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Prof. Dr.med. Heimo Ehmke

"The Role of Angiotensin II and Aldosterone in the Pathomechanism of Hypertension in Mice under a Combined Potassium and Sodium Diet."

Dissertation

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Hannah Hetz
Rottweil

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Prüfungsausschuss, zweite/r Gutachter/in: Prof. Dr. U. Wenzel

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I. Introduction

Nowadays, the typical diet in western, industrialized nations is a high sodium and low potassium diet. Our body, in particular our kidneys, are designed to preserve sodium and excrete potassium, as our Paleolithic ancestors lived on a diet rich in potassium and low in sodium. Their average daily sodium intake was 20mmol compared to a sodium intake of 150mmol nowadays. The daily potassium intake has gone in the opposite direction, decreasing from about 320mmol in former times to 50mmol today. This means a 50-fold increase in the ratio of dietary sodium versus potassium intake. It is known that individual blood pressure levels are most tightly correlated with the Na^+ to K^+ ratio (Huang and Kuo 2007). The causal relation between high salt intake and hypertension was first shown by Dahl and colleagues in a series of metabolic studies published in the 1950s (Dahl and LOVE 1954).

In recent times, salt-sensitive hypertension has become an increasingly relevant health issue. Epidemiologic studies estimate that over 25% of the adult population in industrialized countries are affected by hypertension (Kearney et al. 2005). The treatment of hypertension and its consequences represents a great economical burden for our health care system. For instance, in Germany in the year 2008, medical expenses for hypertension according to ICD-10 definitions amounted to 9.059 million Euros, which constitutes approximately 3.5% of 2008`s total medical expenses (GBE des Bundes 2008).

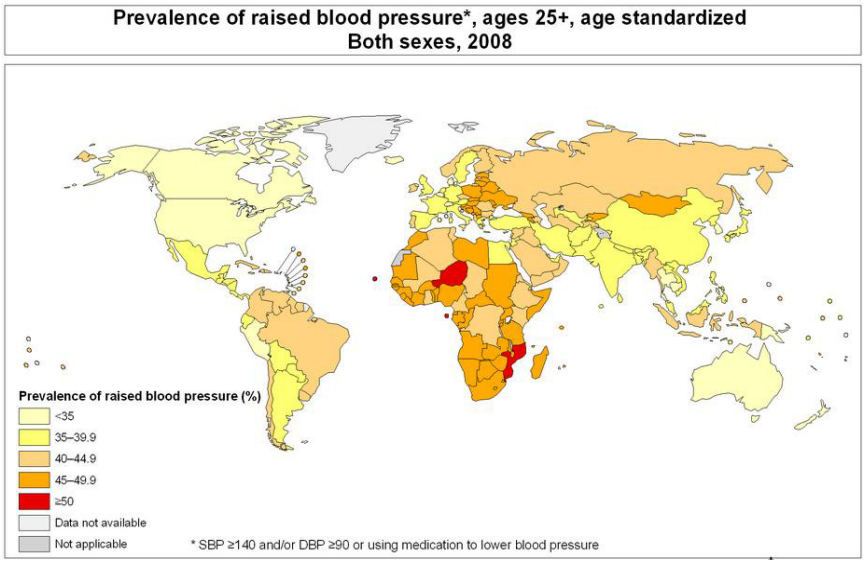


Figure 1: WHO data for the prevalence of hypertension in 2008 for adults aged 25 and above of both sexes. In this figure hypertension is defined as a systolic pressure >140mmHg and/or a diastolic pressure >90mmHg or taking antihypertensive medication. As this map indicates, raised blood pressure is a worldwide health issue with an enormous number of people affected (World Health Organization, www.who.int).

Hypertension in humans is defined as a systolic pressure over 140 mmHg and/or a diastolic pressure over 90 mmHg. Blood pressure measurements have to be repeated on at least two different occasions to confirm the diagnosis. If not treated appropriately, hypertension leads to an increased risk of cardiovascular, cerebrovascular or kidney diseases. As a consequence, hypertension-related deaths are among the leading causes of death worldwide (Ezzati et al. 2002).

Successful treatment of hypertension has proven to be quite difficult. First, development of hypertension is mostly multifactorial with different aspects of disease in every patient. Second, the necessary life-style changes, e.g. losing weight, changing diet, are not easily achieved and sustained. Third, pharmacological therapy often has only limited success in reducing blood pressure as it is a symptomatic and not a causal therapy. In most patients, treatment with two or three different antihypertensive drugs is necessary, with the appertaining side effects and drug interactions. All this is partly due to the fact that the pathomechanisms of salt-sensitive hypertension are not fully understood yet. So, gaining better knowledge of the formation of hypertension through research could also lead to better pharmacologic treatment options in anti-hypertensive therapy.

An important step towards a better understanding of the underlying mechanisms of salt-sensitive hypertension was the discovery of the etiology of pseudohypoaldosteronism type 2 (PHA2). It is a condition characterized by hyperkalemia, hypertension and normal glomerular filtration rate (GFR). In 2001, it was shown by Wilson et al. that PHA2 is caused by mutations in two kinases, WNK1 and WNK4, which belong to a newly discovered family of kinases, called WNK kinases (with no lysine kinases) (Wilson et al. 2001).

Kinases, in general, are enzymes that change the activity of other proteins by adding a phosphate group at specific positions. Therefore, kinases have a highly conserved catalytic domain. The members of this new family of kinases have the lysine necessary for catalysis located in subdomain I instead of subdomain II of the active site. WNK kinases are expressed ubiquitously in body cells. However, there are isoforms that are exclusively expressed in the kidneys. WNK kinases in the kidney regulate the activity, transport and abundance of ion channels, particularly in the distal nephron, which is the main site of renal salt handling. They also interact by

inhibiting or enhancing each other's function. Thus, they have an essential role in regulating salt and water homeostasis. So far, four WNK isoforms are known that are expressed in the kidney.

In PHA2, the gene for WNK1 has a mutation which leads to an increased expression of wild-type WNK1. As wild-type WNK1 inhibits potassium secretion and enhances sodium reabsorption in the distal nephron, overexpression of WNK1 leads to the typical features of PHA2, hypertension and hyperkalemia. The mutation in the WNK4 gene results in an altered function of the kinase where inhibition of sodium reabsorption is abrogated and inhibition of potassium secretion is increased (Wilson et al. 2001).

Up to today, extensive research has been undertaken regarding the role of WNK kinases in salt handling and thus blood pressure regulation. As a consequence, knowledge of physiological and pathophysiological mechanisms has notably increased. To simplify the understanding of the pathophysiology of hypertension, the normal function of the distal nephron with the involved ion channels, signal molecules and receptors is explained.

The distal aldosterone-sensitive nephron consists of three distinct parts, which have different functions regarding sodium and potassium handling. It starts with the distal convoluted tubule (DCT), which is located in the renal cortex (see Fig. 2). The DCT is defined by the expression of the thiazide-sensitive sodium-chloride co-transporter (NCC), and can be further divided into an early segment (DCT1) and a late segment (DCT2). The DCT2 also expresses the epithelial sodium channel (ENaC), as well as the renal outer medullary potassium channel (ROMK), which is present throughout the entire distal nephron (McCormick et al. 2008).

The DCT is followed by the connecting tubule (CNT), in which ENaC and ROMK are expressed. Several connecting tubules end in one collecting duct, which consists of an early part located in the renal cortex (cortical collecting duct, CCD) and a late part crossing the outer and inner medulla (outer medullary collecting duct, OMCD and inner medullary collecting duct, IMCD) and eventually draining the urine into one of the renal calices. The CCD, OMCD and IMCD are the major sites of potassium regulation due to the simultaneous expression of ENaC and ROMK.

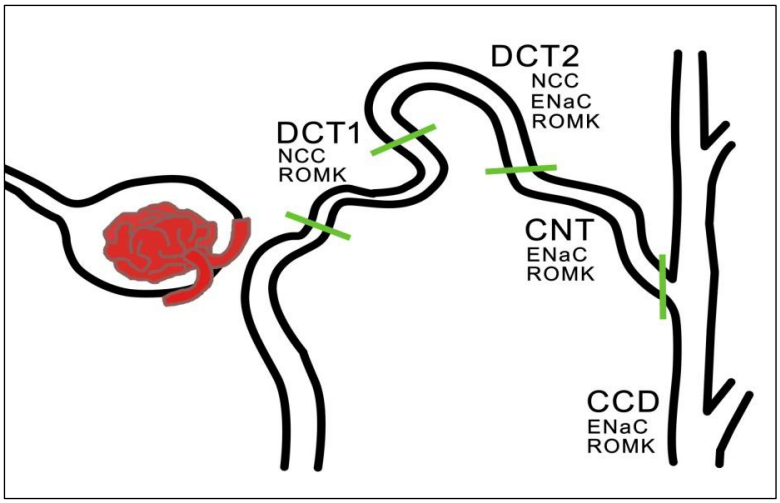


Figure 2: Diagram of the anatomy of a distal nephron with its ion channel distribution along the different segments: The proximal part of the distal convoluted tubule (DCT1) with the sodium-chloride co-transporter (NCC) and the renal outer medullary potassium channel (ROMK), the distal part of DCT (DCT2) with NCC, the epithelial sodium channel (ENaC) and ROMK, the connecting tubule (CNT) with ENaC and ROMK and finally the cortical collecting duct (CCD) with the same ion channels as in the CNT.

The distribution of the main ion channels and transporters in the distal nephron has long been known and is therefore a well-established fact that does not cause controversy anymore. However, the regulation of the same ion channels through kinases and other signal molecules in the distal nephron is the topic of current research and thus, a lot of competing opinions exist. In the following, results of the latest research as to how, in which context and through which molecules these ion channels are regulated, is presented without raising claim to completeness.

The DCT is responsible for the reabsorption of 5-10% of the glomerular filtrate. As it is located just after the macular densa cells, it is the first segment of the nephron which is not subjected to the tubuloglomerular feedback mechanism. Thus, its rate of salt reabsorption affects the final salt concentration in the urine and therefore extracellular fluid balance and blood pressure. The main ion channel mediating sodium reabsorption in the DCT is NCC. It was shown in Xenopus laevis oocyte experiments that NCC is inhibited by wild-type WNK4 (WT-WNK4, see Fig. 3). But when the extracellular signal molecule angiotensin II (ANG II) was added, WNK4 was switched to a state that allows or promotes NCC activation (San-Cristobal et al. 2009). Angiotensin II is part of the renin-angiotensin-aldosterone-system (RAAS) which serves to maintain extracellular volume homeostasis and thus, regulates

arterial blood pressure. In the distal nephron, ANG II acts through a heptahelical G-protein-coupled receptor called angiotensin-1-receptor (AT1R). In hypovolemia, when ANG II levels are high due to RAAS activation, NCC is activated leading to increased electroneutral NaCl reabsorption in the DCT (see Fig. 4). Consequently, delivery of sodium to the collecting duct is diminished, limiting sodium-coupled potassium secretion in that segment. This helps to enable restoration of intravascular volume without appreciable loss of potassium. The mechanism of NCC activation depends both on the presence of WNK4 and on the presence of the intracellular signal molecule STE20/SPS1-related proline/alanine-rich kinase (SPAK), which is predominantly expressed in DCT (Richardson and Alessi 2008). In a cell model, this signal pathway can be inhibited by the AT1R antagonist losartan (San-Cristobal et al. 2009). The downstream effect of AT1R signaling is increased phosphorylation of WNK4, SPAK and NCC, which results in augmented trafficking of NCC to the apical plasma membrane (Sandberg et al. 2007). These findings show that renal AT1R play a fundamental role in sodium chloride (NaCl) reabsorption and therefore, presumably, in the pathogenesis of salt-dependent hypertension.

Secretion of aldosterone, another component of the RAAS, from the outer section of the adrenal cortex in the adrenal glands is stimulated by two distinct physiologic perturbations - hyperkalemia and volume depletion. It has long been known that aldosterone's main effector molecule is the serum- and glucocorticoid-induced kinase 1 (SGK1). Ring et al. showed in a Xenopus oocyte cell model that in the setting of hyperkalemia aldosterone-dependent activation of SGK1 leads to the phosphorylation of WNK4 at a specific site, which mediates the activation of ENaC and ROMK simultaneously (Ring et al. 2007a). This enables efficient potassium secretion through electrogenic sodium reabsorption, mainly in the collecting duct (see Fig. 5). Finally, there seem to be three distinct functional states of WNK4 in the distal nephron (see Fig. 3, 4 and 5). The first state is referred to as the wild-type WNK4 and inhibits NCC, ENaC and ROMK in the absence of any stimulatory or inhibitory signal on the kinase (Ring et al. 2007b, Kahle et al. 2003, Wilson et al. 2003). The second functional state is the one activated by ANG II in the setting of hypovolemia when AT1R signaling leads to phosphorylation of WNK4 and consequently SPAK, causing enhanced sodium reabsorption via activated NCC (Castaneda-Bueno et al. 2012a). This leads to a low tubular sodium load in the CCD

and consequently electrogenic sodium reabsorption and potassium loss is diminished. The third state of WNK4 is activated in hyperkalemia via the aldosterone-SGK1 pathway, which leads to the activation of ENaC and ROMK, permitting potassium secretion without extensive sodium reabsorption (Ring et al. 2007a). This “kinase switch” allows the kidneys a differentiated and appropriate response to distinct metabolic disturbances such as hypovolemia and hyperkalemia and it also explains the so-called “aldosterone paradox”. Because aldosterone levels are elevated both in hypovolemia and hyperkalemia, for a long time it was unclear how the kidneys distinguish between the two different causes for increased aldosterone levels and thus react appropriately to restore a metabolic equilibrium. This enigma has been solved by discovering ANG II as a regulator of kinases and ion channels in the distal nephron in the setting of hypovolemia when RAAS is activated and thus, levels of ANG II and aldosterone are high (Hoorn and Ellison 2012). While aldosterone activates the same SGK1-dependent pathway as in hyperkalemia, the concomitant rise in ANG II levels leads to the activation of NCC and counteracts the activation of ROMK, thereby maximizing sodium reabsorption to reinstall intravascular volume balance without increasing potassium secretion (Castaneda-Bueno et al. 2012b).

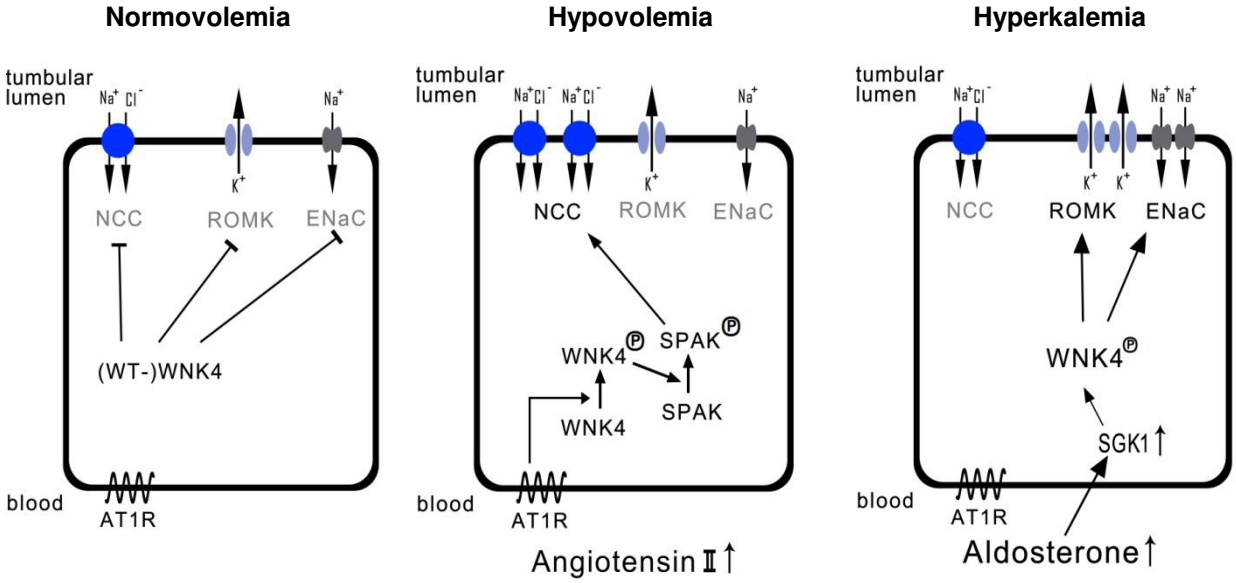


Figure 3, 4 and 5: Integrated cell models for an overview of the role of WNK4 and the involved signaling pathways in normovolemia (left), hypovolemia (middle) and hyperkalemia (right). The arrows in the graphs only indicate the positive or negative effect on kinases and net ion transport through the channels. They do not indicate how this effect is accomplished (e.g. phosphorylation, endocytosis etc.).

In addition to WNK4, another important kinase in regulating renal salt handling, particularly potassium, is WNK1. Two WNK1 isoforms are known: long-WNK1 (L-WNK1), a ubiquitous isoform containing the whole kinase domain, and kidney-specific WNK1 (KS-WNK1), a kinase-defective isoform. While L-WNK1 is expressed in the entire nephron at a low level, KS-WNK1 is highly expressed in the DCT and to a lesser extent in the CNT (Hadchouel et al. 2010). L-WNK1 inhibits wild-type WNK4 and thus, relieves the WNK4-mediated inhibition of NCC (see Fig. 6 and 7). Moreover, L-WNK1 directly inhibits ROMK by enhancing endocytosis of the ion channel (Lazrak et al. 2006). KS-WNK1 antagonizes the ability of L-WNK1 to inhibit ROMK and to activate NCC. It was shown that an increase in dietary potassium intake decreases the ratio of L-WNK1 to KS-WNK1, thus, enhancing potassium secretion and lowering electroneutral sodium reabsorption via NCC. A similar effect is mediated by aldosterone-dependent activation of KS-WNK1, which inhibits degradation of ENaC through inhibition of an ubiquitin-protein ligase, thus, increasing electrogenic sodium reabsorption as a driving force of potassium secretion (Naray-Fejes-Toth et al. 2004).

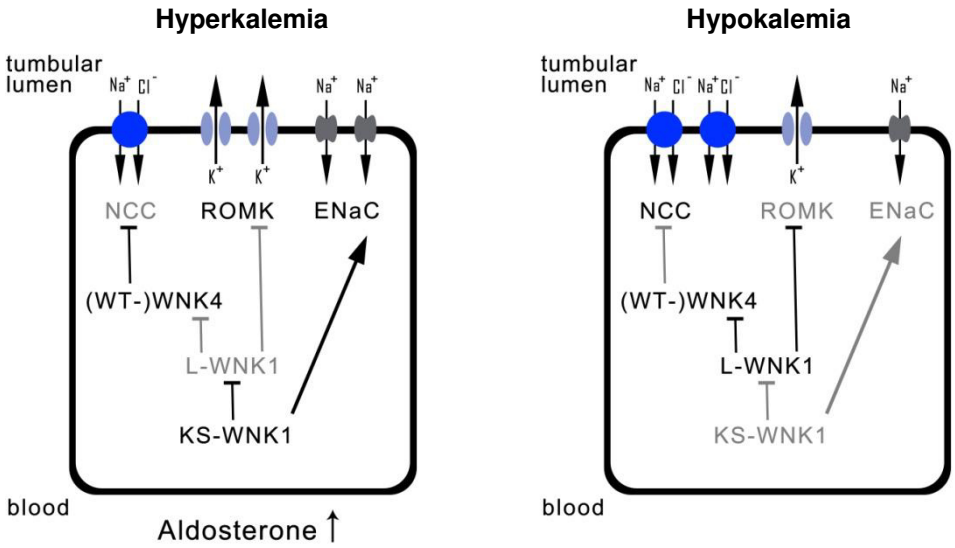


Figure 6 and 7: Integrated cell models for an overview of the role of KS-WNK1 and L-WNK1 and the involved signaling pathways in hyperkalemia, when blood aldosterone levels are high (left), and in hypokalemia (right).

The other two known members of the WNK family, WNK2 and WNK3, seem to be of no importance for renal salt handling. According to current research, WNK2 is only present in the brain and WNK3, although present in the kidneys, does not influence ion channels in the distal nephron (Mederle et al. 2013).

This complex renal system of ion channels and transporters, WNK kinases and other signal molecules regulates the body's water and electrolyte homeostasis and therefore plays a crucial part in blood pressure regulation. Dysfunction or abnormal regulation of any of its components can lead to the development of hypertension. This has been shown for the pathomechanism of PHA2, where mutations in WNK1 and WNK4 lead to altered kinase functioning and consequently to high blood pressure. Besides PHA2, there are many other hypertensive diseases which result from known mutations in renal ion channels or signal molecules. Single nucleotide polymorphisms in the WNK1 gene are likely to account for inter-individual differences in blood pressure response to dietary sodium and potassium intake (Liu et al. 2012, Osada et al. 2009). This predisposition of reacting to a higher NaCl intake with an increase in blood pressure is called sodium sensitivity and appears to be a precursor of hypertension. For a long time, high dietary sodium intake was believed to be the main exogenous factor for developing hypertension. Only in the last few years, has the role of dietary potassium intake on blood pressure become a subject of more intensive research. Several studies have shown that supplementation with potassium, up to the required daily potassium intake, decreases blood pressure in hypertensive patients and reduces salt sensitivity in normotensive people (Morris Jr. et al. 1999, Kaplan et al. 1985).

So far, only how and which renal signaling pathways are activated in hypovolemia or in hyperkalemia have been investigated. It remains unclear, however, how the distal nephron, the major site of salt regulation, reacts to the challenges of our typical Western diet, consisting of high sodium and low potassium. Under high sodium intake, the circulating renin-angiotensin system and thus, ANG II and aldosterone are suppressed, raising the question of how sodium and potassium transport are regulated and which signaling pathways are involved. A second, still unanswered question is as to how the kidneys deal with a high sodium and high potassium diet, two conflicting signals for aldosterone secretion.

To address these questions, a first set of experiments was conducted by Laura Schulte to investigate the effects of low, normal and high potassium intake in combination with high sodium intake on blood pressure, blood and urinary electrolyte levels and gene expression of renal ion channels and kinases (Vitzthum et al. 2014). Therefore, mice were fed a high sodium (3%) diet containing either normal (0.93%), low (0.03%) or high (5.0%) potassium for 10 days. Blood pressure was recorded between day 7 and 10 after the start of the dietary regimen by telemetry, and compared to blood pressure before the onset of diet (baseline conditions: 0.24% Na^+ and 0.93% K^+). Urinary water and electrolyte excretion were investigated in mice housed for 24 hours in metabolic cages from day 9 to 10 of the combined sodium/potassium diet. Blood for determination of plasma electrolytes was collected from the retrobulbar sinus. Expression of L32, NCC, ROMK, ENaC-beta, KS-WNK1, L-WNK1, WNK4, SGK1, and SPAK mRNA was determined by Realtime PCR in whole kidneys as well as in microdissected distal convoluted tubules (DCT) and cortical collecting ducts (CCD). The results of telemetric blood pressure measurements showed an increase in mean arterial pressure (MAP) both in the low and the high potassium diet groups (The blood pressure increase after low K^+ diet averages 5.5±1.5 mmHg and after high K^+ diet 15.7±3.3 mmHg, mean ± SEM, ANOVA; n=5 animals per diet). MAP was not elevated in mice with a normal daily potassium intake. Renal sodium clearances were significantly decreased in the low potassium (10.5±0.9 ml/24h, mean ± SEM, t-test, n= 8) as well as in the high potassium groups (10.5±0.9 ml/24h, mean ± SEM, t-test, n= 8) compared to normal potassium intake (15.7±1.6 ml/24h, mean ± SEM, $p<0.05$, t-test, n= 8). The potassium clearances were elevated in the high potassium group (362.2±111.1 ml/24h, mean ± SEM, t-test, n= 8) and decreased in the low potassium group (10.46±2.8 ml/24h, mean ± SEM, t-test, n= 8) compared to a normal potassium diet (123.2±31.38 ml/24h, mean ± SEM, $p<0.05$, t-test, n= 8). The mRNA expression analysis of the whole kidney shows a significant increase of WNK4 mRNA after 10 days on low potassium / high sodium diet compared to normal potassium diet ($p<0.05$, ANOVA, n= 8 animals per diet). Additionally, SPAK mRNA expression is significantly increased in the low potassium diet ($p<0.01$, ANOVA, n= 8 animals per diet). After 10 days on high potassium / high sodium diet, an increase of ENaC-ß mRNA and KS-WNK1/L-WNK1 mRNA is evident ($p<0.05$, ANOVA, n= 8 animals per

diet). In the distal convoluted tubules, an increase of NCC and WNK4 mRNA expression is detectable in the low potassium / high sodium diet group ($p<0.05$, t-test, n= 8 animals per diet). However, on a high potassium / high sodium diet, an increase of ENaC-ß mRNA is induced ($p<0.05$, t-test, n= 8-11 animals per diet). In the cortical collecting duct, WNK4 mRNA expression is elevated after 10 days on a low potassium / high sodium diet, whereas after 10 days on high potassium / high sodium diet an increase of SGK1 mRNA expression is obvious and the ratio of KS-WNK1 mRNA / L-WNK1 mRNA is increased ($p<0.05$, t-test, n= 8-11 animals per diet).

The data of Laura Schulte shows that under both low and high potassium diet an increase in arterial blood pressure occurred, resulting from excessive sodium retention. The transcriptional responses to low potassium intake were strikingly similar to those triggered by intravascular volume depletion (see Fig. 4). As a consequence of the renal remodeling, potassium excretion was reduced and thus, the acute deleterious effects of hypokalemia were prevented, but on the expense of enhanced electroneutral sodium reabsorption and blood pressure elevation. As shown by the results above, this is achieved through activation of the WNK4-SPAK-NCC pathway in the DCT. High potassium intake, however, induced changes in renal mRNA expression similar to those caused by hyperkalemia, suggesting a major role of aldosterone, and thus, mineralocorticoid receptor stimulation in the pathogenesis of the hypertensive response.

These findings indicate that, in a setting of simultaneous sodium and potassium imbalance, as it is the case in the typical Western diet, potassium homeostasis is maintained, leading to impaired sodium homeostasis and consequently to elevated blood pressure. These results raise the question of the potential upstream regulators for the observed renal remodeling in low and high potassium diet, respectively. Although ANG II is known to be a possible activation signal for the WNK4-SPAK-NCC pathway, so far, its role has only been investigated under hypovolemic conditions when ANG II levels are high due to RAAS activation (van der Lubbe et al. 2011, San-Cristobal et al. 2009). To test the hypothesis that ANG II is also responsible for activation of the WNK4-SPAK pathway in the setting of a Western diet, a similar set of experiments as the one described above, was conducted with the addition of the AT1-receptor-blocker losartan. If ANG II is the upstream signal

molecule in the low potassium/ high sodium diet, then losartan should be able to reverse the effects on renal remodeling and normalize blood pressure. Losartan was also given to mice on the high potassium/ high sodium diet to analyze its effect on the aldosterone-mediated activation of ENaC and ROMK. To investigate whether aldosterone functions as the major activator of the KS-WNK1-ENaC pathway in high potassium/ high sodium diet, effects of the mineralocorticoid receptor inhibitor spironolactone were examined under these conditions. Moreover, spironolactone was given to mice on the low potassium/ high sodium diet to investigate a possible role of aldosterone in this setting.

II. Methods and Material

II.1 Methods

II.1.1 Keeping of animals

The mice were kept in separate cages with free access to food and water. They were kept at a room temperature of about 20°C and were subjected to a circadian rhythm of 12 hours day and 12 hours night. These conditions were maintained throughout the entire experiment.

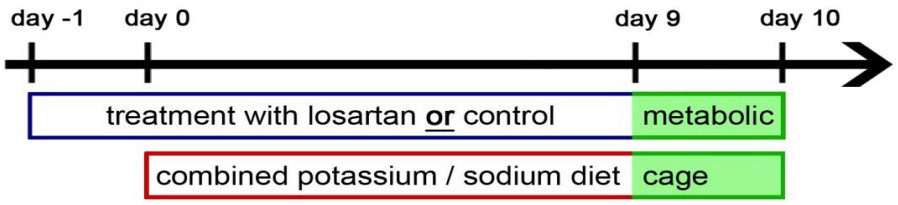
All experiments were approved by local authorities (Ministry for Social Affairs, Family, Health and Consumer Protection, Hamburg, Germany; No.G23/10).

The mice were weighed daily from the first day the medication was given until the last day of the salt diet because the weight was needed to calculate the daily dose of losartan.

II.1.2 Combined potassium/sodium diet

Each experimental group of mice receiving the same salt diet consisted of eight animals, four of which were treated additionally with either losartan or spironolactone. The animals were grouped randomly.

One experimental group was given a ten-day salt diet consisting of 3% sodium and 5% potassium, while the other experimental group was given a ten-day diet of 3% sodium and 0.03% potassium. The potassium/sodium diet started one day after the medication therapy had begun.



Each experiment with a specific potassium/medication combination was conducted twice, except for the experiments with the high potassium/ losartan combination which were carried out three times. This gave totals of eight animals receiving losartan plus 0.03% potassium and the respective control group with eight animals as well as twelve animals receiving losartan plus 5% potassium and twelve animals in the control group. Eight animals received spironolactone plus 0.03% potassium with a control group of eight animals and again a total of eight animals received spironolactone plus 5% potassium with a control group of the same size.

The individual experimental groups are listed in the following tables:

Combined salt diet	**Treatment with losartan**	**Control (no treatment)**
5% potassium plus 3% sodium	n= 12	n = 12
0.03% potassium plus 3% sodium	n = 8	n = 8

Combined salt diet	**Treatment with spironolactone**	**Control (no treatment)**
5% potassium plus 3% sodium	n= 8	n = 8
0.03% potassium plus 3% sodium	n = 8	n = 8

II.1.3 Medication

As previously mentioned, half the animals within each specific diet group were treated with either losartan or spironolactone, both of which are widely used and efficient antihypertensive drugs in humans.

The antihypertensive therapy was started one day before the salt diet and was continued throughout the ten-day salt diet.

II.1.3.1 Losartan

Losartan belongs to a class of drugs known as angiotensin receptor blockers (ARBs). These drugs specifically inhibit the AT1 receptor, one of the two known receptors of the hormone angiotensin II. The AT1 receptor is found in many tissues, for example on smooth muscle cells of blood vessels. In the kidneys, the AT1 receptor is present throughout the entire nephron, both at the apical and basolateral cell pole.

In the experiments, losartan was administered with the drinking water. The target dose in the drinking bottles was 30mg/kg per day for days one to nine of the salt diet and 40mg/kg per day for day ten. In this way, an approximate daily losartan dose of 20mg/kg was applied to each mouse. Therefore, it was necessary to weigh the mice and measure their water intake daily. The concentration of losartan in the drinking water was then calculated as follows: 30 * weight (in gram)/ 1000/ water intake (in ml) = losartan dose (in mg/ml).
A stock solution of losartan with a defined concentration was used and the correct daily dose was obtained by adding tap water. In the event the water bottles leaked, a mean drinking volume of 20ml was used to calculate the daily losartan dose.

As the calculation of the dose was based on the water intake of the last 24 hours, the actual dose the mice received might vary depending on their water intake in the following 24 hours. In order to find out the exact daily amount of losartan taken up by the animals every day, a retrospective calculation was made using the actual water intake of the day (see Fig.8 and 9).

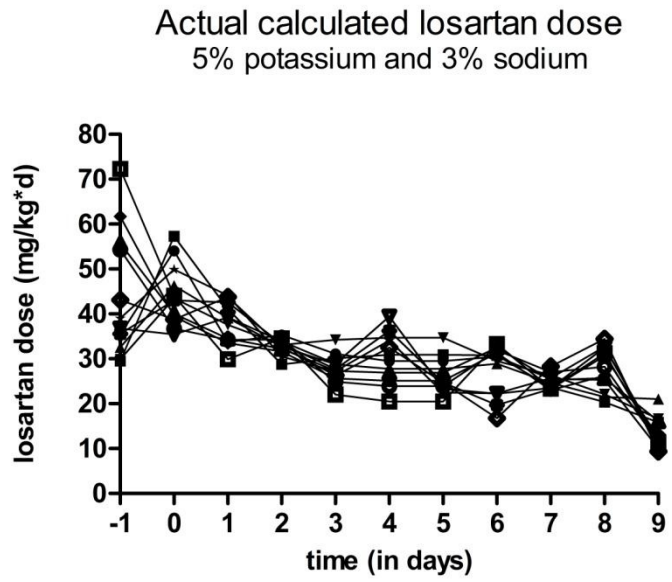


Figure 8: Retrospective calculation of the actual losartan dose from day -1 of the salt diet until day 9 of the diet for each animal receiving the high potassium/high sodium diet.

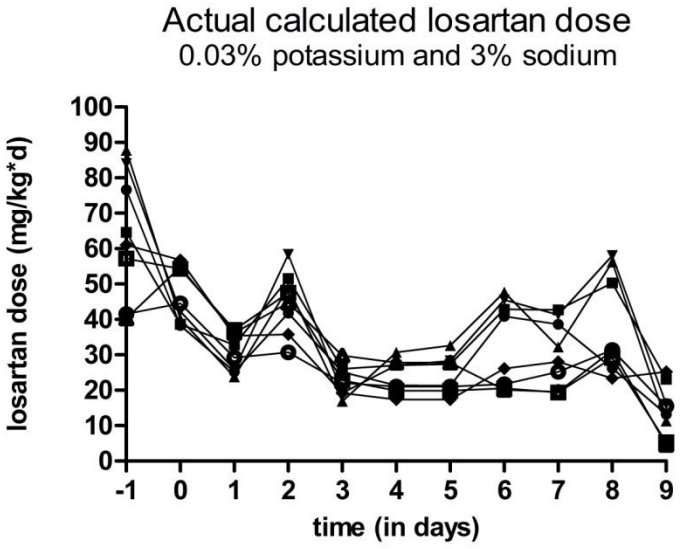


Figure 9: Retrospective calculation of the actual losartan dose from day -1 of the salt diet until day 9 of the diet for each animal receiving the low potassium/high sodium diet. In these experiments the water bottles leaked, so the figure shows the assumed losartan dose based on a mean drinking volume of 20mL.

To test whether the applied losartan dose had an effect on the kidneys, the Renin mRNA expression for both the losartan and control groups was quantified and compared. The quantification was conducted via real-time PCR. As shown in the diagrams below, the Renin expression showed significant differences between the two groups for both the low and high potassium diet, with the losartan treated animals having a significantly higher Renin expression than the untreated animals. The difference in Renin expression is due to the fact that Angiotensin II suppresses Renin release as a negative feedback mechanism (Muller et al. 2002, Kurtz and Wagner 1999). Therefore, losartan prevents this inhibitory effect on Renin secretion leading to an increase in Renin mRNA expression. The second reason for an increase in Renin expression in losartan treated mice is the blood pressure reduction in those animals leading to an increase of the RAAS.

In the 5% potassium group renin mRNA per L32 mRNA was 1.23 (± 0.1, n=12) in the losartan group, and 0.89 (± 0.05, n= 12) in the control group (see Fig.10). The values were significantly different (p= 0.0075).

For the losartan treated mice with the low potassium diet, Renin mRNA per L32 mRNA was 1.03 (± 0.12, n= 7). For the respective control group it was 0.65 (± 0.04, n= 7). The level of significance was p= 0.0096 (see Fig.11).

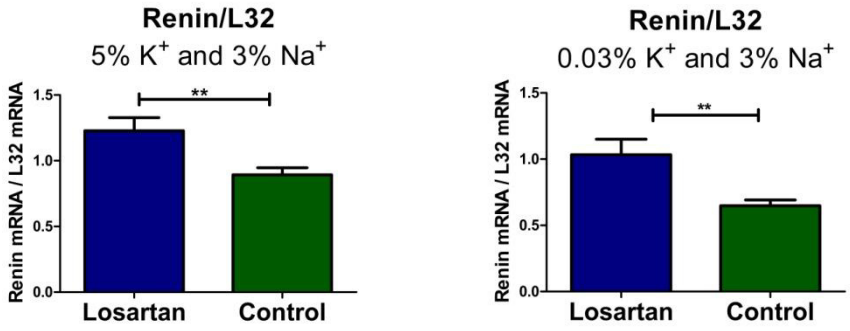


Figure 10 and 11: Comparison of renin mRNA per L32 mRNA between the losartan treated animals (blue column) and the control group (green column). The left diagram shows the results for the high potassium diet, the right diagram for the low potassium diet.

II.1.3.2 Spironolactone

Spironolactone is a member of the mineralocorticoid receptor antagonists and thus, inhibits aldosterone-mediated effects. In the kidney, aldosterone leads to potassium secretion and sodium retention in the collecting duct through stimulation of the renal outer medullary potassium channel (ROMK) and the epithelial sodium channel (ENaC), respectively.

In humans, spironolactone is used as a second line drug in the therapy of hypertension, mostly in combination with other antihypertensive drugs. Furthermore, it is prescribed in congestive heart failure and after a myocardial infarction for its antiproliferative effects.

In our experiments, spironolactone pellets were implanted with a 14-day release of 70mg per pellet. This correlates with a dose of approximately 200mg/kg per day. The pellets were implanted under isoflurane anaesthesia. An incision was made and a pocket was formed in the subcutaneous fatty tissue of the cervical region with forceps. The spironolactone pellet was inserted and the incision sutured.

The control group underwent the same procedure but without the implantation of a pellet (sham surgery).

All animals received analgesia (Carprofen 5mg/kg s.c.) once before the procedure.

Renin mRNA expression was also analyzed for the spironolactone groups. In the 5% potassium group, renin mRNA per L32 mRNA was 1.4 (± 0.12, n= 8) for the spironolactone treated animals, and 1.14 (± 0.06, n= 8) for the control group. The values were not significantly different (see Fig.12).

In the spironolactone treated group of the 0.03% potassium group, renin mRNA per L32 mRNA was 0.88 (± 0.02, n= 7), in the respective control group renin expression was 0.78 (± 0.04, n= 7, see Fig.13). The level of significance was p= 0.045.

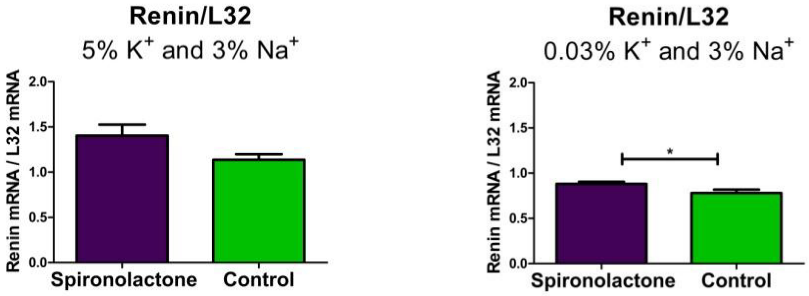


Figure 12 and 13: Comparison of renin mRNA per L32 mRNA between the spironolactone treated animals (purple column) and the control group (green column). The left diagram shows the results for the high potassium diet, the right diagram for the low potassium diet.

II.1.4 Metabolic cages

On day nine of the ten-day diet, the animals were put in metabolic cages for exactly 24 hours. During this period, urine and faeces were collected separately and food and water intake were monitored. The mice had free access to food and water.

The metabolic cages were set up so that the mice were sitting on a grid through which urine and faeces fell into a funnel. The funnel divided urine and faeces and they were collected in separate containers (see Fig.14).

The food was offered as a powder in a special receptacle with a collection tray. The water bottles also had a collection container. In this way, any contamination of urine and faeces through spilt food or water was prevented and food and water intake could be measured precisely.

Only the animals receiving the losartan therapy and the correlating control groups were kept in metabolic cages, not the spironolactone-treated animals or their control group. The reason for this is that the results from urinalysis did not show any significant differences between losartan-treated and control mice, despite differences in blood pressure and gene expression. So, it was decided not to repeat this procedure with the spironolactone experiments. After the 24-hour period in the metabolic cages, the experiment ended. The collected urine and faeces were weighed and then stored at -20°C.

Figure 14: Metabolic cage from Techniplast. The mice sit on a grid above the funnel through which urine and faeces fall into two different containers.

II.1.5 Retrieval of organs and blood

Following the metabolic cages, the animals were given isoflurane anaesthesia and blood was taken from the retrobulbar sinus. In order to be able to do this, the mice were held at the neck with a tight grip to accumulate blood in the retrobulbar venous plexus. Then, a glass capillary was pushed through the medial corner of the eye into the sinus. The blood was collected in a lithium heparin cup and for 10min left to sit at room temperature. After collection, the blood was centrifuged for 10min at 20°C and 2000g to obtain plasma. The plasma was stored at -20°C.

Afterwards, the mice were killed through cervical dislocation and organs (liver, lung, heart, kidneys and adrenal glands) were retrieved and immediately frozen in liquid nitrogen. The organs were stored at -80°C.

II.1.6 Analysis of urine and plasma

II.1.6.1 Urinalysis

After defrosting, urine electrolytes (i.e. sodium, potassium, and chloride) were determined using a potentiometer. This apparatus uses ion selective electrodes to analyze electrolyte concentrations in the samples. It compares the samples with a

control solution, which has a defined sodium, potassium and chloride concentration. From each urine sample 22µL were pipetted on the measuring chip together with 22µL of the control solution.

The urine pH value was determined with the aid of a common urine stick used in clinical routine. Urine osmolarity was measured with an osmometer. Specific gravity was analyzed using a manual refractiometer. The results were used to calculate the exact amount of urine excreted in the metabolic cages from urine weight and specific gravity.

II.1.6.2 Plasma analysis

After defrosting, plasma electrolytes were analysed with the same method as urine electrolytes (see above).

The results from urine and plasma analysis were used to calculate the sodium, potassium and chloride clearances and urine excretion in 24 hours for every animal.

As only the losartan/control group was kept in metabolic cages for 24 hours, urinalysis was solely conducted with these samples.

Formulas used for calculation:

Excretion (mmol/24h) = Urine volume (ml/24h) x Urine electrolyte concentration (mmol/l)

Clearance (ml/24h) = Urine volume (ml/24h) x Urine electrolyte concentration (mmol/l) / plasma electrolyte concentration (mmol/l)

II.1.7 RNA isolation from kidneys

Whole kidneys were used for isolating RNA. To avoid contamination with ribonucleases (RNase), all materials used for RNA isolation were delivered RNase-free, or if not, were washed in DEPC water and treated by autoclave (for 8h at 180°C dry heat) before use. During the entire procedure the samples were cooled on ice and latex gloves were worn.

The kidneys were put in RNase-free tubes with 10ml GTC solution and were then homogenized. From this homogenate 3ml were transferred in new tubes and 300µl sodium acetate were added. The rest of the homogenate was stored at -20°C in case the RNA isolation had to be repeated. After mixing thoroughly, 3ml acidic phenol were added. Again the samples were mixed and 600µl chloroform were added. At this point, the samples were mixed until turning milky to guarantee a proper mixture. The tubes were left on ice for 15min and then centrifuged for 20min at -4°C and 10.000g. The upper phase was pipetted carefully into another tube with 3mL isopropanol and the rest was discarded. After mixing, the samples were kept for at least one hour at -20°C. Afterwards, they were centrifuged for 20min at -4°C and 10.000g. The supernatant was taken off and discarded and the pellets were dissolved in 400µl GTC solution. The RNA containing GTC solution was transferred to Eppendorf tubes and 440µl sodium acetate and 40µl ethanol (100%) were added and mixed. For the second time, the assays were kept for a minimum of one hour at -20°C. They were centrifuged again for 20min at -4°C and 10.000g. The supernatant was taken off and discarded and 500µl ethanol (70%) were added to the pellet. After thorough mixing and centrifugation for 20min at -4°C and 10.000g, the supernatant was removed carefully and discarded. The remaining pellets were dried using a vacuum centrifuge and then dissolved in 100µl DEPC water.

II.1.8 Determination of RNA concentration and purity

For the subsequent reverse transcription of the RNA, it was necessary to determine the RNA concentration with a photometer. Therefore, the RNA was diluted 1:25 with DEPC water and pipetted into cuvettes. With the photometer, the extinction at 260nm was measured, taking into consideration the dilution of the samples. At the same

time, the contamination with proteins was measured by calculating the ratio of absorption at 260nm versus 280nm. The closer the ratio is to two, the smaller the protein contamination.

II.1.9 Quantitative and qualitative RNA analysis via Agilent

Another and far more precise method to determine RNA concentration and integrity is the analysis with the 2100 bioanalyzer using the 6000 RNA Nano kits from Agilent Technologies.

Through gel electrophoresis, the ribosomal RNA of both a ladder and the samples was split up and the 18S and 28S bands were compared in regard to concentration and integrity. The narrower the bands and the more distinct the peaks were, the higher was the RIN (RNA integrity number). Preparation and performance of this method were conducted strictly observing manual instructions.

The RIN of all RNA samples used for RT reaction (II.1.10) ranged from 8.1 to 9.7, with 10 as the highest result possible. If the RIN couldn't be obtained, this RNA sample was excluded from further analysis and interpretation.

The concentrations of the RNA samples were used to calculate the amount of RNA used for real-time PCR.

II.1.10 Reverse transcriptase reaction

Before transcribing the isolated RNA into cDNA, the RNA samples were diluted to obtain a concentration which was close to 0.1µg/µl. For the reverse transcriptase reaction (RT) 0.3µg of each RNA sample were utilized. The respective amount of each RNA sample needed was filled up with DEPC water to 9µL. Then 1µl of Oligo-dT was added, the samples were mixed and put on a heating block for 5min at 65°C. Now, 10µl of the RT mix were added on ice.

The RT mix consisted of (amount per sample):

- 4µl dNTP (2.5mM)

- 4µl buffer

- 1µl reverse transcriptase (enzyme)

- 1µl DEPC water

After mixing, the assays were put on a heating block for one hour at 37°C. Directly afterwards, they were put on a heating block for 2min at 94°C, then the reverse transcription was completed. The cDNA samples were frozen at -20°C until further use.

As a negative control in each experimental series, one RNA sample was run twice, once with the normal RT mixture and once with a mixture which did not contain the enzyme reverse transcriptase (-RT). This sample was later also employed in real-time PCR as a negative control for the specificity of the primers.

II.1.11 Quantification of specific gene expression via real-time PCR

In order to evaluate the effects of the combined potassium/sodium diet and the drug therapy on renal gene expression of certain transporters and intracellular signal molecules, real-time PCR experiments with a Light Cycler were performed using various primers (listed in III.1.10).

For each real-time PCR run, 2µl of all cDNA samples of one experimental group were each added to 18µl of a specific PCR mix, which had already been transferred to cooled Light Cycler capillaries.

The PCR mix consisted of (amount per sample):

- 10µl of a prefabricated mix (Master Mix Roche for SYBR green I)

- 6µl DEPC water

- 1µl sens-primer

- 1µl antisens-primer.

The capillaries were closed with a lid, centrifuged for a few seconds and then incubated in the Light Cycler.

For each Light Cycler run, the following protocol was conducted:

1. Activation of DNA polymerase: 15min at 95°C
2. Separation of double-stranded DNA: 15sec. at 94°C
3. Annealing: 30sec. at 60°C
4. Polymerization: 16sec. at 72°C
5. Amplification: repetition of steps 2 to 4 for 40-50 cycles
6. Melting curve: slow heating up to 94°C
7. Cooling to 40°C.

The Master Mix includes the dye SYBR green I which fluoresces in case it is bound to double-stranded DNA, i.e. the PCR product. After each cycle of amplification, the fluorescence was measured. By comparing the fluorescence of the samples to the fluorescence of a standard series, the relative concentrations of the cDNA samples were calculated.

In each run, one sample with water instead of cDNA and the –RT sample were included, as well as calibrators from one RNA sample.

Through previous experiments, L32 had been established as a reliable housekeeping gene. It was shown that its expression in the kidneys was not affected by the combined potassium/sodium diet nor by the medication. Thus, all relative concentrations from real-time PCR with different targeted genes were divided by those from the L32 run for normalization.

The same real-time PCR results from different genes were divided by the amount of RNA in µg utilized for PCR. In this way, the results from gene expression were comparable among one group with a particular potassium/medication combination, provided that the efficiency of the reverse transcriptase reaction was the same in all samples.

As the results from standardization with L32 and those from standardization with the amount of RNA in µg did not differ in gene expression patterns, the former results are used for further analysis and discussion.

III.1.12 Analysis of data from blood pressure measurements

The data used for analysis was obtained by H. Vitzhtum. Implantation of the telemetric devices was performed by A. Seniuk.

After implanting the telemetric devices, the mice were put on a 10-day baseline diet of 0.93% potassium and 0.24% sodium. Its composition resembles a normal diet. After these ten days, the mice were put on another 10-day diet which consisted of 3% sodium and either 0.03%, 0.93% or 5% potassium. In addition to this second diet, the animals were treated either with losartan or spironolactone, using the application scheme described above. Recorded parameters were activity, heart rate, diastolic, systolic, and mean blood pressure. The parameters were recorded from day 7 until day 10 of the ten-day diet. For data analysis, parameters of baseline diet and of the respective combined salt diet plus medication were compared to identify the effect of the latter.

Data reduction was performed by calculating the moving average per hour with DSI Dataquest A.R.T. Analysis (V 4.0, DataSciences) for each animal. A two-way ANOVA with Bonferroni's Multiple Comparison Test using GraphPad Prism software (Version 5.0) was performed.

II.1.13 Statistics

In all diagrams and texts, the arithmetic mean and its relative standard error (mean ± SEM) are given for every experimental group. The diagrams were created with GraphPad Prism software (Version 5.0) and a t-test was conducted with this program. A value of $p<0.05$ was regarded as significant. The p values in the diagrams were marked as follows:

* : $p < 0.05$

** : $p < 0.01$

*** : $p < 0.001$

II.2 Material

II.2.1 Animals

For all experiments, eight to ten week-old male C57Bl/6j mice from Charles River (Sulzfeld, Germany) were used. For acclimatization, the mice were kept for up to 14 days in our premises before starting the experiments. The mice used for the experiments had weights of 20- 24g.

II.2.2 Food

The mice were fed special salt food from SSNIFF Spezialdiäten GmbH (Soest, Germany). The food was composed of 3% sodium and either 5% or 0.03% potassium. It also contained crude protein (21.6%), crude fat (5.2%), crude fiber (5.0%), crude ash (16.8%), starch (15.0%), sugar (11.6%) and several vitamins.

II.2.3 Medication

II.2.3.1 Spironolactone

The spironolactone pellets were time release pellets for biomedical research from Innovative Research of America (Cat. No. M-161). Each pellet contained 70mg of spironolactone which was released within 14 days.

II.2.3.2 Losartan

Losartan was delivered as a dry chemical by MSD Sharp & Dohme GmbH. It was then dissolved in tap water to obtain a concentration of about 0.3 mg/ml. This solution was further diluted according to the needed daily dose.

II.2.4 Chemicals

II.2.4.1 Chemicals for RNA isolation

Chemical	Specifications
DEPC water	Mix of 1µl diethyl-pyrocarbonate (DEPC) per 1ml sterile, demineralized water which is autoclaved
GTC solution	- 250g GTC - 293ml DEPC water - 17.6ml 0.75M sodium citrate (pH 7), (Merck) - 8.8ml 30% sarcosyl All ingredients are mixed and dissolved in a water bath at 64°C. Before use 360µl mercaptoethanol (Merck) are added per 50ml GTC solution.
Guanidine thiocyanate (GTC)	Fluka
DEPC	Sigma
Sodium citrate	Merck
Acidic phenol (saturated with water)	Roth
Chloroform	Merck
Isopropanol	Merck
Sodium acetate	Merck
Ethanol 100%	Merck
Ethanol 70%	Merck

II.2.4.2 Chemicals for Agilent

All chemicals were delivered in the RNA 6000 Nano Kit from Agilent Technologies.

II.2.4.3 Chemicals for reverse transcriptase reaction

Chemical	Specifications
Oligo-dT	Invitrogen Cat. No. 18418-012 Conc.: 0.5µg/µl
dNTP	Fermentas #R0192 contains aqueous solution of dATP, dCTP, dGTP, dTTP, each at a final concentration of 10mM
Reverse transcriptase	Promega Cat. No. M1705 Conc.: 200U/µl
5x Buffer	Promega
DEPC water	Mix of 1µl diethyl-pyrocarbonate (DEPC) per 1ml sterile, demineralized water which is autoclaved

II.2.4.4 Chemicals for real-time PCR

Chemical	Specifications
SYBR Master Mix	Qiagen
DEPC water	Mix of 1µL diethyl-pyrocarbonate (DEPC) per 1mL sterile, demineralized water which is autoclaved

II.2.4.5 Chemicals for anaesthesia and analgesia

Isoflurane delivered by Abbott GmbH & co. KG (Wiesbaden, Germany).

Carprofen delivered by Pfizer Deutschland GmbH (Berlin, Germany).

II.2.5 Primers for real-time PCR

SGK1 primer

Sequence (5’ → 3’):	
Forward:	cgg gat ccc gag gct gct cga agc ac
Reverse:	gga att cga gga cgg acc cag gtt g

L32 primer

Sequence (5’ → 3’):	
Forward:	gca agt tcc tgg tcc aca atg tca
Reverse:	act cat ttt ctt cgc tgc gta gcc

SPAK primer

Sequence (5’ → 3’):	
Forward:	cag aga gta cct gat cga gaa gc
Reverse:	ctc atc cat ctc gtc atc gct cc

WNK4 primer

Sequence (5’ → 3’):	
Forward:	cgg gat ccg agg ctg tgg atg tgt acg c
Reverse:	gga att ccc gtg cgg atg cag cct tc

L-WNK1 primer

Sequence (5’ → 3’):	
Forward:	cgg gat ccg aaa cca ctg tgg aag tcg
Reverse:	gaa ttc ggt tca gtc act aaa aca atg c

KS-WNK1 primer

Sequence (5’ → 3’):	
Forward:	cgg gat ccc ttc ctt atg ctg tgg gct ag
Reverse:	gga att cgt caa aac tgg ctg gct tca c

Renin primer

Sequence (5’ → 3’):	
Forward:	cgg gat ccg tgc agc cgc ctc tac ctt gct tgt
Reverse:	gga att cgc agc tcg gtg acc tct cca aag g

NCC primer

Sequence (5’ → 3’):	
Forward:	cgg gat aat ggc aag gtc aag tcg
Reverse:	gga att ctg atg cgg atg tca ttg atg g

ROMK primer

Sequence (5’ → 3’):	
Forward:	cgg gat ccg gca ctg aca gaa agg atg
Reverse:	gga att cca ggt caa gta cag ttg tcc

ENaC alpha primer

Sequence (5’ → 3’):	
Forward:	cgg gat ccc ggc atg atg tac tgg cag
Reverse:	gga att cgc ctg gcg agt gta gga ag

ENaC beta primer

Sequence (5’ → 3’):	
Forward:	cgg gat cca tgt ggt tcc tgc tta cgc tg
Reverse:	gga att cgt cct ggt ggt gtt gct gtg

II.2.6 Devices

Device	Manufacturer
Light Cycler	Roche Diagnostics Deutschland GmbH (Mannheim, Germany)
Agilent 2100 bioanalyzer	Agilent Technologies (Waldbronn, Germany)
SPOTCHEM EL SE-1520 Analyzer (potentiometer)	ARKRAY Europe, B.V. (Amstelveen, The Netherlands)
Semi-Mikro-Osmometer	Knauer K-7400
Eppendorf centrifuge 5417C	Eppendorf (Hamburg, Germany)
Heraeus Biofuge stratos	Kendro laboratory products (Osterode, Germany)
SpeedVac (Eppendorf Concentrator 5301)	Eppendorf (Hamburg, Germany)
Autoclave KSG 112	KSG Sterilisatoren GmbH (Germany)
Photometer Ultrospec 3000	Pharmacia Biotech
Vortex (MS2 Minishaker)	Janke&Kunkel, Braun Biotech Int. Certomat MV
Polytron-Homogenisator (Ultra-Turrax T25)	Janke&Kunkel, IKA-Labortechnik
Metabolic cages	Techniplast
Refractiometer	NeoLab

II.2.7 Single-use materials

Material	Manufacturer
Eppendorf cups (1.5ml)	Eppendorf
Falcon tubes (15ml and 50ml)	Sarstedt
Lithium heparin cups	Sarstedt
Glass pipettes (10ml and 25ml)	Falcon
Pipette tips (2µl, 20µl, 200µl, 1000µl)	Eppendorf
Piston-stroke Pipettes	Gilson Pipetman
Light Cycler capillaries (20µl)	Roche
Photometer cuvettes (50-2000µl)	Eppendorf
Phenyl-bonded SPE columns	interchim

III. Results

III.1 Results from losartan experiments

The following table gives an overview of the relevant results from the metabolic cages for both the high and low potassium groups. A more detailed description of the results and the respective figures are shown below.

	High K+ diet 5% K+, 3% Na+		**Low K+ diet** 0.03% K+, 3% Na+	
	Losartan	Untreated	Losartan	Untreated
Food intake (g/24h)	3.2 ± 0.2	2.9 ± 0.2	1.9 ± 0.2	1.9 ± 0.2
Drinking volume (ml/24h)	8.6 ± 0.7	8.1 ± 0.7	6.2 ± 1.4	7.3 ± 1.3
Urine volume (ml/24h)	5.1 ± 0.5	4.6 ± 0.5	3.6 ± 0.9	4.3 ± 0.8
Urine osmolarity (mOsmol/l)	1483 ± 72	1602 ± 96	1167 ± 248	1043 ± 323
Plasma K^+ (mmol/l)	5.7 ± 0.4	5.5 ± 0.2	2.7 ± 0.2	2.5 ± 0.04
Urine K^+ (mmol/24h)	1.8 ± 0.1	1.6 ± 0.1	0.01 ± 0	0.01 ± 0
K^+ clearance (ml/24h)	334.9 ± 36.9	302.6 ± 20.6	4.8 ± 0.8	5.6 ± 0.6
Plasma Na^+ (mmol/l)	147.4 ± 1.5	146.7 ± 1.4	152.3 ± 1.7	153.1 ± 1.4
Urine Na^+ (mmol/24h)	1.9 ± 0.2	1.7 ± 0.1	1.0 ± 0.1	1.22 ± 0.18
Na^+ clearance (ml/24h)	12.9 ± 1.1	11.4 ± 0.9	6.8 ± 1.2	7.9 ± 1.2
Plasma Cl^- (mmol/l)	117.3 ± 1.1	124.3 ± 2.5*	118.0 ± 1.2	117.8 ± 1.9
Urine Cl^- (mmol/24h)	1.4 ± 0.1	1.6 ± 0.1	0.74 ± 0.13	0.87 ± 0.12
Cl^- clearance (ml/24h)	13.2 ± 0.9	11.4 ± 0.6	6.2 ± 1.1	7.4 ± 1.0

Table 1: Overview of the results from the metabolic cages for both the high and low potassium groups. The significant differences between the losartan treated and the untreated animals within each diet are marked as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

III.1.1 Weight during salt diet

For the 5% potassium group, the mean starting weight in the losartan group was 22.5g (± 0.4g, n= 12) and in the control group 22.1g (± 0.4g, n= 12). Thus, on day 0, there was no significant difference in weight between the two groups. On the last day of the ten-day diet, the animals in the losartan group had an average weight of 20.6g (± 0.4g, n=12) and the control group of 19.8g (± 0.4g, n=12). Again, no significant difference in weight was found between the two groups on the last day of the diet.

In the 0.03% potassium group, the starting weight in the losartan group was 23.4g (± 0.3g, n= 8) and in the control group 22.9g (± 0.4g, n= 8). On day 10 of the diet, the weight had decreased to 18.5g (± 0.2g) in the losartan group and to 18.2g (± 0.3g) in the control group. There was no significant difference between the two groups on the last day of the diet.

III.1.2 Food intake in metabolic cage

For the last 24 hours of the ten-day diet the mice were kept in metabolic cages for the exact measurement of urine and fecal excretion as well as water and food intake.

Among the high potassium group, the losartan treated animals had an average food intake of 3.2g (± 0.2g, n= 12) and the control group of 2.9g (± 0.2g, n= 12), showing no significant difference.

The losartan treated animals of the low potassium group had a mean intake of 1.9g (± 0.2g, n= 8), and the respective control group of 1.9g (± 0.2g, n= 8). There was also no significant difference (see Table 1, p.33).

III.1.3 Water intake in metabolic cages

In the last 24 hours of the experiments, the mice were held in metabolic cages where the drinking volume was measured precisely.

In the 5% potassium group, the losartan treated animals drank 8.6ml/24h (± 0.7ml/24h, n= 12) and the control group 8.1ml/24h (± 0.7ml/24h, n= 12). The difference was not significant.

In the 0.03% potassium group, the losartan treated mice had a mean water intake of 6.2ml/24h (± 1.4ml/24h, n= 8). For the control group, the respective drinking volume was 7.3ml/24h (± 1.3ml/24h, n= 8). Thus, there was no significant difference between the two groups (see Table 1, p.33).

III.1.4 Fecal excretion in metabolic cage

For the last 24 hours of the combined potassium/sodium diet, faeces was collected in the metabolic cages and weighed.

For the high potassium group, fecal excretion was 0.7g (± 0.1g, n= 12) in the losartan group and 0.6g (± 0.02g, n= 12) in the control group.

In the low potassium group, the losartan treated mice excreted 0.3g (± 0.03g, n= 8) and the untreated mice 0.4g (± 0.06g, n= 8).

For both diets, fecal excretion was not significantly different.

III.1.5 Urine excretion and urine osmolarity

In the metabolic cages, urine was collected in order to analyze urine volume, electrolytes, osmolarity and pH value. With this information sodium, potassium and chloride clearances were calculated.

Among the high potassium group, the animals which were treated with losartan had a mean urine volume of 5.1ml/24h (± 0.5ml/24h, n= 12) and urine osmolarity of 1483mOsmol/l (± 72mOsmol/l, n= 12). The untreated animals had a urine volume of 4.6ml/24h (± 0.5ml/24h, n= 12) and urine osmolarity of 1602mOsmol/l (± 96mOsmol/l, n= 12). The values are not significantly different.

The losartan group of the low potassium diet had an average urine volume of 3.6ml/24h (± 0.9ml/24h, n= 8) and urine osmolarity of 1167mOsmol/l (± 248mOsmol/l, n= 8). The respective control group excreted 4.3ml/24h (± 0.8ml/24h, n= 8) with a urine osmolarity of 1043mOsmol/l (± 323mOsmol/l, n= 8). The differences between losartan treated and untreated animals were not significant (see Table1, p.33).

III.1.6 Urine pH value

The animals on the low potassium diet had an acidic urine pH of 6.4 (± 0.2, n= 8) in the losartan group and 6.5 (± 0.2, n= 8) in the control group.

However, the animals receiving a high potassium diet had a basic urine pH value of 8.6 (± 0.2, n= 12) in the losartan group and of 8.3 (± 0.2, n= 12) in the control group. No significant difference was found in the above values for both diets.

III.1.7 Plasma electrolytes

To determine the effect of losartan on plasma electrolytes and clearance, plasma levels of sodium, potassium and chloride were analyzed.

The mice of the high potassium group did not develop hyperkalemia both in the losartan and the control group. Mean potassium plasma levels in the losartan group were 5.7mmol/l (± 0.4mmol/l, n= 12) and in the control group 5.5mmol/l (± 0.2mmol/l, n= 12, see Fig.15). Plasma sodium levels for the losartan treated animals were 147.4mmol/l (± 1.5mmol/l, n= 12) and for the untreated animals 146.7mmol/l (± 1.4mmol/l, n= 12, see Fig.16). The chloride levels showed a significant difference with 117.3mmol/l (± 1.1mmol/l, n= 12) in the losartan group and 124.3mmol/l (± 2.5mmol/l, n= 12) in the control group (see Fig.17).

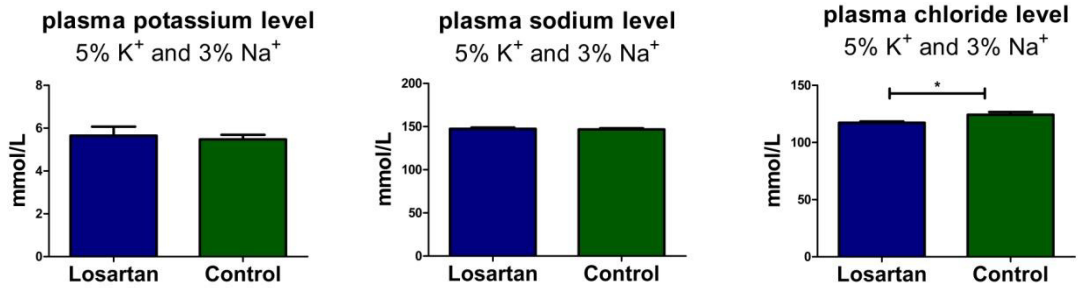


Figure 15, 16 and 17: Plasma electrolyte levels (in mmol/l) of potassium (left), sodium (middle) and chloride (right) for the high potassium/high sodium diet. The losartan group (blue column) and the untreated group (green column) show no significant differences except for the chloride plasma levels (n= 12 per column).

The low potassium and high sodium diet led to hypokalemia in treated and untreated animals. Plasma potassium levels in the losartan group were 2.7mmol/l (± 0.2mmol/l,

n= 8) and in the control group 2.5mmol/l (± 0.04mmol/l, n= 8, see Fig.18). The losartan treated mice had sodium levels of 152.3mmol/l (± 1.7mmol/l, n= 8) and the control group of 153.1mmol/l (± 1.4mmol/l, n= 8, see Fig.19). Plasma chloride levels were also similar with 118.0mmol/l (± 1.2mmol/l, n= 8) in the losartan group and 117.8mmol/l (± 1.9mmol/l, n= 8) in the control group (see Fig.20). Plasma electrolyte levels for both diets are also shown in Table 1 on page 33.

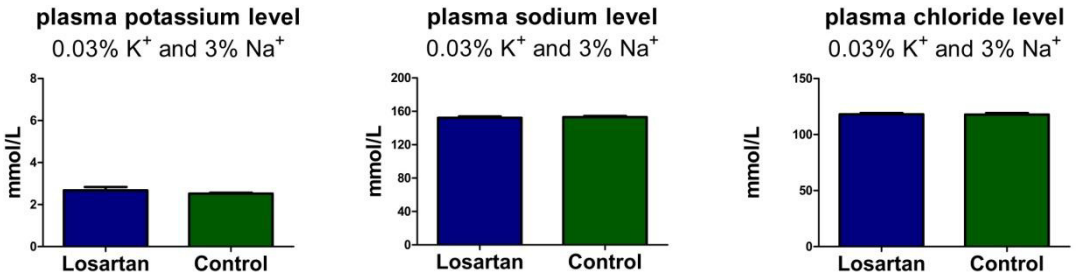


Figure 18, 19 and 20: Plasma electrolyte levels of potassium (left), sodium (middle) and chloride (right) for the low potassium/high sodium diet. The losartan group (blue column) and the untreated group (green column) show no significant differences (n= 8 per column).

III.1.8 Urine electrolyte excretion

Among the 5% potassium group, the losartan treated mice had a mean urine sodium excretion of 1.9mmol/24h (± 0.2mmol/24h, n= 12), while the control group had 1.7mmol/24h (± 0.1mmol/24h, n= 12, see Fig.21). The urine potassium excretion in the losartan group was 1.8mmol/24h (± 0.1mmol/24h, n= 12), and in the control group 1.6mmol/24h (± 0.1mmol/24h, n= 12, see Fig.22). For the same group urine chloride excretion was 1.4mmol/24h (± 0.1mmol/24h, n= 12), and in the losartan group 1.6mmol/24h (± 0.1mmol/24h, n= 12, see Fig.23).

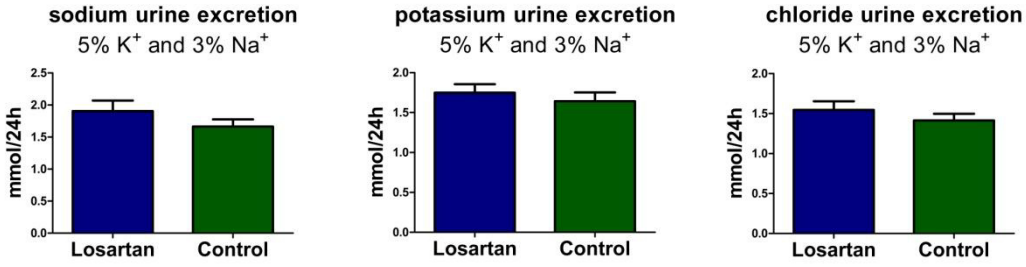


Figure 21, 22 and 23: Urine electrolyte excretion (in mmol/24h) of sodium (left), potassium (middle) and chloride (right) for the 5% potassium experiments. The differences are not significant (n= 12 per column).

The losartan group of the 0.03% potassium diet had an average urine sodium excretion of 1.0mmol/24h (± 0.2mmol/24h, n= 8, see Fig.24), a urine potassium excretion of 0.01mmol/24h (± 0.0mmol/24h, n= 8, see Fig.25), and a urine chloride excretion of 0.74mmol/24h (± 0.13mmol/24h, n= 8, see Fig.26). The untreated mice had a urine sodium excretion of 1.22mmol/24h (± 0.18mmol/24h, n= 8, see Fig.24), a urine potassium excretion of 0.01mmol/24h (± 0.0mmol/24h, n= 8, see Fig.25), and a urine chloride excretion of 0.87mmol/24h (± 0.12mmol/24h, n= 8, see Fig.26). Results of urine excretion are also listed in Table 1 on page 33.

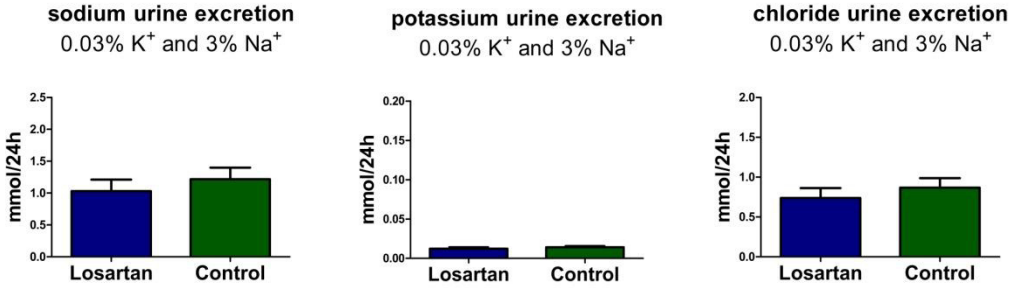


Figure 24, 25 and 26: Urine electrolyte excretion (in mmol/24h) of sodium (left), potassium (middle) and chloride (right) for the 0.03% potassium experiments. The differences between the losartan treated (blue column) and untreated animals (green column) are not significant (n= 8 per column).

III.1.9 Clearances

To find out whether losartan had an impact on renal electrolyte handling, clearances for sodium, potassium and chloride were calculated using the results from plasma electrolyte levels, urine electrolyte excretion and urine volume shown above.

Among the group with the high potassium diet, the losartan treated animals had a sodium clearance of 12.9ml/24h (± 1.1ml/24h, n= 12, see Fig.27), a potassium clearance of 334.9ml/24h (± 36.9ml/24h, n= 12, see Fig.28) and a chloride clearance of 13.2ml/24h (± 0.9ml/24h, n= 12, see Fig.29). The untreated mice had a sodium clearance of 11.4ml/24h (± 0.9ml/24h, n= 12, see Fig.27), a potassium clearance of 302.6ml/24h (± 20.6ml/24h, n= 12, see Fig.28) and a chloride clearance of 11.4ml/24h (± 0.6ml/24h, n= 12, see Fig.29). No significant differences were found.

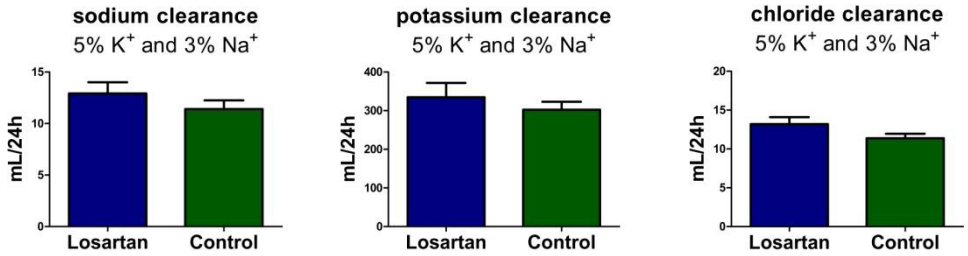


Figure 27, 28 and 29: Clearances (in mL/24h) of sodium (left), potassium (middle) and chloride (right) in the group with the high potassium diet. The results of the losartan treated group (blue column) and the control group (green column) are not significantly different (n= 12 per column).

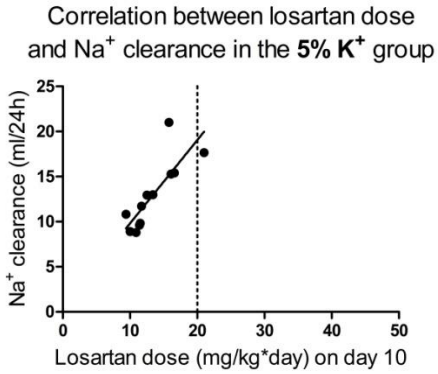


Figure 30: This diagram shows the positive correlation between the calculated losartan dose on the last day of the experiment and the sodium clearance for each animal of the high potassium and high sodium group which was treated with losartan (n=12). The correlation is roughly linear (r^2 = 0.68). The diagram also shows that most animals did not reach the aspired losartan dose of 20 mg/kg*day on the last day.

The losartan treated mice in the low potassium group had a sodium clearance of 6.8ml/24h (± 1.2ml/24h, n= 8, see Fig.31), a potassium clearance of 4.8ml/24h (± 0.8ml/24h, n= 8, see Fig.32), and a chloride clearance of 6.2ml/24h (± 1.1ml/24h, n= 8, see Fig.33). The respective control group had a sodium clearance of 7.9ml/24h (± 1.2ml/24h, n= 8, see Fig.31), a potassium clearance of 5.6ml/24h (± 0.6ml/24h, n= 8, see Fig.32), and a chloride clearance of 7.4ml/24h (± 1.0ml/24h, n= 8, see Fig.33). The differences were not significant (see Table 1, p.33).

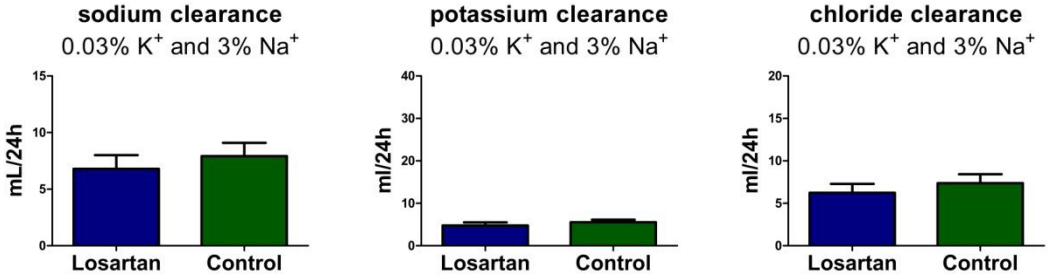


Figure 31, 32 and 33: Clearances (in ml/24h) of sodium (left), potassium (middle) and chloride (right) in the group with the 0.03% potassium diet. The results of the losartan treated group (blue column) and the control group (green column) are not significantly different (n= 8 per column).

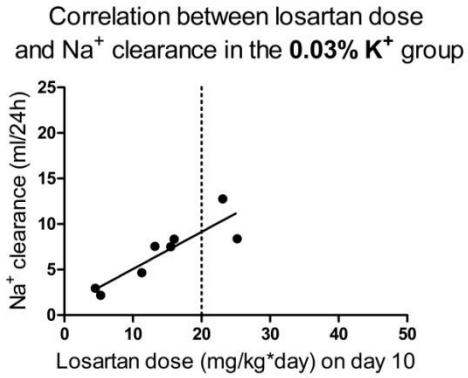


Figure 34: This diagram shows the positive correlation between the calculated losartan dose on the last day of the experiment and the sodium clearance for each animal of the low potassium group which was treated with losartan (n=8). The correlation is roughly linear (r^2 = 0.78). The diagram also shows that most animals did not reach the aspired losartan dose of 20 mg/kg*day on the last day.

III.1.10 Results from gene expression (Real-time results)

III.1.10.1 SGK1

Among the high potassium animals, SGK1 mRNA per L32 mRNA was 1.19 (± 0.09, n= 12) in the losartan group, and 1.38 (± 0.06, n= 12) in the control group (see Fig.35).

The animals in the low potassium plus losartan group were found to have a SGK1 mRNA per L32 mRNA of 0.75 (± 0.07, n= 7), and of 0.76 (± 0.08, n= 7) in the respective control group (see Fig.36). For both diets SGK1 expression was not significantly different.

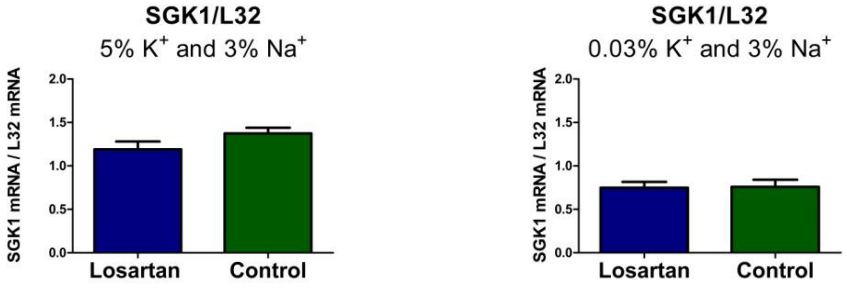


Figure 35 and 36: SGK1 mRNA per L32 mRNA for the high potassium (left) and low potassium (right) diet. The blue columns show the results for the losartan treated group, the green ones for the control groups.

III.1.10.2 SPAK

SPAK mRNA per L32 mRNA in the 5% potassium group was 1.01 (± 0.04, n= 11) for the losartan treated animals, and 1.06 (± 0.04, n= 11) for the control group. The values were not significantly different (see Fig.37).

Among the animals receiving the low potassium diet, SPAK mRNA per L32 mRNA was 0.77 (± 0.03, n= 7) for the losartan group, and 0.78 (± 0.04, n= 7) for the control group. Thus, SPAK expression per L32 was not significantly different (see Fig.38).

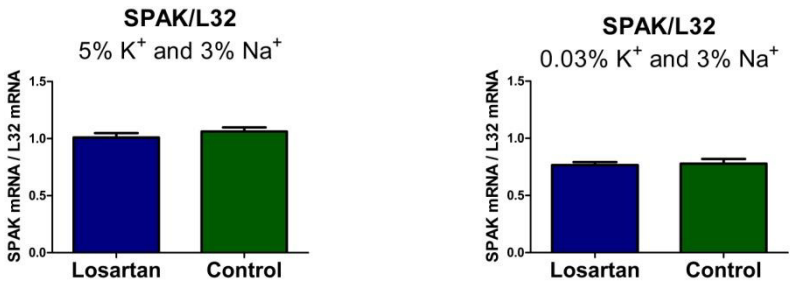


Figure 37 and 38: SPAK mRNA per L32 mRNA for the high potassium (left) and low potassium (right) diet. The blue columns show the results for the losartan treated group, the green ones for the control group.

III.1.10.3 WNK4

WNK4 mRNA per L32 mRNA was 1.28 (± 0.14, n= 11) for the losartan group of the high potassium diet, and 1.43 (± 0.09, n= 11) for the respective control group. Thus, the high potassium diet showed no significant differences in WNK4 expression between the treatment and control group (see Fig.39).

The losartan group of the low potassium diet had a WNK4 per L32 expression of 0.91 (± 0.1, n= 7). WNK4 expression of the control group was 1.14 (± 0.03, n= 7). The values were significantly different (p= 0.049, see Fig.40).

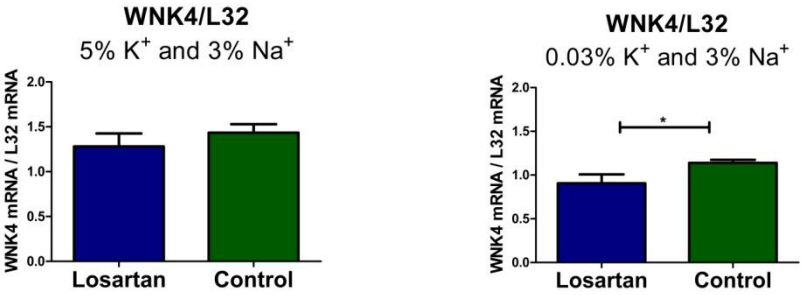


Figure 39 and 40: WNK4 mRNA per L32 mRNA for the high potassium (left) and low potassium (right) diet. The blue columns show the results for the losartan treated group, the green ones for the control group.

III.1.10.4 L-WNK1

Among the mice receiving the 5% potassium and 3% sodium diet the losartan group had a L-WNK1 mRNA per L32 mRNA expression of 1.16 (± 0.08, n= 10), and the control group of 1.28 (± 0.09, n= 11). The values were not significantly different (see Fig.41).

The low potassium experiments showed an L-WNK1 per L32 mRNA expression of 1.03 (± 0.08, n= 7) for the losartan treated animals, and of 1.12 (± 0.08, n= 7) for the control group. There was no significant difference between the two groups (see Fig.42).

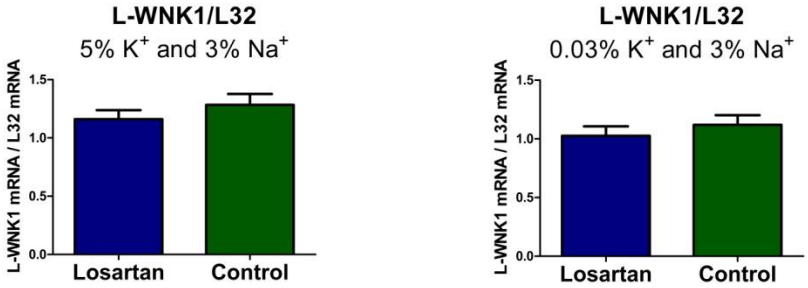


Figure 41 and 42: L-WNK1 mRNA per L32 mRNA for the high potassium (left) and low potassium (right) diet. The blue columns show the results for the losartan treated group, the green ones for the control groups.

III.1.10.5 KS-WNK1

KS-WNK1 mRNA per L32 mRNA was 0.80 (± 0.08, n= 11) for the losartan group of the 5% potassium diet, and 0.86 (± 0.05, n= 10) for the respective control group (see Fig.43).

For the losartan treated mice with the low potassium diet, KS-WNK1 mRNA per L32 mRNA was 1.03 (± 0.14, n= 6), and for the respective control group it was 1.18 (± 0.07, n= 7, see Fig.44). For both diets, the differences were not significant.

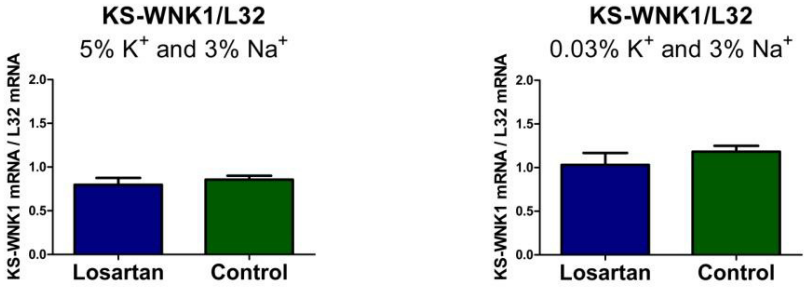


Figure 43 and 44: Expression of KS-WNK1 per L32 for the losartan group (blue columns) and the control group (green columns). The left figure shows the results for the high potassium diet, the right figure for the low potassium diet.

III.1.10.6 KS-WNK1 / L-WNK1

The ratio of KS-WNK1 mRNA per L-WNK1 mRNA in the 5% potassium group was 0.64 (± 0.03, n= 10) for the losartan treated animals and 0.71 (± 0.04, n= 11) for the control group (see Fig.45). The difference was not significant.

Among the 0.03% potassium diet group, the ratio KS-WNK1 / L-WNK1 was 1.04 (± 0.11, n= 7) for the losartan group and 1.3 (± 0.22, n= 8) for the control group (see Fig.46). Again, the difference was not significant.

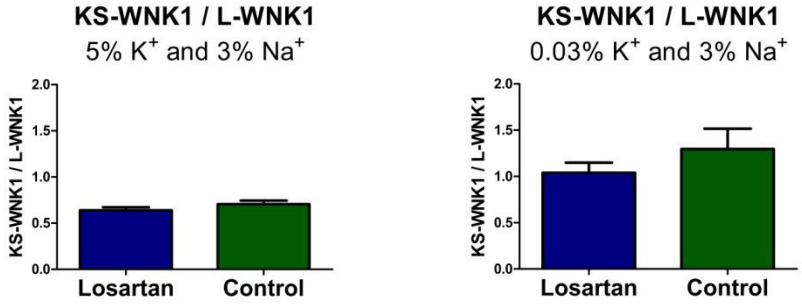


Figure 45 and 46: Expression of KS-WNK1 per L-WNK1 for the losartan group (blue columns) and the control group (green columns). The left figure shows the results for the high potassium diet, the right figure for the low potassium diet.

III.1.10.7 NCC

Among the mice on the 5% potassium diet, NCC mRNA per L32 mRNA was 0.83 (± 0.12, n= 11) for the losartan group, and 0.84 (± 0.1, n= 11) for the untreated group. The mean rates were not significantly different (see Fig.47).

The low potassium experiments showed an NCC per L32 mRNA expression of 0.79 (± 0.06, n= 7) for the losartan treated animals, and of 0.85 (± 0.05, n= 7) for the control group. The difference was not significant (see Fig.48).

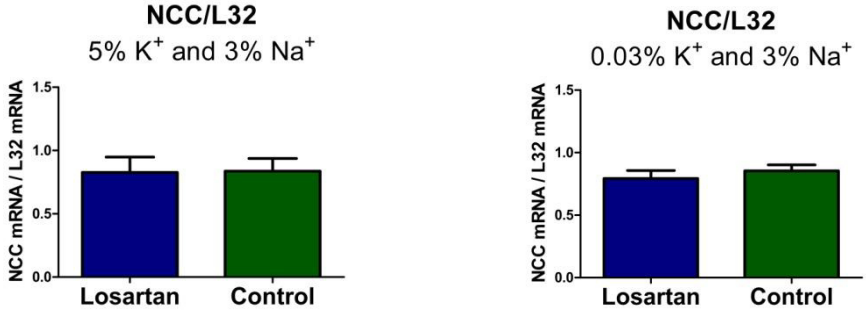


Figure 47 and 48: NCC mRNA per L32 mRNA of the losartan and control group for the high potassium (left) and low potassium (right) diet.

III.1.10.8 ROMK

In the low potassium group the losartan treated mice had a ROMK mRNA per L32 mRNA expression of 1.03 (± 0.13, n= 7), and the control animals of 0.99 (± 0.03, n= 7, see Fig.49). The difference was not significant.

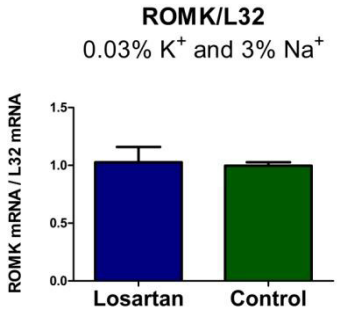


Figure 49: ROMK mRNA per L32 mRNA for the low potassium diet. The blue columns show the results for the losartan treated group, the green ones for the control group.

III.1.10.9 ENaC alpha

Among the animals receiving the 0.03% potassium diet, ENaC alpha mRNA per L32 mRNA was 0.72 (± 0.08, n= 7) for the losartan group, and 1.07 (± 0.16, n= 7) for the control group. Thus, ENaC alpha expression per L32 was not significantly different (see Fig.50).

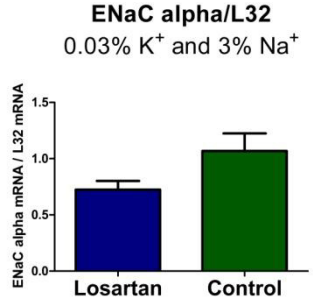


Figure 50: ENaC alpha mRNA per L32 mRNA of the losartan and control group for the low potassium diet.

III.1.10.10 ENaC beta

The low potassium group showed an ENaC beta mRNA per L32 mRNA expression of 1.08 (± 0.06, n= 6) for the losartan treated animals, and of 1.14 (± 0.09, n= 6) for the

control group. There was no significant difference between control and losartan group (see Fig.51).

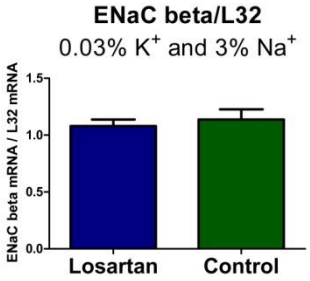


Figure 51: Expression of ENaC beta per L32 for the losartan group (blue columns) and the control group (green columns) in the low potassium diet group. The difference is not significant.

III.1.11 Data from blood pressure experiments

III.1.11.1 Activity

There was no significant difference in activity over the 60-hour period of measurements between baseline and diet plus losartan for all three potassium levels (i.e. 5%, 0.03% and 0.93% potassium). As described before, baseline diet is a ten-day diet with 0.93% potassium and 0.24% sodium which was administered before the mice were put on the combined salt diet and treatment with either losartan or spironolactone. Data obtained during baseline diet is compared to data from combined salt diet plus treatment.

III.1.11.2 Mean arterial pressure (MAP)

In the experiments with losartan plus high potassium diet, mean arterial pressure was significantly higher than in baseline. Compared to the baseline, the MAP during drug plus diet treatment was elevated every night. However, the elevation was only significant at certain times in the first and last night of data recording (see Fig.52). In the experiments with 0.03% potassium plus losartan, the MAP was not significantly different from baseline, except for once when it was significantly lower. At all other times, MAP was almost the same under baseline and under treatment (see Fig.53). Mean arterial pressure was also not significantly different in the 0.93% potassium plus losartan group compared to baseline (see Fig.54).

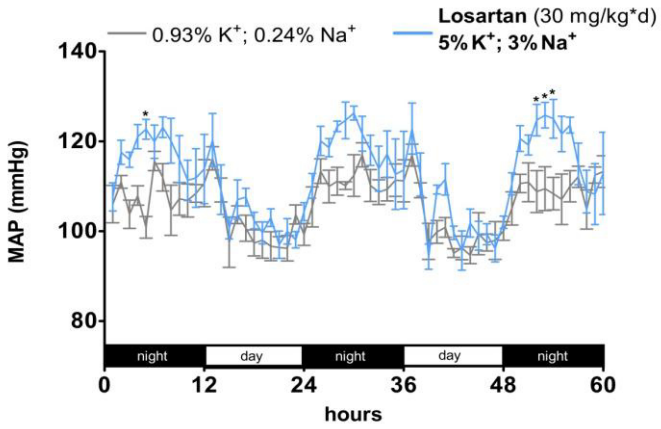


Figure 52: Comparison of MAP (in mmHg) between baseline (grey line) and treatment with losartan plus 5% potassium and 3% sodium (blue line). MAP was significantly higher in experiments with losartan treatment at 5 hours ($p<0.001$), 52 hours, 53 hours and 54 hours ($p<0.05$ each).

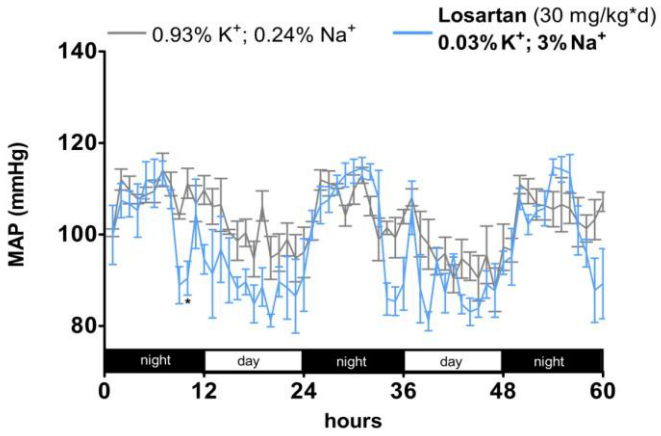


Figure 53: Comparison of MAP (in mmHg) between baseline (grey line) and treatment with losartan plus 0.03% potassium and 3% sodium (blue line). MAP was only significantly lower at 10 hours ($p<0.01$).

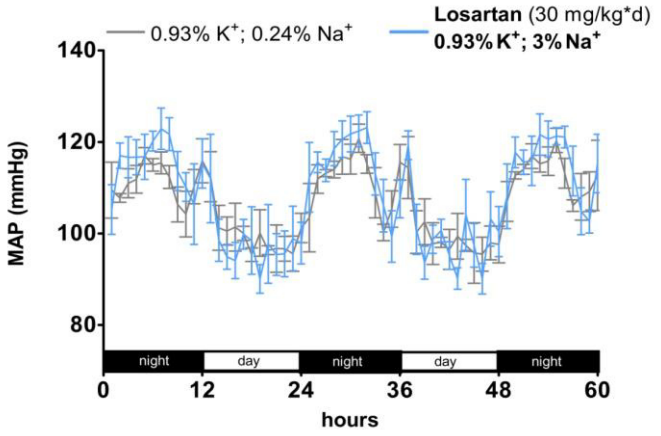


Figure 54: Comparison of MAP (in mmHg) between baseline (grey line) and treatment with losartan plus 0.93% potassium and 3% sodium (blue line). MAP was not significantly different between baseline and treatment.

III.1.11.3 Systolic pressure

Compared to baseline, the losartan plus 5% potassium group had elevated systolic pressure at night throughout the entire 60-hour period. However, this elevation was only significant at one point during the first night (at 5 hours, $p<0.001$). There were no significant differences in systolic pressure over the 60-hour period for neither the 0.03% potassium plus losartan nor the 0.93% potassium plus losartan experiments, compared to the respective baseline measurements.

III.1.11.4 Diastolic pressure

Similar to the results of mean arterial pressure, diastolic pressure was significantly higher in the 5% potassium plus losartan experiments compared to baseline. The increase in diastolic pressure was significant at several times in the first and last night of data recording (at 5 hours ($p<0.001$), 8 hours ($p<0.05$), 52 hours ($p<0.05$), 53 hours ($p<0.05$), 54 hours ($p<0.01$) and 55 hours ($p<0.05$)). In the low potassium group, diastolic pressure was only once significantly lower under losartan compared to baseline (at 10 hours, $p<0.05$). There were no significant differences in diastolic pressure between baseline and losartan plus diet for the normal potassium group.

III.1.11.5 Heart rate

The 5% potassium plus losartan experiment showed a significantly higher heart rate than baseline at several times (see Fig.55). In the 0.03% potassium plus losartan experiments, the heart rate was significantly lower than baseline at several times (see Fig.56). There were no significant differences found in the heart rate between baseline and the 0.93% potassium plus losartan group (see Fig.57).

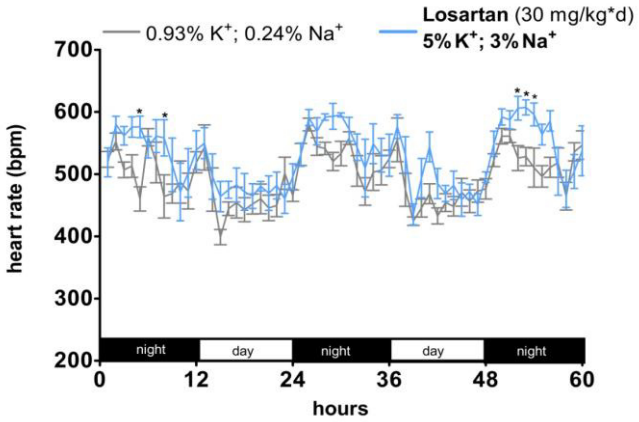


Figure 55: Comparison of heart rate (in beats per minute) between baseline (grey line) and 5% potassium plus losartan (blue line) over a 60-hour period. Heart rate was significantly higher in the losartan experiments at 5 hours ($p<0.001$), 8 hours ($p<0.01$), 52 hours ($p<0.05$), 53 hours ($p<0.05$) and 54 hours ($p<0.05$).

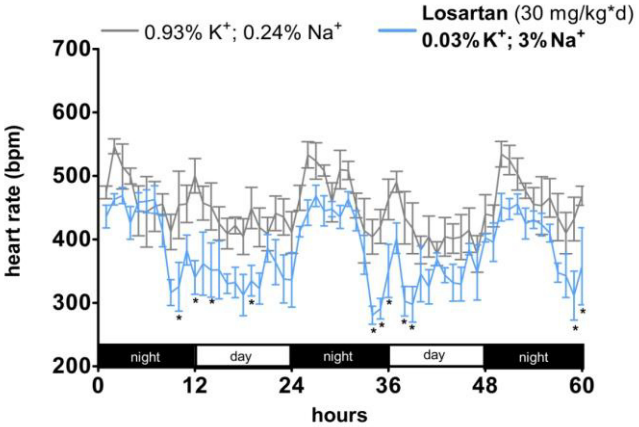


Figure 56: Comparison of heart rate (in beats per minute) between baseline (grey line) and 0.03% potassium plus losartan (blue line) over a 60-hour period. Heart rate was significantly lower in the losartan experiments at 10 hours ($p<0.001$), 12 hours ($p<0.01$), 14 hours ($p<0.05$), 19 hours ($p<0.01$), 34 hours ($p<0.01$), 35 hours ($p<0.001$), 36 hours ($p<0.01$), 38 hours ($p<0.001$), 39 hours ($p<0.01$), 59 hours ($p<0.01$) and 60 hours ($p<0.05$).

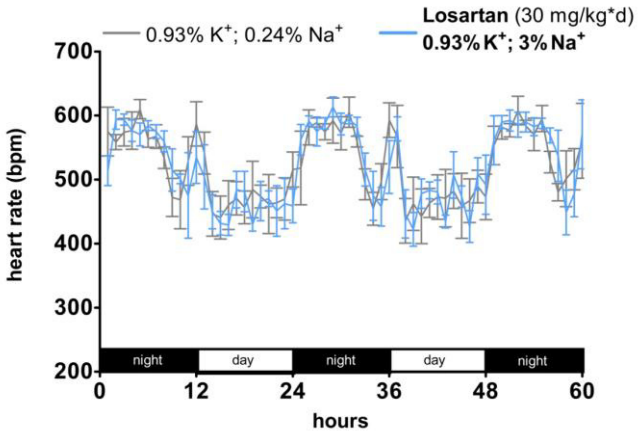


Figure 57: Comparison of heart rate (in beats per minute) between baseline (grey line) and 0.93% potassium plus losartan (blue line) over a 60-hour period. Heart rate was not significantly different.

III.2 Results from spironolactone experiments

III.2.1 Weight during salt diet

The spironolactone treated animals of the high potassium group weighed 22.93g (± 0.22g, n= 8) on the first day of the diet. The untreated mice weighed 23.55g (± 0.49g, n= 8). On the last day of the experiment, the treated animals weighed 22.2g (± 0.2g, n= 8), and the control group's weight was 22.5g (± 0.5g, n= 8). The mean weight of the treated and untreated animals was not significantly different.

In the 0.03% potassium group, the mean weight on day 0 was 25.02g (± 0.22g, n= 7), and in the control group 24.3g (± 0.2g, n= 7). The mean weight on the last day was 21.1g (± 0.5g, n= 7) for the spironolactone treated animals, and the control animals weighed 19.9g (± 0.5g, n= 7). The difference in weight was not significant.

III.2.2 Plasma electrolytes

Plasma potassium levels in the 5% potassium group were found to be significantly higher for the animals that were additionally treated with spironolactone than for the respective control animals. The mean plasma potassium level of the spironolactone group was 5.7mmol/l (± 0.1mmol/l, n=8), and of the control group 4.7mmol/l (± 0.2mmol/l, n= 8, p= 0.002, see Fig.58).

Plasma sodium levels for the same spironolactone group were 149.0mmol/l (± 1.2mmol/l, n= 8), and for the control group 150.4mmol/l (± 1.1mmol/l, n= 8, see Fig.59). The plasma sodium levels were not significantly different.

Plasma chloride levels were 117.9mmol/l (± 0.9mmol/l, n= 8) for the treated animals and 122.0mmol/l (± 1.1mmol/l, n= 8) for the control group. A significant difference was found (p= 0.012, see Fig.60).

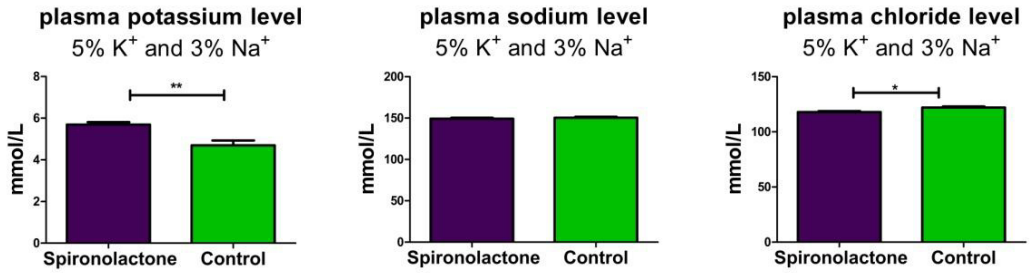


Figure 58, 59 and 60: Plasma potassium, sodium and chloride levels (in mmol/l) for the spironolactone treated (purple column) and untreated animals (green column) of the 5% potassium group.

Among the mice of the 0.03% potassium group, plasma potassium levels showed no significant difference between the spironolactone treated mice and the control group. Both groups developed hypokalemia. Plasma potassium levels of the treated group were 2.6mmol/l (± 0.1mmol/l, n=7), while the control group had levels of 2.5mmol/l (± 0.1mmol/l, n= 7, see Fig.61).

Plasma sodium levels of the spironolactone group were 153.0mmol/l (± 1.7mmol/l, n= 7), and of the control group 152.9mmol/l (± 0.5mmol/l, n= 7, see Fig.62). The values were not significantly different.

Plasma chloride levels were 125.3mmol/l (± 4.1mmol/l, n= 7) in the spironolactone treated group, and 121.3mmol/l (± 1.6mmol/l, n= 7, see Fig.63) in the respective control group. The difference was not significant.

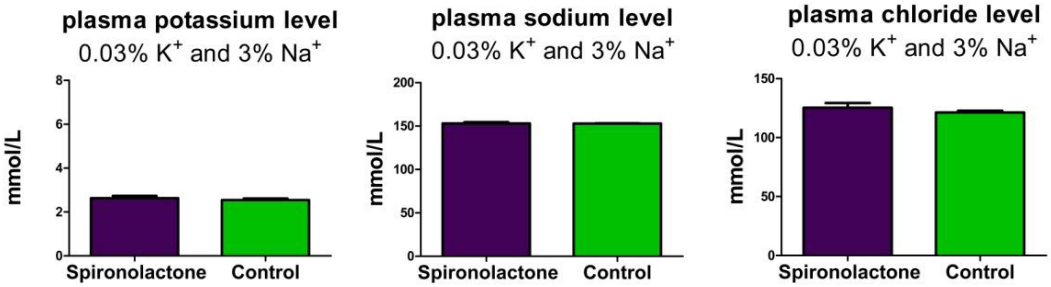


Figure 61, 62 and 63: Plasma potassium, sodium and chloride levels (in mmol/l) for the spironolactone treated animals (purple column) and the untreated animals (green column) of the low potassium diet.

III.2.3 Results from gene expression (Real-time results)

III.2.3.1 SGK1

SGK1 mRNA per L32 mRNA expression in the 5% potassium group was 0.84 (± 0.14, n= 8) for the spironolactone treated animals, and 1.03 (± 0.14, n= 8) for the respective control group. The values were not significantly different (see Fig.64).

The spironolactone group of the low potassium diet had an SGK1 per L32 expression of 0.92 (± 0.06, n= 7), and the control group of 0.95 (± 0.05, n= 7). The difference was not significant (see Fig.65).

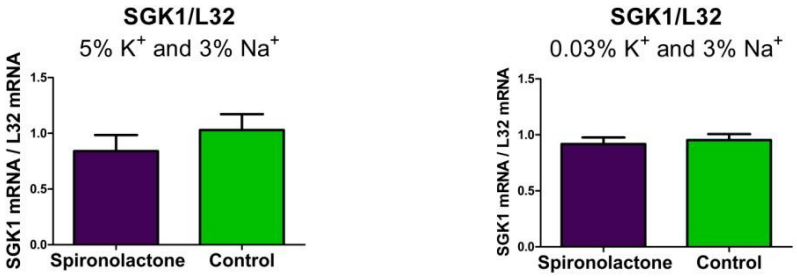


Figure 64 and 65: SGK1 mRNA per L32 mRNA expression of the spironolactone treated group (purple column) and the untreated group (green column). The left figure shows the results for the 5% potassium diet, the right figure for the 0.03% potassium diet.

III.2.3.2 SPAK

SPAK mRNA per L32 mRNA for the 5% potassium group was 1.0 (± 0.02, n= 8) in the losartan group, and 0.97 (± 0.02, n= 8) in the control group. The values were not significantly different (see Fig.66).

Among the animals receiving the0 0.03% potassium diet, SPAK per L32 expression was 0.97 (± 0.02, n= 7) for the losartan group, and 0.87 (± 0.04, n= 7) for the control group. Thus, SPAK expression per L32 was not significantly different in the two groups (see Fig.67).

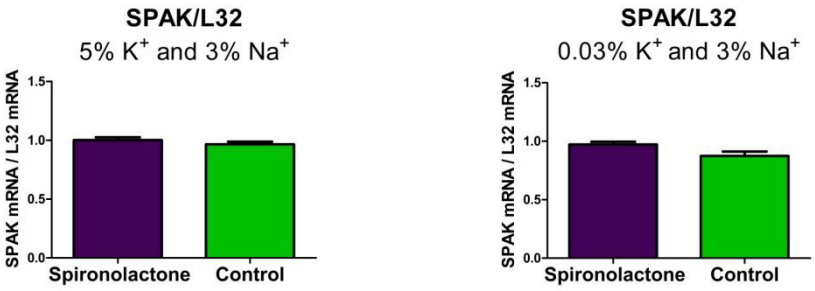


Figure 66 and 67: SPAK mRNA per L32 mRNA for the treated group (purple column) and the untreated group (green column) of the 5% potassium (left) and the 0.03% potassium diet (right).

III.2.3.3 WNK4

The spironolactone treated animals of the 5% potassium group had a WNK4 mRNA per L32 mRNA expression of 0.84 (± 0.05, n= 8), and the control group of 0.83 (± 0.05, n= 8). The values were not significant (see Fig.68).

In the 0.03% potassium group, the WNK4 per L32 expression of the spironolactone group was 1.06 (± 0.05, n= 7). This was significantly higher than 0.89 (± 0.05, n= 7), the expression value for the respective control group (p= 0.032, see Fig.69)

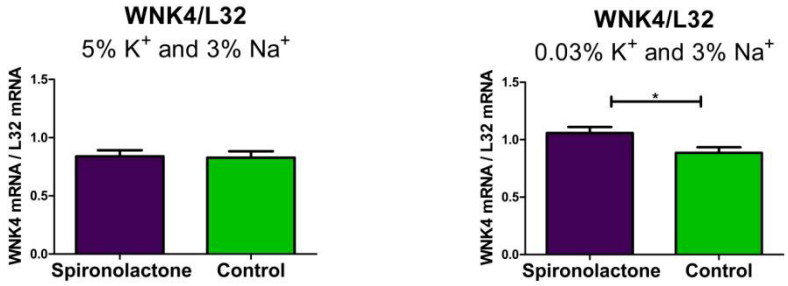


Figure 68 and 69: WNK4 per L32 expression of the spironolactone treated and untreated animals of the 5% potassium (left) and 0.03% potassium (right) group.

III.2.3.4 L-WNK1

Among the mice receiving the 5% potassium diet, the spironolactone treated mice had an L-WNK1 mRNA per L32 mRNA expression of 0.99 (± 0.04, n= 8), and the control group of 0.96 (± 0.05, n= 8). The values were not significantly different (see Fig.70).

The 0.03% potassium experiments showed L-WNK1 per L32 mRNA expressions of 0.84 (± 0.06, n= 7) for the spironolactone group, and of 0.72 (± 0.09, n= 7) for the control group. No significant differences were found between the two groups (see Fig.71).

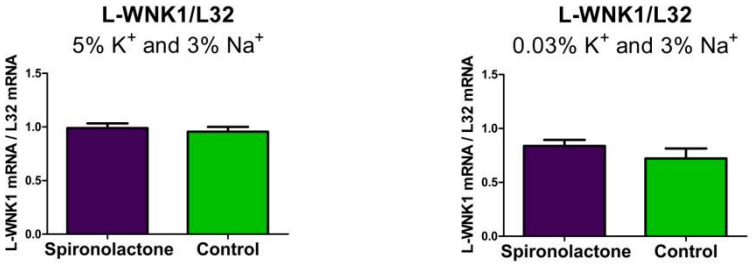


Figure 70 and 71: L-WNK1 mRNA per L32 mRNA for the spironolactone treated group and the control group. The left figure shows the results for the 5% potassium group, the right figure for the 0.03% potassium diet.

III.2.3.5 KS-WNK1

Among the mice of the 5% potassium group, KS-WNK1 mRNA per L32 mRNA was 0.95 (± 0.06, n= 8) for the losartan group, and 0.71 (± 0.07, n= 8) for the untreated group (see Fig.72). The values were significantly different (p= 0.017).

The KS-WNK1 per L32 expression of the 0.03% potassium and spironolactone treated animals was 1.0 (± 0.1, n= 7), and was 1.04 (± 0.06, n= 6) for the respective control group (see Fig.73).

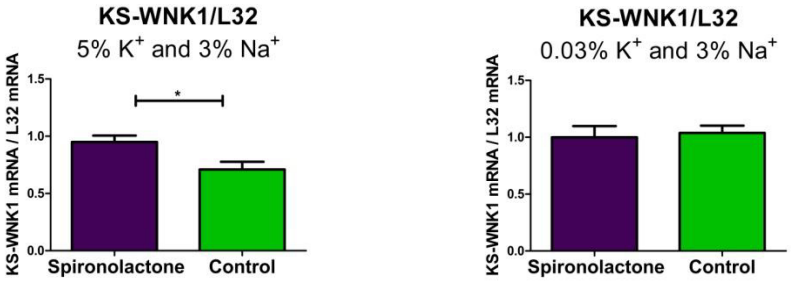


Figure 72 and 73: KS-WNK1 mRNA per L32 mRNA for the spironolactone group (purple column) and the control group (green column) of the 5% potassium (left) and the 0.03% potassium diet (right).

III.2.3.6 KS-WNK1 / L-WNK1

The ratio of KS-WNK1 per L-WNK1 in the 5% potassium group was 0.97 (± 0.07, n= 8) for the spironolactone treated animals and 0.75 (± 0.07, n= 8) for the control group (see Fig.74). The difference was significant (p= 0.041).

Among the animals of the 0.03% potassium group, KS-WNK1 per L-WNK1 ratio was 1.22 (± 0.14, n= 7) for the losartan group and 1.64 (± 0.2, n= 6) for the control group (see Fig.75). The difference was not significant.

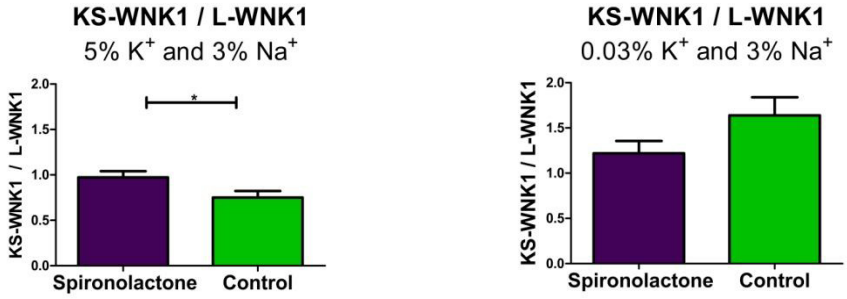


Figure 74 and 75: KS-WNK1 mRNA per L-WNK1 mRNA for the spironolactone group (purple column) and the control group (green column) of the 5% potassium (left) and the 0.03% potassium diet (right).

III.2.3.7 NCC

In the 5% potassium group, NCC mRNA per L32 mRNA expression of the spironolactone treated animals was 0.88 (± 0.03, n= 8), and 0.97 (± 0.03, n= 8) of the control group (see Fig.76).

NCC per L32 was 1.24 (± 0.08, n= 7) in the spironolactone group of the low potassium group, and 1.12 (± 0.03, n= 7) in the respective control group (see Fig.77). The differences in NCC per L32 expression between treated and untreated animals were not significant for either diet.

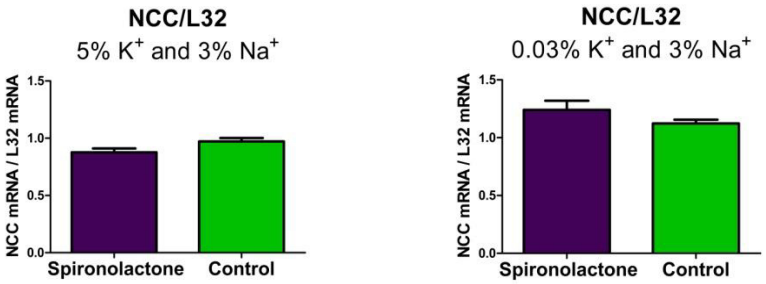


Figure 76 and 77: NCC mRNA per L32 mRNA for the spironolactone treated group and the control group. The left figure shows the results for the 5% potassium group, the right figure for the 0.03% potassium group.

III.2.3.8 ROMK

Among the high potassium group, ROMK mRNA per L32 mRNA was 0.92 (± 0.03, n= 8) in the spironolactone group, and 0.9 (± 0.03, n= 8) in the control group (see Fig.78).

ROMK mRNA per L32 mRNA of the spironolactone treated mice in the low potassium group was 1.7 (± 0.2, n= 7), and 2.06 (± 0.33, n= 7) of the respective control group (see Fig.79). For both diets, ROMK expression was not significantly different.

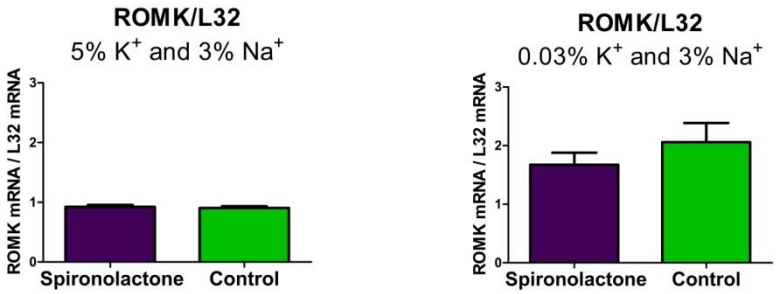


Figure 78 and 79: SPAK mRNA per L32 mRNA for the treated group (purple column) and the untreated group (green column) of the 5% potassium (left) and the 0.03% potassium diet (right).

III.2.3.9 ENaC alpha

ENaC alpha mRNA per L32 mRNA in the high potassium group was 0.8 (± 0.08, n= 8) for the spironolactone treated animals, and 1.0 (± 0.06, n= 8) for the control group. ENaC alpha expression was not significantly different (see Fig.80).

ENaC alpha per L32 expression in the 0.03% potassium group for the spironolactone treated animals was 1.0 (± 0.07, n= 7), and 0.93 (± 0.08, n= 7) for the respective control group. The difference was not significant (see Fig.81).

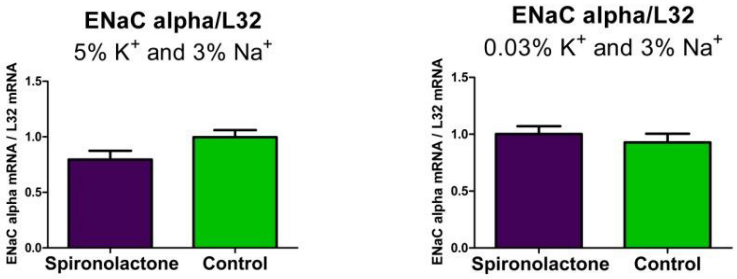


Figure 80 and 81: The left figure shows the ENaC alpha per L32 expression of the treated (purple column) and untreated (green column) group of the 5% potassium diet, the right figure for the 0.03% potassium diet.

III.2.3.10 ENaC beta

Among the mice receiving the high potassium diet, the spironolactone treated group had an ENaC beta mRNA per L32 mRNA expression of 0.81 (± 0.06, n= 8), and the control group of 0.85 (± 0.04, n= 8). The values were not significantly different (see Fig.82).

The low potassium experiments showed ENaC beta per L32 expressions of 1.51 (± 0.07, n= 7) for the spironolactone group, and of 1.28 (± 0.05, n= 7) for the control group. The difference in ENaC beta expression between the two groups was significant (p= 0.02, see Fig.83).

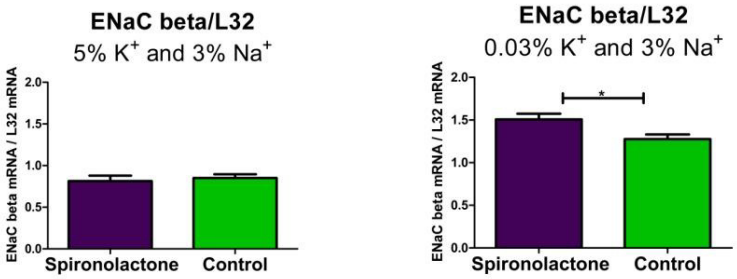


Figure 82 and 83: ENaC beta mRNA per L32 mRNA for the spironolactone treated group (purple column) and the untreated group (green column) of the 5% potassium (left) and the 0.03% potassium diet (right).

III.2.4 Data from blood pressure experiments

III.2.4.1 Activity

The difference in activity between baseline and spironolactone treatment of the 5% potassium diet was only significant at two times over the 60-hour period of data recording. At all other times activity levels were almost the same in both groups. In the 0.03% potassium diet group, activity levels were significantly higher during spironolactone treatment than during baseline conditions in all three nights. There was no significant difference in activity over the 60-hour period of measurements between baseline and spironolactone group for the 0.93% potassium diet.

III.2.4.2 Mean arterial pressure (MAP)

Mean arterial pressure of mice receiving the 5% potassium and 3% sodium diet was significantly higher during additional spironolactone treatment compared to baseline. The significant increases in MAP occurred in all three nights of data recording (see Fig.84). In the experiments with 0.03% potassium plus spironolactone, MAP was twice significantly different from baseline. At all other times, MAP was nearly the same (see Fig.85). MAP was not significantly different between baseline and the spironolactone plus 0.93% potassium diet over the 60-hour period of data recording (see Fig.86).

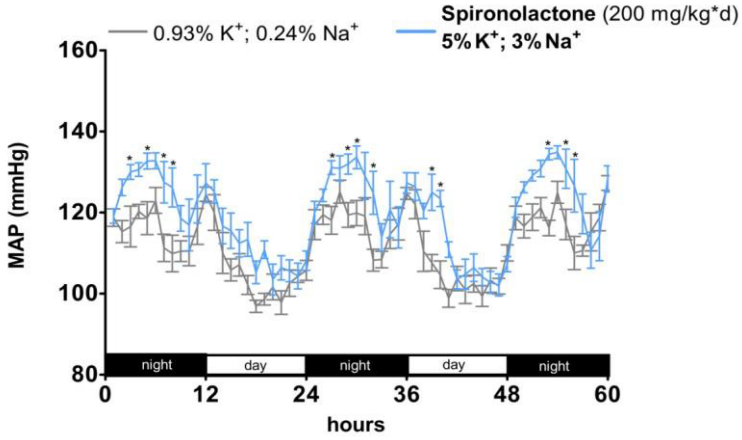


Figure 84: Mean arterial pressure (in mmHg) during baseline (grey line) and spironolactone treatment (blue line) in the 5% potassium and 3% sodium diet (n= 5). MAP was significantly higher at 3 hours ($p<0.05$), 5 hours ($p<0.01$), 7 hours ($p<0.001$), 8 hours ($p<0.001$), 27 hours ($p<0.05$), 29 hours ($p<0.05$), 30 hours ($p<0.05$), 32 hours ($p<0.001$), 39 hours ($p<0.001$), 40 hours ($p<0.001$), 53 hours ($p<0.001$), 55 hours ($p<0.05$) and 56 hours ($p<0.001$).

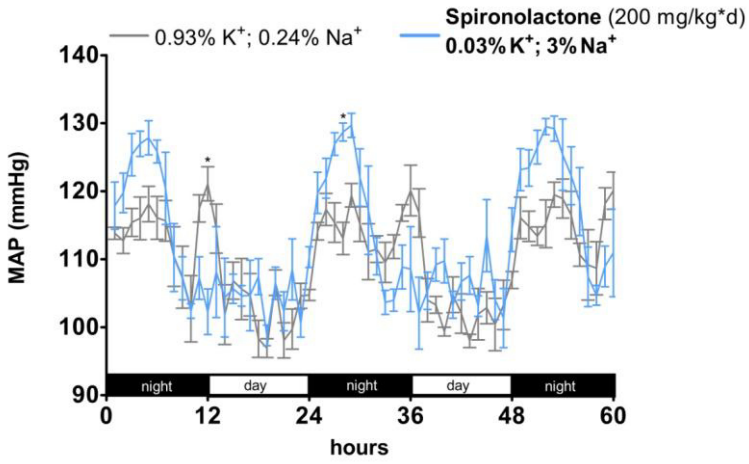


Figure 85: Mean arterial pressure (in mmHg) during baseline (grey line) and spironolactone treatment (blue line) in the 0.03% potassium diet (n= 4). MAP was significantly lower at 12 hours ($p<0.01$) and significantly higher at 28 hours ($p<0.05$) compared to baseline.

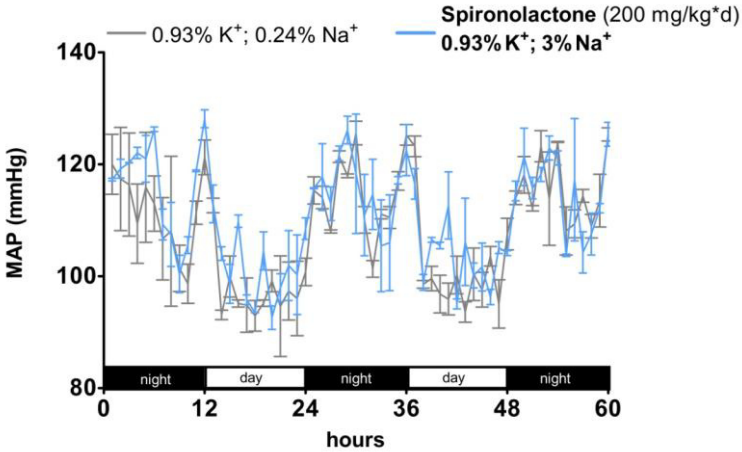


Figure 86: Mean arterial pressure (in mmHg) during baseline (grey line) and spironolactone treatment (blue line) in the 0.93% potassium diet (n= 2). MAP was not significantly different.

III.2.4.3 Systolic pressure

Systolic pressure was significantly different between baseline and 5% potassium plus spironolactone treatment at several times over the 60-hour period (at 3 hours ($p<0.05$), 5 hours ($p<0.05$), 7 hours ($p<0.01$), 8 hours ($p<0.01$), 27 hours ($p<0.05$), 32 hour ($p<0.01$), 39 hours ($p<0.01$), 40 hours ($p<0.001$), 53 hours ($p<0.001$), 55 hours ($p<0.05$) and 56 hours ($p<0.01$)). At several times over the 60-hour period, spironolactone treatment in addition to the 0.03% potassium diet, led to a significantly higher systolic pressure compared to baseline (at 4 hours ($p<0.05$), 27 hours ($p<0.05$), 28 hours ($p<0.05$), 51 hours ($p<0.05$) and 52 hours ($p<0.01$)). There were no significant differences in systolic pressure over the 60-hour period for the 0.93% potassium plus spironolactone experiments, when compared to baseline measurements.

III.2.4.4 Diastolic pressure

Similar to the results of MAP, diastolic pressure was significantly higher in the 5% potassium plus spironolactone experiments compared to baseline. This increase was significant at several times during the 60-hour period (at 3 hours ($p<0.05$), 5 hours ($p<0.05$), 7 hours ($p<0.001$), 8 hours ($p<0.001$), 19 hours ($p<0.05$), 27 hours 8$p<0.05$), 29 hours ($p<0.05$), 30 hours ($p<0.01$), 32 hours ($p<0.001$), 39 hours ($p<0.001$), 40 hours ($p<0.001$), 41 hours ($p<0.05$), 53 hours ($p<0.001$), 55 hours ($p<0.05$) and 56 hours 8$p<0.001$)). Diastolic pressure was significant twice during the 0.03% potassium diet plus spionolactone treatment (at 12 hours ($p<0.01$) and 37 hours ($p<0.05$)). At all other times, it was found to be similar to baseline. Again, there were no significant differences in diastolic pressure between baseline and spironolactone plus 0.93% potassium diet.

III.2.4.5 Heart rate

In the 5% potassium plus spironolactone group, heart rate was found to be significantly higher than baseline once during the 60-hour period (see Fig.87). In the 0.03% potassium plus losartan group, heart rate was significantly lower than baseline at several times throughout the 60-hour period of data recording (see Fig.88). There

were no significant differences in heart rate between baseline and treatment with spironolactone plus 0.93% potassium (see Fig.89).

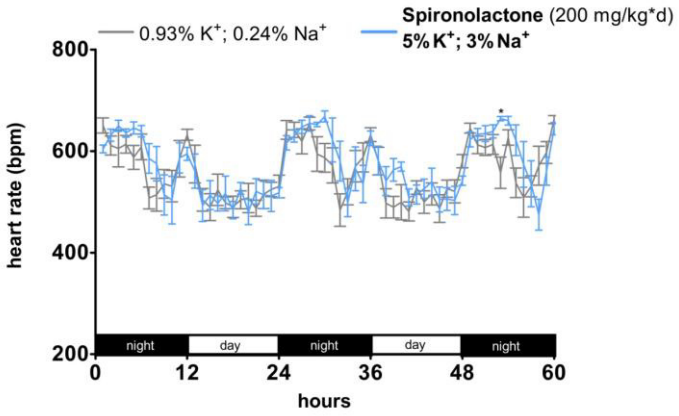


Figure 87: Comparison of heart rate (in beats per minute) between baseline and 5% potassium plus spironolactone over a 60-hour period. Heart rate was significantly different at 53 hours ($p<0.05$).

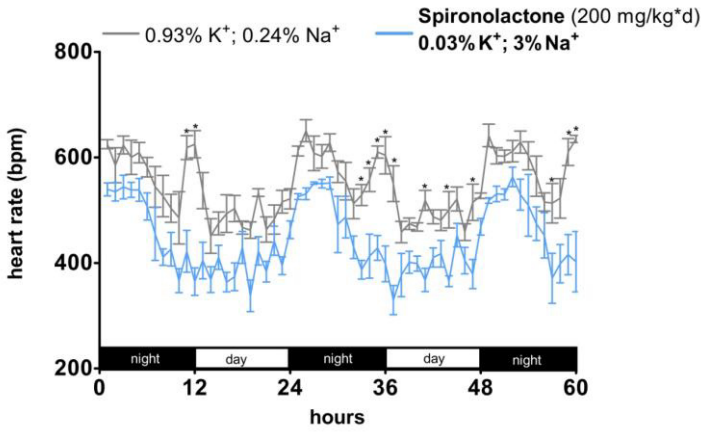


Figure 88: Comparison of heart rate (in beats per minute) between baseline and 0.03% potassium plus spironolactone. Heart rate was significantly different at 11 hours ($p<0.001$), 12 hours ($p<0.001$), 33 hours ($p<0.05$), 34 hours ($p<0.05$), 35 hours ($p<0.001$), 36 hours ($p<0.001$), 37 hours ($p<0.001$), 41 hours ($p<0.05$), 44 hours ($p<0.05$), 47 hours ($p<0.05$), 57 hours ($p<0.05$), 59 hours ($p<0.001$) and 60 hours ($p<0.001$).

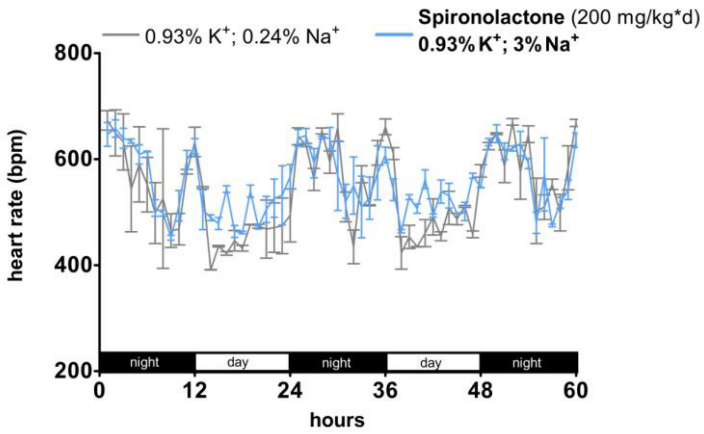


Figure 89: Comparison of heart rate (in beats per minute) between baseline and 0.93% potassium plus spironolactone over a 60-hour period. Heart rate was not significantly different.

IV. Discussion

IV.1 Discussion of results from low potassium diet

In this study, the effects of AT1 receptor and mineralocorticoid receptor inhibition on renal and blood pressure responses during low potassium and high sodium intake were analyzed. In previous experiments by L. Schulte, it was shown that changes induced by low potassium and high sodium intake are similar to those triggered by intravascular volume depletion (Vitzthum et al. 2014). In both settings, an activation of the WNK4-SPAK-NCC pathway leads to enhanced electroneutral sodium reabsorption in the DCT without relevant potassium secretion. It is known that in hypovolemia this renal response is regulated by the enhanced activity of the RAAS. This led to the hypothesis that the same signaling pathways are activated in hypokalemia as they are in hypovolemia and that therefore ANG II is the upstream regulator. To verify this hypothesis, the AT1R blocker losartan was given to mice in combination with a low (0.03%) potassium and high (3%) sodium diet. In a second set of experiments, aldosterone as another component of the renin-angiotensin-aldosterone system was administered to determine its role in renal blood pressure regulation during potassium depletion plus sodium overload.

The results from blood pressure telemetry showed that under treatment with losartan mean arterial pressure was not elevated compared to baseline. In previous experiments by L. Schulte, the combined low potassium/high sodium diet had led to significantly elevated blood pressure. So, AT1R inhibition was able to normalize blood pressure in these animals. In the normal and high potassium group, losartan treatment did not lower blood pressure, so the effect was exclusive to the low potassium group. This strengthens the hypothesis that ANG II plays a key role in the pathogenesis of hypertension in mice with hypokalemia plus sodium overload. But, besides AT1R at the basal pole of tubular cells in the kidneys, AT1R on vascular smooth muscle cells (VSMC), which are involved in the regulation of vascular tone, could also play a role (Kirabo et al. 2011, Do et al. 2009). Binding of angiotensin II to VSMC AT1 receptors leads to vasoconstriction and consequently to an elevation of blood pressure. Thus, it could be stated that the blood pressure lowering effect of losartan is due to an inhibition of ANG II-induced vasoconstriction. If renal salt

handling mechanisms play a relevant role in AT1R-mediated blood pressure reduction, an increase in sodium clearance or urine sodium excretion, as well as corresponding changes in the abundance of renal ion channels and kinases should be found. However, both urine sodium excretion and sodium clearance did not show a significant difference between losartan treated and untreated animals. This lack of detectable changes in urine and plasma electrolyte concentrations under AT1R blockage can have several reasons. On the one hand, the lack of changes in electrolyte excretion in mice treated with losartan might point to the fact that renal mechanisms are not essential for the blood pressure lowering effect of losartan in these animals. Instead, other mechanisms such as ANG II-induced changes in vascular tone could be responsible. Yet it was shown that a low potassium intake increased the expression of AT1R in the kidneys (Wang et al. 2010), favouring a renal involvement in ANG II-induced hypertension. On the other hand, insufficient AT1R inhibition due to an underdose of losartan, in particular in the metabolic cages, might have contorted the results. Due to low water intake in the metabolic cages losartan intake dropped significantly in the last 24 hours of the experiments. The aspired daily losartan dose of 20 mg/kg*day was only reached by two out of eight animals at the end of the experiments. A comparison of the calculated losartan doses in the metabolic cages with the sodium clearances of each animal shows that there is a positive correlation between the two parameters. The higher the losartan dose was, the higher the sodium clearance was (see Fig. 34, page 39). Therefore, it can be reasoned that the decrease in losartan intake in the metabolic cages possibly led to an insufficient inhibition of AT1R and thus, differences in urine and plasma electrolyte concentrations between the two groups were not significant.

In the low potassium group, potassium needs to be preserved to prevent the acute deleterious effects of hypokalemia. This is achieved by switching from electrogenic to electroneutral sodium reabsorption. The result is an enhanced sodium uptake without loss of potassium, leading to intravascular volume overload. Sodium uptake in the distal nephron is further fuelled by a high intratubular sodium load due to a high dietary intake. As mentioned before, electroneutral sodium reabsorption is mediated through activation of NCC and its upstream regulators WNK4 and SPAK. If ANG II is the extracellular signal molecule of this pathway, AT1R inhibition through losartan will result in a noticeable reduction of its components.

Accordingly, the results from renal mRNA analysis showed a significant reduction of WNK4 expression in the losartan treated group compared to the untreated group. This result supports the hypothesis that angiotensin II is the key regulator of the renal response to hypokalemia in sodium-replete mice. However, apart from the reduction in WNK4 expression, no other significant differences in the mRNA expression of the analyzed ion channels and kinases were found. The lack of further significant differences between the losartan treated and the untreated animals might be due to the fact that the differences in mRNA expression, when analyzed on the level of the whole kidney, are not distinct enough to produce significant results. This is supported by the findings in the previous experiments by L. Schulte without losartan where mRNA analysis in microdissected distal tubules and cortical collecting ducts showed additional changes of ion channel and kinase expression which had not been detected in the whole kidney (Vitzthum et al. 2014). Therefore, microdissection studies as part of the losartan experiments would offer a more detailed and precise insight into transcriptional changes in the different segments of the distal nephron. As mRNA expression analysis only detects changes in gene regulation of ion channels and kinases, other regulatory mechanisms such as phosphorylation, increased mRNA stability or increased lysosomal degradation are not assessed with this method. For instance, it has been shown that NCC is mainly regulated by changes in surface expression through an altered rate of lysosomal degradation (Cai et al. 2006). Furthermore, Masilamani et al. demonstrated that NCC mRNA abundance did not increase in response to dietary NaCl restriction despite an increase in NCC protein, suggesting that enhanced gene transcription is not the mode of regulation (Masilamani et al. 2002). Therefore, further analyses would be helpful which take into account the different mechanisms of regulation to get a more detailed picture of the renal remodeling under low potassium and high sodium intake. As mentioned before, insufficient AT1R inhibition may also have contorted the differences in mRNA expression between losartan treated and untreated animals. However, renin mRNA expression was significantly higher in the losartan treated group compared to the control group. Since ANG II is known to inhibit renin secretion, sufficient AT1R inhibition through losartan treatment is expected to result in enhanced renin mRNA expression. To clearly establish that ANG II regulates renal responses to low potassium intake in sodium-replete mice, it is necessary to reassess the results from metabolic cages and gene expression under conditions where losartan intake is

continuously above the preset level. Another important aspect of evaluating signaling pathways is to distinguish between acute and chronic responses to changes in physiological homeostasis. Crowley et al. showed different sites of action for ANG II between the first week and the second and third week of ANG II infusion in mice. While during the initial phase of ANG II-dependent hypertension, the essential pathomechanism is the activation of renal AT1R, which promotes sodium retention, during the following two weeks a sodium-independent contribution of extrarenal AT1R on vascular smooth muscle cells is the main mechanism (Crowley et al. 2011). In the losartan experiments, the duration of the combined low potassium/high sodium diet was ten days. All analyses were conducted at the end of this period. It has to be considered that the results might have been different at an earlier or later point of the experiments and that the chosen duration of the diet might have been too long to detect the renal remodeling in its whole dimension.

Being part of the same biochemical cascade, the effects of angiotensin II and aldosterone are very closely related. For a long time, aldosterone has been known as a potent regulator of renal salt handling. Only recently, the impact of angiotensin II on various renal signaling pathways has been discovered. It has been shown that aldosterone and angiotensin II both interact in the regulation of electrolyte and volume homeostasis and have independent effects (van der Lubbe et al. 2011). In order to assess the influence of aldosterone during potassium depletion plus sodium overload, the MR blocker spironolactone was given in a similar set of experiments as for losartan.

In contrast to AT1R inhibition, MR blockage did not completely normalize blood pressure in the low potassium group. Both MAP and systolic pressure were still significantly elevated compared to baseline, although only during the active nighttime. Furthermore, plasma electrolyte levels, urine electrolyte excretion and clearances did not differ between the spironolactone treated group and the control group. These findings make an involvement of renal mineralocorticoid receptor signaling in the pathogenesis of blood pressure elevation in the low potassium group very unlikely. Results from mRNA analysis showed significant increases of WNK4 and ENaC beta expression in the spironolactone group. A signaling pathway has been identified by Ring et al. in which aldosterone-dependent SGK1 activation leads to the phosphorylation of WNK4 with the result of ENaC and ROMK disinhibition

(Ring et al. 2007). But this pathway is initiated to correct hyperkalemia which was not the case in the losartan experiments. Besides, according to the described signaling pathway, in the presence of spironolactone, ENaC beta and WNK4 expression would be expected to decrease and not to increase. Thus, these results cannot be integrated into the established regulation systems and need to be further investigated. All in all, there is no evidence for a contribution of aldosterone, neither direct nor indirect via ANG II, in the renal remodeling under a low potassium and high sodium diet.

As discussed before, there is some evidence that ANG II is at the origin of renal ion channel and kinase regulation in this setting. These results must be confirmed by further investigations, e.g. microdissection studies of the distal nephron. In future experiments, a constantly sufficient losartan dose has to be ensured in order to achieve a reliable AT1 receptor inhibition, especially during the last 24 hours in the metabolic cages. From the experiments described above, a contribution of extrarenal AT1R to the hypotensive response to losartan cannot be fully excluded. Experiments with a renal AT1R knock-out mouse model could help to assess the influence of extrarenal AT1R on blood pressure reduction under losartan.

If ANG II should be the upstream regulator in the setting of low potassium and high sodium intake, the question emerges as to how the enhanced secretion of ANG II is stimulated in a state of low plasma potassium levels and elevated blood pressure and whether this ANG II is part of the systemic RAAS or a local renal RAAS. For the systemic RAAS, hypotension is known as a physiological disturbance which results in renin secretion from the juxtaglomerular apparatus. Since the combined low potassium/high sodium diet led to elevated blood pressure, it is rather unlikely that systemic ANG II initiated the renal remodeling. The existence of a local RAAS of the kidney has long been established, but the distribution and function of its components along the nephron and its importance for electrolyte and water homeostasis is still incompletely understood (Pohl et al. 2010, Kobori et al. 2007). There is, however, proof that even under conditions where plasma renin activity is markedly suppressed, increased intrarenal and intratubular ANG II concentrations can be found (Shao et al. 2009, Zou et al. 1996). It was shown in previous experiments by L. Schulte that renin mRNA expression in the kidneys is significantly reduced under the combined low potassium diet compared to a normal potassium diet (Vitzthum et al. 2014). But

intrarenal ANG II concentrations under this diet have not been analyzed. So, the role of a local, kidney-specific RAAS in the setting of high sodium and low potassium intake and resulting hypokalemia and elevated blood pressure remains to be elucidated.

IV.2 Discussion of results from high potassium diet

In this experiment, the influence of aldosterone via MR and of ANG II via AT1R on renal mRNA expression and blood pressure during high potassium and high sodium intake was analyzed. Since hyperkalemia is known as one of the main physiological states that lead to enhanced aldosterone secretion, the hypothesis was that aldosterone is the upstream signal for the observed renal remodeling under a combined high salt diet. To verify this hypothesis, the MR blocker spironolactone was given to mice in combination with a high (5%) potassium and high (3%) sodium diet. In another set of experiments, the AT1R inhibitor losartan was administered to determine the role of ANG II in renal blood pressure regulation pathways under this diet.

Data from blood pressure measurements showed that treatment with spironolactone did not completely normalize blood pressure. Both MAP and systolic pressure were still elevated at nighttimes under MR inhibition, as they were in the previous experiments without spironolactone. If aldosterone-mediated electrogenic sodium reabsorption was the only mechanism for blood pressure elevation in these mice, MR blockage would be expected to entirely eliminate blood pressure elevation. Aldosterone-stimulated electrogenic sodium reabsorption takes place in the CCD, where it generates a sufficient electric gradient for enhanced potassium excretion. In accordance with these mechanisms of potassium handling, the spironolactone treated group showed significantly higher plasma potassium levels compared to the control group. As the spironolactone group was not held in metabolic cages, there are no clearances or urine excretion data to further support the aldosterone hypothesis. Also from mRNA analysis there is no definite sign of an involvement of aldosterone. The main aldosterone-dependent pathway to excrete potassium is via SGK1-mediated activation of ROMK and ENaC. But a decrease in SGK1, ROMK and

ENaC mRNA expression under spironolactone compared to the control group was not found.

However, KS-WNK1 expression and KS-WNK1/L-WNK1 ratio were significantly increased in the spironolactone group. This kinase promotes electrogenic sodium reabsorption and potassium excretion by disinhibiting ROMK and activating ENaC. So far, the activation pathway for KS-WNK1 is an aldosterone-dependent upregulation of KS-WNK1 gene expression (Naray-Fejes-Toth et al. 2004). Since aldosterone-dependent effects under MR inhibition are unlikely, the observed hyperkalemia itself remains as a possible activation signal for enhanced KS-WNK1 expression. The upregulation of KS-WNK1 might be an escape mechanism to continue with enhanced potassium excretion despite aldosterone inhibition. Further research is necessary to clarify this hypothesis.

Apart from aldosterone and KS-WNK1, there might be other signaling molecules which are able to activate electrogenic sodium reabsorption. Ecelbarger et al. showed that the pituitary hormone vasopressin (ADH) regulates ENaC, both acutely and chronically. The suggested mode of activation is increased mRNA transcription of ENaC beta and gamma subunits under water restriction (Ecelbarger et al. 2000). In vivo, vasopressin is released during volume depletion and elevated plasma osmolarity. Its main function is to increase water permeability in the distal nephron by promoting trafficking of aquaporin-2 to the apical cell membrane. Since physiological states of vasopressin release are absent under the combined high potassium and high sodium diet, vasopressin signaling seems unlikely to be at the origin of renal remodeling in this setting. To fully investigate the role of vasopressin in the pathogenesis of hypertension in potassium- and sodium-replete mice, experiments with a vasopressin V2 receptor antagonist and the same diet are necessary.

Results under losartan treatment did not show evidence of an involvement of angiotensin II. AT1R inhibition had no effect on blood pressure in the high potassium group. Furthermore, there was no change in plasma or urine electrolytes, clearances or mRNA expression that would indicate that AT1R-mediated renal remodeling plays a role in the development of hypertension under the high potassium and high sodium diet. As discussed previously, insufficient AT1R inhibition due to low losartan doses might have influenced the results. From those animals on the high potassium diet which received losartan treatment, only one mouse had a calculated dose above the

preset level of 20mg/kg*day (see Fig. 30, page 38). Yet renin mRNA expression was significantly higher in the losartan group indicating that AT1R inhibition had some effect.

Based on the results of this study, the hypothesis that aldosterone is at the origin of the renal remodeling leading to hypertension during high potassium and high sodium intake seems to be unlikely. The few changes that were found under treatment with the MR inhibitor spironolactone were partly contradictory and thus not clear enough to establish aldosterone as the main signaling molecule in the pathogenesis of hypertension in this setting. The ideas about the reason for the aldosterone-independent hypertensive response to a high salt diet are only speculative and require further investigations. So far, the exact pathomechanism of hypertension in potassium- and sodium-replete mice remains unidentified.

V. Summary

The typical modern Western diet has a high sodium and low potassium content, as opposed to the diet of our ancestors. This modern food pattern entails serious health issues. Salt-sensitive hypertension has become one of the most widespread diseases in Western industrialized countries. However, still little is known about the pathomechanism responsible for blood pressure elevation under this Western diet. Previous in-vivo experiments with a high (3%) sodium diet in combination with low (0.03%) potassium showed evidence of an activation of the WNK4-SPAK-NCC pathway and led to the hypothesis that angiotensin II might be the activating signal for this renal remodeling. Experiments with a high sodium and high (5%) potassium diet gave rise to the hypothesis that aldosterone is at the origin of the observed renal remodeling. To verify these hypotheses, the AT1 receptor blocker losartan was given to mice on a low (0.03%) potassium diet and the mineralocorticoid receptor inhibitor spironolactone was administered to mice on a high (5%) potassium diet. Since ANG II and aldosterone are known to interact in many regulatory pathways, spironolactone was given to a second group of mice on the low potassium diet and losartan to a second group on the high potassium diet. For all treatment groups, a non-treatment control group was formed that only received the salt diet. Treatment with the respective medication started one day before the beginning of the combined salt diet and the experiments lasted eleven days in total. Animals under losartan treatment were kept in metabolic cages for the last 24 hours of the experiments to measure urine and fecal excretion. Sodium, potassium and chloride concentrations were determined in collected urine and blood, and respective clearances were calculated. Whole kidney mRNA expression analysis was performed with Real-time PCR for all relevant ion channels and kinases in the distal nephron. Additionally, telemetry experiments were conducted to obtain blood pressure data from mice under the same diet and treatment regime as described above. In the low potassium group blood pressure elevation was completely reversed and WNK4 expression was significantly reduced under losartan treatment. But there were no changes in NCC or SPAK mRNA expression under losartan. Therefore, further studies are required to corroborate the importance of ANG II for renal remodeling in response to low potassium and high sodium intake. From this study, contribution of extrarenal AT1R to the hypotensive response to losartan cannot be excluded. Spironolactone

treatment had no influence on blood pressure or mRNA expression in the low potassium group, thus, making aldosterone-dependent mechanisms in this setting very unlikely. In the high potassium experiments, MR inhibition did not entirely correct blood pressure elevation from high salt intake. But spironolactone-treated animals had significantly higher plasma potassium levels, which can be expected when aldosterone-dependent potassium excretion via ROMK is inhibited. Results from mRNA analysis showed higher KS-WNK1 expression and consequently a higher KS-WNK1/L-WNK1 ratio in the treated group, but no other significant changes. These findings are not compatible with any established aldosterone-dependent signaling pathways and therefore make a relevant contribution of aldosterone unlikely. Furthermore, contribution of ANG II can be excluded as losartan treatment in the high potassium group did not result in any changes of blood pressure, mRNA expression or plasma electrolyte levels. Therefore, MR- and AT1R-independent mechanisms must be responsible for hypertension under a high potassium and high sodium diet and further investigations are necessary to identify them.

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VII. List of Abbreviations

ADH	anti-diuretic hormone, vasopressin
ANG II	Angiotensin II
AT1R	Angiotensin-1-receptor
CCD	Cortical collecting duct
cDNA	copy-Deoxyribonucleic acid
CNT	Connecting tubule
DCT	Distal convoluted tubule
ENaC	Epithelial sodium channel
IMCD	Inner medullary collecting duct
KS-WNK1	Kidney-specific WNK1 isoform
L-WNK1	Long WNK1 isoform
MAP	Mean arterial pressure
NCC	Sodium chloride co-transporter
OMCD	Outer medullary collecting duct
PCR	Polymerase chain reaction
PHA2	Pseudohypoaldosteronism type 2
RAAS	Renin-angiotensin-aldosterone system
ROMK	Renal outer medullary potassium channel
SGK1	Serine/threonine-protein kinase
SPAK	STE20/SPS1-related proline/alanine-rich kinase
VSMC	Vascular smooth muscle cells
WNK	With no lysine kinases

VIII. List of Figures

Figure…….Page

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X. Curriculum Vitae

Lebenslauf entfällt aus datenschutzrechtlichen Gründen.

XI. Declaration of academic honesty

Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann. Die vorliegende Arbeit wurde in Teilen in folgender Publikation veröffentlicht: “Vitzthum et al. 2014”.

Unterschrift: .....