Physiology and pathophysiology of the renal Na-K-2Cl cotransporter (NKCC2)

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Human kidneys filter approximately 1.5 kg of NaCl and 180 liters of water each day. The bulk of the filtered load is reabsorbed along the tubular system and the collecting ducts, which results in the formation of ~1.5 liters of urine/day. In total, 20–25% of the filtered NaCl is reabsorbed along the thick ascending limb of the loop of Henle (TAL). NKCC2 facilitates ~20–25% of the reuptake of the total filtered NaCl load. NKCC2 is therefore one of the transport proteins with the highest overall reabsorptive capacity in the kidney. Consequently, even subtle changes in NKCC2 transport activity considerably alter the renal reabsorptive capacity for NaCl and eventually lead to perturbations of the salt and water homeostasis. In addition to facilitating the bulk reabsorption of NaCl in the TAL, NKCC2 transport activity in the macula densa cells of the TAL constitutes the initial step of the tubular-vascular communication within the juxtaglomerular apparatus (JGA); this communication allows the TAL to modulate the preganglionic resistance of the afferent arteriole and the renin secretion from the granular cells of the JGA. This review provides an overview of our current knowledge with respect to the general functions of NKCC2, the modulation of its transport activity by different regulatory mechanisms, and new developments in the pathophysiology of NKCC2-dependent renal NaCl transport.

NKCC2 is encoded by a single gene, but differential splicing of its pre-mRNA gives rise to several splice isoforms, which differ markedly in their transport characteristics and in their localization along the TAL. Three different isoforms, NKCC2B, NKCC2A, and NKCC2F, differ in the variable exon 4, which encodes the amino acids of the second transmembrane domain and parts of the adjacent intracellular loop of the cotransporter. This specific portion of NKCC2 has been shown to be crucial for chloride binding. In addition to these three full-length isoforms, truncated variants of NKCC2 have been reported; these variants differ in the C-terminal portion of the protein and increase the total number of NKCC2 isoforms to at least six (96, 114). In addition, isoforms with tandem repeats of exon 4, such as exon 4A/4F, 4B/4F, and 4B/4A, have been described (20, 43, 67, 161).

Because of the high overall salt transport capacity of NKCC2 and its crucial role in the urinary concentrating mechanism, even subtle modulations in NKCC2 transport activity result in considerable changes in renal salt reabsorptive capacity. Thus inhibitors of NKCC2, known as loop diuretics, constitute the most potent class of diuretics. The profound impact of NKCC2 inhibition on renal salt reabsorption is further enhanced by the limited transport capacity of the portions of the nephron downstream of the TAL, such as the distal convoluted tubule or the collecting duct. The large amount of salt reabsorption mediated by NKCC2 is most likely responsible for the evolution of a complex regulatory network in the TAL: this network modulates NKCC2 expression, differential splicing of its pre-mRNA, surface trafficking, specific transport activity, and, ultimately, the TAL salt-reabsorptive capacity. The regulatory network that controls NKCC2 expression and activity comprises systemic hormones (such as angiotensin II, catecholamines, and vasopressin) and local paracrine/autocrine factors (such as nitric oxide), all of which modulate the complex intracellular signaling network of TAL epithelial cells.

The NKCC2-dependent salt retrieval in the TAL substantially contributes to the overall salt-reabsorptive capacity of the kidney; in addition, NKCC2 transport activity initiates tubulovascular cross talk within the kidney (81, 126). Thus NKCC2-dependent salt transport is the initial step that links the tubular chloride concentration at the macula densa (MD) to the control of the tone of the afferent arteriole and eventually the glomerular filtration rate (GFR) of the respective nephron.
Regulation of NKCC2-Dependent Salt Transport

Regulation of NKCC2 expression, surface trafficking, and transport activity. Considering the key role of NKCC2 in TAL salt reabsorption, it appears logical that the gene expression, the surface trafficking, and the specific activity of the cotransporter in the membrane are tightly regulated. Two major regulatory pathways affect the membrane expression and transport activity of NKCC2: the cAMP and cGMP pathways, with the first being stimulatory and the latter being inhibitory. Several hormones and paracrine factors may modulate the TAL intracellular cAMP levels; the most notably is vasopressin (64, 65, 94, 138), although some data suggested that the cAMP levels in isolated human TALs may not be affected by vasopressin (27). Other hormones, which primarily are not linked to NaCl homoeostasis, are also involved, such as calcitonin, glucagon, parathyroid hormone, and catecholamines (35). Furthermore, factors which activate the calcium-sensing receptor (CaR), such as Ca^{2+}, Mg^{2+}, and amino acids (reviewed in detail in Ref. 45), may modulate the cAMP levels of TAL cells. Most studies addressing the function of the latter hormones in NKCC2 regulation were performed in isolated TALs and in cell culture, and the contribution of these factors in vivo to overall NKCC2 regulation must still be determined. Hormones, such as endothelins, atrial natriuretic peptide, and locally generated nitric oxide, increase TAL intracellular cGMP levels (36), which may activate the cyclic GMP-dependent protein kinase (cGMP-dependent protein kinase) and the specific activity of the cotransporter (5, 34).

NKCC2 gene expression. NKCC2 (BSC1) is encoded by the Slc12a1 gene (solute carrier family 12, member 1). cAMP-responsive elements were identified in the Slc12a1 promoter and are likely to be functionally relevant (68). Although no specific deletion studies have been performed on the cAMP-responsive elements of the NKCC2 promoter, it appears that the stimulation of NKCC2 expression observed in situations of increased intracellular cAMP formation, such as those caused by vasopressin during water restriction, is mediated by the direct effects of cAMP on the Slc12a1 promoter. In addition to being modulated by the transcriptional activity, the overall abundance of NKCC2 protein appears to be dependent on posttranscriptional mechanisms (141). Thus, in mice with the deletion of one allele of the Slc12a1 gene, the transcript levels are 50% of those in wild-type mice, whereas the protein levels are indistinguishable from that of the wild-types, resulting in no apparent phenotype in NKCC2+/− animals (141).

NKCC2 trafficking and phosphorylation. Changes in NKCC2 expression in response to increased cAMP levels are functionally relevant only if they are accompanied by corresponding increases in the surface expression of the cotransporter. An abundance of evidence suggests that the trafficking of NKCC2 to the apical membrane of TAL cells is facilitated by the cAMP pathway (17, 48, 92, 106). Experiments using the PKA inhibitor H-89 suggested that cAMP activates the migration of NKCC2-containing vesicles to the cell surface by a PKA-dependent process because the abundance of NKCC2 in the apical TAL membrane was reduced by ~60% in the presence of H-89 (17). It remains unclear whether the partial inhibition of NKCC2 surface trafficking was related to an inefficient inhibition of PKA or whether it may indicate that the remaining NKCC2 trafficking occurs in a PKA-independent manner.

Considerable efforts have been made in recent years to identify the kinases involved in the phosphorylation of NKCC2. The best characterized kinases are the SPAK (Ste20- and SPS1-related proline- and alanine-rich kinase) and OSMR1 (or oxidative stress-responsive kinase) kinases (111). SPAK and OSMR1 are highly homologous kinases and mediate the hypotonicity-induced phosphorylation of NKCC2 (121). Global SPAK-deficient mice are hypotensive and, surprisingly, showed increased NKCC2 expression and phosphorylation compared with those of wild-type mice, whereas the phosphorylation of the downstream-located Na-CI cotransporter (NCC) was reduced, suggesting that other kinases, possibly OSMR1, compensate for the control of NKCC2 (160). OSMR1 activation and subsequent hyperphosphorylation of NKCC2 in the global SPAK knockout mice were suggested to be mediated by the deletion of an inhibitory TAL-specific and kinase-defective SPAK isoform (SPAK2), resulting in dysinhibition of OSMR1 (54, 90). Conversely to the observations in the global SPAK-deficient mouse strain, a knockin mouse model of SPAK carrying a mutant kinase-dead SPAK showed reduced expression and phosphorylation of NKCC2 (118).

Likely candidates for kinases upstream of SPAK/OSMR1 include the family of WNK (With No Lysine) kinases, such as WNK1, WNK3, and WNK4 (82, 105, 123). Rare hereditary diseases such as pseudohyopaldosteronism type II (PHAII) demonstrate the clinical relevance of WNK kinases with respect to renal function. PHAII is caused by different mutations in WNK kinases, including gain-of-function mutations in WNK1 or missense mutations in WNK4. All these mutations result in volume-dependent hypertension, suggesting that their overall effect on renal salt reabsorption is stimulatory and inhibitory, respectively (29, 40, 72, 156, 157). WNK3 facilitates the phosphorylation of NKCC2 by activation of the SPAK pathway and may mediate the chloride-sensing of TAL cells (109, 115, 123). Although there is solid evidence indicating that WNK3 is a stimulator of NKCC2 activity in vitro, the overall relevance of this kinase in vivo appears to be limited. Thus, in WNK3-deficient mice, no obvious salt-wasting phenotype was observed, which was likely related to the compensatory upregulation of the WNK1/SPAK axis (93, 102). Apparently, the complex network of interacting kinases in TAL cells maintains the regulation of NKCC2, which is crucial for survival, and WNK3 in vivo facilitates the fine-tuning of the tubular reabsorptive function rather than controlling bulk salt reabsorption. Commensurate with the concept of multiple compensatory mechanisms, global SPAK deficiency in mice leads...
to hypotension despite increased NKCC2 expression and elevated NKCC2 phosphorylation, suggesting a compensatory regulation of NKCC2 by OSR1 or other kinases in TAL cells (160). Two different isoforms of WNK1 are expressed in the kidney, the ubiquitous WNK1 isoform L-WNK1 and the kidney-specific WNK1 isoform ks-WNK1. These two isoforms of WNK1 exert opposite effects on NKCC2 (among other renal salt transport systems) (57). L-WNK1 was shown to stimulate the activity of NKCC2, whereas ks-WNK1 has an inhibitor effect (28, 84). Based on the observations in mice with genetic inactivation of both WNK1 isoforms, the effects of the activating L-WNK1 in most conditions appear to outweigh the effects of ks-WNK1 (163). The WNK1+/− animals of this specific strain were hypotensive, suggesting again that the net effect of L-WNK1 and ks-WNK1 on tubular salt transport is stimulatory. An independently generated strain of WNK1+/− mice, however, was normotensive (140), suggesting a marked influence of the genetic background on the phenotype and further emphasizing the large compensatory capacity of TAL cells.

There is accumulating evidence for additional pathways of NKCC2 phosphorylation, including but most likely not limited to pathways involving PKA and the AMP-activated kinase (AMPK); these kinases could provide additional regulatory mechanisms, which may be relevant for the vasopressin- and low-chloride-dependent phosphorylation of the cotransporter, respectively (30, 32, 55, 61, 121). The activity of these kinases may be another explanation for the apparently highly efficient compensatory mechanisms, whose roles increase if the function of one of the classic pathways is compromised. The physiology of these alternative kinases with respect to the phosphorylation of NKCC2 was reviewed in detail recently (5). Furthermore, the normal phosphorylation of NKCC2 requires the presence of Tamm-Horsfall protein (97). Tamm-Horsfall protein is the most abundant protein in urine, although it has no intrinsic kinase activity. Thus, in Tamm-Horsfall protein-deficient mice, the surface expression of NKCC2 in the apical TAL membrane was inconspicuous, but the phosphorylation status of Thr96 and Thr101 was reduced. The reduced phosphorylation was functionally relevant because the diuresis induced by furosemide was reduced in Tamm-Horsfall protein-deficient mice compared with that in wild-type animals (97). The affected kinases and the underlying mechanisms, however, remain unknown.

Solid evidence suggests that the trafficking of NKCC2 to the apical membrane of TAL cells involves an interaction with the vesicle-associated membrane protein 2 (VAMP2). Coimmunoprecipitation and localization studies have shown that VAMP2 is associated with NKCC2 and that both proteins colocalize in the same domains of the apical membrane. Furthermore, the VAMP2 trafficking to the cell surface and the interaction with NKCC2 were cAMP dependent, and silencing of VAMP2 by small interfering (si) RNA abolished the cAMP responsiveness of the NKCC2 surface expression (18).

**Differential splicing of NKCC2: fine-tuning of NKCC2 function.** Differential splicing is a common phenomenon, which generates a large number of different proteins from a limited number of genes (74). Differential splicing affects several ion cotransporters, including NKCC2, which is encoded by the Slc12a1 gene (42, 46). Alternative splicing of the variable exon 4 of the Slc12a1 gene leads to the formation of three different full-length isoforms of the NKCC2 cotransporter protein; these splice isoforms differ in their localization along the TAL and in their transport characteristics (20, 67, 110, 125). Depending on whether exon 3 is linked to exon 4B, 4A, or 4F, three full-length isoforms are generated and are known as NKCC2B, NKCC2A, and NKCC2F. Exon 4 of the Slc12a1 gene encodes 32 amino acids, which are localized in the second transmembrane domain and the adjacent intracellular loop of the cotransporter. This portion of NKCC2 is critical for chloride transport and the binding of loop diuretics (56). In addition to the full-length isoforms, truncated isoforms have been identified. Heterologous expression in *Xenopus laevis* oocytes suggested that the short NKCC2 isoforms are activated under hypotonic conditions and mediate a bumetanide-sensitive and K-independent NaCl transporter (112). Furthermore, during coexpression with the full-length transcripts, a negative regulatory function of the truncated isoforms on the full-length isoforms could be shown (96, 114).

In addition, abnormal splicing events lead to the generation of transcripts with tandem repeats of exon 4, such as 4B/4F, 4A/4F, and 4B/A, with the latter combination being restricted to human NKCC2 (20, 43, 67, 161). The transporter proteins derived from these aberrant transcripts have a dominant-negative effect on NKCC2 activity. Accordingly, NKCC2-dependent ion uptake in oocytes was reduced by 50% when both the NKCC2A and NKCC2A/F isoforms were coexpressed, compared with single NKCC2A expression (14).

**Localization of NKCC2 isoforms along the TAL.** The three full-length isoforms differ in their localization along the TAL. Several localization studies were performed for the NKCC2 isoform transcripts in rabbits, rats, and mice (26). For rabbit and rat kidneys, the NKCC2F isoform was exclusively found in the medullary TAL. In contrast, the NKCC2B isoform was only expressed in the cortex, while the NKCC2A transcript was found in both the medulla and the cortex (110, 161). The overall expression of the NKCC2 mRNA isoforms in the rat was 70:20:10 for NKCC2F, NKCC2A, and NKCC2B, as determined by isoform-specific RNAse protection assays (26). Similar results were obtained for the localization of the NKCC2 isoforms in mice. The NKCC2B transcript was only expressed in the cortical TAL and at low levels in the outer stripe of the outer medulla (125). The NKCC2F isoform was localized to the inner and, to a lesser amount, the outer stripe of the outer medullary TAL. The NKCC2A isoform was expressed in the cortex and the medulla, whereas the medullary localization was restricted to the outer stripe of the outer medulla (103). It shall be noted in this context that the two NKCC2 isoforms found in the medullary TAL (NKCC2F and NKCC2A) might be involved in the activation of nuclear factor of activated T cells type 5 (NFAT5) (60), which is important for protecting cells against a hyperosmotic environment and for preserving the function of the renal medulla (15).

The NKCC2 cotransporter is also expressed in the MD segment of the TAL, where it functions as a NaCl sensor, as discussed in detail later (143). In mice, two NKCC2 transcripts were identified within the MD segment of the TAL: NKCC2A and NKCC2B (104).

Similar to the findings in rodents, the NKCC2 isoforms of the human kidney differ in their localization along the TAL and in their transport characteristics (20). In the human kidney, however, the NKCC2A transcript is the dominant isoform. In general, the expression of NKCC2 in humans declines in the
order NKCC2A > NKCC2F > NKCC2B. Similar to the situation in rodents, NKCC2A is expressed along the entire TAL, but the highest expression levels are found in the outer stripe of the outer medullary TAL (20). As recently reported for rodents (103, 110, 161), the localization of the NKCC2B isoform in the human kidney was also restricted to the cortex. The distribution pattern of the NKCC2F transcript in humans was less distinct. Although it was limited to the medulla in rodents, the NKCC2F transcript was found along the medullary and cortical TAL, including the MD, in humans (20).

_Transport characteristics of NKCC2 isoforms._ The different splicing products of the Slc12a1 gene differ in their transport characteristics (26). Experiments in the early 1970s in isolated, perfused TALs showed a more rapid salt transport in the medullary TAL relative to that in the cortical TAL segments. However, the cortical TAL segments revealed a higher diluting power (16, 124). The molecular basis for this finding is presumably the existence of the different NKCC2 isoforms. Heterologous expression studies using the _X. laevis_ oocyte system were used to study the apparent ion affinities of murine, rabbit, and human NKCC2 isoforms (20, 42, 49, 113). For rodents, the _K_m values of the NKCC2 isoforms varied markedly between different studies and species. For example, in rabbits, _K_m values for chloride of 9, 45, and 111 mM were determined for the isoforms NKCC2B, A, and F, respectively (49). Data from a second study, however, differed considerably compared with the preceding study, with _K_m of NKCC2A for chloride being ~15 mM (43). In mice, the corresponding _K_m values for chloride of isoform NKCC2B, A, and F are much lower: 12, 22, and 29 mM, respectively (113). A recent modeling study reevaluated published NKCC2 transport data and offered a possible explanation for these apparent discrepancies (87). The authors found that the differences in the published _K_m values for Na and Cl transport were inversely correlated with the ambient bath concentrations of K and Rb used in different transected oocyte experiments. These findings suggest that the discrepant findings for the _K_m values in rodents may be related to different experimental protocols (87). However, despite the quantitative discrepancies in the _K_m values, there was a consistent trend for the apparent chloride affinity of rodent NKCC2 isoforms in the order of NKCC2B > NKCC2A > NKCC2F (26). The isoform with the highest chloride affinity, the cortically located NKCC2B, is the isoform with the lowest transport capacity (49, 113). These results are consistent with the differences between the medullary and cortical salt transport activity found by Rocha and colleagues (124), revealing a higher dilution power, but lower transport capacity of the cortical TAL segments compared with the medullary segments.

Loop diuretics inhibit the NKCC2 cotransporter protein at the chloride-binding site (56), thus explaining why NKCC2B-providing cortical TAL cells have a higher sensitivity for loop diuretics than do medullary TAL cells (77). In addition, modeling simulations revealed that when bath conditions were similar to those of the outer medulla (e.g., high osmolality), NKCC2F mediated a greater Na reabsorption in oocytes compared with NKCC2A. Furthermore, the reabsorption of Na by NKCC2F required lower energy consumption compared with that of NKCC2A. Interestingly, the higher energetic efficiency of NKCC2F compared with NKCC2A was abolished when the bath conditions were similar to those of the renal cortex (e.g., low osmolality). These observations suggest that NKCC2F serves as a specialized NKCC2 isoform, offering optimized transport characteristics for the hyperosmolar medullary environment (87).

In humans, NKCC2B has the highest chloride affinity, similar to what was observed in rodents. In contrast, the chloride affinity of the most abundant isoform in humans, NKCC2A, is rather small compared with its rodent equivalent. In summary, the chloride affinity of the human NKCC2 isoforms decline in the given order: NKCC2B > NKCC2F > NKCC2A (20).

The impact of the variable exon 4 on the chloride affinity and transport capacity of the resulting NKCC2 isoform could be further confirmed during investigations of the various amino acid mutations in the sequence encoded by exon 4 (41, 42, 47). Mutations in 6 of 32 amino acids, which were located in the second transmembrane domain and in the adjacent intracellular loop of NKCC2B, led to a marked decline in chloride affinity, which was comparable to that of the NKCC2F isoform (47).

To date, the in vivo transport function of NKCC2 isoforms has been investigated in NKCC2A and NKCC2B knockout mice. For targeted disruption of single isoforms, the alternate exons 4B and A were altered by the introduction of in-frame stop codons, leading to the termination of translation. The result was the absence of both the full-length and the corresponding truncated isoforms (103, 104). The distal tubular chloride concentrations and absorption were assessed by measuring the chloride concentrations and the fluid flow rates in distal tubular fluid samples using in situ microperfused superficial nephrons of NKCC2A- and NKCC2B-deficient mice. For low perfusion flow rates, which correspond to low tubular chloride concentrations in downstream TAL segments, the NKCC2B-deficient mice revealed impaired chloride absorption compared with that of wild-type mice (103). This impairment was not observed in the NKCC2A-deficient mice. Thus, for low perfusion flow rates, no difference in chloride absorption was detected between NKCC2A-deficient and wild-type mice (104). These in vivo data are congruent with the high chloride affinity of the NKCC2B isoform, as determined in vitro (41, 42, 49, 113). Compared with the NKCC2B isoform, NKCC2A has lower chloride affinity, but higher transport capacity in vitro (20, 42, 49, 113). Consistent with these findings, the in vivo situation revealed impaired chloride absorption in NKCC2A-deficient mice at supranormal perfusion flow rates, e.g., for high TAL chloride concentrations. These data suggest that the NKCC2B and NKCC2A isoforms functionally cooperate to facilitate NaCl absorption over a wide range of fluctuating tubular chloride concentrations.

_Differential splicing of NKCC2 pre-mRNA is a regulated process._ As mentioned above, differential splicing of the variable exon 4 of the _Slc12a1_ gene leads to the expression of three different NKCC2 isoforms, which differ substantially in their transport characteristics. This difference gives rise to the question of whether differential splicing may be a measure to adapt to modulations of salt intake and contribute to the adaptive capacity of the kidney to cope with changes in absorptive needs. We recently demonstrated that dietary salt intake modulates NKCC2 differential splicing in a manner such that a salt-restricted diet causes a shift in isoform expression from the low-chloride affinity A-isoform to the high-chloride affinity B-isoform in the cortical and outer medullary TAL, as summarized in Fig. 1 (125). This effect was partially mediated by...
angiotensin II and partially dependent on TAL salt transport activity, as determined in experiments using furosemide in superfused kidney sections, i.e., in the absence of systemic hormones. Furosemide also reduced NKCC2F expression in the inner stripe of the outer medullary TAL (OMIS), the segment in which NKCC2F is the dominant isoform. Thus, when the NKCC2 transport activity in the presence of furosemide is reduced, the differential splicing of NKCC2 changes to an expression pattern that resembles the expression pattern in more downstream portions of the TAL.

Downregulation of NKCC2F during furosemide administration in the OMIS was accompanied by increased levels of NKCC2B and NKCC2A, whose expression in the OMIS under normal conditions is low. Similarly, in a recent study, furosemide reduced the expression level of NKCC2F, whereas it induced the expression of NKCC2A in the mouse kidney (14). Under normal conditions, there is a gradient of the NaCl concentration along the TAL, and the chloride concentrations in the most distal portions of the TAL (in the area of the MD segment) were estimated to be ~35 mM (127). This gradient may be the reason for the low NKCC2F expression levels in the cortical TAL under baseline conditions. Consequently, furosemide transforms the TAL of the OMIS into a state that resembles the situation in the cortical TAL under baseline conditions (125).

These data suggest that the salt transport activity, and thus the intracellular ion composition, of the TAL may influence the function of the splicing machinery. This assumption is further supported by the finding that NKCC2B expression is upregulated in NKCC2A-deficient mice. In the mouse cortical TAL, NKCC2B and NKCC2A are coexpressed, and consequently the genetic inactivation of one isoform will result in a situation similar to the partial transport inhibition by loop diuretics. Although little is known in general about the mechanisms that modulate differential splicing, there are other examples suggesting that the intracellular ion composition and/or osmolarity may influence the splicing machinery (50). The functional impact of the shift of differential splicing from NKCC2A to NKCC2B by a salt-restricted diet was also assessed in a recent modeling study. The results of this analysis suggested that the modulation of differential splicing during low salt enhances the salt reabsorption in the TAL and consequently reduces NaCl delivery to the MD segment (37).

Pathophysiology of NKCC2

Loss-of-function of NKCC2: salt-wasting tubulopathy. The clinical significance of the NKCC2 cotransporter for electrolyte and water homeostasis is demonstrated by the severity of Barter’s syndrome type 1. Barter’s syndrome is a rare salt-wasting nephropathy, which often occurs in consanguineous families and was originally described by Barter et al. in 1962 (79). Barter’s syndrome patients are affected by polyuria, hypokalemic alkalosis, a hyperplasia of the juxtaglomerular apparatus accompanied by elevations in plasma renin activity, and by secondary hyperaldosteronism (9). Despite the marked elevations in plasma renin activity, the patients are usually hypotensive (12).

Barter’s syndromes are heterogeneous diseases and involve a group of severe autosomal-recessive-gene mutations, all of which lead to a reduced or absent ability of salt reabsorption in the TAL (44). To date, five different genes have been shown to be responsible for the different types of Barter’s syndrome (134–136, 148, 153). As described before, NKCC2, the apical ROMK, and the basolateral clcnkb mediate salt reabsorption in the TAL. Mutations in any of the corresponding genes compromise tubular salt reabsorption and account for Barter’s syndrome type 1, type 2, and type 3, respectively.

Barter’s syndrome type 1 is caused by mutations of the apical NKCC2 cotransporter. Simon et al. (135) first linked the NKCC2-coding gene Slc12a1 on chromosome 15 to Barter’s syndrome, when using a candidate gene approach (135). The authors investigated five affected families and identified six independent mutations, including frame shifts and amino acid substitutions (136). Such mutations in the NKCC2 gene lead to extreme salt wasting in the TAL, and this salt loss is not compensated for by a reduction in GFR. This lack of GFR...
compensation is most likely related to the compromised sensor function of MD cells, as outlined in detail later (143). Together with Bartter’s syndrome type 2, which is caused by mutations in the KCNJ1 gene coding for ROMK (136, 150), type 1 is classified as the antenatal, most severe form of the disease. The homozygous patients suffer from severe hypercalciuria, accompanied by nephrocalcinosis; nephrocalcinosis in type 1 Bartter’s patients develops early, sometimes in utero (91). Fetal polypuria causes polyhydramnios. Furthermore, the excessive formation of PGE2 is likely related to the increased likelihood of premature delivery of the child. High levels of PGE2 in the serum, urine, and kidney tissue are predominantly caused by the overexpression of cyclooxygenase-2 (COX-2) and play a key role in the pathology Bartter’s syndrome. Hypokalemia, chronic volume contraction, and high levels of angiotensin II stimulate PGE2 generation (165, 166), and PGE2 in turn stimulates renin secretion, hyperaldosteronism, and hypokalemia (39, 101, 131). Furthermore, PGE2 inhibits vasopressin-mediated water reabsorption in the TAL and thus intensifies the polypuria (98). The polypuria in these patients continues after birth, leading to life-threatening salt wasting and dehydration (133). The nephrocalcinosis further complicates the situation and eventually triggers renal failure (117).

Bartter’s syndromes type 3, 4, and 5 are less severe. An inactivating mutation of the gene encoding for the basolateral chloride channel clcnkb is known as Bartter syndrome type 3, and this mutation accounts for the so-called classic Bartter’s syndrome (134, 149). Type 4 is caused by an inactivating mutation of Barttin (BSND), a regulatory subunit of the clcnkb and clcnka chloride channels (13, 38). In contrast to the other mutations, Bartter syndrome type 5 is caused by a gain-of-function mutation of the calcium-activated receptor in the TAL (148). During normal conditions, the activity of NKCC2 and ROMK is inhibited by high extracellular calcium levels via this calcium-activated receptor (120, 153). The mutated receptor, however, mediates these effects at much lower extracellular calcium levels, leading to salt wasting in the TAL (148).

To study the complex pathophysiology of Bartter’s syndrome type 1 in an animal model, homologous recombination was used to inactivate the Slc12a1 gene in mice (142). The phenotype of NKCC2-deficient mice resembled in many aspects the situation in human patients suffering from Bartter’s syndrome type 1 (142). The homozygous offspring was viable and fertile, suggesting that NKCC2 function was partially preserved (75). In contrast to human patients, NKCC2-deficient mice developed severe bilateral hydronephrosis at the early age of 7 days (142). Hydronephrosis is only occasionally observed in humans, and it is less severe than what was seen in the NKCC2 null mice (132, 142). The authors assumed that hydronephrosis may be related to the back pressure generated by polypuria. As human kidneys are more mature than mouse kidneys at birth (80), the immaturity might be an explanation for the more severe effects of polypuria on the development of hydronephrosis in mice. This assumption was further supported by data obtained from 4-wk-old mice treated with furosemide (142). These wild-type animals developed mild hydronephrosis. When furosemide treatment was started right after birth, hydronephrosis in these mice was more severe, suggesting again that the vulnerability of the kidney depends on its developmental status (142).

Because Bartter’s syndrome type 1 is caused by an autosomal recessive mutation in the Slc12a1 gene, it is commonly found in consanguineous families (79). The heterozygous parents of the patients do not show any symptoms. Congruent with this observation in humans, the phenotype of heterozygous NKCC2 knockout mice was inconspicuous (141). Although the NKCC2 mRNA levels in NKCC2+/− mice were only 50% of those in the wild-type mice, there was no difference in the membrane protein expression and in the distribution of NKCC2 along the TAL. It was suggested that a sodium-mediated posttranslational regulation leads to an increased trafficking of the active transporter to the apical membrane (141).

Similar findings were obtained when single isoforms of the NKCC2 cotransporter were deleted in the mouse model (103, 104). As described above, this was performed to characterize the contribution of the NKCC2A and B isoforms to TAL salt transport. In contrast to NKCC2 null mice, NKCC2A- and the NKCC2B-deficient mice had normal survival rates and largely inconspicuous salt and water homeostasis (103, 104, 142). The mild phenotype of mice lacking specifically NKCC2A and NKCC2B suggests a minor contribution of these single alternative splicing products to the overall salt reabsorption in the TAL (103, 104). The expression of NKCC2F, the isoform with the highest transport capacity for chloride, together with another remaining isoform (either A or B), seems sufficient to largely compensate for the loss of single isoforms A and B (103, 104). Similar to the mild renal phenotype of NKCC2B-deficient mice, a human case of specific NKCC2B deficiency has been documented (147). This male patient showed mild hypokalemic alkalosis, hyperreninemia, hyperaldosteronism, and hypercalciuria. The rather mild phenotype resembled that of the classic Bartter’s syndrome, but the patient had no mutations in the CLCNKB gene. Instead, a heterozygous missense mutation of nucleotide 690 in exon 4B was detected, which caused the substitution of a glycine by an aspartic acid at position 224 (147). The functional relevance of this mutation was assessed in vitro. Consistent with the in vivo findings, the mutation in exon 4B of the patient led to a loss of NKCC2B function (47).
Considering the similarities in the phenotype of the NKCC2B-deficient patient and those affected by Bartter’s syndrome type 3, it appears likely that individuals with similar mutations are sometimes erroneously classified as type 3 Bartter’s patients.

In addition to mutations and/or the deletion of the NKCC2 gene, Bartter’s syndrome-like phenotypes may develop due to impaired activation of NKCC2. As described above, the NKCC2 transporter is activated by phosphorylation and this may be performed by several kinases. A recent study in mice showed the influence of the NKCC2-activating kinase OSR1 in the control of salt homeostasis and blood pressure (83). Homozygous OSR1 knockout animals were lethally affected, while the heterozygous OSR1-deficient animals manifested hypotension accompanied by reduced pNKCC1 levels in aortic tissue and diminished pNKCC2 levels in the kidney (83). In contrast, kidney-specific OSR1 null mice were normotensive. This indicates that a reduction in activated NKCC1, rather than NKCC2 accounts for the attenuated blood pressure in heterozygous OSR1 null mice. When investigating the kidney-specific OSR1 mice, the authors found reduced levels of pNKCC2 and a partially blunted response to furosemide (83). Furthermore, when fed a low-salt diet, the kidney-specific OSR1-deficient mice excreted more sodium than the wild-type controls, indicating a moderate defect in TAL function. Because OSR1 is mainly expressed in the medullary TAL (160), it seems likely that NKCC2, rather than NCC is the main substrate of the kinase. The authors found a mild Bartter’s syndrome-like phenotype, including sodium and potassium wasting and hypercalciuria, which was accompanied by elevations in pNCC abundance (83). The latter might be compensatory and partly responsible for the mild renal phenotype. Consistent with these observations, several different animal models of Bartter’s syndrome showed elevations in NCC expression (19, 122, 151).

Another group described the salt-wasting phenotype of adenyllylcyclase 6 (AC6)-deficient mice (122). AC6 is involved in the cAMP-dependent regulation of NKCC2 expression and its activation in response to vasopressin (122). AC6-deficient mice showed reduced levels of vasopressin-mediated phosphorylation of NKCC2 (pS126-NKCC2). Additionally, the AC6−/− mice revealed hypokalemia and alkalosis, both common features of Bartter’s syndromes (122). Similarly, in heterozygous Gsα-deficient mice (162), the abundance of the NKCC2 protein was significantly reduced, and the urinary concentrating ability was compromised (36). The latter could be related to the reduced expression of NKCC2 and/or compromised cAMP formation in other segments of the nephron, such as the proximal tubule and the collecting duct (36).

Gain-of-function of NKCC2: salt-sensitive hypertension. Because the loss-of-function of NKCC2 inevitably leads to a marked salt-losing phenotype in humans and all other investigated mammalians, it appears reasonable to assume that the gain-of-function of NKCC2 may link the cotransporter to states of overt salt reabsorption, which eventually would result in the development of salt-sensitive hypertension. In contrast to the well-defined correlation between epithelial Na channel activity and hypertension, there is no monogenetic analog of Liddle syndrome for NKCC2. Nevertheless, there is accumulating evidence that changes in cotransporter activity may be linked to hypertension. Considering the massive overall salt-reabsorptive capacity of NKCC2, it is obvious that even very subtle increases in cotransporter activity would result in considerable aberrations of salt homeostasis and, consequently, arterial blood pressure. Such changes in NKCC2 activity may be related to the cotransporter itself (i.e., gain-of-function mutations) or may be related to alterations in the regulatory pathways that regulate NKCC2 trafficking and transport activity. Unlike gain-of-function mutations of NKCC2, rare mutations of the Slc12a1 gene, most of which resulted in a clinically unapparent NKCC2 dysfunction in heterozygous carriers, were identified in a cohort of 3,000 individuals enrolled in the Framingham Heart Study, and these mutations were associated with lower blood pressure (1, 70, 95). Thus the long-term systolic blood pressure among heterozygous carriers of the mutations was ~6 mmHg lower compared with the mean blood pressure of all individuals enrolled in the study (70). Accordingly, carriers of the mutations were overrepresented in the 10% of individuals with the lowest blood pressure (16). Furthermore, ethnic differences in the urinary concentrating ability at baseline and during water restriction are likely partially related to minor differences in NKCC2-dependent salt reabsorption. Thus urinary osmolality is higher and the average urine volume is smaller in individuals of African descent compared with Caucasians (145). Accordingly, Caucasians excrete a water load more rapidly than African-Americans, and this difference does not appear to be related to differences in the vasopressin levels (7, 58, 154). Furthermore, a reduced excretion of calcium in African-Americans compared with Caucasians may indirectly be related to increased NKCC2 activity, which is expected to enhance the driving force for paracellular calcium reabsorption in the TAL (116, 155). In this context, common genetic variations of the CaR in the TAL may be another component that affects the racial differences in NKCC2 activity and the susceptibility to hypertension (71). The CaR is activated by an increase in extracellular Ca and leads to an inhibition of NKCC2 (45).

Similar to the situation in humans, in Dahl salt-sensitive rats, which are a classic animal model of salt-sensitive hypertension, the Rb uptake in isolated TAL segments was markedly increased compared with that in salt-resistant control rats, and this increase was related to NKCC2 transport activity rather than abundance because the NKCC2 levels in the Dahl salt-sensitive rats were actually downregulated (3, 6). The increased activity of NKCC2 in the Dahl salt-sensitive rats was suggested to be related to the hyperphosphorylation of the cotransporter by SPAK and OSR1 (6). It should be noted that NKCC2 is certainly not the only renal candidate gene responsible for the development of (salt-sensitive) hypertension (31); other transporters and ion channels, such as the TAL basolateral chloride channel clcnkb (24) or more downstream sites of sodium reabsorption (for example, the epithelial Na channel), might have similar relevance, in addition to multiple extrarenal factors, which modulate arterial blood pressure.

The Regulatory Function of NKCC2: MD Control of Preglomerular Resistance and Renin Secretion

MD cells are located in the distal end of the TAL. These specialized TAL cells directly contact the extraglomerular mesangium of the corresponding glomerulus (73). The function of MD cells is dual: MD cells sense the salt concentration in the tubular fluid and influence 1) pregglomerular resistance and, consequently, the snGFR and 2) the secretion of renin

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from the juxtaglomerular granular (JG) cells of the afferent arteriole (22, 137, 143). Although the end points of this tubular-vascular communication are different, they share the same initial step, i.e., salt sensing by MD cells. When the tubular salt concentration (more precisely the chloride concentration) (76, 78) increases, the MD cells initiate a signaling cascade that eventually results in a suppression of the renin secretion and a concomitant increase in the tone of the afferent arteriole. The latter response is termed the tubuloglomerular feedback mechanism, and it leads to a decline in the snGFR. The control of the vascular function and renin secretion by MD cells is mediated by the generation of extracellular local factors such as ATP, adenosine, and prostaglandins (10, 23, 51, 139, 144), as recently review in detail (21, 22, 128).

The activity of NKCC2 in the apical membrane of MD cells is most likely the primary mechanism by which MD cells detect the tubular salt concentration (11, 81, 100, 126, 158). Thus inhibition of NKCC2 by loop diuretics abolishes the TGF response and leads to a marked increase in renin secretion (22, 62, 86, 158). The latter, however, at least when the loop diuretic is applied systemically, may be partially mediated by a concomitant inhibition of NKCC1, which is expressed by JG cells (25, 33), and by systemic effects such as volume loss and a reduction in blood pressure (146), which stimulate renin secretion independently of the MD pathway. Loop diuretics inhibit NKCC1 and NKCC2 with similar efficacy, and the pharmacological use of loop diuretics relies on their high tubular concentrations, which are caused by their secretion in the proximal tubule (59). In accordance with a direct stimulatory effect of loop diuretics on renin secretion, NKCC1-deficient mice have elevated plasma renin activity and the acute stimulation of renin secretion by loop diuretics is considerably reduced (25, 152).

Depending on the species investigated, MD cells usually express more than one NKCC2 isoform. Thus in mice, both NKCC2B and NKCC2A are expressed in MD cells (103). In vivo studies using mouse strains with targeted inactivation of NKCC2B and NKCC2A suggested that the coexpression of the two isoforms with different chloride affinities in the MD may have evolved to facilitate salt sensing in a nephron segment in which the chloride concentrations vary substantially (103, 104, 128). In accordance with this assumption, in NKCC2B-deficient mice, the TGF response curves were right-shifted compared with those of wild-type mice; in contrast, in NKCC2A-deficient mice, the TGF response curves were left-shifted, and the maximum response magnitude was reduced when compared with that of wild-type animals. These data suggest that NKCC2B (high chloride affinity) and NKCC2A (low chloride affinity) cooperate in their salt-sensing function and cover a relatively wide range of fluctuating tubular chloride concentrations (26). Similar results were obtained in an evaluation of the role of the different MD NKCC2 isoforms in terms of the tubular control of renin secretion. When mice were administered an intraperitoneal injection of isotonic saline, which is used as a model of volume loading associated with increased distal tubular chloride concentrations (85), the acute suppression of renin secretion was virtually absent in NKCC2A-deficient mice, whereas it was augmented in NKCC2B-deficient mice relative to that in wild-type animals (103, 104). Apparently, when the chloride concentrations at the MD are high, the suppression of renin secretion depends on the functional interaction of NKCC2A as the low-affinity, high-capacity isoform. The interpretation for the increased suppression of renin secretion in NKCC2B-deficient mice compared with controls is not as obvious but may be related to the fact that loss of NKCC2B upstream of the MD may have led to higher chloride concentrations at the MD, as suggested by the previously mentioned measurement of the distal chloride concentration.

Summary and Outlook

The search term “NKCC2” provides >400 hits in PubMed, and over 120 manuscripts on NKCC2 have been published within the last 3 years. The focus during the last few years has been on 1) the intracellular signaling pathways that regulate NKCC2 trafficking and the specific activity of the cotransporter and 2) the role of differential splicing in the fine-tuning of NKCC2 function. Substantial progress has been made in deciphering the cooperation of several kinases and in the identification of the corresponding phosphorylation sites of target proteins, which control NKCC2-mediated TAL salt retrieval. Some of these kinases that appear to be crucial, when assessed in an isolated in vitro setting, seem to function in a redundant network and can be compensated for by other pathways in vivo, as shown recently for SPAK and WNK3 (93, 102, 160). Such redundant control mechanisms may have developed during evolution because even small deviations from the normal control of NKCC2 may have a detrimental outcome on survival and, eventually, on the genetic fitness of an individual.

NKCC2 has long been considered a marker gene of the TAL, but there is growing evidence that NKCC2 is not as kidney specific as was generally believed. Apparently, NKCC2 is also expressed in extrarenal tissues, such as pancreatic β-cells (2) and the gastrointestinal tract (69, 159, 164). The functional relevance of NKCC2 in these extrarenal tissues is largely unknown.

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