THE ALDOSTERONE PARADOX:

differential regulation of the sodium chloride cotransporter

Nils van der **Lubbe**

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Colophon

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Chapter 1

General introduction and aims of the thesis

Based on

The renal WNK kinase pathway: a new link to hypertension
Ewout J. Hoorn, Nils van der Lubbe, Robert Zietse

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The sodium chloride cotransporter SLC12A3:
new roles in sodium, potassium, and blood pressure regulation
Arthur D. Moes, Nils van der Lubbe, Robert Zietse, Johannes Loffing, Ewout J. Hoorn

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INTRODUCTION

Na^+ and K^+ balance and the aldosterone paradox

Maintaining total body Na^+ and K^+ balance is essential to the survival of most species. Hypovolemia (Na^+ deficit) and hyperkalemia (K^+ surplus) elicit different constellations of responses to maintain homeostasis. During hypovolemia, the extracellular fluid volume needs to be maintained to guarantee blood pressure and organ perfusion. Conversely, during hyperkalemia, K^+ secretion is stimulated to avoid cardiac and neuromuscular complications. Both these physiological conditions are regulated by the mineralocorticoid hormone aldosterone which is part of the renin-angiotensin-aldosterone system (RAAS). The observation that a single hormone, aldosterone, has different effects on renal Na^+ and K^+ transport, depending on the physiological situation, has been termed the "aldosterone paradox".[1-5] How the kidney "knows" when to retain Na^+ or secrete K^+ during these two high aldosterone states is unknown. To understand the aldosterone paradox, it is important to briefly review the regulation of Na^+ and K^+ transport in the kidney.

The aldosterone sensitive distal nephron

An important target of RAAS is the kidney. The kidney is in control of Na^+, K^+ and volume balance and is composed of nephrons, the smallest functional units of the kidney. In the last two decades, the major Na^+ and K^+ transport proteins along the nephron have been cloned and characterized. Angiotensin II was shown to mediate $NaHCO_3^-$ reabsorption through the Na^+-H^+ exchanger type 3 (NHE3).[6] In the distal nephron, the Na^+-Cl^- cotransporter (NCC)[7], epithelial Na^+ channel (ENaC)[8], the renal outer medullary K^+ channel (ROMK)[9,10] and the large-conductance Ca^{2+}-activated K (BK) channel[11,12] have all been shown to be sensitive to aldosterone. Therefore this part of the nephron is often referred to as "the aldosterone sensitive distal nephron" (ASDN).[13,14] The ASDN constitutes the distal convoluted tubule (DCT), connecting tubule (CNT), and collecting ducts (CD).[13] NCC is primarily located in the first segment of the DCT (DCT-1), whereas ENaC is located in the second segment (DCT-2), CNT, and CD.[1,2] ROMK is expressed along the entire distal nephron[15] and the BK-channel is located in the CD.[12]

The Na^+-Cl^- cotransporter

NCC is encoded by the *SLC12A3* gene (55-kb, 26 exons) and belongs to the SLC12 family of electroneutral cation Cl^- cotransporters.[16] NCC is primarily expressed in the kidney, but also in intestine and bone.[17,18] In the kidney, NCC is located in the apical plasma membrane of epithelial cells in the DCT (Figure 1). Only 5 to 10% of the filtered load

of Na^+ is reabsorbed in the DCT and this is primarily mediated by NCC.[19] Despite this modest contribution to overall Na^+ reabsorption, the NCC in the DCT together with ENaC in the CNT and the CD fine-tunes the final concentration of NaCl in the urine. It is able to do so because it is not affected by the tubuloglomerular feedback.[16] Due to this quality, NCC plays a pivotal role in extracellular fluid volume and blood pressure control.[16] This is illustrated by the fact that thiazides, which inhibit NCC, are among the most effective antihypertensive drugs.[20] Similarly, monogenetic disorders that result in inactivation (Gitelman's syndrome) or overactivation (Gordon's syndrome) of NCC are also characterized by salt-loss and low-normal blood pressure, or salt-retention and hypertension, respectively.[21]

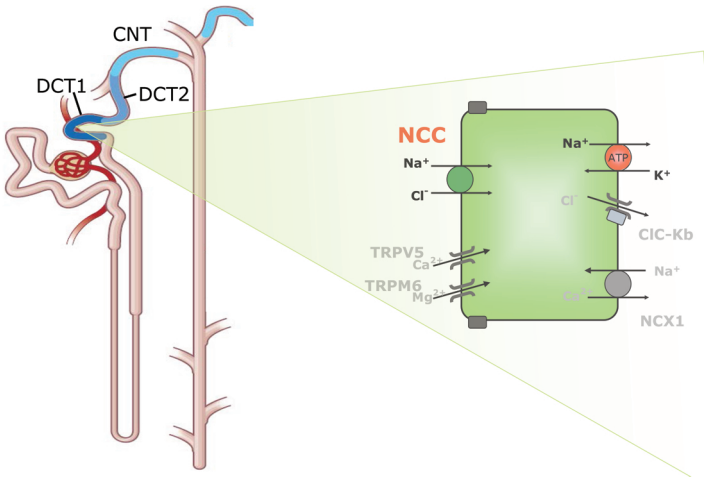

Figure 1: Model of transcellular transport in the early distal convoluted tubule (DCT). A kidney tubule is shown schematically on the left indicating the locations of DCT type 1 and type 2 (DCT1, DCT2) and the connecting tubule (CNT). The sodium chloride cotransporter (NCC) is primarily expressed in DCT1. A model of transcellular transport in DCT1 is shown on the right, including the apical transporters NCC and transient receptor potential channels TRPV5 (a calcium channel) and TRPM6 (a magnesium channel). On the basolateral side the sodium potassium ATPase pump is shown as well as the chloride channel ClC-Kb and the sodium-calcium channel NCX1. This figure was adapted from. [69,70]

Regulation of the Na^+-Cl^- cotransporter

NCC is highly regulated by hormones, including aldosterone, angiotensin II, glucocorticoids, estrogen, insulin, norepinephrine, and vasopressin.[7,22-29] The fact that NCC is regulated by so many different hormones suggests that Na^+ reabsorption through NCC is an important homeostatic control mechanism. Aldosterone was the first hormone recognized to be capable of activating NCC.[7] The regulation of NCC by aldosterone seems logical, because at least the end portion of the DCT expresses the enzyme 11-beta-

hydroxysteroid dehydrogenase II which rapidly inactivates glucocorticoids and hence provides mineralocorticoid-sensitivity to the epithelial cells. Experimentally, aldosterone upregulates NCC both when it is directly infused or when it is secreted in response to a low Na^+ diet.[7,30] The acute effect of aldosterone only involves phosphorylation of NCC[31], whereas the chronic effect also increases the total protein abundance of NCC[7], which likely occurs independent from changes to NCC mRNA levels.[32-34] The N-terminus of NCC contains several conserved phosphorylation sites including threonine 46, 55 and 60 and serine 73 and 91 in humans (Figure 2).[35] In rats these phosphorylation sites correspond to threonine 44, 53, 58 and serine 71 and 89. Phosphorylation of NCC appears to determine its activity, especially at threonine 60 in humans or 58 in rat. Because phosphorylated NCC has thus far only been found in the apical plasma membrane of the DCT, anchoring in the plasma membrane seems necessary for phosphorylation to occur.[22] This suggests that, apart from phosphorylation, the trafficking of NCC from subapical vesicles to the plasma membrane is also important.[36]

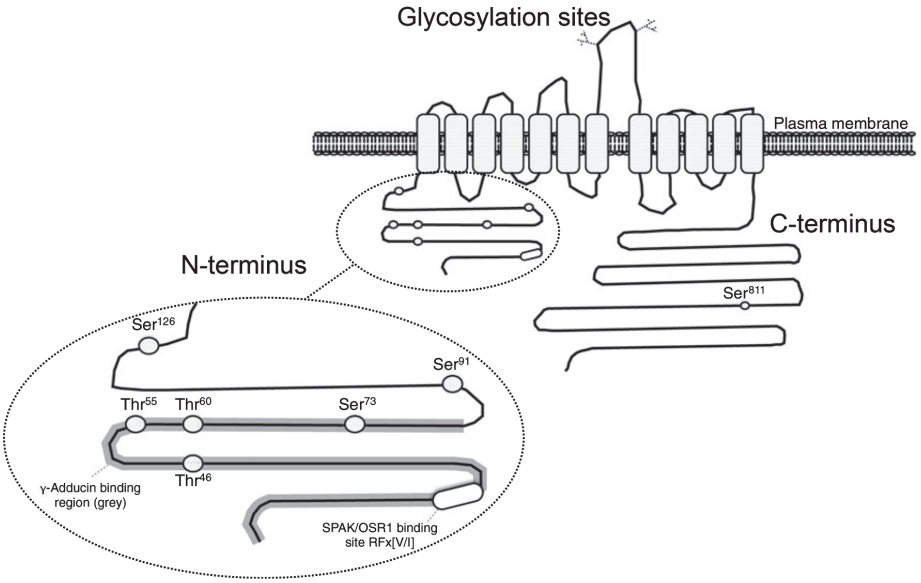

Figure 2: Putative structure of the sodium chloride cotransporter. The twelve transmembrane domains are shown including the hydrophilic loop with the two glycosylation sites. A detailed image of the N-terminus is provided on the left showing the binding sites of γ-adducin and the kinases SPAK/OSR1. This figure was reproduced and adapted from[35] with kind permission.

The Na^+-Cl^- cotransporter signaling cascade

The intracellular signaling cascade that controls NCC activity has largely been unraveled in recent years. This NCC signaling cascade consists of a multikinase network which

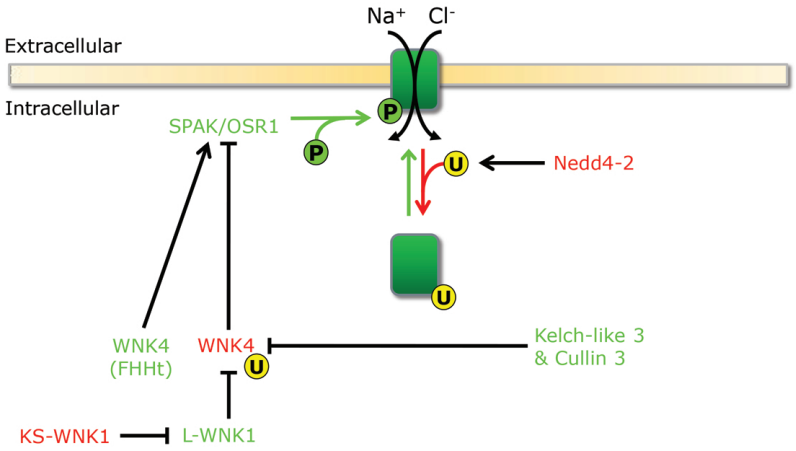

Figure 3: Current model of sodium chloride cotransporter regulation by kinases and ubiquitinases. The various interactions of the NCC regulatory pathway are shown as arrows (stimulatory) or as lines ending with perpendicular lines (inhibitory). Phosphorylation is indicated with the symbol "P", whereas ubiquitylation is shown as "U". SPAK/OSR1, WNK4, kidney-specific WNK1 (KS-WNK1) and long WNK1 (L-WNK1, also called WNK1) are kinases. The role of mutant WNK4 in familial hyperkalemic hypertension (FHHt) is also shown, which overrides the inhibitory effect of wild-type WNK4 on SPAK/OSR1. Nedd4-2 is a ubiquitinase, while Cullin-3 and Kelch-like 3 interact in a ubiquitylation complex that likely ubiquitinates WNK4.

includes the kinases With No K (WNK, with "K" referring to the amino acid lysine), STE20-related, proline alanine-rich kinase (SPAK), oxidative stress responsive protein type 1 OSR1, and the serum- and glucocorticoid-inducible kinase 1 (SGK1) (Figure 3).[37] More recently, proteins involved in ubiquitination including Nedd4-2, Kelch-like 3, and Cullin 3 were also found to regulate NCC.[38-41] Many of these regulatory proteins were identified because mutations in their genes result in familial hyperkalemic hypertension (FHHt, also called pseudohypoaldosteronism type II or Gordon's syndrome). WNKs modulate both the "trafficking" and phosphorylation of NCC.[42] The regulation of NCC trafficking by WNKs involves a sequential inhibitory cascade, in which KS-WNK1 (KS refers to kidney specific) inhibits WNK1, WNK1 inhibits WNK4[43], and WNK4 inhibits NCC.[44] The inhibition of NCC by WNK4 is not caused by endocytosis[45], but rather by promoting lysosomal degradation.[46]

Interestingly, angiotensin II converts WNK4 from an inhibitor to an activator of NCC.[47] In contrast to WNK4, WNK3 stimulates NCC[48], but its actions are less well-defined. WNK3 and WNK4 not only have divergent effects on NCC, they also antagonize each other. Indeed, it appears to be the ratio between WNK3 and WNK4 that determines the net effect on NCC.[49]

The phosphorylation of NCC is mediated by SPAK[50]; several WNKs interact with SPAK and therefore indirectly control the phosphorylation-step of NCC. Interactions between SPAK and WNK1[50], WNK3[51], and WNK4[52] have been reported. However, the brain but not the kidney isoform of WNK3 can activate NCC and does so through a SPAK-independent mechanism.[51] Two isoforms of SPAK have been identified, including full-length SPAK (FL-SPAK) and kidney-specific SPAK (KS-SPAK or SPAK2), of which the latter isoform has low expression levels in the DCT.[53] KS-SPAK, which lacks the kinase domain, inhibits FL-SPAK and OSR1, which are both known to phosphorylate the Na^+- K^+ -$2Cl^-$ cotransporter (NKCC2) in the thick ascending limb upstream of the distal nephron. This may explain why in mice the knockout of SPAK results in decreased NCC phosphorylation (absence of FL-SPAK), but increased NKCC2 phosphorylation (no inhibition of FL-SPAK or OSR1 by KS-SPAK).[53,54]

Both SPAK isoforms are also involved in the stimulatory effect of vasopressin on NCC and NKCC2.[22,24] Namely, vasopressin stimulates FL-SPAK in the DCT to phosphorylate NCC, whereas it attenuates KS-SPAK to allow FL-SPAK and OSR1 to phosphorylate NKCC2.[55]

Although SGK1 was first recognized as an activator of ENaC[56], later reports also showed effects on NCC.[57] SGK1 and NCC do not seem to interact directly, but rather through WNK4 and Nedd4-2.[38,58] SGK1 phosphorylates WNK4 and this phosphorylation step reduces the inhibition of WNK4 on NCC.[58,59] Because SGK1 is sensitive to aldosterone, this pathway appears to be involved in the activation of NCC by aldosterone.[59] The opposite is also true, because SGK1 knockout mice failed to increase NCC activity during a low Na^+ diet.[57]

Recent data indicate that Nedd4-2 is yet another player in the pathway by which aldosterone activates NCC.[38,41] Nedd4-2 was shown to stimulate ubiquitination of NCC and decreased its activity and surface expression *in vitro* and *in vivo*, while SGK1 prevented these effects.[41] The pathophysiological significance of the regulation of NCC by Nedd4-2 was shown by the generation of inducible nephron-specific Nedd4-2 knockout mice. These mice exhibited salt-dependent hypertension that was characterized by upregulation of total and phosphorylated NCC and sensitivity to thiazides. The deletion of Nedd4-2 also affected ENaC and ROMK, which were down- and upregulated, respectively. This may explain the additional characteristics of these mice, namely that they had a normal Na^+/K^+ balance and were not hyperkalemic.

The role of the Na^+-Cl^- cotransporter in response to hypovolemia and hyperkalemia

Besides the direct effects of NCC on the reabsorption of Na^+, recent data indicate that NCC indirectly plays an important role in the regulation of K^+ in the distal nephron. These new data gain insight in the mechanism of the aldosterone paradox, namely,

how aldosterone has different effects on Na^+ and K^+ transport during hypovolemia and hyperkalemia. In search of an explanation for the aldosterone paradox, it is important to consider the factors accompanying hypovolemia. Although both hypovolemia and hyperkalemia are characterized by elevated aldosterone, only hypovolemia is associated with a concomitant rise in angiotensin II. Recently, it has become clear that in addition to its well-established effects on the proximal tubule, angiotensin II can also activate NCC and ENaC.[4,60] Angiotensin II has been shown to abrogate the inhibitory effects of WNK4.[47] This permits NCC to traffic to the plasma membrane[61] and be phosphorylated by SPAK to enhance electroneutral NaCl transport.[26,30,34,47] Increased Na^+ reabsorption in the DCT will reduce the delivery of Na^+ to the collecting duct, limiting Na^+-coupled K^+ secretion in that segment. Moreover, it has recently been shown that angiotensin II inhibits ROMK.[62] Together, these effects favor electroneutral Na^+ reabsorption while preventing K^+ secretion.[63] These recent insights help explain how the aldosterone-sensitive distal nephron responds to hypovolemia. The effects of hyperkalemia on renal tubular transporters have recently been investigated by three studies using high-K^+ (HK) diets. Frindt and Palmer [64] found that a HK diet increased ROMK and decreased NCC in normal rats. In mice, Vallon *et al*.[57] confirmed the suppression of NCC by high dietary K^+ and showed this suppression to be greater in SGK1 knockout mice. Sorensen *et al*.[65] showed that NCC was dephosphorylated as early as 15 min after oral K^+ intake.

NCC plays an important role in the aldosterone paradox. Low dietary Na^+ favors Na^+ reabsorption through NCC, whereas high dietary K^+ inhibits NCC and thereby favors Na^+ reabsorption through ENaC to indirectly promote K^+ secretion.

The Na^+-Cl^- cotransporter in urinary exosomes

The pathogenic role of aldosterone in hypertension and heart failure has been well established. However, determination of the plasma aldosterone concentration is frequently not useful for diagnosis or assessment of the response to treatment. This emphasizes the need for new biomarkers that reflect the biological activity of aldosterone. To search for these biomarkers in urine is especially attractive because the kidneys are aldosterone's main target organ and it allows for non-invasive testing. Of special interest, in this regard, are urinary exosomes. Urinary exosomes are vesicles derived from renal tubular epithelial cells. Exosomes often contain several disease-associated proteins and are thus useful targets for identifying biomarkers of disease. Among these are the aldosterone-sensitive Na^+ transporters, NCC and ENaC and prostasin, a regulatory protein of ENaC that has emerged as a superior marker of ENaC-activity. Recent studies have demonstrated that the expression of these proteins in urinary exosomes is altered in certain physiological and pathophysiological conditions.[66-68] For example, increased abundance of NCC in urinary exosomes has been found in patients with Gordon's syndrome, a monogenetic

disease characterized by hypertension and hyperkalemia due to overactivity of NCC.[67] Furthermore, in healthy volunteers, the presence of urinary prostasin, a known regulator of ENaC, increased in response to a low Na^+ diet, and in turn normalized in response to spironolactone administration.[66] In conclusion, NCC and prostasin in urinary exosomes are promising candidates to be used as biomarkers for aldosteronism.

AIMS OF THE THESIS

In this thesis the following hypotheses were tested:

1: Angiotensin II activates the sodium chloride cotransporter independent of aldosterone (Chapter 2).

2: Aldosterone requires angiotensin II to activate the sodium chloride cotransporter (Chapter 3).

3: The two effects of aldosterone (sodium retention versus potassium secretion) are explained by differential regulation of the sodium chloride cotransporter and the epithelial sodium channel (Chapter 4).

4: The phosphorylated sodium chloride cotransporter is a urinary biomarker for aldosteronism (Chapter 5).

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Chapter 2

Angiotensin II induces phosphorylation of the thiazide-sensitive sodium chloride cotransporter independent of aldosterone

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ABSTRACT

We studied here the independent roles of angiotensin II and aldosterone in regulating the sodium chloride cotransporter (NCC) of the distal convoluted tubule. We adrenalectomized three experimental and one control group of rats. Following surgery, the experimental groups were treated with either a high physiological dose of aldosterone, a non-pressor, or a pressor dose of angiotensin II for 8 days. Aldosterone and both doses of angiotensin II lowered sodium excretion and significantly increased the abundance of NCC in the plasma membrane compared with the control. Only the pressor dose of angiotensin II caused hypertension. Thiazides inhibited the sodium retention induced by the angiotensin II non-pressor dose. Both aldosterone and the non-pressor dose of angiotensin II significantly increased phosphorylation of NCC at threonine-53 and also increased the intracellular abundance of STE20/SPS1-related, proline alanine-rich kinase (SPAK). No differences were found in other modulators of NCC activity such as oxidative stress responsive protein type 1 or with-no-lysine kinase 4. Thus, our *in vivo* study shows that aldosterone and angiotensin II independently increase the abundance and phosphorylation of NCC in the setting of adrenalectomy; effects are likely mediated by SPAK. These results may explain, in part, the hormonal control of renal sodium excretion and the pathophysiology of several forms of hypertension.

INTRODUCTION

The conservation of sodium by the kidney is crucial for maintaining the extracellular fluid volume. The signal for the kidneys to conserve sodium is largely mediated by the renin–angiotensin–aldosterone system. Indeed, the majority of the renal sodium transporters were found to be regulated by this system, including the sodium hydrogen exchanger type 3 by angiotensin II,[1] and both the sodium chloride cotransporter (NCC) and the epithelial sodium channel (ENaC) by aldosterone.[2,3] In recent years, a number of interacting kinase systems have been identified in the distal nephron, which directly or indirectly regulates renal sodium transport. The with-no-lysine kinases (WNK) positively or negatively regulate NCC and ENaC.[4-6] STE20/SPS1-related, proline alanine-rich kinase (SPAK) and oxidative stress responsive protein type 1 (OSR1) have been identified as proteins that are capable of phosphorylating NCC.[7,8] Finally, serum- and glucocorticoid-inducible kinase 1 (SGK1) is pivotal in the regulation of ENaC,[9] and probably also NCC.[10,11] These kinases were found to be sensitive to angiotensin II and aldosterone, which suggested them to have a central position in the receptor–transporter cascade.[4,9,12,13] However, one crucial and unresolved question remains: what is the independent role of each of these hormones on sodium regulation in the distal nephron?[14,15] We hypothesize that angiotensin II has effects independent of aldosterone on the renal sodium transporters and their regulatory kinases in the distal nephron. To test this, we conducted an experiment in which normal rats underwent adrenalectomy and then received either angiotensin II or aldosterone. Our results demonstrate specific effects of angiotensin II on the abundance and phosphorylation of NCC and its regulatory kinases.

METHODS

Animal studies

The animal protocol was approved by the Animal Care Committee of the Erasmus Medical Center (EUR 127-08-02). Male Sprague–Dawley rats (n=20, 15 weeks old, average weight 300 grams) were obtained from Charles River laboratories (Sulzfeld, Germany). Animals were randomly assigned to one of the four groups (Table 1). On day 0, all animals were anaesthetized under 3% isoflurane inhalation and underwent adrenalectomy via a bilateral lumbodorsal incision. Immediately after adrenalectomy (also day 0), all animals received an osmotic minipump (model 2 ml2 Alzet, Cupertino, CA,USA) for 8 days to administer the hormones shown in Table 1. All minipumps also contained dexamethasone (12 µg/kg/day, for glucocorticoid replacement).[16] After surgery, animals

Table 1: Experimental design

	Control	Aldosterone	Angiotensin II non-pressor	Angiotensin II pressor
No. of animals	5	5	5	5
Adrenalectomy	Yes	Yes	Yes	Yes
Infusion	No	Aldosterone (50 µg/kg/day)	Angiotensin II (233 µg/kg/day)	Angiotensin II (700 µg/kg/day)
Dexamethasone (12µg/kg/day)	Yes	Yes	Yes	Yes
Drinking fluid	0.9% NaCl	0.9% NaCl	0.9% NaCl	0.9% NaCl
Hormones present[a]	Angiotensin II	Angiotensin II Aldosterone +	Angiotensin II +	Angiotensin II ++

[a] ´+´ Indicates high physiological concentration, ´++´ indicates supra-physiological concentration.

were placed in metabolic cages (day–night cycle 12 h, temperature 23 °C) from day 0 until day 8. Normal rat chow and drinking fluid (0.9% NaCl) were supplied *ad libitum*. Normal saline was selected to compensate for natriuresis after adrenalectomy. Blood pressure was measured every morning in conscious rats using a computerized tail cuff system after a 7-day acclimatization period (Kent Scientific Corporation, Torrington, CN, USA). On day 8, the animals were killed. A fifth group of sham-operated rats was only used as a normal reference for plasma renin activity, and plasma angiotensin I, II, and aldosterone concentrations. In a separate animal study following the same protocol, we compared urinary sodium excretion in adrenalectomized rats which received an osmotic minipump with vehicle, a non-pressor dose of angiotensin II (233 µg/kg/day), or a non-pressor dose of angiotensin II plus hydrochlorothiazide (18.75 mg/kg/day).[17]

Tissue preparation and plasma measurements

Plasma renin activity, angiotensin I and II, and aldosterone were measured using sensitive radioimmunoassays, as described previously.[18,19] Plasma angiotensin II was expressed as natural logarithm because of a nonlinear distribution. The detection limit for plasma aldosterone was 1 pg/ml. If the measured value was below the detection limit, a value of 1 pg/ml was used as dummy variable for statistics. Immediately after the collection of blood, kidneys were harvested and placed on ice. The right kidney was used for immunoblotting and was placed in an isolation buffer (10 mmol/l triethanolamine, 250 mmol/l sucrose, protease inhibitors (Complete Roche Biochemicals, Roche Diagnostics, Almere, The Netherlands)) and homogenized. The whole kidney homogenate was subjected to differential centrifugation to separate the plasma membrane from the intracellular fraction, as described previously.[20] The pellet (plasma membrane fraction) was resuspended in 1ml isolation buffer. A total of 60 µl of both fractions were

used for quantitative protein assay (Pierce, Thermo Scientific, Rockford, IL, USA) and the remaining samples were stored in 6X Laemmli at -80 °C for immunoblotting.

Immunoblotting

Immunoblotting was performed as described previously.[2] Samples of all 20 rats were immunoblotted simultaneously using two gels in one transfer apparatus including an internal standard. All immunoblots were performed at least three times to confirm linearity and reproducibility. Equal protein loading was confirmed with Coomassie (Supplementary Figure S2 online). Antibodies against the transport proteins, sodium hydrogen exchanger type 3 (1:5000), sodium potassium chloride cotransporter type 2 (1:10,000), NCC (1:500), the α-, β-, and γ-subunits of ENaC (1:100, 1:1000, 1:500), and aquaporin-2 (1:1000) were kindly provided by dr. MA Knepper.[21] Antibodies against phosphorylated NCC at threonine-53 and/or -58 were generated by one of the investigators (RAF, all 1:500).[22] Antibodies against all other proteins were obtained: SPAK (Cell Signaling Technology, Leiden, The Netherlands, 1:300), SGK1 (Millipore, Billerica, MA, USA, 1:2000), and NEDD4-2 (Abcam, Cambridge, UK, 1:4000). Similarly, WNK4 (1:500), OSR1 (1:1000), and an antibody that recognized phosphorylated SPAK at serine-373 and phosphorylated OSR1 at serine-325 (4 µg/ml with 40 µl of the non-phosphorylated peptide) were obtained from the Division of Signal Transduction Therapy, University of Dundee.[8]

Immunohistochemistry

The left kidney was used for immunohistochemistry. The midregion was sectioned into 2- to 3-mm transverse sections and immersionfixed for an additional 1 h, followed by 3X 10 min washes with 0.1 mol/l cacodylate buffer, pH 7.4. The tissue was dehydrated in graded ethanol, incubated overnight in xylene, and embedded in paraffin, and 2-µm sections were cut on a rotary microtome (Leica Microsystems, Herlev, Denmark). Immunolabeling was performed as described previously.[22,23] Labeling was detected using a horseradish peroxidase-conjugated secondary antibody (Dako P448, goat anti-rabbit IgG, Glostrup, Denmark) and visualized with 0.05% 3,30-diaminobenzidine tetrachloride (Kemen Tek, Copenhagen, Denmark). Light microscopy was carried out with a Leica DMRE (Leica Microsystems).

Statistics

All data are expressed as means and s.e. of the mean. Group comparisons were made by analysis of variance using the least significant difference as *post-hoc* test. Blood pressure data were analyzed using repeated measures general linear model, which generates one *P*-value for the overall difference over time between the groups. $P < 0.05$ was considered statistically significant.

RESULTS

Renin–angiotensin–aldosterone system

The experimental set-up is shown in Table 1. Briefly, to analyze the independent effects of angiotensin II, all groups first underwent adrenalectomy and then received vehicle (control group), aldosterone (Aldo group, 50 µg/kg/day), or angiotensin II in a non-pressor (233 µg/kg/day) or a pressor (700 µg/kg/day) dose for 8 days.[24,25] A fifth group of sham-operated rats was used only as a normal reference for plasma renin activity, and plasma angiotensin I, II, and aldosterone concentrations. As expected, plasma renin activity was reduced in the angiotensin II-treated groups compared with control (Figure 1a). Plasma renin activity was also reduced in the aldosterone-treated group, but only compared with the sham-operated animals. Plasma angiotensin I, but not plasma angiotensin II, followed the changes in plasma

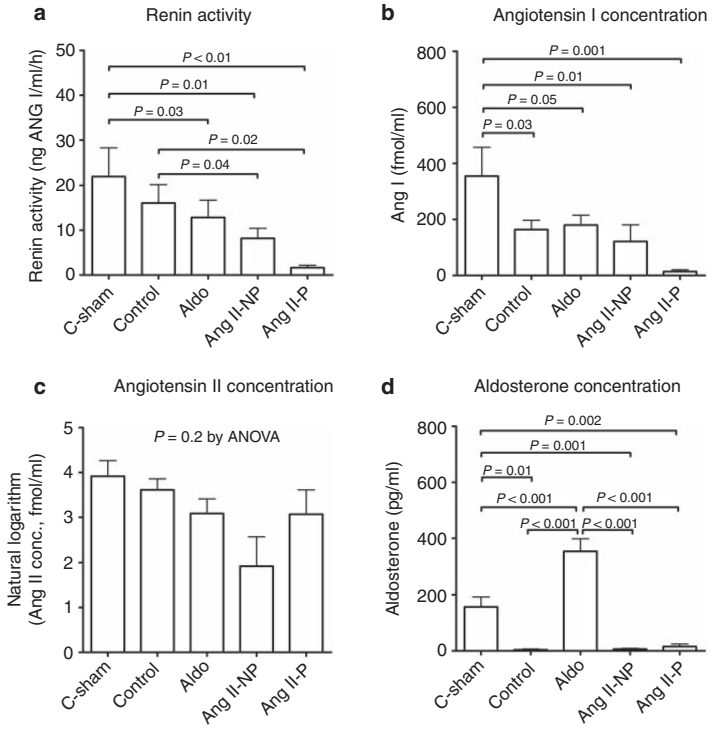


Figure 1: Activity or concentrations of components of the renin–angiotensin–aldosterone system. Plasma renin activity (**a**), and plasma concentrations of angiotensin I (**b**), angiotensin II (**c**), and aldosterone (**d**). Plasma angiotensin II was expressed as natural logarithm because of a nonlinear distribution. Groups were compared by analysis of variance (ANOVA) followed by a *post-hoc* test (see Materials and Methods). A second control group (C-sham) was included as a normal reference (rats that were sham-operated only, no infusion of hormones).

renin activity (Figure 1b and c). Plasma aldosterone showed high physiological levels in the aldosterone-treated group, normal levels in the sham-operated controls, and virtually undetectable levels in the other adrenalectomized groups (Figure 1d).

Effects of aldosterone and angiotensin II on blood pressure, renal function, and sodium excretion

The administration of a pressor dose of angiotensin II resulted in a significant increase in mean arterial blood pressure compared with all other groups (Figure 2a). No significant differences in blood pressure were observed in the other groups. The pressor dose of angiotensin II resulted in higher plasma creatinine concentrations compared with all other groups (Figure 2b). In addition, the aldosterone-treated group also had higher plasma creatinine concentrations than the control group. Both aldosterone and angiotensin II reduced plasma potassium, but only aldosterone caused frank hypokalemia (3.2±0.06 mmol/l versus 4.3–5.2 mmol/l in the other groups, $P < 0.05$ for all). Treatment with

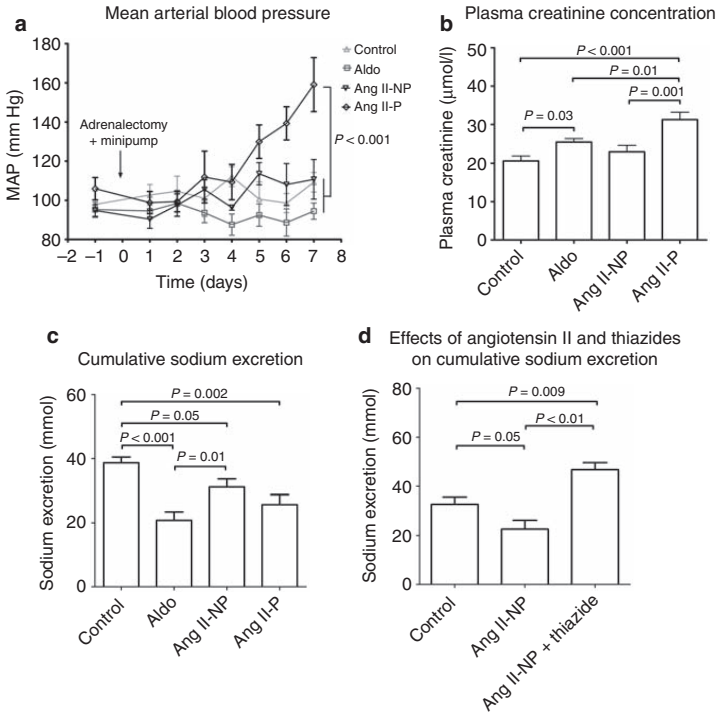


Figure 2: Blood pressure, renal function, plasma potassium, and cumulative urine sodium excretion. Blood pressure (**a**), plasma creatinine (**b**), 6-day cumulative urine sodium excretion (**c**), and the effects of thiazides on 6-day cumulative urine sodium excretion (**d**). Cumulative sodium excretion was calculated based on 6-day urine volume and urine sodium (days 1 and 2 were discarded). Blood pressure was analyzed by a repeated measures general linear model. All other analyses were performed by analysis of variance followed by a *post-hoc* test (see Materials and Methods).

aldosterone and angiotensin II resulted in significantly lower 6-day cumulative sodium excretions compared with control (21±3mmol for Aldo, 31±3 mmol for angiotensin II in a non-pressor, and 26±7 mmol for angiotensin II in a pressor versus 39±2mmol for control; Figure 2c). In addition, the animals treated with aldosterone had a lower cumulative sodium excretion than the animals treated with the non-pressor dose of angiotensin II. A separate study showed that the sodium retention caused by angiotensin II could be inhibited by hydrochlorothiazide (Figure 2d).

Differential centrifugation and analysis by immunoblotting

The abundance of both transport proteins and regulatory proteins was analyzed by immunoblotting. As transport proteins are predominantly localized in plasma membrane, and regulatory proteins in the intracellular compartment, we used differential centrifugation to enrich both fractions (see Materials and Methods). A confirmation of this method is shown in Supplementary Figure S1, illustrating the enrichment of proteins localized in the plasma membrane (P-cadherin, sodium hydrogen exchanger type 3) or intracellular compartment (WNK4). Of all proteins tested and reported below, at least three immunoblots were performed. All results were consistent and showed similar statistics; the most representative immunoblots are shown. A Coomassie loading gel confirmed equal loading among the groups with a variation of < 10% (Supplementary Figure).

Angiotensin II and aldosterone independently increased NCC, ENaC, and aquaporin-2

In these adrenalectomized rats, the non-pressor and pressor dose of angiotensin II increased the plasma membrane abundance of NCC 4.5- and 3.5-fold (Figure 3). Aldosterone increased the plasma membrane abundance of NCC threefold. The plasma membrane abundance of NCC did not differ between the three experimental groups. Interestingly, both aldosterone and angiotensin II increased the plasma membrane abundance of aquaporin-2 three- to fivefold compared with controls. This effect was stronger and dose-dependent in the angiotensin II-treated groups (higher abundance of aquaporin-2 with angiotensin II in a non-pressor versus Aldo and with angiotensin II in a pressor versus angiotensin II in a non-pressor). Only the non-pressor dose of angiotensin II selectively increased the plasma membrane abundance of the β-subunit of ENaC 1.8-fold. Conversely, only aldosterone treatment selectively increased the plasma membrane abundance of the α-subunit of ENaC approximately twofold. The infusion of angiotensin II or aldosterone did not result in significant differences in the plasma

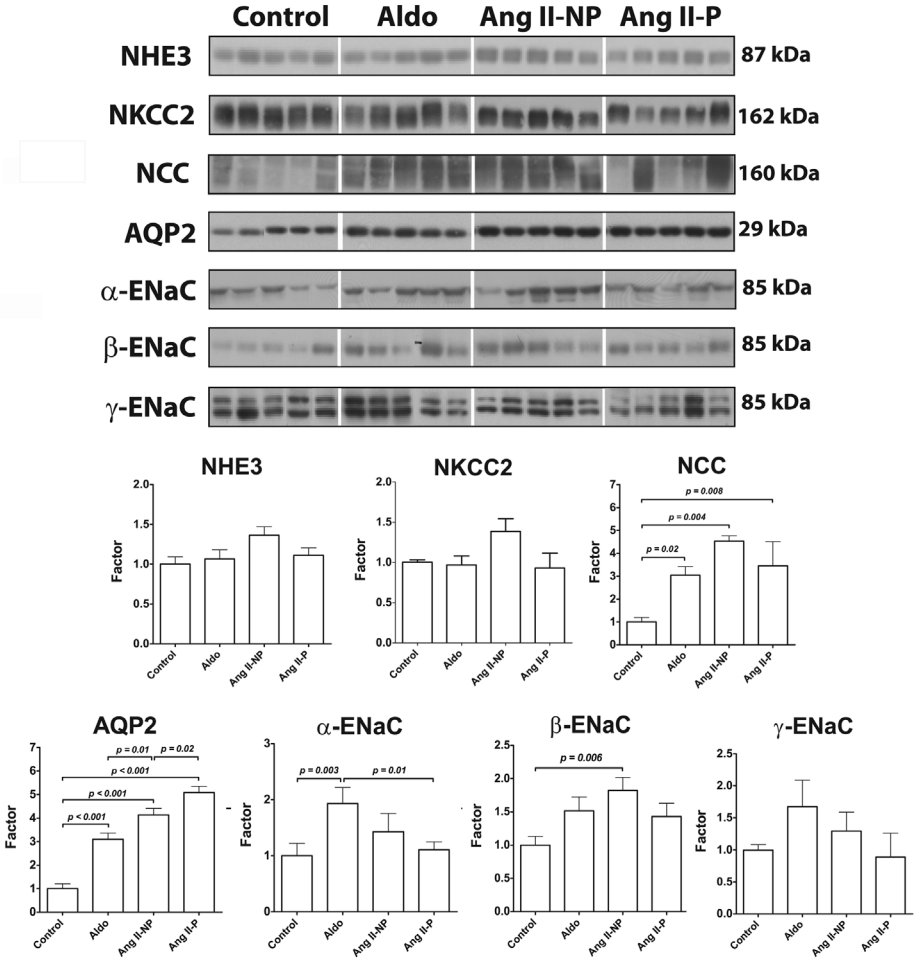


Figure 3: Immunoblots of renal sodium and water transporters. Immunoblots showing the plasma membrane abundance of renal sodium and water transport proteins. These include the sodium hydrogen exchange type 3 (NHE3), the sodium potassium chloride cotransporter type 2 (NKCC2), the sodium chloride cotransporter (NCC), the water channel aquaporin-2 (AQP2), and finally the α-, β-, and γ-subunits of the epithelial sodium channel (ENaC). Groups were compared by analysis of variance followed by a *post-hoc* test (see Materials and Methods).

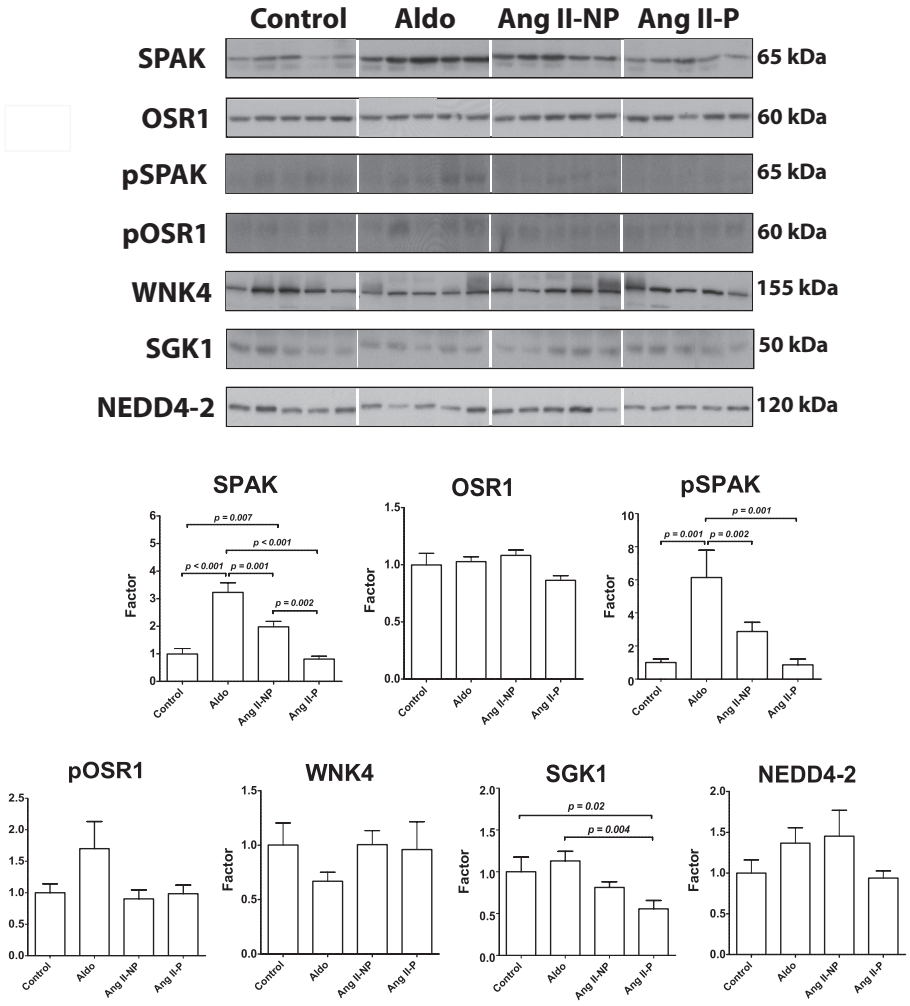


Figure 4: Immunoblots of regulatory proteins. Immunoblots showing the intracellular abundance of regulatory proteins. These include STE20/SPS1-related, proline alanine-rich kinase (SPAK), oxidative stress responsive protein type 1 (OSR1), phosphorylated SPAK at serine-373, phosphorylated OSR1 at serine-325, with-no-lysine kinase 4 (WNK4), serum- and glucocorticoid-inducible kinase 1 (SGK1), and the ubiquitin ligase Nedd4-2. Groups were compared by analysis of variance followed by a *post-hoc* test (see Materials and Methods).

membrane abundance of the sodium hydrogen exchange type 3 and the sodium potassium chloride cotransporter type 2.

Angiotensin II increased SPAK independent of aldosterone

The non-pressor dose of angiotensin II significantly increased the intracellular abundance of SPAK twofold compared with the control group (Figure 4). This effect was not observed with the pressor dose of angiotensin II, which caused a lower intracellular abundance of SPAK than the non-pressor dose. Aldosterone treatment also increased the intracellular abundance of SPAK approximately threefold, which was significantly higher than the intracellular abundance of SPAK in all other groups. Aldosterone increased the phosphorylation of SPAK at serine-373 6.1-fold compared with control. The pressor dose of angiotensin II modestly reduced the intracellular abundance of SGK1 compared with the control group and with the group receiving aldosterone (both approximately 1.4-fold). The intracellular abundance of the other regulatory proteins that were tested, including OSR1, phosphorylated OSR1, WNK4, and NEDD4-2, did not change significantly.

Phosphorylation of NCC by angiotensin II and aldosterone

We analyzed whether angiotensin II also stimulated the phosphorylation of NCC. Indeed, the non-pressor dose of angiotensin II increased phosphorylation of NCC at threonine-53 in plasma membrane 4.3-fold (Figure 5). Aldosterone also increased the phosphorylation of NCC at threonine-53 and to the same degree as the non-pressor dose of angiotensin II. Only aldosterone significantly increased phosphorylation of NCC at threonine-58 (1.7-fold). An antibody that recognized phosphorylation at both threonine-53 and threonine-58 also showed increased phosphorylation of NCC in the plasma membrane by aldosterone and by the non-pressor dose of angiotensin II.

Immunohistochemistry of phosphorylated NCC and aquaporin-2

Figure 6 shows immunohistochemistry for phosphorylated NCC at threonine-53 and for aquaporin-2. Immunohistochemistry showed increased labeling of phosphorylated NCC and aquaporin-2 in the three experimental groups, thereby confirming the immunoblotting results. In addition, Figure 6b shows that aquaporin-2 labeling was predominantly increased in the basolateral membrane.

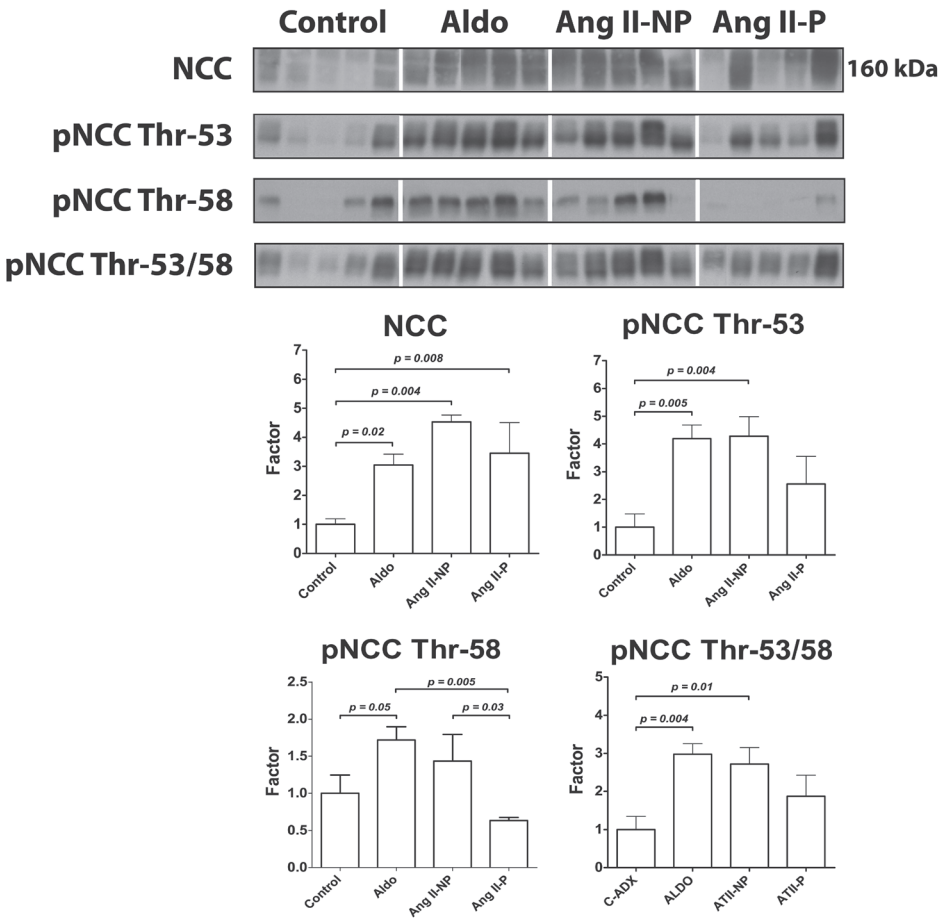


Figure 5: Immunoblots of sodium chloride cotransporter (NCC) and phosphorylated NCC (pNCC). Total abundance and phosphorylation of NCC at threonine (Thr) 53 and 58 are shown. Groups were compared by analysis of variance followed by a *post-hoc* test (see Materials and Methods). Ang II-NP, angiotensin II in a non-pressor; Ang II-P, angiotensin II in a pressor.

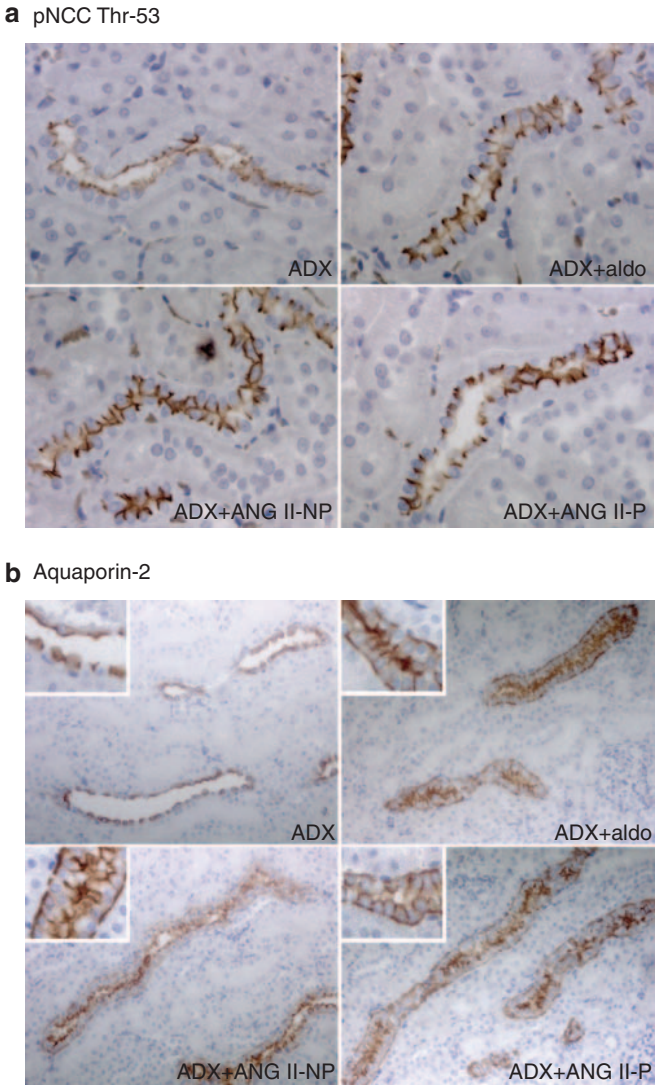


Figure 6: Immunohistochemistry for the phosphorylated sodium chloride cotransporter (pNCC) and aquaporin-2. Immunohistochemistry for (**a**) the pNCC threonine-53 (Thr-53) and (**b**) the water channel aquaporin-2. **a** shows representative images of rat distal tubule, **b** of rat collecting duct cells. In **a** and **b**, the upper left panel shows the control group, which only underwent adrenalectomy (ADX). The three experimental group also underwent adrenalectomy, and were also treated with aldosterone (ADX+Aldo, upper right), a non-pressor dose of angiotensin II (ADX+ANG II-NP, lower left), or a pressor dose of angiotensin II (ADX+ANG II-P, lower right). In **a** and **b**, the more intense staining in the three experimental groups compared with the control group is clearly visible. In **b**, aquaporin-2 staining is especially intense in the basolateral membrane.

DISCUSSION

In the present study, our aim was to test the hypothesis that angiotensin II regulates the NCC independently of aldosterone. Indeed, this is the first controlled in vivo study to show that angiotensin II under a chronic setting increased the abundance and phosphorylation of NCC independently of aldosterone, leading to sodium retention. The observation that sodium retention, secondary to angiotensin II, could be inhibited by thiazides (Figure 2d) suggests that at least part of this effect was mediated via NCC. However, it is not a direct proof of an NCC-mediated effect, because we did not test whether angiotensin II-treated animals were more sensitive to thiazides than control animals; it also remains to be determined whether the effect was direct. Because SPAK has been identified as a protein capable of phosphorylating the N-terminal regulatory regions of NCC,[12] the observed increase in SPAK (Figure 4) suggests its involvement in the phosphorylation of NCC (Figure 5). Although observational, our results provide further insight in the current model of the regulation of NCC (Figure 7). Namely, our data confirm previous suggestions that two separate signaling pathways regulate NCC, one originating from the angiotensin II type 1 receptor and one originating from the mineralocorticoid receptor (Figure 7). The net effect of both signaling pathways is a stimulation of sodium chloride reabsorption by NCC in the distal convoluted tubule. Although trafficking and phosphorylation of NCC by angiotensin II has been shown previously,[26,27]

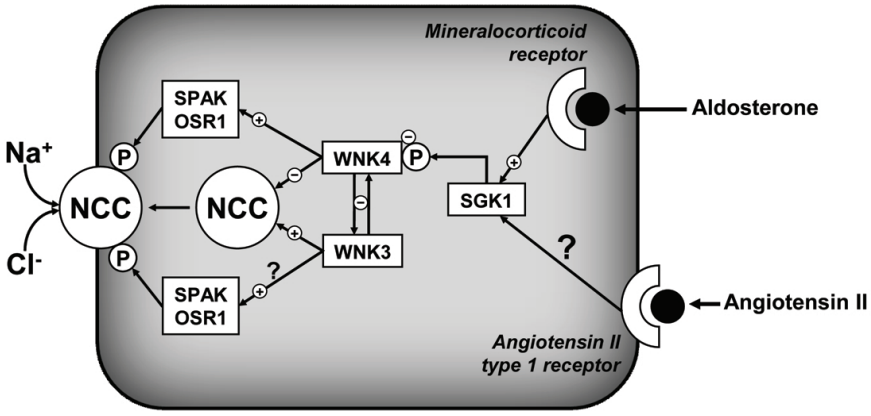


Figure 7: Model for the regulation of the sodium chloride cotransporter (NCC) by increasing plasma membrane abundance and phosphorylation. Effects are stimulatory (plus signs) or inhibitory (minus signs). 'P' denotes phosphorylation. The combined minus sign and phosphorylation sign next to with no-lysine kinase (WNK) 4 means that serum- and glucocorticoid inducible kinase 1 (SGK1) phosphorylates WNK4 and thereby decreases its activity[24] WNK3 was not analyzed in this study. The role of WNK1 and kidney-specific WNK1 is not shown. The model is in part based on the findings in this study and in part on previous literature.[13,17,24-26,28]

this is the first study to demonstrate that these processes can occur independently of aldosterone. Recently, in vitro studies have identified SPAK, WNK4, and SGK1 as important regulatory kinases in the signaling pathways stimulated by angiotensin II and aldosterone.[8,11,13] For example, San-Cristobal *et al.*[13] showed in oocytes of Xenopus and mpkDCT cells that the regulation of NCC by angiotensin II depends on both SPAK and WNK4. Furthermore, Rozansky *et al.*[11] showed in oocytes of Xenopus and HEK293 cells that aldosterone mediates the activation of NCC through SGK1-induced phosphorylation of the C-terminal WNK4. These findings prompted us to also analyze these NCC regulatory proteins. We showed that both angiotensin II and aldosterone increased the intracellular abundance of SPAK, but not OSR1 (Figure 4). The explanation for the fact that the intracellular abundance of SPAK was increased significantly more in the aldosterone-treated rats may be the consequence of the well-known synergistic interaction between (exogenous) aldosterone and (endogenous) angiotensin II in this group (Figure 1).[28] The phosphorylation of SPAK also increased with angiotensin II and aldosterone, but this change was significant only for the latter. Recently, Chiga *et al.*[12] used low- and high-sodium diets in mice to analyze the effects of high and low aldosterone levels (plasma aldosterone ~800 and ~100 pg/ml) on SPAK, OSR1, and their phosphorylated forms. A high plasma aldosterone concentration increased the phosphorylation of SPAK and OSR1, but not their abundances.[12] Although our study confirms that aldosterone increases the phosphorylation of SPAK, we also observed an increase in the intracellular abundance of SPAK, but saw no effect on phosphorylated OSR1 (Figure 4). These differences may be related to the experimental models (diet versus infusion), species (mice versus rats), or tissue preparation (whole kidney homogenates versus intracellular fractions).[12] Despite our confirmation of the importance of SPAK, we observed no change in the intracellular abundance of WNK4 with angiotensin II or aldosterone (Figure 4). This is in agreement with two recent studies which also demonstrated the unchanged WNK4 abundance with low-sodium diet (high aldosterone)[12] and with chronic angiotensin II infusion.[29] However, these results do not preclude a role for WNK4, because it may be regulated through phosphorylation rather than through abundance, as shown by Rozansky *et al*.[11] Because we analyzed whole kidney homogenates, an alternative explanation is an opposite effect on WNK4 abundance in another nephron segment expressing WNK4 (e.g., thick ascending limb or collecting duct). Similar to WNK4, we observed no change in SGK1 with aldosterone or the non-pressor dose of angiotensin II, and even observed a modest reduction in SGK1 with the pressor dose of angiotensin II (Figure 4). Especially, the lack of response in SGK1 with aldosterone is surprising, because Vallon *et al*.[30] recently showed that increased abundance and phosphorylation of NCC with low-sodium diet (high aldosterone) did not occur in SGK1 knockout mice, illustrating the central role of SGK1 in NCC regulation. It has been proposed that angiotensin II also

mediates its effect on the NCC via SGK1,[15,31] but this has not been confirmed experimentally. Although the pressor dose of angiotensin II reduced SGK1 (Figure 4), we are reluctant to conclude that this was primarily because of angiotensin II, as these animals also had additional variables that could have caused this effect (i.e., hypertension and higher plasma creatinine; Figure 2). The same explanations for why WNK4 was unchanged in our model may also apply to SGK1. In addition, the effect of aldosterone on SGK1 may be dose dependent. For example, Hou *et al.*[32] demonstrated an eightfold rise in SGK1 mRNA in normal mice, but their plasma aldosterone concentrations were > 10 times than in our aldosterone-treated rats. Using in situ hybridization, Chen *et al.*[9] also showed an increased abundance of SGK1 in adrenalectomized rats receiving aldosterone, but they also used 2 to 3 times higher dosing than we did. Finally, the plasma aldosterone concentrations of the mice on a low-sodium diet in the study by Vallon *et al.*[30] were almost twice as high as the ones in our aldosterone-treated rats. A separate focus of this study was the effects of angiotensin II and aldosterone on the water channel aquaporin-2. Interestingly, both aldosterone and angiotensin II increased the plasma membrane abundance of aquaporin-2, showing the highest abundance with a pressor dose of angiotensin II (Figure 3). The observed upregulation of aquaporin-2 by angiotensin II may represent a direct effect[33] or an indirect effect because of the ability of angiotensin II to increase vasopressin release.[34] Previously, the role of angiotensin II in aquaporin-2 regulation has been demonstrated *in vivo* in urinary tract obstruction.[23,35] In mpkCCDc14 cells, long-term incubation with aldosterone also increased aquaporin-2 protein abundance by increasing aquaporin-2 mRNA translation.[36] Our immunohistochemistry results (Figure 6) confirm that of De Seigneux *et al.*,[37] who also showed that aldosterone increased the basolateral staining of aquaporin-2 in the cortical collecting duct of rats. They hypothesized that in situations of increased sodium reabsorption, a higher permeability of the basolateral membrane may facilitate water efflux to prevent cell swelling. Except for aquaporin-2, the effects of the pressor dose of angiotensin II tended to be less pronounced than the effects of the non-pressor dose (Figure 3–5). Possible explanations include the presence of hypertension (Figure 2a), reduced renal function (Figure 2b), or a downregulation of the angiotensin II type 1 receptor. As anticipated from previous studies, aldosterone also increased NCC,[3] in addition to α-ENaC[7] (Figure 4). Interestingly, the non-pressor dose of angiotensin II selectively increased β-ENaC. Previously, angiotensin II has been shown to increase the activity of ENaC.[38] However, the ENaC subunits appear differentially regulated by angiotensin II, because an angiotensin receptor blocker decreased α-ENaC, but increased β-ENaC.[39] Therefore, an alternative explanation could be that not angiotensin II but vasopressin increased β-ENaC, which was shown previously[40] and would also be consistent with the observed increase in aquaporin-2. In this study, we focused on the regulation of NCC. Although NCC reabsorbs only 5–10% of

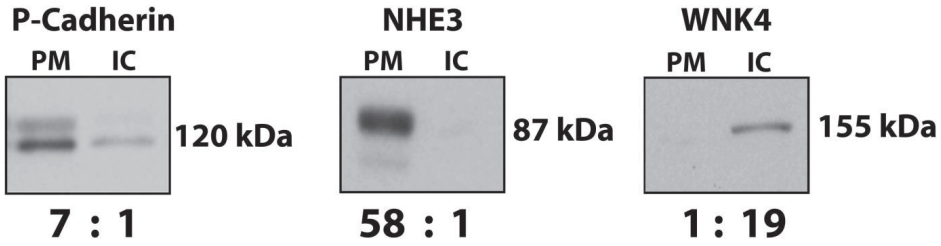
the filtered load of sodium chloride,[27] altered function of this cotransporter has profound effects on total body sodium and blood pressure. This is illustrated by the fact that thiazides, which inhibit NCC, are among the most effective antihypertensive drugs.[41] Similarly, monogenetic disorders that result in inactivation (Gitelman's syndrome) or overactivation (Gordon's syndrome) of NCC are also characterized by salt-loss and low-normal blood pressure, or salt-retention and hypertension, respectively.[42]

Finally, polymorphisms in genes encoding for NCC regulatory proteins, including WNK kinases and SPAK, are associated with variations in blood pressure in populations.[43,44]

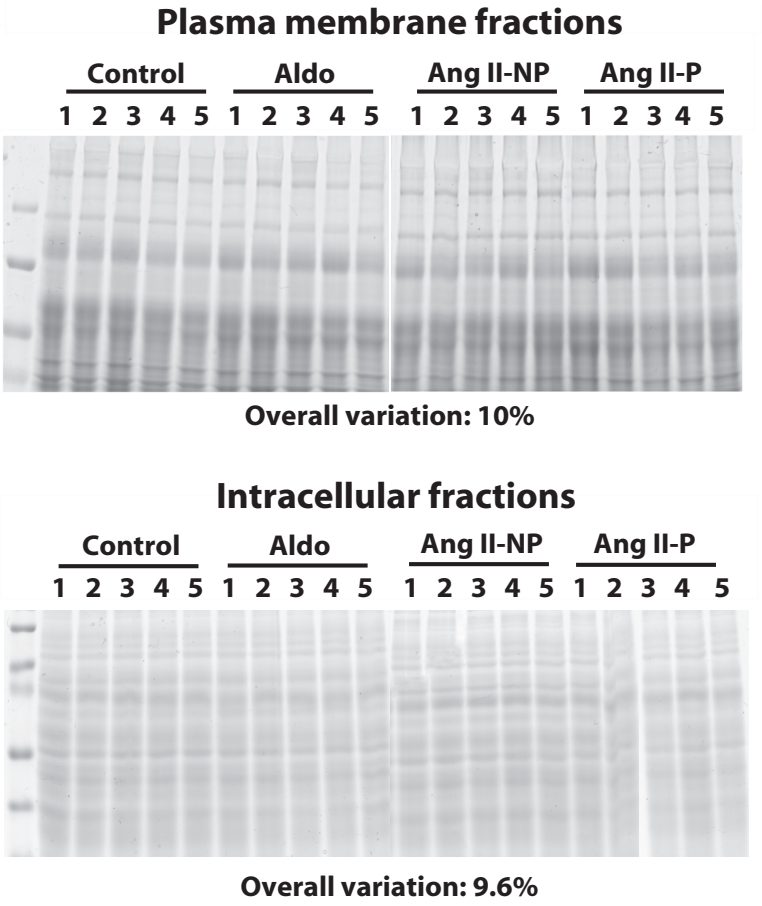
Unravelling the independent effects of angiotensin II and aldosterone on NCC is important both physiologically and pathophysiologically. Physiologically, because angiotensin II and aldosterone had similar effects on sodium and water transporters (Figure 3), both hormones appear to act in concert to maintain extracellular fluid volume during hypovolemia (when both hormones are elevated). Pathophysiologically, the renin–angiotensin–aldosterone system is activated in many disease states, and therefore, angiotensin II and aldosterone appear to individually contribute to sodium retention, which may result in edema and hypertension. These separate effects are important when selecting appropriate pharmacological intervention with angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, or mineralocorticoid receptor blockers. We believe that the strength of this study was the ability to analyze the effects of angiotensin II independent of aldosterone by performing adrenalectomy and then infusing angiotensin II. Adrenalectomy resulted in virtually undetectable aldosterone levels in the angiotensin II-treated groups (Figure 1), thus confirming the validity of this approach. Other evidence supporting our animal model were aldosterone-induced hypokalemia and sodium retention, and angiotensin II-induced sodium retention and hypertension (Figure 2). Animals receiving aldosterone retained most of the sodium, but did not develop hypertension. This suggests that hypertension induced by the pressor dose of angiotensin II was mainly because of other mechanisms such as vasoconstriction, which may also explain the reduction in renal function (Figures 1 and 2). A previous study also showed reduced renal function with a pressor dose of angiotensin II, which was attributed to renal cortical vasoconstriction.[45] The consequent decreased distal sodium delivery may explain why more angiotensin II infusion did not result in more sodium retention. We acknowledge that our study has limitations. Most importantly, because whole kidney homogenates were used, some of the observed changes in protein abundance may not be specific for one nephron segment. In addition, the fact that plasma angiotensin II levels were not higher in animals receiving angiotensin II (Figure 1c) implies that plasma angiotensin II levels do not always correspond with their biological effect. We speculate that this may be explained by differences between endogenous and exogenous angiotensin II, accumulation of angiotensin II in renal

tissue, or differences in angiotensin II degrading enzymes.[46] Finally, we appreciate that phosphorylation is sometimes expressed as the ratio between the phosphorylated and total amount of protein.[12] We chose not to do this, because it remains unclear whether there is a linear relationship between phosphorylation and total protein abundance. In addition, the increase in regulatory kinases suggested that more phosphorylation took place. In summary, in adrenalectomized rats, angiotensin II increased the total and phosphorylated NCC independently of aldosterone. Phosphorylation of NCC by angiotensin II and aldosterone appears to be mediated through SPAK. These results are important for understanding the molecular physiology of sodium transport in the distal nephron, which not only is essential for maintaining the extracellular fluid volume, but also contributes to the pathophysiology of several forms of hypertension.[44,47,48]

SUPPLEMENTARY MATERIAL



Supplemental figure 1. Separation and enrichment of plasma membrane (PM) and intracellular (IC) fractions by differential centrifugation



Supplemental figure 2. Coomassie loading gels for plasma membrane and intracellular fractions showing equal loading with overall variations of 10 and 9.6%.

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Chapter 3

Aldosterone does not require angiotensin II to activate NCC through a WNK4–SPAK–dependent pathway

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ABSTRACT

We and others have recently shown that angiotensin II can activate the sodium chloride cotransporter (NCC) through a WNK4–SPAK-dependent pathway. Because WNK4 was previously shown to be a negative regulator of NCC, it has been postulated that angiotensin II converts WNK4 to a positive regulator. Here, we ask whether aldosterone requires angiotensin II to activate NCC and if their effects are additive. To do so, we infused vehicle or aldosterone in adrenalectomized rats that also received the angiotensin receptor blocker losartan. In the presence of losartan, aldosterone was still capable of increasing total and phosphorylated NCC twofold to threefold. The kinases WNK4 and SPAK also increased with aldosterone and losartan. A dose-dependent relationship between aldosterone and NCC, SPAK, and WNK4 was identified, suggesting that these are aldosterone sensitive proteins. As more functional evidence of increased NCC activity, we showed that rats receiving aldosterone and losartan had a significantly greater natriuretic response to hydrochlorothiazide than rats receiving losartan only. To study whether angiotensin II could have an additive effect, rats receiving aldosterone with losartan were compared with rats receiving aldosterone only. Rats receiving aldosterone only retained more sodium and had twofold to fourfold increase in phosphorylated NCC. Together, our results demonstrate that aldosterone does not require angiotensin II to activate NCC and that WNK4 appears to act as a positive regulator in this pathway. The additive effect of angiotensin II may favor electroneutral sodium reabsorption during hypovolemia and may contribute to hypertension in diseases with an activated renin–angiotensin–aldosterone system.

INTRODUCTION

Angiotensin II and aldosterone are the chief hormones of the renin–angiotensin–aldosterone system. In the last two decades, aldosterone has been recognized as the primary hormone regulating sodium transport along the distal nephron.[1-3] Aldosterone exerts its effects on three parts of the distal nephron, including the distal convoluted tubule (DCT), connecting tubule (CNT), and cortical collecting duct (CCD). In fact, this nephron segment is often referred to as the "aldosterone-sensitive distal nephron (ASDN)".[4,5] Via the mineralocorticoid receptor, aldosterone activates the two main sodium transporters in the ASDN: the thiazide-sensitive sodium chloride cotransporter (NCC) located in the "early" and "late" DCT[6] and the epithelial sodium channel (ENaC) located in the "late" DCT, CNT, and CCD.[2] Conversely, angiotensin II has traditionally been considered to act primarily in the proximal tubule, where it stimulates both the trafficking and phosphorylation of the sodium hydrogen exchanger type 3.[7] The last few years, however, an emerging set of data has indicated that angiotensin II can also activate NCC[8-11] and, to a lesser extent, ENaC.[12] These new insights have raised the question as to the respective roles of angiotensin II and aldosterone in the distal nephron and whether their effects are mutually dependent. This question is of both physiological and clinical relevance because aldosterone performs two completely different functions during hypovolemia (when angiotensin II is also increased) and hyperkalemia (when angiotensin II is not increased). This has been coined the "aldosterone paradox".[13-16] Of further interest is the recent discovery of a kinase network that can favor the sodium-retaining or potassium-secreting function of aldosterone by selectively activating ion channels. This kinase network consists of the Ste20-related kinase SPAK and several members of the WNK (With No K=lysine) kinase family.[5,15,17] Recently, we conducted a study addressing the independent role of angiotensin II in the distal nephron.[11] In adrenalectomized rats, we showed that angiotensin II induced phosphorylation of NCC in the absence of aldosterone. These effects were mediated by SPAK, which is capable of phosphorylating NCC.[18] Similarly, in oocytes and cells, San-Cristobal and colleagues showed that angiotensin II induced phosphorylation of NCC through WNK4 and SPAK.[9] Although WNK4 was previously shown to be a negative regulator of NCC[19], these authors proposed that angiotensin II may convert WNK4 to becoming a positive regulator of NCC.[9] Together, these recent data raise the question whether aldosterone requires angiotensin II to activate NCC and whether the effects of aldosterone and angiotensin II are additive. To address this, adrenalectomized rats were infused with vehicle, normal aldosterone, or high aldosterone in addition to the angiotensin II receptor blocker losartan.

MATERIALS AND METHODS

Animal studies

The animal protocol was approved by the Animal Care Committee of the Erasmus Medical Center (EUR 127-08-02). Three studies were performed in rats (all Sprague-Dawley, 15 weeks old, average weight 370 g; Charles River, Sulzfeld, Germany) (Supplemental Table 1). In the first study, all rats were adrenalectomized (via bilateral lumbodorsal incision) and were then randomized to receive no, normal (50 µg/kg/day), or high aldosterone (100 µg/kg/day). All rats also received losartan (10 mg/kg/day)[3,20,21]; this dose has been shown to inhibit 90 % of the angiotensin receptor type 1 receptors.[22] Blood pressure was measured every morning in conscious rats using a tail-cuff system after a 7-day acclimatization period (Kent Scientific Corporation, Torrington, CN, USA). In the second study, the sensitivity to hydrochlorothiazide or amiloride was tested, as described previously.[23,24] Briefly, rats were adrenalectomized and randomized into six groups. All rats received losartan(10 mg/kg/day). The first three groups also received aldosterone (100 µg/kg/day) for 4 days after which hydrochlorothiazide (25 mg/kg), amiloride (2 mg/kg), or vehicle was injected intraperitoneally. The other three groups served as controls and received the same diuretics or vehicle, but no aldosterone. The diuretics were injected on the day of maximal sodium retention (day 4) and a timed urine was collected 5 h before and after these injections. Diuretic sensitivity was defined as the urine sodium to creatinine ratio. In the third study, the potentially additive effects of angiotensin II and aldosterone were tested. For this, samples from the first study and samples from our previously conducted study [11] were used. Four adrenalectomized rats receiving aldosterone (50 µg/kg/day) were compared to three adrenalectomized rats receiving aldosterone (50 µg/kg/day) and losartan (10 mg/kg/day). In all studies, animals were placed in metabolic cages after adrenalectomy and insertion of minipumps (Alzet, Cupertino, CA, USA). Throughout the study period, they were provided with normal rat chow and drinking fluid (0.9 % NaCl) *ad libitum*; normal saline was selected to compensate for natriuresis after adrenalectomy. All rats also received dexamethasone as glucocorticoid replacement (12 µg/kg/day).[25]

Tissue preparation and plasma and urine measurements

Plasma renin activity and plasma aldosterone were measured as described previously.[11] Urine sodium and creatinine were determined with an automatic analyzer (Modular IPPE, Roche Diagnostics, Almere, The Netherlands). The right kidney was used for immunoblotting and was placed in an isolation buffer (10 mM triethanolamine, 250 mM sucrose, and protease inhibitors [Complete™, Roche Biochemicals, Indianapolis, IN,

USA]) and homogenized. The whole kidney homogenate was subjected to differential centrifugation, as described previously.[26] Sixty microliters of both fractions was used for quantitative protein assay (Pierce, Thermo Scientific, Rockford, IL, USA) and the remaining samples were stored in 6X Laemmli at −80 °C for immunoblotting.

Immunoblotting

Immunoblotting was performed as described previously.[1] For the first study, samples of all rats were immunoblotted simultaneously using two gels in one transfer apparatus including an internal standard. Antibodies against the following transport proteins were obtained: the α-, β-, and γ-subunits of ENaC (all 1:1,000), aquaporin-2 (AQP2) (1:1,000; all StressMarq, Victoria, BC, Canada), NCC and actin (1:500 and 1:100,000; Millipore, Temecula, CA, USA), SPAK (Cell Signaling, Boston MA, USA), and WNK4 (Division of Signal Transduction Therapy, University of Dundee, Dundee, Scotland, UK). Antibodies against pNCC (1:500) were generated by one of the investigators (RAF). Specificity of the SPAK antibody was confirmed using kidney tissue from SPAK−/− mice (data not shown).

Immunohistochemistry

The left kidney was used for immunohistochemistry. The midregion was sectioned into 2- to 3-mm transverse sections and immersion-fixed for an additional 1 h, followed by three times 10-min washes with 0.1 mol/l cacodylate buffer (pH 7.4). The tissue was dehydrated in graded alcohol, incubated overnight in xylene, and embedded in paraffin, and 2-µm sections were cut on a rotary microtome (Leica Microsystems, Herlev, Denmark). Immunolabeling was performed as described previously.[27,28] Labeling was detected using a horseradish peroxidase-conjugated secondary antibody (Dako P448, goat anti-rabbit IgG, Glostrup, Denmark) and visualized with 0.05 % 3,3′-diaminobenzidine tetrachloride (Kemen Tek, Copenhagen, Denmark). Light microscopy was carried out with a Leica DMRE (Leica Microsystems, Herlev, Denmark).

Quantitative PCR

Renal cortex was placed in RNALater (Qiagen, Valencia, CA, USA) and isolated using RNeasy® Mini Kit (Qiagen, Valencia, CA, USA). Five micrograms of RNA was used for first-strand cDNA (SuperScript™ II reverse transcriptase, Invitrogen, Carlsbad, CA, USA). The reaction was inactivated by raising the temperature to 70 °C for 5 min, followed by the addition of RNAse and 15 min incubation at 37 °C. The amplifications were performed using the SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA). The reactions were set for 40 cycles at 60 °C in a Step One Plus System (Applied Biosystem, Foster City, CA, USA). Relative standard curve method was used for calculation. Standard curves enabled target gene quantification and normalization to an endogenous control

(hypoxanthine– guanine phosphoribosyltransferase, Real Time Primers LLC, Elkins Park, PA, USA). All PCR products were checked by sequencing.

Statistics

All data are expressed as the means and standard error of the mean. Group comparisons were made by using a Student's *t* test or analysis of variance with a *post hoc* test, as appropriate. Blood pressure data were analyzed using two-way analysis of variance. Correlations were calculated using Pearson's rho. Because of the wide range, the natural logarithm of the plasma aldosterone concentration was used for these calculations. $P \leq 0.05$ was considered statistically significant.

RESULTS

Animal model to study the effects of aldosterone independent of angiotensin II

Rats were adrenalectomized and then received the angiotensin receptor blocker losartan in addition to vehicle, normal aldosterone, or high aldosterone. This dose of losartan has been shown to inhibit 90 % of angiotensin type 1 receptors.[22] Plasma renin activity was significantly higher in the control group (Fig. 1a). The plasma aldosterone concentrations (Fig. 1b) confirmed that both the adrenalectomy and the delivery of two doses of exogenous aldosterone were successful. Blood pressure was similar in all three groups throughout the experiment (Fig. 1c). The aldosterone-infused groups retained more sodium, whose maximal effect was reached on the fourth day (Fig. 1d). At the end of the experiment, plasma creatinine and urine osmolarity were similar in all three groups (data not shown). Together, these data demonstrate that we successfully established an in vivo model to investigate the sodium-retaining effect of aldosterone independent of angiotensin II and independent of changes in blood pressure and glomerular filtration rate.

Independent effects of aldosterone on transporters and regulatory proteins

Aldosterone infusion increased the abundance and phosphorylation of NCC twofold to threefold (Fig. 2a). A further increase in protein expression with the higher aldosterone dose was observed only for total NCC, but not for phosphorylation at threonine 53 and 58. Both doses of aldosterone also increased the α- and γ-subunits, but not the β-subunit of ENaC (Fig. 2b). The higher dose of aldosterone increased α-ENaC abundance from approximately twofold to fourfold, whereas the increase in γ-ENaC abundance was similar with the normal and high aldosterone doses. Both the 70- and 85-kD subunits of

γ-ENaC increased significantly with aldosterone. The water channel AQP2 also increased approximately threefold with both doses of aldosterone (Fig. 2b). The abundances of the regulatory kinases WNK4 and SPAK increased with both aldosterone doses, whereas the phosphorylated form of SPAK increased significantly only with the normal aldosterone dose (Fig. 2c). Immunohistochemistry confirmed the increase of phosphorylated NCC at threonine 53 and of AQP2 (Fig. 3). It was of interest that aldosterone increased the total expression of AQP2 mainly by inducing basolateral expression. As well as protein expression data, we also studied the effects of aldosterone on the mRNA abundance of transporters and kinases. Although the mRNA abundance of α-ENaC increased significantly with aldosterone (Fig. 4), no significant changes in mRNA abundance were identified for NCC, SPAK, or WNKs. This could not be attributed to the administration of losartan because SPAK mRNA also remained unchanged with aldosterone alone (data not shown).

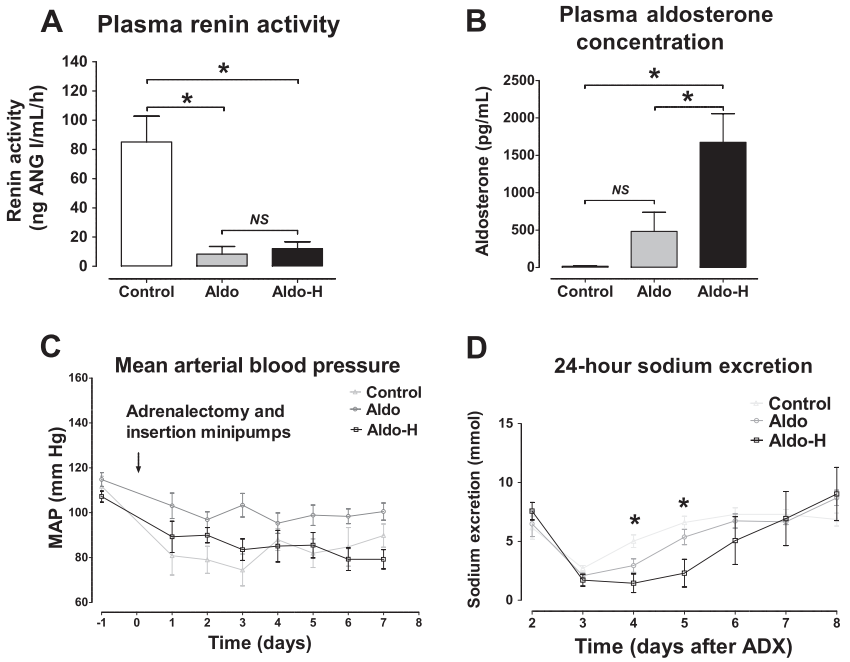

Figure 1: Physiological effects of aldosterone with losartan. Adrenalectomized rats received no aldosterone (*Control, n=5*), normal aldosterone (*Aldo, n=5*), or high aldosterone (*Aldo-H, n=5*) with losartan for 8 days. At the end of the experiment, the plasma renin activity and the plasma aldosterone concentration were measured (**a**, **b**). During the experiment, arterial blood pressure and 24-h urinary sodium excretions were measured (**c**, **d**). By analysis of variance and *post hoc* test. *MAP* mean arterial pressure, *NS* not significant; **P*<0.01

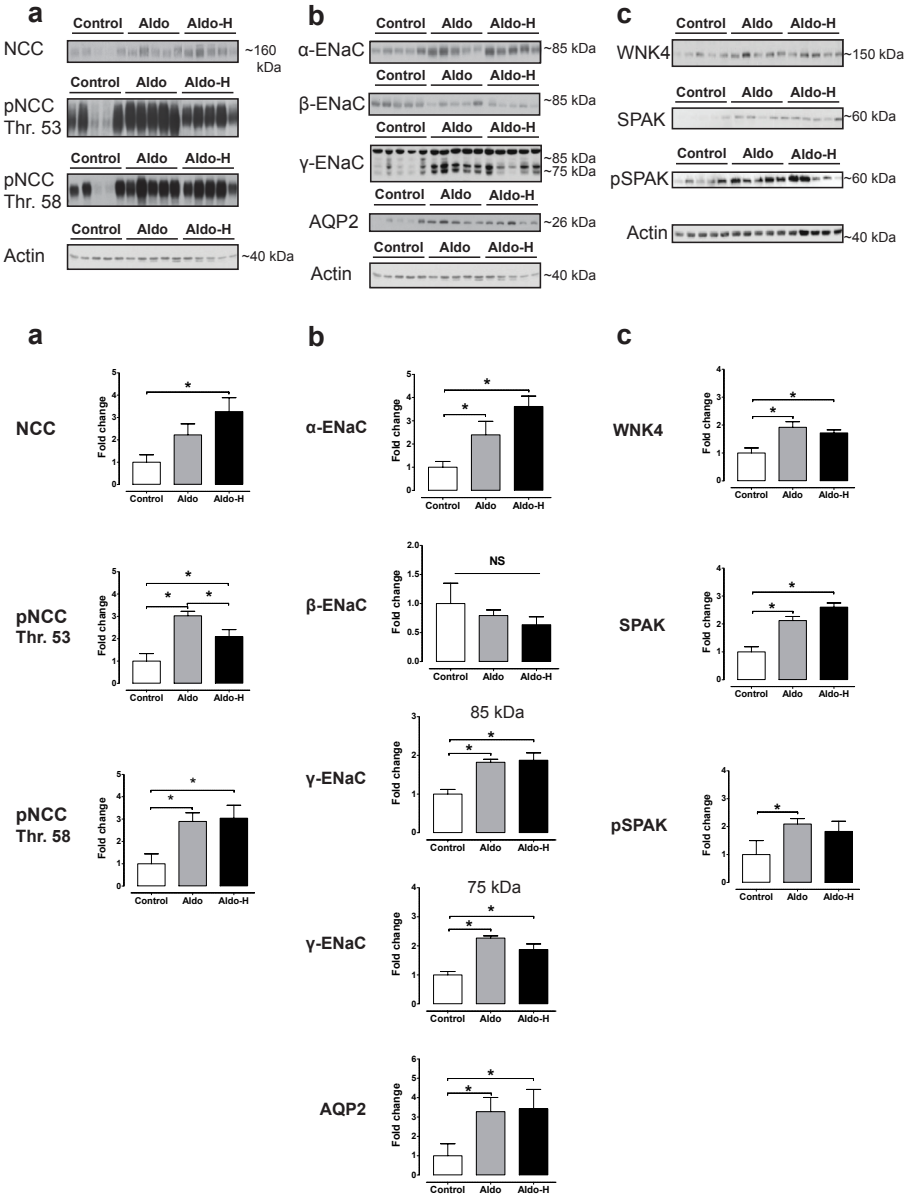

Figure 2: Effects of aldosterone with losartan on renal sodium transporters. Immunoblots showing the effects of aldosterone with losartan on the NCC (**a**), the ENaC and the water channel AQP2 (**b**). In addition, the effects of aldosterone with losartan on two regulatory kinases, WNK4 and SPAK, are shown (**c**). Whole kidney homogenates were differentially centrifuged to obtain plasma membrane fractions (used for all transport proteins) and intracellular fractions (used for the regulatory kinases). Densitometry was normalized for actin. **P*<0.05 by analysis of variance and *post hoc* test.

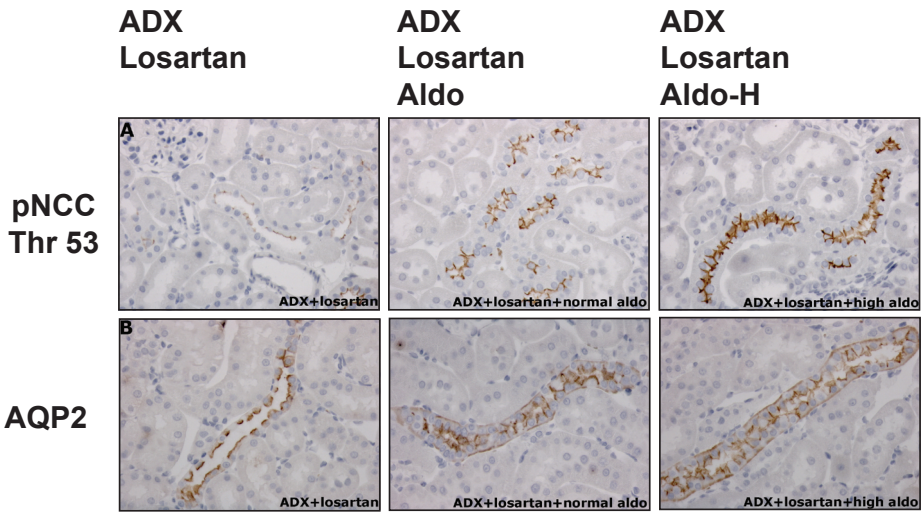

Figure 3: Immunohistochemical analysis of the NCC and AQP2. Immunohistochemistry for NCC phosphorylated at threonine 53 (pNCC) in the DCT (**a**) and the water channel AQP2 in the collecting duct (**b**). **a** and **b** show representative images of kidney sections from adrenalectomized rats treated with losartan only (*left*, ADX+Losartan), aldosterone and losartan (*middle*, ADX + Losartan + Aldo), and a high dose of aldosterone and losartan (*right*, ADX + Losartan + Aldo-H). In **a** and **b**, the more intense staining in the two experimental groups compared to the control group is clearly visible. In **b**, the more intense staining was attributed mainly to the induction of basolateral expression of AQP2.

Aldosterone increased the sensitivity to hydrochlorothiazide and amiloride

A diuretic sensitivity study was conducted in a separate experiment as a measure of the activity of NCC and ENaC during treatment with aldosterone and losartan (Fig. 5). Diuretic sensitivity was defined as the difference in urine sodium to creatinine or urine potassium to creatinine ratio before and after the administration of vehicle or diuretic. As expected, diuretic treatment resulted in significantly higher urine sodium to creatinine ratio in the groups with and without aldosterone. Hydrochlorothiazide increased kaliuresis, whereas amiloride reduced kaliuresis. More importantly, however, the increase in urine sodium to creatinine to hydrochlorothiazide or amiloride was significantly greater in those animals that also received aldosterone, suggesting increased activity of NCC and ENaC. Similarly, the increase in urine potassium to creatinine was also significantly greater in the animals receiving hydrochlorothiazide and aldosterone.

Additive effect of angiotensin II and aldosterone

In the final experiment, we examined whether aldosterone in combination with angiotensin II had an additive effect on renal sodium excretion and the abundance of NCC and ENaC. Seven rats were selected on the basis of similar plasma aldosterone concentra-

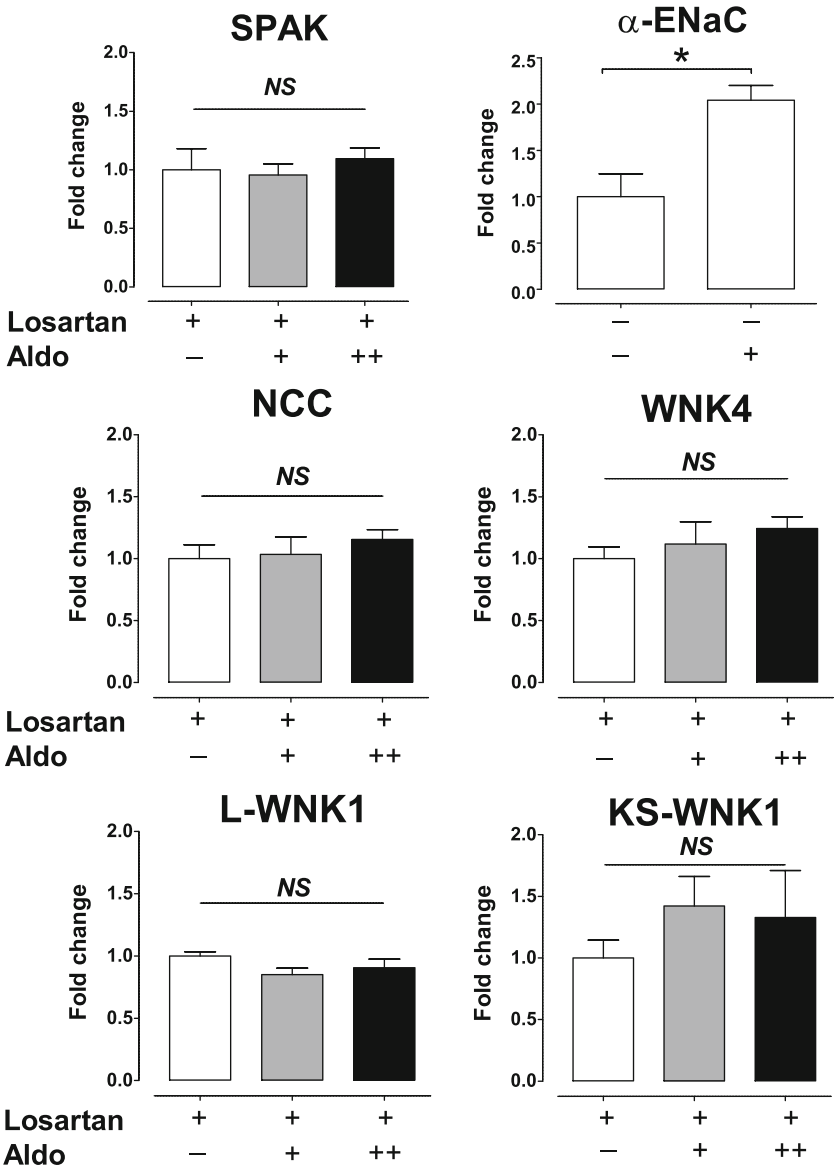

Figure 4: Effects of losartan with aldosterone on mRNA levels of NCC, α-ENaC, SPAK, and WNKs. The transcript abundances of SPAK, NCC, WNK4, L-WNK1, and KS-WNK1 are shown in adrenalectomized rats treated with losartan and no, normal, or high aldosterone. There were no significant changes among groups (using analysis of variance). As positive control, the transcript abundance of α-ENaC is shown for adrenalectomized rats that received no or normal aldosterone but no losartan *(upper right)*. Aldosterone led to a significant increase in α-ENaC mRNA. All data represent the average value of five rats. In these experiments, results were normalized for the abundance of the housekeeping protein hypoxanthine–guanine phosphoribosyltransferase. As a control, specificity of the amplified products was determined using melting curve analysis and by product sequencing. *NS* not significant; **P*=0.05

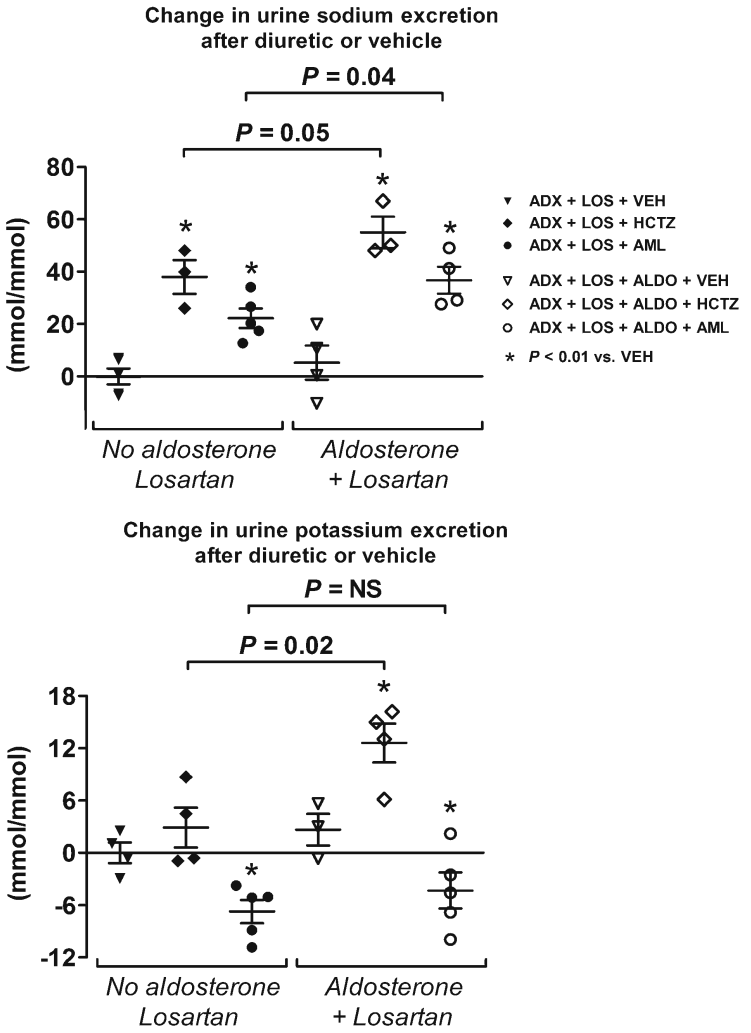

Figure 5: Diuretic sensitivity test. Results of a diuretic sensitivity test expressed as the change in urine sodium to creatinine ratio ($\Delta U_{Nd}/U_{Creat}$) or urine potassium to creatinine ratio ($\Delta U_{K}/U_{Creat}$) before and after the injection of vehicle or diuretic. Each symbol represents one rat. The three groups in the left of the figure (*black symbols*) represent adrenalectomized rats (*ADX*) that received losartan (*LOS*). After 4 days, one of these groups was injected with vehicle (*VEH, black inverted triangle*), whereas the other two groups received a diuretic, including hydrochlorothiazide (*HCTZ, black diamond*) or amiloride (*AML, black circle*). The three groups in the right of the figure (*open symbols*) represent adrenalectomized rats that received aldosterone (*ALDO*) with losartan. After 4 days, one of these groups also received vehicle (*white inverted triangle*), whereas the other two groups received a diuretic, including HCTZ (*white diamond*) or AML (*white circle*). In all groups, diuretic treatment resulted in a significantly higher U_{Nd}/U_{Creat} than vehicle (*P<0.01 using analysis of variance). U_{K}/U_{Creat} increased significantly with HCTZ (except in the group without aldosterone) and decreased significantly with AML. The natriuretic and kaliuretic response to HCTZ in rats that also received aldosterone was significantly greater (P=0.05 and P=0.02, respectively). AML also caused a greater natriuretic response to HCTZ in rats receiving aldosterone (P=0.04).

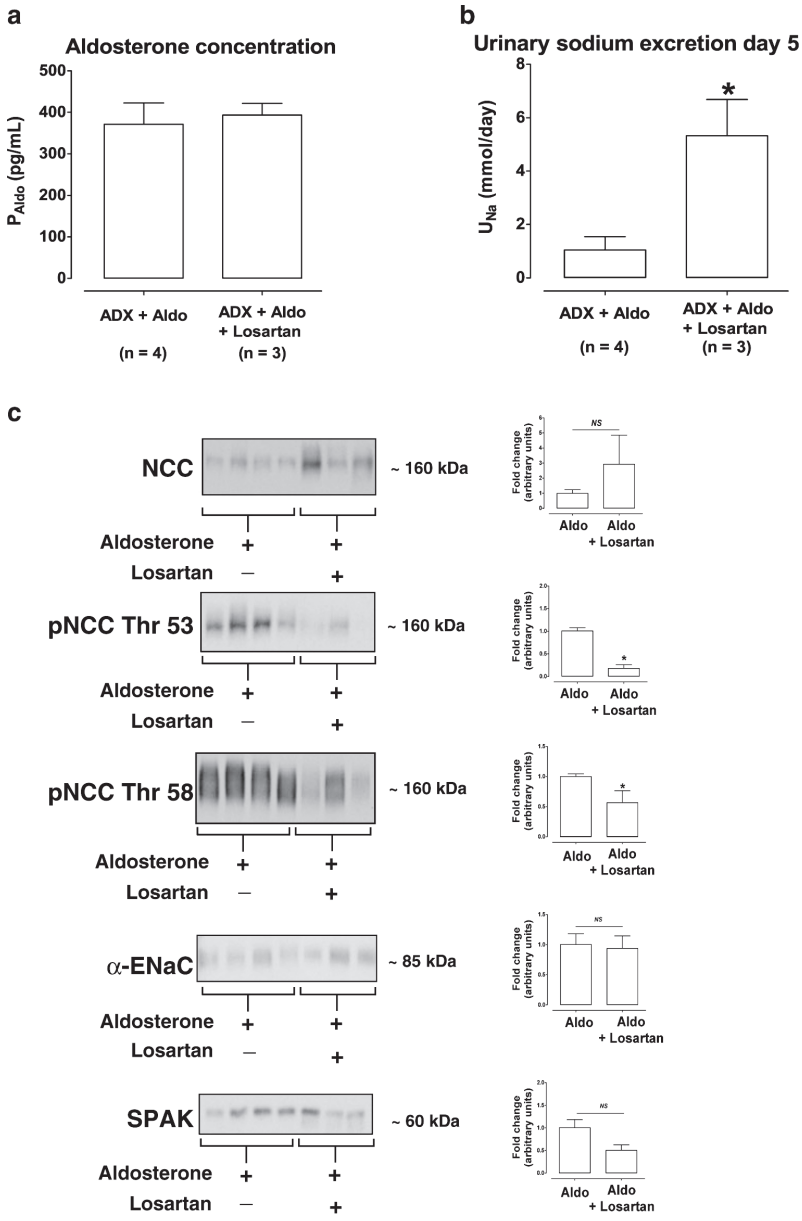

Figure 6: Additive effects of angiotensin II and aldosterone. **a** shows that seven adrenalectomized rats with similar plasma aldosterone concentrations were selected, three of which also received losartan. **b** shows that the addition of losartan resulted in a higher urinary sodium excretion. **c** shows that the addition of losartan reduced the phosphorylation of NCC, while there were no significant effects on total NCC, SPAK, and α- ENaC abundances. Densitometry was normalized for actin. *P<0.05 by unpaired Student's t tests. *ADX* adrenalectomy, *Aldo* aldosterone, *NS* not significant.

tions (Fig. 6). Because only three of these rats also received losartan, this comparison allowed a selective analysis of an angiotensin II effect. Urinary sodium excretion was higher in adrenalectomized rats that received aldosterone and losartan, suggesting that inhibition of angiotensin II action resulted in greater natriuresis. Interestingly, phosphorylation of NCC was markedly reduced in the presence of losartan, both at threonine 53 and 58. Conversely, there was a trend towards a higher total NCC abundance with losartan, but this did not reach significance. Although a trend was observed for lower SPAK abundance with losartan, this was not statistically significant. Finally, the abundance of α-ENaC remained unchanged.

DISCUSSION

In addition to the classical role of aldosterone, there is an increasing body of evidence to suggest that angiotensin II also plays an important role in distal nephron sodium transport.[8-11] Since both hormones are usually present together, it has been difficult to untangle their independent and potentially additive roles in distal nephron sodium transport. Here, we addressed this question by using an in vivo model of adrenalectomy and aldosterone with or without losartan. In the presence of losartan, aldosterone still caused renal sodium retention (Fig. 1), which was associated with increased expression of the distal sodium transporters NCC, pNCC, α-ENaC, and γ-ENaC (Figs. 2 and 3). Although two fixed doses of aldosterone were used, a range of plasma aldosterone concentrations were found. As a more functional measure of NCC and ENaC activity, we also showed that the natriuretic response to hydrochlorothiazide and amiloride was greater with aldosterone and losartan than with losartan alone (Fig. 5). This suggests that increased renal sodium retention with aldosterone and losartan was, at least in part, due to increased NCC and ENaC activity.[24] Next, we focused on the NCC regulatory proteins WNK4 and SPAK, the abundance of which increased with aldosterone and losartan (Fig. 2). Although SPAK has been clearly established as a kinase capable of phosphorylating NCC[29,30], the role of WNK4 in the regulation of NCC is less clear (reviewed in[17]). Several studies have demonstrated that WNK4 can sometimes act as a negative regulator of NCC by diverting post-Golgi NCC to lysosomal degradation.[19,31-33] However, WNK4 hypomorphic mice have reduced phosphorylation of NCC.[34] Similarly, WNK4 appears to mediate the phosphorylation of NCC during treatment with angiotensin II [9], insulin[35], cyclosporine[36], and tacrolimus[37]. Thus, the increase in both WNK4 and SPAK may have been related to their involvement in the phosphorylation of NCC, although more functional data are required to confirm this impression. The SPAK-mediated phosphorylation of NCC also suggests that transporter activity is regulated mainly by posttranscriptional

mechanisms. This was further supported by the absence of changes in the mRNA abundance of either SPAK or NCC (Fig. 4). It is not unusual to see changes in protein expression without changes in mRNA levels. For example, in previous studies, the increase in NCC protein with aldosterone infusion[38] and the decrease in NCC protein during aldosterone escape[39] were not associated with corresponding changes in mRNA. While, like us, O'Reilly and colleagues did not detect differences in mRNA expression of long WNK1 (L-WNK1) or WNK4 in adrenalectomized mice treated with aldosterone for 6 days[40], they did observe an increase in kidney-specific WNK1 (KS-WNK1), while we observed a trend towards increased KS-WNK1 (Fig. 4). Although SPAK, pNCC, NCC, and α-ENaC have all previously been shown to be aldosterone-sensitive[1,2,11,41], this is the first in vivo model to demonstrate that aldosterone does not require angiotensin II for the upregulation of these proteins. Besides angiotensin II, vasopressin is of interest because it was recently shown to be capable of phosphorylating NCC through SPAK.[28,42] Although we did not measure plasma vasopressin levels, the increase in AQP2 expression we observed (Figs. 2 and 3) might either have been indirect (through vasopressin) or direct (through aldosterone). Proof of the latter was provided by experiments in mpkCCDc14 cells, in which long-term incubation with aldosterone increased AQP2 protein abundance by increasing AQP2 mRNA translation.[43] As shown before [44], the increase in AQP2 was due mainly to increased basolateral expression of AQP2 (Fig. 3). It appears unlikely that the AQP2 translocation contributes to water movement because urine osmolality was unaffected and because AQP3 and AQP4 are also constitutively expressed in the basolateral plasma membrane.[45] Interestingly, high sodium intake by itself has also been shown to upregulate ENaC and AQP2 through an effect on collectrin, a homologue of angiotensin-converting enzyme 2 that is expressed in the apical membrane of the collecting duct.[46] Our final question was whether aldosterone and angiotensin II could have an additive effect on sodium transport in the distal nephron. To address this, we selected adrenalectomized and aldosterone-infused rats on the basis of similar plasma aldosterone concentrations (Fig. 6). Indeed, urinary sodium excretion increased with the addition of losartan to aldosterone-infused animals, suggesting a role of angiotensin II in renal sodium retention (Fig. 6). Immunoblot analysis suggested that phosphorylated NCC but not ENaC was involved in the additive effect of angiotensin II because aldosterone with losartan reduced the phosphorylation of NCC at threonine 53 and 58 (Fig. 6). This adds to recent work in which we show that angiotensin II induces phosphorylation of NCC independently of aldosterone.[11] The observation that angiotensin II selectively increases pNCC but not ENaC is likely to be of physiological importance, as it could help explain the "aldosterone paradox".[13-16] During hypovolemia, plasma levels of angiotensin II and aldosterone are elevated. On the basis of our data, this would favor sodium reabsorption by the DCT, limiting the flow and delivery to the CNT and CCD and, therefore, limiting po-

tassium secretion.[47] Conversely, during hyperkalemia, when only aldosterone is elevated, sodium reabsorption by the CNT and CCD is more pronounced, stimulating potassium secretion. According to this model, angiotensin II could function as the "switch" between favoring electroneutral sodium reabsorption by the DCT and favoring electrogenic sodium reabsorption by the CNT and CCD.[14,48] This model is further supported by the interesting recent finding that angiotensin II inhibits the renal outer medullary potassium channel (ROMK).[49] However, the demonstration that a high potassium diet increased aldosterone but decreased NCC[50] suggests that other mechanisms are also involved. For example, a high potassium diet has been shown to increase the KS-WNK1/WNK1 ratio and the abundance of WNK4, which could inhibit NCC and activate ENaC and ROMK.[14,40]
A number of limitations of this study should be mentioned. First, the number of animals in some of the studies was small. Second, the results of our analysis on the additive effects of angiotensin II should be considered preliminary because samples from studies conducted at different times were compared. These results should, therefore, be confirmed in a separate study using a direct comparison (infusion of angiotensin II instead of losartan). Third, although not measured, the supplementation of aldosterone may have decreased plasma angiotensin II levels. Although losartan inhibits the angiotensin II type 1 receptor, it leaves the angiotensin II type 2 receptor unaffected. Therefore, in the control group, higher plasma levels of angiotensin II may have had effects through the angiotensin II type 2 receptor. In this study, we focused on sodium transport by the aldosterone-sensitive distal nephron. Although this part of the kidney reabsorbs only 10–15 % of the filtered load of sodium chloride, altered function of this kidney segment can profoundly affect total body sodium and blood pressure.[51] This is illustrated by the fact that activating mutations of NCC and ENaC lead to renal sodium retention and hypertension.[52,53] Clinically, the renin–angiotensin–aldosterone system is activated in many disease states, including several forms of hypertension, heart failure, liver cirrhosis, and nephrotic syndrome. Pharmacological inhibition of the renin–angiotensin–aldosterone system is the cornerstone of the treatment of these disorders. Unraveling the separate effects of angiotensin II and aldosterone is important when selecting appropriate pharmacological intervention for these diseases with angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, or mineralocorticoid receptor blockers. In summary, by using adrenalectomy, aldosterone, and losartan in rats, we have shown that aldosterone does not require angiotensin II to activate NCC through a WNK4– SPAK-dependent pathway. However, angiotensin II and aldosterone do appear to have additive effects on NCC. This explains a specialized system for the hormonal control of renal salt excretion that is relevant to health and disease.

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Chapter 4

Potassium-induced natriuresis is preserved during sodium depletion and accompanied by inhibition of the sodium chloride cotransporter

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ABSTRACT

During hypovolemia and hyperkalemia, the kidneys defend homeostasis by sodium retention and potassium secretion, respectively. Aldosterone mediates both effects, but it is unclear how the same hormone can evoke such different responses. To address this, we mimicked hypovolemia and hyperkalemia in four groups of rats with a control diet, low sodium diet, high potassium diet or a combined diet. The low sodium and combined diets increased plasma and kidney angiotensin II. The low sodium and high potassium diets increased plasma aldosterone to a similar degree (3-fold), while the combined diet increased aldosterone to a greater extent (10-fold). Despite similar sodium intake and higher aldosterone, the high potassium and combined diets caused a greater natriuresis than the control and low sodium diets, respectively ($p<0.001$ for both). This potassium-induced natriuresis was accompanied by a decreased abundance but not phosphorylation of the sodium chloride cotransporter (NCC). In contrast, the epithelial sodium channel (ENaC) increased in parallel with aldosterone showing the highest expression with the combined diet. The high potassium and combined diets also increased WNK4, but decreased Nedd4-2 in the kidney. Total and phosphorylated SPAK were also increased but were retained in the cytoplasm of distal convoluted tubule cells. In summary, high dietary potassium overrides the effects of angiotensin II and aldosterone on NCC to deliver sufficient sodium to ENaC for potassium secretion. Potassium may inhibit NCC through WNK4 and help activate ENaC through Nedd4-2.

INTRODUCTION

Maintaining total body sodium (Na^+) and potassium (K^+) balance is essential to survival of most species. Hypovolemia (Na^+ deficit) and hyperkalemia (K^+ surplus) elicit different constellations of responses to maintain homeostasis. During hypovolemia, the extracellular fluid volume needs to be maintained to guarantee blood pressure and organ perfusion. Hypovolemia activates the renin-angiotensin system, enhancing aldosterone secretion; aldosterone promotes Na^+ reabsorption in the distal nephron. Conversely, during hyperkalemia, K^+ secretion is stimulated to avoid cardiac and neuromuscular complications; this process is also mediated by aldosterone. The observation that a single hormone, aldosterone, has different effects on renal Na^+ and K^+ transport, depending on the physiological situation, has been termed the "aldosterone paradox".[1,2] How the kidney "knows" when to retain Na^+ or secrete K^+ during these two high aldosterone states is unknown. To understand the aldosterone paradox, it is important to briefly review the regulation of Na^+ and K^+ transport in the aldosterone-sensitive distal nephron.[3,4] The aldosterone-sensitive distal nephron constitutes the distal convoluted tubule (DCT), connecting tubule, and collecting ducts.[3] Four aldosterone-sensitive transport proteins are involved in mediating Na^+ and K^+ transport, namely the NaCl cotransporter (NCC), the epithelial Na^+ channel (ENaC), the renal outer medullary K^+ channel (ROMK), and the big conductance Ca^{2+}-activated potassium (BK) channel.[5-9] These proteins are regulated by phosphorylation via several kinases, including With No Lysine (WNK) kinases and the Ste-20-related kinase (SPAK).[10]ENaC and NCC are also regulated by ubiquitination via Nedd4-2.[11-13]SPAK also regulates the Na^+ K^+ $2Cl^-$ type 2 cotransporter (NKCC2), which is located upstream from the aldosterone-sensitive distal nephron in the thick ascending limb (TAL).[14,15] In search of an explanation for the aldosterone paradox, it is important to consider the factors accompanying hypovolemia. Although hypovolemia and hyperkalemia are both characterized by elevated aldosterone, only hypovolemia is associated with a concomitant rise in angiotensin II. Recently, it has become clear that in addition to its well-established effects on the proximal tubule, angiotensin II can also activate NCC and ENaC.[16-18] Angiotensin II has been shown to abrogate the inhibitory effects of WNK4.[19] This permits NCC to traffic to the plasma membrane[20] and be phosphorylated by SPAK to enhance electroneutral NaCl transport.[17,19,21,22] Increased Na^+ reabsorption in the DCT will reduce the delivery of Na^+ to the collecting duct, limiting Na^+-coupled K^+ secretion in that segment. Moreover, it was recently shown that angiotensin II inhibits ROMK.[23] Together, these effects favor electroneutral Na^+ reabsorption while preventing K^+ secretion.[24] These recent insights help explain how the aldosterone-sensitive distal nephron responds to hypovolemia. The effects of hyperkalemia on renal tubular transporters were investigated by three recent studies using high K^+ diets. Frindt and

Palmer found that a high K^+ diet increased ROMK and decreased NCC in normal rats.[25] In mice, Vallon *et al.* confirmed the suppression of NCC by high dietary K^+ and showed this suppression to be greater in SGK1 knockout mice.[26] Sorensen *et al.* showed that NCC was dephosphorylated as early as fifteen minutes after oral potassium intake.[27] Still unanswered, however, is the question how the kidney will respond to the combination of hypovolemia and hyperkalemia. The combination of the two stimuli could be viewed as the ultimate challenge for the kidney to choose between Na^+ reabsorption and K^+ secretion. Therefore, we mimicked hypovolemia and hyperkalemia by feeding normal rats not only a low Na^+ or high K^+ diet, but also a combination of the two diets. We show that despite maximal activation of the renin-angiotensin system, high dietary K^+ still inhibited NCC and caused a kaliuresis and natriuresis.

MATERIALS AND METHODS

Animal studies

The animal protocol was approved by the Animal Care Committee of the Erasmus Medical Center (EUR 127-11-01). Male Sprague-Dawley rats (15 weeks, average weight 370 g, Charles River, Sulzfeld, Germany) were all fed control diet (Na^+ 0.5%, K^+ 0.8%) and then randomized into four groups (n=8/group). One group was maintained on control diet, while the others were switched to a low Na^+ diet (LS, Na^+ < 0.001%, K^+ 0.8%), high K^+ diet (HK, Na^+ 0.5%, K^+ 5%), or a combination of a low Na^+ and high K^+ diet (LSHK, Na^+<0.001%, K^+ 5%). All diets were purchased from Harlan Laboratories (Madison, WI, USA). Rats were housed in metabolic cages in a temperature- and humidity-controlled room with a 12:12 h light dark cycle to collect 24-hour urine and to measure body weight, and the intake of food and water.[28] Tap water was provided *ad libitum*. Eight days after switching the diets, the animals were killed. In a separate experiment three groups (n = 5/group) were randomized to control diet, HK or HK with angiotensin II for eight days. Angiotensin II was administered in a non-pressor dose (233 µg/kg/day) using osmotic minipumps (Alzet 2ml2, Cupertino, CA, USA) (51); minipumps containing vehicle were implanted in the two other groups. Plasma renin activity, plasma and kidney angiotensin II concentrations, and plasma aldosterone were measured as described previously.[29-31] Urine and plasma electrolytes were determined with an automatic analyzer (Modular IPPE, Roche Diagnostics, Almere, The Netherlands).

Immunoblotting

The right kidney was used for immunoblotting. After harvesting, the kidneys were immediately homogenized on ice in isolation buffer with protease and phosphatase inhibitors. Whole kidney homogenates were then subjected to differential centrifugation to obtain plasma membrane and cytosol fractions, as described previously.[17,32] For the immunoblot analysis of ROMK, cortex was separated from medulla and processed as described previously.[33] Each experimental group was blotted next to a control group. Equal loading was confirmed with staining of alpha actin (1:100.000, Cell Signaling, Boston, MA, USA).Antibodies against the following proteins were obtained: NCC (1:1000), the α-, β-, and γ-subunits of ENaC (all 1:1000, StressMarq, Victoria, BC, Canada), SPAK and SPAK phosphorylated at Ser-373 (both 1:1000, Millipore, Billerica, MA, USA), and Nedd4-2 (1:4000 Abcam, Camebridge UK). Non-commercial antibodies against NCC phosphorylated at Thr-58 (1:500), NKCC2 phosphorylated at Thr-95/100 (1:2000), WNK4 (1:1000), and ROMK (1:2000) were kind gifts from other investigators and were characterized previously.[34-36] All antibodies used for immunoblotting were raised in rabbit.

Immunohistochemistry and immunofluorescence

The left kidney was used for immunohistochemistry. Kidneys were removed and fixed for 120 min at 4°C in 3% paraformaldehdyde in 0.1M Na^+ cacodylate, pH 7.4.[37] The midregion was sectioned into 2- to 3-mm transverse sections and immersion-fixed for an additional 1h, followed by 3 x 10 min washes with 0.1 mol/l cacodylate buffer. The tissue was dehydrated in graded alcohol, incubated overnight in xylene, and embedded in paraffin, and 2 µm sections were cut on a rotary microtome (Leica Microsystems, Herlev, Denmark). Immunolabeling was performed as described previously.[34] Labeling was detected using a horseradish peroxidase-conjugated secondary antibody (Dako P448, goat anti-rabbit IgG, Glostrup, Denmark) and visualized with 0.05% 3,3′-diaminobenzidine tetrachloride (Kemen Tek, Copenhagen, Denmark). Light microscopy was carried out with a Leica DMRE (Leica Microsystems). Confocal microscopy was performed as described in detail previously.[38] Briefly, primary antibodies utilized were against NCC (1:4000), pT58 NCC (1:70,000), total NKCC2 (1:1000), BK (1:800; NeuroMab Facility, University of California, Davis, CA,USA), SPAK (1:2000), pSPAK 154 (1:1000, both SPAK antibodies University of Dundee, UK); anti-pSPAK was pre-incubated with the non-phosphopeptide, as recommended. The NCC and NKCC2 antibodies were raised in rabbit, the SPAK antibodies in sheep, and the BK antibodies in mouse. Goat anti-rabbit Alexa488 or Donkey anti-sheep Alexa555 conjugated secondary antibodies (Invitrogen) were used for visualization of labeling. A Leica TCS SL

confocal microscope with an HCX PL APO 63X oil objective lens (numerical aperture: 1.40) was used for imaging of labeled sections.

Statistics

All data are expressed as means and standard error of the mean (SEM). Group comparisons were made by using a Student's T-test or analysis of variance with a least significant difference post-hoc test, as appropriate. $P \leq 0.05$ was considered statistically significant. All data were analyzed using SPSS (Version 20, IBM).

RESULTS

Differential activation of the renin-angiotensin system

Normal rats were fed control diet, low Na^+ diet (LS), high K^+ diet (HK) or a combination of a low Na^+ and high K^+ diet (LSHK) for eight days. HK and LSHK led to an immediate 3- to 4-fold increase in diuresis that persisted throughout the study (55 ± 3 ml/day in HK and 43 ± 2 ml/day in LSHK vs. 12 ± 1 ml/day in control and 13 ± 1 in LS ml/day). Accordingly, the animals in the HK (71 ± 5 ml/day) and LSHK (62 ± 3 ml/day) groups drank more than those in the control (30 ± 1 ml/day) and LS groups (32 ± 1 ml/day). Because the animals in the HK and LSHK groups ate less food during the first and second day after switching to these diets, the average food intake in the HK (20 ± 1 g) and LSHK (19 ± 1 g) groups was slightly but significantly lower than in the control (24 ± 1 g) or LS (23 ± 1 g) groups. The animals receiving LSHK gained significantly less weight compared with animals on control or LS diets (Figure 1A). Both LS and LSHK increased plasma renin activity and plasma and kidney angiotensin II concentrations compared with control diet (Figure 1C). HK decreased plasma renin activity compared with control diet, but this did not reach statistical significance ($p = 0.09$). LS and HK increased plasma aldosterone similarly compared with control (428 ± 183 and 420 ± 149 vs. 128 ± 26 pg/mL, Figure 1A). LSHK caused mild hyperkalemia (5.9 ± 0.2 mmol/L, Figure 1B) and increased aldosterone to a greater extent (1355 ± 228 pg/mL, Figure 1C). Plasma sodium was similar in all groups (control 142 ± 1 mmol/l, LS 142 ± 1 mmol/l, HK 143 ± 1 mmol/l, LSHK 143 ± 1 mmol/l). Although LS and LSHK both decreased urine Na^+ excretion markedly compared with control diet, urine Na^+ excretion with LSHK was significantly higher than with LS (Figure 1D). HK also increased urine Na^+ excretion compared with control diet. HK and LSHK both induced a marked kaliuresis (Figure 1D).

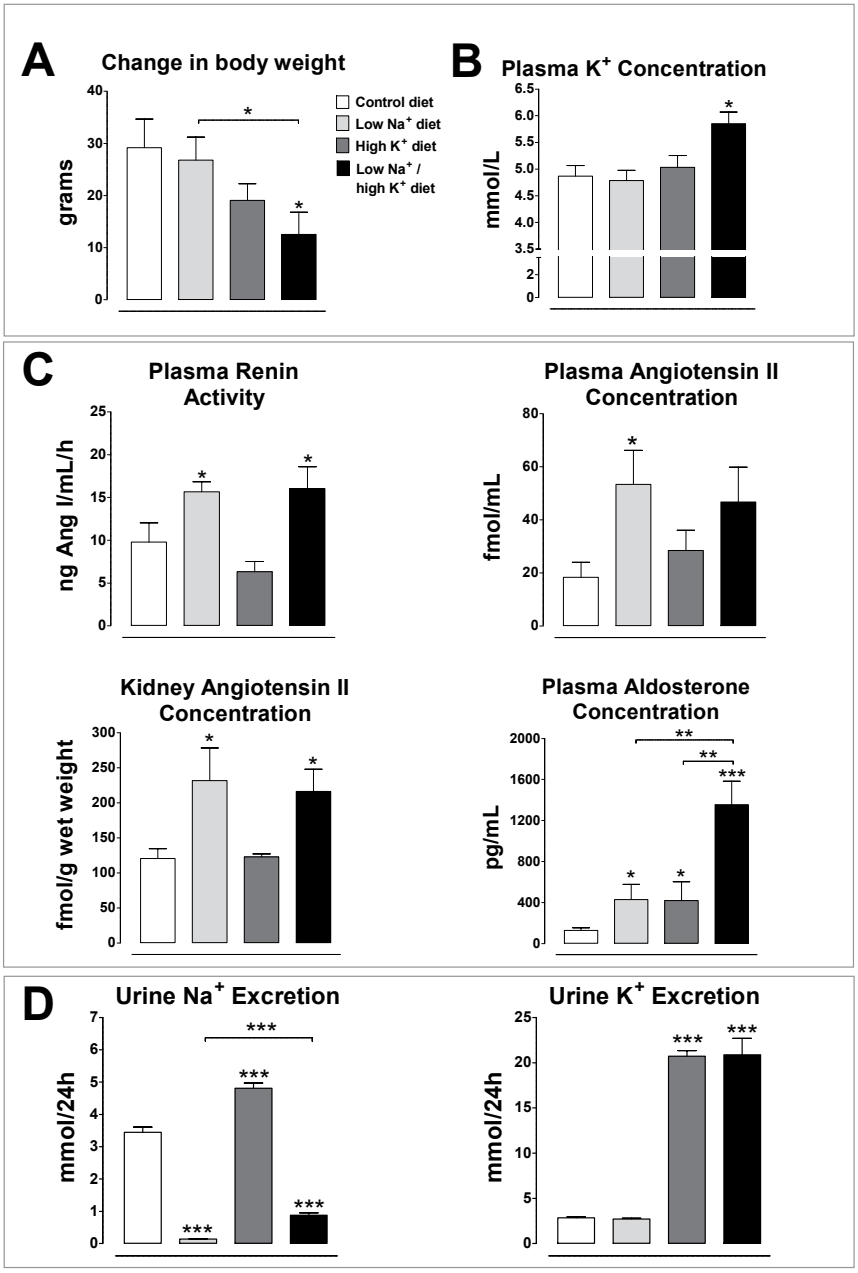


Figure 1: Responses in body weight (***A***), plasma K^+ concentration (***B***), renin-angiotensin system (***C***), and urinary electrolyte excretion (***D***) to the four different diets [control diet, low-Na^+ (LS) diet, high-K^+ (HK) diet, and combined (LSHK) diet]. Averages ± SE are shown; n = 8 animals/group. 24-h urine for Na^+ and K^+ excretion was collected on the last (eighth) day of the experiment. **P* <0.05, ***P* <0.01, and ****P* <0.001 vs. control diet (unless otherwise indicated) using ANOVA with a *post hoc* test.

High dietary K^+ inhibited NCC even in the presence of low dietary Na^+

In the plasma membrane fractions of whole kidney homogenates, LS increased the abundances of total NCC and NCC phosphorylated at threonine 58 (pNCC) approximately 1.7-fold (Figure 2A). Of interest, both HK and LSHK decreased NCC. A similar trend was observed for pNCC, but this was not statistically significant. These changes were confirmed using immunohistochemistry (Figure 2B) and confocal microscopy (data not shown). Furthermore, no changes between the effects of the HK and LSHK diets on NCC or pNCC were observed when these two groups were compared directly in one immunoblot, indicating a comparable reduction in NCC abundance (data not shown). LS decreased NKCC2 (Figure 2C) and this effect was located to the medullary portion of the TAL (Figure 2D). HK and LSHK had no effects on NKCC2. A trend towards more phosphorylated NKCC2 was observed with LSHK, but this was not significant and not confirmed with immunohistochemistry (data not shown).

Both low dietary Na^+ and high dietary K^+ increased ENaC

HK and LSHK increased the abundance of the α-subunit of ENaC, while LS and LSHK increased the abundance of the β-subunit of ENaC (Figure 3). In previous studies using whole kidney homogenates, LS was shown to increase α-ENaC but left β-ENaC unchanged.[6,39] To analyze whether this was due to the fact that we analyzed plasma membrane fractions, we also immunoblotted whole kidney homogenates, which confirmed the results from the previous studies (data not shown). The immunoblot analysis of the γ-subunit of ENaC showed the characteristic four bands ranging from 70–85 kDa (Figure 3C). All dietary manipulations decreased the 85 kDa band and increased the 70 kDa band. Previously, this change has been interpreted as cleavage of γ-ENaC by aldosterone (23). Indeed, the higher the plasma aldosterone concentration (217 Figure 1C), the greater the changes in γ-ENaC with LSHK leading to a 3-fold increase in the 70 kDa isoform (Figure 3).

Effects on potassium channels

All diets increased the abundance of the complex glycosylated band of ROMK, although this did not reach significance for the LSHK diet; the abundance of the unglycosylated band only increased with LS (Figure 3D). BKα labeling was localized to the cytoplasm in the majority of intercalated cells in the cortical collecting duct and outer medullary collecting duct (Figure 4). In contrast to other reports[9] we did not observe any signal above background levels in principal cells of the medullary collecting ducts. Technical reasons (non-specific background in proximal tubule mitochondria) prevented a full quantitative assessment of BKα abundance using immunofluorescent confocal microscopy. However, compared to control diet (Figure 4A) both the HK (Figure 4B) and LSHK (Figure 4C) diets

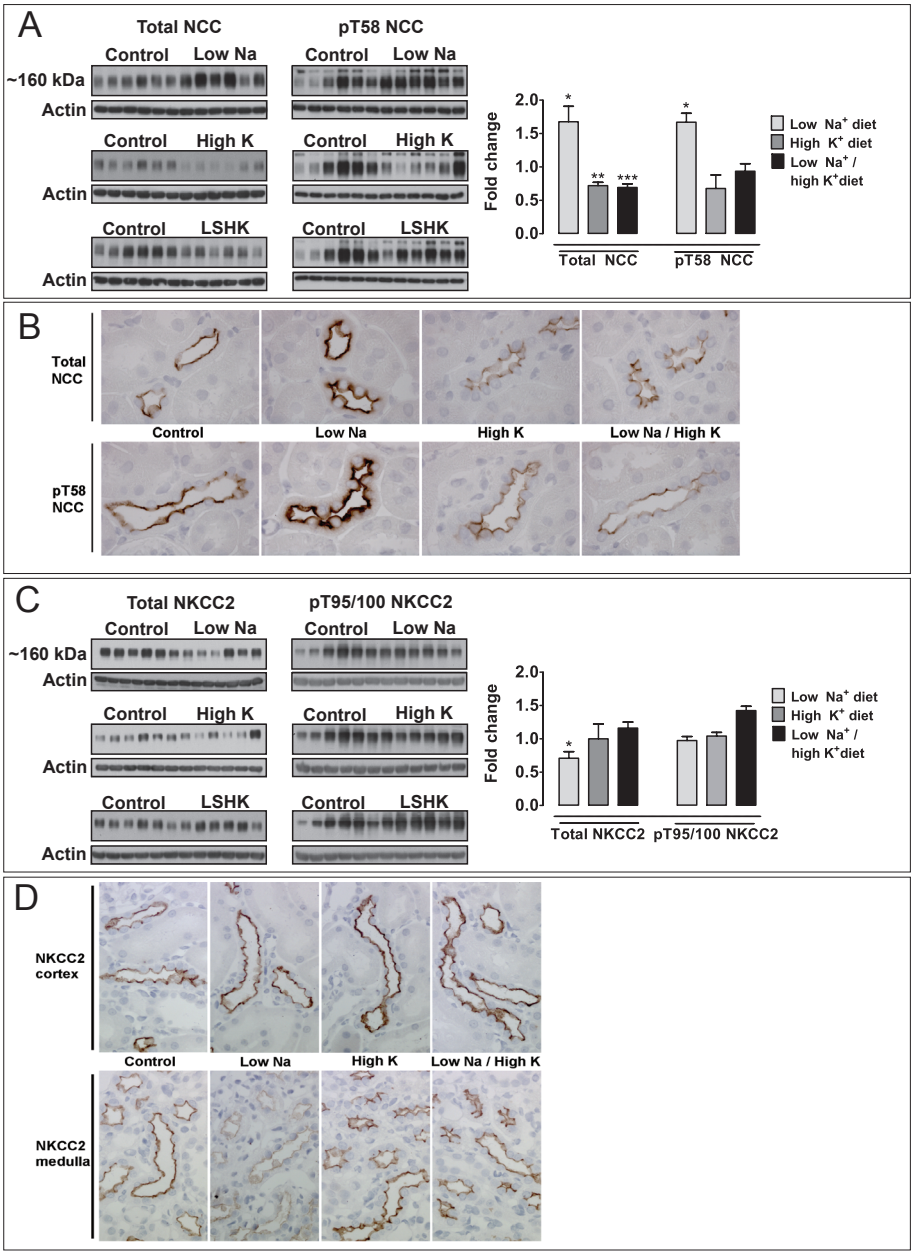


Figure 2: Analysis of changes in the Na^+-Cl^- cotransporter (NCC; ***A*** and ***B***) and Na^+-K^+-$2Cl^-$ type 2 cotransporter (NKCC2; ***C*** and ***D***) to the four different diets. ***A*** and ***C***: immunoblot results; ***B*** and ***D***: immunohistochemistry results. pT58 NCC, NCC phosphorylated at Thr^{58}; pT95/100 NKCC2, NKCC2 phosphorylated at $Thr^{95/100}$.Densitometry is expressed as fold changes ± SE and normalized by actin; n = 6 animals/group.*P <0.05, **P <0.01, and ***P<0.001 vs. the control diet using Student's t-test.

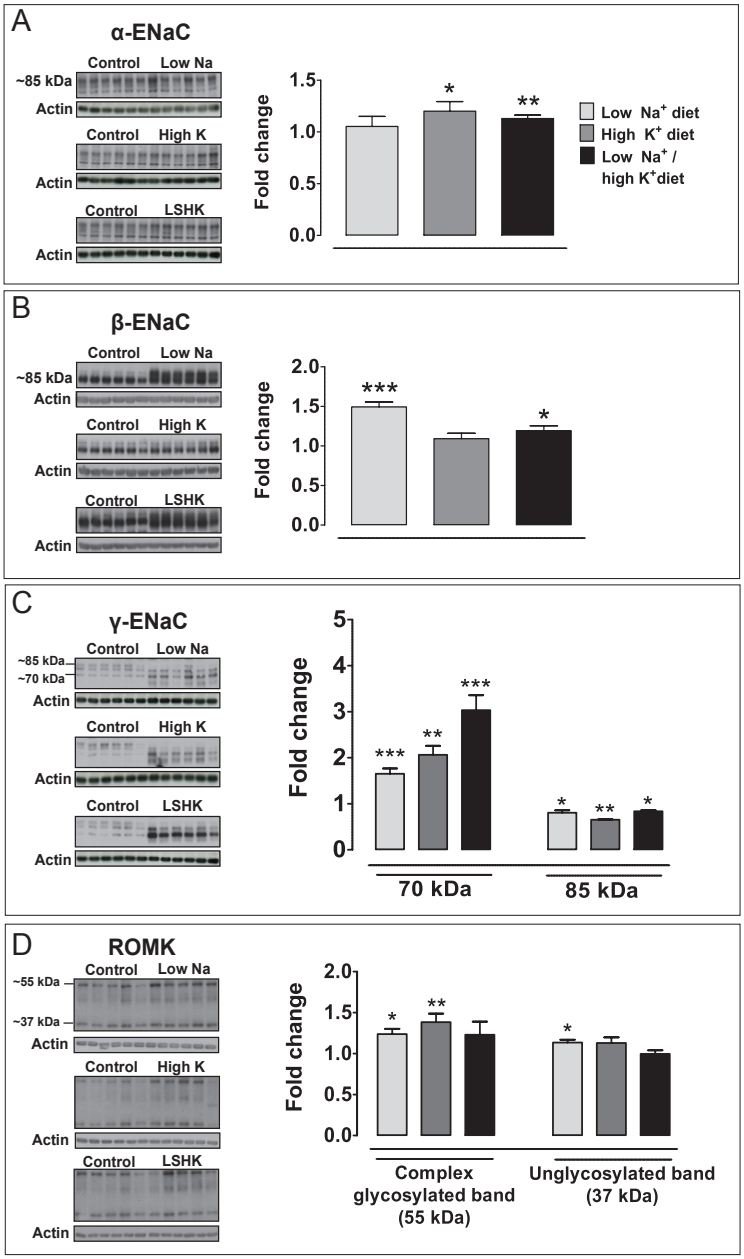


Figure 3: Immunoblots showing changes in the α-subunit (**A**), β-subunit (**B**), and γ-subunit (**C**) of the epithelial Na^+ channel (ENaC) and the renal outer medullary K^+ channel (ROMK; ***D***) to the four diets. Densitometry is expressed as fold changes ± SE and normalized by actin; n = 6 animals/group. For γ-ENaC, densitometry of the 70- and 85-kDa subunits is shown separately; for ROMK, densitometry of the complex glycosylated (~55 kDa) and unglycosylated (~37 kDa) bands is also shown separately. **P* <0.05, ***P* <0.01, and ****P*<0.001 vs. the control diet using Student's *t*-test.

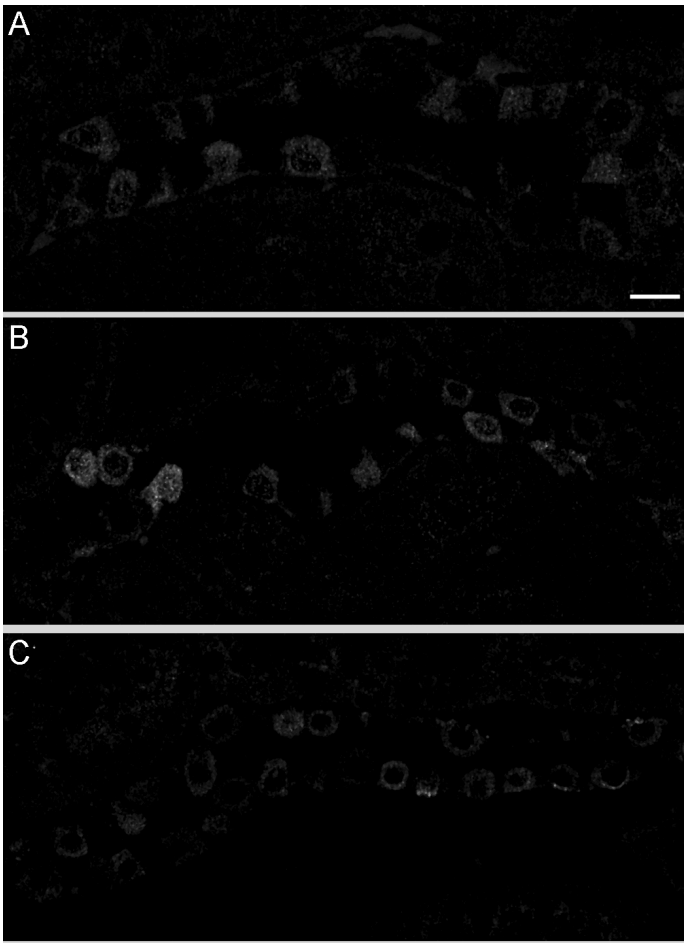


Figure 4: Labeling of the large-conductance $Ca2^{+}$-activated K^{+} channel α-subunit (BK-α). BK-α labeling was localized to the cytoplasm of intercalated cells in the cortical collecting duct and outer medullary collecting duct. Both HK and LSHK diets increased BK-α-positive labeling of intercalated cells.***A***: control diet. ***B***: HK diet. ***C***: LSHK diet. Scale bar = 20 μm.

induced a qualitative increase in cytoplasmic BKα labeling in intercalated cells. No clear apical BKα labeling was detected under any dietary condition.

High dietary K^{+} increased WNK4 and SPAK but reduced Nedd4-2

In the cytosol fractions of the whole kidney homogenates, HK led to a modest (~1.2-fold) but significant increase in WNK4 abundance (Figure 5A). WNK4 showed the same trend with LSHK, but this was not statistically significant ($p = 0.1$). HK also increased the total abundance of SPAK, while both HK and LSHK increased the abundance of SPAK

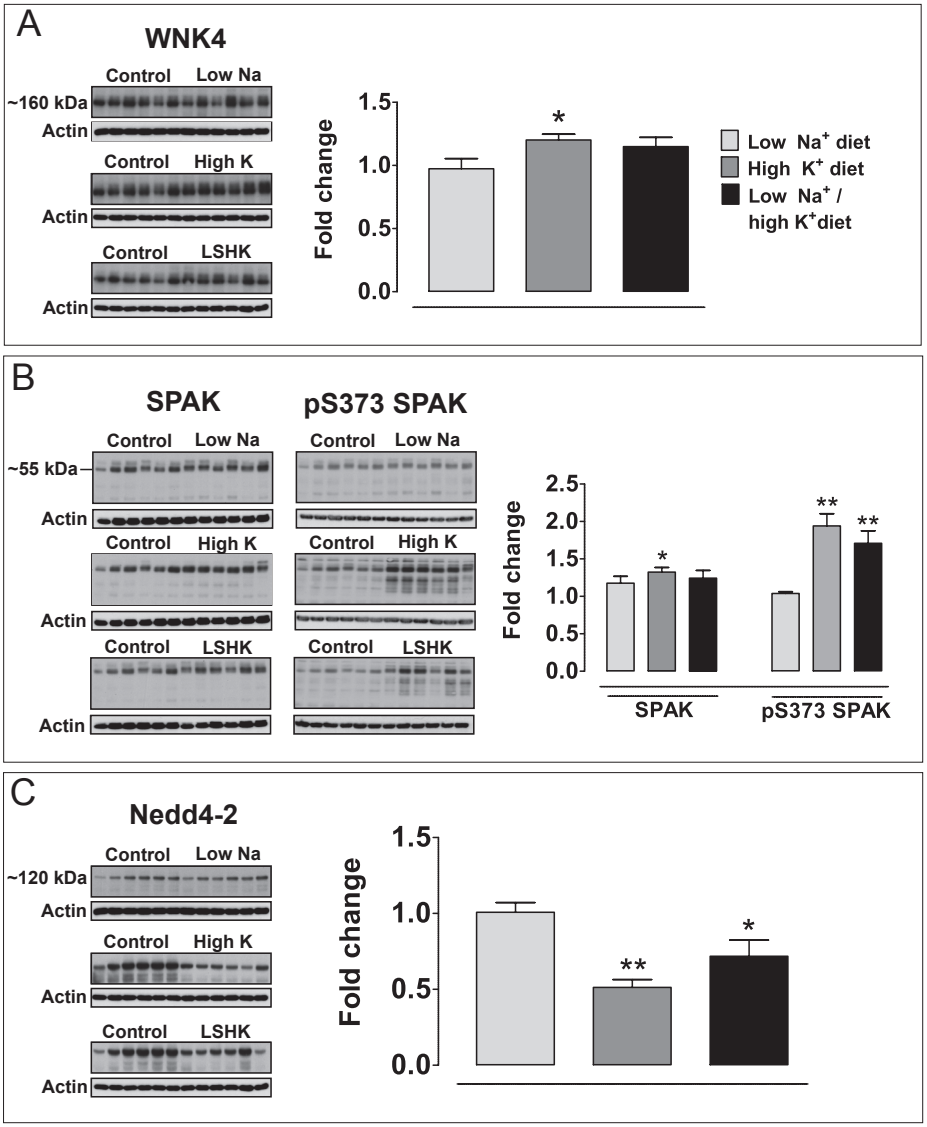


Figure 5: Immunoblots showing changes in WNK4 (***A***), total and phosphorylated Ste-20-related kinase (SPAK; ***B***), and Nedd4-2 (***C***) to the four diets. Densitometry is expressed as fold changes ± SE and normalized by actin; n = 6 animals/group. For SPAK, the 55-kDa band was used for densitometry, which likely represents full-length SPAK.14 The lower bands that are visible in some of the blots likely represent SPAK2 and kidney-specific SPAK.14 pS^{373} SPAK, SPAK phosphorylated at Ser373. **P* < 0.05, ***P* <0.01, and ****P* <0.001 vs. the control diet using Student's *t*-test.

phosphorylated at serine 373 (pSPAK, Figure 5B). Nedd4-2, a ubiquitin ligase regulating ENaC[12], was downregulated approximately 2-fold by both HK and LSHK (Figure 5C).

Differential regulation of SPAK in TAL and DCT

Analysis by confocal microscopy showed that LS increased SPAK primarily at the apical plasma membrane of the DCT where it partially co-localized with NCC (Figure 6, second row). With HK and LSHK, however, SPAK was predominantly localized in the cytoplasm of DCT cells, with minimal overlap with NCC labeling (Figure 6, third and fourth rows). In addition, the speckled expression pattern of SPAK with HK and LSHK suggested SPAK to be located in intracellular vesicles. In some tubules transitions between TAL and DCT can be observed with SPAK on the plasma membrane in the TAL and in vesicles or plasma membrane in the DCT (Figure 6, third and fourth rows). HK also caused a clear redistribution of pSPAK to vesicle-like structures in the cytoplasm (Figure 7A). Similar to SPAK, LS led to more pSPAK at the apical plasma membrane of cells in the DCT (Figure 7B), whereas both HK and LSHK led to more pSPAK at the apical plasma membrane of cells in the medullary TAL (Figure 7C), but not the DCT (data not shown).

Exogenous angiotensin II reversed the inhibition of NCC by high dietary K^+

Because LSHK maximally activated the renin-angiotensin system (Figure 1) but still inhibited NCC (Figure 2), we wanted to investigate whether high dietary K^+ rendered the DCT insensitive to angiotensin II. To do so, we performed a separate animal study in which animals received angiotensin II on top of a high K^+ diet (HK–Ang II). Plasma aldosterone levels were similar between HK and HK–Ang II (399 ± 341 vs. 315 ± 72 pg/mL). Of interest, exogenous angiotensin II was still capable of reversing the reduction in NCC and pNCC induced by HK (Figure 8A). Moreover, the increase in WNK4, SPAK, and pSPAK was also reversed by HK–Ang II (Figure 8B). In contrast, angiotensin II on top of HK further increased the 70 kDa isoform of γ-ENaC (Figure 8A).

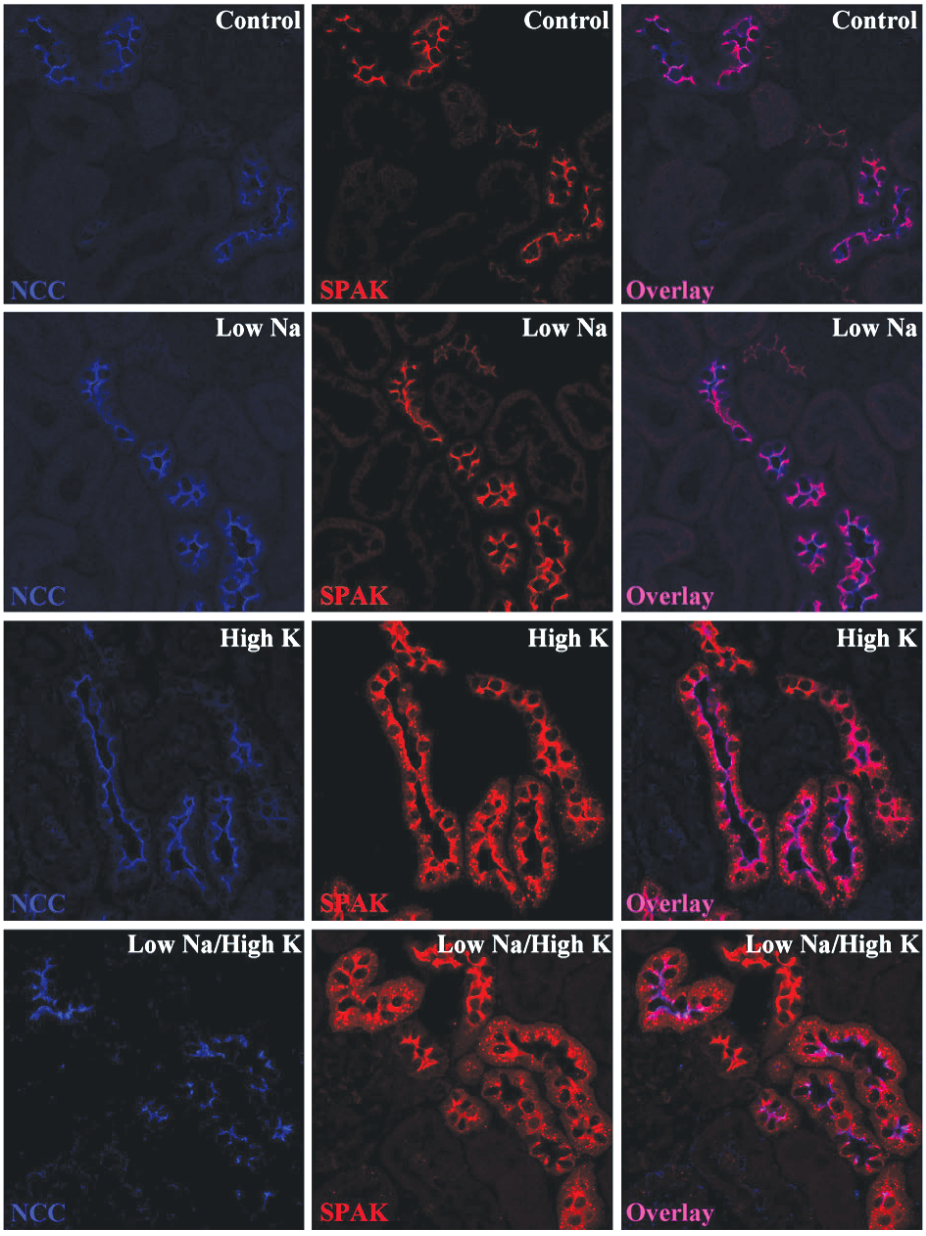


Figure 6: Confocal microscopy showing the localization of SPAK (red, *middle*) in relation to NCC (blue, *left*). The overlay (*right*) clearly shows the colocalization of NCC and SPAK at the apical plasma membrane with the LS diet (purple). In contrast, the HK and LSHK diets showed no colocalization,suggesting that SPAK was redistributed to the cytoplasm in these settings.

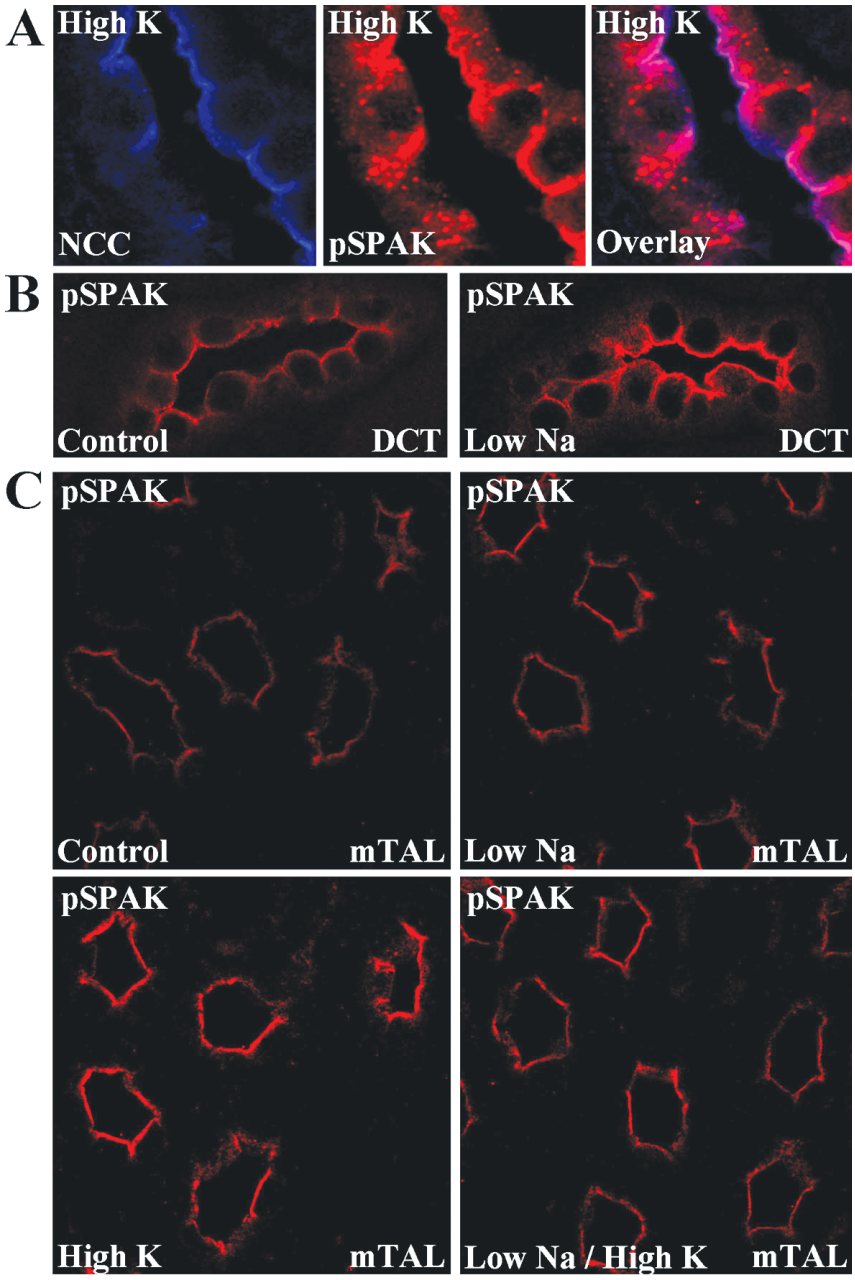
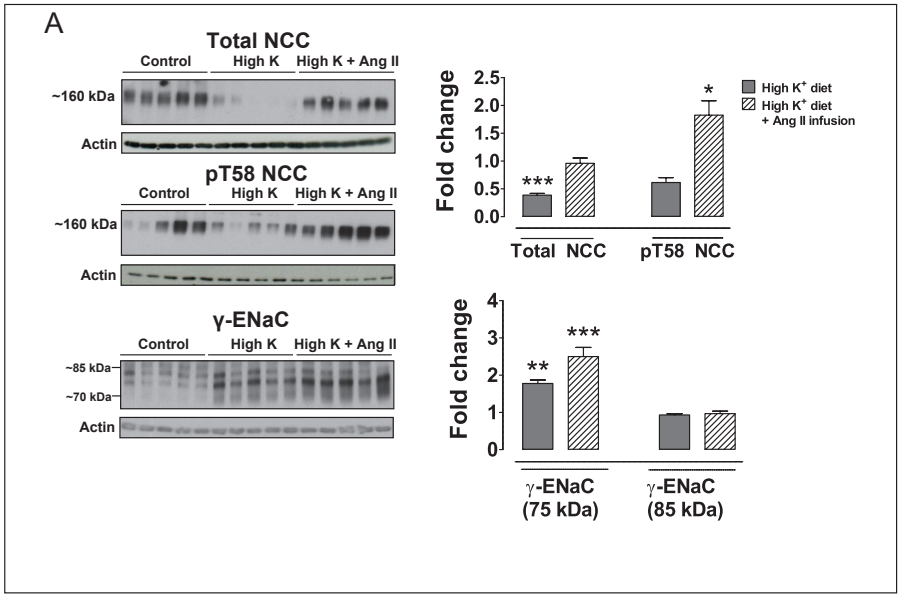


Figure 7: Immunofluorescence microscopy analysis of pSPAK (red). ***A***: double labeling of pSPAK and NCC (blue) in animals on the HK diet, showing that pSPAK was primarily located intracellularly in vesicle-like structures (speckled pattern). ***B***: the LS diet increased the fluorescence intensity of pSPAK compared with the control diet in the distal convoluted tubule (DCT). ***C***: the HK and LSHK diets increased pSPAK in the plasma membranes of cells in the medullary thick ascending limb (mTAL).



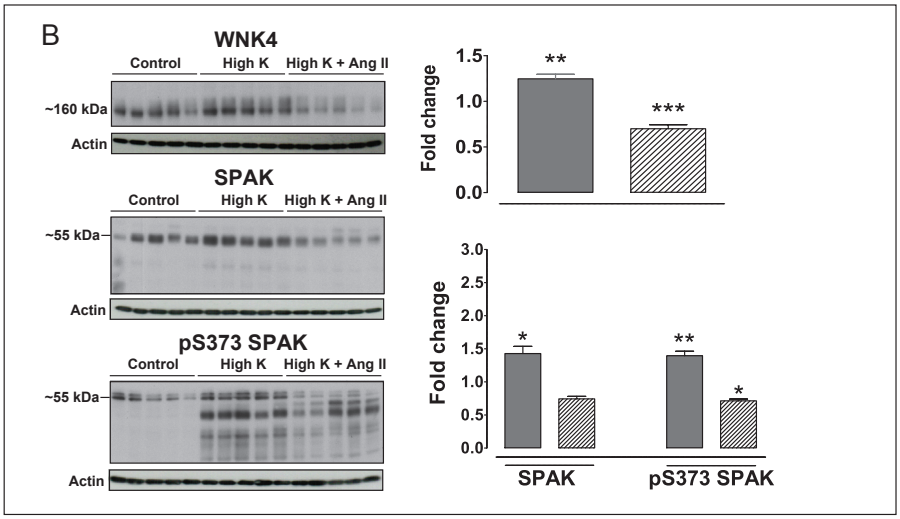


Figure 8: Immunoblots showing the response of NCC and ENaC (***A***) and WNK4 and SPAK (***B***) to the addition of ANG II to the HK diet. Densitometry is expressed as fold changes ± SE and normalized by actin; *n* = 5 animals/group. For SPAK, the 55-kDa band was used for densitometry, which likely represents full-length SPAK[14]. The lower bands that are visible in some of the blots likely represent SPAK2 and kidney-specific SPAK[14]. **P*<0.05, ***P* <0.01, and ****P*<0.001 vs. the control diet using Student's *t*-test.

DISCUSSION

In this study we aimed to clarify the aldosterone paradox, which refers to the question how aldosterone can promote renal Na^+ retention during hypovolemia but K^+ secretion during hyperkalemia.[1,2,40,41] Although angiotensin II and aldosterone normally activate NCC[5,17], high dietary K^+ overrode these effects and even led to a decrease in cotransporter abundance (Figure 2). Remarkably, NCC abundance was still reduced despite the maximal plasma aldosterone concentrations induced by the combined diet. In contrast, the activation of ENaC by angiotensin II and aldosterone was maintained with high dietary K^+. Indeed, the expression of γ-ENaC increased in parallel to the plasma aldosterone concentration (Figure 3). The reduced NCC abundance and phosphorylation by high dietary K^+ likely results in reduced NCC activity, and explains the observed K^+-induced natriuresis. The increased delivery of Na^+ to ENaC will subsequently facilitate K^+ secretion through ROMK and BK[25]. The effects of high dietary K^+ on NCC and ENaC are in line with what Frindt and Palmer reported previously[25]. Cheema-Dhadli *et al.* also reported a higher distal flow rate in animals receiving a KCl load, but ascribed this to an effect on NKCC2[42]. One of the unique aspects of the current study was the use of a combined low Na^+ and high K^+ diet, meaning that the animals had to choose between Na^+ reabsorption and K^+ secretion. Based on the greater natriuresis with the combined diet compared with the low Na^+ diet (Figure 1), it appears that the kidneys maintain K^+ balance at the cost of Na^+ balance. Our results therefore unravel a new differential regulation of NCC and ENaC that is physiologically plausible and partially explains the aldosterone paradox (summarized in Figure 9). It also provides a molecular mechanism for the phenomenon of K^+-induced natriuresis.[43,44]A recent study showed that K^+-induced natriuresis is impaired in NCC knockout mice, further adding to the role of NCC in this mechanism.[27] With regard to potassium channels, in our study the high K^+ but not the combined diet increased ROMK (Figure 3), while BK increased with both diets, but only in the cytoplasm (Figure 4). Recently, Wen *et al.* also found that a high K^+ diet increased BKα in the cytoplasm; apical BKα only increased during a high K^+ alkaline diet.[9] Similar to our results, Rieg *et al.* was also unable to detect apical BK labeling.[7] The absence of apical BKα labeling argues against an important role of these channels in potassium secretion at this time-point in the study. The increase in ROMK by the low Na^+ diet is at odds with its inhibition by angiotensin II.[23,45] Possibly, aldosterone increases the abundance of ROMK, while angiotensin II inhibits channel activity.[23,45] There are several possible mechanisms by which high dietary K^+ could inhibit NCC. In theory, this effect could be mediated by a gut-kidney kaliuretic reflex, a direct effect of the plasma K^+ concentration, or through the renin-angiotensin system.[25,42,46] For example, a previous study showed that infusion of K^+ directly into the renal artery decreased renin and increased natriuresis.[47] Although the

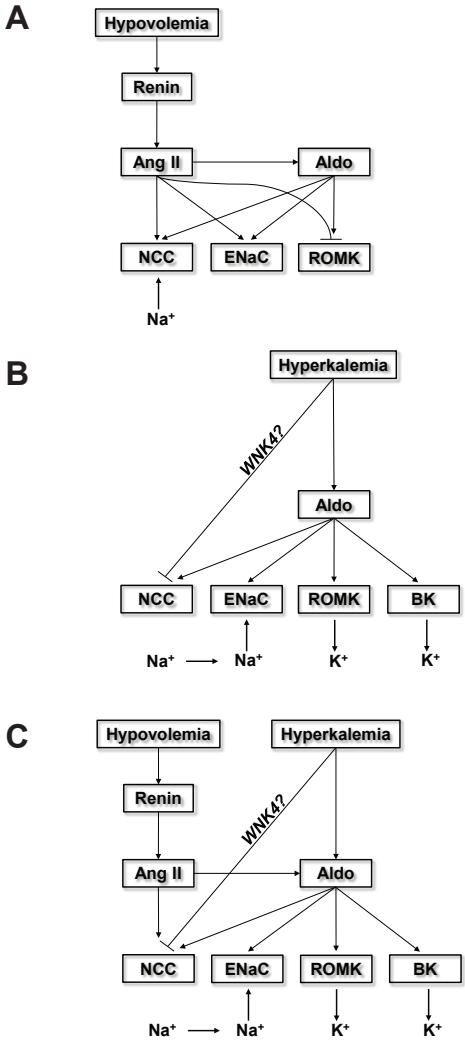


Figure 9: Model of the aldosterone (Aldo) paradox. Arrows indicate a stimulatory effect; dashed arrows indicate a stimulatory effect that is overridden by an inhibitory effect (two perpendicular lines). Arrows to or from Na^+ and K^+ indicate reabsorption and secretion, respectively. Hypovolemia (***A***) activates the renin-angiotensin system so that both ANG II and Aldo activate NCC. ANG II has been shown to inhibit ROMK and therefore overrides the normal activation by Aldo[23]. In this way, hypovolemia favors electroneutral Na^+ reabsorption through NCC while preserving K^+. Hyperkalemia (***B***) directly increases Aldo. Our study shows that this also leads to an inhibition of NCC, possibly mediated by WNK4, therefore overriding the usual stimulatory effect of Aldo and increasing distal Na^+ delivery (indicated by the arrow going to and from Na^+). The effects on ENaC, ROMK, and BK are maintained. Therefore, hyperkalemia stimulates ENaC-coupled kaliuresis. The combination of hypovolemia and hyperkalemia (***C***) still activates the renin-angiotensin system and leads to a further increase in Aldo. The stimulatory effects of ANG II and Aldo on NCC, however, are overridden probably by a direct effect of hyperkalemia. Again, this leads to more delivery of Na^+ to ENaC for coupled K^+ secretion through ROMK.

high K^+ diet also resulted in a slight reduction in plasma renin activity, this did not result in lower angiotensin II levels (Figure 1). We did find that the infusion of angiotensin II on top of a high K^+ diet reversed the inhibition of NCC (Figure 8). However, the combined diet inhibited NCC despite elevated angiotensin II. We are therefore hesitant to conclude that a reduced angiotensin II effect is the mechanism by which dietary K^+ inhibits NCC. Although our analysis of kidney angiotensin II paralleled plasma angiotensin II (Figure 1), these results should be interpreted with caution because whole kidney angiotensin II may differ from renal tubular angiotensin II.[48] It could imply that the combined diet induced other changes than the high K^+ diet alone. We believe it is also important to understand the temporal relationship of the effect of K^+ on NCC. Although our study showed NCC downregulation with chronic exposure to high dietary K^+, a recent study showed that K^+ dephosphorylated NCC acutely (15 minutes).[27] The rapidity of this response seems to argue against mediation by the renin-angiotensin system, although non-genomic effects have also been reported[49]. The fact that we primarily observed changes in total rather than phosphorylated NCC may be related to the duration of the experiment and is also in agreement with previous findings.[26]

Another aim of this study was to see how the different diets would affect some of the accessory proteins that regulate the function of Na^+ transporters and K^+ channels in the aldosterone-sensitive distal nephron. High dietary K^+ increased the abundance of WNK4, as shown previously[50], whereas it decreased Nedd4-2 (Figure 5). Since our antibodies against WNK4 and Nedd4-2 were not suitable for immunofluorescence in rats, we are unable to determine the nephron segments in which these changes occurred. As WNK4 is a negative regulator of both ROMK and ENaC[51,52], it seems logical to suggest that the increase in WNK4 is instead related to NCC. Although the effect of WNK4 on NCC remains controversial[53], several in vitro studies have shown that WNK4 can disrupt the trafficking of NCC to the plasma membrane.[36,54,55] We believe the decrease in Nedd4-2 aided the observed increase in ENaC.[56] Although it was recently shown that Nedd4-2 also regulates NCC, decreased Nedd4-2 would not result in decreased NCC, according to these studies.[11,13]

Another interesting finding was that the low Na^+ diet increased SPAK and pSPAK in the apical plasma membrane of DCT cells (Figure 6), whereas high dietary K^+ appeared to increase SPAK and pSPAK in the apical plasma membrane of TAL cells (Figure 7). The high K^+ diets also resulted in a greater intracellular distribution of SPAK and pSPAK in the DCT (Figure 6). The low Na^+ diet likely increased SPAK to facilitate the phosphorylation of NCC, as demonstrated previously.[21] Although the changes in SPAK with the low Na^+ diet were not evident from our immunoblot analysis (Figure 5), this may be explained by the use of whole kidney homogenates in which both TAL and DCT are present.[14] Why high dietary K^+ increases SPAK in TAL (Figure 7), requires further study, especially because we

observed no changes in NKCC2 (Figure 2). A possible explanation could be the presence of SPAK isoforms, including a kidney-specific and full-length form of SPAK, which inhibit and activate NKCC2, respectively.[57]

Unraveling the renal response to hypovolemia and hyperkalemia is important both from a physiological and clinical perspective. Physiologically, the combination of a low Na^+ and high K^+ diet mimics the diet humans consumed in earliest Paleolithic times.[58] The observation that K^+ still induced a natriuresis despite low dietary Na^+, suggests that K^+-induced natriuresis is an important evolutionary mechanism to maximize kaliuresis. Clinically, the regulation of Na^+ and K^+ in the aldosterone-sensitive distal nephron has direct relevance to understanding salt-sensitive hypertension.[41,59] In fact, a low Na^+ and high K^+ diet is currently recommended in the Dietary Approaches to Stop Hypertension (DASH).[60] One concern could be the very high plasma aldosterone levels we observed with the combined diet (Figure 1). Indeed, the DASH diet also raises plasma aldosterone[61] and one wonders whether this could promote cardiac fibrosis, as observed in for example heart failure.[62] On the other hand, the K^+-induced natriuresis mediated by NCC may explain the blood pressure lowering effect of high dietary K^+.[63,64] We do emphasize, however, that the diets used in this study contained less Na^+ and more K^+ than typically present in human diets.

In summary, this study sheds new light on the aldosterone paradox by showing differential regulation of NCC and ENaC. Low dietary Na^+ favors Na^+ reabsorption through NCC, whereas high dietary K^+ favors Na^+ reabsorption through ENaC to indirectly promote K^+ secretion. High dietary K^+ overrides the effects of angiotensin II and aldosterone on NCC and inhibits this cotransporter to deliver sufficient Na^+ to ENaC for K^+ secretion.

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Chapter 5

The phosphorylated sodium chloride cotransporter in urinary exosomes is superior to prostasin as a marker for aldosteronism

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ABSTRACT

Urinary exosomes are vesicles derived from renal tubular epithelial cells. Exosomes often contain several disease-associated proteins and are thus useful targets for identifying biomarkers of disease. Here, we hypothesized that the phosphorylated (active) form of the sodium chloride cotransporter (pNCC) or prostasin could serve as biomarkers for aldosteronism. We tested this in 2 animal models of aldosteronism (aldosterone infusion or low-sodium diet) and in patients with primary aldosteronism. Urinary exosomes were isolated from 24-hour urine or spot urine using ultracentrifugation. In rats, a normal or a high dose of aldosterone for 2, 3, or 8 days increased pNCC 3-fold in urinary exosomes ($P<0.05$ for all). A low-sodium diet also increased pNCC in urinary exosomes approximately 1.5-fold after 4 and after 8 days of treatment. The effects of these maneuvers on prostasin in urinary exosomes were less clear, showing a significant 1.5-fold increase only after 2 and 3 days of high-aldosterone infusion. In urinary exosomes of patients with primary aldosteronism, pNCC was 2.6-fold higher ($P<0.05$) while prostasin was 1.5-fold higher ($P=0.07$) than in patients with essential hypertension. Urinary exosomal pNCC and, to a lesser extent, prostasin are promising markers for aldosteronism in experimental animals and patients. These markers may be used to assess the biological activity of aldosterone and, potentially, as clinical biomarkers for primary aldosteronism.

INTRODUCTION

Urinary proteins originate from various sources. They may be derived from glomerular filtration, tubular secretion, shedding, glycosylphophatidylinositol anchored protein detachment (eg, Tamm-Horsfall protein), or exosome secretion.[1] Exosomes are low-density membrane vesicles that originate from multivesicular bodies. Urinary exosomes have sparked interest as potential biomarkers for human disease.[1-3] The presence of urinary exosomes and a reproducible method for their isolation were reported in 2004 by Pisitkun and colleagues.[4] Proteomic analysis of these exosomes showed that they contain many disease-related proteins[4,5]; however, the question remained whether the presence of a given protein in urinary exosomes could provide information on physiological or disease processes in the kidney. Studies addressing this question analyzed urinary exosomes in patients with monogenetic diseases, resulting in inactivity or overactivity of renal sodium transport proteins. For example, in Bartter and Gitelman syndrome, in which the sodium potassium chloride cotransporter and the sodium chloride cotransporter (NCC) are genetically inactivated, these proteins were also found to be absent or reduced in urinary exosomes.[5,6] Conversely, Mayan *et al.* found the abundance of NCC to be increased in patients with familial hyperkalemic hypertension, in which mutations in NCC-regulating kinases cause overactivity of this cotransporter.[7] Thus, in these homogeneous groups, the expression of sodium-transport proteins in urinary exosomes correlated with what one would expect from their renal expression. The next step in assessing the potential of exosomes as urinary biomarkers is to analyze their performance in acquired disease. Therefore, in this study, we asked whether various forms of aldosteronism resulted in increased expression of aldosterone-sensitive proteins in urinary exosomes. To address this question, we used animal models of primary and secondary aldosteronism and also studied patients with primary aldosteronism. In the kidney, the 2 main sodium transporters activated by aldosterone are NCC and the epithelial sodium channel (ENaC).[8,9] It therefore appears logical to study NCC and ENaC in urinary exosomes in different forms of aldosteronism. Esteva-Font *et al*, however, found no difference in the abundance of NCC in urinary exosomes of patients with salt-sensitive hypertension.[10] Recently, it has become clear that the phosphorylated form of NCC (pNCC) represents the active form of NCC and that trafficking and phosphorylation of NCC can be regulated independently.[11] Therefore, we propose that pNCC is a better reflection of NCC's biological activity. ENaC is difficult to study in urinary exosomes because it is present in very low quantities.[4] Instead, prostasin has emerged as an interesting surrogate marker of ENaC activity.[12] Prostasin is a serine protease that can increase the activity of ENaC and is also sensitive to aldosterone.[13] Here, we test the hypothesis that pNCC and prostasin in urinary exosomes are markers for aldosteronism.

MATERIALS AND METHODS

Animal studies

All animal protocols were approved by the Animal Care Committee of the Erasmus University Medical Center Rotterdam (EUR 127-11-01 and EUR 127-10-11). Three studies were conducted. In the first study, ten 15-week–old male Sprague-Dawley rats (Charles River) were adrenalectomized via a bilateral lumbodorsal incision and randomized to receive either high-dose aldosterone (100µg/kg/d) or vehicle via osmotic minipump (subcutaneous insertion, Alzet). All rats also received glucocorticoid replacement (dexamethasone, 5 µg/kg/d) and an angiotensin receptor blocker (losartan, 10 mg/kg/d) to inhibit the effects of angiotensin II on pNCC and prostasin.[14,15] The second study was similar to the first one but now included a third group of 5 rats that received a normal dose of aldosterone (50 µg/kg/d).[16] In addition, this study lasted for 8 days instead of 3 days. In the second study, we also harvested the right kidney for immunoblotting analysis. In the third study, 12 rats were randomly assigned to receive a normal (0.5%) or low (0.001% to 0.002%) sodium chloride diet for 8 days (Harlan diets, Harlan Laboratories). In all 3 experiments, animals were housed in metabolic cages to collect 24-hour urine for isolation of urinary exosomes. Urinary exosomes were isolated at various time-points in the 3 studies (days 1, 2, and 3 in the first study; day 8 in the second study; and days 0, 4, and 8 in the third study). Finally, at the end of each experiment, plasma renin and aldosterone, as well as urine sodium, were measured.

Studies in patients

Five patients with primary aldosteronism and 4 patients with essential hypertension were randomly selected from an ongoing study on primary aldosteronism.[17] Briefly, patients were eligible to participate in this study when they had uncontrolled hypertension despite the use of at least 2 antihypertensive drugs. All patients were subjected to volume expansion (2 liters of 0.9% sodium chloride [NaCl] during 4 hours) to analyze whether aldosterone was suppressible. Primary aldosteronism was defined as insuppressible plasma aldosterone (>235 pmol/L) after volume expansion. The patients whose posttest aldosterone was below 235 pmol/L were considered to have essential hypertension. In all patients, spot urine was collected under controlled circumstances for isolation of urinary exosomes. Urine sodium, potassium, and creatinine concentrations were measured as well.

Isolation and immunoblot analysis of urinary exosomes

Urinary exosomes were isolated as reported previously[4,18,19]; a more detailed protocol is provided as an online-only Data Supplement. Briefly, all urine samples were treated with a protease inhibitor before storage at -80°C; no phosphatase inhibitors were used. Urinary exosomes were isolated using a 2-step centrifugation process. First, urine was centrifuged at 17000 X g for 15 minutes at 37°C to remove whole cell membranes and other high density particles. Dithiothreitol was used to disrupt the Tamm-Horsfall polymeric network. Subsequently, the samples were subjected to ultracentrifugation at 200 000 X g for 105 minutes at 25°C. The pellet that formed during ultracentrifugation was suspended in isolation buffer. Finally, the suspended pellets were solubilized in Laemmli buffer for immunoblot analysis. Immunoblotting of the urinary exosomes and kidney samples was performed as described previously (see also online-only Data Supplement).[8] The antibody against NCC phosphorylated at threonine 58 was generated by 1 of the investigators (R.A.F.) and has been characterized previously.[20] All other antibodies were purchased: prostasin (BD Biosciences) and NCC (Stessmarq Biosciences). For the animal studies, the complete volume of the 24-hour urine was used to isolate urinary exosomes, and, therefore, no normalization was used in the analysis. Coomassie blue staining was used to confirm that there were no differences in total protein contents. Conversely, for the patient study, the amount of sample loaded during immunoblotting was normalized by the urinary creatinine concentration.[1]

Statistics

All data are expressed as mean standard error of the mean. Group comparisons were made using the unpaired Student's *t* test or analysis of variances with a *post hoc* test, as appropriate. For analysis, the natural logarithm of the plasma aldosterone concentration was used to yield a normal distribution. $P<0.05$ was considered statistically significant.

RESULTS

Three-day infusion of aldosterone increased pNCC and prostasin in urinary exosomes

Adrenalectomized rats were infused with high-dose aldosterone or vehicle for 3 days. The differences in plasma aldosterone and urinary sodium between the 2 groups confirmed that both the adrenalectomy and the infusion of aldosterone were successful (Figure 1A). The infusion of aldosterone significantly increased pNCC, NCC, and prostasin in urinary exosomes on days 2 and 3 (Figure 1B). The abundance of pNCC in urinary

exosomes increased 2.9±0.4-fold on day 2 and 3.2±0.3-fold on day 3 ($P<0.05$ for day 2 and $P<0.01$ for day 3). The abundance of NCC in urinary exosomes also increased 2.3±0.4-fold on day 2 and 1.8±0.4-fold on day 3 ($P<0.05$ for both). The abundance of prostasin in urinary exosomes increased 1.8±0.2-fold on days 2 and day 3 ($P<0.05$ and $P<0.01$, respectively, Figure 1C).

Effects of an 8-day infusion of aldosterone on pNCC and prostasin in kidney and urinary exosomes

Adrenalectomized rats were infused with vehicle, a normal or a high dose of aldosterone for 8 days. The plasma aldosterone concentrations were significantly different among the 3 groups (Figure 2A). Both the normal and high-aldosterone dose increased the abundance of pNCC and NCC in urinary exosomes (Figure 2B). pNCC increased 3.0±0.4-fold with the normal dose and 2.5±0.5-fold with the high dose ($P<0.01$ and $P<0.05$, respectively); NCC increased only with the normal dose (1.5±0.2-fold, $P<0.05$). Although pNCC and NCC in exosomes showed similar responses to aldosterone compared with the abundances of these proteins in the kidney,[21] no direct correlation was observed (data not shown). In contrast to the 3-day infusion (Figure 1C), the 8-day infusion of aldosterone did not increase prostasin in urinary exosomes (Figure 2C). In the kidney, only the high-aldosterone dose increased prostasin significantly (1.5±0.1-fold, $P<0.05$).

A low-salt diet increased pNCC but not prostasin in urinary exosomes

To induce a physiological increase in plasma aldosterone, 2 groups of rats were fed a normal sodium diet, after which 1 group was switched to a low-sodium diet. The low-sodium diet caused higher plasma renin activity and higher plasma aldosterone after 8 days (Figure 3A). When both groups were on the normal-sodium diet, the abundance of pNCC and NCC in urinary exosomes was similar (Figure 3B). The low-sodium diet increased pNCC in urinary exosomes on day 4 (1.7±0.2-fold) and day 8 (1.4±0.1-fold, $P<0.05$ for both). The low-sodium diet increased NCC in urinary exosomes on day 4 (1.5±0.1-fold) and day 8 (2.0±0.3-fold, $P<0.05$ for both). In contrast, it did not cause significant changes in the abundance of prostasin in urinary exosomes (Figure 3C).

pNCC and prostasin are increased in urinary exosomes of patients with primary aldosteronism

The characteristics of 5 patients with primary aldosteronism and 4 patients with essential hypertension are shown in Figure 4. Both groups had a similar degree of hypertension but had significant differences regarding the plasma aldosterone-to–renin ratio and the urine sodium-to–potassium ratio. The abundance of pNCC in urinary exosomes of patients with primary aldosteronism was higher in patients with essential hypertension

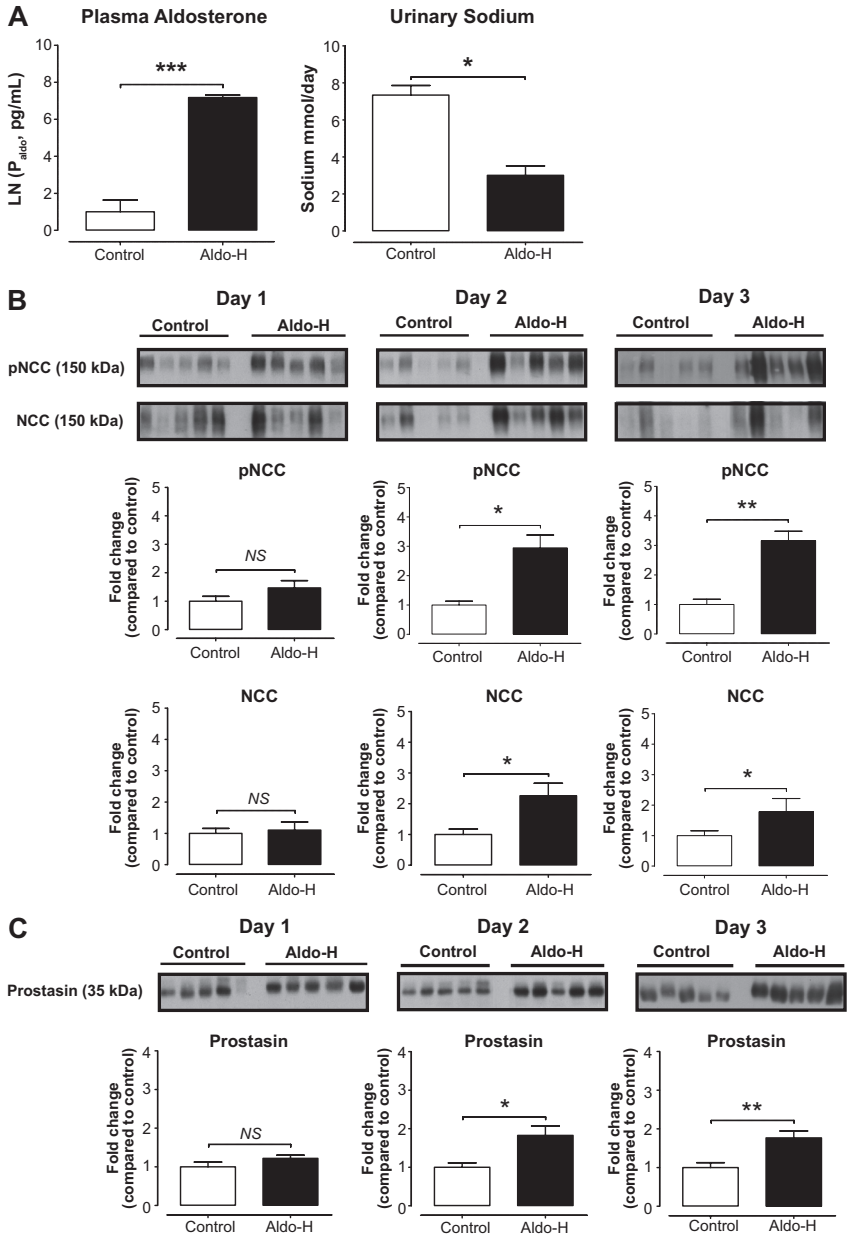

Figure 1: Effects of chronic aldosterone infusion in rats on the abundances of the phosphorylated form of sodium chloride cotransporter (pNCC) and prostasin in urinary exosomes. Adrenalectomized rats were treated with high-dose aldosterone (Aldo-H, 100 μg/kg/d) or vehicle (control) for 3 days. **A**, Plasma aldosterone and urine sodium. **B**, pNCC and NCC in urinary exosomes. **C**, Prostasin in urinary exosomes. **P*<0.05, ***P*<0.01, ****P*<0.001 by Student *t* test.

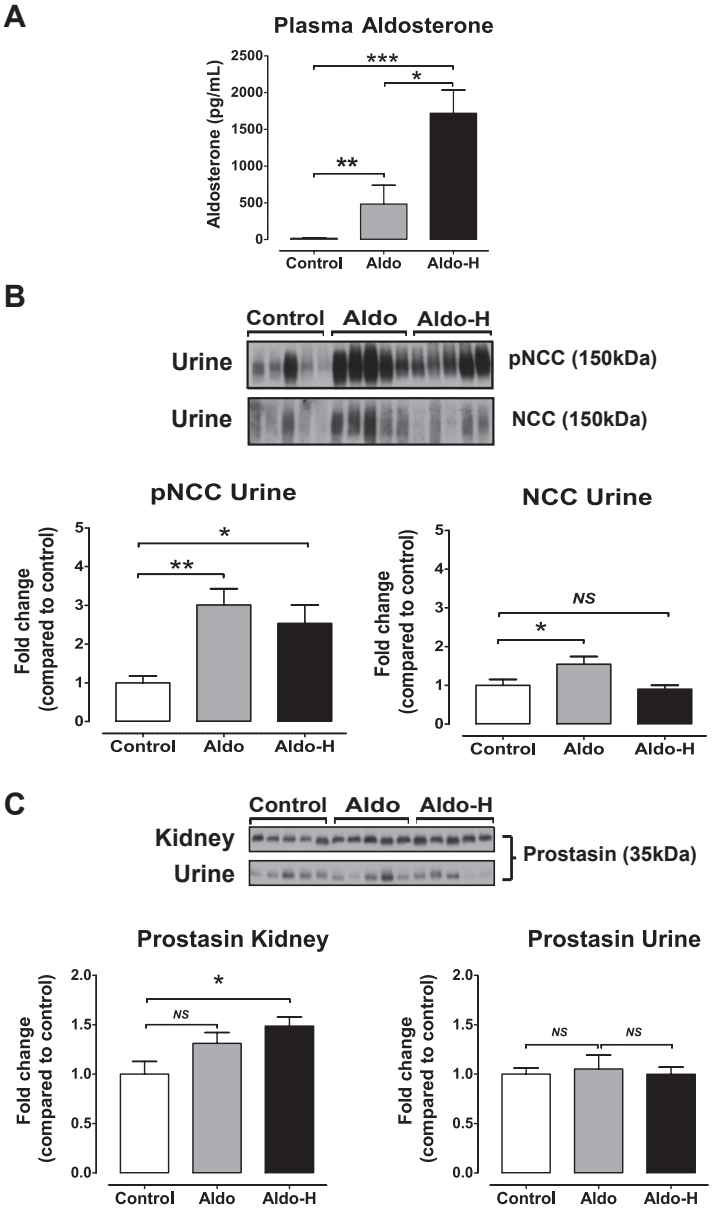

Figure 2: Effects of normal and high-aldosterone infusion in rats for 8 days on plasma aldosterone, phosphorylated form of sodium chloride cotransporter (pNCC), and prostasin in kidney and urinary exosomes. Adrenalectomized rats were infused with vehicle (control), normal (Aldo, 50 μg/kg/d), or high-dose (Aldo-H, 100 μg/kg/d) aldosterone for 8 days. **A**, Plasma aldosterone concentrations. **B**, pNCC and NCC in urinary exosomes. **C**, Prostasin abundance in kidney and urinary exosomes. *P<0.05, **P<0.01, ***P<0.001 by analysis of variance and *post hoc* test.

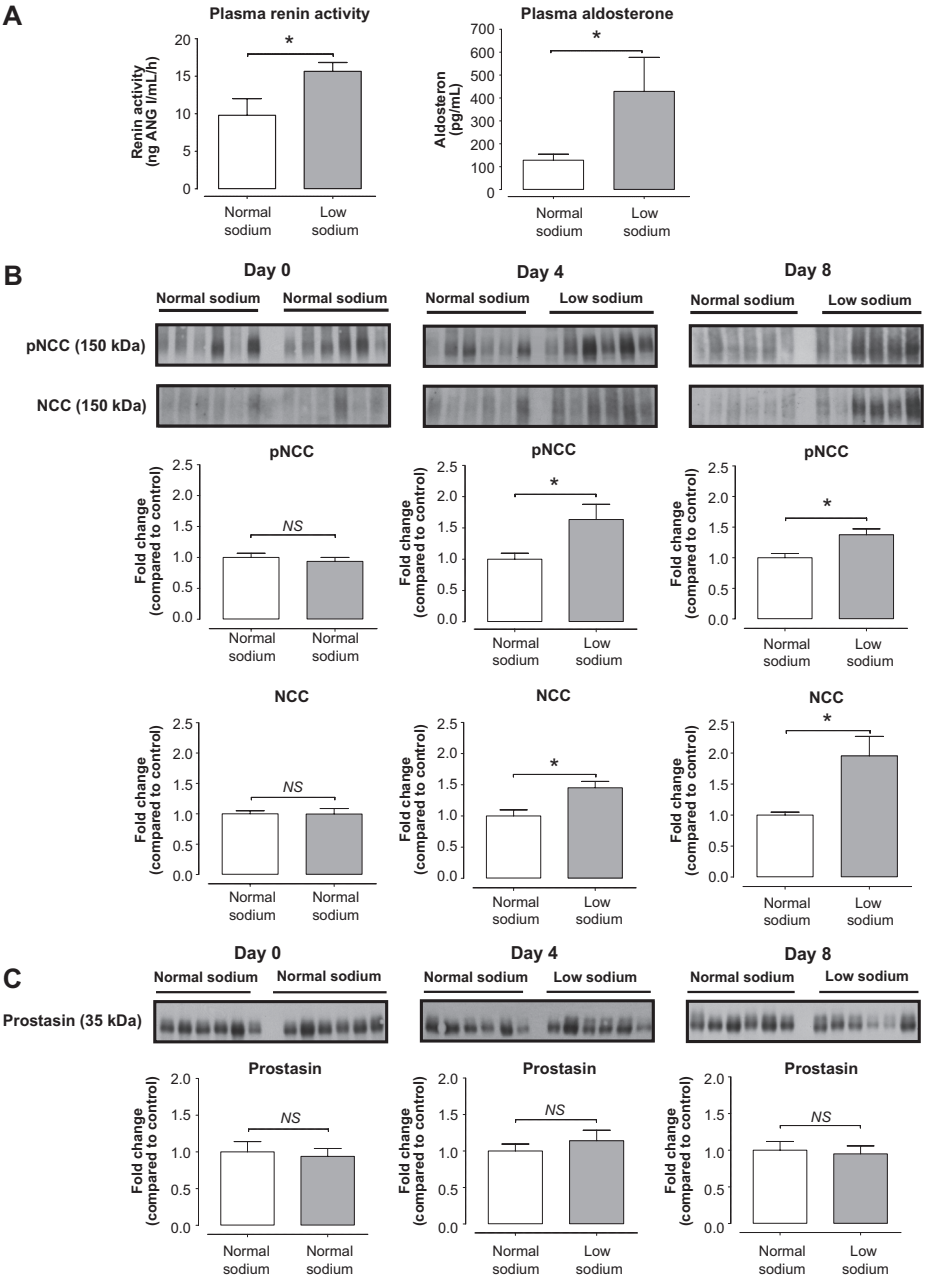

Figure 3: A low-salt diet increased phosphorylated form of sodium chloride cotransporter (pNCC) but not prostasin in urinary exosomes of rats. **A**, Plasma renin activity and plasma aldosterone. **B**, pNCC and NCC in urinary exosomes. **C**, Prostasin in urinary exosomes. *P<0.05 by Student's t test.

(2.6±0.3-fold, *P*<0.05, Figure 4). The abundance of prostasin in urinary exosomes showed a trend toward being higher in the patients with primary aldosteronism (1.5±0.3-fold, *P*=0.07).

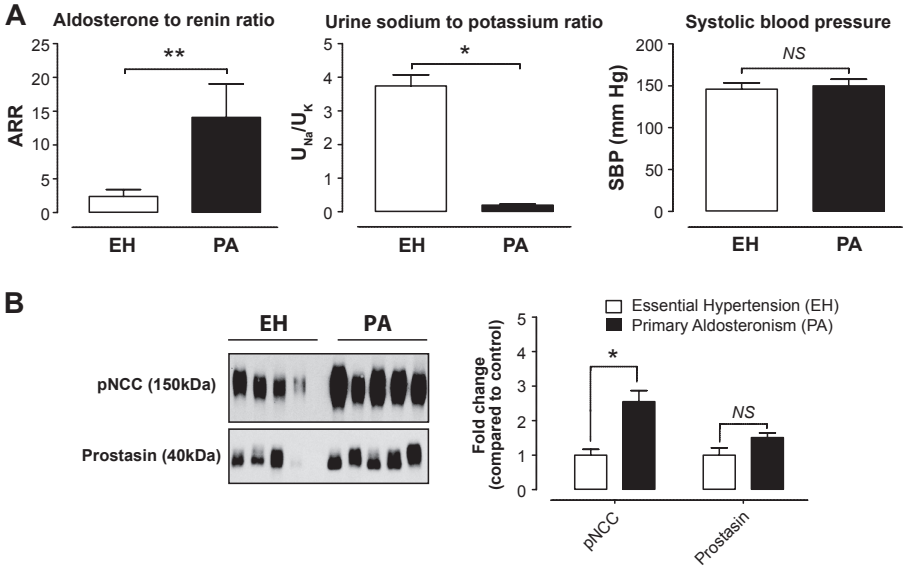

Figure 4: Patients with primary aldosteronism show increased abundance of phosphorylated form of sodium chloride cotransporter (pNCC) and prostasin in urinary exosomes. **A**, Characteristics of patients with essential hypertension (EH) or primary aldosteronism (PA). **B**, Patients with PA show increased abundance of pNCC and prostasin in urinary exosomes. **P*<0.05 by Student's *t* test or Mann-Whitney.

DISCUSSION

In this study, we asked whether NCC and prostasin in urinary exosomes can be used as markers for primary and secondary aldosteronism. In our hands, both total and phosphorylated NCC were superior to prostasin as a marker for aldosteronism. In fact, the abundance of prostasin in urinary exosomes only increased during high-dose, short-term treatment with aldosterone (Figure 1C), whereas the abundance of pNCC in urinary exosomes was higher during aldosteronism, regardless of the duration, dose, or stimulus (Figure 5). Because the changes in pNCC were more pronounced than for NCC, pNCC appears the better marker. In addition, pNCC was increased significantly in urinary exosomes of patients with primary aldosteronism compared with patients with essential hypertension (Figure 4). In contrast, the increase in the abundance of prostasin in urinary exosomes of patients with primary aldosteronism was of borderline significance.

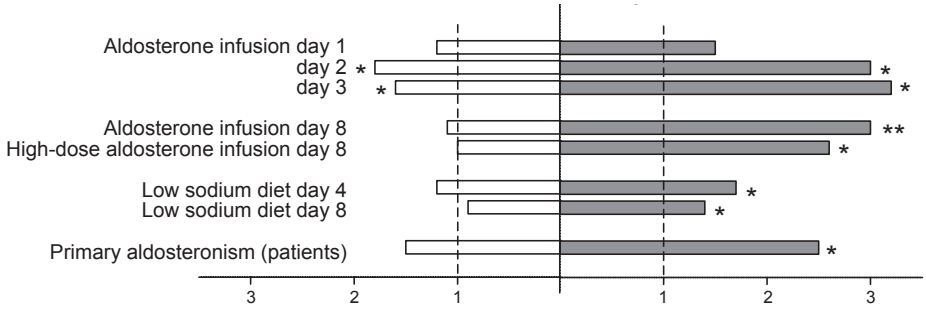

Figure 5: Summary of the performance of urinary exosomal prostasin and phosphorylated form of sodium chloride cotransporter (pNCC) as markers of aldosteronism. The densitometry values of the immunoblot analyses are shown. A value of 1 represents no difference compared with the control group. **P*<0.05, ***P*<0.01 vs control.

Several observations regarding the dynamics of pNCC and prostasin excretion in urinary exosomes during aldosteronism in this study merit discussion. For example, the results of the short-term infusion of aldosterone in rats suggest that it takes at least 1 day for both pNCC and prostasin to increase in urinary exosomes (Figure 1). This may be related to the time it takes aldosterone to increase transcription or posttranslational modification of these proteins.[22] The long-term infusion of aldosterone in rats showed that a lower (physiological) dose of aldosterone was already sufficient to increase pNCC (Figure 2B). In fact, pNCC expression in urinary exosomes was slightly lower with the high dose. This suggests that the stimulatory effect of aldosterone on pNCC in urinary exosomes either saturates or that aldosterone escape has occurred, a defense mechanism known to reduce NCC in the kidney.[23] Although the induction of secondary aldosteronism by a low-sodium diet also increased pNCC in urinary exosomes (Figure 3B), the magnitude of this effect was less than with aldosterone infusion (Figure 5). One could have predicted even higher pNCC in urinary exosomes during a low-sodium diet because this maneuver is likely to increase both plasma angiotensin II and aldosterone. We recently showed that angiotensin II can increase renal pNCC independently of aldosterone and that the combination of angiotensin II and aldosterone leads to an additive effect.[15,21] Apparently, other factors limit pNCC excretion in urinary exosomes during a low-sodium diet. Furthermore, differences in the plasma aldosterone concentrations in the control groups should be taken into consideration (virtually absent in adrenalectomized rats versus ~100 pg/mL in rats on a normal sodium diet, Figures 1A and 3A). Of interest, the increase in NCC in urinary exosomes we observed in this study with a low-sodium diet was similar to the increase Esteva-Font *et al* found for NCC in a separate study.[10] Our study suggests that pNCC was better than prostasin as a marker of aldosteronism, which

could be explained by several factors. First, although prostasin is sensitive to aldosterone and can activate ENaC, this does not necessarily render it a direct marker of ENaC activity and, as such, it may not be a direct marker of distal sodium reabsorption.[12,13] Prostasin is only one of the proteins present in the complex signaling cascade that regulates ENaC.[24] Second, because prostasin is also present in prostate epithelial cells, urinary exosomes may also contain prostasin from this source.[25] This may have limited the specificity of prostasin as a marker for aldosterone actions in the kidney, especially in males. Third, by using 2-dimensional electrophoresis, Olivieri and colleagues have previously shown that several subunits of prostasin exist, only some of which are aldosteronesensitive.[12] Our prostasin results differ from those reported by Narikiyo and coworkers.[13] They found that rats continued to increase urinary prostasin during 7 days of aldosterone infusion (from 1.5- to 4-fold), whereas we were unable to detect increased prostasin in urinary exosomes after 8 days of treatment (Figure 2). This difference may be explained by the use of a 3-fold higher aldosterone dose in the previous study and that their analysis of prostasin was performed on whole urine instead of urinary exosomes. In recent years, the potential to use proteins in urinary exosomes as markers of diseases affecting the kidney has attracted much interest.[1-3,26] Progress has been somewhat hindered by technical and normalization issues, but the proposal of uniform protocols has been a step in the right direction.[18,27] We believe that the strength of this study was to combine a controlled experimental setting with a clinical setting for the analysis of urinary exosomes in aldosteronism. Because of the well-characterized actions of aldosterone on distal tubular sodium transport, aldosteronism appears especially suitable for analysis with urinary exosomes.

Clinically, primary aldosteronism is important because recent studies suggest it to be a common condition among patients with resistant hypertension that is often difficult to diagnose.[28] We do emphasize, however, that the primary aim of this study was to provide proof of principle that the abundance of aldosterone-sensitive proteins in urinary exosomes is increased during aldosteronism. The question whether pNCC in urinary exosomes has diagnostic potential in patients with primary aldosteronism remains to be determined. This will require larger and well-characterized groups of patients to be tested against a golden standard. In addition, the overall differences in NCC and pNCC between conditions were relatively mild even under controlled experimental conditions. Even if the sensitivity and specificity of pNCC in urinary exosomes would outweigh existing tests such as the ARR, the current method of exosome isolation is not suitable for clinical use.[29] Instead, development of an enzyme-linked immunoassay for pNCC would be an attractive alternative. This study suggests several directions for future research. One obvious next step will be to evaluate how pNCC and prostasin in patients with primary aldosteronism respond to treatment with either mineralocorticoid receptor antagonists or adrenalectomy. Narikiyo *et al* showed that

urinary prostasin decreased in 3 patients with primary aldosteronism who had undergone adrenalectomy.[13] Similarly, Olivieri *et al* showed that prostasin decreased in normotensive subjects with aldosteronism owing to a low-sodium diet who were subsequently treated with spironolactone.[12] It would also be informative to know whether other commonly used antihypertensive drugs such as diuretics, angiotensin-converting enzyme inhibitors, or angiotensin receptor blockers affect urinary exosome excretion. Because total NCC also increased, it would be important to known whether NCC mRNA is also present in urinary exosomes and if it behaves similarly. If so, it could be a more sensitive marker because the signal can be amplified. In the kidney, however, previous studies found that the protein and mRNA abundances of NCC did not always correlate.[21,23]Finally, given the recent insights into the effects of angiotensin II on distal tubular sodium transport,[14,15] it would be important to compare urinary exosomal pNCC and prostasin in patients with primary and secondary aldosteronism. In conclusion, in urinary exosomes of animals and patients, pNCC was superior to prostasin as a marker of aldosteronism. These results justify further evaluation of the applicability of urinary exosomes as a diagnostic tool in primary aldosteronism and, possibly, other forms of hypertension. Furthermore, pNCC and, to a lesser extent, prostasin may be used experimentally as a noninvasive method to analyze the biological action of aldosterone in the kidney.

Perspectives

In hypertension, urinary exosomes could be applied as a diagnostic test for primary aldosteronism, to evaluate saltsensitivity or the response to antihypertensive drugs acting on the kidney. Our study can be regarded as proof of principle that analysis of urinary exosomes can be applied to aldosteronism. Subsequent validation studies will be necessary to define their use in experimental or clinical settings.[30]

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Chapter 6

Effects of angiotensin II on kinase-mediated sodium and potassium transport in the distal nephron

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ABSTRACT

Purpose of review

The aim is to review the recently reported effects of angiotensin II (Ang II) on sodium and potassium transport in the aldosterone-sensitive distal nephron, including the signaling pathways between receptor and transporter, and the (patho)physiological implications of these findings.

Recent findings

Ang II can activate the sodium chloride cotransporter (NCC) through phosphorylation by Ste20-related, proline-alanine rich kinase (SPAK), an effect that is independent of aldosterone but dependent on with no lysine kinase 4 (WNK4). A low-sodium diet (high Ang II) activates NCC, whereas a high-potassium diet (low Ang II) inhibits NCC. NCC activation also contributes to Ang-II-mediated hypertension. Ang II also activates the epithelial sodium channel (ENaC) additively to aldosterone, and this effect appears to be mediated through protein kinase C and superoxide generation by nicotinamide adenine dinucleotide phosphate oxidase. While aldosterone activates the renal outer medullary potassium channel (ROMK), this channel is inhibited by Ang II. The key kinase responsible for this effect is c-Src, which phosphorylates ROMK and leaves WNK4 unphosphorylated to further inhibit ROMK.

Summary

The effects of Ang II on NCC, ENaC, and ROMK help explain the renal response to hypovolemia which is to conserve both sodium and potassium. Pathophysiologically, Ang-II-induced activation of NCC appears to contribute to salt-sensitive hypertension.

INTRODUCTION

Physiologically, the renin–angiotensin–aldosterone system (RAAS) operates to increase renal sodium retention during hypovolemia and potassium secretion during hyperkalemia.[1] The paradigm has been that angiotensin II (Ang II) primarily increases sodium reabsorption by the proximal tubule, whereas aldosterone stimulates sodium uptake and potassium secretion in the more distal parts of the nephron. This part of the kidney has therefore also been coined the 'aldosterone-sensitive distal nephron' and comprises the distal convoluted tubule (DCT), connecting tubule, and collecting duct.[2] In the last two decades, the major sodium and potassium transport proteins along the nephron have been cloned and characterized. In the proximal tubule, Ang II was shown to mediate sodium bicarbonate reabsorption through the sodium hydrogen exchanger type 3 (NHE3).[3] In the distal nephron, the sodium chloride cotransporter (NCC)[4], epithelial sodium channel (ENaC)[5], and the renal outer medullary potassium channel (ROMK)[6,7]have all been shown to be sensitive to aldosterone. NCC is primarily located in the first segment of the DCT (DCT-1), whereas ENaC is located in the second segment (DCT-2), connecting tubule, and collecting duct.[8-10] ROMK is expressed along the entire distal nephron.[11]

Key Points

- **Ang II activates NCC through WNK4 and SPAK independently of aldosterone.**
- **Ang II activates ENaC through PKC and NADPH, and this effect is additive to aldosterone**
- **In contrast to aldosterone, Ang II inhibits ROMK and this effect is mediated by c-Src which stimulates endocytosis directly and indirectly through WNK4**
- **The effects of Ang II on NCC, ENaC, and ROMK help explain the renal response to hypovolemia that consist of conserving both sodium and potassium**
- **Ang-II-dependent hypertension has a sodium-sensitive component that is mediated through NCC**

The paradigm that Ang II acts proximally in the kidney has recently been challenged. Several studies now show that Ang II also influences sodium and potassium handling in the distal nephron. Although abundantly located in the proximal tubule, early work using a 125I-Ang-II binding assay already demonstrated the presence of Ang-II binding sites in the thick ascending limb, DCT, and collecting duct.[12] A subsequent study confirmed that the Ang II type 1 receptor (AT1R) was expressed along the entire distal nephron and, interestingly, was located both apically and basolaterally.[13] Using isolated

perfused tubules, Wang and Giebisch[14] were among the first to address the effects of Ang II on sodium and potassium transport in the distal tubule. They found that Ang II increased sodium transport in DCT-1 and DCT-2, whereas it decreased potassium transport. Both an AT1R blocker and amiloride abolished these effects (thiazides were not tested). These data suggest that Ang II may activate NCC and ENaC and inhibit ROMK. Recent studies have addressed the effects of Ang II on these transport proteins. These studies have also analyzed the involved signaling pathways focusing on a recently identified kinase network that regulates NCC, ENaC, and ROMK and includes with no lysine kinase 4 (WNK4), Ste20-related, proline-alanine rich kinase (SPAK), and serum and glucocorticoid inducible kinase 1 (SGK1).[15]

Effects of Ang II on sodium chloride cotransporter

Sandberg *et al.*[16] provided the first evidence that Ang II could regulate NCC. In rats, they showed that infusion of the angiotensin-converting enzyme inhibitor captopril for 20 min caused NCC to move from the apical plasma membrane to subapical cytoplasmic vesicles. This effect was reversed by the infusion of Ang II and not related to changes in blood pressure or glomerular filtration rate. Because the effects of Ang II were acute, aldosterone was unlikely to have played a role. However, to more clearly dissect the effects of Ang II and aldosterone on NCC in a chronic setting, we performed studies in adrenalectomized rats.[17] After adrenalectomy, rats received an infusion of either aldosterone or Ang II for 1 week. Indeed, Ang II increased both total NCC abundance and phosphorylated NCC in plasma membrane fractions independent of aldosterone (Fig. 1). More functionally, we also showed that Ang II caused sodium retention and that this was reversed by thiazides. In a subsequent study, we asked whether aldosterone requires Ang II to activate NCC.[18] Again, we used adrenalectomized rats that now received aldosterone in addition to the AT1R blocker losartan. We showed that in the presence of losartan aldosterone was still capable of increasing NCC (Fig. 1), so that aldosterone alone is sufficient for these effects. However, we also showed that the addition of losartan to the infusion of aldosterone reduced phosphorylated (active) NCC (Fig. 1). This suggests that Ang II may have an additive effect on aldosterone-induced NCC activation. We showed that the kinase SPAK was increased both by aldosterone and Ang II, and was likely responsible for phosphorylating NCC (Fig. 1).[17,18] Previously, an in-vitro study also showed that the kinases SPAK and WNK4 regulate NCC upon stimulation by Ang II.[19] That is, in Xenopus oocytes, Ang II only increased NCC activity when WNK4 was present and when the SPAK-binding motif on NCC was intact. Of interest, in the absence of Ang II, WNK4 inhibited NCC. Ang II, however, did not alter WNK4's previously reported ability to inhibit ROMK.[20] More recently, these findings were confirmed in vivo with the development of WNK4 knockout mice.[21] Both a low-sodium diet and Ang II infusion increased

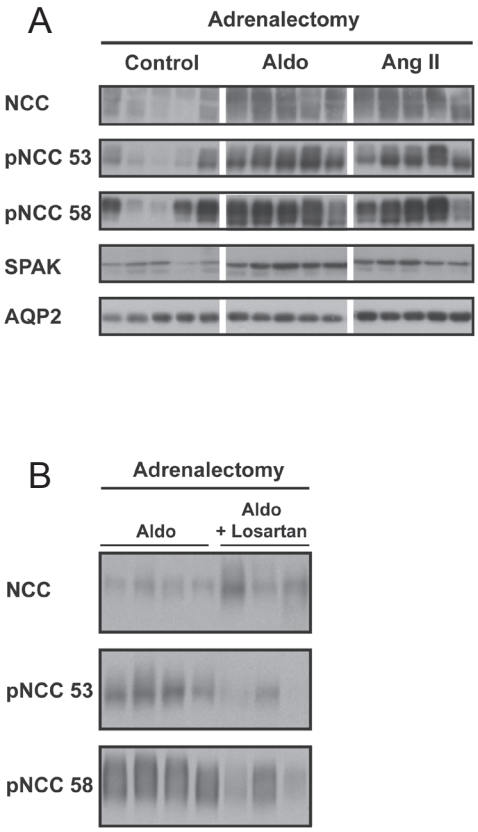

Figure 1: Ang II has independent but additive effects on the sodium chloride cotransporter (NCC). Panel (**a**) shows immunoblots of kidney homogenates from adrenalectomized rats that were infused with vehicle (control), aldosterone or Ang II for 1 week. In the absence of aldosterone (Aldo), Ang II significantly increased both the expression of NCC and its phosphorylation at threonine 53 and 58 (pNCC). This phosphorylation was likely mediated by SPAK. Aldosterone and Ang II also increased the water channel aquaporin-2 (AQP2, see text for further details). Panel (**b**) shows immunoblots of kidney homogenates from adrenalectomized rats that were infused with aldosterone alone or with a combination of aldosterone and losartan. Inhibition of Ang II effects by losartan reduced the phosphorylated NCC at threonine 53 and 58, but not total NCC. Because endogenous Ang II was present in rats only receiving aldosterone, this suggests that Ang II has an additive effect on pNCC. Reproduced with permission.[17,18]

the phosphorylation of SPAK and NCC in wild-type mice but not in WNK4 knockout mice. In summary, Ang II has been shown to activate NCC both acutely and chronically and both in vitro and in vivo through a WNK4–SPAK dependent pathway (see also a recent review[22]).

Effects of Ang II on epithelial sodium channel

In addition to NCC, Ang II activates another sodium transporter in the distal nephron, namely ENaC. This finding was first reported in 2002 by Peti- Peterdi *et al.*[23] using isolated perfused cortical collecting duct segments from rabbit kidney. In this study, the intracellular sodium concentration was assessed by fluorescence microscopy and used as a measure of sodium transport. Using this methodology, they showed that Ang II increased the intracellular sodium concentration when infused either via the luminal perfusate (apical side) or via the bath (basolateral side). These effects could be reversed by the ENaC blocker benzamil and the AT_1R blockers losartan and candesartan. In a subsequent study using similar methods, this group showed that Ang I could also activate ENaC possibly because Ang I was converted to Ang II by angiotensin converting enzyme within the tubular lumen.[24] By using patch clamp electrophysiology, two recent studies also showed that Ang II increased the open probability of ENaC.[25,26] In the first study by Mamenko *et al.*[25]**,** Ang II acutely (1–2 min) increased the open probability of ENaC in freshly isolated split-opened distal nephrons of mice, while more prolonged exposure to Ang II (30 min) also induced a translocation of a-ENaC to the apical plasma membrane. Of interest, aldosterone did not increase the open probability of ENaC acutely, and when mineralocorticoid status was saturated the effect of Ang II still persisted. Thus, Ang II appears to have an additive effect to aldosterone in the activation of ENaC similar to what we showed for NCC.[18] Interestingly, Mamenko *et al.* also showed that Ang II did not increase intracellular calcium but did generate reactive oxygen species. Indeed, inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) by apocynin abolished Ang-II-mediated ENaC activation. The second study by Sun *et al.*[26] also found that Ang II acutely (5–10 min) increased both channel number and open probability of ENaC in rat cortical collecting duct. This study also found that the effect of Ang II on ENaC was independent of intracellular calcium, but depended on protein kinase C (PKC) and NADPH. On the basis of these findings, the authors proposed that the superoxide anions generated by

NADPH indirectly increase ENaC activity because they reduce the inhibitory effect of arachidonic acid on ENaC. This was assumed because superoxide anions are known to inhibit cytochrome P450 epoxygenase, which converts arachidonic acid to epoxyeicosatrienoic acid.[27] Indeed, a confirmatory experiment showed that addition of the superoxide H2O2 reduced the inhibitory effect of arachidonic acid on ENaC.[26]

Effects of Ang II on renal outer medullary potassium channel

The inhibition of ROMK by Ang II is physiologically relevant because it may provide an explanation for the conservation of potassium during hypovolemia.[28] The inhibitory effect of Ang II on ROMK was first reported by Wei *et al.*[29] using patch clamp recordings to

analyze split-opened cortical collecting ducts of rats fed normal or low potassium diets. They found that Ang II dose dependently inhibited ROMK channel activity through AT1R in rats on low but not on normal potassium diets. By using specific kinase inhibitors, the authors showed that Ang II–ROMK signaling was mediated through PKC, the tyrosine kinase c-Src, and NADPH oxidase. More recently, the same group further explored the effects of Ang II on ROMK in HEK293 cells (which express AT_1R endogenously) and in rats fed a low-sodium diet (which will increase Ang II). These studies more clearly elucidated two mechanisms through which Ang II inhibits ROMK, namely by increasing tyrosine phosphorylation of the channel and by stimulating WNK4-induced inhibition of ROMK. The key kinase responsible for these two mechanisms of ROMK inhibition appears to be c-Src. First, tyrosine phosphorylation of ROMK by c-Src stimulates endocytosis of ROMK.[30,31] Second, c-Src is capable of inhibiting SGK1. This will disrupt SGK1- mediated phosphorylation of WNK4, which in its unphosphorylated state can further reduce the surface expression of ROMK.[20,31-33] As such, this provides a mechanism through which Ang II can overrule the actions of aldosterone on SGK1–WNK4–ROMK.

Effects of Ang II on other transport proteins in the distal nephron

With regard to the effects of Ang II on the renal collecting duct, we have so far limited our review to ENaC and ROMK. Ang II, however, has also been shown to regulate the water channel aquaporin-2 (AQP2) and to influence transport processes mediated by the intercalated cells. Although an extensive discussion of these effects is beyond the scope of this review, we believe it is important to provide a brief summary. Ang II has been shown to increase AQP2 in vitro not only through AT_1R, but also through cross-talk with the vasopressin 2 receptor[34]; we confirmed this response in vivo (Fig. 1). In intercalated cells, Ang II has been shown to regulate both the anion exchanger pendrin (expressed by bicarbonate-secreting type B cells) and the apical H^+-ATPase (expressed by acid-secreting type A cells).[35,36] Ang II increased transcellular chloride absorption through pendrin in furosemide-treated mice, but not in mice on a normal diet.[35] This effect was not mediated by an increase in electromotive force, but could be abolished by inhibiting apical H^+-ATPase with bafilomycin. By using immunogold labeling, Ang II was also shown to increase the expression of H^+-ATPase at the apical plasma Membrane.[36] This finding led the authors to propose to a physiologically plausible model in which Ang II stimulates H^+ secretion into the lumen which drives Cl^- absorption through pendrin as well as generates a favorable electrochemical gradient for ENaC-mediated sodium reabsorption.[36,37]

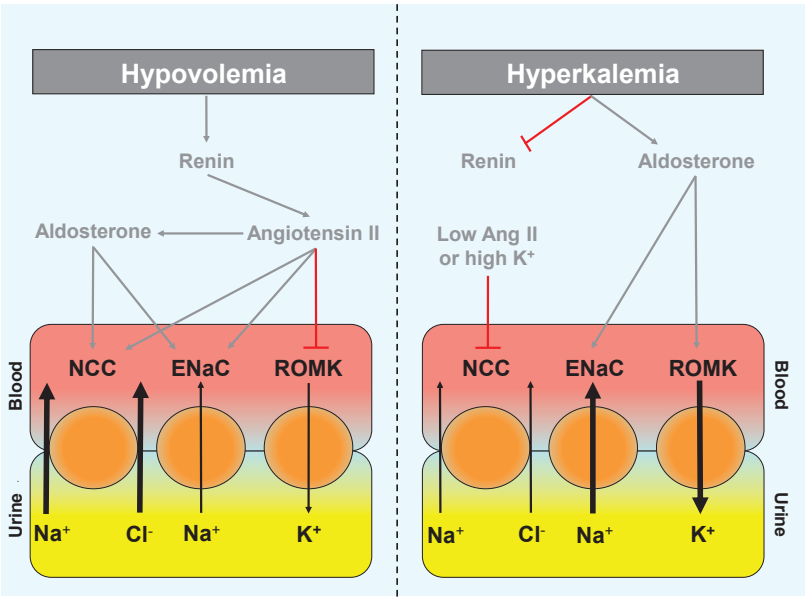

Figure 2: Differential effects of aldosterone during hypovolemia and hyperkalemia (the 'aldosterone paradox'). During hypovolemia (left panel), angiotensin II (Ang II) and aldosterone act synergistically to increase the activity of the sodium chloride cotransporter (NCC) and the epithelial sodium channel (ENaC), while Ang II inhibits the renal outer medullary potassium channel (ROMK). This results in maximal sodium reabsorption to correct hypovolemia, while potassium is being conserved. Hyperkalemia (right panel) directly increases aldosterone secretion independent of renin and Ang II. The absence of Ang II or the direct effects of hyperkalemia cause NCC to be less active and prevent ROMK inhibition. ENaC and ROMK, on the other hand, are maximally activated by aldosterone. These combined effects result in maximal sodium delivery to ENaC to facilitate the coupled secretion of potassium through ROMK. Arrows indicate a stimulatory effect, while two perpendicular lines indicate an inhibitory effect.

Physiological implications

The effects of Ang II on sodium and potassium transport in the distal nephron may help explain the 'aldosterone paradox' (Fig. 2). The term aldosterone paradox was originally proposed by Halperin and Kamel[38] and refers to the question of how the same hormone can cause sodium retention during hypovolemia and potassium secretion during hyperkalemia.[9,39] Both Ang II and aldosterone are elevated during hypovolemia, whereas only aldosterone is increased during hyperkalemia. Therefore, the concerted actions of Ang II and aldosterone may be responsible for the renal sodium reabsorption and potassium conservation observed during hypovolemia. Because Ang II activates NHE3, NCC, and ENaC, sodium reabsorption at multiple nephron sites is achieved. Although we showed that aldosterone alone is sufficient to activate NCC, Ang II appeared to have an additive effect (Fig. 1). Increased sodium reabsorption in the proximal tubule and the DCT will also reduce the delivery of sodium to the collecting duct. This may help conserve

potassium by limiting both sodium-coupled potassium secretion through ROMK and flow-dependent potassium secretion through so called big potassium (BK) channels.[40] In addition, Ang II also directly inhibits ROMK through effects on c-Src, SGK1, and WNK4.[31] Although simultaneous sodium and potassium conservation during hypovolemia is appropriate from a physiological point of view, activation of ENaC but inhibition of ROMK appears at odds with the normal electrical coupling of these transporters. A possible explanation for this dissociation may be that Ang II also activates apical H^+-ATPase in type A intercalated cells.[36] Increased H^+ secretion will generate a current of positive charge that will compensate for the loss of ROMK activity. In addition, increased H^+ secretion will also reduce the intraluminal HCO_3^- concentration thereby increasing CO_2. CO_2 has been shown to activate pendrin which facilitates sodium chloride reabsorption.[41] Together, the effects of Ang II during hypovolemia will contribute to sodium and potassium conservation at the expense of a tendency towards metabolic alkalosis (more bicarbonate reabsorption). Hyperkalemia, on the other hand, stimulates potassium secretion directly. The effects of hyperkalemia are often induced experimentally by feeding animals a high-potassium diet. Of interest, a high-potassium diet inhibits NCC despite elevated levels of aldosterone.[42,43] The inhibition of NCC will increase sodium delivery to more distal parts of the tubule in which sodium reabsorption through ENaC is coupled to potassium secretion through ROMK. Thus, inhibition of NCC will indirectly promote potassium secretion. The mechanism by which a high-potassium diet inhibits NCC, however, remains incompletely understood. A high-potassium diet was shown to inhibit NCC expression rather than phosphorylation.[43] Another study showed that a high-potassium diet increased the ratio between kidney-specific WNK1 and WNK1 and the abundance of WNK4.[44] According to the current model of NCC regulation by WNKs, these effects would be in agreement with an inhibitory effect on NCC.[39] It is unknown, however, how a high-potassium diet induces these changes. One possibility is the lower Ang II concentrations observed during a high potassium diet.[45]

Clinical implications

The emerging effects of Ang II on sodium and potassium transport in the kidney are also relevant clinically with regard to hypertension. Blood Ang II levels are elevated in several forms of essential and secondary hypertension.[46] Ang II plays an important pathogenetic role in the development of hypertension by increasing vasoconstriction, sympathetic nerve activity, and renal tubular sodium and water reabsorption. The relative importance of these effects on different target organs remains a matter of debate[47], but two recent studies have emphasized the importance of Ang-II-mediated proximal and distal sodium reabsorption in blood pressure regulation.[48,49] In the first study by Gurley *et al.*[49], knockout mice were developed which lacked AT_1R only in the proximal tubule (called

'PTKO'). These PTKO mice exhibited lower baseline systolic blood pressures during day and night. The pressor response to acute Ang II infusion in PTKO mice was still intact, ruling out a vascular effect for the lower blood pressure phenotype. Chronic infusion of Ang II, however, protected against hypertension likely by facilitating natriuresis because PTKO mice had a lower cumulative sodium balance than controls. Consistent with this observation, the sodium transporter NHE3 was downregulated during chronic Ang II infusion (but not at baseline), suggesting that this facilitated pressure natriuresis. PTKO mice, however, were not resistant to a salt-dependent rise in blood pressure, suggesting that salt sensitivity is mediated by more distal AT_1R. In this regard, the second study by Ashek *et al.*[48] offers interesting insights. In this study, a transgenic rat was developed with inducible expression of the Ren2 gene. In this animal model, Ang-II-dependent hypertension can be induced through the dietary administration of indole-3-carbinol. A fall in sodium excretion preceded the development of hypertension. Activation of NCC was found to be central to the development of this form of hypertension, because it was responsive to thiazides and losartan but not to amiloride or spironolactone. Accordingly, NCC but not ENaC abundance was increased. In addition, fractional lithium excretion was reduced, suggesting inhibition of proximal tubular sodium reabsorption possibly because of hypertension.[50] In summary, the studies by Gurley *et al.* and Ashlek *et al.* suggest that Ang-II-mediated sodium reabsorption in the kidney contributes importantly to blood pressure regulation[48,49] The effects of Ang II on NHE3 appear mainly relevant for pressure natriuresis, while the effects of Ang II on NCC may be more important for salt sensitivity.

CONCLUSION

Recent studies have indicated that Ang II regulates sodium and potassium handling in the distal nephron. Ang II activates NCC and ENaC, a response that appears additive to the well-established effects of aldosterone on these transporters.[17,25] Conversely, Ang II inhibits the potassium channel ROMK, which is opposite to the effect of aldosterone on this channel.[31] The signaling between AT_1R and these transporters is mediated by interacting kinases and oxidases. For example, Ang II activates NCC through a WNK4–SPAK-dependent pathway and ENaC through a PKC–NADPH-dependent pathway.[19,26] Ang II inhibits ROMK through a c-Src–SGK1–WNK4-dependent pathway.[31] Insights into the effects of Ang II on sodium and potassium handling by the distal nephron help explain the differential renal response to hypovolemia and hyperkalemia, the so-called aldosterone paradox. They also help explain some aspects of hypertension, including pressure natriuresis and the salt sensitivity of Ang-II-dependent hypertension. [48,49]

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Chapter 7

Summary and conclusions

Future directions

Based on

The sodium chloride cotransporter SLC12A3: new roles in sodium, potassium, and blood pressure regulation.

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SUMMARY AND CONCLUSIONS

The distal nephron of the kidney plays a key role in the control of Na^+, K^+ and extracellular volume balance and thereby blood pressure. This part of the nephron acts via Na^+ and K^+ transporters, namely the Na^+-Cl^- cotransporter (NCC), the epithelial Na^+ channel (ENaC), the Renal Outer Medullary K^+ channel (ROMK) and the large-conductance Ca^{2+}-activated K^+ (BK) channel. All these transporters are sensitive to the mineralocorticoid hormone aldosterone, which is part of the renin-angiotensin-aldosterone system (RAAS). Aldosterone is produced by the adrenal gland in two physiological conditions namely hypovolemia and hyperkalemia. During hypovolemia aldosterone induces Na^+ reabsorption; during hyperkalemia, aldosterone induces secretion of K^+. The observation that one hormone, aldosterone, has different effects on the sodium and potassium transport has been termed the "aldosterone paradox". Recent studies have revealed that besides aldosterone, other hormones like angiotensin II can regulate NCC, ENaC and ROMK directly or indirectly via a complex interactive kinase network.

Angiotensin II activates NCC independent of aldosterone (Chapter 2)

In Chapter 2 we hypothesized that angiotensin II can activate NCC independent of aldosterone. To test this, we used normal rats that underwent adrenalectomy to abrogate the endogenous production of aldosterone. All these rats were inserted with osmotic minipumps to chronically deliver angiotensin II, aldosterone or vehicle. During the experiment the rats were housed in metabolic cages to monitor physiological parameters like blood pressure, weight, intake of food and water and the production of 24 h urine. At the end of the experiment rats were sacrificed and blood and kidneys were collected for the measurement of RAAS in the plasma and the abundance of the involved sodium transporters and their related kinases in the kidney. We showed that infusion of both aldosterone and angiotensin II caused a decrease in cumulative 24 h sodium excretion suggesting an increased reabsorption of sodium in the distal nephron. Only the infusion of the pressor-dose of angiotensin II caused hypertension. Aldosterone and angiotensin II increased the abundance and phosphorylation of NCC and the related kinase SPAK. To test whether the increase in sodium reabsorption by angiotensin II was indeed caused by increased NCC activity, we challenged angiotensin II infused rats with hydrochlorothiazide, a specific inhibitor of NCC. Indeed, hydrochlorothiazide could reverse the sodium reabsorption induced by angiotensin II. In conclusion we showed that angiotensin II, independent of aldosterone action, can increase the activity of NCC. These results are important for understanding the molecular physiology of sodium transport in the distal nephron, which not only is essential for maintaining the extracellular fluid volume, but also contributes to the pathophysiology of several forms of hypertension.

Aldosterone does not require angiotensin II to activate NCC (Chapter 3)

In Chapter 3 we hypothesized that aldosterone requires angiotensin II to activate NCC. Again, rats were adrenalectomized and inserted with minipumps to deliver losartan, an angiotensin II receptor blocker. The experimental groups were infused with a physiological and supra-physiological dose of aldosterone. We showed that in the presence of losartan aldosterone was still capable of increasing the abundance and phosphorylation of NCC via a SPAK-WNK4 dependent pathway. In addition we showed that rats receiving aldosterone and losartan showed an increased response to specific inhibitors of NCC and ENaC compared with rats infused with losartan only, adding further proof that the observed Na^+ reabsorption by aldosterone was indeed caused by increased activity of these Na^+ transporters. Finally, we compared rats who received aldosterone and losartan with rats who received aldosterone only. Despite similar plasma aldosterone concentrations, the group of rats with aldosterone only showed increased phosphorylation of NCC suggesting an additive role of angiotensin II and aldosterone. In conclusion aldosterone does not require angiotensin II to activate NCC. This explains a specialized system for the hormonal control of renal salt excretion that is relevant to health and disease.

The two effects of aldosterone (sodium retention versus potassium excretion) can be explained by differential regulation of NCC and ENaC (Chapter 4)

In this study we wanted to gain insight into the two physiological roles of aldosterone, namely how it can induce sodium retention during hypovolemia and potassium secretion during hyperkalemia. We hypothesized that these two effects of aldosterone can be explained by differential regulation of NCC and ENaC. For this study, rats were fed a low Na^+ diet to mimic hypovolemia, a high K^+ diet to mimic hyperkalemia and compared with rats on a normal diet. A fourth group of rats received a combined low Na^+ / high K^+ diet. We showed that during hypovolemia (low Na^+ diet) both aldosterone and angiotensin II increased NCC and ENaC to maximize Na^+ reabsorption. During hyperkalemia (high K^+ diet), aldosterone was increased while the concentration of angiotensin II remained unchanged. Despite the high aldosterone levels NCC was inhibited while ENaC was increased leading to natriuresis and potassium secretion. We don't believe the absence of angiotensin II accounted for the inhibition of NCC because rats on a combined low Na^+/high K^+ diet (with both increased aldosterone and angiotensin II level) still showed inhibition of NCC. WNK4 may be involved in the inhibition of NCC because it was increased in both groups with a high K^+ diet. Summarized, this study sheds new light on the aldosterone paradox by showing differential regulation of NCC and ENaC. During hypovolemia both NCC and ENaC are upregulated to maximize Na^+ reabsorption, during hyperkalemia NCC is inhibited in contrary to ENaC to stimulate K^+ secretion.

NCC and prostasin serve as urinary biomarkers for aldosterone (Chapter 5)

Urinary exosomes are vesicles derived from renal epithelial tubular cells that often contain disease-related proteins. Here, we hypothesized that NCC and prostasin, a serine protease activating ENaC, in urinary exosomes can serve as biomarkers for aldosteronism. We tested this hypothesis in two animal models of aldosteronism and in patients with primary aldosteronism. Phosphorylated NCC (pNCC) and to a lesser extend prostasin were increased in rats infused with aldosterone and rats on a low sodium diet. Patients with primary aldosteronism showed higher pNCC and prostasin in urinary exosomes than patients with essential hypertension. Thus, our study can be regarded as proof of principle that analysis of urinary exosomes can be applied to aldosteronism. Subsequent validation studies will be necessary to define their use in experimental or clinical settings.

Effects of Angiotensin II on sodium transport in the distal nephron (Chapter 6)

The aim of this review was to summarize and to put in perspective the recent findings of angiotensin II on sodium and potassium transport in the aldosterone sensitive distal nephron. Originally it was thought that angiotensin II only regulates sodium reabsorption in the proximal tubule. We and others have shown that angiotensin II also regulates sodium transport in the distal nephron by activating NCC en ENaC and inhibiting of ROMK via a complex kinase-mediated network. In this review we summarize the recent findings of angiotensin II mediated action on Na^+ and K^+ transport in the distal nephron with special interest for the effects on NCC, ENaC, and ROMK.

FUTURE DIRECTIONS

In this thesis we investigated the interdependent roles of aldosterone and angiotensin II on Na^+ and K^+ transporters and their regulatory proteins in the distal nephron. These new insights have partly solved longstanding questions in physiology, but at the same time raise new questions. For example, although the model of NCC regulation is gaining more clarity some of the interactions are still not well understood or even controversial. For example, WNK4 seems to be a negative regulator of NCC under some conditions, but may become a positive regulator in others.[1] The inhibition of NCC by dietary potassium may be mediated by WNK4 [2-4], but leaves the question open through which signal DCT cells "sense" dietary potassium. Pathophysiologically, the role of NCC in "essential" hypertension will likely remain a focus of future studies. The recent linkage of Cullin-3 and Kelch-like 3 to FHHt begs the question whether polymorphisms in these genes exist and whether they may contribute to human hypertension.[5] The discovery that calcineurin inhibitors stimulate NCC to cause hypertension warrants a clinical study to evaluate whether thiazide diuretics are effective drugs to treat this side effect.[6-8] Especially for translational studies it will be important to have a measure of NCC activity *in vivo*. In addition to testing the response to a thiazide diuretic[9], the analysis of NCC in so-called urinary exosomes holds promise.[10] Urinary exosomes are vesicles derived from renal tubular epithelial cells that are thought to reflect the metabolic profile of these cells.[11] We recently showed that the abundance of phosphorylated NCC in urinary exosomes correlated with elevated aldosterone levels in animals and humans. Besides the role of NCC, sodium excretion by the kidney depends on many other sodium transporters, including NHE3, NKCC2, ENaC, and pendrin. Stimuli that activate NCC, sometimes also activate these other transporters, but an opposite, compensatory response may also occur.[12-14] In conclusion, the role of NCC in normal physiology and in the pathophysiology of hypertension is expanding and will likely continue to do so in coming years. To fully grasp the potential of these insights for the treatment of human disease will likely require a more complete understanding of the molecular physiology of this fascinating cotransporter.

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Chapter 8

Nederlandse samenvatting

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Curriculum vitae

List of publications

PhD portfolio

Dankwoord

NEDERLANDSE SAMENVATTING

Het distale nefron van de nier speelt een sleutelrol in de regulatie van bloeddruk door het beïnvloeden van de balans van natrium, kalium en het extracellulair volume. Dit deel van het nefron functioneert via de volgende natrium- en kaliumtransporters: de natrium chloride cotransporter (NCC), het epitheliale natriumkanaal (ENaC), het ROMK-kaliumkanaal en het BK-kaliumkanaal. Al deze transporters zijn gevoelig voor het mineralocorticoïd hormoon aldosteron, dat deel uitmaakt van het renine-angiotensine-aldosteron systeem (RAAS). Aldosteron wordt geproduceerd door de bijnier bij twee fysiologische condities; namelijk bij hypovolemie en hyperkaliëmie. Bij hypovolemie induceert aldosteron retentie van natrium, bij hyperkaliëmie induceert aldosteron juist kaliumsecretie. De observatie dat één hormoon, aldosteron, verschillende effecten heeft op het transport van natrium en kalium, wordt ook wel de "aldosteron paradox" genoemd. Recente studies hebben aangetoond dat behalve aldosteron ook andere homonen (zoals angiotensine II) NCC, ENaC en ROMK kunnen reguleren. Dit gebeurt direct en indirect via een complex interactief netwerk van kinases.

Angiotensine II activeert NCC onafhankelijk van aldosteron (Hoofdstuk 2)

In Hoofdstuk 2 was onze hypothese dat angiotensine II onafhankelijk van aldosteron NCC kan activeren. Om dit te testen gebruikten wij gewone ratten, die een adrenalectomie ondergingen om de endogene productie van aldosteron te stoppen. Bij al deze ratten werd onderhuids een osmotische minipomp geïmplanteerd om chronisch het hormoon angiontensine II, aldosteron, of alleen het oplosmiddel te kunnen infunderen. Tijdens het experiment werden de ratten gehuisvest in een metabole kooi om de fysiologische parameters zoals bloeddruk, gewicht, inname van voedsel en drinken en 24-uurs urine te monitoren. Aan het eind van het experiment werden de ratten geofferd voor het verzamelen van bloed om de plasma RAAS-activiteit te meten en de nieren werden gebruikt voor de bepaling van de expressie van de natrium- en kaliumtransporters en hun gerelateerde kinases. Wij hebben aangetoond dat zowel chronische infusie van aldosteron als angiotensine II een verlaging teweegbracht in de 24-uurs urine natriumexcretie, wat suggereert dat meer natrium werd gereabsorbeerd in het distale nefron. Alleen de infusie van een pressor-dosis van angiotensine II veroorzaakte hypertensie. Zowel aldosteron als angiotensine II verhoogden de expressie en fosforylering van NCC en het gerelateerde kinase SPAK. Hierna rees de vraag of de verhoogde natriumreabsorptie door angiotensine II inderdaad werd veroorzaakt door verhoogde NCC acitiviteit. Om dit te testen stelden wij angiotensine II geïnfundeerde ratten bloot aan hydrochloorthiazide, een specifieke remmer van NCC, en lieten zien dat we de verhoogde natriumreabsorptie weer ongedaan konden maken. Concluderend hebben wij aangetoond dat angiotensine II, onafhankelijk

van aldosteron, de activiteit van NCC kan verhogen. Deze uitkomst is belangrijk om de moleculaire fysiologie van het natriumtransport in het distale nefron te begrijpen. Het distale nefron is niet alleen belangrijk voor het behoud van de extracellulaire volume balans, maar speelt ook een rol in de pathogenese van verschillende vormen van hypertensie.

Aldosteron activeert NCC onafhankelijk van angiotensine II (Hoofdstuk 3)

In Hoofdstuk 3 hypothetiseerden wij dat aldosteron angiotensine II nodig heeft om NCC te activeren. Wederom gebruikten wij ratten die een adrenalectomie ondergingen. Een subcutane minipomp werd geïmplanteerd om losartan, een angiotensine receptor blokker, te infunderen. De twee experimentele groepen ontvingen verder een fysiologische en een suprafysiologische dosis aldosteron. Wij toonden aan dat in losartan behandelde ratten aldosteron nog steeds een verhoogde expressie en fosforylering van NCC kon induceren. De activatie van NCC geschiedde via een enzymnetwerk, waarbij ook de kinases SPAK en WNK4 actief waren. Hierop volgend lieten we zien dat ratten, die zowel losartan als aldosteron ontvingen, een verhoogd effect vertoonden op behandeling met specifieke remmers van NCC en ENaC, wat verder aannemelijk maakt dat de natriumreabsorptie door aldosteron inderdaad werd veroorzaakt door een verhoogde activiteit van deze natriumtransporters. Tot slot vergeleken wij ratten behandeld met aldosteron en losartan, met ratten die alleen met aldosteron werden behandeld. Ondanks dat in beide groepen de plasma aldosteron concentratie gelijk was, had de groep ratten die alleen aldosteron ontvingen een verhoogde fosforylering van NCC in de nier, wat suggereert dat angiotensine II en aldosteron additief op elkaar werken. Concluderend stelden wij dat aldosteron angiotensine II niet nodig heeft om NCC te activeren. Deze uitkomst geeft ons meer inzicht in het hormonale systeem om zoutexcretie te reguleren, wat zowel belangrijk is bij gezondheid als bij ziekte.

De twee effecten van aldosteron (natriumretentie en kaliumsecretie) kunnen worden verklaard door differentiële regulatie van NCC en ENaC (Hoofdstuk 4)

In deze studie wilden we inzicht verkrijgen in de twee fysiologische rollen die aldosteron heeft; namelijk het induceren van natriumretentie bij hypovolemie en kaliumsecretie bij hyperkaliëmie. Onze hypothese was dat deze twee effecten verklaard konden worden door differentiële regulatie van NCC en ENaC. Voor deze studie werden ratten gevoed met een laag natrium dieet en een hoog kalium dieet om respectievelijk hypovolemie en hyperkaliëmie na te bootsen en deze werden vergeleken met ratten die gevoed werden met een normaal dieet. Een vierde groep ratten werd geïntroduceerd die gevoed werd met een gecombineerd laag natrium/hoog kalium dieet. Wij lieten zien dat tijdens hypovolemie (laag natrium dieet) zowel aldosteron als angiotensine II NCC en ENaC activeerden om een maximale natriumreabsorptie te verkrijgen. Tijdens hyperkaliëmie

(hoog kalium dieet) was de plasma aldosteronspiegel verhoogd, maar de spiegel van angiotensine II veranderde niet. Ondanks de hoge plasma aldosteronspiegel werd NCC geremd in tegenstelling tot ENaC, wat resulteerde in natriurese en kaliumsecretie. We geloven niet dat de onverwacht lage angiotensine II- spiegel een rol speelt in de remming van NCC, omdat in het gecombineerde laag natrium/hoog kalium dieet (waarbij zowel de spiegels van angiotensine II als aldosteron verhoogd waren) NCC ook geremd was. Het enzym WNK4 lijkt een rol te spelen bij de remming van NCC, omdat de expressie van dit kinase verhoogd was in beide rattengroepen met het hoge kalium dieet. Samengevat geeft deze studie inzicht in de aldosteron paradox, die verklaard kan worden door differentiële regulatie van NCC en ENaC. Tijdens hypovolemie zijn NCC en ENaC geactiveerd om natrium maximaal te retineren, tijdens hyperkaliëmie is NCC, in tegenstelling tot ENaC, geremd om maximaal kalium te secerneren.

NCC en prostasin in urine kunnen worden gebruikt als biomarkers voor aldosteron (Hoofdstuk 5)

Urine exosomen zijn blaasjes ontstaan uit tubulus epitheelcellen, die ziekte gerelateerde eiwitten kunnen bevatten. Hier was onze hypothese dat NCC en prostasin, een serine protease die ENaC activeert in urine-exosomen, kunnen worden gebruikt als biomarkers voor aldosteron. Wij testten deze hypothese in twee diermodellen met aldosteronisme en in patiënten met primair hyperaldosteronisme. Gefosforyleerd NCC (pNCC) en in mindere mate prostasin waren verhoogd in ratten geïnfundeerd met aldosteron en bij ratten waarbij aldosteron verhoogd was door een laag natrium dieet. Patiënten met primair hyperaldosteronisme hadden meer pNCC en prostasin in urine exosomen dan patiënten met essentiële hypertensie. Onze studie kan worden gezien als bewijs van het principe dat analyse van urine exosomen kan worden gebruikt bij aldosteronisme. Vervolgstudies zijn nodig om het nut hiervan te bepalen in experimentele en klinische settings.

De effecten van angiotensine II op natriumtransport in het distale nefron (Hoofdstuk 6)

Het doel van deze review was om de recente inzichten over de werking van angiotensine II op het natrium- en kaliumtransport in het aldosteron gevoelige distale nefron samen te vatten en te duiden. Oorspronkelijk dacht men dat angiotensine II alleen reabsorptie van natrium in de proximale tubulus reguleerde. Wij en anderen hebben laten zien dat angiotensine II ook natriumtransport in de distale tubulus reguleert, door activatie van NCC en ENaC en remming van ROMK via een complex kinase afhankelijk systeem. In dit review vatten wij de recente ontwikkelingen van de angiotensine II-afhankelijke werking op het natrium- en kaliumtransport in het distale nefron samen, met speciale interesse in NCC, ENaC en ROMK.

ABBREVIATIONS

ANG	Angiotensin
BK	Big conductance calcium-activated potassium
ASDN	Aldosterone sensitive distal nephron
Ca^{2+}	Calcium
CD	Collecting duct
CNT	Connecting tubule
DCT	Distal convoluted tubule
ENaC	Epithelial sodium channel
FHHt	Familial hyperkalemic hypertension
FL	Full-length
HK	High potassium diet
K^+	Potassium
KS	Kidney specific
LS	Low sodium diet
LSHK	Low sodium high potassium diet
Na^+	Sodium
mRNA	Messenger ribonucleic acid
NCC	Sodium chloride cotransporter
NEDD4-2	Neural precursor cell expressed developmentally down-regulated protein 4-2
NHE3	Sodium hydrogen exchanger type 3
NKCC2	Sodium potassium chloride cotransporter isoform 2
OSR1	Oxidative stress responsive protein type 1
pNCC	Phosphorylated sodium chloride cotransporter
pSPAK	Phosphorylated STE20-related, proline alanine-rich kinase
RAAS	Renin-angiotensin-aldosterone system
ROMK	Renal outer medullary potassium channel
SGK1	Serum- and glucocorticoid-inducible kinase 1
SPAK	STE20-related, proline alanine-rich kinase
TAL	Thick ascending limb
Thr	Threonine
WNK	With-no-lysine kinase

CURRICULUM VITAE

Nils van der Lubbe werd geboren op 1 juli 1981 te Leidschendam. Na het voltooien van het Gymnasium aan het Scholengemeenschap Dalton-Vatel in Leidschendam-Voorburg in 1999 startte hij met de opleiding biofarmaceutische wetenschappen aan de Universiteit Leiden. Na het behalen van zijn propedeuse begon hij met de studie geneeskunde binnen dezelfde universiteit. Tijdens zijn wetenschapsstage deed hij onderzoek aan de Universiteit van Barcelona naar variabiliteit in HIV-specifieke epitopen in cytotoxische T-cellen na vaccinatie met autologe denditititische cellen onder leiding van dr. M. Plana. In begin 2007 behaalde hij zijn artsexamen en begon als arts-assistent interne geneeskunde (niet in opleiding) in het Medisch Centrum Haaglanden locatie Westeinde, Den Haag. Eind 2008 begon hij onder leiding van dr. Ewout J. Hoorn en prof. dr. Bob Zietse met wetenschappelijk onderzoek ondersteund door de Nierstichting. Dit vormde uiteindelijk de basis voor dit proefschrift.
Tijdens zijn promotieonderzoek schreef hij zelf een subsidieaanvraag die werd gehonoreerd met een grant van de Netherlands Foundation for Cardiovascular Excellence. Op 1 januari 2013 startte Nils met de opleiding tot internist in het Reinier de Graaf Gasthuis in Delft onder leiding van dr. Ward F.M. Posthuma.

LIST OF PUBLICATIONS

Moes AD, **van der Lubbe N**, Zietse R, Loffing J, Hoorn EJ. The sodium chloride cotransporter SLC12A3: new roles in sodium, potassium, and blood pressure regulation. *Pflugers Arch*. 2014;466:107-18

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PHD PORTFOLIO

PhD candidate:	Nils van der Lubbe
Erasmus MC Department:	Internal Medicine
Research School:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)
Promotor:	Prof. dr. R. Zietse
Supervisor:	Dr. E.J. Hoorn
PhD period:	2008-2012

Courses	Year
Laboratory animal science, EMC	2009
Winter school Dutch kidney foundation, Driebergen	2010
3rd European Masterclass in Renal Physiology & Pathophysiology for the Clinician, Geneva	2010
Biomedical English writing, EMC	2011

Presentations	Year
Linking the WNK's	
Platform AOI's Nephrology (PLAN) day, Rotterdam (oral)	2009
Ang II induces phosphorylation of the thiazide-sensitive NCC independent of aldosterone	
Science days internal medicine EMC, Antwerp (oral)	2010
Dutch nephrology days, Velthoven (oral)	2010
Pre-ASN epithelial transport meeting, Denver (oral)	2010
American Society of Nephrology (ASN) Kidney Meeting, Denver (poster)	2010
Aldosterone does not require angiotensin II to activate NCC through a WNK4–SPAK–dependent pathway	
Science days internal medicine EMC, Antwerp (poster)	2011
Dutch nephrology days, Velthoven (oral)	2011
Pre-ASN epithelial transport meeting, Philadelphia (oral)	2011
American Society of Nephrology (ASN) Kidney Meeting, Philadelphia (poster)	2011

Presentations	Year
The phosphorylated NCC in urinary exosomes is superior to prostasin as a marker for aldosteronism	
Dutch nephrology days, Velthoven (oral)	2012
Benelux kidney meeting, Eindhoven (oral)	2012
Science days internal medicine EMC, Antwerp (poster)	2012
K^+-induced natriuresis is preserved during Na^+ depletion and accompanied by inhibition of NCC	
Pre-ASN epithelial transport meeting, San Diego (oral)	2012
American Society of Nephrology (ASN) Kidney Meeting, San Diego (poster)	2012
Science meeting internal medicine EMC, Antwerp (oral)	2013
Dutch nephrology days (oral)	2013

International conferences	
American Society of Nephrology (ASN) Kidney Meeting, San Diego	2009
American Society of Nephrology (ASN) Kidney Meeting, Denver	2010
American Society of Nephrology (ASN) Kidney Meeting, Philadelphia	2011
American Society of Nephrology (ASN) Kidney Meeting, San Diego	2012
Benelux kidney meeting, Eindhoven	2012

Teaching activities	
Supervising a research project of a medical student (6 months)	2012

Memberships	
Head of the committee Platform AIO's Nephrology (PLAN)	2010-2012

Grants & Prizes	
Netherlands Foundation of Cardiovascular Excellence, research grant, €100.000,-	2011
Science days internal medicine, Antwerp, best poster presentation	2012
Benelux Kidney Meeting, Eindhoven, best oral presentation	2012

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