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Original article

Characterization of the cardiac succinylome and its role in ischemia–reperfusion injury

Jennifer A. Boylston [a], Junhui Sun [a], Yong Chen [b], Marjan Gucek [b], Michael N. Sack [c], Elizabeth Murphy [a,*]

[a] Systems Biology Center, NHLBI, NIH, Bethesda, MD, United States
[b] Proteomics Core Facility, NHLBI, NIH, Bethesda, MD, United States
[c] Center for Molecular Medicine, NHLBI, NIH, Bethesda, MD, United States

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ABSTRACT

Succinylation refers to modification of lysine residues with succinyl groups donated by succinyl-CoA. Sirtuin5 (Sirt5) is a mitochondrial NAD^+-dependent deacylase that catalyzes the removal of succinyl groups from proteins. Sirt5 and protein succinylation are conserved across species, suggesting functional importance of the modification. Sirt5 loss impacts liver metabolism but the role of succinylation in the heart has not been explored. We combined affinity enrichment with proteomics and mass spectrometry to analyze total succinylated lysine content of mitochondria isolated from WT and *Sirt5*$^{-/-}$ mouse hearts. We identified 887 succinylated lysine residues in 184 proteins. 44 peptides (5 proteins) occurred uniquely in WT samples, 289 (46 proteins) in *Sirt5*$^{-/-}$ samples, and 554 (133 proteins) were common to both groups. The 46 unique proteins in *Sirt5*$^{-/-}$ heart participate in metabolic processes such as fatty acid β-oxidation (Eci2) and branched chain amino acid catabolism, and include respiratory chain proteins (Ndufa7, 12, 13, Dhsa). We performed label-free analysis of the peptides common to WT and *Sirt5*$^{-/-}$ hearts. 16 peptides from 9 proteins were significantly increased in *Sirt5*$^{-/-}$ by at least 30%. The adenine nucleotide transporter 1 showed the highest increase in succinylation in *Sirt5*$^{-/-}$ (108.4 fold). The data indicate that succinylation is widespread in the heart and enriched in metabolic pathways. We examined whether the loss of Sirt5 would impact ischemia–reperfusion (I/R) injury and we found an increase in infarct size in *Sirt5*$^{-/-}$ hearts compared to WT littermates (68.5$^+$/_ 1.1% *Sirt5*$^{-/-}$ vs 39.6$^+$/$^-$ 6.8% WT) following 20 min of ischemia and 90-min reperfusion. We further demonstrate that I/R injury in *Sirt5*$^{-/-}$ heart is restored to WT levels by pretreatment with dimethyl malonate, a competitive inhibitor of succinate dehydrogenase (SDH), implicating alteration in SDH activity as causative of the injury.

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1. Introduction

An emerging class of post-translational modifications (PTMs) involves modification of lysine side chains with thioester-coenzyme A metabolites [1–5]. Succinylation refers to the modification of lysine residues with succinyl groups that are donated by the intermediate metabolite succinyl-CoA (SucCoA) [4]. The phenomenon of protein succinylation is conserved across species, and has been identified in *Escherichia coli*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens* [4,6–10]. To date, a cellular succinyltransferase has not been identified, and the reaction of SucCoA with lysine side chains is presumed to occur spontaneously [11]. Conditions of the mitochondrial matrix, where pH is relatively high and SucCoA is concentrated, support this spontaneous reaction, thus it is not surprising that succinylated proteins are enriched in mitochondria [7,9]. Manipulation of cellular SucCoA levels via genetic deletion of TCA enzymes in yeast demonstrates that global protein succinylation positively correlates with SucCoA levels [7]. SucCoA levels reflect the metabolic status of a cell, suggesting that protein succinylation and the intermediate metabolite SucCoA function as a metabolic signaling pathway. The mammalian Sirtuin (Sirt) family of proteins includes seven members, three of which (Sirt3, Sirt4, and Sirt5) are predominantly located in the mitochondria. Sirt5 catalyzes the desuccinylation of lysine residues in a reaction that requires consumption of NAD^+ [12]. The study of Sirt5 and its desuccinylase activity has recently been described, and the general recognition that succinylation is a bona fide PTM and the ability of Sirt5 to regulate it in liver was only recently reported [12]. Succinylation can both increase and decrease protein activity. Succinylation of protein components of the pyruvate and succinate dehydrogenase complexes correlates with increased complex activity [9]. Conversely, succinylation of 3-hydroxy-3-methylglutaryl-CoA synthetase 2 (Hmgcs2) decreases activity of the enzyme, and ketogenesis is altered in the Sirt5 knockout mouse liver [10].

The function of Sirt5 in the heart has not been explored. The unrelenting energy needs of the heart underlie a crucial need for proper metabolic tuning in cardiac tissue. Although metabolic remodeling in liver in the *Sirt5*$^{-/-}$ mouse has been described [9,10,13], there are little or no data on succinylation in the heart. To investigate this, we

* Corresponding author at: NHLBI, NIH, 10 Center Drive, Bethesda, MD, United States.
E-mail address: murphy1@nhlbi.nih.gov (E. Murphy).

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combined affinity enrichment with proteomics to capture an image of lysine succinylation in cardiac mitochondria. The data demonstrate that protein succinylation occurs at baseline in heart mitochondria and suggests that Sirt5 functions as a desuccinylase. The data in this paper provide the first description of the cardiac succinylome. We find that succinylation is widespread in the heart, and proteins succinylated in cardiac mitochondria participate in the processes of oxidative phosphorylation, fatty acid oxidation, ketogenesis, and branched chain amino acid catabolism, among others. We investigated whether the metabolic alterations in the *Sirt5*$^{-/-}$ mice might alter the response of the heart to ischemia-reperfusion injury (I/R), and we found that the *Sirt5* knockout mouse has increase I/R injury.

2. Methods

2.1. Animals

All animals were treated and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health (NIH), Revised 2011), and protocols were approved by the Institutional Animal Care and use Committee. The sexes and ages of mice at time of experimentation are indicated within particular method descriptions. The *Sirt5*$^{-/-}$ mice are on a C57B6 background and have been backcrossed more than 10 generations.

2.2. Mitochondria isolation from mouse hearts

Mitochondria were isolated by differential centrifugation according to standard procedures [14]. Briefly, hearts were minced in mitochondrial isotonic buffer ("Buffer B") consisting of 225 mM mannitol, 75 mM sucrose, 5 mM MOPS, 0.5 mM EGTA, and 2 mM taurine, 5 mM nicotinamide, 1 μM trichostatin A (pH 7.25). Minced heart tissue was homogenized by Polytron. To digest contractile proteins, trypsin (0.001 g/0.1 g wet tissue) in buffer B was added to homogenized hearts for 5 min on ice. Homogenates were centrifuged at 500 ×*g* for 5 min. (4 °C) and the resulting supernatant was centrifuged at 11,000 ×*g* for 5 min. (4 °C) to pellet mitochondria. Final mitochondrial pellets were resuspended in Buffer B.

2.3. Immunoblotting

Protein extracts were quantified with BCA protein assay (Pierce Cat #23,225) or Bradford protein assay (Sigma Cat# B6916). Proteins were reduced with 50 mM DTT, denatured with LDS (lithium dodecyl sulfate), heated, and equivalent amounts of protein were loaded on NuPage 4–12% Bis-Tris gels (Invitrogen) and electrophoresed in 1× MES buffer. Protein gels were transferred to nitrocellulose membranes (0.2 μm pore size). Membranes were blocked with 5% milk. Primary antibodies were incubated in 5% BSA overnight at 4 °C. Secondary antibody incubations were performed in 5% milk at room temperature for 30–45 min. Blots were developed with reagents from the Amersham ECL Select Western blotting kit (Amersham, Cat #RPM2235) and signal was collected with chemiluminescence film (Amersham, Cat #28906834). Sirt5 antibody, Mdh2 antibody, and the antibodies designed to detect acetylated lysine residues were obtained from Cell Signaling Technologies (Sirt5, #8779; Mdh2, #8610; AcK #9441; AcK #9681). The antibody designed to detect succinylated lysine residues was obtained from PTM Biolabs (PTM-401).

2.4. Affinity purification of peptides containing succinylated lysine residues

To measure succinylation we used an approach that has been used previously to measure acetylation [2,15]. This approach employs an antibody against succinyl-lysine (SuK) to precipitate SuK containing peptides, which are then identified by mass spectrometry. Hearts were collected from three male wild type and four *Sirt5*$^{-/-}$ male mice at 6 months of age. Mitochondria were isolated via differential centrifugation in the presence of deacylase inhibitors (5 mM NAM and 1 μM trichostatin A). Isolated mitochondrial pellets (800 μg) were resuspended in 8.0 M guanidine hydrochloride (Sigma, #G9284). Samples were first reduced with 5 mM DTT for 1 h at 60 °C, then alkylated with 15 mM iodoacetamide for 30 min at room temperature in the dark. Alkylation reactions were quenched with DTT. Samples were diluted with 25 mM TEAB such that guanidine hydrochloride concentration was <0.8 M. The samples were then digested overnight at 37 °C with trypsin (Pierce, V5111) at protein:enzyme ratio of approximately 50:1. The tryptic peptides were acidified with formic acid, and desalted on Oasis HLB 1 cm^3 cartridges (Waters #WAT200677) per manufacturer's instructions. Collected eluants were lyophilized overnight and resolubilized in NETN buffer (50 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). Tryptic peptides were enriched via immunoprecipitation with anti-succinyl lysine antibody (PTM Biolabs, PTM-401) immobilized on Protein G agarose beads (Life Technologies, 15,920–010). Peptides and antibody-beads were incubated overnight at 4 °C with end-over-end rotation. The beads were washed 3× with 1 mL NETN buffer, then 2× with 1 mL ETN buffer (50 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA). Bound succinylated peptides were eluted from the agarose with 0.1% TFA. The eluate was cleaned with C18 tips (Varian Omix, A57003100). Peptides were dried and samples resuspended in 0.1% formic acid for mass spec analysis. The samples were analyzed on an LTQ Orbitrap Elite (Thermo Fisher Scientific, San Jose, CA) coupled with an Eksigent nanoLC-Ultra 1D plus system (Dubin, CA). Peptides were separated on a PicoFrit analytical column (250 mm long, ID 75 μm, tip ID 10 μm, packed with BetaBasic 5 μm 300 Å particles, New Objective, Woburn, MA) using a 160-min linear gradient of 5–35% acetonitrile in 0.1% formic acid at a flow rate of 250 nL/min. Mass analysis was carried out in data-dependent analysis mode, where MS1 scanned full MS mass range from m/z 300 to 2000 at 60,000 mass resolution and 10 CID MS2 scans were sequentially carried out in the Orbitrap and the ion trap, respectively. The LC–MS data were searched against the SwissProt database, taxonomy *Mus musculus* (mouse) using Mascot server (Matrix Science, London, UK; version 2.4). Searching parameters were set as follows: precursor mass tolerance at 20 ppm, fragment ion mass tolerance at 0.8 Da, trypsin enzyme with 4 miscleavages, carbamidomethylation of cysteine as fixed modification, and deamidation of asparagine and glutamine, oxidation of methionine, and succinylation of lysine as variable modifications. Peptides were filtered with 1.0% false discovery rate (FDR). Relative quantification of succinylated peptides were performed using QUOIL (QUantification withOut Isotope Labeling), an in-house software program designed as a label-free approach to peptide quantification by LC–MS/MS [16].

2.5. Pathway analysis

Pathway analysis was performed with the Ingenuity Pathway Analysis tool (IPA, Ingenuity Systems, www.ingenuity.com). A dataset containing the Uniprot KB IDs for all proteins identified as succinylated was uploaded to the application. The entire mouse proteome was used as the reference set. A core analysis was performed to identify protein function and a canonical pathway analysis was used to identify the pathways to which the proteins mapped. Significance is measured within IPA by two means: 1.) Determination of a ratio calculated by dividing the number of differentially expressed genes that map to a particular pathway by the total number of genes in that pathway; 2.) Calculation of a p-value by Fisher's exact test to determine the probability that the association between the genes in the dataset and the canonical pathway can be explained by chance.

2.6. Langendorff heart perfusion

Global ischemia/reperfusion was carried out via Langendorff technique with WT and *Sirt5*$^{-/-}$ mice. Mice were anesthetized with