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Phosphoprotein abundance changes in hypertensive cardiac remodeling

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Abstract

There is over-whelming evidence that protein phosphorylations regulate cardiac function and remodeling. A wide variety of protein kinases, e.g., phosphoinositide 3-kinase (PI3K), Akt, GSK-3, TGF β , and PKA, MAPKs, PKC, Erks, and Jaks, as well as phosphatases, e.g., phosphatase I (PP1) and calcineurin, control cardiomyocyte growth and contractility. In the present work, we used global phosphoprotein profiling to identify phosphorylated proteins associated with pressure overload (PO) cardiac hypertrophy and heart failure. Phosphoproteins from hypertrophic and systolic failing hearts from male hypertensive Dahl salt-sensitive rats, trans-aortic banded (TAC), and spontaneously hypertensive heart failure (SHHF) rats were analyzed. Profiling was performed by 2-dimensional difference in gel electrophoresis (2D-DIGE) on phospho-enriched proteins. A total of 25 common phosphoproteins with differences in abundance in (1) the 3 hypertrophic and/or (2) the 2 systolic failure heart models were identified (CI>99%) by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) and Mascot analysis. Among these were (1) myofilament proteins, including alpha-tropomyosin and myosin regulatory light chain 2, cap Z interacting protein (cap ZIP), and tubulin β 5; (2) mitochondrial proteins, including pyruvate dehydrogenase α , branch chain ketoacid dehydrogenase E1, and mitochondrial creatine kinase; (3) phosphatases, including protein phosphatase 2A and protein phosphatase 1 regulatory subunit; and (4) other proteins including proteasome subunits α type 3 and β type 7, and eukaryotic translation initiation factor 1A (eIF1A). The results include previously described and novel phosphoproteins in cardiac hypertrophy and systolic failure.

Keywords

Hypertrophy; Heart failure; Pressure overload; Phospho-profiling; 2D-DIGE; Dahl rats

1. Introduction

Hypertensive heart disease occurs in progressive stages of cardiac remodeling in which concentric hypertrophy precedes the development of systolic failure. Current evidence is

overwhelming that protein phosphorylations play a key role in the cardiac remodeling process.

First, a number of serine–threonine kinases and kinase signaling pathways have been shown to be capable of regulating features of cardiac remodeling. Among these are phosphoinositide 3-kinase (PI3K) (review [1]); Akt, which both regulates and promotes myocyte growth and is antiapoptotic (review [2]); GSK-3, which integrates signals of multiple hypertrophic pathways [3–6]; transforming growth factor- β (TGF- β) receptors, which are major regulators of cardiac fibrosis during the development of cardiac hypertrophy [7,8]; Ca²⁺-calmodulin-dependent protein kinase (CaMKII), which contributes to severe contractile dysfunction, cardiomyocyte apoptosis, and hypertrophic gene expression in heart failure closely correlated with left ventricular ejection fraction in human heart failure (review [9–12]); cAMP-dependent protein kinase (PKA), which increases troponin-I phosphorylation, reduces apoptosis in failing hearts in mice and increases ventricular compliance [13–15]; mitogen-activated protein kinases (MAPKs), including big MAPK (BMK1), extracellular signal regulated kinase (ERK), p38MAPK, c-jun NH₂-terminal kinase (JNK), which regulate myocyte hypertrophy, collagen deposition, and cell apoptosis (review [16]); protein kinase C (PKC), which phosphorylates myofilament proteins, including cTroponinI (cTNI) and cTroponinT (cTNT), and mitochondrial proteins in heart failure, and activates mTOR and S6K1 in cardiac hypertrophy [17]; 70-kDa S6 kinase (p70S6K), which is implicated in the pathogenesis of cardiac hypertrophy caused by long-term inhibition of nitric oxide synthesis and post-infarct remodeling [18,19]; extracellular signal-regulated kinases (Erks) [20,21]; Jak2 [22]; and Pim-1 [23].

Second, protein phosphatases have been linked to heart failure. Protein phosphatase 1 (PP1) activity has been linked to dephosphorylation of cardiac regulatory proteins, including phospholamban, and depressed SR Ca²⁺ pump activity [24–26] [24,27]. The phosphatase calcineurin triggers NFAT and MEF2 transcription factors to regulate MEF2 activity related to cardiac dilation [28,29]. Nuclear factor of activated T-cells (NFAT) is a downstream transcriptional effector for calcineurin [30]. Reduced muscle Lim protein (MLP)-calcineurin signaling predisposes to adverse remodeling after MI [31].

Third, a number of phosphoproteins identified that may be proximal mediators of cardiac remodeling are increasing. Sarcoplasmic reticulum (SR) Ca²⁺ ATPase (SERCA2a) activity is regulated by phosphorylation of Phospholamban (PLN) [32]. Phosphorylation of PLN by either cAMP or cGMP-dependent protein kinase at Ser16 or the Ca²⁺-calmodulin-dependent protein kinase (CaMKII), at Thr17, increases sarcoplasmic reticulum (SR) Ca²⁺ uptake and SR Ca²⁺ load [33]. Reduced phosphorylation of PLN has been linked to depressed cardiac function [34] [35,36]. PLN phosphorylation has also been associated with arrhythmogenicity in heart failure [37]. Hypophosphorylation of Connexin 43 (Cx43), possibly due to enhanced co-localized protein phosphatase type 2A occurs in failing hearts and has been postulated to contribute to gap-junction dysfunction and arrhythmias in heart failure [38,39]. Decreased phosphorylated endothelial nitric oxide synthase (eNOS) has been linked to reduced endothelium dependent relaxation in failing dog hearts [40]. Phosphorylation of class II histone deacetylases (HDACs) has been linked to a reprogramming of cardiac gene expression that accompanies hypertrophy induced by MEF2 by regulating MEF2-HDAC interaction [41]. PKD, a downstream effector of PKC, phosphorylates HDAC5, a transcriptional repressor of cardiac remodeling, to promote hypertrophy [42]. The state of cAMP response element binding protein (CREB) phosphorylation has been linked to both remodeling associated with cardiac hypertrophy and dilation [43] [44–47]. Hyperphosphorylation of the ryanidine receptor (RyR) by PKA and CaMK II has been linked to instability of the RyR channels, leading to improper gating of Ca²⁺ release and leak from the sarcoplasmic reticulum [48]. Activation of extracellular

signal-regulated kinase (ERK) and phosphorylation of EGFR have been linked to cardiac hypertrophy. Amelioration of cardiac remodeling in pressure overload by atorvastatin has been linked to inhibition of ERK and phosphorylation of epidermal growth factor receptor (EGFR) [49]. Others include O transcription factor 3a (FOXO3a) [50,51], P66Shc1 [52], small heat shock protein 27 (HSP27) [53], and eukaryotic initiation factor 4E (eIF4E) [54].

Myofilament proteins are regulated by phosphorylations: An increase in the phosphorylation of myofilaments contributing to myofilament dysfunction in experimental congestive heart failure in rats with aortic banding has been reported; however, the identity of the proteins has not been determined [55]. Phosphorylation of myosin light chain is reduced in end-stage heart failure in humans and may be a compensatory response to increase myocardial contractility [56]. Reduced troponin I (TnI) phosphorylation has been found in end-stage human heart failure [57] and linked to altered myofibrillar function and energy consumption in mice [58]. Cardiac myosin binding protein C (cMyBP-C) phosphorylation occurs in myocardial stunning [59]. Increased troponin I (TnI) and myosin light chain 2 (MLC2) phosphorylation contributes to the frequency-dependent myofilament Ca^{2+} desensitization and negative force-frequency relationship in heart failure [60,61]. Chronic activation of p38alpha MAPK directly depresses sarcomeric function in association with decreased phosphorylation of alpha-tropomyosin [62].

The development of proteomics technology has made it possible to simultaneously analyze the abundance of several hundred phosphoproteins in a tissue or cell without a priori knowledge of function or distribution. Advances in analytic approaches use changes in phosphoprotein expression patterns to discover new phosphorylation signaling pathways and networks. “Phosphorylation profiling” is leading to key insights into the mechanisms of hematopoiesis, oocyte activation, and leukemia. The present studies apply these technologies to identify major patterns of phosphoproteins in the heart occurring in hypertrophy and dilatation/failure.

2. Materials and methods

2.1. Rat models

Male 250–300 g Dahl salt-sensitive (S), Dahl salt-resistant (R) and spontaneously hypertensive heart failure (SHHF) rats were purchased from Harlan Sprague Dawley, and Wistar rats from Charles River. All animal studies were done in accordance with the Institutional Animal Care and Use Committee (IACUC). Dahl R, Dahl S, and SHHF rats were maintained on a 8% NaCl diet ad lib.

2.1.1. Aortic banding (TAC)—Wistar rats were initially anesthetized with etomidate (10 mg/kg BW; i.p.) and intubated with an 18 gauge angiocath sleeve. Surgical anesthesia was maintained using 1.5% isoflurane delivered through a vaporizer with 100% oxygen (compressed gas) connected in series to a rodent ventilator with the stroke volume set at 0.2–0.3 ml/min (based on body weight) and a respiratory rate of 135 per minute. A thoracotomy was performed in mice by a left lateral incision and transection of the first rib and retraction of the thymus. The transverse aorta was isolated and extruded PE-10 tubing tied around the aorta between the right and left carotid artery bifurcations with 6–0 silk suture. The tubing was removed leaving the suture in place and creating a 40% coarctation and an effective pressure gradient across the stenosis. Following the stenosis the thoracotomy was closed in three layers with 6–0 silk sutures and the chest cavity evacuated. Animals will received buprenorphine (0.2 mg/kg; injected into the thoracotomy site (50 μl) at the time of surgery. Six to 8 weeks after banding, LVH develops with increased systolic function, and by 18–20 weeks, LV cavity dilation occurs with reduced systolic function.

Control rats undergo sham surgery. Wistar rats are obtained from Charles River Breeding Laboratories.

2.2. Echocardiography

Rats were lightly anesthetized with isoflurane and placed in a supine or left lateral position with ECG electrodes applied at the paws. A 15-MHz linear transducer and the Acuson Sequoia System (C256) were used to obtain 2DE images. Measurements were made according to the American Society for Echocardiography leading-edge method. 2DE short-axis images at the mid papillary muscle level were obtained for measurement of LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD), and posterior wall thickness (LVPWT). Fractional shortening (FS) $((LVEDD - LVESD)/LVEDD)$ and ejection fraction (EF) $((LVEDD)^3 - (LVESD)^3)/(LVEDD)^3$ were calculated.

Hypertrophy criteria (endpoints) were (1) a significant increase in wall thickness versus age-matched controls; (2) no change in wall thickness over a 2 week interval; and (3) no decrease in FS from previous measurements in the same rat. Systolic heart failure criteria were either (1) FS<0.20 or (2) signs of distress, e.g., tachypnea, loss of appetite, and loss of weight.

2.3. Protein preparation of non-myofilament and myofilament enriched fractions

Cardiac tissue (30 mg) (left ventricular free wall+septum) from each rat was homogenized with a Dounce tissue grinder in 1 ml of Standard Relaxant Buffer (SRB) (75 mM KCl, 10 mM imidazole pH 7.2, 2 mM MgCl₂, 2 mM EDTA, and 1 mM NaN₃) containing 1% v/v protease and phosphatase inhibitors for 30 strokes. The homogenate was spun at 4 °C in an ultracentrifuge for 30 min at 60,000 rpm. The supernatant was harvested and stored as the cytosolic fraction. The pellet was re-suspended in 1 ml of SRB containing 1% v/v Triton X-100 and homogenized for 30 strokes. The homogenate was spun again at 4 °C for 30 min at 60,000 rpm. The supernatant was harvested and stored as the membranous fraction and the myofilament pellet was analyzed separately. The cytosolic and membranous fractions from each rat were incorporated into the Phosphoprotein Purification Kit (QIAGEN). Myofilament fraction was confirmed with Ab to MLC. Phosphoprotein enrichment was performed using Phosphoprotein purification kit supplied by Qiagen Inc according to the manufacturer's instructions. Non-myofilament fractions were analyzed.

2.4. Phosphoprotein profiling and analysis

2.4.1. 2D-DIGE with Cydye staining—2D-DIGE with Cydye staining was performed for in gel comparison of phosphoproteins from hypertrophic and systolic failing hearts to corresponding control hearts. Phosphoenriched proteins were dissolved in 2D lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl, pH 8.8). Protein concentration was determined using the Bio-Rad protein assay method and samples were diluted with 2D lysis buffer to the same protein concentration between 5 and 8 mg/ml. 1.0 µl of diluted Cydye (1:5 diluted with DMF from 1 nmol/µl stock) was added to 30 µg of protein extract. Cy2 was used for control samples and Cy5 was used for hypertrophy and heart failure samples. The mixtures were vortexed, and then incubated under dark conditions on ice for 30 min. One microliter of 10 mM lysine was added to each of the samples to quench the reaction, followed by vortexing and incubation under dark conditions on ice for an additional 15 min. Cy2 and Cy5 labeled samples were mixed together and the appropriate volume of 2x 2D Sample Buffer (8 M urea, 4% CHAPS, 20 mg/ml DTT, 2% pharmalytes, and trace amounts of bromophenol blue) and 100 µl of destreak solution (GE Healthcare) were added to the samples. Rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes, and trace amounts of bromophenol blue) was added to reach a total volume of 250 µl. The samples were mixed, spun, and equal amounts of protein were loaded onto 13

cm IPG strips (pH 3–10 linear) under 1 ml mineral oil. Isoelectric focusing was performed for 12 h at 20 °C with 50 μ A/strip. The focused IPG strips were loaded into the 12% SDS-gels and ran at 15 °C until the dye front runs out of the gels. Image scans were carried out immediately following the SDS-PAGE using Typhoon TRIO (Amersham BioSciences) following the protocols provided. The scanned images were analyzed by Image QuantTL software (GE-Healthcare), and then subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.5 (GE-Healthcare).

A ratio change of the protein differential expression was obtained from in-gel DeCyder software analysis as $R=(\text{treated/control})$ for up-regulated proteins, and calculated as $R=-(\text{control/treated})$ for down-regulated proteins, where a 2-fold up-regulation or down-regulation was represented by 2 and -2 , respectively. Statistical significance was calculated using the DeCyder software and corrected for multiple hypothesis testing using the FDR option. Our preliminary data validates this approach by detecting both previously published phosphoproteins in cardiac remodeling and novel ones.

2.4.2. MALDI-TOF/TOF mass spectrometry—Protein spots chosen for analysis were excised by Ettan Spot Picker (GE Healthcare) and washed multiple times to remove staining dye and other inhibitory chemicals. Gel spots were dried and then rehydrated in digestion buffer containing sequencing grade modified trypsin. Proteins were digested in-gel at 37 °C and digested peptides were extracted from the gel with TFA extraction buffer and shaking. The digested tryptic peptides were desalted using C-18 Zip-tips (Millipore) and then mixed with CHCA matrix (alpha-cyano-4-hydroxycinnamic acid) and spotted into the wells of a MALDI plate. Mass spectra (MS) of the peptides in each sample were obtained using an ABSciex 4700 Proteomics Analyzer and 10–20 of the most abundant peptides in each sample were further subjected to fragmentation and tandem mass spectrometry (MS/MS) analysis. The combined MS and MS/MS spectra were submitted for database search using GPS Explorer software equipped with the MASCOT search engine identifies proteins from the NCBI non-redundant protein database. Proteins that were identified with a confidence interval (C.I.) greater than 99% are reported.

2.5. Immunoprecipitation (IP) of MAPKAPK2

Cell lysates from control and failing hearts were incubated overnight at 4 °C with monoclonal anti-phospho-serine threonine antibody (BD Biosciences) with constant shaking. Fifty microliters of protein A/G agarose beads were added to each reaction and incubated with constant shaking at 4 °C for 2 h. The samples were spun down for 1 min and the pelleted beads were washed sequentially with PBS with 1% triton-X, PBS with 0.5% triton-X, and PBS with 0.1% triton-X. The pellets were re-suspended in SDS sample buffer and subjected to western blotting with polyclonal anti-MAPKAPK2 antibody (Abcam Inc).

3. Results and discussion

3.1. Cardiac phenotyping by echocardiography (Tables 1 and 2)

Rats met hypertrophy and failure at time points consistent with reports in literatures. Dahl rats developed hypertension-induced compensatory cardiac hypertrophy by week 11 and systolic failure by week 20 while Lean male SHHF animals developed hypertension and LVH by 3–5 months of age and reduced systolic function by 16–20 months of age, which is consistent with literature [63,64]. The TAC rats developed left ventricular hypertrophy at 18–20 weeks.

3.2. 2D-DIGE analysis of phosphoproteins from hypertrophied hearts (Figs. 1–3) and systolic failing hearts (Figs. 4 and 5)

Phosphoprotein profiling using 2D-DIGE was performed on enriched phosphoproteins extracted from 1 representative heart from each hypertrophy model, i.e., Dahl salt-sensitive (S), spontaneously hypertensive heart failure prone (SHHF), and TAC rats, and the corresponding age-matched controls, i.e., Dahl salt-resistant (R) and Wistar strains respectively (Figs. 1–3) and each systolic failing model, i.e., Dahl salt-sensitive (S) and spontaneously hypertensive heart failure prone (SHHF) and the corresponding age-matched controls, i.e., Dahl salt-resistant (R) and Wistar strains respectively.

3.2.1. 2D-DIGE analysis of phosphoproteins from hypertrophied hearts

(Tables 3 and 5)—Approximately 105 phosphoproteins were resolved on the gels. In cardiac hypertrophy (Table 3), 28 phosphoprotein spots showed greater abundance and 27 lesser abundance in the Dahl S versus Dahl R rat models; 33 phosphoprotein spots showed greater abundance and 39 lesser abundance in the SHHF model; and 36 phosphoprotein spots showed greater abundance and 33 lesser abundance in the TAC model. Of these, 8 and 7 were common among the ones with greater and lesser abundance, respectively. These phosphoproteins were identified by MALDI-MS. Included among the group of phosphoproteins with greater abundance in the 3 models of hypertrophied hearts studied were enolase 3, tropomyosin alpha-4 chain, troponin C, elongation factor 1-delta, proteasome subunit alpha type-3, proteasome subunit beta type 7, and aconitate hydratase, mito precursor. Proteasome subunits α type-3 and β type-7 regulate ubiquitination. Ubiquitin proteasome dysfunction, which has been identified in failing hearts, has been linked to the progression from hypertrophy to failure [65]. It is speculated that post-translational modifications to the proteasomes account for the dysfunction. PkA has been shown to enhance proteasome assembly and activity in hearts [66]. Aconitate hydratase abundance has been decreased in patients with dilated cardiomyopathy [67] and activity in pacing induced heart failure [68]. In diabetic hearts, phosphorylation of aconitate hydratase by PKC has been shown to regulate its activity [69].

The group of phosphoproteins with less abundance in the 3 models of hypertrophied hearts studied consisted of serum albumin precursor, branched chain ketoacid dehydrogenase E1, cardiac myosin regulatory light chain 2, and protein phosphatase 1 (PP1) regulatory subunit 7 (Sds22). Several can be linked to cardiac remodeling. The main serine/threonine phosphatase activity in the heart is due to PP1 and PP2A. Increases and decreases in PP1 have been linked to depressed and enhanced cardiac contractility, respectively [70]. Cardiac-specific over-expression of the catalytic subunit of PP1 in transgenic mice results in hypertrophy, depresses contractility, and increases propensity to heart failure and likelihood of premature death. Inhibition of PP1 in the transgenic mice by crossing them with transgenic mice which overexpress a functional COOH-terminally truncated form of PP1 inhibitor-2 (I-2) reduces cardiac hypertrophy and normalized both contractility and relaxation [71]. Decreased PP inhibitor-1 has previously reported to be reduced in human heart failure [72]. Sds22 is a regulator of PPA1 and functional studies suggest that it is involved in the most ancient functions of PP1 protein and all mammalian forms bind to PP1 [73,74]. A conserved portion of sds22 folds it into a curved superhelix required for interaction with PP1. Sds22 has been linked to targeting of PP1 to the kinetochore, accumulation of phospho-aurora B, and force generation at the kinetochore-microtubule interface. We have discovered (see Preliminary Data) that it is phosphorylated and the phosphoprotein is less abundant in heart failure. PAS domain kinase, a regulator of glycogen synthesis, has been shown to phosphorylate eIF-1A at Thr432 [75]. eIF-1A gene expression is up-regulated in angiotensin II-induced hypertrophy in mice as well as in end stage cardiomyopathy in patients [76].

3.2.2. 2D-DIGE analysis of phosphoproteins from systolic failing hearts

(Tables 4 and 5)—In systolic heart failure, 14 phosphoprotein spots showed greater abundance and 23 lesser abundance in the Dahl S failing heart, while 16 phosphoprotein spots showed greater abundance and 21 lesser abundance in the SHHF failing heart. Of these, 10 and 15 with greater or less abundance were common to both models of systolic failure studied and were identified by MALDI-MS (Table 4).

Phosphoproteins with greater abundance in failing hearts included alpha tubulin, enolase 3, CapZ interacting protein (CapZIP), troponin I, heat shock protein beta1, myosin light chain 3, and astrocytic phosphoprotein PEA-15. The detection of increased phosphorylation in heart failure of myosin light chain confirms previous studies and validates our results [56,60,61]. Increased abundance of α tubulin has been found in a pressure-overload model of failure as well as in patients with end-stage heart failure due to dilated cardiomyopathy [77]. Human CapZIP has been shown to be phosphorylated at Ser-179 and Ser-244 by MAP kinase-activated protein kinase 2 and at Ser-68, Ser-83, Ser-108 and Ser-216 by other stress-activated kinases. Osmotic shock induced phosphorylation of Ser-179 and triggered the dissociation of CapZIP from CapZ, suggesting that phosphorylation of CapZIP may regulate the ability of CapZ to remodel actin filament assembly [78]. While we found increased troponin I in heart failure, reduced troponin I (TNI) phosphorylation has been reported in end-stage human heart failure [57]. The discrepancy may reside in the sites of phosphorylation since both serine–threonine and tyrosine phosphorylations were detected in our studies.

Among the phosphoproteins less abundant in failing hearts were myosin regulatory light chain, elongation factor 2, serum albumin precursor, MAP kinase-activated protein kinase 2, cAMP-dependent protein kinase I-alpha regulatory subunit, alpha tubulin, pyruvate dehydrogenase E1 alpha subunit, mitochondrial creatine kinase S-type, branched chain ketoacid dehydrogenase E1, and protein phosphatase 2A 65 kDa regulatory subunit (PR65 α). Several of these are of interest. PR65 α is tightly associated with PP2A catalytic subunit, forming a scaffold to which the appropriate B subunit can bind (review [79]). PP2A is a family of highly regulated serine/threonine phosphatases implicated in cell growth, β -adrenergic stimulation of the heart, diastolic dysfunction, and connexin 43 phosphorylation [80]. PP2A dephosphorylates phospholamban and phospholemman. Disruption of PP2A signaling increases excitation–contraction coupling by selectively increasing phosphorylation of the L-type and RyR2 channels [81]. Increased activity of PP2A has been linked to dense microtubule network stabilization by decoration with microtubule-associated protein 4 (MAP4), seen in hypertrophy and failure [82]. Caspase-dependent protein phosphatase 2A activation contributes to endotoxin-induced cardiomyocyte contractile dysfunction [83]. Pyruvate dehydrogenase E1 subunit: An increase in pyruvate dehydrogenase expression has been reported in failing hearts [84]. Pharmacological inhibition of pyruvate dehydrogenase kinase improves cardiac function in ventricular hypertrophy [85]. Phosphorylation of tubulin has been documented; however, phosphorylation does not seem to play a prominent role in the regulation of microtubule dynamics [86]. While we found reduced abundance of the pyruvate dehydrogenase E1 α subunit, an increase in pyruvate dehydrogenase expression has been reported in failing hearts [84]. Thus, the significance of this remains unclear.

Six of the phosphoproteins in systolic failure identified were among those also identified in the hypertrophied hearts. Ones with greater abundance were enolase, troponin I, and tropomyosin alpha. Ones with less abundance were myosin regulatory light chain and branch chain ketoacid dehydrogenase. Based on the present studies, whether phosphorylation sites are different cannot be ruled out.

There were also changes in the abundance of phosphoproteins that were not common between the strains in both cardiac hypertrophy and systolic failure. These were significantly fewer than those in common. We reason that these may be due to (1) simply strain differences; (2) differences in the precise stages of cardiac remodeling studied (even though we attempted to study each model at the same functional/morphological stage, differences in molecular and developmental progression in each stage may have been present, e.g., development of hypertrophy versus compensated hypertrophy versus transition from hypertrophy to systolic failure or progression to end-stage heart failure; and/or (3) simply differences in individual rats.

3.3. MAPKAPK2 abundance by IP in systolic failing hearts

For the confirmation of 2D-DIGE results, IP was performed for MAPKAPK2 on proteins from Dahl R control and Dahl S systolic failing hearts (Fig. 6). Consistent with 2D-DIGE analysis, MAPKAPK2 was less abundant in failing hearts. P38 MAP kinase-mediated phosphorylation and activation of MAPKAPK2 induce cardiac hypertrophy [87]. Decreased phosphorylation of MAPKAPK2 and p38 MAPK activity has been linked to the transition to end-stage human heart failure [88]. Since phosphorylation of CapZIP by MAPKAPK2 triggers its dissociation from CapZ [78], this may be a signaling pathway for myofilament remodeling in heart failure.

4. Conclusion and prospective

In summary, 24 common phosphoprotein spots with greater or less abundance versus control in the models of hypertrophy and systolic failure were identified by MALDI-MS (Table 5). We reasoned that phosphoproteins more or less abundant in (1) the 3 models of hypertrophy and/or (2) both models of failure versus their corresponding controls are good candidates for being important in cardiac remodeling and failure. Thirteen and 22 phosphoproteins meeting this were identified in hypertrophic and failing hearts, respectively (Table 5). These included 10 that had previously been reported in cardiac remodeling and 14 that, based on literature, are both novel and may rationally be linked to cardiac remodeling.

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Fig 1.

2D-DIGE analysis of phosphoproteins from hypertrophic hearts from Dahl S versus age-matched Dahl R rats. Phospho-enriched protein samples from hypertrophy and age-matched control hearts/strains were differentially labeled with Cydyes (failing=Cy5 red, control=Cy2 green) and subjected to 2D-DIGE followed by phosphoprotein profiling (see Materials and methods). Numbered/circled spots are ones common to all 3 models of hypertrophy that were identified (Table 3). Molecular weight markers (kDa) are displayed to the left of each gel and pH markers are displayed underneath each gel.

Fig 2.
2D-DIGE analysis of phosphoproteins from hypertrophic hearts from SHHF versus age-matched Wistar rats. Phospho-enriched protein samples from hypertrophy and age-matched control hearts/strains were differentially labeled with Cydyes (failing=Cy5 red, control=Cy2 green) and subjected to 2D-DIGE followed by phosphoprotein profiling (see Materials and methods). Numbered/circled spots are ones common to all 3 models of hypertrophy that were identified (Table 3). Molecular weight markers (kDa) are displayed to the left of each gel and pH markers are displayed underneath each gel.

Fig 3. 2D-DIGE analysis of phosphoproteins from hypertrophic hearts from Wistar TAC versus from age-matched control rats. Phospho-enriched protein samples from hypertrophy and age-matched control hearts/strains were differentially labeled with Cydyes (failing=Cy5 red, control=Cy2 green) and subjected to 2D-DIGE followed by phosphoprotein profiling (see Materials and methods). Numbered/circled spots are ones common to all 3 models of hypertrophy that were identified (Table 3). Molecular weight markers (kDa) are displayed to the left of each gel and pH markers are displayed underneath each gel.

Dahl S heart failure

Fig 4.

2D-DIGE analysis of phosphoproteins from hearts with systolic failure from Dahl S versus from age-matched Dahl R rats. Phospho-enriched protein samples from failing and age-matched control hearts/strains were differentially labeled with Cydyes (failing=Cy5 red, control=Cy2 green) and subjected to 2D-DIGE followed by phosphoprotein profiling (see Materials and methods). Numbered/circled spots are ones common to both models of systolic failure that were identified (Table 4). Molecular weight markers (kDa) are displayed to the left of each gel and pH markers are displayed underneath each gel.

Fig 5. 2D-DIGE analysis of phosphoproteins from hearts with systolic failure from SHHF versus age-matched Wistar control rats. Phospho-enriched protein samples from failing and age-matched control hearts/strains were differentially labeled with Cydyes (failing=Cy5 red, control=Cy2 green) and subjected to 2D-DIGE followed by phosphoprotein profiling (see Materials and methods). Numbered/circled spots are ones common to both models of systolic failure that were identified (Table 4). Molecular weight markers (kDa) are displayed to the left of each gel and pH markers are displayed underneath each gel.

Fig 6. MAPKAPK2 abundance by IP in control Dahl R (C) and Dahl S systolic failure (F) hearts. Protein samples were isolated from Dahl S failing (F) and Dahl R control (C) hearts (4 each) and immunoprecipitated with monoclonal anti-phospho-serine/threonine Ab and immunoblotted with polyclonal rabbit Ab to MAPKAPK2 (see Materials and methods).

Cardiac parameters for pressure-overload hypertrophic hearts. The age and mean left ventricular posterior wall thickness for each model are listed.

Table 1

Phenotype	n	Age (weeks)	Mean BW (g)	LV Wt. (g)	LV EDD (mm)	LV ESD (mm)	LV PWT (mm)	FS (%)	EF (%)
Hypertrophied hearts									
Dahl S	4	18	377±9	1.6±0.24*	7.7±0.2	5.5±0.3	2.7±0.2*	28 ±0.01	64.0±1.9
Dahl R Ctl.	4	18	415±1	0.85±0.05	7.6±0.5	5.1±0.4	1.8±0.1	32±3.03	67.5±2.1
SHHF	4	17	440±19	1.18±0.04*	7.6±0.7	5.4±0.4	2.5 ±0.2*	30±.02	64.7±3.5
Wistar Ctl	4	17	491±64	0.88±0.06	8.0±0.4	5.6±0.2	1.6±0.1	31±.02	67.3±0.9
TAC	3	17	516±36	1.44±0.2*	8.7±0.4	6.0±0.3	2.05±0.1*	30±.02	65.7±3.5
Non-banded Ctl.	3	17	480±10	0.81±0.13	8.0±0.6	5.5±0.4	1.50±0.02	32±0.10	68.4±1.0

* $P<0.05$ hypertrophy vs. age-matched control hearts.

Spontaneously hypertensive heart failure prone (SHHF), Dahl salt-sensitive (S), thoracic aortic banded rats (TAC), Dahl salt-resistant (R), left ventricular weight (LVWt), left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), left ventricular posterior wall thickness (LV PWT), fractional shortening (FS), and ejection fraction (EF).

Table 2

Cardiac parameters for failing hearts. The age and mean left ventricular ejection fraction for each model is listed.

Phenotype	n	Age (weeks)	Mean BW (g)	LV Wt. (g)	LVEDD (mm)	LVESD (mm)	LV PWT (mm)	FS %	EF (%)
Systolic heart failure									
Dahl S	4	29	362±57*	1.41±0.09	11.1±0.9*	9.6±0.7*	1.6±0.1	13.4±1.2*	34.9±1.3*
Dahl R	4	29	473±45	0.92±0.03	8.9±0.17	6.9±0.5	1.7±0.2	26.0±1.8	60.2±0.9
SHHF	4	72	432±39*	1.46±0.10	10.9±0.1*	9.1±0.8*	1.8±0.1	17.1±3.4*	42.7±7.2*
Wistar	4	72	902±81	1.17±0.38	9.6±0.18	6.9±0.8	1.8±0.1	30.6±3.8	66.4±5.7

* $P<0.05$ failing vs. age-matched control hearts.

Spontaneously hypertensive heart failure prone (SHHF), Dahl salt-sensitive (S), Dahl salt-resistant (R), left ventricular weight (LVWt), left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), left ventricular posterior wall thickness (LV PWT), fractional shortening (FS), and ejection fraction (EF).

Table 3

Phosphoprotein spots which demonstrated either greater or less abundance in hypertrophic hearts identified by MALDI-TOF/TOF mass spectrometry (C.I. > 99%). The fold-change in abundance in hypertrophy compared to control is listed (negative numbers indicate that the phosphoprotein was less abundant in hypertrophy).

Spot number	TAC hypertrophy	Dahl S hypertrophy	SHHF hypertrophy	Accession no.	Protein ID
2	-1.08	-2.86	-1.25	gi 158138568	Serum albumin precursor
6	1.05	1.3	1.3	gi 109468300	Enolase 3
8	-1.52	-1.44	-1.2	gi 149056579	Branched chain ketoacid dehydrog. E1
9	-1.46	1	-1.02	gi 38328248	Tubulin, alpha 1A
16	1.09	1.24	1.41	gi 6981672	Tropomyosin alpha-4 chain
18	1.19	3.5	1.73	gi 207516	Troponin I
19	1.17	1.91	1.91	gi 207516	Troponin I
21	-1.16	-1.26	-2.25	gi 127167	Myosin reg. light chain 2, cardiac
22	-2.82	-1.66	-2.67	gi 127167	Myosin reg. light chain 2, cardiac
27	-1.1	-1.7	-2.2	gi 57634526	Protein phosphatase 1 reg. subunit 7
29	1	1.65	1.32	gi 257096637	Elongation factor 1-delta
31	1.08	1.47	1.53	gi 8394066	Proteasome subunit alpha type-3
32	1.08	1.31	1.37	gi 16758298	Proteasome subunit β type-7
33	-1.48	-1.3	-1.33	gi 65301494	Euk. translation initiation factor 1A
34	1.16	1.53	1.1	gi 40538860	Aconitate hydratase, mito. precursor

Table 4

Phosphoprotein spots which demonstrated either increased or decreased abundance in failing hearts identified using MALDI-TOF/TOF mass spectrometry (C.I. > 99%). The fold change in abundance in failure compared to control is listed (negative numbers indicate that the phosphoprotein was less abundant in failure).

Spot number	Dahl S failure	SHHF failure	Accession no.	Protein ID
1	-1.66	-2.54	gi 8393296	Elongation factor 2
2	-1.45	-1.92	gi 158138568	Serum albumin precursor
3	1.76	1.33	gi 223556	Tubulin, alpha
4	-1.25	-1.24	gi 30017431	MAP kinase-activated protein kinase 2
5	-1.29	-1.09	gi 6981396	cAMP-dependent protein kinase I-alpha reg. subunit
6	1.55	2.29	gi 109468300	Enolase 3
7	-2.56	-1.61	gi 149056579	Branched chain ketoacid dehydrogenase E1, alpha
8	-1.84	-1.53	gi 149056579	Branched chain ketoacid dehydrogenase E1, alpha
9	-1.84	-2.01	gi 38328248	Tubulin, alpha 1A
10	-1.7	-1.46	gi 57657	Pyruvate dehydrogenase E1 alpha form subunit 1
11	-1.4	-1.64	gi 109503594	Pyruvate dehydrogenase E1 subunit alpha, mito.
12	-1.37	-1.64	gi 124430510	Pyruvate dehydrogenase E1 subunit alpha, mito.
13	-2.85	-3.16	gi 125313	Creatine kinase S-type, mitochondria
14	2.74	1.82	gi 157820831	capZ-interacting protein
15	3.83	1.13	gi 62653546	Glyceraldehyde-3-phosphate dehydrogenase-like
16	1.53	1.51	gi 6981672	Tropomyosin alpha-4 chain
17	-1.64	-1.38	gi 6015047	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mito.
18	1.43	1.27	gi 207516	Troponin I
19	1.54	1.46	gi 207516	Troponin I
20	2.21	1.18	gi 1170367	Heat shock protein β -1
21	-2.77	-1.89	gi 127167	Myosin reg. light chain 2, ventricular/cardiac muscle
22	-2.72	-2.24	gi 127167	Myosin reg. light chain 2, ventricular/cardiac muscle
23	1.37	1.57	gi 6981240	Myosin light chain 3
24	2.34	3.91	gi 4505705	Astrocytic phosphoprotein PEA-15
25	-1.35	-1.22	gi 8394027	Protein phosphatase 2A (65 kDa reg. sub.)

Table 5

Summary of phosphoproteins with either increased or decreased abundance in cardiac hypertrophy common to the models of cardiac hypertrophy (Dahl S, SHHF, and TAC), and failure (Dahl S and SHHF).

Hypertrophic hearts	Systolic failing hearts
<i>Cell growth</i>	
Protein phosphatase 1 reg subunit 7↓	Protein phosphatase 2A 65 kD regulatory subunit↓ Eukaryotic translation initiation factor 1A↓ PKA α reg subunit↓ Elongation factor 2↓ Map kinase activated protein kinase-2↓
<i>Excitation/contraction coupling</i>	
Myosin Reg light chain↓*	Troponin 1↑*
Troponin 1↑*	Myosin Reg light chain↓*
Tropomyosin α ↑*	Tropomyosin α ↑* CapZ-interacting protein↑ Tubulin alpha↑
<i>Metabolism</i>	
Branch chain ketoacid dehydrogenase↓*	Glyceraldehyde 3-phosphate dehydrogenase↑
Enolase↑*	Brach chain ketoacid dehydrogenase↓*
Aconitase hydratase↑	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase↓ Creatine kinase S-type↓ Enolase↑*
<i>Miscellaneous</i>	
Serum albumin precursor↓*	Serum albumin precursor↓* Proteasome subunits↑ Heat shock protein β-1↑ Astrocytic phosphoprotein PEA-15↑

↑ =more abundant, ↓ =less abundant,

* present in both hypertrophy and systolic failure.