Effects of Immunosuppressive Drugs on Blood Pressure and Electrolyte Homeostasis

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Effecten van immunosuppressiva op bloeddruk en elektrolyt huishouding

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CHAPTER 1

General introduction and aims of the thesis

The sodium chloride cotransporter

The thiazide-sensitive sodium chloride cotransporter (NCC) is part of the solute carrier family 12 (SLC12), and is encoded by the SLC12A3 gene [1]. The presence of NCC was first reported in 1975 [2]. A decade later Stokes and colleagues discovered that thiazide diuretics are able to block NCC, and finally the cDNA encoding this transporter was isolated [3, 4]. NCC is primarily expressed in the kidney, but also in intestine and bone [5, 6]. In the kidney, NCC is mainly located in the early part of the distal convoluted tubule (DCT1), and to a lesser degree in the later part of the DCT (DCT2; Figure 1) [7]. In DCT2 NCC co-localizes with the epithelial sodium channel (ENaC). The pivotal role of NCC is transport of sodium chloride across the apical plasma membrane of renal epithelial cells [8]. In addition, NCC indirectly affects transcellular magnesium and calcium reabsorption in the DCT through interaction with the transporters transient receptor potential melastatin 6 (TRPM6) and transient receptor potential cation channel subfamily V member 5 (TRPV5), respectively [9, 10]. The activity of NCC is regulated by phosphorylation [11].

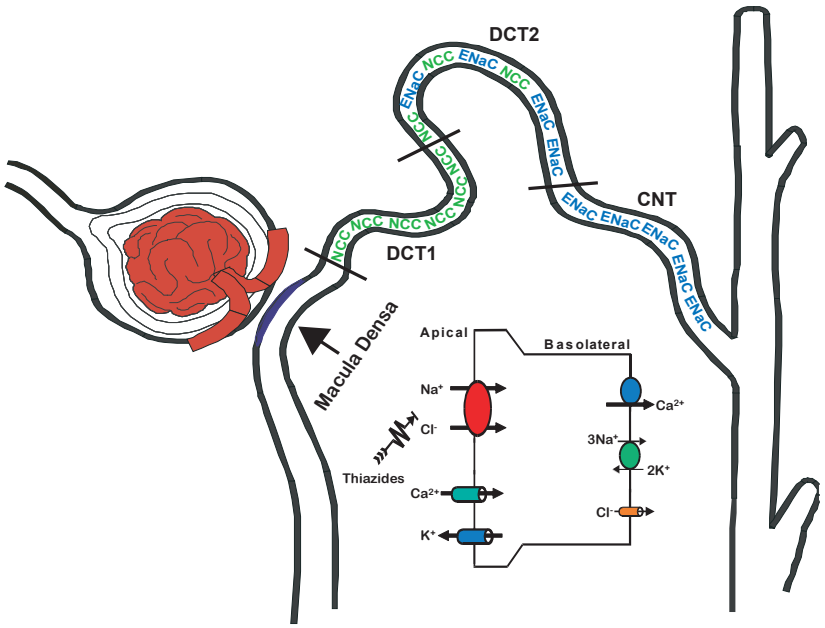

Figure 1 Sodium reabsorption in the distal nephron.

The localization of the apical sodium transporters in the aldosterone sensitive distal nephron is depicted. NCC is expressed in the DCT1 and DCT2, while ENaC is expressed in DCT2 and the connecting tubule (CNT). A schematic overview of a renal epithelial cell from DCT1 is also shown, in which NCC is located on the apical plasma membrane. This figure was reproduced from [1] with kind permission.

Inactive NCC is stored in subapical vesicles located in the cytosol of epithelial cells. In order for NCC to be phosphorylated, it is essential that NCC is first mounted into the apical membrane via trafficking [12, 13]. Although NCC reabsorbs only 5 to 10% of filtered sodium, it is important for the fine-tuning of urinary sodium excretion in response to various hormonal and non-hormonal stimuli. It is able to do so because it is not affected by the tubuloglomerular feedback mechanism in the macula densa (Figure 1) [1]. The mineralocorticoid hormone aldosterone regulates NCC, and is able to activate NCC both through phosphorylation and upregulation of total NCC abundance [14, 15]. Other hormones known to stimulate NCC are angiotensin (ANG) II, glucocorticoids, vasopressin, and insulin [12, 16-18]. More recently, dietary potassium was found to have an inhibitory effect on NCC [19]. Several animal studies showed the effect of oral potassium loading on NCC occurred within minutes, was aldosterone independent, acted through reduction of total NCC abundance or NCC dephosphorylation and still occurred during a low sodium diet [19, 20].

Two rare human monogenetic diseases are known to affect NCC activity, and illustrate the importance of NCC for blood pressure and electrolyte balance. Inactivating mutations of NCC cause the autosomal recessive disorder Gitelman syndrome [21]. This syndrome is characterized by hypokalemia, hypomagnesemia, metabolic alkalosis, hypocalciuria, and low to normal arterial blood pressure. The disease causing the opposite phenotype is called familial hyperkalemic hypertension (FHHt), also known as Gordon syndrome or pseudohypoaldosteronism type II [22]. In contrast to Gitelman syndrome, FHHt is not caused by a mutation in NCC, but by mutations in the signaling cascade of NCC [23-25]. FHHt is characterized by hyperkalemia, hypertension, hypercalciuria, and metabolic acidosis [22]. The side effects of the immunosuppressive drug tacrolimus strikingly resemble the phenotype of FHHt. This observation led to the discovery that the immunosuppressive drug tacrolimus, a calcineurin inhibitor, activates NCC to cause hypertension [26]. Tacrolimus failed to induce hypertension in NCC knockout mice, whereas it caused more severe hypertension in mice overexpressing NCC. Cyclosporine, another calcineurin inhibitor, also activates NCC [27]. Calcineurin inhibitors do not activate NCC directly, but influence the signaling cascade of NCC [27]. Thiazide diuretics inhibit NCC and these drugs were implemented clinically in 1957, long before it became apparent that their primary target is NCC [28]. Thiazide diuretics are still among the most commonly used drugs to treat hypertension worldwide. They are well tolerated and inexpensive.

Sodium and potassium balance and the aldosterone paradox

Maintaining total body sodium and potassium balance is essential to the survival of most species. Hypovolemia (sodium deficit) and hyperkalemia (potassium surplus) cause different responses to maintain homeostasis. During hypovolemia, blood pressure and organ perfusion must be guaranteed. Hypovolemia activates the renin-angiotensin system, enhancing aldosterone secretion in order to retain sodium in the distal nephron. Conversely, during hyperkalemia, potassium secretion is stimulated to avoid cardiac and neuromuscular complications; this process is also mediated by aldosterone. The observation that aldosterone has different effects on renal sodium and potassium transport, depending on the physiological situation, has been termed the "aldosterone paradox" [29, 30]. Aldosterone acts in the distal nephron, which consists of the DCT1, DCT2, connecting tubule (CNT), and collecting duct (Figure 1) [1, 31]. In the aldosterone sensitive distal nephron four apical sodium and potassium transport proteins are involved, including NCC, ENaC (Figure 1), the renal outer medullary potassium channel (ROMK), and the large-conductance Ca2+-activated potassium (BK) channel [14, 32-35]. ANG II is another important hormone involved in controlling sodium and potassium transport in the DCT. ANG II stimulates NCC and ENaC, but inhibits ROMK [36]. Increased sodium reabsorption in the DCT will reduce the delivery of sodium to the collecting duct, limiting sodium-coupled potassium secretion in that segment. Only hypovolemia is accompanied by high ANG II levels. Together, these effects favor electroneutral sodium reabsorption while preventing potassium secretion [37]. Conversely, a high potassium diet increases ROMK [38], but is also able to inhibit NCC [19, 38, 39]. Still unanswered, however, is the question of how the kidneys respond to the combination of hypovolemia and hyperkalemia.

Immunosuppressive drugs

Calcineurin inhibitors

The calcineurin inhibitors (CNIs) tacrolimus and cyclosporine are the most frequently used drugs to prevent rejection after organ transplantation [40]. In addition, CNIs are also used in the treatment of autoimmune diseases such as inflammatory bowel disease, psoriasis and systemic lupus erythematodes [41]. Calci¬neurin is a phos¬phatase that dephosphorylates the cyto¬plasmatic nuclear factor of activated T-cells (NFATc) [42]. Dephosphorylation of NFATc increases the transcrip¬tional activation of early cytokine genes such as IL-2, IL-3, IL-4 and TNF-α. Inhibition of calcineurin therefore prevents the pro-inflamma¬tory response in T cells after interaction with antigen-presenting cells. This immunological mechanism of action

explains the efficacy of CNIs to prevent rejection. CNIs, however, have important side effects including nephro¬toxicity, neurotoxicity and several metabolic disorders. Hypertension is another promi¬nent side effect of CNIs and is estimated to occur in 20–70% of patients using CNIs [43, 44]. Hypertension after kidney transplantation is an independent risk factor for graft failure and is associated with cardiovascular disease and even mortality in the recipient [45-47]. The pathophysiology of hypertension after kidney transplantation is multifactorial. However, treatment with CNIs clearly contributes to the development of hypertension after kidney transplantation. The incidence of post-transplantation hypertension clearly increased after the introduction of cyclosporine [48-50]. Furthermore, CNIs have been shown to induce hypertension in patients without kidney disease, for example in patients with psoriasis or liver transplant recipients [43, 51, 52]. Several mechanisms contribute to CNI-induced hypertension [41, 44]. The vascular effects of CNIs are well known and include systemic and renal vasoconstriction, possibly through endothelin 1, and impaired vasodilation, through reduced nitric oxide [53-55]. This probably explains the efficacy of dihydropyridine calcium channel blockers (CCBs) for the treatment of CNI-induced hypertension [56]. CNIs also affect the renin-angiotensin system and the sympathetic nervous system, which may contribute further to hypertension. Of interest, CNI-induced hypertension is a salt-sensitive form of hypertension [57-61]. More recently, the salt sensitivity of CNI-induced hypertension was linked to the activation of one specific sodium transporter in the kidney. We and others showed that CNIs activate NCC to cause hypertension [26, 27]. These studies were all performed in laboratory animals and suggest that thiazide diuretics might be effective drugs in lowering blood pressure in patients with CNI-induced hypertension. However, no randomized clinical trials have been performed to address this question.

Calcineurin inhibitors and new-onset diabetes after transplantation

Posttransplantation diabetes mellitus (PTDM) is a common and serious complication in kidney transplant recipients (KTRs) [62-66]. PTDM has an incidence of 5-25% in KTRs and is associated with worse graft and recipient outcomes [64, 66-85]. Several risk factors have been identified and include older age, higher body mass index, corticosteroid use, CNI use, and hypomagnesemia [63, 65, 67, 73, 74, 77, 86-95]. CNIs downregulate TRPM6, a channel in the distal convoluted tubule involved in the reabsorption of magnesium, causing hypomagnesemia [96]. Because of the effects of CNIs on TRPM6, hypomagnesemia is a common finding in KTRs [63, 72, 76, 94]. Retrospective studies have implicated CNI-induced hypomagnesemia in the

pathogenesis of PTDM [63, 73, 94]. Another possible factor in the development of PTDM is the transcription factor hepatocyte nuclear factor 1β (HNF1β), which is involved in renal magnesium reabsorption and insulin secretion [97]. Patients with mutations in HNF1β develop both hypomagnesemia and diabetes [97, 98]. Furthermore, single nucleotide polymorphisms (SNPs) in HNF1β are associated with the development of diabetes in the general population [99-104]. To date, only one previous study investigated the HNF1β SNPs and PTDM, but found no association [95].

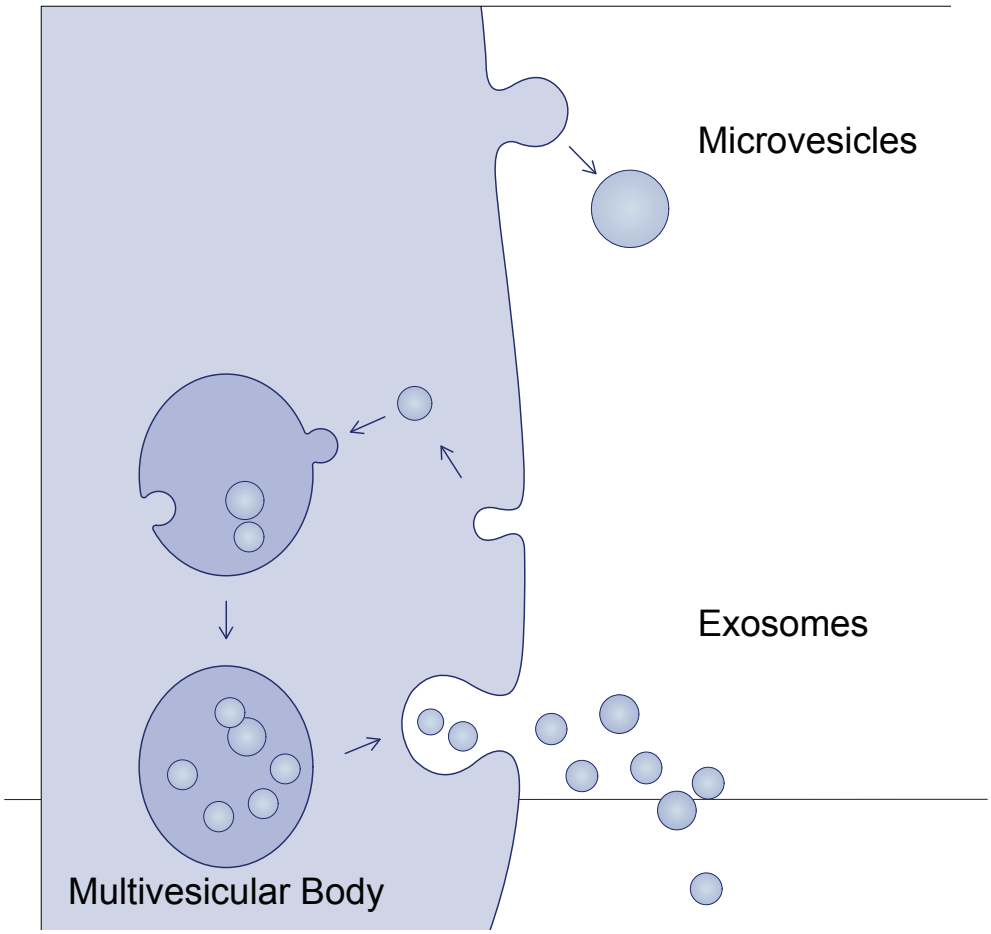

Figure 2 Formation of uEVs, consisting of microvesicles and exosomes.
A cell is depicted with an overview of the two different forms of uEV formation. Microvesicles (top) are formed by outward budding from the plasma membrane. Exosomes (bottom) are formed by endocytosis, followed by fusion with and formation within multivesicular bodies, and finally the release of multivesicular body content into the extracellular space. This figure was reproduced from Mahdi Salih with kind permission.

Mycophenolate mofetil

Mycophenolate mofetil (MMF) is an immunosuppressive drug that was introduced over 20 years ago [105]. MMF is a pro-drug which is converted to mycophenolic acid (MPA) following exposure to esterase in the liver . MPA is the active compound that inhibits inosine 5'-monophosphate dehydrogenase (IMPDH), which subsequently leads to suppression of proliferation of both B and T lymphocytes [105, 106]. MMF is a first-line drug in the field of solid organ transplantation and is currently prescribed to the vast majority of KTRs in Europe and the United States of America [106, 107]. MMF is both used directly following transplantation and as maintenance therapy [108]. MMF is a consistent component of various immunosuppressive regimens [107]. MMF has also been used in many different animal studies studying the antihypertensive effects of immunosuppression. It has been shown that MMF prevented the development of salt-sensitive hypertension during ANG II infusion [109], normalized blood pressure in spontaneous hypertensive rats (SHRs) and Dahl salt-sensitive rats [109, 110], and attenuated hypertension and albuminuria in uninephrectomized rats treated with DOCA-salt [111]. Similarly, in patients with psoriasis or rheumatoid arthritis treatment with MMF attenuated hypertension [112]. It is incompletely understood how pharmacological inhibition of B and T cells with MMF prevents hypertension.

Urinary extracellular vesicles

Extracellular vesicles are nanosized membranous vesicles that have been isolated from various body fluids, including urine [113]. Urinary extracellular vesicles (uEVs) are released from all cells lining the nephron and the urinary tract. uEVs are released by direct shedding from the plasma membrane (microvesicles) or via fusion of intracellular multivesicular bodies with the plasma membrane (exosomes, Figure 2) [113]. The content of uEVs appears to reflect cellular homeostasis. Therefore, uEVs have been studied as non-invasive biomarkers for renal tubular disorders [113-116]. For example, patients with FHHt have an increased NCC abundance in uEVs [117-119]. Patients with Gitelman syndrome, in which NCC is inactivated, exhibit a corresponding decrease in NCC abundance in uEVs [116, 120].

Aims of the thesis

1. To review the recently identified roles of NCC in the regulation of sodium, potassium, and blood pressure (Chapter 2)
2. To analyze the regulation of NCC during potassium-induced natriuresis (Chapter 3)
3. To review the role of pharmacogenetics in CNI-induced hypertension (Chapter 4)
4. To test the anti-hypertensive effects of thiazide diuretics in hypertensive kidney transplant recipients using tacrolimus (Chapter 5)
5. To analyze the effects of CNIs on NCC in uEVs (Chapter 6)
6. To identify the mechanism of the anti-hypertensive effect of MMF in experimental hypertension (Chapter 7)
7. To analyze the relationship between calcineurin inhibitors, serum magnesium, and posttransplantation diabetes mellitus (Chapter 8)

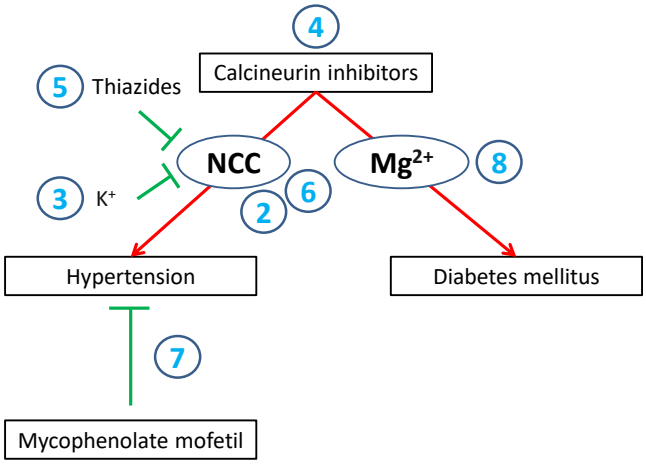

Figure 3 Aims of the thesis.

The numbers refer to the chapters in this thesis. Chapters 2 and 4 are reviews, focusing on NCC and CNIs, respectively. In Chapters 3 and 5 we studied the inhibitory effects of potassium and thiazides on NCC, respectively. In Chapter 6 we analyzed the effects of CNIs on NCC in uEVs. In Chapter 7 we studied the mechanisms underlying the antihypertensive effects of MMF. Finally, in Chapter 8 we analyzed the effect of calcineurin inhibitors on serum magnesium and the subsequent effect of magnesium on posttransplantation diabetes mellitus (PTDM).

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PART I

CHAPTER 2

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

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Abstract

SLC12A3 encodes the thiazide-sensitive sodium chloride cotransporter (NCC), which is primarily expressed in the kidney, but also in intestine and bone. In the kidney, NCC is located in the apical plasma membrane of epithelial cells in the distal convoluted tubule. Although NCC reabsorbs only 5 to 10 % of filtered sodium, it is important for the fine-tuning of renal sodium excretion in response to various hormonal and non-hormonal stimuli. Several new roles for NCC in the regulation of sodium, potassium, and blood pressure have been unraveled recently. For example, the recent discoveries that NCC is activated by angiotensin II but inhibited by dietary potassium shed light on how the kidney handles sodium during hypovolemia (high angiotensin II) and hyperkalemia. The additive effect of angiotensin II and aldosterone maximizes sodium reabsorption during hypovolemia, whereas the inhibitory effect of potassium on NCC increases delivery of sodium to the potassium-secreting portion of the nephron. In addition, great steps have been made in unraveling the molecular machinery that controls NCC. This complex network consists of kinases and ubiquitinases, including WNKs, SGK1, SPAK, Nedd4-2, Cullin-3, and Kelch-like 3. The pathophysiological significance of this network is illustrated by the fact that modification of each individual protein in the network changes NCC activity and results in salt-dependent hypotension or hypertension. This review aims to summarize these new insights in an integrated manner while identifying unanswered questions.

Keywords: Aldosterone; Angiotensin II; Hypertension; Tacrolimus; Thiazide; WNK kinase.

Typical hallmarks of NCC

The presence of a sodium chloride cotransporter (NCC) was first suggested in urinary bladder of the winter flounder [95, 96]. Subsequent studies in this tissue demonstrated that this cotransporter could be inhibited by thiazide diuretics [111] and the cDNA encoding this transporter was isolated [31]. Studies using micropuncture and isolated perfused tubules had identified the same pharmacological and kinetic transport characteristics in the early distal convoluted tubule (DCT) of rat kidney [23, 60]. Indeed, Gamba and colleagues succeeded in isolating cDNA of the sodium chloride cotransporter from rat kidney [30]. NCC is encoded by the SLC12A3 gene (55 kb, 26 exons) and belongs to the SLC12 family of electroneutral cation chloride cotransporters [29]. Besides the kidney, NCC was shown to be expressed in intestine

and bone where it is likely involved in sodium and calcium absorption [4, 20]. It has been suggested that NCC is expressed in various other tissues but this has not been confirmed [29]. In the kidney, NCC is located in the early part of the DCT (also called DCT1) (Fig. 1), but gradually decreases along the later part of the DCT (DCT2), where it co-localizes with the epithelial sodium channel (ENaC) [3]. The NCC mediates the reabsorption of sodium across the apical membrane of the DCT (Fig. 1) [23]. Chronic activation or inhibition of NCC is usually accompanied by morphological changes in the DCT resulting in hypertrophy or atrophy [21, 61, 66, 126]. The gradient required for sodium chloride transport through NCC is generated and maintained by the basolateral sodium–potassium ATPase pump. The potassium that enters the cell via this pump is recycled by basolateral potassium transporters. In addition, potassium may also be secreted apically by the renal outer medullary potassium channel (ROMK) and a potassium chloride cotransporter [133]. In addition, NCC

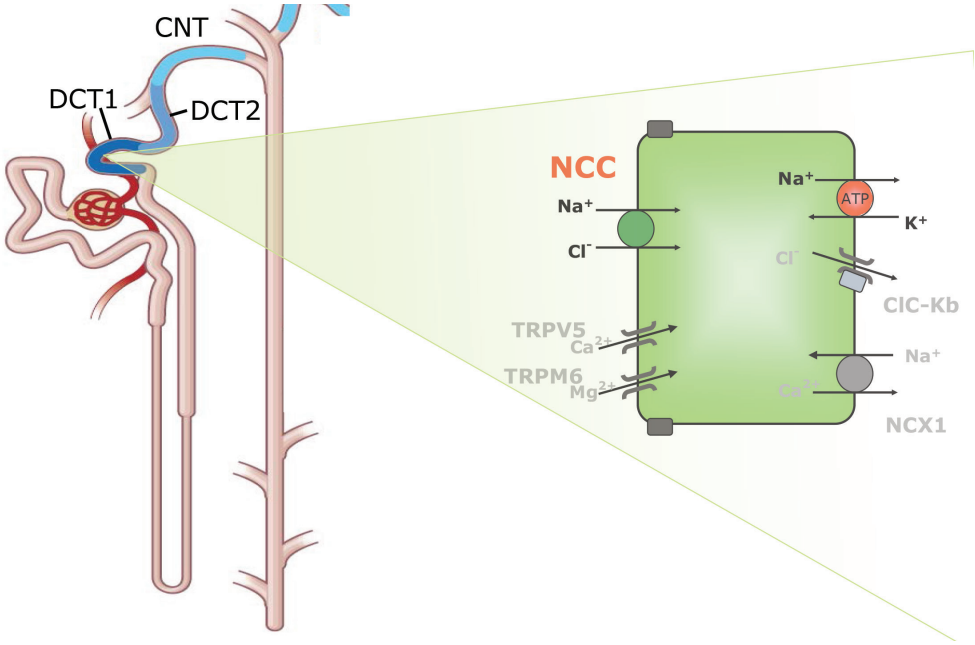

Figure 1 Model of transcellular transport in the early distal convoluted tubule (DCT).
A kidney tubule is shown schematically on the left indicating the locations of DCT type 1 and type 2 (DCT1, DCT2) and the connecting tubule (CNT). The sodium chloride cotransporter (NCC) is primarily expressed in DCT1. 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 [5, 59].

modulates transcellular magnesium and calcium reabsorption in the DCT through interaction with the transporters TRPM6 and TRVP5, respectively (Fig. 1) [16, 85]. Only 5 to 10 % of the filtered load of sodium is reabsorbed in the DCT, and this is primarily mediated by NCC [23]. Despite this modest contribution to overall sodium reabsorption, the NCC in the DCT together with ENaC in the connecting tubule (CNT) and the collecting duct (CD) fine-tunes the final concentration of sodium chloride in the urine. This is possible because it is not affected by tubuloglomerular feedback [29]. Due to this property, NCC plays a pivotal role in extracellular fluid volume and blood pressure control [29].

Structure–function relationship

In humans, NCC is a membrane glycoprotein of 1,021-amino acid residues which resembles the general topology of the sodium–potassium–chloride cotransporters 1 and 2 (NKCC 1 and 2) [70]. NCC is able to form dimers, and it is likely that it functions as a dimer [18, 29]. It consists of 12 putative transmembrane (TM) spanning regions with a central hydrophobic domain (Fig. 2). Between TM7 and TM8, there is a large

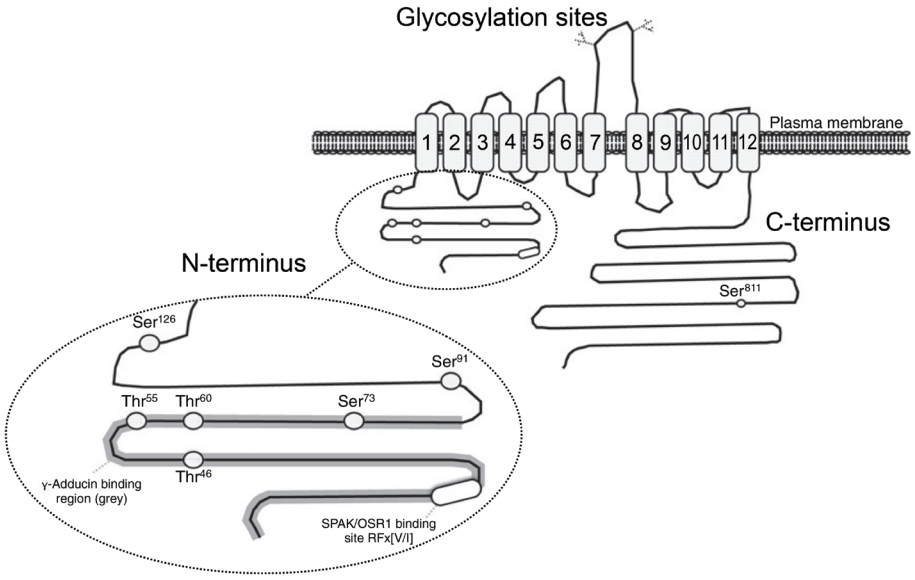

Figure 2 Putative structure of the sodium chloride cotransporter.

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 [19] with kind permission.

extracellular hydrophilic loop that contains two glycosylation sites (N404 and N424) [50]. These glycosylation sites are essential for functioning and surface expression of NCC [40]. When glycosylation is eliminated, thiazide diuretics have much greater access to their binding sites, which suggests that glycosylation blocks the affinity for thiazides [50]. A study that interchanged the transmembrane regions between rat and flounder NCC revealed that affinity-modifying residues for chloride are located within the TM1–7 region [78]. Especially, a highly conserved glycine within TM4 plays a crucial role for the affinity of chloride [78]. The TM8–12 region, distal to the extracellular loop, is sensitive to thiazides. Site-directed mutagenesis was used to show that a single residue in TM11 defines the different affinity for thiazides between mammalian and flounder NCC [12]. Both transmembrane regions appear to have affinity for extracellular sodium. The central domain is flanked by a short amino-terminal domain (N-terminus) and a long carboxy-terminal domain (C-terminus) which are both located intracellularly (Fig. 2) [29]. The N-terminus of NCC contains several conserved phosphorylation sites including threonine 46, 55, and 60 and serine 73 and 91 in humans [19]. In rats and mice, these phosphorylation sites correspond to threonine 44, 53, and 58 and serine 71, 89, and 124. Phosphorylation of NCC appears to determine its activity, especially at threonine 60 in humans or 58 in rat. Indeed, a mutation in threonine 60 is a common cause of Gitelman syndrome [65], the disorder resulting from NCC inactivation (see further). The development of a knock-in mouse model with this mutation exhibits the Gitelman phenotype and did so because mutant NCC was restricted to the cytosol [135]. 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 [91]. This suggests that, apart from phosphorylation, the trafficking of NCC from subapical vesicles to the plasma membrane is also important [42].

Physiological functions

NCC is highly regulated by hormones, including aldosterone, angiotensin II, glucocorticoids, estrogen, insulin, norepinephrine, and vasopressin (Table 1) [13, 53, 82, 83, 91, 108, 118, 124, 125]. The fact that NCC is regulated by so many different hormones suggests that sodium reabsorption through NCC is an important homeostatic control mechanism. Aldosterone was the first hormone recognized to be capable of activating NCC [53], and the DCT is therefore part of what has been called the “aldosterone-sensitive distal nephron” [77], which was defined to comprise the DCT2, the CNT, and the CD [67]. Experimentally, aldosterone upregulates NCC both when it is directly infused or when it is secreted

in response to a low sodium diet [14, 53]. The acute effect of aldosterone only involves phosphorylation of NCC [56], whereas the chronic effect also increases the total protein abundance of NCC [53], which likely occurs independent from changes to NCC mRNA levels [1, 69, 119]. 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 [6]. The discovery that angiotensin II is also capable of activating NCC was more surprising

Table 1 Hormones, metabolic stimuli and drugs influencing NCC activity

	Stimulus	Effect	References
Hormones	Angiotensin II	Stimulatory	[118]
	Aldosterone	Stimulatory¶	[53]
	Glucocorticoids	Stimulatory	[124]
	Vasopressin	Stimulatory	[83, 91, 104]
	Insulin	Stimulatory	[13, 57, 108, 109]
	Estrogen	Stimulatory	[125]
	Norepinephrine	Stimulatory	[82]
Metabolic stimuli	Dietary potassium	Inhibitory	[110, 120]
	Dietary sodium*	Inhibitory	[14, 103]
	Dietary magnesium*	Stimulatory	[25]
	Acidosis*	Stimulatory	[26]
Drugs	Thiazide diuretics	Inhibitory	[16, 23]
	Furosemide*	Stimulatory	[1]
	Tacrolimus	Stimulatory	[44]
	Cyclosporine†	Stimulatory	[76]
	Cisplatin	Inhibitory	[116]

Footnote: ¶ When high dietary K increases aldosterone, NCC may be inhibited; * Some of these effects may be mediated through aldosterone; † In a model of cyclosporine nephrotoxicity, NCC was downregulated, but this may be attributed to kidney failure and reduced renin-angiotensin activity [76].

because its actions were believed to be confined to the proximal tubule [75]. By adrenalectomizing rats and selectively re-infusing aldosterone or angiotensin II, we were able to dissect the stimulatory effects of angiotensin II and aldosterone on NCC [118]. In a subsequent study, we showed that aldosterone did not require angiotensin II for activation of NCC, although the presence of both stimuli led to an additive response [119]. This additive response may be useful to maximize sodium reabsorption during hypovolemia because this is characterized by elevated plasma levels of both angiotensin II and aldosterone [122]. Increased sodium reabsorption in the DCT by NCC will also decrease the delivery of sodium to the CNT and CD [42]. Because in these segments sodium reabsorption is electrochemically coupled to potassium secretion, decreased delivery of sodium will help conserve potassium. In line with this, recent studies have shown that angiotensin II directly inhibits ROMK, further contributing to potassium conservation during hypovolemia [49, 129, 140]. The effects of angiotensin II on NCC and ROMK therefore help to understand the aldosterone paradox, the question how aldosterone increases sodium reabsorption during hypovolemia, but potassium secretion during hyperkalemia [3, 122]. Further insight into the aldosterone paradox comes from the effects of potassium on

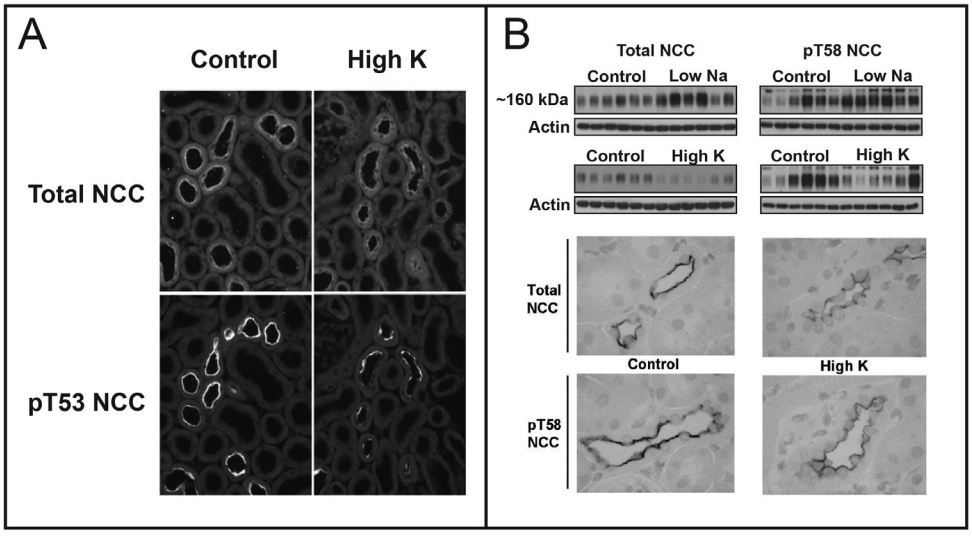

Figure 3 Inhibition of the sodium chloride cotransporter by dietary potassium ("high K").
The results of our recent studies on the inhibitory effect of dietary potassium on NCC are shown [110, 121]. Panel A shows that dietary potassium acutely downregulates phosphorylated NCC but not total NCC. Conversely, panel B shows that a chronic high potassium diet primarily decreased total NCC, but phosphorylated NCC less so. The high potassium diet was 2% and 5%, respectively, and the low sodium diet was < 0.001% [110, 121].

NCC. Recent studies have shown that dietary potassium inhibits NCC [110, 121]. Gastric gavage of potassium in mice increased both urinary potassium and sodium excretion [110]. This response occurred within minutes and was independent from aldosterone because it could also be induced in aldosterone-deficient animals. In the kidney, this was accompanied by dephosphorylation of NCC (Fig. 3a). The increased delivery of sodium to the more distal nephron parts facilitates sodium–potassium exchange and therefore kaliuresis. This "potassium-induced natriuresis" appears to constitute an important physiological response. Namely, potassium-induced natriuresis could still be evoked when a high potassium diet was combined with a low sodium diet [121]. This was accompanied by a reduced abundance of total NCC; phosphorylated NCC was also reduced, although this did not reach statistical significance (Fig. 3b). Taken together, these and other recent results seem to suggest that when the organism is faced with the choice between conserving sodium or secreting potassium, it chooses the latter [27]. The focus of future research will be to identify the signal by which dietary potassium induces NCC downregulation. Similar to high dietary potassium, high dietary sodium also suppresses NCC [14]. This response, however, is not as rapid as the one for high potassium diet [110] and involves a decrease in the plasma membrane abundance of sodium transporters all along the nephron [28, 137]. Furthermore, the effect of high dietary sodium on NCC appears to be mediated through aldosterone [14], although the effect of high dietary sodium on NCC has not been studied in the absence of aldosterone. Similarly, chronic metabolic acidosis also increases aldosterone and therefore NCC [26]. Why other hormones such as insulin [13, 57, 108, 109], vasopressin [83, 91, 104], estrogen [125], and norepinephrine [82] regulate NCC is less clearly defined (Table 1), but warrants further study given the role of these hormones in normal physiology and human diseases such as diabetes mellitus, obesity, and hypertension.

The NCC signaling cascade

Kinases

The intracellular signaling cascade that controls NCC activity has largely been unraveled in recent years (Figure 4). This NCC signaling cascade consists of a multikinase network which includes the kinases WNK, SPAK, OSR1, and SGK1 [130]. More recently, proteins involved in ubiquitylation including Nedd4-2, Kelch-like 3, and Cullin 3 were also found to regulate NCC [2, 7, 68, 99]. 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 syndrome, see also "Relation to disease" below). WNKs appear to modulate

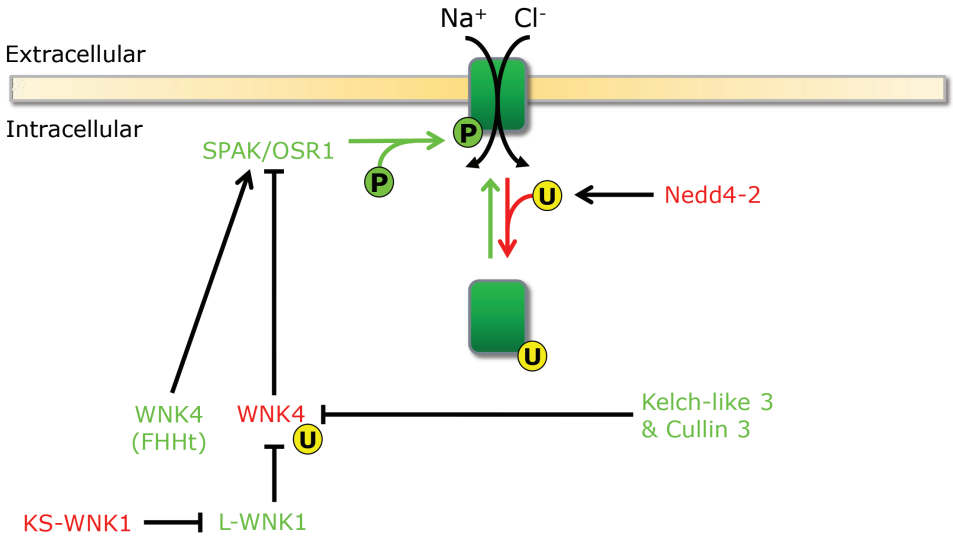

Figure 4 Current model of sodium chloride cotransporter regulation by kinases and ubiquitinases. The various interactions of the NCC regulatory pathway are shown as arrows (stimulatory) or as lines ending with perpendicular lines (inhibitory). Phosphorylation is indicated with the symbol "P", whereas 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. Although WNK3 has been shown to interact with WNK4 and SPAK [90, 135], its precise role in NCC regulation remains less clear and we therefore decided not to include it. See text for further details.

both the "trafficking" and phosphorylation of NCC [41], although most of our knowledge has been derived from studies in oocytes. The regulation of NCC trafficking by WNKs involves a sequential inhibitory cascade, in which KS-WNK1 inhibits WNK1, WNK1 inhibits WNK4 [139], and WNK4 inhibits NCC [134]. The inhibition of NCC by WNK4 is not caused by endocytosis [35], but rather by promoting lysosomal degradation [112]. This inhibition is mediated by the ERK1/2 signaling pathway and the lysosomal targeting receptor sortilin [141, 142]. Interestingly, angiotensin II converts WNK4 from an inhibitor to an activator of NCC [101]. In contrast to WNK4, WNK3 stimulates NCC [97], but its actions are less well-defined. WNK3 and WNK4 not only have divergent effects on NCC but they also antagonize each other. Indeed, it appears to be the ratio between WNK3 and WNK4 that determines the net effect on NCC [138]. The phosphorylation of NCC is mediated by SPAK [79]; several WNKs

interact with SPAK and therefore indirectly control the phoshorylation step of NCC. Interactions between SPAK and WNK1 [79], WNK3 [34], and WNK4 [102] have been reported. However, the brain but not the kidney isoform of WNK3 can activate NCC and does so through a SPAK-independent mechanism [34]. 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 [73]. KS-SPAK, which lacks the kinase domain, inhibits FL-SPAK and OSR1, which are both known to phosphorylate NKCC2. This may explain why in mice the knockout of SPAK results in decreased NCC phosphorylation (absence of full-length SPAK), but increased NKCC2 phosphorylation (no inhibition of FL-SPAK or OSR1 by KS-SPAK) [36, 73]. SPAK deficiency, however, does not completely inhibit NCC phosphorylation [73, 136], suggesting the involvement of other kinases or phosphatases. Both SPAK isoforms are also involved in the stimulatory effect of vasopressin on NCC and NKCC2 [83, 91]. 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 [104]. Although SGK1 was first recognized as an activator of ENaC [127], later reports also showed effects on NCC [115]. SGK1 and NCC do not seem to interact directly, but rather through WNK4 and Nedd4-2 [2, 98]. SGK1 phosphorylates WNK4 and this phosphorylation step reduces the inhibition of WNK4 on NCC [98, 100]. Because SGK1 is sensitive to aldosterone, this pathway appears to be involved in the activation of NCC by aldosterone [100]. The opposite is also true because SGK1 knockout mice failed to increase NCC activity during a low sodium diet [115].

Ubiquitin ligases

Recent data indicate that Nedd4-2 is yet another player in the pathway by which aldosterone activates NCC (Fig. 4) [2, 99]. Nedd4-2 was shown to stimulate ubiquitylation of NCC and decreased its activity and surface expression in vitro and in vivo, while SGK1 prevented these effects [2]. The pathophysiological significance of the regulation of NCC by Nedd4-2 was shown by the generation of inducible nephron-specific Nedd4-2 knockout mice [99]. 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. Similar to Nedd4-2, Kelch-like 3 and Cullin 3 are also involved in ubiquitylation because they are components of an E3 ubiquitin

ligase complex. Although mutations in Kelch-like 3 and Cullin-3 cause hyperkalemic hypertension that is reversible with thiazides, these proteins probably do not interact directly with NCC. Instead, Kelch-like 3 binds and ubiquitinates WNK4, and the subsequent degradation of WNK4 would be expected to increase NCC activity (Fig. 4) [88, 106, 128, 132].

Other signaling molecules

Although the network reviewed above is largely interconnected (Fig. 4), additional NCC regulatory pathways exist. One example is the regulation of NCC by phorbol esters [54]. This effect is mediated through Ras guanyl-releasing protein 1 and ERK1/2, which stimulates ubiquitylation and endocytosis of NCC [55]. Furthermore, NCC phosphorylation is not only controlled by kinases but also by phosphatases including phosphatase 4 [33]. Finally, the process that controls the phosphorylation of NCC may also cause less ubiquitylation, thereby increasing the number of cotransporters available for phosphorylation [51].

Relation to human disease

Gitelman syndrome

The clearest demonstration of the functional relevance of NCC comes from human monogenetic diseases that affect NCC function (Table 2). Inactivating mutations in SLC12A3 cause the so-called Gitelman syndrome [107]. Gitelman syndrome is an autosomal recessive disorder that is characterized by hypokalemia, hypomagnesemia, metabolic alkalosis, hypocalciuria, and low to normal arterial blood pressure. Missense mutations account for approximately 59 % of the mutations in Gitelman syndrome, and compound heterozygosity is common [113, 123]. Gender and the type of mutation contribute to phenotypic variability, with males and patients with homozygous deep intronic mutations exhibiting a more severe phenotype [64, 65]. Novel mutations are still being identified in Gitelman syndrome, and these genetic defects lead to impaired production, processing, insertion, or regulation of the NCC protein (type I, II, III, or IV mutations) [32, 113, 123]. Although Gitelman syndrome is usually a relatively benign disorder that often remains subclinical for many years, a recent report suggests that the electrolyte disorders associated with Gitelman syndrome may result in chronic kidney disease and glucose intolerance [113]. On the other hand, heterozygous mutations in NCC may prevent hypertension and cardiovascular diseases [52] and improve bone density [17] likely because they induce mild sodium excretion and a positive calcium balance. NCC knockout mice recapitulate the phenotype of Gitelman syndrome, although some features

become manifest only when the animals are challenged [73, 80, 105, 136]. In line with the current model of NCC regulation (Fig. 4), genetically modified mice with overexpression of WNK4, deficiency in SPAK or its kinase domain, also exhibit features of Gitelman syndrome [61, 87, 94, 136], although such mutations have not been identified in humans. Gitelman syndrome should be differentiated from the more severe Bartter syndrome, which results from mutations affecting NKCC2, and is characterized by earlier onset of symptoms and hypercalciuria instead of hypocalciuria [48]. An intermediary phenotype is caused by mutations in CLCNKB which encodes a basolateral chloride channel that is expressed in both the thick ascending limb and the DCT (Table 2) [58]. Disturbed basolateral chloride efflux will indirectly impair apical NaCl transport through NKCC2 and NCC.

Familial hyperkalemic hypertension

FHHt is the "mirror image" of Gitelman syndrome because, in addition to hyperkalemia and hypertension, it is characterized by hypercalciuria and metabolic acidosis [37]. Surprisingly, no activating mutations in SLC12A3 have been reported. Overexpression of NCC in transgenic mice also failed to induce hyperkalemic hypertension, but this may have been due to the fact that phosphorylated NCC was

Table 2 Characteristics of mutations causing Gitelman syndrome or familial hyperkalemic hypertension

Disease	Gene	OMIM gene	Human chromosome location	Encoding protein	Major phenotype	Selected references
Gitelman (OMIM 263800)	SLC12A3	600968	16q13	NCC	Hypokalemic hypotension	[107]
	CLCNKB	602023	1p36.13	CLCNKB	Variable	[58]
FHHt* (OMIM 179820)	WNK1	605232	12p13	WNK1	Hyperkalemic hypertension	[131]
	WNK4	601844	17q21	WNK4	Hyperkalemic hypertension	[131]
	KLHL3	605775	5q31	Kelch-like 3	Hyperkalemic hypertension	[7]
	CUL-3	603136	2q36	Cullin-3	Hyperkalemic hypertension	[7, 68]

Abbreviation: * FHHt, familial hyperkalemic hypertension; OMIM, online Mendelian inheritance in man.

not increased [74]. Similarly, increasing NCC activity by inactivating KS-WNK1 was also not sufficient to cause hyperkalemic hypertension because it was associated with a compensatory decrease in ENaC [38]. Instead, familial hyperkalemic hypertension is caused by mutations in the genes encoding WNK1, WNK4, Kelch-like 3, or Cullin-3, which result in overactivity of NCC (Table 2) [7, 68, 131]. Because the WNKs regulate other transporters than NCC alone, these effects may also contribute to the FHHt phenotype [37]. Intronic deletions in the WNK1 gene cause overexpression of WNK1 and therefore more inhibition of WNK4. The inhibition of WNK4 will relieve the inhibition of SPAK and will activate NCC (Fig. 4). Similarly, missense mutations in the WNK4 gene give rise to a mutant protein that no longer inhibits SPAK resulting in NCC activation. Mutations in KLHL3 can be dominant or recessive and homozygous or heterozygous, while the identified mutations in CUL3 were dominant, heterozygous, and often de novo [7]. Mutations in KLHL3 and CUL3 seem to abrogate ubiquitylation of targets normally bound by KLHL3 including WNK4 [106]. Of the different mutations causing FHHt, CUL3 mutations have the most severe phenotype, including the youngest onset of hypertension and the highest serum potassium [7]. Another illustration that NCC activation is the final common pathway of the FHHt mutations is the fact that these disorders are all exquisitely sensitive to treatment with thiazide-type diuretics [71].

Drugs influencing NCC activity

Drugs inhibiting NCC

Several commonly used drugs inhibit or stimulate NCC activity (Table 1). The sensitivity of NCC for thiazide diuretics is well-known and characterizes this cotransporter. Thiazides, however, do not exclusively inhibit NCC, but also the sodium-dependent bicarbonate–chloride cotransporter in the cortical collecting duct [63]. Thiazide diuretics were incidentally discovered while searching for better carbonic anhydrase inhibitors [86]. They were implemented clinically in 1958, long before it became apparent that their primary target is NCC [81]. Thiazide diuretics are the logical drug of choice for diseases with NCC overactivity such as the rare disorder FHHt. However, the clinical indication for thiazide diuretics is much broader. In fact, thiazide diuretics are still among the most commonly used drugs to treat hypertension worldwide. Although the natriuretic effect of these drugs undoubtedly contributes to their antihypertensive effect, this response also seems to be translated to an effect on vascular tone [9–11]. In addition, thiazides may directly cause vasodilation possibly through vascular potassium channel activation [92]. In addition to hypertension, thiazide diuretics can also be used for sodium-

retaining disorders such as heart failure, liver cirrhosis, nephrotic syndrome, and chronic kidney disease [8]. Because thiazide diuretics act in the tubular lumen, their action requires successful delivery to the DCT. This is mediated by active secretion of thiazide diuretics in the proximal tubule through organic anion transporter and multidrug resistance-associated protein 4 [39, 114]. This explains why a reduction in glomerular filtration rate results in reduced efficacy of thiazide diuretics. Patients with Gitelman syndrome also demonstrate a dramatically impaired natriuretic response to thiazides, a feature that may be used diagnostically [15]. The side-effect profile of thiazide diuretics resembles Gitelman syndrome with the exception of hyponatremia, which is only seen with thiazides [22, 24, 47].

Drugs stimulating NCC

Drugs stimulating NCC activity have also been identified recently, and they include the calcineurin inhibitors cyclosporine and tacrolimus (Table 1) [44, 76]. Calcineurin inhibitors are potent immunosuppressive drugs that are used clinically to prevent rejection after transplantation and sometimes in auto-immune disorders. Although cyclosporine and tacrolimus have different intracellular binding proteins, they both activate NCC, and this effect therefore appears to be a class effect [45]. Again, cyclosporine and tacrolimus do not seem to activate NCC directly, but influence the WNKs [76]. This possibility was already suggested by the side-effect profile of these drugs, which is similar to the phenotype of FHHt. Of interest, calcineurin is a protein phosphatase, and protein phosphatases were recently shown to regulate NCC [33]. We were able to illustrate the clinical relevance of NCC activation by tacrolimus by linking it to hypertension [44]. That is, tacrolimus failed to induce hypertension in NCC knockout mice, whereas it caused more severe hypertension in NCC transgenic mice. Furthermore, a thiazide diuretic caused a larger urinary chloride excretion in patients on tacrolimus than in healthy volunteers or patients on sirolimus (a different immunosuppressant). This enhanced chloriuretic response to a thiazide was interpreted as an indication of increased transporter activity [15]. Also, the expression of total and phosphorylated NCC was increased in the kidney biopsies of patients treated with tacrolimus. Of interest, a model of cyclosporine nephrotoxicity showed the opposite effect on NCC, but this was attributed to inactivation of the renin–angiotensin system [62]. Furosemide, which blocks sodium transport in the thick ascending limb but not in the DCT, also increases NCC abundance. This effect is likely caused by the loopdiuretic induced enhanced sodium delivery to the DCT and the activation of the renin– angiotensin system [1]. The upregulation of NCC likely compensates for the furosemide effect and hence may contribute to the

phenomenon of loop-diuretic resistance which frequently develops during chronic treatment. As such it provides the rationale for the combination of a loop-diuretic with a thiazide to overcome diuretic resistance [8]. Furthermore, recent studies in rats suggested that the cytostatic drug cisplatinum decreases renal NCC abundance. However, this effect is likely attributed to a general toxic effect on DCT cells because also the expression of other DCT-specific proteins such as the magnesium channel TRPM6 and the calcium- and magnesium-binding protein parvalbumin were decreased [116].

Perspectives

In this review, we have highlighted several exciting new roles of NCC in sodium, potassium, and blood pressure regulation. 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 (Fig. 4), 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 [72]. The inhibition of NCC by dietary potassium may be mediated by WNK4 [89, 110, 120], 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. 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 [44–46]. 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 [15], the analysis of NCC in so-called urinary exosomes holds promise [43]. Urinary exosomes are vesicles derived from renal tubular epithelial cells that are thought to reflect the metabolic profile of these cells [93]. We recently showed that the abundance of phosphorylated NCC in urinary exosomes correlated with elevated aldosterone levels in animals and humans [117]. Although this review focused on 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 [38, 84, 99]. 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 3

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 Na^+ retention and K^+ 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-Na^+ diet, high-K^+ diet, or combined diet. The low-Na^+ and combined diets increased plasma and kidney ANG II. The low-Na^+ and high-K^+ diets increased plasma aldosterone to a similar degree (3-fold), whereas the combined diet increased aldosterone to a greater extent (10-fold). Despite similar Na^+ intake and higher aldosterone, the high-K^+ and combined diets caused a greater natriuresis than the control and low-Na^+ diets, respectively (P 0.001 for both). This K^+ -induced natriuresis was accompanied by a decreased abundance but not phosphorylation of the Na^+-Cl^- cotransporter (NCC). In contrast, the epithelial Na^+ channel (ENaC) increased in parallel with aldosterone, showing the highest expression with the combined diet. The high-K^+ and combined diets also increased WNK4 but decreased Nedd4-2 in the kidney. Total and phosphorylated Ste-20-related kinase were also increased but were retained in the cytoplasm of distal convoluted tubule cells. In summary, high dietary K^+ overrides the effects of ANG II and aldosterone on NCC to deliver sufficient Na^+ to ENaC for K^+ secretion. K^+ may inhibit NCC through WNK4 and help activate ENaC through Nedd4-2.

Keywords: Aldosterone; Angiotensin II; Hypovolemia; Hyperkalemia: Epithelial sodium channel.

Introduction

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. 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” (3, 27). 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 (28, 58). The aldosterone-sensitive distal nephron constitutes the distal convoluted tubule (DCT), connecting tubule, and collecting ducts (28). Four aldosterone-sensitive transport proteins are involved in mediating Na^+ and K^+ transport, 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 (20, 23, 37, 57, 61). These proteins are regulated by phosphorylation via several kinases, including WNK kinases and Ste-20-related kinase (SPAK) (60). ENaC and NCC are also regulated by ubiquitination via Nedd4-2 (2, 19, 39). 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) (26, 34). 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 ANG II. Recently, it has become clear that in addition to its well-established effects on the proximal tubule, ANG II can also activate NCC and ENaC (22, 51, 53). ANG II has been shown to abrogate the inhibitory effects of WNK4 (40). This permits NCC to traffic to the plasma membrane (41) and be phosphorylated by SPAK to enhance electroneutral Na^+Cl^- transport (6, 40, 51, 52). 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 ANG II inhibits ROMK (64). Together, these effects favor electroneutral Na^+ reabsorption while preventing K^+ secretion (16). These recent insights help explain how the aldosterone-sensitive distal nephron responds to hypovolemia. The effects of hyperkalemia on renal tubular transporters has recently been investigated by three studies using high-K^+ (HK) diets. Frindt and Palmer (11) found that a HK diet increased ROMK and decreased NCC in normal rats. In mice, Vallon et al. (48) confirmed the suppression of NCC by high dietary K^+ and showed this suppression to be greater in serum/glucocorticoid-regulated kinase 1 knockout mice (48). Sorensen et al. (43) showed that NCC was dephosphorylated as early as 15 min after oral K^+ intake. Still unanswered, however, is the question of 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^+ (LS) or HK diet but also

a combination of the two diets (LSHK). We show that despite maximal activation of the renin-angiotensin system, high dietary K still inhibited NCC and caused kaliuresis and natriuresis.

Materials and Methods

Animal experiments

The animal protocol was approved by the Animal Care Committee of the Erasmus Medical Center (EUR 127-11-01). Male Sprague-Dawley rats (15 wk old, average weight: 370 g, Charles River, Sulzfeld, Germany) were all fed the control diet (Na^+: 0.5% and K^+: 0.8%) and then randomized into four groups (n 8 rats/group). The control group was maintained on the control diet, whereas the other groups were switched to a LS diet (Na^+: 0.001% and K^+: 0.8%), HK diet (Na^+: 0.5% and K^+: 5%), or a combination of the LS and HK diets (LSHK; Na^+: 0.001% and K^+: 5%). All diets were purchased from Harlan Laboratories (Madison, WI). Rats were housed in metabolic cages in a temperature- and humidity-controlled room with a 12:12-h light-dark cycle to collect 24-h urine and to measure body weight and the intake of food and water (29). Tap water was provided ad libitum. Eight days after being switched to the diets, animals were euthanized. In a separate experiment, three groups (n 5 rats/group) were randomized to the control diet, HK diet, or HK diet with ANG II for 8 days. ANG II was administered in a nonpressor dose (233 g·kg 1·day 1) using osmotic minipumps (model 2ml2, Alzet, Cupertino, CA) (51); minipumps containing vehicle were implanted in the two other groups. Plasma renin activity, plasma and kidney ANG II concentrations, and plasma aldosterone were measured as previously described (9, 21, 54). Urine and plasma electrolytes were determined with an automatic analyzer (Modular IPPE, Roche Diagnostics, Almere, The Netherlands).

Immunoblot analysis

The right kidney was used for immunoblot analysis. After being harvested, 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 previously described (10, 51). For the immunoblot analysis of ROMK, the cortex was separated from the medulla and processed as previously described (56). Each experimental group was blotted next to a control group. Equal loading was confirmed with staining of actin (1:100,000, Cell Signaling, Boston, MA). Antibodies against the following proteins were obtained: NCC (1:1,000), α-, β-, and γ-subunits of ENaC (all 1:1,000, StressMarq, Victoria, BC, Canada), SPAK and SPAK phosphorylated

at Ser373 (pSPAK) (both 1:1,000, Millipore, Billerica, MA), and Nedd4-2 (1:4,000 Abcam, Camebridge, UK). Noncommercial antibodies against NCC phosphorylated at Thr58 (pNCC; 1:500), NKCC2 phosphorylated at Thr95/100 (pNCC2; 1:2,000), WNK4 (1:1,000), and ROMK (1:2,000) were kind gifts from other investigators and have been previously characterized (33, 35, 63). All antibodies used for immunoblot analysis were raised in the 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 0.1 M Na cacody late (pH 7.4) (4). The midregion was sectioned into 2- to 3-mm transverse sections and immersion fixed for an additional 1 h followed by 3 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- m sections were cut on a rotary microtome (Leica Microsystems, Herlev, Denmark). Immunolabeling was performed as previously described (33). Labeling was detected using a horseradish peroxidase-conjugated secondary antibody (P448, goat anti-rabbit IgG, Dako, 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 previously described in detail (36). Briefly, the primary antibodies used were against NCC (1:4,000), pNCC (1:70,000), total NKCC2 (1: 1,000), BK (1:800, NeuroMab Facility, University of California, Davis, CA), SPAK (1:2,000), and pSPAK (1:1,000) (both SPAK antibodies were from the University of Dundee, Dundee, UK). Anti-pSPAK was preincubated with the nonphosphopeptide, as recommended. NCC and NKCC2 antibodies were raised in the rabbit, SPAK antibodies were raised in sheep, and ~~BK antibodies were raised in the mouse. Goat anti-rabbit Alexa 488- or donkey~~ anti-sheep Alexa 555-conjugated secondary antibodies (Invitrogen) were used for visualization of labeling. A Leica TCS SL confocal microscope with an HCX PL APO 63 oil objective lens (numerical aperture: 1.40) was used for imaging of labeled sections.

Statistics

All data are expressed as means ± SE. Group comparisons were made using a Student's t-test or ANOVA with a least-significant-difference post hoc test, as appropriate. P values of < 0.05 were considered statistically significant. All data were analyzed using SPSS (version 20, IBM).

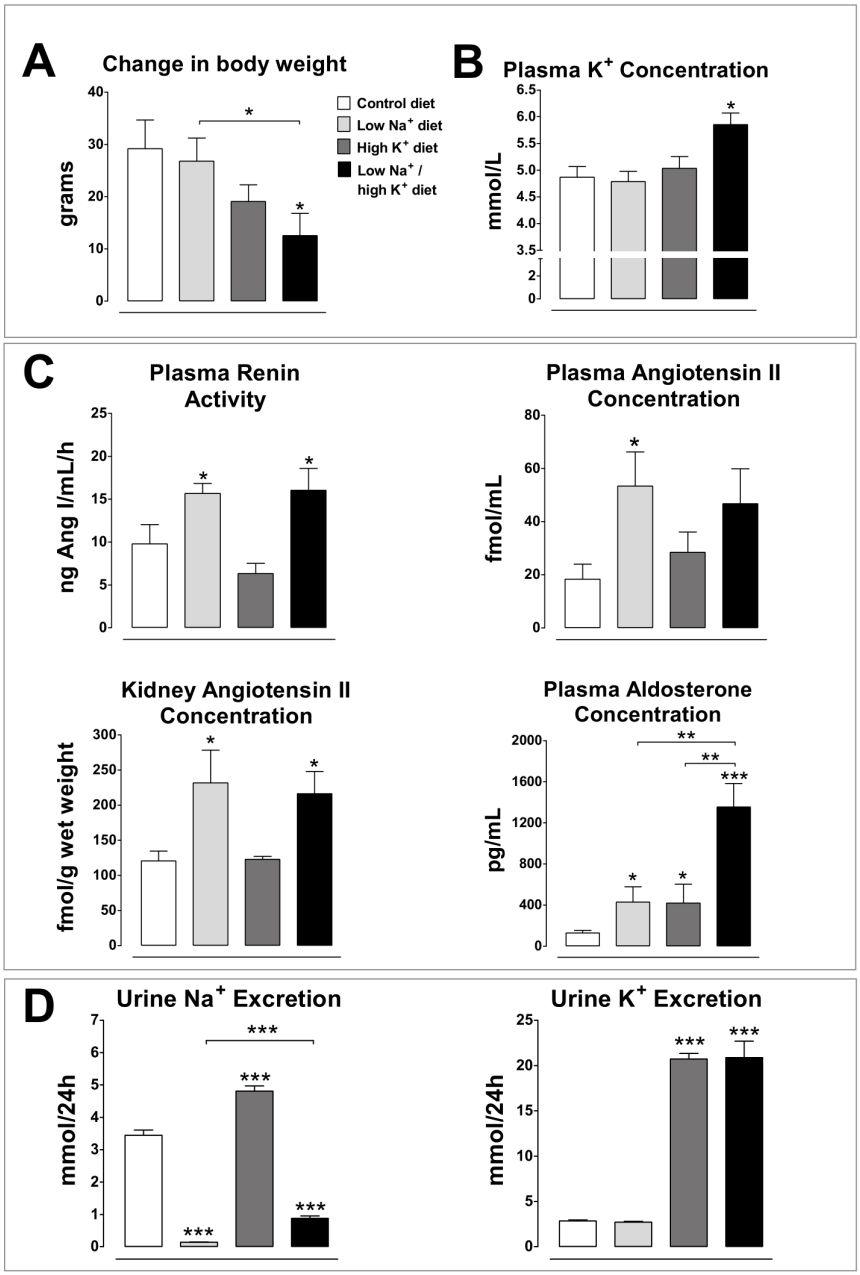


Figure 1 Responses in body weight (A), plasma K^+ concentration (B), renin-angiotensin system (C), and urinary electrolyte excretion (D) to the four different diets [control diet, low-Na^+ (LS) diet, high-K^+ (HK) diet, and combined (LSHK) diet]. Averages ± SE are shown; n = 8 animals/group. Twentyfour-h 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.

Results

Differential activation of the renin-angiotensin system

Normal rats were fed the control diet, LS diet, HK diet, or LSHK diet for 8 days. HK and LSHK diets led to an immediate three- to fourfold increase in diuresis that persisted throughout the study (55 ± 3 ml/day in the HK group and 43 ± 2 ml/day in the LSHK group vs. 12 ± 1 ml/day in the control group and 13 ± 1 ml/day in the LS group). Accordingly, 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 animals in the HK and LSHK groups ate less food during the first and second day after being switched 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. Animals that received the LSHK diet gained significantly less weight compared with animals on the control or LS diets (Fig. 1A). Both LS and LSHK groups had increased plasma renin activity and plasma and kidney ANG II concentrations compared with the control group (Fig. 1C). The HK diet decreased plasma renin activity compared with the control diet, but this did not reach statistical significance (P = 0.09). LS and HK groups had increased plasma aldosterone similarly compared with the control group (428 ± 183 and 420 ± 149 vs. 128 ± 26 pg/ml; Fig. 1A). The LSHK diet caused mild hyperkalemia (5.9 ± 0.2 mmol/l; Fig. 1B) and increased aldosterone to a greater extent (1,355 ± 228 pg/ml; Fig. 1C). Plasma Na^+ was similar in all groups (control group: 142 ± 1 mmol/l, LS group: 142 ± 1 mmol/l, HK group: 143 ± 1 mmol/l, and LSHK group: 143 ± 1 mmol/l). Although both LS and LSHK diets decreased urine Na^+ excretion markedly compared with the control diet, urine Na excretion with the LSHK diet was significantly higher than with the LS diet (Fig. 1D). The HK diet also increased urine Na^+ excretion compared with the control diet. Both HK and LSHK diets induced a marked kaliuresis (Fig. 1D).

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

In the plasma membrane fractions of whole kidney homogenates, the LS diet increased the abundances of total NCC and pNCC by 1.7-fold (Fig. 2A). Of interest, both HK and LSHK diet decreased NCC. A similar trend was observed for pNCC, but this was not statistically significant. These changes were confirmed using immunohistochemistry (Fig. 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). The LS diet decreased NKCC2 (Fig. 2C), and this effect was located to the

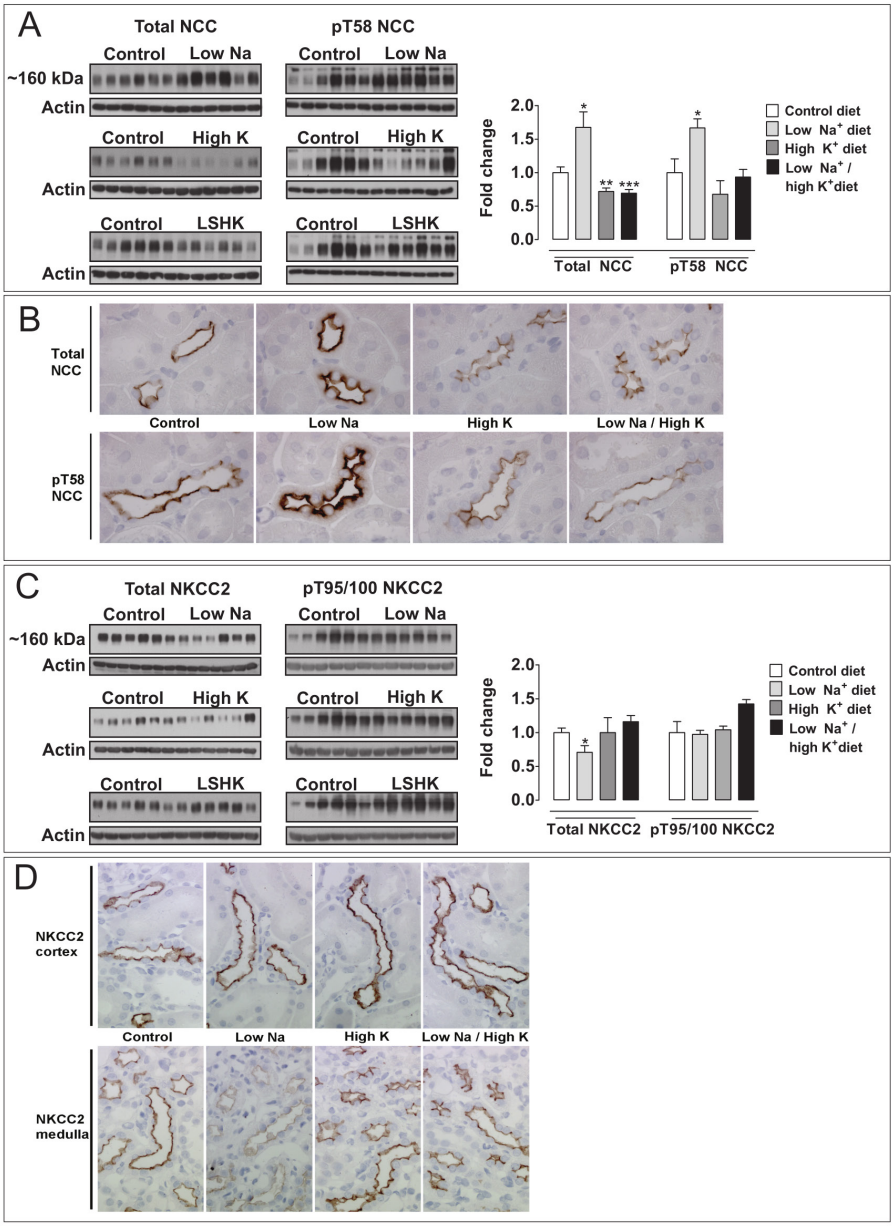


Figure 2 Analysis of changes in the NaCl cotransporter (NCC, panel A and B) and the $Na^+K^+2Cl^-$-cotransporter type 2 (NKCC2, panel C and D) to the four different diets. Results for NCC are shown in panel A (immunoblots) and panel B (immunohistochemistry); results for NKCC2 are shown in panel C (immunoblots) and panel D (immunohistochemistry). Densitometry (n = 6/group) is expressed as fold change ± SEM and normalized by actin. Light grey bar: low Na+ diet; dark grey bar: high K^+ diet; black bar: combined low Na^+ and high K^+ diet. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control diet using Student's T-test.

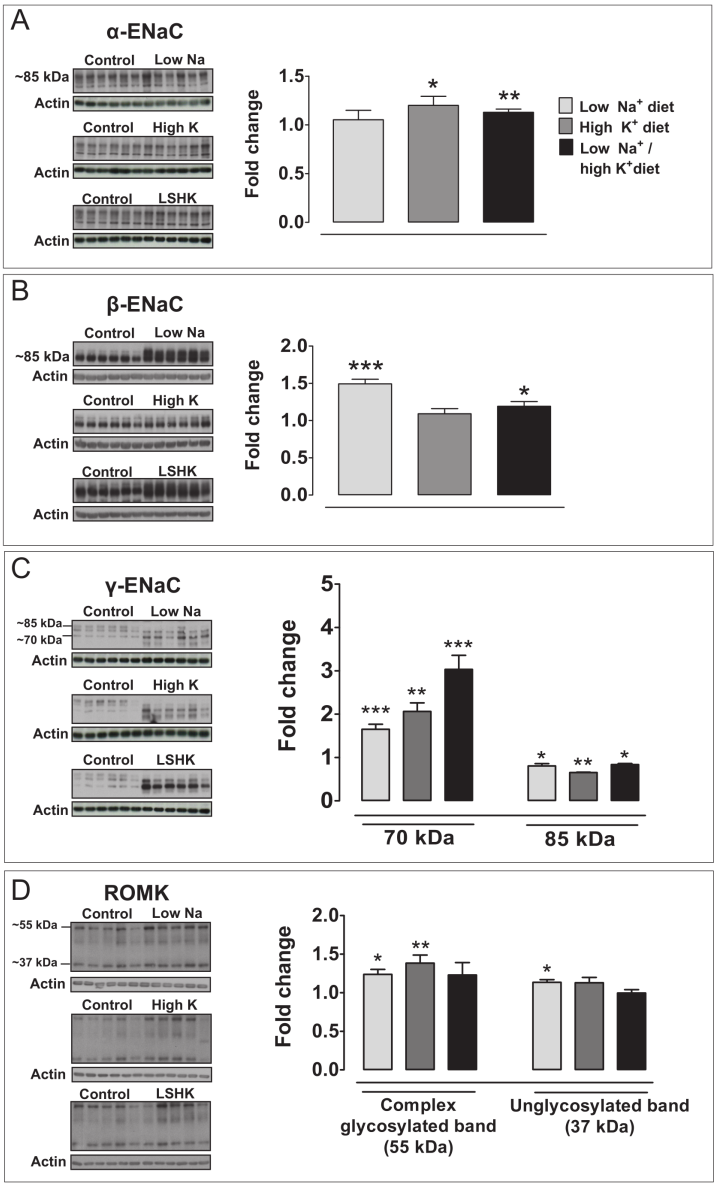


Figure 3 Immunoblots showing changes in the α-subunit (panel A), β-subunit (panel B), and γ-subunit (panel C) of the epithelial Na^+ channel (ENaC) and the renal outer medullary K^+ channel (ROMK, panel D) to the four diets. Densitometry (n = 6/group) is expressed as fold change ± SEM and normalized by actin. 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. Light grey bar: low Na^+ diet; dark grey bar: high K^+ diet; black bar: combined low Na^+ and high K^+ diet. Footnotes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control diet using Student's T-test.

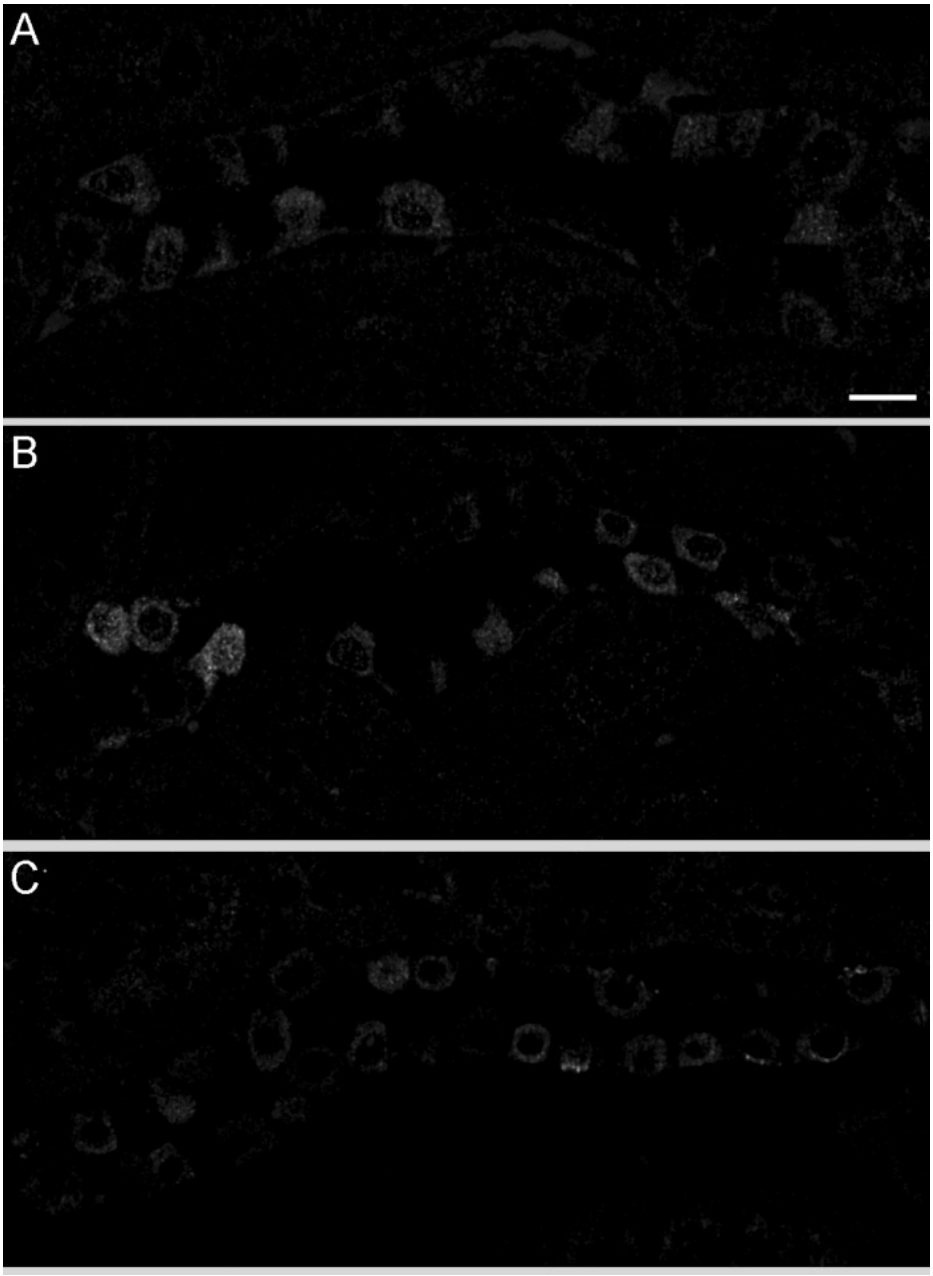


Figure 4 Labeling of the big-conductance $Ca2^{+}$-activated potassium channel α-subunit (BKα). BKα labeling was localized to the cytoplasm of intercalated cells in the cortical collecting duct and outer medullary collecting duct. Both the high K^{+} (HK) and the combined low Na^{+} and high K^{+} (LSHK) diets increased BKα positive labeling of intercalated cells. A) Control diet, B) HK diet C) LSHK diet. Scale bar = 20μM.

medullary portion of the TAL (Fig. 2D). The HK and LSHK diets had no effects on NKCC2. A trend toward more pNKCC2 was observed with the LSHK diet, but this was not significant and not confirmed with immunohistochemistry (data not shown).

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

The HK and LSHK diets increased the abundance of the α-subunit of ENaC, whereas the LS and LSHK diets increased the abundance of the β-subunit of ENaC (Fig. 3). In previous studies (23, 24) using whole kidney homogenates, the LS diet was shown to increase the α-subunit of ENaC but left the β-subunit of ENaC unchanged. 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). Immunoblot analysis of the γ-subunit of ENaC showed the characteristic four bands ranging from 70 to 85 kDa (Fig. 3C). All dietary manipulations decreased the 85-kDa band and increased the 70-kDa band. Previously, this change has been interpreted as cleavage of the γ-subunit of ENaC by aldosterone (23). Indeed, the higher the plasma aldosterone concentration (Fig. 1C), the greater the changes in the γ-subunit of ENaC with the LSHK diet, leading to a threefold increase in the 70-kDa isoform (Fig. 3).

Effects on K^+ 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 the LS diet (Fig. 3D). BK-labeling was localized to the cytoplasm in the majority of intercalated cells in the cortical collecting duct and outer medullary collecting duct (Fig. 4). In contrast to another report (61), we did not observe any signal above background levels in principal cells of the medullary collecting ducts. Technical reasons (nonspecific background in proximal tubule mitochondria) prevented a full quantitative assessment of BK-abundance using immunofluorescent confocal microscopy. However, compared with the control diet (Fig. 4A), both the HK (Fig. 4B) and LSHK (Fig. 4C) diets induced a qualitative increase in cytoplasmic BK- labeling in intercalated cells. No clear apical BK- labeling was detected under any dietary condition.

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

In cytosolic fractions of whole kidney homogenates, the HK diet led to a modest (1.2-fold) but significant increase in WNK4 abundance (Fig. 5A). WNK4 showed the same trend with the LSHK diet, but this was not statistically significant ($P = 0.1$).

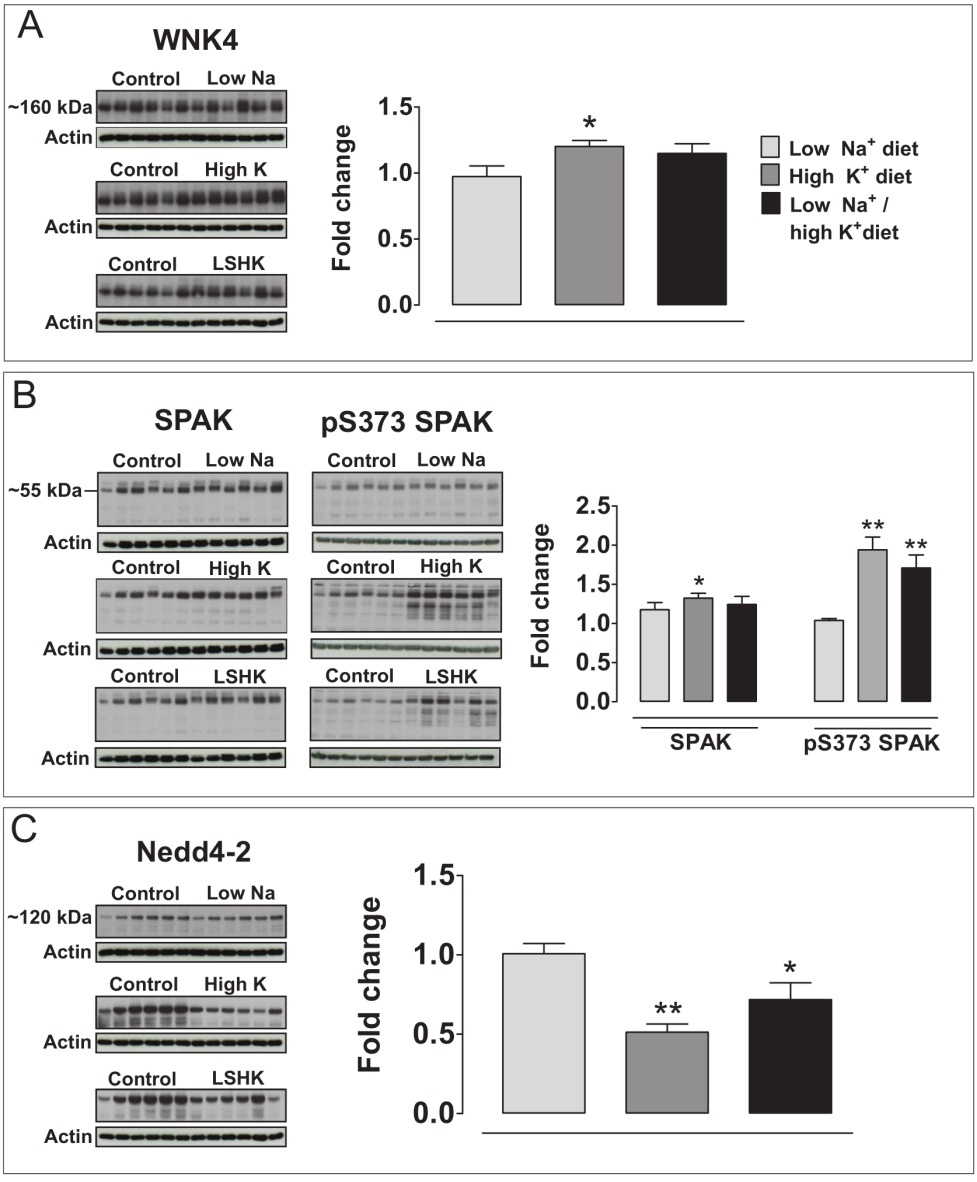


Figure 5 Immunoblots showing changes in WNK4 (panel A), total and phosphorylated SPAK (panel B), and Nedd4-2 (panel C) to the four diets. Densitometry (n = 6/group) is expressed as fold change ± SEM and normalized by actin. For SPAK, the 55 kDa band was used for densitometry, which likely represents full-length SPAK (26). The lower bands that are visible in some of the blots likely represent SPAK2 and kidney-specific SPAK (26). Light grey bar: low Na^+ diet; dark grey bar: high K^+ diet; black bar: combined low Na^+ and high K^+ diet. Footnotes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control diet using Student's T-test.

The HK diet also increased the total abundance of SPAK, whereas both HK and LSHK increased the abundance of pSPAK (Fig. 5B). Nedd4-2, a ubiquitin ligase regulating ENaC (19), was downregulated by approximately twofold by both HK and LSHK diets (Fig. 5C).

Differential regulation of SPAK in TAL and DCT

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

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

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

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 (3, 14, 15, 27). Although angiotensin II and aldosterone normally activate NCC (20, 51), 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

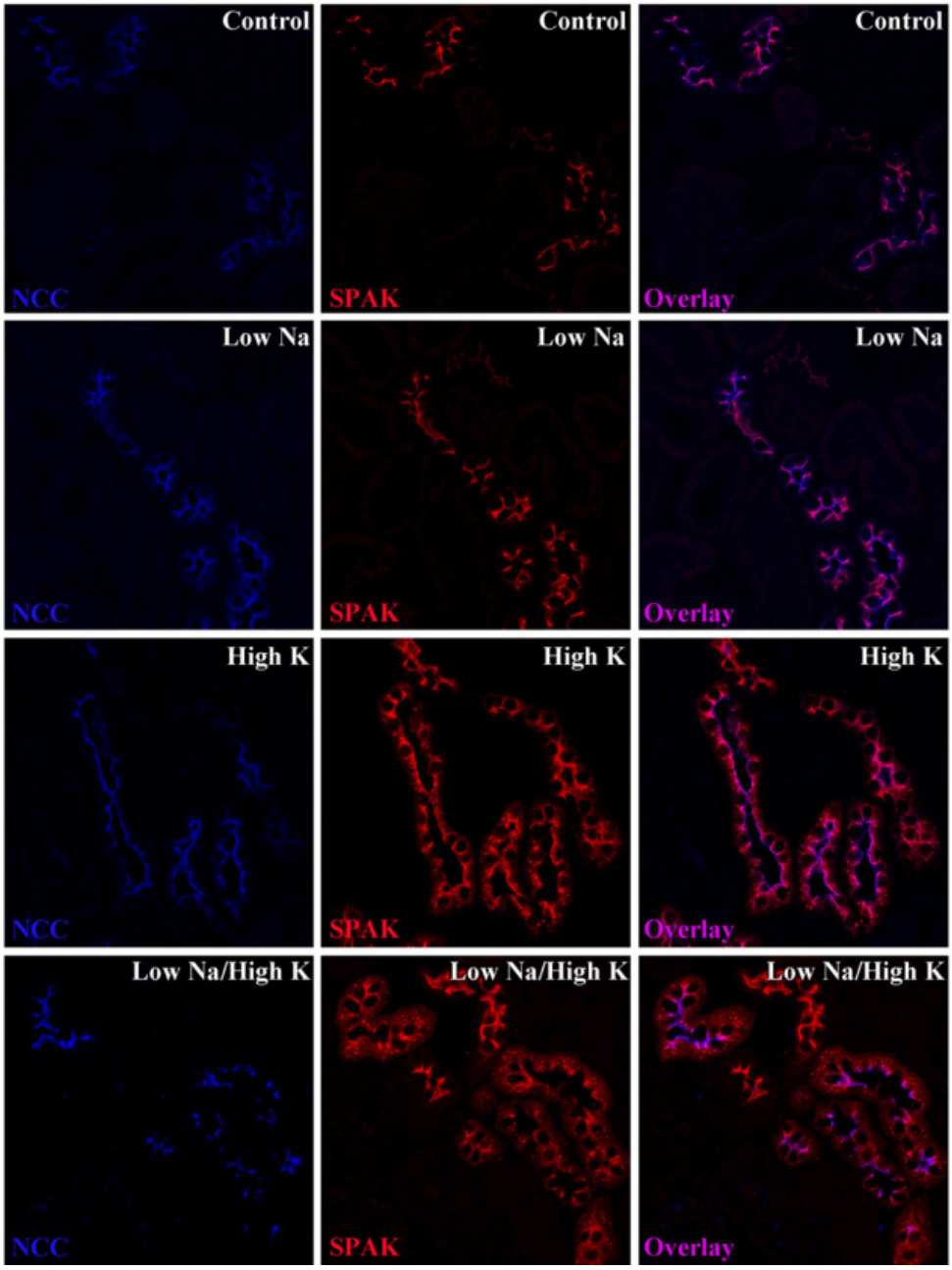


Figure 6 Confocal microscopy showing the localization of the Ste20-related kinase (SPAK, red, middle column) in relation to the Na^+Cl^- cotranspoter (NCC, blue, left column). The overlay (right column) clearly shows co-localization of NCC and SPAK at the apical plasma membrane with the low Na^+ diet (purple). In contrast, the high K^+ and combined diets show no co-localization, suggesting that SPAK was redistributed to the cytoplasm in these settings.

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 (11). The effects of high dietary K^+ on NCC and ENaC are in line with what Frindt and Palmer reported previously (11). Cheema-Dhadli et al. also reported a higher distal flow rate in animals receiving a K^+Cl^- load, but ascribed this to an effect on NKCC2 (5). 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 (49, 50). A recent study showed that K^+-induced natriuresis is impaired in NCC knockout mice, further adding to the role of NCC in this mechanism (43). 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 (61). Similar to our results, Rieg et al. was also unable to detect apical BK labeling (37). 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 (59, 64). Possibly, aldosterone increases the abundance of ROMK, while angiotensin II inhibits channel activity (59, 64).

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 (5, 11, 12). For example, a previous study showed that infusion of K^+ directly into the renal artery decreased renin and increased natriuresis (55). Although the 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

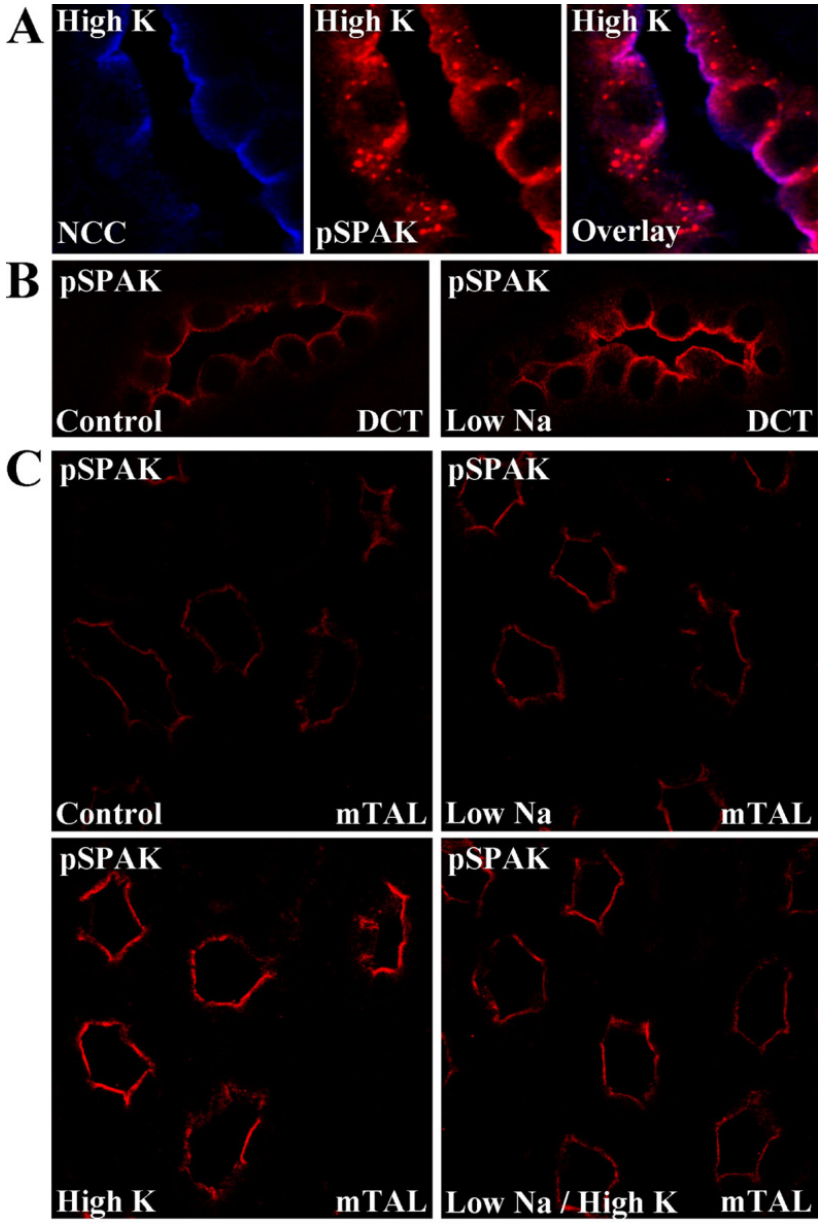
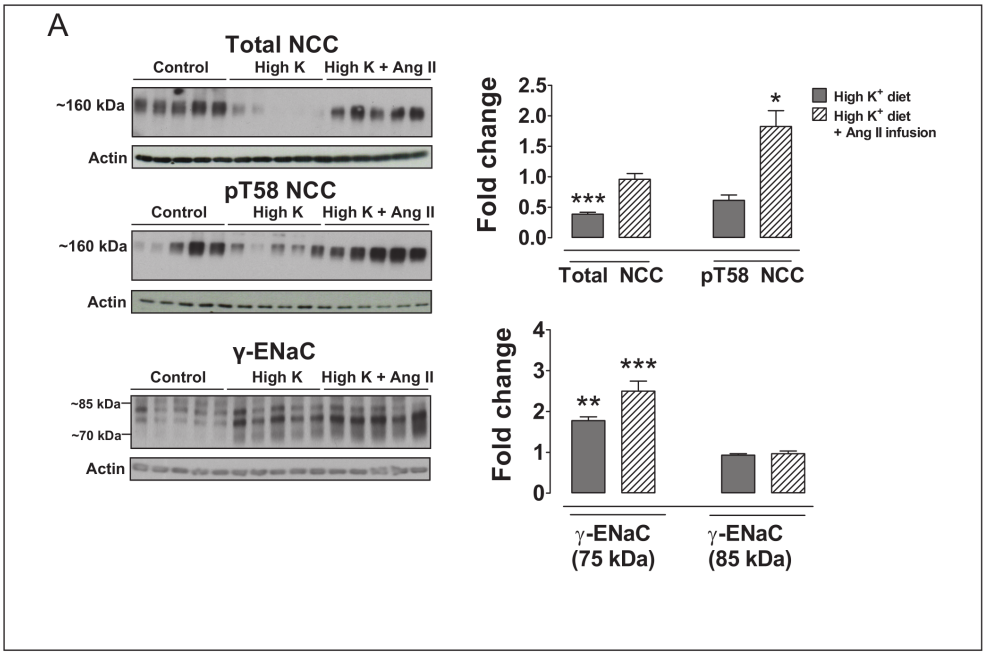


Figure 7 Immunofluorescence microscopy analysis of the phosphorylated form of Ste20-related kinase (pSPAK, red). Panel A shows double labeling of pSPAK and the Na^+Cl^- cotransporter (NCC, blue) in animals on a high K^+ diet, showing that pSPAK was primarily located intracellularly in vesicle-like structures (speckled pattern). Panel B shows that the low Na^+ diet increased the fluorescence intensity of pSPAK compared with the control diet (control) in the distal convoluted tubule (DCT). Panel C shows that the high K^+ and combined diets increased pSPAK in plasma membranes of cells in the medullary thick ascending limb (mTAL).

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 (47). 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) (43). The rapidity of this response seems to argue against mediation by the renin-anigotensin system, although non-genomic effects have also been reported (7). 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 (48).

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 (31), 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 (18, 38), it seems logical to suggest that the increase in WNK4 is instead related to NCC. Although the effect of WNK4 on NCC remains controversial (25), several in vitro studies have shown that WNK4 can disrupt the trafficking of NCC to the plasma membrane (45, 62, 63). We believe the decrease in Nedd4-2 aided the observed increase in ENaC (44). Although it was recently shown that Nedd4-2 also regulates NCC, decreased Nedd4-2 would not result in decreased NCC, according to these studies (2, 39).

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 (6). Although the changes in SPAK with the low Na^+ diet were not evident from our immunoblot



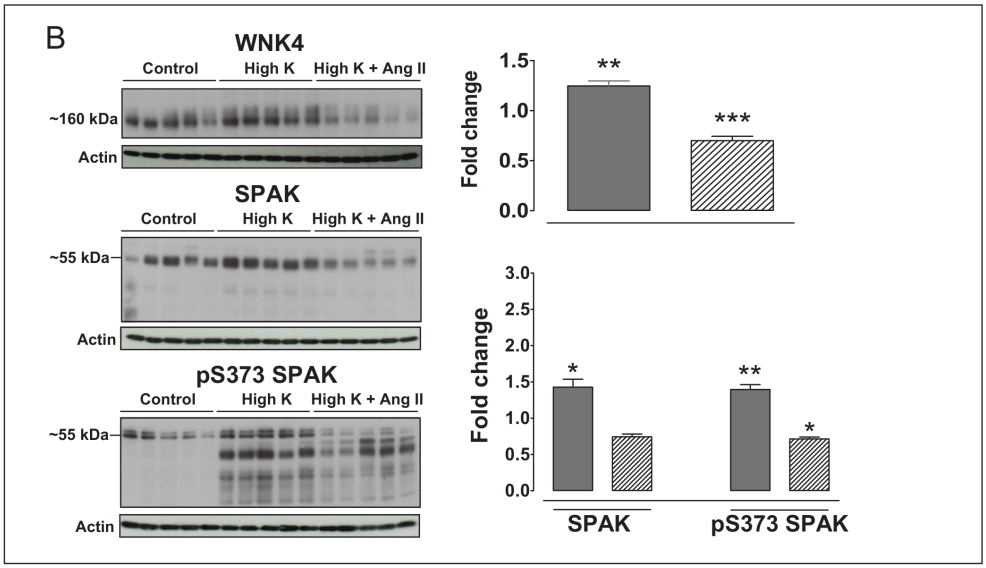


Figure 8 Immunoblots showing the response of the NaCl cotransporter (NCC) and the epithelial Na^+ channel (ENaC) (panel A) and WNK4 and SPAK (panel B) to the addition of angiotensin II (Ang II) to a high K^+ diet. Densitometry (n = 5/group) is expressed as fold change ± SEM and normalized by actin. For SPAK, the 55 kDa band was used for densitometry, which likely represents full-length SPAK (26). The lower bands that are visible in some of the blots likely represent SPAK2 and kidney-specific SPAK (26). Dark grey bar: high K^+ diet; striped bar: high K^+ diet with infusion of angiotensin II. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control diet using Student's T-test.

analysis (Figure 5), this may be explained by the use of whole kidney homogenates in which both TAL and DCT are present (26). 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 (42).

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 (13). 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 (15, 17). In fact, a low Na^+ and high K^+ diet is currently recommended in the Dietary Approaches to Stop Hypertension (DASH) (1). 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 (46) and one wonders whether this could promote cardiac fibrosis, as observed in for example heart failure (30). On the other hand, the K^+-induced natriuresis mediated by NCC may explain the blood pressure lowering effect of high dietary K^+ (8, 32). 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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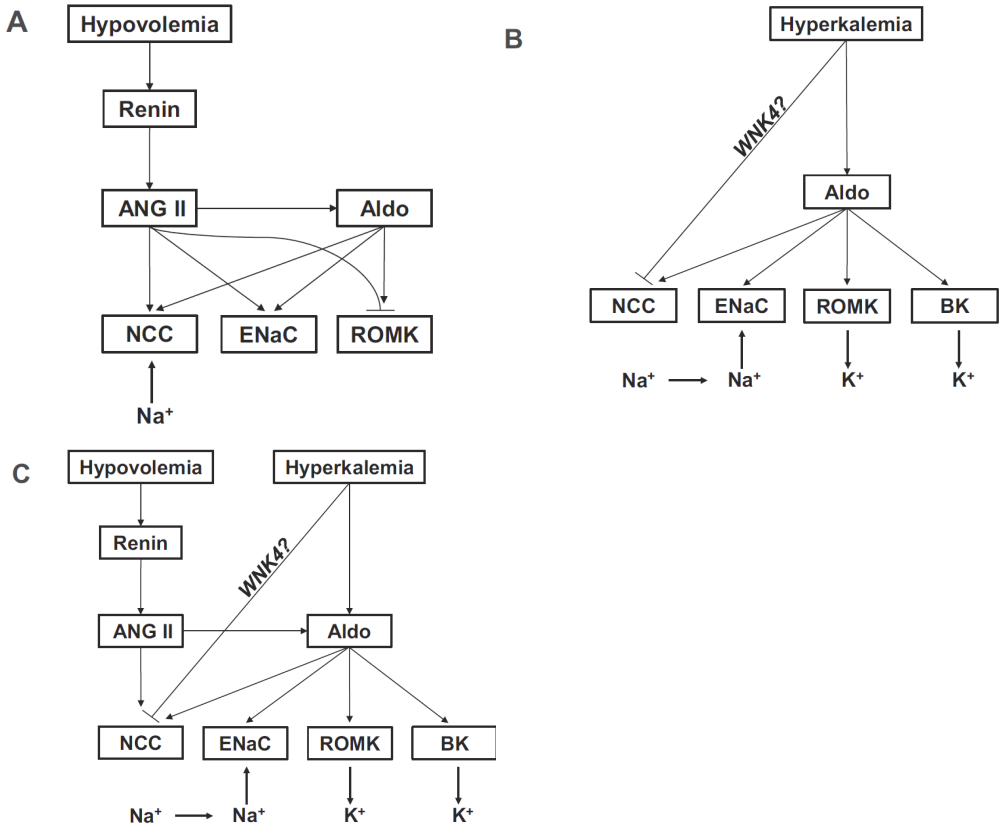

Figure 9: Model of the aldosterone paradox. Arrows indicate stimulatory effect, dashed arrows indicate a stimulatory effect that is overridden by an inhibitory effect (two perpendicular lines). Arrows to or from "Na^+" and "K^+" indicate reabsorption and secretion, respectively. Hypovolemia (panel A) activates the renin-angiotensin system so that both angiotensin II (Ang II) and aldosterone (Aldo) activate the NaCl cotransporter (NCC). Angiotensin II has been shown to inhibit the renal outer medullary K^+ channel (ROMK) and therefore overrides the normal activation by aldosterone (64). In this way, hypovolemia favors electroneutral Na^+ reabsorption through NCC while preserving K^+. Hyperkalemia (panel B) directly increases aldosterone. Our study shows that this also leads to an inhibition of NCC, possibly mediated by WNK4, therefore overriding the usual stimulatory effect of aldosterone and increasing distal Na^+ delivery (indicated by the arrow going to and from "Na^+"). The effects on the epithelial Na^+ channel (ENaC), ROMK, and BK are maintained. Therefore, hyperkalemia stimulates ENaC-coupled kaliuresis. The combination of hypovolemia and hyperkalemia (panel C) still activates the renin-angiotensin system and leads to a further increase in aldosterone. The stimulatory effects of angiotensin II and aldosterone 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.

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Disclosure

The authors declare to have no competing financial interests or other disclosures with regard to this manuscript.

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PART II

CHAPTER 4

Calcineurin inhibitors and hypertension: A role for pharmacogenetics?

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Abstract

Hypertension is a common side-effect of calcineurin inhibitors (CNIs), drugs used to prevent rejection after transplantation. Hypertension after kidney transplantation has been associated with earlier graft failure and higher cardiovascular mortality in the recipient. Recent data indicate that enzymes and transporters involved in CNI pharmacokinetics and pharmacodynamics, are also associated with salt-sensitive hypertension, including CYP3A5, ABCB1, WNK4, and SPAK. These insights raise the question whether polymorphisms in the genes encoding these proteins increase the risk of CNI-induced hypertension. Predicting who is at risk for CNI-induced hypertension may be useful to select specific interventions, including dietary salt restriction, thiazide diuretics or a CNI-free immunosuppressive regimen. This review aims to explore the pharmacogenetics of CNI-induced hypertension, highlighting the knowns and unknowns.

Keywords: ABCB1; CYP3A5; sodium chloride cotransporter; SPAK; transplantation; WNK4.

Background

The calcineurin inhibitors (CNIs) tacrolimus and cyclosporine are the most commonly used drugs to prevent rejection after transplantation [1]. In addition, CNIs are sometimes used to treat autoimmune disease. Their immunological mechanism of action is the inhibition of the pro-inflammatory response in T-cells, which is prompted after interaction with antigen-presenting cells. More specific, CNIs inhibit calcineurin, a phosphatase that dephosphorylates the cytoplasmatic nuclear factor of activated T-cells (NFATc) [2]. Dephosphorylation of NFATc causes it to be translocated into the cell nucleus, where it increases transcriptional activation of early cytokine genes such as interleukin-2, interleukin-3, interleukin-4 and tumor necrosis factor-alpha. The ability of CNIs to prevent the dephosphorylation and subsequent translocation of NFATc, largely explains their immunosuppressive effect. The efficacy of CNIs to prevent rejection, however, comes at the cost of important side-effects. These side-effects include nephrotoxicity, neurotoxicity, and several metabolic disorders. Hypertension is another prominent side-effect of CNIs and is estimated to occur in 20 to 70% of patients using CNIs [3, 4] and more so with cyclosporine than tacrolimus [5]. Studies in kidney transplant recipients have shown that hypertension after transplantation not only increased the risk of cardiovascular complications [6], but was also associated with earlier graft failure [7]. The pharmacokinetics of CNIs are characterized by high inter-

patient variability [8]. Some of this variability has been linked to genetic variation in enzymes or transporters involved in the pharmacokinetics of CNIs, including CYP3A and ABCB1. This raises the question whether CNI-induced hypertension also has a pharmacogenetic component, similar to what has been suggested for CNI-associated neurotoxicity and nephrotoxicity [9, 10]. The aim of this review is to explore the pharmacogenetics of CNI-induced hypertension, addressing both answered and unanswered questions.

CNI-induced hypertension

How do CNIs cause hypertension? CNIs have been shown to affect several systems involved in blood pressure regulation, including the renin-angiotensin system, endothelin-1, nitric oxide, and the sympathetic nervous system (for review see [11]). Most of these studies were performed with cyclosporine, which was shown to increase endothelin-1, angiotensin II, and the activity of the sympathetic nervous system, all of which stimulate vasoconstriction [12-14]. Others, however, found that cyclosporine does not cause vasoconstriction, but rather impairs vasodilation through an effect on nitric oxide [15, 16]. Although many of these effects point to a role for CNIs in changing vascular tone, other data also suggest an important salt-sensitive component to CNI-induced hypertension [17-20]. Of interest, the form of salt-sensitive hypertension induced by CNIs is strikingly similar to a rare genetic cause of hypertension called familial hyperkalemic hypertension (FHHt, also called pseudohypoaldosteronism type 2 or Gordon syndrome) [21]. FHHt can be caused by mutations in WNK (With No Lysine = K) kinases leading to overactivation of the sodium chloride cotransporter (NCC) in the renal distal convoluted tubule [22]. Both FHHt and CNIs cause a form of salt-sensitive hypertension that is further characterized by hyperkalemia, renal tubular acidosis, and hypercalciuria [23]. The striking resemblance between the renal tubular disorders caused by CNIs and FHHt prompted our group to investigate if the adverse effects caused by CNIs could be caused by activation of NCC [18]. First we showed that wild-type mice treated with tacrolimus indeed developed the FHHt phenotype, including salt-dependent hypertension, hyperkalemia, renal tubular acidosis and hypercalciuria [18]. Furthermore, tacrolimus increased the phosphorylation of NCC in the distal convoluted tubule. Phosphorylated NCC is recognized as the active form of the co-transporter [24]. The process of NCC phosphorylation is orchestrated by the kinases WNK3, WNK4, and SPAK (STE20/SPS1-related, Proline Alanine-rich Kinase) [25, 26]. These three kinases were also upregulated by treatment with tacrolimus [18]. To exclude a secondary effect of tacrolimus on NCC we confirmed that calcineurin A-α,

the major isoform of calcineurin, co-localized with NCC in the distal convoluted tubule. To study the functional importance of NCC in causing CNI-induced hypertension we showed that mice overexpressing NCC had an exaggerated hypertensive response to tacrolimus. In contrast, tacrolimus did not cause hypertension in NCC knockout mice. Treatment with hydrochlorothiazide, a pharmacological inhibitor of NCC, reversed tacrolimus-induced hypertension in wild-type mice. These results were then extended to humans. Kidney transplant recipients with CNI-induced hypertension had a greater increase in fractional chloride excretion in response to a thiazide, suggesting increased activity of NCC [27]. Furthermore, a higher abundance of phosphorylated NCC was found in the kidney transplant biopsies of these patients. Although our study focused on tacrolimus, a different group showed that cyclosporine had similar effects on the so-called WNK-SPAK-NCC pathway in a murine cell line and in rats [28]. The recent demonstration that phosphatase knockout mice exhibited lower NCC expression and lower blood pressure further adds to the relationship between calcineurin (which is a phosphatase) and blood pressure regulation [29].

Pharmacokinetics of CNIs

The pharmacokinetics of cyclosporine and tacrolimus are characterized by high inter-patient variability [30]. Following oral intake, CNIs are absorbed from the gut with peak concentrations usually reached within two hours. CNIs are substrates of the drug-efflux pump ABCB1 (also called P-glycoprotein or multidrug-resistance protein 1). Differences in the intestinal expression of ABCB1 between individuals likely explains the variability in CNI absorption [31]. After absorption, CNIs distribute extensively into tissue and the cellular fraction of blood. Cyclosporine and tacrolimus have different intracellular binding proteins that are called cyclophilin and FK506-binding protein 12 (FKBP12), respectively [32]. Erythrocytes have a high concentration of FKBP12 and this explains why whole-blood tacrolimus concentrations are approximately 15 times higher than those in plasma [33]. Distribution of CNIs into tissue also appears to be affected by ABCB1 expression on the surface of various cell types, including lymphocytes, where it controls the access of the drug to its target [34-37]. Tacrolimus is mainly eliminated in bile (>90%) after biotransformation into at least 15 metabolites by the polymorphically-expressed cytochrome P450 (CYP) 3A4 and 3A5 enzymes [38]. CYP3A4 is primarily involved in the metabolism of cyclosporine, whereas CYP3A5 is more important for the metabolism of tacrolimus [39, 40]. Several single-nucleotide polymorphism (SNPs) in CYP3A5 have been characterized [41]. A SNP in the CYP3A5 gene involving

an A to G transition at position 6986 within intron 3 (the CYP3A5*3 variant allele; rs776746) was strongly associated with CYP3A5 protein expression. Individuals carrying at least one CYP3A5*1 allele (considered to be the wild-type allele) were found to express large amounts of CYP3A5 protein, whereas individuals homozygous for the CYP3A5*3 allele did not express significant quantities of CYP3A5 protein. Kidney transplant recipients carrying at least one CYP3A5*1 allele, the so-called expressers, require higher dosing of tacrolimus to reach target concentrations in whole-blood [8]. The CYP3A5*3 allele causes alternative splicing, resulting in protein truncation and a severe decrease of functional CYP3A5. These findings were confirmed by others and several more SNPs in CYP3A5 have been identified since [42, 43]. Allelic frequency differs with ethnicity, with approximately 5-15% of Caucasians expressing functional CYP3A5, whereas around 30% of Asians, and 70% of individuals of African descent are CYP3A5 expressers [41, 44, 45]. Metabolism largely occurs in the liver but considerable pre-systemic biotransformation takes place in the intestine. Less than 5% of CNIs is eliminated by the kidneys and < 1% is eliminated in urine as unchanged drug [46]. ABCB1 expression in the brush border of proximal tubular epithelial cells and more distally in the renal tubule, may contribute to renal elimination, while ABCB1 expression at the canalicular surface of hepatocytes controls excretion into bile [47]. CYP3A5 is the main CYP3A isoform expressed in the kidney and may be involved in local CNI metabolism [33].

CYP3A5, ABCB1 & hypertension

CYP3A5 and ABCB1 have been associated with hypertension independent of their role in the pharmacokinetics of CNIs. Both proteins appear to be involved in the metabolism of mineralocorticoids [44]. CYP3A determines the production of 6β-hydroxycortisol, while ABCB1 plays a role in the transport of aldosterone in the adrenal cortex [44, 48]. Conceivably, changes in CYP3A or ABCB1 activity result in mineralocorticoid excess. Mineralocorticoid excess is a recognized cause of hypertension because it stimulates sodium reabsorption in the distal nephron [49]. Indeed, spontaneously hypertensive rats were found to have enhanced CYP3A activity leading to increased 6β-hydroxylation of cortisol [48]. This process likely contributed to hypertension in these animals because treatment with troleandomycin, a selective inhibitor of CYP3A, reversed hypertension [48]. CYP3A activity in the kidney rather than in the liver correlated best with blood pressure in these rats [50]. Indeed, in renal microsomes of human kidney donors, the CYP3A5*1/*3 genotype resulted in an 8-fold higher CYP3A5 abundance and 18-fold higher catalytic activity than the CYP3A5*3/*3 genotype [51]. The expresser allele CYP3A5*1 is thought to play a

role in the regulation of kidney sodium handling and blood pressure. The presence of this allele likely provided an evolutionary advantage to conserve salt and this may explain the high allele frequency near the equator [41]. Although the precise mechanisms remain unclear, increased CYP3A5 activity in the kidney may result in higher local 6-hydroxycortisol levels, which subsequently stimulates sodium reabsorption [41]. Conversely, the frequency of the non-expresser allele CYP3A5*3 increases with distance from the equator [52]. Studies analyzing whether these

Table 1 Overview of studies analyzing the association between polymorphisms in CYP3A5 and ABCB1 with hypertension

First author and year	Number of subjects	Population	Gene	Association with hypertension	Reference
Givens, 2003	21	Organ donors	CYP3A5	Yes	[51]
Kreutz, 2004	399	Caucasian kidney transplant recipients	CYP3A5	No	[66]
Kreutz, 2005	6777	Caucasian population	CYP3A5	Yes	[57]
Ho, 2005	683	General population	CYP3A5	Variable	[53]
Kivistö, 2005	373	Elderly patients	CYP3A5	Yes	[55]
Fromm, 2005	115	Caucasian students	CYP3A5	Yes	[56]
Bochud, 2006	375	Black hypertensives	CYP3A5	Yes	[54]
Lieb, 2006	1084	Caucasians	CYP3A5	No	[59]
Langaee, 2007	1398	Mixed	CYP3A5	No	[58]
Eap, 2007	373	Black population	CYP3A5 and ABCB1	Yes	[63]
Zolk, 2007	116	Caucasian males	ABCB1	No	[64]
Zhang, 2010	238	Japanese men	CYP3A5	Yes	[60]
Ferraresso, 2011	92	Kidney transplant recipients	CYP3A5	Yes	[67]
Torio, 2012	60	Kidney transplant recipients	CYP3A5	No	[68]
Liu, 2013	1987	Chinese population	ABCB1	Yes	[65]

polymorphisms correlate with blood pressure in different populations have shown variable results, possibly because some studies were underpowered (Table 1). Accordingly, CYP3A5*1/*3 correlated with blood pressure in 25 African-Americans and this association remained after correction for age, sex, and body mass index [51]. In fact, blood pressure of homozygous CYP3A5*1/*1 expressers exceeded that of homozygous non-expressers by as much as 19 mmHg [51]. A much larger study confirmed that CYP3A5 polymorphisms correlated with hypertension in individuals of African descent [53]. That is, the CYP3A5*1 allele was associated with resistent hypertension, although individuals with the CYP3A5*3/*3 genotype had the highest untreated blood pressure [53]. Bochud et al. found that African CYP3A5*1 carriers had higher ambulatory blood pressures than non-carriers; carriers also had a higher age- and sodium-related increase in blood pressure [54]. The relationship between CYP3A5 and blood pressure is less clear-cut in Caucasians. Although Kivistö et al. found that elderly individuals from Finland with the CYP3A5*1/*3 genotype more often had a diagnosis of hypertension [55], Fromm et al. found that individuals with the CYP3A5*3/*3 genotype had a higher 24-h ambulatory blood pressure and lower plasma aldosterone than those with the CYP3A5*1/*3 genotype [56]. Kreutz et al. also found CYP3A5*1 to be associated with lower systolic blood pressure and lower pulse pressure, especially in Caucasian females [57]. Langaee et al. were unable to confirm any of these findings in two cohorts with approximately 700 patients, although non-white CYP3A5*1 carriers had a somewhat better anti-hypertensive response to a calcium channel blocker [58]. Similarly, Lieb et al. failed to identify an association between the CYP3A5*1 allele and blood pressure in over 1,000 untreated Caucasians [59]. A possible explanation for these different results may be that the CYP3A5 genotype alters the response to environmental factors, as illustrated by one study that identified significant interactions between CYP3A5*1 and blood pressure once salt intake was factored in [60].

Although ABCB1 has been studies less extensively than CYP3A5 (Table 1), it may also be associated with blood pressure regulation. ABCB1 was shown to play a role in the transport of aldosterone [61] and the hypertensive response to angiotensin II [62]. It is also important to emphasize that substrates and inhibitors for CYP3A and ABCB1 largely overlap, suggesting that genetic variability in both genes should be analyzed simultaneously. Eap et al. studied both CYP3A5 and ABCB1 polymorphisms in an East-African population and found both genes to play a role in the effect of sodium on blood pressure, the renin-angiotensin system, and the response to treatment with an angiotensin-converting enzyme inhibitor [63]. More specific, the CYP3A5*1 and 3435T alleles reduced the blood pressure lowering effect of lisinopril . Zolk et

al. confirmed that genetic variation in ABCB1 affects the renin-angiotensin system, because Caucasians with the 3435 TT genotype showed a greater increase in plasma aldosterone after infusion of angiotensin II and were less able to excrete an oral sodium load [64]. Liu et al. not only showed a greater prevalence of hypertension in Chinese with the 3435 TT genotype, but also a greater risk of chronic kidney disease [65]. A caveat in studying ABCB1 is that haplotype variability may correlate better with phenotypic effect than a single allele.

Pharmacogenetics of CNI-induced hypertension

Based on the observation that CYP3A and ABCB1 are involved in both CNI metabolism and hypertension, it seems logical to extrapolate this to their involvement in CNI-induced hypertension. This possibility, however, has only been studied for CYP3A5 and only in a limited number of studies. Kreutz et al. studied the CYP3A5*1 allele of both donor and recipient in a large kidney transplant cohort treated with cyclosporine [66]. They found no relationship between the CYP3A5*1 SNP, blood cyclosporine levels, blood pressure or number of antihypertensive drugs [66]. Ferrarresso et al. genotyped 92 kidney transplant recipients receiving cyclosporine or tacrolimus and found that CYP3A5*1 carriers had higher blood pressure one week and 6 months after transplantation [67]. Although Torio et al. found a similar trend for higher blood pressure in CYP3A5*1 carriers 6 and 24 months after kidney transplantation under treatment with CNIs, this difference was not statistically significant, possibly due to low sample size [68]. Even if future studies would confirm that CYP3A5*1 increases the risk of post-transplant hypertension, this still does not prove an interaction between CYP3A5 and CNIs on blood pressure. More definitive proof will be difficult to obtain because it would require demonstrating that CYP3A5*1 donors or recipients treated with CNIs have more hypertension than those without this allele or those not treated with CNIs. If the CYP3A5*1 allele were to increase the risk of CNI-induced hypertension, another question is how this would work mechanistically. Because CYP3A5*1 carriers are rapid metabolizers, they usually require higher doses of tacrolimus to reach target levels. A possible explanation is that although higher doses are needed to obtain similar trough levels in plasma, these higher doses result in a higher area under the curve and therefore a stronger effect on blood pressure. Another possibility is that the CYP3A5*1 allele and CNIs both increase salt-sensitivity but through different mechanisms, resulting in a higher risk of hypertension when both factors are present. In addition to CYP3A5*1, other candidate genes that may increase the risk of CNI-induced hypertension include ABCB1, WNK4, and SPAK (Table 2). The proteins encoded by these genes

have been shown to play a role in CNI metabolism, including its pharmacokinetics (ABCB1) or pharmacodynamics (WNK4, SPAK) [18]. In addition, polymorphisms in these genes have also been associated with hypertension in the general population [63, 65, 69-71]. Even though ABCB1 has not been studied in relation to CNI-induced hypertension, it is an interesting candidate gene on theoretical grounds. Lower expression of ABCB1 results in higher intracellular CNI levels in tubular epithelial cells and more CNI nephrotoxicity [10]. Of interest, ABCB1 is also expressed in the basolateral membrane of the distal convoluted tubule [72], the nephron segment in which NCC is expressed. It is therefore conceivable that patients with lower ABCB1 expression are predisposed to CNI-induced hypertension, because higher intracellular CNI concentrations more easily activate the WNK-SPAK-NCC pathway. Another interesting candidate gene is SGK1 (Serum- and Glucocorticoid-inducible Kinase 1), because this was recently shown to regulate NCC [73] and SNPs in SGK1 are associated with hypertension in the general population [74]. In addition, SGK1 was recently shown to mediate the inflammatory response in T-helper 17 cells to dietary salt [75].

Conclusion & future perspective

Studies are needed to see if polymorphisms in any of the candidate genes discussed in this review truly contribute to CNI-induced hypertension (Table 2). What would be the clinical benefit of identifying a pharmacogenetic component to CNI-induced hypertension? Given the links between CNIs, CYP3A5, ABCB1, WNK, SPAK and salt-sensitivity, dietary salt restriction or treatment with thiazide diuretics may be especially effective interventions in susceptible patients. Another approach could be to select a CNI-free immunosuppressive regimen for these patients, which is becoming more feasible with encouraging results of newer immunosuppressive drugs such as belatacept [76]. In addition, a recent study showed that late withdrawal of CNIs reduced ambulatory blood pressure [77]. The largest group of patients treated with CNIs are kidney transplant recipients and most of the studies discussed in this review have been conducted in this population. It is important to emphasize that the contribution of CNIs to post-transplant hypertension remains difficult to dissect from other causes of hypertension commonly present in kidney transplant recipients [11]. Indeed, post-transplant hypertension is multifactorial and, in addition to CNIs, other contributing factors include pre-existing hypertension, duration of dialysis, donor characteristics, delayed graft function, and the use of glucocorticoids [78, 79]. Still, the prevalence of post-transplant hypertension clearly increased after the introduction of cyclosporine in the mid 1980s [4]. Furthermore,

Table 2 Candidate genes for CNI-induced hypertension

Gene	SNPs associated with hypertension in general population?	Protein involved in CNI pharmacokinetics or –dynamics?	SNPs associated with CNI-induced hypertension?
CYP3A5	Yes	Yes	Possibly
ABCB1	Yes	Yes	Unknown
WNK4	Yes	Yes	Unknown
SPAK	Yes	Yes	Unknown

post-transplant hypertension often coexists with hyperkalemia and acidosis despite normal kidney function, a combination that is highly suggestive of a CNI-effect. CNI-induced hypertension is also clearly recognized in patients in whom other causes of hypertension are less common, including patients with psoriasis and liver or bone marrow transplant recipients [80]. Most of the evidence for treating post-transplant hypertension currently favors calcium channel blockers [81], although evidence in favor of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers is emerging [82, 83]. Given these data, it is important to stress that CYP3A5*1 also predicts the blood pressure response to calcium channel blockers and angiotensin-converting enzyme inhibitors [58, 63]. Based on the activation of NCC by CNIs, thiazide diuretics also seem logical agents to reduce blood pressure. Thiazides, however, are used infrequently after kidney transplantation, probably because of concerns that they reduce glomerular filtration rate. One recent retrospective study showed that thiazide diuretics had a good antihypertensive effect in post-transplant hypertension without detrimental effects on glomerular filtration rate, but often led to potassium balance disorders [84]. We are currently conducting a prospective study to analyze the efficacy and safety of thiazide diuretics in post-transplant hypertension in patients using tacrolimus.

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Executive summary

CNI-induced hypertension

- Hypertension is a common side-effect of calcineurin inhibitors (CNIs), which are used to prevent rejection after transplantation.
- Hypertension after kidney transplantation has been associated with earlier graft failure and higher mortality of the recipient.

Pharmacokinetics of CNIs

- The enzyme CYP3A5 and the drug transporter ABCB1 are involved in the pharmacokinetics of CNIs, but also in the metabolism of mineralocorticoids.
- Recent studies indicate that CNIs activate the sodium chloride cotransporter in the kidney to cause hypertension and that this process is mediated by the kinases WNK4 and SPAK.

CYP3A5, ABCB1, and hypertension

- Single-nucleotide polymorphisms in CYP3A5, ABCB1, WNK4, and SPAK are associated with hypertension in the general population.

Pharmacogenetics of CNI-induced hypertension

- Given their dual role in CNI metabolism and hypertension, CYP3A5, ABCB1, WNK4 and SPAK are candidate genes for CNI-induced hypertension.
- Predicting the risk of CNI-induced hypertension may be useful to select specific interventions, including dietary sodium restriction, thiazide diuretics, or a CNI-free regimen.

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CHAPTER 5

Chlorthalidone Versus Amlodipine for Hypertension in Kidney Transplant Recipients Treated With Tacrolimus: A Randomized Crossover Trial

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Abstract

Background: Chlorthalidone is a very effective antihypertensive drug, but it has not been studied prospectively in kidney transplant recipients with hypertension. Recent data indicate that calcineurin inhibitors activate the thiazide-sensitive sodium chloride cotransporter, providing further rationale to test thiazides in this population.

Study Design: Randomized noninferiority crossover trial (non-inferiority margin -2.8 mmHg).

Setting & Participants: Hypertensive kidney transplant recipients using tacrolimus (median duration, 2.4 years after transplantation; mean estimated glomerular filtration rate, 63 ± 27 ml/min/1.73 m2, systolic blood pressure 151 ± 12 mmHg).

Intervention: Amlodipine (5-10 mg) and chlorthalidone (12.5-25 mg) for 8 weeks (separated by 2-week washout).

Outcomes: Average daytime (9 am – 9 pm) ambulatory SBP.

Measurements: Blood pressure and laboratory parameters.

Results: 88 patients underwent ambulatory blood pressure monitoring, of whom 49 (56%) with average daytime SBP . 140 mm Hg were enrolled. 41 patients completed the study. Amlodipine and chlorthalidone both reduced ambulatory SBP after 8 weeks (mean changes of 150 ± 12 to 137 ± 12 [SD] vs 151 ± 12 to 141 ± 13 mm Hg; effect size, -4.2 [95% CI, -7.3 to 1.1] mm Hg). Despite these similar blood pressure responses, chlorthalidone reduced proteinuria by 30% (effect size, -65 [95% CI, -108 to -35] mg/g) and also reduced physician-assessed peripheral edema (22% to 10%; $P < 0.05$ for both). In contrast, chlorthalidone temporarily reduced kidney function and increased both serum uric acid and glycated hemoglobin levels.

Limitations: Open-label design, short follow-up, per-protocol analysis.

Conclusions: Chlorthalidone is an antihypertensive drug equally effective as amlodipine after kidney transplantation.

Index words: Calcineurin inhibitors (CNIs); hypertension; kidney transplantation; amlodipine; chlorthalidone; blood pressure; ambulatory blood pressure monitoring (ABPM); sodium-chloride cotransporter (NCC); thiazide diuretics; end-stage renal disease (ESRD); kidney function; proteinuria; edema; clinical trial.

Introduction

Several studies have shown that hypertension after kidney transplantation is an independent risk factor for graft failure.[1-3] In addition, hypertension after kidney transplantation associates with increased risk of cardiovascular disease and even

mortality.[2, 4-6] Hypertension after kidney transplantation is multifactorial and factors related to donor, recipient, and transplantation have been implicated.[5, 7] Treatment with calcineurin inhibitors (CNIs) clearly contribute to hypertension after kidney transplantation, as illustrated by the increased incidence of hypertension after the introduction of cyclosporine.[8-10] The hypertensinogenic effect of CNIs has also been demonstrated in patients who are less prone to hypertension than patients with kidney disease, for example in patients with dermatological disease or after liver transplantation.[11-13] Several mechanisms have been shown to contribute to CNI-induced hypertension,[14, 15] including systemic and renal vasoconstriction, possibly through endothelin-1, and impaired vasodilation.[16-18] This may explain the efficacy of dihydropyridine calcium channel blockers (CCB) for the treatment of CNI-induced hypertension.[19] CNI-induced hypertension, however, has also been shown to be salt-sensitive.[12, 13, 20-24] More recently, the salt-sensitivity of CNI-induced hypertension was linked to the activation of one specific sodium transporter in the kidney. We and others showed that CNIs activate the sodium-chloride cotransporter (NCC, encoded by the SLC12A3 gene) to cause hypertension.[25, 26] More recently, the importance of this CNI-NCC pathway was also illustrated by attenuated CNI-induced hypertension in a kidney-specific knock-out mouse for the FK506-binding protein.[27] These findings suggest that thiazide diuretics, which block NCC, may also be effective drugs for CNI-induced hypertension.[25, 27] However, only one retrospective study addressed this question in kidney transplant recipients (all using CNIs). This study showed that thiazide diuretics effectively lowered blood pressure (SBP 147 ± 17 to 139 ± 18 mmHg and DBP 79 ± 9 to 77 ± 11 mmHg), but was associated with higher incidences of hyperkalemia and hypokalemia.[28] The reason that thiazide diuretics are used so infrequently in kidney transplant recipients may be related to concerns regarding efficacy at lower eGFR or adverse effects (gout, glucose intolerance). Therefore, the aim of this study was to analyze the effects of thiazide diuretics in kidney transplant recipients using tacrolimus. Given the roles of vasoconstriction and sodium retention in CNI-induced hypertension, we hypothesized that chlorthalidone is equally effective as amlodipine for the treatment of hypertension after kidney transplantation.

Methods

Patients

The study was approved by the Medical Ethical Committee of the Erasmus MC (MEC-2012-417) and was registered under ClinicalTrials.gov Identifier NCT02644395. The CONSORT checklist for noninferiority and equivalence trials is available as

Supplemental File. The Erasmus MC is a university hospital with an annual volume of approximately 200 kidney transplantations. As part of standard care, glucocorticoids are discontinued three months after transplantation. The inclusion criteria were age ≥ 18 years, treatment with tacrolimus (either the conventional twice-daily formulation (Prograft®, Astellas Pharma) or the once-daily formulation (Advagraf®, Astellas Pharma), eGFR ≥ 30 ml/min/1.73 m2 stable background antihypertensive drugs (i.e., no anticipated change in dose during the study period), and average daytime systolic blood pressure > 140 mmHg during ambulatory blood pressure measurement (ABPM). Patients were invited for ABPM when their office blood pressure was > 140 mmHg or when they were already using a thiazide diuretic or calcium channel blocker (these drugs were discontinued for two weeks prior to ABPM).[29] The exclusion criteria consisted of pregnancy, serum potassium < 3.5 mmol/L, serum sodium < 136 mmol/L, proteinuria > 885 mg/g creatinine (spot urine), the use of diuretics, glucocorticoids, or co-trimoxazole.

Study Design

The study design was a single-center, prospective, randomized, cross-over, open-label, non-inferiority study.

Interventions and Randomization Approach

Eligible patients were randomized to start with chlorthalidone 12.5 mg or amlodipine 5 mg for eight weeks. The eight-week treatment periods were separated by a two-week wash-out period. There were six study visits (at the start and end of each treatment period and two weeks after starting each drug). During the 2-week visit, blood pressure was measured every 5 minutes for 30 minutes using an oscillometric blood pressure monitor. Because oscillometric blood pressure monitors may overestimate systolic blood pressure[30], we used an average mean arterial pressure > 105 mmHg as cut-off to double the dose of the study drug. The patients in the study were analyzed per protocol. The study was stopped in patients who developed a serum sodium < 130 mmol/L or a serum potassium < 3.0 mmol/L during treatment. After completion of the study, patients continued with the drug with the best anti-hypertensive response and/or least side-effects. Patients were enrolled and randomly assigned on a 1:1 basis by the coordinating investigator (A.D.M.) to either receive amlodipine followed by chlortalidone (treatment order 1) or chlortalidone followed by amlodipine (treatment order 2). The randomization was performed by use of sealed, opaque, sequentially-numbered envelopes containing treatment allocation. The random-allocation sequence was generated by

an independent statistician by use of a random number generator on a computer. If a patient was assigned to a particular treatment order it was revealed to the treating physician.

Study Visits and Measurements

Data were collected, monitored, and entered by the coordinating investigator, and stored in a hospital-based electronic study database. The first patient was included 18 January 2013, and the study ended on the last study visit of the last patient on 17 December 2015. ABPMs were performed with the ultralite 90217A (Spacelabs Healthcare, Snoqualmie, USA), and the 30-min BP-recordings were performed with the datascope Accutorr Plus (Mindray, Shenzhen, China). The following criteria for ABPM were used: 24-h recording with measurements at 30-min intervals and with ≥ 70% of expected measurements (20 valid awake, 7 valid asleep).[31] The ABPM-device (with masked screen) was applied at the Erasmus MC by one of the investigators (A.D.M.), and patients returned the device after the 24h measurement period. On the first visit we measured plasma renin concentration (Renin III; Cisbio, Gif-sur-Yvette, France) and plasma aldosterone concentration (Coat-a-Count; Diagnostics Product Corporation, Los Angeles, California, USA). We also collected 24-hour urine to assess dietary salt consumption. On all visits we measured serum creatinine, electrolytes, bicarbonate, uric acid, pre-dose tacrolimus concentrations, and glycated hemoglobin (HbA1c). We also collected spot urines in which urinary protein, creatinine, and electrolytes were measured. Urinary protein and calcium excretion were analyzed as ratio with urine creatinine.[32, 33] All routine serum and urinary measurements were determined using the Cobas 8000 modular analyzer series (Roche, Basel, Switzerland). Tacrolimus was measured with the Dimension Xpand Plus Integrated Chemistry System (Siemens, Munich, Germany). HbA1c was measured using high-performance liquid chromatography (HPLC; HA-8180V, Menarini Diagnostics, Florence, Italy). GFR was estimated using the CKD-EPI 2009 creatinine equation.[34] On all visits a physician also evaluated the presence of the most common side-effects of the drugs (occurring 1 – 10%) and examined patients for the presence of peripheral edema.

Outcomes

The primary outcome was the average daytime SBP as obtained by ABPM (mean of ≥ 20 measurements obtained every 30 minutes over a 12-hour period on a single day). Secondary endpoints included proteinuria, urinary calcium excretion (as measure of thiazide effect), kidney function, and adverse effects.

Sample Size Determination

For the primary outcome, a power calculation for non-inferiority crossover studies showed the requirement of a sample size of 24 patients (assuming $\alpha = 0.05$, $\beta = 0.1$, allowable difference 0 mmHg, non-inferiority margin -2.8 mmHg, and variance 80 mmHg).[35-37] Written informed consent was obtained from all patients before inclusion and randomization. The study was carried out in compliance with the Good Clinical Practice guidelines.

Analysis

Continuous variables are reported as means ± standard deviation, unless indicated otherwise. Because our data consisted of paired and repeated measurements in the two treatment groups, we used a repeated measures general linear model that also included treatment order to analyze primary and secondary outcomes, and laboratory data. Adverse effects were scored as nominal variables and analyzed by $\chi 2$ test. A pre-test was performed to check the assumption of negligible carry-over effects.[38] Data were analyzed using SPSS Statistics (IBM, version 21.0). A P-value < 0.05 was considered statistically significant.

Results

Patient Characteristics

In 49 of the 88 patients (56%) who underwent the initial ABPM, daytime systolic blood pressure (SBP) was > 140 mmHg and they started in the study. Forty-one patients completed the study and were included for analysis. Five patients stopped the study during chlorthalidone treatment (5 for adverse events), one patient during amlodipine treatment (1 for adverse event), and two during wash-out (1 no-show, 1 hyponatremia prior to starting chlorthalidone; **Figure 1**). The baseline characteristics of the participants who completed the study are shown in **Table 1**. Most patients received a living donor kidney transplantation (76%) and had tacrolimus and mycophenolate mofetil as immunosuppressive regimen (88%); none of the patients used glucocorticoids. Dose escalation rates were similar (15 patients or 37% for amlodipine vs. 17 patients or 41% for chlorthalidone, P = 0.8). The average daily drug doses were 6.4 mg amlodipine and 16.4 mg chlorthalidone.

Blood Pressure Response

The primary and secondary outcome parameters are shown in **Table 2** (combined analysis) and **Figures 2 and 3** (divided by randomization groups). Both drugs reduced daytime SBP (from 150 ± 12 to 137 ± 12 mmHg for amlodipine and from 151 ± 12 to

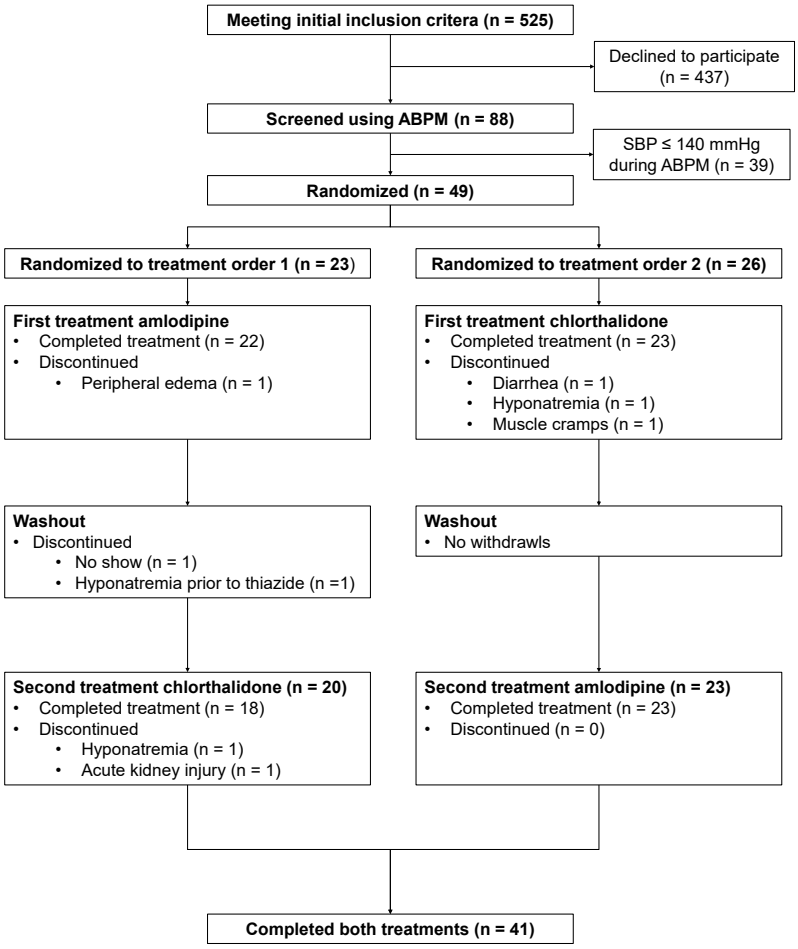

Figure 1 CONSORT (Consolidated Standards of Reporting Trials) diagram.
Of the 49 patients who were randomly assigned, 41 completed the study. Of the 8 patients who stopped the study, 5 stopped during chlorthalidone treatment; 1, during amlodipine treatment; and 2, during the washout. In 4 of the patients who stopped the study, a direct relationship with study drug was considered likely (2 patients, hyponatremia; 1 patient, muscle cramps during chlorthalidone treatment; and 1 patient, edema during amlodipine treatment). Abbreviations: ABPM, ambulatory blood pressure monitoring; SBP, systolic blood pressure.

141 ± 13 mmHg for chlorthalidone). There was no statistical difference in BP response between the two drugs (effect size -4.2, 95% confidence interval -7.3 to 1.1, P = 0.1). Chlorthalidone appeared to cause a carry-over effect (i.e., lower baseline SBP for patients receiving amlodipine as second treatment, **Figure 2A**). **Figure 2** also shows the change in daytime SBP for each individual patient to both drugs. Although most

patients had a decrease in average daytime SBP to both drugs, 4 patients had no response to amlodipine, and 8 patients had no response to chlorthalidone. Twenty-four patients had lower daytime SBP using amlodipine, while 16 patients had lower daytime SBP using chlorthalidone, and one patient responded equally well to both drugs (**Figure 2**). A full overview of the effect of both drugs on 24-hour, daytime, and nighttime blood pressures, pulse pressure, and heart rate is shown in Supplemental **Table S1**. Overall, a trend toward lower blood pressure parameters was observed during amlodipine, but no differences were statistically significant. The concurrent use of other anti-hypertensive drugs did not change the blood pressure response to the study drugs. Baseline urinary sodium and plasma renin did not predict the blood pressure response to the study drugs.

Biochemical Effects

Despite similar blood pressure responses, chlorthalidone significantly reduced proteinuria (median 169 to 116 mg/g, 32 ± 11% reduction vs. 4 ± 10% increase during amlodipine, P = 0.04, **Table 2** and **Figure 3A**). Furthermore, chlorthalidone nearly halved urinary calcium excretion, whereas amlodipine increased it (P < 0.001, **Table 2** and **Figure 3B**). Treatment with chlorthalidone decreased eGFR from 58 ± 22 to 50 ± 18 ml/min/1.73 m2, whereas amlodipine increased it from 54 ± 20 to 58 ± 22 ml/min/1.73 m2 (P < 0.001, **Table 2** and **Figure 3C**). However, in the 23 patients who completed treatment order 2 (first chlorthalidone), eGFR normalized during the wash-out and amlodipine treatment phases (**Figure 3D**). In addition, in five patients who continued using chlorthalidone after the study, eGFR initially decreased (75 ± 22 to 64 ± 20 ml/min/1.73 m2), but stabilized later on (eGFR 65 ± 20 ml/min/1.73 m2 after 21 ± 4 weeks chlorthalidone). Additional effects of chlorthalidone included an increase in serum uric acid (without any attacks of gout) and glycated hemoglobin, while amlodipine increased tacrolimus pre-dose concentrations (Supplemental **Table S2**). No carry-over effects were identified for the biochemical parameters, except for glycated hemoglobin (rise in glycated hemoglobin in patients starting with chlorthalidone which did not return to baseline at the start of amlodipine).

Adverse Effects

Both drugs caused few side-effects, except for physician-assessed edema, which increased during amlodipine (from 10 to 34%), but decreased during chlorthalidone (from 22 to 10%, **Table 3**). Electrolyte disorders were not more common with either study drug, except for hyperuricemia (**Table 3**). During the study period, no acute rejections were diagnosed or treated.

Table 1 Baseline Characteristics

Category and Parameter	First treatment amlodipine (n = 18)	First treatment chlorthalidone (n = 23)	Total (n = 41)
Demographics			
Age, years	59 ± 7	58 ± 13	58 ± 11
Men, n (%)	13 (72)	16 (70)	29 (71)
BMI, kg/m2	28 ± 4	26 ± 5	27 ± 5
Biochemical			
eGFR, ml/min/1.73 m2	60 ± 24	65 ± 29	63 ± 27
ARR, pg/µU	3.26 ± 3.39	5.80 ± 6.16	4.73 ± 5.27
Urinary sodium, mmol/d	160 ± 51	161 ± 61	161 ± 56
Proteinuria, mg/d	229 ± 223	120 ± 95	168 ± 171
Transplantation			
Living donor, n (%)	14 (78)	17 (74)	31 (76)
Time after transplantation, y	4.8 (0.5 – 10.7)	1.8 (0.4 – 16.4)	2.4 (0.4 – 16.4)
TAC + MMF*	15 (83)	21 (91)	36 (88)
Cause of Kidney Disease			
Diabetes mellitus	9 (50)	2 (18)	11 (27)
Hypertension	3 (17)	8 (73)	11 (27)
PKD	1 (6)	6 (26)	7 (17)
Glomerular disease	4 (22)	4 (17)	8 (20)
Blood pressure			
Daytime SBP, mmHg	150 ± 16	152 ± 8	151 ± 12
Anti-hypertensives	15 (83)	21 (91)	36 (88)
α-blocker	0 (0)	4 (17)	4 (10)
β-blocker	12 (67)	17 (74)	29 (71)
ACE-i/ARB	9 (50)	7 (30)	16 (39)

Note: Values for categorical variables are given as number (percentage); values for continuous variables, as mean 6 standard deviation or median [interquartile range].
Abbreviations: ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; ARR, aldosterone-renin ratio; BMI, body mass index; eGFR, estimated glomerular filtration rate; MMF, mycophenolate mofetil; PKD, polycystic kidney disease; SBP, systolic blood pressure; TAC, tacrolimus.
*Other regimens consisted of TAC with mTOR (mammalian target of rapamycin) inhibitor (n 5 3), TAC with azathioprine (n 5 1), or TAC monotherapy (n 5 1).

Discussion

In this cross-over trial we compared the ambulatory blood pressure response of the vasodilatory drug amlodipine with the thiazide diuretic chlorthalidone in 41 hypertensive kidney transplant recipients using tacrolimus. To our best knowledge, this is the first study prospectively analyzing the efficacy and safety of thiazide diuretics in kidney transplant recipients. The rationale to do so was based on the recent insights that part of the hypertensinogenic effect of CNIs is due to the activation of the thiazide-sensitive sodium chloride cotransporter.[25, 26, 39] Both amlodipine and chlorthalidone reduced blood pressure markedly with an average reduction in systolic blood pressure (SBP) of at least 10 mmHg. Despite similar blood pressure responses to both drugs, chlorthalidone reduced proteinuria. Amlodipine caused more peripheral edema, but had little effect on laboratory parameters. In contrast, chlorthalidone had several effects: it (temporarily) reduced eGFR, and increased serum uric acid and glycated hemoglobin.

The changes in blood pressure, eGFR, and proteinuria after chlorthalidone in our study are very similar to those recently reported in a non-transplant population by Cirillo et al.[40] and Agarwal et al.[41] In hypertensive patients with normal or low kidney function (eGFR < 60 ml/min/1.73 m2), Cirillo et al. showed that chlorthalidone caused similar decreases in blood pressure and eGFR, but a greater reduction in body weight in patients with low kidney function.[40] Agarwal et al. conducted a 12-week pilot study to test chlorthalidone in 12 patients with CKD (eGFR 20 – 45 ml/min/1.73 m2). They observed a 40-45% reduction in proteinuria by chlorthalidone, but an increase in serum creatinine with a peak after 8 weeks and return to baseline after 12 weeks. The latter was attributed to intravascular volume depletion, as the rise in serum creatinine was paralleled by a decrease in body weight and total body volume, and an increase in plasma renin and aldosterone. After 12 weeks these changes were mitigated, which led the authors to argue that the blood pressure lowering effect had changed from volume to nonvolume mechanisms.[41] In our study, the improvement in eGFR after discontinuing chlorthalidone and during

Table 2 Effect of Amlodipine and Chlorthalidone on Primary and Secondary Outcome Parameters, Per Protocol Analysis

	Values After Intervention		Comparison	P-value
	Amlodipine	**Chlorthalidone**	**Amlodipine vs. chlorthalidone**	
Primary Outcome				
Daytime SBP, mmHg	137 ± 12	141 ± 13	-4.2 (-7.3 to 1.1)	0.1
Secondary Outcomes				
Proteinuria, mg/g*	169 [80, 260]	116 [55, 235]	65 (35 to 108)	0.04
Urine calcium / creatinine (mmol/mmol)	0.23 ± 0.19	0.10 ± 0.09	0.13 (0.08 to 0.18)	< 0.01
eGFR, ml/min/1.73 m^2	58 ± 22	50 ± 18	8.5 (6.2 to 10.8)	< 0.01

Note: Unless otherwise indicated, values are given as mean 6 standard deviation.

Abbreviations: CI, confidence interval; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure.

*Reported as median [interquartile range] because of non-normal distribution. P value calculated using nonparametric test.

long-term use of chlorthalidone is consistent with this proposed mechanism. This calls into question the clinical significance of the temporary decrease in kidney function. Diuretics may cause acute kidney injury, especially in combination with renin-angiotensin-aldosterone-system (RAAS) blockade. However, a slight reduction in kidney function may predict better renal outcomes, possibly because it indicates amelioration of glomerular hypertension.[42-44] Indeed, the antiproteinuric effect of thiazide diuretics was recently also shown in patients with diabetic nephropathy when added to renin-angiotensin aldosterone blockade and found to be comparable with dietary sodium restriction.[42] Furthermore, diuretic uptitration reduced residual proteinuria better than combined RAAS blockade.[45] Obviously, in kidney transplant recipients an eGFR reduction due to chlorthalidone is less desirable shortly after transplantation, as it will complicate the assessment of possible rejection.

Hypertension after kidney transplantation has been considered a “neglected issue”, because few studies have directly compared antihypertensive agents in this population.[6] Indeed, the majority of studies compared a single antihypertensive drug (mostly CCB or ACE-inhibitor) with no treatment or placebo.[46] When

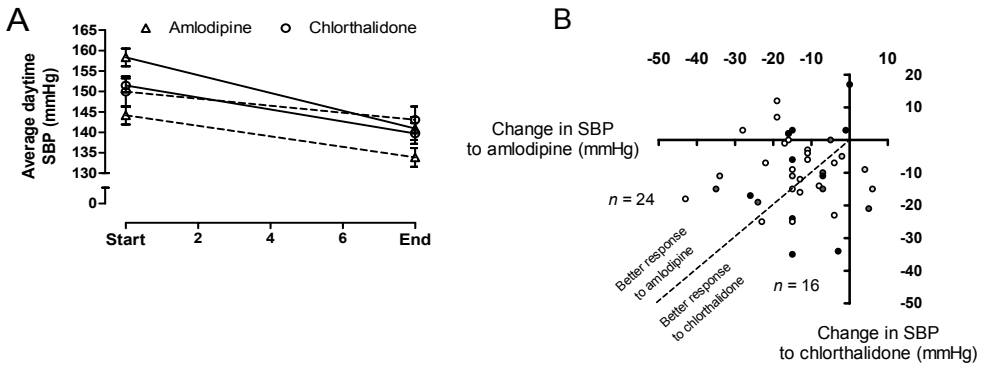

Figure 2. Blood pressure response to chlorthalidone and amlodipine.

(A) Response in average daytime systolic blood pressure (SBP; the primary outcome parameter) to amlodipine and chlorthalidone for the 4 groups (dashed lines indicate second treatment in the crossover design). (B) Individual responses in average daytime SBP to both drugs. Each data point represents 1 patient. Dashed line separates patients in those who had a better SBP response to amlodipine or to chlorthalidone. Open symbol indicates no dose escalation for either drug; light grey, for amlodipine only; dark grey, for chlorthalidone only; and black, for both drugs.

compared directly, ACE-inhibitors more often caused hyperkalemia, a fall in eGFR, and reduction in proteinuria than CCBs.[46] These effects are comparable to thiazide diuretics, although we did not observe more hyperkalemia or hypokalemia, in contrast to a previous retrospective study.[28] Most studies analyzed surrogate endpoints, whereas the choice of antihypertensive therapy should ideally be guided by hard endpoints. Although a meta-analysis suggested that CCBs prevent graft loss, this was in comparison to no treatment or placebo and may therefore be explained through an effect on blood pressure.[46] It remains to be seen whether the observed effects beyond blood pressure of ACE-inhibitors, ARBs, and thiazide diuretics result in better long-term outcomes. One small study suggested better outcomes of a composite endpoint (death and graft function) with the use of ACE-inhibitors.[47] A larger study found a trend towards better graft outcomes with angiotensin II receptor blockade.[48] Thiazide diuretics may be especially effective in patients who, in addition to hypertension, have other renal tubular effects of CNIs, including hyperkalemia and acidosis.[25] In our study population, the blood pressure reduction by chlorthalidone was comparable to that in primary hypertension.[49] Yet, most patients after kidney transplantation are salt-sensitive,[21] providing a rationale for dietary sodium restriction[50] or diuretics.[25] Indeed, the average dietary NaCl intake in this cohort was high (161 mmol/day or 9.3 grams/day).

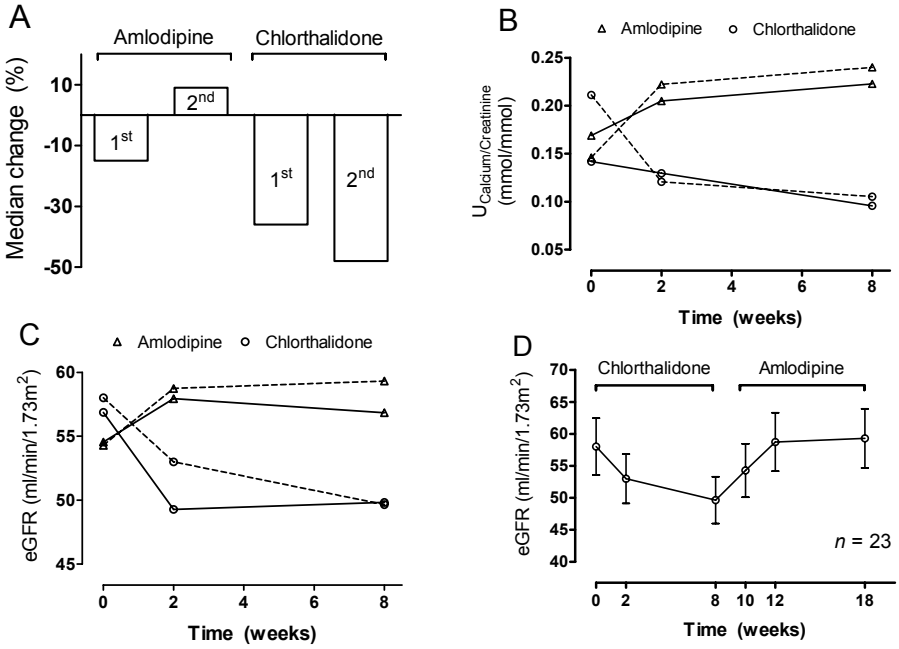

Figure 3. Effects of amlodipine and chlorthalidone onmedian change in (A) proteinuria, (B) urinary calciumexcretion ($U_{calcium/creatinine}$), and (C, D) estimated glomerular filtration rate (eGFR). Dashed lines indicate second treatment in the crossover design (B, C). (D) Restoration of eGFR after treatment with chlorthalidone (in the 23 patients who were randomly assigned to start with this drug).

A number limitations of this study should be mentioned. HowHypertension after kidney transplantation is multifactorial, and we therefore do not know to which degree CNIs contributed to hypertension in our patients. The open-label design is also a limitation of the study. Because follow-up was stopped after study drug discontinuation, no intention to treat analysis was performed. Finally, many eligible patients declined to participate in this study because of the four ABPMs, limiting generalizability. However, we believe the use of ABPM is important, as illustrated by the observation that almost half of the patients with an office SBP > 140 mmHg, did not have hypertension when analyzed with ABPM (**Figure 1**).

In summary, both amlodipine and chlorthalidone are effective anti-hypertensive drugs after kidney transplantation; chlorthalidone may be preferable in patients with proteinuria or edema. Combination therapy with half doses of each agent may also be an interesting approach, but requires future study.

Table 3 Incidence of Adverse Events and Electrolyte Disorders

	Amlodipine			Chlorthalidone		
	1st (n=18)	2nd (n=23)	Combined (n=41)	1st (n=23)	2nd (n=18)	Combined (n=41)
Adverse Events						
Edema	1 (5)	13 (57)	14 (34)	0 (0)	4 (22)	4 (10)*
Headache	2 (11)	3 (13)	5 (12)	6 (26)	6 (33)	12 (29)
Vertigo	1 (5)	6 (26)	7 (17)	8 (35)	6 (33)	14 (34)
Dry mouth	7 (39)	8 (35)	15 (37)	3 (13)	8 (44)	11 (27)
Muscle cramps	4 (22)	8 (35)	12 (29)	5 (22)	11 (61)	16 (39)
Electrolyte Disorders†						
Serum sodium < 136 mmol/L	1 (5)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)
Serum potassium < 3.5 mmol/L	0 (0)	0 (0)	0 (0)	0 (0)	2 (11)	2 (5)
Serum calcium > 10.6 mg/dL	2 (11)	1 (4)	3 (7)	1 (4)	1 (5)	2 (5)
Serum magnesium < 1.4 mEq/L	11 (61)	11 (48)	22 (54)	13 (57)	14 (78)	27 (66)
Serum uric acid > 7.1 mg/dL	4 (22)	4 (17)	8 (20)	12 (52)	13 (72)	25 (61)*

Note: Values are given as number (percentage). Conversion factors for units: calcium in mg/dL to mmol/L, 30.2495; uric acid in mg/dL to mmol/L, 359.48.

*P , 0.05 by X^2 test (amlodipine combined vs chlorthalidone combined).

†Patients who developed serum sodium levels , 136 mmol/L or serum potassium levels , 3.0 mmol/L during the study were discontinued (Fig 1).

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CHAPTER 6

NaCl cotransporter abundance in urinary vesicles is increased by calcineurin inhibitors and predicts thiazide sensitivity

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Abstract

Animal studies have shown that the calcineurin inhibitors (CNIs) cyclosporine and tacrolimus can activate the thiazide-sensitive NaCl cotransporter (NCC). A common side effect of CNIs is hypertension. Renal salt transporters such as NCC are excreted in urinary extracellular vesicles (uEVs) after internalization into multivesicular bodies. Human studies indicate that CNIs also increase NCC abundance in uEVs, but results are conflicting and no relationship with NCC function has been shown. Therefore, we investigated the effects of CsA and Tac on the abundance of both total NCC (tNCC) and phosphorylated NCC at Thr60 phosphorylation site (pNCC) in uEVs, and assessed whether NCC abundance in uEVs predicts the blood pressure response to thiazide diuretics. Our results show that in kidney transplant recipients treated with cyclosporine (n = 9) or tacrolimus (n = 23), the abundance of both tNCC and pNCC in uEVs is 4-5 fold higher than in CNI-free kidney transplant recipients (n = 13) or healthy volunteers (n = 6). In hypertensive kidney transplant recipients, higher abundances of tNCC and pNCC prior to treatment with thiazides predicted the blood pressure response to thiazides. During thiazide treatment, the abundance of pNCC in uEVs increased in responders (n = 10), but markedly decreased in non-responders (n = 8). Thus, our results show that CNIs increase the abundance of both tNCC and pNCC in uEVs, and these increases correlate with the blood pressure response to thiazides. This implies that assessment of NCC in uEVs could represent an alternate method to guide anti-hypertensive therapy in kidney transplant recipients.

Introduction

The calcineurin inhibitors (CNIs) cyclosporine A (CsA) and tacrolimus (Tac) are widely used to prevent rejection of transplanted organs. CNIs inhibit the calcineurin-mediated immune response in T-cells [1]. Although both CsA and Tac exert their principal immunosuppressive effects through inhibition of the same target protein, calcineurin, they differ in cytoplasmic-binding proteins, namely cyclophilins and FKBP12 for CsA and Tac, respectively. CsA and Tac also vary with respect to their immunosuppressive potency [2,3] and side effects [4-6]. A common side effect of CNIs is hypertension, although CsA appears more hypertensinogenic than Tac [6-8]. CNI-induced hypertension may be accompanied by hyperkalemia and metabolic acidosis [9,10]. The clinical characteristics of CNI-treated patients sometimes resemble that of familial hyperkalemic hypertension (FHHt) [11,12], also known as Gordon syndrome [13] or pseudohypoaldosteronism type II [14] (OMIM 145260). FHHt results from mutations in WNK [with no lysine (K)] kinases WNK1 and WNK4 [15], Kelch-like 3 (KLHL3) [16], or Cullin 3 (CUL3) [17], which all lead to a gain-of-

function in the thiazide-sensitive NaCl cotransporter (NCC) resulting in salt retention in the distal part of the nephron [15,18-20].

Several studies have shown that CNIs increase NCC activity possibly contributing to hypertension [21,22]. Melnikov et al. demonstrated that rats treated with CsA develop a phenotype similar to that of FHHt, which they attributed to an increase in WNK4 abundance in the kidney [23]. This phenomenon is supported by in vitro studies showing that the abundance of WNK4 and ultimately of total NCC (tNCC) and phosphorylated, or active, NCC (pNCC), is increased in immortalized mouse distal convoluted tubule (mDCT) cells treated with CsA [23]. Hoorn et al. revealed that Tac-induced hypertension in mice is predominantly mediated by an increase in pNCC abundance, possibly through an effect of the NCC-regulating kinases WNK3, WNK4, and STE20/SPS1-related proline/alanine-rich kinase (SPAK) [21]. Recent evidence suggests that in mice, Tac prevents the high potassium stimulated NCC dephosphorylation [24]. Additionally, it has been demonstrated that Tac acts directly on kidney tubule cells expressing NCC to cause hypertension, and that inhibition of calcineurin is required for this effect [22].

In humans, urinary extracellular vesicles (uEVs), including exosomes, have been extensively characterized and studied as non-invasive biomarkers for renal tubular disorders [25-28]. uEVs are nanosized membranous vesicles released from all cells lining the nephron. Alterations in the expression of different proteins present in the epithelial cells of the distal convoluted tubule (DCT), including NCC, are reflected in the composition of uEVs [29-32]. Patients with FHHt have an increased NCC abundance in uEVs [31-33], while patients with Gitelman syndrome exhibit a decreased NCC abundance in uEVs [28,34]. In humans, minerlocorticoid administration rapidly increased the abundance of tNCC and pNCC in uEVs [35], possibly mediated by a reduced plasma potassium concentration secondary to epithelial sodium channel (ENaC) activation [35,36]. This suggests that NCC abundance in uEVs reflects the actual state of NCC expression in the epithelial cells of the DCT. Accordingly, we previously showed that in patients with primary aldosteronism, pNCC increased similarly in kidney and uEVs [31]. Although protein abundance and characterization in uEVs can potentially be used as bio-markers for some diseases [28,31-34], only a few studies have been conducted to investigate the role of NCC in CNI-induced hypertension in humans. Esteva-Font et al. found a positive correlation between plasma CsA levels and NCC abundance in uEVs of CsA-treated kidney transplant recipients [37]. Rojas-Vega et al. showed an increased abundance of NCC in uEVs of Tac-treated hypertensive kidney transplant recipients [38]. Although these studies showed the stimulatory effect of CNIs on NCC abundance in uEVs of kidney

transplant recipients, they did not explore the relationship between NCC abundance and blood pressure in kidney transplant recipients. Therefore, in the present study, we performed a large-scale study to investigate the effect of CsA and Tac on the abundance of both tNCC and pNCC in uEVs, and assessed whether NCC abundance in uEVs predicts the blood pressure response to thiazide diuretics. Finally, in order to confirm the effect of CNIs on NCC in the kidney, an ex vivo study was conducted in mice cortical tubules exposed to CsA.

Materials and methods

Study design and population

Two groups of kidney transplant recipients using CNIs were studied. Group 1 was recruited at the Radboud university medical center, in Nijmegen, The Netherlands, and consisted of a randomly selected cohort of 45 kidney transplant recipients and 6 healthy volunteers of whom uEVs were isolated and analyzed. The kidney transplant recipients used CsA (n = 9), Tac (n = 23) or a CNI-free immunosuppressive regimen (n = 13) for at least 6 months and were matched for age and gender. Kidney transplant recipients who had been using thiazide diuretics or aldosterone antagonists after transplantation were excluded. Group 2 consisted of Tac-treated hypertensive kidney transplant recipients (median of 2.4 years after kidney transplantation), recruited from a clinical trial studying the anti-hypertensive effect of thiazide-type diuretic chlorthalidone at the Erasmus Medical Center, in Rotterdam, The Netherlands [39]. Patients with an office blood pressure >140/90 mmHg were invited for ambulatory blood pressure measurement. In this group, 18 patients with an average daytime systolic blood pressure >140 mmHg were enrolled and followed for 8 weeks chlorthalidone (12-25 mg once daily) treatment. Patients who responded to chlorthalidone (`responders', decrease of 10 mmHg in average daytime systolic blood pressure, n = 10) were compared with patients who did not respond to chlorthalidone (`non-responders', no change or an increase in average daytime systolic blood pressure, n = 8). All participants gave written informed consent and both cohorts were approved by Medical Ethics Committee (CMO09/073 for Radboud university medical center and MEC-2012-417 for Erasmus Medical Center) and this study was conducted according to the principles expressed in the Declaration of Helsinki.

Urine collection and isolation of extracellular vesicles

In Group 1, second-morning mid-stream urine sample was collected. In Group 2, second-morning mid-stream urine was collected just before starting and after

8 weeks of chlorthalidone treatment. In both groups, immediately after urine collection, the protease inhibitors (50 µmol/L phenylmethylsulfonyl fluoride, 20 µmol/L aprotinin, 10 µmol/L pepstatin A, and 20 µmol/L leupeptin) were added to the urine to reduce protein degradation. All samples were directly stored at -80˚C. uEVs were isolated as reported previously [29–31,40]. In brief, 10 to 40 mL of the collected urine samples were centrifuged at 17,000 × g for 15 minutes at 24˚C in an ultracentrifuge (Sorvall™ WX Floor Ultra Centrifuges, Thermo Scientific, Asheville, NC, USA) with a 70.1Ti rotor. The supernatant was stored at room temperature for 25 minutes. The pellet was resuspended in 50 µL of 3.24 mol/L dithiothreitol and 200 µL isolation solution (10 mmol/L triethanolamine, 250 mmol/L sucrose, HCl pH 7.6) and centrifuged at 17,000 × g for 15 minutes at 24˚C. Next, the supernatant was collected and combined with the supernatant obtained from the previous centrifugation, and the combined supernatants were centrifuged at 170,000 × g for 2.5 hours at 24˚C. Pellets containing uEVs were solubilized in Laemmli sample buffer (0.6% w/v SDS, 3% v/v glycerol, 18 mmol/L Tris-HCl pH 6.8 and 0.003% w/v bromophenol blue). All the samples were preheated for 15 minutes at 65˚C before immunoblotting. Urinary creatinine was measured according to Jaffe's method with the use of a colorimetric assay (Labor und Technik, Berlin, Germany).

Mouse cortical tubule suspension

Animal protocols were approved by the board of the Institute of Biomedicine, University of Aarhus. The animal protocols comply with the European Community guidelines for the use of experimental animals, were approved and performed under a license issued for the use of experimental animals by the Danish Ministry of Justice (Dyreforsøgstilsynet). For this study mice were anesthetized using isoflurane inhalation, followed by cervical dislocation. Both kidneys from a male wildtype C57BL/6J mouse were removed and dissected into approximately 1 mm pieces and placed into 4 mL of enzyme solution containing 1.5 mg/mL collagenase type B (Worthington Labs, Lakewood, NJ, USA) in basic buffer (125 mmol/L NaCl, 30 mmol/L glucose, 0.4 mmol/L KH2PO4, 1.6 mmol/L K2HPO4, 1 mmol/L MgSO4, 10 mmol/L Na-acetate, 1 mmol/L α-ketoglutarate, 1.3 mmol/L Ca-gluconate, 5 mmol/L glycine, 48 µg/mL trypsin inhibitor, and 50 µg/mL DNase, Tris-HCl pH 7.4). Samples were mixed continuously at 37˚C using a benchtop orbital mixer. Samples were incubated for 15 minutes at 22˚C to let the large fragments sink down to the bottom of the tube. Next, 2 mL of the enzyme solution was removed and replaced with 2 mL of basic buffer. After 10 minutes' incubation at 4˚C, an additional 2 mL of basic buffer was added, and samples were incubated for an additional 10 minutes at 22˚C. Large

fragments were allowed to settle, the supernatant was removed and centrifuged at 200 × g for 2 minutes. The pellet was resuspended in 5 mL of basic buffer (buffer B) (120 mmol/L NaCl, 30 mmol/L glucose, 1 mmol/L CaCl2, 1 mmol/L MgCl2, 1 mmol/L Na2HPO4, 1 mmol/L Na2SO4, 15 mmol/L Na-HEPES, Tris-HCl pH 7.4) and centrifuged at 200 × g for 2 minutes. The tubular suspensions were resuspended in buffer B and 500 µL was transferred into individual tubes containing either DMSO (negative control) or different concentrations of CsA (final concentrations of 5, 10, or 20 µmol/L). Hypotonic low chloride (buffer H), which stimulates NCC [41,42], was used as a positive control. Suspensions were incubated with continuous mixing for 30 and 90 minutes at 37˚C. Tubules were centrifuged for 10 minutes at 3,000 × g at 4˚C and pellets were resuspended in 300 µL Laemmli sample buffer containing dithiothreitol (50 mg/mL). Finally, the samples were heated for 15 minutes at 60˚C before immunoblotting.

Immunoblotting

uEV-samples were loaded on a gradient SDS-PAGE gel (4-15% v/v Criterion™ TGX™ Precast Gel, Bio-Rad, The Netherlands). Loading of uEV-sample of each subject was normalized to urinary creatinine concentration to account for uEV concentration differences between the individual samples [26]. Subsequently, immunoblotting was performed on polyvinylidene difluoride membranes (Immobilon-P, Millipore Corporation, Bedford, MA, USA), which were blocked and probed with antigen-specific primary antibodies. Blots were incubated with species-specific fluorescent secondary antibodies and visualized using enhanced chemiluminescence (Thermo Fischer Scientific, Waltham, MA, USA) and gel imaging system (ChemiDoc XRS, Bio-Rad Laboratories, Hercules, CA, USA). Finally, both the dimeric and monomeric forms of the NCC bands on the blots were quantified together with Image Studio Lite software (LI-COR Biosciences, NE, USA) or ImageQuant TL (GE Healthcare Life Sciences, PA, USA). To analyze whether normalization by urinary creatinine resulted in a similar number of uEVs loaded on a gel, the abundance of the uEV-marker CD9 was measured [26,30,43].

Antibodies

The following antibodies were used: anti-total NCC (Millipore, Billerica, MA, USA, #AB3553; 1:2,000); anti-human phosphorylated NCC at Thr60 or (anti-mouse phosphorylated NCC at Thr58; 1:2,000) as previously described [44] and anti-uEV marker CD9 of human origin (C4, Santa Cruz Biotechnology, Inc., CA, USA; 1:500). Secondary antibodies used were peroxidase-conjugated goat anti-rabbit (Sigma-

Aldrich, St. Louis, MO, USA; 1:10,000), and anti-mouse (Sigma-Aldrich, St. Louis, MO, USA; 1:10,000).

Table 1 Clinical and laboratory characteristics of the patients in study Group 1.

Characteristic (mean ± SEM)	CNI-free group (n = 13)	CsA group (n = 9)	Tac group (n = 23)	P-value
Age	53 ± 3	51 ± 2	52 ± 3	0.90[a], 0.96[b]
Male (n (%))	7 (54)	5 (56)	12 (52)	
Body mass index, Kg/m2	25 ± 1	27 ± 2	27 ± 1	0.53[a], 0.38[b]
Cause of ESRD (n)				
Polycystic kidney disease	3	1	5	
Diabetes	0	2	2	
Glomerular disease	2	0	0	
Hypertension/Vascular	0	0	2	
Other	8	6	14	
Plasma sodium, mmol/L	140 ± 1	138 ± 1	139 ± 1	0.44[a], 0.72[b]
Plasma potassium, mmol/L	4.0 ± 0.1	4.1 ± 0.1	4.1 ± 0.1	0.80[a], 0.72[b]
Plasma chloride, mmol/L	106 ± 1	105 ± 1	108 ± 1	0.80[a], 0.29[b]
Plasma creatinine, mg/dL	1.6 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	0.80[a], 0.72[b]
eGFR, mL/min/1.73 m2	46 ± 2	42 ± 3	45 ± 4	0.76[a], 0.97[b]
Systolic blood pressure, mmHg	135 ± 3	137 ± 4	142 ± 3	0.91[a], 0.22[b]
Diastolic blood pressure, mmHg	80 ± 2	84 ± 4	82 ± 2	0.52[a], 0.77[b]
CsA pre-dose level, µg/L		127 ± 14		
Tac pre dose level; µg=L			6.7 ± 0.4	

SEM = standard error of the mean; ESRD = end stage renal disease; data are presented as mean ± SEM.
a = P-value for difference between CsA and CNI-free;
b = P-value for difference between Tac and CNI-free, 95% CI, 95% con®dence interval.

Statistical analysis

Values are expressed as mean ± SEM. In Group 1 the immunoblot data were analyzed by comparing integrated optical densities of bands by one-way ANOVA with Dunnett multiple comparisons post hoc test. In Group 2 the immunoblot data were analyzed using a non-parametric Student's t-test (comparing responders to non-responders). Two-way ANOVA was used to assess changes in uEV protein abundance before and after the chlorthalidone treatment. In the mouse study the immunoblot data were analyzed by comparing integrated optical densities of bands after 30 and 90 minutes exposure to CsA separately compared to its negative control (basic buffer without CsA) by one-way ANOVA with Dunnett multiple comparisons post hoc test. In this study, fold-change of 1 means no change. $P<0.05$ was considered statistically significant. All data were analyzed using Prism 5 software (GraphPad Software Inc, La Jolla, CA, USA).

Results

CNIs increase NCC in uEVs of kidney transplant recipients

Table 1 shows the clinical and laboratory characteristics of 9 CsA, 23 Tac and 13 CNI-free immunosuppressive regimens treated kidney transplant recipients (Group 1). The effect of CNI on NCC abundance in uEVs was assessed using immunoblot analysis. Two immunoreactive bands of ~260 and ~130 kDa representing the dimeric and monomeric forms of the NCC protein were detected in uEVs (Fig 1). Both forms were included in the analysis to determine the effect of CNI treatment on NCC abundance in uEVs. The abundances of tNCC and pNCC in uEVs of both CsA and Tac-treated kidney transplant recipients were significantly higher compared to uEVs isolated from kidney transplant recipients treated with CNI-free immunosuppressive regimens or healthy volunteers (Fig 2A and 2C). No significant difference in tNCC and pNCC abundance was detected between CsA and Tac-treated kidney transplant recipients. The ratio of pNCC to tNCC did not differ between kidney transplant recipients treated with CsA, Tac, CNI-free immunosuppressive regimens, and healthy volunteers (Fig 2E). Females may express more NCC [45], but in our study a gender difference in the abundance of NCC in the uEVs was not demonstrated (S1 Fig). No significant differences in CD9 abundance were observed between the four experimental groups, suggesting comparable uEV numbers (Fig 2B and 2D). Additional normalization by CD9 abundance showed that both tNCC (S2A Fig) and pNCC (S2B Fig) abundance in both CsA- and Tac-treated kidney transplant recipients was significantly higher in comparison to kidney transplant recipients treated with CNI-free immunosuppressive regimens, but not healthy volunteers.

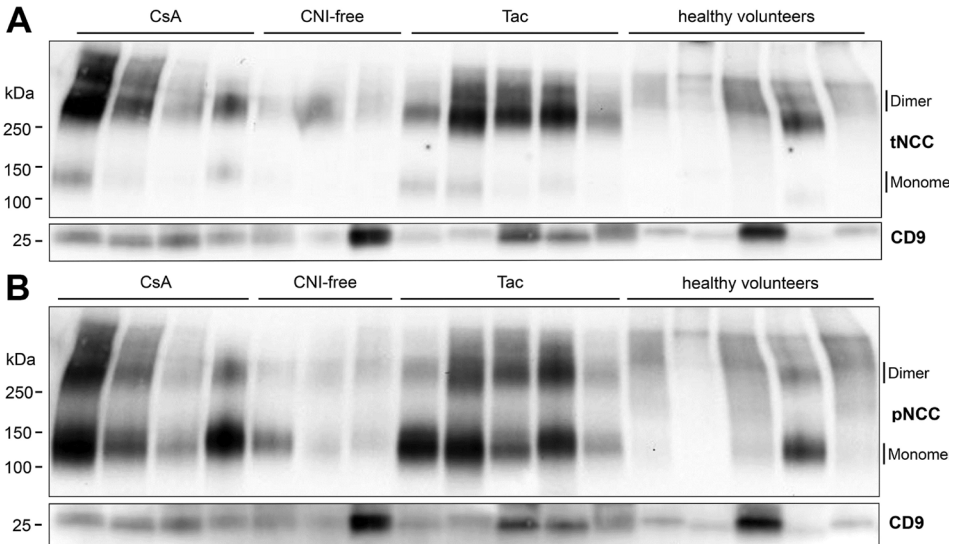


Figure 1 Representative immunoblots of tNCC and pNCC abundance in uEVs of kidney transplant recipients treated with CsA, Tac or CNI-free immunosuppressive regimens and healthy volunteers. Panels A and B show the immunoreactive bands in uEVs of patients treated with CsA (n = 4), Tac (n = 5), CNI-free immunosuppressive regimens (n = 3), and healthy volunteers (n = 5). tNCC (A) and pNCC (B) immunoreactive bands in uEVs of both CsA- and Tac-treated kidney transplant recipients were more abundant compared to kidney transplant recipients treated with CNI-free immunosuppressive regimens and healthy volunteers.

NCC abundance in uEVs predicts the anti-hypertensive response to thiazides

Subsequently, we investigated whether the blood pressure response to thiazide diuretics in hypertensive kidney transplant recipients treated with Tac correlates with NCC abundance in uEVs. To this end, we compared tNCC and pNCC abundances in uEVs of patients with a blood pressure response to a thiazide in comparison to those who did not respond. Table 2 shows the clinical and laboratory characteristics of these patients (Group 2). Pre-treatment abundances of both tNCC and pNCC in uEVs were significantly higher in chlorthalidone responders compared to non-responders (Fig 3). Furthermore, both pNCC and tNCC abundance in uEVs correlated with the blood pressure response (R^2 = 0.27 and 0.30 using log-transformed densitometry data because of non-normal distribution, $P<0.05$ for both, Fig 3). Subsequently, the change in NCC abundance before and after treatment with chlorthalidone in uEVs of both responders and non-responders was compared (Fig 4 and S5 and S6 Figs, show original immunoblots). The increase of tNCC in uEVs was larger in non-responders than in responders ($P<0.05$). While pNCC in uEVs also increased in most responders, there was a decrease in pNCC in the majority of non-

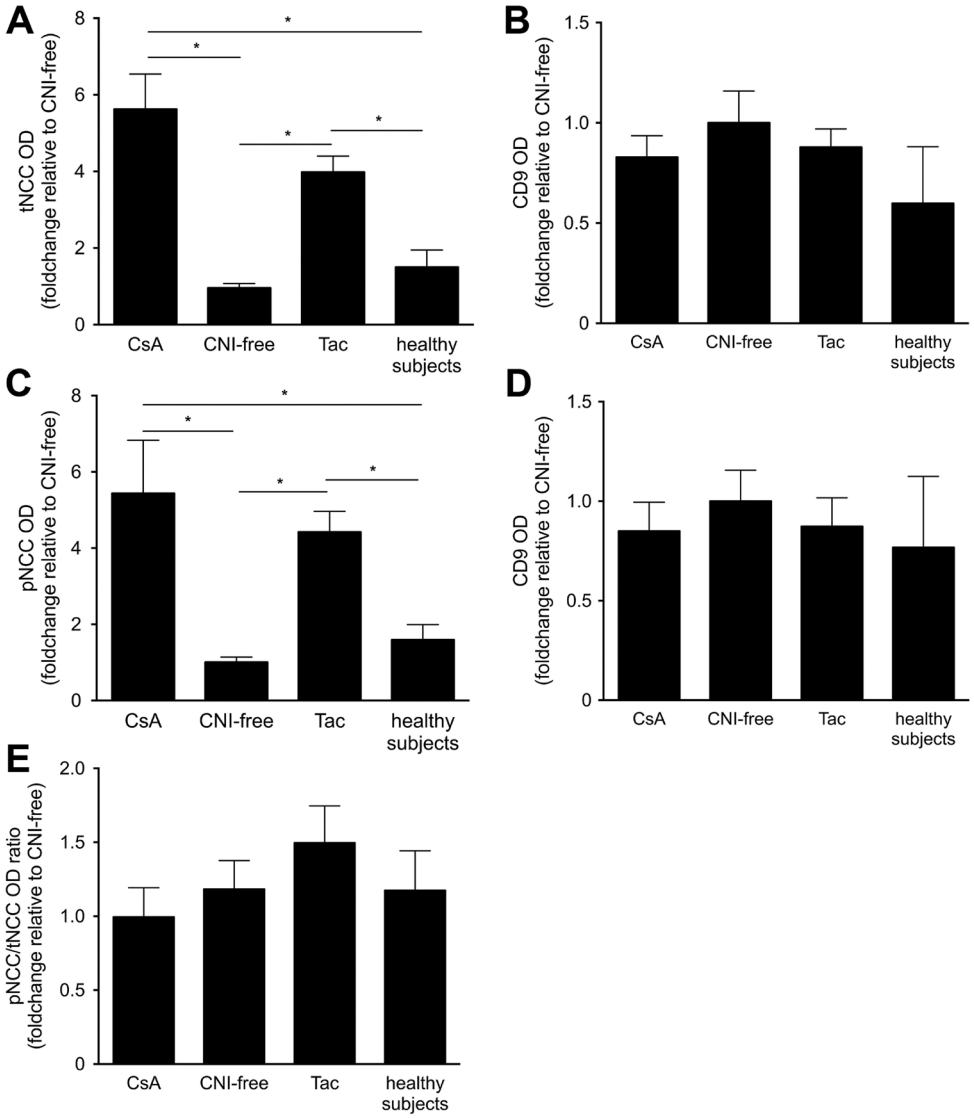


Figure 2 Densitometry of tNCC and pNCC immunoreactive bands in uEVs of all kidney transplant recipients treated with CsA (n = 9), Tac (n = 23) or CNI-free immunosuppressive regimens (n = 13) and healthy volunteers (n = 6). Both tNCC (A) and pNCC (C) abundance in both CsA- and Tac-treated kidney transplant recipients was significantly higher in comparison to kidney transplant recipients treated with CNI-free immunosuppressive regimens and healthy volunteers. Densitometry analysis of CD9 expression of the immunoblots for tNCC (B) and pNCC (D) showed no significant differences between the four groups. The ratio of pNCC to tNCC abundance in uEVs of CsA- and Tac-treated group was not significantly higher in comparison to kidney transplant recipients treated with CNI-free immunosuppressive regimens and healthy volunteers (E). The original immunoblots are shown in Fig 1 and S3 and S4 Figs. Values are mean ± SEM normalized to kidney transplant recipients treated with CNI-free immunosuppressive regimens (one-way ANOVA, *P<0.05, n = 51).

Table 2 Clinical and laboratory characteristics of the patients in study Group 2.

Characteristic (mean ± SEM)	Responders (n = 10)	Non-responders (n = 8)	P-value
Age	61 ± 2	57 ± 3	0.15
Males (n (%))	6 (60)	6 (75)	0.27
Body mass index, Kg/m2	27 ± 2	30 ± 2	0.14
Cause of ESRD (n)			
Polycystic kidney disease	1	0	
Diabetes	2	3	
Glomerular disease	0	3	
Hypertension/Vascular	6	1	
Other	1	1	
Plasma sodium, mmol/L	140 ± 1	140 ± 1	0.40
Plasma potassium, mmol/L	4.5 ± 0.1	5.1 ± 0.2*	<0.01
Plasma chloride, mmol/L	103 ± 1	105 ± 1	0.07
Plasma creatinine, mg/dL	1.2 ± 0.1	1.7 ± 0.2*	0.01
eGFR, mL/min/1.73 m2	55 ± 5	43 ± 6	0.06
Urine K/creatinine, mmol/mmol	5.2 ± 2.1	4.6 ± 1.9	0.28
Systolic blood pressure, mmHg	158 ± 4	151 ± 5	0.15
Diastolic blood pressure, mmHg	86 ± 2	80 ± 3	0.07
Tac pre-dose level; µg=L	5.5 ± 0.4	5.8 ± 0.5	0.30

SEM = standard error of the mean; ESRD = end stage renal disease; data are presented as mean ± SEM. P-value for difference between responders and non-responders, 95% CI, 95% con®dence interval.

responders (P<0.05). As a result, the pNCC to tNCC ratio remained constant in the responders, but markedly decreased in non-responders (P<0.05). This suggests that phospho-occupancy at the measured site was lower in non-responders. No gender differences were found (S7 Fig). Again, no significant differences were found in the abundances of CD9 between groups, suggesting comparable uEV-numbers (Fig 3 and S8 Fig). Additional normalization by CD9 abundance led to similar results (S9 Fig).

Increased pNCC in mouse cortical tubule suspensions exposed to CsA

To study the acute effect of CsA on NCC abundance in kidney, mouse cortical tubules were isolated and exposed to CsA. All mouse cortical tubule suspensions were incubated in basic buffer for 30 or 90 minutes in the absence or presence of CsA (at final concentrations of 5, 10, and 20 µmol/L). No significant changes were observed in tNCC, while the pNCC abundance and the ratio of pNCC to tNCC were significantly increased after 30 minutes of exposure to 10 or 20 µmol/L of CsA, and after 90 minutes of exposure to 5, 10, or 20 µmol/L of CsA (Fig 5). Hypotonic low chloride, which decreases the intracellular chloride concentration thereby activating the WNK-SPAK and OSR1 pathways [41,42], was used as a positive control. pNCC and the ratio of pNCC to tNCC were significantly increased in hypotonic low chloride buffer, whereas tNCC remained unchanged (Fig 5).

Discussion

Our study demonstrates that chronic treatment with CNIs increases both tNCC and pNCC abundance in uEVs of kidney transplant recipients, suggesting increased sodium reabsorption in the distal part of the nephron. Additionally, we show that the increase in pNCC abundance in uEVs of kidney transplant recipients correlates with the blood pressure response to NCC-inhibiting thiazide diuretics. Lastly, we corroborate that CNIs activate NCC in the kidney by showing that short-term exposure to cyclosporine increases pNCC but not tNCC abundance in mouse cortical tubules suspensions. Collectively, these observations indicate that the CNI-induced increase in NCC abundance and activity are involved in the pathogenesis of hypertension in kidney transplant recipients, and suggest that pNCC abundance in uEVs could be used as a biomarker to predict the blood pressure response to thiazide diuretics.

Since the introduction of CNIs in the 1980s, there has been a steep increase in the prevalence of hypertension after kidney transplantation [46,47]. In a study using a kidney specific 12 kDa FK506-binding protein, FKBP12, knockout mice, Tac caused hypertension by inhibiting calcineurin directly in DCT cells expressing NCC [22]. Moreover, NCC knockout mice were protected from tacrolimus-induced hypertension [21], confirming the role of NCC in CNI-induced hypertension. Although both CsA and Tac inhibit calcineurin, they differ in structure and cytoplasmic binding protein, which might explain why hypertension is less common and less severe in patients using Tac than in those using CsA [2,6-8,10,48]. However, it is still not known whether the higher incidence of hypertension in CsA- compared to Tac-treated

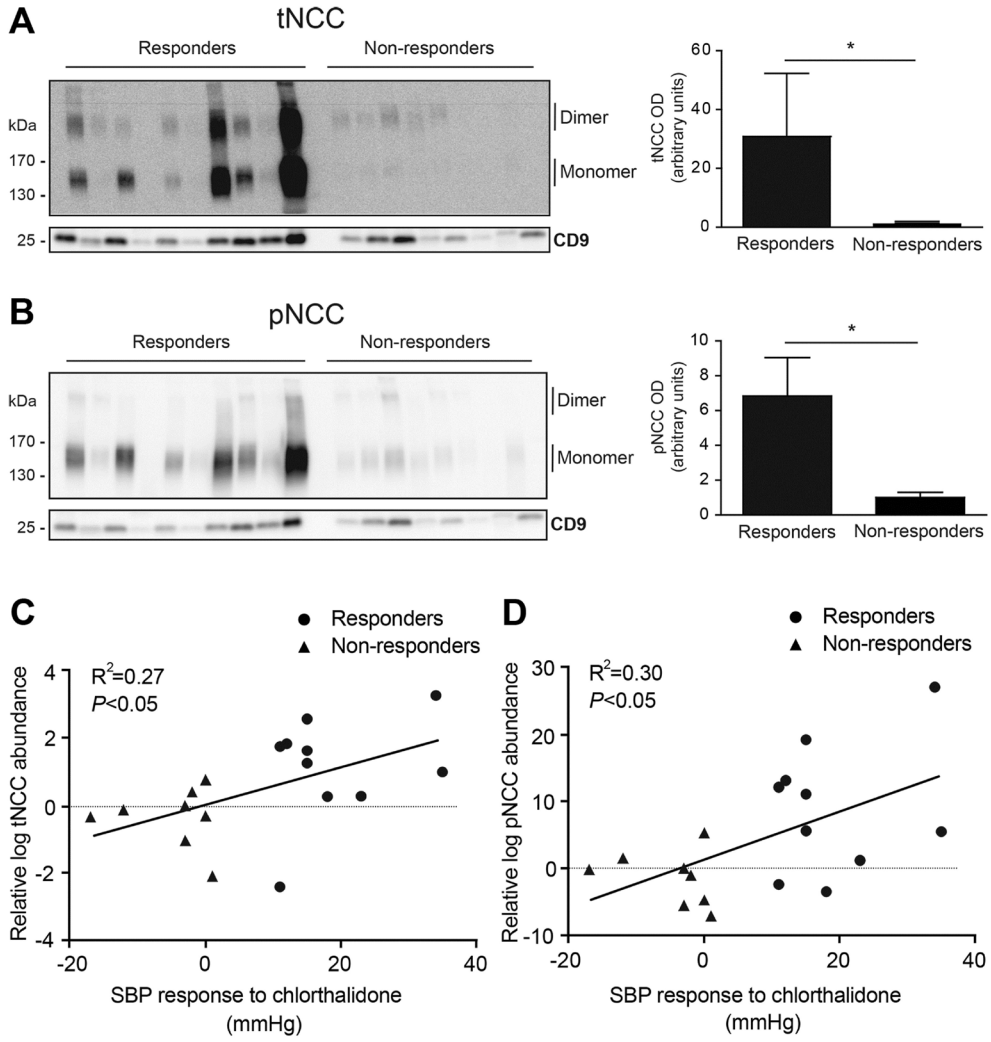
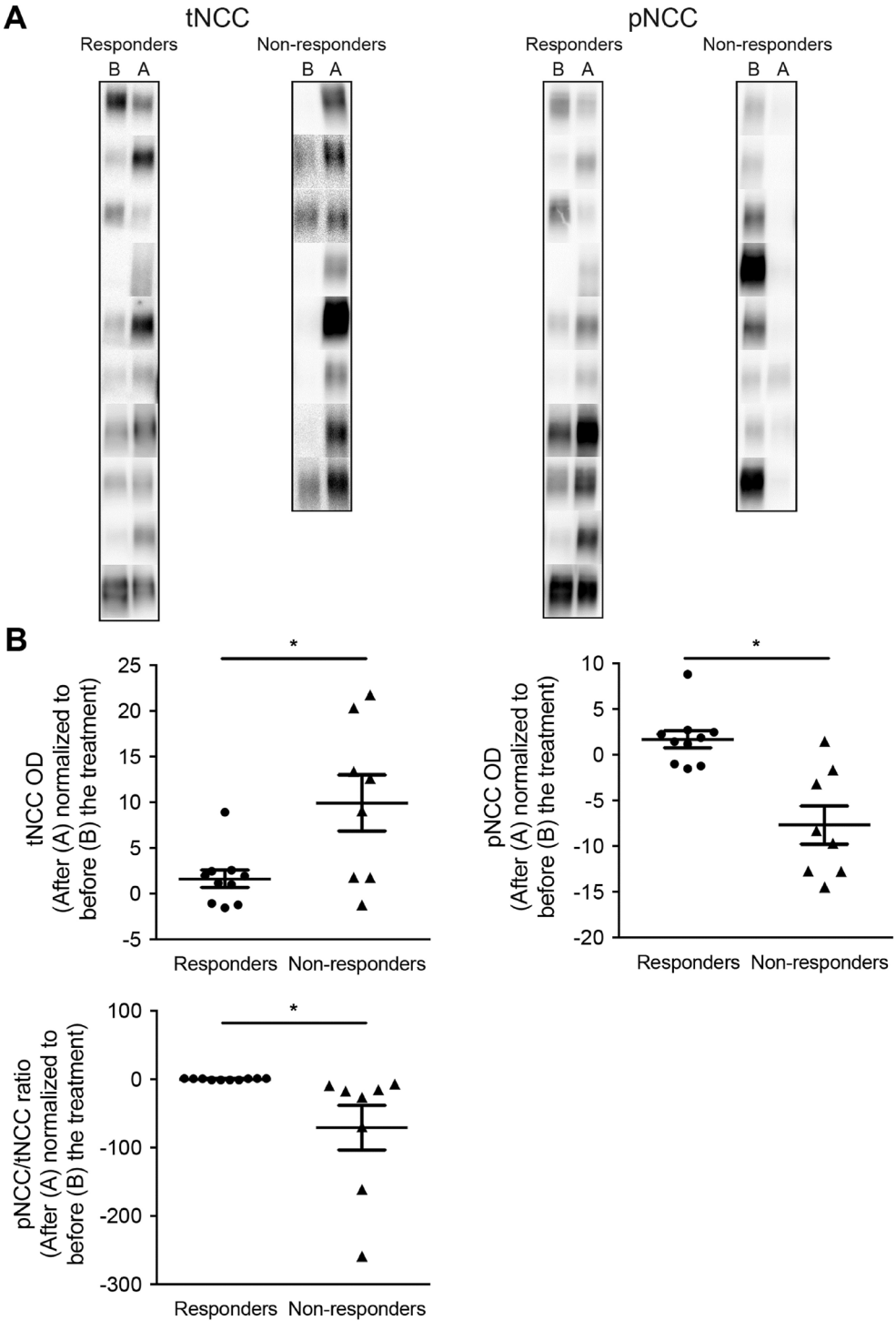


Figure 3 Pre-treatment tNCC and pNCC abundances in uEVs isolated from hypertensive kidney transplant recipients who did or did not respond to chlorthalidone. Shown are immunoblots of tNCC (panel A) and pNCC (panel B) together with CD9 in uEVs of hypertensive kidney transplant recipients using Tac. `Responders' (n = 10) refer to patients who subsequently had a significant anti-hypertensive response (10 mmHg reduction in systolic blood pressure) to 8-week treatment with chlorthalidone. Non-responders (n = 8) did not have an anti-hypertensive response to chlorthalidone (no change or increase in systolic blood pressure). uEVs were isolated before the treatment with chlorthalidone. Both tNCC and pNCC abundance were significantly higher in responders compared to non-responders (non-parametric t-test, *P<0.05, n = 18). Both pNCC and tNCC abundance in uEVs correlated with the blood pressure response (panel C, R2 = 0.27 and panel D, R2 = 0.30 using log-transformed densitometry data because of non-normal distribution, P<0.05 for both). Abbreviations: SBP, ambulatory systolic blood pressure.

kidney transplant recipients is due to a stronger tNCC and pNCC upregulation. Our study demonstrates that CsA does not increase tNCC and pNCC abundances in uEVs of kidney transplant recipients more than Tac (Fig 2). This suggests that factors other than NCC might contribute to the discrepancy in the increased blood pressure between patient groups using CsA and Tac. It is known that CNIs affect tissues other than the kidney, which are also involved in the development of hypertension [22]. CsA and Tac might differ in their ability to cause vasoconstriction [49,50], activate the renin-angiotensin-aldosterone system (RAAS) [21,50], or the sympathetic nervous system [51], all of which can contribute to the development of hypertension.

Several guidelines recommend thiazide-type diuretics as first-line treatment for the management of hypertension in adults [52,53]. Thiazide-type diuretics act by blocking NCC, thus increasing sodium excretion by the kidney [54]. Given the role of NCC in CNI-induced hypertension, thiazide diuretics might be especially effective drugs for hypertensive transplant recipients using CNIs and NCC abundance in their uEVs might predict the blood pressure response to thiazide diuretics. Indeed, uEV analysis in patients selected from our crossover study demonstrates that pre-treatment abundances of tNCC and pNCC in uEVs predict the blood pressure response to thiazide diuretics (Fig 3). This implies that the abundance of pNCC in uEVs of hypertensive kidney transplant recipients using CNIs could be used to predict thiazide sensitivity. Although it may be more pragmatic to simply test the response to a trial of thiazide diuretics, urinary biomarkers may help individualize anti-hypertensive treatment, especially with the development of high-throughput assays [55]. However, acute regulators of NCC activity such as the RAAS and potassium status should also be taken into account when interpreting the obtained results (see below). A relevant question is what explains the higher pNCC and tNCC in uEVs of responders prior to treatment, and the decrease in ratio of pNCC to tNCC in non-responders after thiazide treatment. One could argue that CNI-induced NCC activation was greater in responders. An alternative possibility is that the potassium balance determined thiazide sensitivity. A high potassium diet and hyperkalemia have recently been shown to inhibit NCC [56,57]. Indeed, serum potassium concentrations were higher in non-responders compared to responders. Here, we show that NCC abundance was increased in the majority of ªrespondersº after thiazide treatment (Fig 4), which is consistent with data obtained in animal models [58,59]. Na et al. showed with immunoblot analysis and immunostaining that chronic hydrochlorothiazide treatment in rats increased the abundance of NCC [58]. Moreover, chronic hydrochlorothiazide infusion in mice increased the binding density of [3H] metolazone, an indirect measure of NCC activity, which confirms the


A
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before (B) the treatment)
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before (B) the treatment)
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Responders
Non-responders

Figure 4 pNCC and tNCC abundances in uEVs before and after treatment with chlorthalidone. Panel A shows pNCC and tNCC abundances in uEVs before (B) and after (A) the 8-week treatment period with chlorthalidone in both responders (n = 10) and non-responders (n = 8). The fold-changes in the before-after abundances of pNCC and tNCC in uEVs (as measured by densitometry) of both responders and non-responders are shown in panel B (Fold-change of 1 means no change, *P<0.05). The scatter plots represent the fold change in tNCC, pNCC or their ratio after treatment with chlorthalidone (densitometry values before treatment with chlorthalidone were set to 1). S5 and S6 Figs, show the original immunoblots from which the individual panels in Fig 4A were derived.

increase of NCC after hydrochlorothiazide [60]. Similarly, chronic administration of the diuretics furosemide and amiloride (which blocks the Na-K-Cl cotransporter and ENaC, respectively), increased the abundance of these proteins in rodent kidney [58,61]. These effects might be the result of compensatory mechanisms, potentially mediated by the RAAS or/and potassium balance, which counteract reduced NCC function by the thiazide treatment [62]. Of interest, non-responders had a completely different pattern during thiazide treatment with a decrease in pNCC and ratio of pNCC to tNCC (Fig 4). The explanation for this difference is unclear, but one might speculate that a blood pressure response to thiazides is accompanied by RAAS activation resulting in different NCC excretion patterns in uEVs. Alternatively, differences in potassium balance may explain these results, because a recent study showed that pNCC stimulation by angiotensin II occurs as a compensatory response to renal potassium loss [63].

To further confirm the effect of CNIs on NCC in the kidney, we performed an ex vivo study using mice cortical tubules. This experiment demonstrated that short-term exposure to CsA increases pNCC abundance, while tNCC remained stable (Fig 5). This phenomenon was previously reported by Hoorn et al. [21] in mice and human embryonic kidney 293 cells treated with Tac, and by Melnikov et al. [23] in mDCT cells treated with CsA. Recently, it was demonstrated in mice that treatment with Tac prevents the acute high potassium induced NCC dephosphorylation, while tNCC remained unaffected [24]. In contrast to previous studies, this acute regulatory system mediated by calcineurin is shown to be independent of the WNK-SPAK signaling cascade [24]. Our study demonstrated that chronic administration of CNIs increases the abundance of both tNCC and pNCC in uEVs of kidney transplant recipients. This suggests that the difference in tNCC increase between mice cortical tubules and uEVs might be dependent on the time of stimulation or another signaling molecule involved in the in vivo situation.

A number of limitations of our study should be mentioned. First, several other

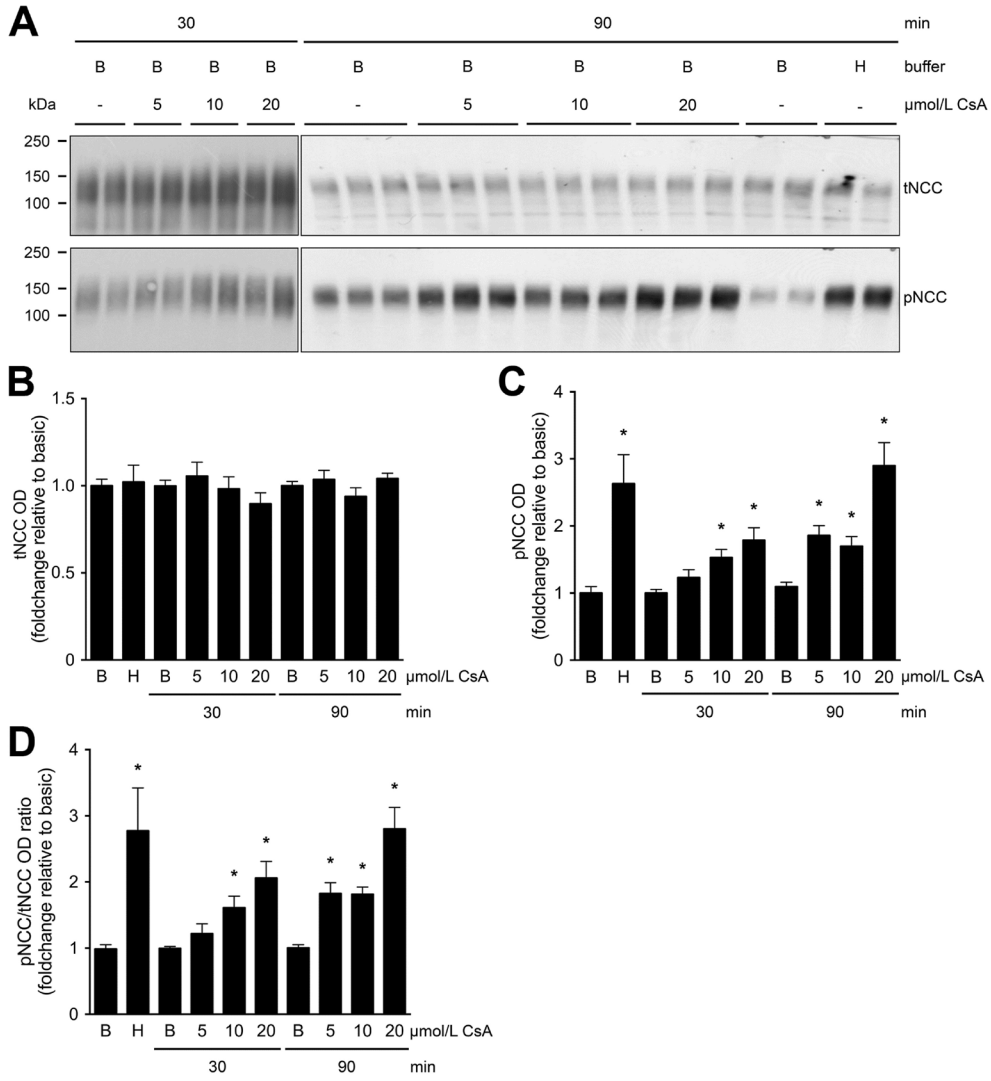

Figure 5 tNCC and pNCC abundance in mouse cortical tubule suspension exposed to CsA. Panel A shows representative immunoblots of tNCC and pNCC abundance in mouse cortical tubule suspension exposed to CsA. Immunoblots of protein homogenates of mouse cortical tubule suspensions were incubated in basic (B) buffer for 30 and 90 minutes, in the absence (-) or presence of CsA at final concentrations of 5, 10, or 20 µmol/L (A). tNCC remained at baseline levels (B), while pNCC (C) and ratio of pNCC to tNCC (D) were significantly increased in the mouse cortical tubule suspension after 30 minutes of exposure to 10 and 20 µmol/L of CsA and 90 minutes of exposure to 5, 10, and 20 µmol/L of CsA. Similarly, pNCC and the ratio of pNCC to tNCC were significantly increased in hypotonic low chloride (H) buffer (C and D). The original immunoblots for 30 and 90 minutes, in the absence (-) or presence of CsA at final concentrations of 5, 10, or 20 µmol/L are shown in Fig 5 and S10 Fig. Values are mean ± SEM normalized to basic (B) condition (one-way ANOVA, *P<0.05, 30 minutes exposure n = 3, 90 minutes exposure n = 4).

factors, in addition to CNIs, may regulate NCC, which may also explain differences in expression of NCC and in thiazide sensitivity. Second, an unresolved question in the uEV-field remains whether the abundance of protein per uEV varies, or that the number of uEVs is regulated. Although we included the uEV-marker CD9, techniques that allow uEV counting will be necessary to address this point more conclusively.

Conclusion

Our findings demonstrate that CNI treatment increases both tNCC and pNCC abundance in uEVs isolated from kidney transplant recipients. We also show that the blood pressure response to chlorthalidone in Tac-treated hypertensive kidney transplant recipients was related to the pNCC abundance in uEVs. This implies that pNCC in uEVs of kidney transplant recipients treated with a CNI might be used to predict blood pressure response to thiazide diuretics. In addition, uEV analysis may have clinical utility as a non-invasive biomarker for a variety of physiological and pathological conditions.

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CHAPTER 7

Mycophenolate Mofetil Attenuates DOCA-Salt Hypertension: Effects on Vascular Tone

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Abstract

Inflammation is increasingly recognized as a driver of hypertension. Both genetic and pharmacological inhibition of B and T cells attenuates most forms of experimental hypertension. Accordingly, the immunosuppressive drug mycophenolate mofetil (MMF) reduces blood pressure in the deoxycorticosterone acetate (DOCA) salt model. However, the mechanisms by which MMF prevent hypertension in the DOCA-salt model remain unclear. Recent studies indicate that immunosuppression can inhibit sodium transporter activity in the kidney, but its effect on vascular tone is not well characterized. Therefore, the aim of the present study was to analyze the vascular and renal tubular effects of MMF in the DOCA-salt model in rats (4 weeks without uninephrectomy). Co-treatment with MMF attenuated the rise in blood pressure from day 11 onward resulting in a significantly lower telemetric mean arterial pressure after 4 weeks of treatment (108 ± 7 vs. 130 ± 9 mmHg, $P < 0.001$ by two-way analysis of variance). MMF significantly reduced the number of $CD3^+$ cells in kidney cortex and inner medulla, but not in outer medulla. In addition, MMF significantly reduced urinary interferon-g excretion. Vascular tone was studied ex vivo using wire myographs. An angiotensin II type 2 (AT_2) receptor antagonist blocked the effects of angiotensin II (Ang II) only in the vehicle group. Conversely, L-NAME significantly increased the Ang II response only in the MMF group. An endothelin A receptor blocker prevented vasoconstriction by endothelin-1 in the MMF but not in the vehicle-group. MMF did not reduce the abundances of the kidney sodium transporters NHE3, NKCC2, NCC, or ENaC. Together, our ex vivo results suggest that DOCA-salt induces AT_2 receptor-mediated vasoconstriction. MMF prevents this response and increases nitric oxide availability. These data provide insight in the antihypertensive mechanism of MMF and the role of inflammation in dysregulating vascular tone.

Introduction

Hypertension is one of the major risk factors for cardiovascular and kidney disease, and therefore an important cause of premature morbidity and mortality. The pathogenesis of hypertension is multifactorial and the result of a complex interplay between dietary and genetic factors, hormones, nerves, and blood-pressure regulating organs, including the nervous system, kidneys, heart, and vasculature. Of interest, an increasing body of evidence suggests that inflammation is another driver of hypertension (McMaster et al., 2015; Wenzel et al., 2016; Crowley and Rudemiller, 2017; Foss et al., 2017).

For example, Svendsen (1976) reported that nude mice, which also have genetic

aplasia of the thymus, became less hypertensive in response to deoxycorticosterone acetate (DOCA) and salt than haired mice. Similarly, subcutaneous administration of rabbit anti-rat thymocyte serum reduced blood pressure in spontaneously hypertensive rats (SHRs) (Bendich et al., 1981). Intravenous injection of splenic cells from DOCA-salttreated rats transferred arterial hypertension to recipient rats (Olsen, 1980). Angiotensin II (Ang II)-induced hypertension is attenuated in genetic mouse models lacking B and T lymphocytes (Guzik et al., 2007; Crowley et al., 2010). Pharmacological inhibition of B and T cells with the immunosuppressive drug mycophenolate mofetil (MMF) can also exert anti-hypertensive effects. Although MMF did not modify hypertension during exogenous Ang II infusion, it did prevent the subsequent development of salt-sensitive hypertension (Rodriguez-Iturbe et al., 2001).MMFdid normalize blood pressure in SHRs and Dahl salt-sensitive rats (Rodriguez- Iturbe et al., 2002; Mattson et al., 2006). Similarly, MMF attenuated hypertension and albuminuria in uninephrectomized rats treated with DOCA-salt (Boesen et al., 2010). The antihypertensive effects of MMF were not only observed in experimental settings, but also in patients with psoriasis or rheumatoid arthritis treated with MMF (Herrera et al., 2006).

It is incompletely understood how genetic or pharmacological inhibition of B and T cells prevents hypertension. Several studies have shown reduced renal infiltration of lymphocytes and macrophages in MMF-treated hypertensive animals (Rodriguez-Iturbe et al., 2002; Franco et al., 2007, 2013). More recently, such infiltrations have been linked to activation of sodium transporters through direct effects or secretion of cytokines (Kamat et al., 2015; Zhang et al., 2016; Liu et al., 2017). However, few studies have analyzed the effects of immunosuppression on vascular function in experimental hypertension.

Here, we hypothesize that B and T cells mediate experimental hypertension not only through effects on renal sodium transport but also on vascular tone. To do so, we induced hypertension with DOCA-salt in rats and co-treated the animals with MMF or vehicle. Using ex vivo myograph analysis of vascular tone, we show that MMF suppresses Ang II type 2 (AT2) receptormediated vasoconstriction and increases nitric oxide (NO) availability.

Materials and Methods

Animal experiments

The animal protocol was approved by the Animal Care Committee of the Erasmus Medical Center (EMC 3101, # 127- 13-01). Sixteen male Sprague-Dawley rats (20 weeks old, average weight 350 g) were obtained from Charles River Laboratories

(Sulzfeld, Germany). Animals were randomized to a treatment and a vehicle group (n = 8/group). All animals were housed in individual cages and maintained on a 12-h light–dark cycle, having ad libitum access to water and standard laboratory rat chow (Na^+ 0.5%, K^+ 0.8%). Radiotelemetry transmitters were implanted in eight rats (n = 4/group) for continuous blood pressure measurement as described before (van Esch et al., 2010). After a 14-day recovery period, 200 mg 60-day continuousrelease DOCA pellets (Innovative Research of America, Sarasota, United States) were implanted subcutaneously in all rats. From this day on, regular drinking water was replaced by 0.9% NaCl for all rats. Rats in the treatment group received a daily dose of 30 mg/kg of MMF (Boesen et al., 2010) via gastric gavage and control rats received an equal volume of water (vehicle). Once a week, animals were placed in metabolic cages for 24 h for the collection of urine and the measurement of food and 0.9% NaCl intake. After 28 days, all animals were killed by left ventricular puncture.

Biochemical Measurements

Urinary sodium, potassium, and total protein were measured by the Department of Clinical Chemistry of the Erasmus Medical Center. Endothelin-1 (ET-1) in plasma and urine was determined by chemiluminescent ELISA (QuantiGlo, R&D Systems, Abingdon, United Kingdom). A Milliplex cytokine/chemokine immunoassay (Merck Millipore, Billerica, MA, United States) was used for the measurement of interferon-g (IFN-g), interleukin-6 (IL-6), chemokine (C-C motif) ligand 5 (CCL5), and tumor necrosis factor-a (TNF-a) in urine.

Tissue Preparation

After blood collection, kidneys were rapidly excised, weighed, and placed on ice. The left kidneys of four rats of each group were immersionfixed in paraformaldehyde and further processed for immunohistochemistry. The right kidneys were used for immunoblotting and were placed in an isolation buffer containing 250 mM sucrose, 10 mM triethanolamine, and a protease inhibitor mix (cOmplete Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Almere, Netherlands) before homogenization. Differential centrifugation of the whole kidney homogenate was performed to obtain a plasma membraneenriched fraction, as described previously (Ecelbarger et al., 1997; van der Lubbe et al., 2011). The pellet (plasma membraneenriched fraction) was resuspended in 1 mL of isolation buffer. A total of 60 mL of both fractions was used for a protein quantification assay (Pierce, Thermo Scientific, Rockford, IL, United States) and the remaining samples were stored in 6° Laemmli buffer at -80 °C until immunoblotting.

Immunohistochemistry

Transverse kidney sections were dehydrated and paraffinembedded, 4-mm sections were cut on a rotary microtome. Staining procedures were performed on the Ventana Benchmark Ultra automatic stainer. For antigen retrieval, deparaffinized sections were incubated with Cell Conditioning 1 medium (Ventana Medical Systems, Tucson, AZ, United States) at 100°C for 32 min. For immunolabeling, slides were incubated in the presence or absence of a polyclonal rabbit anti-$CD3^+$ antibody (1:600, Novus Biologicals, Abingdon, United Kingdom) at 36°C for 32 min, followed by an 8-min incubation with a secondary antibody (Optiview HQ Universal Linker) at 36°C. Detection and visualization was done by subsequent 8-min incubations at 36°C with Optiview HRP Multimer and diaminobenzidine (all from Ventana Medical Systems), after which slides were counterstained with hematoxylin. A rat spleen sample was used as positive control. CD3C cells were counted in 20 randomly selected 400 mm x 200 mm fields in cortex, outer and inner medulla by an investigator blinded to treatment allocation (D.S.). Results are expressed as positive cell counts per mm2.

Immunoblotting

Immunoblotting was performed as described previously (Kim et al., 1998). Antibodies against the following proteins were used: sodium hydrogen exchanger type 3 (NHE3, dilution 1:5000), sodium potassium chloride co-transporter 2 (NKCC2, 1:1000), sodium chloride co-transporter (1:500) and its phosphorylated form at threonine-58 (pNCC, 1:500), and the α- and γ-subunits of the epithelial sodium channel (ENaC, both 1:1000). All antibodies were purchased from StressMarq (Victoria, BC, Canada), except for NCC (Merck Millipore, Billerica, MA, United States) and pNCC (a kind gift by Dr. R. A. Fenton; Pedersen et al., 2010). b-Actin (1:50,000, Abcam) was used for normalization of protein levels. Protein was visualized using horseradish peroxidase-conjugated secondary antibodies (1:3000, Bio-Rad, Veenendaal, Netherlands). Signals were detected by chemiluminescence (Pierce, Rockford, IL, United States), quantified using ImageQuant LAS 4000 (GE Healthcare, Diegem, Belgium), and normalized to actin.

Wire Myograph Studies

Following isolation and after overnight storage, iliac and mesenteric arteries were cut into segments of approximately 2 mm length and mounted in a Mulvany myograph with separated 6-mL organ baths containing carbogen-gassed Krebs– Henseleit buffer at 37°C. First order mesenteric arteries were used for this experiment, and iliac and

mesenteric arteries were used because of their excellent and highly reproducible response to Ang II and ET-1, respectively (Batenburg et al., 2013; Roksnoer et al., 2015). The tension was normalized to 0.9 times the estimated diameter at 100 mmHg of effective transmural pressure. The presence of endothelium was verified by observing relaxation to 1 mM acetylcholine (ACh) after preconstriction with 100 nM of the thromboxane A2 analog U46619 (9,11-dideoxy-11a,9a-epoxy-methanoprostaglandin F2a, Sigma-Aldrich, Zwijndrecht, Netherlands). To determine the maximum contractile response, the tissue was exposed to 100 mM KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min in the absence or presence of 1 mM of irbesartan, 1 mM of PD123319 (Sigma-Aldrich), 100 mM of NG-nitro-L-arginine methyl ester (L-NAME, Sigma- Aldrich), 1 mM of BQ123 (a selective ETA endothelin receptor antagonist), or 1 mM of BQ788 (a selective ETB endothelin receptor antagonist). Thereafter, concentration–response curves (CRCs) were constructed in response to Ang II (iliac arteries) or ET-1 (mesenteric arteries). ACh-induced relaxation was measured in ET-1 CRCs.

Statistical Analysis

All data are expressed as means and standard error of the mean. Blood pressure data were analyzed using repeated measures analysis of variance (ANOVA). CRCs were analyzed as described before (MaassenVanDenBrink et al., 1999) to determine the maximum effect (Emax) and pEC50 (= -10logEC50) values. Contractile responses to Ang II or ET-1 are expressed as a percentage of the contraction to 100 mM KCl. Relaxation responses to ACh are expressed as a percentage of the contraction to U46619. CRC and relaxation response data, immunohistochemical CD3C cell quantification data, and logtransformed cytokine/chemokine data were analyzed by one-way ANOVA, with Dunnett's post hoc test where appropriate. $P < 0.05$ was considered statistically significant.

Results

Biochemical Responses

DOCA-salt induced an increase in fluid intake, diuresis, and urinary Na^+ and K^+ excretion, which was sustained throughout the 4-week period (**Figure 1**). Co-treatment with MMF significantly attenuated fluid intake, diuresis, and urinary NaC and KC excretion. Food intake was unaffected by cotreatment with MMF. The body weight at day 28 did not differ between the MMF and vehicle-treated animals, respectively (492 ± 12 vs. 474 ± 16 grams, P = 0.4). Median ET-1 levels in serum (0.84

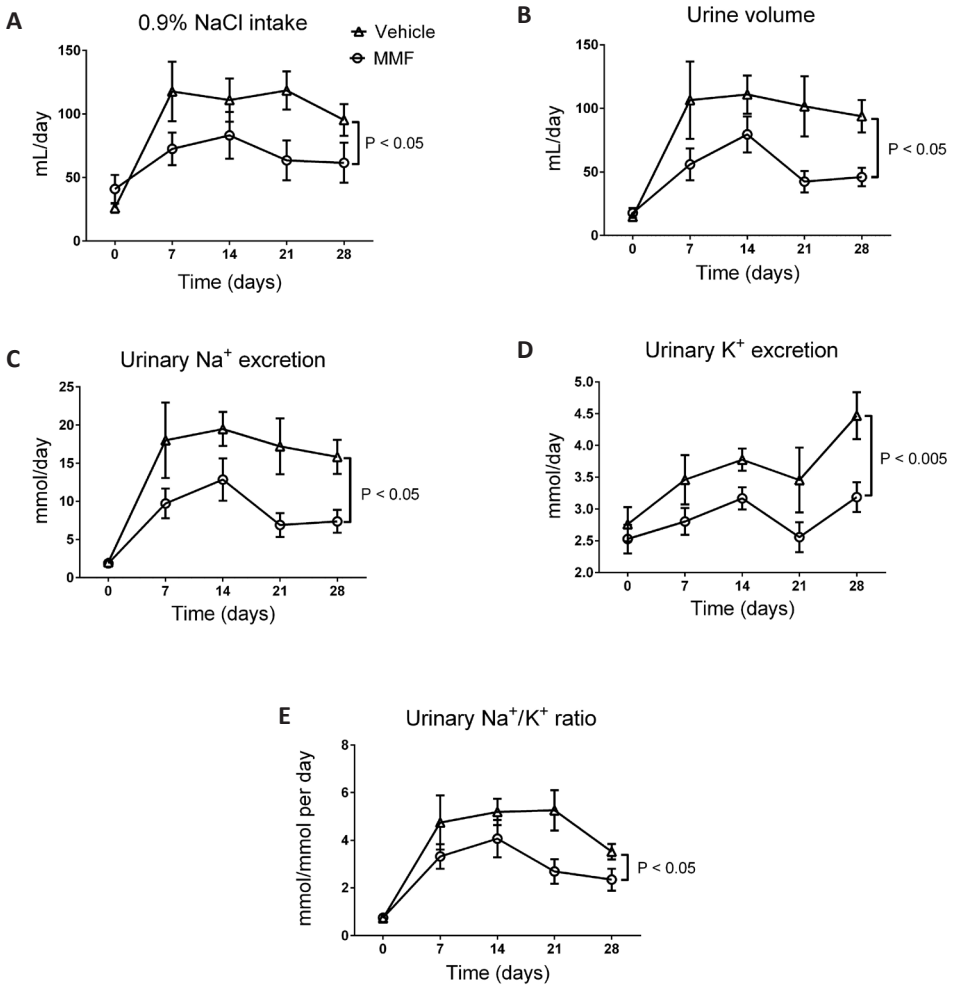


Figure 1 Changes in intake of 0.9% NaCl (A), 24-h urine volume (B), 24-h urinary NaC excretion (C), 24-h urinary KC excretion (D), and the urinary NaC/KC ratio (E) over the total study period of 28 days of DOCA-salt-treated Sprague-Dawley rats co-treated with MMF or vehicle. Averages SEM are shown; n = 6–8 animals/group. Measurements were performed once a week.

vs. 0.85 pg/mL) and urine (0.09 vs. 0.08 pg/mL) and proteinuria (0.16 vs. 0.14 g/L) were not different between the MMF and vehicle-treated animals, respectively.

Blood Pressure Response

DOCA-salt caused a continuing rise in mean arterial pressure (MAP) over the 4-week period (from 101 ± 4 to 130 ± 8 mmHg; **Figure 2**). Co-treatment with MMF attenuated this rise in blood pressure from day 11 onward resulting in a significantly

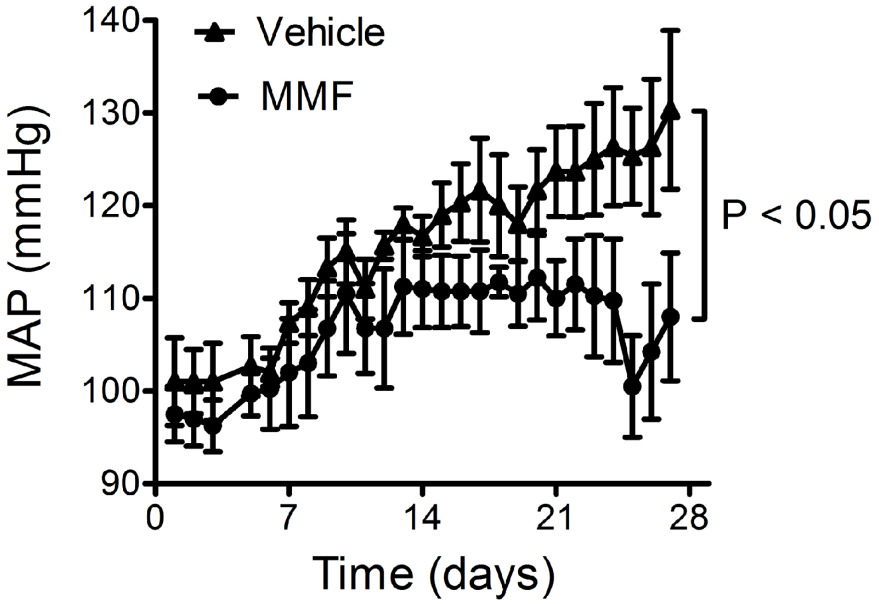


Figure 2 Response of the mean arterial pressure (MAP) to DOCA-salt and co-treatment with MMF or vehicle during the 28-day study period. Averages SEM are shown; n = 4 animals/group.

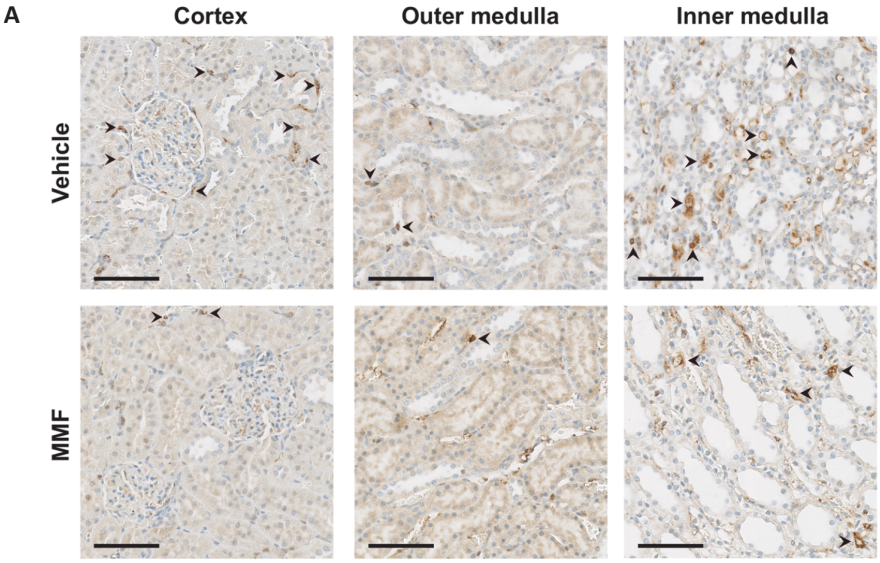
lower MAP after 4 weeks of treatment (108 ± 7 vs. 130 ± 9 mmHg, P < 0.001 by repeated measures ANOVA). Co-treatment with MMF did not alter heart rate (303 ± 7 vs. 304 ± 1 beats/min, P = 0.8).

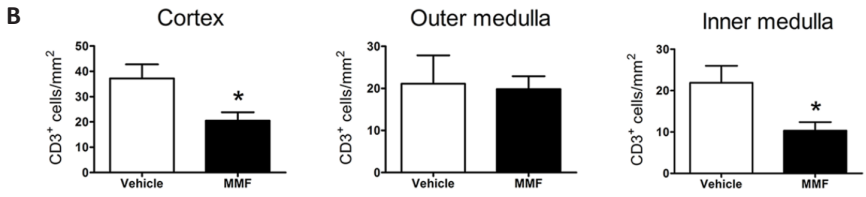
Immunohistochemistry and Urinary Cytokine Excretion

To confirm the anti-inflammatory effect of MMF, we analyzed kidney T-cell infiltration and urinary cytokines (**Figure 3**). MMF significantly reduced the number of CD3C cells in cortex and inner medulla, but not in outer medulla (**Figure 3A**). Urinary IFN-g excretion was significantly suppressed in MMF-treated animals (**Figure 3B**). No statistically significant differences were found in urinary IL-6, CCL5, and TNF-a between the two groups.

Vascular Responses

Ang II exposure led to a concentration-dependent constriction of iliac arteries of vehicle-treated animals (pEC50 = 8.5 ± 0.5, vehicle-treated animals (P = 0.007, **Figure 4A**). NO synthase (NOS) inhibition with L-NAME greatly enhanced the Ang II response in the isolated iliac arteries of MMF-treated animals (Emax = 73.9 ± 12.3, P < 0.0001), but the increase was non-significant in control animals (P = 0.16). Of


A
Cortex
Outer medulla
Inner medulla
Vehicle
MMF


B
Cortex
Outer medulla
Inner medulla
CD3+ cells/mm2
Vehicle
MMF
*

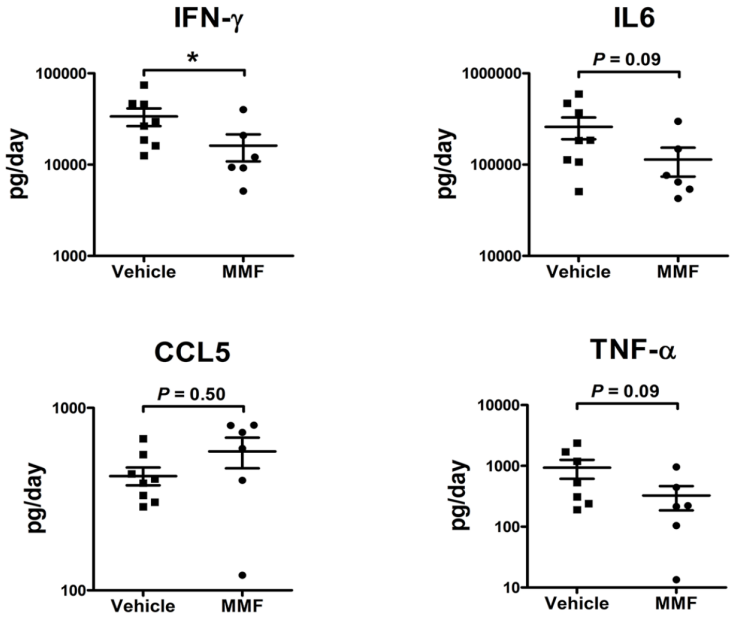

IFN-γ
IL6
CCL5
TNF-α
pg/day
Vehicle
MMF
*
P = 0.09
P = 0.50

Figure 3 Immunohistochemistry data and urinary chemokines/cytokine excretion. (A) Representative images showing localization of CD3C cells in a kidney cortex section of vehicle- and MMF-treated rats with quantification of CD3C cells in cortex, outer medulla, and inner medulla; (B) urine cytokine/chemokine excretion. P < 0.05 vs. vehicle using Student's t-test. IFN-g, interferon-g; IL-6, interleukin-6; CCL5, chemokine (C-C motif) ligand 5; TNF-a, tumor necrosis factor-a.

note, baseline contractility following preincubation with L-NAME was identical in both groups (data not shown), and thus the differences in contractile response after Ang II are not the consequence of L-NAME alone. The Ang II type 1 (AT1) receptor antagonist irbesartan prevented the Ang II-induced constriction in both groups. The AT2 receptor antagonist PD123319 blocked the Ang II induced constriction in iliac arteries of MMF-treated animals, but not in vehicle-treated animals (P < 0.001). ET-1 infusion caused a concentration dependent constriction of mesenteric arteries in vehicle-treated animals (pEC50 = 8.3 ± 0.2, Emax = 235.2 ± 18.7%, n = 7). This constriction was unaffected by MMF treatment (**Figure 4A**). ETA receptor inhibition with BQ123 shifted the ET-1 CRC ±10- fold to the right in mesenteric arteries of MMF-treated animals, but not in vehicle-treated animals (P < 0.0001). ACh relaxed mesenteric arteries after preconstriction with ET-1 in vehicle treated animals (pEC50 = 7.0 ± 0.2, Emax = 22.9 ± 4.4%, n = 5). This response was unaltered by MMF treatment (**Figure 4B**). L-NAME completely prevented ACh-induced relaxation in ET-1-preconstricted arteries of MMF-treated animals, but only partially prevented this relaxation in the arteries of vehicletreated animals (Emax = 89.0 ± 2.2% vs. 62.7 ± 4.6%, P = 0.0009). The latter indicates a diminished NO participation in the ACh response, and thus, given the identical ACh response in both groups, is suggestive for upregulation of non-NO factors to compensate the loss of NO.

Immunoblotting of Kidney Na^+ Transporters

MMF did not change the abundances of NHE3, NKCC2, NCC, the a- and g-subunits of ENaC (**Figure 5**). In addition, the abundance of phosphorylated NCC was unchanged.

Discussion

We show that MMF blunts the development of salt-sensitive hypertension in the DOCA-salt model by reducing vascular resistance, likely through inhibition of T cells. Results of our Mulvany myograph studies point toward a pivotal role for the AT2 receptor. Although DOCA-salt is a low Ang II model, a previous study showed that mineralocorticoids regulate vascular Ang II receptors (Schiffrin et al., 1983).

Under normal circumstances, the AT2 receptor is known to exert either no effect or a vasodilatory effect via the production of vascular NO (Siragy and Carey, 1997;

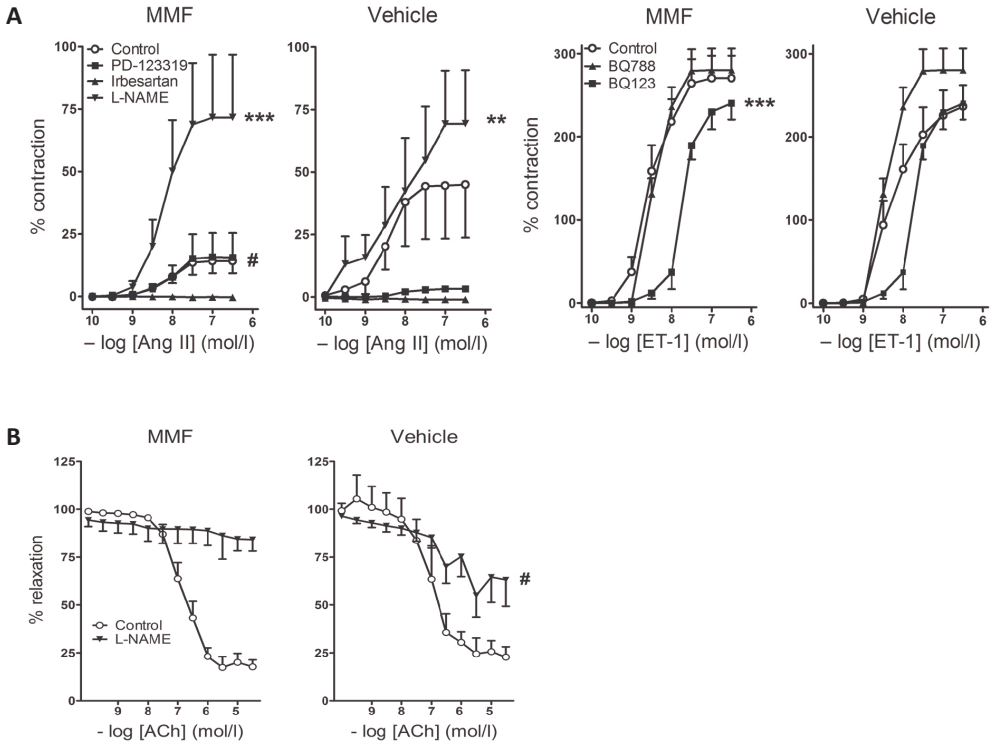


Figure 4 Vascular responses to angiotensin II (Ang II), endothelin-1 (ET-1), and acetylcholine (ACh) in DOCA-salt-treated rats co-treated with MMF or vehicle. (A) Left panel: responses of iliac arteries to Ang II in the absence (control) or presence of irbesartan, PD123319 or L-NAME. Right panel: responses of mesenteric arteries to ET-1 in the absence or presence of BQ123 or BQ788. Contractile responses are expressed as a percentage of the response to 100 mM KCl. (B) ACh-induced vasorelaxation. Relaxation responses are expressed as % reduction of preconstriction (with ET-1). Data are mean SEM of n = 4–8. P < 0.001 (pEC50 or Emax compared with control), #P < 0.001 (Emax compared with vehicle-treated rats).

Batenburg et al., 2004a; Verdonk et al., 2012a). Yet, in isolated arteries of DOCAsalt-treated rats, the AT2 receptor appears to have obtained a constrictor function similar to that of the AT1 receptor. In isolated arteries of rats receiving DOCA-salt and MMF this AT2 receptor-mediated constriction was absent. Moreover, L-NAME fully blocked the ACh-mediated vasodilation after ET-1 preconstriction in the isolated arteries of DOCA-salt C MMFtreated rats, and displayed only a partial blocking effect in the isolated arteries of rats exposed to DOCA-salt alone. Since the ACh response per se was the same in both groups, these data suggest that non-NO relaxant mechanisms (e.g., endotheliumdependent hyperpolarizing factor(s); Batenburg et al., 2004b) come into play after DOCA-salt, which are replaced by NO

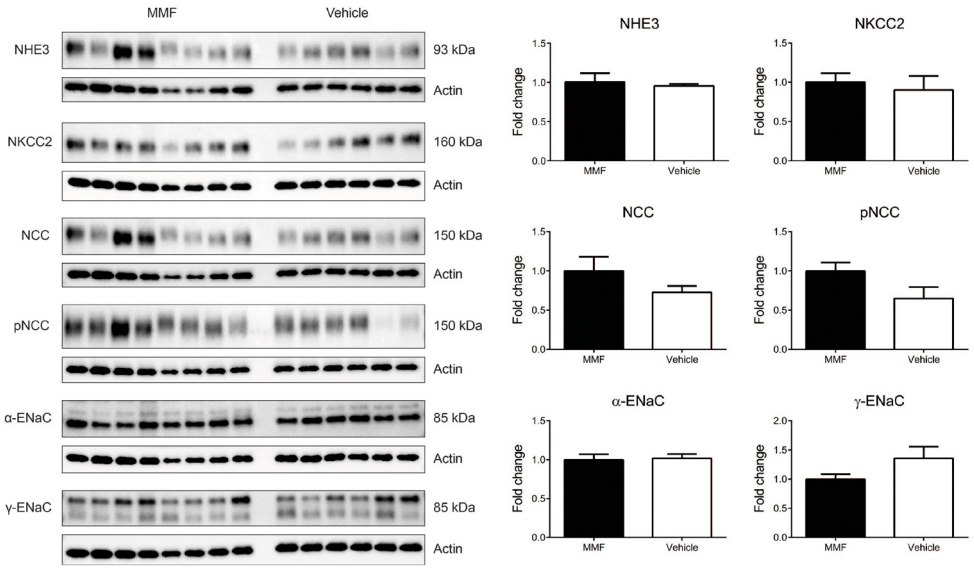


Figure 5 Immunoblots showing protein abundances in of the renal NaC transporters sodium-hydrogen exchanger type 3 (NHE3), Na-K-Cl co-transporter (NKCC2), Na-Cl co-transporter (NCC), NCC phosphorylated at Thr58 (pNCC), a-subunit of the epithelial sodium channel (a-ENaC), and g-ENaC in whole kidney homogenates of DOCA-salt-treated Sprague-Dawley rats co-treated with MMF or vehicle. No significant differences were identified. Data are mean ± SEM, n = 6–8 animals/group.

in the presence of MMF. An MMF-induced NO upregulation is also apparent from the huge increase in Ang II response after L-NAME in the MMF treatment group, which did not occur after DOCA-salt alone. Finally, the ETA receptor antagonist BQ123 blocked ET-1-induced constriction in the isolated arteries of MMF-treated rats, and this effect was less apparent in the isolated arteries of rats treated with DOCA-salt only. This is suggestive for ETA receptor upregulation after DOCA-salt. There was no evidence for vasoconstrictor ETB receptor effects after DOCA-salt, since BQ788 did not block ET-1-induced vasoconstriction. Thus, our data do not support constrictor ETB receptor upregulation, although this has been observed earlier in Ang II-dependent hypertensive rat models (Roksnoer et al., 2015).

When studied in young healthy animals, the AT2 receptor stimulates vasodilation and natriuresis, thereby counteracting the actions of the AT1 receptor, the main mediator of all the wellknown deleterious effects of Ang II (Batenburg et al., 2004a; Padia et al., 2006; Verdonk et al., 2012a). However, when studied in older or hypertensive animals, the AT2 receptor often loses its vasodilatory actions and instead induces vasoconstriction, similar to the AT1 receptor (You et al., 2005;

Pinaud et al., 2007; Moltzer et al., 2010). This loss of function might be explained by reactive oxygen species scavenging by NO (Moltzer et al., 2010; Verdonk et al., 2012a). A further possibility is that AT2 receptors appear on vascular smooth muscle cells under pathophysiological conditions and act in concert with AT1 receptors (e.g., through heterodimerization), as opposed to their predominant, if not exclusive endothelial presence under healthy conditions (van Esch et al., 2006; Moltzer et al., 2010; Verdonk et al., 2012b). We now show that the AT2 receptor also loses its vasodilatory effects during DOCA-salt, illustrated by the fact that the Ang II-induced constrictor responses were blocked by PD123319, an AT2 receptor antagonist (**Figure 4**). Furthermore, we observed that NOS inhibition with L-NAME greatly enhanced the Ang II contractile response in the MMF-treated animals, whereas the increase after vehicle was non-significant, implying that NOS is preserved in the MMF-treated animals. Indeed, in a rat allograft model, MMF prevented endothelial dysfunction and reduced sensitivity to vasoconstrictors by increasing NO availability through endothelial NOS (Freguin-Bouilland et al., 2011).

Our findings are in line with the results of Guzik et al. (2007), who found a reduced hypertensive response and greater vascular relaxation in Ang II-treated RAG-$1^{-/-}$ mice (which lack B and T cells) than wild-type mice. Furthermore, superoxide production is greater in wild-type than in RAG-$1^{-/-}$ mice after 40 days of DOCA-salt (Guzik et al., 2007). Superoxide is known to react with NO, thereby decreasing NO availability and inducing vasoconstriction (Gryglewski et al., 1986). Guzik et al. (2007) investigated the abundance of the AT2 receptor in the aortic wall by Western blot, but found no significant differences between RAG-$1^{-/-}$ and wild type mice. Nevertheless, their previous results and our data support a vasoconstrictive action of the AT2 receptor.

Compared with MMF-treated rats, the rats treated with DOCA-salt alone had higher blood pressure, larger volumes of fluid intake and urine output, and greater excretions of NaC and KC (**Figures 1, 2**). This suggests parallel central effects of DOCA on salt appetite, thirst, and blood pressure, as has been observed previously (Hamlin et al., 1988; Sapouckey et al., 2017). Despite these differences, no changes were observed in any of the renal tubular segments that we studied (**Figure 5**). This is in contrast with recent studies analyzing the role of inflammation on renal sodium transport in hypertensive models. For example, IFN-$g^{-/-}$ or IL-$17A^{-/-}$ mice had a blunted rise in blood pressure after 2 weeks of high-dose Ang II infusion (Kamat et al., 2015). The IFN-$g^{-/-}$ mice, but not the IL- $17A^{-/-}$ mice, had a lower abundance

of the phosphorylated forms of NKCC2 and NCC than wild-type mice. Furthermore, both IFN-g$^{-/-}$ and IL-17A$^{-/-}$ mice had lower abundances of NHE3 in the proximal tubules than wild-type mice (Kamat et al., 2015). A study using cortical collecting duct cells of mice found that treatment with IL-6 increased both the mRNA abundance and protein expression of all ENaC subunits (Li et al., 2010). This was accompanied by an increase in the amiloride-sensitive NaC current. These findings suggest that specific parts of the immune system impair pressure natriuresis and enhance distal tubular NaC reabsorption, ultimately leading to hypertension. Our findings also contrast with a recent study by Liu et al. (2017), who found that in DOCA-salt-treated mice, CD8C T cells upregulated NCC expression through direct contact with distal convoluted tubule cells. We propose the following explanations for these differences. First, we used the DOCA-salt model which has low levels of circulating renin and Ang II, in contrast to the Ang II infusion model (Somers et al., 2000). Second, rats received 0.9% NaCl as drinking water, which may have led to a downregulation of kidney NaC transporters. Accordingly, higher NaCl intake in the vehicle-group (with commensurate suppression of the renin–angiotensin system) may have blunted possible differences between groups. Third, we cannot exclude the possibility that changes in renal transporters were only detectable by analyzing the phosphorylated (activated) forms of these proteins. Finally, the duration of our experiment was twice as long as the more commonly used DOCA-salt model, which also includes uninephrectomy. The longer duration of our model may have led to a new steady state with conversion from a volume to a non-volume hypertension mechanism. Thus, in this study, we cannot prove or reject the hypothesis that the antihypertensive effect of MMF is also mediated through renal salt handling.

A number of strengths and limitations of this study should be mentioned. The strength of this study is that it is the first to study both vascular resistance and kidney NaC transporters within the same model. A weakness is that we have not yet determined the mechanism of the AT2 receptor-mediated constrictor response, the identity of the non-NO component that is upregulated after DOCA-salt, nor the expression of the ET receptors. Finally, future studies should address whether our ex vivo observations also hold true in vivo. For this, rats should be treated with AT2 and ETA receptor antagonists on top of vehicle, DOCA-salt and/or MMF.

Conclusion

In conclusion, our findings provide additional insight into the pathophysiologic

aspects of immune involvement in the development of hypertension. It is known that hypertensive stimuli like old age, high salt diet and atherosclerosis cause stress to the vascular endothelium. This stress causes “sterile inflammation” with activation of both the innate and adaptive immune system, mainly involving infiltration of macrophages and T-cells in the adventitia and perivascular fat with a subsequent production of IL-17, IFN-g, and TNF-a, amongst others (Epelman et al., 2015; McMaster et al., 2015; Wenzel et al., 2016). These actions induce the production of ROS which results in a decreased bioavailability of NO, impaired vasodilation, and increased vascular stiffness, causing hypertension and ultimately end-organ damage. Our results propose an important role for the AT2 receptor, which appears to have a constrictor function under hypertensive stimuli, which is prevented by immunosuppression with MMF.

Author Contributions

AM, DS, KV, and NvdL conducted the experiments. AM, DS, NvdL, AD, RZ, and EH analyzed the data. AM, DS, AD, and EH wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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PART III

CHAPTER 8

Serum magnesium, hepatocyte nuclear factor 1β genotype and post-transplant diabetes mellitus: a prospective study

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Abstract

Background: Retrospective studies suggest that tacrolimus-induced hypomagnesaemia is a risk factor for post-transplant diabetes mellitus (PTDM), but prospective studies are lacking.

Methods: This was a prospective study with measurements of serum magnesium and tacrolimus at pre-specified time points in the first year after living donor kidney transplantation (KT). The role of single nucleotide polymorphisms (SNPs) in hepatocyte nuclear factor 1b (HNF1b) was also explored because HNF1b regulates insulin secretion and renal magnesium handling. Repeated measurement and regression analyses were used to analyse associations with PTDM.

Results: In our cohort, 29 out of 167 kidney transplant recipients developed PTDM after 1 year (17%). Higher tacrolimus concentrations were significantly associated with lower serum magnesium and increased risk of hypomagnesaemia. Patients who developed PTDM had a significantly lower serum magnesium trajectory than patients who did not develop PTDM. In multivariate analysis, lower serum magnesium, age and body mass index were independent risk factors for PTDM. In recipients, the HNF1b SNP rs752010 G > A significantly increased the risk of PTDM [odds ratio (OR) 2.56, 95% confidence interval (CI) 1.05–6.23] but not of hypomagnesaemia. This association lost significance after correction for age and sex (OR 2.24, 95% CI 0.90–5.57). No association between HNF1b SNPs and PTDM was found in corresponding donors.

Conclusions: A lower serum magnesium in the first year after KT is an independent risk factor for PTDM. The HNF1b SNP rs752010 G > A may add to this risk through an effect on insulin secretion rather than hypomagnesaemia, but its role requires further confirmation.

Keywords: calcineurin inhibitors, hypomagnesaemia, post-transplant diabetes mellitus, single nucleotide polymorphism, tacrolimus

Introduction

Post-transplant diabetes mellitus (PTDM) is one of the major metabolic complications after kidney transplantation (KT) [1– 5]. Kidney transplant recipients (KTRs) with PTDM have a reduced long-term graft function [6] and a higher risk of graft failure [6–10], cardiovascular disease [5, 11, 12] and mortality [5, 8–11, 13]. Of the identified risk factors of PTDM, hypomagnesaemia is of special interest, because it is potentially modifiable, and because a link between magnesium and diabetes mellitus is becoming increasingly evident [14]. Magnesium acts as an intracellular

cofactor for several metabolic pathways, including glucose transport and uptake, and insulin sensitivity and secretion [15–21]. In non-transplant populations, lower serum magnesium levels have been firmly linked to a higher risk of diabetes mellitus, although it is unclear if this association is causal [22, 23]. Hypomagnesaemia has also become a common finding in KTRs after the introduction of calcineurin inhibitors (CNIs) [24, 25]. CNIs down-regulate the transient receptor potential melastatin 6, a magnesium channel in the distal convoluted tubule involved in the reabsorption of magnesium [26]. Thus, CNIs cause hypomagnesaemia through renal magnesium loss [25]. CNIs are also a risk factor for PTDM and this risk may be mediated through the effects of CNIs on magnesium [2]. Indeed, in several retrospective studies, CNI-induced hypomagnesaemia has been associated with a higher risk of PTDM [2, 27, 28], although this has not been a consistent finding [3, 29, 30]. Therefore, the main aim of this prospective study was to analyse the roles of tacrolimus and serum magnesium on the risk of developing PTDM.

The second aim of this study was to explore the potential role of the transcription factor hepatocyte nuclear factor 1b (HNF1b) in PTDM. HNF1b plays a role in both insulin secretion and renal magnesium reabsorption [31]. Patients with mutations in the HNF1b gene develop diabetes mellitus andhypomagnesaemia [31, 32], and single nucleotide polymorphisms (SNPs) in HNF1b are associated with diabetes mellitus in the general population [33–36]. Interestingly, a recent study showed that CNIs down-regulate HNF1b, increasing the risk of PTDM 2- to 3-fold in patients with HNF1b mutations [37]. This provided a rationale to explore the role of HNF1b in KTRs receiving tacrolimus.

Methods

Study design and population

This study was embedded within an investigator-initiated, prospective, randomized-controlled, parallel group, open-label, single centre clinical trial [38]. The objective of this randomized controlled trial was to investigate whether adaptation of the tacrolimus starting dose according to CYP3A5 genotype instead of body weight leads to an earlier achievement of the tacrolimus target whole-blood exposure. The design and rationale of the study have been described in detail elsewhere [38]. In brief, between 2010 and 2013, patients aged 18 years who were scheduled to receive a single-organ, blood group AB0-compatible kidney from a living donor in our centre, were screened. The final study population consisted of 237 patients. All patients gave written informed consent before inclusion and randomization. The study pre-specified three whole-blood tacrolimus target ranges: 10.0–15.0 ng/mL

in Weeks 1 and 2; 8.0–12.0 ng/mL in Weeks 3 and 4; and 5.0–10.0 ng/mL after Week 4 post-KTR. The study was approved by the institutional review board of the Erasmus MC (Medical Ethical Review Board number 2010-080) and was registered in the Dutch national trial registry (http://www.trialregister.nl/trialreg/index.asp; number NTR2226, registered 25 February 2010).

Measurements

Pre-specified measurements of serum and urine magnesium, creatinine and non-fasting blood glucose levels were performed prior to transplantation and at 7 days and 1, 3, 6 and 12 months after KT. Blood and urine were collected on the same day. Prior to KT, only blood was collected. The measurements were performed by the Department of Clinical Chemistry using the Cobas 8000 modular analyzer series (Roche). Hypomagnesaemia was defined as serum magnesium level <0.70 mmol/L. The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [39]. Urine magnesium was expressed as ratio with urine creatinine. Whole-blood tacrolimus concentration was measured prospectively at 1, 3, 6 and 12 months after KT using an immunoassay. Because outpatient visits were not always scheduled exactly at these time-points, we allowed the following time windows for the measurements: Month 1 (20–40 days), Month 3 (76–106 days), Month 6 (157– 207 days) and Month 12 (325–405 days). When multiple samples were available within the interval, the measurement closest to the defined time point was used for analysis. Information on age, sex, body mass index (BMI) and primary kidney disease of the patients was derived from the electronic case report forms of the original study.

Genetic analysis

To study the role of HNF1b in the risk of developing PTDM, we used a candidate gene approach to select different SNPs in HNF1b with a known association to diabetes mellitus (rs752010 G > A, rs4430796 A > G and rs7501939 C > T) [33]. To do so, DNA was extracted from peripheral blood leucocytes using the Blood DNA kit (Qiagen, Courtaboeuf, France) in accordance with standard protocols. Genotyping of the HNF1b allele was performed using TaqMan Assay reagents for allelic discrimination (Applied Biosystems, Courtaboeuf, France) with a 7900 Applied Biosystems thermal cycler. All genotyping was performed in duplo according to quality standards of the International Federation of Clinical Chemistry and Laboratory Medicine.

Outcome

The endpoint of this study was PTDM 1 year after transplantation. We chose the 1-year time point because (i) glucocorticoids are discontinued in our centre in all patients 3 months after transplantation and (ii) transient post- transplantation hyperglycaemia is usually resolved 1 year after transplantation, and does not predict PTDM [40]. PTDM was defined according to the American Diabetes Association as a fasting blood glucose >7.0 mmol/L, or a non-fasting blood glucose >11.1 mmol/L [41, 42]. Increased blood glucose levels were confirmed by repeat testing on a different day. In addition, patients who started with glucose-lowering drugs after KT were also considered to have PTDM.

Statistical analysis

Comparisons of the baseline characteristics of patients with PTDM and patients without PTDM were performed with Student's t-tests for continuous variables and Chi-square tests for categorical variables. Missing data in covariates (<10% of total) were handled by multiple imputation using the Multivariate Imputation by Chained Equations package in R [43]. The number of imputed datasets was based on the average percentage of missing values [44], and the number of iterations gradually increased until convergence was achieved. Results are reported for imputed data, except for the baseline and genetic data. To examine the association between serum magnesium levels and whole-blood tacrolimus concentrations, univariate linear regression was used to calculate the betas, and univariate logistic regression was used to calculate odds ratios (ORs). Mixed-design analysis of variance (ANOVA) was used to test for differences in the repeated measurements (trajectories) of serum magnesium, whole-blood tacrolimus and eGFR in patients with and without PTDM. For the regression analyses, we calculated the area under the curve (AUC) for the trajectories of serum magnesium and whole-blood tacrolimus. In the univariate and multivariate logistic regression analyses, we included variables based on our hypothesis (serum magnesium and whole-blood tacrolimus AUC), and those which have previously been reported as risk factors for PTDM (age and BMI) [8]. Comparisons of the genetic characteristics of patients with PTDM and patients without PTDM were made with Chi-square tests. Univariate and multivariate logistic regressions, including dominant and recessive models, were used to examine the association between HNF1b SNPs and PTDM. A dominant model indicates that either one or two copies of the SNP allele are needed, whereas a recessive model indicates that two copies of the SNP allele are needed for the

SNP to have an association with PTDM. Data were analysed using SPSS Statistics for Windows, Version 24.0 (IBM, Armonk, NY, USA) and R version 3.4.4 (R Foundation for Statistical Computing, Vienna, Austria). A two-sided $P < 0.05$ was considered as statistically significant.

Results

Baseline characteristics

The original clinical trial included 237 patients who received their first KT from a living donor (Figure 1) [38]. Of these patients, 50 were excluded because of diabetes mellitus prior to transplantation, and 20 because of discontinuation of tacrolimus, loss-to-follow-up, transplant nephrectomy or death. This resulted in a total study population of 167 patients of whom 29 (17%) met our definition of PTDM. Despite

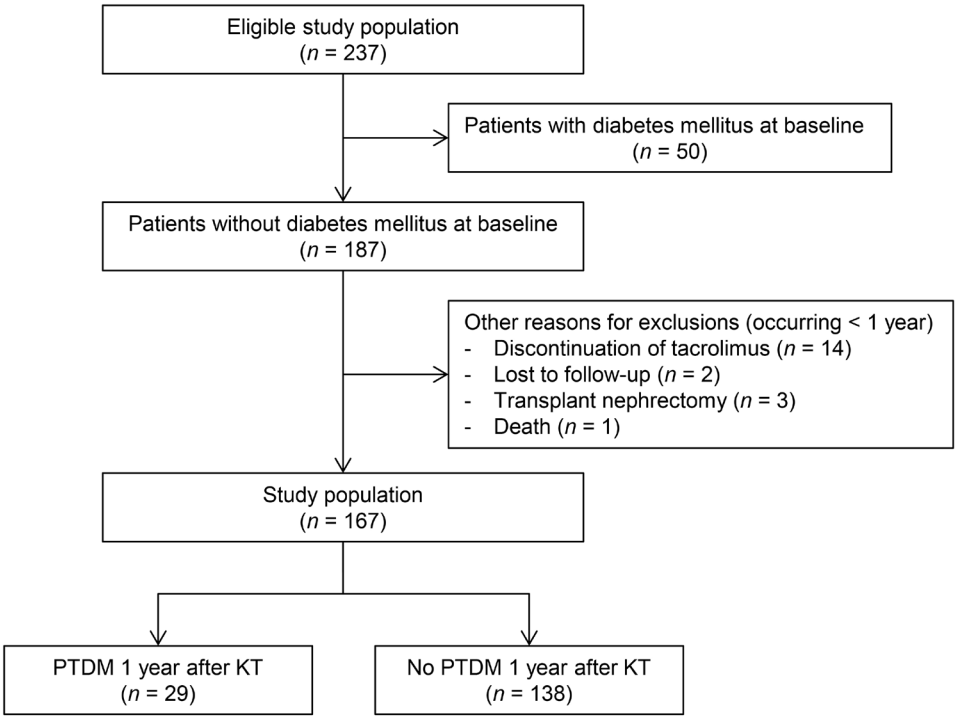

Figure 1 Flowchart of the study population. Of the 237 eligible patients, 50 patients were excluded because of pre-existing diabetes mellitus. Of the remaining 187 patients, 20 additional patients were excluded because of discontinuation of tacrolimus, loss-to-follow-up, transplant nephrectomy or death. This resulted in a total study population of 167 patients, which was further divided into 29 patients with posttransplant diabetes mellitus (PTDM) at 1 year after kidney transplantation (KT) and 138 participants without PTDM.

Table 1 Comparison of baseline characteristics of patients with and without PTDM

	No PTDM (n = 138)	PTDM (n = 29)	P-value
General characteristics			
Age, years	51 ± 15	60 ± 7	< 0.001
Female, n (%)	57 (41.3)	10 (34.5)	0.5
Body mass index, kg/m2	26 ± 4	28 ± 4	0.004
Systolic blood pressure, mmHg	136 ± 16	138 ± 13	0.7
Diastolic blood pressure, mmHg	82 ± 10	81 ± 8	0.4
Primary kidney disease			
Hypertension	36 (26)	12 (41)	0.1
Polycystic kidney disease	27 (20)	5 (17)	0.8
Glomerular disease	33 (24)	6 (21)	0.7
Other causes	41 (30)	6 (21)	0.3
Laboratory values prior to transplantation			
eGFR, ml/min/1.73m2	10 ± 5	10 ± 4	0.9
Blood glucose, mmol/L	5.1 (4.6–5.7)	5.7 (5.2–6.6)	0.003
Serum magnesium, mmol/L	0.86 ± 0.13	0.88 ± 0.13	0.4
Laboratory values at one year			
eGFR, ml/min/1.73m2	48 ± 16	52 ± 17	0.3
Blood glucose, mmol/L	5.5 (4.9–6.2)	6.6 (5.6–8.5)	< 0.001
Serum magnesium, mmol/L	0.72 ± 0.09	0.69 ± 0.09	0.2
Whole-blood tacrolimus, µg/L	6.4 ± 2.7	6.4 ± 3.0	1.0
Urine magnesium/creatinine, mmol/mmol	0.38 ± 0.23	0.36 ± 0.19	0.8

our conservative definition of PTDM, this incidence is in agreement with previous studies [8, 45–47]. The mean age of the study population was 52 6 14 years and 67 (40%) patients were female. Table 1 shows the comparison of the baseline characteristics of patients

Table 2 Association between whole-blood tacrolimus concentration, serum magnesium levels, and the risk of hypomagnesemia

Outcome	β or OR (95% CI)*	P-value
Serum magnesium (mmol/L)	-0.008 (-0.014 to -0.0029)	0.003
Hypomagnesemia (<0.70 mmol/L)	1.28 (1.09 to 1.50)	0.003

Footnote: * Results are given per 1 µg/L increase in whole-blood tacrolimus.

with or without PTDM. Patients with PTDM were significantly older (60 6 7 versus 51 6 15 years, P < 0.001) and had a higher BMI (28 6 4 versus 26 6 4 kg/m2, P 0.004). Patients with PTDM also had a higher blood glucose level prior to KT and 1 year after KT. No differences in whole-blood tacrolimus, serum magnesium or urine magnesium/creatinine were observed. None of the patients had hepatitis C [48].

Relationships between tacrolimus, magnesium and PTDM

We identified significant relationships between whole-blood tacrolimus concentration, serum magnesium levels and the risk of hypomagnesaemia at Year 1 (Table 2). Per microgram per litre increase in whole-blood tacrolimus concentration, the serum magnesium level decreased by 0.008 mmol/L (P 0.003). In addition, per microgram per litre increase in whole-blood tacrolimus concentration, the OR for the development of hypomagnesaemia increased by 1.28 [95% confidence interval (CI) 1.09–1.50, P 0.003]. Because serum magnesium levels did not differ between patients with and without PTDM at Year 1 (Table 1), we pursued a repeated measures (trajectory) analysis. In this analysis, we identified a significant difference between the serum magnesium trajectory of patients with PTDM compared with patients without PTDM (P 0.04; Figure 2) . No significant differences were found between the whole-blood tacrolimus or eGFR trajectories of patients with and without PTDM.

Risk factors for PTDM

Possible risk factors for PTDM were analysed in univariate and multivariate models (Table 3). Based on our hypothesis, we included the AUC of the whole-blood tacrolimus and serum magnesium trajectories in the multivariate analysis in addition to age and BMI. In this multivariate analysis, age (OR 1.05, 95% CI 1.01–1.10), BMI (OR 1.11, 95% CI 1.01–1.23) and the serum magnesium AUC (OR 0.98, 95% CI 0.96–1.00) were identified as independent risk factors for PTDM. Inclusion of the original trial assignment did not change the results.

Figure 2 Serum magnesium, estimated glomerular filtration rate (eGFR), and tacrolimus trajectories during the first year after kidney transplantation in patients with and without posttransplant diabetes mellitus (PTDM).

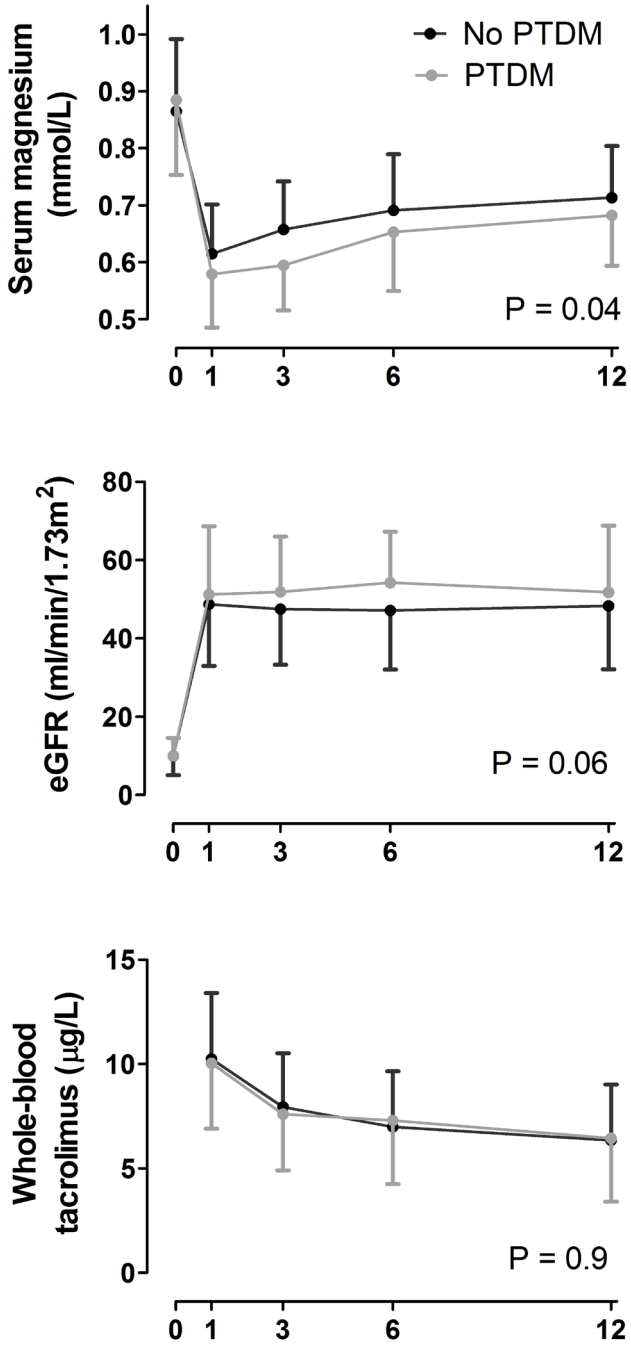

Table 3 Risk factors for the development of PTDM

	Univariable analysis		Multivariable analysis	
Predictor	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
Age, per year	1.06 (1.02-1.10)	0.003	1.05 (1.01-1.11)	0.01
Body mass index, per 1 kg/m2	1.14 (1.04-1.25)	0.006	1.11 (1.01- 1.23)	0.03
Tacrolimus AUC*	1.00 (0.99-1.00)	0.8	1.00 (0.99-1.00)	0.6
Serum magnesium AUC*	0.98 (0.96-1.00)	0.01	0.98 (0.96-1.00)	0.01

Footnote: * AUC, area under the curve. Calculated based on the trajectories in **Figure 2**.

SNPs in HNF1β and the risk of PTDM

We analysed the prevalence of three HNF1b SNPs that were previously implicated in diabetes mellitus in the general population (rs752010 G > A, rs4430796 A > G and rs7501939 C > T) [33]. These SNPs were analysed both in recipients and donors to analyse if the association between the SNPs and PTDM could be explained by a renal or non-renal mechanism. When applying a recessive model, rs752010 G > A was significantly more common in patients with PTDM compared with patients without PTDM (Table 4). No significant differences were found for the other two SNPs (Supplementary data, Table S1). In an unadjusted recessive model, rs752010 G > A in KTRs was also associated with PTDM (Table 5). This association attenuated and became borderline significant when correcting for age and sex. No such associations were identified for the other two HNF1b SNPs (Supplementary data, Table S2). In addition, we found no association between HNF1b SNPs of the donor and the risk of PTDM (data not shown). Finally, we did not find a significant association between HNF1b SNPs and the risk of hypomagnesaemia (data not shown).

Discussion

PTDM is a major metabolic complication of KT, but its pathogenesis is incompletely understood. In this prospective study, we analysed the role of tacrolimus and serum magnesium on PTDM. We show that there is a concentration-dependent effect of the CNI tacrolimus on serum magnesium (Table 2) and that the serum magnesium AUC in the first year after KT is an independent risk factor for the development of PTDM (Table 3). We also added an exploratory analysis on the role of genetic variation in the HNF1b gene on the risk of PTDM. This HNF1b hypothesis was based

Table 4 HNF1β SNP rs752010 G>A in kidney transplant recipients with or without PTDM

HNF1β SNP rs752010 G>A	Genotype	Total population (n = 145)	No PTDM (n = 119)	PTDM (n = 26)	P-value
Genotype	**GG**	30 (21)	25 (21)	5 (19)	0.8
	AG	72 (49)	64 (54)	8 (31)	0.03
	AA	43 (30)	30 (25)	13 (50)	0.01
Dominant	**GG**	30 (21)	25 (21)	5 (19)	0.8
	AG + AA	115 (79)	94 (79)	21 (81)	0.8
Recessive	**GG + AG**	102 (70)	89 (75)	13 (50)	0.01
	AA	43 (30)	30 (25)	13 (50)	0.01

on reports that (i) patients with HNF1b mutations develop hypomagnesaemia and diabetes mellitus [32, 49, 50], (ii) patients with HNF1b mutations are more prone to develop PTDM [37], (iii) CNIs down-regulate HNF1b [37] and (iv) SNPs in HNF1b are associated with diabetes mellitus in the general population [33–36, 51, 52]. Although the univariate analysis showed an association between the HNF1b SNP rs752010 G > A and PTDM, significance was lost when adjusting for age and sex (Table 5). Because our study was not specifically powered to analyse this association, analysis in a larger cohort is of interest.

Van Laecke et al. [2] were the first to identify associations between the use of CNIs, serum magnesium and the risk of developing PTDM. They showed that patients with PTDM had lower serum magnesium levels and that lower serum magnesium levels resulted in a faster development of PTDM. Based on their analysis, they concluded that the association between CNI use and PTDM is largely attributable to CNI-induced hypomagnesaemia. Similarly, Garg et al. [28] showed that a lower serum magnesium level at 1 month after transplantation predicted PTDM as well as pre-diabetes. Huang et al. [27] performed the largest retrospective study to date (948 non-diabetic KTRs) and showed that both a lower post-transplant serum magnesium level and hypomagnesaemia are independent risk factors for PTDM. Stevens et al. [53] showed that a single-dose rabbit anti-thymocyte globulin resulted in less PTDM than a divided-dose, and that this also coincided with less hypomagnesaemia. In contrast, three retrospective studies (with a study population ranging from 205 to 589 patients) were unable to find an association between serum magnesium and

Table 5 Relationship between HNF1β SNP rs752010 G>A in the kidney transplant recipient and PTDM

HNF1β SNP rs752010 G>A	Genotype	Model 1*		Model 2*	
		OR (95%CI)	P-value	OR (95%CI)	P-value
Dominant	**GG**	Ref.	Ref.	Ref.	Ref.
	AG+AA	1.10 (0.38-3.22)	0.9	0.98 (0.32-2.97)	1.0
Recessive	**GG+AG**	Ref.	Ref.	Ref.	Ref.
	AA	2.56 (1.05-6.23)	0.04	2.24 (0.90-5.57)	0.08

Footnote: *Model 1: unadjusted; Model 2: adjusted for age and sex.

PTDM [3, 29, 30]. This discrepancy may be explained by the fact that the studies by Van Laecke et al. [2], Huang et al., [27] and our study analysed multiple serum magnesium values rather than focusing on a single value [28]. This suggests that the duration of the exposure to lower serum magnesium concentrations is important for the risk of PTDM. This may also relate to the fact that serum magnesium is a poor reflection of the intracellular magnesium concentration, which is biologically more relevant [54].

Our research questions primarily focused on the pathogenesis of PTDM, but may also be relevant clinically. Although the association between lower serum magnesium and diabetes mellitus has long been recognized [22], the causality of this association has remained unclear. Hypomagnesaemia may predispose to diabetes mellitus, but hyperglycaemia could also lead to increased urinary magnesium excretion and therefore hypomagnesaemia. To investigate this directionality, we recently analysed whether a lower serum magnesium predicts subsequent pre-diabetes in the general population [23]. Patients with pre-diabetes are less likely to develop hyperglycaemia and therefore urinary magnesium loss. This study showed that lower serum magnesium was independently associated with a higher pre-diabetes risk after a median follow-up of 5.7 years [23]. A clinically more relevant question is whether correction of magnesium deficiency is able to improve glycaemic control. Several clinical trials have shown that oral magnesium supplementation can indeed increase serum magnesium and improve the levels of fasting glucose, insulin and glycated haemoglobin [55–57]. A recent experimental study extended these effects by showing that magnesium supplementation also improves mitochondrial function, reduces oxidative stress and improves diastolic

dysfunction in diabetic mice [58]. One research group performed two small clinical trials with magnesium supplementation in hypomagnaesemic KTRs on tacrolimus [59, 60]. The first trial showed an effect of magnesium supplementation on fasting glucose but not insulin resistance [60], whereas the second trial showed no effects on insulin secretion [59]. Of note, in both trials magnesium oxide was used, which has a relatively low bioavailability and high risk of diarrhoea compared with some of the other magnesium salts (e.g. magnesium chloride, citrate or lactate) [61, 62]. The association between the HNF1b SNP rs752010 G > A and PTDM requires further confirmation. Several other SNPs have been linked to PTDM risk in KTRs, the majority related to b cell function [63]. One recent study also analysed the association between three HNF1b SNPs and PTDM, but did not find an association [64]. However, HNF1b SNPs have been identified as risk factors in several studies on the genetic susceptibility of diabetes mellitus [33–36, 51, 52]. Several of these diabetes mellitus susceptibility genes also predispose to PTDM, especially TCF7L2 [65]. The reason for focusing on HNF1b SNPs in this study was that HNF1b could link CNIs, serum magnesium and PTDM. In this view, patients with HNF1b SNPs would be more sensitive to CNI-induced hypomagnesaemia and therefore PTDM. However, our data do not support this theory, because (i) HNF1b SNPs were not associated with hypomagnesaemia and (ii) HNF1b SNPs in donors were not associated with PTDM risk. The HNF1b SNP rs752010 G > A did show a positive association with PTDM risk in recipients, but lost significance after correction for age and sex. The value of this finding remains uncertain in a study that was not specifically powered to address this association, and it would be of interest to analyse this SNP in a larger cohort of PTDM. If HNF1b increases the risk of PTDM, this is likely mediated through an effect on insulin secretion (recipient effect) rather than an effect on renal magnesium handling (donor effect).

This study has a number of strengths. First, it is the first study to analyse the relationship between tacrolimus, serum magnesium and PTDM in a prospective fashion. Serum magnesium is not always routinely measured [66], and previous studies may, therefore, have suffered from confounding by indication. Secondly, we defined PTDM conservatively as diabetes mellitus 1 year after transplantation to exclude transient forms of PTDM that occur early after transplantation. Thirdly, we analysed the risk factors of PTDM independent of glucocorticoids because these are discontinued 3 months after KT in our centre. Glucocorticoids can also cause renal magnesium loss and contribute to PTDM [26, 67]. Thus, defining PTDM at Year 1 allowed us to more specifically address the role of tacrolimus on serum magnesium and PTDM risk. Finally, analysing HNF1b SNPs in both recipient and donor allowed

us to address the question how HNF1b may contribute to PTDM.
Our study also has a number of limitations. First, this study was conducted within the framework of a previously conducted clinical trial [38]. Therefore, the study was not specifically powered for the research questions of this analysis. However, when designing the trial, this study was already planned with measurements of serum and urine magnesium at pre-specified time points. The relatively small sample size did not allow adjustment for multiple covariates and confounders, which may have limited the external validity of the study. Secondly, because this was a cohort of KTRs using tacrolimus, we did not have patients on ciclosporin or a CNI-free regimen as comparators. Thirdly, the availability of HbA1c or oral glucose tolerance testing may have resulted in a different incidence of PTDM. Finally, reverse causality cannot be excluded. For example, patients who developed PTDM had higher blood glucose prior to KT, as was also observed by Van Laecke et al. [2]. This could imply that these patients were more prone to glycosuria and therefore renal magnesium loss. Conversely, it could also imply that a lower magnesium after KT contributed to the evolution from pre-diabetes to diabetes.
In conclusion, in the first year after KT, a higher tacrolimus whole-blood concentration is associated with lower serum magnesium and an increased risk of hypomagnesaemia. Lower serum magnesium in the first year after KT is an independent risk factor for PTDM. A separate and novel risk factor for
PTDM may be the HNF1b SNP rs752010 G > A in KTRs, although this finding requires confirmation in a larger cohort.

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CHAPTER 9

Summary, Discussion and Future Perspectives

Summary and Conclusions

The studies presented in this thesis were aimed to further elucidate the role of the immune system and immunosuppressive drugs on hypertension, electrolytes, and posttransplantation diabetes mellitus (PTDM). Hypertension and diabetes remain extensively studied subjects because of their high prevalence and their risk on the development of kidney and cardiovascular disease, and therefore premature morbidity and mortality. They are also highly prevalent in kidney transplant recipients (KTRs), partly due to the same risk factors as in the general population, but also due to specific KTR related factors. The immunosuppressive drug tacrolimus has been linked to the development of hypomagnesemia in KTRs, which in turns leads to PTDM. Furthermore, tacrolimus causes hypertension, in part through phosphorylation of the sodium chloride cotransporter (NCC). Urinary extracellular vesicles seem to reflect the abundance and activity of transporters in the renal tubule. Emerging evidence suggests that inflammation also contributes to the development of hypertension. Conversely, potassium has an anti-hypertensive effect through inhibition of renal sodium transporters.

In **Chapter 2** we reviewed novel roles of NCC in sodium, potassium and blood pressure regulation. We further clarified the intracellular signaling cascade that controls NCC activity, which involves the kinases WNK, SPAK, OSR1, and SGK1. Interestingly, in this complex mechanism WNK4 seems to be a negative regulator of NCC under some conditions, but may become a positive regulator in other settings. We also summarized all hormones, metabolic stimuli, and drugs influencing NCC activity. Classic inhibitors of NCC are the thiazide diuretics, which were implemented clinically long before it became apparent that their primary target is NCC. Opposite to thiazide diuretics, calcineurin inhibitors have been shown to activate NCC in animal studies, but this finding had yet to be extrapolated to humans. To do so, the use of urinary extracellular vesicles (uEVs) seems a promising approach to assess NCC activity in vivo, and this was further explored in **Chapter 6**. Also of interest is the recent discovery that dietary potassium is able to inhibit NCC, which leads to an increased delivery of sodium to the potassium-secreting portion of the nephron. This phenomenon of potassium-induced natriuresis may explain the anti-hypertensive effect of potassium, and is further explored in **Chapter 3**.

In **Chapter 3** we also studied the “aldosterone paradox”, more specifically how the kidney responds to the combination of hypovolemia and hyperkalemia. We aimed to gain insight into the two physiological roles of aldosterone, namely sodium retention

during hypovolemia and potassium secretion during hyperkalemia. To address this, we mimicked hypovolemia and hyperkalemia in two groups of rats with a low-sodium diet and a high-potassium diet, and compared them with rats on a normal diet. A fourth group of rats received a combined low sodium/high potassium diet. Low dietary sodium favors sodium reabsorption through NCC by increasing both aldosterone and angiotensin II. A high dietary potassium diet increased aldosterone, but not angiotensin II. Despite increased aldosterone levels, NCC was suppressed to increase sodium delivery to the collecting duct and to indirectly promote potassium secretion. NCC was also suppressed in the combined diet, while both aldosterone and angiotensin II were increased. Increased levels of WNK4 in the high dietary potassium groups may be involved in suppressing NCC. In conclusion, high dietary potassium overrides the effects of ANG II and aldosterone on NCC and inhibits this cotransporter to deliver sufficient sodium to ENaC for potassium secretion.

Chapter 4 provides an overview of the role of pharmacogenetics of CNI-induced hypertension. CNI-induced hypertension is an important side-effect, because it is associated with earlier graft failure and higher cardiovascular mortality in solid organ transplant recipients. Predicting who is at risk for CNI-induced hypertension may be useful when selecting specific interventions, including dietary salt restriction, thiazide diuretics or a CNI-free immunosuppressive regimen. Current candidate genes involved in CNI-induced hypertension in kidney transplant recipients are CYP3A5, ABCB1, WNK4, and SPAK. Polymorphisms in these genes are associated with hypertension in the general population and are involved in the metabolism of CNIs. That is, CYP3A5 and ABCB1 are involved in the pharmacokinetics of CNIs and hypertension, and WNK4 and SPAK have been shown to mediate CNI-induced activation of NCC. However, the direct involvement in CNI-induced hypertension has only been studied for CYP3A5 and only in a limited number of studies. Furthermore, the results of these studies are not uniform. Therefore, future studies are needed to confirm if polymorphisms in any of these candidate genes contribute to CNI-induced hypertension.

In **Chapter 5** we performed a randomized non-inferiority crossover trial comparing the thiazide diuretic chlorthalidone with the calcium channel blocker amlodipine in 41 hypertensive kidney transplant recipients using the CNI tacrolimus. The rationale for this trial were recent data showing that the thiazide-sensitive NCC is activated by tacrolimus in laboratory animals. This suggests that well-tolerated and inexpensive thiazide diuretics may be effective drugs in treating CNI-induced

hypertension. Chlorthalidone and amlodipine both markedly reduced ambulatory systolic blood pressure and did so to a similar degree. Despite similar blood pressure responses, chlorthalidone significantly reduced proteinuria by 30% and edema from 22 to 10%, whereas amlodipine increased peripheral edema. However, chlorthalidone temporarily reduced eGFR and increased serum uric acid and glycated haemoglobin levels. We conclude that both amlodipine and chlorthalidone are effective antihypertensive drugs after kidney transplantation; chlorthalidone may be preferable in patients with proteinuria or edema.

In **Chapter 6** we studied the relation between the NCC abundance and phosphorylation in uEVs and the use of calcineurin inhibitors. To do so, we compared total NCC and phosphporylated NCC in uEVs of kidney transplant recipients treated with a CNI-based or CNI-free immunosuppressive regimen, and healthy volunteers. The first main finding of this study was that both cyclosporine and tacrolimus increased the abundance of total and phosphorylated NCC in uEVs four to five fold compared to CNI-free kidney transplant recipients and healthy volunteers. Furthermore, we studied the interaction between the abundance of NCC in uEVs and the antihypertensive effect of treatment with thiazide diuretics. We found that higher abundances of total NCC (tNCC) and phosphorylated NCC (pNCC) predicted the blood pressure response to thiazide diuretics in hypertensive kidney transplant recipients using tacrolimus. Finally, to further confirm the effect of CNIs on NCC in the kidney, we performed an ex vivo study using mice cortical tubules. Short-term exposure to cyclosporine increased pNCC but not tNCC abundance in mouse cortical tubule suspensions. Together, these findings imply that analysis of NCC in uEVs could represent a novel method to guide anti-hypertensive therapy in kidney transplant recipients using CNIs.

In **Chapter 7** we studied how the immunosuppressive drug mycophenolate mofetil (MMF) attenuates experimental hypertension, including vascular and renal effects. We hypothesized that T cells mediate experimental hypertension through effects on renal sodium transport and vascular tone. To address this, we induced hypertension with DOCA-salt in rats and co-treated the animals with MMF or vehicle. Co-treatment with MMF significantly attenuated the development of salt-sensitive hypertension. Unexpectedly, the abundances of the key renal sodium transporters were unaltered by co-treatment with MMF. The results of our wire myograph studies, however, pointed towards a pivotal role for the AT2 receptor. Under normal circumstances, the AT2 receptor is known to exert either no effect or a vasodilatory effect via the

production of vascular NO. In isolated arteries of DOCA-salt-treated rats, the AT2 receptor appeared to have obtained a constrictor function similar to that of the AT1 receptor, which was absent in rats co-treated with MMF. We therefore conclude that the AT2 receptor obtains a constrictor function under hypertensive stimuli, which is prevented by immunosuppression with MMF.

In **Chapter 8** we performed a study that was embedded within a prospective, randomized-controlled clinical trial consisting of 237 KTRs. We studied serum magnesium and tacrolimus levels the first year after transplantation in order to study their effects on the development of posttransplantation diabetes mellitus (PTDM). Several studies have shown that lower serum magnesium levels are associated with a higher risk of diabetes in the general population. Hypomagnesemia is common in KTRs and might be caused by CNIs through downregulation of TRPM6, a channel which reabsorbs magnesium in the DCT. Indeed, CNI-induced hypomagnesemia has been associated with PTDM, however this finding has not been consistent. We also studied the role of HNF1β in PTDM, which has been linked to diabetes, hypomagnesemia, and PTDM .One hundred and sixty-seven KTRs without prior diabetes completed the one year follow-up and were included in our final analysis. A total of 29 out of 167 KTRs (17%) developed PTDM . The serum magnesium trajectory in the first year after transplantation was significantly lower in patients who developed PTDM and it was an independent predictor of PTDM in a multivariable analysis. The HNF1β SNP rs752010 was twice as common in recipients with PTDM , and predicted the risk of PTDM but not of hypomagnesemia. In conclusion, a lower serum magnesium trajectory in the first year after transplantation is an independent risk factor for the development of PTDM. The rs752010 SNP in HNF1β is also associated with the development of PTDM, but this is not mediated through hypomagnesemia.

Discussion and Future Perspectives

In this thesis we investigated the role of immunosuppressive drugs in hypertension and electrolyte balance. The insights gained by our studies have partly solved longstanding questions in pathophysiology, but at the same time raise new questions.

NCC is one of the key topics studied in this thesis. At the time of writing **Chapter 2** several studies already demonstrated an inhibitory effect of dietary potassium on NCC [1, 2], possibly mediated via WNK4. Since, subsequent publications have appeared unraveling the mechanism by which dietary potassium inhibits NCC [3-5]. For example, NCC phosphorylation and dephosphorylation during changes in extracellular potassium seems to be dependent on the intracellular chloride concentration and also involves the kinases SPAK/OSR1 [3]. In **Chapter 3**, we showed that hyperkalemia decreased NCC independent of aldosterone and angiotensin II. Kir 4.1, a potassium channel expressed at the basolateral plasma membrane, was also shown to mediate the effect of dietary potassium on NCC abundance [5]. Of great interest is the recent study of Wu and colleagues [4], who found that the AT2 receptor regulates NCC through Kir4.1. In this experiment, the AT2 receptor inhibited Kir4.1 and NCC, resulting in potassium excretion. These findings are in line with the known beneficial effects of the AT2 receptor on vascular tone and natriuresis [6, 7].

These findings might link the regulation of NCC by the AT2 receptor to our results of **Chapter 7**. Here were we show that MMF attenuates the development of salt-sensitive hypertension in the DOCA-salt model by reducing vascular resistance, likely through inhibition of T cells. Results of our wire myograph studies point towards a pivotal role for the AT2 receptor. Taking these findings into account, one might hypothesize that long lasting inflammation might also reverse the effects of the AT2 receptor in the kidney, similar to those in the vasculature, resulting in activation of NCC. This is contrary to normal circumstances, where the AT2 receptor inhibits NCC [4]. We were unable to demonstrate an effect of MMF on renal sodium transporters, including NCC. Considering that Wu et al. observed maximal effects after 4 days [4], our 4-week experiment may have been too long to identify any differences in NCC or other renal sodium transporters. Therefore, a similar but shorter experiment as described in **Chapter 3** might be of interest to study the role of the AT2 receptor and Kir4.1, and its effects on renal sodium transporters. In recent years, IFN-γ, IL-17A, and IL-6 were all shown to influence the activity of kidney sodium transporters [8,

9]. Possibly, the duration of our experiment was too long compared to the more commonly used time frame of two weeks [8, 10-12]. The longer duration of our model may have led to a new steady state with conversion from a volume to a non-volume hypertension mechanism. Another possible explanation why we did not observe changes in renal sodium transporters is the absence of performing uninephrectomy, which is commonly used in the DOCA-salt model [10]. We chose not to perform an uninephrectomy, because this better resembles the clinical situation. Finally, further studies are needed to address whether our ex vivo wire myograph findings, pointing to a pivotal role for the AT2 receptor, also hold true in vivo. To confirm our results in vivo, rats should be treated with AT2 and endothelin A (ETA) receptor antagonists on top of vehicle, DOCA-salt and/or MMF. Other interesting aspects to be studied are the role of reactive oxygen species, NO scavenging by ROS, vascular superoxide production, and NADPH oxidase [6, 13, 14].

In **Chapter 5** we showed that the thiazide diuretic chlorthalidone lowered blood pressure to a similar degree as the calcium-channel blocker amlodipine in hypertensive kidney transplant recipients using tacrolimus. The use of thiazide diuretics in these patients led to a temporary reduction in eGFR. An eGFR reduction is less desirable shortly after kidney transplantation because it will complicate the assessment of possible rejection. Considering the recently discovered beneficial effects of potassium on NCC, it would be interesting to study the effects of a high potassium diet or oral potassium supplementation on hypertension in kidney transplant recipients using CNIs. Because CNIs also predispose to hyperkalemia, serum potassium should be monitored carefully with such interventions. Hypertension in kidney transplant recipients is multifactorial, and involves factors related to donor, recipient, and the transplantation surgery [15, 16]. Despite the clear increase in the incidence of hypertension after the introduction of CNIs [17-19], we do not know to which degree CNIs contributed to hypertension in our patients. A similar study in liver or heart transplant recipients using CNIs could therefore provide confirmation, because these patients have a lower incidence of hypertension. Patients with severe psoriasis or eczema who do not have pre-existing co-morbidities like hypertension and who are treated with CNIs would be an even better study population. The challenge would be that these patients are not fully dependent on CNIs and might prefer to switch to another drug when side effects like hypertension appear.

In **Chapter 6** we demonstrated that treatment with CNIs increases both tNCC and pNCC abundance in uEVs isolated from kidney transplant recipients. Furthermore, the blood pressure response to chlorthalidone in hypertensive kidney transplant recipients using tacrolimus was related to the pNCC abundance in uEVs. To which degree CNIs induce hypertension through activation of NCC, vasoconstriction, the RAAS, or the sympathetic nervous system remains unclear. However, the findings in the present study further illustrate the importance of NCC in CNI-induced hypertension. The recent development of a high-throughput assay to analyze uEVs provides interesting possibilities [20]. For example, the pNCC status in uEVs of a kidney transplant recipient might be determined to predict the efficacy of a thiazide diuretic. Furthermore, the analysis of uEVs in response to a high potassium diet or oral potassium supplementation in kidney transplant recipients using CNIs would be of interest. In line is our finding that non-responders had higher serum potassium concentrations, which may have resulted in lower NCC activity prior to initiating treatment with thiazide diuretics.

In **Chapter 8** we showed that higher levels of tacrolimus are associated with both lower serum magnesium levels and an increased risk of hypomagnesemia in the first year after kidney transplantation. Furthermore, we showed that a lower serum magnesium trajectory in the first year after kidney transplantation is an independent risk factor for the development of PTDM. This study is the first to investigate the relationship between CNIs, serum magnesium, and PTDM in a prospective manner. Several retrospective studies analysed this topic previously, however the results are conflicting. Three studies demonstrated that a lower serum magnesium or hypomagnesemia predicted PTDM [21-23], and one study concluded the hypomagnesemia was likely CNI-induced. [21] However, three additional retrospective studies did not find an association between hypomagnesemia and PTDM. [24-26] A possible explanation might be the number of serum magnesium values that were taken into account, which suggests the duration of a low serum magnesium is important. Based on our results, an interesting question is whether the supplementation of magnesium is able to prevent the development of PTDM. Two small clinical trials recently studied this topic in KTRs on CNIs. [27, 28] The first trial included KTRs with hypomagnesemia within 2 weeks after kidney transplantation and focused on glycemic control and insulin sensitivity at 3 months. [27] A significant effect of magnesium oxide supplementation was found on fasting glucose, but not insulin resistance. The second trial included patients with hypomagnesemia at 4 months after kidney transplantation and studied insulin

secretion at 6 months. [28] Magnesium oxide supplementation had no significant effect on insulin secretion, fasting glucose, or HbA1c. Despite these results, it should still be considered to perform a study with magnesium supplementation in KTRs using CNIs. First, magnesium chloride, citrate or lactate supplementation can be used, as they have a higher bioavailability compared to magnesium oxide and cause less diarrhea. [29, 30]. Furthermore, magnesium supplementation could be started directly after or even prior to kidney transplantation, as most cases of PTDM developed within the first month after kidney transplantation in our cohort. Third, magnesium supplementation independent of the presence of hypomagnesemia should be considered, as the effect of tacrolimus trough levels on absolute serum magnesium levels were very small in our study. This suggests subtle decreases in serum magnesium levels within a patient might be sufficient for the development of PTDM, and the clinical cut-off value for hypomagnesemia might be unsuitable as an inclusion criterion.

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Nederlandse samenvatting

De studies in dit proefschrift hadden als doel om de rol van het afweersysteem en afweeronderdrukkende medicijnen op hoge bloeddruk (hypertensie), de zouten in het bloed (elektrolyten) en suikerziekte (diabetes) na transplantatie te onderzoeken. Hypertensie en diabetes zijn onderwerpen die vaak bestudeerd worden vanwege het hoge risico dat zij geven op vroegtijdige morbiditeit en mortaliteit. Beide problemen komen ook veel voor bij niertransplantatiepatiënten, deels door dezelfde risicofactoren als in de algehele populatie, maar deels door specifieke risicofactoren. Over het immunosuppresieve medicijn tacrolimus is gerapporteerd dat het hypomagnesiëmie kan veroorzaken bij niertransplantatiepatiënten en dat zorgt mogelijk voor diabetes na transplantatie. Daarnaast leidt tacrolimus tot hypertensie door het activeren van de natrium chloride cotransporter (NCC) in de tubulus van de nier. Extracellulaire vesikels in de urine (uEV's) lijken de hoeveelheid en activiteit van de transporteiwitten in de niertubulus goed weer te geven. Er bestaat toenemend bewijs dat ontsteking bijdraagt aan het ontstaan van hypertensie. Kalium daarentegen lijkt een gunstig effect te hebben op hypertensie doordat het renaal zouttransport remt.

In **Hoofdstuk 2** geven wij een overzicht waarin nieuwe inzichten over de rol van NCC in natrium-, kalium- en bloeddrukregulatie worden beschreven. We beschrijven in de intracellulaire signaleringscascade die NCC reguleert. WNK, SPAK, OSR1 en SGK1 zijn kinases die hierbij betrokken zijn. Interessant is de observatie dat WNK4 onder sommige omstandigheden NCC kan remmen, maar NCC ook kan activeren. In dit hoofdstuk hebben we ook alle hormonen, metabole factoren en medicijnen samengevat die NCC beïnvloeden. Het bekendst zijn de thiazide diuretica. Dit zijn de meest gebruikte bloeddrukverlagers ter wereld welke al werden gebruikt lang voordat bekend werd dat deze middelen NCC remmen. Calcineurineremmers hebben het omgekeerde effect van thiazide diuretica: ze activeren NCC. Dit effect is aangetoond in dierstudies en moet dus nog worden bevestigd in mensen. Voor deze bevestiging lijkt analyse van blaasjes in de urine, zogenaamde urine extracellulaire vesikels, een veelbelovende methode. Dit hebben wij onderzocht in **Hoofdstuk 6**. Van belang is de recente ontdekking dat kalium in het dieet in staat is om NCC te remmen. Dit leidt tot een hoger aanbod van zout in het gedeelte van het nefron waar kalium kan worden uitgescheiden. Het fenomeen van "kalium geïnduceerde natriurese" kan mogelijk het antihypertensive effect van kalium verklaren. Dit onderwerp hebben wij verder bestudeerd in **Hoofdstuk 3**.

In **Hoofdstuk 3** hebben wij de "aldosteron paradox" bestudeerd. Meer specifiek hebben wij onderzocht hoe de nier reageert op de combinatie van hypovolemie en hyperkaliëmie. We wilden inzicht krijgen in de fysiologische rol van aldosteron op natriumretentie tijdens hypovolemie en kaliumuitscheiding tijdens hyperkaliëmie. Om dit uit te zoeken hebben we hypovolemie en hyperkaliëmie nagebootst in twee groepen ratten door ze een laag natrium dieet of een hoog kalium dieet te geven. Deze twee groepen werden vergeleken met een groep ratten die een normaal dieet kregen. Tot slot hebben wij een vierde groep toegevoegd met een gecombineerd dieet bestaande uit laag natrium en hoog kalium. Bij een laag natrium dieet werd natriumreabsorptie via NCC gestimuleerd door een verhoging in aldosteron en angiotensine II. Bij een hoog kaliumdieet was aldosteron ook verhoogd, maar angiotensine II niet. Ondanks het verhoogde aldosteron was NCC onderdrukt om het natriumaanbod in de verzamelbuis te verhogen. Hierdoor zal de reabsorptie van natrium door ENaC toenemen en daardoor ook de kaliumexcretie in dit deel van de tubulus. Bij het gecombineerde dieet waren zowel aldosteron als angiotensine II verhoogd. Ondanks deze verhoging was ook bij dit dieet NCC onderdrukt. Een grotere hoeveelheid WNK4 in groepen die een hoog kaliumdieet ontvingen is mogelijk betrokken bij het remmen van NCC. Er kan geconcludeerd worden dat een hoog kaliumdieet de effecten van angiotensine II en aldosteron op NCC teniet doet om voldoende kaliumexcretie in de verzamelbuis te waarborgen.

Hoofdstuk 4 geeft een overzicht van de rol van farmacogenetica bij calcineurine remmer (CNI) geïnduceerde hypertensie. Hypertensie is een veelvoorkomende bijwerking van CNI's. Hypertensie na niertransplantatie is geassocieerd met vroeger falen van de transplantaatnier en zelfs met eerder overlijden van de ontvanger. Voorspellen wie een verhoogd risico heeft op CNI-geïnduceerde hypertensie kan belangrijk zijn bij het selecteren van specifieke interventies zoals een zoutbeperking, het gebruik van thiazide diuretica of een CNI-vrij immunosuppressief regime . Momenteel is er een aantal kandidaatgenen welke geassocieerd zou kunnen zijn met hypertensie bij niertransplantatiepatiënten. Dit zijn CYP3A5, ABCB1, WNK4 en SPAK. Polymorfismen in deze genen zijn geassocieerd met zowel hypertensie in de algemene populatie als met CNI geïnduceerde hypertensie. CYP3A5 en ABCB1 zijn zowel betrokken bij hypertensie en de farmacokinetiek van CNI's. Van WNK4 en SPAK is aangetoond dat zij een rol spelen bij de CNI-geïnduceerde activatie van NCC. Alleen de betrokkenheid van CYP3A5 is echter bestudeerd in CNI-geïnduceerde hypertensie. Deze associatie berust slechts op gegevens uit een klein aantal studies, en de resultaten hiervan zijn niet uniform. Daarom zijn toekomstige studies nodig

om te onderzoeken of polymorfismen in één van deze kandidaatgenen bijdragen aan CNI-geïnduceerde hypertensie.

In **Hoofdstuk 5** hebben wij een gerandomiseerde cross-over studie uitgevoerd waarbij wij het thiazide diureticum chloortalidon vergeleken met de vaatverwijder amlodipine. Dit hebben wij gedaan in 41 hypertensieve niertransplantatiepatiënten welke de CNI tacrolimus gebruikten. Dit hebben wij gedaan omdat recente dierstudies laten zien dat tacrolimus de thiazidegevoelige zouttransporter NCC activeert. Dit suggereert dat thiazide diuretica, die goedkoop zijn en goed worden verdragen, een effectieve behandeling zouden kunnen zijn voor CNI-geïnduceerde hypertensie. Chloortalidon en amlodipine veroorzaakten een vergelijkbare daling van de bloeddruk. Ondanks deze vergelijkbare bloeddrukdaling zorgde chloortalidon daarnaast voor een significante daling van eiwitverlies in de urine met 30%. Daarnaast verminderde het voorkomen van perifeer oedeem (daling van 22 naar 10%). Amlodipine verhoogde juist de incidentie van perifeer oedeem. Chloortalidon veroorzaakte wel een tijdelijke verlaging van de geschatte nierfunctie en zorgde voor hogere urinezuurspiegels en hoger HbA1c in het bloed. Onze conclusie van deze studie is dat amlodipine en chloortalidon gelijkwaardige bloeddrukverlagers zijn voor niertransplantatiepatiënten. Chloorthalidon zou wel de voorkeur kunnen hebben bij patiënten met perifeer oedeem of veel eiwitverlies in de urine.

In **Hoofdstuk 6** hebben we allereerst de relatie tussen de hoeveelheid NCC in uEV's en het gebruik van calcineurine remmers bestudeerd. Om dit te doen vergeleken wij patiënten die CNI's gebruikten met patiënten die geen CNI's gebruikten en met gezonde vrijwilligers. De eerste belangrijke bevinding was dat de CNI's ciclosporine en tacrolimus zowel de totale hoeveelheid van NCC als het gefosforyleerde deel vier tot vijf maal verhoogden. Daarnaast hebben wij de interactie tussen de hoeveelheid NCC in uEV's en het antihypertensieve effect van thiazide diuretica bestudeerd. De tweede belangrijke bevinding was dat meer NCC in uEV's het effect van het thiazide diureticum chloortalidon bij niertransplantatiepatiënten met hypertensie voorspelt. Tot slot hebben wij een ex vivo studie verricht in corticale tubulus suspensies van muizennieren om het effect van CNI's op NCC te bevestigen. Experimenten met kortdurende blootstelling aan ciclosporine lieten meer gefosforyleerd NCC zien in de suspensies, maar geen verhoogd totaal NCC. Concluderend impliceren deze bevindingen dat het meten van NCC in uEV's gebruikt zou kunnen worden bij het kiezen van een bloeddrukverlagende therapie bij niertransplantatiepatiënten. Het effect van bijvoorbeeld thiazide diuretica kan dan vooraf voorspeld worden door

het meten van NCC in uEV's.

In **Hoofdstuk 7** hebben wij de effecten van het immuunsuppressieve medicijn mycofenolaatmofetil (MMF) op experimentele hypertensie onderzocht, inclusief effecten op de nier en het vaatbed. Onze hypothese was dat T-cellen hypertensie veroorzaken door effecten op het zouttransport in de nier en op de vaattonus. Om dit te onderzoeken hebben wij hypertensie geïnduceerd bij ratten door gebruik te maken van het zogenaamde DOCA-zout model. De helft van de ratten kreeg hierbij MMF, de andere helft een placebo. Behandeling met MMF remde de ontwikkeling van hypertensie. Opvallend was dat we geen effect van MMF op de verschillende zouttransporters in de nier vonden. De resultaten van onze vaatstudies lieten wel een verlaagde vaattonus zien bij de dieren die behandeld werden met MMF. De angiotensine receptor type 2 (AT2 receptor) lijkt hierbij een cruciale rol te spelen. Het is bekend dat de AT2 receptor onder normale omstandigheden geen effect heeft op de vaattonus of zelfs een vaatverwijdend effect heeft. Dit effect komt tot stand door de productie van stikstof mono-oxide (NO). In geïsoleerde slagaders van ratten die behandeld werden met placebo lijkt de AT2 receptor echter een vaatvernauwende functie aangenomen te hebben welke vergelijkbaar is met die van de AT1 receptor. Dit effect was niet aanwezig in de ratten die behandeld werden met MMF. Daarom concluderen wij dat de AT2 receptor een vaatvernauwende functie ontwikkelt tijdens hypertensie, welke wordt voorkomen door het gebruik van MMF.

In **Hoofdstuk 8** hebben wij een studie uitgevoerd welke onderdeel was van een prospectieve, gerandomiseerde klinische studie bestaande uit 237 niertransplantatie patiënten. Wij hebben bij deze patiënten de waardes van magnesium en tacrolimus in het serum in het eerste jaar na niertransplantatie bestudeerd. Het doel hiervan was om het effect op het ontstaan van post-transplantatie diabetes mellitus (PTDM) te onderzoeken. Meerdere studies hebben reeds laten zien dat een laag serum magnesium is geassocieerd met een hoger risico op het krijgen van diabetes in de algemene populatie. Hypomagnesiëmie, een verlaagd serum magnesium, komt veel voor bij niertransplantatie patiënten. Mogelijk wordt dit veroorzaakt door calcineurine remmers welke het TRPM6 kanaal kunnen remmen. Dit kanaal reabsorbeert magnesium in de tubulus van de nier en remming hiervan verlaagt het serum magnesium. CNI geïnduceerde hypomagensiëmie is inderdaad geassocieerd met het ontstaan van PTDM, echter zijn er ook enkele studies gepubliceerd die deze associatie niet konden vinden. We hebben ook de rol van HNF1β in het ontstaan van PTDM bestudeerd. HNF1β lijkt betrokken te zijn bij diabetes, hypomagnesiëmie en

PTDM. 167 niertransplantatie patiënten zonder reeds bestaande diabetes doorliepen de follow-up van één jaar en werden geïncludeerd in onze uiteindelijke analyse. In totaal ontwikkelden 29 van de 167 patiënten PTDM (17%). Het serum magnesium traject in het eerste jaar na niertransplantatie was significant lager in patiënten die PTDM ontwikkelden. Verder was een lager serum magnesium een onafhankelijke voorspeller voor PTDM in een multivariabele analyse. De HNF1β SNP rs752010 kwam twee keer vaker voor bij niertransplantatie ontvangers en voorspelde het risico op het ontstaan van PTDM, maar niet dat van hypomagnesiëmie. Wij concluderen dat een lager serum magnesium traject in het eerste jaar na niertransplantatie een onafhankelijke risicofactor is voor het ontstaan van PTDM. De rs752010 SNP in HNF1β is ook geassocieerd met het ontstaan van PTDM maar dit lijkt onafhankelijk van hypomagnesiëmie.

List of Abbreviations

ANG	Angiotensin
AT1 receptor	Angiotensin II receptor type 1
AT2 receptor	Angiotensin II receptor type 2
CCBs	Calcium channel blockers
CNIs	Calcineurin inhibitors
CNT	Connecting tubule
DCT	Distal convoluted tubule
DOCA	Deoxycorticosterone acetate
eGFR	Estimated glomerular filtration rate
ENaC	Epithelial sodium channel
FHHt	Familial hyperkalemic hypertension
HK	High potassium diet
HNF-1β	Hepatocyte nuclear factor 1β
LS	Low sodium diet
LSHK	Low sodium high potassium diet
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
NCC	Sodium chloride cotransporter
NFATc	Cytoplasmatic nuclear factor of activated T-cells
NHE3	Sodium hydrogen exchanger type 3
NKCC2	Sodium potassium chloride cotransporter isoform 2
NO	Nitric oxide
KTRs	Kidney transplant recipients
PTDM	Posttransplantation diabetes mellitus
OSR1	Odd-skipped related transciption factor 1
RAAS	Renin-angiotensin-aldosterone system
ROMK	Renal outer medullary potassium channel
ROS	Reactive oxygen species
SGK1	Serum and glucocorticoid-regulated kinase 1
SHRs	Spontaneous hypertensive rats
SLC12	Solute carrier family 12
SNPs	Single nucleotide polymorphisms
SPAK	STE20-related, proline/alanine-rich kinase
TAL	Thick ascending limb
TRPM6	Transporters transient receptor potential melastatin 6
TRPV5	Transient receptor potential cation channel subfamily V member 5

uEVs	Urinary extracellular vesicles
WNK	With no lysine kinase

Curriculum vitae

Arthur Moes werd geboren op 24 juli 1986 te Rotterdam. Na het voltooien van het Atheneum aan het Comenius College te Capelle aan den IJssel begon hij in 2004 met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Daarnaast werkte hij van 2006 tot en met 2010 op de afdeling Interne Geneeskunde in het Erasmus Medisch Centrum. In 2010 deed hij zijn afstudeeronderzoek gesuperviseerd door dr. E.E. Zijlstra in het Queen Elizabeth Central Hospital in Blantyre, Malawi. Hij deed onderzoek naar de oorzaken van niet traumatische dwarslaesies bij volwassen patiënten. Begin 2011 behaalde hij zijn artsexamen en werkte dit jaar als arts niet in opleiding tot specialist bij de Interne Geneeskunde in het IJsselland Ziekenhuis te Capelle aan den IJssel. In 2012 begon hij onder leiding van dr. Ewout J. Hoorn en prof. dr. Bob Zietse als promovendus op de afdeling Interne Geneeskunde, sectie nefrologie. Dit vormde uiteindelijk de basis voor dit proefschrift. Op 1 januari 2016 startte Arthur met de opleiding tot internist in het IJsselland Ziekenhuis met dr. H.E. van der Wiel als opleider. Momenteel is hij aan het vierde jaar van de opleiding bezig en is hij inmiddels weer werkzaam in het Erasmus Medisch Centrum te Rotterdam.

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** Authors contributed equally.*

PhD Portfolio Summary

Name PhD student:	Arthur David Moes
PhD period:	2012-2016
Erasmus MC department:	Internal Medicine Division of Nephrology
Promotor(s):	Prof. dr. E.J. Hoorn Prof. dr. R. Zietse
Supervisor:	-
Research school:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)

PhD training	**Year**	**Workload (ECTS)**
General academic skills		
- Laboratory Animal Science	2012	5.0
- Biomedical English Writing and Communication	2015	3.0
In-depth courses		
- Cardiovascular medicine (COEUR)	2013	1.5
- 5th Rotterdam Course in Electrolyte and Acid-Base disorders	2013	0.3
- Winter School Dutch Kidney Foundation	2013	1.2
- 2nd regional Course Kidney Transplantation	2013	0.6
Symposia and conferences		
- American Society of Nephrology Kidney Week (San Diego, USA)	2012	1.8
- Dutch Nephrology Days **	2014	1.0
- American Society of Nephrology Kidney Week (Atlanta, USA) *	2014	2.5
- NfN Scientific Symposium, Utrecht	2014	0.3
- Lof der Geneeskunst, Rotterdam	2014	0.3
- American Society of Nephrology Kidney Week (San Diego, USA) *	2015	1.9
- Benelux Kidney Meeting, Eindhoven **	2015	0.3
- New Kids on the Block, Amsterdam	2015	0.3
Didactic skills		
- Supervising practicum first year medical students	2014-15	0.4

Other

-	Regional Kindey Biopsy Meeting	2012-13	0.4
-	COEUR lectures: 9	2012-15	0.9
-	PLAN (platform AIOS Nephrology) day **	2012-15	1.6
-	Department of Internal Medicine, Nephrology, and Pharmacology sectormeetings and work discussions **	2012-15	3.8
-	AAV Erasmus MC Scientific Meeting	2013	0.3
-	Annual DIPAK meeting	2013-15	0.9
-	Meeting with physiology lab Radboud Universiteit Nijmegen**	2013-15	2.4
-	Erasmus MC Internal Medicine science days * and **	2013-15	2.6
-	COEUR PhD day	2014	0.3
-	COEUR seminar SALT	2014	0.4
-	Junior Medicine School, Erasmus MC **	2014-15	0.8
Total			**34.8**

** oral presentation(s), * poster presentation(s)

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