through lipid remodeling.* ACS central science, 2019. **6**(1): p. 41-53.
8. Zheng, J. and M. Conrad, *The metabolic underpinnings of ferroptosis.* Cell metabolism, 2020. **32**(6): p. 920-937.
9. Kagan, V.E., et al., *Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis.* Nature chemical biology, 2017. **13**(1): p. 81-90.
10. Doll, S., et al., *ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition.* Nature chemical biology, 2017. **13**(1): p. 91-98.
11. Lee, J.-Y., et al., *Polyunsaturated fatty acid biosynthesis pathway determines ferroptosis sensitivity in gastric cancer.* Proceedings of the National Academy of Sciences, 2020. **117**(51): p. 32433-32442.
12. Shah, R., M.S. Shchepinov, and D.A. Pratt, *Resolving the role of lipoxygenases in the initiation and execution of ferroptosis.* ACS central science, 2018. **4**(3): p. 387-396.
13. Zou, Y., et al., *Cytochrome P450 oxidoreductase contributes to phospholipid peroxidation in ferroptosis.*

Nature chemical biology, 2020. **16**(3): p. 302-309.
14. Banjac, A., et al., *The cystine/cysteine cycle: a redox cycle regulating susceptibility versus resistance to cell death.* Oncogene, 2008. **27**(11): p. 1618-1628.
15. Peng, H., et al., *Thiol reactive probes and chemosensors.* Sensors, 2012. **12**(11): p. 15907-15946.
16. Fontaine, S.D., et al., *Long-term stabilization of maleimide–thiol conjugates.* Bioconjugate chemistry, 2015. **26**(1): p. 145-152.
17. Zhao, S. and L. Li, *Chemical derivatization in LC-MS-based metabolomics study.* TrAC Trends in Analytical Chemistry, 2020. **131**: p. 115988.
18. Kang, Y.P., et al., *Cysteine dioxygenase 1 is a metabolic liability for non-small cell lung cancer.* Elife, 2019. **8**: p. e45572.
19. Soula, M., et al., *Metabolic determinants of cancer cell sensitivity to canonical ferroptosis inducers.* Nature chemical biology, 2020. **16**(12): p. 1351-1360.
20. Dixon, S.J., et al., *Human haploid cell genetics reveals roles for lipid metabolism genes in nonapoptotic cell death.* ACS chemical biology, 2015. **10**(7): p. 1604-1609.
21. Wang, W., et al., *CD8+ T cells regulate tumour ferroptosis during cancer immunotherapy.* Nature, 2019. **569**(7755): p. 270-274.
22. Luo, X., et al., *Oxygenated phosphatidylethanolamine navigates phagocytosis of ferroptotic cells by interacting with TLR2.* Cell Death & Differentiation, 2021. **28**(6): p. 1971-1989.
23. Shimada, K., et al., *Global survey of cell death mechanisms reveals metabolic regulation of ferroptosis.* Nature chemical biology, 2016. **12**(7): p. 497-503.
24. Wang, L., et al., *Spatially resolved isotope tracing reveals tissue metabolic activity.* Nature methods, 2022. **19**(2): p. 223-230.

Figure 1.