Published in final edited form as:

Sci Signal. ; 12(611): . doi:10.1126/scisignal.aax9760.

Metabolic rewiring of the hypertensive kidney

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Abstract

Hypertension is a persistent epidemic across the developed world that is closely associated with kidney disease. Here, we applied a metabolomics, phosphoproteomics and proteomics strategy to analyze the effect of hypertensive insults on kidneys. Our data revealed the metabolic aspects of hypertension-induced glomerular sclerosis, including lipid breakdown at early disease stages and activation of anaplerotic pathways to regenerate energy equivalents to counter stress. For example, branched-chain amino acids and proline, required for collagen synthesis, were depleted in glomeruli at early time points. Furthermore, indicators of metabolic stress were reflected by low amounts of ATP and NADH and an increased abundance of oxidized lipids derived from lipid breakdown. These processes were specific to kidney glomeruli where metabolic signaling occurred through mTOR and AMPK signaling. Quantitative phosphoproteomics combined with

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AUTHOR CONTRIBUTIONS: MMR, OP, AS and GS conceptualized the study. Investigation and experiments were performed by MMR, OP, CG, AP, NPE, BW, JUB, XD, JSR. Analysis was done by MMR, CG, NPE, BW, JUB, XD, JSR. The original draft was written by MMR, and writing, review, editing, and final approval was performed all authors. Resources and supervision were provided by AS and GS.

COMPETING INTERESTS: The authors declare that they have no competing interests.

DATA AND MATERIALS AVAILABILITY: The raw mass spectrometry data of this study has been deposited in PRIDE/ProteomXchange (45) with the following identifiers: PXD007940 (for the proteomics data) and PXD007937 (for the phosphoproteomics data). All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

computational modelling suggested that these processes controlled key molecules in glomeruli and specifically podocytes, including cytoskeletal components and GTP-binding proteins, which would be expected to compete for decreasing amounts of GTP at early time points. As a result, glomeruli showed increased expression of metabolic enzymes of central carbon metabolism, amino acid degradation, and lipid oxidation, findings observed in previously published studies from other disease models and patients with glomerular damage. Overall, multi-layered omics provides an overview of hypertensive kidney damage and suggests that metabolic or dietary interventions could prevent and treat glomerular disease and hypertension-induced nephropathy.

INTRODUCTION

According to the American Heart Association, 116.4 million (46%) adults in the United States have hypertension(1). Although long-term high salt intake increases the risk for hypertension and associated cardiovascular and chronic kidney disease (CKD)(2, 3), the specific mechanisms underlying salt-induced changes in blood pressure and kidney injury are poorly understood. Hypertension causes one-third of chronic kidney disease but its prevention and treatment are largely unmet. Kidney diseases affect more than one out of ten persons in developed countries, potentiate cardiovascular risk, and lead to a large socioeconomic burden.

Kidneys regulate body metabolism by filtering urine through the glomerulus then reabsorbing nutrients, a role that make them a central metabolic organ. The glomerulus is the filtration unit of the kidney that is frequently viewed as a passive filter that limits protein passage through size exclusion yet remain permeable for small molecules. Hypertension is thought to damage glomeruli of the kidney, resulting in increased protein in the urine, a hallmark of kidney disease(4).

To investigate kidney disease, we employed a multi-omic strategy, integrating metabolomics, phosphoproteomics and proteomics. Among all omics dimension, the metabolome is the most downstream and its relevance for the understanding (and regulation of) physiological mechanisms is only beginning to be understood. For example, the metabolome modulates phenotypes by interacting with the other omic levels including the genome, transcriptome, proteome(5, 6), and the posttranslationally modified proteome (7). Here, we investigated in a well-established model of hypertension and proteinuria (the Dahl salt-sensitive (DSS) rat) the metabolome changes that preceded the proteome and phosphoproteome in a sub-tissue specific manner. This naturally occurring model of salt-sensitive hypertension recapitulates many aspects of progressive human hypertension, providing key insight into mechanisms underlying salt-sensitivity(8). In this study, we uncovered key pathways and mechanisms that were controlled by the metabolome and were related to physiological functions and omic perturbations not commonly thought to be metabolically controlled, providing a window into hypertension-induced kidney molecular rewiring and its categorization as a metabolic disease.

RESULTS

Untargeted metabolome analysis of Dahl salt sensitive rats reveals lipid breakdown and branched-chain amino acids in glomeruli, but not in the tubules

DSS rats develop hypertension and salt-induced nephropathy when placed on a high salt diet. The effect of salt on blood pressure can be described in two phases. An initial increase in blood pressure observed in the first week on a high salt diet is followed by the subsequent rise of blood pressure that is accompanied by renal injury. Therefore, we performed an untargeted metabolome analysis of glomeruli and tubules freshly isolated from DSS rats when they were switched from normal (0.4%) to high (4%) salt diets for 7 and 21 days, respectively. Upon induction of hypertension, we found an increase in albuminuria (Fig. 1A). Tissue or glomeruli damage was not detectable at week 1 (Fig. 1B). In contrast, at week 3, strong albuminuria was observed and substantial tissue damage affected both the cortex tubular tissue and the glomeruli. Untargeted metabolomic profiles were generated from both the glomeruli and the cortical fraction, which mainly contained proximal tubules. We performed metabolite extraction and analysis through UPLC/MS experiments for both tissues. In-source fragment annotation using the METLIN-guided In-Source Annotation (MISA) algorithm identified 900 putative metabolite features that were further hierarchically clustered using Euclidean distance(9). The correlation matrix of features across the animals of the study was different between tubule and glomerular datasets (Fig. 1C). Metabolomic analysis also showed that high m/z features (such as complex lipids) around 600–800 Da were generally negatively correlated with proteinuria detected in the same rat (Fig. 1D). Subsequently, metabolites from tubules and glomeruli from rats on the high-salt diets after days 7 and 21 were compared to those from control animals (Data File S1, Supplemental Figure S5). Untargeted analysis revealed that proline and the branched chain amino acid leucine were decreased in glomeruli, but not tubules (Fig. 2A). In tubules, these amino acids were not regulated, but other amino acids, such as aromatic (phenylalanine), cationic and polar amino acids were decreased. Quantification of amino acid derivatives revealed a decrease in the branched chain metabolite acetylleucine, acetylvaline and acetylproline in the glomerulus that was not present in the tubules of the same rats (Fig. 2B). We observed increased glucose 6-phosphate and decreased glyceraldehyde 3-phosphate after three weeks that was again present only in glomeruli but not in tubules (Fig. 2C). In addition, the free fatty acids stearic acid, oleic acid, and linoleic acid, as well as acylcarnitines, were decreased after 3 weeks of high salt, again chiefly in glomeruli (Fig. 2D). Other metabolites, such as choline and creatinine, were strongly regulated in tubules, but not in glomeruli (Fig. 2E). Thus, untargeted metabolite analysis suggested alterations in branched chain amino acid, proline and lipid metabolism in glomeruli, whereas tubules largely showed decreased amino acid content.

Targeted metabolome analysis reveals oxidative stress and energy-equivalent metabolite depletion predominantly in glomeruli

A targeted metabolic analysis of glomeruli and tubules was next performed. Glomeruli showed decreases in products of central carbon metabolism including citrate/isocitrate, α -ketoglutarate, and succinate that occurred at the early time point, suggesting a general depletion of TCA cycle metabolites. Notably, these changes were not significantly altered in

tubules despite a similar trend in many TCA cycle metabolites (Fig. 3A). The pyruvate/lactate ratio was not changed in either tissue (Fig. 3B). Both the NADH/NAD⁺ and GTP/GDP ratios were decreased only in glomeruli after day 7 (Fig. 3C and 3D). The ATP/ADP ratio was significantly reduced at day 21 (Fig. 3E). In addition, the oxidized lipids 5-HETE, 9-HETE and 15-HETE were increased in glomeruli but not in tubules (Fig. 3F). These results suggest that in hypertensive rats, metabolic stress is present chiefly in glomeruli, but not in tubules (Data File S2).

Depleted metabolites trigger mTOR and AMPK rewiring

The observed metabolomic changes could directly contribute to altered phosphoprotein-dependent signaling. Therefore, mass spectrometry-based phosphoproteomics analysis was performed on the same animal model, specifically on glomeruli. 90 phosphopeptides out of the approximately 3000 quantified phosphosites were regulated at day 7, but only 5 phosphopeptides were regulated at day 21 (Fig. 4A and 4B, Data File S3). Notably, after 1 week, we found a decrease in the phosphorylation of an inhibiting site of Prkaa2 (the catalytic subunit of AMP-activated protein kinase (AMPK)), suggesting activation of AMPK, and in the phosphorylation of an activating site of raptor (RPTOR), a protein that activates mTOR Complex 1 (mTORC1). These results suggested that metabolite controlled signaling occurs, because a decreased ADP/ATP ratio (Fig. 3E) would be expected to activate AMPK (as indicated by decreased phosphorylation at Ser⁴⁹¹), and decreased leucine inhibits mTOR, as indicated by a reduced phosphorylation of RPTOR at Ser⁷²².

Quantification of the temporal trajectories of the phosphoproteomics perturbations showed that two clusters of phosphopeptides were increased in a manner that correlated with hypertensive damage. Position-weighted matrices of amino acid frequency over the UniProt background suggested a preference of basophilic sites in this group (Fig. 4C). For a more accurate annotation, we performed enrichment analysis that uncovered an overrepresentation of motifs for AMPK and for related kinases such as GSK and B-HMG-CoA-reductase kinase, the kinase that inhibits the key enzyme in cholesterol synthesis (Fig. 4C). Finally, we performed quantitative phosphoproteomic modeling of the two kinases with the most evidence for activation, namely Raptor and AMPK, using the PHONeMeS algorithm (10) (Fig. 5). The modeling showed a stronger preference for these kinases to target functional cytoskeletal parts at early time points. Notably, targets of the metabolic signaling were the GTP binding protein RCC1 at Ser¹¹, resulting in enhanced activity (11); cofilin at Ser³, a site involved in actin bundling; and PDK1 at an activating site, suggesting that metabolic signaling controls key pathophysiological pathways in the glomerulus.

Proteomics analysis shows that alterations in metabolite-processing enzymes are a conserved feature in glomerular disease

We then asked whether the observed changes were mainly due to changes in protein expression. Proteomic analysis of the same samples revealed that protein abundance was altered to a lesser extent compared to phosphorylation, suggesting that phenotypes were modulated more by changes in metabolic signaling than those in protein abundance. We found that 90 proteins were altered in abundance a statistically significant manner, including collagen type 6, which is proline-rich. Complement proteins were not strongly increased in abundance the glomerulus which was confirmed by complement staining (Supplemental

Figure 1, A). Proteins in the branched chain amino acid catabolic pathway were increased in abundance, including the branched chain aminotransferase BCAT2 and hexokinase 1 at week 3 (Supplemental Figure 1, B, C, Data File S4). Two GTPases were increased in abundance, namely OPA1, a dynamin-like mitochondrial Rho GTPase, and Arpc1b (Supplemental Figure 1, A). The ATPase Asna and the myosin ATPase Myo1b showed increased expression at week 3. The GTP-AMP phosphotransferase AK3 was increased in abundance at week 1. ABCD3, a transporter involved in the transport of branched-chain fatty acids with ATPase activity, was also increased. In addition, the increased abundance of HK1 (at week 3) was preceded by decreases in glucose-6-phosphate at week 1. At the same time, proteins involved in responses to oxidative stress were induced, such as MPST1, a mercaptopyruvate sulfurtransferase. Hierarchical clustering followed by trajectory analysis revealed that extracellular matrix proteins were increased after 3 weeks of hypertension. We next performed metabolic modeling using a KEGG-pathway limited mapping and a network-reaction mapping using MetExplore. Statistical mapping on pathview showed that β -oxidation pathways and branched chain amino acid catabolism were increased (Fig. 6 A, B, Supplemental Figure 2, A and B), consistent with metabolomic data showing a decrease in stearic acid, acylcarnitines and branched-chain amino acids (Fig. 1, E,H). We also mapped fold changes on the MetExplore network tool which links metabolomics data with genome-scale networks(12), which suggested that the branched chain amino acid leucine was a critical carbon source in the system, providing an explanation for the activation of metabolic signaling, including inhibited mTOR signaling (Supplemental Figure 3).

Because widespread molecular rewiring occurred together with or following the metabolite changes, we asked if additional proteins – beyond canonical pathways – could be regulated by metabolites. We used the IBM Watson natural language processing (NLP) platform to identify proteins that were associated with or activated by the known metabolomic perturbations (Figure 6C) (13). We focused on proteins that were regulated by multiple metabolites, according to the IBM Watson NLP. Out of the dysregulated proteins, we found that the activity (as indicated by activating phosphorylation) or abundance of 8 proteins could be explained by the metabolic perturbations. These proteins included VEGF-A, TGF-beta and cell adhesion proteins. The NLP prediction also included the regulation of mTOR and AKT as observed at the phosphoproteomics level. For all proteins, the direction of regulation between metabolites and proteins were consistent (Data file S5).

Finally, to determine whether the observed signatures were conserved across models of albuminuria, including human samples, we compiled previously published glomerular proteomic data from different models of proteinuria. The data included animal models of proteinuria, including the proteinuric rat induced by puromycin-aminonucleoside (PAN) and glomerular proteomic data from mice treated with doxorubicin (14–17). We also included primary urinary cells from a patient with an *ACTN4* mutation that caused proteinuria and FSGS (17). Homologene groups were used to determine the conserved proteins, and we converted fold changes into z-scores which we further processed computationally (Data File S6). Initially, protein data from animal models with similar proteome coverage were assessed. Hierarchical clustering was performed to identify signatures that were common across all animal models (Supplemental Figure Fig. 4A). We found two clusters that were increased in glomeruli of doxorubicin treated mice, as well as in the hypertensive rats

(Supplemental Fig. 4B). Notably, both clusters had over 90% of proteins that were determined to have metabolic activity in any pathway (as defined by GO mapping) (Supplemental Fig. 4C). The clusters showed overrepresentation of expression of enzymes in glycolytic, branched-chain amino acid metabolism, and beta oxidative pathways (Supplemental Fig. 4D, 4E). Most of the reduced proteins were expressed in a podocyte-specific fashion as determined by our previous atlas of podocyte-specific proteins in mice (15), suggesting that these changes occurred in the podocytes. Human primary cells from FSGS patients showed similar patterns of changes in protein expression (Supplemental Fig. 4F).

DISCUSSION

Our analysis of a rat model of hypertensive kidney damage provides deeper understanding of the metabolomic, proteomic, and phosphoproteomic changes that occur in this disease. The study was designed to catch both hypertension-dependent perturbations (1 week after the induction of hypertension) and changes caused by tissue remodeling (3 weeks after the induction of hypertension). Metabolic insults occurred in glomeruli, which comprise 2% of the kidney, whereas tubules showed changes that were chiefly related to altered amino acid handling. The early findings in the glomeruli were depletion of energy equivalents, involving specifically GTP, lipid breakdown, and depletion of distinct amino acid species. These resulted in increased AMPK signaling and phosphorylation-dependent signaling to the cytoskeleton and changes in the proteins that regulate the metabolites. The integrated view of this dataset generates the following insights into metabolic rewiring.

In terms of energy equivalents, hypertensive insults resulted in an early decrease of GTP, and NADH, followed by a decrease in ATP (Fig. 3, C, D, E). The trajectory was consistent with the stress-induced induction of ATPases and GTPases that may be necessary to maintain the transport if membranes are endocytosed and subjected to autophagy (Supplemental Fig. 1, B and C). Small GTPases are highly podocyte-enriched proteins (such as RhoA and Rac1) that are key mediators of podocyte injury, and induction of these proteins may increase actin-branched motility. Notably, the dataset contained increased expression of two increased GTPases, namely OPA1, a dynamin-like mitochondrial Rho GTPase as well as Arpc1, GTP-associated proteins (Supplemental Figure 1, A). The metabolome foreshadows their increased abundance (at week 3). The ATPase Asna, which regulates membrane reorganization, is increased after week 3, when ATP is decreased. Similarly, the myosin ATPase Myo1b is increased at week 3. Notably, the GTP-AMP phosphotransferase AK3 was increased at week 1, suggesting that ADP may be regenerated from GTP sources. ABCD3 is a transporter involved in the transport of branched-chain fatty acids that is also increased and has ATPase activity. Further analyses showed a consistent behaviour: the increased abundance of HK1 (at week 3) is preceded by decreased abundances of the glucose-6-phosphate at week 1. MPST1, a mercaptopyruvate sulfurtransferase, was increased at day 7, suggesting that the early response also involves an antioxidative compound. Notably, these proteomics rewirings were conserved across several animal models of glomerular disease (Supplemental Figure S4, A and B).

The data also revealed strong alteration in phospholipids and lipid species. Globally, putative phospholipids species (a population with masses of 600–800 Da) negatively correlated with albuminuria. In addition, beta oxidation enzymes, including the carnitine palmitoyltransferase Cpt1a were increased in abundance (Fig. 1, D, and 6, A). Both findings occurred in the glomeruli. Overall membrane area is decreased in damaged “effaced” podocytes, which may be caused by increased lipid breakdown. This is suggested by the decrease of higher molecular lipid species (Fig. 1C), and the increase of oxolipids (Fig. 3F) (18). The reduction in N-acetyl-neuramine, a building block for the glycocalyx and a part of the membrane structure of the filtration barrier, may be consistent with this overall decrease in lipid membranes. In addition, we observed a decrease in LysoPC species and an increase in oxidized lipids (HETEs) that result from the breakdown of lipids. These lipids are usually pro-inflammatory(19), but some lipids, such as 15-HETE, are involved in resolving inflammation (14). Together, these findings are consistent with a reduction of membrane component phospholipid species.

The regulation of branched chain amino acids and decrease in ATP correlated with alterations in the phosphoproteome caused by increased signaling of ATP-controlled kinases. AMPK phosphorylation was decreased at an inhibitory site (Fig. 4C). Raptor phosphorylation at an activating site that is targeted by many kinases, including MAPKs (20), was decreased, suggesting a metabolite inhibited mTOR. Notably, based on phosphoproteomics modelling, AMPK-mediated signaling chiefly controls the cytoskeleton: Phosphorylation of cofilin Ser³ was increased, and of PDPK1, a potentially druggable target downstream of AMPK and mTOR (Fig. 5). The general pattern of activation of mechanical components, such as components of the cytoskeleton and focal adhesion structures, is consistent with a phosphoproteomics study identifying AMPK substrates(21). This is of interest, because virtually all disease processes that affect the glomerulus – whether genetic, humoral, chemical or immunological in origin (14, 22–24) – are associated with actin-related and mechanical processes. Therefore, it can be assumed that metabolic dysregulation, mediated chiefly by AMPK and mTOR, could modulate or even trigger cytoskeleton alterations that cause podocyte injury.

Several serum analyses in chronic kidney disease in humans and rats have shown convincing links between amino acid metabolism, lipid composition and oxidative stress (25–29). It is possible that similar changes contribute to the overall observed changes in tissue abundance observed in our study, for instance the observed increased oxidative stress in glomeruli (Fig. 3, C, D,E).

Chakraborty *et al.* reported that the ketone body hydroxybutyrate is decreased in the plasma of the salt sensitive Dahl rat, and that administration of the compound alleviates symptoms of hypertension and improves kidney tissue function(30). This mechanism was independent of the microbiota profile of the animals(30). This finding together with our multi-omics dataset indicate that metabolic changes occur in glomeruli. If used by the kidney, ketone bodies would largely circumvent the processes observed here, reduce overall mitochondrial capacity, and provide sources of energy. In conclusion, the data demonstrate how the complex interplay between metabolites, protein phosphorylation, and proteins drive glomerular disease and raise the possibility for dietary interventions such as lipid-

consuming, ketogenic diet and calorie restriction in humans to prevent the adverse effects of hypertension on the kidney.

MATERIALS AND METHODS

Animals

We used Rapp Dahl SS rats (Onotology: SS/JrHsdMcwi, RS:0002576) that have been inbred for over 50 generations at the Medical College of Wisconsin. This strain is a widely used and physiological model for the study of salt-sensitive hypertension and kidney injury(31–33). Male rats were obtained at weaning from colonies under controlled environmental conditions with parents and offspring. Rats were fed a purified AIN-76A rodent food (Dyets, Inc., #D113755, Bethlehem, PA) containing 0.4% NaCl with water provided ad libitum. At seven to eight weeks of age, the salt content of the chow was either maintained at 0.4% (in the group fed a normal diet) or increased to 4.0% NaCl (Dyets, Inc., #D113756) for either 7 or 21 days. Urine samples were collected for 24 hrs using metabolic cages (40615; Laboratory Products) to measure albuminuria. Albuminuria was quantified using an Albumin Blue 580 (Molecular Probes) fluorescence assay. At the completion of the study, rats were surgically prepared by cardiac perfusion, and blood was flushed out using PBS, and rats humanely euthanized. The kidneys were removed, and part of the left kidneys were formalin-fixed, and paraffin-embedded. Tissue sections were deparaffinized and stained with Hematoxylin-Eosin for analysis of kidney injury and glomerular morphology. The right and the other half of the left kidneys were removed and used for glomeruli and cortex fraction isolation, respectively. C3 immunohistochemistry was performed as described previously (34). Animal use and welfare procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals following protocols reviewed and approved by the MCW Institutional Animal Care and Use Committee.

Isolation of the rat glomeruli

Kidneys of experimental SS rats were perfused to clear them of blood. Kidneys were removed and decapsulated as previously described (31). Briefly, the cortex was isolated, minced in small 1mm³ pieces and mechanically strained through steel sieves with different mesh sizes. These procedures were performed in culture medium solution RPMI1640 (Invitrogen, Inc). Collected glomeruli fractions were obtained by gentle centrifugation. All isolations were performed without the presence of bovine serum albumin (BSA) to minimize protein contamination. The glomerular fraction contained between 90 and 98% glomeruli. After isolation, both glomeruli and cortical samples, which predominantly consists of tubule fractions, were snap frozen and later used for metabolomic and proteomic analyses.

Metabolite extraction

Metabolites were extracted from snap-frozen tissue. Approximately 10 mg of tissue was weighed in and stored on dry ice. 800 µl of ice-cold Methanol/Acetonitrile/Water (2:2:1, by vol.) was added per 10 mg of tissue. Tissue was homogenized in a Methanol/Acetonitrile/Water mixture using a bead-beating procedure (glass beads, for 30 sec), and the homogenate was transferred to a new tube. The beads were washed with 200 µl of Methanol-Acetonitrile-Water (2:2:1, by volume), and the homogenate was incubated for 2 hr at –20 °C. The

insoluble pellet was spun down by centrifugation at 4 °C (16000 x g, 20 min) in a table top centrifuge. The pellet was saved for protein determination. Supernatants were dried down in a speed vac at 4 °C. Pellets were also dried down. Finally, pellets were resuspended using an amount proportional to the protein amount of insoluble pellet using Acetonitrile/Water 1:1 (vol/vol) prior to analysis. Metabolite extracts were directly measured using untargeted and targeted metabolomics analysis. Protein pellets were dissolved using a 2% SDS buffer containing 10mm Tris (at 95 °C) and measured using a commercial BCA assay (Thermo Scientific).

Untargeted metabolomics analysis

Untargeted metabolomics analysis was performed using hydrophilic interaction liquid chromatography (HILIC) fractionation and Reverse phase (RP) chromatography as previously described (35). Samples were analyzed on a Quadrupole-time-of-flight instrument (Impact II, Bruker, Bremen, Germany). The fractionation part was performed by a coupled UHPLC device (Bruker Elute, Bruker, Billerica, MA). Data was acquired over a m/z range 50–1000 Da in positive ion mode. The mass spectrometry was calibrated using Sodium formate (post-run mass calibration). The electrospray source conditions were: end plate offset was 500 V, dry gas temperature, 200 °C, drying gas 6 L/min, nebulizer was 1.6 bar, and capillary voltage was set to 3500 V.

A dual fractionation strategy was used to increase metabolome coverage and minimize ion suppression. RP chromatography was done on an ACQUITY BEH C18 column (1.0 × 100 mm, 1.7 µm particle size, Water Corporation, Milford, MA) and HILIC fractionation was performed using a ACQUITY BEH Amide (1.0 × 100 mm, 1.7 µm particle size, Water Corporation, Milford, MA) column. Flow was 150 µl/min. The gradient for RP was as follows: 99% A for 1 min, 1% A over 9 minutes, 35% A over 13 minutes, 60% A over 3 minutes and held at 60% A for 1 additional minute. The gradient for HILIC consisted of 1% A for 1 minute, 35% A over 13 min, 60% A over 3 min and held at 60% A for 1 additional minute. The injection volume was 2 µl. For identification, putative molecules of interest were fragmented using 3 different collision energies (10, 20 and 40 eV), or ramp collision energies (20–50eV).

Metabolite identification and metabolomics data processing

Metabolomics data was converted from .d (Bruker, Bremen, Germany) format into .mzdata format. Metabolomic data processing was performed using the XC-MS online platform (36) that also performed initial quality controls and multivariate analysis of the data, including principal component analysis, as well as isotope removal and adduct annotation. Primary annotation levels were obtained using the MISA annotation, an in-source fragment annotation algorithm (9). Features were considered for multi-group analysis if $q < 0.05$, maximal intensity was larger than 10000. Metabolites were identified after obtaining MS2 spectra using the following criteria: Accurate masses, authentic standards, and comparison with spectral databases (primarily METLIN (37)) were used in order to identify compounds. A maximum of 2ppm mass error was tolerated. Authentic standard compounds were used for every identified metabolite in order to determine its identity and to confirm its MS2 spectrum in positive and negative ion mode, as well as its retention time. Data were

processed using peak alignment software XC-MS online. Hierarchical clustering was performed using Euclidean distance without K-means preprocessing. Chemicals and reagents were obtained from Sigma.

Targeted metabolomics analysis

Targeted metabolomics analysis were performed on a Triple-Quadrupole (QQQ) mass spectrometer (Agilent Triple quadrupole 6490, San Diego, CA) coupled to a HPLC system (1290 Infinity, Agilent Technologies) coupled to ion-funnel. A Zic-philib (Sequant column ($2.1 \times 150\text{mm}$)) was used for separation. Cycle time was 500 ms. Collision energies and product ions (MS2 or quantifier and qualifier ion transitions) were optimized. ESI source conditions were set as following: Gas temperature 250 °C, gas flow = 12 L/min, Neb = 20 psi, Sheath Gas Temp 350 °C, Cap voltage 2000 V, and Nozzle voltage 1000 V. The gradient was consisting of Buffer A and Buffer B. Buffer a was 95:5 H₂O:ACN 20 mM NH₄OAc, 20 mM NH₄OH, pH=9.4. Buffer B was ACN. The Gradient with A/B ratios were as follows: T0 10:90 T1.5 10:90 T20 60:40 T25: off. 5µl were injected. The used transitions for metabolites can be found in Supplemental Table 2. Metabolite identity was also verified by the addition of deuterated isotope labeled standards. To quantify oxolipids, the manufacturer's suggested transitions were followed (Cayman chemical, Item No 192228).

Sample preparation for proteomic analysis

Glomeruli were solubilized in 8 M urea containing 10 mM Tris as well as protease inhibitor (Roche Complete) as well phosphatase inhibitor cocktail 1x (Thermo scientific). Protein abundance was determined with the BCA assay ® (Thermo scientific) and 50 µg of protein was further processed for proteomic analysis. In brief, proteins were reduced using 5 mM of Dithiothreitol and 10 mM of Iodoacetamide. Trypsin (Promega, mass spectrometry grade) was added at a 1:200 w/w ratio and proteins were digested overnight. The next day, digested peptides were acidified and cleaned up using stop and go in tip clean up (Stagetips).

Sample preparation for phosphoproteomic analysis

Phosphoproteomics of rat glomeruli was essentially performed as previously described (38). 100 µg of glomerular peptides were subjected to desalting using Oasis HLB columns (Waters). Desalted peptides were dried down and resuspended in 20% Acetic acid. Phosphopeptides were enriched using FeNTA phosphopeptide enrichment resin columns (Thermo) as previously described (38) and StageTip clean-up was performed.

Mass spectrometry based proteomics analysis

Peptides were analyzed on a Quadrupole-Orbitrap mass spectrometer as previously described(39). In brief, peptides were separated in a homemade C18 column (Seramag, 50 cm, particle size). A nano-LC fractionation was performed before analysis in the mass spectrometer. For both phosphopeptide and peptide samples, a 2.5 h gradient was used using a binary buffer system consisting of aqueous and organic phase: Buffer A (0.1% formic acid), Buffer B (80% acetonitrile, 0.1% formic acid). Data dependent acquisition was performed using the following parameters: AGC target for MS1 spectra was 1E6. The maximal injection time was 20 ms and resolution was 70 000 (mass range, 200–1200 m/z⁻¹).

MS/MS spectra of the top 10 most intense peaks were obtained by higher-energy collisional dissociation (HCD) fragmentation. MS/MS spectra resolution was set to 35 000 at 200 mz^{-1} , AGC target was 5E5, and maximal injection time was 120 ms, and the isolation window was set to 1.3 Th.

Analysis of proteomic data

Proteomics data from different mouse models was performed using MaxQuant v 1.6. with the Andromeda search algorithm embedded. Default settings were used, such that proteins detected with only one peptide and as a posttranslational modification (site) only were excluded. Peptide spectrum match, protein and peptide FDR were adjusted to <0.01, and a target decoy approach was used against the rat uniprot database (reference proteome) downloaded in January 2017. Match between run option was enabled. Intensity based quantification (iBAQ) was performed as previously described (15). Protein intensities concatenated by the MaxQuant algorithm were quantitatively analyzed using the MaxLFQ algorithm, an algorithm aligning peaks and performing accurate quantification. Phosphopeptide site localization probability was set to be larger than 0.8. Phosphorylation motifs were annotated based on the phosphosite plus (40) dataset downloaded in January 2018, and Fishers exact test was performed for enrichment (FDR value cutoff was smaller than 0.01). Motifs were generated using the iceLogo(41) algorithm using rat UniProt database as a reference set.

Bioinformatic integration of glomerular proteomes of models of albuminuria

Quantitative proteomic data were acquired from glomeruli from published mouse models of Doxorubicin-induced podocyte injury, the PAN rat and human glomerular data and primary urinary cells from patients, as well as podocyte-specific proteomics data. These datasets were downloaded from previous publications (14–17). These data were mapped onto the homologene identifiers in the MGI database downloaded in October 2018 (42). Data were log2 transformed, and the difference between proteinuria and control samples and a p value were obtained. These data were normalized by z-scoring and subjected to hierarchical clustering (Euclidean distance, k-means preprocessing), resulting in definition of 4 major clusters using the perseus 1.4. framework. Differences in cluster expression were also plotted using boxplots.

Phosphoproteomic network analysis

First, the normalized phosphoproteomic data was compared to the baseline (t=0) to obtain the differential phosphorylation after 7 and 21 days. Such analysis was performed by fitting a linear model for each phosphosite and contrast (7d compared to baseline and 21d compared to baseline) by using the R package *limma* (v3.34.9). Significance was estimated using empirical Bayes method from the same package. The kinase-substrate background network was extracted from OmniPath database(43) (as of Feb. 15, 2019). Using this prior knowledge along with the differential phosphorylation, the network analysis was performed using PHONeMeS (10) implemented with integer-linear programming (ILP). This allowed us to extract the phosphorylation cascades from the data at both time points. To run the algorithm, we selected as starting nodes (proteins) PRKAA1, PRKAA2 and MTOR as indicated by the metabolomic data. The p-value threshold was set to 0.1. The resulting

network was visualized using Cytoscape (v3.7.1). The code used to perform the analysis can be found in https://github.com/saezlab/Hypertension_D AHL_rat.

Natural Language processing

The IBM Watson(13) drug discovery platform was used to extract activating relationships between regulated metabolites (as compounds) and genes. The “Explore a Network” function uses natural language processing and searches for genes, drugs, conditions, or chemicals to discover a network of relationships supported by evidence found in medical documents and other data sources. Publication bias is corrected through a non-published mechanism using this approach. The dysregulated metabolites were entered as chemicals, and the results were protein-encoding genes associated with the metabolites. The identifications were filtered using a confidence score of at least 86/100 (default setting), and at least 3 papers showing the relationship. The resulting network was exported and reopened in Cytoscape version 3.6.1 (44). Nodes and edges were colored and rearranged as described in the figure legend. The raw data for the network is presented in Data File S5.

Statistics

GraphPad Prism v 6.00 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. The quantitative triple quadrupole data was log transformed and expressed as mean standard error of the mean (S.E.M) after two-tailed t-tests were carried out. Statistical tests for data without normal distribution (such as a Kruskal-Wallis-Test) were used for non-normal distributed data. Comparisons with $p < 0.05$ were generally assigned to be statistically significant and noted on each graph, unless stated otherwise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We would like to thank Martyna Bruetting for help with the slidescanner, and Jan Becker for help with the complement (C3) staining.

FUNDING: This research was supported by the National Institute of Health grants R35 HL135749 and P01 HL116264 (AS), Department of Veteran Affairs I01 BX004024 (AS), and American Heart Association 16EIA26720006 (AS) and 17SDG33660149 (OP). This research was also supported by the Joint Research Center for Computational Biomedicine (JRC- Combine), which is partially funded by Bayer, to JSR. M.M.R. was supported by the DFG (RI2811/1 and RI2811/2). This research was partially funded by National Institutes of Health grants R35 GM130385, P30 MH062261, P01 DA026146 and U01 CA235493; and by Ecosystems and Networks Integrated with Genes and Molecular Assemblies (ENIGMA), a Scientific Focus Area Program at Lawrence Berkeley National Laboratory for the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, under contract number DE-AC02-05CH11231 to G.S. This research benefited from the use of credits from the National Institutes of Health (NIH) Cloud Credits Model Pilot, a component of the NIH Big Data to Knowledge (BD2K) program to G.S.

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Fig. 1 | Tissue-specific phenotyping and untargeted metabolomics analysis of salt sensitive rats reveals a distinct glomerular response to hypertension.

A. Albuminuria in DSS rats after 1 or 3 weeks on a high-salt diet. Data are generated from N=5 rats for each group. A significant increase in albuminuria was observed ($p < 0.05$ by ANOVA). **B.** Kidney histology of DSS rats after 1 or 3 weeks on a high-salt diet. Images are representative of N=3 rats for each group. Scale bar, 100µm. **C.** Overview of highly abundant metabolites in the tubular and glomerular compartments from DSS rats after 1 or 3 weeks on a high-salt diet obtained through untargeted metabolomics analysis as annotated by the MISA (METLIN-Guided In-source Fragment Annotation) algorithm. **D.** Pearson's correlation of XC-MS annotated features with albuminuria in the same rat. The dashed line indicate a Pearson's correlation coefficient of 0.4 or -0.4. Each dot presents a feature. The dataset of two different chromatographic analyses (hydrophilic interaction liquid chromatography (HILIC) and reverse-phase chromatography (RP)) are presented.

Fig. 2 |. Untargeted metabolomics analysis of the kidney cortex and the kidney glomeruli.

The indicated amino acids (A), amino acid derivatives (B), sugars (C), lipids (D), and other metabolites (E) obtained through untargeted metabolomics analysis of DSS rats after 1 or 3 weeks on a high-salt diet. For A-E, data are from N=6 rats for each group. The bars indicate significance based on a Kruskal-Wallis Test of signal intensities. Data are presented as mean ratios (fold change over control) $\pm$ SEM.

Fig. 3 | Targeted metabolomics analysis of salt-sensitive hypertensive rats reveals metabolic dysfunction in the glomeruli.

A to F. Analysis of TCA cycle metabolites (A), the pyruvate-lactate ratio (B), the NADH/NAD⁺ ratio (C), the GTP/GDP ratio (D), the ATP/ADP ratio (E), and the oxidized lipids 5-HETE, 9-HETE and 15-HETE (F). The color coding scheme in A applies to B-F. *, P<0.05 by one-way ANOVA with Dunetts post-test. For A-F, data are presented as means ± SEM from N=5 rats for each group.

Fig. 4 | Phosphoproteomic analysis reveals metabolome-dependent phosphoproteome rewiring after one, but not three weeks.

A. Overview of the number of phosphosites showing upregulation (“up”) or downregulation (“down”). **B.** Volcano plot of phosphosite intensity quantification after 1 week of hypertension. N=5 rats per group. Significance of the comparison day 7 over control ($-\log$ pvalue of a two-tailed t-test) is plotted against the $\log_2$ ratio of the label free intensities (LFQ). The dashed lines indicate significance after correcting for multiple testing. The color key indicates activating and inhibiting sites (based on phosphosite.org annotation). **C.** Trajectory analysis of high-confidence phosphorylation sites. For A-C, data are from N=4 rats for each group. “n” in the figures indicate the number of proteins in each cluster.

Fig. 5 |. Modelling of metabolite-dependent phosphoproteomic results using Phonemes. Thickness of arrows determines frequency of network observation during modelling. The purple nodes show significantly regulated phosphorylation sites. The green nodes are the source of perturbation (AMPK and MTOR) based on metabolomics results (Figure 4).

Fig. 6. |. Proteome-metabolome integration reveals increased abundance of enzymes involved in branched chain amino acid catabolism, explaining decreased abundance of metabolites across models.

A. Mapping of protein and metabolite abundance on the KEGG pathway “fatty acid degradation”. Logarithmized and normalized fold changes of abundance of metabolites and protein complexes were mapped on the KEGG pathway “fatty acid degradation”. The left square shows the abundance at day 7 and the right square the abundance at day 21 as compared to control. Blue circles represent decreased metabolites. The entire maps are presented in supplemental figure 4. The color code and figure legend also applies to (B).

Members of the degrading enzyme complexes are labeled with their respective gene symbol.

B. Mapping of protein and metabolite abundance on the KEGG pathway “leucine, isoleucine and valine degradation”. Panel legend is the same as in (A). C. Metabolites were fed into a Natural Language Processing software, and active relationships recognized by the program were depicted as a network.

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