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Serendipitous discovery of a novel protein signaling mechanism in heart failure

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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.

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.

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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