SUPPLEMENTARY MATERIAL

Kidney Response to Heart Failure: Proteomic Analysis of Cardiorenal Syndrome

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Methods – detailed description

Chronic heart failure model

Volume overload with consequent HF was induced in male Wistar rats (300-350g) by creating aorto-caval fistula (ACF) using 1.2 mm needle from laparotomy in general anesthesia, as described previously. Control sham-operated animals underwent the same procedure, but without ACF creation. The animals were kept on a 12/12-hour light/dark cycle, and fed normal salt/protein diet (0.45% NaCl, 19-21% protein, SEMED, CR). The investigation conformed to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996), Animal protection laws of the Czech Republic (311/1997) and was approved by the ethics committee of IKEM.

Echocardiography and hemodynamics

Cardiac examinations (controls: n= 6, ACF: n=8) were performed in general anesthesia (ketamin+midazolam mixture) at the study end (21st week) prior to harvesting of renal tissue. Echocardiography was performed with 10 MHz probe (Vivid System 5, GE, USA). End-systolic and end-diastolic LV volume were derived by cubic equation and stroke volume as their difference. Hemodynamics was measured with 2F micro-manometer catheter (Millar Instruments) inserted into the aorta and LV via carotid artery, connected to Powerlab 8 platform for off-line analysis with LabChart software (ADinstruments, Germany). The presence of ACF was verified from laparotomy, the animals were killed by exsanguination and left kidney was harvested into liquid nitrogen for proteomic analysis. The organs were weighted and normalized to body weight.

Renal function studies and biochemistry

In separate set of animals, renal function were measured as previously[1-3]. In 18 weeks after ACF induction, the animals (sham: n=9, ACF: n= 17) were placed in individual metabolic cages and their 24-hour urine was collected for determination of daily albuminuria. Urinary rat albumin was measured by a quantitative sandwich enzyme immunoassay technique, using the commercially available ELISA kit (ERA3201-1, AssayPro, MO, USA) as before[3].

In 21st week, the animals (sham: = 6, ACF: n= 8) were anesthetized and right jugular vein was catheterized for fluid infusion and anesthetic administration. The left femoral artery was catheterized to monitor arterial blood pressure. The left kidney was exposed via a flank incision, isolated from the surrounding tissue and placed in a lucite cup, and the ureter was cannulated with a PE-10 catheter. An ultrasonic transient-time flow probe (1RB, Transonic Systems, Germany) was placed around the left renal artery and RBF was recorded using a computerized acquisition system (PowerLab, ADInstruments). After the surgery, isotonic saline solution containing albumin (1%) and polyfructosan inulin (7.5%, Inutest, Laevosan, Linz, Austria) was infused at constant infusion rate. After completion of the surgical procedures and after establishment of steady state, urine and blood samples were collected collection. Blood samples were collected to allow determination of GFR and renal sodium excretion. Urine volume was measured gravimetrically. Urinary sodium concentration was determined by flame photometry. Polyfructosan in plasma and urine was measured colorimetrically to estimate GFR. Cystatin C was measured by immunoturbidimetric method, serum creatinine by enzymatic spetrophotometric method. Renal tissue angiotensin II (ANG-II) was measured by RIA as described previously in our lab[4].

Materials

KCl and KH_2PO_4 were purchased from Penta (Penta, Czech Republic), DTT, Tween 20 and Tris were from Promega (Madison, USA). All other chemicals were from Sigma-Aldrich unless indicated otherwise.

Kidney sample preparation

Frozen kidney samples were pulverized using liquid nitrogen with mortar and pestle. We created representative pooled samples "Control" and "HF" by mixing equal amounts (10 mg) of pulverized renal tissue from each animal in the respective cohort (n=7). The pooled representative samples were aliquoted and used as a starting material for all proteomic analyses and for western blots.

Filter assisted sample preparation (FASP) and and iTRAQ labeling

The aliquots of Control and HF pooled kidney samples were lysed in a lysis buffer (8.4 M urea, 50 mM DTT, 5 % CHAPS) for 30 minutes at room temperature. Lysates were centrifuged at 20,000-× g for 20 minutes at 22 °C. The supernatants were collected and protein concentration was determined (Bio-Rad Protein Assay, Bio-Rad, CA, USA). Control and HF protein lysates were processed by FASP method[5], each in two technical replicates. Lysates (120 μg) were diluted with MilliQ H_2O to decrease urea concentration to 7M and transferred to 30 kDa filters (Microcon® 30 kDa Centrifugal Filter Unit with Ultracel® 30 membrane) and centrifuged at 7,000-× g for 5 minutes, and washed twice with 400 μl urea buffer (8 M urea in 60 mM HEPES, pH 8.2), at 10,000-× g for 5 minutes. The flow-through fractions were discarded. The filters were then washed 3-times with 400 μl 60 mM HEPES, pH 8.2 at 10,000-× g for 15 minutes. Samples were further reduced and alkylated according to iTRAQ protocol (iTRAQ® Reagents Multiplex Kit, SCIEX) provided by the manufacturer. 20 μl dissolution buffer (TEAB), 1 μl denaturant (SDS) and 2 μl reducing reagent (TCEP)

was added to the filters and incubated at 22 °C for 1 hour. Samples were then alkylated by addition of 1 μl cysteine-blocking reagent (MMTS) at room temperature for 10 minutes. Each sample was digested with 11 μl (0.8 μg/μl) of trypsin solution (Promega, Sequencing grade modified trypsin) overnight at 37 °C.

We labelled the Sham and ACF pooled samples with the four isobaric tags (114, 115, 116 and 117), each sample in two replicates according following schem: 114-HF (replicate 1), 115-Control (replicate 1), 116-HF (replicate 2), 117 Control (replicate 2). The iTRAQ tags were dissolved in 70 μl of ethanol and transferred to each digest. Filters were then incubated for 1 hour at room temperature with gentle shaking. Filters were centrifuged at 8,000-× g for 5 minutes (the flowthrough was collected), and washed twice with 50 μl MilliQ H_2O and once with 20 μl 0.5 M NaCL at 8,000-× g for 5 minutes. All flow-through fractions were collected and pooled into one sample and the volume was reduced by evaporation to roughly 40 microliters in speedvac (Eppendorf Concentrator Plus 5305).

Strong cation exchange chromatography and desalting

To remove unbound tags and to fractionate the peptides before LC-MS, off-line strong cation exchange chromatography was used. iTRAQ-labeled sample was diluted in SCX loading buffer (5 mM KH_2PO_4, pH 3 in 25% acetonitrile) to reach final salt concentration 10 – 15 mM NaCl. Samples were sonicated for 10 minutes. OPTI-TRAP SCX cartridge (Optimized Technologies, OR, USA) in manual macrotap holder was washed with 500 μl SCX-cleaning buffer (350 mM KCl in SCX-loading buffer) and equilibrated with 500 μl SCX-loading buffer. Samples were loaded and washed with 600 μl SCX-loading buffer. Samples were then eluted in four 200 μl fractions in 50 mM, 100 mM, 200 mM and 350 mM KCl in the SCX-loading buffer into new tubes. All four fractions were dried in speedvac and processed separately.

To be desalted, dried samples were resuspended in 300 μl of 0.1 % TFA in MeCN upon sonication. Desalting was performed using manually operated OPTI-TRAP Peptide Concentration & Desalting cartridge (Optimized Technologies, OR, USA). The cartridge was washed with 700 μl 0.1 % TFA in 80 % MeCN, and equilibrated with 600 μl 0.1 % TFA. Samples were loaded and washed with 500 μl 500 μl 0.1 % TFA. Samples were eluted with 200 μl 80 % ACN with 0.1 % TFA into new tubes and dried in speedvac.

LC-MS/MS analysis

Samples obtained by SCX fractionation (4 fractions for each proteomic analysis) were solubilized loaded to 50 cm EASY-Spray column, 50 cm x 75 µm ID, PepMap C18, 2 µm particles, 100 Å pore size with EASY-Spray™ source with PepMap100 Pre-column 5mm x 300µm C18, 2 µm particles 100 Å. A linear gradient from 4 % B to 35 % B (A water 0.1 % FA, B Acetonitrile 0.1 % FA) was applied for 240 minutes at flowrate 300 nl/min using Ultimate 3000 nanoLC (Dionex).

Data were collected on Thermo Orbitrap Fusion™ in MS^3 reporter ion quantification mode. MS^1 were collected each 4 seconds at resolution 120K, maximum possible number of precursors were selected for MS^2 CID fragmentation and detection in ion trap (injection time 60 ms). Top 10 most intensive peaks from MS^2 fragmentation were simultaneously selected and fragmented in HCD. MS^3 masses were acquired in Orbitrap at resolution 60K (injection time 140 ms).

In total we performed three consecutive iTRAQ analyses using the same pooled kidney samples CTRL and HF. The raw data from all three analyses (including 4 SCX fractions each) were analyzed in Proteome Discoverer 2.2. Tolerances were sets: 10 ppm for precursor, 0.6 Da for MS^2 fragments and 20 ppm for MS^3 quantification fragments. Appropriate iTRAQ modification (+144,102) were set as fixed (N term and K) and variable (Y). MethylThio (+45,988) modification of was set as fixed and methionine oxidation

(+15,995) was set as variable. Search engine was Sequest HT (Proteome Discoverer 2.2.Thermo Scientific). Data were searched together against 2018 versions of SwissProt (Rat, Reviewed – 8022 entries) and TrEMBL (Rat, Unreviewed – 28054 entries). Percolator was used for FDR estimation and 1 % FDRs limits for peptides and proteins were used. Quantification data were normalized on total peptide amount, unique and razor peptides were used for quantification. Only the proteins identified by at least two unique peptides were considered for further quantitative analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[6] partner repository with the dataset identifier PXD009296.

Western blot analysis

Pooled samples of Sham and ACF kidneys pulverized under liquid nitrogen with mortar and pestle were lysed in RIPA buffer (Sigma-Aldrich) for 30 minutes at 4 °C (30 mg of sample per 1 ml lysis buffer). Lysates were centrifuged at 20,000-× g for 20 minutes at 22 °C. The supernatants were collected and protein concentrations determined (Bicinchoninic Acid Kit, Sigma-Aldrich). Protein samples (40 μg) were separated using conventional SDS-PAGE (MiniProtean, Bio-Rad, CA) using 10-12% gels, transferred to PVDF membranes and blocked at room temperature for 60 minutes in TBS-T buffer (Dulbecco's Phosphate Buffered Saline, Sigma-Aldrich with 1 % Tween-20) with 5 % nonfat dry milk. Membranes were washed 3 times with TBS-T buffer and incubated with respective primary antibodies at room temperature for 2 hours. Antibodies were anti ACE (Genetex, GTX11734, 1:250); Caveolin 1 (Invitrogen, PA5-17447, 1:1,000); GAPDH (Sigma-Aldrich, G9545, 1:100,000); Periostin, (Abcam, ab92460, 1:1,000); RAGE (Abcam, ab3611, 1:1,000). The membranes were then washed 3 times with TBS-T buffer and then incubated with secondary HRP-conjugated IgG antibodies (Jackson immunoResearch, 711-035-033 and 711-165-152, 1:10,000) at room temperature for 30 minutes. Washed membrane was incubated with ECL detection system

(Western Blotting Luminol Reagent, SantaCruz Biotechnology, sc-2048), chemiluminescence was captured by ChemiDoc™ MP System (Biorad).

Statistical analysis

Values are presented as means ± SD if not stated otherwise. For comparison of physiological variables (table 1-2), unpaired T test was used, with threshold $p< 0.05$. Significance of differential protein expression (adjusted-p-value) was calculated using Benjamini-Hochber method based on reporter ion intensities by the Proteome Discoverer 2.2 software (Thermo Scientific). Only proteins identified with at least two unique peptides were included in quantitative evaluation. A protein was considered to be differentially expressed only if it met all the following criteria: it was identified in at least two of the three separate analyses, the fold-change was 1.5-fold or more and statistical significance (adjusted p-value) of the change was significant in both replicates (i.e. for both 114/115 and 116/117 ratios).

References

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Supplementary Table S1: Full list of differentially regulated proteins in HF kidney compared to controls. For definition of significance of changes, see methods. HF: heart failure. PSMs: Number of peptide spectrum matches.

Accession	Protein name	Gene Symbol	Foldchange HF/CTRL (114/115)	Foldchange HF/CTRL (116/117)	AVG foldchange	Adj. P-Value (114/115)	Adj. P-Value (116/117)	Sequence Coverage [%]	PSMs	Unique Peptides
Proteins upregulated in HF kidney (>1.5 fold)										
P47820	Angiotensin-converting enzyme (ACE)	Ace	100,00	6,94	**53,47**	NA	NA	5	4	4
Q63495	Advanced glycosylation end product-specific receptor (Ager, RAGE)	Ager	15,25	13,50	**14,37**	1,10E-05	1,06E-05	46	75	11
D3ZAF5	Periostin	Postn	6,49	7,12	**6,81**	1,48E-03	1,12E-03	37	72	20
P16303	Carboxylesterase 1D	Ces1d	6,71	6,72	**6,71**	2,54E-02	2,49E-02	35	28	5
Q497C9	Microfibrillar-associated protein 4	Mfap4	5,07	5,66	**5,37**	3,58E-02	2,16E-02	17	19	3
Q6P792	Four and a half LIM domains 1	Fhl1	5,71	4,33	**5,02**	1,77E-03	3,57E-03	36	28	8
F1M957	von Willebrand factor	Vwf	4,64	5,04	**4,84**	4,20E-03	4,27E-03	1	5	2
P08932	T-kininogen 2	Kng1	4,58	4,69	**4,63**	1,59E-02	1,67E-02	50	78	4
P41350	Caveolin-1	Cav1	4,87	4,20	**4,53**	8,19E-03	1,77E-02	52	70	7
G3V8L9	Polymerase I and transcript release factor =Cavin1	Ptrf	4,18	4,29	**4,24**	1,30E-03	1,65E-03	32	40	9
Q4V8H8	EH domain-containing protein 2	Ehd2	3,63	3,44	**3,53**	8,53E-03	9,83E-03	60	119	23
G3V913	Heat shock 27kDa protein 1	Hspb1	3,78	3,23	**3,50**	7,87E-06	8,63E-06	75	33	10
Q9Z1H9	Cavin 3 (Caveolae-associated protein 3)	Prkcdbp	3,06	3,45	**3,25**	3,82E-04	5,35E-04	7	9	2
A0A0G2JTX7	Collagen type VI alpha 5 chain	Col6a5	2,87	2,78	**2,83**	2,97E-02	5,11E-02	10	67	17
A0A0G2JU96	AHNAK nucleoprotein	Ahnak	2,69	2,85	**2,77**	1,10E-05	8,63E-06	31	84	40
D3ZY91	Chloride intracellular channel protein 3	Clic3	2,36	3,08	**2,72**	3,23E-03	8,51E-04	13	13	2
Q08290	Calponin-1	Cnn1	2,96	2,39	**2,68**	5,09E-03	1,00E-02	21	12	4
P14669	Annexin A3	Anxa3	2,62	2,70	**2,66**	2,33E-02	1,36E-02	54	45	13
Q9EPT8	Chloride intracellular channel protein 5	Clic5	2,40	2,20	**2,30**	9,33E-03	6,47E-03	30	23	4
Q6AYF8	Serpinb9	Serpinb9	2,45	2,12	**2,28**	2,59E-02	4,23E-02	17	13	4
D3ZVM5	Heat shock protein family A (Hsp70) member 12B	Hspa12b	2,30	2,17	**2,24**	5,52E-03	7,10E-03	12	12	4
Q3KRE2	Methyltransferase like 7A, isoform CRA_b	Mettl7a	2,54	1,87	**2,21**	4,13E-04	2,28E-03	33	11	5
P55053	Fatty acid-binding protein, epidermal	Fabp5	2,40	1,90	**2,15**	1,75E-02	4,56E-02	22	14	3
C0JPT7	Filamin A	Flna	2,23	2,06	**2,15**	1,10E-05	3,04E-05	54	549	91
A0A0G2K2P5	Tight junction protein ZO- 1	Tjp1	2,09	1,99	**2,04**	6,18E-03	1,67E-02	10	26	12
Q99PD6	Transforming growth factor beta-1-induced transcript 1 protein	Tgfb1i1	2,01	2,03	**2,02**	4,29E-04	5,35E-04	23	26	6

Q66H98	Cavin 2 (Caveolae-associated protein 2)	Sdpr	2,20	1,81	**2,01**	9,56E-03	2,52E-02	34	31	11
Q9ESS6	Basal cell adhesion molecule	Bcam	1,90	2,11	**2,00**	5,37E-04	3,40E-04	30	38	12
Q4KM35	Proteasome subunit beta type-10	Psmb10	2,20	1,60	**1,90**	7,78E-04	5,61E-03	8	6	2
A0A0G2JXN6	Galectin 3	Lgals3	1,98	1,81	**1,90**	2,73E-04	5,35E-04	9	14	3
F1M8E9	Lysozyme	Lyz2	1,76	2,03	**1,89**	1,33E-02	2,03E-03	62	195	6
Q5XIP9	Transmembrane protein 43	Tmem43	1,74	2,01	**1,88**	8,14E-04	3,56E-04	7	8	2
P11762	Galectin-1	Lgals1	1,89	1,86	**1,87**	2,18E-04	3,10E-04	63	35	6
P47942	Dihydropyrimidinase-related protein 2	Dpysl2	1,83	1,89	**1,86**	5,37E-04	5,35E-04	60	104	20
G3V9M6	Fibrillin 1	Fbn1	2,04	1,62	**1,83**	3,25E-03	9,01E-03	1	5	2
P10634	Cytochrome P450 2D26	Cyp2d2	1,82	1,82	**1,82**	4,88E-02	2,19E-02	25	18	6
A0A0G2K014	Lymphocyte cytosolic protein 1	Lcp1	2,00	1,63	**1,82**	1,36E-03	5,59E-03	52	134	20
Q3SWT0	Platelet endothelial cell adhesion molecule	Pecam1	1,97	1,65	**1,81**	3,46E-03	1,23E-02	12	18	6
Q5M7W5	Microtubule-associated protein 4	Map4	1,89	1,73	**1,81**	6,18E-03	2,41E-02	6	22	8
P62856	40S ribosomal protein S26	Rps26	1,82	1,80	**1,81**	6,18E-04	6,08E-04	27	17	3
P14668	Annexin A5	Anxa5	1,61	2,00	**1,81**	5,34E-02	7,02E-03	75	146	10
Q9JKB7	Guanine deaminase	Gda	1,88	1,72	**1,80**	2,52E-04	5,35E-04	54	61	20
Q5XFX0	Transgelin-2	Tagln2	1,64	1,92	**1,78**	2,13E-02	2,42E-02	67	67	12
A0A0G2K6S9	Myosin-11	Myh11	1,87	1,68	**1,77**	1,57E-03	4,94E-03	42	266	48
D3ZNJ5	Indolethylamine N-methyltransferase	Inmt	1,81	1,70	**1,76**	5,69E-04	9,55E-04	45	73	11
P31000	Vimentin	Vim	1,68	1,82	**1,75**	3,58E-02	2,94E-02	72	199	26
G3V888	ATP-citrate synthase	Acly	1,78	1,71	**1,74**	4,72E-03	7,60E-03	25	55	19
G3V9Y1	Myosin, heavy polypeptide 10, non-muscle	Myh10	1,85	1,62	**1,73**	1,06E-02	2,85E-02	38	253	41
Q9ER28	Endothelial type gp91-phox	Cybb	1,80	1,58	**1,69**	7,53E-03	1,84E-02	2	6	2
D3ZFU9	Myosin light chain kinase	Mylk	1,79	1,53	**1,66**	2,10E-04	6,86E-04	16	37	21
P13832	Myosin regulatory light chain RLC-A	Myl12a	1,60	1,71	**1,65**	3,31E-02	1,12E-02	57	29	4
F1LNH3	Collagen type VI alpha 2 chain	Col6a2	1,71	1,56	**1,63**	3,25E-03	4,23E-03	26	184	21
Q6S3A0	Plectin	Plec	1,67	1,56	**1,62**	1,10E-05	5,08E-05	14	106	44
Q8R3Z7	EH-domain-containing 4	Ehd4	1,64	1,59	**1,61**	1,87E-02	4,23E-02	52	78	14
P02764	Alpha-1-acid glycoprotein	Orm1	1,60	1,61	**1,60**	1,54E-02	7,77E-03	14	20	4
D3ZUL3	Collagen type VI alpha 1 chain	Col6a1	1,60	1,53	**1,57**	9,11E-05	2,43E-04	29	255	23
A0A0G2K9I6	Ceruloplasmin	Cp	1,59	1,54	**1,57**	2,99E-05	5,08E-05	40	118	32
B4F7E8	Niban-like protein 1	Fam129b	1,60	1,52	**1,56**	6,69E-03	4,66E-03	12	11	6
Q3ZB99	Tight junction protein 2	Tjp2	1,54	1,54	**1,54**	4,07E-03	7,01E-03	9	12	8

P62425	60S ribosomal protein L7a	Rpl7a	1,49	1,58	**1,54**	2,59E-02	2,16E-02	13	10	2
M0RCL5	Histone H2A	LOC10091	1,56	1,51	**1,54**	2,39E-02	2,44E-02	55	66	2
Q1PBJ1	Lactadherin	Mfge8	1,52	1,53	**1,52**	1,34E-03	1,61E-03	36	47	14
Proteins downregulated in HF kidney (<0,66 fold)										
Q4FZV0	Beta-mannosidase	Manba	0,66	0,64	**0,65**	1,71E-02	2,34E-02	16	21	10
D3ZTX4	Maltase-glucoamylase	Mgam	0,65	0,62	**0,64**	2,79E-02	3,99E-02	24	194	32
Q5I0M3	Complement component factor h-like 1	Cfhr1	0,62	0,59	**0,61**	4,88E-02	2,64E-02	7	24	2
P07379	Phosphoenolpyruvate carboxykinase, cytosolic	Pck1	0,43	0,44	**0,43**	2,10E-04	4,71E-04	76	290	36
P02761	Major urinary proteins/Alpha Alpha-2u globulin	NA	0,43	0,39	**0,41**	6,79E-05	5,08E-05	80	2053	4