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REVIEW ARTICLE

Enzymatic and metabolic regulation of lysine succinylation

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Abstract Lysine succinylation (Ksucc), defined as a transfer of a succinyl group to a lysine residue of a protein, is a newly identified protein post-translational modification[1–3]. This chemical modification is reversible, dynamic, and evolutionarily conserved [4] where it has been comprehensively studied in both bacterial and mammalian cells[5–7]. Numerous proteins involved in the regulation of various cellular and biological processes have been shown to be heavily succinylated[5–7]. Emerging clinical data provides evidence that dysregulation of Ksucc is correlated with the development of several diseases, including cardiovascular diseases and cancer[7–9]. Therefore, an in-depth understanding of Ksucc and its regulation is important not only for understanding its physiological function but also for developing drug therapies and targeted agents for these diseases. In this review, we highlight some of the recent advances in understanding the role of Ksucc and desuccinylation under physiological and pathological conditions.

Introduction

Post-translational modifications (PTMs) are covalent modifications introduced to amino acids of proteins either enzymatically or non-enzymatically.[10–13] These modifications affect protein structure to modulate their stability, localization, and activity. Furthermore, PTMs are tightly regulated in cells, enabling long and persistent effects on cell structure and function. Thus, PTMs are considered one of the most efficient biological mechanisms

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to increase the functional diversity of the proteome. To date, more than 300 types of PTMs are known to occur physiologically, of which, the common ones are phosphorylation, methylation, acetylation, glycosylation, and succinylation.[12] A growing body of research suggests that dysregulation of PTMs plays crucial roles in numerous biological functions and diseases.[10,14–16]

Lysine succinylation (Ksucc) is one of the major post-translational modifications and occurs on a wide range of proteins.[1–3,5,7,9,13] Lysine is an essential amino acid for humans and is one of three amino acids that has a positively charged side chain at physiological pH. Thus, lysine residue side chains can be involved in noncovalent interactions such as van der Waals interactions, hydrogen bonds, and electrostatic interactions with negatively charged residues.[17] Subsequently, this makes lysine essential for protein–protein interactions and the formation of protein complexes. Given the importance of lysine in the regulation of protein structure and function, it is not surprising that Ksucc has a significant impact on protein folding and function.

Ksucc is the addition of a succinyl group (-CO-CH_2-CH_2-CO_2H) to a lysine residue of a protein. Succinylation of a lysine residue neutralizes its positive charge (+1) and gives it a net negative charge (−1). Moreover, the addition of a succinyl group to a protein residue introduces a relatively large structural moiety (100 kDa) compared to lysine methylation and acetylation.[3] Accordingly, the shift in the charge and the structural alteration by Ksucc may have significant effects on the function of the protein. Mass spectrometry (MS) based high-throughput proteomics is a powerful technique to perform global PTM analysis.[12–14] Recent studies using MS based proteomic analysis have generated large amounts of data identifying numerous lysine succinylated-cytosolic, mitochondrial, and nuclear proteins, including metabolic enzymes involved in fatty acid synthesis, fatty acid oxidation, amino acid degradation, mitochondrial respiration, and the tricarboxylic (TCA) acid cycle.[7,18–20] Therefore, Ksucc likely plays a critical role in regulating various cellular metabolic pathways.

History of the discovery of Ksucc

Approximately 60 years ago, succinylation was known to inhibit antibody formation and was utilized for testing allergic skin responses in animals that were sensitive to dinitrophenyl-polyline. However, the mechanisms underlying this antibody inhibition had remained unclear until 1976, when a study investigated the effects of succinylation on the conformation and immunological activity of ovalbumin.[21] They found that succinylation, via the addition of succinic anhydride to ovalbumin solution *in vitro*, altered the electrophoretic mobility and isoionic pH of ovalbumin and caused a conformational shift in the protein.

One of the biggest breakthroughs in succinylation research in recent times, succinylation was identified as a naturally occurring post-translational modification of lysine residues in bacteria.[1–3] Notably, succinyl-CoA was identified as a putative substrate for Ksucc. Since then, numerous studies have investigated the roles of Ksucc in cellular physiology, and Ksucc has been shown to occur extensively in both prokaryotes and eukaryotes.[7,18,20,22] Given that lysine residues on many diverse proteins are subjected to succinylation, it is implied that Ksucc is crucial for the functional regulation of proteins and in the control of various signaling and regulatory pathways.

Regulation of Ksucc by desuccinylation

The biological functions of Ksucc were poorly understood until significant progress was made in Ksucc research with the identification of sirtuin 5 (SIRT5) as a desuccinylase.[4] SIRT5 is a family member of proteins that acts predominantly as nicotinamide adenine dinucleotide (NAD)-dependent protein lysine deacetylases and desuccinylases.[4,23] SIRT5 was initially shown to deacetylate and upregulate the activity of carbamoyl phosphate synthase 1 (CPS1), which is a key enzyme in urea cycle.[24] However, it has been shown that SIRT5 also possesses a desuccinylase function and SIRT5's desuccinylase activity is much higher than its deacetylase activity *in vitro*.[4] To further confirm this, SIRT5 was knocked out in mice to examine whether CPS1 is a desuccinylation target of SIRT5.[4] Their results confirmed that SIRT5 knockout increased the level of succinylation on CPS1 at K1291, thus suggesting that Ksucc can be regulated by SIRT5 *in vivo*. This study established SIRT5 as a lyine desuccinylase that catalyzes the removal of a succinyl group from lysine residues on proteins using NAD^+ as a co-factor.[4] Although SIRT5 was initially thought to be a mitochondrial protein, recent reports have shown SIRT5 localized to the cytosol as well,[25] and many cytosolic and mitochondrial proteins are targets of SIRT5. It has been shown that loss of SIRT5 leads to hyper-succinylation of a variety of mitochondrial proteins including CPS1, malate dehydrogenase (MDH), and several enzymes involved in fatty acid metabolism, including 3-ketoacyl-CoA thiolase, acyl-coenzyme A synthetase medium-chain family member 1, enoyl-CoA delta isomerase 1, and the trifunctional enzyme α subunit. A total of 386 sites on 140 proteins involved in major metabolic pathways (ketogenesis, fatty acid β-oxidation, TCA cycle, and ATP-synthesis) were identified as hyper-succinylated (at least two-fold increase in succinylation as compared to WT mice) in SIRT5 knockout mice.[26] Taken together, these studies suggest that succinylation is an abundant lysine modification, and SIRT5 is a crucial regulator of Ksucc on a number of metabolic enzymes. However, more work is required to better understand the effects of reversible Ksucc on these enzymes and subsequent cellular physiology.

Regulation of Ksucc by succinyl-CoA

While a mechanism of desuccinlyation had been identified, the mechanism regarding succinylation of substrates remained to be identified. Ksucc can occur by a non-enzymatic chemical reaction, originating directly from succinyl-CoA. This suggests that the abundance of succinyl-CoA would be one of the main governing factors of Ksucc. It has been shown that succinyl-CoA could non-enzymatically succinylate BSA and ovalbumin *in vitro* in a concentration-dependent manner, demonstrating that succinylation depends on intracellular succinyl-CoA levels.[2] Succinyl-CoA

can be generated from the TCA cycle, lipids, and amino acid metabolism and synthesized by the enzyme succinyl-CoA synthetase and the corresponding acyl salt succinate.[27] Because of the high concentration of succinyl-CoA in mitochondria, it has therefore been hypothesized that protein succinylation in mitochondria may occur non-enzymatically. In addition, although succinyl-CoA is primarily formed in mitochondria, a previous study showed that succinate in the cytosol can be converted back to succinyl-CoA.[28] This suggests a mechanism to how Ksucc can occur on cytosolic and nuclear proteins as well.[2] To substantiate this, a recent study showed that loss of succinate dehydrogenase (SDH) leads to global lysine hyper-succinylation in multiple cellular compartments due to the accumulation of succinyl-CoA. More than one-third of nucleosomes, including histone and non-histone chromatin components, were lysine succinylated, suggesting that TCA cycle dysfunction, due to SDH loss, has significant effects on chromatin succinylation and subsequent gene expression.[29] Another study showed that nicotinamide adenine dinucleotide phosphate-isocitrate dehydrogenase ($NADP^+$-IDH) R132H mutation results in mitochondrial hyper-succinylation.[8] Stable overexpression of $NADP^+$-IDH R132H mutant results in a 280% increase in cellular succinyl-CoA levels leading to hyper-succinylation in the mitochondria and further resulting in mitochondrial membrane depolarization, impaired respiration, and tumor cell proliferation.[8] Taken together, these studies support the notion that succinyl-CoA, succinate, or another succinyl-metabolite may drive succinylation within and outside the mitochondria (Fig 1A).

Enzymatic succinylation of lysine

Intracellular succinyl-CoAlevels can regulate lysine succinylation in cells, however, the possibility that there might be a lysine succinyltransferase (KSTase) in cells cannot be excluded. Several recent studies have indicated that enzyme-catalyzed lysine succinylation exists in cells. Wang Y et al, showed that lysine acetyltransferase 2A (KAT2A) acts as KSTase and succinylates histone H3 on lysine 79 (H3K79) to promote tumor cell proliferation.[20] Tyrosine 645 (Y645) in KAT2A interacts with α-ketoglutarate dehydrogenase complex (α-KGDH) in the nucleus to access succinyl-CoA generated locally by the α-KGDH complex and hence, this complex serves as a local source of succinyl-CoA for KAT2A-dependent histone succinylation. Preventing α-KGDH from entering the nucleus or mutating Y645 in KAT2A to alanine reduced the binding ability and catalytic activity of KAT2A toward succinyl-CoA, resulting in decreased H3K79 succinylation[20].

Another study has demonstrated that carnitine palmitoyltransferase (CPT) 1A, a mitochondrial outer membrane protein involved in fatty acid oxidation, has the moonlight activity as a KSTase *in vivo* and *in vitro*.[18] Using SILAC-based quantitative lysine succinylation proteomic analysis, they identified that 171 lysine sites on 101 proteins (out of 550 lysine sites on 247 proteins total) were succinylated in a CPT1A expression-dependent manner in cells. Importantly, this study showed that the canonical CPTase activity and the novel KSTase activity of CPT1A can be separated by mutation of CPT1A G710E and that CPT1A G710E mutant promotes cell proliferation under metabolic stress conditions without affecting intracellular succinyl-CoA levels, suggesting that the KSTase activity of CPT1A may succinylate downstream substrate proteins to promote proliferation[18]. Although this study further identified enolase 1 as a downstream substrate of CPT1A, whether the KSTase activity of CPT1A contributes to tumor growth remains unknown (Fig 1B).

Effects of Ksucc on target proteins

Ksucc, like all PTMs, can affect localization, stability, structure, or function of its downstream targets. Of note, Ksucc has been observed within the catalytic pocket of target proteins, and target proteins display differences in function following succinylation.[6,18,22] There also appear to be multiple mechanisms of regulation of Ksucc on target proteins. Of the identified enzymes with KSTase or

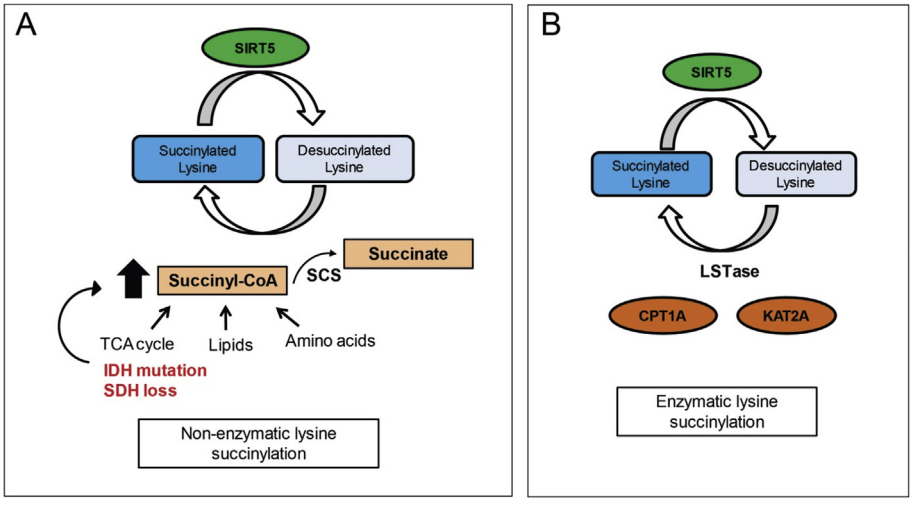

Figure 1 Metabolic regulation of Ksucc by succinyl-CoA **(A)**. Enzymatic regulation of Ksucc **(B)**.

desuccinylase activity, the motif sequences recognized and targeted differ. CPT1A recognizes a LVxxK motif, while SIRT5 recognizes lysine residues immediately flanked by nonpolar amino acids, including alanine, glycine, and phenylalanine.[18,26] The differences in motif sequence suggest additional, unidentified mechanisms of regulation may also be present. The subcellular localization of target proteins also suggests differences in the regulatory mechanisms. KAT2A has been reported to succinylate nuclear histone 3, a majority of CPT1A targets are cytosolic, and non-enzymatic succinylation and SIRT5 desuccinylation are most prevalent in the mitochondria.[18,20] The differences in the motif sequence, as well as the differences in subcellular localization of the target proteins highlights the complexity of Ksucc regulation and suggest the possibility of additional KSTases and desuccinylases that remain to be identified.

A second mechanism that may influence Ksucc is the presence of other PTMs. It has been reported that some Ksucc sites directly overlap with lysines ubiquitinated, methylated, acetylated and malonylated.[6,20] The overlap between Ksucc and lysine acetylation has been investigated due to the similarities between these two PTMs. Park et al reported that, of the over 2000 Ksucc and 2000 lysine acetylation sites examined, only 282 appeared to overlap in mouse MEFs.[6] Rardin et al, however, observed that of the 1200 Ksucc sites observed in mouse liver mitochondria, 79% of the sites are reported sites of deacetylation by SIRT3.[26] Thus it appears that the extent of overlap between lysine acetylation and Ksucc varies between model systems. Second, H3K79 succinylation, a substrate of KAT2A, is also a target for methylation by DOT1L.[20,30] Wang et al reported ~7200 genes which possess H3K79 succinylation, however the effect of this succinylation in place of methylation on gene expression remains to be fully elucidated.[20] Ksucc also occurs within flanking distance to sites of other PTMs, including phosphorylation, ubiquitination, and lipid modifications.[6] This suggests that Ksucc may alter protein structure to facilitate these protein modifications, or these PTMs may facilitate Ksucc. More research is needed to examine the crosstalk between Ksucc and other PTMs.

Aberrant Ksucc and its implication in disease

Growing evidence suggests that Ksucc is a common protein modification that is important in many cellular processes, including metabolism, gene transcription, DNA damage response, and protein folding, stability, and function. Thus, it requires further research to understand the role of Ksucc in cellular physiology and pathology. The tight balance between Ksucc and desuccinylation events is optimal for gene regulation and signaling pathways. It is no surprise that dysfunction in this balance is therefore associated with a variety of diseases, including cancer and heart disease.

Ksucc in cancer

The physiological function of SIRT5 in cancer has long been obscure. Although SIRT5 is speculated to play a role in neoplasia, researchers are still struggling to establish the complex role of SIRT5 in cancer development and progression. SIRT5 has been shown to exhibit tumor suppressor as well as tumor promoter functions under different cellular conditions. A recent study found that PKM2 is succinylated at K498 and succinylation increases PKM2 activity. SIRT5 binds to, desuccinylates and inhibits the activity of PKM2, thus contributing to tumorigenesis. Inhibition of SIRT5 and/or substitution of endogenous PKM2 with a succinylation mimetic mutant K498E decreased cell proliferation and tumor growth through desuccinylation of PKM2, thus suggesting that SIRT5-mediated desuccinylation of PKM2 enhanced tumorigenecity.[31] On the other hand, another study showed that overexpression of SIRT5 decreased succinylation and inhibited tumor growth of cells harboring $NADP^+$-IDH1 R132H mutant that causes hyper-succinylation, suggesting that hyper-succinylation promotes tumorigenesis and SIRT5-mediated desuccinylation inhibits tumor growth.[8] In addition, expression of the KSTase activity-deficient Y645A KAT2A mutant decreased tumor cell proliferation and tumor growth in mice, suggesting that KAT2A-mediated histone succinylation contributes to tumor development.[20] Finally, a recent study showed that CPT1A-mediated succinylation increased human gastric cancer invasion. They found that S100A10, a family of calcium-binding cytosolic proteins, was succinylated at K47 in a CPT1A-dependent manner and the levels of succinylated S100A10 were increased in human gastric cancer. CPT1A binds to and succinylates S100A10 and overexpression of a succinylation mimetic mutant, K47E S100A10, increased cell invasion and migration, suggesting that CPT1A-mediated succinylation promotes gastric cancer invasion and migration.[22] These are just a few examples implying that Ksucc may have a dual role in cancer. Hence, further detailed studies are needed to dissect the role and functional significance of Ksucc in cancer.

By the recent extensive use of MS-based proteomics techniques, many succinylation sites on proteins and sequence variants have been identified in cancers. One study examined whether the essential enzymes of the TCA cycle and pentose phosphate pathway (PPP) have any Ksucc modifications in gastric cancer patients.[32] Their proteome analysis showed that Ksucc levels were increased in disease development, suggesting that aberrant changes in Ksucc and desuccinylation may regulate cancer cell metabolism. Although it seems that Ksucc and SIRT5 are differentially regulated in cancer tissues compared with their normal tissues, the details of this regulation remain inconclusive. Therefore, it would be intriguing to identify and investigate which succinylated enzymes are involved in tumor growth.

Ksucc in heart disease

Studies on Ksucc regulation in cancer opened up directions to discover novel activities for lysine succinylated proteins and their regulation by SIRT5 in heart disease. Using proteomics, it has been shown that Ksucc predominantly accumulates in the mouse heart when SIRT5 is deleted. 124 succinylated cardiac proteins that were regulated by SIRT5, of which ECHA, a protein involved in fatty acid oxidation, was identified to have the most succinylation sites (28 Lys residues) in the SIRT5 knockout heart. SIRT5-mediated desuccinylation was shown to activate ECHA, and as a result, SIRT5 knockout mice exhibited defective fatty acid

oxidation and decreased ATP production.[33] Furthermore, another study showed that SIRT5 knockout mice were more susceptible to ischemia-reperfusion (I/R) injury compared to wild type.[19] Four succinylated lysine residues in the SDH subunit DHSA and one succinylated residue in the subunit DHSB were identified in this study. The loss of SIRT5 activity increased succinylation of DHSA, promoted SDH activity, and increased ischemia-reperfusion, suggesting that the increase in I/R injury in SIRT5 knockout mice may be mediated by lysine succinylated SDH.

Another recent study examined the impact of lysine acetylation and succinylation on maturational changes in energy metabolism in the newborn heart.[34] This study showed that, although the overall acetylation is decreased by age, the overall succinylation continued to increase even in adult hearts. It was suggested that the increase in succinylation is possibly due to an age-dependent decrease in SIRT5 expression. Taken together, these studies implicate a distinct role of Ksucc and SIRT5-mediated desuccinylation in cardiac metabolism. However, as discussed above, there is a need to intensify efforts towards a mechanism-based approach to understand how Ksucc contributes to heart disease.

Conclusion

Ksucc has emerged as a major PTM that occurs in a wide range of proteins both in prokaryotes and eukaryotes. Thus, Ksucc and SIRT5-mediated reversible succinylation can be critical regulators of cellular processes, and the tight balance between these two processes may function to keep cellular homeostasis. Because Ksucc targets a significant number of cellular and metabolic processes, aberrations in Ksucc and desuccinylation have been associated with several diseases. Thus, it is necessary to have an in-depth understanding of the substrates targeted by Ksucc and SIRT5, their biological roles, and downstream targets.

Although several molecular consequences of Ksucc have been described, it is still not clear as to why the cell succinylates certain proteins and what factors determine the fate of Ksucc. It is also important to measure succinyl-CoA levels in subcellular compartments and to understand what factors may influence the levels of succinyl-CoA in subcellular compartments. In addition, it is interesting to speculate that the roles of Ksucc and SIRT5-mediated desuccinylation are differentially regulated in healthy versus diseased cells and may depend on many of the factors that influence disease progression. Therefore, future research needs to address these possibilities to advance our understanding of the regulation of Ksucc and SIRT5-mediated desuccinylation.

Author Contributions

AS, EKW, and TH wrote and edited the review.

Conflict of interest

The authors declare no conflict of interest.

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