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Comparative proteomic analysis in microdissected renal vessels from hypertensive SHR and WKY normotensive rats

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ABSTRACT

Systemic hypertension leads to renal damage known as hypertensive nephrosclerosis without obvious clinical symptoms in the initial stages and it has a profound impact on the renal vascular physiology. Despite its major role in End Stage Renal Disease, many aspects of hypertensive nephrosclerosis remain unknown. In order to elucidate the biological pathways and macromolecules deregulated by hypertension, renal vessels were obtained by Laser Capture Microdissection (LCM) from Spontaneously Hypertensive Rats (SHR) and age-matched controls (20 weeks). Proteomic analysis was performed aiming to detect molecular alterations associated with hypertension at the renal vessels before the onset of vascular damage. This analysis identified 688 proteins, of which 58 were differentially expressed (15 up-regulated and 43 down-regulated) in SHR. Many of these proteins are involved in vascular tone regulation by modulating the activity of endothelial Nitric Oxide Synthase (eNOS) (Xaa-Pro aminopeptidase 1 (XPP1), N(G) N(G)-dimethylarginine dimethylaminohydrolase 1 (DDAH1), Dehydropteridine reductase (DHPR)) or in blood pressure control by regulating the renin-angiotensin system (Glutamyl aminopeptidase/Aminopeptidase A (AMPE), Aminopeptidase N (AMPN)). Moreover, pathway enrichment analysis revealed that the eNOS activation pathway is deregulated only in SHR. Our study demonstrates that hypertension causes early proteomic changes in the renal vessels of SHR. These changes are relevant to vascular tone regulation and consequently may be involved in the development of vascular damage and hypertensive nephrosclerosis. Further validation and interference studies to investigate potential therapeutic impact of these findings are warranted.

Keywords: Laser Capture Microdissection; Vascular proteomics; essential hypertension; Endothelial dysfunction; Spontaneously hypertensive rats.

1. Introduction

Chronic hypertension is the second leading cause of End Stage Renal Disease (ESRD) [1]. It leads to renal damage known as hypertensive nephrosclerosis without specific clinical presentation in the initial stages. Despite its major role in ESRD, many aspects of hypertensive nephrosclerosis remain unknown. For instance, alterations in the vascular wall may be involved in the pathogenesis of hypertension at an early stage, while at the same time may be considered a consequence of hypertension at a late stage [2]. Endothelial function is impaired in hypertensive individuals in several arterial beds, including the renal arteries and their branches. Whether endothelial dysfunction is a cause or consequence of hypertension has been the subject of many studies [3]. The mechanisms that lead to endothelial dysfunction may be associated with a decrease of endothelium-derived relaxing factors (EDRFs) (mainly nitric oxide) and/or an increase of various endothelium-derived constricting factors (EDCFs).

Spontaneously Hypertensive Rat (SHR) is a well-established animal model of hypertension [4]. The importance of this model has been attributed to the similarity of its pathophysiology with essential hypertension in humans [2,5]. In a recent publication, we used proteomic analysis of renal parenchyma to identify processes and organelles affected from the early stages of the development of hypertension. Among many molecules and pathways identified, we showed over-expression of the chloride channel CLIC4 in the brush border of proximal tubules and

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we suggested that this molecule could be involved in the pathogenesis of hypertension and could be used as a useful early marker jof renal tubular alterations during hypertension [6]. Other investigators have focused their attention in vascular wall of SHR and found inconsistent results regarding the role of nitric oxide (NO) and endothelial dysfunction in the development of hypertension. Despite hypertension, endothelium-dependent vasodilatory responses were similar or even augmented in SHR compared with the control normotensive animals [7]. Age, gender, the vascular type studied and methodological aspects may all contribute to the variability among various reports.

In this communication we focused our attention to the arterial wall in the kidney of SHR at an early stage of hypertension before the development of hypertensive nephrosclerosis. To this end, we combined Laser Capture Microdissection (LCM) technology and high resolution GeLC-MS/MS proteomic analysis followed by a bioinformatics approach to compare the arterial wall between SHR and their normotensive counterparts Wistar-Kyoto (WKY) rats [8]. Our results demonstrate that significant proteomic changes occur in the renal vessels of SHR compared to WKY prior to the development of structural damage and that these changes are relevant to vascular tone regulation, which eventually lead to the development of hypertensive nephrosclerosis.

2. Material and Methods

2.1. Experimental animals:

The Male WKY and SHR rats were purchased from Charles River Laboratories (Germany) and housed in the animal facility of the National Center for Scientific Research Demokritos (EL25 BIO 019020022). Male SHR rats were outbred WKY rats with marked elevation of blood pressure (Charles River from NIH in 1973). The animals were maintained in polycarbonate cages and were fed with standard rodent diet with free access to water and with a 12 h light-dark cycle. Mean blood pressure was measured using a computerized rat tail-cuff technique (Kent Scientific co, USA) as described previously [6]. Following blood pressure measurements, the animals were deeply anesthetized with ether and the kidneys were removed as reported in our previous study [6]. The experimental protocols were approved by the Institutional Animal Care and were carried out in agreement with the ethical recommendations of the European Communities Council Directive of 22 September 2010 (2010/63/EU).

2.2 Laser Capture Microdissection

Laser Capture Microdissection was performed using a PALM MicroBeam Laser System (Carl Zeiss Microscopy GmbH, Germany). The laser capture system is equipped with an UV-A laser and a computer. Frozen unfixed renal tissue samples from 20 weeks of age SHR and WKY rats (N=4 for each group) were cut into 14 μm cryosections, mounted on glass slides coated with a biochemically inert membrane (PALM Membrane slides NF 1.0 PEN, Carl Zeiss Microscopy, Germany) and lightly stained with Gill's hematoxylin according to standard procedures. Renal vessels and a limited number of glomeruli which exhibited prominent afferent and efferent arterioles were outlined with the drawing tool on the computer view of the field. The laser cut the selected area of renal parenchyma, following the drawn outline, and blew the microdissected sections off the slide by a higher energy laser pulse, to be captured onto 500-μl AdhesiveCap Touch opaque tube (Carl Zeiss Microscopy, Germany) placed over the sample area. Approximately 10,000,000 – 14,000,000 μm2 of microdissected segments were collected from each sample.

2.3. GeLC-MS analysis:

2.3.1. Sample preparation for LC-MS/MS (Liquid Chromatography coupled with tandem Mass Spectrometry):

Microdissected segments of renal parenchyma were homogenized and proteins were extracted in sample buffer (7M Urea, 2M Thiurea, 4% CHAPS, 1% DTE). Protein concentration was determined by the Bradford assay. Equal amounts of protein from each sample (6 μg) were analyzed by SDS PAGE. The electrophoresis was terminated when the samples just entered into the separating gel. In this way each sample was represented by a single band including its total protein content and processed for LC-MS/MS analysis. In brief, Coomassie Colloidal Blue-stained bands were excised from the gels and cut in small pieces (1-2 mm). Gel pieces were destained in 40% Acetonitrile, 50 mM NH4HCO3, reduced in 10 mM DTE, 100 mM NH4HCO3, and alkylated in 50 mM IAA, 100 mM NH4HCO3. Samples were dried and trypsinized overnight with 600 ng trypsin in 10 mM NH4HCO3. Peptide extraction was performed with one wash of the trypsinized gel pieces with 50 mM NH4HCO3, followed by two washes with 50% acetonitrile, 5% formic acid for 15 min at room temperature. Extracted peptides were dried and kept at -80oC.[9]

2.3.2. LC-MS/MS

Dried peptides were solubilized in 10 μL mobile phase A (0.1% formic acid) and separated on a nano HPLC Dionex Ultimate 3000 RSLS system (Dionex™, Camberly, UK). Five μL of each sample were loaded on a Dionex 0.1×20 mm, 5 μm C18 nanotrap column at a flow rate of 5 μL/min in 98% mobile phase A (0,1% formic acid) and 2% mobile phase B (100% acetonitrile, 0,1% formic acid). At a next step the sample was injected into an Acclaim PepMap C18 nanocolumn 75 μm×50 cm (Dionex™, Sunnyvale, CA, USA), at a flow rate of 0,3 μL/min. The samples were eluted with a gradient of solvent B: 2% B-80% B within 120 min LC run

time. The eluted peptides were ionized using a Proxeon nanospray ESI source, operating in positive ion mode, and injected into an Orbitrap Elite FTMS (ThermoFinnigan, Bremen, Germany). The mass-spectrometer was operated in MS/MS mode scanning from 380 to 2000 amu. The resolution of ions in MS1 was 60,000 and 15,000 for HCD MS2. The top 10 multiply-charged ions were selected from each scan for MS/MS analysis using HCD at 35% collision energy. Data analysis was performed with Proteome Discoverer 1.4 software package (ThermoScientific, Hemel Hempstead, UK), using the SEQUEST search engine and the Uniprot rat reviewed database including 7,928 entries. The search was performed using carbamidomethylation of cysteine as static and oxidation of methionine as dynamic modifications. Two missed cleavage sites, a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.05 Da were allowed. SEQUEST results were filtered for false-positive identifications.

2.3.3. Quantification and statistical analysis

Reliable protein identifications (e.g. based on at least one high confidence peptide, FDR <1%) which were present in at least 3 of the available 4 samples were retained for quantification analysis performed at the peptide level. The intensity for each protein in each sample was normalized in ppm (quotient of intensity for the particular protein to the sum of all intensities of all proteins of the specific sample multiplied with 106). The average normalized intensity for each protein was then determined for all the samples of the two groups (SHR and WKY).

As differentially expressed proteins selected for further analysis, were considered those with a fold change of > 1.5 (up-regulated in SHR compared to WKY) or < 0.66 (down-regulated in SHR compared to WKY), and a p-value of ≤ 0.05 (Mann Whitney).

A comparison of all identified proteins with the online available Rat IMCD Proteome database (https://hpcwebapps.cit.nih.gov/ESBL/Database/IMCD_Proteome_Dev/) was performed.

A heat map of differentially expressed proteins was generated using the online tool available in the link: http://www.heatmapper.ca/. The Row Z-score was calculated for each row using the formula:

$$z_i = (x_i - \bar{x})/s$$

2.4. Bioinformatics analysis

In order to unveil the pathways that may be associated with the identified proteins in both SHR and WKY animals, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using Cytoscape software [10] with the ClueGO plugin [11] using Rattus Norvegicus as organism. P-value (corrected with Bonferroni) ≤ 0.05 was set as the threshold.

3. Results

In the present study, we used LCM technology and GeLC-MS/MS analysis in order to characterize the proteomic profile of renal vessels in the SHR model compared to control. Detailed information on the experimental animals, such as blood pressure measurements and histopathology are described in our previous study [6]. The experimental approach we followed is outlined in Figure 1.

3.1. GeLC-MS/MS analysis reveals differentially expressed proteins in renal vessels of hypertensive rats compared to control

Proteomics techniques were applied to investigate the differences in the proteomic profiles of the renal vessels between SHR and WKY rats. 688 proteins were identified based on at least one high confidence peptide; of these, 550 proteins were detected in both SHR and WKY rats, 71 proteins were found exclusively in WKY and 67 exclusively in SHR rats. We refer to exclusively identified proteins as those identified in at least three biological replicates of one condition and in no replicates of the other. A comprehensive list of the identified proteins including the fold change is shown in the Supplementary Table 1. A comparison of all 688 identified proteins in renal vessels with the available Rat IMCD Proteome database revealed 517 overlapping proteins.

Of the 550 proteins identified in both SHR and WKY samples, 58 proteins were differentially expressed, of which 15 proteins were up-regulated and 43 were down-regulated in SHR (listed in Supplementary Table 2). A volcano plot representing the distribution of identified proteins according to p-value and fold change is shown in Figure 2a. Plotting the negative log10 function of the unadjusted p-value against the log2 function of fold change, visualizes all the identified

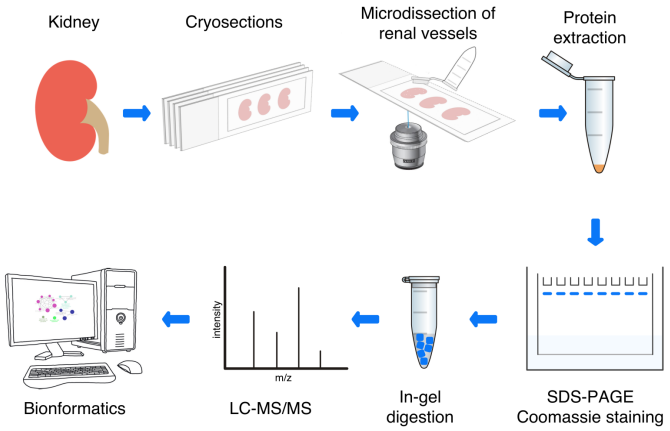

Figure 1 | A. Graphical overview of the experimental approach. Renal vessels were microdissected from kidney tissue cryosections using an UV-A laser. Extracted proteins were analyzed by SDS-PAGE and the resulting single bands were stained with Coomassie Colloidal Blue and excised from the gel. Proteins were in-gel digested and the resulting peptides were analyzed by LC-MS/MS. Identified molecules were used for further bioinformatics analysis.

proteins and the statistically significant up-regulated and down-regulated proteins. Figure 2 (b) presents a heat map of the statistically significant differentially expressed proteins.

3.2. Bioinformatics pathway analysis

Pathway enrichment analysis was performed for all proteins identified in each group in order to uncover significant biological processes relevant for blood vessels functions. 114 and 111 KEGG pathways were identified in WKY and SHR respectively and 106 pathways were common in both groups. Among the common pathways identified were pathways associated with normal functions of vascular cells e.g metabolism, oxidation, signaling, membrane trafficking, detoxification of Reactive Oxygen Species (ROS), VEGFR2 mediated cell proliferation and smooth muscle contraction. These data are presented in Supplementary Figures 1 and 2 for SHR and WKY respectively and a comprehensive list is given in the Supplementary Tables 4 and 5. It is worth mentioning that the eNOS (endothelial nitric oxide synthase) activation pathway was identified only in SHR samples. eNOS is an enzyme responsible for the generation of nitric oxide (NO) in the vascular endothelium, a key player in the regulation of the vascular tone [12].

3.3. Prominent proteomics findings:

GeLC-MS/MS analysis revealed a large number of identified proteins in renal vessels of SHR and WKY animals. The most prominent findings relevant to vascular tone and blood pressure regulation, along with the corresponding P-value and fold change are presented in Table 1. These proteins may play significant roles in the context of vascular physiology.

4. Discussion

Our study aimed to identify early proteomic changes in the renal vessels of SHR involved with pathogenesis of hypertension. The applied protocol consisted of LCM coupled to high resolution GeLC-MS/MS analysis. The high number of identified proteins verifies that this method was highly efficient and capable of identifying canonical proteomic alterations in microdissected renal parenchyma segments of SHR animals compared to controls. Furthermore, a comparison of our data with the available Rat IMCD Proteome database revealed 75% (517 out of 688 proteins of our dataset) overlap of identified proteins in renal vessels with the IMCD database. A recent report on LCM coupled with LC-MS/MS in the study of mouse brain sections following a protocol comparable to our approach resulted in the identification of a similar number of proteins (on average 322 proteins) in the microdissected tissue samples [13]. However, the application of the SP3 method (single-pot solid-phase-enhanced sample preparation) yielded a significantly higher number of protein identifications, on average 5744.

In the SHR model, alterations in vascular structure and

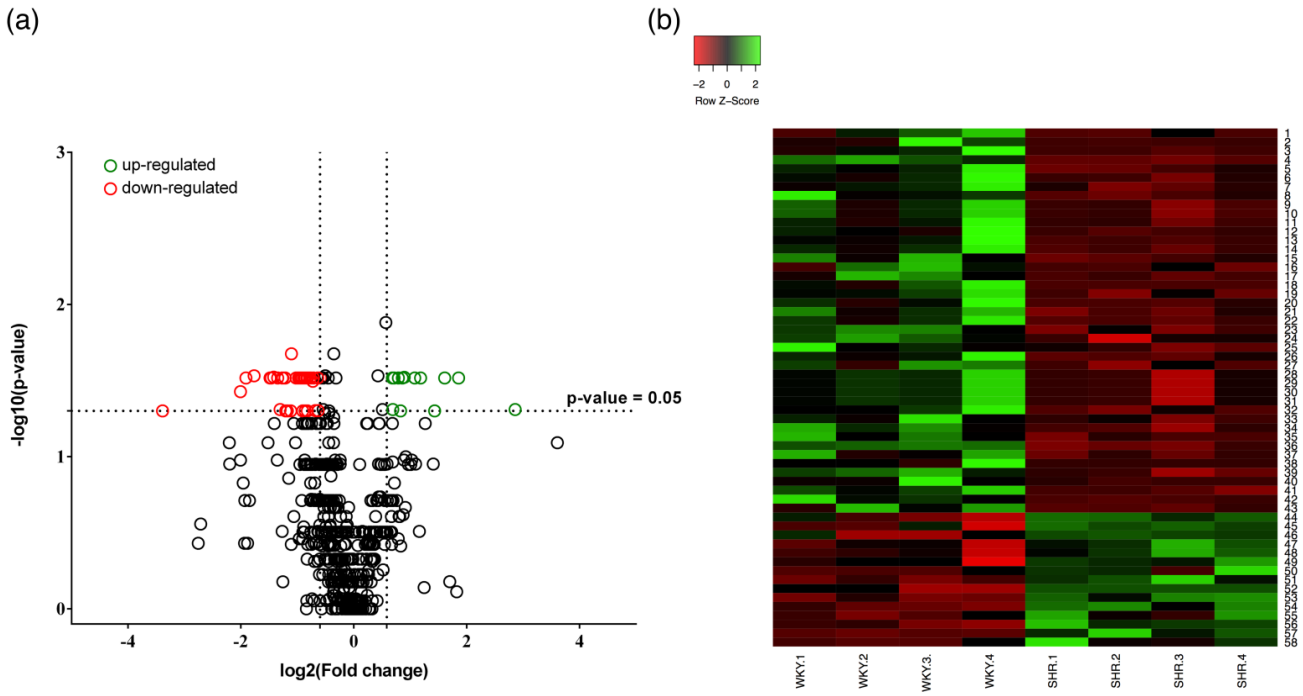

Figure 2 | (a) Volcano plot of proteomic data, presenting the negative log10 of P-value against the log2 of the fold change. All the proteins with fold change <1 have negative X-values and the proteins with fold change >1 have positive X-values. The proteins with fold change > 1.5 and P-value ≤ 0.05 are considered significantly up-regulated in SHR in comparison to controls. The proteins with fold change < 0.66 and P-value ≤ 0.05 are considered significantly down-regulated in SHR. (b) Heat map of 58 differentially expressed proteins. A list of proteins along with their normalized area and the corresponding protein name used in the heat map is presented in the Supplementary Table 3.

Table 1 | Differentially expressed proteins relevant to vascular tone and blood pressure regulation.

Gene	Protein	p-value	Fold change (SHR/WKY)	Function	Reference
Qdpr	Dihydropteridine reductase	0.03	3.05	BH4 regeneration	[32,33]
Ddah1	N(G), N(G)-dimethylarginine dimethylaminohydrolase 1	0.05	1.77	ADMA hydrolase	[34]
Cfl1	Cofilin-1	0.03	1.6	Cytoskeletal remodeling	[35]
Xpnpep1	Xaa-Pro aminopeptidase 1	0.05	0.64	Bradykinin degradation	[23,24]
Pah	Phenylalanine-4-hydroxylase	0.04	0.62	Tyrosine generation	[36]
Enpep	Glutamyl aminopeptidase/ Aminopeptidase A	0.05	0.53	Metabolism of AngII	[20]
Anpep	Aminopeptidase N	0.03	0.37	Metabolism of AngIII	[19]
Calb1	Calbindin	0.05	0.46	Calcium-binding protein	[37]
Calb2	Calretinin	0.05	0.55	Calcium-binding protein	[38]
Phb	Prohibitin	0.03	0.24	ROS formation	[39,40]
Phb2	Prohibitin-2	0.03	0.43	Mitochondrial function	[41]

functions related to hypertension are not present in young animals but are observed in adult animals (older than 25 weeks) [7]. In the present study, focusing on an early interval, we provide evidence that early molecular changes occur in the renal vessels of SHR animals at 20 weeks of age. The proteomic analysis yielded 58 proteins differentially expressed between SHR and WKY animals that play important roles in the context of vascular dysfunction.

Many of the interesting differentially expressed proteins identified in our study are relevant to vascular tone regulation. Thus, proteins involved with NO and vasodilation and affecting eNOS include Xaa-Pro aminopeptidase 1 (XPP1), N(G) N(G)-dimethylarginine dimethylaminohydrolase 1 (DDAH1), Dehydropteridine reductase (DHPR), whereas proteins affecting vasoconstriction and blood pressure regulation by renin-angiotensin system [14] include Glutamyl aminopeptidase/ Aminopeptidase A (AMPE), Aminopeptidase N (AMPN). These molecules may be involved in the pathogenesis of hypertensive nephropathy.

Angiotensin metabolism is critical for blood pressure regulation since in addition to Ang II, peptide fragments derived from this hormone also have diverse and important physiological roles. Ang III has been shown to be a major effector peptide; in the intrarenal Renin–Angiotensin system it has been reported to increase angiotensinogen levels and TGF-β, fibronectin and monocyte chemoattractant protein-1 gene expression [15,16]. Furthermore, Ang III formation is critical for the AT2 receptor-mediated natriuresis in rats contributing in reduction of blood pressure [17] and endothelial metabolism of Ang II to Ang III boosts the vasorelaxation response in adrenal cortical arteries [18]. In our analysis, both AMPE and AMPN responsible for Ang III and Ang IV formation respectively [19,20], were down-regulated in SHR most likely enhancing the vasoconstrictor effect of Ang II, decreasing the Ang III-induced sodium excretion [17], and thus inducing hypertension. These results are consistent with the high mean blood pressure (160 - 170 mmHg) measured in SHR animals used in our study [6].

Nevertheless, despite the established high blood pressure, our analysis revealed several differentially expressed molecules that may have a protective role against hypertension. In other words, defensive mechanisms may be activated in the renal vessels of SHR at an early phase of the development of hypertension, possibly to counteract the vasoconstricting effects of Ang II and other constricting substances. In this regard, other investigators have studied the role of NO in hypertension and kidney damage in SHR and found elevated levels of iNOS in SHR, but not in WKY rats [21,22]. Molecules such as XPP1, DDAH1 and DHPR may affect the synthesis of endothelium-derived relaxing factors (EDRFs) increasing NO levels and vasodilatation. In this regard, XPP1 was found to be down-regulated in SHR compared to WKY reducing the degradation of bradykinin which stimulates endothelial cells to produce and release EDRFs that cause blood vessels to dilate and therefore reduce blood pressure [23,24]. Other protective molecules, such as DDAH1 and DHPR appear to be up-regulated in SHR presumably as a defense mechanism to hypertension.

DDAH 1 is a methylarginine-metabolizing enzyme that reduces asymmetric dimethylarginine (ADMA) levels. ADMA is an endogenous compound derived from the proteolysis of proteins containing methylated arginine residues that inhibits nitric oxide synthase (NOS) catalytic activity. Moreover, ADMA is accumulated in plasma during CKD [25] and reduced renal tubular ADMA synthesis protects against progressive kidney function decline [26]. DHPR is an enzyme that catalyzes the regeneration of tetrahydrobiopterin (BH4). BH4 is a cofactor of a set of enzymes including NOS and plays an essential role in neurovascular homeostasis and has anti-inflammatory activity [27]. DHPR converts BH2 to BH4 which is necessary for NO production by NOS. If BH4 is insufficient, NOS becomes uncoupled and generates superoxide [28,29]. The up-regulated DHPR in SHR model favors the regeneration of BH4 allowing the enzymatic synthesis of NO.

Collectively, the validity of our proteomic results is supported by the relevance of the proteins identified as differentially expressed in the context of hypertension. The trend observed for the differentially expressed proteins is supported by related literature and existing knowledge on regulatory mechanisms of blood pressure (e.g. renin-angiotensin system [14-17]). An overview of these molecules and their putative action on vascular tone and consequently blood pressure regulation is presented in Figures 3 and 4.

Using LCM for renal research provides an important advantage. It gives us the ability to separate different compartments and sub-compartments of renal parenchyma to almost total purity. This is not the case with already described methods that employ different types of sieves in order to separate glomeruli from tubules [30]. In addition, these methodologies are expensive and cannot discriminate between different types of tubular compartments (e.g. proximal vs. distal) [31]. LCM has been used in our study for proteomic analysis but it can also be useful for in depth transcriptomic analysis of different renal compartments in the future. Therefore, use of LCM material in future studies will lead to a more in depth understanding of renal pathophysiology.

5. Concluding Remarks

In summary, combining LCM technology and high resolution GeLC-MS/MS analysis in the renal blood vessels of SHR, a well-established model of hypertension, we offer insights on proteomic profiles of renal vessels with regard to hypertension. Significant early proteomic changes occur in the renal blood vessels of SHR compared to WKY before the onset of vascular dysfunction and structural damage. Whether these changes are of decisive importance in the development of hypertensive nephropathy and endothelial dysfunction due to hypertension needs to be investigated further. Moreover, our data suggest that AMPE, AMPN, XPP1, DDAH1, DHPR are moderators and mediators of renal vascular tone modulation contributing in blood pressure regulation. These molecules may be potentially important therapeutic targets for the hypertensive nephropathy and should be studied further.

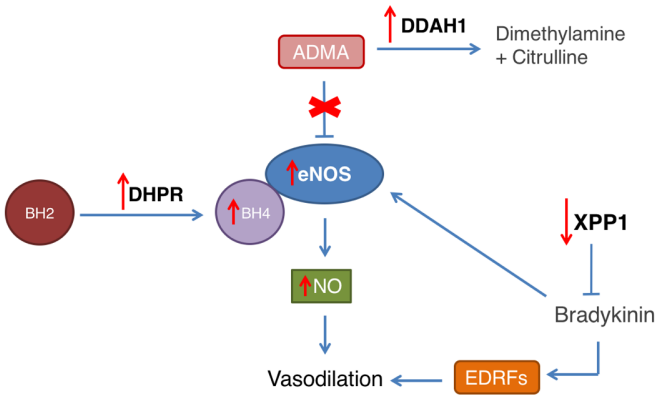

Figure 3 | Putative activation of vasodilation mechanisms via NO and EDRFs production. ADMA: asymmetric dimethylarginine, BH2: dihydrobiopterin, BH4: tetrahydrobiopterin, DDAH1: dimethylarginine dimethylaminohydrolase 1, DHPR: dehydropteridine reductase, EDRFs: endothelium-derived relaxing factors, eNOS: endothelial nitric oxide synthase, NO: nitric oxide, XPP1: Xaa-Pro aminopeptidase 1.

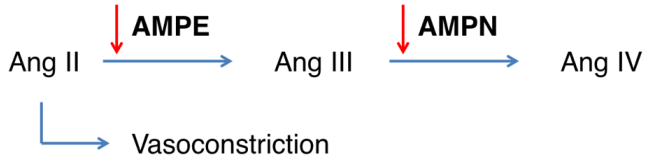

Figure 4 | Putative activation of vasoconstriction mechanism via Angiotensin II (AngII). Down-regulated Aminopeptidases A (AMPE) and N (AMPN) in SHR lead to impaired Ang II and Ang III degradation respectively, enhancing possibly the levels and the vasoconstrictor effect of Ang II.

6. Supplementary material

Supplemetary Figure 1: Pathway analysis of all identified proteins in SHR

Supplementary Figure 2: Pathway analysis of all identified proteins in WKY

Supplementary Table 1: List of all identified proteins

Supplementary Table 2: List of statistically significant differentially expressed proteins in renal vessels of SHR compared to WKY

Supplementary Table 3: List of differentially expressed proteins presented in Figure 2 (b) along with their normalized area per sample and the corresponding ascending number.

Supplementary Table 4: SHR pathway analysis

Supplementary Table 5: WKY pathway analysis

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [40] partner repository with the dataset identifier PXD008792.

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References

1] Kopp JB; Rethinking hypertensive kidney disease: arterionephrosclerosis as a genetic, metabolic, and inflammatory disorder. Current opinion in nephrology and hypertension 2013;22:266-272.

2] Hultstrom M, Development of structural kidney damage in spontaneously hypertensive rats. Journal of hypertension 2012;30:1087-1091.

3] Bernatova I, Endothelial dysfunction in experimental models of arterial hypertension: cause or consequence? BioMed research international 2014;2014:598271.

4] Dornas WC, Silva ME, Animal models for the study of arterial hypertension. Journal of biosciences 2011;36:731-737.

5] Mullins LJ, Conway BR, Menzies RI, Denby L, Mullins JJ: Renal disease pathophysiology and treatment: contributions from the rat. Disease models & mechanisms 2016;9:1419-1433.

6] Hatziioanou D, Barkas G, Critselis E, Zoidakis J, Gakiopoulou H, Androutsou ME, Drossopoulou G, Charonis A, Vlahakos DV: Chloride Intracellular Channel 4 Overexpression in the Proximal Tubules of Kidneys from the Spontaneously Hypertensive Rat: Insight from Proteomic Analysis. Nephron 2017

7] Bernatova I, Conde MV, Kopincova J, Gonzalez MC, Puzserova A, Arribas SM: Endothelial dysfunction in spontaneously hypertensive rats: focus on methodological aspects. Journal of hypertension Supplement : official journal of the International Society of Hypertension 2009;27:S27-31.

8] Datta S, Malhotra L, Dickerson R, Chaffee S, Sen CK, Roy S: Laser capture microdissection: Big data from small samples. Histology and histopathology 2015;30:1255-1269.

9] Makridakis M, Vlahou A: GeLC-MS: A Sample Preparation Method for Proteomics Analysis of Minimal Amount of Tissue. Methods in molecular biology 2018;1788:165-175

10] Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T: Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome research 2003;13:2498-2504.

11] Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, Fridman WH, Pages F, Trajanoski Z, Galon J: ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 2009;25:1091-1093.

12] Siragusa M, Fleming I: The eNOS signalosome and its link to endothelial dysfunction. Pflugers Archiv : European journal of physiology 2016;468:1125-1137.

13] Dilillo M, Pellegrini D, Ait-Belkacem R, de Graaf EL, Caleo M, McDonnell LA: Mass Spectrometry Imaging, Laser Capture Microdissection, and LC-MS/MS of the Same Tissue Section. Journal of proteome research 2017;16:2993-3001.

14] Ferrao FM, Lara LS, Lowe J: Renin-angiotensin system in the kidney: What is new? World journal of nephrology 2014;3:64 -76.

15] Ruiz-Ortega M, Lorenzo O, Egido J: Angiotensin III increases MCP-1 and activates NF-kappaB and AP-1 in cultured mesangial and mononuclear cells. Kidney international 2000;57:2285-2298.

16] Ruiz-Ortega M, Lorenzo O, Egido J: Angiotensin III up-regulates genes involved in kidney damage in mesangial cells and renal interstitial fibroblasts. Kidney international Supplement 1998;68:S41-45.

17] Padia SH, Kemp BA, Howell NL, Fournie-Zaluski MC, Roques BP, Carey RM: Conversion of renal angiotensin II to angiotensin III is critical for AT2 receptor-mediated natriuresis in rats. Hypertension 2008;51:460-465.

18] Kopf PG, Campbell WB: Endothelial metabolism of angiotensin II to angiotensin III, not angiotensin (1-7), augments the vasorelaxation response in adrenal cortical arteries. Endocrinology 2013;154:4768-4776.

19] Danziger RS: Aminopeptidase N in arterial hypertension. Heart failure reviews 2008;13:293-298.

20] Reaux A, Iturrioz X, Vazeux G, Fournie-Zaluski MC, David C, Roques BP, Corvol P, Llorens-Cortes C: Aminopeptidase A, which generates one of the main effector peptides of the brain renin-angiotensin system, angiotensin III, has a key role in central control of arterial blood pressure. Biochemical Society transactions 2000;28:435-440.

21] Chou TC, Yen MH, Li CY, Ding YA: Alterations of nitric oxide synthase expression with aging and hypertension in rats. Hypertension 1998;31:643-648.

22] Huang CF, Hsu CN, Chien SJ, Lin YJ, Huang LT, Tain YL: Aminoguanidine attenuates hypertension, whereas 7-nitroindazole exacerbates kidney damage in spontaneously hypertensive rats: the role of nitric oxide. European journal of pharmacology 2013;699:233-240.

23] Dendorfer A, Wolfrum S, Wagemann M, Qadri F, Dominiak P: Pathways of bradykinin degradation in blood and plasma of normotensive and hypertensive rats. American journal of physiology Heart and circulatory physiology 2001;280:H2182

-2188.

24] Ersahin C, Simmons WH: Inhibition of both aminopeptidase P and angiotensin-converting enzyme prevents bradykinin degradation in the rat coronary circulation. Journal of cardiovascular pharmacology 1997;30:96-101.

25] Mihout F, Shweke N, Bige N, Jouanneau C, Dussaule JC, Ronco P, Chatziantoniou C, Boffa JJ: Asymmetric dimethylarginine (ADMA) induces chronic kidney disease through a mechanism involving collagen and TGF-beta1 synthesis. The Journal of pathology 2011;223:37-45.

26] Tomlinson JA, Caplin B, Boruc O, Bruce-Cobbold C, Cutillas P, Dormann D, Faull P, Grossman RC, Khadayate S, Mas VR, Nitsch DD, Wang Z, Norman JT, Wilcox CS, Wheeler DC, Leiper J: Reduced Renal Methylarginine Metabolism Protects against Progressive Kidney Damage. Journal of the American Society of Nephrology : JASN 2015;26:3045-3059.

27] Rivera JC, Noueihed B, Madaan A, Lahaie I, Pan J, Belik J, Chemtob S: Tetrahydrobiopterin (BH4) deficiency is associated with augmented inflammation and microvascular degeneration in the retina. Journal of neuroinflammation 2017;14:181.

28] Grobe AC, Wells SM, Benavidez E, Oishi P, Azakie A, Fineman JR, Black SM: Increased oxidative stress in lambs with increased pulmonary blood flow and pulmonary hypertension: role of NADPH oxidase and endothelial NO synthase. American journal of physiology Lung cellular and molecular physiology 2006;290:L1069-1077.

29] Kim HL, Park YS: Maintenance of cellular tetrahydrobiopterin homeostasis. BMB reports 2010;43:584-592.

30] Liu X, Fan Q, Yang G, Liu N, Chen D, Jiang Y, Wang L: Isolating glomeruli from mice: A practical approach for beginners. Experimental and therapeutic medicine 2013;5:1322-1326.

31] Takemoto M, Asker N, Gerhardt H, Lundkvist A, Johansson BR, Saito Y, Betsholtz C: A new method for large scale isolation of kidney glomeruli from mice. The American journal of pathology 2002;161:799-805.

32] Ponzone A, Spada M, Ferraris S, Dianzani I, de Sanctis L: Dihydropteridine reductase deficiency in man: from biology to treatment. Medicinal research reviews 2004;24:127-150.

33] Lee CK, Han JS, Won KJ, Jung SH, Park HJ, Lee HM, Kim J, Park YS, Kim HJ, Park PJ, Park TK, Kim B: Diminished expression of dihydropteridine reductase is a potent biomarker for hypertensive vessels. Proteomics 2009;9:4851-4858.

34] Tojo A, Welch WJ, Bremer V, Kimoto M, Kimura K, Omata M, Ogawa T, Vallance P, Wilcox CS: Colocalization of demethylating enzymes and NOS and functional effects of methylarginines in rat kidney. Kidney international 1997;52:1593-1601.

35] Wang QZ, Gao HQ, Liang Y, Zhang J, Wang J, Qiu J: Cofilin1 is involved in hypertension-induced renal damage via the regulation of NF-kappaB in renal tubular epithelial cells. Journal of translational medicine 2015;13:323.

36] Flydal MI, Martinez A: Phenylalanine hydroxylase: function, structure, and regulation. IUBMB life 2013;65:341-349.

37] Lee CT, Ng HY, Lee YT, Lai LW, Lien YH: The role of calbindin -D28k on renal calcium and magnesium handling during treatment with loop and thiazide diuretics. American journal of physiology Renal physiology 2016;310:F230-236.

38] Chang IY, Yoon SP: The changes of calretinin immunoreactivity in paraquat-induced nephrotoxic rats. Acta histochemica 2012;114:836-841.

39] Zheng H, Lu GM: Reduction of prohibitin expression contributes to left ventricular hypertrophy via enhancement of mitochondrial reactive oxygen species formation in spontaneous hypertensive rats. Free radical research 2015;49:164-174.

40] Zhou TB, Qin YH, Lei FY, Huang WF, Drummen GP: Prohibitin attenuates oxidative stress and extracellular matrix accumulation in renal interstitial fibrosis disease. PloS one 2013;8:e77187.

41] Ising C, Bharill P, Brinkkoetter S, Brahler S, Schroeter C, Koehler S, Hagmann H, Merkwirth C, Hohne M, Muller RU, Fabretti F, Schermer B, Bloch W, Kerjaschki D, Kurschat CE, Benzing T, Brinkkoetter PT: Prohibitin-2 Depletion Unravels Extra-Mitochondrial Functions at the Kidney Filtration Barrier. The American journal of pathology 2016;186:1128-1139.