ONLINE-SUPPLEMENT

Proteomics Methodology

To analyze changes in protein between revascularized and non-revascularized subendocardium we used 2D in gel differential electrophoresis (2D-DIGE) (1). The details of the methods we have used in our laboratory to prepare samples from swine with hibernating myocardium, quantify results and identify myocardial proteins using MALDI-TOF (matrix assisted laser desorption ionization – time of flight) mass spectrometry are described in our previous publications(2-5). Salient aspects of the methods and identification of proteins in myocardial tissue are summarized below.

Total Protein Sample Preparation

Total protein was extracted from approximately 0.2 g of myocardium by polytron homogenization in 8M urea/2% CHAPS/2% ß-mercaptoethanol. The homogenate was placed in a sonicating water bath, centrifuged, and further purified by precipitation with chloroform/methanol.

Protein Electrophoresis and Spot analysis

Samples were labeled with CyDye DIGE Fluor prior to electrophoresis. Cy3 and Cy5 were randomly used to label revascularized (n=12) and non-revascularized (n=12) samples to prevent dye bias, while Cy 2 was used to label the pooled sham control sample. Pharmalytes (pH 3-10NL) were added to the samples to a concentration of 1%, dithiothreitol (DTT) was added to a final concentration of 65mM, and rehydration buffer [8M urea/4% CHAPS/1% Pharmalytes (pH 3-10NL)/13mM DTT] was added to a total volume of 450µl. The samples were applied to immobilized pH gradient (IPG) strips (24cm, pH 3-10 nonlinear), allowed to absorb by active rehydration, and focused on an IPGphor isoelectric focusing system. Strips were then

equilibrated for 15 minutes in 6M urea/ 50mM Tris pH 8.8/ 30% glycerol/ 2% SDS/ 5mg/ml DTT followed by 15 minutes in 6M urea/ 50mM Tris pH 8.8/ 30% glycerol/ 2% SDS/ 45mg/ml iodoacetamide. Proteins were separated by molecular weight on 12.5% gels in an Ettan DALT SDS-PAGE system (GE Healthcare).

Gels were scanned with a Typhoon 9410 imager, cropped (ImageQuant v5.2), and imported into DeCyder software version 6.5 (GE Healthcare) for spot identification and normalization. We manually extracted intensity data and calculated an average volume ratio and an unpaired student's t-test derived p value for each spot, with $p < 0.05$ considered significant. Note that average volume ratios for decreasing spot intensities are represented in DeCyder as a number less than -1 as the software takes the negative reciprocal of the average volume ratio. These were converted back to a simple ratio for our analyses, and are represented as a number between 0 and 1.

In Gel Digestion and Protein Identification by Mass Spectrometry

Spots were either picked by hand or by an automated Ettan robotic spot picker (GE Healthcare) from 2D-gels loaded with 500-1000 ug of total protein and stained with Sypro Ruby (Molecular Probes). Gel pieces were washed for 30 minutes to 1 hour in 100-200 µl of 200 mM ammonium bicarbonate and 50% acetonitrile at 37° C, and then dried under vacuum in either a SpeedVac evaporator, or a desiccator. Trypsin (Promega, Madison, WI) solution (20 µg/ml) was added to the gel pieces, and they were allowed to rehydrate for 20 minutes at room temperature. Gel pieces were allowed to incubate overnight at 37° C in 40 µl of 40 mmol/L^{-1} ammonium bicarbonate /10% acetonitrile. Digests were concentrated and purified with ZipTips (Millipore, Bedford, MA), eluted in approximately 1.0 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile in 0.1% trifluoroacetic acid, and spotted on a ground

steel MALDI-TOF target plate. Additionally, some gel pieces were digested using the automated Proteineer Digest and Prep Station (Proteineer DP) from Bruker Daltonics (Billerica, MA, USA). The instrument was run with default settings using digestion reagents provided in Proteineer DP specific chemical kits. Samples were spotted directly by the instrument onto a 400 µm, 384 sample capacity Anchor Chip (Bruker Daltonics).

Spectra were obtained using a Bruker Daltonics Biflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bellerica, MA USA). Spectra in the range of 500-3200 Da were obtained in reflector mode by the summation of 50-200 laser shots. Peaks were identified and labeled manually. External calibration of peptide masses was achieved through use of a standard peptide mix available from Bruker Daltonics. MALDI-TOF-TOF data was obtained using a Bruker Daltonics Autoflex MALDI-TOF instrument in TOF-TOF mode. Proteins were identified by peptide mass fingerprinting using Mascot (www.matrixscience.com). Mascot search parameters included mammalian taxonomy, carbamidomethyl fixed modification, variable methionine oxidation, 0-2 missed cleavages, and 20-200 ppm mass error. Both the NCBI and Swiss Protein databases were searched. Protein identifications were made if the MOWSE score was equal to or greater than the minimum score required by Mascot for statistical significance for the respective database.

Supplemental References

1. Unlu M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 1997;18:2071-2077.

2. Page B, Young R, Iyer V et al. Persistent regional downregulation in mitochondrial enzymes and upregulation of stress proteins in swine with chronic hibernating myocardium. Circ Res 2008;102:103-112.

3. Page BJ, Young RF, Suzuki G, Fallavollita JA, Canty JM, Jr. The physiological significance of a coronary stenosis differentially affects contractility and mitochondrial function in viable chronically dysfunctional myocardium. Basic Res Cardiol 2013 108:354.

4. Qu J, Young R, Page B et al. A reproducible ion current-based approach for 24-plex comparison of the tissue proteomes of hibernating vs. normal myocardium in swine models. J Proteome Res 2014 13:2571-2584.

5. Tu C, Li J, Young R et al. Combinatorial peptide ligand library treatment followed by a dual-enzyme, dual-activation approach on a nanoflow liquid chromatography/orbitrap/electron transfer dissociation system for comprehensive analysis of swine plasma proteome. Anal Chem 2011;83:4802-13.

Supplemental Table: Echocardiographic Indices of Left Ventricular Structure and Function in Revascularized and Non-Revascularized Animals

	LVDd (mm)	LVDs (mm)	FS (%)	EF (%)
Revascularized				
Baseline	**46.3±1.6**	**32.9±1.9**	**29.5±1.9**	**59.1±3.6**
2-Hours Post-PCI	**45.2±1.1**	**32.7±1.8**	**28.1±2.6**	**56.4±4.7**
1-Month Post-PCI	**50.4±1.3**	**35.1±1.9**	**30.7±2.3**	**59.9±4.4**
Non-Revascularized				
Baseline	**50.0±1.7**	**35.8±2.1**	**29.0±2.1**	**63.1±3.3**
1-Month	**54.7±1.8**	**37.8±1.8**	**31.0±2.0**	**66.0±2.9**

Values are mean±SEM.
Baseline measurements were collected 3-months after surgical placement of a LAD stenosis.
LV – Left Ventricular; LVDd – Left Ventricular Diastolic Dimension; LVDs – Left Ventricular Systolic Dimension; FS – Fractional Shortening; EF – Ejection Fraction.