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Phosphoproteome mapping of cardiomyocyte mitochondria in a rat model of heart failure

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Abstract

Mitochondria are complex organelles essential to cardiomyocyte survival. Protein phosphorylation is emerging as a key regulator of mitochondrial function. In the study reported here, we analyzed subsarcolemmal mitochondria harvested from rats who have received 4 weeks of aldosterone/salt treatment to simulate the neurohormonal profile of human congestive heart failure. Our objective was to obtain an initial qualitative inventory of the phosphoproteins in this biological system. Subsarcolemmal mitochondria were harvested and the phosphoproteome was analyzed with a gel-free bioanalytical platform. Mitochondrial proteins were digested with trypsin, and the digests were enriched for phosphopeptides with Immobilized Metal Ion Affinity Chromatography (IMAC). The phosphopeptides were analyzed by ion trap LC-MS/MS, and the phosphoproteins identified via database searches. Based on MS/MS and MS³ data, we characterized a set of 42 phosphopeptides that encompassed 39 phosphorylation sites. These peptides mapped to 26 proteins, for example long-chain specific acyl-CoA dehydrogenase (LCAD), Complex III subunit 6, and mitochondrial import receptor TOM70. Collectively, the characterized phosphoproteins belong to diverse functional modules, including bioenergetic pathways, protein import machinery, and calcium handling. The phosphoprotein panel discovered in this study provides a foundation for future differential phosphoproteome profiling towards an integrated understanding of the role of mitochondrial phosphorylation in heart failure.

Keywords

mitochondria; rat; heart; phosphoproteome; mass spectrometry

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Conflict of Interest

The authors declare that they have no competing interests.

Introduction

The disabling symptoms and signs of the congestive heart failure (CHF) syndrome have their pathophysiologic origins rooted in neurohormonal activation. This includes the renin-angiotensin-aldosterone system (RAAS) whose effector hormones contribute to pathologic cardiac remodeling. This adverse remodeling includes the loss of necrotic cardiomyocytes and their replacement by stiff type I fibrillar collagen as scar tissue [1–3]. Oxidative stress, generated by mitochondria, occurs when the rate of reactive oxygen species generation overwhelms their rate of detoxification by endogenous antioxidant defenses, and is integral to the appearance of programmed cardiomyocyte necrosis [4].

Mitochondria are complex, multifunctional organelles whose repertoire includes the provision of intracellular energetics and maintenance of redox balance, each of which determine cardiomyocyte survival. The compromised functional integrity and lost synthesis of high energy phosphates by these subcellular organelles eventuates in the necrosis of these obligate aerobic cells. We have used a rat model of aldosterone/salt treatment (ALDOST) to simulate the neurohormonal profile of human CHF and to interrogate the pathophysiologic link between RAAS activation and nonischemic, hormone-mediated cardiomyocyte necrosis [3]. A mitochondriocentric signal-transducer-effector pathway (MSTE) was identified involving the subsarcolemmal (SSM) population of mitochondria [1, 5]. Its features include: calcitropic hormone-driven calcium overloading; their induction of oxidative stress and loss of ATP synthesis; and opening of the inner membrane permeability transition pore (mPTP) to allow osmotic swelling and organellar degeneration with ensuing cell death. Cotreatment with mitochondria-targeted interventions, either an antioxidant or mPTP opening inhibitor, have proven cardioprotective [5, 6].

To further elucidate the involvement of the MSTE pathway during ALDOST-associated cardiomyocyte necrosis and its crosstalk with other components of the molecular mitochondrial machinery, we turned to a global-scale approach that includes transcriptomics and proteomics [7–9]. Such methodology is uniquely suited to capture perturbations within cellular, subcellular and molecular compartments of cardiomyocytes. In this context phosphoproteomics is an emerging resource to interrogate molecular mechanisms of pathologic cardiac remodeling [10]. In particular, phosphorylation is increasingly recognized as a critical regulator of mitochondrial function [11–13] and a growing number of phosphoproteins have been identified in various mammalian species [14–17]. The continued development of analytical technologies and applications of bioinformatic tools now enables probing mitochondrial phosphorylation on a global scale as well as the interrogation of the functionality of these proteins. Mitochondrial phosphoproteomics studies often proceed in two phases. The first phase aims at qualitative phosphoproteome discovery, i.e., qualitative description of the mitochondrial phosphoproteome in the biological system under study [15], to provide information (specific phosphopeptide sequences and site assignments) that serves as a foundation for the second phase. The second phase involves development of tailored multiple-reaction monitoring (MRM) workflows and their application for quantitative assessment of mitochondrial phosphorylation dynamics [18, 19].

Herein and using the chronic ALDOST rat model, we have completed a pilot qualitative study of the phosphoproteome in subsarcolemmal mitochondria. We obtained an initial catalog of phosphoproteins and their sites of phosphorylation using a gel-free bioanalytical workflow to characterize 39 sites in 26 proteins that belong to critical functional modules of these organelles.

Materials and methods

Animal Model

Eight-week-old male Sprague-Dawley rats were used as approved by our institution's Animal Care and Use Committee. The ALDOST treatment duration was 4 weeks. In brief and following uninephrectomy, an osmotic minipump was implanted subcutaneously releasing aldosterone (0.75 g/h) to raise its plasma concentrations to those found in human CHF and which suppress plasma renin activity and angiotensin II [20]. In addition to standard laboratory chow, rats received drinking water fortified with 1% NaCl and 0.4% KCl to prevent hypokalemia. Cardiac pathology in the form of widely scattered microscopic scars, a footprint of cardiomyocyte necrosis, first appears in both atria and ventricles of the previously intact heart at wk 4 of ALDOST [3, 21].

Isolation of Mitochondria

The subsarcolemmal population of cardiac mitochondria (SSM) was isolated from the myocardium as previously reported [22]. In brief, whole heart homogenates were subjected to differential centrifugation, with interfibrillar mitochondria (IFM) discarded after 1,500g and only the SSM harvested at 10,000g. The purity of mitochondrial preparation was assessed by flow cytometry and mitochondria-specific dye, Mito Tracker Red (Invitrogen), as previously described [22]. Mitochondria from a total of nine animals (6 ALDOST and 3 untreated controls) were used for phosphoproteome mapping. Three pooled samples (two ALDOST pools and one control pool) were analyzed separately. Each pooled sample was prepared by combining three individual mitochondrial preparations of the same type (ALDOST or control).

Protein solubilization and digestion

The mitochondrial proteins were extracted with a solubilization buffer containing 400 mM ammonium bicarbonate (pH 8), 8 M urea, and phosphatase inhibitors (PhosStop, Roche). Ninety microliters of this buffer were added to each mitochondrial preparation and the mixtures were incubated under shaking for 1 h; during this time, the samples were sonicated three times with a sonicator probe. The proteins were reduced with DTT (5 mM final concentration) and alkylated with iodoacetamide (20 mM final concentration, incubated at r.t. in the dark for 20 min). Prior to digestion, the samples were diluted with water to a final urea concentration of 2 M. Protein concentration in the samples was determined with the 2-D Quant kit (GE Healthcare); the protein amounts in each mitochondrial sample were 250–350 µg. Sequencing-grade trypsin (Promega) was added to each sample at a protease-to-protein ratio of 1:100 and the samples were incubated O/N at 37 °C. After digestion the mixtures were acidified with TFA and pooled as described above. The pooled samples were

subjected to C18 solid phase extraction (SPE) using a home-packed mini-column. After elution from the SPE column the peptides were dried in a vacuum centrifuge.

Phosphopeptide enrichment

Phosphopeptide enrichment via Immobilized Metal Ion Affinity Chromatography (IMAC) was performed with the Phosphopeptide Isolation kit (Pierce) using a procedure described earlier [23]. The eluted phosphopeptide mixtures were acidified and the solution volume was reduced in a vacuum centrifuge. Finally, the enriched digests were purified with ZipTip C18 (Millipore) using manufacturer's procedures. The phosphopeptides bound to the C18 column were eluted with 3 μ L of 50% ACN/50% water/0.1% TFA and diluted with 6 μ L of 0.1% formic acid.

LC-MS/MS

The LC-MS/MS analyses were performed with an LTQ linear ion trap mass spectrometer (Thermo Scientific) interfaced with a Famos/Ultimate nanoflow LC system (Dionex). The separations were performed with a fused silica microcapillary column/spray needle (15 cm length, 75 μ m i.d., New Objective) using a 90-min linear gradient from 0% to 90% mobile phase B at a flow rate of 200 nL/min. Mobile phase B was 10% water/90% methanol/0.05% formic acid; mobile phase A was 98% water/2% methanol/0.05% formic acid. For each sample two analyses were performed in the data-dependent acquisition mode. The first analysis consisted of MS and MS/MS cycles; the second analysis included MS³ triggered when a phosphopeptide-diagnostic neutral loss ($[M+2H-98]^{2+}$ at 49 m/z below the precursor ion mass, or $[M+3H-98]^{3+}$ at 32.8 m/z below the precursor, was detected in the MS/MS spectrum [24].

Bioinformatics

The LC-MS/MS datasets were used to interrogate the UniProt protein sequence database (subset of rat proteins, created January 2012) using the SEQUEST search engine (Proteome Discoverer 1.3 software suite, Thermo Scientific). The search parameters were: full trypsin specificity; phosphorylation (or loss of water in MS³) as a dynamic modification on S, T, and Y; dynamic modification of oxidized M; and static modification of carbamidomethylated C. The search results were filtered to include peptides retrieved with XCorr-versus-charge values ≥ 3.1 and ≥ 4.0 for doubly and triply charged precursor ions, respectively; this filtering procedure produced phosphopeptide set with a calculated false discovery rate (FDR) of 1%. A few lower-scoring phosphopeptides were included in the set if supporting evidence (such as an MS³ spectrum, etc.) was available. The phosphosite localization probability was calculated with the phosphoRS algorithm [25]. All MS/MS and MS³ spectra for the individual phosphopeptides were inspected manually to confirm the peptide sequence and the location of the phosphorylation site.

In-silico predictions of potential kinase families were produced by NetworKIN [26]. Information on protein function was compiled from annotations in the UniProt and PhosphoSite knowledgebases and from searches of primary literature.

Results and discussion

Summary of bioanalytical strategy

In this discovery study, we applied a gel-free bioanalytical workflow to obtain a first description of the phosphoproteome in rat cardiomyocytes in ALDOST. This description encompasses protein identities, sequences of the phosphopeptides originating from these proteins, and locations of the exact sites of phosphorylation.

The bioanalytical workflow chosen for interrogation of the mitochondrial phosphoproteome involved four major steps: (1) solubilization and in-solution digestion of the SSM proteomes; (2) enrichment of the digests by IMAC, which selectively captured phosphorylated peptides; (3) analyses of the enriched digests by LC-MS/MS, which included enhanced sequence- and site-diagnostic phosphopeptide fragmentation (MS/MS and MS³); (4) identification of phosphopeptides/proteins and assignment of phosphorylation sites.

It should be noted that the bioanalytical strategy was designed in the context of planned large-scale phosphoproteomics studies that will involve multiple biological replicates and time points. To ensure a reasonable throughput of these future studies, we have omitted from our workflow multiple dimensions of separation at the protein and/or peptide level. Although we and others have previously demonstrated that additional fractionation results in increased phosphoproteome coverage [15, 27, 28], the concomitant decrease in throughput would be incompatible with multiple sample analyses. Isolation of mitochondria (vs. phosphoproteomics of whole cells) already provides an effective enrichment of phosphoproteins in terms of their functional relevance.

Mitochondrial phosphoproteins and sites of phosphorylation were identified by database searches and verified by manual inspection of the MS/MS and MS³ spectra. One of the signature features inspected in the MS/MS spectra was a prominent product ion due to the neutral loss of elements of phosphoric acid from the phosphopeptide molecular ions [29]; this fragmentation pathway is favored in the ion trap mass spectrometer. Representative MS/MS and MS³ spectra for one of the phosphopeptides from our dataset are depicted in Figure 1. Figure 1a shows the MS/MS spectrum for the phosphopeptide IFsSEHDIFR, which displays a major signal at m/z 617.2, arising from the loss of phosphoric acid from the doubly-charged precursor ion. In addition, a number of product ions from the b- and y-series are present in the MS/MS spectrum and determine the amino acid sequence of the phosphopeptide. The MS³ spectrum of the primary product ion $[M+2H-98]^{2+}$ (Figure 1b) confirms the amino acid sequence of IFsSEHDIFR and location of the phosphorylation site in this peptide. Inclusion of neutral-loss triggered MS³ in the LC-MS/MS data acquisition provided increased confidence in sequence/site assignment.

Phosphoproteome panel discovered in SSM

Results for the rat cardiomyocyte mitochondrial phosphoproteome analyses are summarized in Table 1. Overall, interrogation of the IMAC-enriched digests of mitochondrial samples with LC-MS/MS and MS³ resulted in the characterization of 42 distinct phosphopeptides that contained a total of 39 phosphorylation sites and mapped to 26 proteins. Details for the

characterization of each phosphopeptide/site are provided in Supplementary Table 1. For all phosphorylation sites that were characterized, in-silico analysis was performed to predict potential kinase families that may be responsible for phosphorylation of a particular site based on consensus sequence motifs. As shown in Figure 2, the highest number of the phosphosites is predicted as putative targets of casein kinase II (CKII), protein kinase C (PKC) and protein kinase A (PKA). These results are similar to mitochondrial phosphoproteome findings reported previously [26] and suggest a potential role of these kinases in mitochondrial protein phosphorylation.

It should be pointed out that the aim of this pilot study was to provide the first description of the phosphoproteome in rat heart mitochondria, and thus the study was designed for qualitative phosphoproteome description/site discovery. The data were obtained for a total of three biological samples of rat heart mitochondria (Supplementary Table 1) to demonstrate that phosphopeptides/sites can be characterized in this biological system with the chosen bioanalytical methodology. The study was not intended to assess differential phosphorylation between ALDOST and control but to serve as foundation for our future quantification studies with tailored MRM workflows [19].

In terms of phosphoproteome size, it has been postulated that, analogously to whole proteome, the mitochondrial phosphoproteome is much smaller than the phosphoproteome of whole eukaryotic cells; this has been confirmed experimentally in a recent study that applied high-resolution mass spectrometry to comparative examination of protein phosphorylation extent in diverse biological systems [30]. Our phosphoprotein panel, which encompasses 39 sites in 26 proteins, is not expected to represent a complete catalog of the cardiomyocyte mitochondrial phosphoproteome; certainly, a portion of the phosphoproteins escaped detection due to limitations in our bioanalytical workflow. Nevertheless, our panel consists of phosphoproteins that are highly relevant to mitochondrial function, as discussed in more detail below.

Functional relevance of the characterized phosphoproteins

The phosphoproteins were mapped in all main locations of the mitochondria: outer mitochondrial membrane (OMM), intermembrane space, inner mitochondrial membrane (IMM), and matrix. In addition, our panel includes phosphoproteins that localize to the sarcoplasmic reticulum (SR), which is known to be in close association with the mitochondria. The characterized phosphoproteins belong to multiple functional modules including bioenergetics, transport, and calcium regulation. Selected examples are discussed in more detail below.

The panel in Table 1 includes phosphoproteins that are constituents of several bioenergetic pathways, in keeping with the critical importance of mitochondria as the principal source of cellular energy. Several of these proteins are classical cases of phosphorylation-dependent mitochondrial enzymes that have long been established. For example, the activity of the pyruvate dehydrogenase complex (PDC) is well known to be regulated by phosphorylation/dephosphorylation via PDKs and pyruvate dehydrogenase phosphatases [31]. The PDC is the last enzyme in the glycolytic pathway; it is a multi-subunit complex that catalyzes the decarboxylation of pyruvate to form acetyl-CoA, which is used as a substrate for the TCA

cycle. We have mapped three phosphorylation sites in pyruvate dehydrogenase E1 subunit alpha. Because phosphorylation-dependent modulation of the PDC complex is well studied, it could serve as a reference standard in functional phosphoproteomics studies of proteins/sites with unknown mechanistic impact [14].

Another phosphoprotein characterized in our study is the long-chain specific acyl-CoA dehydrogenase (LCAD) with serine 54 identified as the site of modification (Figure 1). This enzyme is involved in the first step of the mitochondrial fatty acid beta-oxidation pathway (FAO) and catalyzes the initial dehydrogenation of long-chain fatty acids. In the normal adult heart, FAO is the preferred pathway to generate energy that sustains cardiac function. Aberrations in substrate utilization, specifically a decline in FAO and shift towards glucose oxidation, may be found in association with heart failure [32].

We have mapped the sites of phosphorylation in two members in OXPHOS. This functional element responsible for production of ATP is composed of protein complexes located in the inner membrane, including the electron transport chain (ETC) complexes I-IV and ATP synthase (complex V). In particular complexes I and III of ETC are major sites of reactive oxygen species generation as a by-product of normal metabolism. As we have shown in our ALDOST model, the pathophysiological scenario leading to cardiomyocyte necrosis includes aberrant production of reactive oxygen species and increased oxidative stress that eventually overwhelms protective antioxidant scavengers with ensuing opening of the mPTP pore to result in cell death [1, 5]. Our pilot results include characterization of two phosphorylation sites in cytochrome b c-1 complex subunit 6 (complex III subunit 6) and of one site in ATP synthase subunit alpha.

Phosphorylation of several protein transport machinery proteins (TOM70, HSP90, and TIMM8a) revealed in our investigation suggests a possible role of this modification in protein translocation into the mitochondria [33]. The mitochondrial import protein subunit TOM70, found to be phosphorylated at serine 94, is a component of a translocase (TOM) complex on the outer mitochondrial membrane and enables protein translocation across the outer membrane. The chaperone heat shock protein HSP90 participates in the targeting of precursor proteins to the TOM assembly. The mitochondrial import inner membrane translocase subunit TIMM8A, located in the intermembrane space, operates in conjunction with the TIM protein import machinery that facilitates entry of proteins into the mitochondrial matrix.

Finally, our collection of phosphoproteins encompasses proteins involved in calcium handling, for example sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) and phospholamban. These proteins are known to be located in the sarcoplasmic reticulum (SR). In cardiomyocytes, the subsarcolemmal mitochondrion and the SR are in close proximity to each other. Physical and functional interfacing of the two organelles has been demonstrated [34]. In keeping with this interaction, numerous SR proteins were identified in a recent large-scale inventory of cardiac mitochondrial proteome [35]. The SR is responsible for calcium storage/release and hence for regulation of intracellular calcium homeostasis and cardiac contractility. SERCA2 mediates the uptake of calcium into SR and it has been one of the key proteins implicated in the molecular mechanisms associated with impaired

contractility of the failing heart. Phospholamban (PLN) inhibits the activity of SERCA2 in a phosphorylation-dependent manner. Other participating partners and their roles are being characterized as a detailed picture of the SERCA2/PLN regulatory complex is developing [36]. The mitochondriocentric signal-transducer-effector pathway to nonischemic cardiomyocyte necrosis during ALDOST is initiated by intracellular and intramitochondrial calcium overloading, which is mediated by calcitropic hormones [37].

In conclusion, this study presents an initial inventory of the phosphoproteins in cardiomyocyte subsarcolemmal mitochondria. The characterized phosphoproteins are directly relevant to various aspects of mitochondrial function. Results from this phosphoproteome discovery study can be used to design stable-isotope labeled standard phosphopeptides to develop tailored MRM workflows for targeted quantification of specific phosphorylation sites. Therefore, our findings provide an important foundation for future phosphoproteome studies in the ALDOST heart failure model, directed towards functional understanding of mitochondrial phosphorylation at the systems level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ALDOST	Aldosterone/salt treatment
CHF	Congestive heart failure
ETC	Electron transport chain
FAO	Fatty acid oxidation
IMAC	Immobilized metal ion affinity chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MRM	Multiple reaction monitoring
MSTE	Mitochondriocentric signal-transducer-effector pathway
SSM	Subsarcolemmal mitochondria
TFA	Trifluoroacetic acid

References

1. Kamalov G, Bhattacharya SK, Weber KT. Congestive heart failure: where homeostasis begets dyshomeostasis. *J Cardiovasc Pharmacol.* 2010; 56:320–328. [PubMed: 20588190]

2. Sukhanov S, Semprun-Prieto L, Yoshida T, Michael TA, Higashi Y, Galvez S, Delafontaine P. Angiotensin II, oxidative stress and skeletal muscle wasting. *Am J Med Sci.* 2011; 342:143–147. [PubMed: 21747283]
3. Sun Y, Zhang J, Lu L, Chen SS, Quinn MT, Weber KT. Aldosterone-induced inflammation in the rat heart : role of oxidative stress. *Am J Pathol.* 2002; 161:1773–1781. [PubMed: 12414524]
4. Kung G, Konstantinidis K, Kitsis RN. Programmed necrosis, not apoptosis, in the heart. *Circ Res.* 2011; 108:1017–1036. [PubMed: 21493924]
5. Shahbaz AU, Kamalov G, Zhao W, Zhao T, Johnson PL, Sun Y, Bhattacharya SK, Ahokas RA, Gerling IC, Weber KT. Mitochondria-targeted cardioprotection in aldosteronism. *J Cardiovasc Pharmacol.* 2011; 57:37–43. [PubMed: 20966765]
6. Cheema Y, Sherrod JN, Zhao W, Zhao T, Ahokas RA, Sun Y, Gerling IC, Bhattacharya SK, Weber KT. Mitochondriocentric pathway to cardiomyocyte necrosis in aldosteronism: cardioprotective responses to carvedilol and nebivolol. *J Cardiovasc Pharmacol.* 2011; 58:80–86. [PubMed: 21558884]
7. Ahokas RA, Warrington KJ, Gerling IC, Sun Y, Wodi LA, Herring PA, Lu L, Bhattacharya SK, Postlethwaite AE, Weber KT. Aldosteronism and peripheral blood mononuclear cell activation: a neuroendocrine-immune interface. *Circ Res.* 2003; 93:e124–e135. [PubMed: 14576195]
8. Cheema Y, Zhao W, Zhao T, Khan MU, Green KD, Ahokas RA, Gerling IC, Bhattacharya SK, Weber KT. Reverse remodeling and recovery from cachexia in rats with aldosteronism. *Am J Physiol Heart Circ Physiol.* 2012; 303:H486–H495. [PubMed: 22730385]
9. Gerling IC, Sun Y, Ahokas RA, Wodi LA, Bhattacharya SK, Warrington KJ, Postlethwaite AE, Weber KT. Aldosteronism: an immunostimulatory state precedes proinflammatory/fibrogenic cardiac phenotype. *Am J Physiol Heart Circ Physiol.* 2003; 285:H813–H821. [PubMed: 12860567]
10. Sun Z, Hamilton KL, Reardon KF. Phosphoproteomics and molecular cardiology: Techniques, applications and challenges. *J Mol Cell Cardiol.* 2012; 53:354–368. [PubMed: 22691689]
11. Covian R, Balaban RS. Cardiac mitochondrial matrix and respiratory complex protein phosphorylation. *Am J Physiol Heart Circ Physiol.* 2012; 303:H940–H966. [PubMed: 22886415]
12. Edwards AV, Cordwell SJ, White MY. Phosphoproteomic profiling of the myocyte. *Circ Cardiovasc Genet.* 2011; 4:575. [PubMed: 22010164]
13. O'Rourke B, Van Eyk JE, Foster DB. Mitochondrial protein phosphorylation as a regulatory modality: implications for mitochondrial dysfunction in heart failure. *Congest Heart Fail.* 2011; 17:269–282. [PubMed: 22103918]
14. Boja ES, Phillips D, French SA, Harris RA, Balaban RS. Quantitative mitochondrial phosphoproteomics using iTRAQ on an LTQ-Orbitrap with high energy collision dissociation. *J Proteome Res.* 2009; 8:4665–4675. [PubMed: 19694452]
15. Deng N, Zhang J, Zong C, Wang Y, Lu H, Yang P, Wang W, Young GW, Wang Y, Korge P, Lotz C, Doran P, Liem DA, Apweiler R, Weiss JN, Duan H, Ping P. Phosphoproteome analysis reveals regulatory sites in major pathways of cardiac mitochondria. *Mol Cell Proteomics.* 2011; 10:M110.000117. [PubMed: 20495213]
16. Feng J, Zhu M, Schaub MC, Gehrig P, Roschitzki B, Lucchinetti E, Zaugg M. Phosphoproteome analysis of isoflurane-protected heart mitochondria: phosphorylation of adenine nucleotide translocator-1 on Tyr194 regulates mitochondrial function. *Cardiovasc Res.* 2008; 80:20–29. [PubMed: 18558627]
17. Hopper RK, Carroll S, Aponte AM, Johnson DT, French S, Shen RF, Witzmann FA, Harris RA, Balaban RS. Mitochondrial matrix phosphoproteome: effect of extra mitochondrial calcium. *Biochemistry.* 2006; 45:2524–2536. [PubMed: 16489745]
18. Lam MP, Scruggs SB, Kim TY, Zong C, Lau E, Wang D, Ryan CM, Faull KF, Ping P. An MRM-based workflow for quantifying cardiac mitochondrial protein phosphorylation in murine and human tissue. *J Proteomics.* 2012; 75:4602–4609. [PubMed: 22387130]
19. Lam MP, Lau E, Scruggs SB, Wang D, Kim TY, Liem DA, Zhang J, Ryan CM, Faull KF, Ping P. Site-specific quantitative analysis of cardiac mitochondrial protein phosphorylation. *J Proteomics.* 2013; 81:15–23. [PubMed: 23022582]
20. Brilla CG, Pick R, Tan LB, Janicki JS, Weber KT. Remodeling of the rat right and left ventricles in experimental hypertension. *Circ Res.* 1990; 67:1355–1364. [PubMed: 1700933]

21. Sun Y, Ramires FJ, Weber KT. Fibrosis of atria and great vessels in response to angiotensin II or aldosterone infusion. *Cardiovasc Res.* 1997; 35:138–147. [PubMed: 9302358]
22. Gandhi MS, Deshmukh PA, Kamalov G, Zhao T, Zhao W, Whaley JT, Tichy JR, Bhattacharya SK, Ahokas RA, Sun Y, Gerling IC, Weber KT. Causes and consequences of zinc dyshomeostasis in rats with chronic aldosteronism. *J Cardiovasc Pharmacol.* 2008; 52:245–252. [PubMed: 18806605]
23. Chen L, Fang B, Giorgianni F, Gingrich JR, Beranova-Giorgianni S. Investigation of phosphoprotein signatures of archived prostate cancer tissue specimens via proteomic analysis. *Electrophoresis.* 2011; 32:1984–1991. [PubMed: 21739434]
24. Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc Natl Acad Sci U S A.* 2004; 101:12130–12135. [PubMed: 15302935]
25. Taus T, Kocher T, Pichler P, Paschke C, Schmidt A, Henrich C, Mechtler K. Universal and confident phosphorylation site localization using phosphoRS. *J Proteome Res.* 2011; 10:5354–5362. [PubMed: 22073976]
26. Zhao X, Leon IR, Bak S, Mogensen M, Wrzesinski K, Hojlund K, Jensen ON. Phosphoproteome analysis of functional mitochondria isolated from resting human muscle reveals extensive phosphorylation of inner membrane protein complexes and enzymes. *Mol Cell Proteomics.* 2011; 10:M110.
27. Chen L, Giorgianni F, Beranova-Giorgianni S. Characterization of the phosphoproteome in LNCaP prostate cancer cells by in-gel isoelectric focusing and tandem mass spectrometry. *J Proteome Res.* 2010; 9:174–178. [PubMed: 20044836]
28. Beranova-Giorgianni S, Zhao Y, Desiderio DM, Giorgianni F. Phosphoproteomic analysis of the human pituitary. *Pituitary.* 2006; 9:109–120. [PubMed: 16807684]
29. Boersema PJ, Mohammed S, Heck AJ. Phosphopeptide fragmentation and analysis by mass spectrometry. *J Mass Spectrom.* 2009; 44:861–878. [PubMed: 19504542]
30. Gnad F, Forner F, Zielinska DF, Birney E, Gunawardena J, Mann M. Evolutionary constraints of phosphorylation in eukaryotes, prokaryotes, and mitochondria. *Mol Cell Proteomics.* 2010; 9:2642–2653. [PubMed: 20688971]
31. Linn TC, Pettit FH, Reed LJ. Alpha-keto acid dehydrogenase complexes. X. Regulation of the activity of the pyruvate dehydrogenase complex from beef kidney mitochondria by phosphorylation and dephosphorylation. *Proc Natl Acad Sci U S A.* 1969; 62:234–241. [PubMed: 4306045]
32. Neubauer S. The failing heart--an engine out of fuel. *N Engl J Med.* 2007; 356:1140–1151. [PubMed: 17360992]
33. Smits P, Smeitink J, van den HL. Mitochondrial translation and beyond: processes implicated in combined oxidative phosphorylation deficiencies. *J Biomed Biotechnol.* 2010; 2010:737385. [PubMed: 20396601]
34. Dorn GW, Scorrano L. Two close, too close: sarcoplasmic reticulum-mitochondrial crosstalk and cardiomyocyte fate. *Circ Res.* 2010; 107:689–699. [PubMed: 20847324]
35. Zhang J, Li X, Mueller M, Wang Y, Zong C, Deng N, Vondriska TM, Liem DA, Yang JI, Korge P, Honda H, Weiss JN, Apweiler R, Ping P. Systematic characterization of the murine mitochondrial proteome using functionally validated cardiac mitochondria. *Proteomics.* 2008; 8:1564–1575. [PubMed: 18348319]
36. Kranias EG, Hajjar RJ. Modulation of cardiac contractility by the phospholamban/SERCA2a regulatome. *Circ Res.* 2012; 110:1646–1660. [PubMed: 22679139]
37. Borkowski BJ, Cheema Y, Shahbaz AU, Bhattacharya SK, Weber KT. Cation dyshomeostasis and cardiomyocyte necrosis: the Fleckenstein hypothesis revisited. *Eur Heart J.* 2011; 32:1846–1853. [PubMed: 21398641]

Fig. 1.

Representative MS/MS and MS³ spectra obtained in cardiomyocyte mitochondrial phosphoproteome analyses. Based on these spectra, phosphorylation site S54 in the phosphopeptide IFsSEHDIFR was characterized. This peptide belongs to long-chain specific acyl-CoA dehydrogenase. **a** MS/MS spectrum of the [M+2H]²⁺ of IFsSEHDIFR. **b** MS³ of the [M+2H-98]²⁺ product ion (*m/z* 617.2).

Fig 2.

Potential kinases predicted for the phosphorylation sites characterized in subsarcolemmal mitochondria from rat cardiomyocytes. The in-silico analysis was performed with NetworKIN.

Table 1

Phosphopeptides, phosphosites and proteins characterized in subsarcolemmal mitochondria isolated from rat cardiomyocytes.

No	UniProt Accession Number	Protein Name	Peptide Sequence ^I	Site
1	P26284	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	YGMGTsVER	S232
			YGmGTsVER	S232
			YHGHsMSDPGVSYR	S293
			YHGHsmSDPGVSYR	S293
			YHGHsMSDPGVsYR	S293/S300
2	Q00972	[3-methyl-2-oxobutanoate dehydrogenase [lipoamide]] kinase, mitochondrial (BCKD-kinase)	StSATDTHHVELAR	T32
3	P15650	Long-chain specific acyl-CoA dehydrogenase, mitochondrial (LCAD)	IFsSEHDIFR	S54
4	Q5M9I5	Cytochrome b c-1 complex subunit 6	sQTEEDCTEELFDLHAR	S59
			VSSRSQtEEDCTEELFDLHAR	T61
5	P15999	ATP synthase subunit alpha, mitochondrial	FESAFLSHVVSQHQsLLGNIR	S521
6	Q75Q39	Mitochondrial import receptor subunit TOM70	AsPALGSGPDGSGDSLEMSSLDLR	S94
7	Q9WVA1	Mitochondrial import inner membrane translocase subunit Tim8 A	SKPVFSESLsD	S96
8	P34058	Heat shock protein HSP-90 beta	IEDVGsDEEDDSGKDKK	S255
9	P11507	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2)	EFDELSPsAQR	S663
10	P61016	Cardiac phospholamban	RAstIEMPQQR	S16
			RAstIEMPQQR	S16/T17
11	P23562	Band 3 anion transport protein	VLEIPDRDsEEEEHVIEQIAYR	S18
12	P19357	Solute carrier family 2, facilitated glucose transporter member 4 (GLUT-4)	RTPsLLEQEVKPSLEYLGPDEND	S488
13	P08050	Gap junction alpha-1 protein (connexin-43)	QAsEQNWANYSAEQNR	S306
			MGQAGSTIsNSHAQPFDFPDDNQNAK	S328
			MGQAGSTIsNSHAQPFDFPDDNQNAK	S328/S330
			VAAGHELQPLAIVDQRPSsR	S365
14	P09605	Creatine kinase S- type, mitochondrial (S-MtCK)	VAAGHELQPLAIVDQRPSsRAssR	S365/S368/S369
			LGyILTCPSNLGTGLR	S319
15	Q05962	ADP/ATP translocase 1 (ANT 1)	LLLQVQHAsK	S42
			YFPtQALNFAFK	T84
			AAyFGVyDTAK	Y195
16	O08589	Phospholemman	TGEPDEEETFRSsIR	S83
17	P02563	Myosin-6	RNtLLQAELEELR	T1680
18	P60905	DNAJ homolog subfamily C member 5	sLSTSGESLYHVLGLDK	S8
19	P04692	Tropomyosin alpha-1 chain	AISEELDHAlNDMTsI	S283
20	Q9Z2L0	Voltage-dependent anion-selective channel protein 1 (VDAC1)	LTFDSSFsPNTGKK	S104

No	UniProt Accession Number	Protein Name	Peptide Sequence ^I	Site
			DVFtKGYGFGLIK	T19
21	Q9ER34	Aconitate hydratase, mitochondrial	WVVIGDENYGEGsSREHAALPR	S669
22	B1WBW4	Armadillo repeat- containing protein 10	sAEDLTEGSYDAILSAEQLEK	S43
23	Q68FT1	Ubiquinone biosynthesis protein COQ9, mitochondrial	YTDQsGEEEEEDYESEEQIHR	S80
24	P53987	Monocarboxylate transporter 1	SKEsLQEAGKSDANTDLIGGSPK	S213
25	P11960	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial	IGHHsTSDDSSAYR	S333
			sVDEVNYWDK	S343
			sVDEVNYWDKQDHPISR	S343
			IGHHsTSDDSSAYRsVDEVNYWDK	S333/S343
26	B0LPN4	Ryanodine receptor 2	RIQTsQVSIDAAHGYSR	S2798

^I Phosphorylation site(s) on S, T, Y, are indicated with lower-case letters. Lower-case m denotes an oxidized M residue.