ROLE OF LYMPHATICS IN RENAL SODIUM HANDLING:

IMPLICATIONS FOR HYPERTENSION

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Role of Lymphatics in Renal Sodium Handling: Implications for Hypertension.

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Hypertension is the leading risk factor for cardiovascular diseases and strokes [1]. A portion of these patients exhibit SSHTN with high cardiovascular risk and are more difficult to pharmacologically control. The etiology of chronic hypertension includes renal immune cell accumulation and sodium retention. The lymphatic system maintains interstitial homeostasis and an expansion of lymphatic vessels is necessary to resolve inflammation. We have previously demonstrated that specifically increasing renal lymphatic density prevents HTN in mice [2]. These mice, KidVD+, undergo kidney specific lymphangiogenesis through the overexpression of VEGF-D [3]. The HTN prevention in the KidVD mice is attributed to a common role for lymphatics: a reduction in renal immune cell numbers. We have recently identified that these mice with expanded renal lymphatics demonstrate increased sodium excretion [2]. The aim of this study is to identify the mechanism by which enhancement of renal lymphatics correlates with urinary Na^+ excretion, and sensitivity to hypertensive stimuli. To improve the diagnostics and increase treatment efficacy, the mechanism of lymphatic expansion and Na+ retention within the kidney lymphatics may thus prove to be a potential therapeutic target for patients afflicted with SSHTN.

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NOMENCLATURE

HTN	Hypertension
SSHTN	Salt-Sensitive Hypertension
L-NAME	nitro-L-Arginine Methyl Ester hydrochloride
HSD	High Salt Diet
KidVD	Kidney-specific VEGF-D overexpression mice
BP	Blood Pressure
VEGF-D	Vascular endothelial growth factor
VEGFR-3	Vascular endothelial growth factor receptor-3
DLOH	Descending Loop of Henle
ALOH	Ascending Loop of Henle
PCT	Proximal Convoluted Tubule
DCT	Distal Convoluted Tubule
NHE3	Na+ H+ Exchanger 3
NKCC	Na+ K+ Cl- Cotransporter
NCC	Na+ Cl- Symporter
ENaC	Epithelial Sodium Channel
IL-17A	Cytokine interleukin 17A
IFN- γ	Cytokine interferon gamma
IAL	Inflammation-Associated Lymphangiogenesis

CHAPTER I

INTRODUCTION

Hypertension

Hypertension is a leading cause and contributor to a majority of deaths in the United States [1]. Hypertension, otherwise known as chronic high blood pressure, is characterized by an increased blood volume traversing through the vasculature or an increased vessel constriction. The kidneys' balance of Na+ excretion and reabsorption in the nephron plays a key role in blood volume control. The excretion of Na+ in the urine is referred to as natriuresis and the commensurate excretion of water is known as diuresis. An imbalance of renal solute regulation can lead to hypertension. Specifically, chronic hypertension can be maintained by the kidney only fractionally excreting excess Na^+ in urine. Hypertension has also been correlated to high salt intake which alters the osmolarity in tissue and blood fluids. An increased molar amount of Na+ will require a higher fluid volume in order to maintain constant Na+ concentrations and prevent cell lysis [4]. The increase in salt intake can also stimulate physiological changes in extrarenal areas such as the liver, dermis, and lymphatic vessels. These changes have been hypothesized to be due to changes in tissue Na+ storage, downstream of reabsorption [5].

The exact etiology of hypertension has numerous factors involved that can be a unique combination for each patient[1]. However, some researchers have postulated that renal immune cell infiltration and subsequent renal Na^+ transporter activation is involved in elevating blood pressure [6]. Although there are multiple treatments, some patients are unresponsive to these therapies or develop a resistant. In defining the role lymphatic expansion in the prevention of hypertension, further research may translate into impactful treatments for at-risk patients.

Renal Inflammation and Immunology in Hypertension

Chronic renal inflammation has also been linked to HTN. Inflammation involves the release of numerous cytokines that attract a variety of effector cells and induce extravasation. This leads to an increase in local tissue fluid or swelling known as edema. Uncontrolled inflammation is capable of decreasing the function of the kidney due to the local pressure buildup and other factors. The most popular route for immune cells is the lymphatic network, therefore the lymphatic system has been identified to play a role in the hypertensive response [3]. This mechanism is considered a positive feedback loop: the release of cytokines in the kidneys attracts a local upregulation of angiotensin II production [6, 7]. Angiotensin II antagonizes natriuresis by activation of Na^+ transporters in the nephrons. In addition, IFN-γ and IL-17A and other unidentified cytokines have been shown to play a large role in regulating the Na^+ transporter activities [6]. Circulating macrophages in lymphatics are also involved in the migration of sodium and electrolyte balance [8].

Nephron Anatomy & Physiology

The nephron is the physiological unit of the kidney and balances the blood electrolytes during filtration. The kidney is divided into the deep renal medulla and the superficial renal cortex, the cortex housing the distal portion of the nephron. Filtration begins when blood from the renal artery flows into the nephron at the Bowman's Capsule in the renal cortex. Here, the larger components of blood are filtered out by the Glomerulus. The filtrate subsequently passes into the Proximal Convoluted Tubule (PCT) where reabsorption begins. The PCT contains Na+/K+/ATPases at the basolateral membrane of the tubular epithelial cells and NHE3, a Na+/H+ antiporter at the apical membrane [9]. Approximately 2/3 of sodium reabsorption occurs at the PCT [6].

The filtrate then flows into the Descending Loop of Henle (DLOH), which initially resides in the renal cortex, but curves deeper into the increasingly saltier environment of the inner renal medulla. The DLOH is permeable to water but impermeable to ions, thus allowing for the high concentration of Na+ in the medulla to drive water diffusion into the interstitial space. The high salt gradient of the medulla is maintained by the vasa recta, the group of capillaries that transports reabsorbed fluid back into circulation.[10] As filtrate travels further in the nephron, it reaches a thicker part of the tubule known as the Ascending Loop of Henle (ALOH). The ALOH has numerous Na+ transporters, including NKCC-1 and NKCC-2, that reabsorbs Na+ and other ions from the filtrate. The ALOH relies on both passive and active transport of Na+, and is responsible for approximately 25% of the total Na+ reabsorption [9].

The filtrate then passes into distal convoluted tubule (DCT) which contains NCC an important sodium chloride co-transporter. The DCT plays a role in the balance of K+, Na+, Ca^{2+} and blood pH. This portion of the nephron is key in the finer control of blood pressure and volume regulation as 8% of the NaCl is reabsorbed here [9]. The filtrate then flows into the collecting duct where the ENaC family of proteins reabsorbs additional Na+ and water prior to release of the filtrate into the renal pelvis, with subsequent urine excretion.

Renal Na+ Transporters

Our studies investigated the functions of the distal and proximal nephron transporter proteins: NKCC-1, NKCC-2, NCC, NHE3, as well as ENaC. NKCC-1 and NKCC-2 are located in the ALOH. Upon activation, NKCC-1 and NKCC-2 function as basolateral and apical symporters, respectively. They transport Na+, K+, and Cl- from the filtrate into the epithelial cells of the ALOH and ultimately back into circulation. NKCC-1 resides mainly in the thin ALOH and NKCC-2 is found in the thick ALOH [9].

The DCT contains NCC, which is also known as the Thiazide-sensitive Na+ Cl- cotransporter. NCC reabsorbs Na+ from the glomerular filtrate through the apical membrane of the tubular epithelial cells. Both the NKCC transporters and NCC are activated upon phosphorylation.

ENaC is also known as the Amiloride-sensitive Na+ channel and is located in the collecting ducts. This protein is activated upon cleavage into ENaC- γ, ENaC- α, and ENaC- β. Previous studies have shown a correlation of increased activity of these transporters and thus an increase in renal filtration rate in mice with a HSD [6]. All of the transporters can be regulated using transcriptional and post-transcriptional regulation as well, and these mechanisms need to be fully characterized.

Lymphatic Physiology

The lymphatic system has recently been considered to play a role in regulation of blood pressure [11]. The overall functions of the lymphatic system are in providing a route for immune cell, lipid, protein, and interstitial fluid flow. Namely, the lymphatics provide a conduit for clearance of interstitial fluid and passage to the lymph nodes [12]. This allows for antigens to be presented to the cells of the adaptive immunity to optimally attack any invaders in the case of infection. The lymphatic system can also deliver new immune cells to sites of injury and can clear them from the inflamed area, and in this way can either perpetuate or attenuate inflammation. The capacity that the lymphatics carryout is tissue-specific and is also dependent on the timing of lymphangiogenesis [12]. Lymphangiogenesis is defined as the growth of new lymphatic vessels, this growth can be stimulated by both VEGF-C and VEGF-D with the latter being more of an initial instigator of vessel growth. In some chronic inflammatory diseases, lymphangiogenesis does worsen the disease by allowing for an increased infiltration of immune

cells and fluid, this is known as IAL (Inflammation-Associated Lymphangiogenesis). Recent studies have also identified a role of the trafficking immune cells in secreting VEGF-C in states of inflammation, leading to further IAL [13]. Other studies have also demonstrated the role of dermal lymphatics in the regulation of excess Na+ storage in the skin upon a chronic HSD or SSHTN models. They have shown that increased Na+ content in the skin is correlated with increased blood pressure [14].

Renal Lymphatics

Studies have indicated little presence of a lymphatic network in the renal medulla, however it has been proven that there are lymphatics present in the renal cortex [15]. The renal medulla contains segments of the DLOH, ALOH, and collecting ducts whereas the renal cortex contains the entire lengths of the PCT, DCT, and bowman's capsule. The differences in function of each segment, as previously discussed, creates differences between the medullary and cortical interstitial fluid, the medulla being more concentrated. This then poses the question of why there is an apparent lack of lymphatics in the medulla, and what the direct role of renal lymphatics is in kidney function. Several researchers have discovered the link between interstitial albumin protein clearance by the lymphatics and the differential concentrations of albumin in the medulla and cortex that contribute to the proper oncotic gradients needed for their respective functions [15]. Although the renal medulla rarely contains lymphatic capillaries, it does utilize the vasa recta to transport interstitial fluid [10, 15, 16].

Numerous studies have indicated the correlation between renal lymphatic dysfunction and renal interstitial edema [15, 17, 18]. Without proper functioning of the renal lymphatics there is a decreased ability of the kidney to clear out the accumulating interstitial fluid which exacerbates the edema. Similarly, in the case of congestive heart failure, the excess pressure load

on the renal lymphatics leads to lymphatic dysfunction [15, 19, 20]. Researchers have shown that this perpetuates renal edema and increased Na+ retention. Litigation of the renal lymphatics results in renal inflammation and fibrosis [21], and interestingly, an elevation in blood pressure [22]. Overall, the proper functioning of the renal lymphatic network is likely to be key to maintaining a normally functioning kidney and the homeostatic balance of Na+ reabsorption and natriuresis.

Lymphatic Expansion

Lymphatic expansion due to lymphangiogenesis includes both increased lymphatic branching and lymphatic width. As previously discussed, the lymphatic network plays a role in the regulation of electrolyte balance [5]. In addition, lymphatic expansion is linked to migration of macrophages into the renal cortex and medulla [2]. Our KidVD (Kidney-specific VEGF-D overexpression mice) expression system is activated upon addition of Doxycycline hylate, triggering the genetic overexpression of VEGF-D and therefore instigating lymphangiogenesis in the renal tissues [2, 3]. These mice have been shown to be resistant to hypertension, in part through limiting immune cell accumulation [2]. As hypertension is also a condition of sodium retention, these mice provide a clever model to identify if increased renal lymphatic density also alters sodium handling in the kidney.

CHAPTER II

METHODS

Experimental Cohorts

KidVD+ and wildtype (WT) mice were used for all experiments. All mice were fed on an *ad libitum* diet of their respective chow and water. The Chronic Na+ group was given 4% NaCl chow with 1% NaCl water with 200 mg/L of doxycycline hylate (Sigma-Aldrich) for three weeks. The baseline cohort was similarly administered 200 mg/L of doxycycline hylate in their water.

The acute Na+ loading group received 200 mg/L of doxycycline in their water for three weeks followed by an intraperitoneal injection of isotonic saline solution at 5% body weight. These mice then underwent a 10-hour acclimation period, followed by placement in urine collection cages where their urine was collected hourly for 6 hours.

The SSHTN cohort was administered L-NAME in their water *ad libitum* for 2 weeks, followed by a week-long wash-out period with normal water [3]. At week 4, a subsequent week-long dose of 200mg/L doxycycline was administered. Then at week 5, 1% NaCl was added to the doxycycline water. All mice were sacrificed by administration of isofluorane and cervical dislocation. Liver tissue, both kidneys, back skin (hair removed with Nair), ears, cardiac tissue, skeletal muscle tissue, and blood were collected.

Urine Analysis

Urine excretion data collected from the from the acute Na+ loading group was analyzed by the UT Southwestern Charles and Jane Pak Center for Mineral Metabolism and Clinical Research. Measurements were obtained using the Beckman AU400 Autoanalyzer.

Dermal Lymphatic Quantifications

Ear specimens were pealed in half, separating the anterior and posterior sides, and were fixed in 10% buffered formalin solution (Sigma, St. Louis, MO) for approximately 2 hours. They were then rinsed in 1x PBS, and the posterior half of the ear was soaked in 0.3% detergent (0.1% Tween and 0.2% Triton X). The samples were then incubated in Lyve-1 (polyclonal goat; R&D Systems, Minneapolis, MN) at a 1:400 dilution by volume in AquaBloc (EastCoastBio, North Berwick, ME) for 3 days at 4℃. After rinsing, the ears were incubated for 3 days in donkey IgG anti-goat 488 (Life Technologies, Carlsbad, CA) using the same dilution process as the primary antibody. The interior portion of the ear was imaged using a fluorescence stereoscope at 5x magnification. Images of ears were taken of the edge to center of ear. Total branches, length, and widths were collected for a standardized area using ImageJ software.

Tissue Sodium Quantifications

Wet weights of transversely-sliced kidney tissue halves were obtained. Samples were boiled for 4 days at 110℃ to remove water content. Dried samples were weighed and subsequently oxidized using 500 µL of 12 N HCl at 82℃ for 3 days. The samples were resuspended in 500 µL of deionized water and sonicated for 10 minutes. Samples were centrifuged for 10 minutes at 11g, then 100 µl of each sample was analyzed using the Horiba Scientific, LAQUAtwin, compact water quality meter, sodium sensor. Calculations were performed to normalize each sample's original weight to the concentration output. Skin sodium quantifications were performed in a similar manner. Skin processing, however, differed by the incubation times: the back-skin samples (30-200 mg) were boiled for 12 days and oxidized for 5 days under temperature conditions previously mentioned (see Appendix A for further detail).

Tissue Collagen Quantification

Weight of collagen in the dehydrated and oxidized kidney and skin samples was obtained using the hydroxyproline assay (see Appendix A). 100 µL of sample was combined with 250 µL of 1.4% Chloramine-T in 0.5 M sodium acetate/10% isopropanol. Mixture incubated for 20 minutes at room temperature, and then 250 µL of Ehrlich's solution (1M p-dimethylaminobenzaldehyde in 70% isopropanol/30% perchloric acid) was added and incubated for 15 minutes at 65℃. Concentrations were measured using a spectrophotometer at 550 nm.

Protein Extraction and Immunoprecipitation

Transverse halves of renal tissue homogenate was purified and protein lysates were extracted, following the BCA assay, to obtain the protein concentrations. Volume for immunoprecipitation was kept constant at 30 µL for each sample, and protein concentrations were approximately 1.2mg/mL. Antibodies used were for NKCC-1, NKCC-2, NCC, ENaC- α sodium transporters (see Appendix B). Each primary antibody was rabbit derived and used at a 1:1000 dilution in Odyssey Blocking Buffer (LI-COR, Lincoln, NE). The secondary antibodies were used at a 1:5000 dilution similarly. The blots were then re-probed in a similar manner using the phospho-tyrosine antibody afterwards on same blot to determine activation of each protein.

Statistical Methods

The Student's T-test was utilized to determine significant relationships between 2 variables. The significance level was held at $p = 0.05$ for each comparison.

CHAPTER III

RESULTS

Increased Fractional Excretion in KidVD+ Mice

There was a statistically significant increase in the excretion of Na+ across the KidVD+ chronic Na+ loading cohort as compared to the KidVD- for both males and females as demonstrated in Figure 1. In addition, there was a statically significant increase in fractional excretion in the chronic Na+ loading mice as compared to the chow fed mice (see Figure 1).

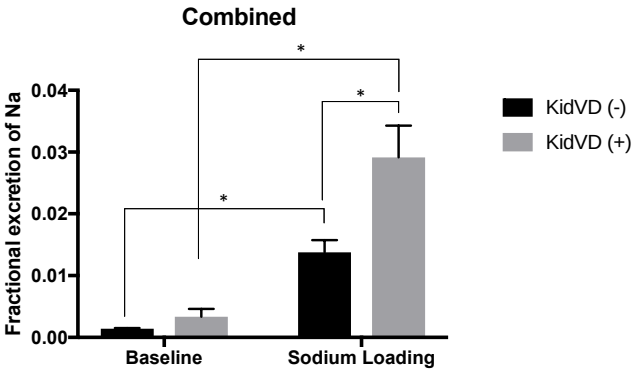

Figure 1. Fractional Na+ Excretion in urine. The Combined male and female fractional excretion data collected from the chow mice and the acute Na+ loading mice on a percent added to diet basis. *$P < 0.05$.

Absence of Na+ Accumulation in Skin and Kidney Tissues

There was no statistically significant difference in skin or kidney tissue Na+ concentrations between the Chow fed KidVD- and KidVD+ mice as shown in Figures 2A and 2B. The KidVD- acute Na+ loading cohort had a statistically significant level of kidney Na+ as

compared to the KidVD+ acute Na+ Loading cohort as seen in Figure 2A. The KidVD+ SSHTN mice had an elevated level of Na+ in the kidney tissues, however, the Chronic Na+ mice showed no change in kidney Na+ between the two genotypes, demonstrated in Figure 2A. The skin samples had a relatively constant Na+ concentration across all cohorts and genotypes ranging from approximately 0.1 mM to 0.16 mM (see Figure 2B).

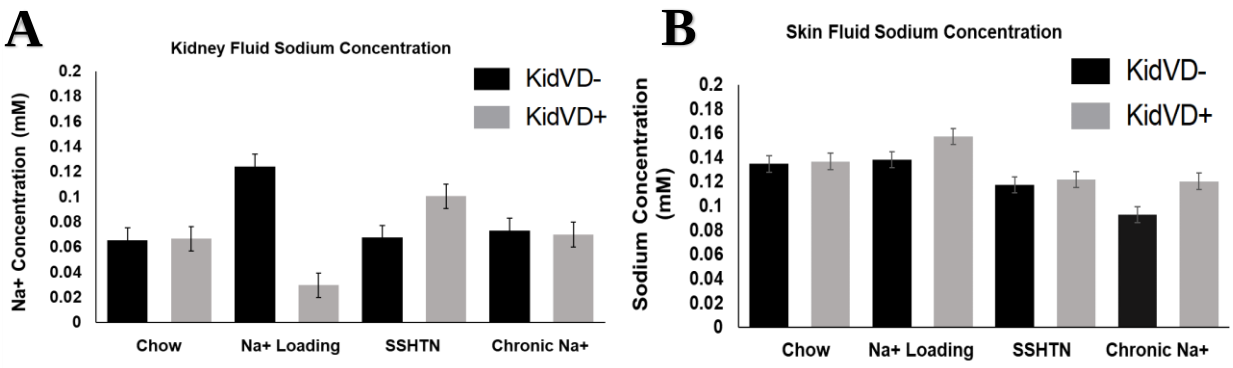

Figure 2. Tissue Sodium concentrations. Concentrations of tissue sodium content was analyzed for kidney and skin tissues and was normalized to sample weight. A. Kidney fluid sodium concentration. B. Skin fluid sodium concentration. *P < 0.05.

Absence of Dermal Lymphangiogenesis in KidVD+ Mice

There was a statistically significant decrease in dermal lymphatic density in the SSHTN KidVD+ mice as compared to the SSHTN KidVD- mice as demonstrated by both Figures 3A and 3B. There was a statistically significant increase in the total lymphatic vessel length per area for both KidVD+ and KidVD- mice in the SSHTN cohort as compared to their chow fed counterparts (see Figure 3A). There was also a statistically significant increase in the number of branchpoints per area in the SSHTN KidVD+ and KidVD- mice as compared to the chow fed mice (see Figure 3B). The SSHTN KidVD+ mice showed a lesser amount of vessel density

increase as compared to the SSHTN KidVD- mice in both the total number of branchpoints and total vessel length per area shown in Figure 3A and 3B.

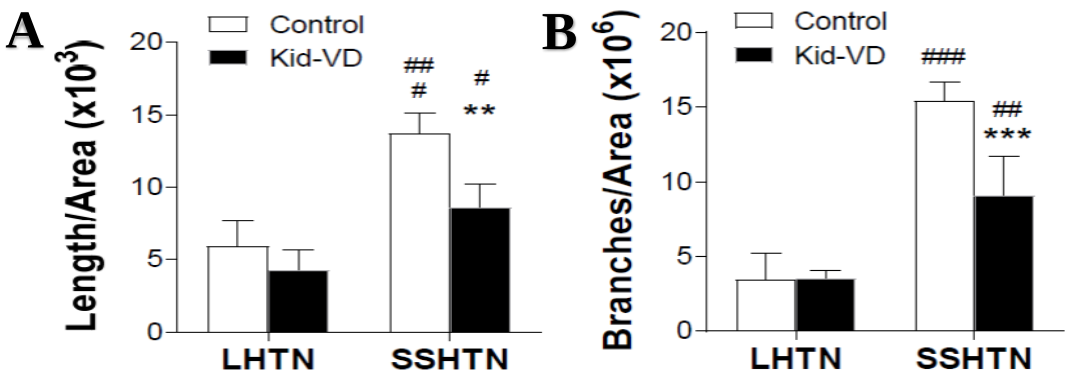

Figure 3. Dermal lymphatic vessel quantification. A. Displays the average vessel Length per unit area analyzed in both the chow fed mice (LHTN) and SSHTN mice. B. Displays the average number of branchpoints per unit area analyzed in both chow fed mice (LHTN) and SSHTN mice. Both sets of data are from the previous study conducted by the Rutkowski lab [2, 3]. *P < 0.05.

Tissue Collagen Levels Unchanged

There was no significant changes in the relative amounts of collage in the kidney and skin tissues of the mice across all cohorts demonstrated in Figures 4A and 4B. Significant collagen levels can be linked to tissue fibrosis, and this was not evident in any of the mice.

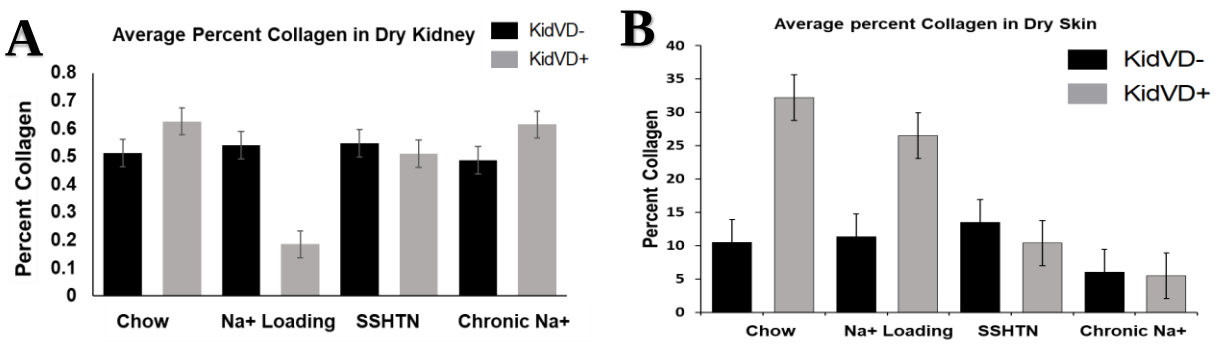

Figure 4. Tissue collagen analysis. A. Relative amount of total kidney collagen. B. Relative amount of total skin tissue collagen. Both samples were normalized to dry weights of the original tissue sample. *P < 0.05.

Elevated Expression Levels of Na+ Transporters in KidVD- Mice

There was an overall downregulation in gene expression of renal Na+ transporters in the KidVD+ mice as compared to KidVD- mice all on the chronic Na+ diet as seen in Figure 5A. There was a statistically significant decrease in genetic expression of NKCC-2, NCC, NHE3, and ENaC- γ. In addition, there was a decrease in NKCC-1, ENaC-α and ENaC-β in the KidVD+ mice. This result was synonymous with the protein expression of NCC as seen in Figure 5B. The expression of NCC in the chronic Na+ loading KidVD+ mice was statistically significantly lower as compared to the chronic Na+ loading KidVD- mice.

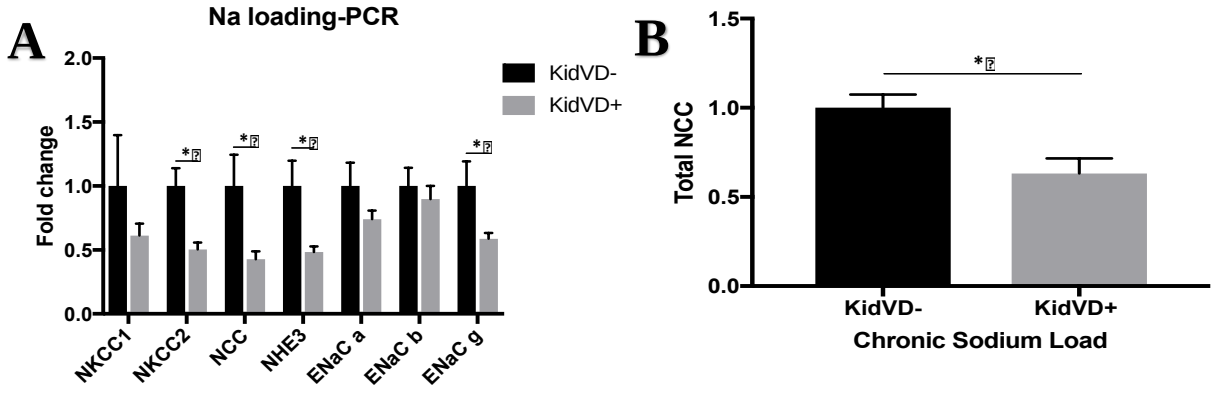

Figure 5. Renal Na+ transporter expression analysis. A. Genetic expression of renal Na+ transporters in KidVD- and KidVD+ acute Na+ loading mice quantitated by RT-qPCR. B. Relative expression of NCC in KidVD- and KidVD+ mice of the Chronic Na+ cohort. *P < 0.05.

CHAPTER IV

CONCLUSION

Protective Mechanisms in the KidVD Model

In conclusion, the increase in fractional Na+ excretion in the KidVD+ mice is explanatory of the HTN resistant phenotype that has been previously described [2]. This finding was parallel with the decreased expression of renal Na+ transporters, with NCC playing a significant role in the balance of reabsorption and excretion of Na+ in these mice. For the acute Na+ loading KidVD- mice, this mechanism for increased Na+ retention, see Figure 1, was confirmed by the increased expression of renal Na+ transporters. Increased Na+ retention is osmotically followed by an increase in water retention, leading to an increase in blood fluid volume and ultimately results in HTN. The acute Na+ loading KidVD- mice also demonstrated an increase in dermal lymphatic density. These results are parallel to the findings from Speed et al. which demonstrated that with chronic HSD, there was an increased dermal tissue remodeling due to the excess storage of Na+ in dermal tissues [14].

Our results are indicative of a similar dermal tissue remodeling event linked to changes in Na+ handling, and most likely dermal Na+ storage. However, the tissue sodium measurements did not demonstrate any significant increase in skin Na+ storage (see Figure 3). Although our findings on Na+ storage in dermal tissue were insignificant across all cohorts and genotypes, the Titze group has previously shown that rats with HSD have utilized the skin as a Na+ storage depot and the skin playing a newly characterized role in electrolyte homeostasis [5]. This group has also characterized much of the Na+ handling mechanisms in the periphery, and have also

utilized more intensive methods to both visualize and quantify Na+ storage throughout the body [5, 23].

Future Directions

Enhancing renal lymphatics has proved an interesting preventative route for pre-clinical treatment of HTN. It has been suggested that increased renal lymphatics allows for an increased ability for clearance of immune cells and interstitial fluid. However, this mechanism is controversial, and the efficacy of enhanced lymphatics is dependent on both the timing of lymphangiogenesis in relation to hypertensive stimulus and the degree of efficiency of the new vessels in clearing fluid [11, 24]. There are also studies that have indicated the importance of a renal medulla vasculature with characteristics similar to both blood vessels and lymphatics, known as the vasa recta, which provide clearance of interstitial fluid from the renal medulla [10]. Like lymphatics, the vasa recta also depend on R3 signaling [10]. Further investigation using the KidVD model may provide clarity on the role that the vasa recta play in comparison to the cortical lymphatics in providing a protective mechanism against HTN and renal edema.

Future studies will allow for the elucidation of the specific mechanisms by which the kidney and infiltrating immune cells use in regulating the expression and activity of the renal Na+ transporters. These mechanisms have a high potential for becoming a therapeutic target in patients who suffer from HTN, by preventing excess Na+ reabsorption and storage. In discouraging excess Na+ reabsorption, the fluid volume necessary for osmotic balance will decrease, therefore preventing high blood pressure. In addition, further characterization of specific cytokines and chemokines involved in renal inflammation, and ultimately HTN, will allow for further evolution of treatment options for HTN patients.

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APPENDIX

Appendix A. Antibody Information

Antibody	Company	Lot
NCC	Abcam	3030545
α -ENac	Invitrogen	TE264898
NKCC-1	Abcam	GK3233837-1
NKCC-2	Abcam	GR128934-7
P-tyrosine	Abcam	2593884

Note: This table contains the descriptions of all antibodies used for protein extraction and Co-immunoprecipitation.

Appendix B. Hydroxyproline Assay Protocol

(assume collagen = 12.5% hydroxyproline)

For collection of **tissue Na+ concentrations perform steps 1, 5-7, using 500uL of HCl in step 5 and 500uL to resuspend in step 7. In the heating steps, higher temperatures may be advisable and longer periods of heating, use your own discretion for the size of your tissue. After step 7, centrifuge the samples to clear all debris and then use 50-100uL of sample to test for sodium using the Horiba Scientific, LAQUAtwin, compact water quality meter, sodium sensor and filter paper. Proceed to the subsequent steps, using half of the volume required for all reagents and samples. If needed for the skin tissues (higher collagen content, samples may all be oversaturated and unable to measure), make the standard a serial dilution starting at 2mg/mL, and then dilute all samples and the standards by a factor of 10 prior to analysis with spectrometer. The *best* range is not yet known.

1. Weigh and record tissue mass (100-500mg)
2. Homogenize***** in distilled water
3. Precipitate with 1/5 water volume 50% trichloroacetic acid on ice for 30 minutes.
4. Spin and remove liquid.
5. Resuspend precipitate in 1 mL of 12N (36%) hydrochloric acid. Transfer to tall glass tube.
6. "Boil" off HCl at 110°C overnight – in the fume hood!!! Samples will blacken. They must be dry when finished. Continue heating until dry if necessary. Caps off.
7. Resuspend – it will not go wholly back into solution – in 1mL distilled water****** by sonication. Black goop stuck to the walls is lipid; don't worry about it.

Samples in water are stable at room temperature. Let them sit.

Prepare serial dilutions of trans-4-hydroxy-L-proline (Sigma) in water starting at 0.5mg/mL.

Prepare (must be fresh!) 1.4% solution of chloramine T in 0.5M sodium acetate/10% isopropanol.

8. In duplicate Eppendorf tubes, add 200µL of samples and standards to 500 µL prepared chloramine T solution. Dispense in a hood.
9. Incubate 20 minutes at room temperature.

While incubating, **prepare** Ehrlich's solution: 1.0 M p-dimethylaminobenzaldehyde in 70% isopropanol/30% percholoric acid.

10. Add 500 µL of Ehrlich's solution to each of the sample and standard tubes. Invert to mix.
11. Incubate at 65°C (a water bath works well for this) for 15 minutes. Higher concentration standards will start to turn pink-purple.
12. Remove from heat and transfer to 96-well plate.
13. Measure absorbance at 550nm. Standard curve should be linear.
14. Collagen concentration = hydroxyproline concentration/0.125

15. Calculate total collagen mass/total mass. Alternatively, after homogenization, take a small aliquot for BCA and report collagen/protein.

$$Collagen(mg) = \frac{hydroxpro(mg/mL)}{0.125} \cdot \frac{volume H_2O blackstuff\ (\mu L)}{200 \mu L}$$

Typical values (g collagen/100g tissue): liver 0.26, kidney 0.37, spleen 0.73, tendon 56.5

***** For collagen rich and lipid poor tissues, the sample can be placed directly into HCl without homogenization. Sufficient HCl should be used to cover the sample. Adipose needs to have the lipid removed.
****** For adipose, where matrix is so little of the tissue weight, use more tissue in Step 1 and less water in Step 6.

Other note: the solutions you're using are pretty nasty. Wear a lab coat, goggles, and work in a hood. The solutions are also very concentrated – make up only enough chloramine T and Ehrlich's for use (500µL each per sample); discard the unused.
ADDITIONAL NOTES (A REYNA)

1. If using tissues from -80C they must first be weight "wet"
-Measure weight of standard glass test tube
-Add tissue sample and weigh again
-Subtract these values to get the "wet" weight
2. These then need to be completely dried. Place at 80C up to overnight if needed (For kidney it only took 3 hrs.)

Then continue with regular protocol.