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Quantitative phosphoproteomics using acetone-based peptide labeling: Method evaluation and application to a cardiac ischemia/reperfusion model

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Abstract

Mass spectrometry (MS) techniques to globally profile protein phosphorylation in cellular systems that are relevant to physiological or pathological changes have been of significant interest in biological research. In this report, an MS-based strategy utilizing an inexpensive acetone-based peptide labeling technique known as reductive alkylation by acetone (RABA) for quantitative phosphoproteomics was explored to evaluate its capacity. Since the chemistry for RABA-labeling for phosphorylation profiling had not been previously reported, it was first validated using a standard phosphoprotein and identical phosphoproteomes from cardiac tissue extracts. A workflow was then utilized to compare cardiac tissue phosphoproteomes from mouse hearts not expressing FGF2 vs. hearts expressing low molecular weight fibroblast growth factor-2 (LMW FGF2) to relate low molecular weight fibroblast growth factor-2 (LMW FGF2) mediated cardioprotective phenomena induced by ischemia/reperfusion (I/R) injury of hearts, with downstream phosphorylation changes in LMW FGF2 signaling cascades. Statistically significant phosphorylation changes were identified at 14 different sites on 10 distinct proteins including some with mechanisms already established for LMW FGF2-mediated cardioprotective signaling (e.g. connexin-43), some with new details linking LMW FGF2 to the cardioprotective mechanisms (e.g. cardiac myosin binding protein C or cMyBPC), and also several new downstream effectors not previously recognized for cardio-protective signaling by LMW FGF2. Additionally, one of the phosphopeptides, cMyBPC/pSer-282, identified was further verified with site-specific quantification using an SRM (selected reaction monitoring)-based approach that also relies on isotope labeling of a synthetic phosphopeptide with deuterated acetone as an internal standard. Overall, this study confirms that the inexpensive acetone-based peptide labeling can be used in both exploratory and targeted quantification phosphoproteomic studies to identify and verify biologically-relevant phosphorylation changes in whole tissues.

Keywords

Fibroblast growth factor-2; relative quantification; phosphoproteome; mass spectrometry; external tagging; reductive alkylation; cardioprotection; phosphorylation

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1. Introduction

Protein phosphorylation is considered as one of the pivotal post-translational modifications (PTMs) central for normal cellular function as well as disease initiation and progression [1–3]. Hence, details about phosphorylation flux in cellular components have become critical to link physiological or pathophysiological changes to the underlying biochemistry, and thus target therapeutic interventions. However, global profiling of phosphorylation is not trivial due to the sheer number; the relatively low site occupancy of phosphorylation on proteins, and the high complexity of phosphorylation signaling in complex cellular proteomes when they are perturbed [2–3]. Even so, mass spectrometry (MS) techniques have been the “method of choice” to cope with such challenges as it allows profiling of proteomes in a global fashion.

MS methodologies to link phosphorylation changes in proteins with pathophysiological changes are still evolving [2–3, 4]. To perform such correlations, it is prudent that two phosphoproteomes, one from a control condition (e.g. normal cellular function) and the other from an experimental condition (e.g. a particular pathological state or gene knockout) are compared quantitatively [5–6]. Thus proteins with phosphorylation changes can be identified and then related to specific kinase/phosphatase signaling networks. For such comparisons, MS strategies such as stable isotope labeling with amino acids in cell culture (SILAC) are often used [7–8]. However, the SILAC approach is not ideal for relative quantification of phosphoproteomes in tissue extracts due to the cost associated with metabolic labeling of a whole animal. Isotope coded affinity tag (ICAT) approaches and related “iTRAQ”-type approaches are often used for relative quantification of proteins from cells or tissues [9–10]. Unfortunately, the high cost of labeling reagents limits such approaches to small quantities of samples (e.g 100 µg) and a minimal number of biologically distinct comparisons. In lieu of such approaches, less expensive and simultaneously effective labeling strategies have been reported including trypsin digestion in H₂¹⁸O [11] and dimethyl tagging with ¹³C [12] to name a few. The primary challenge associated with both of these methods is the limited mass shift for the “heavy” isotope thus resulting in some interference in the relative quantitation for the “light” and “heavy” pairs.

Another strategy for relative quantification of peptides known as reductive alkylation by acetone or RABA, uses inexpensive isotopically-labeled acetone (CD₃COCD₃) and non-labeled acetone (CH₃COCH₃) for “heavy” and “light” labeling of peptides, respectively [13]. Such a methodology would be particularly useful if site-specific post-translational modifications (PTMs) were to be quantified in a relative fashion. However, amenability of this labeling technique for quantitative phosphoproteomic profiling has not been explored. Also, it is yet to be applied to a biological model to evaluate its potential in revealing statistically significant or biologically reproducible and relevant information.

The present study demonstrates that relative quantification of phosphopeptides using MS can be achieved using a workflow that relies on RABA-labeling chemistry and TiO₂-chromatography. Furthermore, data are presented to show that the RABA-labeling approach also offers an inexpensive alternative for the generation of heavy isotope-labeled reference standards for direct mass spectrometry-based quantitation across samples using selective reaction monitoring (SRM).

2. Materials, Animals and Methods

2.1 Materials

Acetone, d6-acetone- (cat. no. 540331, 99.9 atom % deuterium), Guanidine:hydrogen chloride (GHC1), ammonium bicarbonate (NH₄HCO₃), Phosphatase inhibitor cocktail 2 (cat.

no. P5726), dithiothreitol (DTT), iodoacetamide (IAA), formic acid (HCOOH, FA), trifluoroacetic acid (TFA), α -casein from bovine milk (cat. no. C6780, α -casein minimum 70%), acetonitrile (CHROMASOLV[®], for HPLC, gradient-grade, >99.9%), water (CHROMASOLV[®]-Plus, for HPLC), α -cyano-4-hydroxycinnamic acid (CHCA), sodium cyanoborohydride (NaBH₃CN), glycine, and glycolic acid were purchased from Sigma-Aldrich (St. Louis, MO). For proteomics sample preparation work-flow, deionized water was obtained from an in-house Milli-Q system (Millipore, Bedford, MA). All centrifugation steps were complete in an IEC Micromax RF microfuge at 14,600 RCF (Relative Centrifugal Force). Modified trypsin was obtained from Promega (Madison, WI). Oligo R3 reversed-phase material was obtained from Applied Biosystems (Foster City, CA). For the packing of Oligo R3 reversed phase material, Bio-select extraction columns (reversed phase C4) were obtained from GRACE-VYDAC (W.R. Grace & Co., Deerfield, IL). For TiO₂-chromatography, Titansphere TiO₂-beads were obtained from GL Sciences Inc Japan. Ammonium hydroxide (trace-metal grade, assay: 20–22% as NH₃) was obtained from Fisher Scientific (Hampton, NJ). The phosphopeptide, RT(pS)DSHEDAGTLDFSSLLK, was synthesized by Thermo Scientific Open Biosystems for the SRM-based targeted quantitation experiments.

2.2 *Fgf2*KO and HMW FGF2KO mice

A pathogen-free facility was used to house all mice, and mice were handled according to standard protocols, animal welfare regulations, and the NIH *Guide for the Care and Use of Laboratory Animals*. Approvals for protocols were obtained from the University of Cincinnati Institutional Animal Care and Use Committee. *Fgf2*KO (referred throughout the manuscript as LMW FGF2 “non-expressed”) mice and HMW FGF2KO (LMW “expressed”) mice were generated, on a mixed 50% 129 and 50% Black Swiss background, in the laboratory of Dr. Thomas Doetschman by Dr. Ming Zhou and Dr. Mohammed Azhar, respectively, using a tag and exchange construct [14–16].

2.3 Isolation of mouse hearts, inducing I/R injury and protein content determination

Age- (10 weeks–14 weeks) and sex-matched mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg). After mice were unconscious and unresponsive to painful stimulus, isolated work-performing heart preparation and global low-flow ischemia procedures were followed as previously described [17]. Each heart was subjected to low-flow ischemia for 60 minutes followed by 5 minutes of reperfusion (sample size = 5 hearts from LMW FGF2 “expressed” and 5 hearts from LMW “non-expressed”). The reperfusion time window, 5–6 min, was chosen because previous studies indicate sufficient percent recovery of contractile functions are attained during a 5–6 min time-window for the *Fgf2*KO as well as in kinase activation [14,18]. The hearts were then snap frozen in liquid nitrogen, pulverized and homogenized on ice in a buffer containing 25 mM Hepes, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1% glycerol, 1 mM sodium orthovanadate, 25 mM β -glycerolphosphate, 50 mM sodium fluoride, 0.5 mM okadaic acid, 100 mM calpain inhibitor, Pefabloc Stock 1 and 2 (Roche), Roche phosphatase inhibitor (1 tablet/10 mL), Roche complete mini EDTA-free protease inhibitor cocktail (1 tablet/10 mL), and 1 mM PMSF. Residual particulate material removed by centrifugation for 15 min and the supernatant was pipetted into a clean Eppendorf tube. Biorad DC protein assay was followed on the supernatants for protein content determination, according to previous reports [17].

2.4 Trypsin digestion

500 μ g aliquots of protein were precipitated with 8 volumes of cold acetone (–20 °C) in 1.5 mL Eppendorf tubes. After centrifugation (14,600 RCF, 5 min), supernatants were discarded and pellets were washed three times using –20 °C acetone (100 μ L for each wash). Sample

tubes were then kept open in a fume-hood for 2 min to ensure any residual acetone vaporization. The pellets were reconstituted in 3 M Guanidine:HCl in 100 mM NH_4HCO_3 (90 μL) containing phosphatase inhibitor cocktail (2 μL). The solutions were subsequently reduced with DTT (1 mM final concentration, incubated at 37 °C, for 45 min) and then alkylated with iodoacetamide (5.5 mM final concentration, incubated at 37 °C, for 30 min). The solutions were finally diluted with ddH₂O to 1 mL before trypsin-based digestion. 100 μg of modified trypsin was dissolved in 300 μL of 0.1 M NH_4HCO_3 , and 10 μg aliquots were added into each 500 μg protein sample (i.e. 1:50 weight ratio). Samples were then incubated overnight at 37 °C, and the digestion was quenched by adding 20 μL of formic acid (to bring the pH of solutions to less than 5). After centrifugation, the supernatants were recovered for further processing. Similarly, 2.3 mg of bovine α -casein was subjected to trypsin digestion and from this stock a standard solution (2.3 $\mu\text{g}/\mu\text{L}$) was prepared. Dilutions of this standard solution were used to spike into samples as an internal standard either to follow the workflow (50 ng) or to monitor procedural errors (23 ng).

2.5 Desalting prior to RABA labeling and TiO₂-chromatography using Oligo R3 beads

Oligo R3 reversed-phase material was dispersed in ACN/H₂O/TFA 70/29.9/0.1 (v/v/v) to make a 60 mg/mL slurry as previously described [19], and divided into 500 μL aliquots each containing 30 mg of Oligo R3 beads in 1.5 mL Eppendorf tubes. The beads prepared for peptide desalting by sequential vortex, spin and removal of the supernatant followed by two washes with 200 μL of 0.1% TFA in ddH₂O. Peptide solutions were added onto washed Oligo R3 beads and incubated for 30 min at room-temperature using end-over-end rotation. GRACE-VYDAC BIOSELECT-C4 columns (CAT. NO. 214SPE1000) were adapted onto an extraction manifold (Waters Manifold, Massachusetts, USA), washed sequentially with 1) ACN (500 μL), 2) ACN/TFA/H₂O 70/0.1/29.9 (v/v/v, 200 μL), 3) 0.1% TFA (500 μL) and 4) dd H₂O (500 μL), and then packed with peptide-bound Oligo R3 beads by a gentle application of vacuum into the extraction manifold vacuum chamber. Subsequently, the peptide-bound beads were washed with 500 μL ddH₂O and eluted by sequentially passing 200 μL of ACN/TFA/H₂O 70/0.1/29.9 (v/v/v) two times, and then 200 μL of ACN/TFA/H₂O 90/0.1/9.9 (v/v/v) two times. All elution fractions were collected into 1.5 mL Eppendorf tubes. These solutions were directly used for the subsequent RABA-reactions.

For the Oligo R3 desalting step prior to TiO₂-chromatography, the amount of beads, wash and elution steps were increased by 2X to account for twice as much peptide due to mixing of the heavy and light labeled samples. Elution fractions were subsequently subjected to vacuum centrifugation.

2.6 RABA-based differential peptides labeling and mixing

Labeling of peptides was adapted from the previously described method [13] with some modifications. Briefly, two solutions (RABA-reaction mix) containing NaBH₃CN (3.6 mg/100 μL) in d0-acetone and d6-acetone were freshly prepared. NaBH₃CN preparation and RABA-labeling were all carried out inside a chemical fume hood. RABA-reaction mix (100 μL) was added to each sample where, d0-acetone containing RABA-reaction mix was for the LMW FGF2 non-expressing samples, and d6-acetone containing mix was for the LMW FGF2 expressing samples. Samples were subjected to gentle vortex, and incubated at room temperature for 1 hr in the dark. Similarly, two additional RABA-labeling reactions were performed to ensure near complete labeling of peptides. The reactions were quenched by adding glycine (1 M, 150 μL) as previously described [13]. The samples were then dried using a vacuum centrifuge system. The dried samples were dissolved in dd H₂O (400 μL) and each biological replicate of the “light” and “heavy” labeled peptide solutions were mixed in separate 1.5 mL Eppendorf tubes.

2.7 Separation of RABA-labeled phosphopeptides using TiO₂-chromatography

Phosphopeptide enrichment of the RABA-labeled mixtures was carried out using an optimized strategy adapted from previous reports [19–20]. Briefly, Titansphere TiO₂-beads were dispersed in ACN/H₂O/TFA 80/15/5 (v/v/v) to make a 100 µg/µL slurry then divided into 10 µL aliquots each containing 1 mg of TiO₂ beads in 0.5 mL Eppendorf tubes. Each vial was subjected to a vortex and spin with supernatants discarded, followed by 2 additional washes with 200 µL of the same solvent. Dried RABA-labeled peptide mixtures (500 µg) were reconstituted in 200 µL of 1 M glycolic acid in ACN/H₂O/TFA 80/15/5 (v/v/v,) and loaded onto the pre-washed Titansphere TiO₂-beads. The RABA-labeled peptides were allowed to interact with the TiO₂ for 30 min at room temperature using end-over-end rotation. The TiO₂ beads were then sequentially washed with 400 µL ACN/H₂O/TFA 80/15/5 (v/v/v) with a spin and removal of the supernatant followed by an additional 200 µL wash with the same solvent. Finally the phosphopeptides captured on the TiO₂-beads were eluted 2X with 200 µL of 5% NH₄OH. 2.5 µL aliquots of the elution fractions were removed from each sample, desalted by ZipTip(C-18µ) as described by the manufacturer (Millipore) and evaluated by MALDI-TOF-MS to monitor the process. The remaining NH₄OH elution fractions were dried by vacuum centrifugation prior to nanoLC-MS/MS analysis.

2.7 MALDI-MS analysis

MALDI-MS analysis was performed on a 4800 MALDI-TOF/TOF™ instrument (AB Sciex, Foster city, CA). Mass spectra were obtained in positive reflector mode. MALDI-matrix solution was prepared by dissolving -cyano-4-hydroxy-cinnamic acid (CHCA, 5 mg) in 10 mM ammonium phosphate (monobasic) in ACN/FA/H₂O 60/0.1/39.9 (v/v/v, 1 mL). In order to perform MALDI-MS analyses, desalted (using Oligo R3 reversed phase material) or ZipTip™(C-18µ) isolated peptides in solution (0.5 µL) were mixed with MALDI-matrix solution (1 µL), and spots were placed on a calibrated MALDI plate.

2.8 Nano-LC-MS/MS analysis

Nano-LC-MS/MS analyses were performed on a TripleTOF™ 5600 (ABSciex, Toronto, ON, Canada) coupled to an Eksigent (Dublin, CA) nanoLC.ultra nanoflow system. Dried phosphopeptide samples were reconstituted in FA/H₂O 0.4/99.6 (v/v,) and loaded onto IntegraFrit Trap Column (outer diameter of 360 µm, inner diameter of 100, and 25 µm packed bed) from New Objective, Inc. (Woburn, MA) at 2 µl/min in FA/H₂O 0.4/99.2 (v/v) for 10 min to desalt and concentrate the samples. For the chromatographic separation of peptides, the trap-column was switched to align with the analytical column, Acclaim PepMap100 (inner diameter of 75 µm, length of 15 cm, C18 particle sizes of 3 µm and pore sizes of 100 Å) from Dionex-Thermo Fisher Scientific (Sunnyvale, CA). The peptides were eluted using a varying mobile phase (MP) gradient from 95% phase A (FA/H₂O 0.4/99.6, v/v) to 40% phase B (FA/ACN 0.4/99.6, v/v) for 70 min, from 40% phase B to 85% phase B for 5 min and then keeping the same MP-composition for 5 more minutes at 300 nL/min.

Nano-LC mobile phase was introduced into the mass spectrometer using a NANOSpray® III Source (AB Sciex, Toronto, On, Canada). Ion source gas 1 (GS1) was zero grade air while ion source gas 2 (GS2) and curtain gas (CUR) were both nitrogen. The gas settings were kept at 7, 0 and 25, respectively in vendor specified arbitrary units. Interface heater temperature and ion spray voltage was kept at 150 °C, and at 2.3 kV. The mass spectrometer method was operated in positive ion mode set to go through 4156 cycles for 90 minutes, where each cycle consisted of one TOF-MS scan (0.25 secs accumulation time, in a 400 to 1600 m/z window) followed by twenty information dependent acquisition (IDA) mode MS/MS-scans on the most intense candidate ions selected from initially performed TOF-MS scan during each cycle, having a minimum of 150 counts. Each product ion scan was operated under vendor specified high-sensitivity mode with an accumulation time of 0.05

secs and a mass tolerance of 50 mDa. Former MS/MS-subjected candidate ions were excluded for 10 secs after its first occurrence, and data were recorded using Analyst®-TF (1.5.1) software.

2.9 Data Analyses

Identification of Phosphopeptide Pairs—The nano-LC-MS/MS data (*.wiff file) from the enriched phosphopeptides were analyzed for protein identification using ProteinPilot™ software (version 4.2, revision 1297) that integrates the Paragon™ algorithm, searched against a SwissProt database of *Mus Musculus* protein sequences which was modified to carry the protein, bovine β -casein, on a local 12-processor server. A custom “sample-type” was selected that specifies fixed-modifications, either propyl(d0) or propyl(d6), on N-terminus and/or on lysine (K), along with other variable biological modifications as specified defaults in the ProteinPilot software. The vendor defined phosphorylation emphasis on serine/threonine/tyrosine was also used as a special factor. The resulting *.group file was then used to generate a spreadsheet as a peptide summary report. Only those phosphopeptides identified with a minimum of 95% confidence in identity (calculated by probability algorithms of ProteinPilot™ software), and phosphorylation as a modification along with RABA-labeling were selected for further evaluation. Unique phosphopeptides were then selected based on sequence, modifications, mass to charge ratio (m/z-value) and charge (z). From the 95% confidence phosphopeptides, a set of expected phosphopeptide pairs was tabulated for both light and heavy RABA labeled peptides. Note: for the five biologically distinct phosphoproteomic comparisons, phosphopeptide pairs were identified from a merged search performed on all five LC-MS/MS analyses and a single set of phosphopeptide pairs was tabulated for all five replicates as described above.

Relative Quantification of Phosphopeptide Pairs—The relative quantification values for each of the phosphopeptide pairs were calculated and reported as base two logarithmic “heavy”-labeled to “light”-labeled extracted ion chromatogram (XIC) area ratios ($\log_2[H/L]_{\text{Pair}}$). The XICs were generated using PeakView™ software (version 1.1.0.0) from AB Sciex and were subjected to a two point Gaussian smooth prior to capturing the peak areas. The $\log_2[H/L]$ value was also calculated for the internal standard of β -casein phosphopeptides (designated as $\log_2[H/L]_{\text{IS}}$) such that the normalized logarithmic values for each phosphopeptide pair ($\log_2[H/L]_{\text{Pair}}$) is represented by equation (A):

$$\log_2[H/L]_{\text{Pair}} = \log_2\left[\frac{[H/L]_{\text{Pair}}}{[H/L]_{\text{IS}}}\right] \quad (\text{A})$$

To establish the technical variability of the system, the normalized $\log_2[H/L]_{\text{Pair}}$ values were captured from two identical cardiac tissue extracts (technical replicates) and a frequency plot of the ratio variability was captured. For the five biologically distinct, pairwise comparisons from LWM FGF2 non-expressing and expressing tissues, the normalized heavy to light ratios ($\log_2[H/L]_{\text{Pair}}$) were captured independently for each phosphopeptide pair from each biological replicate. Relative quantitative differences that fell outside the technical variability frequency plot (described above) were considered as viable quantitative phosphorylation changes. Such changes observed in at least in four out of the five biological replicates were selected for further evaluation. This included determining the average and standard error for the normalized relative quantitative differences ($\log_2[H/L]_{\text{Pair}}$) across all 5 samples sets and a more detailed evaluation of the phosphopeptide fragmentation spectrum to validate the site(s) of phosphorylation.

2.10 SRM (selected reaction monitoring) based quantification of phosphoserine 282 from cardiac myosin binding protein C (MyBP-C) using a synthetic phosphopeptide standard

RABA Labeling of the synthetic phosphopeptide—280-

RT(pS)DSHEDAGTLDFSSLLK-298 from cardiac myosin binding protein C—10 nmoles of the synthetic phosphopeptide was labeled with the d6-acetone as described in section 2.6. Complete conversion to the heavy labeled peptide was monitored by MALDI-TOF MS and aliquots of the final product were dried by vacuum centrifugation. Dried samples were reconstituted in ddH₂O to a final stock concentration of 1 pmol/μL of d6-RABA labeled phosphopeptide.

Sample preparation for the Selective Reaction Monitoring (SRM) based

workflow—Preparation of 500 μg of cardiac tissue through, protein digestion, OligoR3 desalting, d0-RABA labeling of peptides, and phosphopeptide separation using TiO₂-chromatography was performed in the same manner as described above with the exception that the d6-RABA labeled synthetic phosphopeptide was used instead of the alpha casein as the internal standard. Three, LMW FGF2 “non-expressed” and three, LMW FGF2 “expressed” cardiac tissue extracts originating from mouse hearts subjected to 60 min of ischemia followed by 5 min of reperfusion were used for the evaluation.

SRM based nano-LC-MS/MS analyses—The d6-acetone labeled synthetic phosphopeptide was analyzed using nano-LC-MS/MS under various conditions to determine the optimal charge-state, collision energy and MS/MS transition for maximum selectivity and sensitive of detection. The final optimized SRM parameters included: positive ion nanospray set to go through 10806 cycles for 90 minutes, where each cycle performing one TOF-MS scan type (0.05 secs accumulation time, in a 100 to 1800 m/z window) followed by two MS/MS-scan (0.05 secs accumulation time, in a 100 to 1800 m/z window) events on the candidate precursor ions m/z, 748.4 (3+) and 752.4 (3+) representing triply protonated (d0-RABA-R)T(pS)DSHEDAGTLDFSSLL(d0-RABA-K) from each cardiac sample and (d6-RABA-R)T(pS)DSHEDAGTLDFSSLL(d6-RABA-K) for the internal quantitation standard. Product ion scans were operated under vender specified high sensitivity mode with a collision energy value of 46 arbitrary units (vender defined), an accumulation time of 0.05 sec and a mass tolerance of 50 mDa. Data were recorded using Analyst®-TF (1.5.1) software.

3. Results and Discussion

This research study evaluates and demonstrates the application of a simple and inexpensive differential peptide labeling method, reductive alkylation by acetone (RABA), to address relative quantitation of phosphoproteomes from tissue extracts. RABA is an external chemical tagging approach that uses d0-acetone and d6-acetone along with NaCNBH₃ to differentially tag peptides at primary amines. It has shown promise to relatively quantify peptides in standard proteins even in a background of complex peptide mixtures [13]. A RABA-based relative quantitative MS study involves, “light”-chemical labeling (adding 42 Da of mass) and “heavy”-chemical labeling (adding 48 Da of mass) to peptides that are identically generated from a normal tissue and an experimental tissue. Once labeled, the samples can be mixed together based on equal protein content so that when analyzed in a mass spectrometer, the peptides can be identified by MS-based peptide fragmentation sequencing and quantified based on the relative abundance of the “light” and “heavy” labeled peptide pairs. However, this method has yet to be evaluated for relative quantification of phosphopeptides. Since this method alters molecular structures of peptides at primary amines, and it is not clear whether this reductive chemistry would affect

phosphorylation, it is also necessary to examine its amenability for labeling and quantification of phosphopeptides.

3.1 RABA-based chemistry for relative quantification of phosphopeptides

The first goal of this study was to assess whether RABA-labeling chemistry affects phosphorylation on peptides and whether TiO₂-chromatography can be used to separate RABA-labeled phosphopeptides from the non-phosphorylated peptides. To accomplish this, two complex cardiac peptide solutions (500 µg each), spiked with γ -casein tryptic peptides (50 ng for each) were subjected to differential RABA-labeling, mixed together, and subjected to the TiO₂-chromatography to selectively enrich the phosphopeptides. Bovine γ -casein was employed as an internal standard since it has known phosphopeptides and non-phosphopeptides that can be readily monitored using MALDI-MS. The phosphopeptide enrichment, identification and relative quantification strategy is illustrated in Figure 1(A). Details associated with sample preparation are indicated in the Methods section.

As demonstrated in Figure 1B(i), γ -casein tryptic peptides, 91-YLGYLEQLLR-100 and 8-HQGLPQEVNENLLR-22 represented by m/z values at 1267.75 and 1760.00, respectively, are shifted by 42 Da and 48 Da due to the differential RABA labeling and mixing [Figure 1B(ii)], and can be seen as peptide pairs in the mixture. The mass spectral responses with appreciable 1:1 quantitative ratios reflect effective quantitative RABA-labeling and mixing. As explained in the methods section, after binding the differentially RABA-labeled mixture onto TiO₂-beads, and eluting using NH₄OH (5%), the MALDI-MS of TiO₂ elution fraction [Figure 1B(iii)], clearly indicates that the γ -casein phosphopeptide (m/z 1660.84, VPQLEIVPN(pS)AEER, in Figure 1B(i), has been RABA-labeled and markedly enriched as judged by the peptide pair at 1702.76 m/z and 1708.76 m/z [Figure 1B(iii)]. Simultaneously, the non-phosphopeptide pairs were largely eliminated. This clear appearance of differentially-mass labeled and quantitatively equally mixed phosphopeptide pairs from γ -casein demonstrate that differential RABA-labeling exerts negligible chemical influence on peptide phosphorylation, and is also compatible with TiO₂-chromatography that enables separation of RABA-labeled phosphopeptides from the non-phosphopeptides.

In order to further investigate the variability of this workflow for quantitative comparisons among complex phosphoproteomes in tissue extracts, and to establish statistical merits for confident relative quantifications, phosphopeptide pairs originating from cardiac tissues were quantitatively compared using the same workflow as in Figure 1A. For this experiment, the γ -casein tryptic peptides were added to identical tryptic digestion samples from cardiac tissue extracts. After RABA-labeling, phosphopeptide enrichment, nano-LC-MS/MS analysis and identification of the phosphopeptide pairs from the cardiac tissue, the γ -casein phosphopeptides that had been spiked into the cardiac digests were used as a normalization standard to account for procedural error associated with sample preparation. As defined in Equation (A) in the Methods section under data analysis, $\log_2[H/L]^*_{\text{Pair}}$ values that represent “heavy” and “light” labeled phosphopeptide ratios normalized with $\log_2[H/L]$ IS ratio from the γ -casein phosphopeptide standard, were tabulated. An average $\log_2[H/L]^*_{\text{Pair}}$ value of 0.05 ± 0.02 indicates minimal variability from the ideal $\log_2[H/L]^*_{\text{Pair}}$ equals zero, thus indicating minimal bias to either “heavy” or “light” peptide labeling using d6-acetone or d0-acetone. The occurrence frequency distribution of “heavy” to “light” ratios of phosphopeptide pairs, $\log_2[(H/L)]^*_{\text{Pair}}$, illustrated in Figure 1C, indicate that almost 95% of the pairs evaluated were distributed between -0.40 and $+0.40$ of $\log_2[(H/L)]^*_{\text{Pair}}$ values. It also indicates that 100% of the pairs, that theoretically should be representing equal amounts, are distributed in their $\log_2[(H/L)]^*_{\text{Pair}}$ values between -0.60 and $+0.60$. Thus, these technical variability data would infer that when applying this workflow for “non-identical” phosphoproteomic comparisons, any phosphopeptide pairs with $\log_2[(H/L)]^*_{\text{Pair}}$

changes beyond ± 0.60 would represent changes in phosphorylation between the two groups being compared.

Mass spectrometry (MS)-based techniques are widely utilized for phosphoproteomic comparisons [2–4, 21]; however, most of the reports have focused on comparative phosphorylation using SILAC-based metabolic labeling of cultured cells [7]. Applications of MS-based techniques that can effectively compare two differentially-regulated tissue phosphoproteomes are still evolving [19–25], due in part to the additional technical challenges associated to tissues protein extraction and labeling as compared to the more controlled conditions of cultured cells. In addition, since many biological model systems now take advantage of knockout and transgenic mice, the need for profiling phosphorylation from tissue extracts is only expected to continue to increase. As such, the RABA-based external labeling strategy may offer a cost effective opportunity for these studies. Since we already evaluated the potential of RABA-labeling chemistry for relative quantification of phosphopeptides in complex peptide mixtures, we next applied the RABA-based strategy to compare cardiac tissue phosphoproteomes to explore the capacity of the workflow to return biological targets with statistical significance by applying it across a set of biologically distinct replicate comparisons in a cardiac ischemia/reperfusion model system.

3.2 Application of the workflow to identify downstream targets in LMW FGF2 mediated cardio-protective signaling networks in an ischemia/reperfusion model system

Previous biological experiments have shown that the presence of endogenous LMW FGF2 isoform in mouse hearts result in better recovery of cardiac function after an ischemia and reperfusion (I/R)-injury, as compared to the model with no LMW FGF2 [17–18]. The LMW FGF2 protein isoform has also been shown to participate in multiple biochemical pathways by activating signaling cascades such as nitric oxide (NO), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) to protect the heart from (I/R)-injury [17–18, 26–29]. However, downstream mediators relevant to these signaling pathways are yet to be fully elucidated. Since many of these cardio-protective functions are regulated by phosphorylation changes, a secondary goal in evaluating the RABA-labeling method was to advance the understanding of LMW FGF2-mediated phosphorylation changes in downstream targets that are relevant to cardioprotection from I/R injury, because detailing such potential targets could lead to new avenues for therapeutics against cardiac I/R injury.

LMW FGF2 “non-expressed” mice and LMW FGF2 “expressing” mice had been generated and characterized previously (see method section for details) and thus offered an appropriate model system to evaluate the changes in the phosphoproteome using the RABA labeling method. Specifically, these two types of hearts have shown considerable pathophysiological differences in percent recovery of post-I/R contractile function, that is 70% of contractile function recovery for LMW FGF2 “expressed” mouse hearts compared to only about 35% recovery for the total LMW FGF2 “non-expressed” mouse hearts [17–18, 29]. Thus, the hypothesis is that these differences in the recovery of cardiac contractile function are mediated by LMW FGF2 and may be linked to differential downstream phosphorylation events.

In order to further evaluate the RABA-labeling strategy for phosphoproteomics and to perhaps gain some mechanistic insight into the pathophysiological differences observed in post-ischemic cardiac function, multiple biological replicates were prepared. For phosphorylation changes in parallel, the workflow (Figure 1A) was simultaneously applied to five hearts from LMW FGF2 “non-expressed” mice and five hearts from the LMW FGF2 “expressing (i.e., five biologically distinct pairwise comparative replicates) as illustrated in Figure 2A. . As described in the methods section, a merged database search was performed on the combined LC-MS/MS profiles of phosphopeptides generated from all five

comparisons. This analysis resulted in identifying over 2300 unique phosphopeptides, representing 234 proteins that were singly or multiply phosphorylated. In each case, these phosphopeptide pairs carried high confidence values (>95%), and were initially assigned as phosphopeptides by the Paragon probabilistic algorithms used by the ProteinPilot software. However, all differentially-phosphorylated peptide candidates were subsequently confirmed by manual annotation of the fragmentation spectra and are illustrated as supplemental information including documentation of the diagnostic fragment ions designating the site(s) of phosphorylation (Supplemental Figures 1–16).

To validate each replicate and to monitor procedural errors, each replicate was first evaluated by relatively quantifying the RABA-labeled α -casein-phosphopeptide pair observed at m/z 679.356 and 683.387 as the internal standard (IS). The MS/MS profile of “heavy” labeled α -casein-phosphopeptide is illustrated in Supplemental Figure 1. Figure 2B summarizes the MS responses, corresponding extracted ion chromatograms (XICs) of fully labeled α -casein-phosphopeptide pair obtained for each replicate and the $\log_2[H/L]_{IS}$ for each pair obtained from the XIC profiles. It should be noted that XICs were utilized for quantification purposes as they provide a better representation for peptide quantity than peak intensities from the peptide spectrum. Using the peak area from the XICs was also important due to concerns about possible variations in retention time for deuterated peptides. While this retention time shift was not observed as a major issue (see the quantitative overlays of the XICs in Figures 2, 5 and Supplemental Figures 17–29), nonetheless, use for the peak areas from the XIC for quantitation effectively overcome this issues. Although α -casein tryptic peptides were “spiked-in” in equal amounts some minor deviation from its ideal 1:1 quantitative ratio is apparent when $\log_2[H/L]_{IS}$ values are compared among the five replicates. However, this relative quantification of the internal standard in Figure 2B provides a baseline reference so that comparison of phosphorylation differences between the tissue extracts can be calculated by normalization to the internal standard.

Similar figures were also generated for the high-confidence (>95%) phosphopeptide pairs identified during the merged search. These profiles are subsequently referred as “quantitative figures.” These quantitative figures were generated using PeakView™ software and were required to satisfy several stringent criteria. That is, confident extractions of XICs for each pair were required to carry a clear MS response compared to the background, and the MS-responses relevant to each pair were also required to have no interferences by neighboring peptide-responses at least in a mass tolerance range of ± 0.01 m/z . Such XICs were then required to be retrieved in at least four out of the five biological replicates to be considered as possible candidates for differential phosphorylation. Phosphorylation differences outside the range of the 95% frequency of technical variation (between -0.40 and $+0.40$ of $\log_2[H/L]_{Pair}$ values) as determined in Figure 1C, that were apparent in four or five biological replicates were then selected to obtain normalized $\log_2[H/L]^*_{Pair}$ values as described in the methods section. Collectively from the 5 biological replicates, 14 phosphopeptides representing 10 different proteins met these criteria (Supplemental Table 1) and were further evaluated.

To further illustrate the quality of these phosphorylation changes, we have singled out one example to follow through with a more detailed explanation, but have provided the data for all the other pairs in the supplemental information. Figure 2C represents a phosphorylation change identified on cMyBP-C/Ser-282 (cardiac-type myosin-binding protein C, phosphorylation on Ser-282), with “light” and “heavy” RABA-labeled phosphopeptide pairs observed at, m/z values, 561.521 and 564.542 representing 280-RT(pS)DSHEDAGTLDFSSLLK-298 with modifications RABA@N-term, RABA(K)@298 and charge state 4+. From both the XICs and the mass spectra, the relative increase in phosphorylation on cMyBP-C/Ser-282 is noted for the LMW FGF2 expressing samples in 4

of the 5 biological replicates with an average $\log_2[H/L]_{\text{Pair}}$ value with standard error calculated to be 1.32 ± 0.44 , thus showing nearly a 2-fold increase over the previously determined expected technical variability (Figure 1C). Similarly, the rest of the phosphopeptide pairs that showed significant changes were evaluated and the list of the identified candidate proteins, site(s) of phosphorylation, and the change in phosphorylation levels (i.e. higher or lower) are summarized in Supplemental Table 1. Fragmentation spectra and quantitative figures for each of the phosphorylation sites are provided in Supplemental Figures (1–29) as summarized on the Supplemental Table 1.

3.3 Biological relevance of the observed changes

One general observation from the phosphorylation changes identified and listed in Supplemental Table 1 is that all the proteins have shown a higher degree of phosphorylation for LMW FGF2 expressed mouse hearts compared to non-expressed tissue. This general up-regulation of phosphorylation in LMW FGF2 “expressed” hearts is not unexpected given our current knowledge of the central role of phosphorylation in normal cardiac function [30] and likely provides some meaningful connections regarding how LMW FGF2 modulates improved recovery for post-ischemic function following I/R injury [18]. Supplemental Table 1 includes several such phosphorylation changes that are already known to be mediated by FGF2 isoforms. For instance, it has been shown in cardiomyocytes (but not cardiac tissue) that gap junction alpha-1 protein (connexin-43 or Cx43) associates with and is phosphorylated by PKC ϵ upon FGF2 treatment [30]. Although its exact mechanism in protecting the heart is yet to be fully detailed, it has been suggested that Cx43 might be contributing to cardioprotection by reducing the metabolic coupling between myocytes and preventing the propagation of contraction. Even so, reports have not specifically indicated the site of phosphorylation on Cx43, and by which isoform of FGF2 that these cardioprotective mechanisms were initiated. The results from the current study are consistent with a model where the LMW FGF2 isoform is responsible for the PKC ϵ activation which results in a higher degree of phosphorylation on Cx43 at Ser-297.

Additionally, a higher degree of protein phosphorylation identified on cardiac myosin binding protein C (cMyBP-C) in LMW FGF2 expressed tissues is also consistent with the known role of cMyBP-C in normal cardiac functions. Thus, phosphorylation changes in cMyBP-C observed in the current study can be effectively related to LMW FGF2-mediated cardioprotective mechanisms after I/R injury. Other reports indicate links between PKC and cMyBP-C phosphorylation, and that phosphorylation of cMyBP-C is required for normal cardiac function [32–33]. More specifically, studies using transgenic mice with multiple phosphorylation sites of cMyBP-C that contain genetically substituted alanine (to mimic an unphosphorylated state) for phosphorylatable serines have shown that the contractility as well as sarcomeric structure of these hearts was depressed compared to normal hearts [33]. Similarly, when all the phosphorylatable serines were substituted with aspartic acid to mimic phosphorylated serines, these hearts have exhibited cardioprotective phenomena when they were subjected to I/R injury, thus demonstrating the critical role of phosphorylation on cMyBP-C in cardioprotection. In addition, it has also been shown that LMW FGF2 expressing mouse hearts carry a better contractile recovery after ischemia (i.e. 70% for LMW FGF2 expressing mice) compared to when LMW FGF2 is absent in mice [17–18, 29]. Hence, according to the present study, mouse hearts with better recovered contractility (i.e. LMW FGF2 expressed mouse hearts) show a quantitatively higher degree of phosphorylation on cMyBPC, at multiple locations (Ser-273, Thr-281, Ser-282 and Ser-285) compared to when LMW FGF2 is absent. Furthermore, it is known that LMW FGF2 isoforms exert cardioprotective phenomena after I/R injury through protein kinase C (PKC) activation [34], and cMyBP-C is a known substrate for PKC- and PKA-mediated phosphorylation at multiple sites (Ser-273, Ser-282 and Ser-302) that modulate cardiac

contractility for normal heart functions [32–33]. Thus, the current results link LMW FGF2-mediated cardioprotection after I/R injury to cardioprotection via signaling pathways that involve activation of PKC signaling resulting in up-regulation of phosphorylation on cMyBP-C at multiple locations [35], as a possible mechanism for cardioprotection mediated by LMW FGF2. Figure 3 illustrates a putative signaling pathway that correlate the findings in the current study and findings in previously published literature reports, but additional biological validation studies will be need to confirm this model.

3.4 SRM (Selected reaction monitoring)-based methodology to confirm cMyBPC/Ser282 phosphorylation in LMW FGF2 cardioprotective signaling

To further assert endogenous Ser282 phosphorylation induction by LMW FGF2 on cMyBP-C for cardioprotection, an alternate mass spectrometry methodology, based on selected reaction monitoring (SRM) was performed. SRM-based methodologies allow for selective and quantitative measurements of analytes from complex mixtures by measuring a unique transition of ions from the precursor m/z to their specific fragment ions [4]. Such approaches become particularly useful when phosphoproteomic quantifications are required to be site specific and to further elaborate the details in phosphoproteomic signaling. In this approach, a heavy isotope labeled peptide is added to the complex mixture as a quantitative internal standard. Since this heavy peptide will have the same ionization and fragmentation properties as the target phosphopeptide of interest, normalization of the peak areas for the SRM transition of the target peptide to the internal standard allows for quantitation the target peptide. However, due to high cost associated with synthetic peptides with ^{13}C and ^{15}N containing amino acid residues, a synthetic peptide labeled with d6-acetone was employed as the internal reference standard in the SRM methodology to specifically quantify the phosphopeptide, 280-RT(pS)DSHEDAGTLDFSSLLK-298 representing cMyBP-C with phosphorylation at Ser-282. As detailed in the methods section, the synthetic phosphopeptide, 280-RT(pS)DSHEDAGTLDFSSLLK-298, was first subjected to d6-acetone based RABA labeling to generate the SRM internal standard peptide. Then, from a standard solution prepared from the fully d6-labeled phosphopeptide, a series of LC-MS/MS analyses were performed to identify the optimal charge-state, collision energy and site-specific MS/MS transition to perform SRM-based site specific phosphopeptide quantification. Figure 4 illustrates the optimal MS/MS fragmentation of the doubly d6-RABA labeled (both at N-terminus, and at ϵ -amino group of Lys) synthetic phosphopeptide. The $[\text{b}_4\text{-H}_3\text{PO}_4]^+$ ion distinguishes phosphorylation on cMyBP-C/Ser-282 from phosphorylation on cMyBP-C/Ser-284, 294, 295 and Thr-290. Phosphorylation on cMyBP-C/Thr-281 was previously shown to be represented by a different peptide, 281-(pT)SDSHEDAGTLDFSSLLK-298 during the phosphoproteomics profiling of the 5 comparative tissue replicates (see Supplemental Table 1). Since this peptide elutes at a different retention time, the $[\text{b}_4\text{-H}_3\text{PO}_4]^+$ fragment ion from 280-RT(pS)DSHEDAGTLDFSSLLK-298 represents the most intense “site-specific” transition to quantify pSer-282 from the precursor ion at m/z 752.4 Da (the 3+ charge state).

As detailed in the methods section and illustrated in Figure 5A, tryptic digests from 3 biologically distinct mouse hearts that had been subjected to 60 min of ischemia and 5 min of reperfusion for each cardiac type (LMW FGF2 “expressed” and “non-expressed”) were “spiked” with the synthetic “RABA-heavy” labeled phosphopeptide, 280-(d6-RABA-R)T(pS)DSHEDAGTLDFSSLL(d6-RABA-K)-298. Then, after subjecting the rest of the peptides from each cardiac samples to d0-RABA labeling using d0-acetone and separating the phosphopeptide using TiO_2 -chromatography, “light” and “heavy” labeled responses of $[\text{b}_4\text{-H}_3\text{PO}_4]^+$ ions— m/z (z): 484.255 (1+) and 490.255 (1+) were monitored by subjecting precursor ions, m/z (z): 748.4 (3+) and 752.4 (3+) for fragmentation using the optimized method described above for the heavy labeled reference peptide. Figure 5B illustrates the

XICs and mass spectra for $[b4-H_3PO_4]^+$ fragment ions with $m/z(z+)$: 484.255 (1+) and 490.255 (1+) indicating a higher abundance of 484.255 (1+) in LMW FGF2 “expressed” samples compared to LMW FGF2 “non-expressed” samples. The average relative abundance of $[b4-H_3PO_4]^+$ fragment ions for the triplicate LMW FGF2 “expressed” samples was 3.441 ± 0.198 , and 1.114 ± 0.286 for the triplicate LMW FGF2 “non-expressed” samples as shown in Figure 5C. These triplicate samples returned a statistically significant p value of 0.002 (Figure 5D). These quantitative data were consistent with the higher degree of phosphorylation of cMyBP-C/pSer-282 by LMW FGF2 for cardioprotection after an I/R-event detected in the initial discovery phosphoproteome comparisons and also demonstrates the robustness of the acetone-based peptide labeling to generate the reference standards for SRM analyses in targeted quantitative phosphoproteomic studies.

4. Conclusions

In summary, using mass spectrometry and a standard phosphoprotein (α -casein), we have demonstrated that RABA-based chemistry is a viable peptide-labeling approach for relative quantification of phosphopeptides. Our analysis shows that RABA-labeling chemistry does not alter phosphorylation on peptides and is compatible with TiO_2 -based phosphopeptide enrichment chromatography. This validation of RABA-labeling chemistry for relative quantification of phosphopeptides prompted us to evaluate its utility for phosphoproteomics profiling to study LMW FGF2-mediated phosphorylation changes downstream of various signaling cascades that are relevant to cardioprotection. Application of this cost-effective MS-based strategy to compare I/R-induced tissue phosphoproteomes of LMW-FGF2 “non-expressed” hearts to LMW FGF2 “expressed” hearts in five biologically distinct replicates successfully identified 234 phosphoproteins with high confidence.

Most importantly, this study revealed biologically and statistically significant phosphorylation changes with known mechanistic details for FGF2-mediated cardioprotective signaling, new details linking LMW FGF2 to the cardioprotective mechanisms, and also several new potential downstream effectors. Furthermore, SRM-based evaluation employing a d6-RABA heavy labeled synthetic phosphopeptide further confirmed the site specific phosphorylation and provided a targeted quantification of cMyBP-C/pSer-282 across several biological replicates, thus providing validation that the RABA-labeling approach is well suited to perform both discovery-based comparative phosphorylation and targeted quantitative phosphoproteomic studies in a cost-effective manner. Further studies are being developed to validate the mechanistic connectivity of these phosphorylation changes between LMW FGF2 signaling and cardioprotection and will be the subject of other cardiac function manuscripts. Overall, these observations clearly demonstrate the utility for implementing this cost effective strategy for relative quantification of phosphopeptides in a challenging biological tissue model, thus it is expected that this method can also be applied to a wide variety of phosphorylation studies in wide variety of biological model systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Profiling strategy for global phosphorylation from tissue extracts. **(A)** Workflow: equal amounts of protein from cardiac tissue extracts were subjected to trypsin digestion, internal standard inclusion, differential peptide labeling using reductive alkylation by acetone (RABA), mixing, phosphopeptide enrichment using TiO_2 -chromatography, and LC-MS/MS analysis all as described in the Method section. **(B)** MALDI-MS based validation of the RABA-labeling and phosphopeptide enrichment using α -casein tryptic peptides as the internal standard in a complex background of tryptic peptides originating from cardiac tissue extracts. **(i)** Spectrum of α -casein tryptic peptides, highlighting peptides 91-YLGYLEQLLR-100, 8-HQGLPQEVLENLLR-22, and 106-VPQLEIVNP(S)AEER-119 at m/z 1267.75, 1760.00 and 1660.84, respectively. **(ii)** Spectrum of equal amounts of tryptic digests from cardiac tissue extracts spiked with α -casein tryptic peptide after RABA-labeled and mixing them together highlighting that the responses at m/z values 1267.75 and 1760.00 from **(i)** are now shifted by 42 Da and 48 Da corresponding to the light and heavy RABA labels. **(iii)** Spectrum after TiO_2 -enrichment of the mixture highlighting the RABA-labeled pair of α -casein phosphopeptides, at 1702.76 and 1708.76 that is significantly enriched compared to the non-phosphorylated peptide pairs that are virtually eliminated. **(C)** Frequency Distribution highlighting the technical of “heavy” to “light” phosphopeptide pair ratios from identical cardiac tissue lysates subjected to the full workflow in panel A. $\text{Log}_2 [\text{H/L}]^*_{\text{Pair}}$ represent the ratio of Heavy to Light phosphopeptide pair normalized to the internal standard of casein phosphopeptide H/L ratio as described in the Methods section.

Figure 2.

Application of the workflow for quantitative phosphoproteomic profiling of tissue extracts. **(A)** Experimental design: five hearts from each, LMW FGF2 “non-expressed” and LMW FGF2 “expressed” were subjected to I/R injury followed by quantitative phosphoproteomic comparisons via the workflow described in Figure 1(A). **(B)** Relative quantification of the alpha casein phosphopeptide pairs as a normalization standard from each replicate where, m/z 679.356 and 683.385 represents 104-YKVPQLEIVN(pS)AEER-119 with modifications RABA@N-term, RABA(K)@2 and charge state 3+. Biologically distinct replicates from different mouse hearts are denoted by “replicate numbers”. Below each replicate number are the averaged MS spectra and extracted ion chromatograms (XICs) for the phosphopeptide pairs. $\text{Log}_2[\text{H/L}]_{\text{IS}}$ values are indicated to denote procedural errors associated to each replicate. **(C)** Relative quantification of one example phosphopeptide with an apparent phosphorylation change between the two types of cardiac tissue. Phosphopeptide pair—m/z 561.521 and 564.542 represents 280-RT(pS)DSHEDAGTLDFSSLLK-298 with modifications RABA@N-term, RABA(K)@2 and charge state 4+. $\text{Log}_2[\text{H/L}]_{\text{Pair}}$ represent the H/L of a the phosphopeptide pair normalized to the internal standard of casein phosphopeptide H/L ratio as described in the Methods section. Average $\text{Log}_2[\text{H/L}]_{\text{Pair}}$ with standard error (SE) is indicated to signify the magnitude and variability of an identified phosphorylation change.

Figure 3.

One putative signaling pathway for cardioprotection initiated by LMW FGF2 via cMyBP-C phosphorylation. This model is consistent with both the current study and various literature reports [17, 31–34]. The arrows indicate the direction of signaling and an increased level of phosphorylation.

Figure 4.

(**A**) Amino acid residue structure of the phosphopeptide representing cMyBP-C/pSer-282, indicating the most intense fragment ions observed from the optimized LC-MSMS analyses (see Methods section for details). (**B**) MS/MS fragmentation spectrum obtained for the precursor ion at m/z 752.4 Da (3+), representing the reference standard phosphopeptide, (d6-RABA-R)T(pS)DSHEDAGTLDFSSLL(d6-RABA-K)-298. Fragmentation of the precursor ions were performed by application of collision energy, 46.0 (optimal value for best responses) in vendor-defined arbitrary units.

Figure 5.

SRM (selected reaction monitoring) based experimental workflow for the quantification of cardiac Myosin-binding protein C, phosphorylation at Ser-282 in ischemia/reperfusion injury induced hearts of LMW FGF2 "non-expressed" and LMW FGF2 "expressed" mice. **(A)** Experimental Workflow for lysates from three biologically distinct replicates from LMW FGF2 "non-expressed" and LMW FGF2 "expressed" mouse hearts that had been subjected to I/R injury (for details see Methods section). **(B)** Extracted ion profiles and mass spectra corresponding to the [b4-H₃PO₄]⁺ fragment ions — m/z(z+): 484.255 (1+) from each cardiac sample (d0) and 490.255 (1+) for the spiked in Internal Standard (d6), indicating relative abundance of the phosphopeptide, 280-(d0-RABA-R)T(pS)DSHEDAGTLDFSSLLK-298 for the two cardiac types. **(C)** Relative abundance and standard error (SE) values obtained from each extracted ion profile for the two cardiac types. **(D)** Bar-graphs of average relative abundance of the [b4-H₃PO₄]⁺ ion, from the two groups. Statistically significant increase in relative abundance of phosphopeptide, 280-R)T(pS)DSHEDAGTLDFSSLLK-298 was observed for LMW FGF2-expressing mouse hearts compared to the LMW FGF2 non-expressing mouse hearts, n = 3 for each group.