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Site-specific quantitative analysis of cardiac mitochondrial protein phosphorylation☆

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ABSTRACT

We report the development of a multiple-reaction monitoring (MRM) strategy specifically tailored to the detection and quantification of mitochondrial protein phosphorylation. We recently derived 68 MRM transitions specific to protein modifications in the respiratory chain, voltage-dependent anion channel, and adenine nucleotide translocase. Here, we have now expanded the total number of MRM transitions to 176 to cover proteins from the tricarboxylic acid cycle, pyruvate dehydrogenase complex, and branched-chain alpha-keto acid dehydrogenase complex. We utilized the transition set to analyze endogenous protein phosphorylation in human heart, mouse heart, and mouse liver. The data demonstrate the potential utility of the MRM workflow for studying the functional details of mitochondrial phosphorylation signaling. This article is part of a Special Issue entitled: From protein structures to clinical applications.

1. Introduction

Mitochondrial functions are continuously modulated by metabolic and signaling cues, including covalent protein modifications via reversible phosphorylation. Phosphorylation of pyruvate dehydrogenase (PDH) constitutes a classic metabolic switch that shunts pyruvate in or out of the glucose oxidation pathway [1,2]. More recently, phosphorylation signaling was shown to modulate cardioprotection and ischemic injury through the reperfusion-injury salvage kinase (RISK) pathway [3,4], and to serve as an allosteric regulator of respiratory chain complex activities [5,6] and supercomplex assembly [7]. Large-scale proteomics discovery experiments by our laboratory [8] and others [9,10] have unveiled a complex network of hundreds of protein phosphorylation events in mitochondria. For example, the mitochondrial voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT) contain over 15 known phosphorylation sites, collectively targeted by multiple kinases including PKA, PKC, and GSK3β. The physiological functions of most of the recently discovered mitochondrial phosphorylations remain unknown.

Site-specific quantitative data on phosphorylation levels will help elucidate the functions of these novel phosphorylation modifications under various phenotypes, but technical challenges have hampered progress. Protein phosphorylation is reversible and transient, and frequently occurs at only partial stoichiometry, or occupancy, of the phosphoproteins. Phosphorylated peptides are thus more difficult to detect and

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quantify than unmodified peptides, and demand a combination of specialized enrichment methods and sensitive, specific mass spectrometric techniques [11,12]. Recently, we developed a multiple-reaction monitoring (MRM) workflow to quantify mitochondrial protein phosphorylation [11]. In the workflow, peptide ion transitions were chosen that not only specifically identify phosphopeptides with high sensitivity, but also demarcate the locations of individual phosphorylations when multiple modifiable residues exist in close proximity. We optimized the MRM detection parameters for individual transitions, and utilized RP–TiO_2–RP chromatography for phosphopeptide enrichment. In total, we manually collated over 60 parent ion/fragment ion transitions that unequivocally identify endogenous phosphorylation sites of interest. Isotope-labeled synthetic peptides corresponding to known phosphorylation sites were used as internal standards and assayed simultaneously with endogenous peptides to quantify the phosphopeptides in a site-specific manner.

In this study, we expanded the coverage of the MRM assays considerably and utilized them to analyze endogenous phosphorylations in mouse and human mitochondria. We observed unique phosphorylation patterns between species (mouse versus human), organs (heart versus liver), and metabolic states (fed versus fasted mice) that suggested differential regulation by phosphorylation in these systems. We further discuss some considerations for the development of phosphorylation-specific MRM transitions and their biological implications.

2. Materials and methods

2.1. Animals and human samples

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council and approved by UCLA. C57BL/6 mice, 8–10 weeks of age (Harlan Laboratories) were housed in a 12 h/12 h light–dark cycle with controlled temperature and humidity and free access to standard lab chow and water. For fasting experiments, food was withheld from three groups of two mice for 48 h before euthanasia. The control group had *ad libitum* access to food during the same period. Experimental procedures involving human tissues were approved by the UCLA Human Subjects Protection Committee and Institutional Review Board. Patients gave written informed consent. Anterior left ventricular wall samples were collected during heart transplantation from 3 individual heart failure patients between 05/05/2010 and 08/18/2010.

2.2. Mitochondrial protein isolation and digestion

Mitochondrial samples were isolated from the liver and heart of euthanized mice as previously described [13,14]. Organs were excised, homogenized (Dounce homogenizer, 10 strokes for liver, 20 strokes for heart) in sucrose buffer (250 mmol·L^{-1} sucrose, 10 mmol·L^{-1} HEPES, 10 mmol·L^{-1} Tris–HCl, 1 mmol·L^{-1} EGTA, protease inhibitors (Roche Complete, 1×), phosphatase inhibitors (Sigma Phosphatase Inhibitor Cocktail II and III, 1×), and 10 mmol·L^{-1} dithiothreitol (Sigma), pH 7.5), then centrifuged (800 rcf, 4 °C, 7 min) to remove debris. The supernatant was re-centrifuged (4000 rcf, 4 °C, 20 min). The pellets were washed, centrifuged again, then resuspended in 19% (v/v) Percoll (Sigma) in the sucrose buffer, overlaid on 30% and 60% Percoll, and centrifuged (12,000 rcf, 4 °C, 20 min). Purified mitochondria were collected from the bottom layer, washed twice, and pelleted (4000 rcf, 4 °C, 20 min). The pellet was lysed by sonication in 10 mmol·L^{-1} Tris–HCl, pH 7.4. Protein concentration was assayed by the bicinchoninic acid procedure. Proteins were denatured (80 °C, 5 min) in 0.1% Rapigest (Waters), reduced and alkylated by dithiothreitol and iodoacetamide (Sigma), digested with 50:1 (w/w) sequencing-grade trypsin (Promega) (37 °C, overnight), treated with 0.1% trifluoroacetic acid (30 min), and centrifuged (14,000 rcf, ambient temperature, 15 min). Peptides from the supernatant were extracted by C18 spin columns (Thermo Pierce) according to the manufacturer's instructions.

2.3. Phosphopeptide enrichment and liquid chromatography

Peptide separation was carried out on an Agilent 1200 nano-LC system connected to an Agilent Phosphochip II HPLC-chip. The chip contained integrated microfluidics and a sandwiched Zorbax 300SB-C18 5-μm (RP)-TiO_2-RP trapping column connected to an analytical RP column. The binary buffer system consisted of buffer A (99.1% water, 0.6% acetic acid, 0.5% formic acid, v/v/v) and buffer B (99.1% acetonitrile, 0.6% acetic acid, 0.5% formic acid, v/v/v). Phosphopeptides from the trapping column were eluted with 16 μL of Phosphochip elution buffer (Agilent). Peptide separation on the analytical column was accomplished by ramping buffer B%(0 min, 5%; 90 min, 40%) at a flow-rate of 300 nL·min^{-1}.

2.4. Multiple-reaction monitoring

Transitions were monitored on an Agilent 6460 triple-quadrupole mass spectrometer equipped with a ChipCube ion source as described [11]. Collision energies for each transition were chosen after ramping from 0 to 50 V at 4-V intervals using the *Optimizer for Peptides* software (Agilent). Fragmentor voltage was similarly optimized by ramping from 50 to 200 V. Dwell time ranged from 20 to 500 ms to cover at least 8 data points per LC peak. For endogenous peptide analysis, 4 μg of mitochondrial digests were co-injected with 1 pmol each of crude synthetic unmodified peptides and 20 to 200 fmol each of crude synthetic phosphorylated peptides (Thermo PEPotec SRM).

2.5. Data analysis

All chromatograms were inspected manually using the *Mass Hunter Qualitative Analysis* software. Areas under MRM peaks were integrated at full-width prior to smoothing.

3. Results and discussion

We previously reported 62 MRM transitions for quantifying mitochondrial phosphorylation [11]. In this study, we expanded the number of developed MRM transitions to a total of 176, corresponding to 54 phosphorylated mitochondrial peptides and their unmodified counterparts (Table 1). The new MRM assay covers additional phosphorylation sites in ANT, the tricarboxylic acid cycle proteins, the PDH complex, and the