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Serendipitous discovery of a novel protein signaling mechanism in heart failure

Andrea M. Vitello, Yanmei Du, Peter M. Buttrick, Lori A. Walker *

University of Colorado, Department of Medicine, Division of Cardiology, Aurora, CO, USA

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ABSTRACT

A number of protein signaling mechanisms are known to be involved in the progression of heart failure, yet the mechanism(s) by which the heart fails remains poorly understood. Therefore, we undertook a global approach to this question and used an antibody microarray to identify proteins differentially expressed in dysfunctional right ventricles in a bovine model of heart failure and the results were validated using cardiac tissue from both bovine and human heart failure. We found that protein disulfide isomerase 3, PDIA3, a protein that resides in the lumen of the endoplasmic reticulum, is significantly upregulated in both animal and human models of right and left heart failure. Altered expression of this protein has not previously been described in models of heart failure. In our initial microarray analysis, we found that CSK (c-Src kinase) was among the proteins upregulated in failing bovine ventricle. To further elucidate the role of CSK in heart failure, we studied the expression of its downstream target, Src, and found that Src expression and phosphorylation were markedly upregulated in failing ventricles. However, we also noted a smaller immunologically reactive protein that was only seen in experimental animals. In order to positively identify the smaller, Src-reactive protein, we used 2-dimensional gel electrophoresis and mass spectrophotometry. Surprisingly, we identified this protein as PDIA3, a protein that did not belong to the Src family of proteins. Upon sequence examination we found that PDIA3 contains a short C-terminal sequence with strong homology to Src and that it was this short sequence to which the antibody was generated. PDIA3 participates in MHC class I presentation and is implicated in the progression of valvular dysfunction in rheumatic heart disease, as well as calcium modulation in the sarcoplasmic reticulum. The molecule resides in the lumen of the endoplasmic reticulum and participates in disulfide bond formation during protein folding by interacting with calnexin and calreticulin. This interaction may indirectly effect SERCA (sarco/endoplasmic reticulum Ca^{2+}-transport ATPase) activity and by extension contribute to the calcium dysregulation that characterizes progressive heart failure. Further studies are needed to elucidate the role that PDIA3 may play in the progression of heart failure.

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1. Introduction

A number of cellular mechanisms and signaling pathways are involved in the pathogenesis of heart failure including those implicated in fibrosis, remodeling, and apoptosis. Studies suggest that genes involved in these mechanisms are expressed in an effort to compensate for the injury or stress to the heart following myocardial infarction, viral infection, or pressure overload and to preserve its hemodynamics, however these compensatory effects can ultimately lead to ventricular dysfunction.

Heart failure is characterized, in part, by dysfunctional calcium regulation. At various stages in disease progression calcium entry into the cell can be either increased or decreased, reflecting altered expression, regulation and compartmentalization of the calcium regulating elements in the cell and SR membranes. This dysregulation in turn influences both myocyte contractility and a variety of calcium dependent signaling processes that have been linked both to cellular hypertrophy and to apoptosis [1,2]. Consequently, explicating the process that regulates calcium influx and egress from the cell has been central to our understanding of progressive heart failure.

In addition, progressive heart failure is driven, in part, by apoptotic cell loss. Although cellular necrosis occurs at the site of ischemic injury, it is thought that apoptosis ultimately is the more prominent form of cellular death in the subsequent progression to heart failure, specifically contributing to adverse remodeling and increased risk for symptomatic heart failure [3], and ultimately, mortality. By targeting the mechanisms involved in

Abbreviations: PDI, protein disulfide isomerase; PDIA3, ERp57, ERp60, ERp61, PDI-Q2, Glucose Regulated Protein 58 (Grp58) Hormone-Induced Protein-70 (HIP-70) 1,25D3-MARRS; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; ER, endoplasmic reticulum; UPR, unfolded protein response; CSK, c-Src kinase; TUNEL, terminal dUTP nick end labeling-positive; MHC, major histocompatibility complex.

* Corresponding author. Address: University of Colorado, Department of Medicine, Division of Cardiology, B8600 Aurora, CO 80045, USA. Fax: +1 303 724 5451.
E-mail address: lori.walker@ucdenver.edu (L.A. Walker).

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apoptosis, it may be possible to ameliorate or prevent the progression to heart failure.

The protein disulfide isomerases (PDI) are a family of the proteins involved in the inhibition of apoptosis, and catalyze oxidation, reduction, and isomerization of disulfide bonds [4–7]. There are twenty members in the PDI family in the human endoplasmic reticulum. PDI is a member of the unfolded protein response system (UPR), and participates in oxidation and isomerization of nascent peptides, and regulates receptor function, cell–cell interactions, gene expression, and actin filament polymerization [4]. As such, this class of molecules is potentially intimately involved in pathways that have been implicated in progressive heart failure.

Here we report that PDIA3 is upregulated in heart failure and discuss potential consequences of this protein's expression. In the endoplasmic reticulum (ER) PDIA3 interacts with membrane bound calnexin and calreticulin, but its role in the pathogenesis of heart failure is unknown.

2. Methods

2.1. Tissue extraction

Tissue was obtained from human control and failing (ischemic and non-ischemic dilated cardiomyopathy) left ventricles (LV) and from neonatal bovine control and failing (chronic pressure overload) right ventricles (RV). Tissues were homogenized in 8 M urea, 2.5 M thiourea, 2 mM EDTA, 4% CHAPS, 2 mM TBP, protease inhibitors and DTT for Western blot analysis and in Protein Extraction buffer (Clontech) for antibody arrays.

2.2. Antibody microarrays

Tissue samples (100 μg) were labeled with either cyanine 3 or cyanine 5 fluorophores (Amersham) and mixed to provide the following comparisons: control RV vs. control LV, control RV vs. failing RV, control LV vs. failing LV, failing RV vs. failing LV. Each protein mixture was incubated with a slide-based antibody microarray (Clontech). Slides were washed and microarrays were imaged using a Perkin Elmer microarray scanner. Cyanine 5 and cyanine 3 fluorescence was determined for each spot and cy5/cy3 ratios were calculated. Changes in the cyanine 5 to cyanine 3 ratios were analyzed for 505 different proteins.

2.3. Western blot

Fifty micrograms of protein was loaded for 1-dimensional SDS–PAGE and Western blot analysis. Proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA and incubated with the following primary antibodies: rabbit monoclonal antibody Src (36D10) (Cell Signaling #2109), rabbit antibody phospho-Src (Tyr527) (Cell Signaling #2105), rabbit antibody non-phospho-Src (Tyr527) (Cell Signaling #2107), and rabbit/ mouse antibody PDIA3 (ERp57) (USBiological). After washing the membranes, they were incubated with anti-rabbit IgG-HRP secondary antibody (Sigma-Aldrich A9169) and visualized using enhanced chemiluminescence.

2.4. 2-Dimensional gel electrophoresis and in-gel extraction and digestion

Bovine RV homogenates (50 μg) were first separated using nonlinear isoelectric focusing (pH 3–10) and subsequently separated by molecular weight using 10% SDS–PAGE. Gels were run in parallel and one gel was stained with Coomassie brilliant blue and the others were Western blotted and Src-reactive spots were identified. The Src-reactive spots were cut out of the Coomassie stained gels and washed with 25 mM NH_4HCO_3/50% ACN. Samples were dried and digested with proteomic grade trypsin overnight at 4 °C. Peptides were isolated and sent to the University of Colorado Cancer Center Mass Spectrometry core facility for analysis.

2.5. Chromatography and electrospray ionization mass spectrometry analysis

Samples were analyzed by microcapillary HPLC tandem mass spectrometry (μLC–MS/MS) using an LTQ XL mass spectrometer (Thermo, San Jose, CA). Samples (2 μL) were injected onto a reverse-phase column via a cooled (8 °C) autosampler (Eksigent, Dublin, CA) connected to an HPLC system (Agilent 1100, Agilent Technologies, Santa Clara, CA) that was set at 70μL/min before the split and ~350 nL/min after the split. HLPC buffers used were Buffer A: 94.9% water, 5% acetonitrile, and 0.1% formic acid and Buffer B: 94.9% acetonitrile, 5% water, and 0.1% formic acid. A 60-min HPLC gradient was used to separate peptides. The gradient changed from 5% to 30% acetonitrile over 40 min followed by organic and aqueous washes on a house-packed 10 cm microcapillary HPLC column with a pulled 5 μm nanospray tip for nanoelectrospray ionization. The column was packed in-house with reverse-phase stationary phase Synergi 4u, 100 A C_{18} (Phenomenex, Torrance, CA). The column was heated to 40 °C using a column heater that was constructed in-house.

Mass spectrometry data acquisition was performed in date-dependent mode on the Xcalibur instrument software (v. 2.0.6, Thermo, San Jose, CA) with a single MS1 scan (30 ms) followed by up to three data dependent collision induced dissociation scans (MS/MS, 30 ms each). Data were converted from the Thermo *.raw data file format to the *.mgf format using an in-house script. After conversion, data were searched against the bovine IPI database (v. 3.64) using Mascot® (v. 2.2.07, Matrix Science Ltd., Boston, MA). For searches, mass tolerances were set at ±0.60 Da for both MS peaks and MS/MS fragment icons. Trypsin enzyme specificity was applied allowing one missed cleavage in the database searches. Modifications searched included fixed carbamidomethyl modification of cysteine and the variable modifications oxidation of methionine, protein N-terminal acetylation, and peptide N-terminal pyro-glutamic acid formation. Results form the Mascot searches were analyzed and sorted using Scaffold® (v. 3.00, Proteome Software, Portland, OR).

3. Results

Using protein antibody microarray techniques we identified a number of proteins that were differentially regulated in both human and bovine failing right and left ventricles. We were able to examine over 500 proteins in each tissue sample and Table 1 details a number of proteins that were differentially regulated in failing human hearts. There was substantial overlap between right and left ventricles and between bovine and human heart failure [8]. Among the proteins that were found to be upregulated in heart failure was c-terminal Src kinase (CSK). CSK is a tyrosine kinase that negatively regulates c-Src. Src has been implicated in angiotensin-II signal transduction, cardiac hypertrophy, and remodeling [9].

To investigate the role of increased CSK expression we initially sought to characterize its downstream target, Src. Src was upregulated in failing ventricles, along with an immunologically reactive band that was slightly smaller (Fig. 1). Initially it was hypothesized that this smaller immunoreactive band represented either a truncated form of Src or one of the other nine isoforms in the Src family. Interestingly, in the total Src western blots there was a doublet

Table 1
Relative abundance was determined by calculating the cy5 to cy3 ratio for each protein. The numbers in bold represent significant values in that sample comparison group. A value greater than 1 indicates an increase in expression in the first group relative to the second group examined (for example, in FRV vs. FLV a value of 1.5 indicates that a protein was over-expressed in the FRV compared to the FLV). Upper and lower limits of significance are given at the bottom of the table.

Proteins	FRV vs. FLV	CRV vs. CLV	CRV vs. FRV	CLV vs. FLV
p96	**1.513**	0.982	1.193	1.325
CSK	**0.758**	0.935	0.995	1.01
IL-1b	0.898	0.968	**0.824**	0.926
DMPK	0.889	0.908	**0.803**	1.024
Caspase-9/ICE-LAP6/Apaf-3	1.078	0.977	1.145	**1.409**
FADD/Mort-1	1.024	1.031	1.081	**1.444**
NF-kB	1.06	1.008	1.192	**0.803**
Doublecortin	0.933	0.94	1.105	**0.763**
PARP	1.015	0.973	1.31	**0.758**
Nexilin	0.993	1.015	1.126	**0.75**
Flotillin2/ESA	**1.506**	1.015	1.198	1.009
Cytochrome c/Apaf	**0.52**	1.147	1.229	0.914
Upper limit of significance	1.3445	1.3455	1.4391	1.3579
Lower limit of significance	0.7963	0.7969	0.8524	0.8043

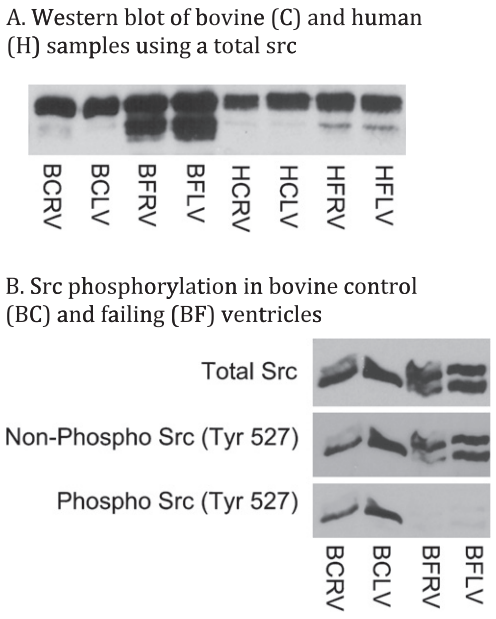


Fig. 1. Western blot analysis of control and failing human (H) and bovine (C) ventricular homogenates. Proteins were separated by 10% SDS–PAGE, transferred and blotted with a pan-specific or phospho-specific anti-src antibody (Cell Signaling). Proteins were visualized using enhanced chemiluminescence. C; control, F; failing.

seen in each of the failing ventricular samples and not in any of the control samples.

The activity of Src is regulated at two tyrosine residues. Phosphorylation at Tyr 416 activates Src, whereas Tyr 527 phosphorylation leads to inactivation via a conformational change and intramolecular binding to its SH2 domain [10,11]. We therefore blotted the same tissue samples with phospho-Src (Tyr 527) and non-phospho-Src (Tyr 527) (Fig. 1b) as well as with another Src isoform Lck (data not shown). There was a slight upregulation of both phospho-Src and non-phospho-Src in failing left ventricles compared with control left ventricles, with the persistence of a doublet signal in failing ventricles. There was no Lck reactivity with the lower band in the doublet (data not shown).

In order to identify the lower band, we separated the protein homogenates further using 2-dimensional (2D) gel electrophoresis. Both control and failing bovine right ventricle 2D gels were run in duplicate (Fig. 2). One set of gels was used for Western blot analysis and the other for isolating the protein of interest using in-gel digestion and peptide extraction. The membranes were blotted with the total Src antibody and the lower molecular weight band was localized on the second set of Coomassie stained gels using the Western blot as a guide. The isolated spots were cut from the gel, digested with trypsin and sent to the mass spectrometry core facility for protein identification.

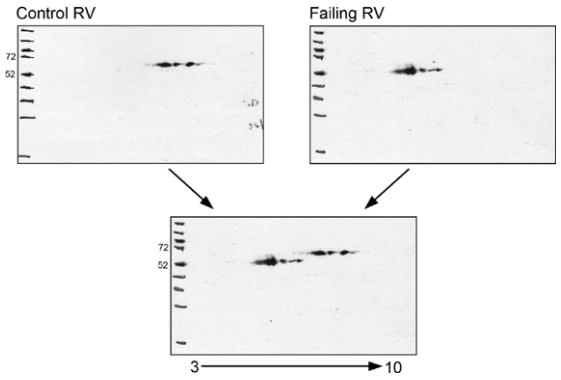
Mass spectrometry identified 19 unique peptides with 41% coverage of protein disulfide isomerase A3 (PDIA3) (Fig. 2b). Because the band was immunologically reactive with the anti-src antibody, we compared the sequences of Src and PDIA3. Surprisingly there was only 8% homology between Src and PDIA3. By examining the sequence surrounding Tyr527, we were able to identify the putative epitope that was used to generate the Src antibody. Blast analysis of that sequence against full-length PDIA3 demonstrated a region of significant overlap in the c-terminus of PDIA3. So, although Src and PDIA3 have very little sequence homology overall, the putative antibody epitope has high sequence homology to PDIA3 (Fig. 2C).

To confirm that the immunologically reactive lower band was PDIA3, we ran western blots with an antibody generated against PDIA3 (ERp57, USBiologicals). These blots confirmed that there was a marked up-regulation of PDIA3 seen in the failing bovine right ventricles (Fig. 3). In addition, blots were run using failing human left ventricular tissue (from patients with both ischemic and non-ischemic cardiomyopathy) and similar up-regulation of PDIA3 was seen in these samples as well (data not shown).

4. Discussion

PDIA3 is a member of the endoplasmatic reticulum stress signaling pathway also known as unfolded protein response (UPR) and is activated in response to cellular stress [15]. There are twenty known members of this protein family that have been found in humans. In its oxidized form PDI binds to proteins with thiol groups and uses its disulfide bond to oxidize thiols on target proteins, resulting in a structural change and an activation or deactivation of the target protein. Similarly, reduced PDI binds to proteins with disulfide bridges and can either reduce the disulfide bridge or change the disulfide bridge [16]. PDI participates in chaperone, isomerase, and redox-activities. These proteins are abundant in the ER, although they are occasionally seen elsewhere [17]. For example, cell surface localized PDI is implicated in regulation of leukocyte adhesion, platelet adhesion and nitric oxide signaling pathways [17]. A member of this family, PDIA3, is a thiol-dependent oxidoreductase that facilitates formation of inter- and intramolecular disulfide bonds [12]. PDIA3 has two active

A. 2-dimensional gel electrophoresis of bovine control and failing ventricles



B. Mass spec identification of Protein Disulfide Isomerase A3 (PDIA3)

PDIA3 protein, 56,931.8 Da
19 unique peptides, 26 unique spectra, 76 total spectra, 207/505 amino acids (41% coverage)

MRLRRLALFP GLALLLAAAR LAAASDVLEL TDDNFESRIT DTGSSGLMLV
EFFAPWCGHC KKLAPEYEAA ATRLKGIVPL AKVDCTANTN TCNKYGVSGY
PTLKIFRDGE ESGAYDGPRT ADGIVSHLKK QAGPASVPLK SEEEFEKFIS
DKDASVVGFF KDLFSEAHSE FLKAASNLRD NYRFAHTNVE SLVNKYDDDG
EGITLFRPSH LTNKFEDKTV AYTEQKMTSG KIKRFIQENI FGICPHMTED NKDLLQGKDL
LIAYYDVDYE KNAKGSNYWR NRVMMVAKKF LDAGKKLHFA VASRKTFSHE
LSDFGLESTT GEIPVVAVRT AKGEKFVMQE EFSRDGKALE RFLEDYFDGN
LKRYLKSEPI PESNDGPVKV VVAENFDEIV NNENKDVLIE FYAPWCGHCK
NLEPKYKELG EKLRKDPNIV IAKMDATAND VPSPYEVRGF PTIYFSPANK
KQNPKKYEGG RELSDFISYL KREATNPPVI QEEKPKKKKK AQEDL

C. Putative src-antibody epitope sequence alignment with PDIA3

Putative epitope	FLEDYFTSTEPQYQPGE
PDIA3 a.a. 352	FLQDYFDGNLKRYLKSE

Fig. 2. Two-dimensional gel electrophoresis of bovine right ventricular samples and identification of PDIA3. (A) Proteins were separated first by charge over a 3–10 non-linear pH gradient and then by molecular weight using 10% SDS–PAGE. Proteins were transferred to nitrocellulose and blotted using a pan-specific anti-src antibody. Duplicate gels were stained with coomassie brilliant blue and the relevant protein was cut from the gel, digested and the peptides were analyzed using mass spectrometry. (B) Mass spec identified 19 unique peptides (highlighted) with 41% coverage of protein disulfide isomerase A3 (PDIA3). (C) Sequence similarity of the amino acids surrounding Tyr527 in src with a region of PDIA3.

thioredoxin-like domains, and shares 33% amino acid sequence identity to PDI. It has been found in liver, lung, placenta, pancreas, kidney, heart, skeletal muscle, and brain [13]. It has been most well studied in the formation of MHC class I heavy chain and MHC I peptide loading. As a stress-responsive protein, its expression increases following glucose depletion and protects cells against ER-stress induced apoptosis [5,14]. Furthermore, it has also been shown to have a role in calcium modulation. PDIA3 (ERp57) has also been found in the cytosol interacting with STAT3 and in the nucleus bound to DNA [17]. Although the function of ERp57 in these settings is still speculative, it may have implications in gene transcription and transduction.

Oxidative stress plays an important role in heart failure. The NADPH oxidases (Nox), xanthine oxidase, mitochondria and nitric oxide synthetases (NOS) are sources of reactive oxygen species and reactive nitrogen species. Specifically, Nox4 has been shown to increase mitochondrial levels of superoxide and lead to apoptosis, mitochondrial dysfunction and LV dysfunction as a result of pressure overload [18]. Superoxide radicals in heart failure have been linked to the calcium leak from SR, and coronary endothelial dysfunction and decreased blood flow [18].

Both hypoxic and oxidative stresses induce the expression of PDI [19], and this stress is attenuated in neurons and cardiomyocytes overexpressing PDI [7,19]. It is thought that the thioredoxin-like

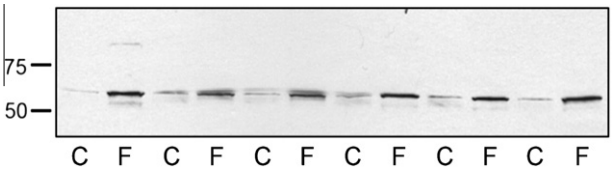


Fig. 3. Bovine right ventricular samples were separated on 10% SDS-PAGE, transferred to nitrocellulose and Western blotted with an antibody against PDIA3 (ERP57, USBiologicals). Western blot of control (C) or failing (F) bovine right ventricular samples.

domains in PDI confer this protection as evidenced by the fact that there are a significantly reduced number of terminal dUTP nick end labeling-positive (TUNEL) cells after transfection with PDI [20]. Severino et al. showed that adenovirally mediated overexpression of PDI in a murine model of MI has a role in limiting apoptosis, reducing cardiac infarct size, preventing cardiac dilatation and adverse remodeling [7]. The up-regulation of this protein seen here in both bovine and human heart failure suggests that it may play a cardioprotective role in the dysfunctional myocardium.

In addition, heart failure is characterized, in part, by calcium dysregulation. It has been proposed that decreased expression of sarcoplasmic reticulum calcium ATPase (SERCA) and reduced

affinity of SERCA for calcium are responsible for this dysregulation in the failing myocardium [21]. SERCA2a is the most prominent isoform of the sarcoplasmic reticulum calcium ATPase found in the heart. Through its expression levels, SERCA2a along with phosphpholamban modulate the contractile properties of the heart. SERCA2a has an overall lower affinity for calcium than the ubiquitous SERCA2b isoform that is conferred by a 49 amino acid 2b-tail and has a 2-fold higher turnover rate [21]. The lectin-like chaperones, calnexin and calreticulin, are proposed to interact with the 2b-tail and increase Ca^{2+} affinity seen in SERCA2b.

It is attractive to hypothesize that PDIA3 modulates the activity of SERCA2b though its interactions with calnexin and calreticulin [22]. The presumed mechanism for this interaction is that calnexin and calreticulin act as calcium sensors in the ER and use PDIA3 as a cofactor for their activities. In the presence of high intraluminal calcium content in the ER, calnexin and calreticulin bind to the c-terminal glycosylation site of SERCA, then PDIA3 complexes with calnexin and calreticulin facilitates the formation of intramolecular disulfide bonds within SERCA2b thereby inhibiting its activity.

Our study is the first to demonstrate increased levels of PDIA3 in human failing heart samples. The fact that it is seen in both animal and human models (including ischemic and non-ischemic) and that it is likely linked to cellular mechanisms of calcium regulation and apoptosis suggests a central role for this protein in the progression of heart disease although the precise biologic functions are not yet known. Indeed, whether the protein serves a compensatory or decompensatory function is not clear. Therefore, future studies are needed both describing the temporal changes in protein expression following a pathologic insult as well as studies in which levels of expression are modulated, using adenoviral or siRNA strategies or transgenesis.

Acknowledgments

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