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Original Paper

Kidney Response to Heart Failure: Proteomic Analysis of Cardiorenal Syndrome

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Key Words
Heart failure • Kidney function • Cardiorenal syndrome • Proteomics • Angiotensin-II

Abstract
Background/Aims: Chronic heart failure (HF) disrupts normal kidney function and leads to cardiorenal syndrome that further promotes HF progression. To identify potential participants in HF-related injury, we analyzed kidney proteome in an established HF model. ***Methods:*** HF was induced by chronic volume overload in male HanSD rats using aorto-caval fistula. After 21 weeks, cardiac and renal functions (in-situ kidney study) and renal proteomics were studied in sham-operated (controls) and HF rats, using iTRAQ labeling and LC-MS with Orbitrap Fusion, leading to identification and quantification of almost 4000 proteins. ***Results:*** Compared to controls, HF rats had cardiac hypertrophy, systemic and pulmonary congestion. Kidneys of HF rats had reduced renal blood flow, sodium excretion and urine production. While glomerular filtration rate, serum cystatin C and creatinine were still normal compared to controls, HF kidneys showed albuminuria and markedly increased tissue angiotensin-II levels (5-fold). HF kidneys (versus controls) displayed differential expression (>1.5-fold) of 67 proteins. The most upregulated were angiotensin-converting enzyme (ACE, >20-fold), advanced glycosylation product-specific receptor (RAGE, 14-fold), periostin (6.8-fold), caveolin-1 (4.5-fold) and other proteins implicated in endothelial function (vWF, cavins 1-3, T-kininogen 2), proinflammatory ECM activation (MFAP4, collagen-VI, galectin-3, FHL-1, calponin) and proteins involved in glomerular filtration membrane integrity (CLIC5, ZO-1). Carboxylesterase-1D (CES1D), an enzyme that converts ACE inhibitors or sacubitril into active drugs, was also upregulated in HF kidneys. ***Conclusion:*** Chronic HF leads to latent kidney injury, associated with deep changes in kidney protein composition. These alterations may act in concert with intrarenal renin-angiotensin system activation and may serve as markers and/or targets to tackle cardiorenal syndrome.

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Introduction

Chronic heart failure (HF) affects 2-3% of adults and is the dominant cause of morbidity, mortality and health care expenditures in developed countries. Patients with chronic HF often have impaired renal function. Kidney dysfunction in HF is the critical determinant of cardiac compensation and of the rate of HF progression [1, 2]. The mechanistic relations between failing heart and kidneys are bidirectional and complex. In some situations, kidney dysfunction precede the development of HF, but the opposite – chronic HF-induced kidney injury (cardiorenal syndrome type II) [3] is more frequent. In the later scenario, reduced effective cardiac output due to HF leads to decreased renal blood flow (RBF) that is coupled with elevated venous pressure. Circulatory alterations, along with other signals, disrupt normal kidney function and lead to further neurohumoral activation, elevated oxidant stress and retention of fluids, electrolytes and toxins. These factors are increasing hemodynamic load and have negative effects on cardiac function, which further perpetuates HF state [2]. Despite the kidney injury is secondary due to the cardiac disease; it actively amplifies and propagates cardiac dysfunction [4]. Mitigation of HF-induced kidney injury could have favorable clinical impact on the course of HF.

Chronic high-output HF induced by a shunt between infrarenal aorta and inferior vena cava (aorto-caval fistula-ACF) [5, 6] is particularly useful model for studies of cardiorenal interactions. Chronic volume overload leads to profound biventricular cardiac hypertrophy, neurohumoral activation, systemic congestion, edema formation and death [6-13]. Despite systemic cardiac output is increased by high shunt flow, the "effective" cardiac output is actually reduced and redistributed away from kidneys [10, 14], leading to reduction of renal blood flow (RBF), beyond the extent explainable by lower systemic blood pressure [5]. Renal hypoperfusion than leads to reduced urinary flow and sodium retention [10]. Reduced RBF in ACF model was explained by elevated angiotensin II (ANG-II) levels and attenuated effect of endogenous vasodilators, such a nitric oxide [15] and natriuretic peptides [5]. However, more detailed biochemical and molecular basis of HF-induced induced kidney injury are unexplored in this and other animal models.

Comprehensive proteome analysis is a novel tool for exploring the changes in protein composition of tissues without a priori assumption, capable to identify potential biomarkers and therapeutic targets. This approach was previously used for analysis of HF-induced changes in the heart [13], but there are no previous studies of HF-induced changes in kidney proteome. The goal of the study was to identify characteristic changes of kidneys in animals with ACF-induced heart failure.

Materials and Methods

For details, see Supplement 1 (for all supplementary material see www.karger.com/doi/10.1159/000493657).

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 [8]. Randomly selected sham-operated animals (controls) 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, Animal protection laws of the Czech Republic (311/1997) and was approved by local ethics committee.

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). 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 (AD-instruments, Germany). The presence of ACF at the end of study was verified from laparotomy and it was present in all ACF-operated animals. 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 [10, 16, 17]. In 18 weeks after ACF induction, the animals (controls: n=9, HF: 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 the commercially available ELISA (ERA3201-1, AssayPro, MO, USA) [17]. In a randomly selected subgroup of animals surviving till 21st week, control (n=6) and HF (n=8) animals were anesthetized and right jugular vein was catheterized for fluid and drug 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 cup, and the ureter was cannulated. 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 was infused at constant infusion rate. After establishment of steady state, urine and blood samples were collected for measurements of glomerular filtration rate (GFR). Urine volume was measured gravimetrically. Polyfructosan in plasma and urine was measured colorimetrically to estimate GFR. Urinary sodium and potassium concentration was determined by flame photometry. 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 [18].

Kidney sample preparation for proteomic analysis

Frozen kidney samples were pulverized using liquid nitrogen with mortar and pestle. For all analyses we created pooled samples "Control" and "HF" by mixing 10 mg of pulverized renal tissue from each animal in the respective cohort (n=7). The pooled samples were lysed in a lysis buffer (8.4 M urea, 50 mM DTT, 5 % CHAPS) and protein concentration of supernatants was determined.

Filter assisted sample preparation (FASP) and iTRAQ labeling

Protein lysates of "HF" and "Controls" were processed by filter assisted sample preparation essentially as FASP method as described [19] each in two technical replicates according the following scheme: 114-HF (replicate 1), 115-Control (replicate 1), 116-HF (replicate 2), 117 Control (replicate 2). The iTRAQ labelling was integrated into the FASP workflow as described [20]. In total three iTRAQ analyses were performed according the same scheme.

Strong cation exchange chromatography and desalting

To remove unbound tags and to fractionate the peptides before LC-MS, off-line manual strong cation exchange chromatography was performed and peptides were eluted into 4 fractions and desalted before LC-MS analysis manual macrotap holder.

LC-MS/MS analysis

Samples were solubilized and separated using 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 Linear gradient of acetonitrile 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. Data from all fractions were analyzed using Proteome Discoverer 2.2 (Thermo Scientific) software. 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. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009296.

Table 1. Baseline characteristics – organ weights and cardiac function LV. LV: left ventricle, BW: body weight, HF: heart failure. Values are mean±SD, t-test used for comparison

Parameter	controls n=8	HF n=12	p
Organ weight			
Body weight (BW), g	560 ± 28	615 ± 58	0.014
Heart weight, g	1.60 ± 0.09	3.59 ± 0.56	< 0.0001
Heart weight/BW, g.kg^{-1}	2.86 ± 0.09	5.85 ± 0.82	< 0.0001
Left ventricle weight/BW, g.kg^{-1}	1.98 ± 0.09	3.37 ± 0.49	< 0.0001
Lung weight/BW, g.kg^{-1}	3.44 ± 0.17	5.00 ± 0.87	< 0.0001
Right and left kidney weight, g	3.51 ± 0.32	3.34 ± 0.28	0.24
Cardiac function			
Heart rate,min^{-1}	473 ± 16	372 ± 40	< 0.0001
LV Systolic blood pressure, mmHg	136 ± 25	111 ± 10	0.03
LV end-diastolic diameter, mm	6.45 ± 0.59	12.5 ± 1.39	< 0.0001
LV end-systolic diameter, mm	2.72 ± 0.41	7.89 ± 1.82	< 0.0001
LV posterior wall thickness, mm	2.39 ± 0.29	1.84 ± 0.20	0.0001
Interventricular septum thickness, mm	2.79 ± 0.30	2.09 ± 0.22	< 0.0001
LV fractional shortening, %	57.9 ± 4.02	37.5 ± 7.88	0.0001
Cardiac output, ml.min^{-1}	120 ± 35	532 ±109	< 0.0001
LV end-diastolic pressure, mmHg	5.98 ± 2.18	11.4 ± 4.66	0.004

Western blot analysis

Kidney lysates (RIPA buffer) (40 μg) were separated using conventional SDS-PAGE (MiniProtean, Bio-Rad, CA), transferred to PVDF membranes, blocked and incubated with primary antibodies for 2 hours washed and re-incubated with secondary HRP-conjugated IgG antibodies. Washed membrane was incubated with ECL detection system, 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, Fig. 1 and 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) based on triplicate iTRAQ analysis. 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 in both replicates (i.e. for both 114/115 and 116/117 ratios) and the statistical significance (adjusted p-value >0.05) was reached for both replicates.

Results

The effects of chronic volume overload from ACF on organ weights and cardiac function are summarized in the Table 1. Due to fluid retention, HF animals were heavier. Lung weights were increased in HF compared to controls due to pulmonary congestion, confirming decompensated HF. Heart weight adjusted to body weight was more than twice in HF than in control group due to biventricular eccentric hypertrophy with enlargement of ventricular volumes and relative thinning of left ventricular walls. Due to presence of systemic arteriovenous shunt, cardiac output was markedly increased, yet fractional shortening, an index of ventricular contractility, was already reduced in HF group, demonstrating presence of cardiac dysfunction. Left ventricular end-diastolic pressure almost doubled in HF group, confirming the presence of congestive HF. Weight of kidneys was similar between groups.

The impact of ACF on renal function is summarized in Fig. 1. Renal function studies demonstrated reduced renal blood perfusion (by 50%), diminished urine formation, reduced sodium and enhanced potassium excretion compared to controls, reflecting response to renal hypoperfusion. Gross markers of kidney function - glomerular filtration rate, serum creatinine and cystatin C were normal in HF group compared to controls, but HF kidneys displayed albuminuria, indicating the presence of subclinical HF-induced kidney injury. Mean blood pressure during renal studies was lower in HF animals compared to controls

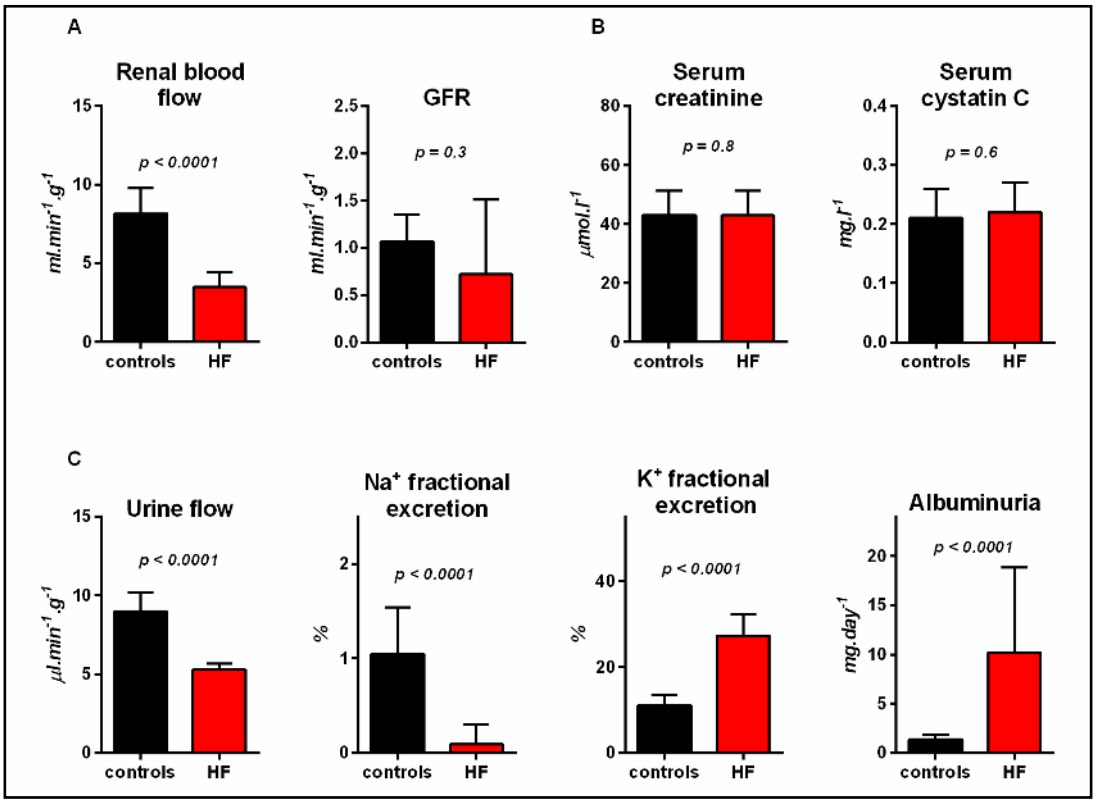
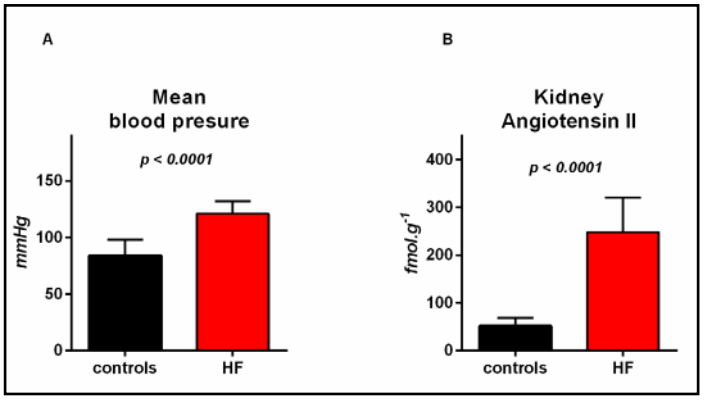


Fig. 1. Renal functions in male SD rats 21 weeks after sham procedure (controls) or after aorto-caval fistula leading to chronic heart failure (HF). The impact on renal hemodynamics and glomerular filtration (A), blood surrogates of renal function (B), urine production and composition (C). Values are mean±SD, t-test used for comparison.

Fig. 2. Blood pressure and renal angiotensin-II in controls and in HF animals. Invasively-measured mean blood pressure during renal function studies (A) and renal tissue angiotensin-II concentration (B). Values are mean±SD, t-test used for comparison.



(Fig. 2A). Tissue angiotensin II concentration in HF kidney was almost five times higher than in control group (Fig. 2B).

The impact of HF on renal proteome

To comprehensively map renal proteome and to identify HF-induced changes in protein expression, we used quadruplex iTRAQ labeling. To suppress potential inter-individual variability among animals, we analyzed pooled samples of renal tissue homogenates obtained from Control and HF animals. To ensure statistical robustness, we performed three independent analyses including sample extraction, labeling, fractionation and LC-MS analysis. Moreover, we included two replicates of Control and HF pooled samples into each

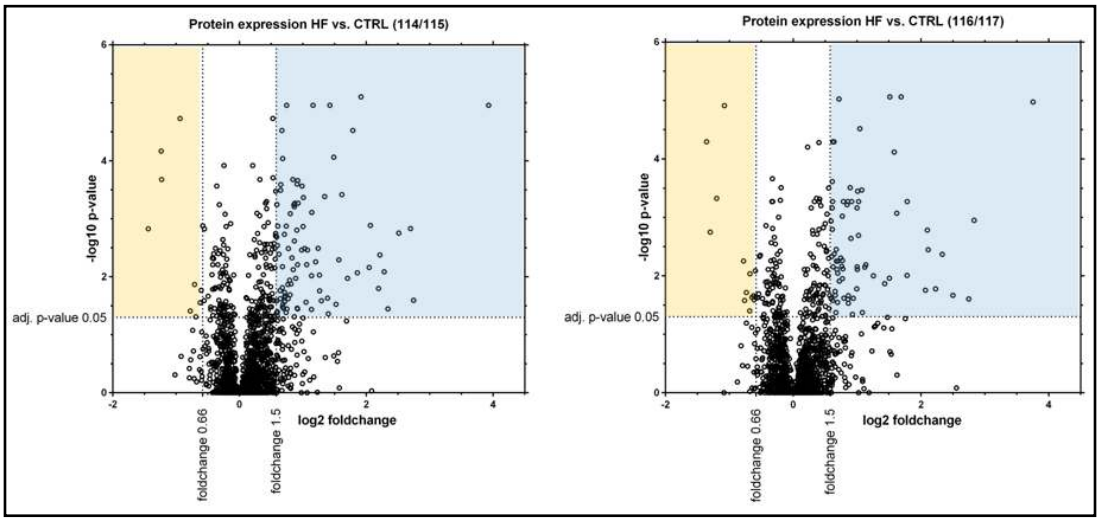


Fig. 3. Volcano graphs of differential expression of proteins in HF and control kidneys. Graph plots log2 fold-change (HF vs Controls) of individual proteins against statistical significance (-log10 adjusted p-value of the difference). Each dot represent individual protein. Dashed lines indicate values above and below of statistical significance and 1.5 fold-change.

of the three analyses using following scheme of iTRAQ tag assignment: 114-HF (replicate 1), 115-Control (replicate 1), 116-HF (replicate 2), 117-Control (replicate 2).

Our triplicate proteomic analysis (3 analyses, 4 SCX fractions each) identified 3979 master proteins with FDR 1%. 3040 of the proteins were identified by 2 or more unique peptides; only those were further considered in the quantitative evaluation. Fig. 3 shows a volcano graphs for both comparisons (114/115 and 116/117) with fold-change (HF vs Control) plotted against statistical significance. We identified 67 significantly (at least 1.5-fold change, adjusted p-value<0.05) differentially expressed proteins; abbreviated list of proteins is presented in the Table 2, full list in Supplementary Table S1. Functional annotation analysis of the differentially expressed proteins using DAVID (The Database for Annotation, Visualization and Integrated Discovery, v6.8) identified enriched G.O. terms "focal adhesion" (p-value 0.00002), "tight junction" (p-value 0.0026) and "extracellular matrix-receptor interaction" (p-value 0.005).

The differential expression of four key upregulated proteins - Angiotensin-converting enzyme (ACE), advanced glycosylation product-specific receptor (RAGE), periostin and caveolin-1 in pooled samples from HF and control animals was validated by other method - western blotting (Fig. 4).

The functional significance of individual proteins is discussed below and its association with angiotensin II is summarized in Fig. 5. In the whole proteome dataset, angiotensinogen and renin were both detected, but were not significantly different between HF and controls. Individual angiotensin variants were not observed due to their low molecular weight and because of their sequence identity with angiotensinogen.

Table 2. Abbreviated list of differentially regulated proteins in HF kidney compared to controls. For definition of significance of changes, see methods. HF: heart failure. ECM: extracellular matrix

Accession	Protein name	Relevance for kidney function	Gene Symbol	Fold-change
Proteins upregulated >1.8 fold in HF kidney				
P47820	Angiotensin-converting enzyme (ACE)	renin-angiotensin system activation	Ace	> 20
Q63495	Advanced glycosylation end product-specific receptor (RAGE,Ager)	inflammation	Ager	14,37
D3ZAF5	Periostin	ECM protein, fibrosis	Postn	6,81
P16303	Carboxylesterase 1D	ACE inhibitor activation	Ces1d	6,71
Q497C9	Microfibrillar-associated protein 4	ECM protein, fibrosis	Mfap4	5,37
Q6P792	Four and a half LIM domains 1	fibrosis, epithelial-mesenchymal transition	Fhl1	5,02
F1M957	von Willebrand factor	endothelial function	Vwf	4,84
P08932	T-kininogen 2	endothelial function	Kng1	4,63
P41350	Caveolin-1	caveolar protein, endothelial function	Cav1	4,53
G3V8L9	Cavin 1 (Polymerase I and transcript release factor)	caveolar protein, endothelial function	Ptrf	4,24
Q4V8H8	EH domain-containing protein 2	caveolar protein, endothelial function	Ehd2	3,53
G3V913	Heat shock 27kDa protein 1	stress response	Hspb1	3,50
Q9Z1H9	Cavin 3 (Caveolae-associated protein 3)	caveolar protein, endothelial function	Prkcdbp	3,25
A0A0G2JTX7	Collagen type VI alpha 5 chain	ECM protein, fibrosis	Col6a5	2,83
A0A0G2JU96	AHNAK nucleoprotein	unknown	Ahnak	2,77
D3ZY91	Chloride intracellular channel protein 3	podocytes, glomerular filtration membrane	Clic3	2,72
Q08290	Calponin-1	fibrosis	Cnn1	2,68
P14669	Annexin A3	signal transduction	Anxa3	2,66
Q9EPT8	Chloride intracellular channel protein 5	podocytes, glomerular filtration membrane	Clic5	2,30
Q6AYF8	Serpinb9	apoptosis, inflammation	Serpinb9	2,28
D3ZVM5	Heat shock protein family A (Hsp70) member 12B	endothelial function, ROS response	Hspa12b	2,24
Q3KRE2	Methyltransferase like 7A, isoform CRA_b	metabolism	Mettl7a	2,21
P55053	Fatty acid-binding protein, epidermal	binding of lipids and prostanoids	Fabp5	2,15
C0JPT7	Filamin A	ECM protein, mechanosensing	Flna	2,15
A0A0G2K2P5	Tight junction protein ZO- 1	podocytes, glomerular filtration membrane	Tjp1	2,04
Q99PD6	TGF beta-1-induced transcript 1 protein	fibrosis	Tgfb1i1	2,02
Q66H98	Cavin 2 (Caveolae-associated protein 2)	caveolar protein, endothelial function	Sdpr	2,01
Q9ESS6	Basal cell adhesion molecule	podocytes, filtration membrane	Bcam	2,00
Q4KM35	Proteasome subunit beta type-10	proteasome	Psmb10	1,90
A0A0G2JXN6	Galectin 3	ECM protein, fibrosis	Lgals3	1,90
F1M8E9	Lysozyme	innate immunity	Lyz2	1,89
Q5XIP9	Transmembrane protein 43	nuclear membrane component	Tmem43	1,88
P11762	Galectin-1	ECM, fibrosis, glomerular membrane	Lgals1	1,87
P47942	Dihydropyrimidinase-related protein 2	metabolism	Dpysl2	1,86
G3V9M6	Fibrillin 1	ECM protein	Fbn1	1,83
P10634	Cytochrome P450 2D26	lipids and prostanoids metabolism	Cyp2d2	1,82
A0A0G2K014	Lymphocyte cytosolic protein 1	actin binding, cytoskeletal component	Lcp1	1,82
Q3SWT0	Platelet endothelial cell adhesion molecule	endothelial function	Pecam1	1,81
Q5M7W5	Microtubule-associated protein 4	cytoskeletal mechanosensing	Map4	1,81
P62856	40S ribosomal protein S26	component of ribosome	Rps26	1,81
P14668	Annexin A5	signal transduction, apoptosis	Anxa5	1,81
Proteins down-regulated in HF kidney				
Q4FZV0	Beta-mannosidase	unknown	Manba	0,65
D3ZTX4	Maltase-glucoamylas	unknown	Mgam	0,64
Q5I0M3	Complement component factor h-like 1	innate immunity	Cfhr1	0,61
P07379	Phosphoenolpyruvate carboxykinase, cytosolic	metabolism	Pck1	0,43
P02761	Major urinary proteins/Alpha Alpha-2u globulin	small molecule (pheromone) transport	NA	0,41

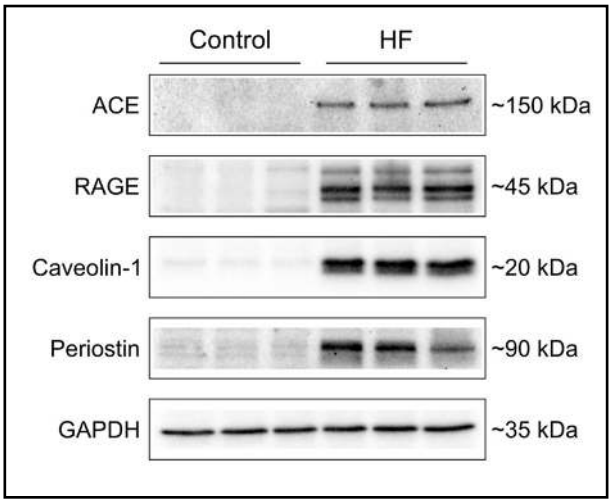


Fig. 4. Verification of expression of key differentially expressed proteins using western blots in control (left) and HF (right) kidney homogenates (triplicates of group-pooled samples). ACE: angiotensin—converting enzyme, RAGE: Advanced glycosylation end-product specific receptor, GADPH: Glyceraldehyde 3-phosphate dehydrogenase was used as internal loading control. For details and antibodies see Methods and Supplement 1.

Discussion

This study is the first systematic description of changes in renal proteome induced by presence of experimental chronic HF. Despite HF animals did not have "overt" renal dysfunction with azotemia, they have subclinical kidney injury with albuminuria and proteomic analysis already displayed extensive list of differential expressed proteins, some of them previously linked to other kidney pathologic states. This finding indicates that HF-induced kidney injury is not a transient adaptation to low renal perfusion state, but it is associated with deeper changes molecular structure of renal tissue. Interestingly, several proteins from this list is associated with enhanced intrarenal ANG-II signaling, which seems as critical mediator or HF-induced kidney changes [21, 22] and particularly in this model [23, 24]. In addition, our analysis points to other, previously unidentified pathways that act in concert with intrarenal renin-angiotensin system activation and may serve as markers and/or potential targets for therapeutic intervention of cardiorenal syndrome.

While renal perfusion and urine formation were reduced, the whole kidney glomerular filtration was still preserved in ACF animals. Sodium retention and enhanced potassium loss in HF kidney is consistent with response to low kidney perfusion due to presence of HF, mediated by renin-angiotensin system activation [22, 25]. The extent of disturbance of intrarenal hemodynamics in ACF kidney is similar to changes observed in HF model induced by myocardial infarction [22, 26], which indicates that ACF is indeed a relevant model to study cardiorenal interactions. Presence of subclinical renal injury is highlighted by the presence of albuminuria in HF animals, consistent with observation in humans with HF [27].

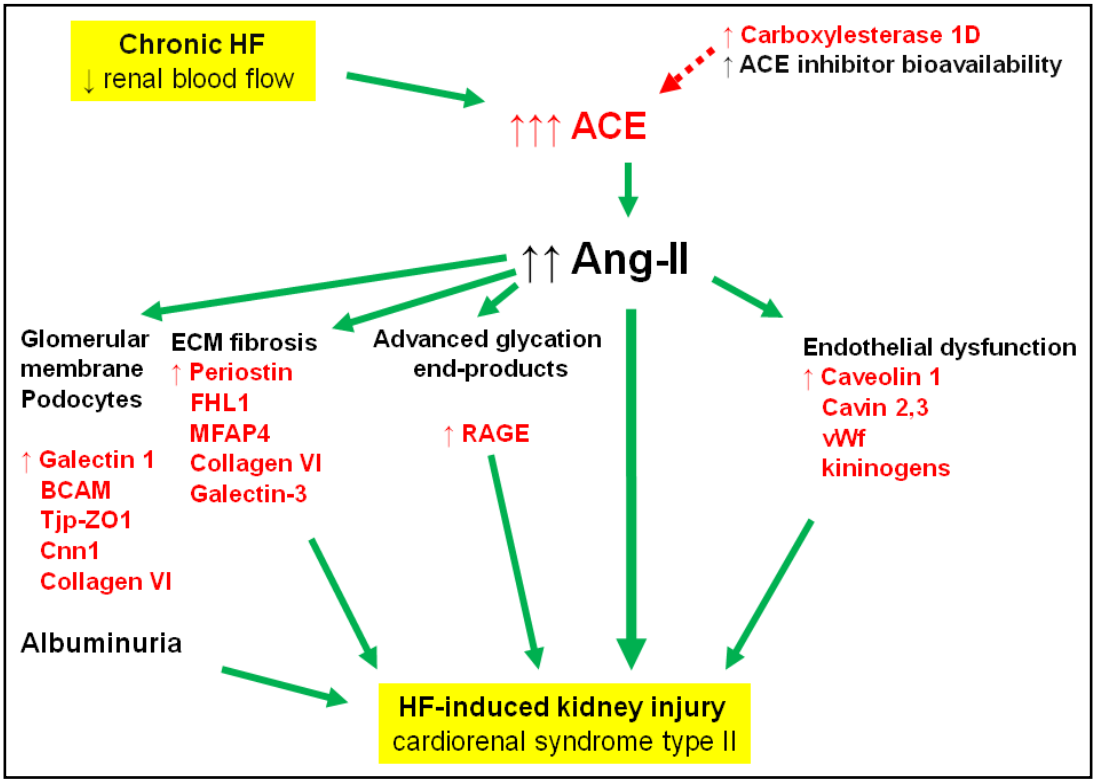


Fig. 5. Central Figure. Proposed sequence leading from chronically reduced renal perfusion to HF-induced renal dysfunction in experimental model, involving direct effect of angiotensin-II and pathways intertwined with ANG-II signaling. For abbreviations, see Table 2. Solid green arrow: positive effect, dashed red arrow: negative effect.

Renal angiotensin converting enzyme (ACE) and angiotensin II

The most differentially expressed (>20-fold upregulation) protein in HF kidneys was angiotensin-converting enzyme (ACE), the rate-limiting enzyme responsible for conversion of angiotensin-I into vasoactive angiotensin-II (ANG-II). Previous studies also demonstrated increased kidney expression of ACE in renal blood vessels, glomeruli and proximal tubules in rats with ACF [28, 29], confirming our observation. Upregulated ACE drives local ANG-II generation [29] and we observed almost 5-times higher kidney ANG-II levels than in controls. Renal microcirculation studies in rats with cardiac dysfunction due to infarct [22] or ACF [25, 26] demonstrated that glomerular and tubular functions are profoundly influenced by ANG-II and these alterations are reversible by ACE inhibition [22]. In our recent study, ACE inhibitor enhanced renal blood flow, improved urine generation, decreased renal ANG-II, decreased HF symptoms and profoundly improved survival in rats with HF due to ACF, despite not having beneficial effects on myocardial function or systemic blood pressure [10]. Enhanced ANG-II signaling appears to be the dominant molecular feature of HF-induced kidney injury (Fig. 5). Moreover, other proteins upregulated in HF kidney, such as advanced glycosylation product-specific receptor (RAGE), periostin and caveolins also show potential mechanistic links with enhanced ANG-II action.

ACE is functionally connected with kininogens, glycoproteins that are converted by kallikrein into bradykinins. Bradykinins, which are degraded by ACE, have vasodilatatory and natriuretic properties and partly mediate cardioprotective and nefroprotective actions of ACE inhibitors. We observed 4.6-fold upregulation of T-kininogen 2 in HF kidney which may be a contraregulatory response to ANG-II-mediated vasoconstriction or enhanced degradation of kinins by ACE.

Intriguing finding, potentially highly relevant for HF pharmacotherapy, is marked (6.7-fold) upregulation of carboxylesterase 1 (CES1) in HF kidneys. Carboxylesterase 1 is a serine esterase, responsible for the hydrolysis of ester- and amide-bond-containing substrates, including xenobiotics and drugs. Most of ACE inhibitors and sacubitril are ester pro-drugs that are hydrolyzed to their active metabolites just by CES1 [30]. Low CES1 activity-variant carriers may have worse therapeutic response to ACE inhibitors [31]. Elevated kidney CES1 expression in HF kidney, as observed for the first time in our study, may therefore amplify physiological effects of ACE inhibitors or sacubitril-valsartan in HF kidneys.

Advanced glycosylation product-specific receptor (RAGE)

The second most strongly upregulated protein (14-fold) was *Advanced glycosylation product-specific receptor* (RAGE). RAGE is a multiligand receptor from immunoglobulin family that binds amyloids, S100 proteins and advanced glycation end-products (AGEs) that accumulate in tissues with aging, diabetes or renal insufficiency [21, 32]. In kidneys, RAGE is expressed in podocytes, endothelial and mesangial cells and RAGE activation triggers pro-inflammatory response and promotes nephropathy [33]. Administration of ANG-II to non-diabetic rats causes RAGE activation, indicating that a cross-talk between RAGE system and ANG-II signaling participates in the pathogenesis of non-diabetic renal diseases [34]. Our study is the first to provide evidence supporting the involvement of RAGE in pathogenesis of HF-related kidney injury. Because blocking RAGE prevented progression of experimental chronic kidney disease [35], an interference with RAGE may offer a means how to tackle cardiorenal syndrome [36, 34].

Caveolae, endothelial dysfunction and angiotensin-II signaling

Among proteins markedly upregulated in HF kidneys were proteins critical for formation of caveoleae - the main caveolar structural protein caveolin 1 (CAV-1, 4.5-fold upregulated), three adapter proteins cavins 1, 2,3 and two additional caveoleae components - EH-domain containing proteins 2 and 4. Caveolae are cholesterol-rich membrane invaginations that are abundant on endothelial, epithelial and smooth muscle cells and are critical for receptor-mediated uptake, transcytosis and vascular tone regulation. Enhanced CAV-1 expression in

glomerular endothelial cells was documented in various glomerular renal diseases [37, 38]. In endothelial cells, CAV-1 directly interacts and regulates endothelial nitric oxide synthase (eNOS); sequestering of eNOS into the caveolae reduces nitric oxide (NO) generation. Impairment of NO system contributes to decrease in renal blood flow in ACF animals, despite eNOS is not downregulated [15]. Our data suggest that enhanced CAV-1 expression in ACF kidneys may contribute to endothelial dysfunction due to attenuation in the NO-mediated renal vasodilatory responses. Caveolae and caveolar proteins also play critical role in angiotensin II receptor-mediated uptake, transendothelial transport and downstream vasoconstrictive and profibrotic ANG-II effects [39]. Upregulation of CAV-1 can enhance ANG-II delivery to the point of action and its biological activity (Fig. 5).

Increased abundance of von Willebrand factor (vWF, upregulated 4.8-fold) can also reflect endothelial dysfunction in HF kidney. In eNOS knock-out mice, lack of NO led to increased kidney vWF release, early mesangial and later intraluminal vWF deposition with development of age-dependent thrombotic microangiopathy [31]. Endothelial function and integrity can be also affected by cytoskeleton-interacting proteins that are involved in mechanosensing, such as filamin A, vimentin and plectin that were upregulated in HF kidney.

Inflammatory response and fibrosis in the HF kidney

Kidneys with ACF had extensive upregulation of extracellular matrix (ECM) proteins – periostin, collagen VI, galectin-3, microfibrillar-associated protein 4 (MFAP4), fibrillin, transgelin-2 and others. Marked upregulation of periostin (6.8-fold) and collagen VI (3 upregulated variants) [40] in the kidney suggests profibrotic activation of ECM due to presence of HF. Periostin interacts with collagen and fibronectin, stimulates fibrogenesis and modulates TGF β-signaling pathway and was found upregulated in hypertensive kidney injury [41]. Periostin may serve as a potential therapeutic target, because periostin inhibition using antisense oligonucleotides was protective against renal inflammation and fibrosis in several models of kidney injury [42]. Renal upregulation (5-fold) of Four-and-half LIM domain protein 1 (FHL1) and calponin-1 [43] may suggest interstitial fibrosis due to endothelial mesenchymal transition.

HF kidney displayed upregulation of galectins – carbohydrate-binding proteins with diverse regulatory biological activities. Increased expression of galectin-3 (1.8-fold) is associated with renal or cardiac fibrosis and elevated circulating galectin-3 levels is an independent marker of poor prognosis in HF and chronic kidney disease. Pharmacological or genetic inhibition of galectin-3 alleviates organ fibrosis, which suggest its active involvement [44].

Glomerular filtration membrane defects and proteinuria

Glomerular filtration barrier is highly complex structure consisting of endothelial layer, basal membrane and podocytes. Its dysfunction leads to the loss of selectivity of glomerular filtration barrier with increased filtration of larger proteins, saturation of tubular protein degradation and ensuing enhanced loss of larger circulating proteins, mainly albumin, into the urine. Mechanisms how HF itself can induce albuminuria are incompletely understood, but elevated renal venous pressure [45], ANG-II-induced changes in renal hemodynamics [46] or alterations in glomerular electrostatic barrier function [47] were implicated. The list of proteins upregulated in HF kidney contained several proteins that take part in glomerular filtration barrier, such as tight junction protein 1 (ZO-1), galectin-1 [48], basal cell adhesion molecule (BCAM) and chloride intracellular channel protein 5 (CLIC5) [49]. We can hypothesize that upregulation of these proteins in ACF HF kidney may reflect compensatory response to glomerular filtration barrier damage, indicating that proteinuria in HF is not only due to hemodynamic changes, but also due to altered filtration barrier [47].

Proteins downregulated in ACF kidneys

From the five downregulated proteins, the most pronounced was the suppression of major urinary protein/alpha-2u globulin. This cluster of proteins includes several highly homologous extracellular proteins belonging among lipocalins. High homology of individual major urinary proteins and their poor annotation in rat precludes unequivocal identification of the particular coding gene. However, major urinary proteins serve as carriers of small molecules, including odorants and pheromones. In rodents (but not in humans) these lipocalins are secreted by the kidneys into urine in large quantities and play role in social behavior, mating and response to xenobiotics [50]; significance of these changes for kidney function in HF are not known.

Study limitations

This analysis is very in-depth, but it represents a one-time cross-sectional observation, not allowing causal inferences about changes in protein expression and organ function. We present data from the late stage (21 weeks) after ACF induction when all animals are in profound HF. It would be informative to analyze also earlier phases to better understand causal consequences. We have no morphological or immunohistochemistry data to link HF-induced changes of proteome to kidney ultrastructure. We do not know if removal of volume overload would revert changes in renal proteome. We tested changes in the whole kidney, but not separately cortex or medulla or specific renal cell populations. We cannot also discriminate whether specific protein is more expressed in the kidney, or is only more deposited from the circulation.

Conclusion

This analysis illustrates that presence of HF leads to extensive changes in renal proteome, despite glomerular filtration is still preserved. In addition to established role in enhanced intrarenal ANG-II signaling due to ACE upregulation, we identified several potential participants than can contribute to HF-induced kidney injury, either separately or in concert with renal RAS activation (such as RAGE, Periostin, Caveolin-1, von Willebrand factor and Galectin-3) or that can influence response of kidneys to common HF drugs (carboxyesterase 1).

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Disclosure Statement

The authors declare they have no conflicts of interest.

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