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Title: The role of renal transporters and novel regulatory interactions in the TAL that control blood pressure.

Abstract: Hypertension (HTN), a major public health issue is currently the leading factor in the global burden of disease, where associated complications account for 9.4 million deaths worldwide every year (98). Excessive dietary salt intake is among the environmental factors that contribute to HTN, known as salt sensitivity. The heterogeneity of salt sensitivity and the multiple mechanisms that link high salt intake to increases in blood pressure are of upmost importance for therapeutic application. A continual increase in the kidney's reabsorption of sodium (Na^+) relies on sequential actions at various segments along the nephron. When the distal segments of the nephron fail to regulate Na^+, the effects on Na^+ homeostasis are unfavourable. We propose that the specific nephron region where increased active uptake occurs as a result of variations in Na^+ reabsorption is at the thick ascending limb of the loop of Henle (TAL). The purpose of this review is to urge the consideration of the TAL that contributes to the pathophysiology of salt sensitive HTN. Further research in this area will enable development of a therapeutic application for targeted treatment.

Keywords: Thick ascending limb of the loop of Henle, hypertension, salt sensitivity, sodium, kidney.

Introduction

Essential hypertension (HTN) is a highly hereditable trait of complex aetiology, where multiple genetic and environmental factors contribute to blood pressure variation. The study of genetic architecture has proven useful to detect a small number of genes, loci, and single nucleotide polymorphisms (SNPs) that have appreciable effects on blood pressure (40, 94, 123). Among the environmental factors that contribute to HTN, excessive dietary salt intake is one of the common and important risk factor (31). The link between dietary salt intake and HTN is well established and a reduction in salt intake is known to lower blood pressure (BP) (29, 137). Albeit, individuals respond differently to dietary salt; the increase in BP with an increase in salt intake occurs in some individuals, whereas others show no significant change in BP (179). This phenomenon is known as salt sensitivity, which is estimated to be present in 51% of the hypertensive and 26% of the normotensive populations (67, 68). Although salt sensitivity is a well-established phenomenon in experimental and human hypertension, the pathophysiological mechanisms are not fully elucidated (7). The heterogeneity of salt sensitivity and the multiple mechanisms that link high salt intake to increases in BP are important for clinical application. To this end many studies over the decades have focused on elucidating the pathophysiological mechanisms of salt sensitivity and the associated risk of developing HTN using; epidemiological studies (70, 148), clinical trials (1, 2, 53, 106, 180, 181), *in vivo* studies (11, 33, 36), and more recently systems approaches by means of metabolomics (110, 167); proteomics (58, 115); and transcriptomics (reviewed in (39, 125)). Even so, historical research on salt handling and blood pressure control has been vigorously perused demonstrating long term regulation of BP relies on the synchronised regulation of Na^+ (sodium), K^+ (potassium), and Cl^- (chloride) movement in the kidney to maintain blood volume and water balance (60, 70) (97).

The ability of the kidneys to absorb large quantities of Na^+ relies on sequential actions at various segments along the nephron, each with highly specialised transport capacities (outlined in Figure 1). Na^+ transport along the nephron is flow dependant, and a basic form of communication between parts of the nephron occurs as a

result of changes in Na^+ delivery by altered glomerular filtration rate (GFR). These alterations effect Na^+ reabsorption in the upstream segments of the nephron, provoking and a change in delivery/absorption of Na^+ to the downstream nephron (133). Suggesting the regulation of Na^+ reabsorption is highly controlled at the distal nephron, and failure of these downstream segments have more adverse effects on Na^+ homeostasis that alterations that occur in the proximal tubule. Proposing, that sustained increases of Na^+ reabsorption at the distal segments of the nephron is generated by a higher natriuretic pressure, and the specific nephron region where increased active uptake occurs as a result of variations in Na^+ reabsorption is at the thick ascending limb of the loop of Henle (TAL). Accordingly, this review will discuss the evidence implicating variations of Na^+ reabsorption at the TAL which are detrimental to sodium homeostasis and blood pressure control. This review will direct future studies to consider the TAL when investigating the pathophysiological mechanisms of salt sensitivity and HTN which will aid the search for precise therapies for essential hypertension.

ION TRANSPORT ALONG THE THICK ASCENDING LIMB OF THE LOOP OF HENLE

Anatomy and morphology of the loop of Henle

The loop of Henle encompasses the thin descending limb, the thin ascending limb, and the TAL (Figure 2). Within the inner stripe of the outer medulla there are short looped nephrons that derive from superficial and mid-cortical nephrons. These segments have a short descending limb and close to the hairpin turn of the loop, these tubules merge into the TAL. In contrast, the long-looped nephrons (originating from juxtamedullary glomeruli) have a long ascending thin limb where the TAL begins at the boundary between the inner and outer medulla. The TAL proceeds immediately after the thin ascending limb of long-looped nephrons. Aquaporin 1 (AQP1) is expressed abundantly in the proximal tubule; however, expression of AQP1 is also used as a marker to determine the junction between thin limbs of the loops of Henle and the TAL (124). The TAL begins after an AQP1 negative segment of short-limbed nephrons, immediately following the AQP1 positive thin descending limb (119, 124). Near the end of the TAL, the nephron passes between its afferent and efferent arterioles and meets its parent glomerulus; the macula densa (MD) constitutes the tightly packed epithelial cells at the junction of the TAL and the distal convoluted tubule (DCT) (119).

Characteristics of the thin descending limb include: high water permeability and low permeability for Na^+ and urea, with exceedingly low expression of Na^+-K^+-ATPase. Therefore, this segment is unlikely to contribute markedly to renal Na^+ reabsorption. Thin ascending limbs reabsorb some Na^+, but this absorption is likely passive as Na^+-K^+-ATPase expression is low (150). The TAL is the major Na^+ reabsorbing segment of the nephron accounting for approximately 20%–25% of filtered sodium chloride (NaCl). Features of the TAL include: maintenance of the extracellular fluid (ECF), regulation of arterial haemodynamics, and regulation of NaCl homeostasis contributing to the regulation of the urinary concentrating mechanisms. It is essential for the kidney to osmotically concentrate the urine above isotonicity; this is dependent on Na^+ reabsorption in the absence of measurable water permeability (124). Specifically, active NaCl absorption by the TAL dilutes luminal fluid to drives the counter current gradient that generates the axial osmolality gradient in the outer medulla region (119). The MD cells sense luminal NaCl concentrations and share most transport properties with TAL cells even though they are distinct in aspects of tubuloglomerular feedback (TGF). An increase of luminal NaCl at the MD generates a signalling cascade that induces vasoconstriction of the afferent arterioles which

reduces GFR. The augmented uptake of NaCl at the MD via the Na^+-K^+-Cl^- transporter (NKCC2) mediates TGF (71, 133). These signals cause the release of ATP into the juxtaglomerular interstitium and the subsequent generation of the nucleoside adenosine that constricts afferent arterioles (93). Figure 3 outlines the main ion transporters found at each nephron segment.

Thick ascending limb – Principal ion transporters.

At least 80% of NaCl uptake across the apical membrane of TAL cells is mediated by a co-transport process in which the influx of Na^+ drives the uptake of Cl^- and K^+. To achieve net salt absorption, apical electroneutral co-transport of Na^+, K^+, and Cl^- via NKCC2 is complemented by recycling of K^+ via ROMK, efflux of Cl^- via basolateral Cl^- channels (CLC- Kb/Barttin), coupled transport of Na^+ and H^+ via NHE3 transporters, basolateral extrusion of Na^+ by Na^+-K^+ -ATPase, and the final adjustment to urine concentration, K^+ homeostasis, acid base balance with Na^+ reabsorption is accomplished by ENaC (8).

The Na^+ /H^+ exchangers (NHEs) are typical Na^+ coupled transporters that mediate the counter transport of one extracellular Na^+ for one cytosolic proton (H^+) at the luminal apical membrane in the TAL. NHE3 is confined mainly to the apical membrane of renal cells (6), and functions by direct reabsorption of filtered Na^+ along with indirect reabsorption of bicarbonate (HCO_3-) and chloride and secretion of ammonium. The NHE3 anti-porters are positioned at the luminal apical membrane in the proximal convoluted tubule and the TAL and are responsible for reabsorbing up to 70% of filtered Na^+. NHE3 transports Na^+ into the cell in exchange for H^+ into the tubular lumen by the electrochemical gradient produced by Na^+-K^+-ATPase. The secreted H^+ combines with HCO_3^- to form H_2O and CO_2 and the latter diffuses into cells. Carbonic anhydrase (CA) allows rapid conversion of H_2CO_3 into H_2O and CO_2. Secreted H^+ generates OH^- inside the cell which is then converted into HCO_3^- by combining with CO_2. The HCO_3^- leaves the cell via the basolateral Na^+ -$3HCO_3^-$ co-transporter (adapted from (149)) (Figure 4A).

NKCC2 is localised at the apical membrane of the TAL. The function of this co-transporter is to reabsorb Na^+ without reabsorbing water. Na^+, K^+, and Cl- are transported into the cell across the apical membrane at a stoichiometry of 1:1:2 via the electrochemical gradient of the sodium pump. Na^+ and Cl^- are transported into the peritubular fluid while K^+ re-cycles back into the tubular lumen via ROMK (8). NKCC2 mediates approximately 90-100 % of Cl^- reabsorption, and 30- 50 % of all Na^+ transport, furthermore its function is linked with paracellular Na^+, Ca^+, and Mg^+ flux (73). For each Na^+ ion transported into the cell by NKCC2, 1 Na^+ ion is absorbed via paracellular pathways. Inhibition of NKCC2 results in decreased K^+ exit through ROMK halting NaCl reabsorption and paracellular transport of Ca^+ and Mg^+ (72, 76). ROMK channels supply a sufficient amount of K^+ to the NKCC2 co-transporter to maintain reabsorptive transport of NaCl. As Na^+ transport of NKCC2 is rate limited by the availability of luminal K^+ in the TAL, changes in activity of ROMK has regulatory effects of NaCl reabsorption (46) (Figure 4B). Previous immunolocalisation experiments revealed that NKCC2 is expressed at the apical cell surface of the TAL and MD but also in subapical vesicles, where intracellular trafficking regulates NKCC2 membrane expression. Vasopressin has been shown to shuttle the subapical vesicles of NKCC2 to the apical surface, leading to enhanced activity and expression levels (54). The transporter undergoes constitutive exocytosis at the TAL (27) suggesting NKCC2 trafficking is a dynamic continuous event rather than a triggered action. It was reported by Ares *et al* that endocytosis and recycling of

NKCC2 in the absence of stimuli results in the retrieval of the protein from the plasma membrane at a rate that is closely matched to that of exocytosis insertion (8). These reports suggest that under basal conditions and in the absence of stimuli dynamic trafficking of NKCC2 occurs. Three NKCC2 isoforms (A, B, and F) are derived by differential splicing of exon 4 of the *SLC12A1* gene. These isoforms differ in their distribution along the nephron and account for 20%, 10% and 70%, for A, B, and F respectively, of the total NKCC2 transcript abundance in mice (28). NKCC2A is located in the medullary and cortical TAL and exhibits immediate affinity for Cl^-, whereas NKCC2B shows highest affinity for Cl^- and is primarily located in the macula densa cells and cortical region. NKCC2F has the lowest affinity for ion transport, however, is mainly located in the medullary TAL. The relative distribution of these isoforms and affinity for ion transport matches the luminal concentration of NaCl along the TAL. Mice lacking NKCC2A revealed lower urine osmolality and decreased Cl^- reabsorption with enhanced expression of NKCC2B indicating compensation (135).

The basolateral Na^+/K^+-ATPase is the primary exit pathway for Na^+ at the basolateral membrane of TAL cells. This transporter is composed of α, β, and γ subunits where studies have demonstrated the α subunit at the TAL participates in the natriuretic response to salt load (101). Suppression of Na+ K+/ATPase activity markedly increases intracellular sodium (127). Studies have shown that rodents fed high salt diets display reduced levels of Na+ K+/ATPase in the renal tissues (105) and pigs administered deoxycorticosterone plus 1% NaCl in the drinking water develop reduced natriuresis increasing Na+ K+/ATPase inactivity by 30 fold and resulting in augmented arterial pressure (63). The activity of Na^+/K^+-ATPase generates a Na^+ gradient that enables apical entry of Na^+, K^+, and Cl^- via NKCC2. Ouabain can inhibit the activity of Na^+/K^+-ATPase, leading to the collapse of the lumen positive potential. This in turn abolishes the transepithelial Na^+-Cl^- transport at the TAL (75). Cl^- channels primarily mediate basolateral exit of Cl^- from TAL cells, there are two chloride channels co-expressed at the TAL; CLC-K1 and CLC-K2 (denoted CLC-NKA and CLC-NKB in humans), with the dominant Cl^- channel in the TAL being encoded by CLC-NKB (178). The characterisation of "Barttin" which is co-expressed with CLC-K2 in several nephron segments, including the TAL has aided the physiological understanding of the TAL. The human CLC-NKA and CLC-NKB paralogs do not function correctly in the absence of Barttin co-expression. CLC-NKB co-expressed with Barttin is highly selective for Cl^- (44).

Paracellular transport

Crossing the tubular epithelium can be performed in a single step (paracellular) or a twostep processes (transcellular). The paracellular route of ionic flow occurs when the substance goes through the matrix of the tight junctions that link each epithelial cell to its neighbouring cell, an example of paracellular movement is the reabsorption of calcium and magnesium. The TAL reabsorbs approximately 50%–60% of filtered magnesium and approximately 20% of filtered calcium, exclusively *via* the paracellular pathway (119). A lumen-positive transepithelial voltage (V_{te}) drives the paracellular reabsorption of Mg^{2+} and Ca^{2+}. The generation of V_{te} can be caused by the active transport due to apical K^+ secretion through ROMK and basolateral Cl^- exit through ClC-Kb/barttin channels (78). Therefore, the paracellular channels function by being charge and size selective. In particular, the charge and size selectivity of the tight junctions of the epithelia is predominantly by the claudins, a large gene family of tetraspan transmembrane proteins. Claudins are the key components of the paracellular pathway. Defects in claudin 16 and 19 result in a range of renal diseases, including hypomagnesemia,

hypercalciuria and nephrolithiasis. The claudin 16 channel provides cation permeability to the tight junction and the claudin 19 channel increases the cation selectivity of the tight junction alongside the diffusional V_{te} by selectively blocking anion permeation. Additionally, claudin 19 interacts with claudin 16 to increase the overall cation selectivity of the tight junction. Removal of either of these claudins results in the tight junctions loosing cation selectivity, creating renal defects in Mg^{2+} and Ca^{2+} reabsorption (78). Genetic studies in human suffering hereditary hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC), have demonstrated that claudin 16 and claudin 19 play an associated role in the cation selectivity of TAL tight junctions. *In vivo* the knockdown of claudin 16 increases Na^+ absorption in the collecting duct, accompanied by increases of ECF volume after treatment with amiloride (77, 79). Mice lacking Claudin-19 exhibit increased natriuresis with marked elevations in serum aldosterone, both strains replicating the phenotypes of human FHHNC (79). Collectively, these studies illustrate the vital role of claudin-16 and claudin-19 at the tight junctions of the TAL, representing contributing significantly to the selectivity of transepithelial absorption of Na^+, Ca^{2+}, and Mg^{2+}.

REGULATION OF ION TRANSPORT AT THE THICK ASCENDING LIMB BY ALTERED LUMINAL FLOW

Tubular luminal flow in the nephron constantly changes, where the rate of luminal flow varies acutely in response to changes of e.g. glomerular hemodynamics and/or changes in upstream fluid reabsorption. Increases in luminal flow are associated with enhanced Na^+ reabsorption at major sites along the nephron (24-26, 128) and chronic increases in luminal flow are observed in HTN. Thus it is important to understand the cellular mechanisms in which each nephron segment senses and responds to changes in flow by appropriately altering transport rates. *In vivo* microperfusion studies have provided much of the evidence for flow dependent NaCl reabsorption along the TAL. The mechanisms of signalling events and interactions between soluble mediators and mechanical forces at the TAL have yet to be defined. Nevertheless, the actions of furosemide on NaCl reabsorption enforces that flow dependence of loop Na^+ transport is mediated mainly by the TAL (153, 185). Wright and colleagues report a 4-fold increase in flow rate leads to a doubling of NaCl reabsorption, demonstrating that fractional reabsorption of NaCl falls with increasing flow rates (185). Flow dependence of TAL NaCl transport relies on the activity of NKCC2 (57). At times of low luminal flow, NaCl concentrations decrease along the TAL. If flow rate elevates, the drop in NaCl concentration is reduced, leading to higher luminal concentrations of NaCl that augment NaCl transport. In these conditions, minimum NaCl concentrations are not reached, resulting in flow-dependent increases in NaCl concentration at the TAL (133). The regulation of function along the TAL and NaCl transport capacities at this nephron segment are regulated by numerous biological factors, such as; autacoids, hormones, and eicosanoids. Increased intracellular cAMP stimulates transepithelial NaCl transport in the TAL, specifically; vasopressin (AVP), parathyroid hormone (PTH), glucagon, calcitonin, and β-adrenergic receptor signalling (49). This activation can subsequently be modulated by a number of negative influences, notably prostaglandin E_2 (PGE_2), extracellular Ca^{2+}, hormones and autocoids working via cGMP-dependent signalling, including nitric oxide, all having powerful negative effects on NaCl transport within the TAL (8).

Mechanisms that activate NKCC2

The most extensively studied activating modulator of NaCl transport in the TAL is vasopressin (AVP), mediated via the V2 receptor (122). Vasopressin activates NKCC2 in perfused mouse TAL segments exerting longer-term effects on NKCC2 expression and function at the apical surface. Previous immunohistochemical localisation experiments revealed that NKCC2 is expressed not only at the apical cell surface but also in subapical vesicles, where intracellular trafficking regulates NKCC2 membrane expression. Vasopressin has been shown to facilitate the shuttling of NKCC2 in subapical vesicles to the apical membrane, leading to enhanced activity and expression levels (54). *In vivo* studies have demonstrated that the phosphorylation of NKCC2 is associated with a cluster of N-terminal threonines residues (54). Rats treated with desmopressin showed induced phosphorylation of these residues, which are substrates for the homologous STE20/SPS1-related proline/alanine-rich kinase (SPAK) and oxidative stress–responsive kinase 1 (OSR1) kinases (54). These kinases mediate hypotonicity and low chloride induced activation of NKCC2 and sodium reabsorption. Studies have shown that NKCC2 phosphorylation is mainly driven by OSR1 abundance whereas SPAK abundance is responsible for NCC phosphorylation (99, 116, 190). SPAK and OSR1, are activated by upstream WNK (with no lysine [K]) kinases and the N terminus of NKCC2 contains binding sites for SPAK and OSR1 (35) (51), where OSR1 kinase has been reported as responsible for N-terminal phosphorylation of NKCC2 and is critical for activity of the transporter. Reduced N-terminal NKCC2 phosphoprotein in mice with targeted TAL-specific deletion of OSR1 results in the loss of function of the TAL. Lin *et al* generated global and tubule specific ORS1 knockout mice, and found global knockout was embryonically lethal whereas tubule specific OSR1+/- mice displayed lower BP associated and reduced phosphorylated NKCC2 and NCC (99). The tubule specific knockout mice had reduced Na^+ reabsorption at the TAL and a blunted response to furosemide with significantly reduced pNKCC2. They also demonstrated that total expression of SPAK and pSPAK was increased in parallel to NCC despite unchanged NKCC2 expression. Suggesting that, ORS1 is necessary for BP and renal sodium reabsorption via activation of NKCC2. The only upstream stimulators of SPAK/OSR1 are the family of with-no-lysine kinases (WNKs); WNK1, WNK2, WNK3, and WNK4. Phenotypic studies of WNK "knock-in" mice have demonstrated the role of the upstream WNK kinases in which mutant SPAK or OSR1 cannot be activated by upstream WNK kinases; these mice have reduced phosphorylation of NKCC2 and NCC, with associated salt-sensitive hypotension (119, 141). These studies report that WNK kinases appear to regulate SPAK/OSR1 and NKCC2 in chloride-dependent fashion, phosphorylating and activating SPAK/OSR1 and the transporter in response to changes in intracellular Cl^- activity as a result of altered luminal flow and NaCl uptake at the TAL (136). The effect of hypotonicity/low Cl- on NKCC2 is enhanced/activated by WNK3 i.e. a decreased intracellular Cl- induces phosphorylation of NKCC2 to enhance its activity. The loss of WNK3 expression is compensated by WNK1 (117). One group used wild type (WT) and WNK3-/- mice and studied GFR, renin levels, urine output, urine osmolarity during a normal diet and a salt restricted diet. WNK1 was markedly increased in WNK3-/- mice compared with WT during a normal diet. During a salt restricted diet, levels of pSPAK/OSR1, pNKCC2, and pNCC were upregulated in WNK3-/- mice. These mice also displayed increased diuresis in response to hydrochlorothiazide. Therefore, WNK3 may function as a Cl- sensor in TAL cells (130).

WNKs are thought to regulate renal outer medulla ROMK by interacting with intersectin and influencing clathrin mediated endocytosis. He *et al* have demonstrated that ROMK is inhibited by WNK1 and WNK4 due to increased endocytosis of the channel (69). WNK4 phosphorylates tight junction proteins claudins 1-4 to regulate

the paracellular chloride permeability (86, 187). This paracellular chloride permeability in cells expressing mutant WNK4 is much greater than that of cells expressing WT WNK4 proteins. Demonstrating that HTN in patients with WNK4 mutations is caused by increased NaCl reabsorption (18, 87, 188). The endocytosis occurs via clathrin coated vesicles, amongst these, intersectin which contains Src-homology 3 domains that can intercat with PXXP proline rich motifs (88). The interaction between WNK1 and WNK4 was specific to intersectin via the WNK proline rich motifs but not their kinase activity. When intersectin was knocked out the endocytosis of ROMK was prevented, providing a molecular mechanism for stimulation of ROMK endocytosis and reduced expression by WNK kinases.

Tumour necrosis factor (TNF) as a negative regulator of the TAL

TNF is produced by and affects the functions of several renal cell types including proximal tubule (PT), TAL, and collecting duct (CD) as well as podocytes and mesangial cells (34, 52, 83, 91, 107, 109, 113, 147). While TNF is best known for its pro-inflammatory actions, this cytokine also exhibits immunosuppressive, immunomodulatory, and regulatory effects in diverse tissues including kidney, lung, and colon, where active Na^+ transport is important for fluid clearance and other transport mechanisms (5, 21, 30, 42, 43, 114, 146). Data from TNF knockout mice and the clinical use of anti-TNF drugs have exposed intrinsic effects of this cytokine that preclude the neutralization of TNF in heart failure and SLE, for example (42, 82, 89, 112, 134, 169, 184, 193). Context-dependent effects of TNF also are observed in blood pressure regulation where studies have documented the pro-hypertensive effects of TNF in experimental models involving inflammation, however, TNF does not increase blood pressure in all models of hypertension and does not elevate blood pressure *per se* when given to normal animals (4, 41, 45, 61, 92, 111, 120, 144, 164, 173, 175). Collectively, these studies infer the importance of determining the mechanisms that subserve both the beneficial and deleterious effects of TNF in individual cell types as an approach that may facilitate the development of cell-targeted therapies.

Moreover, it is important to determine if the autocrine inhibitory effects of TNF on TAL cells involving NKCC2 *in vitro*, which are consistent with the TNF-dependent polyuria and natriuresis observed in various studies, are operational *in vivo* (14, 43, 45, 64, 154, 155, 171). Defining the role of TNF *in vivo* for individual cell types is noteworthy since there are conflicting accounts, for instance, of TNF effects on amiloride-sensitive Na^+ transport, where it inhibits transport *in vivo* (108) while stimulating it *in vitro* (176). Similarly, the sensitizing effect of TNF to increase ENaC activity in distal tubules from diabetic rats contrasts with its effects in control rats or mice where TNF inhibits ENaC activity (12, 38, 108). The heterogeneity of tubular epithelial cells may necessitate distinct regulatory mechanisms for each cell type by molecules such as TNF that affect the expression and activity of assorted transporters including NHE8, SGLT2, NKCC2, ENaC, and Na+-K+-ATPase (12, 14, 38, 109, 176, 186). Renal transporters must be tightly regulated and coordinated to maintain Na^+ and blood pressure homeostasis and new models are being generated to help advance the field by uncovering the *in vivo* effects of TNF derived from an individual nephron segment, the TAL, where variations in Na+ reabsorption have been linked to hypertension (47, 85). Understanding how autocrine cascades in the TAL operate in normal and diseased states may provide insight towards the development of new 'loop diuretic' therapies that could offer fine-tuning of Na^+ reabsorption via discrete targeting of regulatory molecules.

UROMODULIN

Protein structure

Uromodulin (UMOD) is a kidney protein exclusively synthesised at the TAL and is encoded by the *UMOD* gene. Based on the cDNA sequence, the UMOD precursor is composed of 640 amino acid residues and motifs include signal sequence residues 1 – 24, 1 epidermal growth factor like and 2 calcium binding epidermal growth factor like domains (residues 31 – 64, 65 – 107, and 108 – 149), 1 zona pellucid domain at residues 334 - 585 (this is essential for polymerisation), 8 potential N-glycosylation sites, and 1 stretch of hydrophobic amino acids similar that acquire glycosylphosphatidylinositol (GPI) attachment site (residue 614). There are 48 cysteine residues involved in disulphide bond formation (62). It was proposed an additional epidermal growth factor like domain spanning residues 281 – 336 was present in the region between the 2nd calcium binding epidermal growth factor domain and zona pellucida domain. However, a new domain D8C (residues 199-287), common to families of proteins including liver specific zona pellucida, glycoprotein 2, UMOD and several other uncharacterised proteins was described by Yang *et al* (189).

UMOD is GPI anchored to the apical plasma membrane, thus its biosynthesis and intracellular trafficking proceed through a secretory pathway. During biosynthesis, the UMOD precursor is translocated to the ER (endoplasmic reticulum). The signal peptide is then cleaved and the protein glycosylated on 7 of the 8 potential N-glycosylation sites. Disulphide bridges are formed and glypiation on its C terminus occurs, the Golgi apparatus further modifies the N-glycan moieties. The mature glycan moieties and the GPI modifications act to route the protein to the apical membrane of epithelial cells in the TAL, this is when UMOD is finally GPI anchored and facing the tubular lumen, here it is said to form supramolecular structures to ensure its proposed physiological properties are performed (81). The protein is released for the lumen side of the membrane by specific but currently unidentified protease(s). Proteolytic cleavage was originally thought to occur after residue F548 (50), but later thought F587 (151). Proteolytic cleavage of the GPI anchor still remains to be determined, however, once cleaved form the apical membrane UMOD forms polymers in the urine and becomes the most abundant protein in mammalian urine.

Physiological and pathological roles of UMOD

Due to UMODs structure it has one peculiar feature; it has a tendency to aggregate with gel like properties in solution when NaCl concentrations are close to 100 mmol/l or CaCl (calcium chloride) is 1 mmol/l (15, 23) which may be responsible for the binding properties of the protein. This along with the GPI anchoring and multidomain structure on the luminal side of the apical membrane, in conjunction with rich and highly variable post translational protein modification, and a large presence of polymerised protein in the urine suggests that UMOD may play multiple roles with site specific physiological functions. However, over 60 years of research has not elucidated the biological role of UMOD. Sorting of the apical membrane proteins requires GPI anchors, N-glycosylation, and polymerisation, with the large turnover of UMOD and its half-life of 16 hours it is assumed that the biosynthesis plays either a direct or indirect role in the formation of the apical membrane targeted cargo vesicles and vesicle trafficking. GPI anchored proteins associate with lipid raft domains that play a role in organising the apical membrane and signalling transduction pathways (142). When the apical

membrane is highly ordered it allows for close packing of GPI anchored proteins on the surface of cell membranes, in the case of UMOD this may promote formation of the complex gel like structures providing a water barrier at the luminal membrane of the TAL cells. This physical barrier to water permeability may play a role in ion transport to maintain counter current gradients in the interstitium (80).

As the TAL are nephron segments characterised by high electrolyte and water impermeability it was proposed that UMOD plays a role in salt transports and acts as a water barrier at this level, a process crucial for urine concentration. Bleyer *et al* reported low urine osmolality was consistent in subjects with *UMOD* mutations (19). A transgenic mouse model for Uromodulin associated kidney diseases (UAKD) confirmed urinary concentrating deficit (16). Water reabsorption by the tubule is regulated by transmembrane systems of aquaporin and ion channels (3), Bachmann and colleagues studied the renal effects of UMOD deficiency in *UMOD* knockout mice and reported the inability of these mice to concentrate urine possibly due to a decrease in cyclooxygenase-2 (COX-2) expression (10). COX-2 inhibition prevents regulation of key renal water and sodium transport proteins including aquaporin 2 (AQP2), NHE3, and NKCC2 (126), confirming the role of UMOD in urine osmolality. More commonly in the clinic, UMOD is associated with renal cystic diseases. These are a major group of inherited renal conditions, representing the leading cause of end stage renal disease. Cystic kidney disease (CKD), in both the dominant and recessive variants, accounts for the clinical conditions. Hart *et al* and Rampoldi *et al* discovered autosomal dominant mutations in *UMOD* lead to medullary cystic kidney disease type 2 (MCKD2), familial juvenile hyperuricemic nephropathy (FJHN), and glomerular cystic kidney disease (GCKD) (66, 143). These conditions are characterised by urinary concentration deficits, urinary salt wasting, hyperuricemia, gout, medullary cysts, interstitial nephritis, glomerular cysts, hypertension, and end stage renal failure. MCKD2 is autosomal dominant disorder is mainly characterised by hypertension and end stage renal failure, in the fourth decade of life, with renal complications including tubular membrane disintegration, tubular atrophy, with cyst development at the corticomedullary border and interstitial cell infiltration associated with fibrosis. MCKD2 has clinical and morphological overlap with the autosomal dominant FJHN. GCKD is characterised by a cystic dilatation of Bowmen's capsule and collapse of the glomerulus (191). All three disorders have significant clinical overlap and arise from *UMOD* mutations and are often referred to as UAKD and are said to cause the so called "uromodulin storage disease" (177, 183).

To date there have been more than 58 *UMOD* mutations reported, that mainly localise to exon 3 and 4 of the *UMOD* gene, with the majority being missense mutations or small inframe deletions (95). Early genome wide linkage mapping in Italian, Czech, and Belgian families revealed loci for MCKD and FJHN on chromosome 16 in the regions of 16p11.2 and 16p12 (32, 166) in close proximity to the *UMOD* gene. These conditions are associated with mutations that lead to amino acid changes at cysteine sites causing defective protein folding and immature UMOD being retained at the ER and not released at the apical membrane and remains intracellular (16, 143). Accumulation of misfolded UMOD in the ER causes ER stress and degradation by increased synthesis of chaperones and foldases that in turn activate the misfolded protein (90). This unfolded protein response may trigger apoptosis and autophagy or alternatively lead to cell activation via MAP kinases and NF-$_{k}$B leading to the eventual progressive renal failure seen in MCKD2, FJHN, and GCKD (96). It is known that the transcription factor hepatic nuclear factor 1-β (HNFIβ) positively regulates UMOD expression and binds to

the promoter elements of the gene. Inactivation of HNFIβ *in vivo* is associated with decreased *UMOD* transcription (100). Interestingly mutations of HNFIβ are associated with features of MCKD2, FJHN, and GCKD (160). This transcription factor is also known to regulate nephrocystins which prompted work by Zaucke *et al* in 2010 to investigate if UMOD is linked to cillary cystogenesis (191). They reported 7 novel *UMOD* mutations (missense or deletion mutations) between exon 4 - 5 and that UMOD is expressed in primary cilia of renal tubules and the number of UMOD positive tubules declines in UAKD. The mutations caused localisation of UMOD in the mitotic spindle poles co-localised with nephrocystin-1, suggesting a novel cause of cystic kidney disease pathologies of MCKD2, FJHN, and GCKD.

In the absence of UMOD there is susceptibility to calcium oxalate stones due to the lack of gel like properties of the protein, thus altered salt concentration at the apical membrane is apparent (165). Mo *et al* showed that *UMOD* knockout mice spontaneously formed intra-renal crystals predominantly in the interstitial space and in the collecting duct of the deep medulla and papilla (118). They reported that the stones consisted primarily of calcium phosphate in the form of hydroxyapatite and strongly resemble the stones found in humans caused by idiopathic calcium oxalate stones. The inhibitory effect of UMOD in stone aggregation had been described in cases of calcium oxalate (13, 37). There is consensus that inhibition of stone formation in normal urine is caused mainly by urinary macromolecules rather than low molecular weight components, and this property has been associated with polyanionic structures (48). UMOD is said to be a polyanionic macromolecule due to the large extent of sialylation and presence of sulphate groups bound to the N-linked glycans thus playing a crucial role in regulating stone formation (172). Urinary tract infections (UTI's) are mainly caused by *E Coli*, and critically depends on filamentous appendages on the bacterial surface called fimbriae (13). Colonisation is mediated by binding of lectin like adhesins present on *E coli* fimbriae to carbohydrate structures carried by glycoprotiens exposed at the cell surface. *E coli* fimbriae are classified according to their sugar specificity: type 1, type P, and type S, Pak *et al* illustrated that UMOD binds with type 1 *E coli* fimbriae *in vitro*, and Raffi *et al* described UMOD as a general host defence mechanism against urinary tract infections (132, 140) *in vivo* with a UMOD knockout mouse model, supporting the concept that urinary UMOD represents a protective mechanism against UTI's. In terms of urinary excretion, in adults, there appears to be considerable variation in daytime excretion values, but does correlate with urinary volumes brought about with diuresis in those drinking in response to thirst (103). A positive correlation between urinary UMOD and dietary salt intake revealed that in subjects with high salt sensitivity i.e. exaggerated BP response to high salt intake, there is a greater excretion of UMOD in the urine compared low salt intake (168). UMOD excretion seems to increase gradually from birth to adulthood, where it remains stable until a decline after the sixth decade of life (129, 161), with urinary UMOD/creatinine ratio also remains stable from the age of 4 until the age of 70. Reduced urinary excretion of UMOD has also been correlated with GFR and corresponds to declining kidney function in virtually all chronic kidney diseases (138). In an attempt to elucidate why urinary UMOD excretion is altered in disease Ma *et al* studied potential mechanisms using human *UMOD* mutations in polarised Madin Darby canine kidney cells (MDCK). They report that cysteine mutations inside and out of the domain are able to specifically bind and trap UMOD preventing it from exiting the ER and translocating to the cell surface. However, they found specifically cysteine altering mutation in the cysteine rich domain had more sever deficits in ER exit and surface translocation, initiating increased apoptosis explaining partly in some diseases the marked reductions in urinary UMOD (104).

Uromodulin, blood pressure, and ion transport

Genome-wide association studies recently linked more common genetic variants in the *UMOD* promoter with the risk of HTN (131). Our previous GWAS identified a locus upstream of the *UMOD* gene transcriptional start site, which was associated with altered BP (131). It was reported that the minor G allele of rs13333226 when adjusted for estimated glomerular filtration rate (eGFR) was associated with a 7 % lower risk of developing hypertension. This GWAS discovery followed by functional validation studies have now resulted in a re-focussing of interest in UMOD and its role in BP regulation (56, 170). Results from our recent studies in *UMOD*-/- mice suggest a biological link between *UMOD* and ion transport in the TAL. In the absence of *UMOD* there is augmented sodium excretion in the *UMOD-/-* mice, thought to be a consequence of reduced expression of NKCC2. This modulated Na^+ reabsorption by reduced NKCC2 leads to exaggerated natriuresis and lower arterial pressure in the *UMOD-/-* mice, consistent with findings in humans where salt wasting phenotypes and hypotension are characteristics of Bartter's syndrome (139, 157, 174). In a complimentary set of experiments, Trudu *et al* demonstrated that uromodulin-transgenic mice over expressing UMOD manifested salt-sensitive hypertension, due to activation of the SPAK kinase and activating N-terminal phosphorylation of NKCC2 (170). Biochemical and histological analysis of UMOD has been used to assess changes in biosynthesis of proteins related to NaCl transport along the nephron in *UMOD-/-* mice. Bachmann *et al* revealed upregulation of major distal transporters (Na^+/K^+ -ATPase, NKCC2, NHE3, ROMK, and ENaC) and downregulation of juxtaglomerular apparatus components (10, 121). They reported that the augmented total NKCC2 expression was in fact as a result of increased intracellular expression of NKCC2, where it remained unphosphorylated and inactive in *UMOD-/-* mice (121). This group concluded that in the absence of *UMOD* the reduced NKCC2 activity results in impaired NaCl reabsorption at the TAL, implying a permissive role of *UMOD* in the modulation of Na^+ transport. ROMK resides in the apical membrane in the TAL and processes NaCl reabsorption via the recycling of K^+ ions into the tubular lumen. It forms a functional unit with NKCC2, the rate limiting transporter. Disrupted ROMK function at the TAL limits NaCl reabsorption resulting in the salt wasting phenotypes observed in Bartter's syndrome. Renigunta, *et al* identified UMOD as a ROMK interacting protein, regulating ROMK function by increasing its expression at the cell surface of the TAL apical membrane (145). Furthermore, this group observed a decrease in ROMK immunoreactivity in the plasma membrane enriched fractions of *UMOD*-/- mice kidney compared to *UMOD*+/+ counterparts. They demonstrated that *UMOD* ablation results in large accumulation of ROMK in the sub apical vesicles resulting in delayed or decreased surface expression leading to salt wasting. This accumulation of ROMK is a result of the fluid entering the TAL having high concentrations of Na^+ and Cl^- with low concentrations of K^+. Therefore, efficient reabsorption of Na^+ and Cl^- would be prevented as the stochiometric flux of Na^+ and K^+ into the cell (via NKCC2) would quickly deplete K^+ levels in the luminal fluid. This stimulation is normally avoided by the recycling of K^+ entering the cell back into the lumen. Thus, the absence of K^+ recycling results in markedly impaired NaCl reabsorption which may be occurring in the *UMOD-/-* mice. A physiological regulator of NKCC2 transport rate is the Cl- ion, in times of low luminal Cl- the activity of NKCC2 is attenuated (22, 57). Mutig *et al* have shown that activation of NKCC2 is facilitated by UMOD in a Cl^- sensitive manner (121). These findings suggest inactivation of NKCC2 and ROMK explaining the phenotypes similar to Bartter we reported previously in these

mice (56). To date the differential expression analysis of the major Na^+ handling transporters in the kidney of *UMOD*+/+ and *UMOD*-/- mice before and after salt loading has not been explored.

PATHOPHYSIOLOGY OF THE TAL

Given its role in renal physiology, it is not surprising that the TAL plays an important part in the pathophysiological role of disease. An extensive understanding of the TAL in renal ion transport systems will encourage precision medicine to provide stratified treatments for individuals. Single gene mutations present in Mendelian forms of HTN all increase Na^+ reabsorption at the distal segments of the nephron, but not at the TAL (97, 182). Monogenic blood pressure disorders where TAL ion gene expression is altered result in hypotension, suggesting a role for variants in the TAL to lower rather than raise blood pressure, reducing the risk for HTN. Re-sequencing of three salt-handling genes (*SLC12A3* (NCC), *SLC12A1* (NKCC2), and *KCNJ1* (ROMK)) in a cohort of over 5000 subjects of the Framingham Heart study showed the large influence rare variants have on BP (84). The authors documented 30 mutations in these 3 genes that were inferred to have functional consequences. Carriers of any of the rare variants in the three salt-handling genes (with minor allele frequency ≤ 1 %) had mean reductions of 6.3 mmHg in SBP and 3.4 mm Hg in DBP, compared with the entire cohort. Mutation carriers had mean SBP values 6.6 mm Hg less than their non-carrier siblings. These are large effects when compared with those of common variants, for which the effect size is usually 1 mm Hg or less (84). Authors conclusively demonstrated that these variants were associated with lower BP (approximately 6-10 mmHg) and were protective against increased risk for hypertension, emphasising the contribution of sodium handling in blood pressure regulation in the general populations.

Studies so far suggest that variations in Na^+ uptake by the TAL influence an individual's chronic level of blood pressure. Loss-of-function mutations in TAL Na^+-Cl^- transport are associated with Bartter's syndrome. Lifton and colleagues reported that Bartter's syndrome results from mutations in NKCC2 or its regulators and is referred to as type 1 (156-158) . Cl^- carried into the cell by NKCC2 exits through the Cl^- channels CLCNKA and CLCNKB with facilitation by BSND (barttin gene). Mutations in CLCNKB and BSND result in, respectively, types 3 and 4 Bartter's syndrome. Bartter's syndrome type 3 is caused by mutations of the Cl^- channel CLCNKB (156). NaCl entering the cell via NKCC2 at the apical membrane must exit the cell via the Na^+ -K^+ /ATPase at the basolateral membrane; however this mutation prevents normal Cl^- exit due to inhibition of membrane localisation. Thus, the functional activity of NKCC2 and blood pressure control is determined by clearing excess Cl^- from TAL cells. Additionally, these patients may have an increase in urinary Ca^{2+} excretion and approximately 20% are hypomagnesemic (59). Upon binding of the Ca^{2+} sensing receptor (CASR) on the basolateral surface in TAL leads to inhibition of NKCC2 (74) resulting in a more negatively charged lumen and less recycling of K^+. These actions facilitate excretion of Ca^{2+}, serving as a means for correcting and/or preventing a state of hypercalcemia. Loss of function mutations in ROMK also result in Bartter's syndrome (Type 2). The mutations results in salt wasting as: fluid entering the TAL has increased concentrations of Na^+ and Cl^- with low K^+ thus the stochiometric flux of Na^+ and K^+ via NKCC2 rapidly depletes K^+ in the luminal fluid which prevents efficient reabsorption of Na^+ and Cl^- (157). This chain of events can be avoided by K^+ entering back into the luminal fluid, implicating ROMK as the necessary channel for the recycling of K^+ to

maintain Na^+ and Cl^- reabsorption, demonstrating the importance of synchronised actions of these channels in blood pressure regulation.

Gitelman syndrome presents as a form of hypotension and renal sodium wasting caused by monogenic mutations. This condition is genetically homogeneous and is caused by loss of function mutations of the NCC transporter (Na-Cl co-transporter in the distal convoluted tubule (DCT)) (159). Patients usually present in adulthood with lower BP than the general population and display neuromuscular symptoms. These individuals have low serum Mg^+ and low urinary Ca^+ (17). The sodium wasting at the DCT activates the renin-angiotensin-aldosterone system (RAAS), however in this condition there is augmented ENaC activity, protecting sodium homeostasis at the expense of increased H^+ and K^+ secretion. In order for Na^+ reabsorption in TAL to reach a level sufficient to raise blood pressure it requires help from another nephron site. For example if Na^+ reabsorption by NKCC2 was increased, the distal nephron should in response to the renin-angiotensin-aldosterone system reduce Na^+ downstream. Should these compensatory mechanisms not occur, a higher blood pressure could develop. When investigating the role of the TAL to increase blood pressure, the distal nephron or proximal tubule should be considered. Simultaneously, greater Na^+ reabsorption elsewhere could require a more active TAL before manifesting in an increase in blood pressure.

ROLE OF THE TAL IN SALT SENSITIVE HYPERTENSION

A role for TAL in regulating blood pressure in humans is difficult to establish in the clinic setting due to limitations essential for medical assessments. If TAL function increased, the increments in Na^+ reabsorption necessary to raise BP would be relatively small and only enough over time to exceed any adjustments either proximally or distally. For example, ENaC in the collecting duct reabsorbs only a small fraction of filtered Na^+ (2%), thus Na^+ reabsorption in TAL would need only to exceed this small fractional range to affect blood pressure. A more active TAL could explain greater proximal tubular reabsorption of Na^+. This would result in reduced TGF followed by increased glomerular filtration thereby increasing delivery of Na^+ to the proximal tubule that then increases absolute amounts of Na+ reabsorption (20). Indeed, Aviv *et al* extensively reviewed clinical studies demonstrating the TAL as a pivotal part of the nephron contributing to the heightened susceptibility salt sensitive hypertension (9). A plausible mechanism responsible was proposed as a consequence of increased NKCC2 activity leading to an expansion of extracellular volume, decreased urinary K^+ excretion and increased Na^+ reabsorption with greater water conservation. An increase in Na^+ uptake by NKCC2 would enhance TGF (less Na^+ reaching the macula densa) leading to glomerular hyperfiltration, delivering more Na^+ to the proximal tubule resulting in an increase in the absolute amount of Na^+ reabsorbed in the proximal tubule and downstream in TAL (85). In 2004, Sonalker *et al* illustrated that spontaneously hypertensive rats (SHR) had 4 fold higher expression levels of total NKCC2 (163). This group later measured membrane bound and total NKCC2 in the outer medulla in these rats by subcellular fractionation and found no difference in the surface to intracellular ratio of NKCC2 (162). Yet, progression from normotension to hypertension (5 – 8 weeks of age) in SHR was accompanied by a two fold increase in the surface to intracellular ratio of NKCC2 (54). Additionally, in Dahl salt sensitive rats, NKCC2 dependent TAL transport, phosphorylation, and cell surface NKCC2 were enhanced upon salt loading with increased BP (65). Indicating that salt stimuli modulates NKCC2 trafficking beyond essential requirements altering Na^+ uptake, ECF volume, and increasing arterial pressure. Zhou *et al* studied the effects of ROMKi B (a selective ROMK inhibitor) on systemic hemodynamics, renal function and

structure, and vascular function in Dahl salt-sensitive rats on a high salt diet (192). They reported a potential utility of ROMKi B as a novel antihypertensive agent, particularly for the treatment of the salt-sensitive hypertension patient population as this inhibitor promoted increased natriuresis without any adverse effects on electrolytes and acid–base balance, kidney function, and structure.

Other TAL ion transporters may also contribute to salt sensitive hypertension; a high sodium intake increases the capacity of the TAL to absorb $HCO3^-$. Good *et al* examined the role of the apical NHE3 and basolateral NHE1 Na(+)/H(+) exchangers in TAL tubules from salt sensitive rats drinking normal tap water or 0.28 M NaCl for 5-7 days (55). They reported that high sodium intake increased $HCO3^-$ absorption rate by 60% and this was mediated by an increase in apical NHE3 activity. Inhibiting basolateral NHE1 eliminated 60% of the $HCO3^-$ absorption. Demonstrating that during a high salt diet, these rats have increases in NHE3 activity dependent on NHE1. Studies in NHE3 KO mice report chronic volume depletion and hypotension (152). Furthermore, NHE3-/- mice display blunted proximal fluid reabsorption without altered distal delivery of fluid (102). This compensation is largely attributable to decreased GFR due to activation of TGF suggesting normalisation of fluid delivery to the distal tubule is achieved through alterations in filtration rate and/or downstream transport processes. Reabsorption of filtered Na^+ along the PCT in proportion to GFR suggests that rates of Na^+ and fluid not being reabsorbed will change in proportion to the rate of filtration, denoting that despite glomerulotubular balance (GTB), delivery rate of Na^+ to the tubular segments beyond the PCT will increase whenever GFR increases. Thus, Na^+ homeostasis is regulated by TGF, a counter regulatory mechanism that senses an increase in NaCl delivery to the late nephron segments. These studies support a role of the TAL enabling the kidneys to regulate acid-base balance during changes in sodium and volume balance.

Future direction

A considerable body of evidence links salt sensitive hypertension with the role of the TAL. Further research in this area will enable development of a therapeutic application for targeted treatment. This is crucial because despite major advances in cardiovascular health, hypertension remains the risk factor contributing most to the overall burden of disease globally and there is a paucity of novel antihypertensive drugs in clinical trials or pharmaceutical development pipeline.

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Figure captions

Figure 1. Schematic of a nephron segment.

The nephron consists of a renal corpuscle, a proximal tubule, a loop of Henle, a distal tubule, and a collecting duct system. The renal corpuscle consists of glomerular capillaries and Bowman's capsule. The proximal tubule initially forms several coils followed by a straight segment that descends into the medulla. The next segment is the loop of Henle, which consists of a straight section of the proximal tubule, the descending thin limb (which ends in a hair pin turn), the thin ascending limb, and the thick ascending limb. Near the end of the thick ascending limb, the nephron passes between its afferent and efferent arterioles. The short segment of the thick ascending limb is called the macula densa. The distal tubule begins a short distance beyond the macula densa and extends to the point in the cortex where two or more nephrons join to form the cortical collecting duct. The collecting ducts enter the medulla and become the outer medulla collecting ducts, and then the inner medullary collecting ducts. In terms of ion transport the proximal tubule is responsible for 60 – 70 % of the filtered Na^+, whereas 15 – 25 % is absorbed by the loop of Henle. The distal tubule reabsorbs 5 – 10 % and the collecting ducts only 1 -2 % (149).

Figure 2. Organisation of the Loop of Henle.

Structures are as follows: 1, thin descending limb of the loop of Henle; 2, thin ascending limb of the loop of Henle; and 3, thick ascending limb of the loop of Henle. See main text for details relevant to the labelled structures.

Figure 3. Schematic of a nephron segment indicating ion transporter positions.

PCT: Proximal convoluted tubule; TAL: Thick ascending limb of the loop of Henle; and DCT: Distal convoluted tubule.

Figure 4. Schematic of ion transport in the TAL by actions of (A) NHE3, (B) NKCC2.

ATP (adenosine triphosphate), CA (carbonic anhydrase), Ca^+ (calcium), CO_2 (carbon dioxide), Cl- (chloride), H^+ (hydrogen), H_2O (water), H_2CO_3 (carbonic acid), (HCO_3^- (bicarbonate), K^+ (potassium), Mg^+ (magnesium), Na^+ (sodium), NKCC2 (Na^+-K^+-Cl^- transporter), and ROMK (renal outer medullary potassium channel).