Supplemental Digital Content 3

Supplemental laboratory methods

Staining of the right atrial tissue cross-sections and image analyses

Due to the limitations in size of the right atrial tissue samples, we used immunohistochemistry because this method allows detection of levels of several proteins in the tissue sample, in contrast to biochemical analyses of enzymatic activities that require larger tissue specimens. Myocardial tissue samples were frozen immediately in O.C.T. Compound (Tissue-Tek, Sakura Finetek Europe BV, Alphen aan den Rijn, The Netherlands) in liquid nitrogen and transferred to −80°C until sectioning. Frozen sections of 10 μm were prepared and stored at −80°C until the staining procedure. Frozen sections were incubated with specific antibodies overnight at 4°C, followed by the incubation with secondary antibodies for 45 min at room temperature, and the staining was developed using Vecta Stain ABC Kits (catalog ## PK6102 and PK6101, Vector Laboratories, Inc., Burlingame, CA) for blocking the sections, biotinylation, and colorimetric detection of the markers according to manufacturer's instruction. Rabbit polyclonal anti-c-Fos antibody (# ab102699, Abcam, Cambridge, MA) was used in dilution 1:40; rabbit polyclonal anti-early growth response protein (Egr-1) antibody (# bs1076R, Bioss, Woburn, MA) in dilution 1:100; mouse monoclonal anti-O-Linked N-Acetylglucosamine antibody (RL2) (# MA1-072, 1:100, Thermo Fisher Scientific Inc., Waltham, MA), rabbit monoclonal antibodies against glyceraldehyde 3-phosphate dehydrogenase (# 5174S, 1:500, Thermo-Scientific, Waltham, MA); Hexokinase I (# 2024S, 1:500), Hexokinase II (# 2867S, 1:500), and pyruvate dehydrogenase (PDH; 1:100) were from Cell Signaling (Danvers, MA). Mouse monoclonal anti-thrombospondin-1 (TSP-1) Ab 4 (clone 6.1) from Thermo Scientific (1:100) was used to assess TSP-1 protein levels. Images of stained sections were acquired using a Leica DMR 4000B upright microscope (Heidelberg, Germany) fitted with a single-slide x-, y-, and z-motorized

stage, a 20X (dry) objective, a FITC fluorescence filter cube, and a Retiga 2000R CCD digital camera (QImaging, Burnaby, British Columbia, Canada). High magnification image fields were raster-scanned across each cross-section (Oasis 4i controller, Objective Imaging, Kansasville, MI) and stitched together to form a single high-resolution, large field-of-view (FOV) image (~289 tiles/mosaic). Each image field was background corrected prior to stitching to ensure continuity and minimize chromatic variability. For quantitative analysis, large FOV images were batch processed using customized macros and algorithms generated for Image-Pro Plus 6.1 (Media Cybernetics, Silver Spring, MD). Data for quantitative analyses of large FOV images have been collected by a blinded investigator, and quantification of staining intensity and area was done using Image-Pro Plus 6.1 (Media Cybernetics). The large blood vessels were traced and deleted from the images. The total area of the section, stained area, and the intensity of staining of each cross-section were then exported to Excel (Microsoft, Redmond, WA). Representative images from the laboratory analyses are shown in Fig. 1 and 2.

Our analysis normalized to area rather than number of cells in order to avoid errors due to cardiomyocyte polyploidy and variability related to presence of other cell types (fibroblasts, leukocytes, etc.), although normalization to area is subject to error due to fibrosis in the tissue and the variable amount of matrix. Out of the two methods, we have chosen normalization to the area because we feel that this method better reflects the goal of the analyses, which is to examine the total cumulative change in myocardium function.

Results from the laboratory analysis are shown in Table 1.

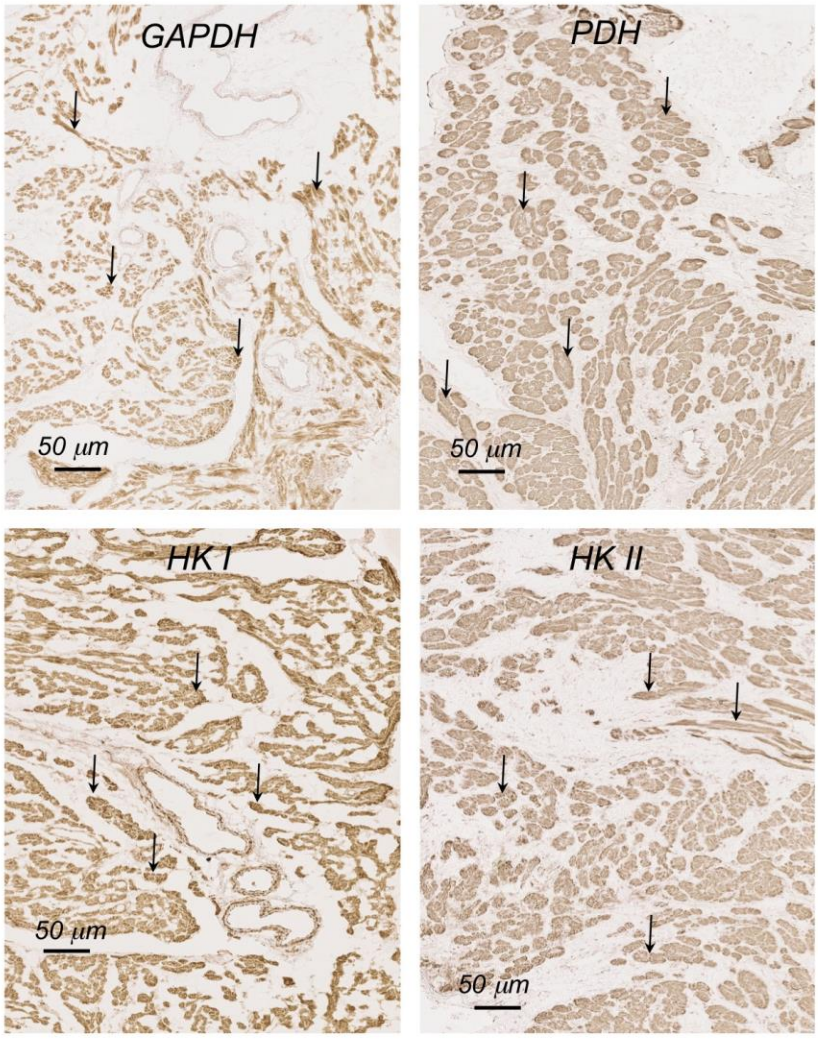

Fig. 1. Right atrial tissue analysis. The figure is representative of the images collected for quantitative tissue analysis of key regulatory enzymes of the glycolytic (hexokinase I (HK I), hexokinase II (HK II), glyceraldehyde 3-phosphate dehydrogenase, GAPDH) and pyruvate oxidation pathways (pyruvate dehydrogenase, PDH) (brown staining, black arrows indicate stained area).

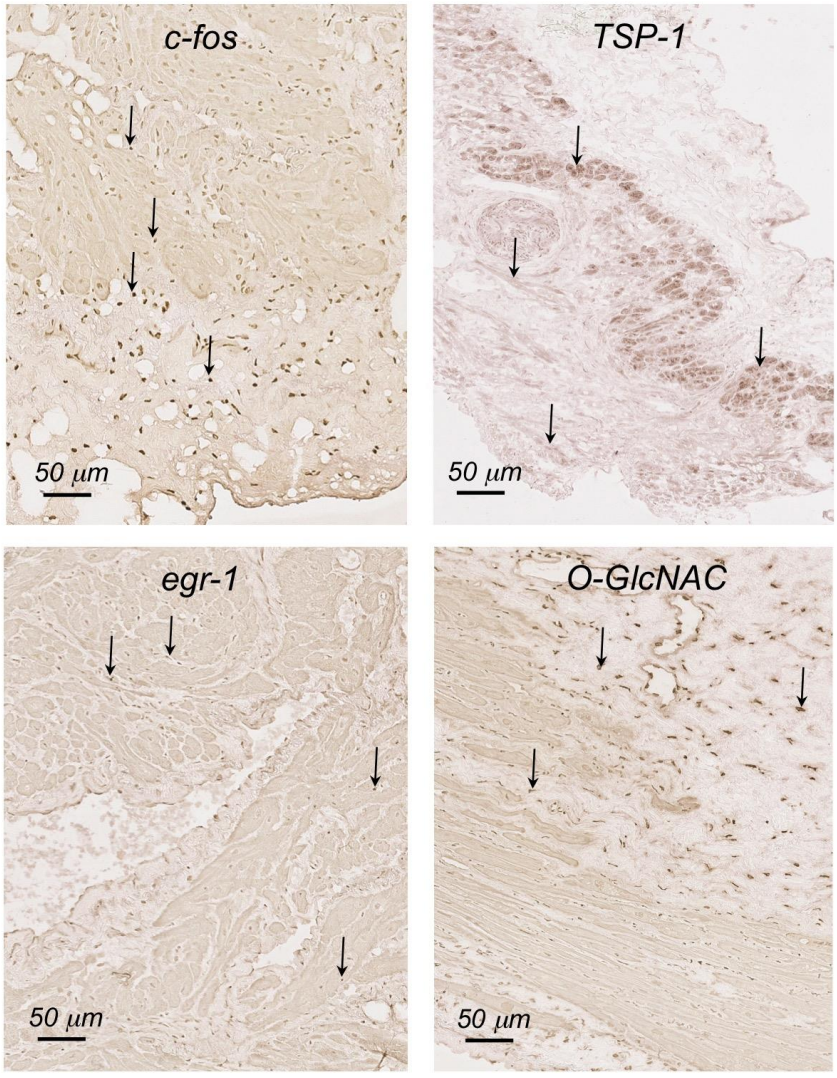

Fig. 2. Right atrial tissue analysis. The figure is representative of images used to quantify the staining of proto-oncogenes c-fos and early growth response protein-1 (egr-1), thrombospondin-1 (TSP-1), and O-linked N-acetylglucosamine transferase (O-GlcNAc). Arrows indicate the stained areas.

Table 1. Supplemental Laboratory Results

Variable	***N***	***HNC (N = 20)***	***N***	***Standard (N = 22)***	**Ratio of means (95% CI) ***	***P****
Signs of adverse cellular and biochemical effects of hyperglycemia						
Thrombospondin-1						
Treatment × time						0.81
Overall		798 [323, 925]		524 [245, 729]	1.3 (0.7, 2.22)	0.41
preaortic clamp	20	812 [313, 925]	22	463 [245, 722]	1.1 (0.4,3.09)	0.79
postaortic clamp	20	757 [326, 920]	19	599 [215, 740]	1.3 (0.7,2.40)	0.40
Change: post − pre	20	−97 [−214, 212]	19	−52 [−207, 132]		
c-Fos						
Treatment × time						0.80
Overall		51 [35, 83]		72 [42,123]	0.6 (0.3,1.22)	0.17
preaortic clamp	20	46 [38, 106]	22	66 [35, 123]	0.7 (0.3,1.42)	0.28
postaortic clamp	20	58 [32, 79]	19	72 [42, 131]	0.6 (0.3,1.30)	0.19
Change: post − pre	20	−3 [−57, 32]	19	12 [−47, 36]		
Early growth response protein-1						
Treatment × time						0.19

Overall		187 [98, 346]		214 [145, 361]	0.8 (0.5,1.33)	0.45
preaortic clamp	20	171 [115, 338]	22	210 [128, 351]	1.0 (0.6,1.58)	0.88
postaortic clamp	20	202 [89, 351]	19	250 [148, 510]	0.7 (0.4,1.19)	0.17
Change: post − pre	20	−24 [−72, 122]	19	83 [33, 163]		
O-linked N-acetylglucosamine transferase						
Treatment × time						0.30
Overall		297 [229, 377]		223 [159, 293]	1.2 (0.8, 1.69)	0.32
preaortic clamp	20	295 [204, 351]	22	237 [176, 308]	1.1 (0.8,1.62)	0.59
postaortic clamp	20	297 [232, 450]	19	219 [150, 263]	1.3 (0.9,2.04)	0.16
Change: post − pre	20	59 [-56, 136]	19	−14 [−100, 63]		
Regulatory enzymes of the glycolytic pathway						
Pyruvate Dehydrogenase						
Treatment × time						0.67
Overall		156 [79, 567]		268 [129, 691]	0.7 (0.3, 1.28)	0.21
preaortic clamp	20	170 [82, 515]	22	319 [130, 671]	0.6 (0.3,1.29)	0.19
postaortic clamp	20	141 [52, 595]	19	258 [111, 909]	0.8 (0.3,2.05)	0.58
Change: post − pre	20	0 [−186, 141]		−65 [−318, 130]		

Hexokinase I						
Treatment × time						0.43
Overall		1,121 [219,1,693]		1067 [711,1,506]	0.9 (0.4,1.92)	0.77
preaortic clamp	20	990 [478, 1,388]	22	1,182 [711, 1,603]	1.1 (0.4,2.50)	0.90
postaortic clamp	20	1,251 [211, 1,997]	19	938 [654, 1,506]	0.7 (0.2,1.92)	0.45
Change: post − pre	20	221 [−180, 808]	19	−1 [−360, 415]		
Hexokinase II						
Treatment × time						0.71
Overall		1,289 [745,1,683]		910 [296,1,374]	2.0 (0.9,4.43)	0.09
preaortic clamp	20	1,271 [847, 1,830]	22	1,019 [229, 1,640]	2.3 (0.7,7.56)	0.15
postaortic clamp	20	1,306 [677, 1,522]	19	745 [296, 1,329]	1.8 (0.7,4.74)	0.23
Change: post − pre	20	−85 [−752, 382]	19	−110 [−376, 249]		
Glyceraldehyde 3-phosphate dehydrogenase						

Treatment × time						0.06
preaortic clamp	20	182 [62, 309]	22	711 [97, 959]	0.4 (0.1,1.05)	0.06
postaortic clamp	20	594 [111, 1,056]	19	533 [36, 1,036]	1.2 (0.5,3.20)	0.70
Change: post − pre	20	54 [−30, 713]		−9 [−328, 316]		

Data is presented as median [interquartile range]; *The Repeated Measures ANCOVA, and comparing groups on at each time (pre vs. postaortic clamp) if $P_{\text{Treatment} \times \text{time}} < 0.15$ or the collapsed times (main effect model) if no interaction. Laboratory outcomes were log-transformed in the model.

Laboratory measures from right atrial tissue analysis in hyperinsulinemic normoglycemic clamp (HNC) and standard therapy groups in patients with aortic stenosis. The distribution of laboratory measures before (pre) and after (post) aortic cross-clamping which examined the adverse cellular and biochemical effects of hyperglycemia and cardioplegic arrest are shown. If no interaction between time and treatment was found, we collapsed time and fit a main effect model. If interaction between time (pre *vs.* post) and treatment was significant ($P<0.15$), we compared groups at each time point.